Lipase catalyzed esterification of sugars with alkyl side chain containing amino acids

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By

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Declaration

I hereby declare that the thesis entitled, **"Lipase catalyzed esterification of sugars with alkyl side chain containing amino acids"** submitted for the degree of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore** is the result of the work carried out by me under the guidance of **Dr. S. Divakar** in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore, during the period 2004-2007.

I further declare that the results of this work have not been submitted for the award of any other degree or fellowship.

Date:
Place: Mysore   (B.R Somashekar)

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Certificate

I hereby declare that the thesis entitled, **"Lipase catalyzed esterification of sugars with alkyl side chain containing amino acids"** submitted by **Mr. B. R. Somashekar** for the degree of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore** is the result of the work carried out by him under my guidance in the Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore during the period 2004-2007.

Date:
Place: Mysore   (S. Divakar)
                                                          Guide

….………. To My Beloved Parents and Brother

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B. R. Somashekar

List of Patents and Publications

Patents

1. K Lohith, **B R Somashekar**, B Manohar and S Divakar. An improved process for the preparation of amino acid esters of disaccharides. Indian Patent, 285/NF/2006, 2006.

Publications

1. G R Vijayakumar, K Lohith, **B R Somashekar** and S Divakar. Lipase catalyzed synthesis of L-alanyl, L-leucyl and L-phenylalanyl esters of D-glucose using unprotected amino acids. **Biotechnology Letters** 2004; 26**:** 1323–1328.

2. Vasudeva Kamath, P S Rajani, K Lohith, **B R Somashekar** and S Divakar. Angiotensin Converting Enzyme inhibitory activity of amino acid esters of carbohydrates. **International Journal of Biological Macromolecules** 2006; 38: 89-93.

3. K. Lohith, G R Vijayakumar, **B R Somashekar**, R Sivakumar and S Divakar. Amino acyl esters of carbohydrates and glycosides as potent Angiotensin Converting Enzyme Inhibitors. **European Journal of Medicinal Chemistry** 2006; 41:1059-1072.

4. **B R Somashekar** and S Divakar. Lipase catalyzed synthesis of L-alanyl esters of carbohydrates. **Enzyme and Microbial Technology** 2007; 40: 299-309.

5. **B R Somashekar**, K. Lohith, B Manohar and S Divakar. Inhibition of *Rhizomucor miehei* and *Candida rugosa* lipases by D-glucose in the esterification reaction between L-alanine and D-glucose. **Journal of Bioscience and Bioengineering** 2007; 103: 122-128.

6. **B R Somashekar** and S Divakar. Synthesis of L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates using lipase from *Candida rugosa*. 2007. Submitted for publication.

Abstract

Amino acyl esters of carbohydrates are used as sweetening agents, surfactants, microcapsules in pharmaceutical preparations, active nucleoside amino acid esters, antibiotics and in the delivery of biological active agents. Chemical acylation of carbohydrates regio-selectively is complex due to the presence of multiple hydroxyl groups, which require protection and deprotection. However enzymatic reactions can overcome this drawback. Hitherto, very few references are available on the lipase catalyzed esterification of amino acyl esters of sugars. Most of the earlier workers used proteases and N-protected and carboxyl group activated amino acids for synthesizing aminoacyl esters of carbohydrates. All these reactions were conducted in shake flasks using lesser quantity of substrates and larger quantity of enzymes. The present work deals with lipases catalyzed preparation of amino acyl esters of carbohydrates using unprotected and unactivated amino acids and carbohydrates.

Chapter **ONE** deals with literature survey on mainly lipase catalyzed synthesis in organic media. Biotechnological applications of lipase catalysis in different food and pharmaceutical industries are discussed. A brief description on the lipase structure and catalytic mechanism on esterification is made. Parameters regulating lipase activity in organic media like nature of substrates, nature of solvents, effect of salt, thermal stability of lipases, water activity and immobilization are discussed. Diverse application of lipases like esterification using reverse micelles, supercritical carbon dioxide, micro oven, ionic liquids assisted reactions, kinetic studies and resolution of racemic mixture are presented. The chapter ends with a brief description on the scope of the present investigation.

Chapter **TWO** deals with materials and methods. Chemicals employed and their sources are listed. Methods of preparation of L-amino acyl esters of carbohydrates and the other related appropriate aspects of the same are discussed in detail.

Chapter **THREE** describes results from optimization of reaction parameters for the lipase catalyzed synthesis of L-alanyl **16a-e**, L-valyl **25a-e** and L-leucyl **34a-e** esters of D-glucose. Lipases from *Rhizomucor miehei* (RML), porcine pancreas (PPL) and *Candida rugosa* (CRL) were employed. The reaction conditions were optimized in terms of incubation period, solvent, enzyme concentrations, substrate concentrations, buffer (pH and concentration) and enzyme reusability. Under the experimental conditions employed, all the three lipases exhibited good esterification potentialities. Both RML and PPL showed maximum conversion yields of L-alanyl-D-glucose **16a-e** (30 % and 18 % respectively) at 40 % (w/w D-glucose) of enzyme, L-valyl-D-glucose **25a-e** (59 % and 62 % respectively) at 10 % (w/w D-glucose) of enzyme and L-leucyl-D-glucose **34a-e** (85 % and 18 % of respectively) at 40 % (w/w D-glucose) of enzyme employed. CRL showed a maximum conversion of 84 % of L-valyl-D-glucose **25a-e** at 30 % (w/w D-glucose) enzyme concentration. The present work showed enhanced activity of RML and CRL in presence of buffer salts. Optimum pH was found to be pH 4.0 for RML and pH 5.0 for PPL in case of L-alanyl-D-glucose**,** pH 7.0 for CRL in case of L-valyl-D-glucose and pH 5.0 for RML in case of L-leucyl-D-glucose reactions. Higher equivalents of D-glucose were found to inhibit RML in case of L-alanyl-D-glucose reaction. However, in case of L-valyl-D-glucose and L-leucyl-D-glucose reactions, free amino acids and D-glucose were not found to be inhibitors of RML and CRL. In the synthesis of L-alanyl-D-glucose **16a-e,** RML could be reused upto four cycles where as PPL could be used only upto two cycles.

Chapter **FOUR** describes the syntheses and characterization of L-alanyl **1**, L-valyl **2**, L-leucyl **3** and L-isoleucyl **4** esters of carbohydrates - D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14** and D-sorbitol **15**. Esterification was carried out by reacting 0.002 mol unprotected L-amino acid (**1-4**) and 0.001 mol of carbohydrate (**5-15**) along with 100 ml CH$_2$Cl$_2$: DMF (90:10 v/v, 40 ^{o}C) in presence of 40 % (w/w carbohydrate employed) of lipases under reflux for a period of three days. *Rhizomucor miehei* lipase (RML) in presence of 0.1 mM (0.1 ml of 0.1 M) of pH 4.0 acetate buffer, *Candida rugosa* lipase (CRL) in presence of 0.1 mM (0.1 ml of 0.1 M) of pH 7.0 phosphate buffer and crude porcine pancreas lipase in presence of 0.1 mM (0.1 ml of 0.1 M) of pH 5.0 acetate buffer were employed to impart 'pH tuning' to the enzyme. All the three lipases employed showed broad substrate specificity towards amino acids as well as carbohydrates. Esterification yields were obtained in the range of 3 – 78 %. Two dimensional HSQCT NMR confirmed the formation of 1-*O*-, 2-*O*-, 3-*O*-, 4-*O,* 5-*O*-, 6-*O*- and 6′-*O*- mono esters and 1,6-di-*O*-, 2,5-di-*O*, 2,6-di-*O*-, 3,5-di-*O*-, 3,6-di-*O*, 4,6-di-*O*- and 6,6′-di-*O*-diesters to varying extents depending on the carbohydrate employed. Nature of the products clearly indicated that primary hydroxyl groups of the carbohydrates (1-*O*-, 5-*O*-, 6-*O*- and 6′-*O*-) esterified predominantly over the secondary hydroxyl groups (2-*O*-, 3-*O*- and 4-*O*-). Among the secondary hydroxyl groups, 4-*O*- ester was formed only in case of D-mannose (**18b**, **36b** and **46b**). Carbohydrates containing hydroxyl groups in axial position like C2 in D-mannose and D-ribose and C4 in D-galactose have not reacted, indicating that esterification with axial secondary hydroxyl groups are difficult, especially with alkyl amino acyl donors. In case of L-alanyl-D-glucose **16a-e**, only β-anomer of D-glucose reacted, the D-glucose employed being a 40: 60 mixture of α and β

anomers respectively. Lesser incubation periods gave rise to only monoesters. The anomeric hydroxyl groups of carbohydrate molecules did not react because of rapid glycosidic ring opening and closing process. Aldohexoses (D-glucose, D-mannose and D-galactose), ketohexose (D-fructose), pentose (D-ribose) and the disaccharides (maltose) showed better conversions with all the four amino acids. Least conversions were observed for carbohydrate alcohols and sucrose esters. L-Valyl esters (25 – 78 %) as well as L-leucyl esters (21 – 65 %) showed better conversion than L-alanyl esters (3 – 78 %) and L-isoleucyl esters (9 – 55 %). Among the lipases employed, *Candida rugosa* lipase and porcine pancreas lipase have shown better conversions than *Rhizomucor miehei* lipase. L-Alanine **1**, L-valine **2** and L-leucine **3** with D-glucose **5** and L-alanine **1** and L-isoleucine **4** with D-mannose **7** gave five diastereomeric esters. Both D-arabinose **9** and D-ribose **10** showed three diastereomeric esters with all the amino acids (**1-4**) employed. Lactose **11** did not react with L-valine and L-leucine and D-sorbitol **15** did not react with L-alanine, L-valine and L-isoleucine. L-Alanyl-sucrose **24**, L-valyl-D-mannose **27**, L-valyl-sucrose **32**, L-leucyl-maltose **40**, L-leucyl-sucrose **41** and L-isoleucyl-sucrose **52**, formed only 6-*O*- esters. Carbohydrates like lactose **11**, D-mannitol **14** and D-sorbitol **15** reacted selectively depending on the amino acid indicating that they may not be good nucleophiles, probably due to more hydrogen bonding propensity for D-mannitol and D-sorbitol and steric hindrance in case of lactose. About 99 L-amino acyl esters of carbohydrates were prepared out of which 97 esters have not been reported before. So far unreported esters are L-alanyl-D-glucose **16a,d,e**, L-alanyl-D-galactose **17a-c**, L-alanyl-D-mannose **18a-e**, L-alanyl-D-fructose **19a-c**, L-alanyl-D-arabinose **20a-c**, L-alanyl-D-ribose **21a-c**, L-alanyl-lactose **22a-c**, L-alanyl-maltose **23a-c**, L-alanyl-sucrose **24**, L-valyl-D-glucose **25a-e**, L-valyl-D-galactose **26a-c**, L-valyl-D-mannose **27**, L-valyl-D-fructose **28a-**

c, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-valyl-maltose **31a,b**, L-valyl-sucrose **32**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-galactose **35a,b**, L-leucyl-D-mannose **36a-c**, L-leucyl-D-fructose **37**, L-leucyl-D-arabinose **38a-c**, L-leucyl-D-ribose **39a-c**, L-leucyl-maltose **40**, L-leucyl-sucrose **41**, L-leucyl-D-mannitol **42a,b**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b**, L-isoleucyl-D-galactose **45a-c**, L-isoleucyl-D-mannose **46a-e**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-arabinose **48a-c**, L-isoleucyl-D-ribose **49a-c**, L-isoleucyl-lactose **50a-c**, L-isoleucyl-maltose **51a-c**, L-isoleucyl-sucrose **52** and L-leucyl-D-mannitol **53a,b**.

Chapter **FIVE** describes kinetic study on the esterification of D-glucose **5** with L-alanine **1** catalyzed by lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL). A detailed investigation showed that both the lipases followed Ping-Pong Bi-Bi mechanism wherein L-alanine and D-glucose bind in subsequent steps releasing water and L-alanyl-D-glucose also in subsequent steps, with competitive substrate inhibition by D-glucose at higher concentrations leading to the formation of dead-end lipase-D-glucose complexes. An attempt to obtain the best fit of this kinetic model through curve fitting yielded in good approximation, the apparent values of four important kinetic parameters, RML: k_{cat} = 0.29 ± 0.028 x 10^{-3} M h^{-1} mg^{-1}, $K_{m\ L-alanine}$ = 4.9 ± 0.51 x 10^{-3} M, $K_{m\ D-glucose}$ = 0.21 ± 0.018 x 10^{-3} M, $K_{i\ D-glucose}$ = 1.76 ± 0.19 x 10^{-3} M; CRL: k_{cat} = 0.75 ± 0.08 x 10^{-3} M h^{-1} mg^{-1}, $K_{m\ L-alanine}$ = 56.2 ± 5.7 x 10^{-3} M, $K_{m\ D-glucose}$ = 16.2 ± 1.8 x 10^{-3} M, $K_{i\ D-glucose}$ = 21.0 ± 1.9 x 10^{-3} M.

Chapter **SIX** describes potentiality of some of the amino acyl esters of carbohydrates as inhibitors towards Angiotensin Converting enzyme (ACE) activity. The esters tested are: L-alanyl-D-glucose **16a-e**, L-alanyl-lactose **22a-c**, L-valyl-D-glucose **25a-e**, L-valyl-D-fructose **28a-c**, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-

valyl-maltose **31a,b**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-fructose **37**, L-leucyl-D-ribose **39a-c**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-ribose **49a-c** and L-isoleucyl-maltose **51a-c**. Amino acyl esters of carbohydrates tested for ACE inhibition activity showed IC_{50} values for ACE inhibition in the 0.7 mM to 6.0 mM range. Among them, L-isoleucyl-D-glucose **44a,b** (IC_{50}: 0.7±0.067 mM), L-leucyl-D-fructose **37** (IC_{50}: 0.9±0.08 mM), L-isoleucyl-maltose **51a-c** (IC_{50}: 0.9±0.09 mM) and L-valyl-D-mannitol **33** (IC_{50}: 1.0±0.092 mM) showed the best ACE inhibitory activities.

The present investigation has thus demonstrated the potentiality of RML, PPL and CRL to synthesize biologically and nutritionally active amino acyl esters of carbohydrates (**16a-e − 53a,b**) using unprotected and unactivated L-amino acids and carbohydrates.

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List of abbreviations and symbols

A	Absorbance
α	Alpha
ACE	Angiotensin Converting Enzyme
Å	Angstrom
β	Beta
BSA	Bovine serum albumin
CRL	*Candida rugosa* lipase
^{13}C	Carbon-13
J	Coupling constant
CMC	Critical Micellar Concentration
k_{cat}	Catalytic efficiency of the enzyme
^{o}C	Degree centigrade
δ	Delta
DMSO-d_6	Deuteriated Dimethyl sulfoxide
CH_2Cl_2	Dichloromethane
DCF	Dichloroflurocein
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
eV	Electron volt
EC	Enzyme commission
γ	Gamma
g	Gram
Hz	Hertz
HMQCT	Heteronuclear Multiple Quantum Coherence Transfer
HSQCT	Heteronuclear Single Quantum Coherence Transfer
HPLC	High Performance Liquid Chromatography
h	Hour
IR	Infra Red
K_i	Inhibitor constant
v	Initial velocity

KDa	Kilodalton
K_{mA}	Michelis-Menten constant for the lipase-L-alanine complex
$K_{m\,L\text{-}alanine}$	Michelis-Menten constant for the lipase-L-alanine complex
K_{mB}	Michelis-Menten constant for the lipase-D-glucose complex
$K_{m\,D\text{-}glucose}$	Michelis-Menten constant for the lipase-D-glucose complex
K_i	Dissociation constant for the lipase-inhibitor (D-glucose) complex
$K_{i\,D\text{-}glucose}$	Dissociation constant for the lipase-inhibitor (D-glucose) complex
MS	Mass Spectrometry
V_{max}	Maximum velocity
MHz	Mega hertz
K_m	Michelis Menton constant
μg	Microgram
μl	Microlitre
mg	Milligram
ml	Milliliter
mmol	Millimole
min	Minute
M	Molarity
mol	Mole
ε	Molar extinction coefficient
$[M]^+$	Molecular ion
nm	Nanometer
N	Normality
nmol	Nano mole
NMR	Nuclear Magnetic Resonance
[α]	Optical rotation
ppm	Parts per million
%	Percentage
π	Pi
PAGE	Polyacrylamide gel electrophoresis
PPL	Porcine pancreas lipase
KBr	Potassium bromide

^1H	Proton
R_f	Retention factor
R	Regression coefficient
t_{ret}	Retention time
RML	*Rhizomucor miehei* lipase
σ	Sigma
SDS	Sodium dodecyl sulfate
TEMED	N, N, N', N'-Tetramethylethylenediamine
TMS	Tetra methyl silane
TLC	Thin layer chromatography
2D	Two-Dimensional
UV	Ultra Violet
v/v	Volume by volume
a_w	Water activity
cm^{-1}	Wave per centimeter
w/w	Weight by weight

Chapter 1
Introduction

1. Introduction

The enormous variety of biochemical reactions that takes place in living systems are all mediated by a series of enzymes (Michal, 1999). Of the estimated 25,000 enzymes present in nature, only about 2800 have been characterized and about 400 enzymes, mainly hydrolases, transferases and oxido-reductases have been identified as commercially potential ones. But only 50 different kinds of enzymes find application on an industrial scale (Winterhalter and Schreier, 1993; Schreier and Winterhalter, 1993; Berger, 1995). The enzymes of commercial importance in food industry are amylases, proteases, pectinases, cellulases, hemicellulases, lipases and lactases. About 75 % of the enzymes commercially used are hydrolytic enzymes. These enzymes are used as tools in hydrolysis, synthesis, analysis, biotransformation and affinity separation (Sharma *et al.,* 2001; Klibanov, 1986; Bosley and Calyton, 1994; Vulfson, 1993).

1.1. Enzymes in organic synthesis

Enzymes have given better solution for the problems encountered in chemical synthesis. Chemical approach in synthesis needs drastic conditions, like use of acids or alkaline media as catalysts, high temperature, hazardous chemicals, heavy metals, protection and deprotection of the reactants which in most cases lead to multi-step processes, high energy consumption, coloring of products, low regio and stereo selectivity, more number of byproducts, economically not so feasible conditions and results in environmental pollution. Some of the synthesized products are harmful in nature and require more purification. Hence, their use in food and pharmaceutical industry is very limited. However, enzymes are biocatalysts that catalyze reactions under mild conditions which do not require drastic conditions like high temperature, use of hazard chemicals and protection and deprotection of reactants. Using enzymes in non

1

aqueous media initiated by Alexander Klibanov resulted in wide applications in organic synthesis (Klibanov, 1986).

1.2. Lipases

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases are hydrolases which catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues. They differ from esterases by their substrate specificity. While lipases acts on water insoluble long chain fatty acyl glycerides, esterases prefer water soluble short chain fatty acyl esters. Lipases contain a hydrophobic oligopeptide lid which is not present in esterases covering the entrance of its active site. It is this hydrophobic lid which requires interfacial activation at lipid-water interface (Martinelle *et al.,* 1995). In presence of hydrophobic interfaces, lipases undergo conformational change by acquiring an open structure in which the active site residues become accessible to substrates. However, in the absence of interfaces, lipase molecules exhibit a closed structure in which the lid covers the active site, making it inaccessible to substrates (Brzozowski *et al.,* 1990). The catalysis by lipase includes a steep substrate concentration gradient at the interface, better orientation of a scissile ester bond, reduction in the water shell around the substrate ester molecules and the conformational change of the enzyme (Derewenda and Sharp, 1993). Lipases can tolerate organic solvents in the reaction mixture. Therefore, lipases find promising position in organic chemical processing (Kiran and Divakar, 2001; Kiran *et al.*, 2001a; Therisod and Klibanov, 1986; Berglund and Hutt, 2000, Harikrishna and Karanth, 2001), detergent

formulations (Jaeger and Reetz, 1998), synthesis of biosurfacants (Plou *et al.* 1999; Sarney *et al.* 1996; 1995), oleochemical industry (Bornscheur, 2000; Undurranga *et al.,* 2001), dairy industry (Vulfson, 1994), paper manufacture (Jaeger and Reetz, 1998), nutrition (Pabai *et al.,* 1995a,b; Undurraga *et al.,* 2001) and cosmetics and pharmaceutical processing (Berglund and Hutt, 2000). Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding (Liese *et al.,* 2000). Other biotechnological applications of lipases are shown in Table 1.1. Lipases are employed to get poly unsaturated fatty acids (PUFAs) which are then used along with their mono- and diglycerides for the synthesis of nutraceuticals, pharmaceuticals like anti-cholesterolemics, anti-inflammatories and thrombolytics (Gill and Valivety, 1997; Belarbi *et al.,* 2000). *Candida rugosa* lipase is employed in the paper industry to remove the pitch from the pulp (Sharma *et al*., 2001). The main reason for the use of lipases is the growing interest and demand for the products prepared through natural resource, which are environmentally compatible. Because of their versatility in application, lipases are regarded as enzymes with high commercial potential. Lipase catalyzed esterification in organic solvents offers synthetic challenges, which if dealt with successfully, can result in the generation of several useful compounds.

Table 1.1 Biotechnological applications of lipases (Vulfson, 1994)

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery	Flavor improvement and shelf-life elongation	Bakery products
Beverages	Improved aroma	Beverages
Food	Quality improvement, transesterification	Mayonnaise, dressings, and whippings, health foods
Meat and fish	Flavor development and removal of fats	Meat and fish products
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks and chemicals
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality by removing wax.
Cleaning	Synthesis and hydrolysis	Removal of cleaning agents like surfactants
Food dressing	Quality improvement	Mayoannaise, dressing and whipping
Pharmaceuticals	Transesterification, hydrolysis	Speciality lipids, digestive aids
Health food	Transesterification	Health foods

1.2.1. *Candida rugosa* lipase

Candida rugosa lipase (CRL) is the first example of a native interface-activatable lipase in 'open' form (Schrag *et al.,* 1996). CRL is a member of the α/β-hydrolase fold family, which consists of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices (Fig.1.1). CRL is made up of a single polypeptide chain with 534 amino acid residues with a molecular weight of 57 kDa. CRL appears in five isoforms, which have been cloned and sequenced (Kawaguchi *et al.,* 1989, Longhi *et*
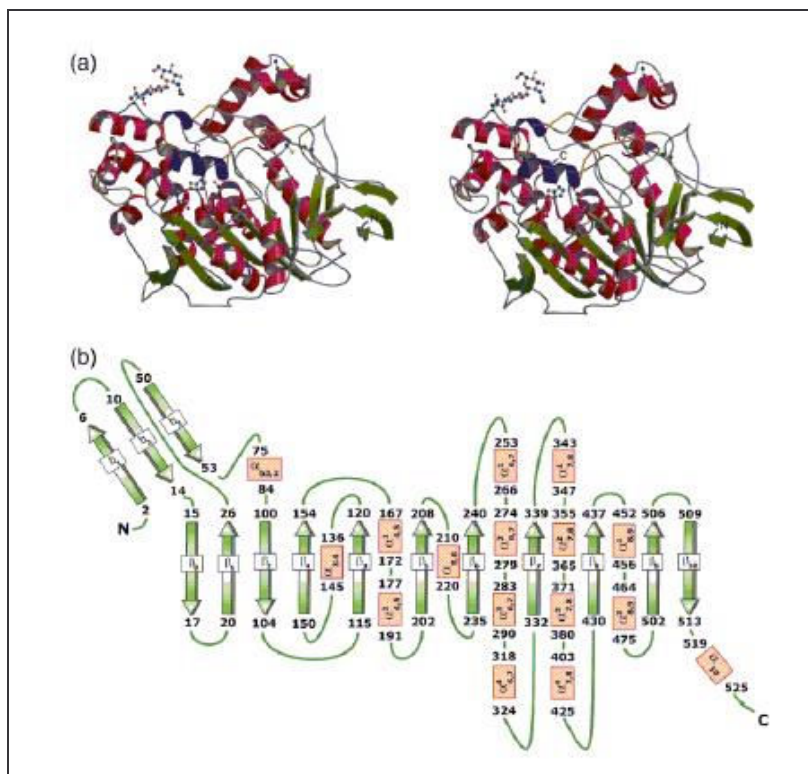
Fig. 1.1. Overall structure of *Candida rugosa* lipase. (a) Ribbon representation with a-helices, β-strands and coils colored in red, green and gray, respectively. The helical and coil segments forming the flap region are shown in dark blue and orange, respectively. The catalytic triad residues (Ser-209, Glu-341 and His-449), the disulfide bridges and the Asn-attached N-acetyl-glucosamine moieties are shown in ball-and-stick representation. (b) A representation of the lipase 2 topology with the secondary structure elements identified (β, strands; α, helix). (adopted from Mancheño *et al.,* 2003).

al., 1992). In CRL, the active site triad consists of Ser-209, Glu-341 and His-449 and nearer to this active site and three surface loops (62-92, 122-129 and 294-305) are present which are very important for catalytic activity (Fig. 1.1). Ser-209 is embedded in a characteristic super secondary structure motif, found in all lipase. In the open conformation observed for CRL, the lid extends nearly perpendicular to the protein surface forming a large depression that surround the active site. The hydrophilic area consists of uncharged polar residues. The face of the flap facing the active site is hydrophobic in nature which is mainly composed of aliphatic side chain amino acid residues. The flap facing opposite the active site is hydrophilic in character. The geometry of loop 13 and 4 and the active site of CRL suggests that the oxyanion hole O_γ is formed by the backbone amide of Gly-123, Gly-124 and Ala-210, which are involved in hydrogen bond formation with the substrates (Fig. 1.1). Presence of two acyl binding 'pockets' in the active site of CRL depicts the substrate specificity for carbon chain lengths, a small pocket which can bind well with short chain acids and a bigger pocket which can bind well with longer chain acids (Parida and Dordick, 1993). Experimentally, this was proved by showing that the mechanism depends on the chain length of the acyl moiety and independent of the type of reaction catalysed and this behaviour is similar when the reaction was carried out in different solvent systems also.

1.2.2. Porcine pancreas lipase

Pancreatic lipases are high active lipases where one molecule of lipase can cleave nearly 7000 ester bonds per second under optimal conditions (Scharpe *et al.,* 1997). Most of the work conducted with pancreatic lipase were from porcine pancreas. Porcine pancreas is the richest source for pancreatic lipases which was also the first purified lipase (Peschke, 1991). Pancreatic lipase is a single chain glycoprotein of 48 kDa
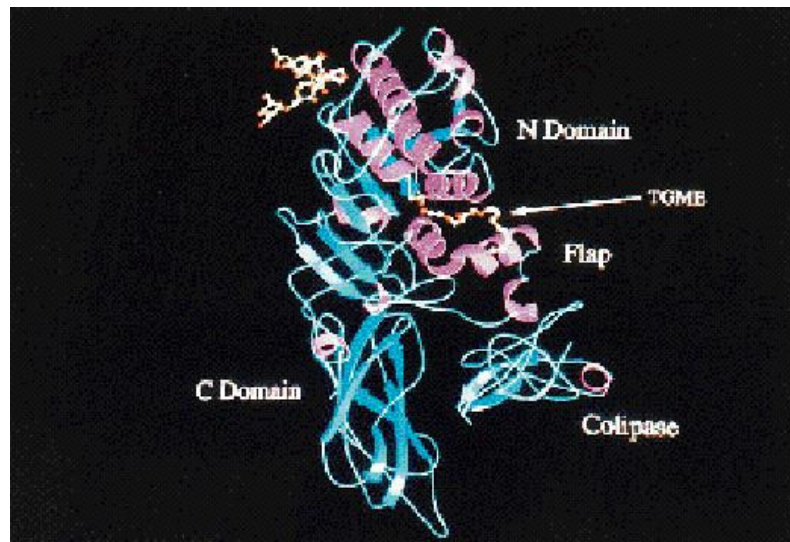
Fig. 1.2. Schematic ribbon diagram of the porcine lipase-colipase structure. The glycan chain, connected to the lipase N-terminal domain (N domain), is drawn as a stick model. One tetra ethylene glycol monooctyl ether inhibitor molecule, located in the open active site, is represented by *balls* and *sticks*. Colipase interacts with the lipase C-terminal domain (C domain) and with the flap (adopted from Hermoso *et al.,* 1996).

molecular weight and it is a serine hydrolase (Winkler and Gubernator, 1994; Scharpe *et al.,* 1997). It consists of a central predominant parallel β-sheet having helical connection (van Tilbeurgh, 1992). From pH titration and photo-oxidation studies, histidine residue was found to be responsible for the catalytic activity (Winkler *et al.,* 1990), the catalytic triad consisting of Ser-152, His-263, and Asp-176. Donner (1976) purified and measured some of the physical parameters: molecular weight 52000, sedimentation coefficient (s degrees 20, w) 4.0 S, diffusion coefficient (D degrees 20, w) $6.7 \times 10^{-7} cm^2 s^{-1}$, Stokes' radius (r) 30.3 Å, partial specific volume (v) $0.72 \ cm^3 \ g^{-1}$, frictional ratio (f/f$_0$) 1.23 and isoelectric point 5.18. Lipase was bound by prolipase at its edge with the plane of the prolipase roughly perpendicular to the C-terminal β-sheet domain of the lipase molecule (Fig. 1.2). Prolipase is a flattened molecule with dimension of about 33 Å x 24 Å x 16 Å consisting of mainly three finger shaped regions formed by residues between 26-39, 47-64 and 67-87 held together by disulfide bonds. It was found that majority of hydrophobic amino acids are found in the region opposite of the lipase binding site. The catalytic active site also contains a surface helix (residue 248-258) which is a part of the amphiphatic lid covering the active site (van Tilbeurgh, 1992).

1.2.3. *Rhizomucor miehei* lipase

Rhizomucor miehei lipase (RML) is made up of a single polypeptide chain with 269 amino acid residues with a molecular weight of 29 kDa (Brandy *et al.,* 1990, Brzozowski *et al*., 1991). Brandy *et al.,* (1990) identified that RML is an α/β type protein with a central eight-stranded mixed β-pleated sheet folded on to a highly amphiphatic N-terminal helix and a number of loops and helices including a semi-external, long kinked α-helix, with three disulphide bonds which are responsible in stabilizing the two terminal strands. The catalytic triad is constituted by Ser-144, His-257
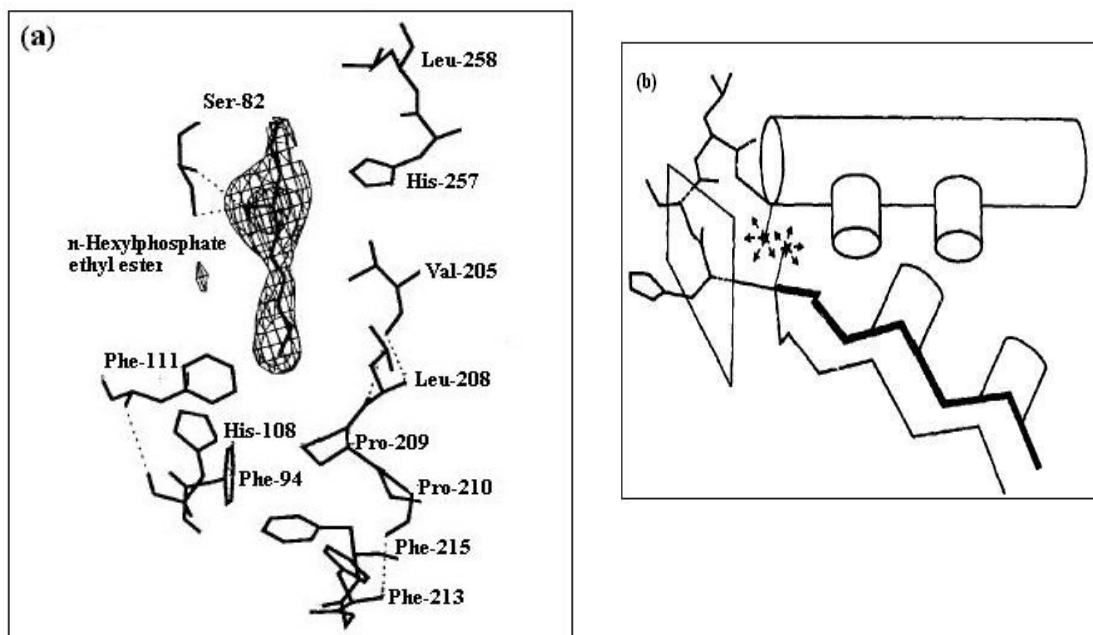
Fig. 1.3 (a) The hydrogen-bonding network in the active site of the *Rhizomucor miehei* lipase. The crystal structure (at 3 Å resolution) of a complex of RML lipase with n-hexylphosphonate ethyl ester in which the enzyme's active site is exposed by the movement of the helical lid. The catalytic Ser-144 is immediately beneath the phosphorus atom of the inhibitor. His-257 is clearly displaced towards the ethyl oxygen consistent with the proposed orientation of the substrate and the mechanism of hydrolysis. Hydrogen bond contacts between O_γ atom of Ser-82 and its amide NH are indicated (adopted from Brzozowski *et al.,* 1991)

(b) A schematic drawing showing the packing within the β-εSer-α-motif. The helix and strand pack against each other with four amino acids (tinted) forming the interface. The residues nearer the turn are in closer contact and therefore their side chains are restricted to those of smaller hydrophobic amino acids. The plane of the central peptide of the turn is perpendicular to the axis of the motif, which forces the catalytic Ser to adopt a strained ε conformation. The two stars show the positions that β-carbons of amino acids other than Gly would occupy if the two invariant Gly residues of the GX_1SX_2G pentapeptide were mutated (adopted from Derewenda *et al.,* 1993).

and Asp-203, which is buried under a single 17 residue long surface loop (82-96 residue) called lid, that occludes the active site in the native structure (Derewenda *et al.,* 1992). Brzozwski *et al.,* (1991) studying an atomic model of inhibitor-RML complex showed that a direct covalent bond formation between nucleophilic O_γ of Ser-144 and substrate is formed (Fig. 1.3a). The sequence as Gly^{142}-His^{143}-Ser^{144}-Leu^{145}-Gly^{146}, a pentapeptide corresponds to a tight turn between the fourth strand of the central β-sheet and a buried α-helix (Fig.1.3b). The catalytically active Ser found in the middle of the turn is in the rarely found ε-conformation ($\phi=62$; $\varphi=121^o$). This structural motif consisting of a β-strand followed by a tight turn containing the active Ser and an α-helix was called as β-εSer-α-motif (Fig.1.3b; Derewenda *et al.,* 1993). Carbonyl oxygen of the substrate may be stabilized by the interaction of NH group of Leu-145 and Gly-146 and the hydroxyl of Ser-82 through hydrogen bonding and this Ser-82 possesses favorable conformation for the oxy-anion interaction (Brzozowski *et al.,* 199).

The inhibition study of lipase by serine protease inhibitors like di-isopropyl phosphofluoridate indicated that a Ser residue might be involved in the catalytic mechanism of the enzyme. All known amino acid sequence of neutral lipases share a consensus pentapeptide GX_1SX_2G (where X represents any amino acid, G represents glycine and S represents serine) which contain an essential Ser residue(Derewenda *et al.,* 1993).

1.3. Lipase specificity

One of the major advantage of enzymes is their ability to exhibit regioselectivity and stereospecificity in reactions catalyzed by them. Lipases show positional and substrate specificity. Based on positional specificity, lipases can be divided into five different classes (Camp *et al.,* 1998).

Lipases of first group catalyze hydrolysis of fatty acyl tri-glycerides independent of their type or position. Lipases from *Candida cylindraceae*, *Cornybacterium acnes*, *Staphylococcus aurus* come under this group (Camp *et al.,* 1998) which do not exhibit regioselectvity.

Lipases of second group are known as 1,3-specific lipases. Lipases from *Aspergillus niger*, *Rhizopus delemar*, *Rhizomucor miehei*, *Candida rugosa* and *porcine pancreas* catalyze reaction at *sn*-1 and *sn*-3 positions of triacyl glycerides (Macrea, 1985).

The third group of lipases cover lipases with different rates of hydrolysis of mono acyl, diacyl and triacyl glycerides. Some of these lipases are found in the tissues of rats and humans.

The fourth group of lipases catalyze the exchange of specific type of fatty acids. Extracellular lipases from the fungus *Geotrichium candidum* preferentially releases unsaturated *cis-n*-9 fatty acid groups (Macrea, 1985).

The fifth group of lipases contains special enzymes that show a faster rate of hydrolysis of fatty acids placed at the *sn*-1 position than the *sn*-3 position or vice versa. This is commonly referred as stereospecificity. Lipoprotein lipases from milk, adipose tissues and post heparin plasma preferentially cleave the ester bond in *sn*-1 and human and rat lingual lipases which react preferentially with the fatty acids at *sn*-3 position (Jensen *et al.,* 1983) are examples of this group.

1.4. Reactions catalyzed by lipases

The ranges of substrates with which lipases react and also the range of reactions they catalyze are probably far more than any other enzyme studied till date. Lipases catalyze three types of reactions (**Scheme 1.1**).

1.4.1. Hydrolysis

In aqueous media when there is large excess of water, ester hydrolysis is the dominant reaction.

1.4.2. Esterification

Under low water conditions such as in nearly anhydrous solvents, esterification can be achieved. If the water content of the medium is controlled, relatively better product yields can be obtained.

1.4.3. Transesterification

The acid moiety of an ester is exchanged with another one. If the acyl donor is a free acid, the reaction is called **acidolysis** and if the acyl donor is an ester, the reaction is called **interesterification**. In **alcoholysis**, the nucleophile alcohol acts as an acyl acceptor.

1. Hydrolysis
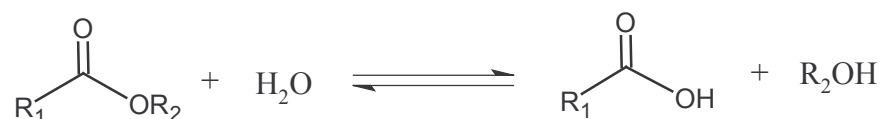
2. Esterification

3. Transesterification

a. Acidolysis
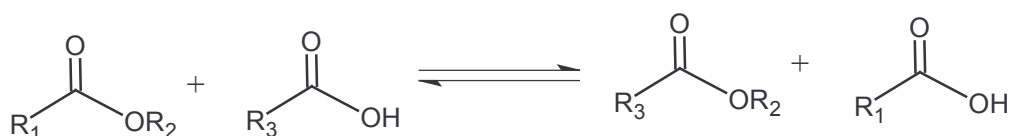
b. Alcoholysis
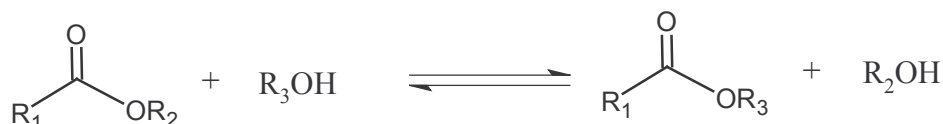
c. Interesterification
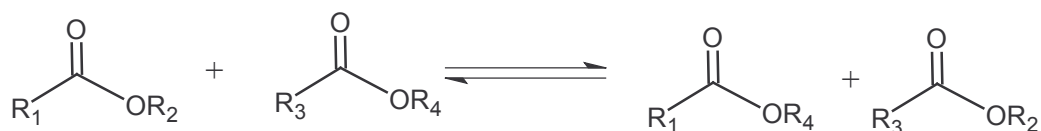
Scheme 1.1. Types of reactions catalyzed by lipases

1.5. Catalytic mechanism of lipase mediated esterification in organic media

Lipases show lipid splitting nature and the mechanism is same as that of serine proteases (Pleiss *et al.,* 1998). Catalytic triad in lipases is constituted by Ser, His and Asp / Glu residues. The serine residue in the active center is activated by histidine and aspartic / glutamic acid residues, together forming the catalytic triad. The substrate acid forms a tetrahedral acyl-enzyme intermediate by reaction with the OH group of the catalytic serine residue. The resulting excess negative charge that develops on the carbonyl oxygen atom is stabilized by the oxyanion hole (Brzozwski *et al.,* 1991). The

tetrahedral intermediate I, forms a serinate ester with elimination of water molecule. Subsequent nucleophilic attack of alcohol to the acyl-enzyme intermediate leads to tetrahedral intermediate II. Finally, the product ester is released and the enzyme is free for the next molecule to attack. Grochulski *et al.,* (1993), Cygler *et al.,* (1994) and Schrag and Cygler (1997) proposed a mechanism for the ester formation which is depicted in **Scheme 1.2**.

Step I. Acylation step

Initially serine hydroxyl group forms a tetrahedral intermediate complex I with acyl donor, the negative charge that is formed in the tetrahedral intermediate is stabilized by hydrogen bonding with the acid given which are responsible for the oxyanion hole formation.

Step II. Formation of acyl enzyme complex

After the formation of the tetrahedral intermediate I, an acyl-enzyme complex is formed through covalent bond with Ser residue by losing one molecule of water.

Step III. Nucleophilic attack by alcohol (Carbohydrate)

Nucleophile alcohol, attacks the carbonyl center of the tetrahedral intermediate forming a tetrahedral complex II forming an enzyme-acid-alcohol complex.

Step IV. Release of ester (L-amino acyl ester of carbohydrate)

Finally, the ester is released and the enzyme will be ready for the next molecule to attack.

Step I

Tetrahedral intermediate I

Step II

$-H_2O$

Step III

Step IV

Tetrahedral intermediate II
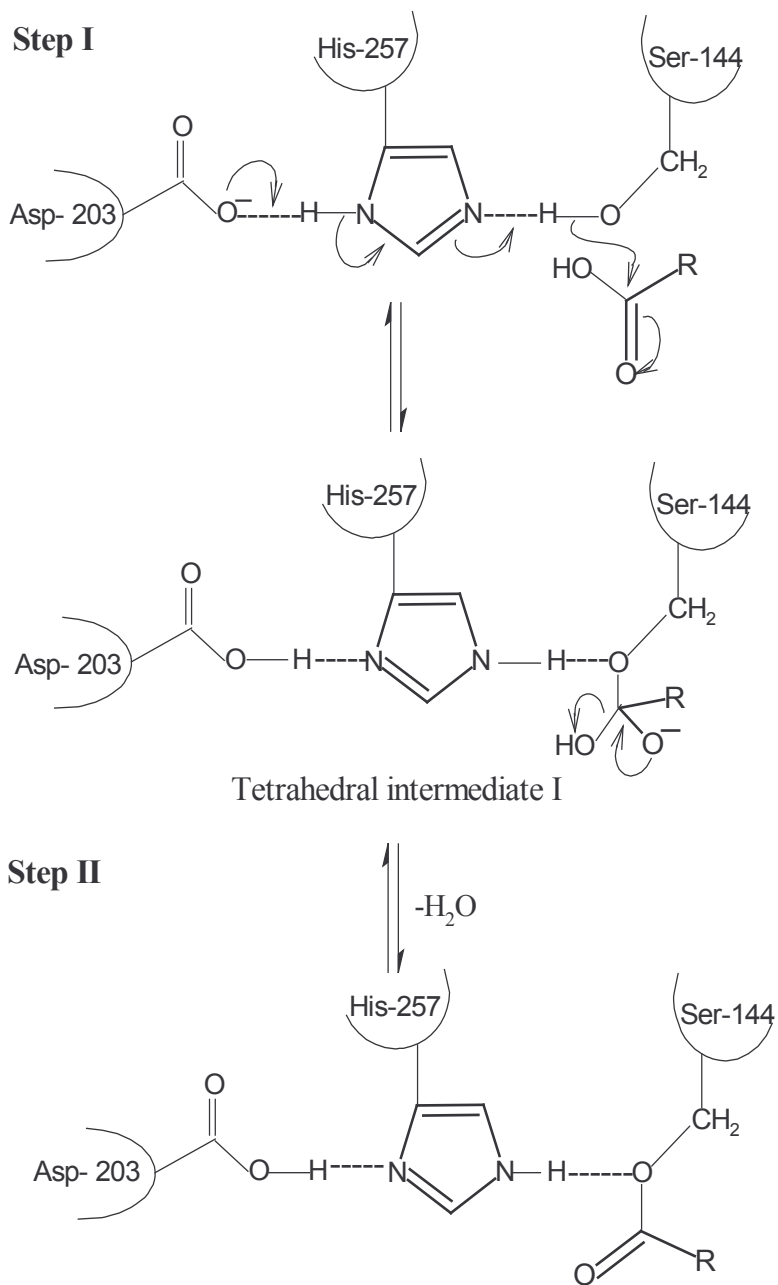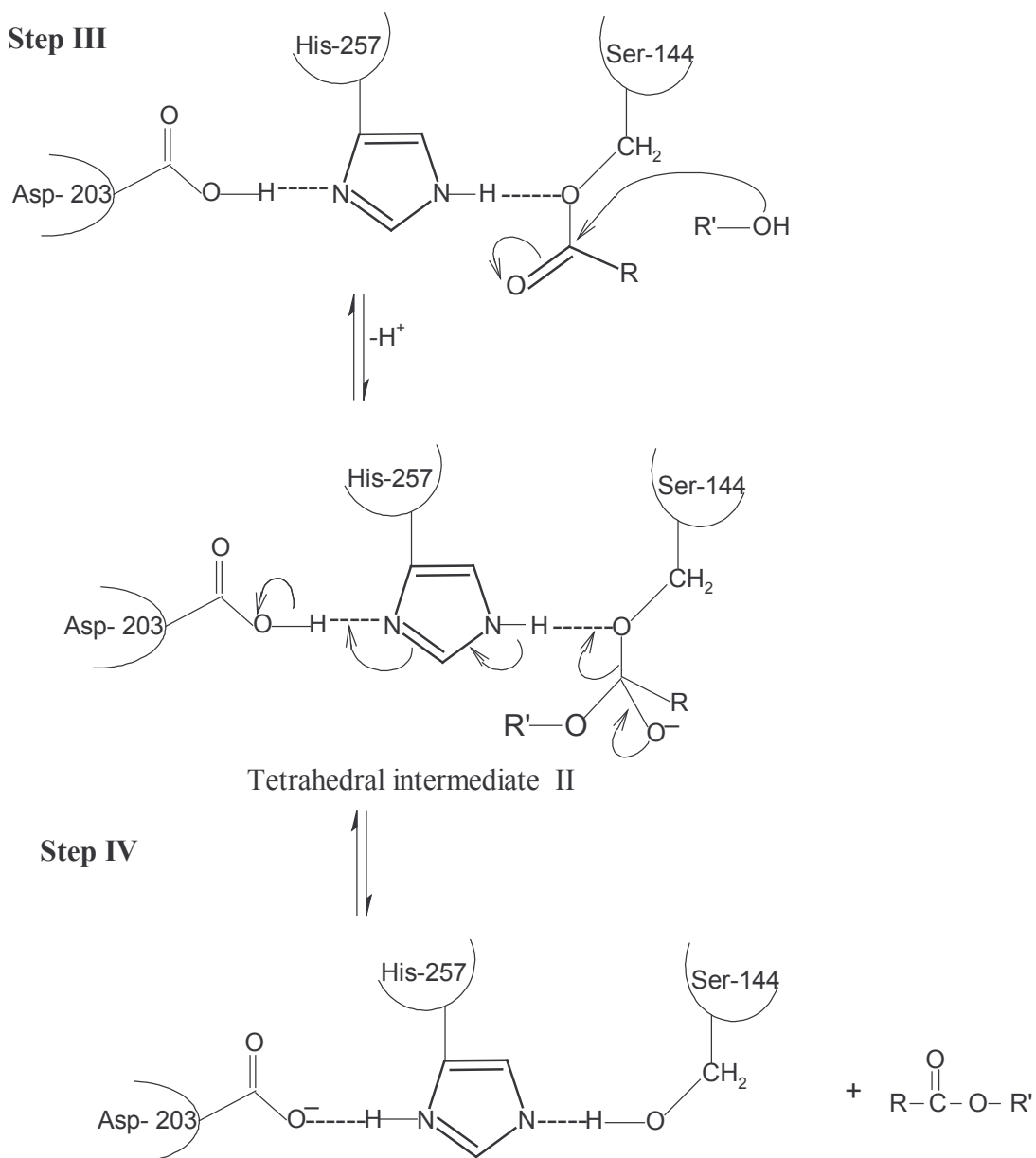
Scheme 1.2. Catalytic mechanism of lipase mediated esterification in organic media

1.6. Advantages of lipase catalysis over chemical catalysis

Lipases are found to be advantageous over chemical synthesis. The following are the advantages of using lipases as biocatalysts.

1. Stereospecificity towards substrate.

2. Milder reaction conditions under which the synthetic process can be operated.

3. Non-generation of by-products associated with the use of several chemical

procedures.

4. Improved product-yield and better product quality.

5. Exploitation of the stereo and regio-specificities shown by lipases to produce high value chiral synthons.

6. Success in immobilization techniques that have enabled the reuse of lipases leading to economically viable processes.

7. Good conversion yields.

8. Lipases are highly thermostable, they exhibit activity even at $100\ ^{o}C$.

9. Use of non-polar solvents which impart stability to lipase rather than in water, renders insolubility of the enzyme, solubility of substrates and products in organic solvents resulting in homogenous reaction conditions, easy product workout procedures and easy removal of water formed as a by-product.

Table 1.2 lists some of the commercially important flavor, fragrance, surfactant and sweetener esters prepared through lipase mediated catalysis.

Table 1.2. Lists some of the commercially important esters synthesized by lipase mediated catalysis

Source of lipase	Name of the compound	Applications	References
1. Flavour esters			
Pseudomonas fluorescence	Isoamyl acetate	Banana flavour	Takahashi *et al.*, 1988
Candida antarctica			Langrand *et al.*, 1990
Rhizomucor miehei			Rizzi *et al.*, 1992; Chulalaksananukul *et al.*, 1993;
			Razafindralambo *et al.*, 1994; Divakar *et al.*, 1999
Rhizomucor miehei			Harikrishna *et al.*, 2000; 2001
Candida cylindraceae, PPL,			Welsh *et al.*, 1990,
Aspergillus niger			Gubicza *et al.*, 2000
Novozyme 435			Afife Guvenc *et al.*, 2002
Rhizomucor miehei, Candida			Romero *et al.*, 2005a; 2005b;
antarctica			Kanwar and Goswami, 2002
Pseudomonas pseudomallei			Ngrek 1947

Organism	Ester	Flavour	Reference
Porcine liver lipase			Kumar *et al.*, 2005
Candida antarctica	Isoamyl butyrate	Banana flavour	Langrand *et al.*, 1988; Langrand *et al.*, 1990
Rhizomucor miehei			Mestri and Pai, 1994b
Candida cylindraceae, PPL,			Welsh and Williams 1990
Aspergillus niger			
Candida antarctica,			Gubicza *et al.*, 2000
Geotrichum sp. and *Rhizopus* sp.			Macedo *et al.*, 2004
Candida antarctica	Isoamyl propionate	Banana flavour	Langrand *et al.*, 1988
Rhizomucor miehei	Isoamyl isovalerate	Apple flavour	Chowdary *et al.*, 2002
Rhizomucor miehei	Isobutyl isobutyrate	Pineapple flavour	Hamsaveni *et al.*, 2001
Rhizomucor miehei	Methyl propionate	Fruity flavour	Perraud and Laboret, 1989
Candida cylindracea	Ethyl butyrate	Pineapple flavour	Gubicza *et al.*, 2000
			Gillies *et al.*, 1987

Candida cylindracea, PPL and	Butyl isobutyrate	Sweet fruity odor	Yadav and Lathi, 2003
Aspergillus niger			Welsh and Williams, 1990
Rhizomucor miehei, PPL	Protocatechuic aldehyde		Divakar, 2003
Staphylococcus warneri	Short chain alcohol esters of C_2-C_{18} acids	Fruity odor	Talon *et al.*, 1996.
Staphylococcus xylosus	Short chain fatty acid esters	Fruity odor	Mestri and Pai, 1994a
			Macedo *et al.*, 2003; Xu *et al.*, 2002
Candida antarctica	Long chain alcoholic esters of lactic acids	Flavor	From et al., 1997; Torres and Otero 1999;
Novozym 435 *Rhizomucor miehei*			Parida and Dordick, 1991
Candida rugosa	Methyl benzoate	Exotic fruity and berries flavor	Bousquet *et al.*, 1999
			Leszczak and Minh, 1998

Novozym 435	Tetrahydrofurfuryl butyrate	Fruity favor	Yadav and Devi, 2004
Rhizomucor miehei	Cis-3-hexen-1-yl acetate	Fruity odor	Chiang *et al.*, 2003
2. Fragrance esters			
Rhizomucor miehei, PPL	Tolyl esters	Honey note	Burdock, 1994 Suresh Babu *et al.*, 2002; Manohar and Divakar, 2002
Candida cylindracea, PPL	Anthranilic acid esters of $C_2 - C_{18}$ alcohols.	Flowery odor of jasmine	Kittleson and Pantaleone, 1994 Suresh Babu and Divakar, 2001 Manohar and Divakar, 2004a
PPL	4-t-Butylcyclohexyl acetate	Woody and intense flowery notes	Manohar and Divakar, 2004b

Organism	Product	Odor	Reference
Rhizomucor miehei, PPL, *Pseudomonas cepacia*	Geranyl methacrylate	Floral fruity odor	Athawale *et al.,* 2002
Candida antarctica SP435	Citronellyl acetate	Fruity rose	Claon and Akoh, 1994b
Pseudomonas fragi	Citronellyl propionate	odor	Mishio *et al.,* 1987
	Citronellyl valerate		Marlot *et al.,* 1985
	Geranyl butyrate		
	Geranyl propionate		
	Geranyl valerate		
Candida rugosa	Farnesol butyrate	Fruity odor	Akoh *et al.,* 1992; Shieh *et al.,* 1996
	Farnesol propionate		
	Farnesol valerate		
	Phytol butyrate		
	Phytol propionate		
	Phytol valerate		

Novozym SP	Citronellyl laurate		Yadav and Lathi, 2004
Rhizomucor miehei	α-Terpinyl acetate	Fruity, characteristic	Rao and Divakar, 2002
	α-Terpinyl propionate	lavendar and bergamot-like fragrance	
Rhizomucor miehei	α-Terpinyl esters of fatty acids		Rao and Divakar, 2001
Aspergillus niger, Rhizopus			Iwai *et al.*, 1980
delemar, Geotrichum candidum,	α-Terpinyl esters of short chain acids		Claon and Akoh, 1994a
Pencillium cyclopium	Terpinyl esters of triglycerols		

3. Surfactant esters

Novozym 435	Oleic acid esters of short chain alcohols	Surfactants	Dorm *et al.*, 2004
Rhizomucor miehei	Butyl oleate	Surfactants	Knez *et al.*, 1990

Rhizomucor miehei, PPL	2-O- Alkanoyl lactic acid esters of C_2-C_{18} alcohols	Surfactants	Kiran and Divakar, 2001a; Kiran *et al.*, 1998

4. Surfactant and sweetener esters

Mucor javanicus, *Pseudomonas cepacia*, Subtilisin,	N-Acetyl- L-leucyl -D-glucose	Surfactants	Maruyama *et al.*, 2002
	N-Acetyl-L-methionyl –D –glucose		
	N-Acetyl- L-tyrosinyl -D –glucose		
	N-Acetyl-L-tryptophanyl-D –glucose		
Subtilisin	N-Acetyl-L-phenylalanyl-D- glucose	Surfactants	Maruyama *et al.*, 2002;
	N-Acetyl-L-phenylalanyl–D –galactose		Riva *et al.*, 1988
	N-Acetyl-L-phenylalanyl–fructose		
	N-Acetyl-L-phenylalanyl – mannose		
	N-Acetyl-L-phenylalanyl – lactose		
	N-t-Boc-L-phenylalanyl – glucose		

	N-Acetyl-L-methionyl – methyl-β-galactopyranoside	Surfactants	Park *et al.*, 1996
Optimase M-440, Proleather, APG 380	N-t-Boc-L-phenylalanyl – galactose	Surfactants	
	N-t-Boc-L-phenylalanyl – fructose		
	N-t-Boc-L-phenylalanyl –methyl α-D-glucopyranoside		
Optimase M-440	N-t-Boc-L-phenylalanyl – sorbitol	Surfactants	
	N-t-Boc-L-phenylalanyl – sucrose		
	N-t-Boc-L-phenylalanyl – cellobiose		
	N-t-Boc-L-phenylalanyl – raffinose		
	N-t-Boc-L-phenylalanyl – trehalose		
	N-t-Boc-L-phenylalanyl – maltose		
	N-t-Boc-L-phenylalanyl – lactose		

Optimase M-440	N–t-Boc-L-leucyl – sucrose	Surfactants	Jeon *et al.*, 2001
	N–t-Boc-L-tyrosinyl – sucrose		
	N–t-Boc-L-methionyl – sucrose		
	N–t-Boc-L-aspartyl– sucrose		
	Di-N–t-Boc-L-lysyl – sucrose		
	N–t-Boc-L-phenylalanyl – xylitol		
	N–t-Boc-L-phenylalanyl – arabitol		
	N–t-Boc-L-phenylalanyl – mannitol		
	N–t-Boc-L-phenylalanyl–N-acetyl-D-glucosamine		
Rhizomucor miehei, PPL, *Candida rugosa*	L-Alanyl, L-valyl, L-leucyl and L-isoleucyl ester of carbohydrates	Surfactants, Sweeteners	Vijayakumar *et al.*, 2004; Somashekar and Divakar 2007

	L-Prolyl, L-phenylalanyl, L-tryptophanyl and L-histidyl esters of carbohydrates 1-*O*-ester, 2-*O*-ester, 3-*O*- ester, 4-*O*- ester, 5-*O*-ester, 6-*O*- ester, 6'-*O*- ester, 2,5-di-*O*- ester, 3,5-di-*O*- ester, 2, 6-di-*O*- ester, 3, 6-di-*O*- ester, 6,6'-di-*O*- ester		Lohith *et al.*, 2003; Lohith and Divakar, 2005 ; 2007
Subtilisin	N-Acetyl-L-alanyl -methyl-β-D-galactopyranoside	Surfactants, Sweeteners	Riva *et al.*, 1988 Suzuki *et al.*, 1991
Rhodotorula lactosa	2-*O*- ester, 3-*O*- ester, 4-*O*- ester, 6-*O*- ester		

Enzyme	Product	Application	Reference
PPL	6-*O*-Butyl glucose	Surfactants	Therisod and Klibanov, 1986
Subtilisin	6-*O*- Acetyl glucose		Kirk *et al.*, 1992
	6-*O*- Capryloyl glucose		Zaks and Dodds, 1997
	6-*O*- Acetyl galactose		Klibanov, 1986
	6-*O*- Acetyl maltose		Dordick, 1989
	6-*O*- Acetyl fructose		Schlotterbeck *et al.*, 1993
	1-*O*- Acetyl fructose		Boyer *et al.*, 2001.
Lipozyme, *Rhizomucor miehei*	Fructose oleate	Surfactant	Khaled *et al.*, 1991
Candida antarctica	Fatty acid esters of glycosides		Adlerhorst *et al.*, 1990
Candida rugosa	Butyl oleate		Zaidi *et al.*, 2002
	Oleyl butyrate		
	Oleyl oleate		

Humicola lanuginose	6-*O*- Lauroyl sucrose	Surfactants	Ferrer *et al.*, 1999;
Candida antarctica B	6-*O*-Lauroyl glucose		Ferrer *et al.*, 2005
	6-*O*-Lauroyl maltose		
	6-*O*-Palmitoyl maltose		
	1,6-di-*O*-Lauroyl sucrose		
	6,6-di-*O*-Lauroyl sucrose		
	6′-*O*- Palmitoyl maltose		
Candida antarctica	β-Methylglucoside methacrylate / acrylate	Surfactants	Kim *et al.*, 2004

1.7. Important parameters regulating the lipase activity in organic solvents

1.7.1. Nature of substrate

Lipases display various degrees of selectivities towards the substrates with which they interact (Bloomer, 1992). Steric hindrance (branching, unsaturation and chain length) and electronic effects of the substrates are the two major forces that determine selectivity (Bevinakatti and Banerjee, 1988). In esterification reactions, many lipases display high selectivities on long and medium chain fatty acids than the short chain and branched ones (Alhir *et al*., 1990). Most lipases display selectivities towards carboxylic acids. *Geotrichum candidum* lipase reacts only with fatty acids containing a *cis* bond at the 9th position (Schrag *et al*., 1996). Generally alcohols like ethanol and geraniol have been reported to be inhibitory in esterification and transesterification reactions (Chulalaksananukul *et al*., 1990 and 1992; Miller *et al*., 1988). Molar ratio of the substrates plays an important role in esterification, which can be improved by increasing either alcohol or acid but in most of the cases, the alcohols may be inhibitory and acids may cause acidification of microaqueous interface resulting in inactivation of lipases (Dorm *et al*., 2004; Guvenc *et al*., 2002; Zaidi *et al*., 2002). It is difficult to generalize the effect of chain length on esterification, because it depends on the lipase preparation and the specificity of the enzymes. Esterification increased with increase in chain length when the reaction was catalyzed by lipases from *Staphylococcus warneri* and *Staphylococcus xylosus* (Talon *et al*., 1996). In case of Lipolase 100T, esterification decreased with increase in chain length and found to be independent of chain length when esterification was catalysed with Novozyme 435 (Kumar *et al*., 2005). *Rhizomucor miehei* lipase exhibited fatty acid chain length optima towards C_8 and C_{12} in an esterification reaction with glycerol and its diol analogues. However, *Candida antartica* B lipase exhibited fatty acid chain length optima only towards C_8. Kirk *et al.,* (1992)

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showed that *Candida antartica* B lipase is not very selective in esterification reaction with *n*-octanol in hexane. Zhang *et al.,* (2005) found high specificity of pancreatic lipase towards butyric and valeric acids and a significantly lower esterification rate with lower (propionic) and higher (C_6-C_{14}) carbon number acids. The specificity increased in longer chain fatty acids (palmitic and stearic), but remained significantly lower than that observed for the best-suited substrates. For the same chain length, unsaturation leads to decrease in specificity as demonstrated for oleic acid compared to stearic acid (Zhang *et al.,* 2005).

Use of acetic acid as an acyl donor was attempted with little or no success (Takahashi *et al.,* 1988) in the preparation of acetates. Compared to its higher homologues (propionates, butyrates), acetic acid is a potent inhibitor (Segel, 1993) by preferentially reacting with the serine residue at the active site of lipase (Huang *et al.,* 1998). Iwai *et al.,* (1980) did not observe any reaction between acetic acid and geraniol using lipases from four different microorganisms. Langrand *et al.,* (1988) showed that acetic acid esters were difficult to synthesize in high yields due to lipase inactivation by acid. Although, few researchers have focused their attention on transesterification to obtain high yields of acetates (Langrand *et al.,* 1990; Rizzi *et al.,* 1992; Chulalaksananukul *et al.,* 1993), reports on maximizing acetate production by direct esterification are scanty. Also, low molecular weight substrates are more water-soluble and as such may react differently than do high molecular weight (less water soluble) substrates in non-aqueous systems.

In most cases, lipase showed enantio preference towards (*R*)-enantiomer than (*S*)-enantiomer (Cardenas *et al.,* 2001). There were also reports that lipase from *Fusarium oxysporum*, *Ophiostoma sulphureoochraceum*, *Staphylococcus halstedii* and *Fusarium*

poae displayed preference for the (*S*)-stereomer in the acylation of (*R*) and (*S*)-glycidol (Cardenas *et al.,* 2001).

1.7.2. Nature of solvent

Enzymes are known to be active and stable at physiological pH. Hence information regarding the rate of reaction, kinetics and catalytic mechanism of the enzyme has been derived from investigations conducted in aqueous buffer solutions (Dixon and Webb, 1979; Welsh and Williams, 1990). However, when enzymes are directly introduced in organic solvents, they display incredible changes in their properties (Klibanov, 1986). Organic solvents employed influence reaction rate, maximum velocity (V_{max}) or specific activity (K_{cat}), substrate affinity (K_m), specificity constants (K_{cat}/K_m) (Zaks and Klibanov, 1986), enantio-selectivity (Sakurai *et al*., 1988), lipase stability (Kung and Rhee, 1989) and stereo and regio-selectivities (Parida and Dordick, 1991 and 1993; Nakamura *et al*., 1991; Rubio *et al*., 1991). Several efforts have also been made to rectify the kinetic affinity parameters for substrate-solvent interactions (Van Tol *et al*., 1992; Reimann *et al*., 1994). The enzyme activity in different solvents could be due to variable degree of enzyme hydration imposed by the solvents and not to their direct effect on the enzyme or substrates. Generally use of organic solvents in enzyme catalysed synthesis exhibit more advantages than in aqueous media (Klibanov, 1986).

1. Non polar / hydrophobic substrates can be employed for the reaction.

2. Enhances the reaction rate.

3. Shifting of thermodynamic equilibria towards synthesis of ester by maintaining

expensive and difficult.

5. Since enzymes are insoluble in organic solvents, can be easily recovered from the reaction mixture by simple filtration.

6. Water dependent reactions such as hydrolysis and racemization can be avoided.

7. Inhibition of enzymes by hydrophobic substrates or products is minimized due to dispersion in the organic solvents, which minimizes substrate concentration at enzyme surface.

8. Microbial contamination resulting in enzyme inhibition as well as degradation/

Investigations on quantification of solvent effects on enzyme catalysis were carried out (Brink and Tramper, 1985; Laane *et al*., 1987). The efficiency of lipase was found to increase with log P value of solvent. Brink and Tramper (1985) tried to explain the influence of many water immiscible solvents on biocatalysis by employing the Hildebrand parameter, δ, as a measure of solvent polarity. They concluded that enhanced reaction rates could be expected when the polarity of the organic solvents was low ($\delta \approx$ 8) and its molecular weight > 150. But later, it was demonstrated that δ was a poor measure of solvent polarity. Laane *et al*., (1987) quantified solvent polarity on the basis of log P values. The efficiency of lipase was found to increase with log P value of solvent. The log P value of a solvent could be defined as the logarithmic value of the partition coefficient of the solvent in *n*-octanol/water two-phase system. Generally, biocatalysis is low in solvents of log P < 2, is moderate in solvents with a log P value between 2 and 4 and high in non-polar solvents of log P > 4. *Rhizomucor miehei* lipase was shown to follow these rules when esterification reactions were conducted in different solvents (Laane *et al*., 1987). The conversion of lipase in solvent improved

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from tetrahydrofuran (log P = 0.49), toluene (log P = 2.5), hexane (log P = 3.5), n-heptane (log P = 4.0) and *n*-nonane (log P = 5.1). In general, enzymes are more stable when suspended in non-polar solvents that have low solubility for water than in polar solvents (Acros *et al.,* 2001). In presence of hydrophilic solvents (log P < 2) lipozyme showed no esterification. Hence, polar solvents may remove the essential water from the enzyme and disrupt the active confirmation (Gargouri *et al*., 2002; Adachi and Kobayashi, 2005). They also probably form hydrogen bonding between solvent molecule and acids which are present on the surface of the enzyme. Solvents whose log P values were >2, dissolve to a lesser degree in water, leaving the enzyme suitably hydrated in its active conformation and so were able to support product synthesis (Soo *et al*., 2003). But a lipase which exhibits increasing activity with increased DMSO content (a polar solvent), has also been isolated (Bloomer, 1992). Effect of log P values of organic solvents was studied by correlating with K_{cat} and K_m. K_{cat} showed strong correlation with log P but not K_m. K_{cat} was not affected by different solvent composition with constant log P whereas K_m was reported to change remarkably (Hirakawa *et al*., 2005). Most of the lipases readily under go inactivation in presence of polar solvents, which is very much essential to dissolve certain substrates like sugars. Hence, the idea of solvent mixtures of non polar solvents with small amount of polar solvents was also employed in lipase catalysis (Ferrer *et al*., 1999). Ongino *et al*., 1999 screened solvent tolerant microorganism *Psuedomonas aeroginosa* which secretes LST-03 lipase which is much more stable in the presence of organic solvents than in its absence. While it is generally accepted that non-polar solvents are better than polar ones for lipase catalyzed esterification reactions, a clear consensus is yet to be reached regarding the issue of solvent effects on enzyme catalysis in general.

1.7.3 Effect of salt

Catalytic activity of the enzyme is based on protein ionization which is pH depend. Any change in pH is detrimental to 3D-structure of protein which in turn is detrimental to enzyme activity. It is well known that enzyme hydration play a crucial role in minimizing solvent-induced conformational rigidity (Patridge *et al.,* 1998) which is one of the cause for reducing catalytic activity in non-aqueous media (Klibanov, 1986). Along with the relevant factors such as degree of hydration and nature of solvent, ionization state of the enzyme also play a key role in overall reactions of the active conformation of the enzyme (Zacharis *et al*., 1997). Use of buffers in the reaction mixture or lyophilizing enzyme from buffers of known pH can reset enzyme ionization state and ensure pH memory. (Valivety *et al.,* 1990; Xu and Klibanov, 1996). Quiros (2002) tuned *Candida antartica* lipase B with solid state organic buffers like ET_3N, 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropane sulfonic acid and 3-[cyclohexyl-amino]-2-hydroxy-1-propane sulfonic acid and showed improved enantioselectivity and catalytic activity. Xu and Klibanov (1996) found the catalytic activity of substilisin Carlsberg lyophilized in buffers like phenylborate and *p*-toulene sulfonic acid and their corresponding sodium salt improved catalytic activity by 100 folds than without buffers in organic media. Fontes *et al.,* (2003) found that to achieve full activation of substilisin, the catalytic triad should be negatively charged which was achieved by replacing H^+ by a counter Na^+ adding salt hydrates of hydrogen phosphate, pyrophosphate and acetate. Addition of simple inorganic salts like KCl prior to lyophilization has dramatically enhanced V_{max}/K_m for several hydrolases in non-aqueous media (Lindsay *et al.,* 2002). This process was optimized by using numerous combinations of salt and lyophilization period (Ru *et al.,* 1999, 2000 and 2001).

Addition of salt hydrates to the reaction mixture accelerates the transfer of water between the salt hydrates pair and the enzyme by disturbing the ionic character of the enzyme microenvironment and thereby influencing catalytic activity (Fontes *et al.,* 2002)

~~1.7.11 Thermal Stability~~

At least over a certain range of temperature, the reaction catalyzed by enzymes behave like ordinary chemical reactions - as the temperature is increased, the rate increases. The same will begin to suffer thermal inactivation at higher temperatures. The actual temperature at which an enzyme passes through its maximum activity as well as stability is also influenced by environmental factors such as purity of enzyme, substrate, presence of activators or inhibitors, pH, solvent and presence of metal ions. The thermostability of lipases varies considerably according to their orgin: animal and plant lipase are less thermo stable than microbial extracellular lipase. Zhu *et al.,* (2001) reported that thermal stability of a lipase is obviously related to its structure. At least in some cases, thermal denaturation appears to occur through intermediate states of unfolding of the polypeptide (Zhu *et al.,* 2001). Of them, the two major ones are the nature of the organic medium employed and water content in the microenvironment of the enzyme (Halling, 1994). There are a few reports on the thermostability of lipases in aqueous media. Lipase from *Psuedomonas fluorescens* 33 was found to retain 10-20 % higher activity in the presence of casein and Ca^{2+} at higher temperatures (60 °C to 90 °C) at only 10 min of incubation (Kumura *et al.*, 1993). Novozym SP 435 is thermally stable at 60 °C (Yadav and Lathi, 2004). Thermostabilities of some serine esterases like chymotrypsin and lipase from *Candida rugosa* and *Rhizomucor miehei* have been studied as a function of hydration of the enzymes using differential scanning calorimetry (Turner *et al.*, 1995). It was found that the denaturation temperature (T_m)

was 30 °C to 50 °C higher in anhydrous environments than in aqueous solutions. Volkin *et al.,* (1991) reported that enzymes are more thermostable in hydrophobic solvents rather than in hydrophilic solvents. In hydrophilic solvents, stripping of water from the enzyme leads to denaturation. Porcine pancreas lipase was reported to retain higher esterification activity in dry organic environment (2 M heptanol solution in tributyrin) at a temperature of 100 °C when a low concentration of water (0.015 %) was maintained in the reaction system (Zaks and Klibanov, 1985). Half-life for the enzyme was found to be more than 12 h at 100 °C. However, when concentration of water was increased to 3 %, loss of activity was almost instantaneous (half life = 2 min). Porcine pancreas lipase (PPL) in non-aqueous media showed that longer periods of incubation of PPL at especially 80 ºC did not affect the active conformation of PPL even after incubation for a period upto 10 days (Kiran *et al.*, 2001b) at very low water content. Immobilization of the enzyme and addition of salt hydrates are known to enhance the thermostability of lipases in organic media (Kvittengen *et al.*, 1992; Halling, 1992). Iso *et al.,* (2001) conducted experiments at a temparature range from 40 ºC to 70 ºC on both free and immobilised *Pseudomonas flurorescens* lipase where free lipase showed highest conversion at 60 ºC and decreased activity at 70 ºC. However, immobilized lipase showed increased activity at 70 ºC and can be reused several times without much loss of activity. Immobilization shifts the temperature optima from 35 ºC of the free lipase to 40 ºC to 45 ºC. It could be attributed to a low restriction in the diffusion of the substates and products at higher temperature as well improved resistance of protein to thermal denaturation (Deng *et al.,* 2004). Storage stability of native and covalently immobilized lipase B from *Candida antartica* was studied by Arrayo *et al.,* 1999 under wet conditions at 50 ºC who found that immobilized lipases are thermally more stable than

native ones with half life values ranging from 0.5 to 14 h. Under the same conditions, native *Candida rugosa* lipase B is more stable than *Candida antartica* lipase as the former protein structure contains three disulfide bonds (Cys-22 to Cys-64, Cys-216 to Cys-258 and Cys-293 to Cys-311) and the latter contains only two (Cys-60 to Cys-97 and Cys-268 to Cys-277, Arroyo *et al.,* 1999). Thermal stability can also be improved by preparing a surfactant-lipase complex (Goto *et al*., 2005; Maruyama *et al*., 2002; Wu *et al*., 2002). It is a common practice now to carry out lipase catalyzed esterification reactions at around 80 °C to 90 °C (Trani *et al*., 1991). Noel and Combes (2003) conducted series of experiments at different temperatures to study the thermal effect of RML and concluded that thermal deactivation occurs due to the aggregation of enzymes of denatured proteins rather than protein unfolding.

1.7.6. Significance of water in lipase catalysis

Water plays a significant role in the reversible reaction catalyzed by lipase, involving hydrolysis and esterification (Hahn-Hägardal, 1986; Gayot *et al*., 2003). While a critical amount of water is necessary for maintaining the active conformation of the enzyme, excess water facilitates hydrolysis (Halling, 1989 and 1994; Zaks and Klibanov, 1985 and 1988; Valivety *et al*., 1993; Cameron *et al*., 2002). The bound water structure is very essential in stabilizing the 3D-conformation of a lipase in non-aqueous media. There are 22 buried water molecules in case of *Rhizomucor miehei* lipase (Brady *et al*., 1990) where water on the enzyme is bound to charged and polar residues on the surface as a monolayer (Tramper *et al*., 1992; Kvittengen, 1994; Halling, 1994; Orrenius *et al*., 1995; Vermue and Tramper, 1995; Zaks and Klibanov, 1988; Gorman and Dordick, 1992). Presence of excess water affects the catalytic activity from both kinetic and thermodynamic points of view (Chulalaksananukul *et al*., 1990; Marty *et al*., 1992). The

concentration of water in organic solvents is inversely proportional to the thermostability of lipases (Zaks and Klibanov, 1984). It was shown that for PPL, hydrophobic solvents served better than hydrophilic ones for catalysis (Zaks and Klibanov, 1988; Parida and Dordick, 1991). Substrate concentrations and water activity can control the product distribution. Hence, only monoester of ethylene glycol can be prepared by using either low water activity or employing higher concentrations of alcohols and *vice versa* for the diesters (Chand *et al*., 1997). Presence of certain amount water (optimum water, 5%) with crude lipase from horse liver, enhanced the reaction rate by three fold and also stereoselectivity by 50% (Gutman and Shapira, 1991). Humeau, *et al*., (1998) and Osorio *et al*., (2001) reported that beyond the critical water concentration, esterification decreases because, the size of the water layer which is formed around the enzyme, retards the transfer of acyl donor to the active site of the enzyme. Yadav and Devi (2004) conducted experiments at various speed of agitation and found that there is no effect of speed of agitation (Guvenc *et al*., 2002) on esterification. The water layer surrounding the enzyme makes the enzyme to be more flexible (act as molecular lubricant) by forming multiple hydrogen bonds and interacting with the organic solvent, causing denaturation. Organic substrates and products with poor solubility in aqueous medium diffuse with difficulty through the intra-particle water layer to the active centers of the enzyme. Thus the activity of the enzyme would depend on water-induced inactivation or partition of components between the bulk solvent and the microenvironment (Yadav and Devi, 2004; Yadav and Lathi, 2004). Almost all lipases are active at low water activity but there is large difference in optimal water activity between the lipases (Ma *et al*., 2002; Gargouri *et al*., 2002).

There are several techniques available to monitor the water activity like Karl-

Fischer titration (Halling, 1994) and specialized sensors (Blanco *et al*., 1989). In

esterification reactions, water formed, can be removed from the reaction mixture leading

to higher yields (Bloomer *et al*., 1992), by passing the reaction mixture through a bed of

desiccants (Halling, 1990; Gubicza *et al*., 2000). In a non-polar solvent, excess water

adds to the already existing hydration shell on the enzyme constituting the microaqueous

inter-phase. Partitioning of the acid, alcohol and product between microaqueous inter-

phase and solvent phases plays a significant role in regulating esterification. The

solubility of the acid and its dissociation result in a proton build-up at the inter-phase. In

lipase catalyzed esterification, the various equilibria involved at the microaqueous inter-

phase are shown in **Scheme 1.3** (Aires-Barros *et al*., 1989).

Organic phase   HA   ROH   Est

$$K_d{}^{HA} \qquad K_d{}^{ROH} \qquad K_d{}^{Est}$$

Microaqueous $A^- + H^+ \rightleftharpoons$ HA   +   ROH $\rightleftharpoons$ Est + H$_2$O
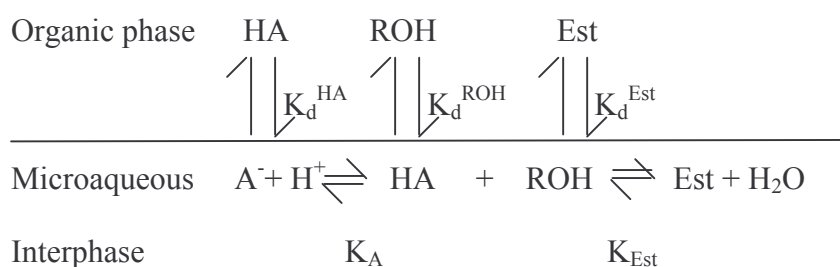
Interphase K_A K_{Est}

Scheme 1.3. Lipase catalyzed esterification at the microaqueous inter-phase

where HA = acid, ROH = alcohol, Est = ester, $K_d{}^{HA}$, $K_d{}^{ROH}$ and $K_d{}^{Est}$ = distribution

coefficients of acid, alcohol and ester respectively; K_A = dissociation constant of acid,

K_{Est} = equilibrium constant of esterification. Since water is present in micro-quantities

and is inaccessible, direct measurement of microaqueous pH is not possible (Valivety *et*

al., 1990b). Attempts have been made to measure the pH in non-aqueous systems by

Cambou and Klibanov (1984), who reported the use of an indicator, which changed

colour with pH. A reliable method has been developed by Valivety *et al*., (1990b) in

which an hydrophobic indicator (fluorescein ester with 3,7,11- trimethyldodecanol) is

used, which remains completely in the organic phase, but responds to pH changes in an

adjacent aqueous phase. Thermodynamic factors operating at the microaqueous enzyme-water-solvent phase in non-polar solvents were also investigated in terms of the water of reaction, partitioning of acid between the microaqueous phase and the organic solvent, dissolution and dissociation of the acid, the resultant number of H^+ present in the microaqueous phase and the extent of esterification (Kiran *et al*., 2002).

1.7.6. Immobilization

The use of enzymes for analytical, clinical or any synthetic purposes has been limited because of certain disadvantages, such as their instability and lack of availability. Immobilization of enzymes on solid support renders both practical and economic advantages such as easy handling, enzyme recovery at any stage of the reaction and re-utilization. Immobilization of lipase facilitates large area of inter-phase for the reaction to occur. The advantages of immobilizing lipases are repetitive use of a given batch of enzymes, better process control, enhanced stability, enzyme - free products (Welsh and Williams, 1990; Rahman *et al*., 2005), increased stability of polar substrates, shifting of thermodynamic equilibria to favour ester synthesis over hydrolysis, reduction in water dependent side reactions such as hydrolysis, elimination of microbial contamination and potential to be used directly within a chemical process. After immobilization, there are changes in activity, optimum pH, temperature, affinity towards substrate and stability of the enzyme, which are all depended upon the source of the enzyme as well as type of support material and immobilisation (Klnc *et al*., 2006; Sato *et al*., 1999). In presence of organic solvents, immobilized lipase showed enhanced activity (Ye *et al*., 2005). Ye *et al*., (2005) reported that at lesser water content, free lipase preparations showed increase in activity than the immobilized lipase preparation. With the increment of the amount of water added, conformational limitation on the enzyme as a result of covalent

bond formation between the enzyme and the matrix, led to increase in activity due to immobilization.

Immobilization of enzymes has been performed by variety of methods which has been broadly classified as physical where interaction between support and enzyme exists, and chemical where covalent bonds exist between support and enzyme. (Bullock 1995; Tischer and Wedekind, 1999). Physical immobilization method includes:

(i)   containment of an enzyme within a membrane reactor,

(ii)   adsorption (physical, ionic) on a water-insoluble matrix,

(iii)   inclusion (or gel entrapment),

(iv)   microencapsulation with a solid membrane,

(v)   microencapsulation with a liquid membrane, and

(vi)   formation of enzymatic Langmuir-Blodgett films

Chemical immobilization methods includes

(i) covalent attachment to a water-insoluble matrix,

(ii) crosslinking with use of a multifunctional, low molecular weight reagent, and

(iii) co-crosslinking with other neutral substances, e.g. proteins.

In case of non-covalent immobilization, lipases can be adsorbed with very good activity to a weak anion exchange resin (Eigtved, 1989; Ison *et al*., 1990). For covalent immobilization, usually, support matrices like silica beads are activated with glutaraldehyde (Ulbrich *et al*., 1991). For non-covalent immobilization, both ionic and hydrophobic interactions between lipase and surface are important (Eigtved, 1989; Ison *et al*., 1990; Malcata *et al*., 1990). Polymers such as polyvinyl alcohol (PVA), carboxymethyl cellulose (CMC), poly ethylene oxide (PEO) and CMC-PVA blends can also be used for lipase immobilization (Vecchia *et al*., 2005). Morphology of film

surfaces analyzed by scanning electron microscopy indicated that lipases were preferentially located on the polymer surface (Crespo *et al*., 2005). Vecchia *et al.,* (2005) have immobilized 10 different lipases on polyvinyl alcohol, carboxymethyl cellulose and polyvinyl alcohol-carboxymethyl cellulose blends (50:50% m/m) and among them *Mucor javanicus* (MJL) and *Rhyzopus oryzae* (ROL) exhibited highest activity. This immobilized enzyme can be reused at least 10 times for a span of 80 days. *Candida antartica* B (Novozym 435) was immobilized on mesoporous silica with octyl triethoxysilane and it retained its activity even after 15 reaction cycles (Blanco *et al.,* 2004). Porcine pancreatic lipase immobilized on chitosans exhibited nearly twofold higher and that on chitins exhibited four-fold higher activity than that of the free enzyme. (Klnc, 2006). Porcine pancreas lipase and *Candida cylindracea* lipase immobilized on celite and amberlite IRA-938 showed activity at 7-10 ^{o}C and at acidic pH than that of free enzyme (Sagiroglu *et al*., 2004). Calcium carbonate was found to be the most suitable adsorbent when crude *Rhizopus oryzae* lipase was immobilized on different supports and it exhibited long-chain fatty acid specificity (Ghamgui *et al*., 2004). Lipase from *Pseudomonas cepacia* was gel-entrapped by polycondensation of hydrolyzed tetramethoxy silane and isobutyl trimethoxy silane and it was subjected to repeated use without loosing much activity (Noureddini *et al*., 2005). A major disadvantage of adsorption is the leaching of protein out of the carrier during on line utilization, which can be attributed to the relative weakness of binding forces. In presence of specific components such leaching will lead to loss of catalytic activity and to contamination of reaction products, especially at higher salt concentrations, unless the reaction mixture is hydrophobic and nonpolar in character (Paiva *et al*., 2000). For covalent immobilization, usually, support matrices like silica beads are activated with glutaraldehyde (Ulbrich *et*

al., 1991). Porcine pancreatic lipase immobilized by covalent attachment to C030F cellulose Nadir filtration membrane (of cut off value 30 kg mol^{-1}) are found to be less active than the lipase adsorbed on a plain nylon surface. This may be due to modification of protein tertiary structure by covalent binding (Manjon *et al*.,1991).

1.8. Diverse applications of lipases in reaction

1.8.1. Esterification in reverse micelles

Enzymatic reactions in water-in-oil microemulsions with reverse micelles offer many advantages over those in micelles (oil-in-water) or in organic solvents, like solubilization of lipases and both hydrophobic/hydrophilic substrates at higher concentrations, better control over water activity and large interfacial area leading to enhanced reaction rates in a thermodynamically stable single phase (Stamatis *et al*., 1999). Various reactions like synthesis of flavour esters (Borzeix *et al*., 1992), macrocylic lactones (Rees *et al*., 1995), and resolution of chiral alcohols (Rees and Robinson, 1995) have been attempted in reverse micelles. Krieger *et al*., (2004) highlighted some of the recent developments on the use of lipases in reverse micelles. There have been some efforts to continuously recover the product as well as enzyme for reuse, which, are the major problems of enzyme catalysis in reverse micelles. Reverse micelles can exchange biocatalyst, water, substrates and products with the bulk organic solvent (Krieger *et al.,* 2004). Murakata *et al.,* (1996) have reported high esterification activity of entrapped lipases on lipophilic substrates. The effective diffusion coefficient of lauric acid varied with the lecithin microemulsion-based organogels (MBGs) composition, while that of butyl alcohol remained constant in the esterification of lauric acid with butyl alcohol catalyzed by *Candida rugosa* lipase (Nagayama *et. al*., 2002). High initial reaction rate was obtained in extremely low water content when the

esterification of oleic acid with octyl alcohol catalyzed by *Rhizopus delemar* lipase was investigated in reverse micellar system of sugar ester DK-F-110 (Naoe *et al.*, 2001). Kinetic studies were carried out to study the esterification of octanoic acid with 1-octanol, catalyzed by *Candida lypolytica* (CL) lipase, in water-in-oil microemulsions formed by water/ bis-(2-ethylhexyl) sulfosuccinate sodium (AOT)/isooctane (Zhou *et al.*, 2001). An esterification reaction of hexanol and hexanoic acid in the cyclohexane/dodecylbenzenesulfonic acid (DBSA)/water microemulsion system using *Candida cylindracea* lipase demonstrated that DBSA itself can act as a kind of acid catalyst (Han and Chu, 2005).

1.8.2. Esterification in supercritical carbon dioxide

Use of super critical carbon dioxide (SCCO$_2$) as a solvent and reaction medium is an emerging approach which is growing rapidly in recent years. Super critical carbon dioxide has several advantages over organic solvents: it is cheap, non-toxic, non-inflammable, used at near ambient critical temperature (31.1 °C) and moderate critical pressure (Srivastava *et al.*, 2003). The solvent properties of SCCO$_2$ can be readily modified by adjusting the pressure and temperature, the diffusivity of solutes in CO$_2$, which is higher than in organic solvents and easy recovery of CO$_2$ from the reaction products minimizing the need for costly downstream processing. When CO$_2$ is used along with organic solvents, it has an additional advantage of being an environment-friendly process (Clifford, 1994).

Fatty acid methyl esters were prepared from methanol and seed oils in flowing CO$_2$ by employing immobilized *Candida antartica* lipase (Holmberg *et al.*, 1989). Transesterification of soybean oil with glycerol, 1,2-propanediol and methanol by immobilized *Candida antartica* lipase for the synthesis of monoglycerides has also been

reported (Jackson and King, 1997). Isoamyl acetate was synthesized using lipases from *Candida antartica* and *Rhizomucor miehei* in SCCO$_2$ where *Rhizomucor miehei* gave 100% esterification with acetic anhydride (Romero *et al*., 2003). Enantioselective enzymatic hydrolysis of 3-hydroxy-5-phenyl-4-pentenoic acid ethyl ester in a biphasic buffer/SCCO$_2$-system was also carried out (Hartmann *et al*., 2001) where one ester enantiomer was preferably hydrolyzed, the other remained in the supercritical phase. *Candida cylindracea* lipase catalyzed enantioselective esterification of racemic 2-(6-methoxy-2-naphthyl) propionic acid in microaqueous isooctane showed that alcohol concentration influences enzyme performance, not because of stripping water from the enzyme (Wu and Liu, 2000). Production of ethyl esters from ethanol and cod liver oil by an immobilized lipase from *Candida antartica,* in SCCO$_2$ has also been described (Gunnlaugsdottir *et al*., 1998).

1.8.3. Esterification in micro oven assisted reactions

Recently, the use of microwave technology in organic synthesis is becoming an `increasingly popular method` because of short reaction time, enhanced selectivity, purity of the resulting products and enhancement of reaction yields (Loupy *et al*., 1998). Adlerlhorst *et al*., 1990 synthesized monoesters of glucopyranosides in large scale using lipases from *Candida antartica* and *Rhizomucor miehei* in dry media. Immobilised lipase from *Pseudomonas* and Novozyme SP 435 showed increased enantioselectivity in transesterification reaction of racemic sec-phenethyl alcohol with isopropenyl acetate with microwave activation than with convensional heating (Carrillo-Munoz *et al.,* 1996) Gelo-pujic *et al*., (1996) investigated the esterification of methyl-D-glucopyranoside, D-glucose and α,α-trehalose with dodecanoic acid in dry media by employing immobilized *Candida antartica* under focused microwave irradiation. Rajan and Abraham (2004)

studied *Thermomyces lanuginosa* lipase catalyzed esterification of starch from maize and cassava with lauric acid using microwave heating and found a substitution percentage of 53 % with a degree of substitution of 1.55 in case of maize starch and a substitution percentage 39 % with a degree of substitution of 1.1 in the case of cassava starch. Parker *et al.,* 1996 found that an irradiated hydrated cutinase in organic media using microwaves enhanced the reaction rate 2-3 folds over classical heating. A selective esterification of γ-linolenic acid in a mixture of free fatty acids with *n*-butanol was reported with lipase from *Rhizomucor miehei* under microwave irradiation (Vacek *et al.,* 2000). Lipase from porcine pancreas is demonstrated to catalyze acylation reactions in organic media under microwave irradiation. Reaction rates and enantioselectivities are significantly enhanced 1-14 and 3-9 folds, respectively. It was found that micro wave assisted reactions enhanced enzyme selectivity and retained its activity without much loss.

1.8.4. Esterification in ionic liquids

Biocatalytic transformations in ionic liquids have also become the subject of intense research. Different combinations of cationic and anionic solvents made possible to dissolve enzymes and carbohydrates that would not dissolve adequately in organic solvent directly in ionic liquids (Park and Kazlauskas 2001; Swatloski *et al.,* 2002; Patel *et al.,* 2002). Different enzymes such as hydrolases (proteases and lipases) and oxidoreductases (peroxidases and dehydrogenases) retained their activity in ionic liquids. Transesterification reaction of ethyl butanoate with butan-1-ol catalysed by immobilised *Candida antartica* lipase B gave good yields and enantio selectivity in 1-butyl-3-methyl-imidazolium tetraflouroborate, [bmim][BF_4] and 1-butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF_6] than in *tert*-butanol and *n*-butanol which were better

than native lipase (Lau *et al*., 2000). The same was observed with *Pseudomonas cepacia* lipase (Kim *et al*., 2001). The native *Pseudomonas cepacia* lipase showed a high initial rate in [bmim][PF$_6$] as compared to [bmim][BF$_4$] and dichloromethane and the difference in activity in the two ionic liquids was attributed to their hydrophobicity (Nara *et al.,* 2002). Relatively hydrophobic solvents are capable of removing the essential water from the enzyme surface, leading to insufficient hydration of the enzyme, which may, in some cases, exert a strong influence on the enzyme and decrease its activity (Yang and Russel, 1996). The ionic liquids with the enzyme were recycled for five consecutive runs without any substantial diminution in the lipase activity. Enantioselective esterification of (±)-menthol with propionic anhydride using *Candida rugosa* lipase in ionic liquids ([bmim][PF$_6$] and [bmim][PF$_4$]) showed 2.5 times higher activity and enantioselectivity in recycling application than in hexane (Yuan *et al*., 2006). Nara (2004) studied lipase-mediated regioselective biotransformations involving hydrolysis and alcoholysis of 3,4,6-tri-*O*-acetyl-D-glucal in tetrahydrofuran (THF) and two different ionic liquids, namely [bmim]PF$_6$ and [bmim]BF$_4$ showing   marked regioselectivity towards the formation of 4,6-di-*O*-acetyl-D-glucal in [bmim]PF$_6$ with 84% product formation after 6 h with 98% selectivity in hydrolysis and 48% after 8 h with 98% selectivity in alcoholysis (Nara *et al*., 2004). The main limitation of usage of ionic liquid in organic synthesis is recovery of reaction products. To separate products one should use solvents that are immiscible with ionic liquids which give biphasic systems. However even that extract will contain small amount of ionic liquid as well as catalysts (Gordon, 2001). Also, the partitioning of the solute between the phases limits the extent of solute

1.8.5. Kinetic studies of lipase catalyzed esterification reactions

Kinetic study of enzyme reactions help in determining the rate of a reaction and how this rate changes in response to different conditions which can be intimately related to the path followed by the reaction and is therefore indicative of its reaction mechanism. It also describes the intricate details of enzyme inhibition and how the rate of enzymatic reactions vary with enzyme and concentration, incubation period, pH, solvent and temperature. When lipases were used in organic solvents, it was found that they followed a complex two-substrate Ping-Pong Bi-Bi mechanism (Zaks and Klibanov, 1986). A Ping-Pong Bi-Bi mechanism, which stands for two-substrate two-product reaction, is a sequential one where both substrates do not bind to the enzyme simultaneously before the product is formed. The amount of lipase available and the rate of breakdown of the enzyme-substrate complex govern the overall rate of reaction. If the organic acid employed is inhibitory in nature, then it remains bound to the enzyme strongly and no acyl transfer occurs. In some cases, even if the acyl transfer occurs, product formed may remain bound to the enzyme resulting in inhibition.

Lipase-catalyzed esterification between oleic acid and ethanol (Chulalaksananukul *et al.,* 1990) and transesterification between geraniol and propyl acetate (Chulalaksananukul *et al.,* 1992) were found to follow Ping-Pong Bi-Bi mechanism where both ethanol and geraniol were found to be inhibitory. Similar Ping-Pong Bi-Bi mechanism was found to be followed in the kinetics of esterification of lauric acid by (-)-menthol catalyzed by lipase from *Penicillium simplicissium* with (-) menthol being inhibitory (Stamatis *et al*., 1993). In a transesterification reaction between isoamyl alcohol and ethyl acetate catalyzed by Lipozyme IM20, the substrates, ethyl acetate and isoamyl alcohol and one of the products (ethanol) were found to be inhibitory. Of the

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others. *n*-Octanol is inhibitory to RML and CRL in the transesterification between vinyl acetate and *n*-octanol (Yadav and Trivedi, 2003) following the Ternary Complex Bi-Bi mechanism, in which *n*-octanol binds twice or once to lipase to yield a dead-end lipase-*n*-octecule complex and a second molecule of *n*-octanol binds to give another dead-end lipase-*n*-octanol complex. For citronellyl laurate synthesis, RML follows the Ordered Bi-Bi mechanism wherein β-citronellol binds to the enzyme to yield the β-citronellol-enzyme complex, which again binds to lauric acid to form the ternary enzyme-β-citronellol-lauric acid complex. Finally, the complex decomposes to give β-citronellyl laurate and water as products in this process (Yadav and Lathi, 2004). A series of dead-end RML-lauric acid complexes were also reported in this process.

A kinetic model has also been proposed, taking into account, the effect of solvent (γ_s – substrate solvation) and a_w, which are thermodynamic parameters. In this model, the hydration state of the enzyme molecule was examined and equilibrium kinetic constants were expressed in terms of thermodynamic activity. Predictions based on this model were found to be in good agreement with experimental observations (Lee, 1995; Janssen *et al.*, 1999). Mathematical analyses of experimentally observed initial rates yielded various parameters, $K_{m(lactic\ acid)}$ = 0.059 M, $K_{m(stearic\ acid)}$ = 0.04 M, $V_{max(lactic\ acid\text{-}stearic\ acd)}$ =0.00163 M/h and $K_{i(lactic\ acid)}$ =0.079 M^{-1} in the kinetic study of the reaction between stearic acid and lactic acid (Kiran and Divakar, 2002). Thus, with improved kinetic models being proposed, one can predict the enzyme behaviour in a more efficient manner.

1.8.6. Lipase catalyzed resolution of racemic esters

There is an increasing demand for preparing optically active pure compounds in pharmaceutical industry. Various methods are available to synthesize the optically active compounds and among them enzymatic methods are more attractive (Sheldon, 1996). Lipases have also been extensively used in the resolution of racemic alcohols and carboxylic acids through asymmetric hydrolysis of the corresponding esters (Cambou and Klibanov, 1984b). Chirally pure hydroxyalkanoic acids which find wide applications as drug intermediates have been obtained from racemic (±)-hydroxyalkanoic esters (Scilimati *et al*., 1988; Feichte *et al*., 1989; Engel *et al*., 1991). Molecular modelling studies have revealed that enzyme behaviour towards racemic substrates can be predicted (Hult and Norin, 1993). Rantwijk and Sheldon (2004) critically reviewed resolution of chiral amines through enantioselective acylation by three different serine hydrolases - lipases, subtilisin and *Penicillin* acylase and recommended *Candida antartica* lipase because of its high enantioselectivity and stability. Resolution of some enantiomeric alcohols like (*R*,*S*)-2-octanol, (*R*,*S*)-2-(4-chlorophenoxy) propionic and (*R*,*S*)-2-bromo hexanoic acids were carried out using lipases from *Candida rugosa* and *Pseudomonas* species where *R*-alcohol was obtained with an enantiometric excess of about 98% (Crespo *et al.,* 2005). Optically active (*S*)-α-cyano-3-phenoxybenzyl (CPB) acetate was obtained from racemic cyanohydrins by transesterification using lipase from *Alcaligenes* sp. in organic media (Zhang *et al.,* 2005). Lipase isolated from porcine pancreas immobilized in DEAE-Sepharose gave pure (*S*)-(-) glycidol from (*R*)-(-)-glycidyl butyrate when the reaction was carried out in pH 7.0 and 10 % dioxane at 25 °C (Palomo *et al.,* 2003). Bocola *et al.,* 2003 reported that in kinetic resolution of (*R*)- and (*S*)-1-phenylethylamine using *Candida antartica* lipase B preacylated with an ethoxyacetyl group. The reaction showed a remarkable stereo discrimination with an enantiomeric

ratio E > 1000 in favour of (*R*)-1-phenylethylamide. The differential activation free energy showed that the fast-reacting (*R*)-amine is discriminated with respect to the slow-reacting (*S*)-amine by a free energy difference of 19.4 kJ/mol (Bocola *et al*., 2003). From molecule crystal structure analysis, it was found that fast reacting (*R*)-phospho-transition state analogue appears to exhibit perfect surface and shape complementarity to the *Candida antartica* lipase B binding pocket (Bocola *et al*., 2003).

1.9. Scope of the present investigation

The above described features have clearly established that, as one of the most thoroughly studied hydrolyzing enzymes in synthetic reactions, lipases have come a long way in establishing themselves as an important synthetic tool in bio-organic reactions. The main scope of the present work described in this thesis is to use the lipases in esterification reactions to prepare commercially important esters. Amino acyl esters of carbohydrates, find use in a wide variety of applications. They are used in industry as detergents, microcapsules in pharmaceutical preparations, in the delivery of biologically active agents, antibiotics, sweetening agents, emulsifying agents, active nucleoside amino acid esters, antitumor agents, plant growth inhibitors and peptide prodrugs (Kirk *et al.,* 1992; Zaks & Dodds, 1997; Glinskii and Guennadi, 1998; Jeric-Horvat, 2001; Perng *et al.,* 1998). Hence, the present work deals with the syntheses of the amino acyl esters of carbohydrates and that too, involving amino acids with alkyl side chain like L-alanine, L-valine, L-leucine and L-isoleucine.

Literature survey indicates that lipase catalysed synthesis of amino acyl esters of carbohydrates are very few (Park *et al.,* 1999; 1996; Boyer *et al*., 2001; Maruyama *et al.,* 2002; Vijayakumar *et al.,* 2004; Lohith and Divakar, 2004). The available literature deals with N-protected and carboxyl group activated amino acids and carbohydrates. The

reported protocols exhibit some severe limitations. Most of researchers have employed proteases for their work (Boyer *et al*., 2001; Park *et al*., 1999; 1996; Riva *et al.,* 1988). Reactions were carried out with health hazardous solvents like dimethyl sulphoxide, pyridine and dimethylformamide. Most of the reports are from experiments carried out in shake flask levels with large amount of enzyme being employed in such reactions (Riva *et al*., 1988). Lipases have been reported to give insignificant results in preparing amino acyl esters of carbohydrates (Park *et al*., 1996).

The experimental protocols developed in the present work described in this thesis have efficiently dealt with the above mentioned disadvantages. Commercially available lipases, which are also economically viable, were employed in the present work. Use of unprotected and unactivated amino acids and carbohydrates have been employed for the reactions, which effectively reduce the number of acylation and deacylation steps. Low boiling solvents were employed for the reactions such that solvents can be easily recovered. The work out procedures are easy and the product recovery involved very few unit operations. Smaller amounts of enzyme and larger amounts substrates were employed to obtain high yields than the reported. All the reactions were carried out in presence of solvent mixtures using an experimental set up, which maintained a very low water activity, very much essential for high enzyme activity and for carrying out reactions in a large-scale level as well.

Thus, the present work describes synthesis of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of aldohexoses (D-glucose, D-galactose and D-mannose), ketohexose (D-fructose), pentoses (D-arabinose and D-ribose), disaccharides (lactose, maltose and sucrose) and sugar alcohols (D-sorbitol and D-mannitol) using lipases from *Rhizomucor miehei, Candida rugosa* and porcine pancreas in organic solvents. The effects of various

parameters such as enzyme, substrate, buffer pH, concentrations, enzyme reusability, kinetics and stereo selectivity were investigated. The isolated product esters were also tested for probable pharmaceutical and biological activities as well. Thus the full potentialities of lipases are brought out in this study which describes the synthesis of several of the above mentioned amino acyl esters of carbohydrates for the first time.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Lipases

In the present work, three different lipases were employed - two from fungal sources, *Rhizomucor miehei* lipase (RML) and *Candida rugosa* lipase (CRL) and one from animal source, porcine pancreatic lipase (PPL).

Table 2.1. List of lipases and their sources

Lipase	Source	Employed for
Porcine pancreas lipase (Type II, Steapsin, crude preparation)	Sigma Chemical Co., St. Louis. MO, USA.	Preparation of L-alanyl and L-valyl esters of D-glucose
Rhizomucor miehei lipase (Lipozyme IM20 immobilized on weak anion exchange resin)	Novo-Nordisk, Bagsvaerd, Denmark and Boenhringer Mannheim, Germany.	Preparation of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of D-glucose, D-galactose, D-mannose, D-fructose, D-arabinose, D-ribose, lactose, maltose, sucrose, D-mannitol and D-sorbitol and esterification kinetics.
Candida rugosa lipase	Sigma Chemical. Co. MO, USA.	Preparation of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of D-glucose, D-mannose, D-galactose, D-arabinose, D-ribose, lactose, maltose, sucrose, D-mannitol and D-sorbitol and esterification kinetics.

2.1.2. L-Amino acids

L-Alanine, L-valine, L-leucine and L-isoleucine from Hi-Media Ind. Ltd. were employed as such.

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2.1.3. Carbohydrates

D-Glucose and sucrose from SD fine chemicals (Ind.) Ltd.; D-galactose and D-fructose from Hi-Media Ind. Ltd.; D-mannose, D-arabinose, D-ribose and D-mannitol from LOBA Chemie Pvt. Ltd. India; maltose from Sigma Chemical. Co. MO, USA; lactose from SISCO Research Laboratories Pvt. Ltd. India and D-sorbitol from Rolex Laboratory Reagent India Ltd. were procured and employed as such.

2.1.4. Solvents

Solvents such as chloroform, dichloromethane, *n*-hexane, *n*-heptane, pyridine, dimethylformamide, *n*-butanol, acetic acid, butyric acid, acetone and diethyl ether purchased from SD fine Chemicals (Ind.) Ltd. were employed after distilling once. Acetonitrile and methanol of HPLC grade were obtained from Qualigens fine chemicals (Ind) Ltd. and were used as such. Deuterium oxide and dimethyl sulfoxide-d_6 from Aldrich Chemicals limited were used for preparing samples for Nuclear Magnetic Resonance Spectroscopy.

2.1.5. Other chemicals

Table 2.2 List of chemicals and suppliers

Chemicals	Manufacturer
Sodium acetate (CH_3COONa), Sodium dihydrogen phosphate (Na_2HPO_4)	Ranbaxy Laboratories Ltd. India,
Di-sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), sodium hydroxide, potassium dihydrogen phosphate, acetic acid, hydrochloric acid, sulphuric acid, iodine (resublimed), molecular sieves 4Å, copper sulphate, silica gel and sodium potassium tartarate.	SD fine Chemicals (Ind.) Ltd.
Ninhydrin, sodium benzoate, sodium dodecyl sulfate, 1-naphthol, hippuric acid, gum acacia and triton x-100	LOBA Chemie Pvt. Ltd. India.

Hippuryl-L-histidyl-L-leucine tetrahydrate, bovine serum albumin, tributytrin, hemoglobin from bovine serum, Sephadex G-10 and Sephadex G-25	Sigma Chemical. Co. MO, USA
Bio Gel P-2	Bio-Rad Laboratories, USA.
Potassium bromide, Folin-Cicolteau reagent and HEPES buffer (N-[2-hydroxyethyl]-piperazine-N′-[2-Ethane sulphonic acid])	SISCO Research Laboratories Pvt. Ltd. India
Karl Fischer Reagent	Qualigens Fine Chemicals Ltd. India

2.2. Methods

2.2.1. Lipase activity

Both esterification as well as hydrolytic activities of the lipases were determined.

2.2.1.1. Hydrolytic activity

Hydrolytic activities of different lipases were determined by the tributyrin method (Vorderwulbecke *et al.,* 1993). Hydrolytic activity of lipase is defined as μmol of butyric acid released from tributyrin per min with one mg of immobilized lipase preparation. The specific activity is expressed as μmol of butyric acid released per min per mg of protein present in the lipase (Table 2.3).

A stock solution was prepared consisting of 10 ml of tributyrin, 90 ml of 0.01 M sodium phosphate buffer (pH 7.0), 0.2 g sodium benzoate, 0.5 g of gum acacia and 50 μl 10% SDS. It was emulsified by stirring and the pH was adjusted to 7.0 with concentrated NaOH. From this stock solution, 4 ml was pipetted out into stoppered conical flasks (S), containing 8 ml, 0.01 M sodium phosphate buffer (pH 7.0) to obtain a solution with a final concentration of 0.113 M tributyrin. Known quantities of lipases (5 –20 mg) were added to this solution and incubated at 37 ^{o}C in a Heto-Holten shaker water bath for

different intervals of time. After incubation, the reaction mixture in the flask was titrated with 0.04 N NaOH until a pH of 9.5 was reached. A blank (B) was also performed without adding the enzyme. The hydrolytic activity was determined by using the following equation.

$$\text{Hydrolytic activity} = \frac{(S\text{-}B) \times N}{1000 \times E \times T} \quad \text{µmol/(min.mg of lipase preparation or protein)}$$

(S-B) = difference in volume of NaOH in ml between sample (S) and blank (B),

N= normality of NaOH,

E = amount of lipase preparation or protein in mg and

T= incubation period in min.

2.2.1.2. Esterification activity

Esterification activities of four different lipases from porcine pancreas, *Rhizomucor miehei*, *Candida rugosa* and Chirozyme were determined by butyl butyrate method (Kiran *et al*., 2000). Esterification activity of lipase is defined as µmol of butyl butyrate formed from *n*-butanol and butyric acid per min with one mg of immobilized lipase preparation. The specific activity is expressed as µmol of butyric acid released per min per mg of protein present in the lipase (Table 2.3).

A stock solution containing 0.33 M *n*-butanol and 0.16 M butyric acid was prepared. In a 25 ml stoppered conical flak, 3 ml of the stock solution was dispersed in 5 ml of *n*-heptane. The reaction mixtures were incubated with known quantities of lipases for different intervals (30, 60, 90 and 120 min) in a Heto-Holten shaker water bath at 50 °C. After incubation, the contents of the flasks were titrated with standard 0.02 N NaOH. A blank (without the enzyme) was also performed. Table 2.3 shows the esterification

activities of four different lipases. The esterification activity was evaluated by using the following equation.

$$\text{Esterification activity} = \frac{(B-S) \times N}{1000 \times E \times T} \quad \mu\text{mol/(min.mg of lipase preparation or protein)}$$

(B-S) = difference in volume of NaOH in ml between blank (B) and sample (S),

N= normality of NaOH,

E= actual amount of the lipase preparation or protein in mg and

T= incubation period in min.

Table 2.3. Esterification and hydrolytic activities of different lipases [a].

Lipase	Esterification activity [b]		Hydrolytic activity [c]	
	Activity μmol/(min.mg of lipase preparation)	Specific activity μmol/(min.mg of enzyme protein)	Activity μmol/(min.mg of lipase preparation)	Specific activity μmol/(min.mg of enzyme protein)
Porcine pancreas	0.06	0.17	0.32	0.97
Rhizomucor miehei	0.46	7.27	0.10	1.59
Candida rugosa	0.03	7.64	-	-
Chirozyme	0.35	0.32	-	-
Porcine pancreas (partially purified)	0.02	0.2	0.02	2.45

[a] For each enzyme the activity results were obtained from an average of three individual experiments for different time intervals. Error in activity measurements were within ± 10 %.
[b] Butyl butyrate method, lipase employed : 5, 10 and 15 mg, Incubation temperature : 50 °C.
[c] Tributyrin method, lipase employed : 5, 10, 15 and 20 mg, Incubation temperature : 37 °C.

2.2.2. Protein estimation

Protein content of all the above mentioned five lipases were determined by using Lowry's method (Lowry *et al.,* 1951). In order to leach out the protein from the
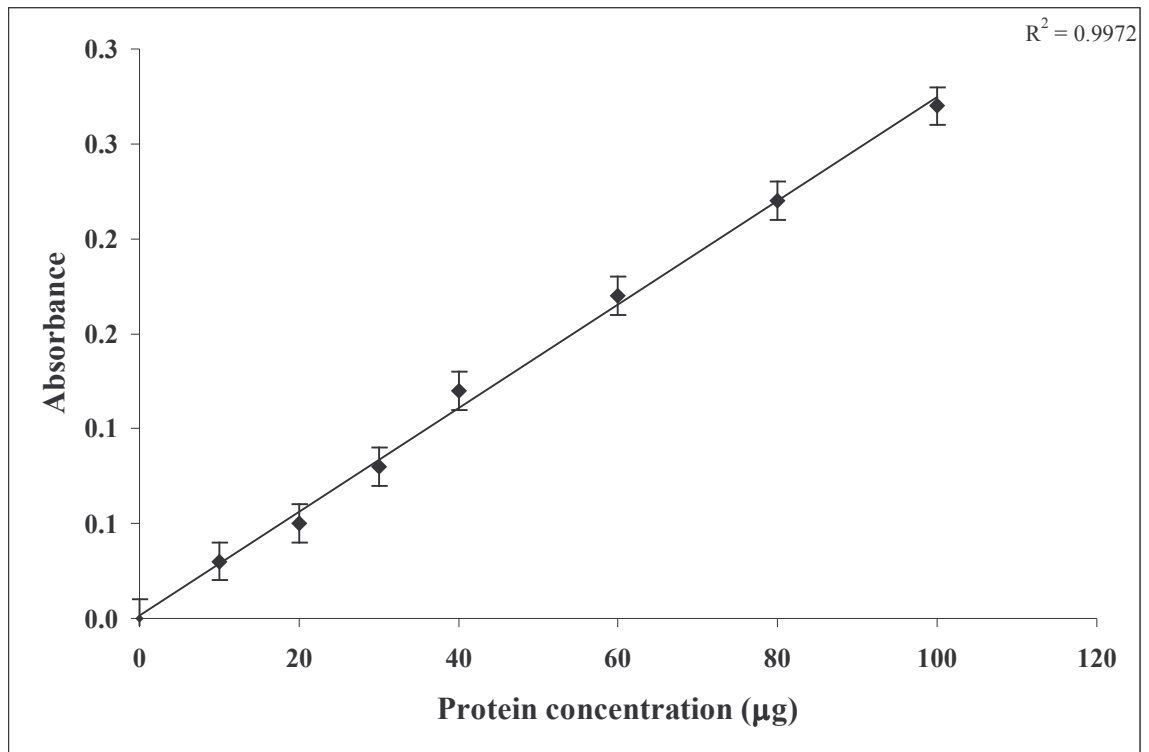
Fig. 2.1. Calibration curve for protein estimation by Lowry's method. A stock solution of 300 μg of 3 ml BSA solution was prepared. From the stock solution 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 ml solutions were pipetted out and the total volume was made upto 1 ml with distilled water after treatment with solutions as described in the text (Section 2.2.2) Absorbance was measured at 750 nm.

immobilized matrix or carrier, 20 mg lipase preparations were stirred in 50 ml of 0.5 M NaCl at 4 °C for 12 h and from this, known volumes of the samples were taken for protein estimation.

Solution **A** – 1 % of copper sulphate in water, solution **B** – 1 % of sodium potassium tartarate in water and solution **C** – 2 % of sodium carbonate solution in 0.1 N NaOH were prepared. Lowry's working solution **I** was prepared by mixing one part each of solution **A** and **B** and 98 parts of **C**. A 1:1 diluted solution of commercially available Folin-Cicolteau reagent with distilled water served as working solution **II**. To the protein sample in 1 ml water, 5 ml of Lowry's working solution **I** was added and incubated for 10 min at room temperature. A 0.5 ml of working solution **II** was then added followed by incubation at room temperature for 30 min and absorbance was measured at 750 nm using a Shimadzu UV – 1601 spectrophotometer. Calibration curve for protein concentrations was prepared by employing bovine serum albumin (BSA) in the concentration range 0 - 100 μg in 6.5 ml of the sample (Fig. 2.1). Using this calibration plot protein content of four different lipases were determined and values are shown in Table 2.4.

Table 2.4. Protein content of different lipase preparations [a]

Lipase	Protein content (%)
Porcine pancreas	32.8
Rhizomucor miehei	6.3
Candida rugosa	35.3
Chirazyme	3.2
Porcine pancreas (partially purified)	8.3

[a] Lowry's method, Lipase employed - 20 mg in 50 ml 0.5 M NaCl, Absorbance measured at 750 nm. Values are an average of three different concentrations of lipases. Errors in measurement will be within ± 5%.

2.2.3. Preparation of buffers

Decimolar concentrations of CH_3COONa buffer for pH 4.0 and 5.0, Na_2HPO_4 for pH 6.0 and 7.0 and $Na_2B_4O_7 \cdot 10H_2O$ for pH 8.0 buffers were prepared by dissolving appropriate amounts of respective salts in distilled water and the pH was adjusted with dilute HCl or NaOH using Controlled dynamics pH meter, model – APX175 E/C, India.

2.2.4. Esterification procedure

Preparation of L-amino acyl esters of carbohydrates was carried out by adopting a bench-scale level procedure (Divakar *et al.,* 1999). Esterification was carried out in presence of 0.018 – 0.225 g of lipases (expressed in terms of % w/w based on the carbohydrate employed). Unprotected L-amino acids (0.001 - 0.005 mol) and carbohydrates (0.001 – 0.005 mol) in 100 ml of organic media containing CH_2Cl_2: DMF (v/v 90:10) or hexane: $CHCl_3$:DMF (v/v/v 45:45:10) were taken in a flat bottomed two necked flask and refluxed for a period of 3 - 120 h. The enzymes were 'pH tuned' in some experiments by adding known volumes of 0.1 M buffer solutions of specified pH value to 100 ml (solvent) of the reaction mixture. The condensed vapours of solvent which formed an azeotrope with water during reflux was passed through a desiccant (sodium aluminosilicate molecular sieves of 4Å) before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction (Lohith and Divakar, 2005). This set up maintained a very low water activity of $a_w= 0.0054$ throughout the reaction period which was determined by Karl Fischer titration of the reaction mixture using Karl Fischer reagent by examining the aliquots of the reaction mixture for the water content during the course of the reaction. The reaction mixture after distilling off the solvent was then added to 20 - 30 ml of water, stirred and filtered to remove the lipase. The filtrate was evaporated on a water bath to get the unreacted carbohydrate,

unreacted L-amino acids and the product esters which were then analyzed by HPLC. The amino acids employed were L-alanine **1**, L-valine **2**, L-leucine **3** and L-isoleucine **4** and the carbohydrates employed were D-glucose **5**, D-mannose **6**, D-galactose **7**, D-fructose **8**, D-arabinose **9**, D- ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14** and D-sorbitol **15**.

2.2.5. High Performance Liquid Chromatography

The reaction mixture was monitored by employing a Shimadzu LC10AT high-performance liquid chromatography instrument (Kyoto, Japan) connected to a μ-Bondapack aminopropyl column (10 μm particle size, 3.9 x 300 mm length) with acetonitrile:water (v/v 80:20) as mobile phase at a flow rate of 1 ml/min and refractive index detector. Also, a LiChrosorb RP-18 column (5 μm particle size, 4.6 x 150 mm length) with acetonitrile:water (v/v 20:80) as a mobile phase at a flow rate of 1 ml/min and UV detector at 210 nm was employed. Since different equivalents of L-amino acids were employed, the conversion yields were determined based on the peak areas of L-amino acid and L–amino acid ester of the carbohydrate and expressed in mmol as well as in percentage values with respect to the L-amino acid concentration employed. The error in HPLC yields will be within ± 10 %.

2.2.6. Separation of L-amino acyl esters of carbohydrates

The esters formed were separated by size exclusion chromatography using Sephadex G-10/Sephadex G-25/Bio Gel P-2 in a column of size 100 cm x 1.0 cm. About 30 g of gel (bed volume of 70-80 ml) was packed and 150 mg of the reaction mixture was dissolved in distilled water and loaded onto these columns and eluted with water at a flow rate of 1 ml/h. Separation was observed by performing thin layer chromatography by spotting individual fractions on silica plate. Silica plates were prepared by dissolving

8 g of silica gel (mesh 60-120) in 20 ml of water which were spreaded uniformly over 20 x 20 cm glass plate. After air and oven drying, chromatography was performed using *n*-butanol: acetic acid: water (v/v/v 70:20:10) as mobile phase. The spots were identified by spraying ninhydrin (for NH_2 group detection) and 1-naphthol (for reducing sugar detection) and kept in hot air oven at 100 ^{o}C for 20 minutes to obtain colored spots. Ninhydrin reagent was prepared by dissolving 400 mg of ninhydrin in absolute alcohol. Sugar spray was prepared by dissolving 1.46 g of 1-napthol in 41 ml of ethanol and then to that was added 3.4 ml of water and 5.6 ml of concentrated sulfuric acid. The fraction containing product esters were evaporated on a water bath and subjected to spectral characterization.

2.2.7. UV-Visible spectroscopy

A Shimadzu UV–1601 spectrophotometer (Kyoto, Japan) was used for UV characterization of the isolated esters. Samples were prepared in water at 0.1 - 2.0 mM range.

2.2.8. Infra Red spectroscopy

A Nicolet 5700 FTIR instrument (Madison, USA) was used for recording IR spectra for the isolated esters. A 2.0 to 3.0 mg of ester sample was prepared as KBr pellet and the IR spectrum was recorded.

2.2.9. Nuclear Magnetic Resonance Spectroscopy
2.2.9.1. ^{1}H NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 500.13 MHz was used to record ^{1}H NMR spectra in DMSO-d_6 with 40 mg of the sample dissolved in 0.5 ml of solvent. About 50-200 scans were accumulated with a recycle period of 2-3 seconds to obtain good spectra. The spectra were recorded at 35 ^{o}C with TMS as internal standard for measuring the chemical shift values to within ± 0.01 ppm.

A region from 0 –10 ppm was scanned for all the samples.

2.2.9.2. ^{13}C NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 125 MHz was used to record ^{13}C NMR. Samples were dissolved in 0.5 ml of DMSO-d_6 and recorded at 35 °C. A region from 0-200 ppm was scanned and about 500-6000 scans were accumulated for each spectrum. TMS was taken as the internal standard.

2.2.9.3. Two-dimensional HSQCT

Two dimensional Heteronuclear Multiple Quantum Coherence Transfer spectra (2-D HMQCT) and Heteronuclear Single Quantum Coherence Transfer spectra (2-D HSQCT) were recorded at 500 MHz on a Brüker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C). Proton and carbon 90° pulse widths were 12.25 and 10.5 μs respectively. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard. About 40 mg of the sample dissolved in DMSO-d_6 was used for recording the spectra in magnitude mode with sinusoidal shaped z-gradients of strength 25.7, 15.42 and 20.56 G/cm with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps with a computer memory size of 4K. The spectra were processed using unshifted and π/4 shifted sine bell window function in F_1 and F_2 dimensions respectively.

2.2.10. Mass spectrometry

Mass spectra of the isolated esters were recorded on a Q-TOF Waters Ultima instrument (No.Q-Tof GAA 082, Waters corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source. Version 4.0 data acquisition software was used. The spectra were recorded in positive ion mode using spray voltage at 3.5 kV and a source temperature of 80 °C. Mass spectra were recorded under electron impact

ionization at 70 eV electron energy. Samples were prepared in the concentration range of 0.5 - 1.0 mg/ml in distilled water and injected by flow injection analysis at a flow rate of 10 μl/min. The recorded mass of the sample was in the range of 100-1000.

2.2.11. Polarimetry

Optical rotations of the isolated esters were measured at 20 °C using Perkin-Elmer 243 polarimeter (Überlingen, Germany). A 0.2 – 1.5 % solutions of the ester in distilled water was employed for the measurements. Optical rotations were determined from

$$[\alpha]_D^{20\,^{\circ}C} = \frac{[\alpha]_{obs} \times 100}{C \times l}$$

$[\alpha]_D$ = specific rotation in degrees in sodium lamp at 590 nm

$[\alpha]_{obs}$ = observed rotation,

C= concentration of the esters in g/100 ml and

l = path length in dm.

2.2.12. Determination of Critical micellar concentration (CMC)

Critical micellar concentration for L-alanyl-β-D-glucose was determined by using Coomassie Brilliant Blue G 250 reagent (Rosenthal and Koussale, 1983) by dissolving 100 mg of the dye in 50 ml of 95 % ethanol. To this solution, 100 ml of 85 % (w/w) phosphoric acid was added and the resulting solution was diluted to a final volume of 1L using distilled water. Thus, final concentrations were 0.01 % (w/v) Coomassie Brilliant Blue G 250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid.

Aliquots of sample concentrations in the range of 0 - 15 mM were prepared and made up to 1 ml by adding Coomassie brilliant blue G 250 reagent. The reaction mixture was shaken well and the absorption was measured at 470 nm. A plot of concentrations of

the sample versus absorption was constructed, from which CMC was determined as the concentration of the ester corresponding to a change in the slope of the absorption versus concentration plot.

2.2.13. Water activity

Water activity of the reaction media was measured using Mettler Toledo DL-50 auto titrator (Vogel, 1961; Grünke, 2003). Known amount of the solvents were titrated against the Karl-Fischer reagent. Karl-Fischer reagent contains mixture of pyridine, iodine and sulfur dioxide dissolved in methanol. The main reaction can be described in two steps. In the first step, formation of an intermediate 'pyridine sulfurtrioxide' takes place and this is later converted into 'pyridine-N-sulfuric acid' by the action of pyridine, sulfur dioxide and methanol. In the second step, pyridine-N-sulfuric acid is oxidized by iodine in presence of water.

The Karl-Fisher titrator contains a polarizing double platinum electrode and the voltage between the electrodes is measured against added titrant (Karl-Fischer reagent). As long as traces of water is present in the titration cell, all iodine molecules are immediately reduced to iodide by the KF reagent. The electrical resistance of the solution stays at a high level. After titrating all the water molecules, a small excess of iodine leads to a strong decrease in the resistance which is considered as the end point. The stoichiometry in the reaction is 1:1 $I_2:H_2O$ where one molecule of iodine is equivalent to one molecule of water. A 5.0 g of Karl-Fischer reagent can react with 1.0 g of water. Amount of water was determined by the amount of Karl-Fischer reagent consumed by the solvent. Table 2.5 shows the water activities of some organic solvents.

Table 2.5. Water activities for different organic solvents by Karl-Fisher method

Solvent [a]	Water activity (a_w)
Acetone	0.008
Benzene	0.142
Chloroform	0.028
Dichloromethane	0.054
Heptane	≈ 0
Hexane	0.183
Methyl isobutyl ketone	0.043

a – volume of solvent taken – 40 ml, Errors in measurement were within ± 10%.

2.2.14. Preparation of N-Acetyl alanine

A 5.0 g (56 mmol) of L-alanine was dissolved in 15 ml (186 mmol) of pyridine in 100 ml round bottomed flask. The reaction mixture was allowed to stir for 10 min. To this stirring solution 26.5 ml (280 mmol) of acetic anhydride was added drop wise. The reaction mixture was allowed to react for 24 h at room temperature and then the whole reaction mixture was poured into ice. Unreacted pyridine and acetic anhydride were removed by evaporation using a flash evaporator. N-Acetyl alanine was then extracted by using 4-methyl penta-2-one repeatedly for three times and then recrystallized using dry acetone. 4.0 g of N-acetyl alanine was obtained, yield (54 %); Mpt: 127 °C; specific rotation: –4.44 °.

2.2.15. Extraction of Angiotensin Converting Enzyme (ACE) from porcine lung

ACE was partially purified from porcine lung by following the method of Andujar-Sanchez *et al.,* (2003). A 100 g of porcine lung was minced and homogenized using a blender with 10 mM HEPES buffer (pH 7.0) containing 0.4 M NaCl at a volume ratio of 5:1 (v/w of pig lung) at 4 °C. The homogenate was centrifuged at 9000 g for 60 min. The supernatant was discarded and the precipitate was washed twice with 200 ml of

10 mM HEPES buffer (pH 7.0) containing 0.4 M NaCl. The final precipitate was resuspended in 200 ml of 10 mM HEPES buffer (pH 7.0) containing 0.4 M NaCl, 10 μM ZnCl$_2$ and 0.5 % (w/v) triton-X-100 and stirred over night. The solution was centrifuged to remove the pellets. The supernatant was dialyzed against water using a dialysis bag of molecular weight cut off of 10 kD and later lyophilized. A yield of 2.3 g crude ACE was obtained. The protein content determined by Lowry method (Section 2.2.2) was found to be 8.3 %. The specific activity of the enzyme was found to be 0.243 μmol/min/mg of enzyme protein.

2.2.16. Angiotensin Converting Enzyme (ACE) inhibition assay

ACE inhibition assay for the esters prepared were performed by following Cushman and Cheung (1971) method. Aliquots of ester solutions in the concentration range 0.2 to 2.5 mM (0.1 ml to 1.0 ml of 5.0 mM stock solution) were taken and to this 0.1 ml of ACE solution (0.1% in 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl) was added. To this solution, 0.1 ml of 5.0 mM hippuryl-L-histidyl-L-leucine (HHL) was also added and the total volume was made upto 1.25 ml by adding phosphate buffer (1.05 ml to 0.15 ml of 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl). The solution was incubated on a Heto-Holten shaking water bath for 30 min at 37 $^{\circ}$C. Blanks were performed without the enzyme by taking only the ester solution (0.1 to 1.0 ml) along with 0.1 ml of 5.0 mM HHL. The total volume was made upto to 1.25 ml by adding the same buffer (1.15 ml to 0.25 ml). The reaction was terminated by adding 0.25 ml of 1 M HCl. Hippuric acid formed in the reaction was extracted with 1.5 ml of ethyl acetate. One ml of ethyl acetate layer was evaporated to dryness and treated with equal amount of distilled water and the absorbance was measured at 228 nm for hippuric acid. The hippuric acid formed in 1.5 ml of ethyl acetate was determined from a calibration
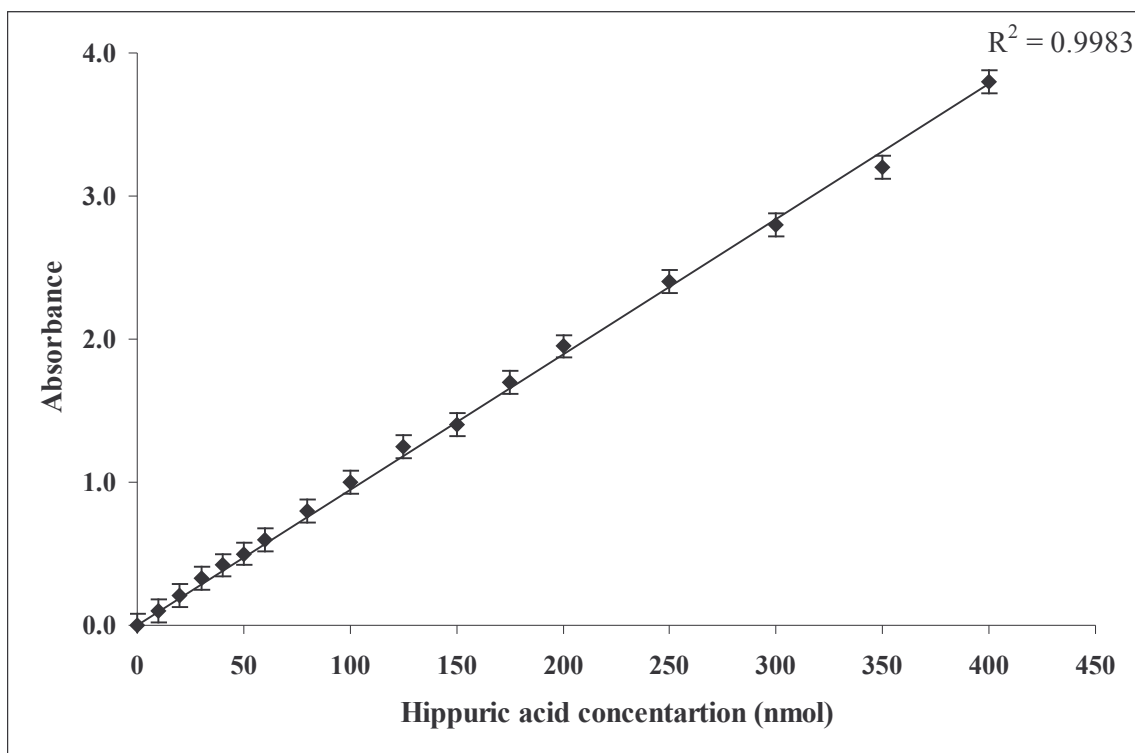
Fig. 2.2. Calibration curve for hippuric acid estimation by spectrophotometric method. A stock solution of 44.8 mg hippuric acid in 25 ml water was prepared, from which different aliquots of concentrations 0 to 350 nmol were pipetted out and made up to 1 ml. Absorbance was measured at 228 nm after following the procedure described in the text (Section 2.2.16).

curve prepared using a standard hippuric acid solutions of 0 – 400 nmol in 1 ml of distilled water (Fig. 2.2). Specific activity was expressed as μmol of hippuric acid formed per min per mg of enzyme protein.

$$\text{Specific activity} = \frac{A_{ts} - A_{blank}}{T \times S \times E}$$

A_{ts} = absorbance of test solution,

A_{blank} = absorbance of blank solution,

T = incubation period in min,

S = slope value of the calibration plot (1.006×10^{-2} Abs units/nmol of hippuric acid),

E = amount of enzyme in mg of protein.

2.2.17. Protease activity

The hydrolyzing activity of the protease (in ACE) was determined using bovine hemoglobin as substrate (Dubey and Jagannadham, 2003). To 0.5 ml of ACE enzyme solution, 0.5 ml of 0.6 % (w/v) bovine hemoglobin solution was added and the reaction was allowed to proceed for 30 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of 10 % trichloro acetic acid and allowed to stand for 10 min. The resulted precipitate was removed by centrifugation at 20000 g for 15 min. A 0.5 ml of supernatant was taken and mixed with equal volume of 0.5 M NaOH and the color developed was measured by absorbance at 440 nm. A control assay without the enzyme was carried out and used as reference (blank). Protease inhibition activity to compare with ACE inhibition activity was tested by adding 0.5 ml of the amino acyl esters of carbohydrates solution to 0.5 ml of ACE enzyme solution. To this was added, 0.5 ml of hemoglobin solution and incubated at 37 °C for 30 min. The specific activity of protease in crude ACE preparation was found to be 0.0436 unit/min/mg of enzyme protein.

One unit of enzyme activity was expressed as the amount of enzyme under given assay conditions that gives rise to an increase in one unit of absorbance at 440 nm per min of digestion. Number of units of activity per mg of protein was taken as the specific activity of the enzyme.

$$\text{Specific activity} = \frac{A_{sample} - A_{blank}}{T \times E}$$

A_{sample} = Sample absorbance at 440 nm

A_{blank} = Blank absorbance at 440 nm

T = Time in min

E = amount of enzyme in mg of protein.

2.2.18. Extraction of porcine pancreas lipase

The crude PPL was extracted by adopting the procedure of Verger *et al.* (1969). Porcine pancreas bought from local slaughter house was minced into small pieces. About 100 g pancreas was homogenized with 300 ml of chloroform:butanol mixture in the ratio of 9:1 (v/v) at 25 °C. The solvent was decanted and washed with 200 ml of a fresh solvent mixture of 4:1 (v/v) chloroform:butanol mixture and drained completely. The lipid free pellets were then washed with 200 ml of cold acetone and finally with 150 ml diethyl ether to get a fine powder which was then lyophilized. A yield of 11 g crude PPL was obtained. The protein content determined by Lowry method (Section 2.2.2) was found to be 8.3 %. The specific hydrolytic activity and esterification activity of the PPL were found to be 2.45 and 0.2 μmol/min/mg of enzyme protein respectively.

2.2.19. Immobilization of porcine pancreas lipases

Immobilization of the isolated porcine pancreas lipase was carried out according

to the method described by Won *et al.,* (2005) by entrapping lipase on calcium-alginate beads. A 200 ml of 1 g PPL solution was mixed with 800 ml of 1 % sodium alginate. The mixture was stirred thoroughly to ensure complete mixing. The mixed solution was drawn with a syringe into 1 L of 50 mM $CaCl_2$ solution through a needle of 0.5 mm diameter. The beads formed were allowed to harden for 30 minutes. The $CaCl_2$ solution was separated from beads by filtration. They were then washed twice with 50 mM Tris-HCl buffer (pH 7.2). The calcium alginate beads obtained were then lyophilized. A yield of 3.54 g immobilized PPL was obtained. The protein content determined by Lowry method (Section 2.2.2) was found to be 0.64 %. The hydrolytic activity and specific hydrolytic activity of the immobilized PPL were found to be 6.67 nmol/min/mg of enzyme protein and 1.04 μmol/min/mg of enzyme protein respectively. The esterification activity and specific esterification activity of the immobilized PPL were found to be 0.74 nmol/min/mg of enzyme protein and 0.115 μmol/min/mg of enzyme protein respectively.

2.2.20. Identification of lipases and ACE by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check the purity of lipases according to the method described by Laemmli (1970) in a discontinuous buffer system.

The following reagents were prepared.

A. Acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved in 100 ml water (30 % solution) filtered and stored in a dark brown bottle at 4 ^{o}C.

B. Separating gel buffer (18.1 g) was dissolved in water and the pH of the solution adjusted to 8.8 with HCl. Then the solution was made upto 100 ml and stored at 4 ^{o}C.

C. Stocking gel buffer – Tris-HCl (3.0 g) was dissolved in water, pH of the solution adjusted to 6.8 with HCl (6.0 N) and made upto 100 ml in water.

D. Sodium dodecyl sulphate (SDS), 10 g was dissolved in 100 ml water

E. Ammonium persulphate was freshly prepared by dissolving 50 mg in 0.5 ml of distilled water.

F. Tank buffer – Tris-HCl (0.3 g), glycine (1.44 g) and SDS (0.15 g) were dissolved in 150 ml of water.

G. Staining solution – A 0.2 g of Coomassie brilliant blue R 250 was dissolved in a mixture of methanol:acetic acid:water (v/v/v 25:15:60). The reagent was filtered and stored in room temperature.

H. Destaining solution – Methanol:acetic acid:water (v/v/v 25:15:60).

I. Sample buffer was prepared in solution C diluted to 1:4 containing SDS (w/v 4 %), β-mercaptoethanol (v/v 10 %), glycerol (v/v 20 %) and bromophenol blue (w/v 0.1 %).

Preparation of separating gel – A 3.2 ml of A, 0.92 ml of B, 2.71 ml of distilled water, 0.05 ml of D and 0.03 ml of solution E were mixed and then degassed which was then poured between the assembled glass plates sealed with agar (w/v 2 %). The gels were layered with 0.5 ml of distilled water and allowed to polymerize at room temperature for 30 min.

A stock solution was prepared by mixing the solutions of 0.66 ml of A, 1.0 ml of C, 2.25 ml of distilled water, 0.05 ml of D, 0.01 ml of TEMED and 0.03 ml of E and poured above the polymerized gel. The gel thus prepared was of the size 10.5 x 9.0 cm and thickness 0.8 mm.

Lipase and ACE samples were prepared by dissolving 25 µg of protein in solution 'I' (50 µL). The samples were heated in a boiling water bath for 10 min, then samples were loaded into the wells immersed in solution F (tank buffer) and were run at
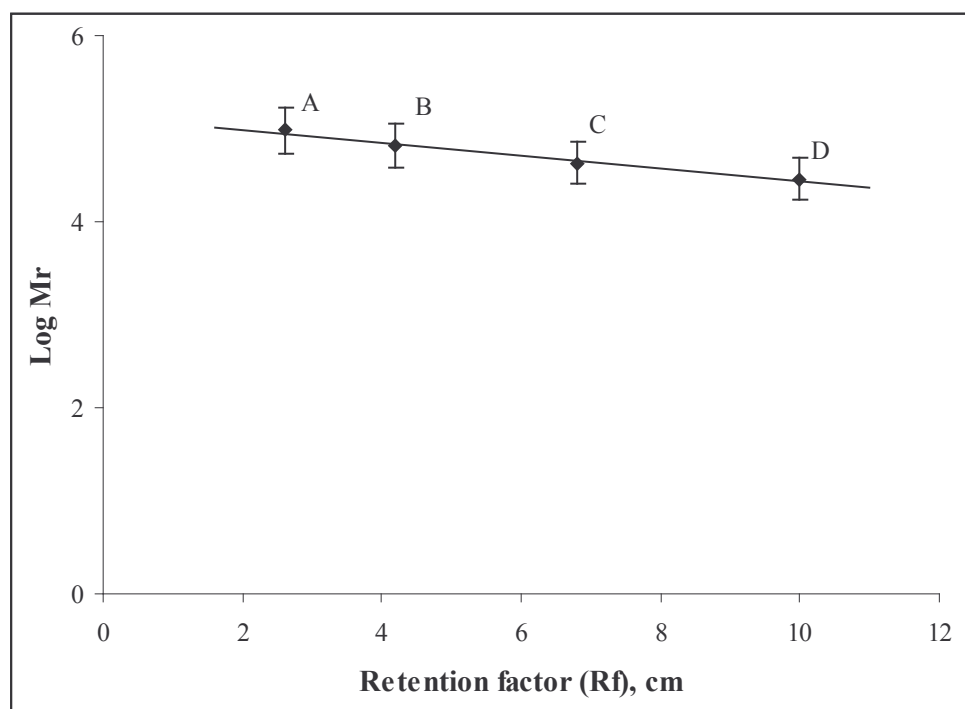
Fig. 2.3 Log M_r versus R_f plot. (A) Phosphorylase (97.4 kDa), (B) BSA (66.3 kDa), (C) Ovalbumin (43.0 kDa), (D) Carbonic anhydrase (29.0 kDa).

a constant voltage of 40 Volts until the tracking dye, bromophenol blue was just (0.5 cm) above the lower end of the gel. Medium range protein markers phosphorylase (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and soyabean trypsin inhibitor (20.0 kDa) were used as a solution having each protein at a concentration of 0.5 to 0.8 mg/ml. The markers were 1:1 diluted with solution I and boiled prior to use. Later the gel was stained for protein with reagent 'G' for 6 h at room temperature followed by destaining in reagent H.

A plot was constructed by taking R_f values of the molecular marker on X-axis and log M_r values of each molecular marker on Y-axis (Fig 2.3). From this plot molecular weight of the unknown protein was determined. Lipases from *Rhizomucor miehei*, porcine pancreas and *Candida rugosa* and molecular weight markers were subjected to SDS-PAGE and stained with Coomassie brilliant blue R 250 (Fig. 2.4A). Lane 1 is the crude porcine pancreas extracted from pig lung, showing large number of bands and one band corresponding to a molecular mass of 54.9 kDa along with other protein contaminants of different molecular masses. Lane 2 is a commercial PPL showing a major band of molecular mass 54.9 kDa, also containing some protease of molecular masses less than 50 kDa. Lane 3 representing *Candida rugosa* lipase, showed a band corresponding to a molecular mass of 57.5 kDa. The band with a molecular mass of 30.2 kDa was obtained for *Rhizomucor miehei* lipase in lane 4. Molecular masses of the all the three lipases showed good correspondence to literature reports (Pernas *et al.,* 2002; Brady *et al.,* 1990; Winkler and Gubernator, 1994).

Angiotensin Converting Enzyme (ACE) showed a molecular mass of 152 kDa (along with the other protein contaminations, Fig. 2.4B, Lane 1) and this band showed a good correspondence to the reported molecular mass of 147 kDa by Hopper *et al.,* (1987).
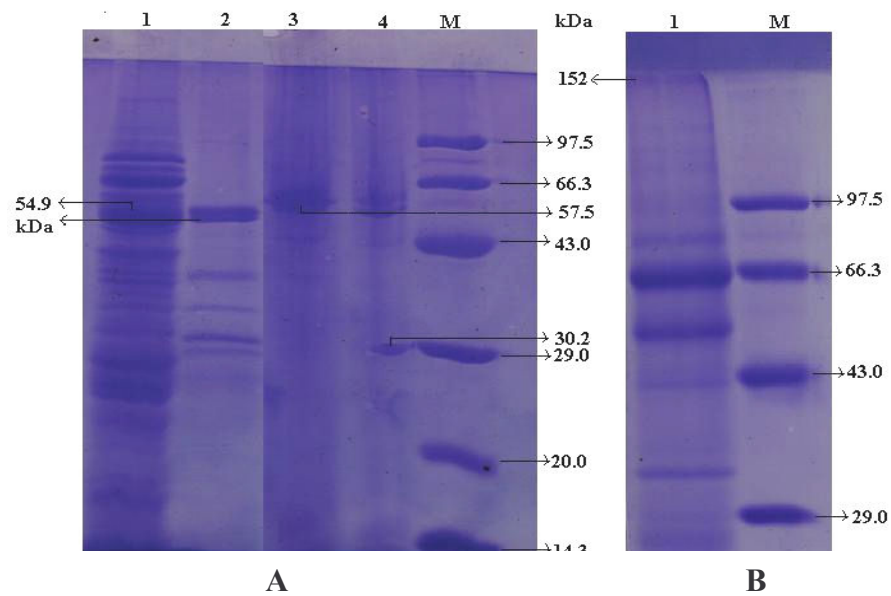
Fig. 2.4. SDS-PAGE for (A) lipases; Lane 1 –crude PPL isolated from porcine pancreas; Lane 2 – PPL from Sigma; Lane 3- CRL from Sigma; Lane 4 –RML from Novo-Nordisk; Lane M for M_r standard proteins: Phosphorylase (97.4 kDa), BSA (66.3 kDa), Ovalbumin (43.0 kDa), Carbonic anhydrase (29.0 kDa) and soyabean trypsin inhibitor (20.0 kDa). (B) Lane 1 for ACE isolated from pig lung.

Chapter 3
Optimization of reaction parameters for the synthesis of L-alanyl, L-valyl and L-leucyl esters of D-glucose

3.1 Introduction

Amino acyl esters of carbohydrates are used as sweetening agents, surfactants, microcapsules in pharmaceutical preparations, active nucleoside amino acid esters, antibiotics and in the delivery of biological active agents (Dordick, 1989; Tamura *et al*., 1985; Kirk *et al.,* 1992; Zaks and Dodds, 1997; Vulfson *et al.,* 1993). Chemical acylation of carbohydrates regio-selectively is complex due to the presence of multiple hydroxyl groups, which require protection and deprotection (Tamura *et al*., 1985; Haines, 1981). When enzymes are used in organic media, they exhibit specificity (Wescott and Klibanov, 1994), thermostability (Ayala *et al.,*1986; Wheeler and Croteau, 1986), molecular memory (Stahl *et al.,* 1991; Dabulis and Klibanov, 1993) and capacity to catalyze reverse reactions (Kuhl *et al.,* 1990; West *et al.,*1990).

Hitherto, very few references are available on the lipase catalyzed esterification of amino acyl esters of sugars. Most of the earlier workers used proteases and N-protected and carboxyl group activated amino acids for synthesizing aminoacyl esters of carbohydrates (Riva *et al.,* 1988; Park *et al.,* 1996, 1999; Jeon *et al.,* 2001). Therisod and Klibanov (1986) used subtilisin to acylate carbohydrates with activated carboxylic acids in anhydrous organic solvents. Riva *et al.,* (1998) carried out subtilisin catalyzed synthesis of N-acetyl-L-alanyl-methyl-β-D-galactopyranoside in anhydrous DMF, with an yield of 70% (4-*O*- 16 % and 6-*O*- 84 *%)* and N-acetyl-D-alanyl-methyl-β-D-galactopyranoside with an yield of 35 % (2-*O*- 10 %, 3-*O*- 10 %, 4-*O*- 12 % and 6-*O*- 68 %). Suzuki *et al.,* (1998) synthesized L-alanyl-D-glucose by using D-glucose, methyl-L-alaninate hydrochloride and intact cells of *Rhodotorula lactosa.* There are no reports on the synthesis of L-valyl-D-glucose esters enzymatically. There are some reports on the chemical synthesis of methyl 2-*O*-[N-*t*-boc]-L-valyl-D-glucose, methyl-2,3-di-*O*-L-leucyl-α-D-glucose and methyl-3-*O*-[N-*t*-boc]-L-valyl-glucose and diesters such as ethyl-

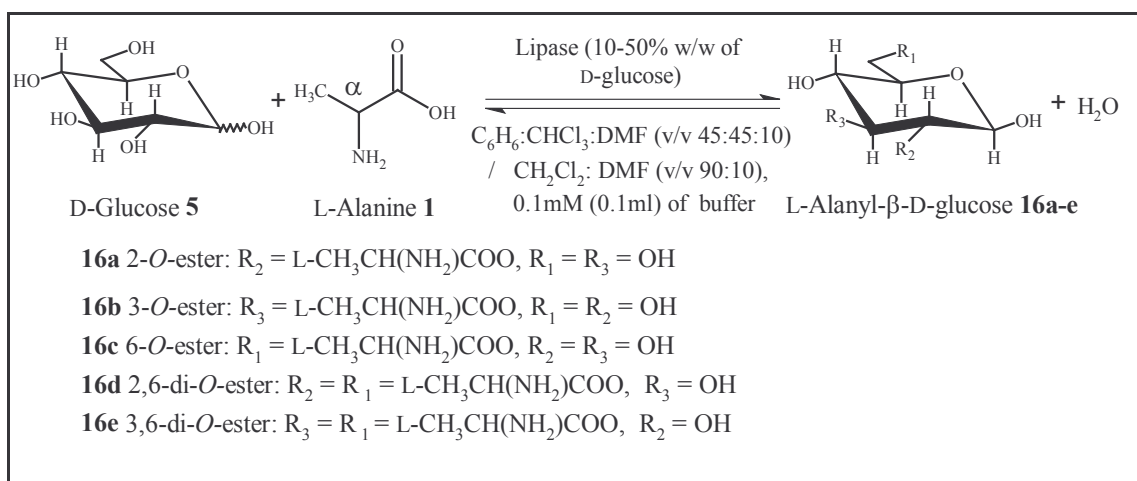2,3-di-*O*-[N-*t*-boc]-L-valyl-D-glucose, methyl-2,3-di-*O*-L-valyl-D-glucose, methyl-4,6-di-*O*-L-valyl-D-glucose and methyl-2,3-di-*O*-L-isoleucyl-α-D-glucose (Tamura *et al*., 1985) whose synthesis involved protection and deprotection. Park *et al.,* (1999) has carried out transesterification with Optimase M 440 to synthesize *t*-boc-leucyl-sucrose by using tert-butoxy-carbonyl-L-leucyl-cyanomethyl   ester   /   tert-butoxy-carbonyl-L-leucyl-trifluro ethyl ester and sucrose. Maruyama *et al.,* (2002) have investigated the synthesis of N-acetyl-L-leucyl-D-glucose in *t*-butanol containing 10 % dimethyl sulfoxide by transesterification between N-acetyl-L-leucyl-cyanomethyl ester and D-glucose. Park *et al.,* (1999) reported that lipase from porcine pancreas and Lipozyme IM20 gave very low yields (< 2 %), compared to proteases which gave conversion yields ranging from 15 – 98 % when N-protected and carboxyl group activated amino acid was used for the acylation of D-glucose in pyridine. All these reactions were conducted in shake flasks using lesser quantity of substrates and larger quantity of enzymes.

In the present work, optimization of reaction parameters for the lipase catalyzed synthesis of L-alanyl, L-valyl and L-leucyl esters of D-glucose is described. Attempts to synthesize the same using N-acetyl-L-alanine resulted in very little conversion with the above mentioned enzymes. Hence, unprotected and unactivated L-amino acids and carbohydrates were employed to synthesize L-alanyl, L-valyl and L-leucyl acyl esters of carbohydrates using lipases from *Rhizomucor miehei* (RML), *Candida rugosa* (CRL) and porcine pancreas (PPL) as biocatalysts.

3.2. Present work

3.2.1. Synthesis of L-alanyl-D-glucose

Esterification was carried out in presence of lipase by incubating D-glucose and L-alanine in an organic media (**Scheme 3.1**). Lipases form *Rhizomucor miehei* (RML) and porcine pancreas (PPL) were employed for the reaction. The extent of esterification was monitored by HPLC (Fig. 3.1).

16a 2-*O*-ester: R$_2$ = L-CH$_3$CH(NH$_2$)COO, R$_1$ = R$_3$ = OH

16b 3-*O*-ester: R$_3$ = L-CH$_3$CH(NH$_2$)COO, R$_1$ = R$_2$ = OH

16c 6-*O*-ester: R$_1$ = L-CH$_3$CH(NH$_2$)COO, R$_2$ = R$_3$ = OH

16d 2,6-di-*O*-ester: R$_2$ = R$_1$ = L-CH$_3$CH(NH$_2$)COO, R$_3$ = OH

16e 3,6-di-*O*-ester: R$_3$ = R$_1$ = L-CH$_3$CH(NH$_2$)COO, R$_2$ = OH

Scheme 3.1 Lipases catalyzed regioselective synthesis of L-alanyl-D-glucose esters in anhydrous organic media

Since different equivalents of L-alanine were employed, the conversion yields were determined based on the peak areas of L-alanine and L-alanyl esters of carbohydrates and were expressed relative to the L-alanine concentration employed (Fig. 3.1). The error in HPLC yields were within 10 %. The esters formed were separated by size exclusion chromatography using Sephadex G-10/Bio Gel P-2 as column materials and eluted with water and subjected to spectral characterization by UV, IR, optical activity, MS and NMR (shown in detail in Section 3.3.1.1). The esterification reaction between unprotected and unactivated L-alanine and D-glucose was studied in detail using RML and PPL in terms of incubation period, lipase concentrations, substrate concentrations, buffer (pH and concentration) and enzyme reusability. The esterification reactions described in present work did not occur without the use of enzymes.
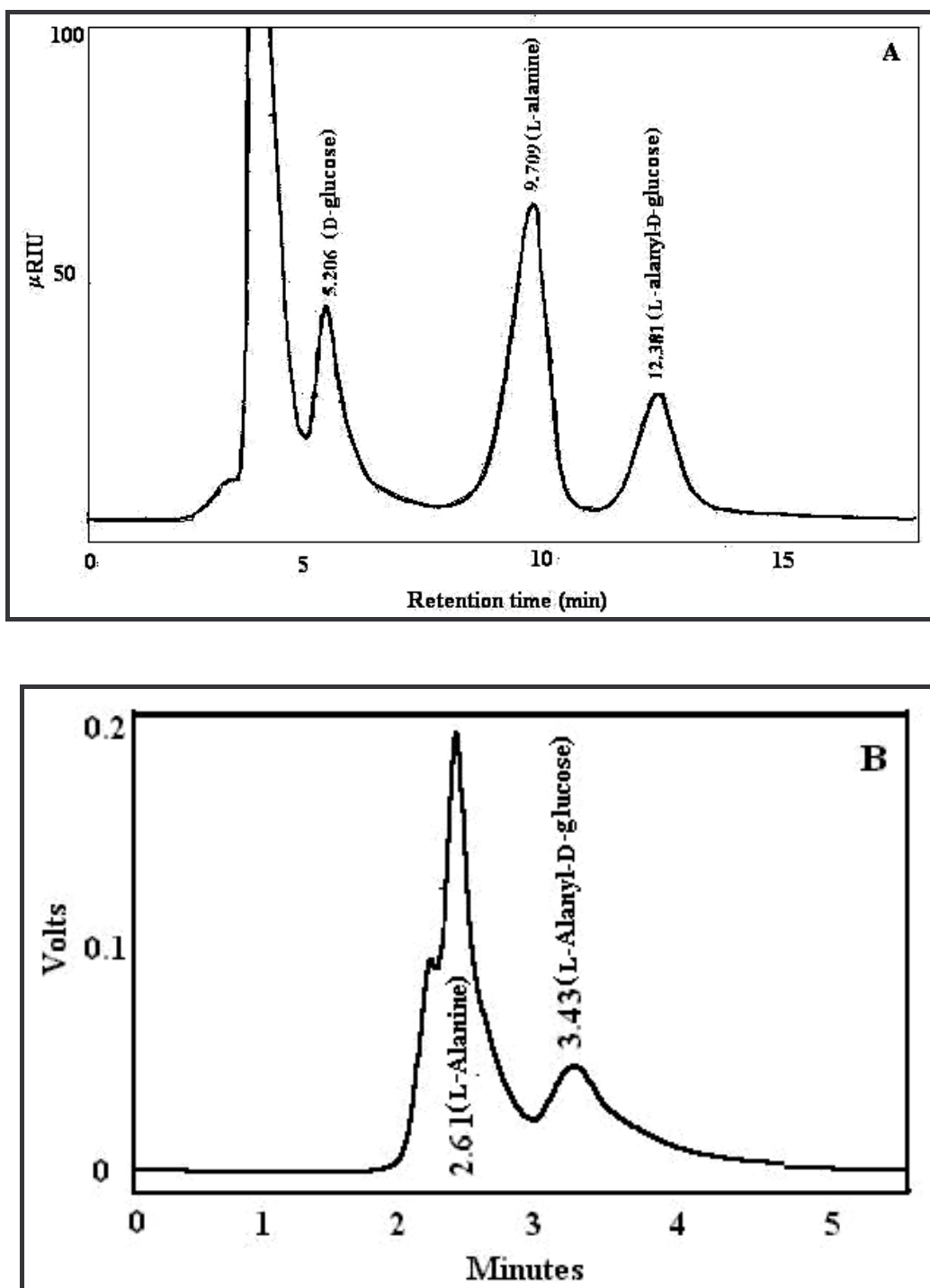
Fig. 3.1. HPLC chromatograph for the reaction mixture of L-alanine and D-glucose. (A) Column – aminopropyl, mobile phase – acetonitrile: water (80:20 v/v), flow rate - 1 ml/min, detector – Refractive Index. (B) Column – C-18, mobile phase – acetonitrile: water (v/v 20:80), flow rate- 1 ml/min, detector – UV at 210 nm. Errors in yields are within ± 10 %.

### 3.2.1.1. Esterification profile

In presence of RML (30 % w/w D-glucose), the conversion yields showed an increase in esterification from 5 % at 3 h to 26 % at 72h and thereafter remained constant up to 120 h at 26 % (Fig. 3.2). From the initial slope value of the plot, the rate of esterification was found to be 0.004 mmol h^{-1}.

3.2.1.2. Effect of lipase concentration

In case of RML (Table 3.1), maximum esterification (18 %) was achieved at 50 % w/w D-glucose (1 mmol D-glucose and 2 mmol L-alanine for incubation up to 72h). In case of PPL (Table 3.1), maximum yield of 18 % was achieved at 40 % w/w D-glucose (1 mmol D-glucose and 2 mmol L-alanine for an incubation period of 72 h). In presence of 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 4.0), RML at 40 % w/w D-glucose showed (Table 3.1) a maximum esterification of 30 % (for 1 mmol D-glucose and 2 mmol L-alanine for incubation up to 72 h). While lesser amounts of enzymes ($\leq$ 20 % RML and PPL employed) could be inhibited by L-alanine (2 mmol) and D-glucose (1 mmol), >30 % RML and PPL could favour better conversion as the enzyme/substrate ratio is enhanced.

Lipase from porcine pancreas purified partially (Section 2.2.18) was also employed in the range of 100 mg to 500 mg (Table 3.2) in presence of 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 5.0). Esterification increased from 100 mg (41 %) to 200 mg (78 %) and thereafter decreased upto 500 mg (39 %).
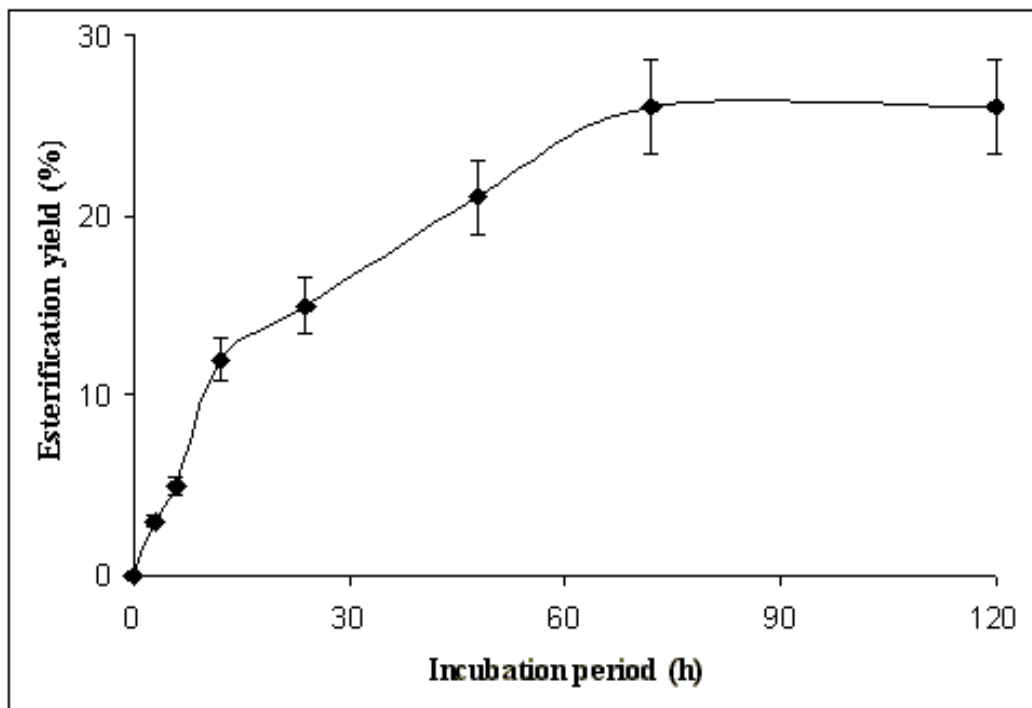
Fig. 3.2. Reaction profile for L-alanyl-D-glucose synthesis. Reaction conditions: CH_2Cl_2:DMF (v/v 90:10), RML –54 mg (30 % w/w D-glucose), L-alanine - 2 mmol, D-glucose - 1.0 mmol, Buffer - 0.1 mM (0.1 ml of 0.1M) acetate buffer (pH 4.0).

Table 3.1 Effect of lipase concentration on the synthesis of L-alanyl-D-glucose [a]

Lipase concentration (% w/w D-glucose)	*Rhizomucor miehei* lipase (RML) [b] Yield % (mmol)	Porcine pancreas lipase (PPL) [c] Yield % (mmol)	*Rhizomucor miehei* lipase (RML) [d] Yield % (mmol)
10	2 (0.04)	14 (0.27)	7 (0.14)
20	1 (0.02)	15 (0.29)	10 (0.21)
30	3 (0.05)	17 (0.35)	26 (0.53)
40	9 (0.17)	18 (0.36)	30 (0.60)
50	18 (0.37)	15 (0.29)	22 (0.44)

[a] D–glucose-1 mmol and L–alanine - 2 mmol. Conversion yields from HPLC determined with respect to L-alanine. Error in yield measurements will be ± 10 – 15 %. This applies to all the yields given in the subsequent tables also.
[b] Solvent - $CHCl_3$:hexane:DMF - (v/v/v 45:45:10) at 60 ^{o}C.
[c] Solvent - CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C.
[d] Carried out in presence of buffer with 100 ml of solvent system b containing 0.1 mM (0.1 ml of 0.1M) acetate buffer (pH 4.0) .

Table 3.2 Effect of partially purified PPL concentration on the synthesis of L-alanyl-D-glucose [a]

Partially purified PPL concentration (% w/w of D-glucose)	Yield % (mmol)
55.6	41 (0.41)
111.1	78 (0.78)
166.7	64 (0.68)
222.2	50 (0.50)
277.8	39 (0.39)

[a] D–glucose - 1 mmol and L–alanine - 1 mmol; Solvent CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C. Carried out in presence of buffer with 100 ml of solvent system b containing 0.2 mM (0.2 ml of 0.1 M) acetate buffer (pH 5.0).

3.2.1.3. Effect of buffer salts

While earlier workers have not studied the effect of buffer salts on this esterification reaction, the present study investigated the same, in connection with both the stabilization of the enzyme in non-polar solvents and also the use of zwitter ionic amino acid in the reaction. Carrying out this esterification reaction in presence of buffers

of certain pH not only imparted 'pH memory' or 'pH tuning' to the enzyme, but also provided the optimum water activity necessary for better performance of the enzyme. Besides, addition of buffer salts of certain concentration also affected the ionic activities of especially the micro-aqueous layer around the enzyme, where the buffer salts are concentrated during the course of the reaction. All these have been found to be operative in these esterification reactions.

In presence of buffer salts, conversion yield increased in case of RML. In presence of 0.1 mM (0.1 ml of 0.1 M) pH 4.0 buffer, RML showed maximum esterification of 26 % (at 1 mmol D-glucose and 2 mmol L-alanine for a period of 72 h, Table 3.3). However, PPL, in presence of 0.1 mM (0.1 ml of 0.1 M) pH 5.0 buffer showed maximum esterification of 17 % only (Table 3.3), similar to the conversion yield obtained in the absence of buffer salts.

The effect of buffer salt concentration was studied by using different concentrations (0.05 mM to 0.5 mM) of pH 4.0 buffer in case of RML and pH 5.0 buffer in case of PPL. In case of RML, the maximum conversion yield of 26 % (at 1 mmol D-glucose and 2 mmol L-alanine for 72 h) was obtained when 0.1 mM (0.1 ml) pH 4.0 buffer was employed (Table 3.3). In case of PPL, 0.5 mM (0.5 ml) pH 5.0 buffer showed the maximum conversion yield (Table 3.3) of 17 % (at 1 mmol D-glucose and 2 mmol L-alanine for 72 h incubation).

Table 3.3 Effect of buffer salts (pH and buffer concentration) on the synthesis of L-alanyl-D-glucose [a]

	RML				PPL		
pH [b]	Yield % (mmol)	pH 4.0 [c] concn mM	Yield % (mmol)	pH [d]	Yield % (mmol)	pH 5.0 [e] concn mM	Yield % (mmol)
4.0	26 (0.53)	0.05	25 (0.49)	4.0	9 (0.18)	0.05	13 (0.25)
5.0	7 (0.13)	0.1	26 (0.53)	5.0	17 (0.33)	0.1	17 (0.33)
6.0	20 (0.39)	0.2	8 (0.16)	6.0	15 (0.31)	0.2	11 (0.23)
7.0	8 (0.16)	0.3	7 (0.15)	7.0	11 (0.23)	0.3	10 (0.21)
8.0	10 (0.19)	0.4	17 (0.34)	8.0	No yield	0.4	12 (0.24)
-	-	0.5	18 (0.35)	-	-	0.5	17 (0.34)

[a] D-glucose - 1mmol and L-alanine - 2 mmol; Incubation period - 72 h; RML – 30 % (w/w D-glucose). 100 ml of the solvent containing specified volumes, concentration and pH of the buffer
[b] Solvent – 100 ml CHCl$_3$:hexane:DMF (v/v/v 45:45:10) at 60 ^{o}C; Buffer - 0.1 mM (0.1 ml of 0.1 M) appropriate pH buffer
[c] Solvent – 100 ml CH$_2$Cl$_2$:DMF   (v/v 90:10) at 40 ^{o}C; Buffer – 0.05 ml to 0.5 ml of 0.1 M acetate (pH 4.0).
[d] Same solvent system as c. Buffer - 0.1 mM (0.1 ml of 0.1 M) appropriate pH.
[e] Solvent – Same solvent system as c. 0.05 ml to 0.5 ml of 0.1 M acetate (pH 5.0).

3.2.1.4. Effect of substrate concentration

When L-alanine was varied from 1 mmol to 5 mmol at a constant 1 mmol D-glucose, there was in general, an increase in esterification (Fig. 3.3) from 0.68 mmol (68 % with respect to 1 mmol L-alanine - 0.54 mmol monoesters and 0.14 mmol diesters) to 1.12 mmol (28 % with respect to 4 mmol L-alanine – 0.88 mmol monoesters and 0.24 mmol diesters) in presence of 30 % (w/w D-glucose) RML, 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 4.0) for 72 h incubation. The yields were determined with respect to L-alanine concentrations which were usually greater than that of D-glucose (1 mmol). In terms of D-glucose, one D-glucose molecule forms mono as well as diesters with L-alanine. Hence, in presence of higher equivalents of L-alanine, yields >1 mmol (D-glucose concentration) are reported.
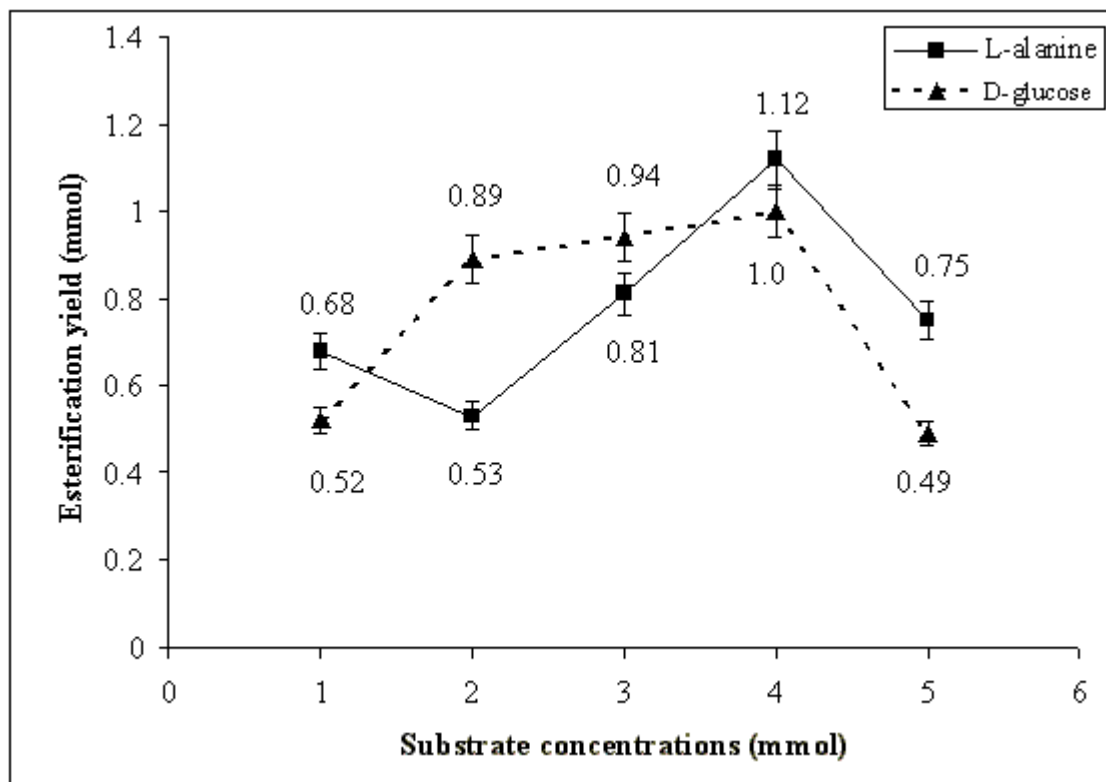
Fig 3.3. Effect of substrate concentration on synthesis of L-alanyl-D-glucose. RML – 30 % (w/w D-glucose); Solvent - CH_2Cl_2:DMF - (v/v 90:10) at 40 $^{\circ}$C; Buffer – 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 4.0). L-alanine (■) – 1 – 5 mmol at 1 mmol D-glucose; D-glucose (▲) – 1 – 5 mmol at 1 mmol L-alanine and a constant enzyme concentration of 54 mg.

Similarly, when D-glucose was varied from 1 mmol to 5 mmol, at a constant 1 mmol L-alanine, there was a steep increase in esterification from 52 % (0.52 mmol - 0.41 mmol monoesters and 0.11 mmol diesters) at 1mmol D-glucose to > 99 % (> 0.99 mmol - 0.78 mmol monoesters and 0.21 mmol diesters) at 4 mmol D-glucose. In both the cases, esterification decreased after 4 equivalents which could be due to inhibition at higher concentrations of L-alanine and D-glucose.

3.2.1.5. Reusability of lipases

Reusability of both RML and PPL employed were studied at optimized conditions. After completion of each reaction (cycle, 72 h), the enzyme was separated from the reaction mixture by filtration, air dried and reused in the next reaction. After each cycle, total esterification activity (μmol/min) of the enzyme was determined. In case of RML, there was a steady loss of 15 % to 22 % of enzyme concentration after each cycle (Table 3.4) both in presence as well as absence of 0.2 mM (0.2 ml of 0.1 M) acetate buffer (pH 4.0). In the absence of buffer salts, the esterification activity decreased from 24 % (1st cycle: total enzyme activity - 99.4 μmol/min) to 8 % (4th cycle- total enzyme activity - 23.5 μmol/min). The yields in 2nd and 3rd cycles were 17 % (total enzyme activity - 66.4 μmol/min) and 10 % (total enzyme activity - 43.1 μmol/min) respectively. In presence of buffer salts (pH 4.0), the esterification activity decreased from 17 % (1st cycle: total enzyme activity – 99.4 μmol/min) to 5 % (4th cycle - enzyme activity of 23 μmol/min). The yields in 2nd and 3rd cycles were 11 % (total enzyme activity - 66.9 μmol/min) and 8 % (total enzyme activity - 42.2 μmol/min) respectively.

However, in case of PPL, there was a drastic loss of enzyme concentration from 20 % to 60 % after each cycle, as PPL was partially soluble in water and the reaction was stopped after the 2nd cycle due to reduction in enzyme (Table 3.4). In the absence of

buffer salts, esterification activity of the PPL decreased from 19 % (1st cycle- total enzyme activity - 15 μmol/min) to 3 % (4th cycle-total enzyme activity - 0.7 μmol/min). The 2nd and 3rd cycle yields were 12 % (total enzyme activity - 5.6 μmol/min) and 10 % (total enzyme activity - 3.3 μmol/min) respectively. However, in presence of buffer salts (pH 5.0), the esterification activity decreased slightly from 11 % (1st cycle- total enzyme activity - 15 μmol/min) to 6 % (2nd cycle-total enzyme activity - 4.1 μmol/min).**Table 3.4   Reusability of lipase in presence and absence of buffer salts [a]**

	RML		PPL	
No. of reactions (cycles)	**% Yield (Total enzyme activity, μmol/min)**	**% Yield [b] (Total enzyme activity, μmol/min)**	**% Yield (Total enzyme activity, μmol/min)**	**% Yield [c] (Total enzyme activity, μmol/min)**
1	24 (99.4)	17 (99.4)	19 (15)	11 (15)
2	17 (66.4)	11 (66.9)	12 (5.6)	6 (4.1)
3	10 (43.1)	8 (42.2)	10 (3.3)	-
4	8 (23.5)	5 (24.6)	3 (0.7)	-

[a] D-glucose - 4 mmol and L-alanine - 8 mmol; Incubation period –72 h; Solvent – 100 ml CH$_2$Cl$_2$:DMF - (v/v 90:10) at 40 °C. Total enzyme activity of the recovered enzyme employed for the reaction. Percentage yields are an average from two independent experiments.
[b] in presence of 0.2 mM (0.2 ml of 0.1M) acetate buffer (pH 4.0) in each cycle.
[c] in presence of 0.2 mM (0.2 ml of 0.1 M) acetate buffer (pH 5.0) in each cycle.

3.2.1.6. Synthesis of L-alanyl-D-glucose at gram scale using crude PPL and immobilized PPL

Synthesis of L-alanyl-D-glucose was carried out at large scale by employing 1 g L-alanine, 2 g D-glucose in 200 ml of CH$_2$Cl$_2$:DMF (v/v 90:10) in presence of 1 g of crude PPL (50 % w/w D-glucose, described in Section 2.2.18) / 1.5 g of immobilized PPL (described in Section 2.2.19) and 1.15 mM (2.3 ml of 0.1 M) acetate buffer (pH 5.0) for a period of 72 h. Conversion yield of 47 % with crude PPL and 58 % with immobilized PPL were obtained.

3.2.2. Synthesis of L-valyl-D-glucose

Esterification was carried out in presence of lipase by incubating D-glucose and L-valine in an organic media (**Scheme 3.2**). Lipases form *Rhizomucor miehei* (RML) and porcine pancreas (PPL) were employed for the reaction. The extent of esterification was monitored by HPLC (Fig. 3.4). The esters formed were separated by size exclusion chromatography using Sephadex G-10/Bio Gel P-2 as column materials and eluted with water and subjected to spectral characterization by UV, IR, optical activity, MS and NMR (shown in detail in Section 3.3.1.2).

The esterification reaction between unprotected and unactivated L-valine and D-glucose was studied in detail using RML and PPL in terms of incubation period, lipase concentrations, buffer (pH and concentration) and substrate concentrations.

Lipase (10–50% w/w of D-glucose)

CH_2Cl_2: DMF (v/v 90:10), 0.1mM (0.1ml) of buffer

D-Glucose **5** + L-Valine **2** → L-Valyl-D-glucose **25a-e** + H_2O

25a 2-*O*-ester: R_2 = L-$(CH_3)_2CHCH(NH_2)COO$, $R_1 = R_3 = OH$
25b 3-*O*-ester: R_3 = L-$(CH_3)_2CHCH(NH_2)COO$, $R_1 = R_2 = OH$
25c 6-*O*-ester: R_1 = L-$(CH_3)_2CHCH(NH_2)COO$, $R_2 = R_3 = OH$
25d 2,6-di-*O*-ester: $R_2 = R_1$ = L-$(CH_3)_2CHCH(NH_2)COO$, $R_3 = OH$
26e 3,6-di-*O*-ester: $R_3 = R_1$ = L-$(CH_3)_2CHCH(NH_2)COO$, $R_2 = OH$
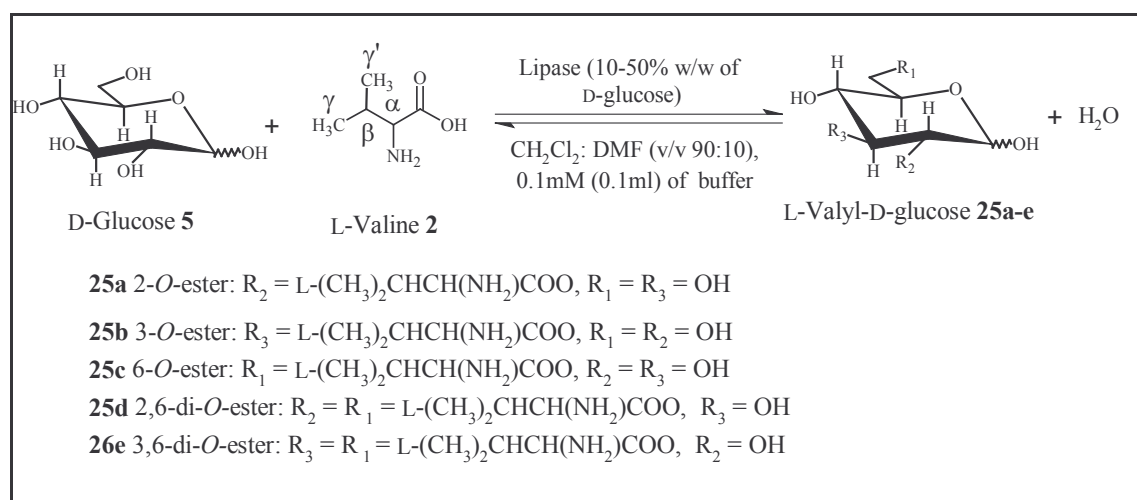
Scheme 3.2 Lipases catalysed synthesis of L-valyl-D-glucose esters in anhydrous organic media

3.2.2.1. Esterification profile

For CRL (40 % w/w D-glucose) catalyzed esterification of L-valine with D-glucose (1 mmol each) in presence of 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0), it was found that there was a linear increase in esterification upto 72 h and thereafter decreased at 120h (Fig. 3.5). Esterification yield obtained after each incubation period was: 52 % (0.52 mmol) at 6 h, 56 % (0.56 mmol) at 12 h, 57 % (0.57 mmol) at 24 h, 61 % (0.61
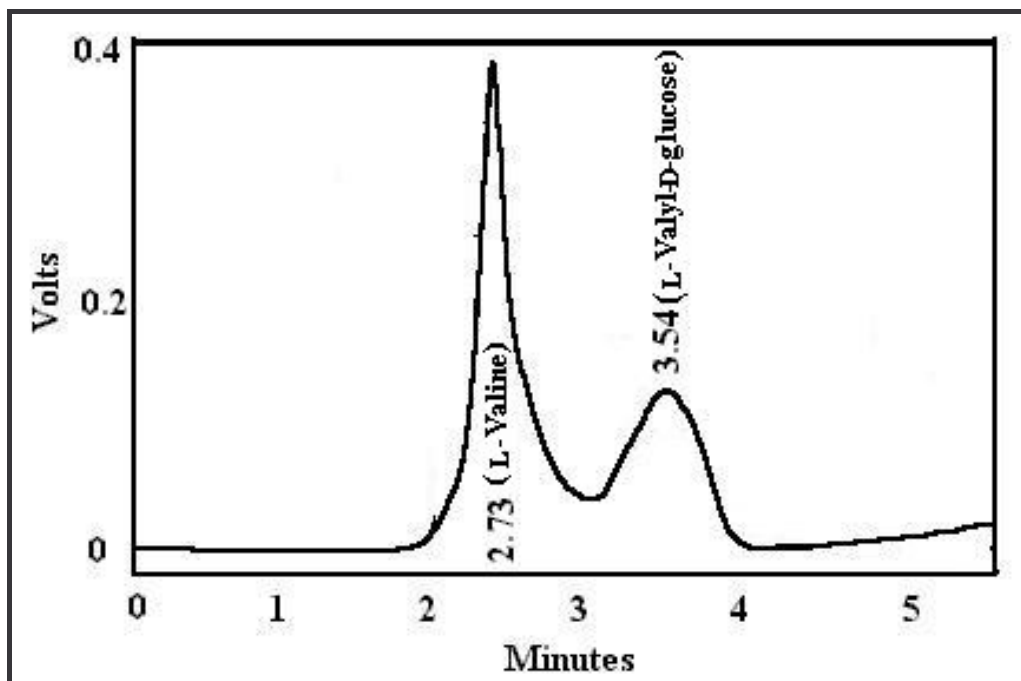
Fig. 3.4. HPLC chromatograph for the reaction mixture of L-valine and L-valyl-D-glucose. Column – C-18, mobile phase – acetonitrile: water (v/v 20:80), flow rate- 1 ml/min, detector – UV at 210nm. Errors in yields are within ± 10 %.

mmol) at 48 h, 68 % (0.68 mmol) at 72 h, 52 % (0.52 mmol) at 96 h and 47 % (0.47 mmol) at 120 h. From the initial slope value, the initial rate of esterification was found to be 0.0425 mmol h^{-1}

3.2.2.2. Effect of buffer salts

Treatment of buffer salts stabilizes three-dimensional structure of enzymes against denaturation in organic solvents by dissolving in the micro aqueous layer thereby imparting effective 'pH memory' or 'pH tuning' to the enzyme. A buffer of known pH and concentration was treated to the reaction mixture to study the effect of imparting 'pH tuning' to the enzyme on esterification.

Effect of buffer salts at a fixed CRL concentration of 40 % (w/w of D-glucose) and 1:1 millimolar equivalent of D-glucose and L-valine on the synthesis of L-valyl-D-glucose showed that CRL in presence of 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0) showed the highest yield of 68 % (0.68 mmol) which was 48 % higher than in its absence (Table 3.5). With increase in buffer concentration above 0.1 mM (0.1 ml) there was a steady decrease in esterification. It was found that 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0) showed the highest yield of 68 % (0.68 mmol).

Table 3.5   Effect of buffer salts (pH and buffer concentration) on the synthesis of L-valyl-D-glucose [a]

pH [b]	Yield % (mmol)	pH 7.0 [c] concn mM	Yield % (mmol)
4.0	47 (0.47)	0.05	58 (0.58)
5.0	43 (0.43)	0.1	68 (0.68)
6.0	59 (0.59)	0.2	48 (0.48)
7.0	64 (0.64)	0.3	49 (0.49)
8.0	54 (0.54)	0.4	44 (0.44)
-	-	0.5	43 (0.43)

[a] D-glucose - 1mmol and L-valine - 1 mmol; Incubation period - 72h;   CRL – 40 % (w/w D-glucose); 100 ml of the solvent containing specified volumes, concentration and pH of the buffer. [b] Solvent - 100 ml CH$_2$Cl$_2$:DMF   (v/v 90:10) at 40 °C . Buffer - 0.1 mM (0.1 ml of 0.1 M) appropriate pH buffer.
[c] Solvent – 100 ml. Buffer - 0.05 ml to 0.5 ml of 0.1 M phosphate (pH 7.0).
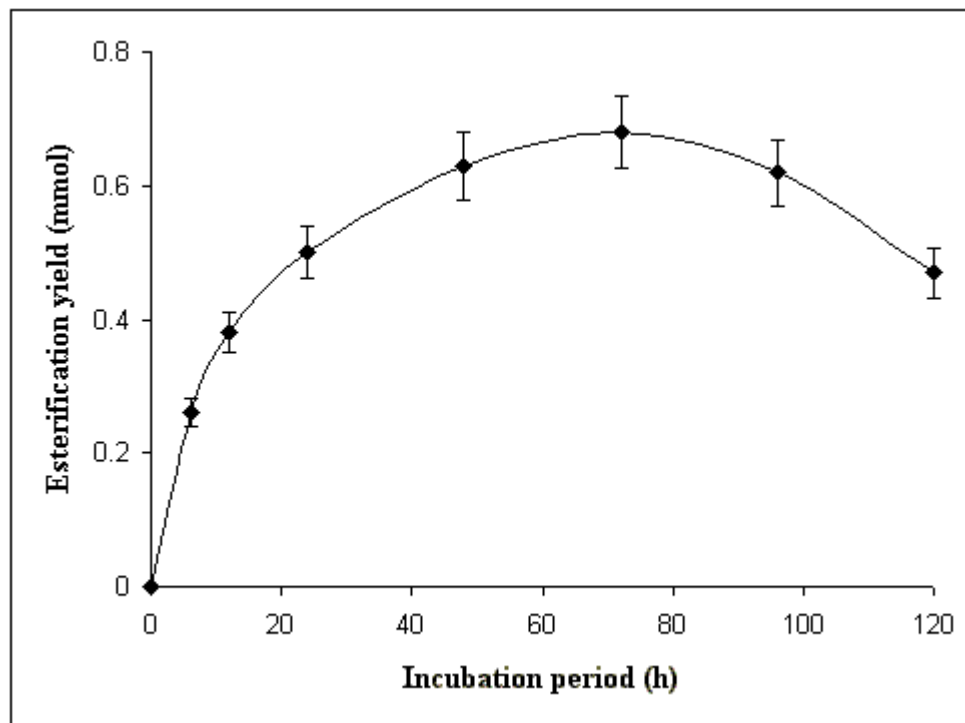
Fig. 3.5. Effect of incubation period on esterification of L-valyl-D-glucose. CRL – 40 % (w/w D-glucose); D-glucose - 1 mmol and L-valine - 1 mmol; Solvent - CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C. Buffer - 100 ml of solvent containing 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0).

3.2.2.3. Effect of lipase concentration

With increase in CRL concentration, in the lipase range 10 % to 50 % (w/w D-glucose) at 1 mmol of both D-glucose and L-valine, there was a steady increase in esterification upto 40 % enzyme concentration in the absence of the buffer, with a maximum yield of 0.19 mmol (Table 3.6). However, in presence of 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0), with the increase in CRL concentration, there was a steady increase in esterification upto 30 % enzyme concentration (84 %, 0.84 mmol) which decreased thereafter upto 50 % of lipase concentration (Table 3.6). Both 10 % (w/w D-glucose) RML and PPL in presence of 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 5.0) showed a maximum yield of 59 % (0.59 mmol) and 62 % (0.62 mmol) respectively which thereafter decreased upto 50 % of lipase concentration (Table 3.6).

Table 3.6 Effect of lipase concentration on the synthesis of L-valyl-D-glucose [a]

Lipase concentration (% w/w D-glucose)	*Candida rugosa* lipase (CRL) Yield % (mmol)	*Candida rugosa* lipase (CRL) [b] Yield % (mmol)	*Rhizomucor miehei* lipase (RML) [c] Yield % (mmol)	Porcine pancreas lipase (PPL) [c] Yield % (mmol)
10	2 (0.02)	73 (0.73)	59 (0.59)	62 (0.62)
20	6 (0.06)	77 (0.77)	41 (0.41)	45 (0.45)
30	14 (0.14)	84 (0.84)	31(0.31)	33 (0.33)
40	25 (0.25)	68 (0.68)	24 (0.24)	27 (0.27)
50	10 (0.10)	65 (0.65)	25 (0.25)	36 (0.36)

[a] D-glucose - 1 mmol and L–valine - 1 mmol; Solvent - CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C.
[b] Carried out in presence of buffer with 100 ml of solvent containing 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0).
[c] Carried out in presence of buffer with 100 ml of solvent containing 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 5.0).

3.2.2.4. Effect of L-valine and D-glucose concentration

Effect of L-valine and D-glucose concentrations at a fixed CRL concentration of 40 % (w/w D-glucose) on the synthesis of L-valyl-D-glucose was investigated (Fig. 3.6). When L-valine concentrations were varied in the range 1 to 5 mmol at a fixed 1 mmol of
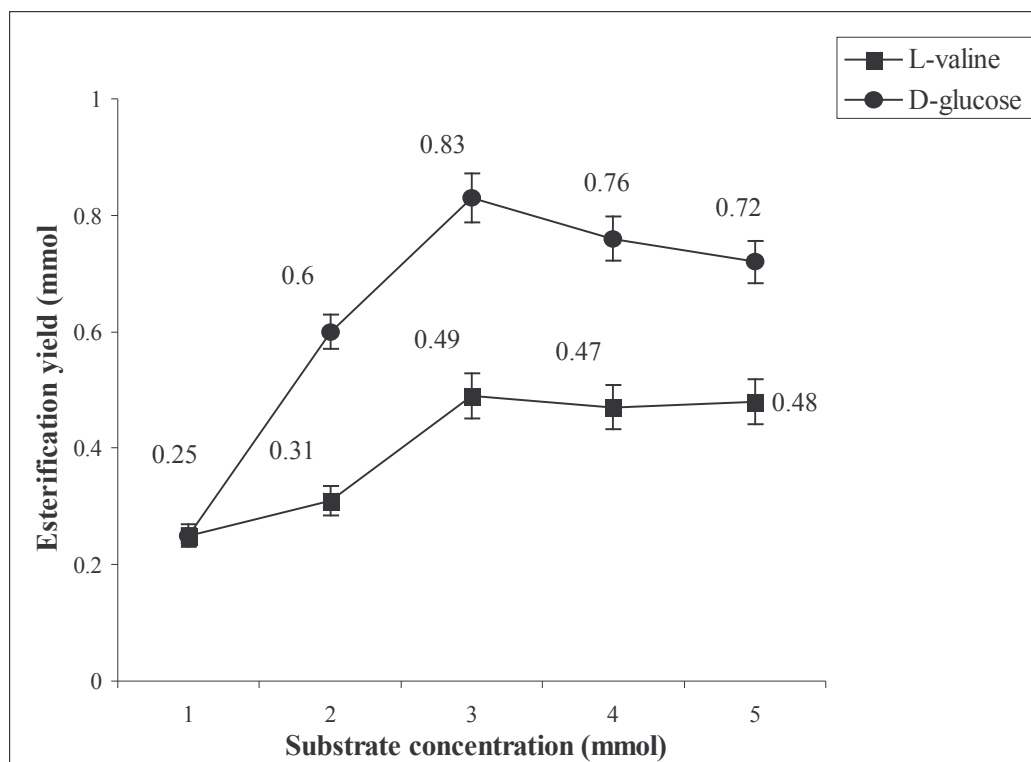
Fig. 3.6. Effect of substrate concentration on synthesis of L-valyl-D-glucose. CRL – 40 % (w/w D-glucose); Solvent - CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C. L-Valine (■) – 1 – 5 mmol at 1 mmol D-glucose; D-glucose (●) – 1 – 5 mmol at 1 mmol L-valine and a constant enzyme concentration of 72 mg.

D-glucose, there was an increase in esterification from 1 mmol L-valine (yield - 0.25 mmol) to 3 mmol L-valine (yield - 0.49 mmol) and thereafter remained constant upto 5 mmol L-valine. Similarly, when D-glucose was varied from 1 mmol to 5 mmol at a fixed 1 mmol of L-valine, there was a steep increase in esterification from 1 mmol D-glucose (yield - 0.25 mmol) to 3 mmol D-glucose (yield - 0.83 mmol) and thereafter decreased upto 5 mmol D-glucose (yield - 0.72 mmol). In both the cases, esterification increased upto 3 equivalents, indicating that L-valine and D-glucose are not inhibitors to CRL.

3.2.3. Synthesis of L-leucyl-D-glucose

Esterification was carried out in presence of lipase from *Rhizomucor miehei* (RML) and porcine pancreas (PPL) by incubating D-glucose and L-alanine in an organic media (**Scheme 3.3**). The extent of esterification was monitored by HPLC. The esters formed were separated by size exclusion chromatography using Sephadex G-10 / Bio Gel P-2 as column materials and eluted with water and subjected to spectral characterization by UV, IR, optical activity, MS and NMR (shown in detail in Section 3.3.1.3). The esterification reaction between unprotected and unactivated L-leucine and D-glucose was studied in detail using RML and PPL in terms of lipase concentrations, buffer (pH and concentration) and substrate concentrations.

34a 2-*O*-ester: R$_2$ = L-(CH$_3$)$_2$CHCH$_2$CH(NH$_2$)COO, R$_1$ = R$_3$ = OH
34b 3-*O*-ester: R$_3$ = L-(CH$_3$)$_2$CHCH$_2$CH(NH$_2$)COO, R$_1$ = R$_2$ = OH
34c 6-*O*-ester: R$_1$ = L-(CH$_3$)$_2$CHCH$_2$CH(NH$_2$)COO, R$_2$ = R$_3$ = OH
34d 2,6-di-*O*-ester: R$_2$ = R$_1$ = L-(CH$_3$)$_2$CHCH$_2$CH(NH$_2$)COO, R$_3$ = OH
34e 3,6-di-*O*-ester: R$_3$ = R$_1$ = L-(CH$_3$)$_2$CHCH$_2$CH(NH$_2$)COO, R$_2$ = OH
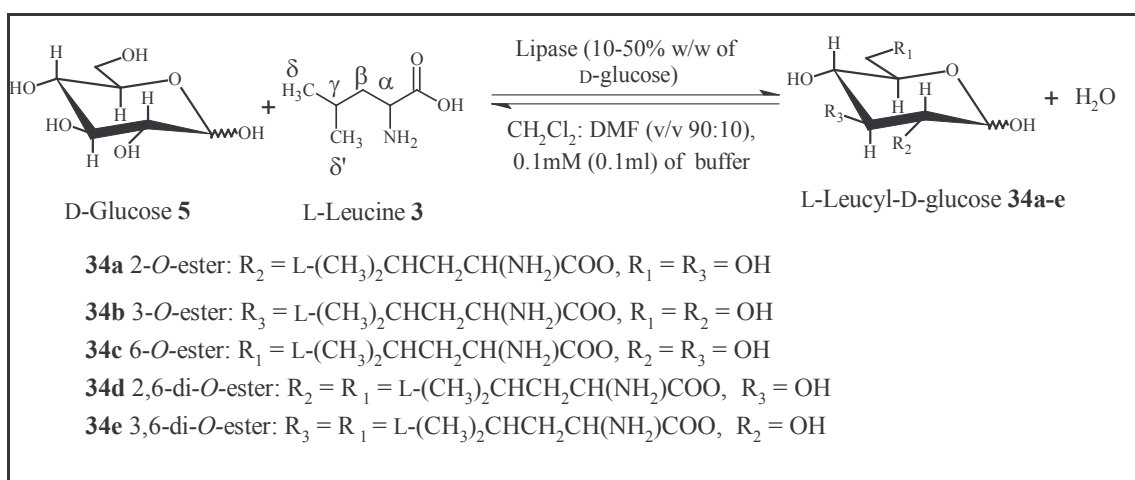
Scheme 3.3. Lipases catalyzed synthesis of L-leucyl-D-glucose esters in anhydrous organic media

3.2.3.1. Effect of *Rhizomucor miehei* lipase concentration

Effect of increasing lipase concentration on the synthesis of L-leucyl-D-glucose ester was studied by employing various RML concentrations ranging from 10 % to 50 % (w/w D-glucose, Table 3.7). The results showed that the yields obtained were very low. The enzyme showed the highest yield of 10.8 % (0.22 mmol) at 20 % enzyme concentration. With the increase in enzyme concentration from 10 % (yield - 0.11 mmol, 5.7 %) to 20 % (yield - 0.22 mmol, 10.8 %) there was an increase in esterification and beyond 30 % there was decrease (30 % - 0.04 mmol, 0.2 % yield; 50 % - 0.03 mmol, 0.3 % yield) in esterification probably due to hydrolysis of the esters formed.

Table 3.7   Effect of lipase concentration on the synthesis of L-leucyl-D-glucose[a]

RML concentration (% w/w D-glucose)	*Rhizomucor miehei* lipase (RML) Yield % (mmol)
10	5.7 (0.11)
20	10.8 (0.22)
30	0.2 (0.04)
50	0.3 (0.06)

[a] D–glucose - 1 mmol and L–leucine - 2 mmol. Conversion yields from HPLC determined with respect to L-leucine. Error in yield measurements will be $\pm$ 10 – 15 %. Solvent - CH_2Cl_2:DMF (v/v 90:10) at 40 $^{\circ}$C. Buffer – 100 ml of solvent containing 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 4.0).

3.2.3.2. Effect of L-leucine concentration

Effect of L-leucine concentrations at fixed RML and PPL concentrations of 30 % (w/w D-glucose) on the synthesis of L-leucyl-D-glucose ester was studied by increasing the L-leucine concentration from 1 to 5 molar equivalent (Table 3.8). The results showed that better conversions could be achieved with both the enzymes at 5 equivalents of L-leucine.

Table 3.8 Effect of L-leucine on the synthesis of L-leucyl-D-glucose [a]

L-Leucine (mmol)	Yield % (mmol)	
	RML	PPL
1	0.2 (0.04)	18 (0.31)
2	7 (0.14)	4 (0.8)
3	6 (0.12)	9 (0.18)
4	8 (0.16)	6 (0.12)
5	36 (0.72)	24 (0.48)

[a] Lipase – 40 % (w/w D-glucose); L-leucine – 1 – 5 mmol at 1 mmol D-glucose; Solvent – CH_2Cl_2:DMF - (v/v 90:10) at 40 ^{o}C.

3.2.3.3. Effect of buffer salts

Depending on the method of preparation, the enzyme always possesses a micro-aqueous layer around it. Buffer salts dissolve in such a micro aqueous layer and stabilize the enzyme structure against denaturation and subsequent loss of activity in non-polar solvents. A buffer of known pH and volume was added to the reaction mixture and their effect on imparting 'pH memory' or 'pH tuning' to the enzyme was studied. This reaction also gave better yields in presence of buffer salts than in their absence. Effect of buffer salts in terms of pH in the range 4.0 to 8.0 with RML at 40 % (w/w D-glucose) on the synthesis of L-leucyl-D-glucose ester at 1:2 molar equivalent of D-glucose and L-leucine (Table 3.9) showed that 0.2 mM (0.1 ml of 0.1 M) acetate buffer (pH 5.0) gave the highest yield of 63 % (0.63 mmol). The yields were: 16 % (0.31 mmol) at pH 4.0, 16 % (0.33 mmol) at pH 6.0, 14 % (0.27 mmol) at pH 7.0 and 7 % (0.14 mmol) at pH 8.0. The effect of various buffer volumes in the range 0.05 mM (0.05 ml) to 0.6 mM (0.6 ml) of phosphate buffer (pH 7.0) showed that the esterification yields increased with increase in the buffer concentration from 0.05 mM (0.05 ml) to 0.6 mM (0.6 ml) with 0.6 mM (0.6 ml) phosphate buffer (pH 7.0) showing the highest yield of 85 % (1.7 mmol).

Table 3.9   Effect of buffer salts (pH and buffer concentration) on the synthesis of L-leucyl-D-glucose [a]

pH [b]	Yield % (mmol)	pH 7.0 [c] concn mM	Yield % (mmol)
4.0	16 (0.31)	0.05	5 (0.1)
5.0	63 (1.26)	0.1	21 (0.42)
6.0	16 (0.33)	0.2	14 (0.28)
7.0	14 (0.27)	0.4	32 (0.64)
8.0	7 (0.14)	0.6	85 (1.70)

[a] D-glucose – 1 mmol and L-leucine - 2 mmol; Incubation period - 72 h; RML – 40 % (w/w D-glucose) 100 ml of the solvent containing specified volumes, concentration and pH of the buffer
[b] Solvent – 100 ml C_6H_6:$CHCl_3$:DMF (v/v/v 45:45:10) at 60 °C. Buffer - 0.2 mM (0.2 ml of 0.1 M) appropriate pH buffer.
[c] Solvent – 100 ml. Buffer - 0.05 ml to 0.6 ml of 0.1 M phosphate (pH 7.0).

3.2.4. Selectivity:

An attempt to improve the selectivity of ester formation was also made by reducing the incubation period in case of L-alanyl-D-glucose synthesis using RML (at 1 mmol D-glucose and 2 mmol L-alanine, RML – 40 % w/w D-glucose, incubation period - 12 h, solvent CH_2Cl_2:DMF - (v/v 90:10) at 40 °C buffer – 100 ml of solvent containing 0.1 mM acetate buffer, pH 4.0). A conversion yield of 15 % was obtained. From two-dimensional HSQCT NMR the formation of three monoesters (6-*O*- 42 %, 3-*O*- 33 % and 2-*O*- 25 %) with only β-anomer of D-glucose were found, the D-glucose employed being a 40:60 mixture of α and β anomers respectively.
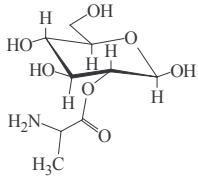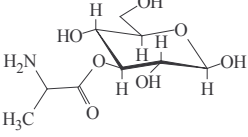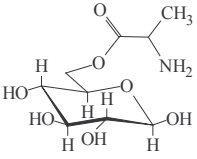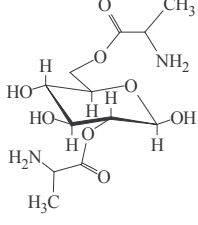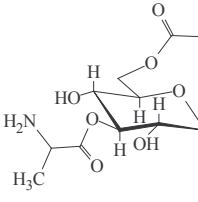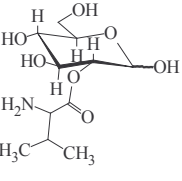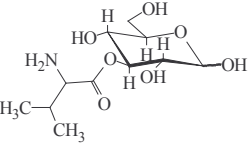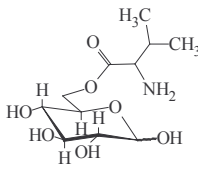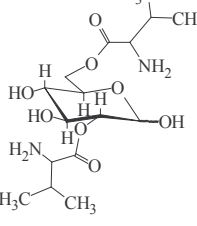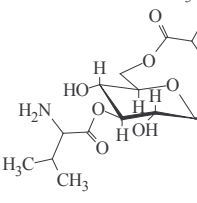
3.2.5. Determination of Critical Micellar Concentration (CMC)

Surfactant property of the amino acyl esters of carbohydrate was evaluated by determining the critical micellar concentration (CMC) value for L-alanyl-β-D-glucose spectroscopically at 470 nm (Rosenthal and Koussale, 1983) by following the method explained in Section 2.2.12.   The CMC of L-alanyl-β-D-glucose was found to be 2.25 mM (0.056 %).

3.3. Products of L-alanyl, L-valyl and L-leucyl esters of D-glucose

The isolated esters were subjected to UV, IR, MS and 2-D HSQCT NMR characterization (Section 3.3.1.1 – 3.3.1.3). Table 3.10 shows the ester yields form HPLC, types of esters formed and percentage proportions of the individual esters yields from HPLC by both RML and CRL.

Table 3.10 Synthesis of L-alanyl, L-valyl and L-leucyl esters of D-glucose[a]

L-amino acyl esters of D-glucose (% proportions [e])			Yield (%)

16a 2-*O*-L-alanyl-β-D-glucose (20) **16b** 3-*O*-L-alanyl-β-D-glucose (12) **16c** 6-*O*-L-alanyl-β-D-glucose (47)

30 [b] (mono esters- 24, diesters-6)

16d 2,6-di-*O*-L-alanyl-β-D-glucose (15) **16e** 3,6-di-*O*-L-alanyl-β-D-glucose (6)

25a 2-*O*-L-valyl-D-glucose (10) **25b** 3-*O*-L-valyl-D-glucose (12) **25c** 6-*O*-L-valyl-D-glucose (31)

68 [c] (mono esters- 36, di esters-32)

25d 2,6-di-*O*-L-valyl-D-glucose (23) **25e** 3,6-di-*O*-L- valyl-D-glucose (24)

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34a 2-*O*-L-leucyl -
D-glucose (16)

34a 3-*O*-L-leucyl-
D-glucose (21)

34a 6-*O*-L-leucyl-
D-glucose (42)

63 [d]
(mono
esters- 49,
diesters-14)

34d 2,6-di-*O*-L-leucyl-D-glucose (11)

34e 3,6-di-*O*-L- leucyl-D-glucose (10)

[a] L-Amino acid – 2 mmol and D-glucose – 1 mmol. Conversion yields were from HPLC with respect to L-amino acid concentration. Solvent – 100 ml of CH_2Cl_2:DMF (v/v 90:10) at 40 ^{o}C, incubation period – 72 h.
[b] RML – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) acetate buffer pH 4.0.
[c] CRL – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) phosphate buffer pH 7.0,
[d] RML – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) acetate buffer pH5.0.
[e] Percentage proportions of individual esters were determined from the peak areas or from their cross peaks of the Carbon-13 C6 signals in the 2D HSQCT spectrum.

3.3.1. Spectral data for L-alanyl, L-valyl and L-leucyl esters of D-glucose

3.3.1.1. L-Alanyl-D-glucose (16a-e): Solid; HPLC t_{ret}: 3.4 min; R_f: 0.22; UV (H_2O, λ_{max}): 227.0nm ($\sigma\rightarrow\sigma^*$ $\varepsilon_{227.0}$ − 1150.8 M^{-1}), 294.0nm ($n\rightarrow\pi^*$ $\varepsilon_{294.0}$ - 763.8 M^{-1}); IR (KBr, stretching frequency): 3371cm^{-1} (NH), 3410cm^{-1} (OH), 2297 cm^{-1} (CH), 1653 cm^{-1} (CO); MS (*m/z*) : 274 $[M+Na]^{+}$.

2D HSQCT (DMSO-d_6) **2-*O*- ester (16a)** : ^{1}H NMR δ (500.13 MHz) : 2.95 (αCH), 1.07 (βCH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 52.1 (αCH), 15.7 (βCH$_3$), 102.8 (C1β), 82.6 (C2β), 77.9 (C3β), 68.8 (C4β), 60.5 (C6β) ppm; **3-*O*- ester (16b)** : ^{1}H NMR δ : 2.87 (αCH), 3.93 (H-3β), 3.58 (H-4β), 3.36 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.4 (αCH), 83.3 (C3β), 69.3 (C4β), 57.3 (C6β) ppm; **6-*O*- ester (16c)** : ^{1}H NMR δ : 2.95 (αCH), 1.30 (βCH$_3$), 3.86 (H-2β), 3.76 (H-5β), 3.82 (H-6) ppm; ^{13}C NMR δ : 50.2 (αCH), 15.1 (βCH$_3$), 171.4 (CO), 101.8 (C1β), 75.0 (C2β),

70.1 (C5β), 63.5 (C6β) ppm; **2,6-di-*O*- ester (16d)** : ^{1}H NMR δ : 3.36 (αCH), 1.30 (βCH$_3$), 3.78 (H-2β), 3.47 (H-6) ppm; ^{13}C NMR δ : 49.5 (αCH), 16.4 (βCH$_3$), 100.8 (C1β), 76.5 (C2β), 62.7 (C6β) ppm; **3,6-di-*O*- ester (16e)** : ^{1}H NMR δ: 1.30 (βCH$_3$), 3.78 (H-3β), 3.82 (H-6) ppm; ^{13}C NMR δ : 51.4 (αCH), 16.7 (βCH$_3$), 81.6 (C3β), 63.1 (C6β) ppm.

3.3.1.2. L-Valyl-D-glucose (25a-e): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H$_2$O, λ$_{max}$): 200.0 nm (σ→σ* ε$_{200.0}$ − 2630 M^{-1}), 295.0 nm (n→π* ε$_{295.0}$ - 2089 M^{-1}); IR (KBr, stretching frequency): 2971 cm^{-1} (NH), 3407 cm^{-1} (OH), 2950 cm^{-1} (CH), 1622 cm^{-1} (CO); MS (*m/z*) : 302 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*- ester (25a)**: ^{1}H NMR δ (500.13 MHz) : 3.08 (αCH), 1.35 (γCH$_3$), 3.75 (H-2α), 3.67 (H-3α), 3.78 (H-4α), 4.22 (H-5α), 3.50 (H-6) ppm; ^{13}C NMR δ (125 MHz): 51.5 (αCH), 19.4 (γCH$_3$), 77.4 (C2α), 74.6 (C3α), 71.3 (C4α), 71.3 (C5α), 60.0 (C6α) ppm; **3-*O*- ester (25b)** : ^{1}H NMR δ: 3.10 (αCH), 1.15 (βCH$_3$), 3.85 (H-2α), 3.90 (H-3α), 4.01 (H-3β), 3.34 (H-6) ppm; ^{13}C NMR δ : 52.3 (αCH), 18.3 (γCH$_3$), 71.3 (C2α), 82.6 (C3α), 83.1 (C3β), 60.4 (C6α) ppm; **6-*O*- ester (25c)**: ^{1}H NMR δ : 3.20 (αCH), 3.35 (βCH), 1.55 (γCH$_3$), 4.95 (H-1α), 4.24 (H-1β), 3.90 (H-3α), 3.74 (H-4α), 3.41 (H-5α), 3.15 (H-6) ppm; ^{13}C NMR δ : 53.4 (αCH), 29.9 (βCH), 19.8 (γCH$_3$), 94.9 (C1α), 103.4 (C1β), 76.2 (C3α), 69.5 (C4α), 69.0 (C5β), 63.4 (C6α) ppm; **2,6-di-*O*- ester (15d)**: ^{1}H NMR δ : 3.20 (αCH), 3.85 (H-2α), 3.56 (H-6) ppm; ^{13}C NMR δ: 51.4 (αCH), 76.4 (C2α), 61.5 (C6α) ppm ; **3,6-di-*O*-ester (25e)**: ^{1}H NMR δ : 3.15 (αCH), 1.45 (γCH$_3$), 3.70 (H-3α), 3.52 (H-6) ppm; ^{13}C NMR δ : 53.4 (αCH), 19.5 (γCH$_3$), 82.4 (C3α), 61.8 (C6α) ppm.

3.3.1.3. L-Leucyl-D-glucose (34a-e): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H$_2$O, λ$_{max}$): 230.0 nm (σ→σ* ε$_{230.0}$ - 724 M^{-1}), 297.0 nm (n→π* ε$_{297.0}$ - 363 M^{-1}); IR (KBr,

stretching frequency): 3383 cm^{-1} (NH), 3360 cm^{-1} (OH), 2240 cm^{-1} (CH), 1657 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^{+}$.

2D-HSQCT (DMSO-*d$_6$*) : **2-*O*-ester (34a)**: ^{1}H NMR δ (500.13 MHz): 3.85 (H-2α) ppm; ^{13}C δ (125 MHz): 75.5 (C2α) ppm; **3-*O*-ester (34b)**: ^{1}H NMR δ : 3.15 (αCH), 3.85 (H-3α), 3.96 (H-3β) ppm; ^{13}C NMR δ : 50.0 (αCH), 83.5 (C3α), 83.6 (C3β) ppm;. **6-*O*-ester (24c)**: ^{1}H NMR δ : 3.10 (α CH), 1.56 (βCH$_2$), 1.82 (γCH), 0.82 (δ, δ′ CH$_3$), 4.99 (H-1α), 3.81 (H-2α), 3.45 (H-3α), 3.68 (H-4α), 3.55 (H-5α), 3.80 (H-6) ppm ; ^{13}C NMR δ: 53.2 (αCH), 40.5 (βCH$_2$), 23.5 (γCH), 25.0 (δ, δ′ CH$_3$), 173.6 (CO), 92.5 (C1α), 70.3 (C2α), 75.8 (C3α), 70.2 (C4α), 71.0 (C5α), 65.0 (C6α) ppm; **2,6-di-*O*-ester (34d)**: ^{1}H NMR δ : 3.21 (H-2α), 3.59 (H-6) ppm; ^{13}C NMR δ : 76.3 (C2α), 65.0 (C6α) ppm. **3,6-di-*O*-ester (34e)**: ^{1}H NMR δ: 3.68 (H- 3α), 3.59 (H-6) ppm; ^{13}C δ: 81.5 (C3α), 65.0 (C6α) ppm.

3.4. Discussion

The optimum conditions determined for these esterification reactions by studying the effect of variables like incubation period, enzyme and substrate concentrations, pH and buffer concentration clearly explain the behaviour of the lipases. Most of the parameters show that esterification increases upto a certain point, and thereafter they remain as such or decrease a little. This complex esterification reaction is not controlled by kinetic factors or thermodynamic factors or water activity alone.

Use of lower enzyme concentrations did not result in thermodynamic yields. The thermodynamic binding equilibria regulate the concentrations of the unbound substrates at different enzyme and substrate concentrations and thereby conversion as the reaction proceeds with time. At lesser enzyme concentrations, for a given amount of substrates (enzyme/substrate ratio low), rapid exchange between bound and unbound forms of both

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the substrates with the enzyme (on a weighted average based on binding constant values of both the substrates) leaves substantial number of unbound substrate molecules at the start of the reaction which decrease progressively as conversion takes place (Romero *et al.,* 2005a; Marty *et al.,* 1992). This becomes more so, if one of them binds more firmly to the enzyme than the other (higher binding constant value) as the respective enzyme/substrate ratios change (during the course of the reaction) unevenly till the conversion stops due to total predominant binding (inhibition). At intermediatory enzyme concentrations, such a competitive binding results in a favorable proportions of bound and unbound substrates to effect quite a good conversion. At higher enzyme concentrations, most of the substrates would be in the bound form leading to inhibition and lesser conversion (higher enzyme/substrate ratios). Also, the esterification reaction requires larger amount of enzyme compared to hydrolysis. While this leads to lesser selectivity, they also give rise to varying bound and unbound substrate concentrations till the conversion ends. For a given amount of enzyme and substrates there is no increase in conversion beyond 72 h to 120 h. Longer incubation periods of especially lesser enzyme concentrations could also result in partial enzyme inactivation. However, not all the enzymes are inactivated before the end of the reaction.

By imparting 'pH memory' or 'pH tuning', the catalytic activity of the lyophilized substilisin Carlsberg in the pH range 5.0 - 11.0, in organic solvents like acetonitrile and 3-pentanone was reported to be enhanced (Xu and Klibanov, 1996). The enzymatic activity of substilisin cross linked crystals in anhydrous 3-pentanone was accelerated by the addition of organic soluble (a mixture of a suitable acid and sodium salt) buffers (Xu and Klibanov, 1996). The enantio selectivity of *Candida antarctica* lipase B in organic media was increased by 'pH tuning' of the enzyme by the addition of

certain buffer salts which altered the protonation state of the enzyme and selectively tuned enantioselectivity and catalytic activity (Quiros et al., 2002). Similarly, the present work also showed enhanced activity of RML and CRL in presence of buffer salts. However, buffer salts did not enhance esterification with PPL.

Besides imparting 'pH memory' or 'pH tuning', added water is essential for the integrity of the three-dimensional structure of the enzyme molecule and therefore its activity (Dordick, 1989). Zaks and Klibanov (1988) reported that at low water activities, lower the solvent polarity, the higher the enzyme activity. Beyond the critical water concentration, esterification decreases because the size of the water layer formed around the enzyme retards the transfer of acyl donor to the active site of the enzyme (Humeau *et al.,* 1998, Camacho-Paez *et al.,* 2003) and also the water layer surrounding the enzymes makes enzyme to be more flexible by forming multiple hydrogen bonds and interacting with organic solvent causing denaturation (Valiveti *et al.,* 1991). Increase in buffer volume affected this esterification reaction significantly. It could increase the water activity of the system in the initial stages by increasing the thickness of the microaqueous layer around the enzyme. Higher volumes of the buffer in the microaqueous layer could also cause slight inactivation of the enzyme due to increase in salt concentration beyond a critical point. Patridge *et al.,* (2001) reported that when an enzyme is suspended in a low-water organic solvent, the counter ions are in closer contact with the opposite charges on the enzyme because of the lower dielectric constant of the medium. Thus, protonation of the ionizable groups on the enzyme could be controlled by the type and availability of these ions as well as hydrogen ions resulting in a 'pH memory' or 'pH tuning'. The third factor is the increase in ionic strength which could play a favourable role in esterification. Optimum pH were found to be pH 4.0 for

RML and pH 5.0 for PPL in case of L-alanyl-D-glucose, pH 7.0 for CRL in case of L-valyl-D-glucose and pH 5.0 for RML in case of L-leucyl-D-glucose reactions clearly indicate a slight unfavorable conformational change in the enzyme at about pH 4.0 to 6.0 leading to lesser conversion beyond pH 4.0 and 5.0 for RML, 5.0 for PPL and 7.0 for CRL.

The experimental setup employed in the present work is such that it maintained a low water activity (a_w= 0.0054) due to azeotropic distillation and recycling the solvent back into the reaction system after passing through a bed of desiccant. Even the water of reaction formed could also be used to constitute the microaqueous layer around the enzyme and the excess water could be removed by azeotropic distillation. The same could occur even with the addition of added enzyme (with little water content) and buffer volume. The added carbohydrate molecule could also reduce the water content of the reaction mixture. Adachi and Kobayashi (2005) have reported that the hexose which is more hydrated decreased the water activity in the system and shifts the equilibrium towards synthesis. All these factors lead to maintenance of an equilibrium concentration of water around the enzyme all the time. Hence, thermodynamic binding equilibria interplayed by inactivation and inhibition along with maintenance of an optimum water activity could be governing this reaction as reflected by the extent of conversion under different reaction conditions of added buffer, enzyme and substrate concentrations.

As monosaccharides contain five hydroxyl groups, 31 diastereomeric esters (mono, di, tri, tetra and penta) are possible for both the anomers. In case of L-alanyl, L-valyl and L-leucyl esters of D-glucose (Section 3.3.1.1. 3.3.1.2 and 3.3.1.3), only *6-O-* was the major ester produced (47 %, 31 % and 42 % respectively, Table 3.10). The anomeric composition of D-glucose employed for the reaction was 40:60 (α:β) and the

equal peak areas of anomeric H-1 chemical shift values observed at 4.24 and 4.0 ppm indicated that either both the anomers have reacted to equal extent (1:1) or D-glucose had undergone mutarotation in case of L-valyl and L-leucyl esters of D-glucose. In case of L-alanyl-D-glucose, only β-anomer of D-glucose was found to react, the D-glucose employed being a 40:60 mixture of α and β anomers respectively. Only monoesters were formed with only β-anomer of D-glucose, when an attempt was made to improve the selectivity of ester formation by decreasing the incubation period in case of L-alanyl-D-glucose synthesis using RML.

Commercial crude PPL preparations contain variety of estero/lipo lytic enzymes with low PPL concentrations (Segura *et al.,* 2006, Birner-Grunberger *et al.*, 2004) which could also perform facile esterification. Hence, a small amount of esters formed from esterases along with those of lipases in the present reaction cannot be ruled out. Since the reactions were carried out at a low temperature of 40 – 60 ^{o}C, the formation of peptide was less than 3 %, even though unprotected L-amino acid was used for the reaction. NMR data clearly indicated that no Maillard reaction occurred. Under these reaction conditions, formation of Maillard reaction products are quite likely. For instance, Maillard and Pictet Spengler phenolic condensation products were reported in the reaction between phenolic amino acids and D-glucose in phosphate buffer at different pH from 5.0 to 9.0 at 90 ^{o}C (Manini *et al.,* 2005). Similarly Maillard products from the reaction between D-glucose and N-*t*-boc-L-lysine incubated with aminoguanidine in phosphate buffer (pH 7.4) at 70 ^{o}C was also reported (Reihl *et al.,* 2004). No such Maillard reaction type products were detected by mass as well as NMR in the present investigation. RML, CRL and PPL showed significant esterification (up to 84 %) when unprotected L-amino acid was used. When N-acetyl-L-alanine was used in the present

work, both RML and PPL gave < 5 % yield. Riva *et al.,* (1988) have reported two monoesters (4-*O*- and 6-*O*- ester) and no diester for L-alanine, using subtilisin, a protease. Our present study has shown that comparable esterification yields to others could be achieved by employing PPL, CRL and RML instead of protease. Thus, our study clearly indicates that unprotected L-amino acid could be used for esterification of carbohydrates.

3.5. Experimental

3.5.1. Esterification procedure

Preparation of L-alanyl, L-valyl and L-leucyl esters of D-glucose was carried out by adopting a bench-scale level procedure (Divakar *et al.,* 1999). Esterification was carried out in presence of 0.018 – 0.090 g of lipases (expressed in terms of % w/w D-glucose employed). Unprotected L-amino acids (L-alanine **1**, L-valine **2** and L-leucine **3,** 0.001- 0.005 mol) and D-glucose **5** (0.001 – 0.005 mol) in 100 ml of organic media containing CH_2Cl_2:DMF (v/v 90:10) or hexane:$CHCl_3$:DMF (v/v/v 45:45:10) were taken in a flat bottomed two necked flask and refluxed for a period of 3 - 120 h. The enzymes were imparted with or 'pH memory' or 'pH tuning' in some experiments by adding known volumes of 0.1 M buffer solutions of specified pH value to 100 ml (solvent) of the reaction mixture. The condensed vapors of solvent which formed an azeotrope with water during reflux was passed through a desiccant (sodium aluminosilicate molecular sieves of 4 Å) before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction (Lohith and Divakar, 2005). This set up maintained a very low water activity of a_w= 0.0054 throughout the reaction period which was determined by Karl Fischer titration of the reaction mixture using Karl Fischer reagent by examining aliquots for the water content during the course of the reaction.

The reaction mixture after distilling off the solvent was then treated with 20-50 ml of water, stirred and filtered to remove the lipase. The filtrate was evaporated on a water bath to get the unreacted D-glucose, unreacted L-amino acids and the product esters which were then analyzed by HPLC. The conversion yields were determined with respect to peak areas of the L-amino and that of the esters. The esters formed were separated by size exclusion chromatography using Sephadex G-10/Bio-Gel P-2 as column materials and eluted with water. The product esters separated were subjected to spectral characterization by UV, IR, mass, specific rotation and 2D-NMR (Sections 3.3.1.1, 3.3.1.2 and 3.3.1.3). Although, the esters were separated from unreacted amino acids and D-glucose by this procedure, the individual esters in the mixture of esters formed could not be separated. This could be due to the similar polarity of the ester molecules.

3.5.2. High Performance Liquid Chromatography (HPLC)

A Shimadzu LC 10AT HPLC instrument connected to a μ-Bondapack aminopropyl column (10 μm particle size, 3.9 x 300 mm length) was employed for analyzing the reaction mixture. Acetonitrile:water (80:20 v/v) as a mobile phase at a flow rate of 1ml/min was used with Refractive Index detector. Also LiChrosorb RP-18 column (5 μm particle size, 4.6 x 150 mm length) was employed with an UV detector at 210 nm using acetonitrile: water (v/v 20:80) as a mobile phase at a flow rate of 1 ml/min.

3.5.3. Spectral characterization

A Shimadzu UV – 1601 spectrophotometer (Kyoto, Japan) was used for recording UV spectra of the isolated esters in water at 0.1 - 2.0 mM concentrations. A Nicolet 5700 FTIR instrument (Madison, USA) was used for recording the IR spectra with a 1.0 - 3.0 mg of ester sample as KBr pellet. Specific rotation of the isolated esters

were measured at 25 ^{o}C using Perkin-Elmer 243 polarimeter (Überlingen, Germany) with a 0.2 – 1.5 % solution of the esters in water. Mass spectra of the isolated esters were recorded using a Q-TOF Waters Ultima instrument (No.Q-Tof GAA 082, Waters corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source.

3.5.4. Nuclear Magnetic Resonance Spectroscopy

3.5.4.1. ^{1}H NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 500.13 MHz was used to record ^{1}H NMR. A 40 mg of sample was dissolved in 0.5 ml of DMSO-d_6 solvent. About 50 - 200 scans were accumulated with a recycle period of 2 - 3 seconds to obtain good spectra. The spectra were recorded at 35 ^{o}C with TMS as internal standard for measuring the chemical shift values to within ± 0.001 ppm. A region from 0 – 10 ppm was scanned for all the samples.

3.5.4.2. ^{13}C NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 125 MHz was used to record ^{13}C NMR. Samples were dissolved in 0.5 ml of DMSO-d_6 and recorded at 35 ^{o}C. A region from 0 - 200 ppm region was scanned and about 500 – 6000 scans were accumulated for each spectrum to get good spectrum. TMS was taken as an internal standard.

3.5.4.3. Two-dimensional HSQCT

Two dimensional Heteronuclear Multiple Quantum Coherence Transfer spectra (2-D HMQCT) and Heteronuclear Single Quantum Coherence Transfer spectra (2-D HSQCT) (Fallanden, Switzerland) were recorded at 500 MHz on a Brüker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard. About 40 mg of the sample dissolved in DMSO-d_6 was used for recording the spectra.

Chapter 4
Syntheses of carbohydrate esters of ʟ-alanine, ʟ-valine, ʟ-leucine and ʟ-isoleucine

4.1 Introduction

Carbohydrates are biologically interesting molecules whose modification can bestow useful additional functionality to sugar moiety. Introduction of an amino acid into the carbohydrate moiety results in an additional functionality like an amino group besides converting an hydroxyl group into an ester. Addition of amino acids to sugar moiety can also improves the bioavailability of amino acids in biological systems. Presence of hydroxyl as well as amino groups in the molecule help in the polycondensation reactions (Park *et al.,* 1999). Recently Shiraki *et al.,* (2004) reported that amino acid esters prevent thermal inactivation and aggregation of lysozyme. Besides these uses, other application of amino acyl esters of carbohydrates have been mentioned in previous chapters.

Hitherto, very few references are available on the lipase catalyzed acylation of amino acids with carbohydrates, most of which have been described in Section 3.1.

A comprehensive investigation has been carried out on the lipase catalyzed syntheses of L-amino acid esters of eleven carbohydrate molecules. In the present investigation, lipases from *Candida rugosa* (CRL), *Rhizomucor miehei* (RML) and porcine pancreas (PPL) were employed towards syntheses of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates. Aldohexoses (D-glucose, D-galactose and D-mannose), ketohexose (D-fructose), pentoses (D-arabinose and D-ribose), disaccharides (lactose, maltose and sucrose) and sugar alcohols (D-mannitol and D-sorbitol) are the carbohydrate molecules employed. Attempts to synthesize the same using N-acetyl derivatives of these amino acids resulted in a very low conversion with the above mentioned enzymes. Hence, unprotected and unactivated L-amino acids and carbohydrates were employed. The results are presented below.

4.2. Present investigation

Syntheses and characterization of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates

Syntheses of L-amino acid esters of different carbohydrates were carried out between different amino acids (L-alanine **1**, L-valine **2**, L-leucine **3** and L-isoleucine **4**) and carbohydrates (D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14**, D-sorbitol **15**) using *Rhizomucor miehei* lipase (RML), *Candida rugosa* lipase (CRL) and porcine pancreas lipase (PPL) in presence of organic solvent. A 0.1 mM (0.1 ml of 0.1M) acetate buffer (pH 4.0) in case of RML, 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0) in case of CRL and 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 5.0) in case of PPL were employed in the reaction mixture to impart the 'pH memory' or 'pH tuning' to the lipase. The reaction mixture containing L-amino acid **1-4** (1-2 mmol), carbohydrate **5-15** (1 mmol), lipase (40 % in case of RML and CRL w/w based on respective carbohydrate and 111 % in case of PPL) and the solvent were refluxed for a period of 72 h (experimental procedure is described in Section 2.2.4). The esterification reaction described in this present work did not occur without the use of enzymes. Extent of esterification was analyzed by HPLC and the esters were isolated by passing through Sephadex G-10/Bio Gel P2 and eluting with water. The isolated esters were then subjected to characterization by UV, IR, Mass, optical rotation and 2D-HSQCT NMR spectroscopy. NMR assignments of amino acids as well as carbohydrates and esters were made according to literature reports (Suzuki *et al.,* 1991; Riva *et al.,* 1988; Park *et al.,* 1996, 1999). Some of the assignments, especially ^{13}C signals mentioned in the sections 4.2.1.1 – 4.2.1.9 for L-alanyl esters, 4.2.2.1 – 4.2.2.9 for L-valyl esters, 4.2.3.1 – 4.2.3.10 for L-leucyl esters and 4.2.4.1 - 4.2.4.10 for L-isoleucyl esters are interchangeable. Only resolvable signals are

shown. Non-reducing end carbohydrate signals in disaccharides are primed. Since, the esters are surfactant molecules, they appear to aggregate in the solvent and usually give broad signals, thus, making it difficult to resolve the coupling constant values in the ^{1}H NMR spectra accurately. Mass data for the monoesters are shown. However, although NMR data clearly indicated the presence of di-*O-* esters, they were not detected in the mass spectra, which could be due to instantaneous decomposition. The percentage proportions of the individual esters formed were determined by considering the peak areas of the C6, C5 (in case of pentoses) of ^{13}C signals or cross peaks from 2D-NMR. Although column chromatography using Sephadex G-10/Bio Gel P2 separated unreacted amino acids and carbohydrates from the esters, the individual esters could not be separated in many cases due to similar polarity of the ester molecules. However, NMR data from individual esters could be detected unequivocally.

4.2.1. Syntheses of L-alanyl esters of carbohydrates 16a-e to 24

L-Alanine is a polar and a non-essential amino acid containing methyl group as a side chain. Esterification of L-alanine with carbohydrates was carried out using CRL, RML and crude PPL under optimal conditions (Section 2.2.4, **Scheme 4.1**). The reaction mixture consists of 1-2 mmol L-alanine **1** and 1 mmol carbohydrates (D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14**, D-sorbitol **15**) along with 40 % RML (w/w carbohydrate) / 40 % CRL (w/w carbohydrate) / 111 % PPL (w/w carbohydrate) and incubated in 100 ml of CH_2Cl_2 and DMF (v/v 90:10, 40 ^{o}C) containing 0.1 mM (0.1 ml of 0.1 M) of acetate buffer, pH 4.0 (RML) or 0.1 mM (0.1 ml of 0.1 M) of phosphate buffer, pH 7.0 (CRL) or 0.2 mM (0.2 ml of 0.1 M) of acetate buffer, pH 5.0 (crude PPL). The reaction mixture was analyzed by HPLC using a C-18 column with acetonitrile:water (v/v 20:80) mobile phase and detected at 210 nm using a UV detector (Fig. 4.1). Ester formation was

also monitored by TLC and spots were detected by spraying ninhydrin (for NH$_2$ group detection) and 1-naphthol (for reducing sugar detection). The retention times (t_{ret}) by HPLC and retention factor (R_f) values by TLC are mentioned in sections 4.2.1.1 – 4.2.1.9.
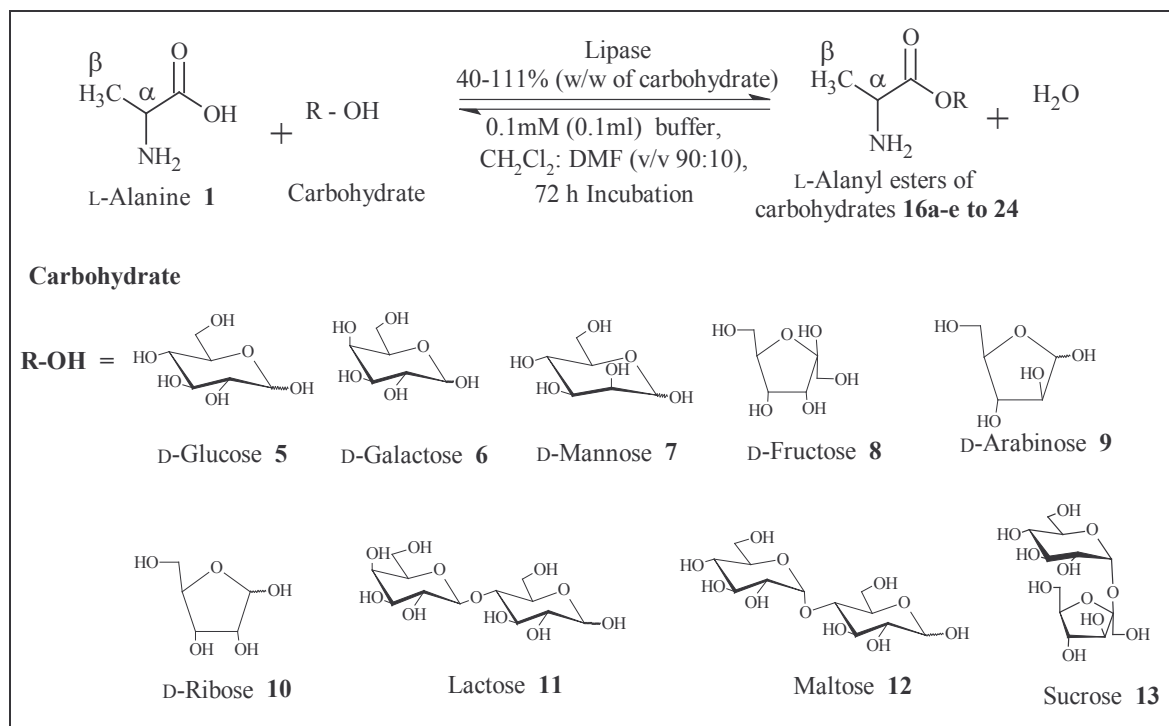
Scheme 4.1 Lipase catalyzed syntheses of L-alanyl esters of carbohydrates

The isolated esters were subjected to UV, IR, MS, optical rotation and 2D-HSQCT NMR characterization (Sections 4.2.1.1 – 4.2.1.9). The spectral data for the isolated esters are shown in Sections 4.2.1.1 – 4.2.1.9. Table 4.1 shows chemical shift values for free carbohydrates. Table 4.2 shows the ester yields form HPLC, types of esters formed and percentage proportions of the individual esters by RML and Table 4.3 shows the ester yields from HPLC by both CRL and PPL.
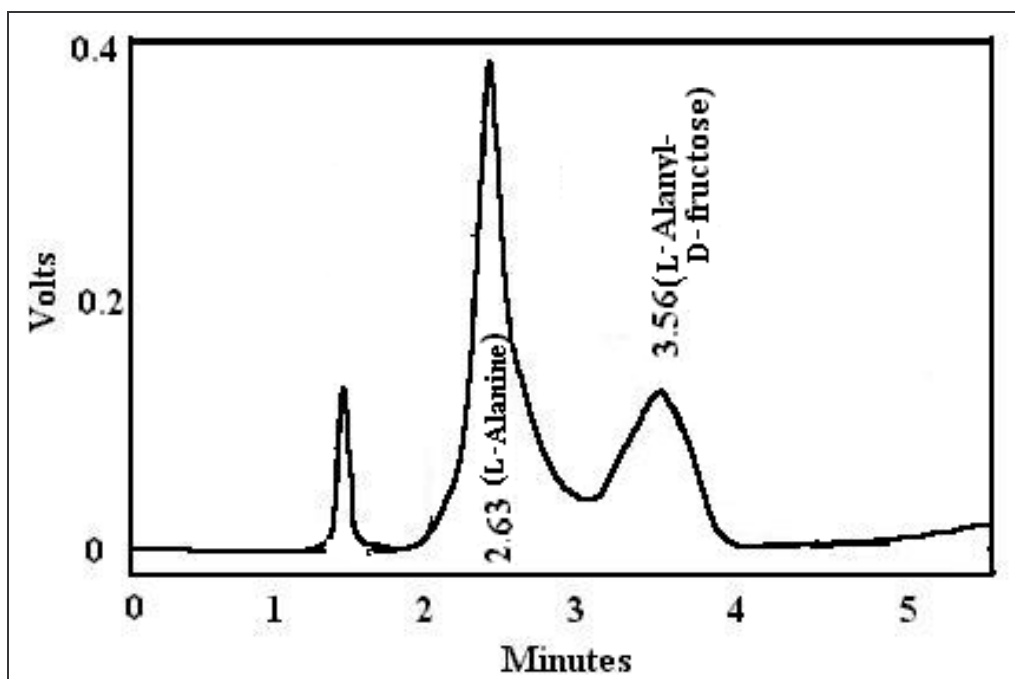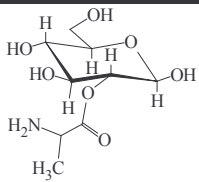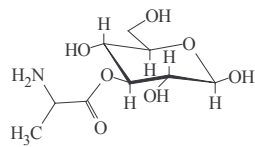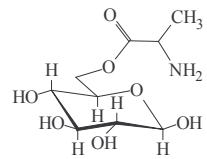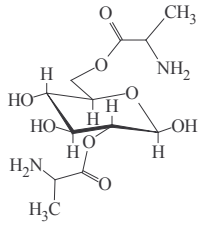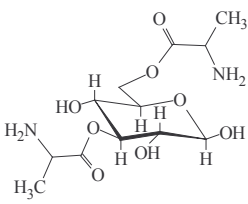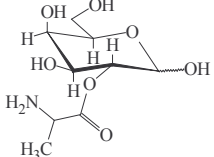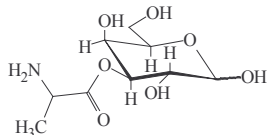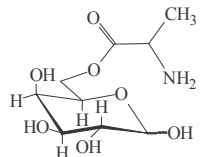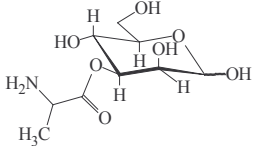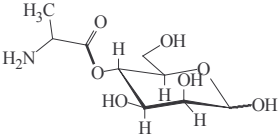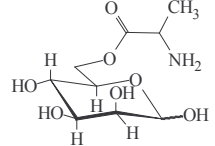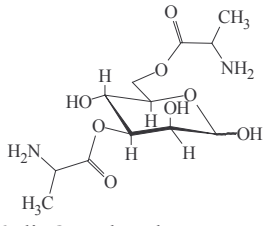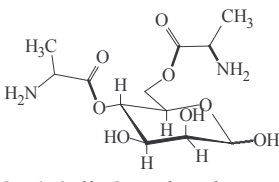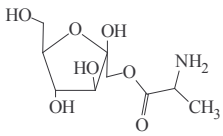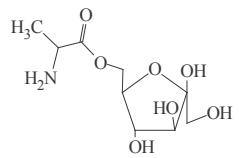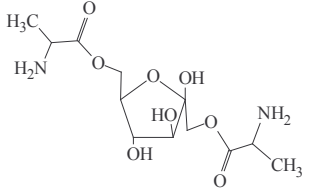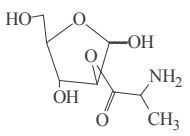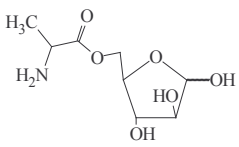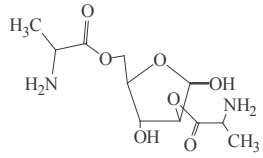
Fig. 4.1. HPLC chromatogram: reaction mixture of L-alanine and D-fructose esterification catalyzed by RML. Column – C-18; mobile phase – acetonitrile: water (v/v 20:80); flow rate- 1 ml /min; detector – UV at 210 nm; errors in conversion yields are within ± 10 %.
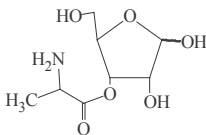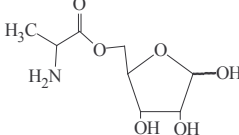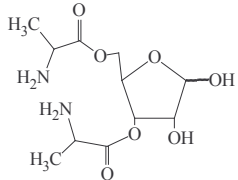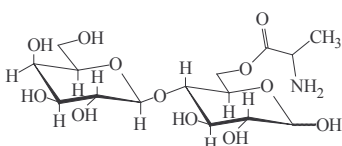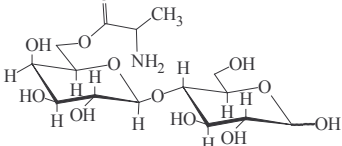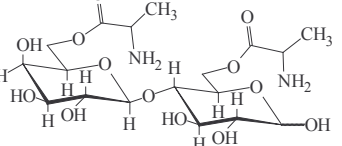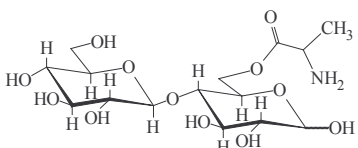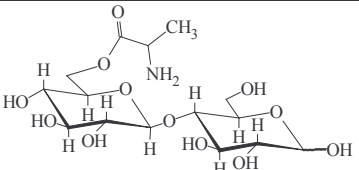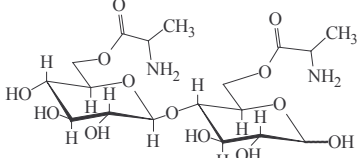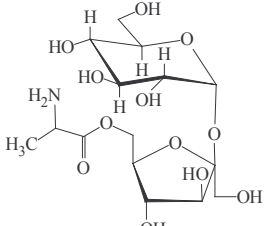
Table 4.1 Chemical shift values for free carbohydrates [a]

Carbohydrate		Chemical shift values in ppm (J Hz)																							
		¹H H-1	¹³C C1	¹H H-2	¹³C C2	¹H H-3	¹³C C3	¹H H-4	¹³C C4	¹H H-5	¹³C C5	¹H H-6	¹³C C6	¹H H-1'	¹³C C1'	¹H H-2'	¹³C C2'	¹H H-3'	¹³C C3'	¹H H-4'	¹³C C4'	¹H H-5'	¹³C C5'	¹H H-6'	¹³C C6'
D-Glucose	α	4.95 (6.2)	92.3	3.14 (6.2)	72.5	3.44 (5.01)	72.0	3.07 (5.01)	70.7	3.58 (6.49)	72.0	3.53 (11.3, 6.5)	61.4												
	β	4.30 (6.2)	97.0	2.92 (6.2)	75.0	3.06 (5.01)	76.9	-	-	3.45 (4.1, 1.3)	76.9	3.62 11.3, 6.5	61.6												
D-Galactose	α	4.14 (6.2)	92.7	3.50 (6.2)	68.4	3.59 (5.01)	69.0	3.70 (5.0, 4.1)	70.0	3.35 (4.1, 1.3)	70.5	3.31 (1.3, 11.3)	60.7												
	β	4.83 (6.2)	97.6	--	--	3.15 (5.01)	73.7	3.10 (5.0, 4.1)	72.3	3.25 (4.1, 1.3)	74.8	3.32 (1.3, 11.3)	60.8												
D-Mannose	α	4.89 (6.2)	94.0	3.54 (6.2)	71.3	3.55 (6.2, 5.0)	70.0	3.36 (5.0, 4.1)	67.4	3.50 (4.1, 1.3)	73.0	3.63 (1.3, 11.3)	61.5												
	β	4.54 (6.2)	93.9	3.32	71.5	3.26 (6.2, 5.0)	73.7	3.37 (5.0, 4.1)	67.0	3.02 (4.1, 1.3)	77.0	3.46 (1.3, 11.3)	61.4												
D-Fructose	β	3.80 (12.2)	63.9	-	97.5	3.56 (5.8)	67.9	3.64 (5.8, 4.0)	69.9	3.57 (4.0, 2.4)	68.7	3.50 (2.4, 12.0)	62.6												
D-Ribose	α	4.75 (5.1)	93.2	3.23 (5.1, 5.0)	70.6	3.33 (5.0, 4.97)	68.8	3.71 (4.97, 2.4)	66.7	3.51 (2.4, 12.0)	62.8														
	β	4.31 (2.6)	94.0	3.31 (2.6, 4.7)	71.5	3.38 (4.7, 4.9)	68.0	3.54 (4.97, 2.4)	67.5	3.29 (2.4, 12.0)	62.8														
D-Arabinose	α	4.92 (1.7)	92.3	4.32 (1.7, 5.8)		4.6 (5.8, 4.0)	-	4.08 (4.0, 2.4)	-	3.73 (2.4, 12.0)	60.4														
	β	4.33 (4.5)	96.2	3.65 (4.5, 7.2)	69.1	4.02 (7.2, 4.0)	68.9	3.37 (4.0, 2.4)	67.1	3.64 (2.4, 12.0)	60.7														
Lactose	α	4.90 (6.2)	91.9	3.70 (6.2)	69.8	3.54 (6.2, 5.0)	71.4	3.27 (5.0, 4.1)	80.8	3.55 (4.1, 1.3)	72.1	3.64 (1.3, 11.3)	60.5	4.19 (6.2)	103.7	3.30 (6.2)	70.7	3.18 (6.2, 5.0)	73.2	3.62 (5.0, 4.1)	68.2	2.93 (4.1, 1.3)	75.4	3.52 (1.3, 11.3)	60.9
	β	4.34	96.3	3.31 (6.2)	74.2	3.53 (6.2, 5.0)	74.7	3.28 (5.0, 4.1)	81.1	3.44 (4.1, 1.3)	74.9	3.72 (1.3, 11.3)	60.6												
Maltose	α	4.80 (6.2)	92.0	2.85 (6.2)	73.9	3.29 (6.2, 5.0)	76.4	3.15 (5.0, 4.1)	79.7	3.30 (4.1, 1.3)	73.3	3.50 (1.3, 11.3)	60.8	4.90 (6.2)	100.3	2.94 (6.2)	72.0	3.10 (6.2, 5.0)	73.0	3.51 (5.0, 4.1)	69.5	3.62 (4.1, 1.3)	72.7	3.60 (1.3, 11.3)	60.2
	β	4.20 (6.2)	96.9	3.10 (6.2)	74.3	3.31 (6.2, 5.0)	76.0	3.19 (5.0, 4.1)	79.4	3.38 (4.14, 1.3)	76.4	3.34 (1.3, 11.3)	60.9												
Sucrose Glc	α													5.18 (6.2)	91.7	3.65 (6.2)	72.7	3.20 (6.2, 5.0)	71.5	3.11 (5.0, 4.1)	69.8	3.47 (4.1, 1.3)	72.8	3.54 (1.3, 11.3)	60.5
	Fru β	3.41 (12.2)	62.0	-	103.9	3.78 (5.8)	77.1	3.88 (5.8, 4.0)	74.1	3.41 (4.0, 2.4)	82.4	3.55 (2.4, 12.0)	62.0												
D-Sorbitol		3.41 (12.0, 7.3)	62.5	3.54 (7.3, 4.3)	73.6	3.68 (4.3)	68.9	3.39 (4.3)	72.2	3.48 (4.3, 7.3)	71.4	3.56 (7.3, 12.0)	63.3												
D-mannitol		3.40 (12.0, 7.3)	63.7	3.47 (7.3, 4.3)	71.2	3.54 (4.3)	69.6	3.54 (4.3)	69.6	3.47 (4.3, 7.3)	71.2	3.61 (7.3, 12.0)	63.7												

[a] 40 mg of carbohydrate in 0.5 ml of DMSO-d_6 (Bock and Pedersen 1983; Bock et al. 1984)

Table 4.2 Syntheses of L-alanyl esters of carbohydrates [a]

L-Alanyl esters of carbohydrates (% proportions [b])			Yield (%)
16a 2-*O*-L-alanyl-β-D-glucose (20)	**16b** 3-*O*-L-alanyl-β-D-glucose (12)	**16c** 6-*O*-L-alanyl-β-D-glucose (47)	30 (mono esters- 24, diesters-6)
16d 2,6-di-*O*-L-alanyl-β-D-glucose (15)	**16e** 3,6-di-*O*-L-alanyl-β-D-glucose (6)		
17a 2-*O*-L-alanyl-D-galactose (33)	**17b** 3-*O*-L-alanyl-D-galactose (32)	**17c** 6-*O*-L-alanyl-D-galactose (35)	21 (only mono esters)
18a 3-*O*-L-alanyl-D-mannose (25)	**18b** 4-*O*-L-alanyl-D-mannose (25)	**18c** 6-*O*-L-alanyl-D-mannose (30)	49 (mono esters-39, diesters-10)
18d 3,6-di-*O*-L-alanyl-D-mannose (9)	**18e** 4,6-di-*O*-L-alanyl-D-mannose (11)		
19a 1-*O*-L-alanyl-D-fructose (34)	**19b** 6-*O*-L-alanyl-D-fructose (34)	**19c** 1,6-di-*O*-L-alanyl-D-fructose (32)	52 (mono esters-35, diester-17)
20a 2-*O*-L-alanyl-D-arabinose (33)	**20b** 5-*O*-L-alanyl-D-arabinose (34)	**20c** 2,5-di-*O*-L-alanyl-D-arabinose (33)	9 [c] (mono esters-6, diester-3)

21a 3-*O*-L-alanyl-D-ribose (16)   **21b** 5-*O*-L-alanyl-D-ribose (32)   **21c** 3,5-di-*O*-L-alanyl-D-ribose (52)

48[c]
(mono esters-23, diester-25)

22a 6-*O*-L-alanyl-lactose (34)   **22b** 6′-*O*-L-alanyl-lactose (34)   **22c** 6,6′-di-*O*-L-alanyl-lactose (32)

20
(mono esters-14, diester-6)

23a 6-*O*-L-alanyl- maltose (34)   **23b** 6′-*O*-L-alanyl- maltose (34)

56
(mono esters-38, diester-18)

23c 6,6′-di-*O*-L-alanyl-maltose (32)

24 6-*O*-L-alanyl-sucrose

8
(only mono ester)

[a] L-Alanine – 2 mmol, carbohydrates – 1 mmol, RML – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) acetate buffer (pH 4.0), CH$_2$Cl$_2$:DMF (v/v 90:10) at 40 °C, Incubation period – 72 h. Conversion yields were from HPLC with respect to L-alanine concentration.

[b] Percentage proportions of individual esters were determined from the peak areas or from their cross peaks of the Carbon-13 C6 and C5 (in case of pentoses) signals in the 2D HSQCT spectrum.

[c] Several cross peaks, due to opening and/or degradation of the five membered ring during esterification.

Table 4.3 Preparation of L-alanyl ester of carbohydrate using lipases from *Candida rugosa* and porcine pancreas

L-Alanyl ester of carbohydrate	CRL[a] %Yield (mmol)	Crude PPL [b] %Yield (mmol)
L-Alanyl-D-glucose **16a-e**	25 (0.50)	78 (0.78)
L-Alanyl-D-galactose **17a-c**	22 (0.44)	72 (0.72)
L-Alanyl-D-mannose **18a-e**	33 (0.66)	10 (0.10)
L-Alanyl-D-fructose **19a-c**	12 (0.24)	67 (0.67)
L-Alanyl-D-arabinose **20a-c**	3 (0.06)	27 (0.27)
L-Alanyl-D-ribose **21a-c**	12 (0.24)	38 (0.38)
L-Alanyl- lactose **22a-c**	27 (0.54)	58 (0.58)
L-Alanyl- maltose **23a-c**	17 (0.34)	28 (0.28)
L-Alanyl- sucrose **24**	8 (0.16)	8 (0.83)

[a] L-Alanine – 2 mmol, carbohydrates – 1 mmol, CRL – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) phosphate buffer pH 7.0, CH_2Cl_2:DMF (v/v 90:10) at 40 $^{\circ}$C, Incubation period – 72 h. Conversion yields were from HPLC with respect to L-alanine concentration.
[b] L-Alanine – 1 mmol, carbohydrates – 1 mmol, crude PPL – 111% (w/w carbohydrate),buffer – 0.2 mM (0.2 ml of 0.1 M) acetate buffer pH 5.0, CH_2Cl_2: DMF (v/v 90:10) at 40°C, Incubation period – 72 h.

Spectral data for L-Alanine (1): Solid; Mpt: 258 $^{\circ}$C; HPLC t_{ret}: 2.6 min; R_f: 0.30;UV (H$_2$O, λ_{max}): 190.0 nm ($\sigma\rightarrow\sigma^*$ $\varepsilon_{190.0}$ − 111.9 M^{-1}), IR (KBr, stretching frequency): 3415 cm^{-1} (OH), 2945 cm^{-1} (CH), 1715 cm^{-1} (CO); optical rotation (c 1.0, H$_2$O): $[\alpha]_D$ at 25°C = +3.33°.

2D HSQCT (DMSO-d_6) : ^{1}H NMR δ (500.13 MHz) : 3.35 (αCH), 1.25 (βCH$_3$) ppm; ^{13}C NMR δ (125 MHz) : 53.0 (αCH), 15.5 (βCH$_3$), 170.5 (CO) ppm.

4.2.1.1. L-Alanyl-D-glucose (16a-e): Solid; HPLC t_{ret}: 3.4 min; R_f: 0.22; UV (H$_2$O, λ_{max}): 227.0nm ($\sigma\rightarrow\sigma^*$ $\varepsilon_{227.0}$ − 1150.8 M^{-1}), 294.0nm (n$\rightarrow\pi^*$ $\varepsilon_{294.0}$ − 763.8 M^{-1}); IR (KBr, stretching frequency): 3371cm^{-1} (NH), 3410 cm^{-1} (OH), 2297 cm^{-1} (CH), 1653 cm^{-1} (CO); MS (*m/z*) : 274 [M+Na]$^+$.

2D HSQCT (DMSO-d_6) **2-*O*- ester (16a)** : ^{1}H NMR δ (500.13 MHz) : 2.95 (αCH), 1.07 (βCH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6) ppm; ^{13}C NMR δ (125 MHz)

Fig. 4.2. UV spectra for L-alanyl-β-D-glucose **16a-e** from RML catalyzed reaction. (A) L-alanine (B) L-alanyl-D-glucose
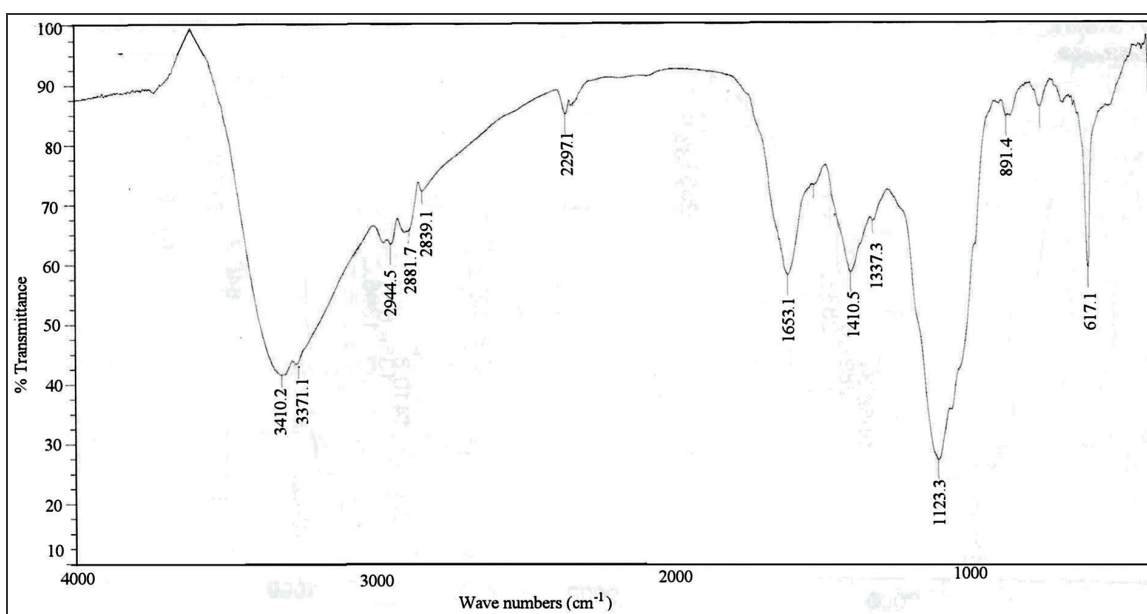
Fig. 4.3. A typical IR spectrum of L-alanyl-β-D-glucose **16a-e** by RML catalyzed reaction. A 2.0 mg of ester sample was prepared as KBr pellet and used.

Fig. 4.4. A typical mass spectrum of L-alanyl-β-D-glucose **16a-e**

: 52.1 (αCH), 15.7 (βCH$_3$), 102.8 (C1β), 82.6 (C2β), 77.9 (C3β), 68.8 (C4β), 60.5 (C6β) ppm; **3-*O*- ester (16b)** : ^{1}H NMR δ: 2.87 (αCH), 3.93 (H-3β), 3.58 (H-4β), 3.36 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.4 (αCH), 83.3 (C3β), 69.3 (C4β), 57.3 (C6β) ppm; **6-*O*- ester (16c)** : ^{1}H NMR δ : 2.95 (αCH), 1.30 (βCH$_3$), 3.86 (H-2β), 3.76 (H-5β), 3.82 (H-6) ppm; ^{13}C NMR δ : 50.2 (αCH), 15.1 (βCH$_3$), 171.4 (CO), 101.8 (C1β), 75.0 (C2β), 70.1 (C5β), 63.5 (C6β) ppm; **2,6-di-*O*- ester (16d)** : ^{1}H NMR δ : 3.36 (αCH), 1.30 (βCH$_3$), 3.78 (H-2β), 3.47 (H-6) ppm; ^{13}C NMR δ : 49.5 (αCH), 16.4 (βCH$_3$), 100.8 (C1β), 76.5 (C2β), 62.7 (C6β) ppm; **3,6-di-*O*- ester (16e)** : ^{1}H NMR δ: 1.30 (βCH$_3$), 3.78 (H-3β), 3.82 (H-6) ppm; ^{13}C NMR δ : 51.4 (αCH), 16.7 (βCH$_3$), 81.6 (C3β), 63.1 (C6β) ppm.

A typical UV, IR, mass and 2D-HSQCT NMR spectra for L-alanyl-D-glucose **16a-e** are shown in Figures 4.2, 4.3, 4.4 and 4.5 respectively.

4.2.1.2. L-Alanyl-D-galactose (17a-c): Solid; HPLC t_{ret}: 3.4 min; R_f: 0.19; UV (H$_2$O, λ$_{max}$): 200.0 nm (σ→σ* ε$_{200.0}$ − 2630.3 M^{-1}), 295.0 nm (n→π* ε$_{295.0}$ − 2089.3 M^{-1}); IR (KBr, stretching frequency): 2889 cm^{-1} (NH), 3407 cm^{-1} (OH), 2950 cm^{-1} (CH), 1622 cm^{-1} (CO); MS (*m/z*) : 274 [M+Na]$^+$.

2D HSQCT (DMSO-d_6) **2-*O*- ester (17a)** : ^{1}H NMR δ (500.13 MHz) : 2.95 (αCH), 3.38 (H-2α), 3.36 (H-2β), 3.55 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.5 (αCH), 76.4(C2α), 76.5 (C2β), 60.7(C6β) ppm; **3-*O*- ester (17b)** : ^{1}H NMR δ : 3.75 (H-3α), 3.60 (H-3β), 3.35 (H-6) ppm; ^{13}C NMR δ : 81.6 (C3α), 82.6 (C3β), 60.7 (C6α) ppm; **6-*O*- ester (17c)** : ^{1}H NMR δ : 3.05 (αCH), 4.90 (H-1α), 4.85 (H-1β), 3.85 (H-3α), 3.30 (H-6) ppm; ^{13}C NMR δ : 52.0 (αCH), 175.0 (CO), 95.4 (C1α), 101.8 (C1β), 70.8 (C3α), 63.1 (C6α) ppm.
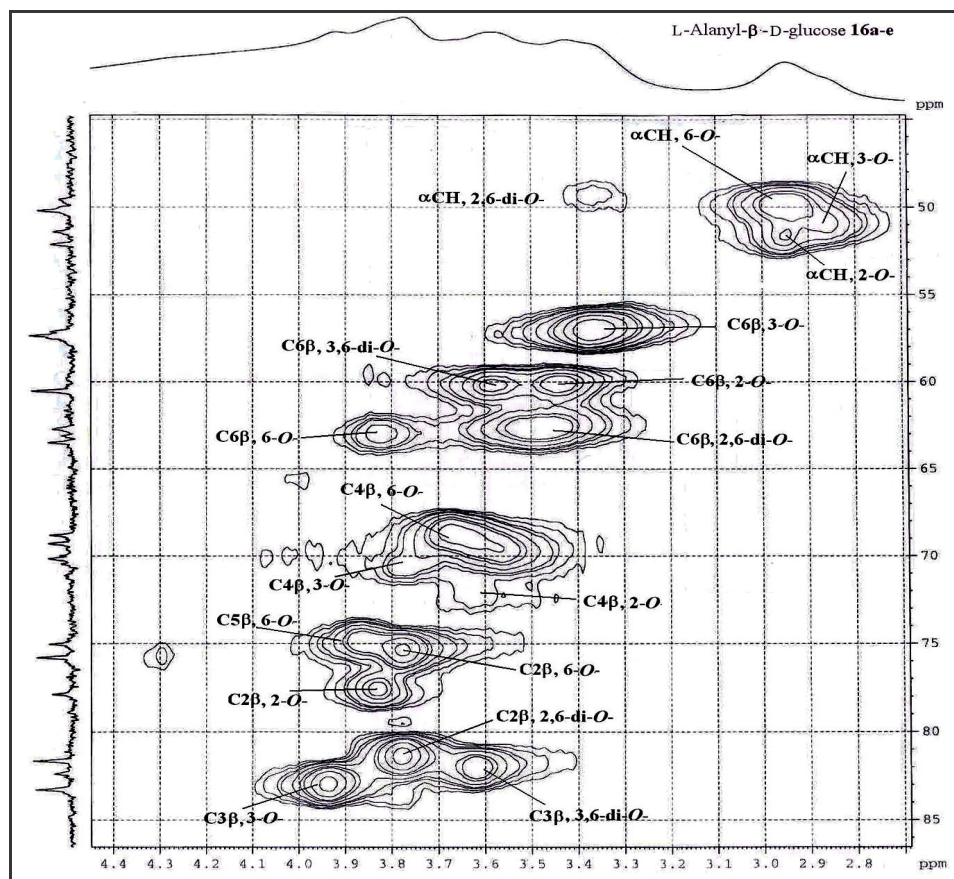
Fig. 4.5. Two-dimensional HSQCT NMR spectrum for L-alanyl-β-D-glucose **16a-e** of RML catalyzed reaction. Isolated through repeated HPLC injections in an aminopropyl column eluted with acetonitrile:water (v/v 80:20) as mobile phase and detected by Refractive Index detector.

A typical 2D-HSQCT NMR spectrum for L-alanyl-D-galactose **17a-c** is shown in Figure 4.6.

4.2.1.3. L–Alanyl–D–mannose (18a-e) : Solid; HPLC t_{ret}: 3.4 min; R_f: 0.19; UV(H$_2$O, λ_{max}): 194.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{194.0}$ $-$ 2630.3 M^{-1}), 295.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{295.0}$ $-$ 1047.1 M^{-1}); IR (KBr, stretching frequency): 2887 cm^{-1} (NH), 3387 cm^{-1} (OH), 2816 cm^{-1} (CH), 1626 cm^{-1}(CO); MS (*m/z*) : 274 [M+Na]$^+$.

2D HSQCT (DMSO-d_6) **3-O- ester (18a)**: ^{13}C NMR δ (125 MHz) : 51.5 (αCH), 15.2 (βCH$_3$), 74.0 (C2α), 89.2 (C3α), 60.1 (C6α) ppm; **4-O- ester (18b)** : ^{13}C NMR δ : 49.7 (αCH), 14.7 (βCH$_3$), 75.32 (C4β), 60.1 (C6α) ppm; **6-O- ester (18c)** : ^{13}C NMR δ : 52.1 (αCH), 15.9 (βCH$_3$), 172.0 (CO), 95.5 (C1α), 101.5 (C1β), 69.7 (C2α), 69.0 (C3α), 68.4 (C4α), 74.9 (C5α), 63.1 (C6α) ppm; **3,6-di-O- ester (18d)** : ^{13}C NMR δ : 82.0 (C3α), 82.9 (C3β), 62.8 (C6α) ppm; **4,6-di-O- ester (18e)** : ^{13}C NMR δ : 77.2 (C4α), 62.5 (C6α) ppm.

A typical ^{13}C NMR spectrum for L-alanyl-D-mannose **18a-e** is shown in Figure 4.7.

4.2.1.4. L–Alanyl–D–fructose (19a-c) : Solid; HPLC t_{ret}: 3.5 min; R_f: 0.19; UV (H$_2$O, λ_{max}): 200.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{200.0}$ $-$ 2630.3 M^{-1}), 295.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{295.0}$ $-$ 2089.3 M^{-1}); IR (KBr, stretching frequency): 2889 cm^{-1}(NH), 3407 cm^{-1}(OH), 2950 cm^{-1}(CH), 1622 cm^{-1}(CO); MS (*m/z*) : 274 [M+Na]$^+$.

2D HSQCT (DMSO-d_6) **1-O- ester (19a)** : ^{1}H NMR δ (500.13 MHz) : 3.05 (αCH), 3.40 (H-1α), 4.85 (H-2β), 3.85 (H-3α), 3.30 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 52.0 (αCH), 175.0 (CO), 63.5 (C1α), 102.4 (C2β), 70.8 (C3α), 62.8 (C6α) ppm; **6-O- ester (19b)** : ^{1}H NMR δ : 3.75 (H-3α), 3.60 (H-3β),   3.30 (H-6) ppm; ^{13}C NMR δ : 81.6 (C3α), 82.6 (C3β), 63.6 (C6α) ppm; **1,6-di-O- ester (19c)** : ^{1}H NMR δ : 2.95 (αCH), 3.12 (H-
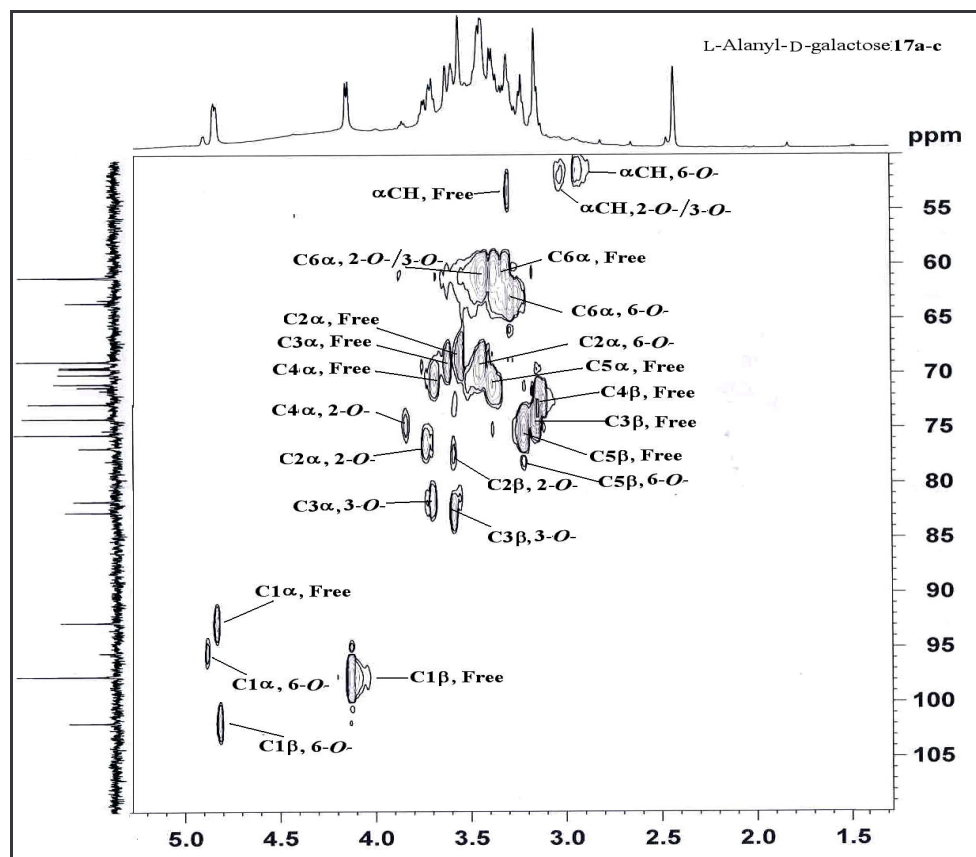
Fig. 4.6. Two-dimensional HSQCT NMR spectrum for L-alanyl-D-galactose **17a-c** of RML catalyzed reaction.
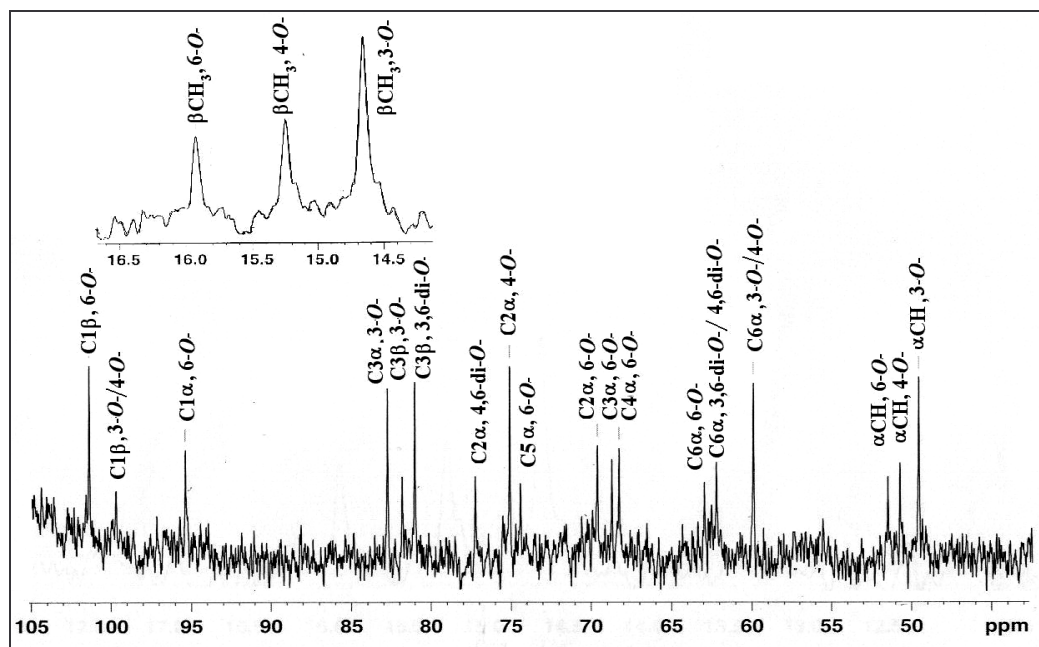
Fig. 4.7. ^{13}C NMR spectrum for L-alanyl-D-mannose **18a-e** catalyzed by RML. Isolated through Sephadex G-10 using water as eluant.

1α), 3.38 (H-2α), 3.36 (H-2β), 3.55 (H-6) ppm; ^{13}C NMR δ : 51.5 (αCH), 63.6 (C1α),

76.4 (C2α), 76.5 (C2β) ppm.

A typical mass spectrum for L-alanyl-D-fructose **19a-c** is shown in Figure 4.8.

4.2.1.5. L–Alanyl–D–arabinose (20a-c): Solid; HPLC t_{ret}: 3.2 min; R_f: 0.23; UV (H$_2$O, λ

$_{max}$): 200.0 nm (σ→σ* $\varepsilon_{200.0}$ − 1584.9 M^{-1}), 276.0 nm (n→π* $\varepsilon_{276.0}$ − 933.3 M^{-1}); IR

(KBr, stretching frequency): 3397 cm^{-1} (NH), 3443 cm^{-1} (OH), 2940 cm^{-1} (CH), 1629

cm^{-1} (CO); MS (*m/z*) : 219 [M-2]$^+$ and 293 [M+1]$^+$.

2D HSQCT (DMSO-d_6) **2-O- ester (20a)** : ^{1}H NMR δ (500.13 MHz) : 3.15 (αCH), 1.22

(βCH$_3$), 5.0 (H-1α), 4.93 (H-1β), 3.60 (H-2α), 3.15 (H-3α), 3.35 (H-5) ppm; ^{13}C NMR

δ (125 MHz) : 48.2 (αCH), 17.2 (βCH$_3$), 172.5 (CO), 96.0 (C1α), 102.0 (C1β),

77.8(C2α), 72.0 (C3α), 63.5 (C5α) ppm; **5-O- ester (20b)** : ^{1}H NMR δ (500.13 MHz)

3.92 (αCH), 1.20 (βCH$_3$), 4.30 (H-1α), 4.18 (H-1β), 3.35 (H-2α), 3.25 (H-3α), 3.60 (H-

4α), 3.60 (H-5) ppm; ^{13}C NMR δ : 48.2 (αCH), 16.0 (βCH$_3$), 97.1 (C1α), 104.0 (C1β),

72.9 (C2α), 72.0 (C3α), 67.8 (C4α), 65.1 (C5α) ppm; **2,5-di-O- ester (20c)** : ^{1}H NMR δ

: 1.30 (βCH$_3$), 3.45 (H-2α), 3.30 (H-5) ppm; ^{13}C NMR δ : 17.2 (βCH$_3$), 76.9 (C2α),

65.1 (C5α) ppm.

A typical UV and mass spectra for L-alanyl-D-arabinose **20a-c** are shown in Figures 4.9

and 4.10 respectively

4.2.1.6. L–Alanyl–D–ribose (21a-c): Solid; HPLC t_{ret}: 3.2 min; R_f: 0.22; UV (H$_2$O, λ

$_{max}$): 224.0 nm (σ→σ* $\varepsilon_{224.0}$ − 3801.9 M^{-1}), 294.0 nm (n→π* $\varepsilon_{294.0}$ − 1288.2 M^{-1}); IR

(KBr, stretching frequency): 3402 cm^{-1} (NH), 3242 cm^{-1} (OH), 2887 cm^{-1} (CH), 1625

cm^{-1} (CO); MS (*m/z*): 221 [M]$^+$.

2D HSQCT (DMSO-d_6) **3-O- ester (21a)**: ^{1}H NMR δ (500.13 MHz): 1.25 (αCH), 3.12

(βCH$_3$), 3.50 (H-2α), 3.67 (H-3α), 3.63 (H-4α), 3.64 (H-5) ppm; ^{13}C NMR δ (125
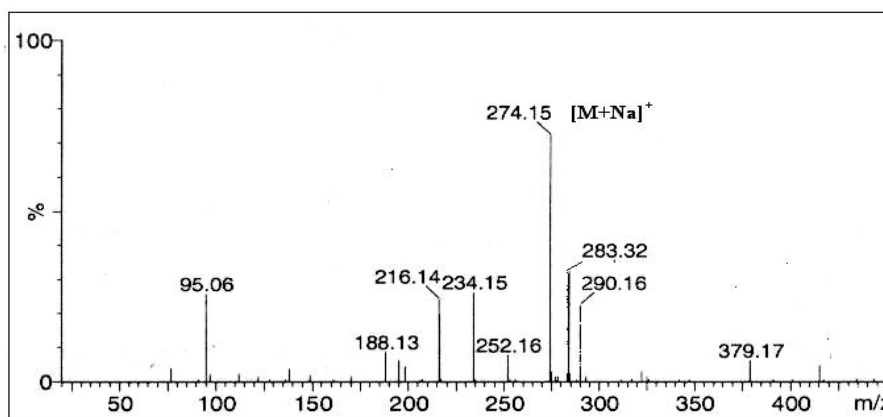
Fig. 4.8. A typical mass spectrum of L-alanyl-D-fructose **19a-c**.
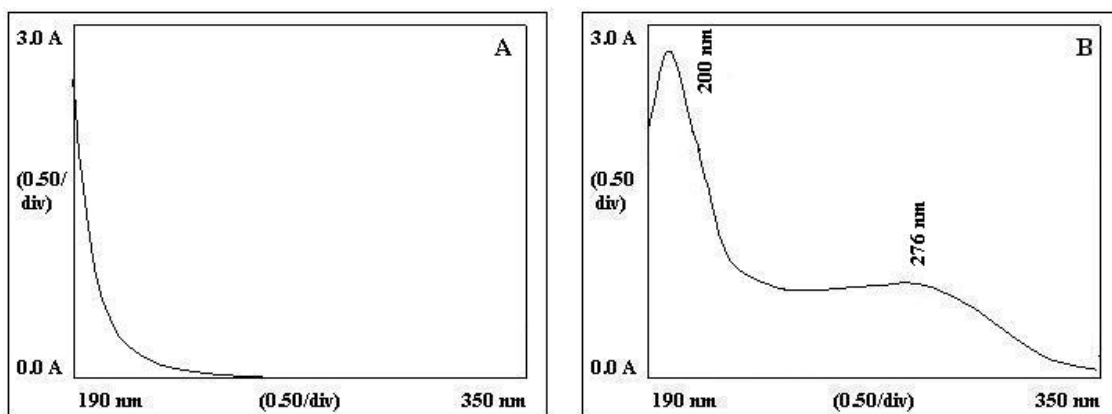

Fig. 4.9. UV spectra for L-alanyl-D-arabinose **20a-c** from RML catalyzed reaction. (A) L-alanine. (B) L-alanyl-D-arabinose.
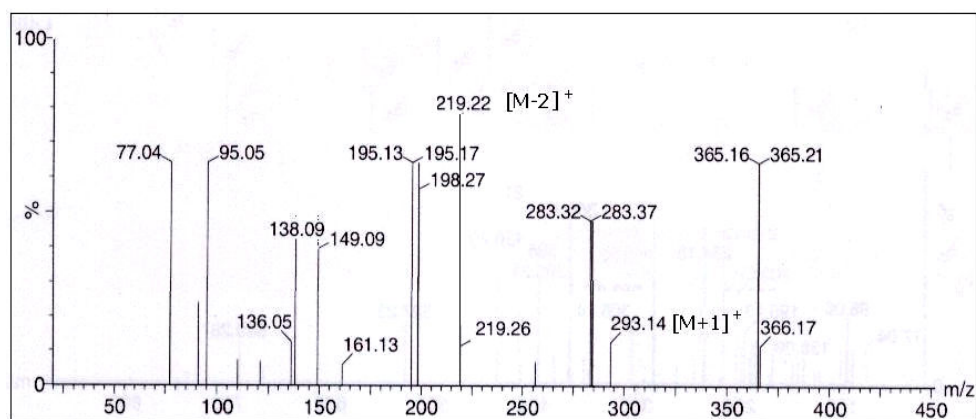

Fig. 4.10. A typical mass spectrum for L-alanyl-D-arabinose **20a-c**.

MHz): 48.2 (αCH), 15.9 (βCH$_3$), 67.2 (C2α), 75.7 (C3α), 68.1 (C4α), 60.6 (C5α) ppm;

5-O- ester (21b) : ^{1}H NMR δ : 3.39 (αCH), 1.25 (βCH$_3$), 4.95 (H-1α), 4.20 (H-1β), 3.27

(H-3α), 3.88 (H-4α), 3.61(H-5) ppm; ^{13}C NMR δ : 53.0 (αCH), 18.5 (βCH$_3$), 173.5

(CO), 101.6 (C1α), 103.9 (C1β), 75.0 (C3α), 71.0 (C4α), 63.4 (C5α) ppm; **3,5-di-*O-***

ester (21c) : ^{1}H NMR δ : 1.20 (βCH$_3$), 3.45 (H-3α), 3.79 (H-4α), 3.52 (H-5) ppm; ^{13}C

NMR δ : 18.5 (βCH$_3$), 74.9 (C3α), 63.4 (C5α) ppm.

A typical UV and mass spectra for L-alanyl-D-ribose **22a-c** are shown in Figures 4.11

and 4.12 respectively.

4.2.1.7. L–Alanyl–lactose (22a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.10; UV (H$_2$O, λ $_{max}$):

220.0 nm (σ→σ* ε$_{220.0}$ − 436.5 M^{-1}), 294.0 nm (n→π* ε$_{294.0}$ − 239.9 M^{-1}); IR (KBr,

stretching frequency): 3378 cm^{-1} (NH), 3378 cm^{-1} (OH), 2946 cm^{-1} (CH), 1624 cm^{-1}

(CO); MS (*m/z*) : 436 [M+Na]$^+$.

2D HSQCT (DMSO-d_6) **6-O- ester (22a)** : ^{1}H NMR δ (500.13 MHz) : 3.55 (αCH), 1.25

(βCH$_3$), 4.78 (H-1α), 4.82 (H-1β), 2.95 (H-2α), 3.25 (H-2β), 2.95 (H-3α), 4.05 (H-

4α,β), 3.15 (H-5α), 3.35 (H-5β), 3.80 (H-6), 4.90 (H′-1β), 3.90 (H′-2), 2.85 (H′-3), 3.70

(H′-4), 3.60 (H′-5), 3.40 (H′-6) ppm; ^{13}C NMR δ (125 MHz) : 51.0 (αCH), 15.5 (βCH$_3$),

173.0 (CO), 98.0 (C1α), 100.2 (C1β), 70.3 (C2α), 72.4 (C2β), 74.3 (C3α), 81.0 (C4α,β),

73.3 (C5α), 73.4 (C5β), 61.2 (C6α,β), 100.2 (C′1β), 76.5 (C′2), 75.1 (C′3), 68.5 (C′4),

78.5 (C′5), 60.6 (C′6) ppm; **6′-*O*- ester (22b)**: ^{1}H NMR δ : 3.35 (αCH), 3.85 (H-4α),

3.70 (H′-6) ppm; ^{13}C NMR δ : 53.5 (αCH), 81.5 (C4α), 64.0 (C′6) ppm. **6,6′-di-*O*- ester**

(22c) : ^{1}H NMR δ : 3.85 (H′-6) ppm; ^{13}C NMR δ : 67.5 (C′6) ppm.

A typical mass spectrum for L-alanyl-lactose **22a-c** is shown in Figure 4.13.

4.2.1.8. L-Alanyl-maltose (23a-c) : Solid; HPLC t_{ret}: 3.3 min; R_f: 0.11;UV (H$_2$O, λ $_{max}$):

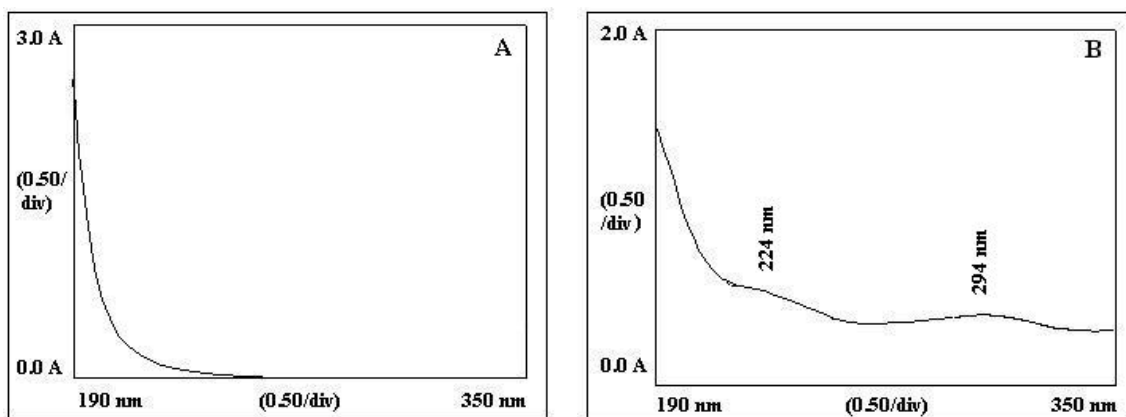228.0 nm (σ→σ* ε$_{228.0}$ − 114.8 M^{-1}), 294.0 nm (n→π* ε$_{294.0}$− 56.2 M^{-1}); IR (KBr,

Fig. 4.11. UV spectra for L-alanyl-D-ribose **21a-c** from RML catalyzed reaction. (A) L-alanine. (B) L-alanyl-D-ribose.
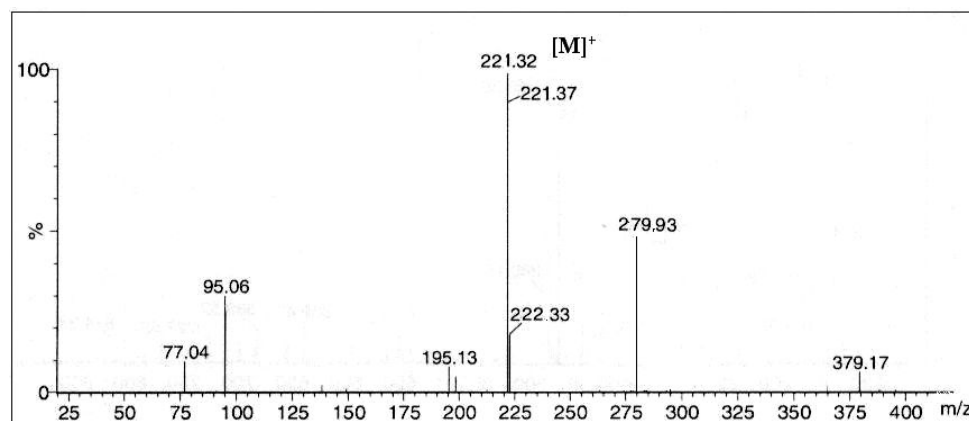

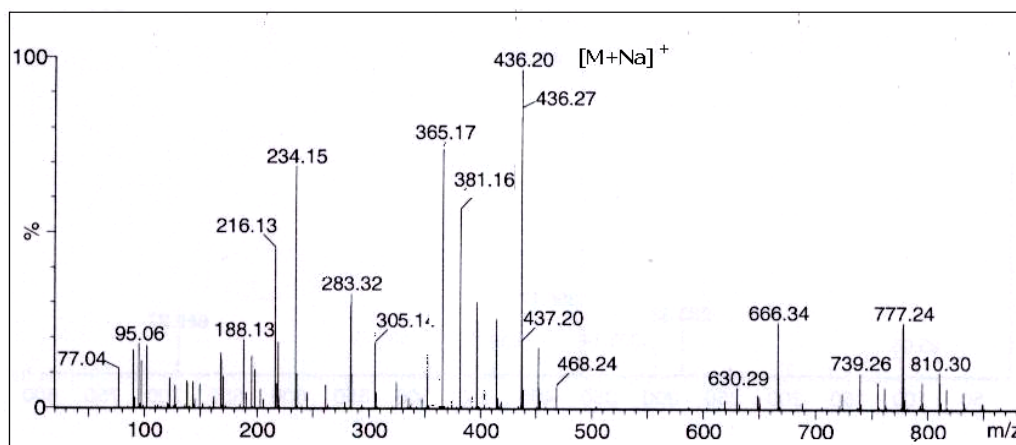**Fig. 4.12.** A typical mass spectrum for L-alanyl-D-ribose **21a-c**.

Fig. 4.13. A typical mass spectrum for L-alanyl-lactose **22a-c**.

stretching frequency): 3283 cm^{-1} (NH), 3380 cm^{-1} (OH), 2937 cm^{-1} (CH), 1626 cm^{-1} (CO); MS (*m/z*) : 436 [M+Na]$^+$.

2D HSQCT (DMSO-*d₆*) **6-O- ester (23a):** ^{1}H NMR δ (500.13 MHz): 3.55 (αCH), 1.25 (βCH₃), 4.80 (H-1α), 4.20 (H-1β), 4.05 (H-2α,β), 3.30 (H-3α), 3.85 (H-4α,β), 3.65 (H-5α,β), 3.50 (H-6), 4.90 (H′-1α), 2.95 (H′-2), 3.10 (H′-3), 3.50 (H′-4), 3.60 (H′-5), 3.60 (H′-6) ppm; ^{13}C NMR δ (125 MHz): 50.0 (αCH), 175.5 (CO), 92.0 (C1α), 96.7 (C1β), 79.7 (C2α,β), 76.4 (C3α), 81.5 (C4α,β), 77.2 (C5α,β), 67.5 (C6α,β), 100.7 (C′1α), 70.3 (C′2), 71.8 (C′3), 69.9 (C4′), 72.4 (C′5), 60.6 (C′6) ppm; **6′-O- ester (23b):** ^{1}H NMR δ : 3.35 (αCH), 3.95 (H′-6) ppm; ^{13}C NMR δ) : 53.0 (αCH), 67.0 (C′6α) ppm; **6,6′-di-O-ester (23c)**: ^{1}H NMR δ : 3.75 (H′-6) ppm; ^{13}C NMR δ: 63.0 (C′6) ppm.

A typical IR and 2D-HSQCT NMR spectra for L-alanyl-maltose **23a-c** are shown in Figures 4.14 and 4.15 respectively.

4.2.1.9. L–Alanyl–sucrose (24): Solid; Mpt : 155 ^{o}C; HPLC t_{ret}: 3.3 min; R_f: 0.13; UV (H₂O, λ $_{max}$): 224.0 nm (σ→σ* ε$_{224.0}$ − 2344.2 M^{-1}), 294.0 nm (n→π* ε$_{294.0}$ − 1288.2 M^{-1}); IR (KBr, stretching frequency): 3100 cm^{-1} (NH), 3319 cm^{-1} (OH), 2958 cm^{-1} (CH), 1625 cm^{-1} (CO); optical rotation (*c* 0.5, H₂O) : [α]$_D$ at 25 ^{o}C = -17.4^o; MS (*m/z*): 436 [M+Na]$^+$.

2D HSQCT (DMSO-*d₆*) **6-O- ester (24):** ^{1}H NMR δ (500.13 MHz): 3.59 (αCH), 1.30 (βCH₃), 3.41 (H-1β), 3.79 (H-3β), 3.54 (H-4β), 3.47 (H-5β), 3.67 (H-6), 4.35 (H′-1α), 3.31 (H′-2), 3.45 (H′-3), 3.31 (H′-4), 3.58 (H′-5), 3.54 (H′-6) ppm; ^{13}C NMR δ (125 MHz): 49.5 (αCH), 18.0 (βCH₃), 172.0 (CO), 64.0 (C1β), 104.2 (C2β), 77.33 (C3β), 82.71 (C4β), 74.5 (C5β), 67.0 (C6β), 96.0 (C′1α), 71.0 (C′2), 75.5 (C′3), 71.0 (C4′), 74.0 (C′5), 60.71 (C′6) ppm.

A typical 2D-HSQCT NMR spectrum for L-alanyl-sucrose **24** is shown in Figures 4.16.
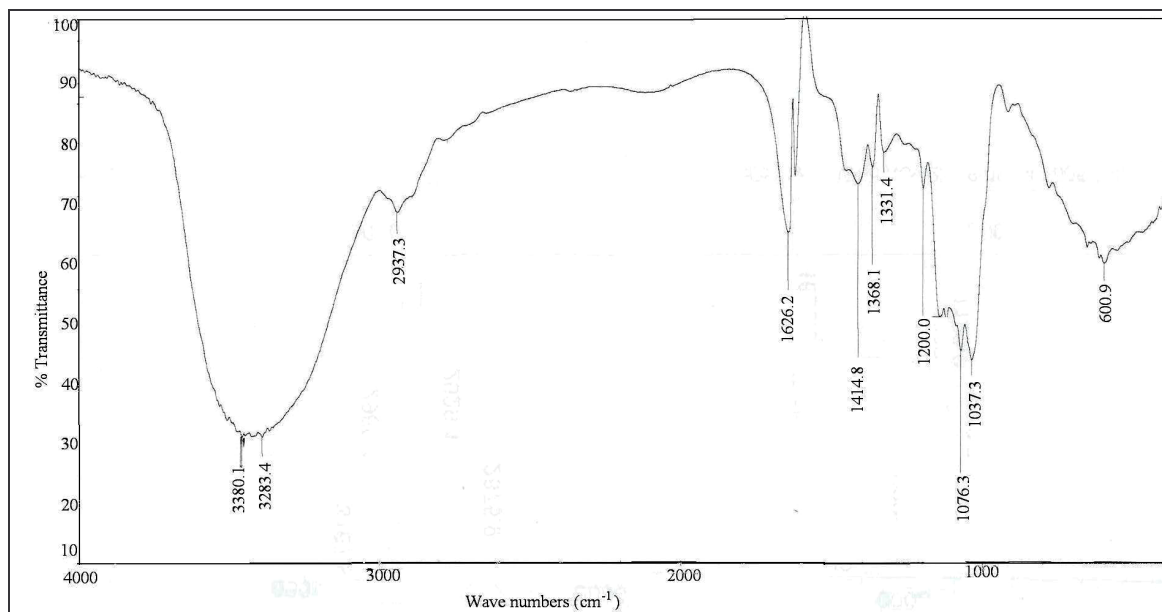
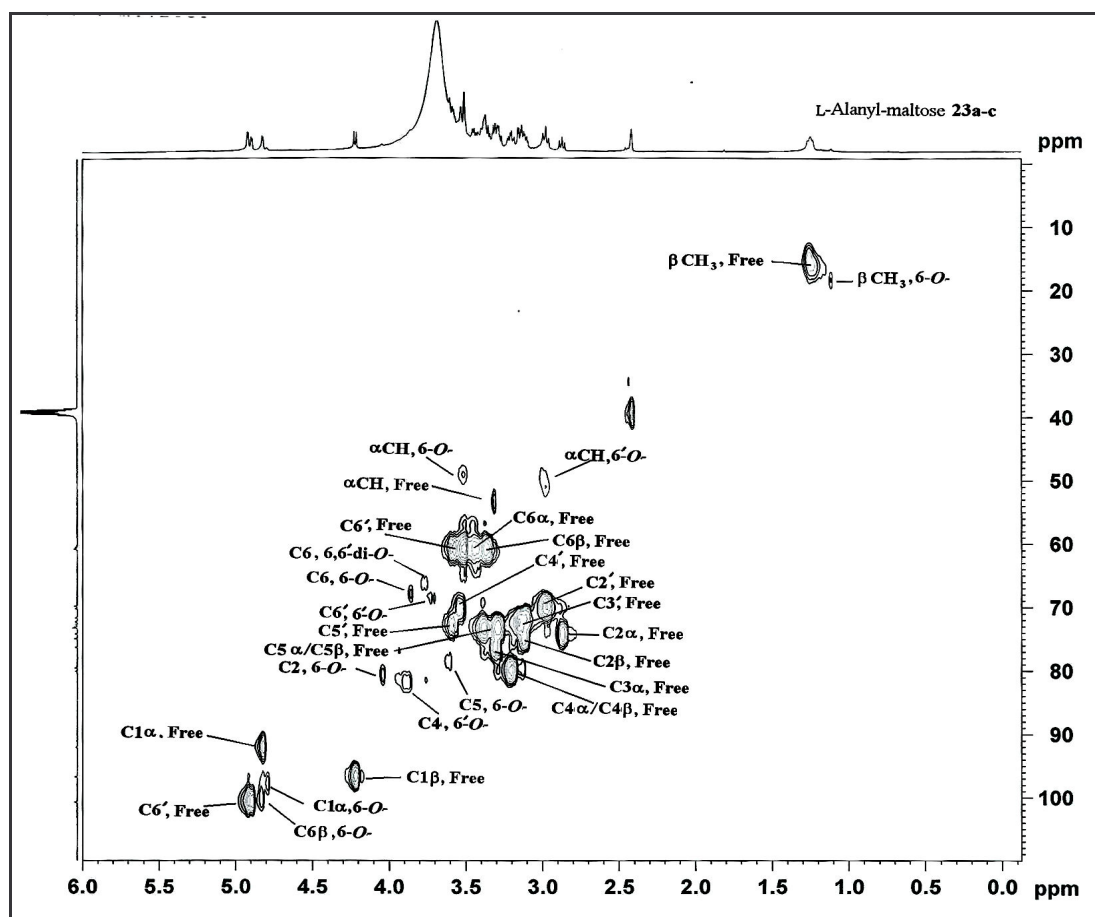**Fig. 4.14.** A typical IR spectrum of L-alanyl-D-maltose **23a-c** of CRL catalyzed reaction.
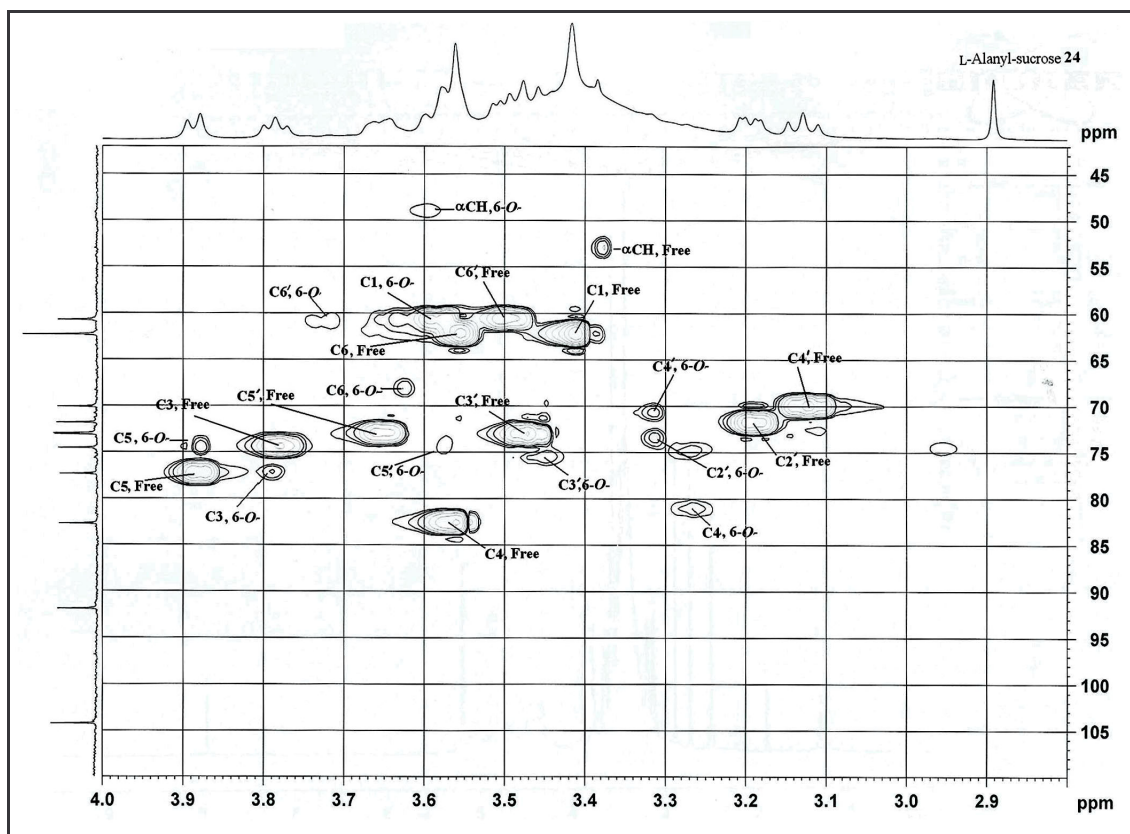

Fig. 4.15. Two-dimensional HSQCT NMR spectrum for L-alanyl-maltose **23a-c** prepared through RML catalysis.

Fig. 4.16. Two-dimensional HSQCT NMR spectrum for L-alanyl-sucrose **24** of RML catalyzed reaction.

4.2.2. Syntheses of L-valyl esters of carbohydrates 25a-e to 33

L-Valine (L-2-amino-3-methyl butanoic acid) is a polar and an essential dietary amino acid containing isopropyl group as a side chain. Using optimum conditions, an attempt was made to prepare L-valyl esters of different carbohydrates (**Scheme 4.2**). Esterification of L-valine with carbohydrates was carried out using CRL and crude PPL under optimal conditions (Section 2.2.4). The reaction mixture consists of 1-2 mmol L-valine **2** and 1 mmol carbohydrates (D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14**, D-sorbitol **15**) along with 40 % CRL (w/w carbohydrate) / 111 % of crude PPL (w/w carbohydrate) and incubated in 100 ml of CH_2Cl_2 and DMF (v/v 90:10, 40 $^{\circ}$C) containing 0.1 mM (0.1 ml of 0.1M) of phosphate buffer, pH 7.0 (CRL) or 0.2 mM (0.2 ml of 0.1M) of acetate buffer, pH 5.0 (crude PPL). The reaction mixture was analyzed by HPLC using C-18 column with acetonitrile:water (v/v 20:80) as mobile phase and detected at 210 nm (Fig. 4.17). Ester formation was also monitored by TLC and spots were detected by spraying ninhydrin (for NH_2 group detection) and 1-naphthol (for reducing sugar detection). The retention times (t_{ret}) by HPLC and retention factor (R_f) values by TLC are mentioned in sections 4.2.2.1 – 4.2.2.9.
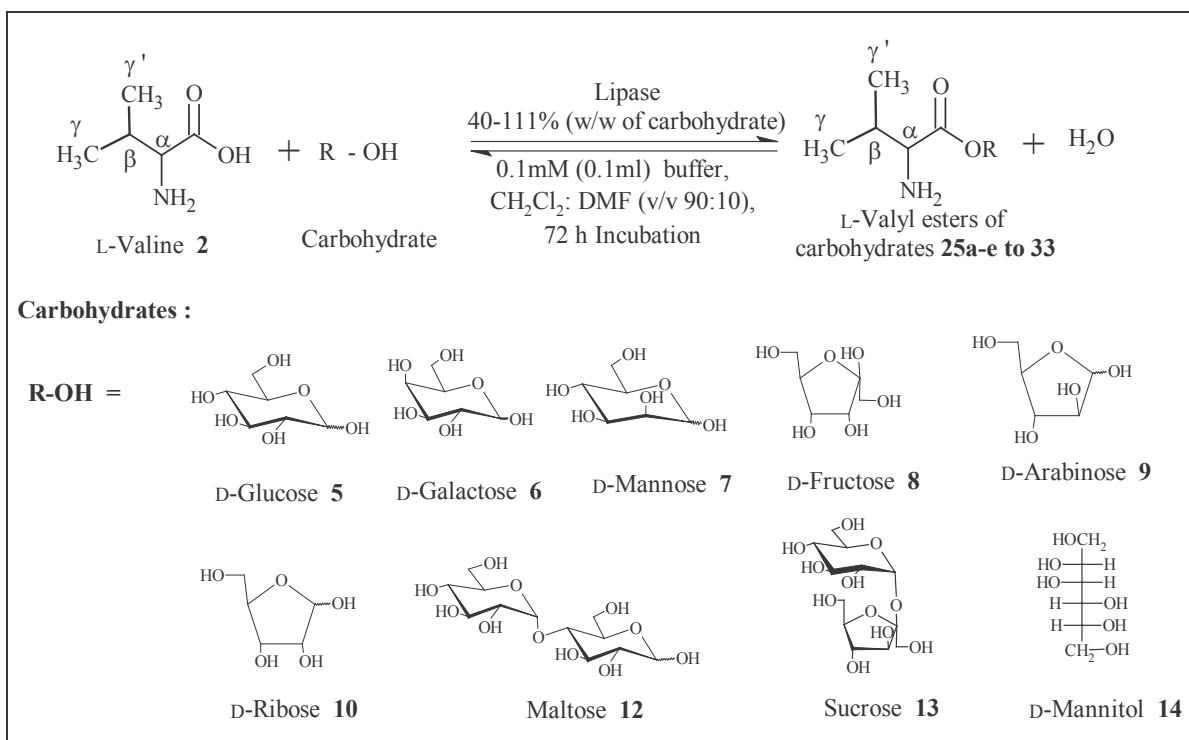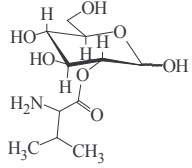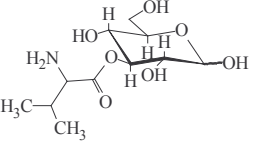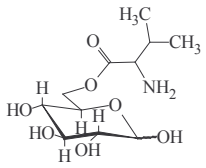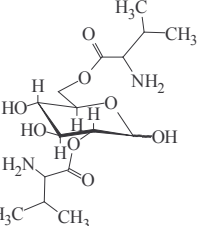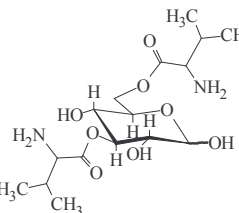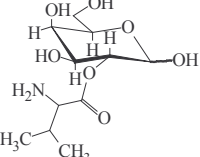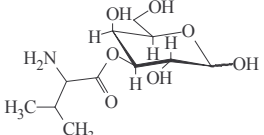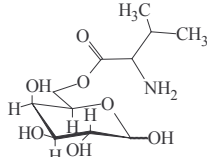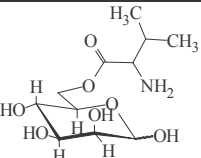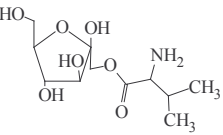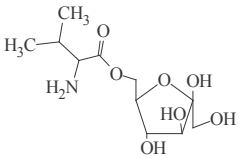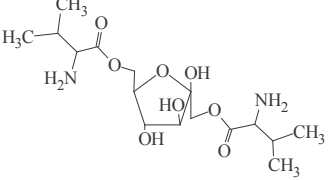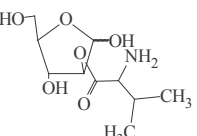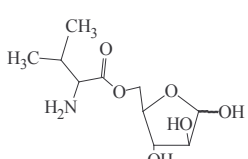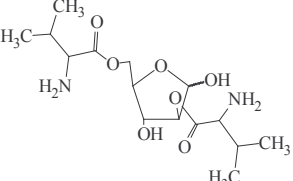
Scheme 4.2 Lipase catalyzed syntheses of L-valyl esters of carbohydrates
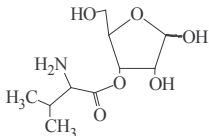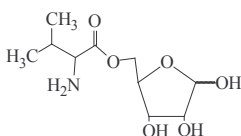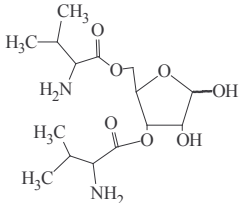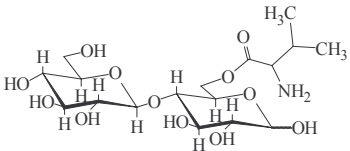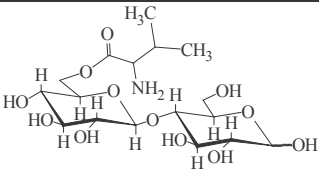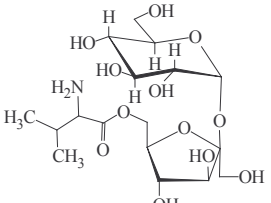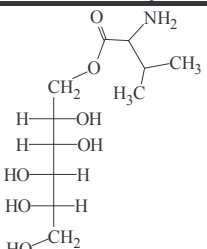
The isolated esters were subjected to UV, IR, MS, optical rotation and 2D- NMR characterization (Sections 4.2.2.1 – 4.2.2.9). The spectral data for the isolated esters are shown in Sections 4.2.2.1 – 4.2.2.9. Table 4.4 shows the HPLC ester yields, types of esters formed and percentage proportions of the individual esters from CRL catalyzed reaction and Table 4.5 shows the HPLC ester yields obtained through crude PPL catalysis.
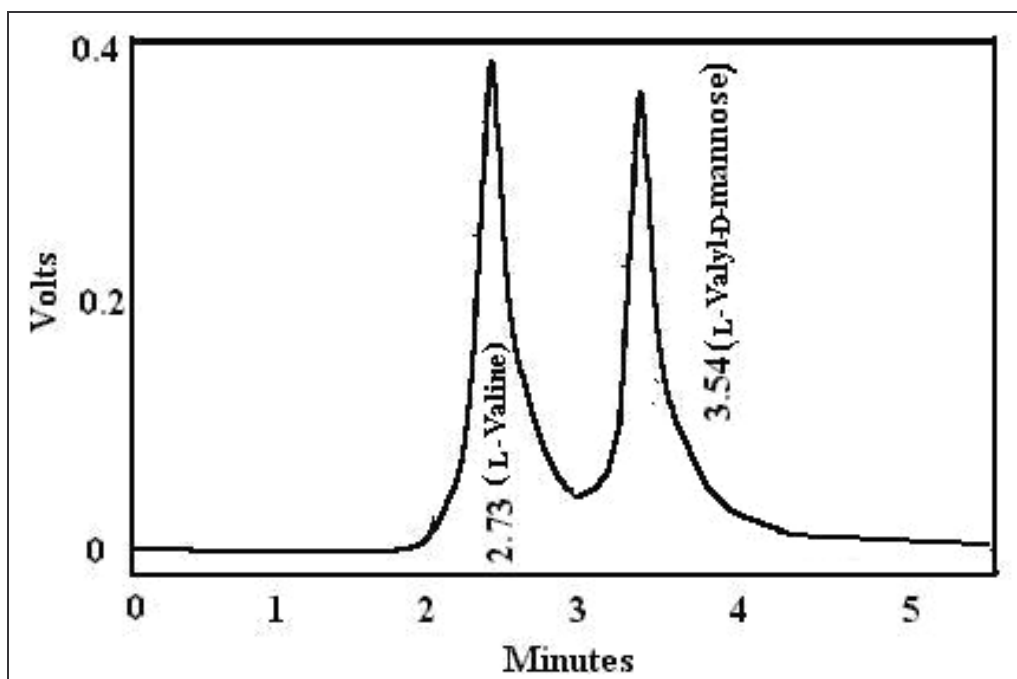
Fig. 4.17. HPLC chromatogram: reaction mixture of L-valine and D-mannose esterification catalysed by CRL. Column – C-18; mobile phase – acetonitrile:water (v/v 20:80); flow rate - 1 ml/min; detector – UV at 210 nm; errors in conversion yields are within ± 10 %.

Table 4.4. Syntheses of L-valyl esters of carbohydrates [a]

L-Valyl esters of carbohydrates (% proportions [b])			Yield (%)
25a 2-*O*-L-valyl-D-glucose (10)	**25b** 3-*O*-L-valyl-D-glucose (12)	**25c** 6-*O*-L-valyl-D-glucose (31)	68 (mono esters- 36, di esters- 32)
25d 2,6-di-*O*-L-valyl-D-glucose (23)	**25e** 3,6-di-*O*-L- valyl-D-glucose (24)		
26a 2-*O*-L-valyl –D-galactose (48)	**26b** 3-*O*-L-valyl-D-galactose (26)	**26c** 6-*O*-L-valyl-D-galactose (26)	30 (only mono esters)
27 6-*O*-L-valyl-D-mannose			51 (only mono ester)
28a 1-*O*-L-valyl-D-fructose (29)	**28b** 6-*O*-L-valyl-D-fructose (34)	**28c** 1,6-di-*O*-L-valyl-D-fructose (37)	34 (mono esters-21, diester-13)
29a 2-*O*-L-valyl-D-arabinose (32)	**29b** 5-*O*-L-valyl-D-arabinose (25)	**29c** 2,5-di-*O*-L-valyl-D-arabinose (43)	25 [c] (mono esters-14, diester-11)

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30a 3-*O*-L-valyl-D-ribose (26)	**30b** 5-*O*-L-valyl-D-ribose (26)	**30c** 3,5-di-*O*-L-valyl-D-ribose (48)	33 [c] (mono esters-17, diester-16)
31a 6-*O*-L-valyl-maltose (49)	**31b** 6′-*O*-L-valyl-maltose (51)		47 (only mono esters)
32 6-*O*-L-valyl- sucrose			60 (only mono ester)
33 1-*O*-L-valyl-D-mannitol			52 (only mono ester)

[a] L-Valine – 2 mmol, carbohydrates – 1 mmol, CRL – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1M) phosphate buffer pH 7.0, CH$_2$Cl$_2$:DMF (v/v, 90:10) at 40 ^{o}C, incubation period – 72 h. Conversion yields were from HPLC with respect to L-valine concentration.

[b] Percentage proportions of individual esters were determined from the peak areas or from their cross peaks of the Carbon-13 C6 and C5 (in case of pentoses) signals in the 2D HSQCT spectrum.

[c] Several cross peaks, due to opening and/or degradation of the five membered ring during esterification

Table 4.5 Preparation of ʟ-valyl esters of carbohydrates using crude porcine pancreas lipase [a]

ʟ-Valyl ester of carbohydrate	%Yield (mmol)
ʟ- Valyl-ᴅ-glucose **25a-e**	31 (0.31)
ʟ- Valyl-ᴅ-galactose **26a-c**	26 (0.26)
ʟ- Valyl-ᴅ-mannose **27**	43 (0.43
ʟ- Valyl-ᴅ-fructose **28a-c**	47 (0.47)
ʟ- Valyl-ᴅ-arabinose **29a-c**	31 (0.31)
ʟ- Valyl-ᴅ-ribose **30a-c**	53 (0.53)
ʟ- Valyl-maltose **31a,b**	79 (0.79)
ʟ- Valyl-sucrose **32**	58 (0.58)
ʟ- Valyl-ᴅ-mannitol **33**	52 (0.52)

[a] ʟ-Valine – 1 mmol, carbohydrates – 1 mmol, Crude PPL – 111 % (w/w carbohydrate), buffer – 0.2 mM (0.2 ml of 0.1 M) acetate buffer pH 5.0, CH_2Cl_2:DMF (v/v 90:10) at 40 °C, Incubation period – 72 h. Conversion yields were from HPLC with respect to ʟ-valine concentration.

Spectral data for ʟ-Valine (2): Solid; Mpt : 298 °C; HPLC t_{ret}: 2.7 min; R_f: 0.32; UV (H_2O, λ_{max}): 195.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{195.0}$ - 116 M^{-1}); IR (KBr, stretching frequency): 3415 cm^{-1} (OH), 2945 cm^{-1} (CH), 1605 cm^{-1} (CO); optical rotation (*c* 1.0, H_2O) : $[\alpha]_D$ at 25 °C = +3.33 °.

2D-HSQCT (DMSO-d_6) : ^{1}H NMR δ (500.13 MHz) : 3.30 (αCH), 1.90 (βCH), 0.98 (γ, γ′CH$_3$) ppm; ^{13}C NMR δ (125 MHz) : 53.2 (αCH), 29.2(βCH), 18.1 (γ, γ′CH$_3$), 170.5 (CO) ppm.

4.2.2.1. ʟ-Valyl-ᴅ-glucose (25a-e): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H_2O, λ_{max}): 200.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{200.0}$ – 2630 M^{-1}), 295.0 nm (n→π* $\varepsilon_{295.0}$ - 2089 M^{-1}); IR (KBr, stretching frequency): 2971 cm^{-1} (NH), 3407 cm^{-1} (OH), 2950 cm^{-1} (CH), 1622 cm^{-1} (CO); MS (*m/z*) : 302 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*- ester (25a)**: ^{1}H NMR δ (500.13 MHz) : 3.08 (αCH), 1.35 (γCH$_3$), 3.75 (H-2α), 3.67 (H-3α), 3.78 (H-4α), 4.22 (H-5α), 3.50 (H-6) ppm; ^{13}C NMR
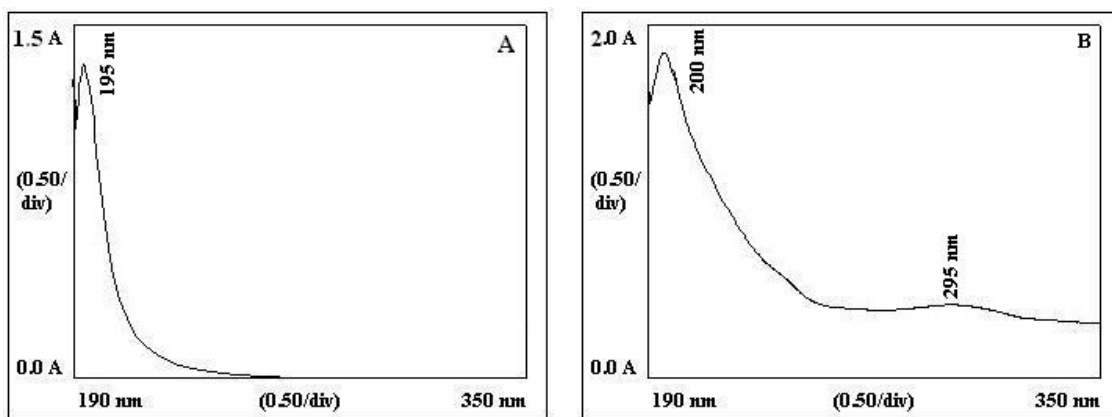
Fig. 4.18. UV spectra for L-valyl-D-glucose **25a-e** from CRL catalyzed reaction. (A) L-valine (B) L-valyl-D-glucose.
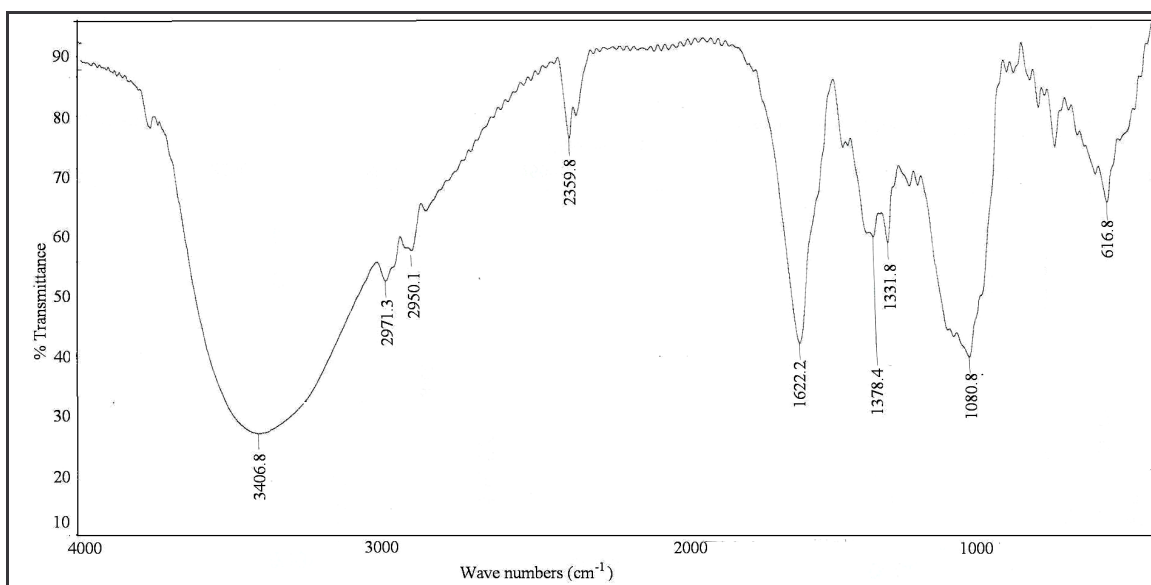
Fig. 4.19. A typical IR spectrum of L-valyl-D-glucose of CRL catalysed reaction **25a-e**.
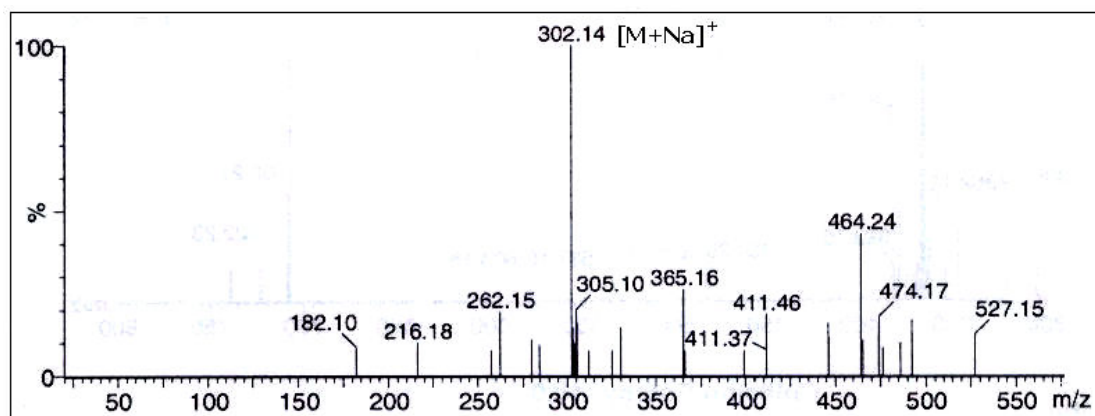
Fig. 4.20. A typical mass spectrum for L-valyl-D-glucose **25a-e**.

δ (125 MHz): 51.5 (αCH), 19.4 (γCH$_3$), 77.4 (C2α), 74.6 (C3α), 71.3 (C4α), 71.3 (C5α), 60.0 (C6α) ppm; **3-*O*- ester (25b)** : ^{1}H NMR δ: 3.10 (αCH), 1.15 (βCH$_3$), 3.85 (H-2α), 3.90 (H-3α), 4.01 (H-3β), 3.34 (H-6) ppm; ^{13}C NMR δ : 52.3 (αCH), 18.3 (γCH$_3$), 71.3 (C2α), 82.6 (C3α), 83.1 (C3β), 60.4 (C6α) ppm; **6-*O*- ester (25c)**: ^{1}H NMR δ : 3.20 (αCH), 3.35 (βCH), 1.55 (γCH$_3$), 4.95 (H-1α), 4.24 (H-1β), 3.90 (H-3α), 3.74 (H-4α), 3.41 (H-5α), 3.15 (H-6) ppm; ^{13}C NMR δ : 53.4 (αCH), 29.9 (βCH), 19.8 (γCH$_3$), 94.9 (C1α), 103.4 (C1β), 76.2 (C3α), 69.5 (C4α), 69.0 (C5β), 63.4 (C6α) ppm; **2,6-di-*O*- ester (15d)**: ^{1}H NMR δ : 3.20 (αCH), 3.85 (H-2α), 3.56 (H-6) ppm; ^{13}C NMR δ: 51.4 (αCH), 76.4 (C2α), 61.5 (C6α) ppm ; **3,6-di-*O*-ester (25e)**: ^{1}H NMR δ : 3.15 (αCH), 1.45 (γCH$_3$), 3.70 (H-3α), 3.52 (H-6) ppm; ^{13}C NMR δ : 53.4 (αCH), 19.5 (γCH$_3$), 82.4 (C3α), 61.8 (C6α) ppm.

A typical UV, IR and mass spectra for L-valyl-D-glucose **25a-e** are shown in Figures 4.18, 4.19, and 4.20 respectively.

4.2.2.2. L-Valyl-D-galactose (26a-c): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.21; UV (H$_2$O, λ$_{max}$): 225.0 nm (σ→σ* ε$_{225.0}$ −871 M^{-1}), 295.0 nm (n→π* ε$_{295.0}$ − 407 M^{-1}), IR (KBr, stretching frequency): 3309 cm^{-1} (NH), 2944 cm^{-1} (CH) and 1621 cm^{-1} (CO); MS (*m/z*): 302 [M+ Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*- ester (26a)**: ^{1}H NMR δ (500.13 MHz) : 3.30 (αCH), 1.90 (βCH), 0.98 (γ, γ′CH$_3$), 3.83 (H-2α), 3.65 (H-2β), 3.54 (H-6) ppm; ^{13}C NMR δ (125 MHz): 53.2 (αCH), 29.2 (βCH), 18.1 (γ, γ′CH$_3$), 171.2 (CO), 76.4 (C2α), 77.6 (C2β), 60.6 (C6α) ppm; **3-*O*- ester (26b)**: ^{1}H NMR δ : 3.80 (H-3α), 3.67 (H-3β), 3.54 (H-6) ppm; ^{13}C NMR δ : 81.6 (C3α), 82.7 (C3β), 60.6 (C6α) ppm; **6-*O*- ester (26c)**: ^{1}H NMR δ : 4.98 (H-1α), 4.89 (H-1β), 3.56 (H-2α), 3.71 (H-3α), 3.31 (H-3β), 3.78 (H-4α), 3.23
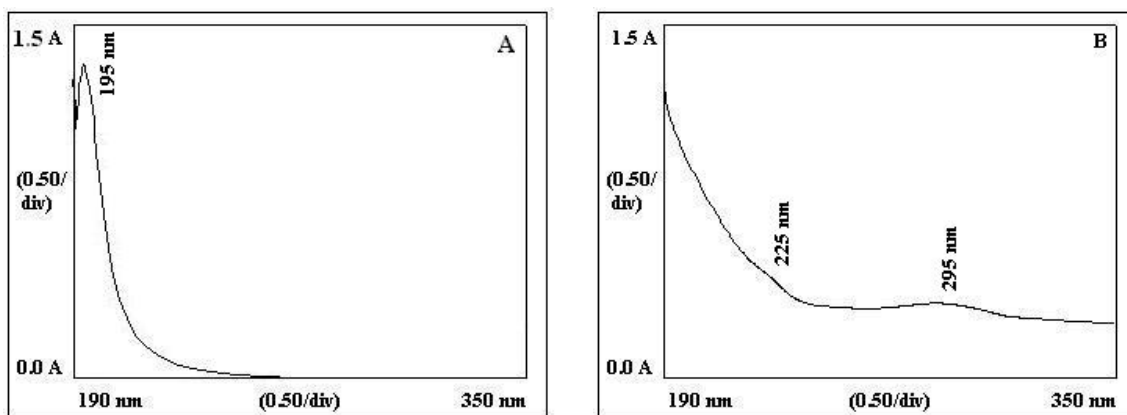
Fig. 4.21. UV spectra for L-valyl-D-galactose **26a-c** from CRL catalysed reaction. (A) L-valine; (B) L-valyl-D-galactose.
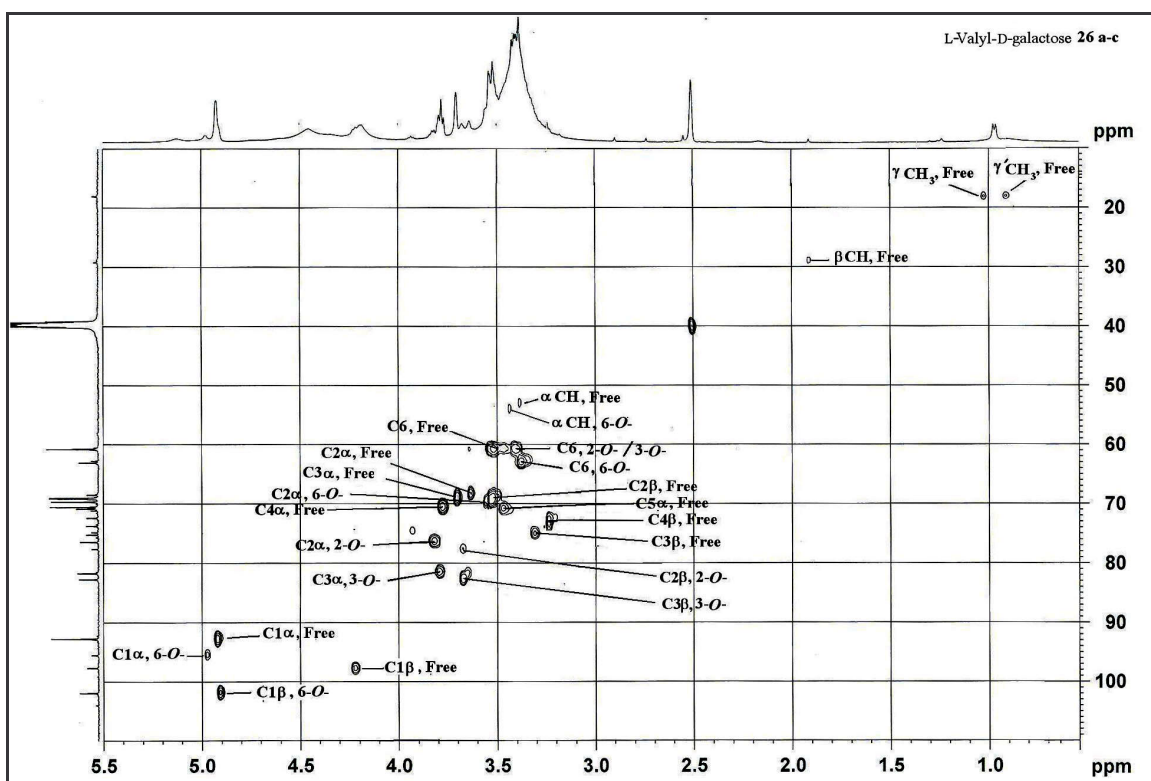
Fig. 4.22. Two-dimensional HSQCT NMR spectrum for L-valyl-D-galactose **26a-c** obtained through CRL catalysis.

(H-4β), 3.44 (H-5α), 3.35 (H-6) ppm; ^{13}C NMR δ : 95.5 (C1α), 101.9 (C1β), 69.6

(C2α), 69.1 (C3α), 73.7 (C3β), 70.5 (C4α), 72.3 (C4β), 70.5 (C5α), 63.1 (C6α) ppm.

A typical UV and 2D-HSQCT NMR spectra for L-valyl-D-galactose **26a-c** are shown in

Figures 4.21 and 4.22 respectively.

4.2.2.3. L-Valyl-D-mannose (27): Solid; Mpt : 98 °C; HPLC t_{ret}: 2.0 min; R_f: 0.59; UV

(H$_2$O, λ_{max}): 227.0 nm (σ→σ* ε$_{227.0}$ - 447 M^{-1}), 290.0 nm (n→π* ε$_{290.0}$ - 603 M^{-1}); IR

(KBr, stretching frequency): 3410 cm^{-1} (NH), 3354 cm^{-1} (OH), 2932 cm^{-1} (CH), 1644

cm^{-1} (CO); optical rotation (c 0.5, H$_2$O) : [α]$_D$ at 25 °C = -20 ° ; MS (m/z) : 302

[M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **6-O- ester (27)** : ^{1}H NMR δ (500.13 MHz): 3.90 (αCH), 2.16

(βCH), 1.02 (γ, γ′CH$_3$), 4.31 (H-1α), 3.63 (H-2α), 3.32 (H-3α), 3.51 (H-4α), 3.45 (H-

5α), 3.63 (H-6) ppm; ^{13}C NMR δ (125 MHz): 53.0 (αCH), 29.2 (βCH), 18.2 (γ, γ′CH$_3$),

171.0 (CO), 96.8 (C1α), 67.5 (C2α), 73.5 (C3α), 67.5 (C4α), 75.5 (C5α), 63.5 (C6α)

ppm.

A typical mass spectrum for L-valyl-D-mannose **29** is shown in Figure 4.23.

4.2.2.4. L-Valyl-D-fructose (28a-c): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.20; UV (H$_2$O, λ_{max}):

223.0 nm (σ→σ* $\lambda_{223.0}$ - 53 M^{-1}), 288.0 nm (n→π* ε$_{288.0}$ - 23 M^{-1}); IR (KBr, stretching

frequency): 3352 cm^{-1} (NH), 3290 cm^{-1} (OH), 2946 cm^{-1} (CH), 1623 cm^{-1} (CO); MS

(m/z) : 304 [M^{+2}+Na]$^+$;

2D-HSQCT (DMSO-d_6) : **1-O-ester (28a)**: ^{1}H NMR δ (500.13 MHz) : 3.38 (αCH), 2.14

(βCH), 1.06 (γ, γ′CH$_3$), 3.78 (H-1α), 3.32 (H-3α), 3.55 (H-3β), 3.78 (H-4α), 3.45 (H-

4β), 3.22 (H-5α), 3.32 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 52.5 (αCH), 29.2 (βCH),

18.4 (γ, γ′CH$_3$), 171.2 (CO), 64.1 (C1α), 104.3 (C2β), 70.8 (C3α), 83.9 (C3β), 72.5

(C4α), 76.0 (C4β), 73.2 (C5α), 64.0 (C6α) ppm; **6-O-ester (28b)** : ^{1}H NMR δ : 3.63 (H-

Fig. 4.23. A typical mass spectrum of L-valyl-D-mannose **27**.
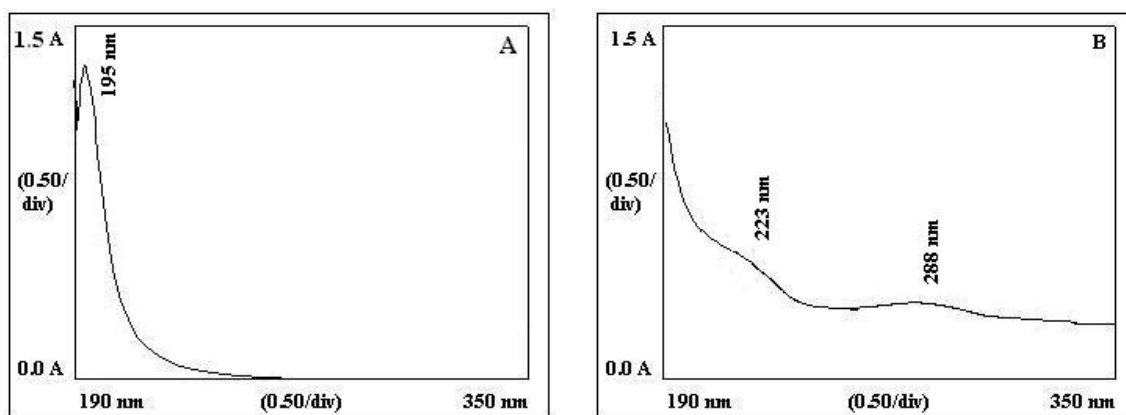
Fig. 4.24. UV spectra for L-valyl-D-fructose **28a-c** from CRL catalyzed reaction (A), L-valine; (B) L-valyl-D-fructose.
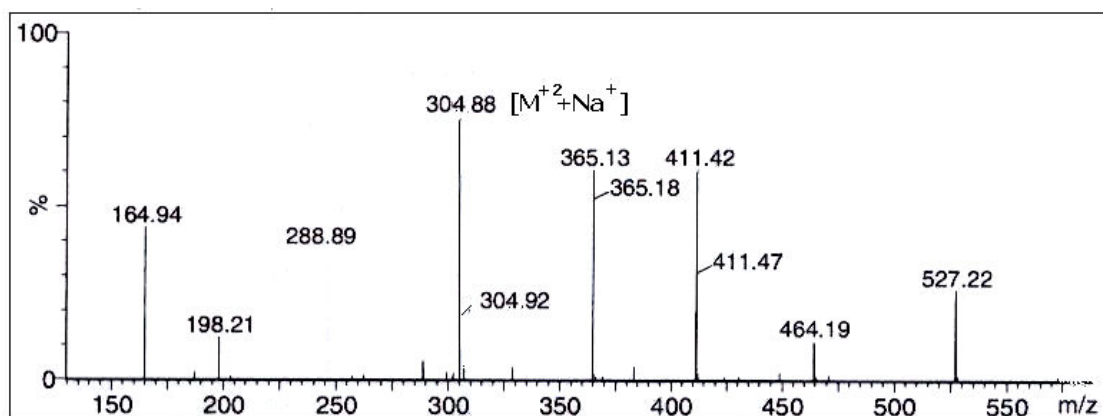
Fig. 4.25. A typical mass spectrum of L-valyl-D-fructose **28a-c**.

1α), 3.73 (H-4β), 3.72 (H-5β), 3.18 (H-6) ppm; ^{13}C NMR δ : 63.0 (C1α), 75.9 (C4β), 81.2 (C5β), 64.0 (C6α) ppm; **1,6-di-*O*-ester (28c)**: ^{1}H NMR δ : 3.83 (H-4β), 3.28 (H-6α) ppm; ^{13}C NMR δ : 76.1 (C4β), 63.4 (C6α) ppm.

A typical UV and mass spectra for L-valyl-D-fructose **28a-c** are shown in Figures 4.24 and 4.25 respectively.

4.2.2.5. L-Valyl-D-arabinose (29a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.23; UV (H$_2$O, λ$_{max}$): 224.0 nm (σ→σ* ε$_{224.0}$ - 1585 M^{-1}), 281.0 nm (n→π* ε$_{281.0}$ - 933 M^{-1}); IR (KBr, stretching frequency): 3339 cm^{-1} (NH), 3290 cm^{-1} (OH), 2963 cm^{-1} (CH), 1598 cm^{-1} (CO); MS (*m/z*) : 273 [M^{+1}+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (29a)** : ^{1}H NMR δ (500.13 MHz): 1.05 (αCH), 4.99 (H-1α), 4.91 (H-1β), 3.65 (H-2α), 3.19 (H-3α), 3.72 (H-5) ppm; ^{13}C NMR δ (125 MHz):, 15.5 (αCH), 95.9 (C1α), 102.1 (C1β), 77.0 (C2α), 71.9 (C3α), 60.5 (C5α) ppm; **5-*O*-ester (29b)**: ^{1}H NMR δ : 3.39 (αCH), 2.08 (βCH), 1.02 (γ, γ′CH$_3$), 4.32 (H-1α), 4.21 (H-1β); 3.28 (H-2α), 3.33 (H-3α), 3.69 (H-4α), 3.64 (H-5) ppm; ^{13}C NMR δ: 53.0 (αCH), 29.5 (βCH), 19.3 (γ, γ′CH$_3$), 172.0 (CO), 96.8 (C1α), 103.9 (C1β), 74.8 (C2α), 70.8 (C3α), 67.6 (C4α), 65.0 (C5α) ppm; **2,5-di-*O*-ester (29c)**: ^{1}H NMR δ : 3.45 (H-2α), 3.26 (H-3α), 3.68 (H-5α), 3.35 (H-5β) ppm; ^{13}C NMR δ : 75.6 (C2α), 73.4 (C3α), 63.3 (C5α), 65.0 (C5β) ppm.

A typical UV and mass spectra for L-valyl-D-arabinose **29a-c** are shown in Figures 4.26 and 4.27 respectively.

4.2.2.6. L-Valyl-D-ribose (30a-c): Solid; HPLC t_{ret}: 3.4 min; R_f: 0.22; UV (H$_2$O, λ$_{max}$): 230.0 nm (σ→σ* ε$_{230.0}$ - 1412 M^{-1}), 297.0 nm (n→π* ε$_{297.0}$-1072 M^{-1}); IR (KBr, stretching frequency): 3349 cm^{-1} (NH), 3137 cm^{-1} (OH), 2934 cm^{-1} (CH), 1588 cm^{-1} (CO); MS (*m/z*) : 273 [M^{+1}+Na]$^+$.
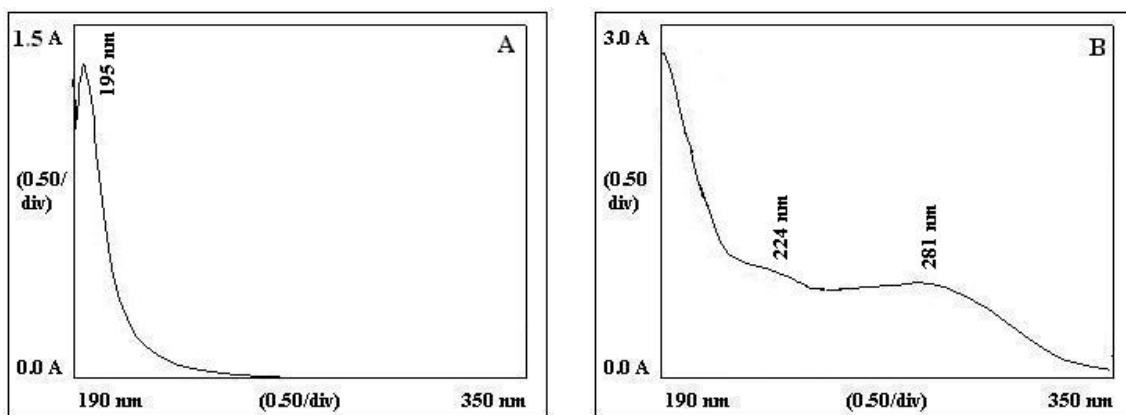
Fig. 4.26. UV spectra for L-valyl-D-arabinose **29a-c** from CRL catalyzed reaction (A) L-valine; (B) L-valyl-D-arabinose.
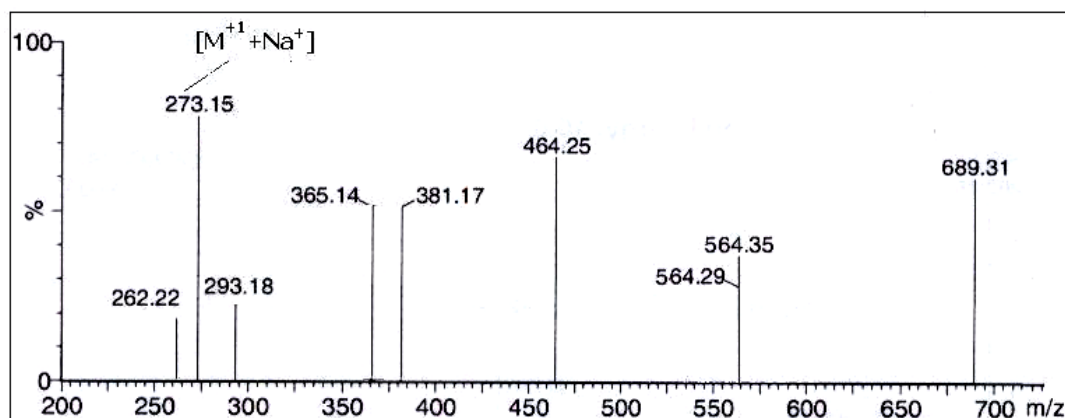
Fig. 4.27. A typical mass spectrum of L-valyl-D-arabinose **29a-c**.
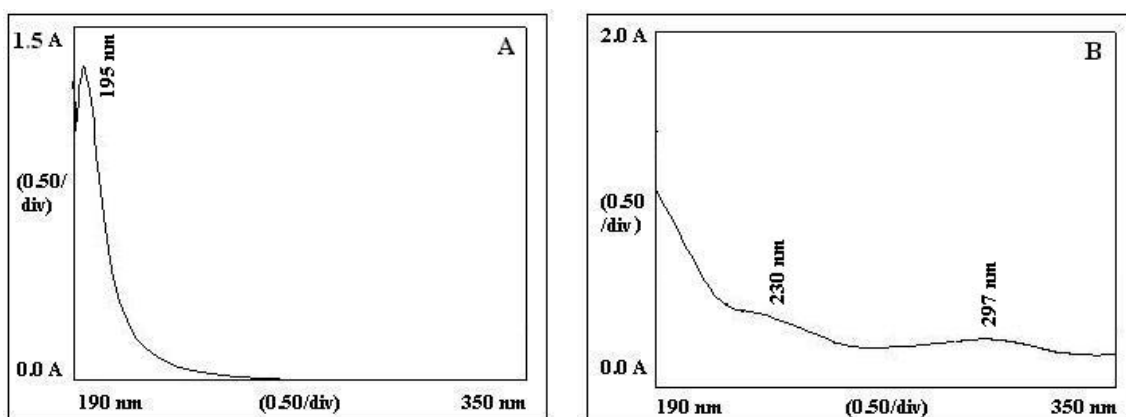
Fig. 4.28. UV spectra for L-valyl-D-ribose **30a-c** from CRL catalyzed reaction (A) L-valine; (B) L-valyl-D-ribose.

2D-HSQCT (DMSO-d_6) : **3-*O*-ester (30a)**: ^{1}H NMR δ (500.13 MHz) : 3.38 (αCH), 1.82 (βCH), 0.68 (γ, γ′CH$_3$), 3.98 (H-3α), 3.72 (H-5) ppm; ^{13}C NMR δ (125 MHz): 53.2 (αCH), 30.5 (βCH), 18.4 (γ, γ′CH$_3$), 174.3 (CO), 77.1 (C3α), 61.2 (C5α) ppm; **5-*O*-ester (30b)**: ^{1}H NMR δ : 2.08 (βCH), 1.03 (γCH$_3$), 4.20 (H-1α), 4.93 (H-1β), 3.22 (H-2α), 3.31 (H-2β), 3.31 (H-3α), 3.54 (H-3β), 3.81 (H-4α), 3.62 (H-5) ppm; ^{13}C NMR δ : 29.7 (αCH), 18.3 (γCH$_3$), 173.5 (CO), 101.5 (C1α), 93.8 (C1β), 72.1 (C2α), 73.4 (C2β), 70.4 (C3α), 68.6 (C3β), 65.1 (C4α), 63.9 (C5α) ppm; **3,5-di-*O*-ester (30c)**: ^{1}H NMR δ: 4.42 (H-1α), 4.62 (H-1β), 3.67 (H-3α), 3.34 (H-5) ppm; ^{13}C NMR δ : 97.0 (C1α), 94.0 (C1β), 74.6 (C3α), 65.4 (C5α) ppm.

A typical UV and mass spectra for L-valyl-D-ribose **30a-c** are shown in Figures 4.28 and 4.29 respectively.

4.2.2.7. L-Valyl-maltose (31a,b): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.13; UV (H$_2$O, λ$_{max}$): 221.0 nm (σ→σ* ε$_{221.0}$ - 1622 M^{-1}), 291.0 nm (n→π* ε$_{291.0}$ - 776 M^{-1}); IR (KBr, stretching frequency): 3419 cm^{-1} (NH), 3267 cm^{-1} (OH), 2936 cm^{-1} (CH), 1634 cm^{-1} (CO); MS (*m/z*) : 464 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **6-*O*-ester (31a)**: ^{1}H NMR δ (500.13 MHz) : 3.12 (αCH), 2.25 (βCH), 1.05 (γ, γ′CH$_3$), 4.92 (H-1α), 5.0 (H-1β), 3.32 (H-2α), 3.41 (H-3α), 4.14 (H-4α), 3.39 (H-5α), 3.93 (H-6), 4.90 (H-1′α), 3.27 (H-2′), 3.35 (H-3′), 3.63 (H-4′), 3.68 (H-5′), 3.67 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 51.0 (αCH), 31.1 (βCH), 18.8 (γCH$_3$), 175.5 (CO), 97.0 (C1α), 100.5 (C1β), 73.0 (C2α), 76.3 (C3α), 80.5 (C4α), 78.8 (C5α), 66.5 (C6), 100.7 (C1′α), 67.5 (C2′), 67.0 (C3′), 70.2 (C4′), 72.3 (C5′), 60.5 (C6′) ppm; **6′-*O*-ester (31b)**: ^{1}H NMR δ : 2.90 (αCH), 3.98 (H-4α), 3.07 (H-2′), 3.32 (H-3′), 3.83 (H-6′) ppm; ^{13}C NMR δ : 52.0 (αCH), 80.6 (C4α), 71.5 (C2′), 70.5 (C3′), 68.0 (C6′) ppm.

A typical 2D-HSQCT NMR spectrum for L-valyl-maltose **31a,b** is shown in Figure 4.30.
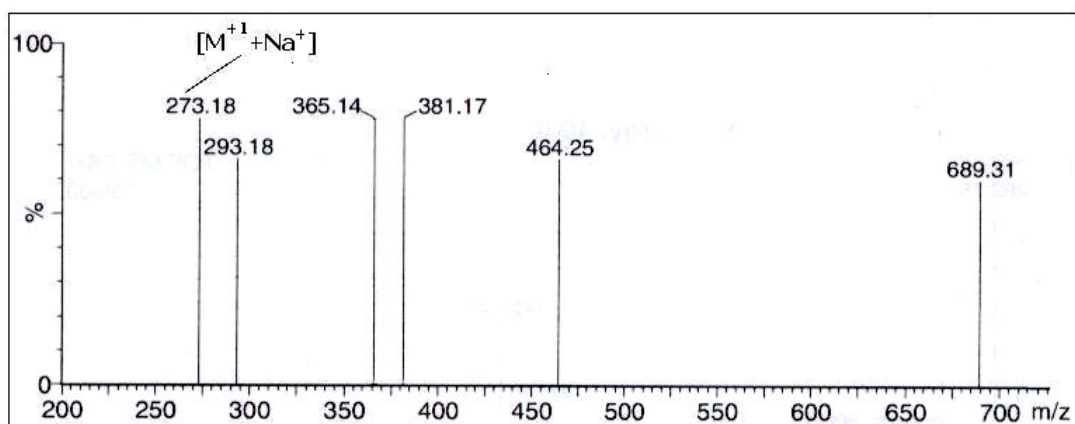
Fig. 4.29. A typical mass spectrum of L-valyl-D-ribose **30a-c**.
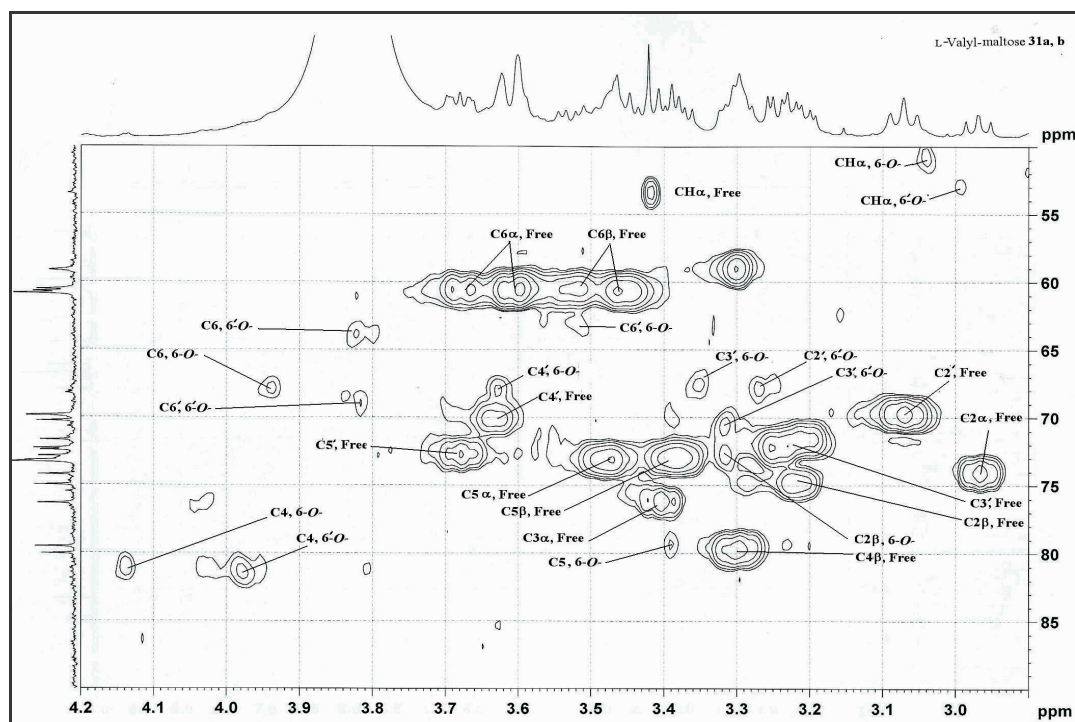
Fig. 4.30. Two-dimensional HSQCT NMR for L-valyl-maltose **31a,b** obtained through CRL catalysis.
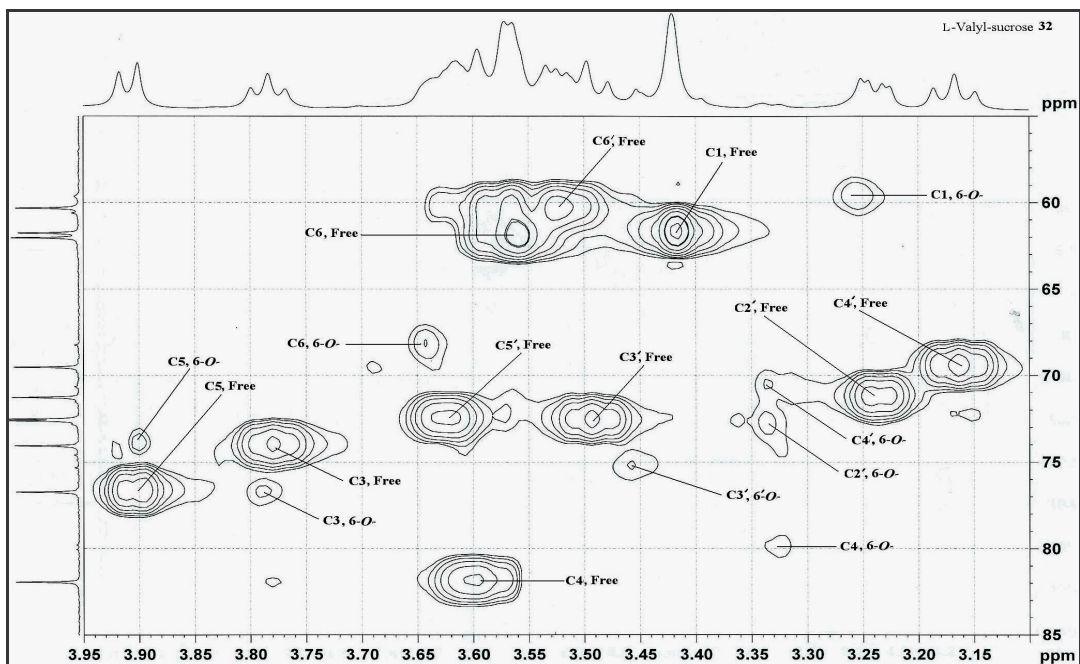
Fig. 4.31. Two-dimensional HSQCT NMR for L-valyl-sucrose **32** obtained through CRL catalysis.
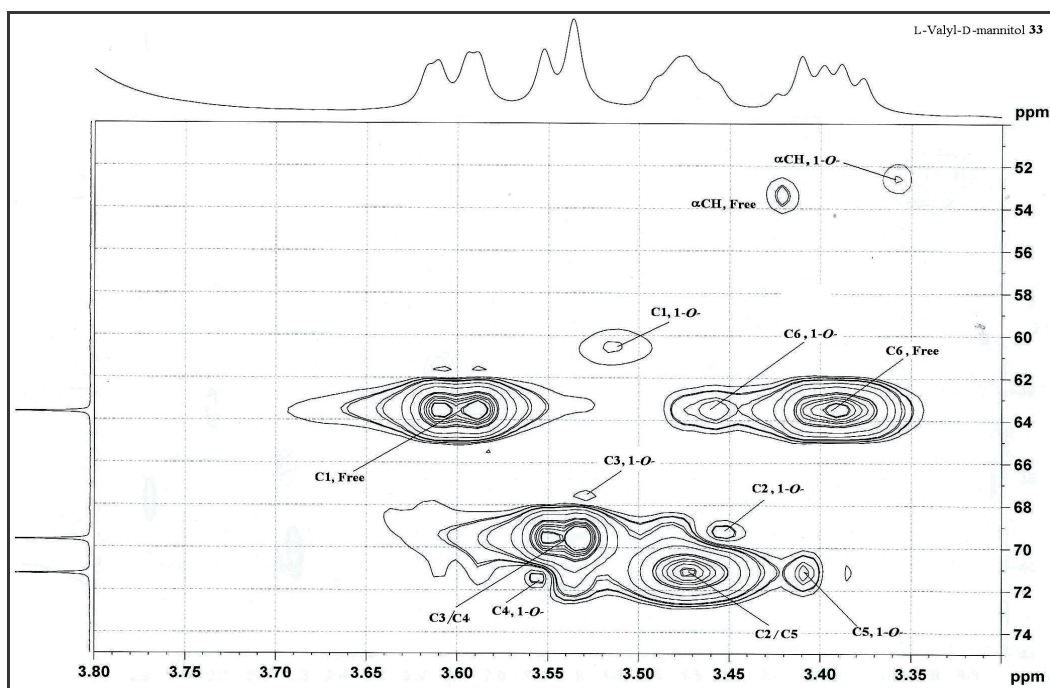
Fig. 4.32. Two-dimensional HSQCT NMR for L-valyl-D-mannitol **33** obtained through CRL catalysis.

4.2.2.8. L-Valyl-sucrose (32): Solid; Mpt:184 °C; HPLC t_{ret}: 3.4 min; R_f: 0.13; UV (H$_2$O, λ_{max}): 225.0nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{225.0}$ - 195 M^{-1}), 275.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{275.0}$ - 91 M^{-1}); IR (KBr, stretching frequency): 3520 cm^{-1} (NH), 3411 cm^{-1} (OH), 2940 cm^{-1} (CH), 1588 cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O) : [α]$_D$ at 25 °C = +36 °; MS (*m/z*) : 464 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **6-O-ester (32)**: ^{1}H NMR δ (500.13 MHz) : 3.13 (αCH), 2.42 (βCH), 1.05 (γ, γ'CH$_3$), 3.26 (H-1α), 3.79 (H-3β), 3.33 (H-4β), 3.90 (H-5β), 3.64(H-6), 4.92 (H-1'α), 3.34 (H-2'), 3.46 (H-3'), 3.34 (H-4'), 3.62 (H-5'), 3.52 (H-6') ppm; ^{13}C NMR δ (125 MHz) : 53.5 (αCH), 28.8 (βCH), 18.5 (γ, γ'CH$_3$), 172.0 (CO), 59.5 (C1β), 103.3 (C2β), 74.1 (C3β), 80.0 (C4β), 74.3 (C5β), 68.2 (C6β), 91.8 (C1'α), 72.9 (C2'), 75.2 (C3'), 70.6 (C4'), 72.6 (C5'), 60.3 (C6') ppm .

A typical 2D-HSQCT NMR spectrum for L-valyl-sucrose **32** is shown in Figure 4.31.

4.2.2.9. L-Valyl-D-mannitol (33): Solid; Mpt: 132 °C; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H$_2$O, λ_{max}): 225.0nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{225.0}$ -372 M^{-1}), 270.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{270.0}$ - 178 M^{-1}); IR (KBr, stretching frequency): 3294 cm^{-1} (OH), 2957 cm^{-1} (CH), 1630 cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O) : [α]$_D$ at 25 °C = +6.6 °; MS (*m/z*) : 304 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6): **1-O-ester (33):** ^{1}H NMR δ (500.13 MHz): 3.12 (αCH), 2.05 (βCH), 0.92 (γ, γ'CH$_3$), 3.53 (H-1), 3.46 (H-2), 3.53 (H-3), 3.56 (H-4), 3.41 (H-5), 3.46 (H-6) ppm; ^{13}C NMR δ (125 MHz): 55.8 (αCH), 29.8 (βCH$_2$), 19.1 (γ,γ'CH$_3$), 60.6 (C1), 69.5 (C2), 67.5 (C3), 71.2 (C4), 71.0 (C5) 63.6 (C6) ppm.

A typical 2D-HSQCT NMR spectrum for L-valyl-D-mannitol **33** is shown in Figure 4.32.

4.2.3. Syntheses of L-leucyl esters of carbohydrates 34a-e to 43

L-Leucine (L-2-amino-4-methyl pentanoic acid) is a polar and an essential dietary amino acid containing 2-methyl propyl group as a side chain. Compared to L-alanine (solubility 127.3 g/l at 25 °C) and L-valine (solubility 88.5 g/l at 25 °C), the solubility of

L-leucine in water is low (24.26 g/l at 25 ^{o}C). However, the carbohydrate esters can be expected to exhibit higher solubility in water. Using optimum conditions, L-leucyl esters of different carbohydrates (**Scheme 4.3**) were prepared using CRL and crude PPL (Section 2.2.5). The reaction mixture consists of 1 – 2 mmol L-leucine and 1 mmol carbohydrates (D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14**, D-sorbitol **15**) along with 40% CRL (w/w carbohydrate)/111% of crude PPL (w/w carbohydrate) incubated in 100 ml of CH_2Cl_2 and DMF (v/v 90:10, 40 ^{o}C) containing 0.1 mM (0.1 ml of 0.1 M) of phosphate buffer, pH 7.0 (CRL) or 0.2 mM (0.2 ml of 0.1 M) of acetate buffer, pH 5.0 (crude PPL). The reaction mixture was analyzed by HPLC using a C-18 column with acetonitrile:water (v/v 20:80) as a mobile phase and detected at 210 nm (Fig. 4.33). Ester formation was also monitored by TLC as described in Section 4.2.2. The retention times (t_{ret}) by HPLC and retention factor (R_f) values by TLC are mentioned in sections 4.2.3.1 –4.2.3.10.
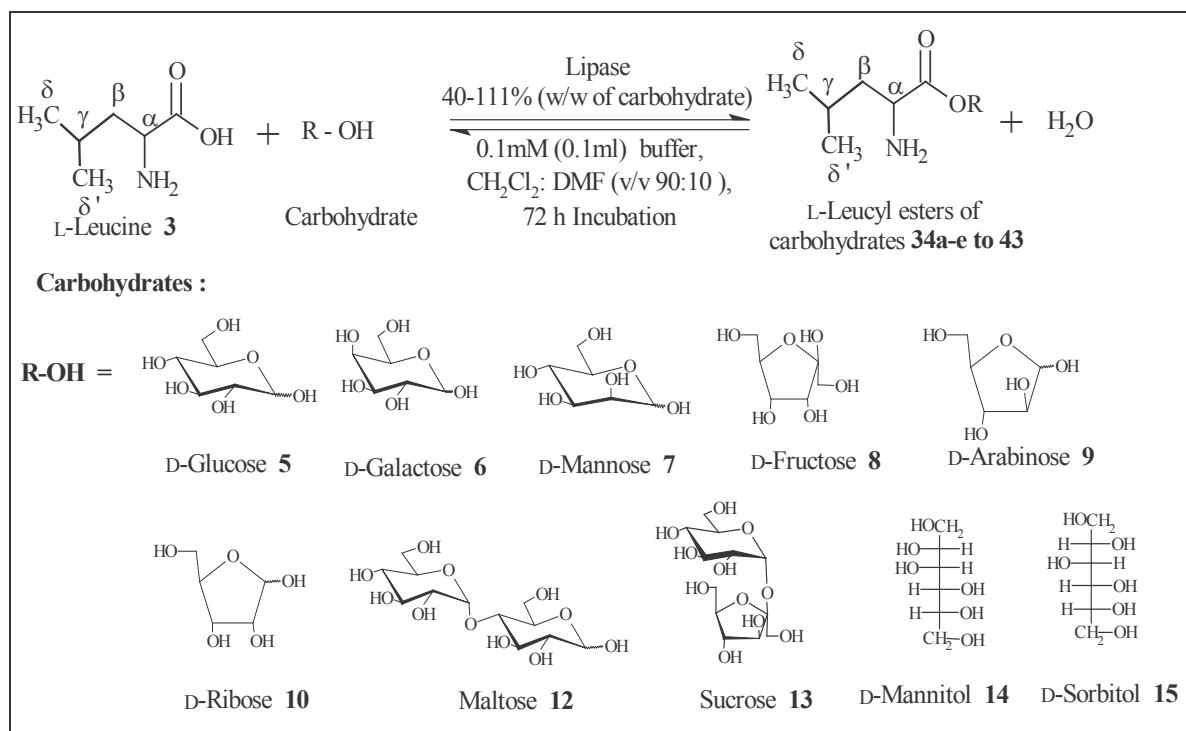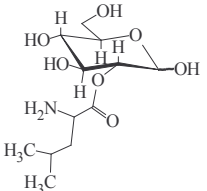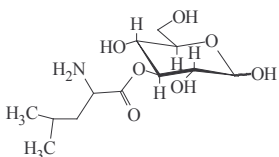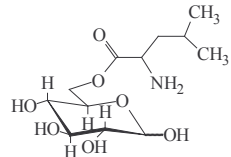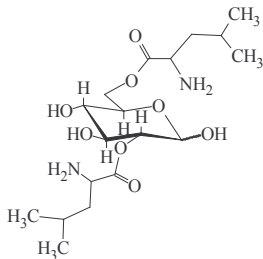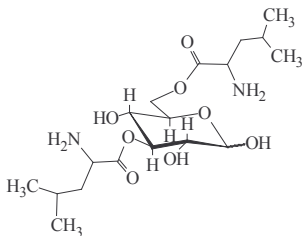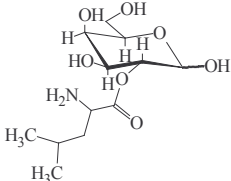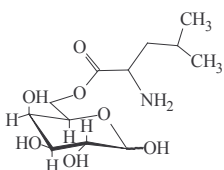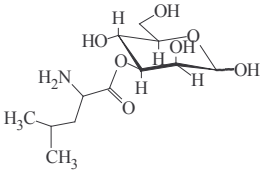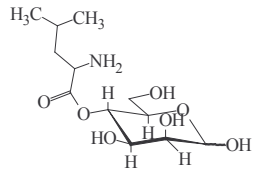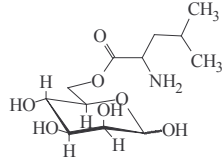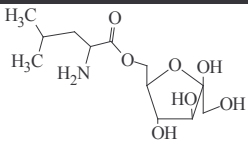
Scheme 4.3 Lipase catalyzed syntheses of L-leucyl esters of carbohydrates
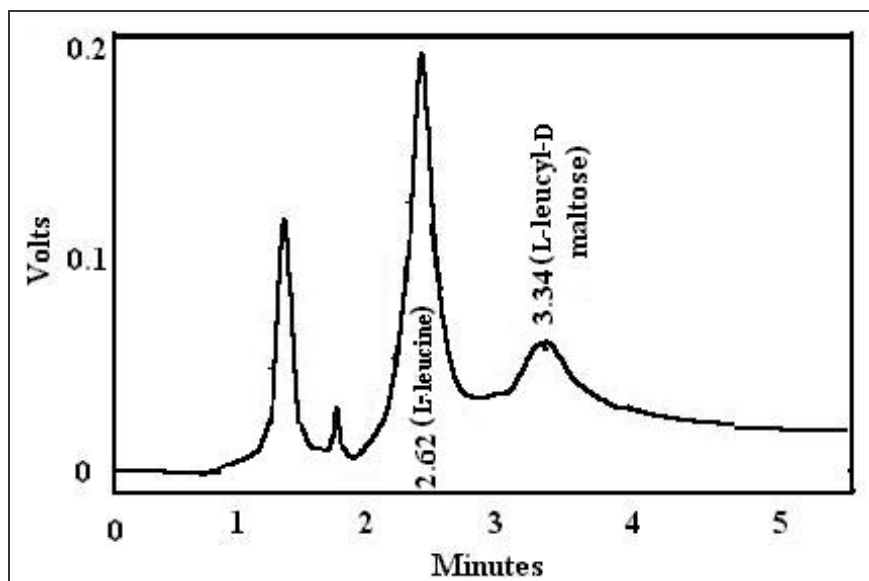
Fig. 4.33. HPLC chromatogram for reaction mixture of L-leucine and maltose esterification reaction catalysed by CRL. Column – C-18; mobile phase – acetonitrile:water (v/v 20:80); flow rate - 1 ml/min; detector – UV at 210 nm; errors in conversion yields are within ± 10 %.

The isolated esters were subjected to UV, IR, MS, optical rotation and 2D- NMR characterization (Sections 4.2.3.1 – 4.2.3.10). The spectral data for the isolated esters were shown in Sections 4.2.3.1 – 4.2.3.10. Table 4.6 shows the HPLC ester yields, types of esters formed and percentage proportions of the individual esters from CRL catalysis and Table 4.7 shows the HPLC ester yields from crude PPL catalysis.

Table 4.6 Syntheses of L-leucyl esters of carbohydrates[a]

L-Leucyl esters of carbohydrates (% proportions [b])			Yield (%)
34a 2-*O*-L-leucyl -D-glucose (17)	**34a** 3-*O*-L-leucyl-D-glucose (20)	**34a** 6-*O*-L-leucyl-D-glucose 42)	43 (mono esters- 34, diesters-9)
34d 2,6-di-*O*-L-leucyl-D-glucose (10)	**34e** 3,6-di-*O*-L- leucyl-D-glucose (11)		
35a 2-*O*-L-leucyl-D-galactose (48)	**35b** 6-*O*-L-leucyl-D-galactose (52)		21 (only mono esters)
36a 3-*O*-L-leucyl-D-mannose (28)	**36b** 4-*O*-L-leucyl-D-mannose (30)	**36b** 6-*O*-L-leucyl-D-mannose (42)	31 (only mono esters)
37 6-*O*-L-leucyl-D-fructose			48 (only mono ester)

38a 2-*O*-L-leucyl-D-arabinose (24) **38b** 5-*O*-L-leucyl-D-arabinose (33) **38c** 2,5-di-*O*-L-leucyl -D-arabinose (43)

42 [c] (mono esters-24, diester-18)

39a 3-*O*-L-leucyl-D-ribose (16) **39b** 5-*O*-L-leucyl-D-ribose (32) **39c** 3,5-di-*O*-L-leucyl-D-ribose (52)

38 [c] (mono esters-18, diester-20)

40 6-*O*-L-leucyl-maltose

44 (only mono ester)

41 6-*O*-L- leucyl-sucrose

38 (only mono ester)

42a 1-*O*-L-leucyl-D-mannitol (56) **42b** 1,6- di-*O*-L- leucyl-D-mannitol (44)

45 (mono ester-25, diester-20)

43 1-*O*-L-leucyl-D-sorbitol

25 (only mono ester)

[a] L-Leucine – 2 mmol, carbohydrates – 1 mmol, CRL – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1M) phosphate buffer (pH 7.0), CH$_2$Cl$_2$:DMF (v/v 90:10) at 40 ^{o}C, incubation period – 72 h. Conversion yields were from HPLC with respect to L-leucine concentration.

[b] Percentage proportions of individual esters were determined from the peak areas or from their cross peaks of the Carbon-13 C6 and C5 (in case of pentoses) signals in the 2D HSQCT spectrum.

[c] Several cross peaks, due to opening and/or degradation of the five membered ring during esterification.

Table 4.7 Preparation of L-leucyl esters of carbohydrates using crude porcine pancreas lipase [a]

L-Leucyl ester of carbohydrate	%Yield (mmol)
L- Leucyl-D-glucose **34a-e**	58 (0.58)
L- Leucyl-D-galactose **35a,b**	46 (0.46)
L- Leucyl-D-mannose **36a-c**	44 (0.44)
L- Leucyl-D-fructose **37**	52 (0.52)
L- Leucyl-D-arabinose **38a-c**	39 (0.39)
L-Leucyl-D-ribose **39a-c**	57 (0.57)
L- Leucyl-maltose **40**	64 (0.64)
L- Leucyl-sucrose **41**	65 (0.65)
L- Leucyl-D-mannitol **42a,b**	50 (0.50)
L- Leucyl-D-sorbitol **43**	54 (0.54)

[a] L-Leucine – 1 mmol, carbohydrates – 1 mmol, Crude PPL – 111 % (w/w carbohydrate), buffer – 0.2 mM (0.2 ml of 0.1 M) acetate buffer (pH 5.0), CH_2Cl_2: DMF (v/v 90: 10) at 40 $^{\circ}$C, Incubation period – 72 h. Conversion yields were from HPLC with respect to L-leucine concentration.

Spectral data for L-leucine (3): Solid; Mpt: 293-295 $^{\circ}$C; HPLC t_{ret}: 2.6 min; R_f: 0.32; UV (H_2O, λ_{max}): 190.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{190.0}$ - 132 M^{-1}), IR (KBr, stretching frequency): 3415 cm^{-1} (OH), 2945 cm^{-1} (CH), 1595 cm^{-1} (CO); optical rotation (c 1.0, H_2O): $[\alpha]_D$ at 25 $^{\circ}$C = -15.2 $^{\circ}$.

2D-HSQCT (DMSO-d_6) : ^{1}H NMR δ (500.13 MHz) : 3.41 (αCH), 1.56 (βCH$_2$), 1.64 (γCH), 0.85 (δ, δ' CH$_3$) ppm; ^{13}C NMR δ (125 MHz) : 53.2 (αCH), 39.4 (βCH$_2$), 24.0 (γCH), 21.8 (δ, δ' CH$_3$), 173.0 (CO) ppm.

4.2.3.1. L-Leucyl-D-glucose (34a-e): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H_2O, λ_{max}): 230.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{230.0}$ - 724 M^{-1}), 297.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{297.0}$ - 363 M^{-1}); IR (KBr, stretching frequency): 3383 cm^{-1} (NH), 3360 cm^{-1} (OH), 2240 cm^{-1} (CH), 1657 cm^{-1} (CO); MS (m/z) : 316 [M+Na]$^+$.

Fig. 4.34. UV spectra for L-leucyl-D-glucose **34a-e** from CRL catalyzed reaction (A) L-leucine; (B) L-leucyl-D-glucose.
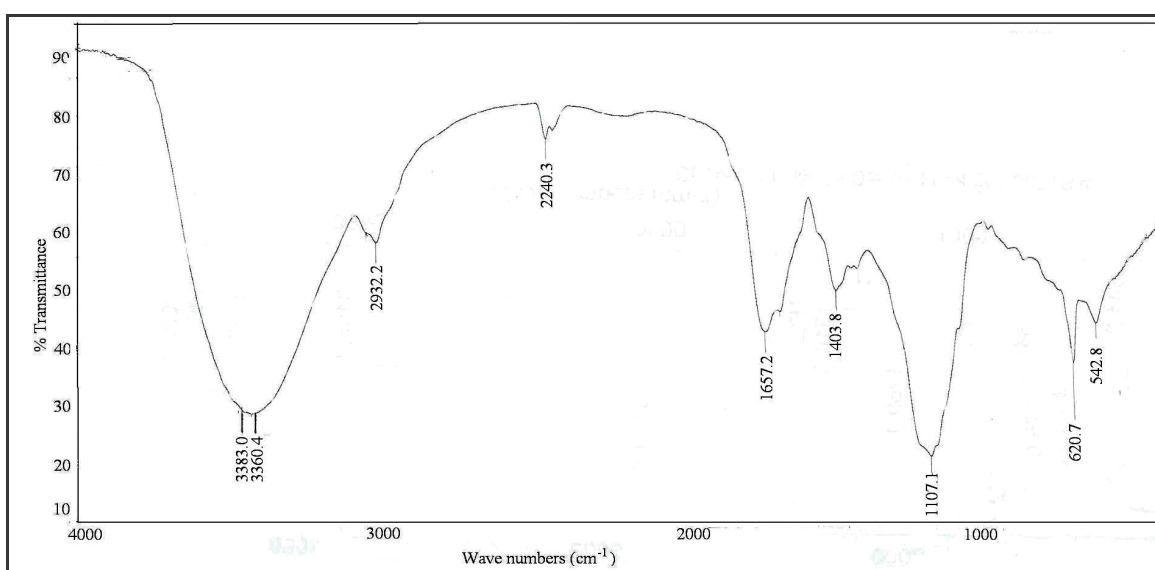
Fig. 4.35. A typical IR spectrum of L-leucyl-D-glucose **34a-e** of CRL catalysed reaction. A 2.5 mg of ester sample was prepared as KBr pellet.

Fig. 4.36. A typical mass spectrum of L-leucyl-D-glucose **34a-e**.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (34a)**: ^{1}H NMR δ (500.13 MHz): 3.85 (H-2α) ppm; ^{13}C δ (125 MHz): 75.5 (C2α) ppm; **3-*O*-ester (34b)**: ^{1}H NMR δ : 3.15 (αCH), 3.85 (H-3α), 3.96 (H-3β) ppm; ^{13}C NMR δ : 50.0 (αCH), 83.5 (C3α), 83.6 (C3β) ppm;. **6-*O*-ester (24c)**: ^{1}H NMR δ : 3.10 (α CH), 1.56 (βCH$_2$), 1.82 (γCH), 0.82 (δ, δ′ CH$_3$), 4.99 (H-1α), 3.81 (H-2α), 3.45 (H-3α), 3.68 (H-4α), 3.55 (H-5α), 3.80 (H-6) ppm ; ^{13}C NMR δ: 53.2 (αCH), 40.5 (βCH$_2$), 23.5 (γCH), 25.0 (δ, δ′ CH$_3$), 173.6 (CO), 92.5 (C1α), 70.3 (C2α), 75.8 (C3α), 70.2 (C4α), 71.0 (C5α), 65.0 (C6α) ppm; **2,6-di-*O*-ester (34d)**: ^{1}H NMR δ : 3.21 (H-2α), 3.59 (H-6) ppm; ^{13}C NMR δ : 76.3 (C2α), 65.0 (C6α) ppm. **3,6-di-*O*-ester (34e)**: ^{1}H NMR δ: 3.68 (H- 3α), 3.59 (H-6) ppm; ^{13}C NMR δ: 81.5 (C3α), 65.0 (C6α) ppm.

A typical UV, IR, mass and 2D-HSQCT NMR spectra for L-leucyl-D-glucose **34a-e** are shown in Figures 4.34, 4.35, 4.36 and 4.37 respectively.

4.2.3.2. L-Leucyl-D-galactose (35a,b): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H$_2$O, $λ_{max}$): 226.0 nm (σ→σ* $ε_{226.0}$ - 2291 M^{-1}), 295.0 nm (n→π* $ε_{295.0}$ - 1349 M^{-1}); IR (KBr, stretching frequency): 3426 cm^{-1} (NH), 3317 cm^{-1} (OH), 2815 cm^{-1} (CH), 1589 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (35a)**: ^{1}H NMR δ (500.13 MHz) : 3.74 (H-2α), 3.70 (H-6) ppm; ^{13}C NMR (125 MHz) δ : 171.4 (CO), 72.5 (C2α), 59.5 (C6α) ppm; **6-*O*-ester (35b)**: ^{1}H NMR δ : 3.50 (αCH), 1.54 (βCH$_2$), 1.44 (γCH), 0.95 (δ, δ′CH$_3$), 5.0 (H-1α), 4.49 (H-1β), 3.66 (H-2α), 3.68 (H-3α), 3.74 (H-4α), 3.42 (H-4β), 3.62 (H-5α), 3.48 (H-5β), 3.65 (H-6) ppm; ^{13}C NMR δ: 54.0 (αCH), 39.2 (βCH$_2$), 24.0 (γCH), 21.6 (δ, δ′ CH$_3$), 172.1 (CO), 92.0 (C1α), 96.1 (C1β), 62.5 (C2α), 68.0 (C3α), 68.2 (C4α), 72.5 (C4β), 70.0 (C5α), 74.5 (C5β), 62.5 (C6α) ppm.

Fig. 4.37. Two-dimensional HSQCT NMR for L-leucyl-D-glucose **34a-e** obtained through CRL catalysis.
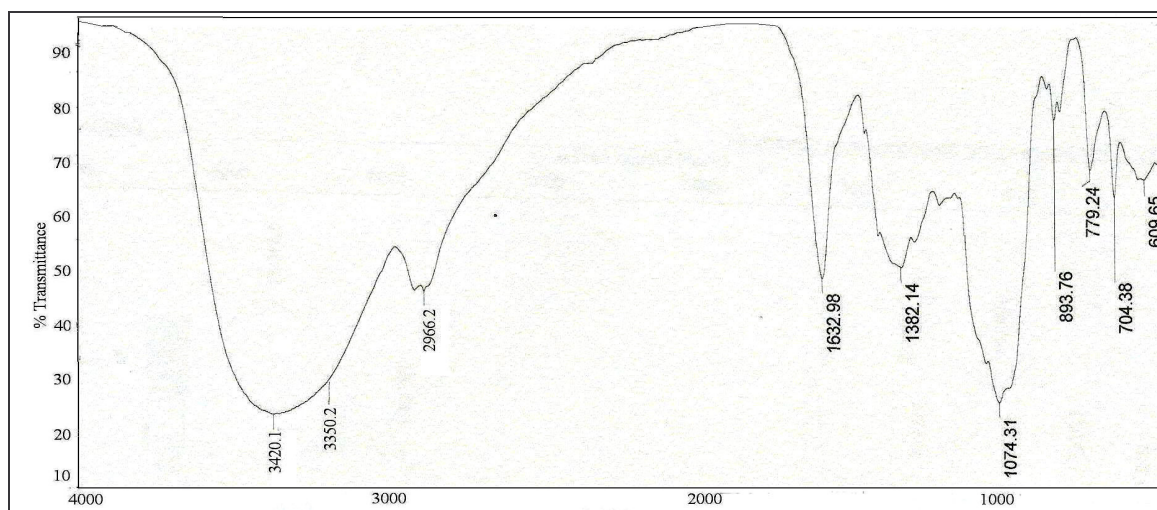
Fig. 4.38. A typical IR spectrum of L-leucyl-D-galactose of RML catalyzed reaction **35a,b**.

A typical IR and mass spectra for L-leucyl-D-galactose **35a,b** are shown in Figures 4.38 and 4.39 respectively.

4.2.3.3. L-Leucyl-D-mannose (36a-c): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H_2O, λ_{max}): 230.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{230.0}$ - 1122 M^{-1}), 292.0 nm (n$\rightarrow \pi^*$ $\varepsilon_{292.0}$ - 120.2 M^{-1}); IR (KBr, stretching frequency): 3425 cm^{-1} (NH), 3329 cm^{-1} (OH), 2950 cm^{-1} (CH), 1591 cm^{-1} (CO); MS (*m/z*) : 316 [M+ Na]$^+$.

2D-HSQCT (DMSO-d_6) **3-*O*-ester (36a)**: ^{1}H NMR δ (500.13 MHz): 3.05 (αCH), 3.86 (H-3α), 3.97 (H-3β), 3.55 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.5 (αCH), 81.4 (C3α), 82.5 (C3β), 61.7 (C6α) ppm; **4-*O*-ester (36b)** : ^{1}H NMR δ : 3.86 (H-4α), 3.78 (H-4β), 3.61 (H-6) ppm; ^{13}C NMR δ: 73.8 (C4α), 75.0 (C4β), 61.6 (C6α) ppm; **6-*O*-ester (36c)**: ^{1}H NMR δ: 3.47 (αCH), 1.59 (βCH$_2$), 1.64 (γCH), 1.0 (δ, δ′CH$_3$), 4.91 (H-1α), 4.75 (H-2α), 3.65 (H-3α), 3.43 (H-4α), 3.63 (H-5α), 3.80 (H-6) ppm; ^{13}C NMR δ : 53.9 (αCH), 39.4 (βCH$_2$), 24.0 (γCH), 21.1 (δ, δ′CH$_3$), 172.6 (CO), 95.3 (C1α), 68.0 (C2α), 68.1 (C3α), 66.3 (C4α), 72.3 (C4β), 70.5 (C5α), 63.2 (C6α) ppm.

A typical UV and mass spectra for L-leucyl-D-mannose **36a-c** are shown in Figures 4.40 and 4.41 respectively.

4.2.3.4. L-Leucyl-D-fructose (37): Solid; Mpt: 128 ^{o}C; HPLC t_{ret}: 3.5 min; R_f: 0.22; UV (H_2O, λ_{max}): 223.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{223.0}$ -1023 M^{-1}), 284.0 nm (n$\rightarrow \pi^*$ $\varepsilon_{284.0}$ - 489.8 M^{-1}); IR (KBr, stretching frequency): 3266 cm^{-1} (NH), 3219 cm^{-1} (OH), 2951 cm^{-1} (CH), 1630 cm^{-1} (CO); optical rotation (*c* 0.5, H_2O): [α]$_D$ at 25 ^{o}C = -6.7 ^{o}; MS (*m/z*) : 332 [M+K]$^+$.

2D-HSQCT (DMSO-d_6): **6-*O*-ester (37)**: ^{1}H NMR δ (500.13 MHz) : 3.47 (αCH), 1.56 (βCH$_2$), 1.64 (γCH), 1.0 (δ, δ′CH$_3$), 3.58 (H-1α), 3.61 (H-3α), 3.87 (H-3β), 3.77 (H-4β), 3.65 (H-5α), 3.83 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 52.8 (αCH), 39.4 (βCH$_2$), 24.0

126
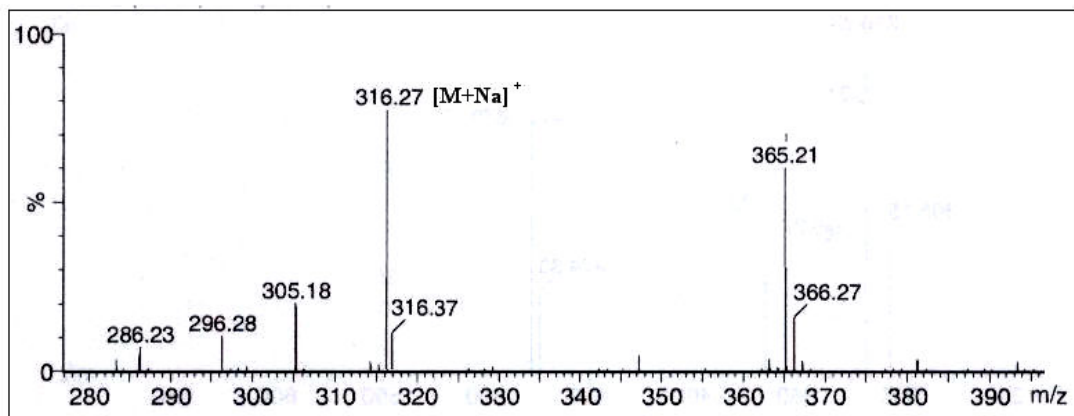
Fig. 4.39. A typical mass spectrum of L-leucyl-D-galactose. **35a,b**
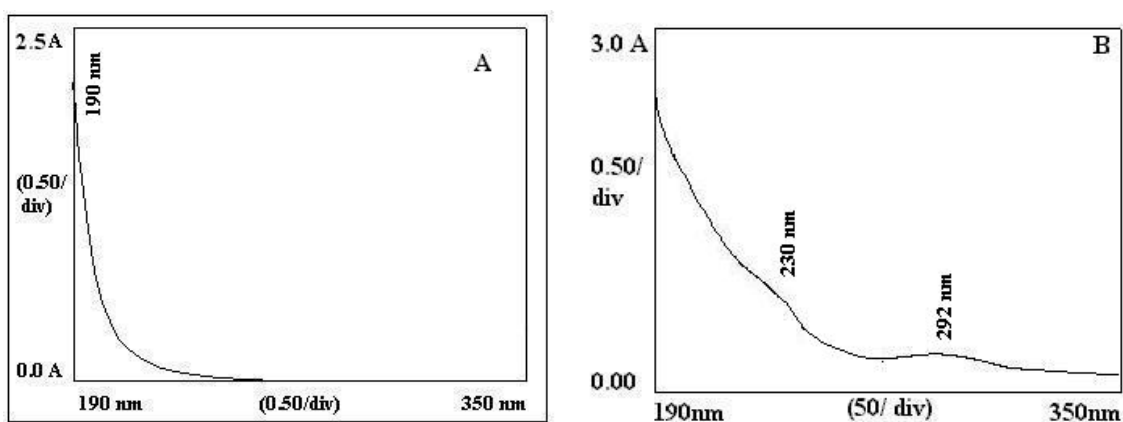
Fig. 4.40 UV spectra for L-leucyl-D-mannose **36a-c** from CRL catalyzed reaction (A) L-leucine; (B) L-leucyl-D-mannose.
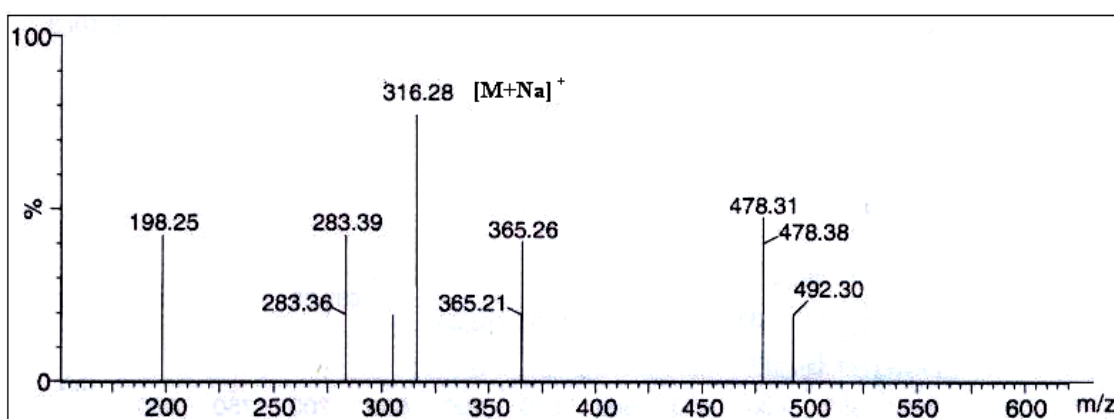
Fig. 4.41. A typical mass spectrum of L-leucyl-D-mannose **36a-c**.

(γCH), 22.2 (δ, δ′CH$_3$), 173.1 (CO), 61.5 (C1α), 104.2 (C2α), 69.0 (C3α), 82.0 (C3β), 75.4 (C4β), 66.8 (C5α), 63.5 (C6α) ppm.

A typical UV and mass spectra for L-leucyl-D-fructose **37** are shown in Figures 4.42 and 4.43 respectively.

4.2.3.5. L-Leucyl-D-arabinose (38a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.24; UV (H$_2$O, λ_{max}): 210.0 nm (σ→σ* $\varepsilon_{210.0}$ - 7080 M^{-1}), 295.0 nm (n→π* $\varepsilon_{295.0}$ - 3236 M^{-1}); IR (KBr, stretching frequency): 3275 cm^{-1} (NH), 3094 cm^{-1} (OH), 2958 cm^{-1} (CH), 1609 cm^{-1} (CO); MS (*m/z*): 287 [M^{+1}+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (38a)** : ^{1}H NMR δ (500.13 MHz): 3.41(αCH), 2.51 (βCH$_2$), 1.64 (γCH), 0.85 (δ, δ′ CH$_3$), 4.32 (H-1α), 4.89 (H-1β), 3.76 (H-2α), 3.45 (H-2β), 3.70 (H-3α), 3.68 (H-5) ppm; ^{13}C NMR δ (125 MHz): 53.0 (αCH), 39.4 (βCH$_2$), 24.0 (γCH), 22.2 (δ, δ′ CH$_3$), 173.0 (CO), 96.6 (C1α), 92.0 (C1β), 75.0 (C2α), 75.2 (C2β), 69.5 (C3α), 62.6 (C5α) ppm; **5-*O*-ester (38b)**: ^{1}H NMR δ : 4.91 (H-1α), 4.99 (H-1β), 3.47 (H-2α), 3.60 (H-3α), 3.67 (H-4α), 3.64 (H-5) ppm; ^{13}C NMR δ: 101.6 (C1α), 94.2 (C1β), 74.2 (C2α), 69.5 (C3α), 67.3 (C4α), 64.8 (C5α) ppm; **2,5-di-*O*-ester (38c)**: ^{1}H NMR δ : 4.18 (H-1α), 3.66 (H-2α), 2.96 (H-3α), 3.35 (H-5) ppm; ^{13}C NMR δ : 103.5 (C1α), 77.2 (C2α), 74.2 (C3α), 64.8 (C5α) ppm.

A typical 2D-HSQCT NMR spectrum for L-leucyl-D-arabinose **38a-c** is shown in Figure 4.44.

4.2.3.6. L-Leucyl-D-ribose (39a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.23; UV (H$_2$O, λ_{max}): 226.0 nm (σ→σ* $\varepsilon_{226.0}$ - 170 M^{-1}), 295.0 nm (n→π* $\varepsilon_{295.0}$ - 891 M^{-1}); IR (KBr, stretching frequency): 3332 cm^{-1} (NH), 3120 cm^{-1} (OH), 2960 cm^{-1} (CH), 1585 cm^{-1} (CO); MS (*m/z*) : 286 [M+Na]$^+$ and 400 (diester) [M^{+1}+Na]$^+$.
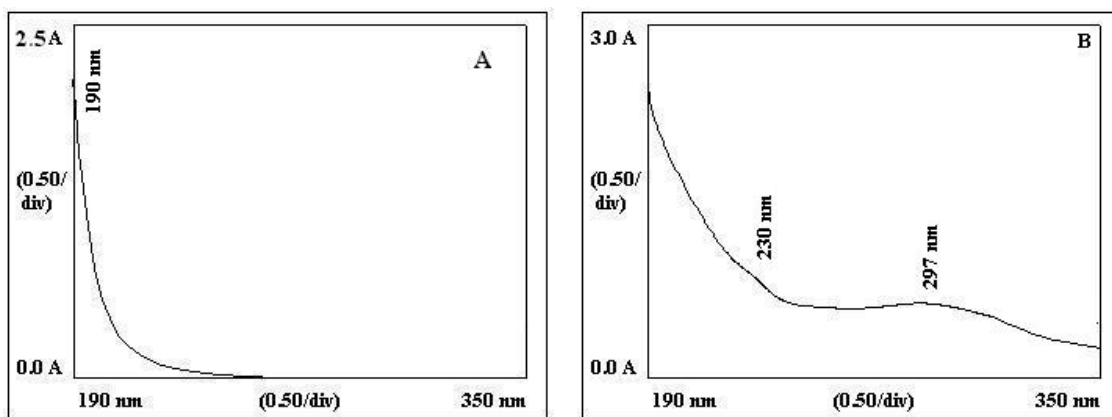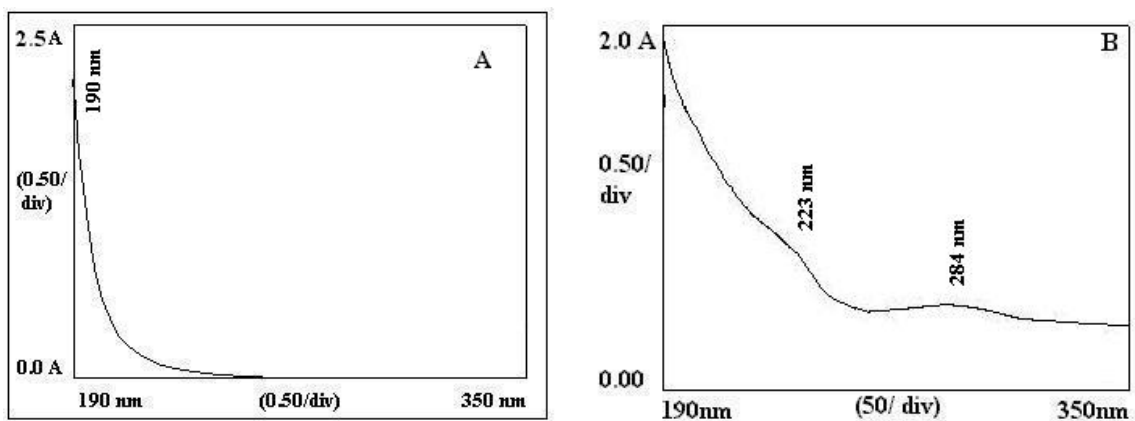
Fig. 4.42 UV spectra for L-leucyl-D-fructose **37** from CRL catalyzed reaction (A) L-leucine; (B) L-leucyl-D-fructose.
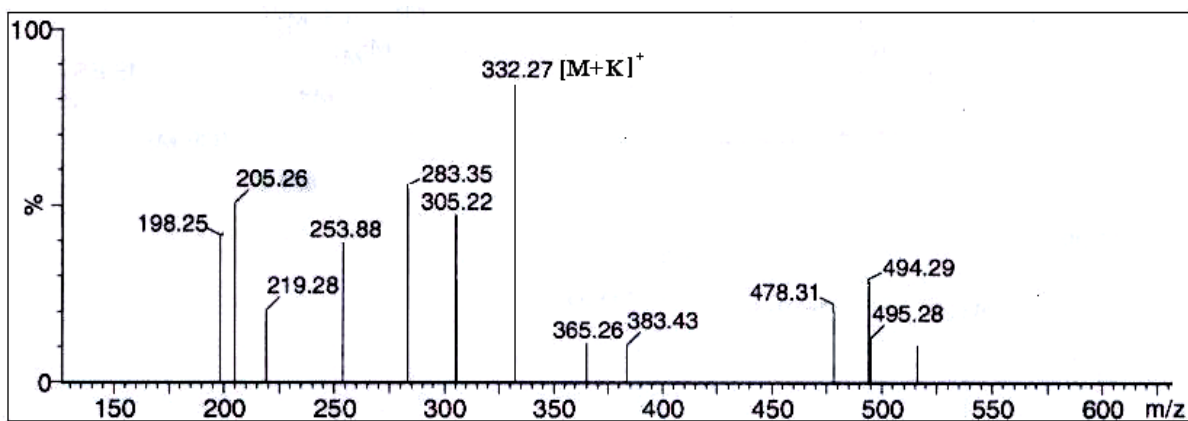
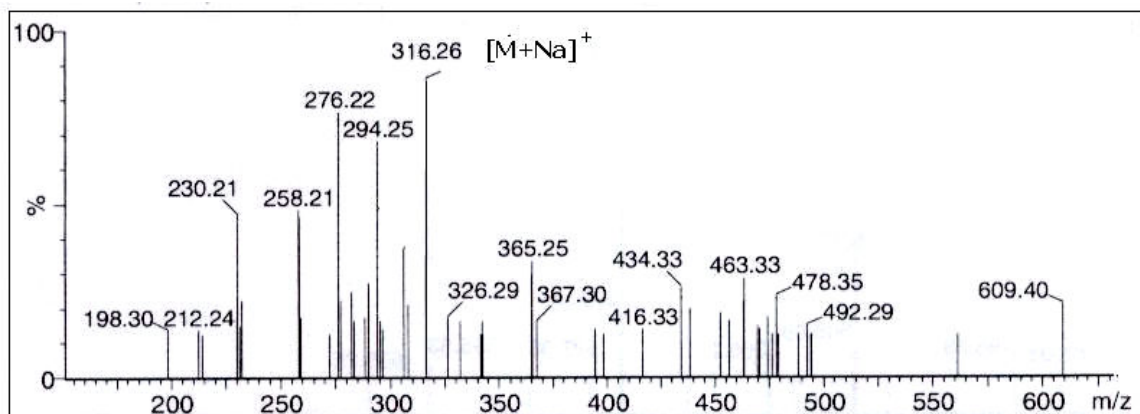**Fig. 4.43.** A typical mass spectrum of L-leucyl-D-fructose **37**.

2D-HSQCT (DMSO-d_6) : **3-*O*-ester (39a)** : ^{1}H NMR δ (500.13 MHz): 3.41(αCH), 1.56 (βCH$_2$), 1.64 (γCH), 0.85 (δ, δ′CH$_3$), 4.89 (H-1α), 4.85 (H-1β), 3.18 (H-2α), 3.60 (H-3α), 3.68 (H-4α), 3.64 (H-5) ppm; ^{13}C NMR δ (125 MHz): 53.0 (αCH), 39.4 (βCH$_2$), 24.0 (γCH), 22.2 (δ, δ′CH$_3$), 173.0 (CO), 92.3 (C1α), 92.6 (C1β), 71.8 (C2α), 76.0 (C3α), 67.2 (C4α), 60.8 (C5α) ppm; **5-*O*-ester (39b)**: ^{1}H NMR δ : 4.92 (H-1α), 4.22 (H-1β), 3.45 (H-2α), 3.48 (H-3α), 3.72 (H-4α), 3.59 (H-5a) and 3.68 (H-5b) ppm; ^{13}C NMR δ : 101.5 (C1α), 97.0 (C1β), 75.8 (C2α), 69.0 (C3α), 70.8 (C4α), 63.0 (C5α) ppm; **3,5-di-*O*-ester (39c)**: ^{1}H NMR δ: 3.27 (H-2α), 3.77 (H-3α), 3.77 (H-4α), 3.34 (H-5a) and 3.37 (H-5b) ppm; ^{13}C NMR δ : 74.7 (C2α), 76.2 (C3α), 71.0 (C4α), 64.8 (C5α) ppm.

A typical mass spectrum for L-leucyl-D-ribose **39a-c** is shown in Figure 4.45.

4.2.3.7. L-Leucyl-maltose (40): Solid; Mpt : 157 ^{o}C; HPLC t_{ret}: 3.3 min; R_f: 0.13; UV (H$_2$O, λ$_{max}$): 224.0 nm (σ→σ*$_{224.0}$ - 617 M^{-1}), 281.0 nm (n→π*$_{281.0}$ – 191 M^{-1}); IR (KBr, stretching frequency): 3419 cm^{-1} (NH), 3300 cm^{-1} (OH), 2956 cm^{-1} (CH), 1642 cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O): [α]$_D$ at 25 ^{o}C = +54.6 ^{o}; MS (*m/z*) : 478 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **6-*O*-ester (40)** : ^{1}H NMR δ (500.13 MHz): 3.60 (αCH), 1.58 (βCH$_2$), 1.69 (γCH), 0.87 (δ, δ′CH$_3$), 4.91 (H-1α), 4.19 (H-1β), 2.97 (H-2α), 3.23 (H-2β), 3.40 (H-3α), 3.23 (H-4β), 3.32 (H-5α), 3.33 (H-6), 5.0 (H-1′α), 3.06 (H-2′), 3.23 (H-3′), 3.63 (H-4′), 3.67 (H-5′), 3.59 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 51.0 (αCH), 39.5 (βCH$_2$), 24.0 (γCH), 22.4 (δ, δ′ CH$_3$), 173.5 (CO), 94.0 (C1α), 103.5 (C1β), 73.2 (C2α), 75.4 (C2β), 76.3 (C3α), 79.9 (C4β), 70.5 (C5α), 62.9 (C6α), 102.2 (C1′α), 70.2 (C2′), 71.3 (C3′), 69.8 (C4′), 72.3 (C5′), 62.5 (C6′) ppm.

A 2D-HSQCT NMR spectrum for L-leucyl-maltose **40** is shown in Figure 4.46.

4.2.3.8. L-Leucyl-sucrose (41) Solid; Mpt: 125 ^{o}C; HPLC t_{ret}: 3.3 min; R_f: 0.13; UV (H$_2$O, λ$_{max}$): 223.0 nm (σ→σ* ε$_{223.0}$ - 324 M^{-1}), 275.0 nm (n→π* ε$_{275.0}$ - 174 M^{-1}); IR

Fig. 4.44. Two-dimensional HSQCT NMR for L-leucyl-D-arabinose **38a-c** obtained through CRL catalysis.
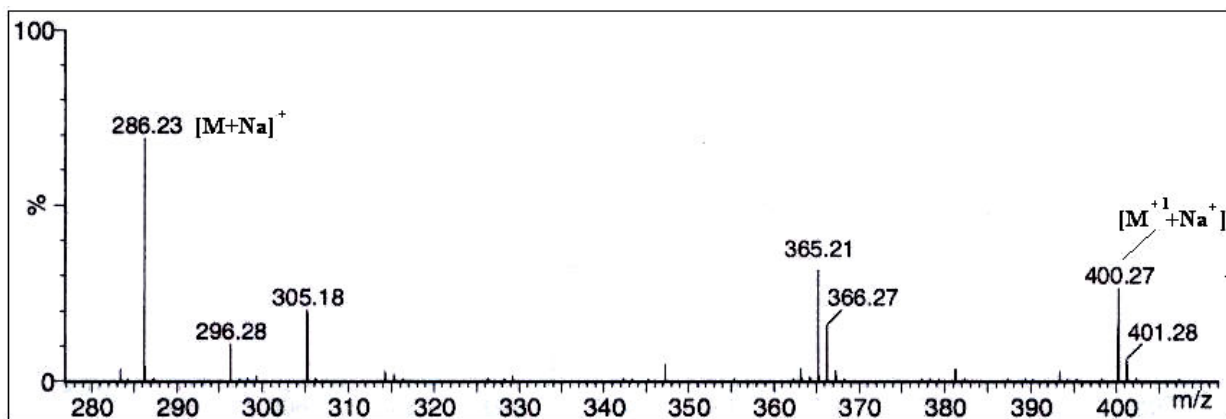
Fig. 4.45. A typical mass spectrum of L-leucyl-D-ribose **39a-c**.

(KBr, stretching frequency): 3598 cm^{-1} (NH), 3455 cm^{-1} (OH), 3105 cm^{-1} (CH), 1616 cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O): [α]$_D$ at 25 ^{o}C = -27.1 ^{o}; MS (*m/z*): 478 [M+Na]$^+$.

2D-HSQCT (DMSO-*d$_6$*) : **6-*O*-ester (41)**: ^{1}H NMR δ (500.13 MHz) :3.45 (αCH), 1.55 (βCH$_2$), 1.68 (γCH), 0.50 (δ, δ′ CH$_3$), 3.39 (H-1β), 3.76 (H-3β), 3.58 (H-4β), 3.89 (H-5β), 3.64 (H-6), 5.16 (H-1′α), 3.23 (H-2′), 3.41 (H-3′), 3.49 (H-4′), 3.59 (H-5′), 3.51 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 53.0 (αCH), 39.5 (βCH$_2$), 24.1 (γCH), 22.0 (δ, δ′CH$_3$), 172.0 (CO), 64.5 (C1β), 103.4 (C2β), 74.0 (C3β), 84.1 (C4β), 79.0 (C5β), 68.2 (C6β), 92.1 (C1′α), 71.3 (C2′), 71.6 (C3′), 74.5 (C4′), 72.8 (C5′), 64.5 (C6′) ppm.

A typical 2D-HSQCT NMR spectrum for L-leucyl-sucrose **41** is shown in Figure 4.47.

4.2.3.9. L-Leucyl-D-mannitol (42a,b) Solid; HPLC *t$_{ret}$*: 3.3 min; *R$_f$*: 0.19; UV (H$_2$O, λ$_{max}$): 224.0 nm (σ→σ* ε$_{224.0}$ - 2344 M^{-1}), 294.0 nm (n→π* ε$_{294.0}$ - 1288 M^{-1}); IR (KBr, stretching frequency): 3294 cm^{-1} (NH), 3068 cm^{-1} (OH), 2957 cm^{-1} (CH), 1630 cm^{-1} (CO); MS (*m/z*) : 320 [M^{+2}+Na]$^+$.

2D-HSQCT (DMSO-*d$_6$*) : **1-*O*-ester (42a)**: ^{1}H NMR δ (500.13 MHz) : 3.58 (αCH), 1.57(βCH$_2$), 1.67 (γCH), 0.71 (δ, δ′CH$_3$), 3.51 (H-1), 3.45 (H-2), 3.54 (H-3), 3.56 (H-4), 3.39 (H-5), 3.46 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.5 (αCH), 39.5 (βCH$_2$), 24.0 (γCH), 21.8 (δ,δ′ CH$_3$), 171.5 (CO), 60.4 (C1), 75.4 (C2), 72.6 (C3), 71.3 (C4), 71.2(C5), 63.5 (C6) ppm; **1,6-di-*O*-ester (42b)**: ^{1}H NMR δ : 1.02 (δ,δ′ CH$_3$), 3.61 (H-1), 3.32 (H-2), 3.32 (H-5), 3.64 (H-6) ppm; ^{13}C NMR δ : 21.8 (δ,δ′ CH$_3$), 60.4 (C1), 72.8 (C2), 70.5 (C5), 68.2 (C6) ppm.

A typical mass spectrum for L-leucyl-D-mannitol **42a,b** is shown in Figure 4.48.

4.2.3.10. L-Leucyl-D-sorbitol (43): Solid; Mpt : 155 ^{o}C; HPLC *t$_{ret}$*: 3.3 min; *R$_f$*: 0.19; UV (H$_2$O, λ$_{max}$): 203.0 nm (σ→σ ε$_{203.0}$ - 4677 M^{-1}), 285.0 nm (n→π* ε$_{285.0}$ - 182 M^{-1});
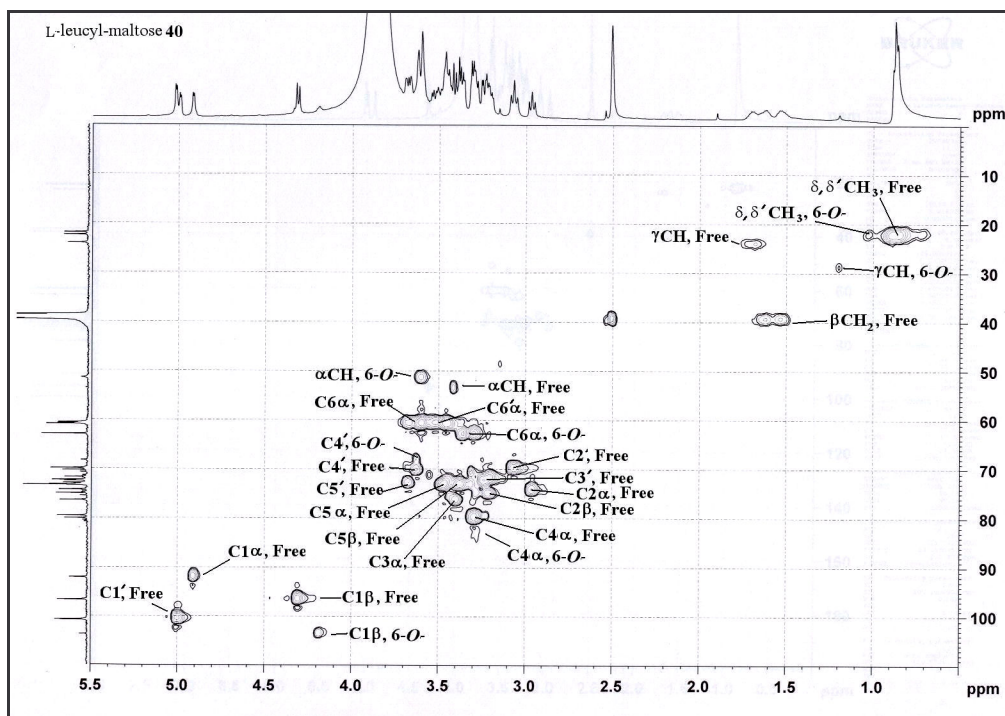
Fig. 4.46. Two-dimensional HSQCT NMR for L-leucyl-maltose **40** obtained through CRL catalysis.
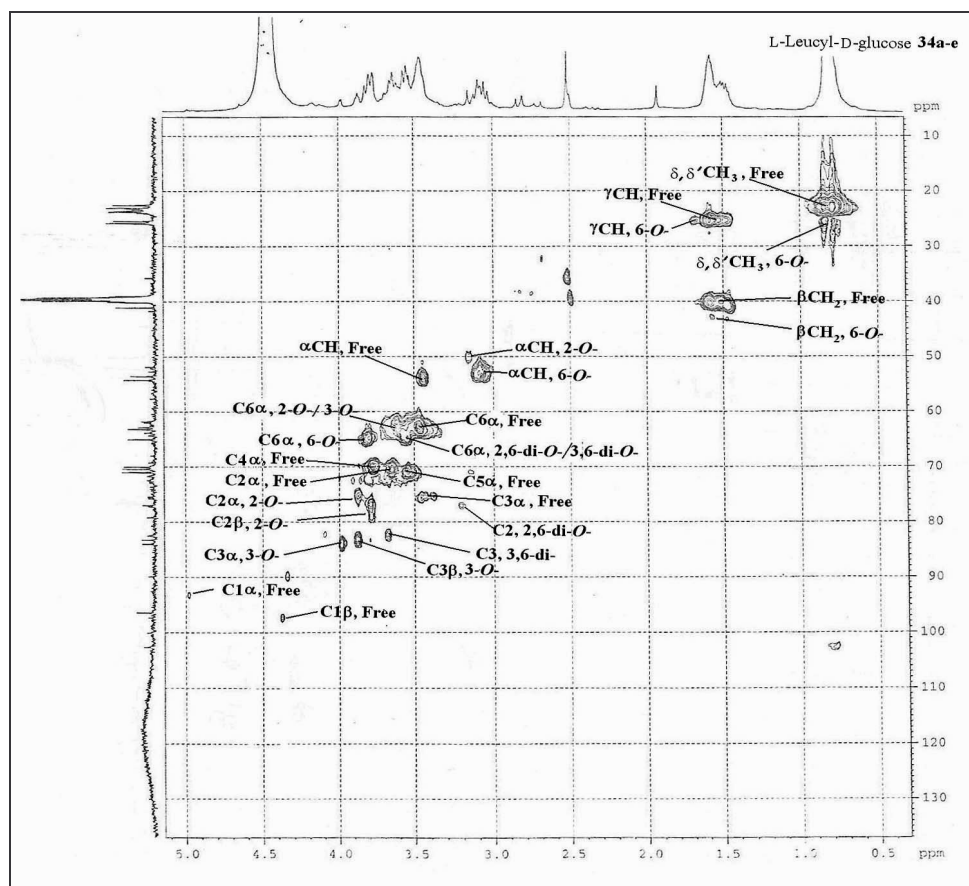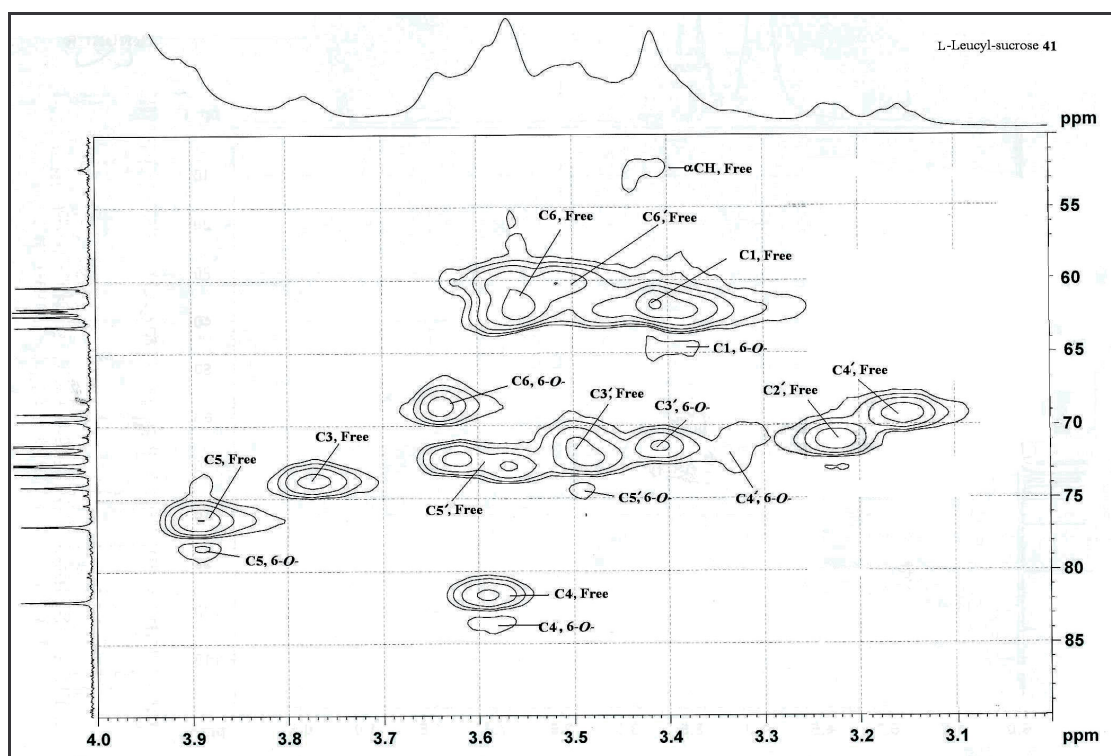

Fig. 4.47. Two-dimensional HSQCT NMR for L-leucyl-sucrose **41** obtained through CRL catalysis.
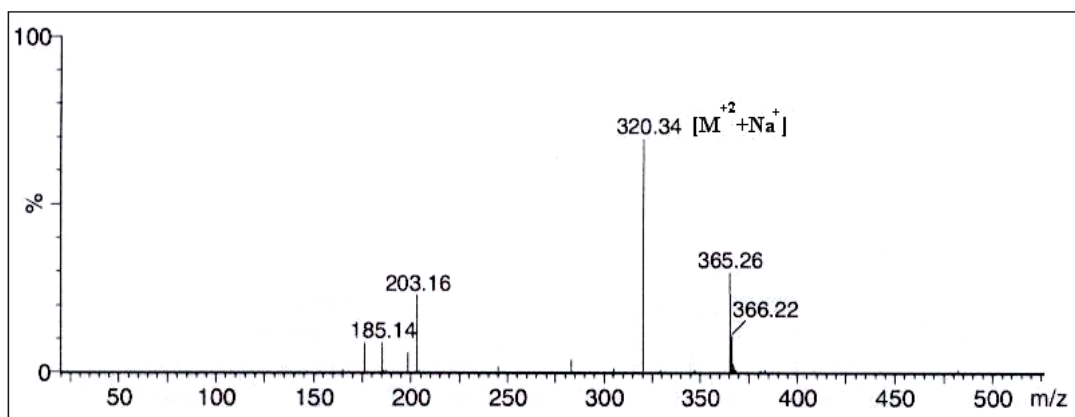
Fig. 4.48. A typical mass spectrum of L-leucyl-D-mannitol **42**
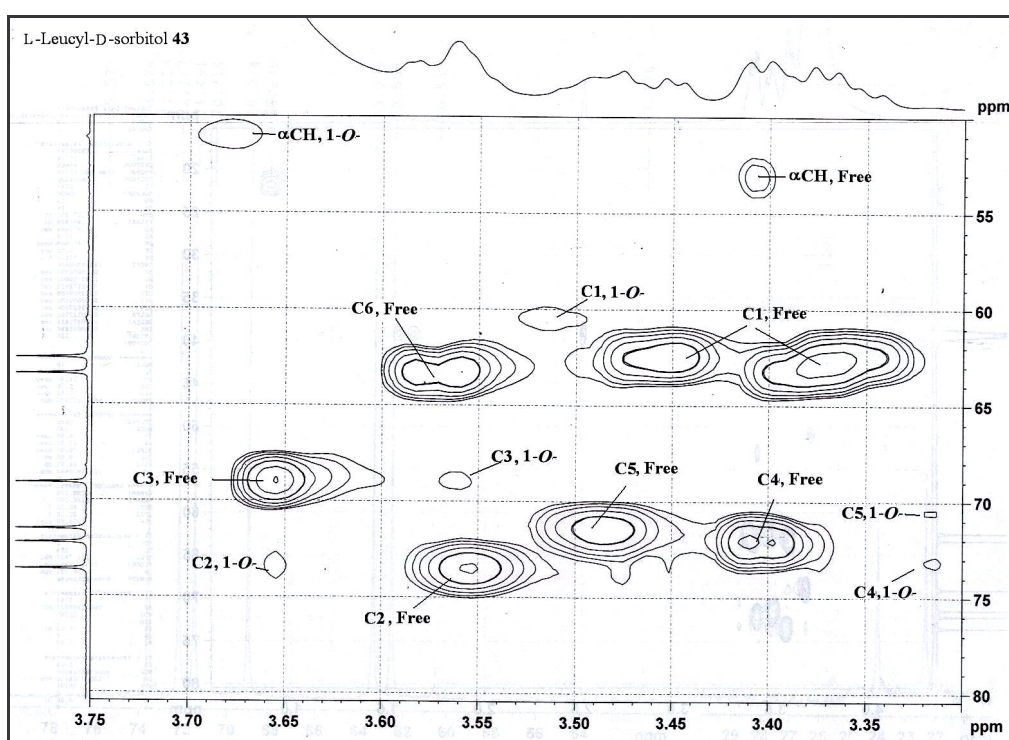
Fig. 4.49. Two-dimensional HSQCT NMR for L-leucyl-D-sorbitol **43** obtained through CRL catalysis.

IR (KBr, stretching frequency): 3250 cm^{-1} (NH), 3150 cm^{-1} (OH), 2917 cm^{-1} (CH), 1606 cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O) : [α]$_D$ at 25 ^{o}C = -5.3 ^{o}; MS (*m/z*): 316 [M^{-2} +Na]$^+$.

2D-HSQCT (DMSO-*d$_6$*) **1-*O*-ester (43)**: ^{1}H NMR δ (500.13 MHz): 3.68 (αCH), 1.59 (βCH$_2$), 1.72 (γCH), 1.04 (δ, δ′CH$_3$), 3.51 (H-1), 3.66 (H-2), 3.56 (H-3), 3.32 (H-4), 3.32 (H-5), 3.57 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 51.3 (αCH), 35.9 (βCH$_2$), 24.0 (γCH), 22.0 (δ,δ′ CH$_3$), 171.6 (CO), 60.5 (C1), 73.8 (C2), 68.8 (C3), 74.2 (C4), 70.5 (C5), 63.4 (C6) ppm.

A typical 2D-HSQCT NMR spectrum for L-leucyl-D-sorbitol **43** is shown in Figure 4.49.

4.2.4. Syntheses of L-isoleucyl esters of carbohydrates 44a,b to 53a, b

L-Isoleucine (L-2-amino-3-methyl pentanoic acid) is a polar and an essential dietary amino acid containing 3-methyl propyl group as a side chain. Here also, the solubility of L-isoleucine is low (41.2 g/L at 25 ^{o}C) like of that of L-leucine. Using optimum conditions, L-isoleucyl esters of different carbohydrates were prepared using CRL and crude PPL (Section 2.2.4, **Scheme 4.4**). The reaction mixture consists of 1 – 2 mmol L-isoleucine **4** and 1 mmol carbohydrates (D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14**, D-sorbitol **15**) along with 40 % CRL (w/w carbohydrate) / 111% of crude PPL (w/w carbohydrate) incubated with 100 ml of CH$_2$Cl$_2$ and DMF (v/v 90:10, 40 ^{o}C) containing 0.1 mM (0.1 ml of 0.1M) of phosphate buffer, pH 7.0 (CRL) or 0.2 mM (0.2 ml of 0.1M) of acetate buffer, pH 5.0 (crude PPL). The reaction mixture was analyzed by HPLC with C-18 column using acetonitrile:water (v/v 20:80) as a mobile phase and detected at 210 nm (Fig. 4.50). Ester formation was also monitored by TLC as described in Section 4.2.1. The retention times (*t$_{ret}$*) by HPLC and retention factor (*R$_f$*) values by TLC are mentioned in sections 4.2.4.1 –4.2.4.10.
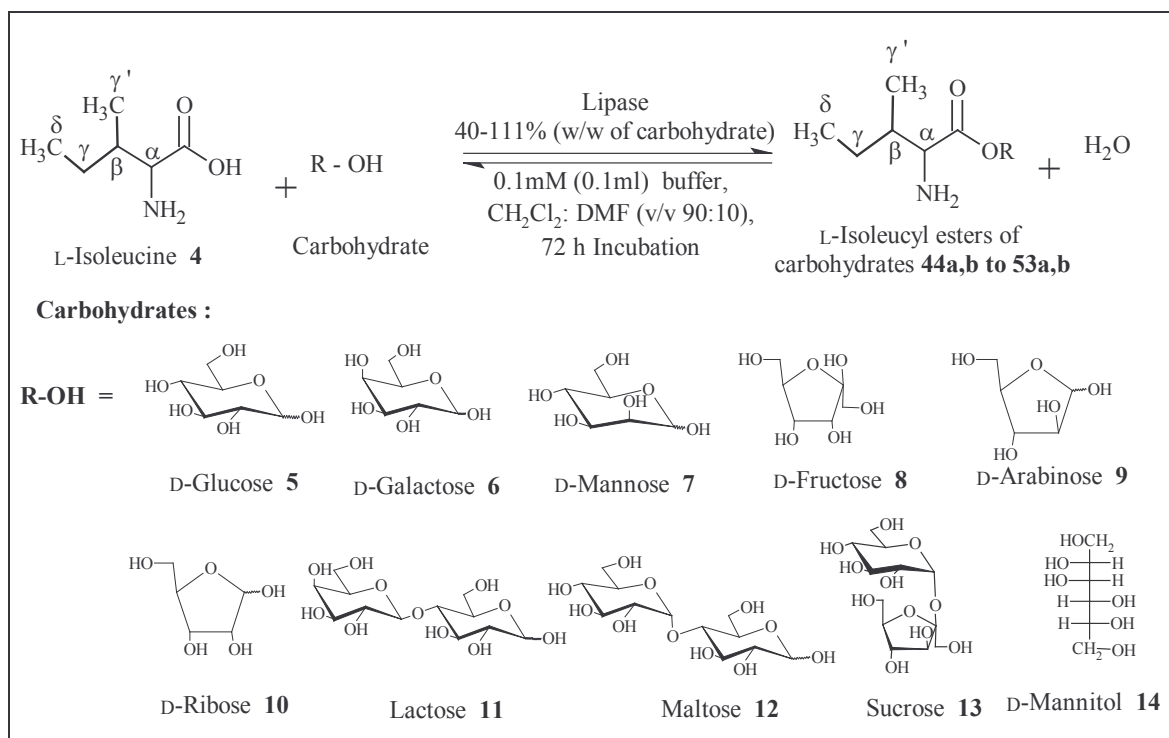
Scheme 4.4 Lipase catalyzed synthesis of L-isoleucyl esters of carbohydrates

The isolated esters were subjected to UV, IR, MS, optical rotation and 2D- NMR characterization (Sections 4.2.4.1 – 4.2.4.10). The spectral data for the isolated esters were shown in Sections 4.2.4.1 – 4.2.4.10. Table 4.8 shows the HPLC ester yields, types of esters formed and percentage proportions of the individual esters from CRL catalyzed reaction and Table 4.9 shows the HPLC ester yields from crude PPL catalyzed reaction.
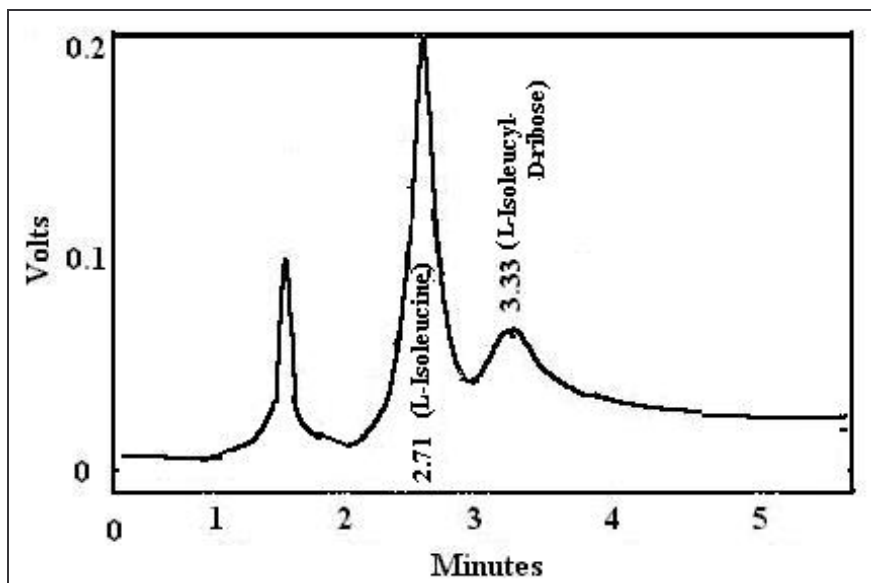
Fig. 4.50. HPLC chromatogram for reaction mixture of L-isoleucine and D-ribose esterification catalyzed by CRL. Column – C-18; mobile phase – acetonitrile: water (v/v 20:80); flow rate - 1 ml/min; detector – UV at 210 nm; errors in conversion yields are within ± 10 %.

Table 4.8 Syntheses of L-isoleucyl esters of carbohydrates[a]

L-Isoleucyl esters of carbohydrates (% proportions [b])			Yield (%)
44a 3-*O*-L-isoleucyl-D-glucose (42)	**44b** 6-*O*-L-isoleucyl-D-glucose (58)		47 (only mono esters)
45a 2-*O*-L-isoleucyl-D-galactose(78)	**45b** 3-*O*-L-isoleucyl-D-galactose(10)	**45c** 6-*O*-L-isoleucyl-D-galactose(12)	46 (only mono esters)
46a 3-*O*-L-isoleucyl-D-mannose (19)	**46b** 4-*O*-L-isoleucyl -D-mannose (13)	**46c** 6-*O*-L-isoleucyl-D-mannose (13)	55 (mono esters-25, diesters-30)
46d 3,6-di-*O*-L-isoleucyl -D-mannose (27)	**46e** 4,6-di-*O*-L-isoleucyl-D-mannose (28)		
47a 1-*O*-L-isoleucyl-D-fructose (36)	**47b** 6-*O*-L-isoleucyl-D-fructose (30)	**47c** 1,6-di-*O*-L- isoleucyl-D-fructose (34)	43 (mono esters-28, diester-15)
48a 2-*O*-L-isoleucyl-D-arabinose (24)	**48b** 5-*O*-L-isoleucyl-D-arabinose (33)	**48c** 2,5-di-*O*-L-isoleucyl-D-arabinose (43)	55 [c] (mono esters-31, diester-24)

49a 3-*O*-L-isoleucyl-D-ribose (52) **49b** 5-*O*-L-isoleucyl-D-ribose (20) **49c** 3,5-di-*O*-L-isoleucyl-D-ribose (28)

53 [c]
(mono esters-38, diester-15)

50a 2-*O*-L-isoleucyl -lactose (39) **50b** 6-*O*-L-isoleucyl-lactose (40) **50c** 6′-*O*-L-isoleucyl-lactose (21)

45
(only mono esters)

51a 2-*O*-L-isoleucyl-maltose (38) **51b** 6-*O*-L-isoleucyl-maltose (40) **51c** 6′-*O*-L-isoleucyl-maltose (22)

54
(only mono esters)

52 6-*O*-L-isoleucyl – sucrose

22
(only mono ester)

53a 1-*O*-L-isoleucyl -D-mannitol (62) **53b** 1,6-di-*O*-L- isoleucyl-D-mannitol (38)

52
(mono esters-32, diester-20)

[a] L-Isoleucine – 2 mmol, carbohydrates – 1 mmol, CRL – 40 % (w/w carbohydrate), buffer – 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0), CH_2Cl_2:DMF (v/v 90:10) at 40 ^{o}C, incubation period – 72 h. Conversion yields were from HPLC with respect to L-isoleucine concentration.

[b] Percentage proportions of individual esters were determined from the peak areas or from their cross peaks of the Carbon-13 C6 and C5 (in case of pentoses) signals in the 2D HSQCT spectrum.

[c] Several cross peaks, due to opening and/or degradation of the five membered ring during esterification.

Table 4.9 Preparation of L-isoleucyl esters of carbohydrates using crude porcine pancreas lipase [a]

L-Isoleucyl ester of carbohydrate	%Yield (mmol)
L-Isoleucyl-D-glucose **44a,b**	30 (0.30)
L-Isoleucyl-D-galactose **45a-c**	21 (0.21)
L-Isoleucyl-D-mannose **46a-e**	22 (0.22)
L-Isoleucyl-D-fructose **47a-c**	25 (0.25)
L-Isoleucyl-D-arabinose **48a-c**	9 (0.09)
L-Isoleucyl-D-ribose **49a-c**	--
L-Isoleucyl-lactose **50a-c**	39 (0.39)
L-Isoleucyl-maltose **51a-c**	32 (0.32)
L-Isoleucyl-sucrose **52**	17 (0.17)
L-Isoleucyl-D-mannitol **53a,b**	28 (0.28)

[a] L-Isoleucine – 1 mmol, carbohydrates – 1 mmol, Crude PPL – 111% (w/w carbohydrate), buffer – 0.2 mM (0.2 ml of 0.1 M) acetate buffer pH 5.0, CH_2Cl_2:DMF (v/v 90:10) at 40 $^{\circ}$C, Incubation period – 72 h. Conversion yields were from HPLC with respect to L-isoleucine concentration.

Spectral data for L-isoleucine (4) : Solid; Mpt : 284 $^{\circ}$C; HPLC t_{ret}: 2.7 min; R_f: 0.33; UV (H_2O, λ_{max}): 195.0 nm ($\sigma \to \sigma^*$ $\varepsilon_{195.0}$ - 126 M^{-1}), IR (KBr, stretching frequency): 3415 cm^{-1} (OH), 2945 cm^{-1} (CH), 1584 cm^{-1} (CO); optical rotation (*c* 1.0, H_2O) : $[\alpha]_D$ at 25 $^{\circ}$C = +8.6 $^{\circ}$.

2D-HSQCT (DMSO-d_6) : ^{1}H NMR δ (500.13 MHz) : 3.03 (αCH), 1.72 (βCH), 0.84 (γCH$_2$), 1.38 (γ'CH$_3$), 0.84 (δCH$_3$) ppm; ^{13}C NMR δ (125 MHz): 52.5 (αCH), 36.8 (βCH), 15.1 (γCH$_2$), 25.0 (γ'CH$_3$), 11.2 (δCH$_3$), 171.4 (CO) ppm.

4.2.4.1. L-Isoleucyl-D-glucose (44a, b): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.23; UV (H_2O, λ_{max}): 227.0 nm ($\sigma \to \sigma^*$ $\varepsilon_{227.0}$ - 1862 M^{-1}), 278.0 nm (n$\to \pi^*$ $\varepsilon_{278.0}$ - 1047 M^{-1}); IR (KBr, stretching frequency): 3420 cm^{-1} (NH), 3350 cm^{-1} (OH), 2966 cm^{-1} (CH), 1631 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^+$.
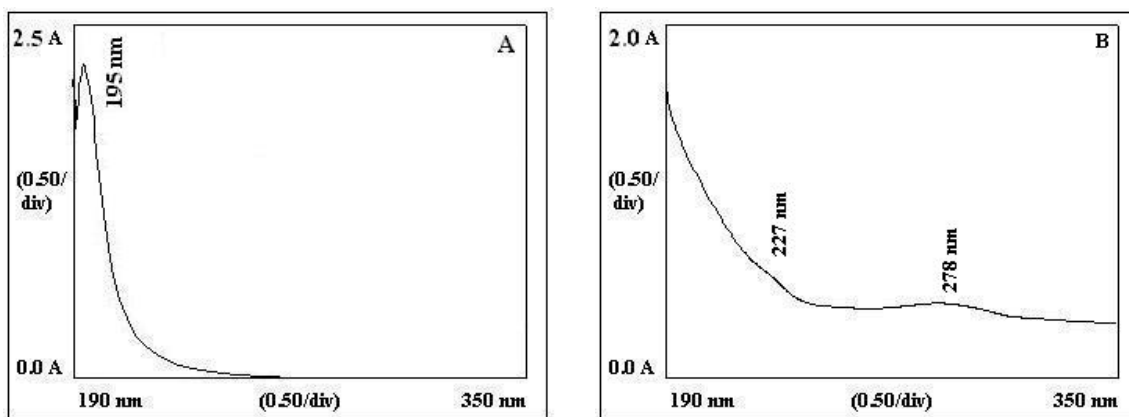
Fig. 4.51 UV spectra for L-isoleucyl-D-glucose **44a,b** from CRL catalyzed reaction (A) L-isoleucine; (B) L-isoleucyl-D-glucose.
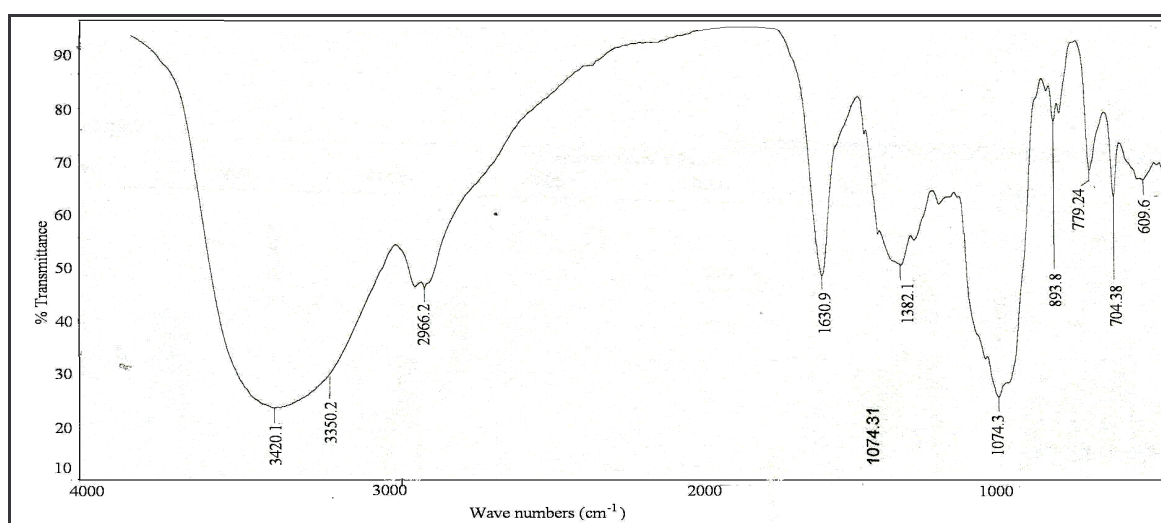
Fig. 4.52. A typical IR spectrum of L-isoleucyl-D-glucose **44a,b** of CRL catalyzed reaction. A 1.5 mg of ester sample was prepared as KBr pellet and used.
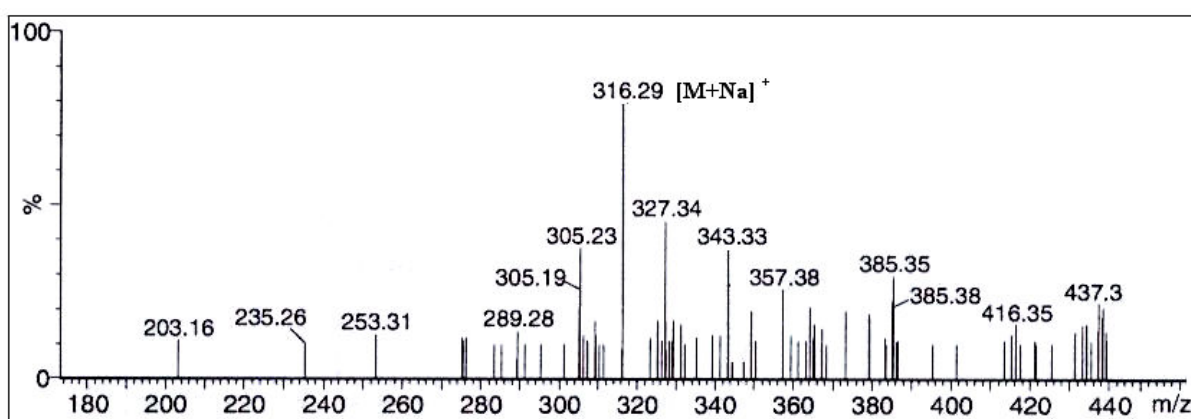
Fig. 4.53. A typical mass spectrum of L-isoleucyl-D-glucose **44a,b**.

2D-HSQCT (DMSO-d_6) : **3-*O*-ester (44a)**: ^{1}H NMR δ (500.13 MHz) : 3.13 (αCH), 1.61 (βCH), 0.61 (γCH$_2$), 1.08 (γ′CH$_3$), 0.59 (δCH$_3$), 5.0 (H-1α), 4.4 (H-1β), 4.01 (H-3α), 3.88 (H-3β), 3.46 (H-4α), 3.58 (H-6) ppm; ^{13}C NMR δ (125 MHz): 51.0 (αCH), 35.3 (βCH), 14.0 (γCH$_2$), 25.6 (γ′CH$_3$), 11.2 (δCH$_3$), 171.4 (CO), 91.8 (C1α), 95.8 (C1β). 82.0 (C3α), 81.9 (C3β), 67.5 (C4α), 63.0 (C6α) ppm; **6-*O*-ester (44b)**: ^{1}H NMR δ : 3.11 (αCH), 1.61 (βCH), 1.08 (γ′CH$_3$), 0.61 (γCH$_2$), 0.59 (δCH$_3$), 5.0 (H-1α), 4.4 (H-1β), 3.59 (H-2α), 3.48 (H-3α), 3.64 (H-4α), 3.63 (H-5α), 3.82 (H-6) ppm; ^{13}C NMR δ : 53.1 (αCH), 35.3 (βCH), 25.2 (γ′CH$_3$), 14.0 (γCH$_2$), 11.2 (δCH$_3$), 91.8 (C1α), 95.8 (C1β), 70.0 (C2α), 72.2 (C3α), 69.0 (C4α), 69.2 (C4α), 63.6 (C6α) ppm.

A typical UV, IR, mass and 2D-HSQCT NMR spectra for L-isoleucyl-D-glucose **44a,b** are shown in Figures 4.51, 4.52, 4.53 and 4.54 respectively.

4.2.4.2. L-Isoleucyl-D-galactose (45a-c): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.23; UV (H$_2$O, λ_{max}): 224.0 nm (σ→σ* $\varepsilon_{224.0}$ - 1622 M^{-1}), 290.0 nm (n→π* $\varepsilon_{290.0}$ - 776 M^{-1}), IR (KBr, stretching frequency): 3309 cm^{-1} (NH), 2944 cm^{-1} (CH), 1621 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (45a)**: ^{1}H NMR δ (500.13 MHz): 3.81 (H-2α), 3.45 (H-5α), 3.40 (H-6) ppm; ^{13}C NMR δ (125 MHz): 76.2 (C2α), 70.5 (C5α), 60.4 (C6α) ppm; **3-*O*-ester (45b)**: ^{1}H NMR δ : 3.58 (H-3α), 3.58 (H-3α), 3.66 (H-3β), 3.64 (H-6) ppm; ^{13}C NMR δ : 66.3 (C2α), 81.3 (C3α), 82.2 (C3β), 60.4 (C6α) ppm; **6-*O*-ester (45c)**: ^{1}H NMR δ : 2.79 (αCH), 1.78 (αCH), 0.94 (γCH$_2$), 1.35 (γ′CH$_3$), 0.93 (δCH$_3$), 4.97 (H-1α), 4.87 (H-1β), 3.51 (H-2α), 3.69 (H-3α), 3.76 (H-4α), 3.22 (H-4β), 3.59 (H-5α), 3.30 (H-5β), 3.40 (H-6) ppm; ^{13}C NMR δ : 53.5 (αCH), 36.5 (βCH), 15.2 (γCH$_2$), 25.0 (γ′CH$_3$), 11.2 (δCH$_3$), 95.0 (C1α), 101.6 (C1β), 68.8 (C2α), 68.5 (C3α), 70.5 (C4α), 72.6 (C4β),75.0 (C5α), 62.8 (C6α) ppm.
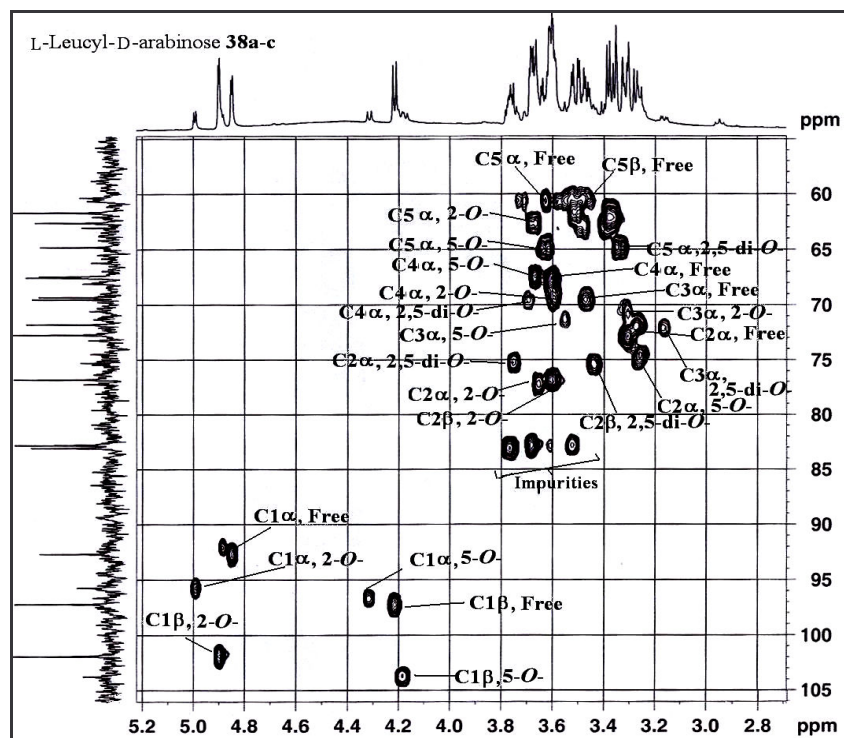
Fig. 4.54. Two-dimensional HSQCT NMR for L-isoleucyl- D-glucose **44a,b** obtained through CRL catalysis.
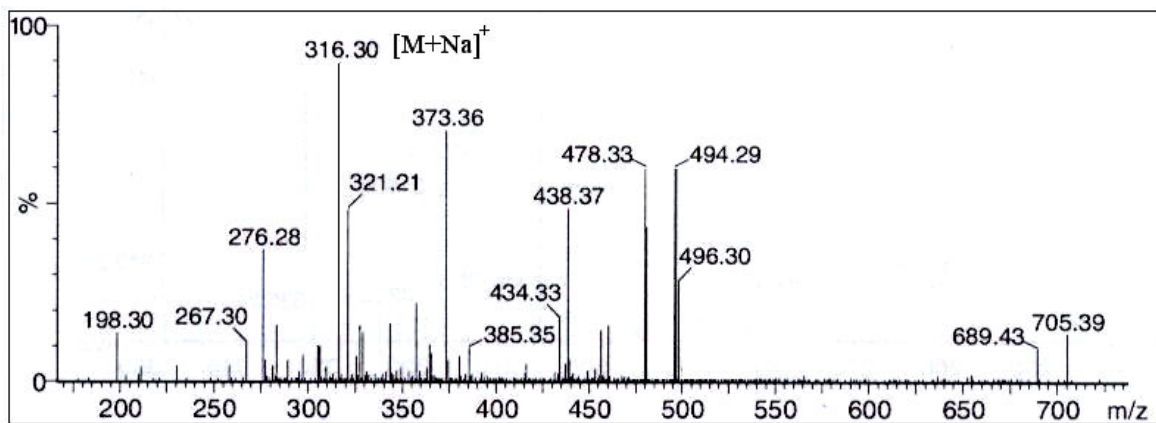
Fig. 4.55. A typical mass spectrum of L-isoleucyl-D-galactose **45a-c**.

A typical mass spectrum for L-isoleucyl-D-galactose **45a-c** is shown in Figure 4.55.

4.2.4.3. L-Isoleucyl-D-mannose (46a-e): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.23; UV (H$_2$O, λ_{max}): 226.0 nm ($\sigma\rightarrow\sigma^*$ $\varepsilon_{226.0}$ - 1737.8 M^{-1}), 281.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{281.0}$ - 891 M^{-1}); IR (KBr, stretching frequency): 3385 cm^{-1} (NH), 3267 cm^{-1} (OH), 2966 cm^{-1} (CH), 1641 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **3-O- Ester (46a)**: ^{1}H NMR δ (500.13 MHz) : 3.72 (H-3α), 3.85 (H-3β), 2.92 (H-4α), 3.41 (H-6) ppm; ^{13}C NMR δ (125 MHz): 80.5 (C3α), 83.1 (C3β), 65.9 (C4α), 60.5 (C6α) ppm; **4-O-ester (46b)**: ^{1}H NMR δ : 2.72 (αCH), 3.80 (H-4α), 3.90 (H-4β), 3.41 (H-6) ppm; ^{13}C NMR δ : 52.7 (αCH), 75.0 (C4α), 77.1 (C4β), 60.5 (C6α) ppm; **6-O-ester (46c)**: ^{1}H NMR δ : 2.68 (αCH), 1.72 (βCH), 0.77 (γCH$_2$), 1.38 (γ'CH$_3$), 0.79 (δCH$_3$), 4.31 (H-1α), 3.64 (H-2α), 3.58 (H-3α), 3.06 (H-4α), 3.58 (H-5α), 3.79 (H-6) ppm; ^{13}C NMR δ : 52.4 (αCH), 36.8 (αCH), 25.0 (γ'CH$_3$), 15.0 (γCH$_2$), 11.3 (δCH$_3$), 103.0 (C1α), 69.0 (C2α), 69.8 (C3α), 65.8 (C4α), 69.9 (C5α), 63.1 (C6α) ppm; **3,6-di-O-ester (46d)**: ^{1}H NMR δ : 3.55 (H-3α), 3.41 (H-6) ppm; ^{13}C NMR δ : 82.1 (C3α), 62.9 (C6α) ppm; **4,6-di-O-ester (46e)**: ^{1}H NMR δ : 3.75 (H-4α), 3.52 (H-6) ppm; ^{13}C NMR δ : 76.0 (C4α), 62.9 (C6α) ppm.

A typical UV and 2D-HSQCT NMR spectra for L-isoleucyl-D-mannose **46a-e** are shown in Figures 4.56 and 4.57 respectively.

4.2.4.4. L-Isoleucyl-D-fructose (47a-c): Solid; HPLC t_{ret}: 3.5 min; R_f: 0.23; UV (H$_2$O, λ_{max}): 226.0nm ($\sigma\rightarrow\sigma^*$ $\varepsilon_{226.0}$ – 309 M^{-1}), 302.0 nm (n$\rightarrow\pi^*$ $\varepsilon_{302.0}$ -110 M^{-1}); IR (KBr, stretching frequency): 3378 cm^{-1} (NH), 3217 cm^{-1} (OH), 2900 cm^{-1} (CH), 1632 cm^{-1} (CO); MS (*m/z*) : 316 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **1-O-ester (47a)**: ^{1}H NMR δ (500.13 MHz): 3.15 (αCH), 1.65 (βCH), 0.87 (γCH$_2$), 1.35 (γ'CH$_3$), 0.83 (δCH$_3$), 3.25 (H-1α), 3.32 (H-3α), 3.32 (H-4α),
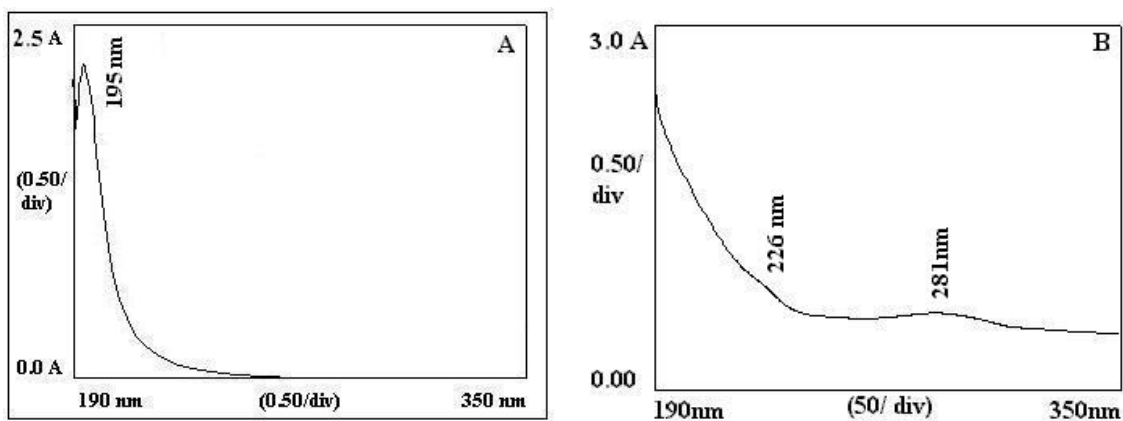
Fig. 4.56 UV spectra for L-isoleucyl-D-mannose **46a-e** from CRL catalyzed reaction (A) L-isoleucine; (B) L-isoleucyl-D-mannose.
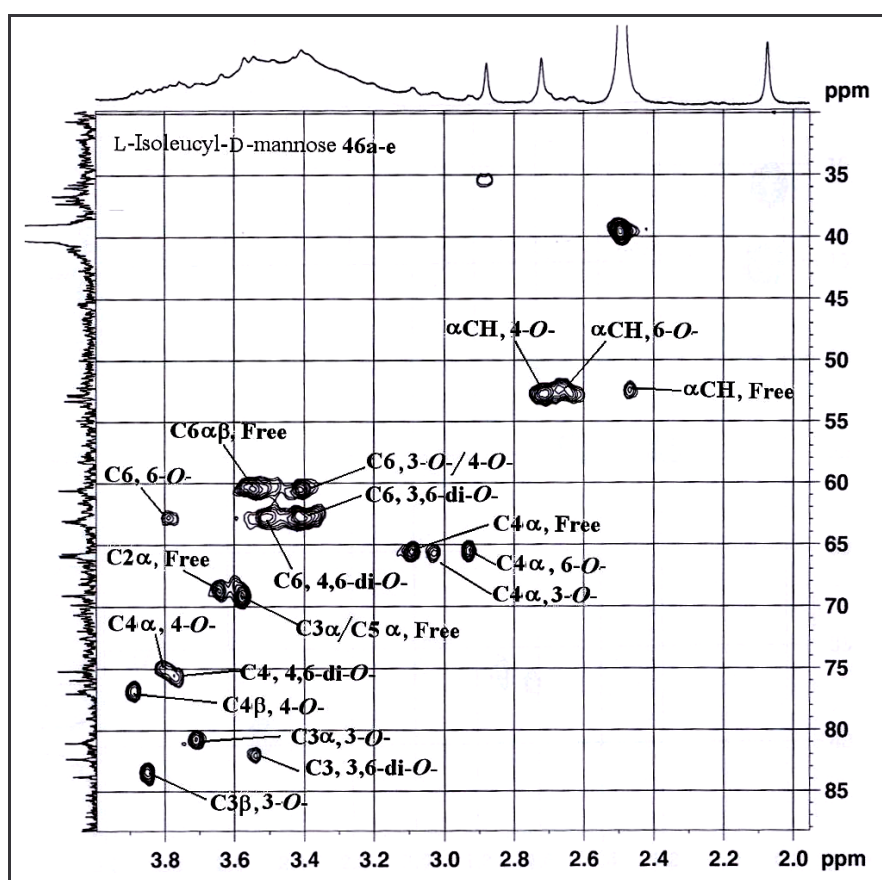
Fig. 4.57. Two-dimensional HSQCT NMR for L-isoleucyl-D-mannose **46a-e** obtained through CRL catalysis.

3.73 (H-4β), 3.72 (H-5β), 3.50 (H-6) ppm; ^{13}C NMR δ (125 MHz): 57.0 (αCH), 36.9

(βCH), 14.8 (γCH$_2$), 24.8 (γ′CH$_3$), 11.4 (δCH$_3$), 63.7 (C1α), 104.0 (C2α), 72.8 (C3α),

70.5 (C4α), 75.8 (C4β), 81.0 (C5β), 62.4 (C6α) ppm; **6-*O*-ester (47b)**: ^{1}H NMR δ : 3.77

(H-5α), 3.78 (H-6) ppm; ^{13}C NMR δ : 71.5 (C5α), 63.1 (C6) ppm; **1,6-di-*O*-ester (47c)**:

^{1}H NMR δ : 3.28 (H-1α) ppm; ^{13}C NMR δ: 64.4 (C1α) ppm.

A typical 2D-HSQCT NMR spectrum for L-isoleucyl-D-fructose **47a-c** is shown in

Figure 4.58.

4.2.4.5. L-Isoleucyl-D-arabinose (48a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.21; UV (H$_2$O,

$λ_{max}$): 225.0 nm (σ→σ* $ε_{225.0}$ - 661 M^{-1}), 297.0 nm (n→π* $ε_{297.0}$ - 363.1 M^{-1}); IR (KBr,

stretching frequency): 3320 cm^{-1} (NH), 3168 cm^{-1} (OH), 2929 cm^{-1} (CH), 1629 cm^{-1}

(CO);

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (48a)**: ^{1}H NMR δ (500.13 MHz) : 3.70 (αCH), 1.65

(βCH), 1.35 (γCH$_2$), 0.87 (γ′CH$_3$), 0.83 (δCH$_3$), 4.32 (H-1α), 4.89 (H-1β), 3.76 (H-2α),

3.45 (H-2β), 3.70 (H-3α), 3.68 (H-5) ppm; ^{13}C NMR δ (125 MHz): 54.8 (αCH), 36.5

(βCH), 14.8 (γCH$_2$), 24.8 (γ′CH$_3$), 11.4 (δCH$_3$), 96.6 (C1α), 92.0 (C1β), 75.0 (C2α),

75.2 (C2β), 69.5 (C3α), 62.6 (C5α) ppm;. **5-*O*-ester (48b)**: ^{1}H NMR δ : 4.91 (H-1α),

4.99 (H-1β), 3.27 (H-2α), 3.60 (H-3α), 3.67 (H-4α), 3.64 (H-5) ppm; ^{13}C NMR δ: 101.6

(C1α), 94.2 (C1β), 74.2 (C2α), 69.5 (C3α), 67.3 (C4α), 64.8 (C5α) ppm; **2,5-di-*O*-ester**

(48c): ^{1}H NMR δ : 4.18 (H-1α), 3.66 (H-2α), 2.96 (H-3α), 3.35 (H-5) ppm; ^{13}C NMR δ :

103.5 (C1α), 77.2 (C2α), 74.2 (C3α), 64.8 (C5α) ppm.

A typical UV spectrum for L-isoleucyl-D-arabinose **48a-c** is shown in Figure 4.59.

4.2.4.6. L-Isoleucyl-D-ribose (49a-c): Solid; HPLC t_{ret}: 3.3 min; R_f: 0.21; UV (H$_2$O,

$λ_{max}$): 225.0 nm (σ→σ* $λ_{225.0}$ - 1585 M^{-1}), 284.0 nm (n→π* $λ_{284.0}$ - 871 M^{-1}); IR (KBr,
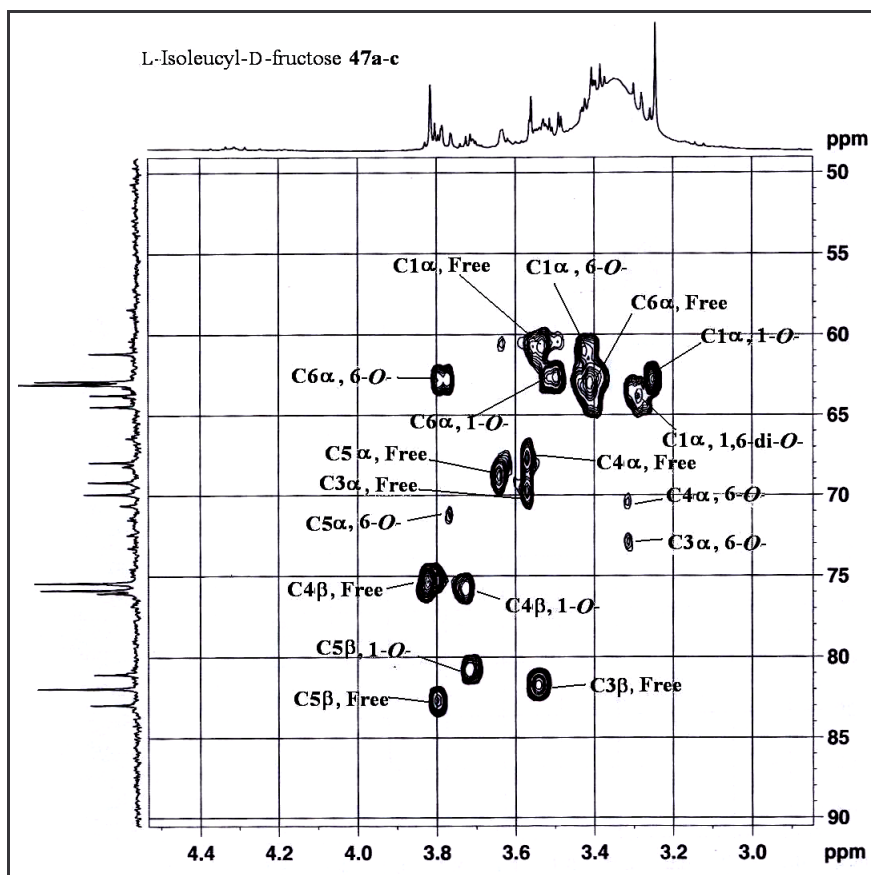
Fig. 4.58. Two-dimensional HSQCT NMR for L-isoleucyl-D-fructose **47a-c** obtained through CRL catalysis.
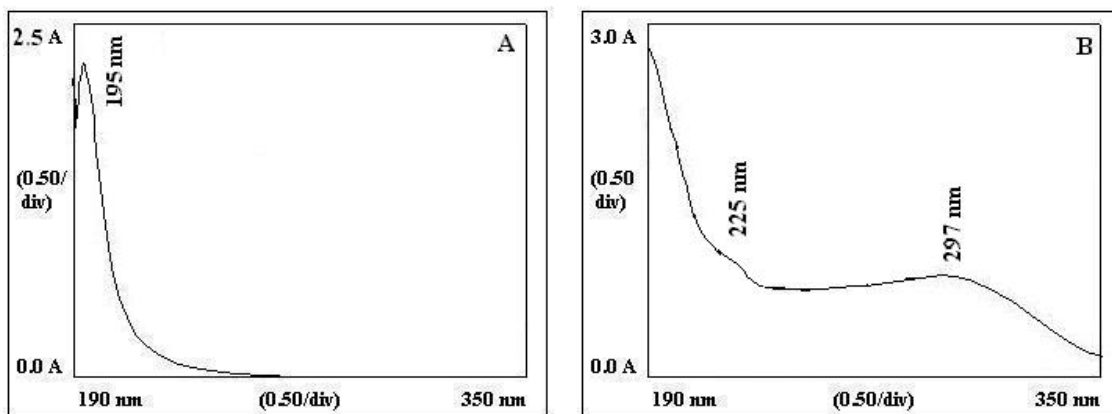
Fig. 4.59. UV spectra for (A) L-isoleucine (B) L-isoleucyl-D-arabinose **48a-c**, CRL catalyzed reaction; concentration – 2.6 mM.

stretching frequency): 3384 cm^{-1} (NH), 3233 cm^{-1} (OH), 2936 cm^{-1} (CH), 1631 cm^{-1} (CO); MS (*m/z*) : 283 [M^{-2}+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **3-*O*- ester (49a)**: ^{1}H NMR δ (500.13 MHz) : 2.86 (αCH), 1.65 (βCH), 0.87 (γCH$_2$), 1.35 (γ′CH$_3$), 0.83 (δ CH$_3$), 4.90 (H-1β), 3.98 (H-3α), 3.92 (H-3β), 3.64 (H-5) ppm; ^{13}C NMR δ (125 MHz): 51.1 (αCH), 36.5 (βCH), 14.8 (γCH$_2$), 24.8 (γ′CH$_3$), 11.4 (δ CH$_3$), 91.9 (C1α), 76.5 (C3α), 77.7 (C3β), 60.4 (C5α) ppm; **5-*O*-ester (49b)**: ^{1}H NMR δ: 5.05 (H-1α), 3.65 (H-2α), 3.50 (H-3β), 3.87 (H-4α), 3.72 (H-4β), 3.58 (H-5) ppm; ^{13}C NMR δ : 95.9 (C1α), 73.1 (C2α), 66.5 (C3β), 70.5 (C4α), 70.7 (C4β), 63.2 (C5α) ppm; **3,5-di-*O*-ester (49c)**: ^{1}H NMR δ: 4.64 (H-1α), 3.60 (H-3α), 3.76 (H-4α), 3.52 (H-5) ppm; ^{13}C NMR δ : 93.5 (C1α), 75.5 (C3α), 70.3 (C4α), 63.3 (C5α) ppm.

A 2D-HSQCT NMR spectrum for L-isoleucyl-D-ribose **49a-c** is shown in Figure 4.60.

4.2.4.7. L-Isoleucyl-lactose (50a-c): Solid; HPLC t_{ret}: 3.4 min; R_f: 0.12; UV (H$_2$O, λ$_{max}$): 227.0 nm (σ→σ* ε$_{227.0}$ – 81 M^{-1}), 279.0 nm (n→π* ε$_{279.0}$ - 37 M^{-1}); IR (KBr, stretching frequency): 3520 cm^{-1} (NH), 3344 cm^{-1} (OH), 2900 cm^{-1} (CH), 1640 cm^{-1} (CO); MS (*m/z*) : 478 [M+Na]$^+$.

2D-HSQCT (DMSO-d_6) : **2-*O*-ester (50a)**: ^{1}H NMR δ (500.13 MHz) : 4.28 (αCH), 1.78 (βCH), 0.84 (γCH$_2$), 1.30 (γ′CH$_3$), 0.81 (δ CH$_3$), 4.96 (H-1α), 4.32 (H-1β), 4.0 (H-2α), 3.82 (H-2β), 3.56 (H-3α), 2.96 (H-3β), 3.27 (H-4α), 3.17 (H-5α), 3.28 (H-5β), 3.49 (H-6), 5.0 (H-1′α), 3.06 (H-2′), 3.23 (H-3′), 3.63 (H-4′), 3.67 (H-5′), 3.64 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 53.8 (αCH), 36.5 (βCH), 15.3 (γCH$_2$), 24.5 (γ′CH$_3$), 11.2 (δ CH$_3$), 172.5 (CO), 92.0 (C1α), 96.5 (C1β), 84.5 (C2α), 85.0 (C2β), 71.5 (C3α), 74.8 (C3β), 81.4 (C4α), 81.1 (C4β), 72.3 (C5α), 75.1 (C5β), 63.0 (C6α), 100.7 (C1′α), 70.3 (C2′), 71.8 (C3′), 69.9 (C4′), 72.4 (C5′), 60.6 (C6′) ppm; **6-*O*-ester (50b)**: ^{1}H NMR δ: 3.85 (H-6), 3.50 (H-6′) ppm; ^{13}C NMR δ: 66.5 (C6α), 68.0 (C6′) ppm; **6′-*O*-ester (50c)**: ^{1}H NMR
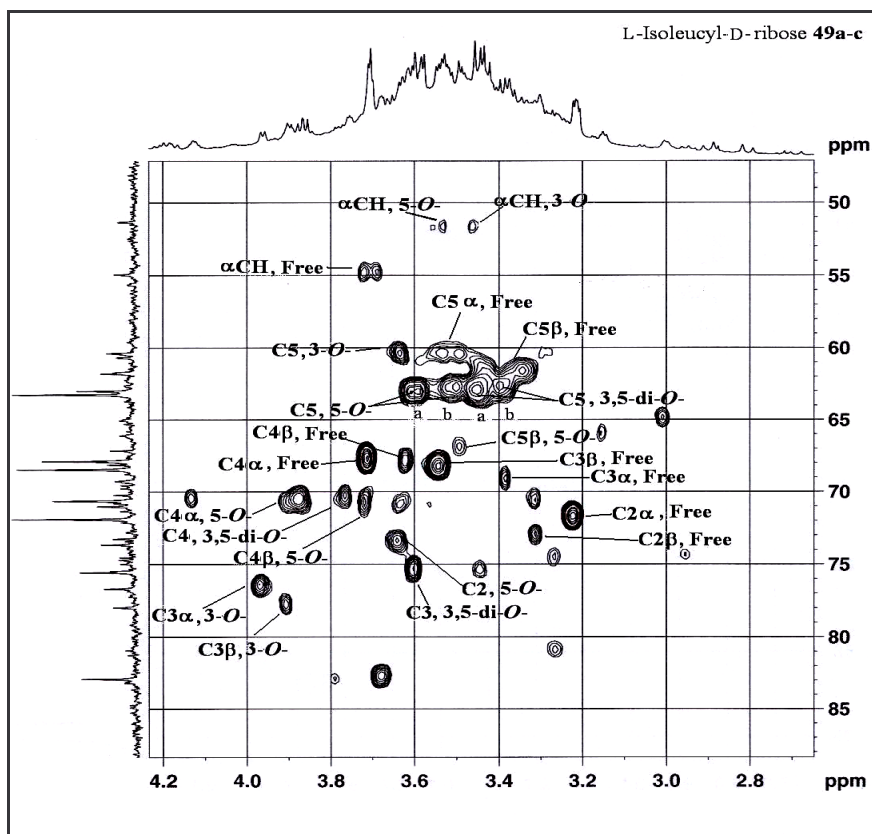
Fig. 4.60. Two-dimensional HSQCT NMR for L-isoleucyl- D-ribose **49a-c** obtained through CRL catalysis.
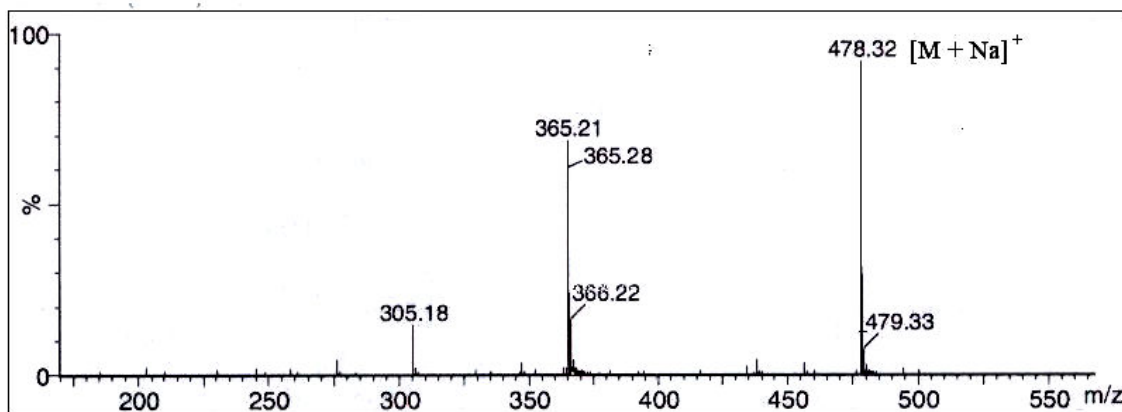
Fig. 4.61. A typical mass spectrum of L-isoleucyl-lactose **50a-c**.

δ : 3.50 (H-6), 3.54 (H-4′), 3.85 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 61.5 (C6α), 70.0 (C4′), 66.9 (C6′) ppm.

A typical mass and 2D-HSQCT NMR spectra for L-isoleucyl-lactose **50a-c** are shown in Figures 4.61 and 4.62 respectively.

4.2.4.8. L-Isoleucyl-maltose (51a-c): Solid; Mpt. 122 ^{o}C; HPLC t_{ret}: 3.4 min; R_f: 0.12; UV (H$_2$O, λ$_{max}$): 224.0 nm (σ→σ* ε$_{224.0}$ - 525 M^{-1}), 276.0 nm (n→π* ε$_{276.0}$ - 234.4 M^{-1}); IR (KBr, stretching frequency) : 3450 cm^{-1} (NH), 3386 cm^{-1} (OH), 2937 cm^{-1} (CH), 1646 cm^{-1} (CO); MS (*m/z*) : 478 [M+Na]$^{+}$.

2D-HSQCT (DMSO-d_6) : **2-O-ester (51a)**: ^{1}H NMR δ (500.13 MHz) : 4.25 (αCH), 1.78 (βCH), 0.84 (γCH$_2$), 1.30 (γ′CH$_3$),0.81 (δCH$_3$), 4.91 (H-1α), 4.32 (H-1β), 4.05 (H-2α), 3.96 (H-2β), 3.40 (H-3α), 3.29 (H-4β), 3.42 (H-5α), 3.38 (H-5β), 3.48 (H-6), 5.0 (H-1′α), 3.06 (H-2′), 3.23 (H-3′), 3.63 (H-4′), 3.67 (H-5′), 3.64 (H-6′) ppm; ^{13}C NMR δ (125 MHz) : 53.8 (αCH), 36.5 (βCH), 15.3 (γCH$_2$), 24.5 (γ′CH$_3$), 11.2 (δCH$_3$), 172.5 (CO), 92.0 (C1α), 96.5 (C1β), 81.2 (C2α), 81.4 (C2β), 76.3 (C3α), 79.9 (C4β), 72.4 (C5α), 72.8 (C5β), 63.0 (C6α), 100.7 (C1′α), 70.3 (C2′), 71.8 (C3′), 69.9 (C4′), 72.4 (C5′), 60.6 (C6′) ppm; **6-O-ester (51b)**: ^{1}H NMR δ : 3.21 (H-4β), 3.32 (H-5α), 3.62 (H-6), 3.70 (H-4′) ppm; ^{13}C NMR δ : 79.5 (C4β), 70.5 (C5α), 68.0 (C6α), 69.5 (C4′) ppm; **6′-O-ester (51c)**: ^{1}H NMR δ : 3.55 (H-6), 3.62 (H-6′) ppm;^{13}C NMR δ (125 MHz) : 63.0 (C6α), 67.2 (C6′) ppm.

A typical UV and mass spectra for L-isoleucyl-maltose **51a-c** are shown in Figures 4.63 and 4.64 respectively.

4.2.4.9. L-Isoleucyl-sucrose (52): Solid; Mpt. 162 ^{o}C; HPLC t_{ret}: 3.4 min; R_f: 0.12; UV (H$_2$O, λ$_{max}$): 226.0 nm (σ→σ* ε$_{226.0}$ - 324 M^{-1}), 275.0 nm (n→π* ε$_{275.0}$ - 174 M^{-1}); IR (KBr, stretching frequency): 3598 cm^{-1} (NH), 3455 cm^{-1} (OH), 3105 cm^{-1} (CH), 1616
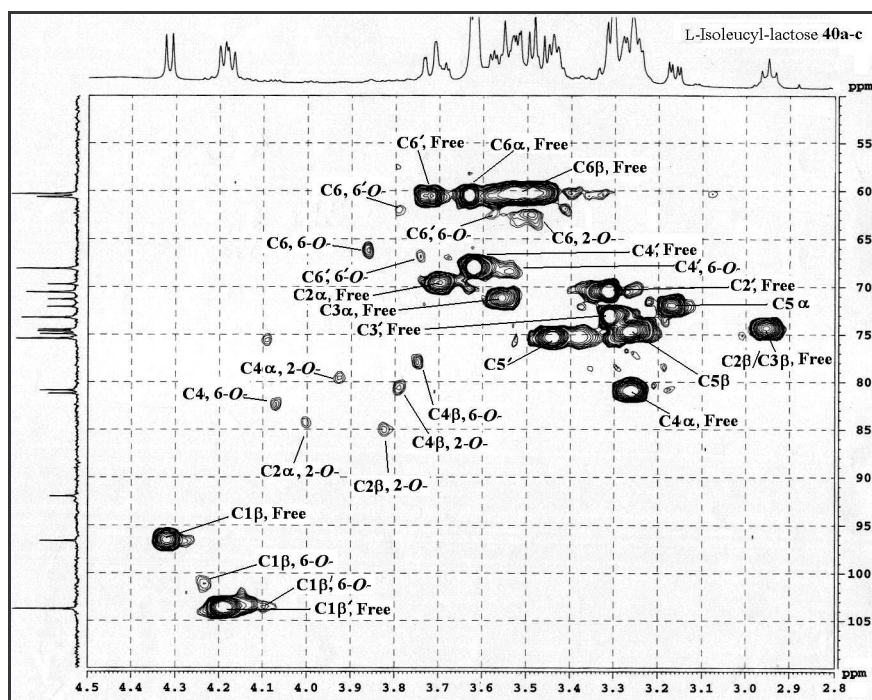
Fig. 4.62. Two-dimensional HSQCT NMR for L-isoleucyl-lactose **50a-c** obtained through CRL catalysis.
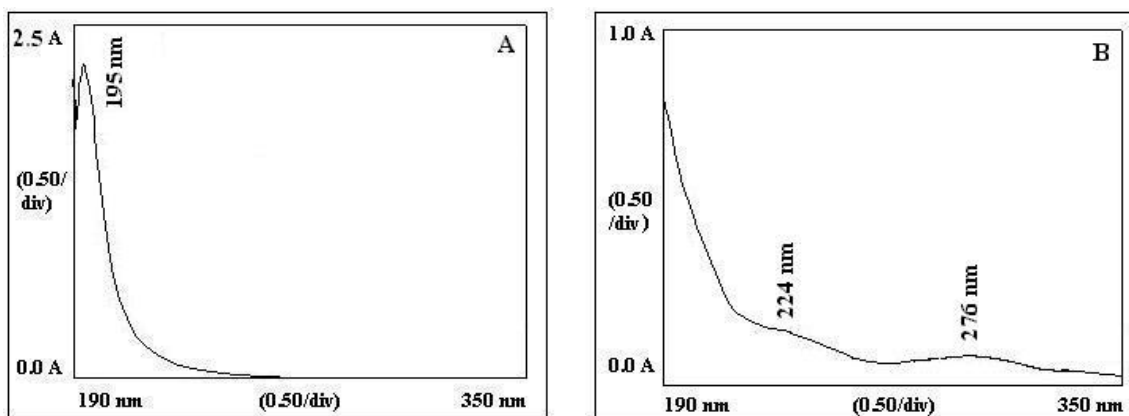
Fig. 4.63 UV spectra for L-isoleucyl-maltose **51a-c** from CRL catalyzed reaction. (A) L-isoleucine (B) L-isoleucyl-maltose.

cm^{-1} (CO); optical rotation (*c* 0.5, H$_2$O): [α]$_D$ at 25 ^{o}C = -28.3 ^{o}; MS (*m/z*) : 478 [M+Na]$^+$.

2D-HSQCT (DMSO-*d$_6$*) : **6-*O*- ester (52)**: ^{1}H NMR δ (500.13 MHz): 4.25 (αCH), 1.78 (βCH), 0.84 (γCH$_2$), 1.30 (γ′CH$_3$), 0.81 (δ CH$_3$), 3.41 (H-1β), 3.31 (H-3β), 3.27 (H-4β), 3.87 (H-5β), 3.63 (H-6), 4.89 (H-1′α), 3.19 (H-2′), 3.44 (H-3′), 3.32 (H-4′), 3.70 (H-5′), 3.63 (H-6′a) and 3.71 (H-6′b) ppm; ^{13}C NMR δ (125 MHz) : 54.8 (αCH), 36.9 (βCH), 24.5 (γCH$_2$), 15.3 (γ′CH$_3$), 11.2 (δCH$_3$), 172.5 (CO), 62.3 (C1β), 103.5 (C2β), 73.0 (C3β, 81.0 (C4β), 74.5 (C5β), 67.2 (C6), 92.0 (C1′α), 71.8 (C2′), 75.0 (C3′), 70.5 (C4′), 69.5 (C5′), 60.7 (C6′) ppm.

A typical mass spectrum for L-isoleucyl-sucrose **52** is shown in Figure 4.65.

4.2.4.10. L-Isoleucyl-D-mannitol (53a, b): Solid; HPLC *t$_{ret}$*: 3.3 min; *R$_f$*: 0.15; UV (H$_2$O, λ$_{max}$): 214.0 nm (σ→σ* ε$_{214.0}$ - 87 M^{-1}), 281.0 nm (n→π* ε$_{281.0}$ - 22 M^{-1}); IR (KBr, stretching frequency): 3425 cm^{-1} (NH), 3068 cm^{-1} (OH), 2957 cm^{-1} (CH), 1630 cm^{-1} (CO); MS (*m/z*) : 319 [M^{+1}+Na]$^+$.

2D-HSQCT (DMSO-*d$_6$*) : **1-*O*-ester (53a)**: ^{1}H NMR δ (500.13 MHz) : 3.15 (αCH), 1.78 (βCH), 1.30 (γCH$_2$), 0.84 (γ′CH$_3$), 0.81 (δCH$_3$), 3.49 (H-1a), 3.53 (H-1b), 3.41 (H-2), 3.48 (H-3), 3.54 (H-4), 3.32 (H-5), 3.45 (H-6) ppm; ^{13}C NMR δ (125 MHz) : 58.2 (αCH), 39.5 (βCH), 24.5 (γCH$_2$), 15.3 (δ′CH$_3$), 11.2 (δCH$_3$), 172.5 (CO), 60.8 (C1), 71.2 (C2), 69.0 (C3), 71.0 (C4), 70.8 (C5), 63.8 (C6) ppm; **1,6-di-*O*- ester (53b)**: ^{1}H NMR δ : 3.63 (H-1), 3.45 (H-2), 3.63 (H-3), 3.32 (H-5), 3.29 (H-6) ppm; ^{13}C NMR δ : 60.8 (C1), 75.2 (C2), 68.0 (C3), 73.0 (C5), 63.5 (C6) ppm.

A typical UV and 2D-HSQCT NMR spectra for L-isoleucyl-D-mannitol **53a,b** are shown in Figures 4.66 and 4.67 respectively.
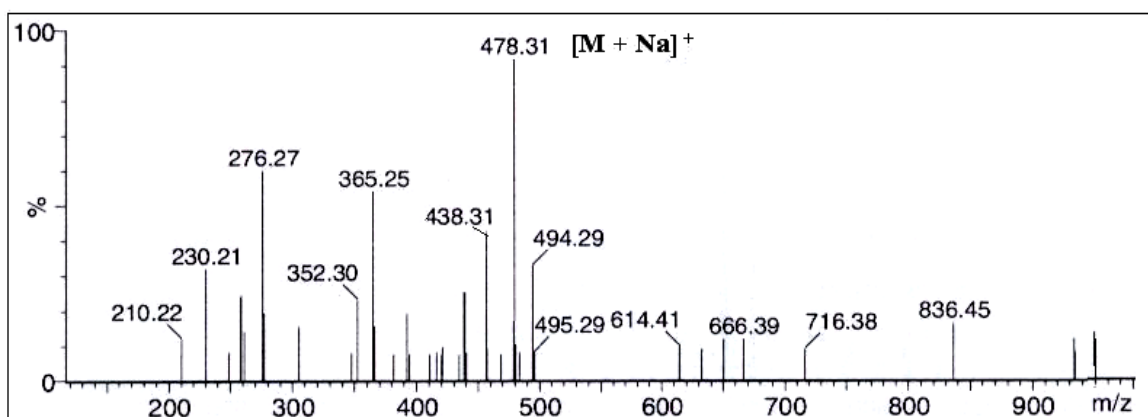
Fig. 4.64. A typical mass spectrum of L-isoleucyl-maltose **51a-c**.
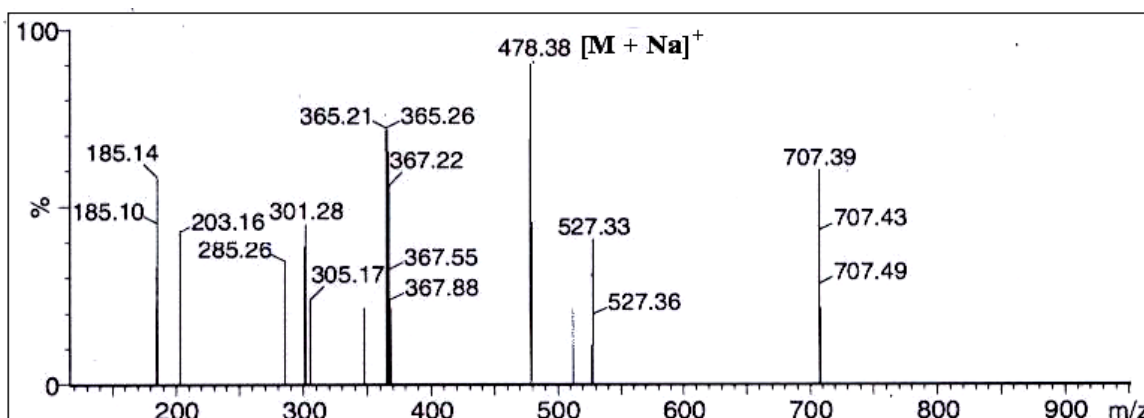
Fig. 4.65. A typical mass spectrum of L-isoleucyl-sucrose **52**.
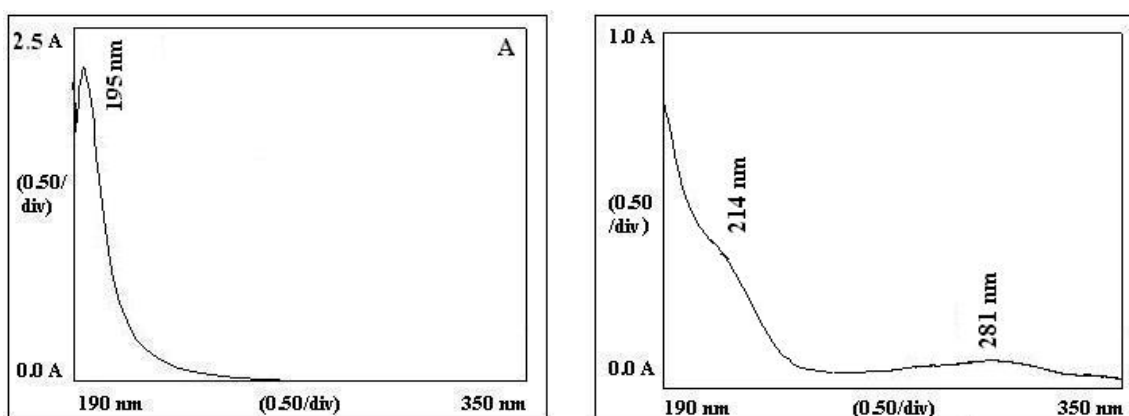
Fig. 4.66 UV spectra for L-isoleucyl-D-mannitol **53a,b** from CRL catalyzed reaction (A) L-isoleucine; (B) for L-isoleucyl-D-mannitol.
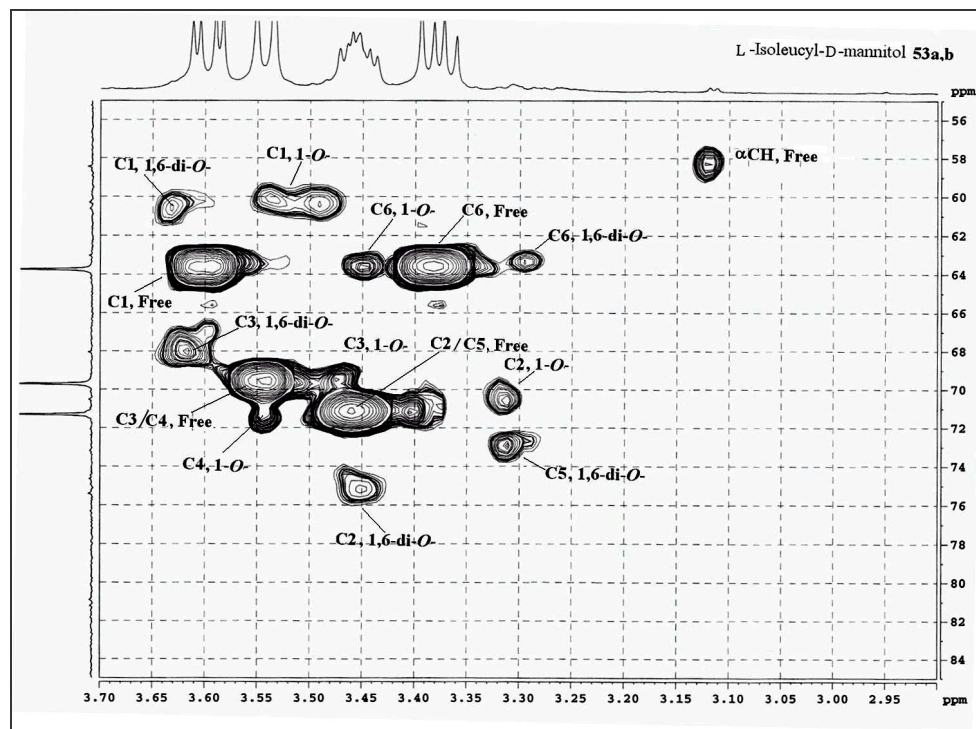
Fig. 4.67. Two-dimensional HSQCT NMR for L-isoleucyl-D-mannitol **53a,b** obtained through CRL catalysis.

4.3. Spectral characterization of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates

Two-dimensional HSQCT NMR spectroscopy of all the four amino acyl esters of carbohydrates gave good information on the nature and proportion of the esters formed (Table 4.10). Two-dimensional HSQCT NMR data showed that upfield chemical shift values for α-CH from L-alanyl and β-CH₂ from L-valyl, L-leucyl and L-isoleucyl units indicated that the respective amino acids reacted with the carbohydrates and multiple cross peaks indicated that the reaction occurred at more than one hydroxyl group of the carbohydrate molecules employed.

4.3.1. L-Alanyl esters of carbohydrates 16a-e to 24

UV spectra of L-alanyl esters of carbohydrates showed shifts in the σ→σ* band in the 194-228 nm range (190 nm for L-alanine **1**) and IR carbonyl stretching frequencies in the 1606 – 1653 cm⁻¹ range (1715 cm⁻¹ for L-alanine **1**) indicating that L-amino acid carboxylic group had been converted into their corresponding carbohydrate esters. In the 2D HSQCT spectra of the L-alanyl esters of carbohydrates, the following ester formation were confirmed from their respective chemical shift values: from D-glucose (**5**), 2-*O*-ester **16a** to C2β at 82.6 ppm and the corresponding H-2β cross peak at 3.62 ppm, 3-*O*-ester.**16b** to C3β at 83.3 ppm (H-3β at 3.93 ppm), 6-*O*- ester **16c** to C6β at 63.5 ppm (H-6 at 3.82 ppm), 2,6-di-*O*- ester **16d** to C6β at 62.7 ppm (H-6 at 3.47 ppm) and C2β at 76.5 ppm (H-2β at 3.78 ppm) and 3,6-di-*O*- ester **16e** to C6β at 63.1 ppm (H-6 at 3.82 ppm) and C3β at 81.6 ppm (H-3β at 3.78 ppm); from D-galactose (**6**), 2-*O*- ester **17a** to C2α at 76.4 ppm (H-2α at 3.38 ppm) and C2β at 76.5 ppm (H-2β at 3.36 ppm), 3-*O*-ester **17b** to C3α at 81.6 ppm (H-3α at 3.75 ppm) and C3β at 82.6 ppm (H-3β at 3.60 ppm) and 6-*O*- ester **17c** to C6α at 63.1 ppm (H-6 at 3.30 ppm); from D-mannose (**7**), 3-

O- ester **18a** to C3α at 89.2 ppm, 4-*O*-ester **18b** to C-4β at 75.3 ppm, 6-*O*- ester **18c** to

C6α at 63.1 ppm, 3,6-di-*O*- ester **18d** to C6α at 62.8 ppm and C3α at 82.9 ppm and 4,6-

di-*O*- ester **18e** to C6α at 62.5 ppm and C4α at 77.2 ppm; from D-fructose (**8**), 1-*O*- ester

19a to C1α at 63.1 ppm (H-1α at 3.40 ppm), 6-*O*- ester **19b** to C6α at 63.6 ppm (H-6 at

3.30 ppm) and 1,6-di-*O*- ester **19c** to C1 and C6 at around 63.6 ppm (H-1 and H-6 at

around 3.12 ppm); from D-arabinose (**9**), 2-*O*- ester **20a** to C2α at 77.8 ppm (H-2α at

3.60 ppm), 5-*O*- ester **20b** to C5α at 65.1 ppm (H-5 at 3.60 ppm) and 2,5-di-*O*- ester **20c**

to C2α at 76.9 ppm (H-2α at 3.45 ppm) and C5α at 65.1 ppm (H-5 at 3.30 ppm); from D-

ribose (**10**), 3-*O*- ester **21a** to C3α at 75.7 ppm (H-3α at 3.67 ppm), 5-*O*- ester **21b** to

C5α at 63.4 ppm (H-5 at 3.61 ppm) and 3,5-di-*O*- ester **21c** to C3α at 74.9 ppm (H-3α at

3.45ppm) and C5α at 63.4 ppm (H-5 at 3.52 ppm); from lactose (**11**), 6-*O*- ester **22a** to

C6 at 61.2 ppm (H-6 at 3.80 ppm) of glucose moiety, 6′-*O*- ester **22b** to C-6′at 64.0 ppm

(H-6′at 3.70 ppm) of galactose moiety and 6,6′-di-*O*- ester **22c** to C6 and C6′ at around

67.5 ppm (H–6 and H–6′ at around 3.85 ppm); from maltose (**12**), 6-*O*- ester **23a** to C6 at

67.5 ppm (H-6 at 3.50 ppm) of reducing end glucose moiety, 6′-*O*- ester **23b** to C6′at

67.0 ppm (H-6′at 3.95 ppm) of the non reducing end glucose moiety and 6,6′-di-*O*- ester

23c to C6 and C6′ at around 63.0 ppm (H–6 and H–6′ at around 3.75 ppm); and from

sucrose (**13**), 6-*O*-ester **24** to C6β at 67.0 ppm (H-6β at 3.67 ppm) of the fructose moiety.

In D-glucose, only β-D-glucose esterified as observed from C1 at 102.6 (2-*O*-ester),

101.8 (6-*O*-ester) and 100.8 (2,6-di-*O*-ester) from a 40:60, mixture of α and β anomers

of the D-glucose employed. Mass spectra also confirmed the formation of the above

mentioned esters.

4.3.2. L-Valyl esters of carbohydrates 25a-e to 33

UV spectra of L-valyl esters of carbohydrates showed shifts in the σ→σ* band in the 200-230 nm range (195 nm for L-valine **2**) and IR carbonyl stretching frequencies in the 1588 -1644 cm^{-1} range (1605 cm^{-1} for L-valine **2**) indicating that L-amino acid carboxylic group had been converted into their corresponding carbohydrate esters. In the 2D HSQCT spectra of the L-valyl esters of carbohydrates, the following ester formation were confirmed from their respective chemical shift values: from D-glucose (**5**), 2-*O*-ester **25a** to C2α at 77.4 ppm and the corresponding H-2α cross peak at 3.75 ppm, 3-*O*-ester **25b** to C3α at 82.6 ppm (H-3α at 3.96 ppm) and C3β at 83.1 ppm (H-3β at 4.01 ppm) , 6-*O*- ester **25c** to C6 at 63.4 ppm (H-6 at 3.15 ppm), 2,6-di-*O*- ester **25d** to C6α at 61.5 ppm (H-6 at 3.56 ppm) and C2α at 76.4 ppm (H-2α at 3.85 ppm) and 3,6-di-*O*-ester **25e** to C6α at 61.8 ppm (H-6 at 3.52 ppm) and C3α at 82.4 ppm (H-3α at 3.70 ppm); from D-galactose (**6**), 2-*O*- ester **26a** to C2α at 76.4 ppm (H-2α at 3.83 ppm) and C2β at 77.6 ppm (H-2β at 3.65 ppm), 3-*O*- ester **26b** to C3α at 81.6 ppm (H-3α at 3.80 ppm) and C3β at 82.7 ppm (H-3β at 3.67 ppm) and 6-*O*- ester **26c** to C6α at 63.1 ppm (H-6 at 3.35 ppm); from D-mannose (**7**), 6-*O*- ester **27** to C6α at 63.5 ppm (H-6 at 3.63 ppm); from D-fructose (**8**), 1-*O*- ester **28a** to C1α at 64.1 ppm (H-1α at 3.78 ppm), 6-*O*-ester **28b** to C6α at 64.0 ppm (H-6 at 3.18 ppm) and 1,6-di-*O*- ester **28c** to C1 and C6α at around 63.4 ppm (H-1 and H-6 at around 3.28 ppm); from D-arabinose (**9**), 2-*O*- ester **29a** to C2α at 77.0 ppm (H-2α at 3.65 ppm), 5-*O*- ester **29b** to C5α at 65.0 ppm (H-5 at 3.64 ppm) and 2,5-di-*O*- ester **29c** to C2α at 75.6 ppm (H-2α at 3.45 ppm) and C5α and C5β at 63.3 ppm and 65.0 ppm (H-5α at 3.35 ppm and H-5β at 3.35 ppm) respectively; from D-ribose (**10**), 3-*O*- ester **30a** to C3α at 77.1 ppm (H-3α at 3.98 ppm), 5-*O*- ester **30b** to C5α at 63.9 ppm (H-5 at 3.62 ppm) and 3,5-di-*O*- ester **30c** to C3α at 74.6 ppm

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(H-3α at 3.67 ppm) and C5α at 65.4 ppm (H-5 at 3.34 ppm); from maltose (**12**), 6-*O*-ester **31a** to C6α,β at 66.5 ppm (H-6 at 3.93 ppm) of reducing end glucose moiety, 6'-*O*-ester **31b** to C6′at 68.0 ppm (H-6′at 3.83 ppm) of the non reducing end glucose moiety; from sucrose (**13**), 6-*O*-ester **32** to C6β at 68.2 ppm (H-6β at 3.64 ppm) of the fructose moiety; and from D-mannitol (**14**), 1-*O*- ester **33** to C1 at 60.6 ppm and the corresponding H-1 cross peak at 3.53 ppm. Mass spectra also confirmed the formation of the above mentioned esters.

4.3.3. L-Leucyl esters of carbohydrates 34a-e to 43

UV spectra of L-leucyl esters of carbohydrates showed shifts in the σ→σ* band in the 203-230 nm range (190 nm for L-leucine **3**) and IR carbonyl stretching frequencies in the 1580 -1657 cm⁻¹ range (1605 cm⁻¹ for L-leucine **3**) indicating that L-amino acid carboxylic group had been converted into their corresponding carbohydrate esters. In the 2D HSQCT spectra of the L-leucyl esters of carbohydrates, the following ester formation were confirmed from their respective chemical shift values: from D-glucose (**5**), 2-*O*-ester **34a** to C2α at 75.5 ppm and the corresponding H-2α cross peak at 3.85 ppm, 3-*O*-ester **34b** to C3α at 83.5 ppm (H-3α at 3.85 ppm) and C3β at 83.6 ppm (H-3β at 3.96 ppm), 6-*O*- ester **34c** to C6α at 65.0 ppm (H-6 at 3.80 ppm), 2,6-di-*O*- ester **34d** to C6α at 65.0 ppm (H-6 at 3.59 ppm) and C2α at 76.3 ppm (H-2α at 3.21 ppm) and 3,6-di-*O*-ester **34e** to C6α at 65.0 ppm (H-6 at 3.59 ppm) and C3α at 81.5 ppm (H-3α at 3.68 ppm); from D-galactose (**6**), 2-*O*- ester **35a** to C2α at 72.5 ppm (H-2α at 3.74 ppm), 6-*O*- ester **35b** to C6α at 62.5 ppm (H-6 at 3.65 ppm); from D-mannose (**7**), 3-*O*- ester **36a** to C3α at 81.4 ppm (H-3α at 3.86 ppm) and C3β at 82.5 ppm (H-3β at 3.97 ppm), 4-*O*-ester **36b** to C4α at 73.8 ppm (H-4α at 3.86 ppm) and C4β at 75.0 ppm (H-4β at 3.78 ppm) and 6-*O*- ester **36c** to C6α at 63.2 ppm (H-6 at 3.80 ppm); from D-fructose (**8**), 6-

O- ester **37** to C1α at 63.5 ppm (H-1α at 3.83 ppm); from D-arabinose (**9**), 2-*O-* ester **38a** to C2α at 75.0 ppm (H-2α at 3.76 ppm) C2β at 75.2 ppm (H-2β at 3.45 ppm) and 5-*O-* ester **38b** to C5α at 64.8 ppm (H-5 at 3.64 ppm), 2,5-di-*O-* ester **38c** to C2α at 77.2 ppm (H-2α at 3.66 ppm) and C5α at 64.8 ppm (H-5 at 3.35); from D-ribose (**10**), 3-*O-* ester **39a** to C3α at 76.0 ppm (H-3α at 3.60 ppm), 5-*O-* ester **39b** to C5α at 63.0 ppm (H-5a at 3.59 ppm and H-5b at 3.68 ppm), 3,5-di-*O-* ester **39c** to C3α at 76.2 ppm (H-3α at 3.77 ppm) and C5α at 64.8 ppm (H-5a at 3.34 ppm and H-5b at 3.37 ppm); from maltose (**12**), 6-*O-* ester **40** to C6 at 62.9 ppm (H-6 at 3.33 ppm) of reducing end glucose moiety; from sucrose (**13**), 6-*O*-ester **41** to C6β at 68.2 ppm (H-6 at 3.64 ppm) of the fructose moiety; from D-mannitol (**14**), 1-*O-* ester **42a** to C1 at 60.4 ppm (H-1 at 3.51 ppm), 1,6-di-*O*-ester **42b** to C1 at 60.4 ppm (H-1 at 3.61 ppm) and C6 at 68.2 ppm (H-6 at 3.64 ppm); and from D-sorbitol (**15**), 1-*O-* ester **43** to C1 at 60.5 ppm (H-1 at 3.51 ppm). Mass spectra also confirmed the formation of the above mentioned esters.

4.3.4. L-Isoleucyl esters of carbohydrates 44a-e to 53a, b

UV spectra of L-leucyl esters of carbohydrates showed shifts in the σ→σ* band in the 214-227 nm range (195 nm for L-isoleucine **4**) and IR carbonyl stretching frequencies in the 1616 -1646 cm^{-1} range (1584 cm^{-1} for L-isoleucine **4**) indicating that L-amino acid carboxylic group had been converted into their corresponding carbohydrate esters. In the 2D HSQCT spectra of the L-isoleucyl esters of carbohydrates, the following ester formation were confirmed from their respective chemical shift values: from D-glucose (**5**), 3-*O-* ester **44a** to C3α at 82.0 ppm (H-3α at 4.01 ppm) and C3β at 81.9 ppm (H-3β at 3.88 ppm), 6-*O-* ester **44b** to C6α at 63.6 ppm (H-6 at 3.82 ppm); from D-galactose (**6**), 2-*O-* ester **45a** to C2α at 76.2 ppm (H-2α at 3.81 ppm), 3-*O-* ester **45b** to C3α at 81.3 ppm (H-3α at 3.58 ppm) and C3β at 82.2 ppm (H-3β at 3.66 ppm), 6-*O-*

ester **45c** to C6α at 62.8 ppm (H-6 at 3.40 ppm); from D-mannose (**7**), 3-*O*- ester **46a** to

C3α at 80.5 ppm (H-3α at 3.72 ppm) and C3β at 83.1 ppm (H-3β at 3.85 ppm), 4-*O*-

ester **46b** to C4α at 75.0 ppm (H-4α at 3.80 ppm) and C4β at 77.1 ppm (H-4β at 3.90

ppm), 6-*O*- ester **46c** to C6α at 63.1 ppm (H-6 at 3.79 ppm), 3,6-di-*O*- ester **46d** to C6α

at 62.9 ppm (H-6 at 3.41 ppm) and C3α at 82.1 ppm (H-3α at 3.55 ppm) and 4,6-di-*O*-

ester **46e** to C6α at 62.9 ppm (H-6 at 3.52 ppm) and C4α at 76.0 ppm (H-4α at 3.75

ppm); from D-fructose (**8**), 1-*O*- ester **47a** to C1α at 63.7 ppm (H-1α at 3.25 ppm), 6-*O*-

ester **47b** to C6α at 63.1 ppm (H-6 at 3.78 ppm) and 1,6-di-*O*- ester **47c** to C1α and C6α

at around 64.4 ppm (H-1 and H-6 at around 3.28 ppm); from D-arabinose (**9**), 2-*O*- ester

48a to C2α at 75.0 ppm (H-2α at 3.76 ppm) and C2β at 75.2 ppm (H-2β at 3.45 ppm), 5-

O- ester **48b** to C5α at 64.8 ppm (H-5 at 3.64 ppm) and 2,5-di-*O*- ester **48c** to C2α at

77.2 ppm (H-2α at 3.66 ppm) and C5α at 64.8 ppm (H-5 at 3.35 ppm); from D-ribose

(**10**), 3-*O*- ester **49a** to C3α at 76.5 ppm (H-3α at 3.98 ppm) and C3β at 77.7 ppm (H-3β

at 3.92 ppm), 5-*O*- ester **49b** to C5α at 63.2 ppm (H-5 at 3.58 ppm) and 3,5-di-*O*- ester

49c to C3α at 75.5 ppm (H-3α at 3.60 ppm) and C5α at 63.3 ppm (H-5 at 3.52 ppm);

from lactose (**11**), 2-*O*- ester **50a** to C2α at 84.5 ppm (H-2α at 3.40 ppm), C2β at 85.0

ppm (H-2β at 3.82 ppm) of glucose moiety, 6-*O*- ester **50b** to C6 at 66.5 ppm (H-6 at

3.85 ppm) of glucose moiety and 6′-*O*- ester **50c** to C-6′ at 66.9 ppm (H-6′ at 3.85 ppm)

of galactose moiety; from maltose (**12**), 2-*O*- ester **51a** to C2α at 81.2 ppm (H-2α at 4.05

ppm) C2β at 81.4 ppm (H-2β at 3.96 ppm) of reducing end glucose moiety, 6-*O*- ester

51b to C6 at 68.0 ppm (H-6 at 3.62 ppm) of reducing end glucose moiety and 6′-*O*- ester

51c to C6′ at 67.2 ppm (H-6′ at 3.62 ppm) of the non reducing end glucose moiety; from

sucrose (**13**), 6-*O*- ester **52** to C6β at 67.2 ppm (H-6 at 3.63 ppm) of the fructose moiety;

and from D-mannitol (**14**), 1-*O*- ester **53a** to C1 at 60.8 ppm (H-1 at 3.51 ppm) and 1,6-

di-*O*- ester **53b** to C1 at 60.8 ppm (H-1 at 3.63 ppm) and C6 at 63.5 ppm (H-6 at 3.29 ppm). Here also, mass spectra also confirmed the formation of the above mentioned esters.

4.4. Discussion

Two dimensional HSQCT NMR confirmed the formation of 1-*O*-, 2-*O*-, 3-*O*-, 4-*O,* 5-*O*-, 6-*O*- and 6′-*O*- mono esters and 1,6-di-*O*-, 2,5-*di-O-,* 2,6-di-*O*-, 3,5-di-*O,* 3,6-di-*O-,* 4,6-di-*O*- and 6,6′-di-*O*- diesters to varying extents depending on the carbohydrate employed (Table 4.2, 4.4, 4.6, 4.8 and 4.10). Lipases from *Candida rugosa*, *Rhizomucor miehei* and porcine pancreas showed broad substrate specificity towards amino acids as well as carbohydrates. The present work describes preparation of 99 L-amino acyl esters of carbohydrates of which 97 esters have not been reported before. So far unreported esters are L-alanyl-D-glucose **16a,d,e**, L-alanyl-D-galactose **17a-c**, L-alanyl-D-mannose **18a-e**, L-alanyl-D-fructose **19a-c**, L-alanyl-D-arabinose **20a-c**, L-alanyl-D-ribose **21a-c**, L-alanyl-lactose **22a-c**, L-alanyl-maltose **23a-c**, L-alanyl-sucrose **24**, L-valyl-D-glucose **25a-e**, L-valyl-D-galactose **26a-c**, L-valyl-D-mannose **27**, L-valyl-D-fructose **28a-c**, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-valyl-maltose **31a,b**, L-valyl-sucrose **32**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-galactose **35a,b**, L-leucyl-D-mannose **36a-c**, L-leucyl-D-fructose **37**, L-leucyl-D-arabinose **38a-c**, L-leucyl-D-ribose **39a-c**, L-leucyl-maltose **40**, L-leucyl-sucrose **41**, L-leucyl-D-mannitol **42a,b**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b,** L-isoleucyl-D-galactose **45a-c,** L-isoleucyl-D-mannose **46a-e**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-arabinose **48a-c**, L-isoleucyl-D-ribose **49a-c**, L-isoleucyl-lactose **50a-c**, L-isoleucyl-maltose **51a-c**, L-isoleucyl-sucrose **52**, L-isoleucyl-D-mannitol **53a,b**.

D-Glucose **5**, D-galactose **6** and D-mannose **7**, D-fructose **8** and maltose **12** showed better conversions with all the four amino acids. Least conversions were

observed for carbohydrate alcohols and sucrose esters. Among the four amino acids investigated, L-alanine showed lesser conversion (3 – 78 %) to esters compared to the other three amino acids (9 – 78 %). L-Alanine being smaller compared to the other amino acids employed would not be binding firmly to the active site. L-Valyl esters (25 - 78 %) as well as L-leucyl esters (21 – 65 %) showed better conversion followed by L-isoleucyl esters (9 – 55 %, Tables 4.4 - 4.9). Among the lipases employed, *Candida rugosa* lipase and porcine pancreas lipase have shown better conversion than *Rhizomucor miehei* lipase

Except lactose **11** and D-sorbitol **15**, all the carbohydrates employed have reacted with all the three amino acids (**1-4**). L-Alanine **1**, L-valine **2** and L-leucine **3** with D-glucose **5** and L-alanine **1** and L-isoleucine **4** with D-mannose **7** gave five diastereomeric esters. Both D-arabinose **9** and D-ribose **10** have shown three diastereomeric esters with all the amino acids (**1-4**) employed. Lactose with L-valine and L-leucine and D-sorbitol with L-alanine and L-valine and L-isoleucine did not undergo any reaction. L-Alanyl-sucrose **24**, L-valyl-D-mannose **27**, L-valyl-sucrose **32**, L-leucyl-maltose **40**, L-leucyl-sucrose **41** and L-isoleucyl-sucrose **52** formed only 6-*O*- ester.

Nature of the products clearly indicated that primary hydroxyl groups of the carbohydrates (1-*O*-, 5-*O*-, 6-*O*- and 6′-*O*-) esterified predominantly over the secondary hydroxyl groups (2-*O*-, 3-*O*- and 4-*O*-). Among the secondary hydroxyl groups, 4-*O*-ester was formed only in case of D-mannose (**18b**, **36b** and **46b**). Carbohydrates containing axial hydroxyl groups in axial position like C2 in D-mannose and D-ribose and C4 in D-galactose, have not reacted indicating that esterification with axial secondary hydroxyl groups are difficult, especially with alkyl amino acyl donors.

The anomeric hydroxyl groups of carbohydrate molecules did not react because of rapid glycosidic ring opening and closing process. Loss of specificity could be due to

use of larger amount of enzymes (about 40% w/w carbohydrate), which gave a large number of esters.

Carbohydrates like lactose, D-mannitol and D-sorbitol reacted selectively depending on the amino acid indicated that they may not be good nucleophiles. This could be due to more hydrogen bonding propensity for D-mannitol and D-sorbitol and steric hindrance in case of lactose. In case of aldopentoses (D-arabinose and D-ribose), NMR spectrum clearly indicated degradation and/or ring opening during the reaction. In case of L-alanyl-D-ribose and L-valyl-D-ribose, opening of the five membered ring during esterification was noticed by observation of a large number of signals in the 3.0 - 5.0 ppm (^{1}H) and 63 - 75 ppm (^{13}C) region which could be due to excess strain on the ring due to introduction of bulky amino acid groups to D-ribose OH groups.

Since the reactions were carried out at a low temperature of 40-60 ^{o}C, the formation of peptide was less than 3 %, even though unprotected L-alanine was used for the reaction. NMR data clearly indicated that no Maillard reaction occurred. RML and PPL showed significant esterification (up to 68 %) when unprotected L-alanine was used. When N-protected amino acid N-acetyl-L-alanine, was used in the present work, both RML and PPL gave < 5 % yield. Our present study has shown that comparable esterification yields to others could be achieved by employing PPL, CRL and RML instead of protease.

Thus this study has shown that unprotected and unactivated amino acids containing hydrophobic alkyl side chain can serve as good acyl donors in the esterification reaction catalyzed by lipases from *Candida rugosa*, *Rhizomucor miehei* and porcine pancreas.

Table 4.10. Percentage yields and proportions of L-alanyl esters of carbohydrates from RML and L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates CRL catalyzed reactions [a]

Carbohydrate	L-Alanyl		L-Valyl		L-Leucyl		L-Isoleucyl	
	Yield[a]	% Proportions	Yield[b]	% Proportions	Yield[b]	% Proportions	Yield[b]	% Proportions
D-glucose	30	**16a:** 2-*O*-Ester (20) **16b:** 3-*O*-Ester (12) **16c:** 6-*O*-Ester (47) **16d:** 2,6-di-*O*-Ester (15) **16e:** 3,6-*di-O*-Ester (6)	68	**25a:** 2-*O*-Ester (10) **25b:** 3-*O*-Ester (12) **25c:** 6-*O*-Ester (31) **25d:** 2,6-di-*O*-Ester (23) **25e:** 3,6-di-*O*-Ester (24)	43	**34a:** 2-*O*-Ester (17) **34b:** 3-*O*-Ester (20) **34c:** 6-*O*-Ester (42) **34d:** 2,6-di-*O*-Ester (10) **34e:** 3,6-di-*O*-Ester (12)	47	**44a:** 3-*O*-Ester (42) **44b:** 6-*O*-Ester (58)
D-galactose	21	**17a:** 2-*O*-Ester (33) **17b:** 3-*O*-Ester (32) **17c:** 6-*O*-Ester (35)	30	**26a:** 2-*O*-Ester (48) **26b:** 3-*O*-Ester (26) **26c:** 6-*O*-Ester (26)	21	**35a:** 2-*O*-Ester (48) **35b:** 6-*O*-Ester (52)	46	**45a:** 2-*O*-Ester (78) **45b:** 3-*O*-Ester (10) **45c:** 6-*O*-Ester (12)
D-mannose	49	**18a:** 3-*O*-Ester (25) **18b:** 4-*O*-Ester (25) **18c:** 6-*O*-Ester (30) **18d:** 3,6-di-*O*-Ester (9) **18e:** 4,6-di-*O*-Ester (11)	51	**27:** 6-*O*-Ester	31	**36a:** 3-*O*-Ester (28) **36b:** 4-*O*-Ester (30) **36c:** 6-*O*-Ester (42)	55	**46a:** 3-*O*-Ester (19) **46b:** 4-*O*-Ester (13) **46c:** 6-*O*-Ester (13) **46d:** 3,6-di-*O*-Ester (27) **46e:** 4,6-di-*O*-Ester (28)
D-fructose	52	**19a:** 1-*O*-Ester (34) **19b:** 6-*O*-Ester (34) **19c:** 1,6-di-*O*-Ester (32)	34	**28a:** 1-*O*-Ester (29) **28b:** 6-*O*-Ester (34) **28c:** 1,6-di-*O*-Ester (37)	48	**37:** 6-*O*-Ester	43	**47a:** 1-*O*-Ester (36) **47b:** 6-*O*-Ester (30) **47c:** 1,6-di-*O*-Ester (34)
D-arabinose	9	**20a:** 2-*O*-Ester (33) **20b:** 5-*O*-Ester (34) **20c:** 2,5-di-*O*-Ester (33)	25	**29a:** 2-*O*-Ester (32) **29b:** 5-*O*-Ester (25) **29c:** 2,5-di-*O*-Ester (43)	42	**38a:** 2-*O*-Ester (24) **38b:** 5-*O*-Ester (33) **38c:** 2,5-di-*O*-Ester (43)	55	**48a:** 2-*O*-Ester (24) **48b:** 5-*O*-Ester (33) **48c:** 2,5-di-*O*-Ester (43)
D-ribose	48	**21a:** 3-*O*-Ester (16) **21b:** 5-*O*-Ester (32) **21c:** 3,5-di-*O*-Ester (52)	33	**30a:** 3-*O*-Ester (26) **30b:** 5-*O*-Ester (26) **30c:** 3,5-di-*O*-Ester (48)	38	**39a:** 3-*O*-Ester (16) **39b:** 5-*O*-Ester (32) **39c:** 3,5-di-*O*-Ester (52)	53	**49a:** 3-*O*-Ester (52) **49b:** 5-*O*-Ester (20) **49c:** 3,5-di-*O*-Ester (28)
lactose	20	**22a:** 6-*O*-Ester (34) **22b:** 6'-*O*-Ester (34) **22c:** 6,6'-di-*O*-Ester (32)	--	--	--	--	45	**50a:** 2-*O*-Ester (39) **50b:** 6-*O*-Ester (40) **50c:** 6'-*O*-Ester (21)
maltose	56	**23a:** 6-*O*-Ester (34) **23b:** 6'-*O*-Ester (34) **23c:** 6,6'-di-*O*-Ester (32)	47	**31a:** 6-*O*-Ester (49) **31b:** 6'-*O*-Ester (51)	44	**40:** 6-*O*-Ester	54	**51a:** 2-*O*-Ester (38) **51b:** 6-*O*-Ester (40) **51c:** 6'-*O*-Ester (22)
sucrose	8	**24:** 6-*O*-Ester	60	**32:** 6-*O*-Ester	38	**41:** 6-*O*-Ester	22	**52:** 6-*O*-Ester
D-mannitol	--		52	**33:** 1-*O*-Ester	45	**42a:** 1-*O*-Ester (56) **42b:** 1,6-di-*O*-Ester (44)	52	**53a:** 1-*O*-Ester (62) **53b:** 1,6-di-*O*-Ester (38)
D-sorbitol	--	--	--	--	25	**43:** 1-*O*-Ester	--	--

[a] Confirmation of esters and their percentage proportions were determined by 2D-HSQCT NMR. Conversion yields are an average from two experiments. [b] Yields from HPLC. Errors in yield measurements will be within ± 10%

4.5. Experimental

4.5.1. Esterification procedure

Esterification was carried out in a flat bottom two necked flask by reacting 0.002 mol unprotected L-amino acid (L-alanine **1**, L-valine **2**, L-leucine **3** and L- isoleucine **4**) and 0.001 mol of carbohydrate (D-glucose **5,** D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-sorbitol **14** and D-mannitol **15**) along with 100 ml CH_2Cl_2:DMF (90:10 v/v, 40 ^{o}C) in presence of 0.75 - 0.18 g (50 % w/w carbohydrate employed) of lipases under reflux for a period of three days. *Rhizomucor miehei* lipase (RML) in presence of 0.1 mM (0.1 ml of 0.1M) of acetate buffer (pH 4.0), *Candida rugosa* lipase (CRL) in presence of 0.1 mM (0.1 ml of 0.1M) of phosphate buffer (pH 7.0) and crude porcine pancreas lipase in presence of 0.2 mM (0.2 ml of 0.1 M) of acetate buffer (pH 5.0) were employed to impart 'pH memory' or 'pH tuning' to the enzyme. The condensed vapour of solvents which formed an azeotrope with water was passed through a desiccant (molecular sieves of 4Å made up of sodium aluminosilicate were used as desiccants) before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction (Lohith and Divakar 2005). This set up maintained a very low water activity of a_w= 0.0054 throughout the reaction period. After completion of the reaction, the solvent was distilled off and 20- 30 ml of warm water was added, stirred and filtered to remove the lipase. The filtrate was evaporated to get a mixture of the unreacted carbohydrate, unreacted L-amino acids and the product esters, which were then analyzed by HPLC. The conversion yields were determined with respect to peak areas of the L-amino acid and that of the esters. The esters formed were separated by size exclusion chromatography using Sephadex G-10 and Bio Gel P-2 as column materials and eluted with water. Although, the esters were separated from unreacted amino acids and carbohydrates by this procedure, the

individual esters in the mixture of esters formed could not be separated. This could be due to the similar polarity of the ester molecules. The product esters separated were subjected to spectral characterization by UV, IR, mass, specific rotation and 2D-NMR.

4.5.2. High Performance Liquid Chromatography

A Shimadzu LC 10AT HPLC (Kyoto, Japan) connected to LiChrosorb RP-18 column (5 μm particle size, 4.6 x 150 mm length) with acetonitrile:water (v/v 20:80) as a mobile phase at a flow rate of 1 ml/ min was employed using an UV detector at 210 nm.

4.5.3. Spectral characterization

A Shimadzu UV – 1601 spectrophotometer (Kyoto, Japan) was used for recording UV spectra of the isolated esters in water at 0.1 - 2.0 mM concentrations. A Nicolet 5700 FTIR instrument (Madison, USA) was used for recording the IR spectra with a 1.0 - 3.0 mg of ester sample as KBr pellet. Specific rotation of the isolated esters were measured at 25 ^{o}C using Perkin-Elmer 243 polarimeter (Überlingen, Germany) with a 0.2 – 1.5 % solution of the esters in water. Mass spectra of the isolated esters were recorded using a Q-TOF Waters Ultima instrument (No.Q-Tof GAA 082, Waters corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source.

4.5.4. Nuclear Magnetic Resonance Spectroscopy

4.5.4.1. ^{1}H NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 500.13 MHz was used for recording ^{1}H NMR. A 40 mg of sample dissolved in 0.5 ml of DMSO-*d₆* solvent was employed. About 50-200 scans were accumulated with a recycle period of 2-3 seconds to obtain good spectra. The spectra were recorded at 35 ^{o}C with TMS as internal standard for measuring the chemical shift values to within ± 0.001 ppm. A region from 0 – 10 ppm was scanned for all the samples.

4.5.4.2. ^{13}C NMR

A Brüker DRX-500 MHz spectrometer (Fallanden, Switzerland) operating at 125 MHz was used to record the ^{13}C NMR. Samples were dissolved in 0.5 ml of DMSO-d_6 and recorded at 35 ^{o}C. A region from 0-200 ppm region was scanned and about 500 – 6000 scans were accumulated for each spectrum to get a good spectrum. TMS was taken as an internal standard.

4.5.4.3. Two-dimensional HSQCT

Two dimensional Heteronuclear Multiple Quantum Coherence Transfer spectra (2-D HMQCT) and Heteronuclear Single Quantum Coherence Transfer spectra (2-D HSQCT) (Fallanden, Switzerland) were recorded at 500 MHz on a Brüker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 for MHz ^{13}C). Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard. About 40mg of the sample dissolved in DMSO-d_6 was used for recording the spectra.

Chapter 5

Competitive inhibition by D-glucose in Rhizomucor miehei and Candida rugosa lipases in the esterification reaction between L-alanine and D-glucose

5.1. Introduction

Lipase is a single-domain molecule that belongs to the family of α/β-hydrolase proteins (Derewenda *et al.,* 1992; Grochulski *et al.,* 1993). Most of the lipases reported contain Ser-His-Asp/Glu catalytic triads in their active site (Grochulski *et al.,* 1993) with the exception of esterases from *Streptomyces scabies,* which contain only Ser-144 and His-283 (Wei *et al.,* 1995).

Kinetic studies of esterification (Yadav and Trivedi, 2003; Kiran and Divakar, 2002; Janssen *et al.,* 1999; Lortie *et al.,* 1993; Rizzi *et al.,* 1992), racemization (Duan *et al.,* 1997) and hydrolysis (Van-Tol *et al.,* 1992) using lipases have been performed. In some esterifications, lipases follow the Ping-Pong Bi-Bi mechanism (Kiran and Divakar, 2002; Zhang *et al.,* 2005; Zaidi *et al.,* 2002; Yadav and Lathi, 2004). This mechanism involves binding acid with alcohol in successive steps, which is followed by the release of water and the ester products in succession. The kinetic behavior of CRL in the esterification of long-chain fatty acids with alcohols (Zaidi *et al.,* 2002) and tetrahydrofurfuryl alcohol with butyric acid (Yadav and Devi, 2004) follows the Ping-Pong Bi-Bi mechanism, in which the binding of an acid leads to an acyl enzyme complex followed by the release of water molecules. The subsequent binding of an alcohol leads to the transfer of the acyl group to the alcohol, which results in ester formation. Thereafter, an ester product is released. *n*-Octanol is inhibitory to RML and CRL in the transesterification between vinyl acetate and *n*-octanol (Yadav and Trivedi, 2003) following the Ternary Complex Bi-Bi mechanism, in which *n*-octanol binds twice or once to lipase yield a dead-end lipase-*n*-octanol complex and a second molecule of *n*-octanol that binds to this dead-end lipase-*n*-octanol complex to give another dead-end lipase-*n*-octanol complex.

In citronellyl laurate synthesis, RML follows the Ordered Bi-Bi mechanism wherein β-citronellol binds to the enzyme to yield the β-citronellol-enzyme complex, which again binds to lauric acid to form the ternary enzyme-β-citronellol-lauric acid complex. Finally, it decomposes to give β-citronellyl laurate and water as products in this process (Yadav and Lathi, 2004). A series of dead-end RML-lauric acid complexes were also reported in this process.

In this chapter, kinetics of esterification between L-alanine and D-glucose to form L-alanyl-D-glucose with RML and CRL is carried out and the results from these investigations are described below.

5.2. Present investigation

To graphically evaluate the apparent values of the kinetic parameters, initial rate (specific reaction rate) was determined from the concentration of L-alanyl-D-glucose at different incubation periods, and typical time courses of RML and CRL- catalyzed reactions are shown in Fig. 5.1.

For the concentrations of D-glucose and L-alanine, individual experiments in duplicate (30 x 2 lipases) were performed for incubation periods of 3 h, 6 h, 12 h, 24 h and 36 h. Initial rate (specific reaction rate, v) was determined from the initial slope of the plot of the amount of esters formed (M) versus incubation period (h) and expressed as $M\,h^{-1}$ (mg protein)$^{-1}$. R^2 obtained from least-squares analysis for the initial rate in each cases was found to be within 0.88 - 0.95. Each plot shown in this work was constructed from all experimentally determined values; a few initial rates were obtained by curve-fitting.

The initial rates (v) for RML were found to be in the range of 15-176 x $10^{-6}\,M\,h^{-1}$ (mg protein)$^{-1}$. CRL experiments showed the initial rates to be in the range of 20-460 x
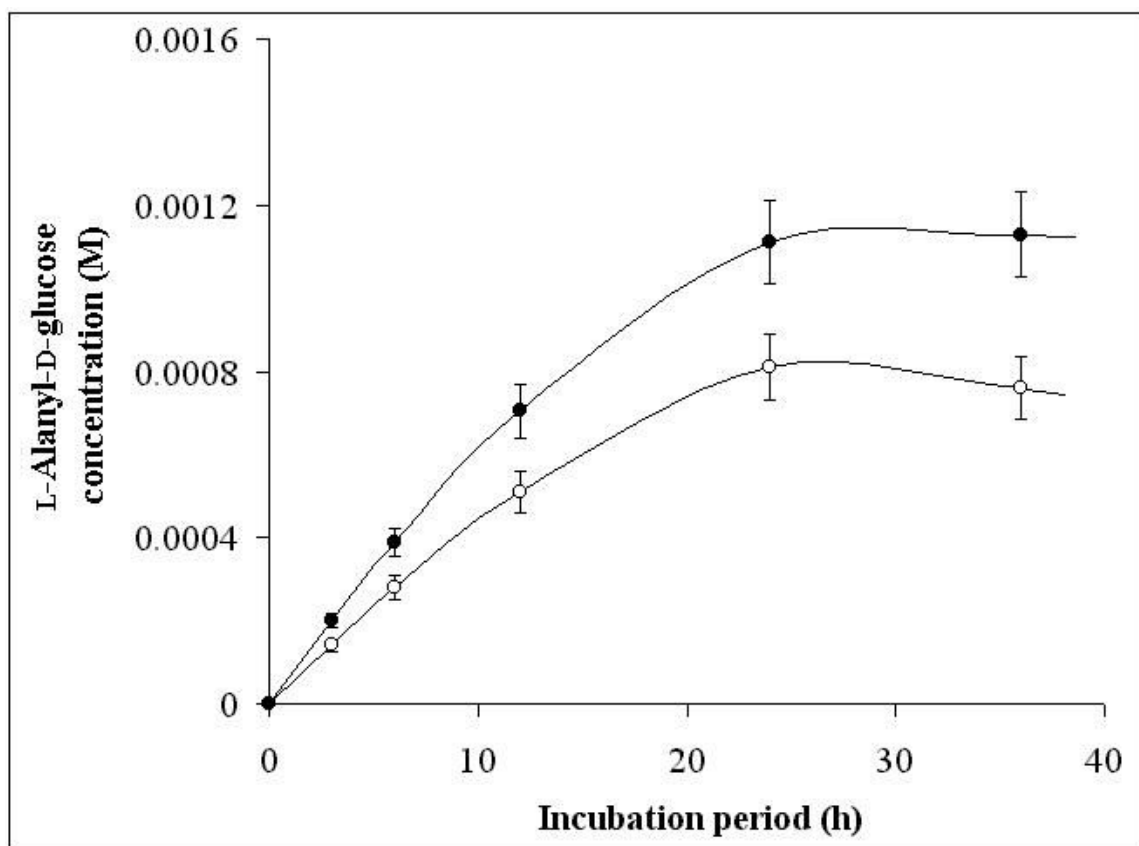
Fig.5.1. Time courses of esterification reactions – concentrations of L-alanyl-D-glucose versus incubation periods; RML (90 mg) or CRL (90 mg) was allowed to react with 0.005 M L-alanine and 0.020 M D-glucose in 100 ml of mixture of dichloromethane /dimethylformamide (v/v 90:10) mixed with 0.1 mM (0.1 ml of 0.1 M) sodium acetate buffer (pH 4.0) for RML or 0.1 mM (0.1 ml of 0.1 M) sodium phosphate buffer (pH 7.0) for CRL.

10^{-6} M h^{-1}(mg protein)$^{-1}$. At the initial periods of incubation, the reaction is relatively fast owing to the shift in equilibrium towards esterification. The process slows down at incubation periods longer than 24 h, resulting in a stable steady-state equilibrium. The effects of external mass transfer phenomena - internal and external diffusions (Yadav and Devi, 2004; Marty *et al.,* 1992), if any, on the RML and CRL enzymes employed were not tested in this work.

5.2.1. *Rhizomucor miehei* and *Candida rugosa* lipase catalysis

Using initial rates, double reciprocal plots were constructed to graphically evaluate the apparent values of k_{cat}, $K_{m\ L\text{-}alanine}$, $K_{m\ D\text{-}glucose}$ and K_i: RML – Fig. 5.2 (1/v versus 1/[D-glucose]) and Fig. 5.3 (1/v versus 1/[L-alanine]); CRL – Fig. 5.4 (1/v versus 1/[D-glucose]) and Fig. 5.5 (1/v versus 1/[L-alanine]). Figure 5.6 shows a replot of the slopes from Fig. 5.3 (RML), and Fig. 5.7 shows the replot of the slopes from Fig. 5.5 (CRL). Figure 5.2 from RML reactions and Fig. 5.4 from CRL reactions show a series of curves obtained for different fixed concentrations of L-alanine for varying D-glucose concentration, in which slight increases in initial rate are observed at lower D-glucose concentrations. At higher D-glucose concentrations, the rates markedly decrease. Also, increasing L-alanine concentration increases the initial rates at all D-glucose concentrations. Figure 5.3 from RML reactions and Fig. 5.5 from CRL reactions show a series of parallel lines for different fixed low D-glucose concentrations at varying L-alanine concentration. These change to lines with different slopes at higher D-glucose concentrations.

The plots in Figs. 5.2, 5.3, 5.4 and 5.5 show that the kinetics could be best described by Ping-Pong Bi-Bi model in which L-alanine and D-glucose bind in
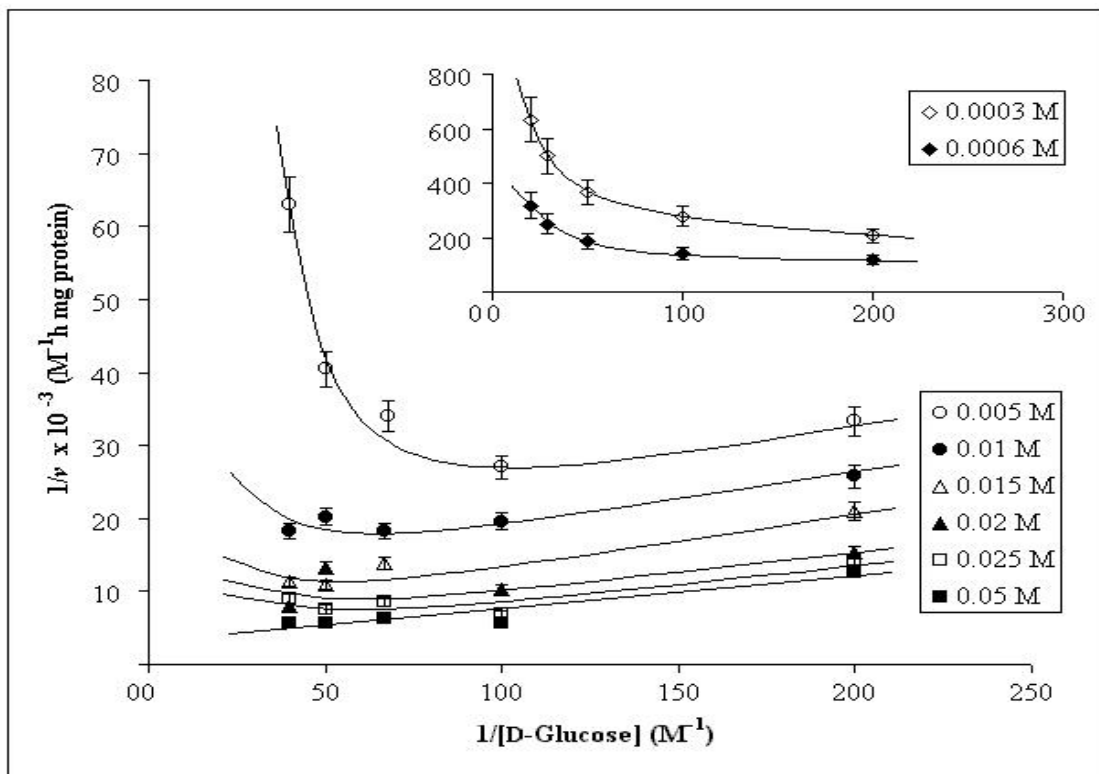
Fig. 5.2. Double reciprocal plots for RML-catalyzed L-alanyl-D-glucose reaction: $1/v$ versus 1/[D-glucose] plots; a series of curves show the effect of varying D-glucose concentration at different fixed L-alanine concentrations in the range of 0.005 - 0.05 M. The inset shows plots obtained by the computer simulation for 0.0003 M and 0.0006 M L-alanine concentrations

subsequent steps releasing water and L-alanyl-D-glucose. This also happens in subsequent steps, (**Scheme 5.1**) with competitive substrate inhibition that lead to dead-end inhibition (Segel, 1993). Both RML and CRL were found to be inhibited by D-glucose.
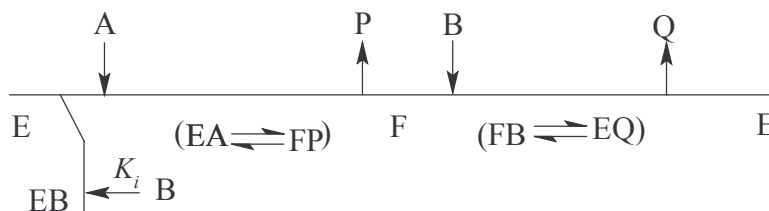
A = L-alanine, P = H$_2$O, B = D-glucose, E = Lipase -*Rhizomucor miehei* lipase/ *Candida rugosa* lipase, F = lipase-L-alanyl complex, EA = lipase-L-alanine complex, FP = lipase-L-alanyl-water complex, EB = lipase-D-glucose complex, K_i = dissociation constant of lipase-D-glucose complex, FB = L-alanyl-lipase-D-glucose complex, EQ = lipase-L-alanyl-D-glucose complex, Q = L-alanyl-D-glucose.

Scheme 5.1. Ping-Pong Bi-Bi mechanism of RML and CRL-catalysed synthesis of L-alanyl-D-glucose showing inhibition by D-glucose.

This model could be described by the following rate equation,

$$\frac{v}{V_{max}} = \frac{[A]\,[B]}{K_{mA}[B]\,(1+[B]\,/\,K_i)\; + K_{mB}\,[A] + [A]\,[B]}$$

.... (5.1)

where v is initial rate, V_{max} is maximum velocity, [A] is L-alanine concentration, [B] is D-glucose concentration, K_{mA} is Michaelis-Menten constant for the lipase-L-alanine complex, K_i is dissociation constant for the lipase-inhibitor (D-glucose) complex and K_{mB} is Michaelis-Menten constant for the lipase-D-glucose complex. Because the initial rates are in M h^{-1} (mg protein)$^{-1}$, V_{max} is expressed as $k_{cat}= V_{max}$/enzyme concentration.

The apparent values of the four important kinetic parameters $K_{i\,D\text{-}glucose}$, $K_{m\,L\text{-}alanine}$, $K_{m\,D\text{-}glucose}$ and k_{cat} were graphically evaluated. The intercepts of the positive slopes of the curves in Fig. 5.2 and Fig. 5.4 on the Y-axis, particularly, at the highest L-alanine
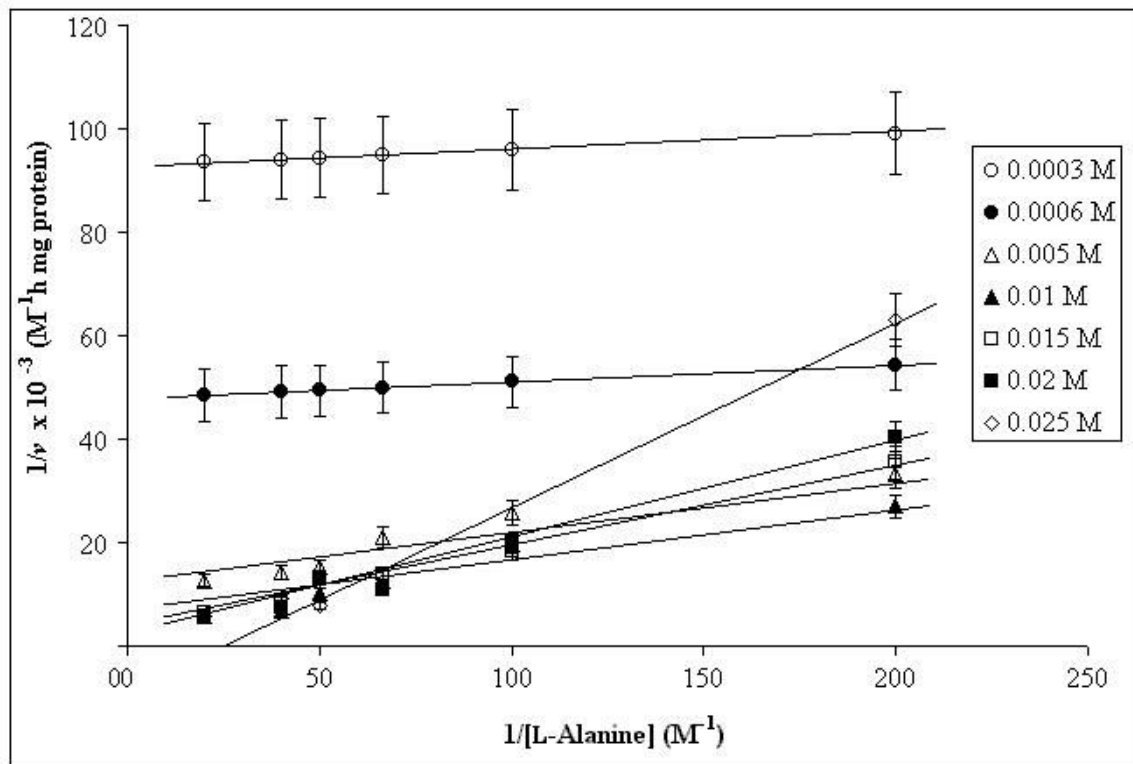
Fig. 5.3. Double reciprocal plots for RML-catalyzed L-alanyl-D-glucose reaction: $1/v$ versus 1/[L-alanine] plots; a series of plots shows the effects of varying L-alanine concentration at different fixed D-glucose concentrations in the range of 0.005 - 0.025 M, plots shown for 0.0003 and 0.0006 M D-glucose concentrations are from computer simulation.

concentration (0.05 M / 0.1 M) employed, gave 1/ k_{cat} for RML and CRL (Table 5.1).

Figure 5.6 (RML) and Fig. 5.7 (CRL) shows a replot of the slopes from Fig. 5.3 and Fig.

5.5 versus [D-glucose], respectively, for which slope = $K_{m\ L\text{-alanine}}$ /($k_{cat}\ K_i$), Y intercept =

$K_{m\ L\text{-alanine}}$/ k_{cat} and   X intercept = -K_i, where K_i represents the dissociation constant for

the lipase-D-glucose complex. $K_{m\ D\text{-glucose}}$ was obtained using equation 5.2 derived by

rearranging equation 5.1,

$$K_{mB} = \frac{k_{cat}\ [B]}{v} - \frac{K_{mA}\ [B]}{[A]} - \frac{K_{mA}\ [B]^2}{[A]\ K_i}$$

$$\dots\dots\dots\dots (5.2)$$

where, K_{mB} = Michelis-Menten constant for the lipase-D-glucose complex.

Table 5.1. Apparent values of kinetic parameters for RML and CRL-catalysed synthesis of L-alanyl-D-glucose.

Name of the lipase		k_{cat} x10^3 (M h^{-1}mg^{-1})	K_{mA} x10^3 (M)	K_{mB} x10^3 (M)	K_i x 10^3 (M)
RML	a	0.29 ± 0.028	4.9 ± 0.51	0.21 ± 0.018	1.76 ± 0.19
	b	0.4 ± 0.038	11.2 ± 1.23	10.0 ± 0.96	5.5 ± 0.59
CRL	a	0.75 ± 0.08	56.2 ± 5.7	16.2 ± 1.8	21.0 ± 1.9
	b	1.0 ± 0.11	56.2 ± 5.4	16.1 ± 1.5	21.0 ± 2.3

A = L-alanine, *B* = D-glucose, a=graphical method, b=curve-fitted values.

To confirm that the kinetics of the RML- and CRL- catalyzed syntheses of L-alanyl-D-glucose follow the above-mentioned model, the apparent values of the four important kinetic parameters k_{cat}, K_i, K_{mA} and K_{mB} were also estimated through curve fitting using equation 5.1.

The range of values tested for these parameters and the constraints employed for the iteration procedure are as follows: k_{cat} < 1 M h^{-1} mg^{-1}, $K_{i\ D\text{-glucose}}$ > $K_{m\ D\text{-glucose}}$, $K_{m\ D\text{-glucose}}$ < $K_{m\ L\text{-alanine}}$, and $K_{m\ L\text{-alanine}}$ < 10 M.
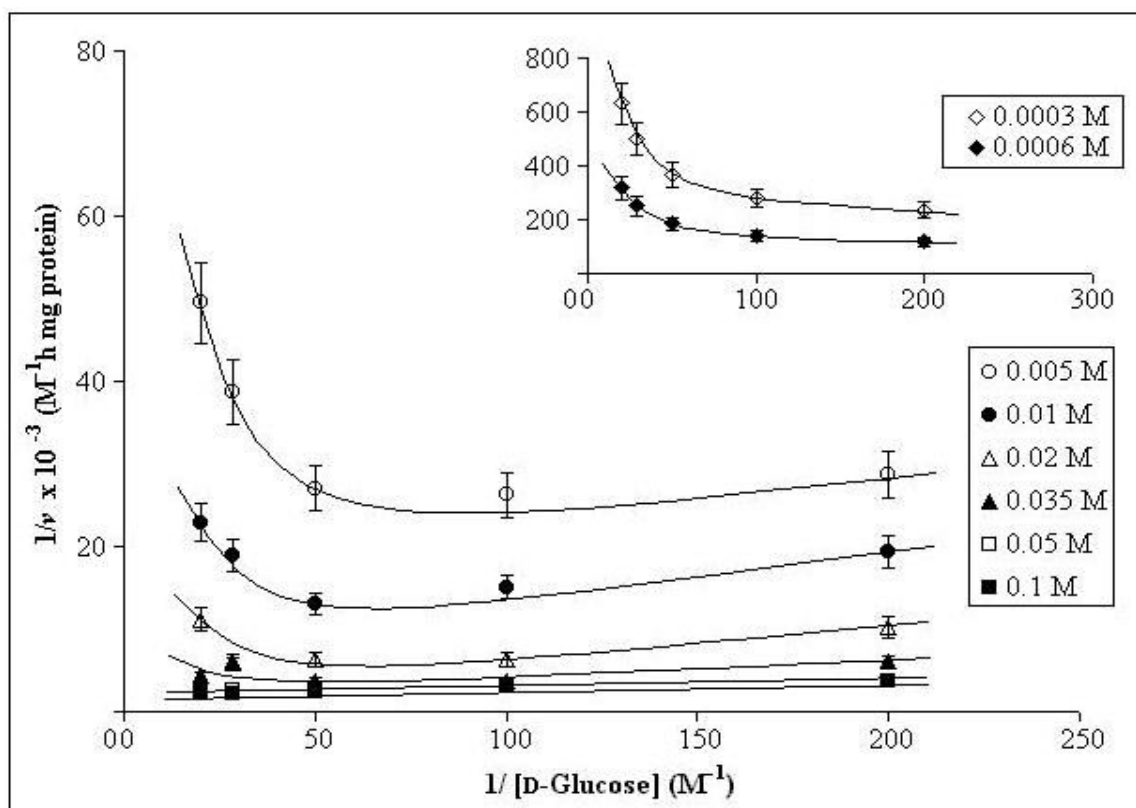
Fig. 5.4. Double reciprocal plots for CRL-catalyzed L-alanyl-D-glucose reaction: $1/v$ versus 1/[L-alanine] plots $1/v$ versus 1/[D-glucose] plots; a series of plots show the effect of varying D-glucose concentration at different fixed L-alanine concentrations in the range of 0.005 - 0.1 M, the plots shown for 0.0003 M and 0.0006 M L-alanine concentrations are from computer simulation.

The iteration procedure for the curve-fitting method involved non-linear optimization through minimizing the sum of squares of deviations between v_{exptl} and v_{pred}, such that values for the four kinetic parameters mentioned above correspond to the best fit achieved.

Table 5.1 lists graphical as well as the curve-fitted values for comparison. Table 5.2 and Table 5.3 show the comparison between experimental and predictive initial rate obtained under different reaction conditions. Although computer simulated v_{pred} values showed R^2 values of 0.84 for RML and 0.86 for CRL, the discrepancy between v_{exptl} and v_{pred} appeared to be significant at several substrate concentrations. This could be due to (i) the constraints employed in the iteration procedure (curve fitting method), which limits the flexibility required to examine the real system in solution (ii) the error in the experimental graphical methods based on HPLC measurements, which itself could involve errors of the order of $\pm$ 10 % and (iii) the heterogeneous experimental conditions employed involving undissolved carbohydrate and enzyme, on one hand, and, partly due to dissolved amino acid, on the other, in the mixture of dichloromethane and dimethylformamide.
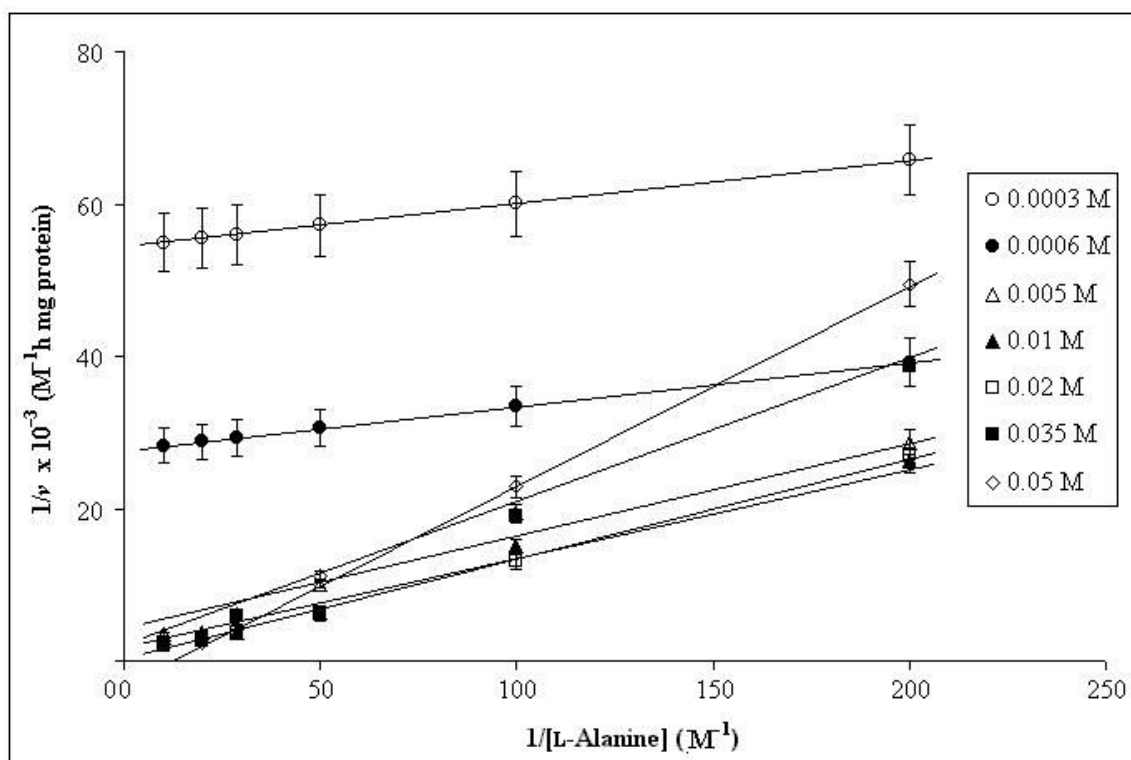
Fig. 5.5. Double reciprocal plots for CRL-catalyzed L-alanyl-D-glucose reaction: $1/v$ versus 1/[L-alanine]; a series of plots show the effect of varying L-alanine concentrations at different fixed D-glucose concentrations in the range of 0.005-0.05 M. The plots shown for 0.0003 and 0.0006 M D-glucose concentrations are from the computer simulation.

Table 5.2. Experimental and predicted initial rate values for the synthesis of L-alanyl-D-glucose by RML

L-Alanine (M)	D-Glucose (M)	$v_{experimental}$ x 10^6 (M h^{-1} mg^{-1}) [a]	$v_{predictive}$ x 10^6 (M h^{-1} mg^{-1}) [b]
0.005	0.005	30	51
0.005	0.01	37	45
0.005	0.015	28	37
0.005	0.02	25	31
0.005	0.025	15	27
0.01	0.005	38	73
0.01	0.01	51	72
0.01	0.015	55	64
0.01	0.02	49	56
0.01	0.025	55	49
0.015	0.005	48	84
0.015	0.01	46	91
0.015	0.015	72	84
0.015	0.02	91	75
0.015	0.025	88	67
0.02	0.005	65	92
0.02	0.01	97	104
0.02	0.015	41	99
0.02	0.02	76	91
0.02	0.025	127	83
0.025	0.005	71	97
0.025	0.01	146	114
0.025	0.015	116	112
0.025	0.02	132	104
0.025	0.025	111	96
0.05	0.005	79	109
0.05	0.01	176	142
0.05	0.015	158	149
0.05	0.02	176	147
0.05	0.025	176	141

[a] Graphical method.

[b] Curve-fitted values.

$$\frac{K_{m\ L\text{-}alanine}}{k_{cat}}\left[1+\frac{[\text{D-glucose}]}{K_{i\ D\text{-}glucose}}\right]$$
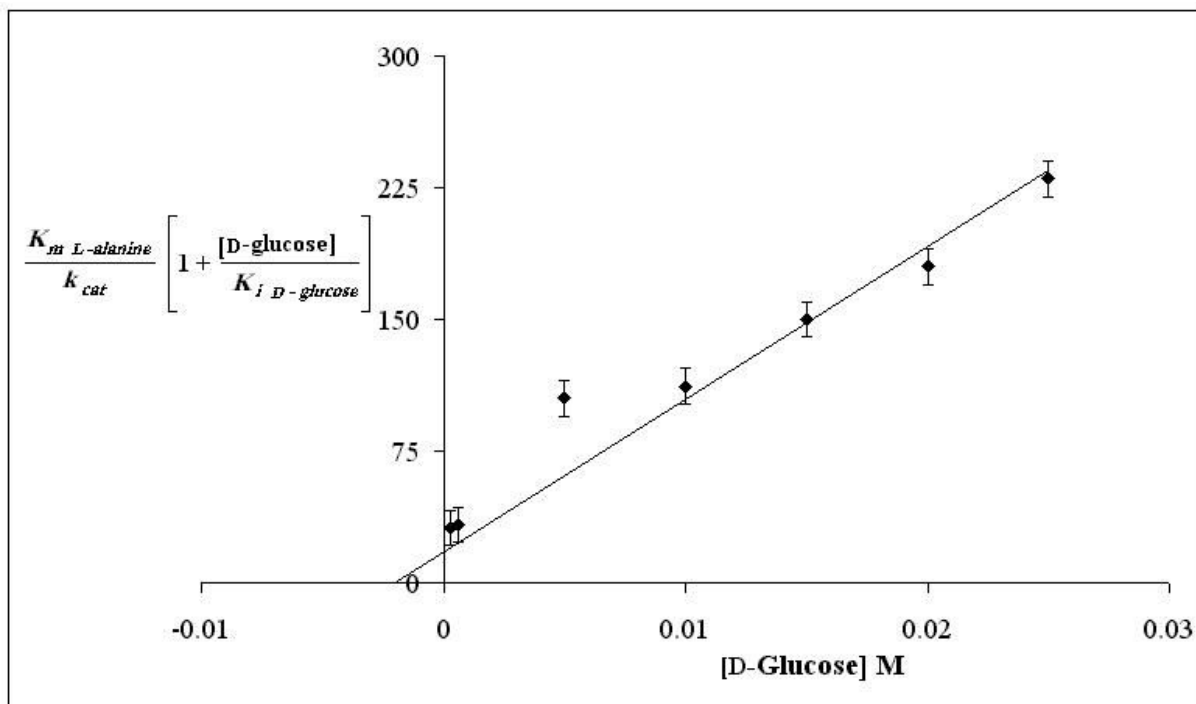
Fig. 5.6. Replot of slopes obtained from Fig. 5.3 versus [D -glucose] (RML).

$$\frac{K_{m\ L\text{-}alanine}}{k_{cat}}\left[1+\frac{[\text{D-glucose}]}{K_{i\ D\text{-}glucose}}\right]$$
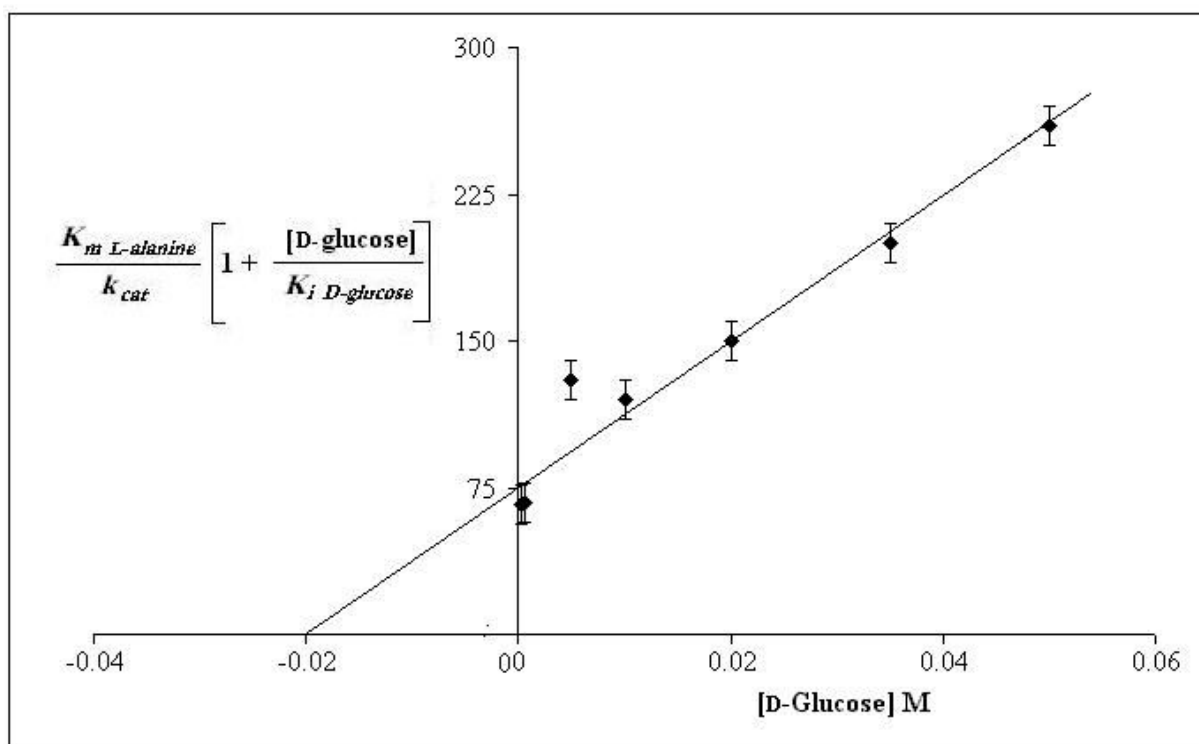
Fig. 5.7. Replot of slopes obtained from Fig. 5.5 versus [D-glucose] (CRL).

Table 5.3. Experimental and predicted initial rate values for the synthesis of L-alanyl-D-glucose by CRL

L-Alanine (M)	D-Glucose (M)	$v_{experimental}$ [a] x 10^6 (M h^{-1} mg^{-1})	$v_{predictive}$ [b] x 10^6 (M h^{-1} mg^{-1})
0.005	0.005	35	55
0.005	0.01	38	52
0.005	0.02	37	42
0.005	0.035	26	32
0.005	0.05	20	25
0.01	0.005	52	89
0.01	0.01	66	92
0.01	0.02	76	78
0.01	0.035	53	61
0.01	0.05	44	49
0.02	0.005	99	130
0.02	0.01	157	148
0.02	0.02	158	137
0.02	0.035	162	112
0.02	0.05	90	92
0.035	0.005	164	161
0.035	0.01	275	201
0.035	0.02	267	202
0.035	0.035	168	174
0.035	0.05	228	148
0.05	0.005	269	178
0.05	0.01	314	234
0.05	0.02	347	250
0.05	0.035	370	224
0.05	0.05	404	195
0.1	0.005	269	203
0.1	0.01	320	291
0.1	0.02	362	345
0.1	0.035	449	338
0.1	0.05	460	310

[a] Graphical method.

[b] Curve-fitted values.

5.3. Discussion

This kinetic data clearly shows the inhibitory nature of D-glucose towards both RML and CRL. With increasing L-alanine concentration (Fig. 5.2 for RML and Fig. 5.4 for CRL), the initial rate increases with decreasing D-glucose concentration. With increasing D-glucose concentration up to the minimum $1/v$, the initial rate decreases, and the plots tend to become closer to the $1/v$ axis (Y-axis).

Figure 5.3 (RML) and Fig. 5.5 (CRL) also show the same behavior, in which at low D-glucose concentrations, the plots appear parallel probably as long as $K_i > K_{mB}$ concerned. However, at high fixed D-glucose concentrations, the slopes of the plots drastically vary. Thus, in these reactions, the kinetic data clearly shows the inhibitory nature of D-glucose. The competition between L-alanine and D-glucose for the active site (binding site) of lipases (RML/CRL) could result in a predominant binding of D-glucose at high concentrations, displacing L-alanine, and thus leading to the formation of the dead-end lipase-D-glucose complex.

For the RML reaction, K_{mA} (4.9 ± 0.51 x 10^{-3} M) is always higher than K_{mB} (0.21 ± 0.018 x 10^{-3} M, Table 5.1), which shows that L-alanine is bound to RML less firmly than D-glucose (K_{mA}/K_{mB} = 23.3). A similar behavior is also observed with CRL (Table 1) K_{mA} (56.2 ± 5.7 x 10^{-3} M), K_{mB} (16.2 ± 1.8 x 10^{-3} M), K_{mA}/K_{mB} = 3.5. However, the respective values are very much higher for CRL than for RML, indicating that CRL can yield better conversions than RML. Between RML and CRL, the K_i for D-glucose is lower for RML (5.5 ± 0.59 x 10^{-3} M) than for CRL (21.0 ± 2.3 x 10^{-3} M), indicating that the RML is inhibited by D-glucose far more efficiently than CRL. This could also explain the better conversion observed with CRL than with RML.

Both RML and CRL contain amino acids in their active sites capable of forming hydrogen bonds with suitable donor molecules. The catalytic triad in RML consists of

Ser-144, His-257 and Asp-203 (Brady *et al.,* 1990). CRL contains Ser-209, Glu-341 and

His-449 (Grochulski *et al.,* 1993, 1994). Brzozwski *et al.* (1991) showed in an atomic

model of the inhibitor *n*-hexylchlorophosphonate ethyl ester–RML complex that in the

oxyanion hole, which is directly responsible for the substrate binding, a direct covalent

bond formation between the nucleophilic O_γ of Ser-144 and the phosphorous atom of *n*-

hexylchlorophosphonate ethyl ester is possible. In CRL, the oxyanion hole O_γ (Ser-209)

is formed by the amide backbones of Gly-123, Gly-124 and Ala-210 through the

hydrogen bonding between the amide -CO-NH- and the hydroxyl of Ser-209, which is

stabilized by the helix dipole (Grochulski *et al.,* 1994). D-Glucose possesses five

hydroxyl groups and L-alanine possesses carboxyl and amino groups capable of forming

hydrogen bonds with polar side chains of amino acids. Ser-144 hydroxyl and Asp-203

carboxyl groups of RML and Ser-209 and Glu-341 of CRL (Grochulski *et al.,* 1993)

residues are very good candidate molecules for exhibiting hydrogen-bonding

interactions.

Between D-glucose and L-alanine, the former possesses more hydrogen-bonding

functional groups than the amino or carboxyl groups of L-alanine. Ser-144 in RML and

Ser-209 in CRL can form hydrogen bonds with the amino N atom of L-alanine as well as

the O atom of D-glucose. Because the $K_{m\ L\text{-}alanine}$ values are higher than the of $K_{m\ D\text{-}glucose}$

values for both enzymes, D-glucose could strongly binding to these enzymes than L-

alanine.

Zaidi *et al.,* (2002) reported that the interaction between nylon-immobilized CRL

and alcohol through hydrogen-bonding could block the nucleophilic site of the enzyme

engaged in acylation, leading to inhibition. A similar behavior can also be envisaged

between D-glucose hydroxyl groups and the above-mentioned oxygen of serine and the

carboxylate groups of glutamic acids. Hence, this kinetic study could clearly explain the

inhibition of both RML and CRL by D-glucose. Also, for the first time, it has been found that D-glucose could be inhibitor to both the lipases at higher concentrations.

5.4. Experimental section

5.4.1. Kinetic experiments

Kinetic experiments were conducted by refluxing L-alanine and D-glucose along with 90 mg of RML or CRL in 100 ml of dichloromethane and dimethylformamide (v/v 90:10) solvent mixture (Somashekar and Divakar, 2007; Somashekar *et al.,* 2007) containing 0.1 mM (0.1 ml of 0.1 M) sodium acetate buffer (pH 4.0) for RML or 0.1 mM (0.1 ml of 0.1 M) sodium phosphate buffer (pH 7.0) for CRL. Unprotected and unactivated molecules of L-alanine and D-glucose were employed as substrates. The temperature of the reaction mixture was maintained at the reflux temperature of dichloromethane (40 ^{o}C). Experiments with RML were conducted by maintaining the concentration of one of the substrates constant in the range of 0.005 - 0.05 M and varying the concentration of the other in the same concentration range and *vice versa*. With CRL, the concentration range employed was 0.005 - 0.1 M. Product workout involved distilling off the solvent, heating to denature the enzyme, stirring and filtering to remove the lipase. The filtrate was then evaporated to obtain a residue containing L-alanine, D-glucose and the ester. The residue was subjected to high performance liquid chromatography. The retention times of L-alanine and L-alanyl-D-glucose were found to be 2.6 min and 3.4 min, respectively. No D-glucose was detected at 210 nm. The molar concentrations of the ester products formed were determined from the L-alanyl-D-glucose peak area with reference to that of free unreacted L-alanine in the reaction mixture. The error in yield measurement was within ± 10%.

5.4.2. High Performance Liquid Chromatography (HPLC)

A Shimadzu LC 10AT HPLC instrument connected to a LiChrosorb RP-18 column (5 μm particle size, 4.6 x 150 mm length) was employed for analyzing the reaction mixture. Acetonitrile: water (v/v 20:80) as a mobile phase at a flow rate of 1ml/min was used with an UV detector at 210 nm. The retention times of L-alanine and L-alanyl-D-glucose were found to be 2.6 min and 3.4 min, respectively. No D-glucose was detected at 210 nm.

Chapter 6

Angiotensin Converting Enzyme inhibition activity of L-alanyl, L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates

6.1. Introduction

Angiotensin Converting Enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc containing nonspecific dipeptidyl carboxypeptidase widely distributed in mammalian tissues (Li *et al.,* 2004). Angiotensin-converting enzyme (ACE) regulates the blood pressure by modulating renin-angiotensin system as shown in **Scheme 6.1** (Vermeirssen *et al.,* 2002). This enzyme increases the blood pressure by converting the decapeptide angiotensin I into the potent vaso-constricting octapeptide, angiotensin II. Angiotensin II brings about several central effects, all leading to a further increase in blood pressure. ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin (blood pressure-lowering nanopeptide) and therefore inhibition of ACE results in an overall antihypertensive effect (Li *et al.,* 2004; Johnston, 1992).
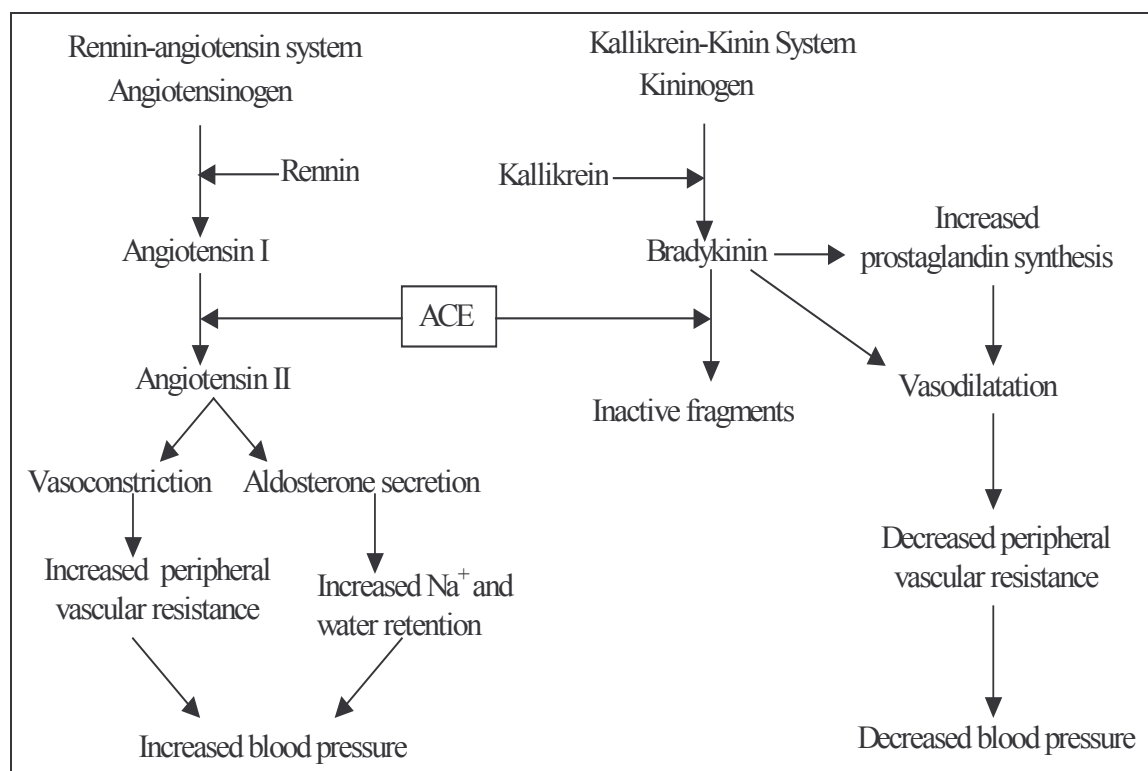
Scheme 6.1 Role of angiotensin converting enzyme (ACE) in regulating blood pressure (Li *et al.,* 2004)

Several synthetic drugs and bio-molecules are available for ACE inhibition. Captopril is a successful synthetic anti-hypertensive drug and similar such molecules like

enalapril, perindopril, ceranopril, ramipril, quinapril and fosinopril also show ACE inhibitory activities (Hyuncheol *et al.,* 2003; Dae-Gill *et al.,* 2003; Chong-Qian *et al.,* 2004). The mechanism of ACE inhibition by captopril is shown in **Scheme 6.2** (De-Lima, 1999). The hypothetical representation of inhibitors (hydrolyzed products of peptides) binding to the ACE is shown in **Scheme 6.3** and also reported that glycine, valine and leucine at the carboxyl terminus of the peptide inhibitor are the potent inhibitors (De-Lima, 1999; Wu *et al.,* 2002; Kim *et al.,* 2001).
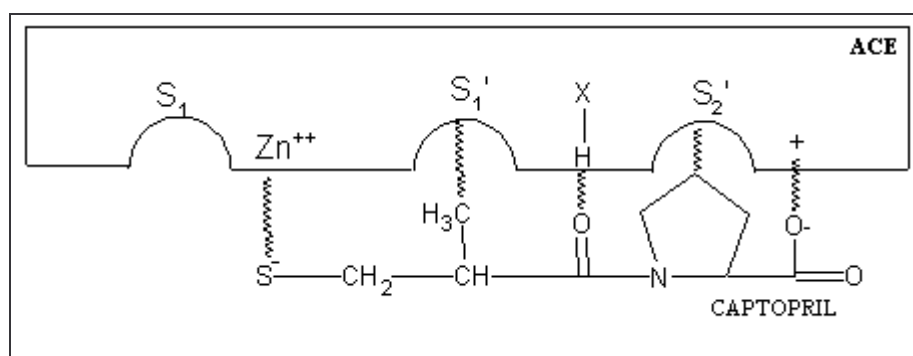
Scheme 6.2. Hypothetical representation of ACE inhibition by captopril binding to the active sites **(De-Lima 1999)**
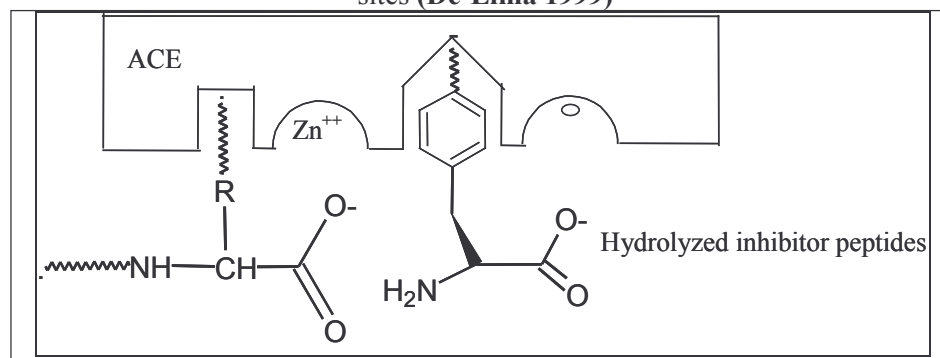
Scheme 6.3. Hypothetical representation of ACE active sites and binding of inhibitors (De-Lima 1999)

Some naturally occurring 'biologically active peptides' also act as ACE inhibitors. Deloffre *et al.,* (2004) reported that a neuro-peptide from leach brain showed ACE inhibition with an IC_{50} value of 19.8 μM. The N-terminal dipeptide (Tyr-Leu) of β-lactorphin was found to be the most potent inhibitor (Mullally *et al.,* 1996). Many peptide inhibitors are derived from different food proteins like Asp-Leu-Pro and Asp-Gly

from soy protein hydrolysis (Wu *et al.,* 2002) and Gly-Pro-Leu and Gly-Pro-Val from bovine skin gelatin hydrolysis (Kim *et al.,* 2001). Cooke *et al.,* (2003) prepared 4-substituted phenylalanyl esters of alkyl or benzyl derivatives, which exhibited ACE inhibitory activity.

Amino acyl esters of carbohydrates find wide variety of applications in food and pharmaceutical industries. Amino acyl esters have not been shown so far to exhibit ACE inhibition activity. Since most of the ACE inhibitory drugs are peptides, it was envisaged that the amino acyl esters of carbohydrates also could possess ACE inhibition activities as they contain amino acyl groups as part of their structure. Hence, this chapter deals with exploration of ACE inhibition activities for some enzymatically synthesized L-alanyl **1**, L-valyl **2**, L-leucyl **3** and L-isoleucyl **4** esters of carbohydrates **16a-e** to **53a,b** using lipases in organic media.

6.2. Present investigation

Some selective synthesized esters (each one from aldohexoses **5,6**, ketose **8**, pentose **10**, disaccharide **11, 12** and carbohydrate alcohol **14, 15** esters of L-alanine **1**, L-valine **2**, L-leucine **3** and L-isoleucine **4**) were tested for the inhibitory activities of ACE isolated from porcine lung (Section 6.4.1). Thus amino acyl esters - L-alanine **1**, L-valine **2**, L-leucine **3** and L-isoleucine **4** esters of D-glucose **5**, D-galactose **6**, D-fructose **8**, D-ribose **10**, lactose **11** maltose **12**, D-mannitol **14** and D-sorbitol **15** were subjected to ACE inhibition activities studies. The enzymatic reactions were carried out under optimized conditions worked out for these reactions (Section 6.4.2). ACE inhibition activity of the above mentioned amino acyl esters of carbohydrates were carried out by the Cushman and Cheung method (1984). Since hippuryl-L-histidyl-L-leucine (HHL) mimics the carboxyl dipeptide of angiotensin I and is resistant to angiotensinase, it has been

routinely used as the substrate for screening ACE inhibitors (Cushman and Cheung, 1969).

Underivatized L-amino acids and carbohydrates were also tested for ACE inhibition as such as controls and they did not show any ACE inhibitory activities. Only esters showed activities. Isolated ACE inhibitor tested for lipase and protease activity (Table 6.1) showed a small extent of protease activity (13.3 %) compared to ACE activity but no lipase activity. In presence of L-isoleucyl-D-glucose prepared, the isolated ACE showed 8.9 % protease activity (Table 6.1) compared to the ACE activity. This confirmed that the ACE inhibition observed in the presence of amino acyl esters prepared is more due to ACE inhibition rather than protease inhibition.

Table 6.1. Protease inhibition assay for D-glucose ester [a]

System	Protease activity Unit min^{-1}mg^{-1} enzyme protein [b]	Percentage of protease activity with respect to ACE activity [c]
Control ACE- 0.5 ml + 0.5 ml of 0.6% hemoglobin + 0.5 ml Buffer	0.0436	13.3
L-Isoleucyl-D-glucose - 0.5 ml ester + ACE -0.5 ml + 0.5 ml of 0.6% hemoglobin	0.0267	8.9

[a] Conditions: ACE – 0.5 ml (0.5mg), All the solutions were prepared in 0.1 M Tris-HCl (pH 7.5), incubation period – 30 min, temperature – 37 $^{\circ}$C, 0.5 ml of 10 % trichloro acetic acid added to arrest the reaction; Blank performed without enzyme and ester; Absorbance measured at 440 nm; ester – 0.5 ml of 0.8 mM; [b] Average absorbance values from three individual experiments; [c] Percentage protease activity with respect to an ACE activity of 0.327 μmol/(min.mg protein).
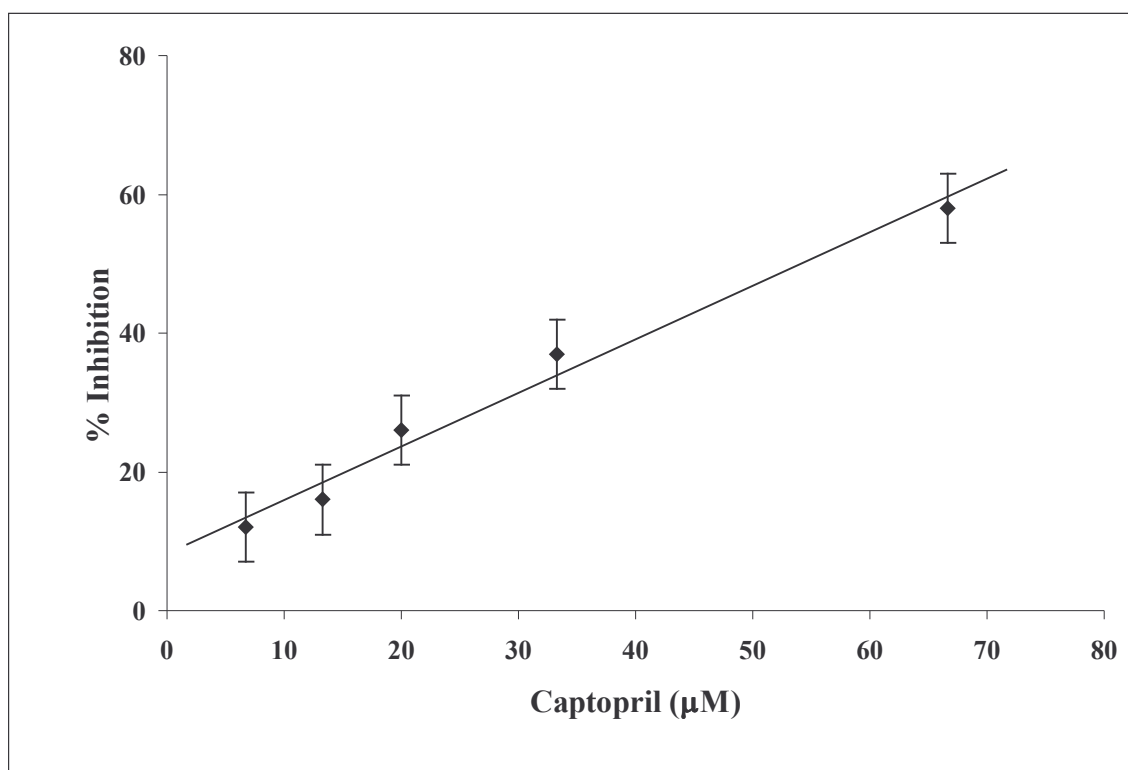
Fig. 6.1. A typical ACE inhibition plot for captopril. Concentration range – 6.7 - 66.7 μM Substrate – 0.1 ml hippuryl-histidyl-leucine (5 mM), Buffer – 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl, Incubation period – 30 min, temperature - 37 ^{o}C. IC$_{50}$ value - 0.060 ± 0.006 mM.

Table 6.2. IC$_{50}$ values for ACE inhibition by amino acyl esters of carbohydrates [a]

Amino acyl ester of carbohydrates	Conversion Yield (%)[b]	Products (% Proportion) [c]	IC$_{50}$ value (mM) [d]
L-Alanyl-β-D-glucose	30	**16a** 2-*O*-L-alanyl-β-D-glucose (47) **16b** 3-*O*-L-alanyl-β-D-glucose (12) **16c** 6-*O*-L-alanyl-β-D-glucose (20) **16d** 2,6-di-*O*-L-alanyl-β-D-glucose (15) **16e** 3,6-di-*O*-L-alanyl-β-D-glucose (6)	3.1±0.30
L-Alanyl-D-ribose	48	**21a** 3-*O*-L-alanyl-D-ribose (16) **21b** 5-*O*-L-alanyl-D-ribose (32) **21c** 3,5-di-*O*-L-alanyl-D-ribose (52)	2.7±0.25
L-Alanyl-lactose	20	**22a** 6-*O*-L-alanyl-lactose (34) **22b** 6′-*O*-L-alanyl-lactose (34) **22c** 6,6′-di-*O*-L-alanyl-lactose (32)	2.0±0.20
L-Valyl-D-glucose	68	**25a** 2-*O*-L-valyl-D-glucose (10) **25b** 3-*O*-L-valyl-D-glucose (12) **25c** 6-*O*-L-valyl-D-glucose (31) 25d **2,6-di-*O*-L-valyl-D-glucose (23)** 25e **3,6-di-*O*-L-valyl-D-glucose (24)**	6.0±0.59
L-Valyl-D-fructose	34	**28a** 1-*O*-L-valyl-D-fructose (29) **28b** 6-*O*-L-valyl-D-fructose (34) **28c** 1,6-di-*O*-L-valyl-D-fructose (37)	2.8±0.28
L-Valyl-maltose	42	**31a** 6-*O*-L-valyl-maltose (49) **31b** 6′-*O*-L-valyl-maltose (51)	3.1±0.30
L-Valyl-D-mannitol	56	**33** 1-*O*-L-valyl-D-mannitol	1.0±0.09
L-Leucyl-D-glucose	43	**34a** 2-*O*-L-leucyl-D-glucose (17) **34b** 3-*O*-L-leucyl-D-glucose (20) **34c** 6-*O*-L-leucyl-D-glucose (48) 34d **2,6-di-*O*-L-leucyl-D-glucose (8)** **34e** 3,6-di-*O*-L-leucyl-D-glucose (7)	2.8±0.27
L-Leucyl-D-fructose	48	**37** 6-*O*-L-leucyl-D-fructose	0.9±0.08
L-Leucyl-D-ribose	38	39a **3-*O*-L-leucyl-D-ribose (16)** 39b **5-*O*-L-leucyl-D-ribose (32)** **39c** 3,5-di-*O*-L-leucyl-D-ribose (52)	1.5±0.14
L-Leucyl-D-sorbitol	60	**43** 1-*O*-L-leucyl-D-sorbitol	2.7±0.25
L-Isoleucyl-D-glucose	47	**44a** 3-*O*-L-isoleucyl -D-glucose (42) **44b** 6-*O*-L-isoleucyl -D-glucose (58)	0.7±0.07
L-Isoleucyl-D-fructose	42	47a **1-*O*-L-isoleucyl-D-fructose (36)** 47b **6-*O*-L-isoleucyl-D-fructose (30)** **47c** 1,6-di-*O*-L-isoleucyl-D-fructose (34)	0.9±0.09
L-Isoleucyl-D-ribose	53	**49a** 3-*O*-L-isoleucyl-D-ribose (52) **49b** 5-*O*-L-isoleucyl-D-ribose (20) **49c** 3,5-di-*O*-L-isoleucyl-D-ribose (28)	3.8±0.37
L-Isoleucyl-maltose	54	**51a** 2-*O*-L-isoleucyl- maltose (38) **51b** 6-*O*-L-isoleucyl -maltose (40) **51c** 6′-*O*-L-isoleucyl -maltose (22)	0.9±0.09

[a] Respective amino acids and carbohydrates as controls showed no ACE inhibition activity; [b] Conversion yields were from HPLC within ± 10 % errors in HPLC yield measurements; [c] Product proportions determined from ^{13}C, 2D HSQCT NMR C6 peak areas (C5 cross peaks in case of ribose) or their cross peaks; [d] IC$_{50}$ values compared to that of captopril 0.060 ± 0.006 mM determined by Cushman and Cheung method.
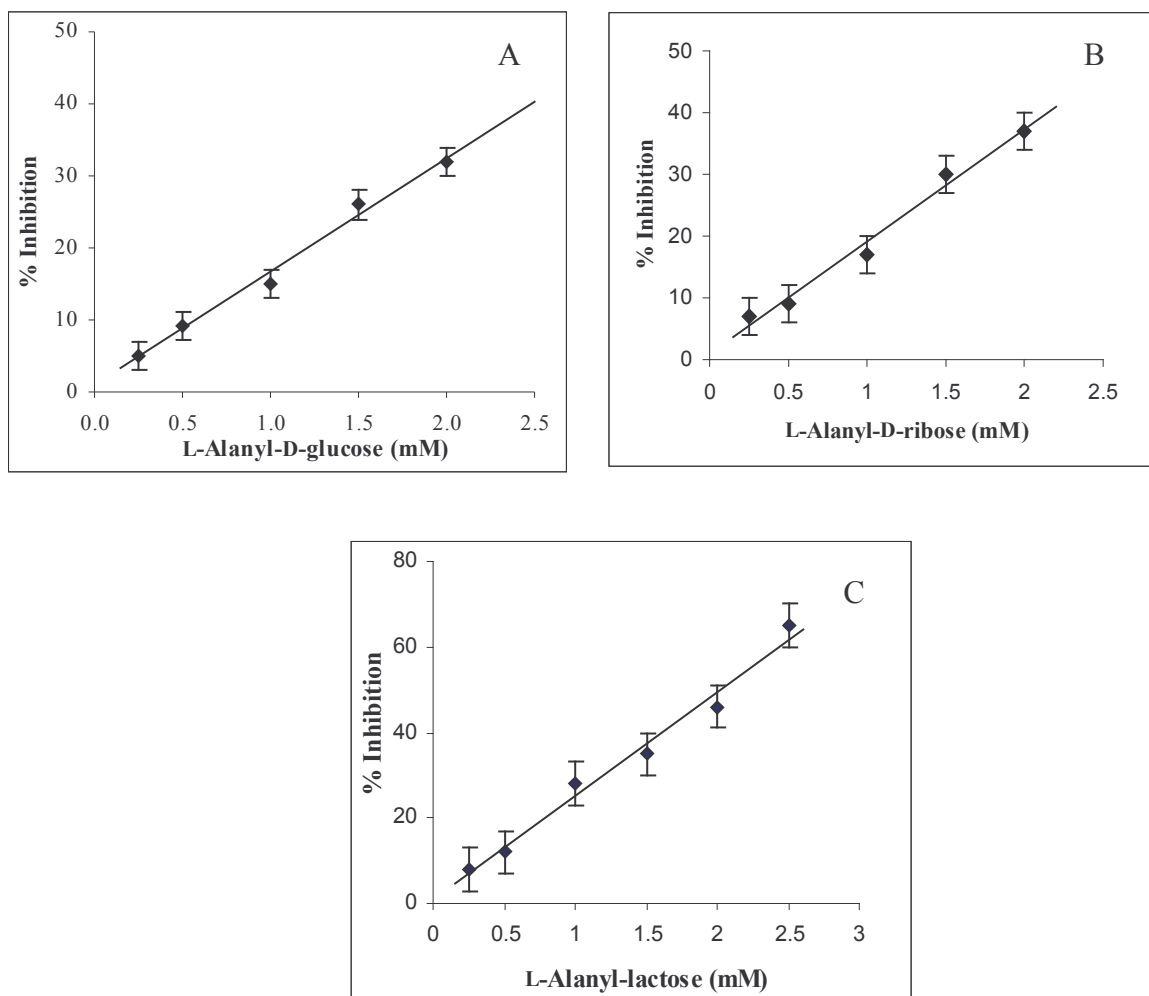
Fig. 6.2. ACE inhibition plots for L-alanyl esters of carbohydrates, (A) L-alanyl-D-glucose **16a-e**, concentration range – 0.25 – 2.0 mM, substrate – 0.1 ml hippuryl-histidyl-leucine (5 mM), Buffer – 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl, Incubation period – 30 min, Temperature-37 ^{o}C, (B) L-alanyl-D-ribose **21a-c**, concentration range – 0.25 - 2.0 mM. (C) L-alanyl-D-lactose **22a-c**, concentration range – 0.25 – 2.5 mM.

Figure 6.1 shows a typical ACE inhibition plot for captopril which showed an IC$_{50}$ value of 0.060 ± 0.006 mM. ACE inhibition plots for all the tested esters, such as carbohydrate esters of L-alanine (Fig. 6.2), L-valine (Fig. 6.3), L-leucine (Fig. 6.4) and L-isoleucine (Fig. 6.5) are shown. Table 6.2 shows the compounds tested, their conversion yields from the respective enzymatic reactions, proportions and nature of the esters formed and ACE inhibitory activities for these compounds.

The compounds were characterized by two-dimensional Heteronuclear Single Quantum Coherence Transfer (2D-HSQCT) NMR spectra recorded for the samples (Chapter 4). From NMR it was confirmed that mono and di esters, in different proportions were detected (Table 6.2). In some cases like L-valyl-maltose **31a,b**, L-valyl-D-mannitol **33**, L-leucyl-D-fructose **37**, L-leucyl-D-mannitol **42** and L-isoleucyl-D-glucose **44a,b** only monoesters were found to be formed. A 1-*O*- monoester was formed in case of L-valyl-D-fructose **28a**, L-valyl-D-mannitol **33**, L-leucyl-D-sorbitol **43** and L-isoleucyl-D-fructose **47a.** A 2-*O*- monoester was found to be formed in case of L-alanyl-β-D-glucose **16a,** L-valyl-D-glucose **25a**, L-leucyl-D-glucose **34a** and L-isoleucyl-maltose **51a**. A 3-*O*- monoester was found to be formed in case of L-alanyl-β-D-glucose **16b,** L-valyl-D-glucose **25b** and L-leucyl-D-glucose **34b** and L-isoleucyl-D-glucose **44a**. A 6′-*O*-monoester was found to be formed in case of L-alanyl-lactose **22b**, L-valyl-maltose **31b** and L-isoleucyl-maltose **51c**. All the esters invariable showed formation of 6-*O*-monoester (**16c**, **22a**, **25c**, **28b**, **31a**, **34c**, **37**, **44b**, **47b** and **51b**) except L-alanyl-D-ribose **21a-c**, L-leucyl-D-ribose **39a-c** and L-isoleucyl-D-ribose **49a-c** where the primary C-5 hydroxyl group reacted to form 5-*O*- ester (**21b, 39b** and **49b**). Diesters such as 1,6-di-*O*-, 2,6-di-*O*-, 3,6-di-*O*-, 3,5-di-*O*- and 6,6′-di-*O*- were found to be formed in case of L-alanyl-β-D-glucose **16d,e,** L-alanyl-D-ribose **21c**, L-alanyl-lactose **22c,** L-valyl-D-glucose

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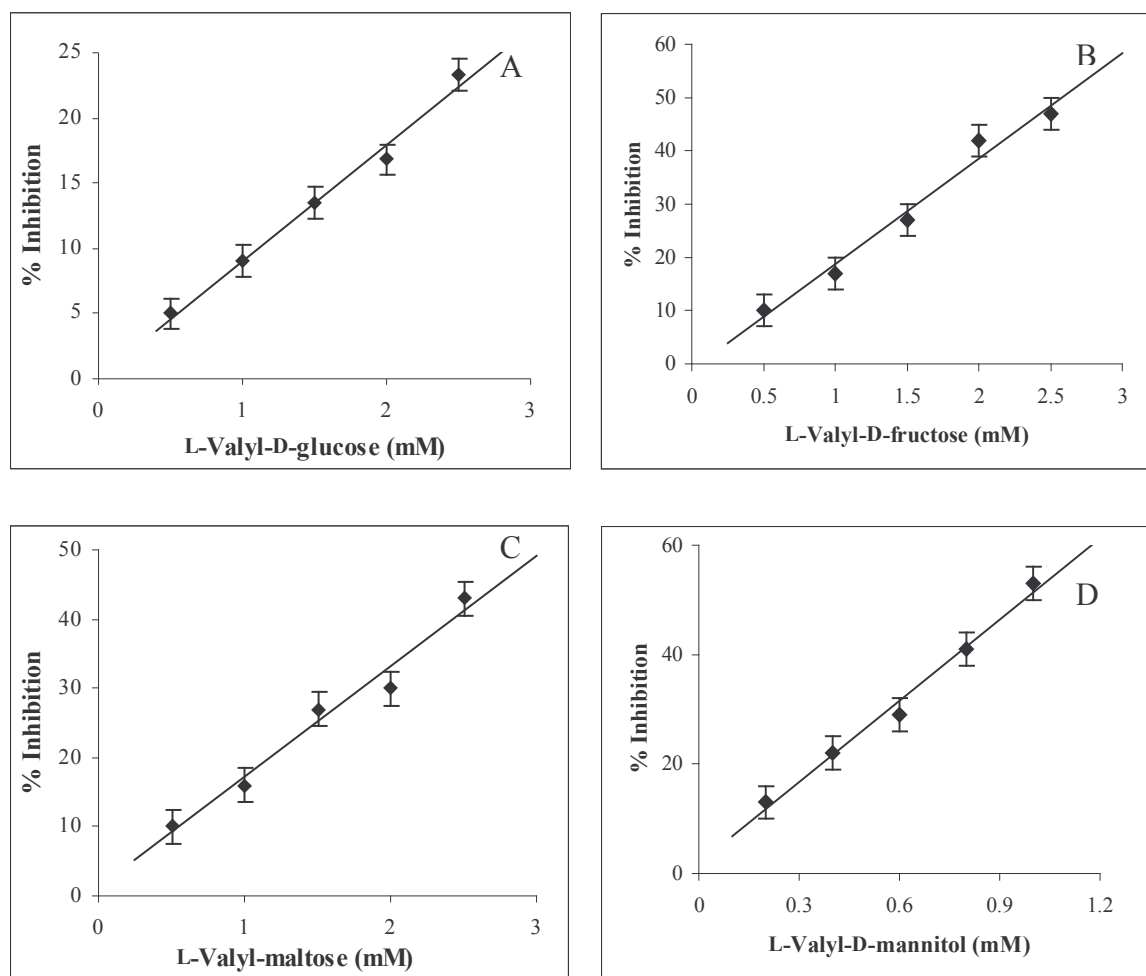
Fig. 6.3. ACE inhibition plots for L-valyl esters of carbohydrates (A) L-valyl-D-glucose **25a-e**, concentration range – 0.5 – 2.5 mM, substrate – 0.1 ml hippuryl-histidyl-leucine (5 mM), Buffer – 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl, Incubation period – 30 min, Temperature-37 °C. (B) L-valyl-D-fructose **28a-c**, concentration range – 0.5 – 2.5 mM. (C) L-valyl-maltose **31a,b**, concentration range – 0.5 – 2.5 mM. (D) L-valyl-D-mannitol **33,** concentration range – 0.2 – 1.0 mM.

25d,e, L-valyl-D-fructose **28c**, L-leucyl-D-glucose **34d,e**, L-leucyl-D-ribose **39c**, L-isoleucyl-D-fructose **47c** and L-isoleucyl-D-ribose **49c**. It was not possible to separate the individual esters from their reaction mixtures even through chromatography on Sephadex G-10 or Bio Gel P2. Thus the activities described are for the mixtures of these mono and diesters.

6.3. Discussion

Among the esters, L-isoleucyl-D-glucose (0.7±0.07 mM) was found to exhibit the best inhibitory activity. With increase in alkyl side chain branching, D-glucose esters of L-alanine (3.1±0.30 mM), L-valine (6.0±0.59 mM), L-leucine (2.8±0.27 mM) and L-isoleucine (0.7±0.07 mM) showed better inhibition (lesser IC_{50} values), than the other esters, which could be directly correlated to increase in hydrophobicity (Table 6.2). IC_{50} values ≤ 1.0 mM were detected for L-valyl-D-mannitol (1.0±0.09 mM), L-isoleucyl-D-glucose (0.7±0.07 mM), L-isoleucyl-D-fructose (0.9±0.09 mM), L-isoleucyl-maltose (0.9±0.09 mM) and L-leucyl-D-fructose (0.9±0.08 mM). Although, amino acyl esters were separated from the reaction mixture by column chromatography, it was difficult to separate the individual esters. Hence, the actual potency of the individual esters could not be unequivocally established in the present work.

The present work for the first time has shown the ACE inhibitory potency of the above mentioned amino acyl esters prepared enzymatically. Since milder reaction conditions were employed, the products formation did not suffer due to side reactions. Captopril is N-[(S)-3-mercapto-2-methylpropionyl]-L-proline containing prolyl unit as essential for ACE inhibition (De-Lima 1999). Some naturally occurring 'biologically active peptides' such as N-terminal dipeptide (Tyr-Leu) of β-lactorphin, Asp-Leu-Pro and Asp-Gly from soy protein and Gly-Pro-Leu and Gly-Pro-Val from bovine skin gelatin
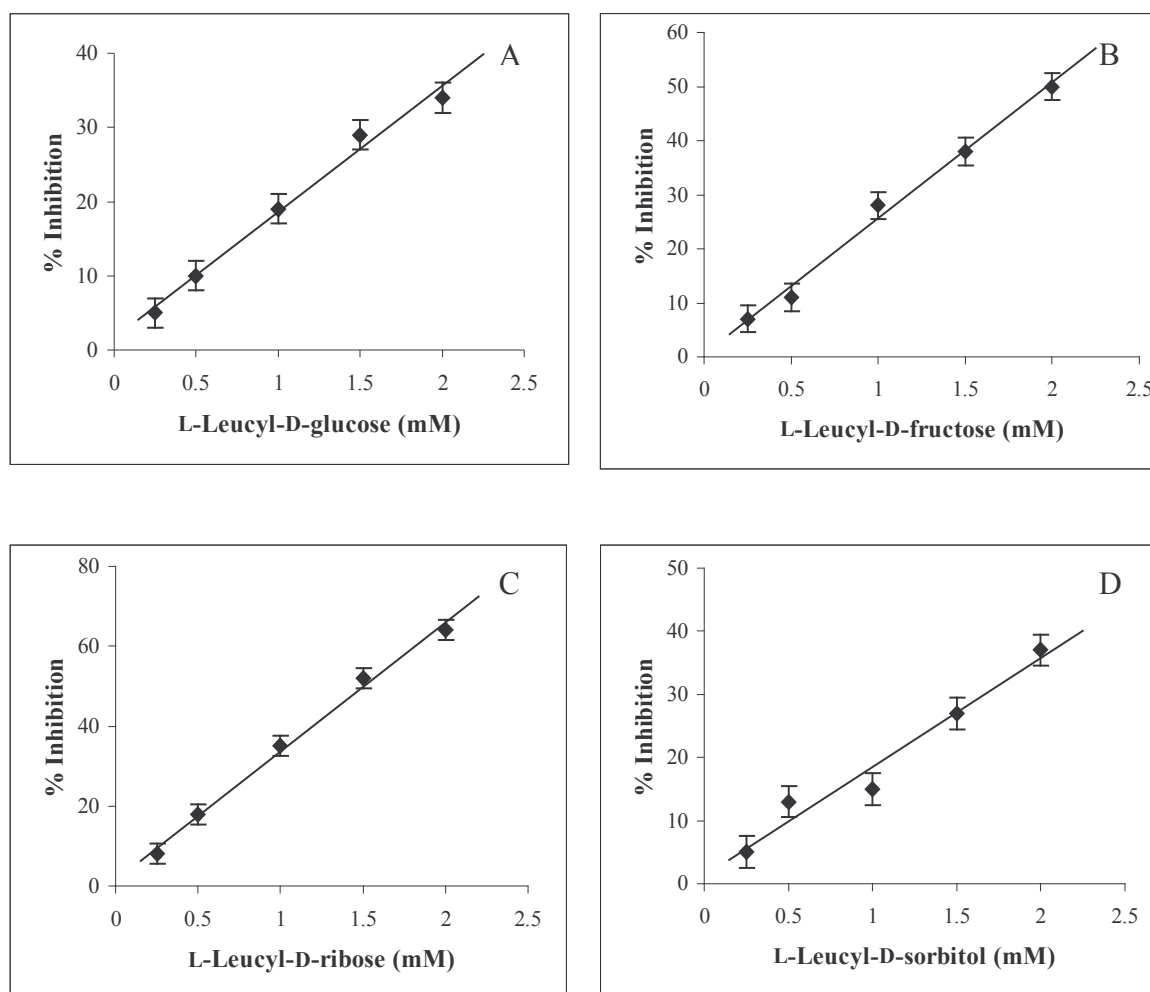
Fig. 6.4. ACE inhibition plots for L-leucyl esters of carbohydrates. (A) L-leucyl-D-glucose **34a-c**, concentration range − 0.25 − 2.0 mM, substrate − 0.1 ml hippuryl-histidyl-leucine (5 mM), Buffer − 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl, Incubation period −30 min, Temperature-37 ^{o}C. (B) L-leucyl-D-fructose **37**, concentration range − 0.25 − 2.0 mM, (C) L-leucyl-D-ribose **39a-c**, concentration range − 0.25 − 2.0 mM, (D) L-leucyl-D-sorbitol **41**, concentration range − 0.25 − 2.0 mM.

hydrolysis also act as ACE inhibitors (Deloffre *et al.,* 2004; Mullally *et al.,* 1996; Wu *et al.* 2002; Kim *et al.,* 2001). Although, the aliphatic amino acyl esters of D-glucose, D-fructose, D-ribose and lactose were prepared and tested, mere presence of a alkyl unit does not give rise to a high level of ACE inhibition. Overall it was clear that alkyl side chains can be accommodated in the hydrophobic S_1 and S_2 subsites of angiotensin I converting enzyme (Michaud *et al.,* 1997; De-Lima, 1999). The free amino group in the amino acid esters can also serve as good ligands for Zn^{2+} in the ACE active site. Carbohydrates in esters could also bind to the hydrophobic and/or hydrophilic subsites of angiotensin I converting enzyme, as they possess both hydrophobic and hydrophilic groups in their structure. Although the ACE preparation in the present work from pig lung is ACE I (Andujar-Sanchez *et al.,* 2003), it showed a low protease inhibitory activity but no lipase activity. This indicates that the amino acyl esters of carbohydrates inhibit ACE rather than the proteases as protease inhibition activity can be construed some times to be ACE inhibition. The results indicate that the esters hold promise as the potential inhibitors for ACE.

6.4. Experimental

6.4.1. Extraction of ACE from pig lung

ACE was extracted from pig lung (Section 2.2.15) using the method described by Andujar-Sanchez *et al.,* (2003). A 100 g of pig lung was minced and homogenized using a blender with 10 mM HEPES buffer (pH 7.0) containing 0.4 M NaCl at a volume ratio of 5:1 (v/w of pig lung) at 4 $^\circ$C. The homogenate was centrifuged at 9000 g for 60 min. The supernatant was discarded and the precipitate was washed twice with 200 ml of 10 mM HEPES buffer (pH 7.0) containing 0.4 M NaCl. The final precipitate was resuspended in 200 ml of 10 mM HEPES buffer pH 7.0 containing, 0.4 M NaCl, 10 μM
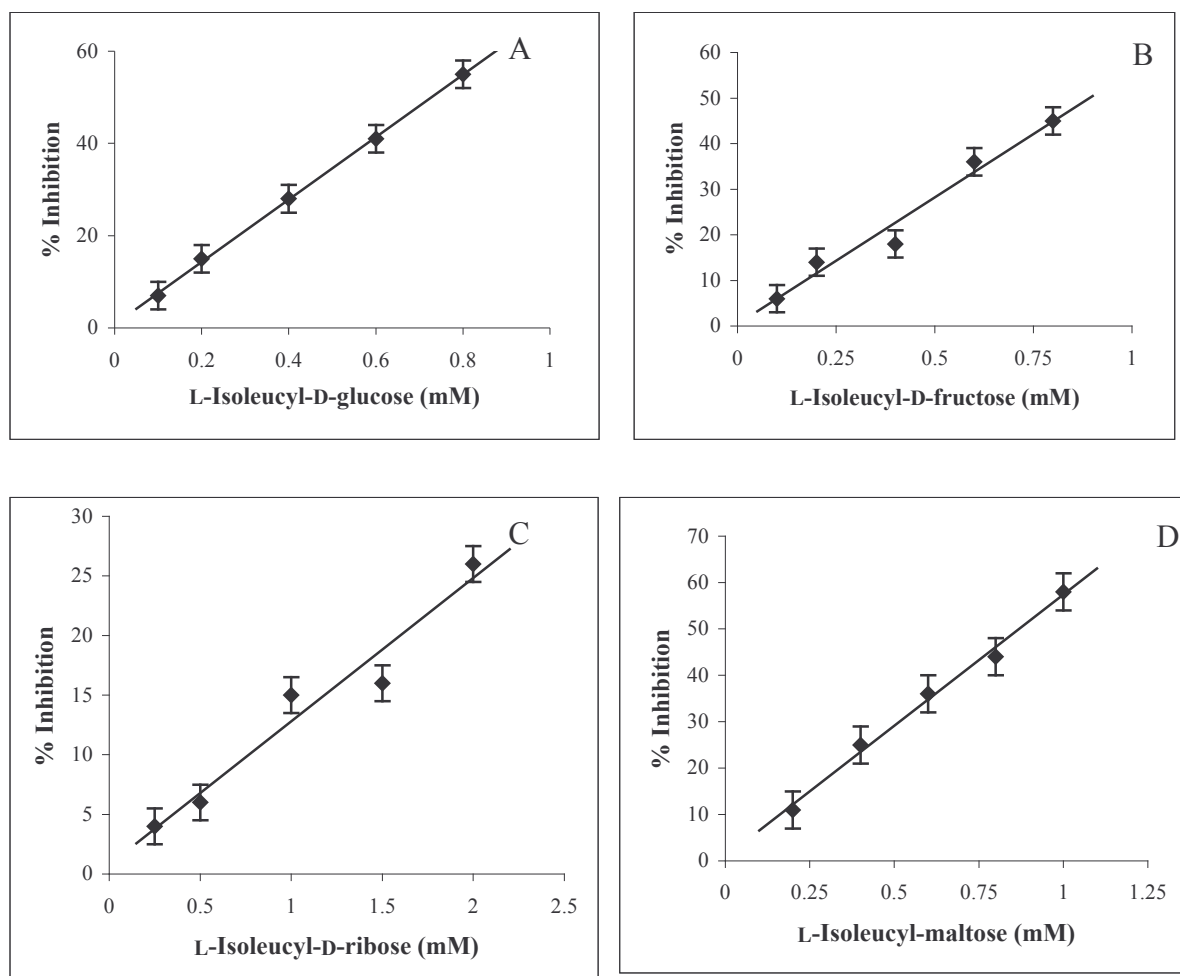
Fig. 6.5. ACE inhibition plots for L-isoleucyl esters of carbohydrates (A) L- isoleucyl-D-glucose **44a,b**, concentration range – 0.2 – 0.8 mM, substrate – 0.1 ml HHL (5 mM), Buffer – 100 mM phosphate buffer (pH 8.3) containing 300 mM NaCl, Incubation period –30 min, Temperature-37 ^{o}C. (B) L-isoleucyl-D-fructose **47a-c**, concentration range – 0.2 – 0.8 mM, (C) L-isoleucyl-D-ribose **48-c**, Concentration range 0.25 – 2.0 mM, (D) L-isoleucyl-maltose **51a-c**, concentration range – 0.2 – 1.0 mM.

ZnCl$_2$, 0.5 % (w/v) Triton-X-100 and stirred over night at 4 ^{o}C. The solution was centrifuged to remove the pellets. The supernatant was dialyzed against water and later lyophilized. The protein content of ACE determined by Lowry's method was found to be 8.3 %. The specific activity of the enzyme was found to be 0.243 μmol/min/mg of enzyme protein.

6.4.2. Esterification Procedure

A general procedure employed for the esterification reaction is as follows. Esterification was carried out in a flat bottom two necked flask by reacting 0.001- 0.008 mol unprotected L-amino acid (L-alanine, L-valine, L-leucine and L- isoleucine) and 0.001 – 0.002 mol of carbohydrate (D-glucose, D-galactose, D-fructose, D-ribose, lactose and D-mannitol) along with 100 ml CH$_2$Cl$_2$:DMF (v/v 90:10, 40 ^{o}C) or hexane:CHCl$_3$:DMF (v/v/v 45:45:10, 61 ^{o}C) in presence of 0.60 - 0.180 g of lipases (40 to 50 % w/w carbohydrate employed) under reflux for a period of three days. *Rhizomucor miehei* lipase in presence of 0.1 mM (0.1ml of 0.1 M) acetate buffer (pH 4.0) towards L-alanyl esters of carbohydrates syntheses and *Candida rugosa* lipase (CRL) in presence of 0.1 mM (0.1 ml of 0.1 M) phosphate buffer (pH 7.0) towards L-valyl, L-leucyl and L-isoleucyl esters of carbohydrates syntheses were employed. The condensed vapour of solvents which formed an azeotrope with water was passed through a desiccant before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction (Lohith and Divakar, 2005). This set up maintained a very low water activity of a$_w$= 0.0054 throughout the reaction period. After completion of the reaction, the solvent was distilled off 20 - 30 ml of warm water was added, stirred and filtered to remove the lipase. The filtrate was evaporated to get a mixture of the unreacted carbohydrate, unreacted L-amino acids and the product esters, which were then analyzed by HPLC. A Shimadzu LC10AT HPLC connected to LiChrosorb RP-18

column (5 μm particle size, 4.6 x 150 mm length) with acetonitrile: water (v/v 20:80) as a mobile phase at a flow rate of 1 ml/min was employed using an UV detector at 210 nm. The conversion yields were determined with respect to peak areas of the L-amino acid and that of the esters. The esters formed were separated by size exclusion chromatography using Sephadex G-10 and Bio Gel P-2 as column materials and eluted with water. The product esters separated were subjected to spectral characterization by UV, IR, mass, specific rotation and 2D-NMR.

6.4.3. Angiotensin Converting Enzyme (ACE) inhibition assay

ACE inhibition assay for the esters prepared were performed by the Cushman and Cheung method (Cushman and Cheung, 1971). Aliquots of ester solutions in the concentration range 0.2 to 2.5 mM (0.1 ml to 1.0 ml of 5.0 mM stock solution) were taken and to this 0.1 ml of ACE solution (0.1% in 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl) was added. To this solution, 0.1 ml of 5.0 mM hippuryl-L-histidyl-L-leucine (HHL) was also added and the total volume made upto 1.25 ml by adding phosphate buffer (1.05 ml to 0.15 ml of 0.1 M, pH 8.3 containing 300 mM NaCl). The solution was incubated on a Heto-Holten shaking water bath for 30 min at 37 $^{\circ}$C. Blanks were performed without the enzyme by taking only the ester solution (0.1 to 1.0 ml) along with 0.1 ml of 5.0 mM HHL. The total volume was made upto to 1.25 ml by adding same buffer (1.15 ml to 0.25 ml). The reaction was terminated by adding 0.25 ml of 1M HCl. Hippuric acid formed in the reaction was extracted with 1.5 ml of ethyl acetate. One ml of the ethyl acetate layer was evaporated to dryness and treated with equal amount of distilled water and absorbance was measured at 228 nm for hippuric acid. The hippuric acid formed in 1.5 ml of ethyl acetate was determined from a calibration plot prepared by using a standard hippuric acid in 1 ml of distilled water in the concentration range 0-400 nmol and measuring its absorbance at 228 nm. Specific

activity was expressed as millimoles of hippuric acid formed per min per mg of enzyme protein.

$$\text{Specific activity} = \frac{A_{ts} - A_{blank}}{T \times S \times E}$$

A_{ts} = absorbance of test solution,

A_{blank} = absorbance of blank solution,

T = incubation period in min,

S = slope value of the calibration plot (1.006×10^{-2} Abs units/nmol of hippuric acid),

E = amount of the enzyme in mg protein.

Percentage inhibition was expressed as the ratio of the specific activity of ACE in the presence of the inhibitor to that in the absence of the inhibitor, the latter being considered as 100 %. IC_{50} value was expressed as the concentration of the inhibitor required for 50 % reduction in ACE specific activity. Molecular weights of the esters employed in the calculations are weighted averages of molecular weights of esters detected by NMR spectroscopy.

A Shimadzu UV–1601 spectrophotometer was employed for the measurement of absorbance readings at 228nm.

6.4.4. Protease and lipase assay

Protease activity for the ACE inhibitor (Section 2.2.2) was determined by the method described by Dubey and Jagannadham (2003) and lipase activity (Section 2.2.1.1) by the tributyrin method (Vorderwulbecke *et al.,* 1992) in presence of L-isoleucyl-D-glucose (0.8 mM in 0.1M Tris-HCl buffer, pH 7.5). Specific protease activity was expressed as the increase in absorbance at 440 nm per min per mg of the protein employed. Similarly specific lipase activity was determined as μmol of butyric acid formed per min per mg of the protein employed.

Conclusions

The important findings of the present investigation are:

1. The esterification potentialities of lipases from *Rhizomucor miehei* (RML), *Candida rugosa* (CRL) and porcine pancreas (PPL) were explored in detail in the syntheses of L-alanyl **1**, L-valyl **2**, L-leucyl **3** and L-isoleucyl **4** esters of carbohydrates - D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14** and D-sorbitol **15**, using unprotected and unactivated amino acids and carbohydrates.

2. An experimental set-up was employed which maintained a very low water activity (a_w= 0.005) throughout the reaction. The set-up involved refluxing an appropriate amount of L-amino acid and carbohydrate in presence of buffer salts and lipases in the specified low boiling solvent mixture. The condensed vapors of the solvent was passed through a desiccant before being returned into the reaction mixture, which facilitated complete removal of water of reaction. This set-up facilitated use of larger concentrations of substrates and lesser amounts of the enzymes which resulted in higher conversions.

3. The present study investigated the effect of buffer salts on this esterification reaction which rendered 'pH tuning' of the enzyme, besides providing optimum water activity necessary for the better performance of the enzyme. All the three lipases RML, CRL and PPL showed higher conversions when a small amount of buffer salt was employed.

4. Two dimensional HSQCT NMR confirmed the formation of 1-*O*-, 2-*O*-, 3-*O*-, 4-*O*, 5-*O*-, 6-*O*- and 6′-*O*- mono esters and 1,6-di-*O*-, 2,5-di-*O*-, 2,6-di-*O*-, 3,5-di-*O*-, 3,6-di-*O*-, 4,6-di-*O*- and 6,6′-di-*O*- diesters to varying extents depending on the carbohydrate employed. Nature of the products clearly indicated that primary

hydroxyl groups of the carbohydrates (1-*O*-, 5-*O*-, 6-*O*- and 6'-*O*-) esterified predominantly over the secondary hydroxyl groups (2-*O*-, 3-*O*- and 4-*O*-). Among the secondary hydroxyl groups, 4-*O*- ester was formed only in case of D-mannose (**18b**, **36b** and **46b**). Carbohydrates containing axial hydroxyl groups in axial position like C2 in D-mannose and D-ribose and C4 in D-galactose have not reacted, indicating that esterification with axial secondary hydroxyl groups are difficult, especially with alkyl amino acyl donors. In case of L-alanyl-D-glucose **16a-e**, only β-anomer of D-glucose reacted, the D-glucose employed being a 40:60 mixture of α and β anomers respectively. Lesser incubation periods gave rise to only monoesters. The anomeric hydroxyl groups of carbohydrate molecules did not react because of rapid glycosidic ring opening and closing process.

5. Aldohexoses (D-glucose, D-mannose and D-galactose), ketohexose (D-fructose), pentose (D-ribose) and the disaccharides (maltose) showed better conversions with all the four amino acids employed. Least conversions were observed for carbohydrate alcohols and sucrose esters. L-Valyl esters (25 - 78 %) as well as L-leucyl esters (21 - 65 %) showed better conversion than L-alanyl esters (3 - 78 %) and L-isoleucyl esters (9 - 55 %). Among the lipases employed, *Candida rugosa* lipase and porcine pancreas lipase have shown better conversions than *Rhizomucor miehei* lipase. L-Alanine **1**, L-valine **2** and L-leucine **3** with D-glucose **5** and L-alanine **1** and L-isoleucine **4** with D-mannose **7** gave five diastereomeric esters. Both D-arabinose **9** and D-ribose **10** have shown three diastereomeric esters with all the amino acids (**1-4**) employed. Lactose **11** did not react with L-valine and L-leucine and D-sorbitol **15** with L-alanine, L-valine and L-isoleucine. L-Alanyl-sucrose **24**, L-valyl-D-mannose **27**, L-valyl-sucrose **32**, L-leucyl-maltose **40**, L-leucyl- sucrose **41** and L-isoleucyl-sucrose **52** formed only 6-*O*-ester. Carbohydrates like lactose **11**, D-mannitol **14** and

D-sorbitol **15** reacted selectively depending on the amino acid indicating that they may not be good nucleophiles, probably could be due to more hydrogen bonding propensity for D-mannitol and D-sorbitol and more steric hindrance in case of lactose. Loss of specificity could be due to use of larger amount of enzymes (about 40 % w/w carbohydrate), which gave a large number of esters. In the present esterification no maillard reaction product was found to be formed. Although underivatised amino acids were employed, less than 3 % peptide formation was detected and that too in few cases only.

6. About 99 L-amino acyl esters of carbohydrates (**16a-e** – **53a,b**) have been synthesized in the present work of which 97 esters are reported for the first time. The new esters reported are: L-alanyl-D-glucose **16a,d,e**, L-alanyl-D-galactose **17a-c**, L-alanyl-D-mannose **18a-e**, L-alanyl-D-fructose **19a-c**, L-alanyl-D-arabinose **20a-c**, L-alanyl-D-ribose **21a-c**, L-alanyl-lactose **22a-c**, L-alanyl-maltose **23a-c**, L-alanyl-sucrose **24**, L-valyl-D-glucose **25a-e**, L-valyl-D-galactose **26a-c**, L-valyl-D-mannose **27**, L-valyl-D-fructose **28a-c**, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-valyl-maltose **31a, b**, L-valyl-sucrose **32**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-galactose **35a,b**, L-leucyl-D-mannose **36a-c**, L-leucyl-D-fructose **37**, L-leucyl-D-arabinose **38a-c**, L-leucyl-D-ribose **39a-c**, L-leucyl-maltose **40**, L-leucyl-sucrose **41**, L-leucyl-D-mannitol **42a,b**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b,** L-isoleucyl-D-galactose **45a-c,** L-isoleucyl-D-mannose **46a-e**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-arabinose **48a-c**, L-isoleucyl-D-ribose **49a-c**, L-isoleucyl-lactose **50a-c**, L-isoleucyl-maltose **51a-c**, L-isoleucyl-sucrose **52** and L-isoleucyl-D-mannitol **53a,b**.

7. A kinetic study on the esterification of D-glucose with L-alanine catalyzed by lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) investigated in detail

showed that both the lipases followed Ping-Pong Bi-Bi mechanism wherein L-alanine and D-glucose bind in subsequent steps releasing water and L-alanyl-D-glucose also in subsequent steps, with competitive substrate inhibition by D-glucose at higher concentrations leading to the formation of dead-end lipase-D-glucose complexes.

8.   Among the amino acyl esters synthesized, about 15 amino acyl esters of carbohydrates were tested for angiotensin converting enzyme (ACE) inhibition activity. Among them, L-isoleucyl-D-glucose **44a,b** (IC_{50}: 0.7±0.067 mM), L-leucyl-D-fructose **37** (IC_{50}: 0.9±0.08 mM), L-isoleucyl-maltose **51a-c** (IC_{50}: 0.9±0.09 mM) and L-valyl-D-mannitol **33** (IC_{50}: 1.0±0.092 mM) showed the best ACE inhibitory activities. Other esters showed IC_{50} values in the range 1.5 mM – 6.0 mM.

Summary

Amino acyl esters of carbohydrates are used as sweetening agents, surfactants, microcapsules in pharmaceutical preparations, active nucleoside amino acid esters, antibiotics and in the delivery of biological active agents. Chemical acylation of carbohydrates regio-selectively is complex due to the presence of multiple hydroxyl groups, which require protection and deprotection. However enzymatic reactions can overcome this drawback. Hitherto, very few references are available on the lipase catalyzed esterification of amino acyl esters of sugars. Most of the earlier workers used proteases and N-protected and carboxyl group activated amino acids for synthesizing aminoacyl esters of carbohydrates. All these reactions were conducted in shake flasks using lesser quantity of substrates and larger quantity of enzymes. The present work deals with lipases catalyzed preparation of amino acyl esters of carbohydrates using unprotected and unactivated amino acids and carbohydrates.

Chapter **ONE** deals with literature survey on mainly lipase catalysed synthesis in organic media. Biotechnological applications of lipase catalysis in different food and pharmaceutical industries are discussed. A brief description on the lipase structure and catalytic mechanism on esterification is made. Parameters regulating lipase activity in organic media like nature of substrates, nature of solvents, effect of salt, thermal stability of lipases, water activity and immobilization are discussed. Diverse application of lipases like esterification using reverse micelles, supercritical carbon dioxide, micro oven, ionic liquids assisted reactions, kinetic studies and resolution of racemic mixture are presented. The chapter ends with a brief description on the scope of the present investigation.

Chapter **TWO** deals with materials and methods. Chemicals employed and their sources are listed. Methods of preparation of L-amino acyl esters of carbohydrates and the other related appropriate aspects of the same are discussed in detail.

Chapter **THREE** describes results from optimization of reaction parameters for the lipase catalyzed synthesis of L-alanyl **16a-e**, L-valyl **25a-e** and L-leucyl **34a-e** esters of D-glucose. Lipases from *Rhizomucor miehei* (RML), porcine pancreas (PPL) and *Candida rugosa* (CRL) were employed. The reaction conditions were optimized in terms of incubation period, solvent, enzyme concentrations, substrate concentrations, buffer (pH and concentration) and enzyme reusability. Under the experimental conditions employed, all the three lipases exhibited good esterification potentialities. Both RML and PPL showed maximum conversion yields of L-alanyl-D-glucose **16a-e** (30 % and 18 % respectively) at 40 % (w/w D-glucose) of enzyme, L-valyl-D-glucose **25a-e** (59 % and 62 % respectively) at 10 % (w/w D-glucose) of enzyme and L-leucyl-D-glucose **34a-e** (85 % and 18 % of respectively) at 40 % (w/w D-glucose) of enzyme employed. CRL showed a maximum conversion of 84 % of L-valyl-D-glucose **25a-e** at 30 % (w/w D-glucose) enzyme concentration. The present work showed enhanced activity of RML and CRL in presence of buffer salts. Optimum pH was found to be pH 4.0 for RML and pH 5.0 for PPL in case of L-alanyl-D-glucose**,** pH 7.0 for CRL in case of L-valyl-D-glucose and pH 5.0 for RML in case of L-leucyl-D-glucose reactions. Higher equivalents of D-glucose were found to inhibit RML in case of L-alanyl-D-glucose reaction. However, in case of L-valyl-D-glucose and L-leucyl-D-glucose reactions, free amino acids and D-glucose were not found to be inhibitors of RML and CRL. In the synthesis of L-alanyl-D-glucose **16a-e,** RML could be reused upto four cycles where as PPL could be used only upto two cycles.

Chapter **FOUR** describes the syntheses and characterization of L-alanyl **1**, L-valyl **2**, L-leucyl **3** and L-isoleucyl **4** esters of carbohydrates - D-glucose **5**, D-galactose **6**, D-mannose **7**, D-fructose **8**, D-arabinose **9**, D-ribose **10**, lactose **11**, maltose **12**, sucrose **13**, D-mannitol **14** and D-sorbitol **15**. Esterification was carried out by reacting 0.002 mol

unprotected L-amino acid (**1-4)** and 0.001 mol of carbohydrate (**5-15**) along with 100 ml

CH_2Cl_2: DMF (90:10 v/v, 40 ^oC) in presence of 40 % (w/w carbohydrate employed) of

lipases under reflux for a period of three days. *Rhizomucor miehei* lipase (RML) in

presence of 0.1 mM (0.1 ml of 0.1 M) of pH 4.0 acetate buffer, *Candida rugosa* lipase

(CRL) in presence of 0.1 mM (0.1 ml of 0.1 M) of pH 7.0 phosphate buffer and crude

porcine pancreas lipase in presence of 0.1 mM (0.1 ml of 0.1 M) of pH 5.0 acetate buffer

were employed to impart 'pH tuning' to the enzyme. All the three lipases employed

showed broad substrate specificity towards amino acids as well as carbohydrates.

Esterification yields were obtained in the range of 3 – 78 %. Two dimensional HSQCT

NMR confirmed the formation of 1-*O*-, 2-*O*-, 3-*O*-, 4-*O,* 5-*O*-, 6-*O*- and 6′-*O*- mono

esters and 1,6-di-*O*-, 2,5-di-*O*, 2,6-di-*O*-, 3,5-di-*O*-, 3,6-di-*O*, 4,6-di-*O*- and 6,6′-di-*O*-

diesters to varying extents depending on the carbohydrate employed. Nature of the

products clearly indicated that primary hydroxyl groups of the carbohydrates (1-*O*-, 5-*O*-

, 6-*O*- and 6′-*O*-) esterified predominantly over the secondary hydroxyl groups (2-*O*-, 3-

O- and 4-*O*-). Among the secondary hydroxyl groups, 4-*O*- ester was formed only in

case of D-mannose (**18b**, **36b** and **46b**). Carbohydrates containing hydroxyl groups in

axial position like C2 in D-mannose and D-ribose and C4 in D-galactose have not reacted,

indicating that esterification with axial secondary hydroxyl groups are difficult,

especially with alkyl amino acyl donors. In case of L-alanyl-D-glucose **16a-e**, only β-

anomer of D-glucose reacted, the D-glucose employed being a 40: 60 mixture of α and β

anomers respectively. Lesser incubation periods gave rise to only monoesters. The

anomeric hydroxyl groups of carbohydrate molecules did not react because of rapid

glycosidic ring opening and closing process. Aldohexoses (D-glucose, D-mannose and D-

galactose), ketohexose (D-fructose), pentose (D-ribose) and the disaccharides (maltose)

showed better conversions with all the four amino acids. Least conversions were

observed for carbohydrate alcohols and sucrose esters. L-Valyl esters (25 – 78 %) as well as L-leucyl esters (21 – 65 %) showed better conversion than L-alanyl esters (3 – 78 %) and L-isoleucyl esters (9 – 55 %). Among the lipases employed, *Candida rugosa* lipase and porcine pancreas lipase have shown better conversions than *Rhizomucor miehei* lipase. L-Alanine **1**, L-valine **2** and L-leucine **3** with D-glucose **5** and L-alanine **1** and L-isoleucine **4** with D-mannose **7** gave five diastereomeric esters. Both D-arabinose **9** and D-ribose **10** showed three diastereomeric esters with all the amino acids (**1-4**) employed. Lactose **11** did not react with L-valine and L-leucine and D-sorbitol **15** did not react with L-alanine, L-valine and L-isoleucine. L-Alanyl-sucrose **24**, L-valyl-D-mannose **27**, L-valyl-sucrose **32**, L-leucyl-maltose **40**, L-leucyl-sucrose **41** and L-isoleucyl-sucrose **52**, formed only 6-*O*- esters. Carbohydrates like lactose **11**, D-mannitol **14** and D-sorbitol **15** reacted selectively depending on the amino acid indicating that they may not be good nucleophiles, probably due to more hydrogen bonding propensity for D-mannitol and D-sorbitol and steric hindrance in case of lactose. About 99 L-amino acyl esters of carbohydrates were prepared out of which 97 esters have not been reported before. So far unreported esters are L-alanyl-D-glucose **16a,d,e**, L-alanyl-D-galactose **17a-c**, L-alanyl-D-mannose **18a-e**, L-alanyl-D-fructose **19a-c**, L-alanyl-D-arabinose **20a-c**, L-alanyl-D-ribose **21a-c**, L-alanyl-lactose **22a-c**, L-alanyl-maltose **23a-c**, L-alanyl-sucrose **24**, L-valyl-D-glucose **25a-e**, L-valyl-D-galactose **26a-c**, L-valyl-D-mannose **27**, L-valyl-D-fructose **28a-c**, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-valyl-maltose **31a,b**, L-valyl-sucrose **32**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-galactose **35a,b**, L-leucyl-D-mannose **36a-c**, L-leucyl-D-fructose **37**, L-leucyl-D-arabinose **38a-c**, L-leucyl-D-ribose **39a-c**, L-leucyl-maltose **40**, L-leucyl-sucrose **41**, L-leucyl-D-mannitol **42a,b**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b**, L-isoleucyl-D-galactose **45a-c**, L-isoleucyl-D-mannose **46a-e**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-arabinose

48a-c, L-isoleucyl-D-ribose **49a-c**, L-isoleucyl-lactose **50a-c**, L-isoleucyl-maltose **51a-c**, L-isoleucyl-sucrose **52** and L-leucyl-D-mannitol **53a,b**.

Chapter **FIVE** describes kinetic study on the esterification of D-glucose **5** with L-alanine **1** catalyzed by lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL). A detailed investigation showed that both the lipases followed Ping-Pong Bi-Bi mechanism wherein L-alanine and D-glucose bind in subsequent steps releasing water and L-alanyl-D-glucose also in subsequent steps, with competitive substrate inhibition by D-glucose at higher concentrations leading to the formation of dead-end lipase-D-glucose complexes. An attempt to obtain the best fit of this kinetic model through curve fitting yielded in good approximation, the apparent values of four important kinetic parameters, RML: $k_{cat} = 0.29 \pm 0.028$ x 10^{-3} M h^{-1} mg^{-1}, $K_{m\ L\text{-}alanine} = 4.9 \pm 0.51$ x 10^{-3} M, $K_{m\ D\text{-}glucose} = 0.21 \pm 0.018$ x 10^{-3} M, $K_{i\ D\text{-}glucose} = 1.76 \pm 0.19$ x 10^{-3} M; CRL: $k_{cat} = 0.75 \pm 0.08$ x 10^{-3} M h^{-1} mg^{-1}, $K_{m\ L\text{-}alanine} = 56.2 \pm 5.7$ x 10^{-3} M, $K_{m\ D\text{-}glucose} = 16.2 \pm 1.8$ x 10^{-3} M, $K_{i\ D\text{-}glucose} = 21.0 \pm 1.9$ x 10^{-3} M.

Chapter **SIX** describes potentiality of some of the amino acyl esters of carbohydrates as inhibitors towards Angiotensin Converting enzyme (ACE) activity. The esters tested are: L-alanyl-D-glucose **16a-e**, L-alanyl-lactose **22a-c**, L-valyl-D-glucose **25a-e**, L-valyl-D-fructose **28a-c**, L-valyl-D-arabinose **29a-c**, L-valyl-D-ribose **30a-c**, L-valyl-maltose **31a,b**, L-valyl-D-mannitol **33**, L-leucyl-D-glucose **34a-e**, L-leucyl-D-fructose **37**, L-leucyl-D-ribose **39a-c**, L-leucyl-D-sorbitol **43**, L-isoleucyl-D-glucose **44a,b**, L-isoleucyl-D-fructose **47a-c**, L-isoleucyl-D-ribose **49a-c** and L-isoleucyl-maltose **51a-c**. Amino acyl esters of carbohydrates tested for ACE inhibition activity showed IC$_{50}$ values for ACE inhibition in the 0.7 mM to 6.0 mM range. Among them, L-isoleucyl-D-glucose **44a,b** (IC$_{50}$: 0.7±0.067 mM), L-leucyl-D-fructose **37** (IC$_{50}$: 0.9±0.08 mM), L-

isoleucyl-maltose **51a-c** (IC$_{50}$: 0.9±0.09 mM) and L-valyl-D-mannitol **33** (IC$_{50}$: 1.0±0.092 mM) showed the best ACE inhibitory activities.

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Publications

Lipase catalyzed synthesis of L-alanyl, L-leucyl and L-phenylalanyl esters of D-glucose using unprotected amino acids

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Abstract

Enzymatic synthesis of L-alanyl, L-leucyl and L-phenylalanyl esters of D-glucose was carried out in a non-polar solvent using lipases from *Rhizomucor miehei* and porcine pancreas. The unprotected amino acids at millimolar concentrations were used in presence of 10 to 50% (w/w) glucose of the lipases to give ester yields up to >99%. The reaction mixture on analysis by 2-D NMR showed that the product is a mixture of 6-O-, 3-O- and 2-O-monoesters and 2,6-di-O- and 3,6- di-O-esters.

Introduction

Amino acid esters of glucose are used as detergents, as sweetening agents, as emulsifying agents, as microcapsules in pharmaceutical preparations, as bio-active nucleoside amino acid esters, as antibiotics and in the delivery of biological active agents (Margolin 1993, Zaks & Dodds 1997, Kirk *et al.* 1992). Earlier reports on the synthesis of amino acyl esters of sugars using lipases involved use of amino acids which are N-protected and carboxyl group activated (Suzuki *et al.* 1991, Riva *et al.* 1988, Therisod & Klibanov 1986, Park *et al.* 1999, Boyer *et al.* 2001, Maruyama *et al.* 2002).

Riva *et al.* (1988) carried out the reaction between N-acetyl-L-phenylalanyl chloroester and D-glucose in pyridine using subtilisin which resulted in 73% conversion to the three mono esters (80% of 6-O-, 15% of 3-O- and 5% of 2-O-). Park *et al.* (1999) reported that 98% of 6-O-t-Boc-L-phenylalanyl glucose was formed when the reaction was carried out between N-t-Boc-L-phenylalanyl trifluroester and glucose in pyridine using subtilisin. They also reported that lipase from porcine pancreas and Lipozyme IM20 gave insignificant results. Reactions were conducted in shake flasks using lesser quantity of substrate and larger

quantity of enzymes. Specific microbial enzymes like Optimase M-440 and subtilisin were employed, in some cases with longer periods of incubation up to 7 d.

We report here on an enzymatic method using *Rhizomucor miehei* lipase (RML) and porcine pancreas lipase (PPL) for the preparation of L-alanyl, L-leucyl and L-phenylalanyl esters of D-glucose (Scheme 1). This investigation employed free unprotected amino acids and the results are presented below.

Materials and methods

Materials

Porcine pancreas lipase Type II, Steapsin (PPL), was from Sigma and Lipozyme IM20 (*Rhizomucor miehei*) (RML) immobilized on weak anion exchange resin was from Novo Nordisk, Denmark. L-Alanine, L-leucine and L-phenylalanine from HiMedia Ind. Ltd. were used as such. Solvents were employed after distilling once before use. Both *Rhizomucor miehei* and porcine pancreas lipases employed showed esterification activities of 0.46 μmol min^{-1} mg^{-1} enzyme preparation and 0.06 μmol min^{-1} mg^{-1} enzyme preparation respectively (Kiran *et al.* 2000).

R= -CH₃, -CH₂CH(CH₃)₂, -CH₂Ph.
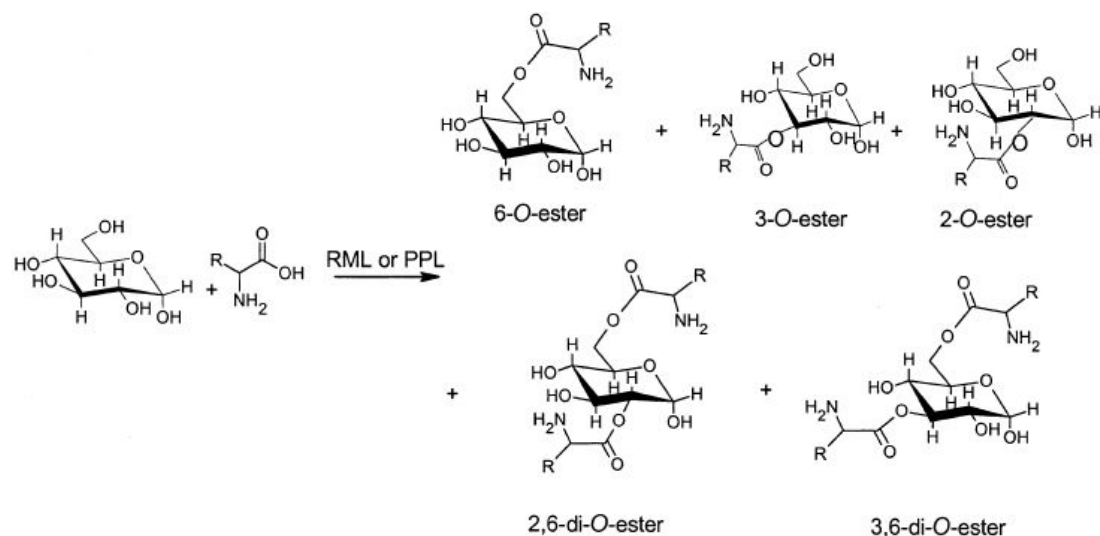
Scheme 1.

Esterification procedure

Typically about 1–10 mmol L-amino acid (L-alanine/L-leucine/L-phenylalanine) and D-glucose (1–2.5 mmol) was refluxed with 100 ml dichloromethane/dimethylformamide (90:10 v/v) or hexane/chloroform/dimethylformamide (45:45:10 v/v) or chloroform/dimethylformamide (90:10 v/v) in presence of 0.018–0.225 g lipases (10%–50% w/w D-glucose) for 3 d. Solvents which formed an azeotrope with water were refluxed and the condensed solvent vapors were passed through a desiccant before being returned into the reaction mixture, thereby facilitating complete removal of water (Lohith *et al.* 2003, Divakar *et al.* 1999), thereby maintaining a very low water activity of $a_w = 0.0054$ throughout the reaction. The reaction mixture after distilling off the solvent was then added to 20 ml water, stirred and filtered to remove the lipase. The filtrate was evaporated on a water bath to get the unreacted glucose, unreacted aminoacid and the product esters which were analysed by HPLC. In reactions which involved use of buffer salts, buffers were added to the reaction mixture to impart 'pH memory' to the enzyme. 0.1 M acetic acid/acetate buffer at pH 4 and pH 5, 0.1 M Na_2HPO_4/NaH_2PO_4 at pH 6 and pH 7 and 0.1 M $Na_2B_4O_7/HCl$ at pH 8 buffers were employed.

Product monitoring and characterization

Analytical HPLC was performed using an aminopropyl column (3.9 × 300 mm length) and acetonitrile/water at 80:20 (v/v) as the mobile phase at 1 ml min⁻¹ and a refractive index detector. Conversion yields were determined from HPLC peak areas of D-glucose esters and unreacted D-glucose. Error measurements in HPLC yields will be ±5%. Retention times are (min): glucose 5.2, L-alanine 9.4, L-alanyl-D-glucose 11.9; L-leucine 6.4, L-leucyl-D-glucose 8.3; L-phenylalanine 6.2, L-phenylalanyl-D-glucose 9.1. L-Phenylalanyl-D-glucose esters were separated from unreacted D-glucose and L-phenylalanine by subjecting the reaction mixture to repeated HPLC.

Products were characterized by recording one-dimensional ¹H and ¹³C NMR and two-dimensional NMR spectra. ¹H and ¹³C NMR spectra were recorded on a Bruker DRX 500 MHz spectrometer (500.13 MHz proton and 125 MHz carbon frequencies) operating at 20 °C. Proton and carbon 90° pulse widths were 12.25 and 10.5 μs respectively. Samples (50 mg) were dissolved in 1:1 DMSO-d₆ and D₂O mixture and signals were referenced to TMS to within ±0.01. Two dimensional Heteronuclear Single Quantum Coherence Transfer Spectra (2-D HSQCT) were recorded in magnitude mode with the sinus-

oidal shaped Z gradients of strength 25.7, 15.42 and 20.56 G cm^{-1} in the ratio of 5:3:4 applied for a duration of 1 ms each with a gradient recovery delay of 100 μs to defocus unwanted coherences which was incremented in 256 steps. The 2D data was accumulated on 4K size computer memory. The spectra were processed using unshifted and $\pi/4$ shifted sine bell window function in F$_1$ and F$_2$ dimensions respectively.

NMR data for L-alanyl-D-glucose esters: *6-O*-ester: ^{1}H NMR δ_{ppm}: (500.13 MHz) 3.35 (αCH), 1.54 (βCH$_3$, J $=$ 7.81), 4.8 (H-1β), 3.4 (H-4α), 3.25 (H-4β), 4.05 (H-6α), 3.84 (H-6β); ^{13}C NMR δ_{ppm}: (125 MHz) 53.3 (αCH), 16 (βCH$_3$), 175.4 (CO), 96.8 (C$_{1}\beta$), 71 (C$_{4}\alpha$), 65.4 (C$_{6}\beta$); *3-O*-ester ^{1}H δ_{ppm}: 3.9 (αCH), 4.2 (H-3α), 4.25 (H-3β); ^{13}C δ_{ppm}: 52.4 (αCH), 175.2 (CO), 84 (C$_{3}\alpha$), 84.1 (C$_{3}\beta$); *2-O*-ester ^{1}H δ_{ppm}: 3.79 (αCH), 4.05 (H-2α), 4.06 (H-2β); ^{13}C δ_{ppm}: 51.8 (αCH), 76.5 (C$_{2}\alpha$), 79.5 (C$_{2}\beta$); *2,6-di-O*-ester ^{1}H δ_{ppm}: 3.24 (H-2α), 3.75 (H-6α) ^{13}C δ_{ppm}: 75.7 (C$_{2}\alpha$), 62.1 (C$_{6}\alpha$); *3,6-di-O*-ester ^{1}H δ_{ppm}: 3.4 (H-3α), 3.76 (H-6α) ^{13}C δ_{ppm}: 77 (C$_{3}\alpha$), 59.9 (C$_{6}\alpha$); NMR data for L-leucyl-D-glucose esters: *6-O*-ester: ^{1}H δ_{ppm}: 3.08 (αCH), 2.51 (βCH$_{2a}$), 1.57 (γ-CH), 0.81 (δ, ε-CH$_3$), 3.86 (H-6α); ^{13}C δ_{ppm}: 53.5 (αCH), 36 (βCH$_2$), 25.3 (γCH), 22.5 (δ-CH$_3$), 23 (ε-CH$_3$), 173.6 (CO), 102.5 (C$_{1}\alpha$), 65 (C$_{6}\alpha$); *3-O*-ester ^{1}H δ_{ppm}: 3.15 (αCH), 1.5 (γ-CH), 0.77 (δ, ε-CH$_3$), 3.86 (H-2β), 3.97 (H-3α), 3.88 (H-3β); ^{13}C δ_{ppm}: 50 (αCH), 25 (γCH), 23.3 (δ-CH$_3$), 23.1 (ε-CH$_3$), 84 (C$_{3}\alpha$), 83.2 (C$_{3}\beta$); *2-O*-ester ^{1}H δ_{ppm}: 2.8 (αCH), 0.75 (δ, ε-CH$_3$), 3.79 (H-2α); ^{13}C δ_{ppm}: 46.1 (αCH), 23.4 (δ-CH$_3$), 23.9 (ε-CH$_3$), 76.9 (C$_{2}\alpha$); *2,6-di-O*-ester ^{1}H δ_{ppm}: 3.45 (H-2α), 3.44 (H-6α); ^{13}C δ_{ppm}: 75.5 (C$_{2}\alpha$), 62.8 (C$_{6}\alpha$); *3,6-di-O*-ester ^{1}H δ_{ppm}: 3.68 (H-3α), 3.45 (H-6α); ^{13}C δ_{ppm}: 82.5 (C$_{3}\alpha$), 63.1 (C$_{6}\alpha$); NMR data for L-phenylalanyl-D-glucose esters: *6-O*-ester ^{1}H δ_{ppm}: 2.66 (αCH, J $=$ 6.8,3.4), 2.89(βCH$_{2a}$); 2.76 (βCH$_{2b}$), aromatic-7.2 (H$_{2,6}$), 7.25 (H$_{3,5}$), 7.27 (H$_4$), 4.24 (H-1α), 3.47 (H-2α), 3.51 (H-2β), 3.55 (H-4α), 3.63 (H-5α), 3.72 (H-6α); ^{13}C δ_{ppm}: 51.85 (αCH), 36.91 (βCH$_2$), aromatic-137.92 (C$_1$), 126.23 (C$_2$, C$_6$), 128.07 (C$_3$, C$_5$), 129.17 (C$_4$), 174 (C$=$O), 102.46 (C$_{1}\alpha$), 69.57 (C$_{2}\alpha$), 69.56 (C$_{2}\beta$), 69.6 (C$_{4}\alpha$), 68.92 (C$_{5}\alpha$), 70.2 (C$_{5}\beta$), 62.97 (C$_{6}\alpha$, C$_{6}\beta$); *3-O*-ester ^{1}H δ_{ppm}: 2.63 (αCH), 3.12 (βCH$_{2a}$), 3.69 (H-3α), 3.81 (H-3β), 3.52 (H-6α); ^{13}C δ_{ppm}: 52.3 (αCH), 37.46 (βCH$_2$), aromatic-137.8 (C$_1$), 173.2 (C$=$O), 101.09 (C$_{1}\alpha$), 81.33 (C$_{3}\alpha$), 83.49 (C$_{3}\beta$), 60.93 (C$_{6}\alpha$); *2-O*-ester ^{1}H δ_{ppm}: 2.55 (αCH), 3.02 (βCH$_{2a}$), 3.8 (H-2α), 3.79 (H-2β), 3.38 (H-6α); ^{13}C δ_{ppm}: 52.8 (αCH), 37.91 (βCH$_2$), 172 (C$=$O), 96.72 (C$_{1}\alpha$), 75.1 (C$_{2}\alpha$),

Table 1. Effect of L-leucine concentration on the synthesis of L–leucyl-D-glucose[a].

Amount (equiv.)	Conversion yield[b] (%) with:	
	PPL	RML
1	17.8	0.2
2	3.6	6.6
3	8.7	6.0
4	6.2	8.2
5	24.4	36.0

[a]D-Glucose (1 mmol) was employed throughout. Temperature $-40\,^{\circ}$C; time 72 h; solvent-DMF/dichloromethane mixture (10: 90 v/v).
[b]Conversion yields correspond to conversion of L-leucine to L-leucyl-D-glucose ester with respect to D-glucose as measured by HPLC in presence of PPL (porcine pancreas lipase) and RML (*Rhizomucor miehei* lipase).

77.32 (C$_{2}\beta$), 62.8 (C$_{6}\alpha$); *2,6-di-O*-ester ^{1}H δ_{ppm}: 3.42 (H-6α), 3.63 (H-2α), 3.65 (H-2β); ^{13}C δ_{ppm} 76.21 (C$_{2}\alpha$, C$_{2}\beta$), 63.2 (C$_{6}\alpha$); *3,6-di-O*-ester ^{1}H δ_{ppm}: 3.51 (H-6α), 3.64 (H-3α), 3.54 (H-3β); ^{13}C δ_{ppm} 81.33 (C$_{3}\alpha$), 82.4 (C$_{3}\beta$), 63 (C$_{6}\alpha$).

Results

L-Leucyl-D-glucose ester

Esterification of D-glucose with L-leucine using RML and PPL was studied in detail.

Effect of enzyme concentration

Effect of increasing enzyme concentration on the synthesis of L-leucyl-D-glucose ester was studied by employing various RML concentrations ranging from 10% to 50% (w/w) D-glucose. The results showed that the yields obtained were very low. The enzyme showed the highest yield of 10.8% at 20% enzyme concentration. With the increase in enzyme concentration from 10% (5.7% yield) to 20% (10.8% yield) there was an increase in esterification and beyond 30% there was decrease (30%–0.2% yield, 50%–0.3% yield) in esterification probably due to hydrolysis of the esters formed.

Effect of L-leucine concentration

Effect of L-leucine concentrations at a fixed RML and PPL concentrations of 30% (w/w of D-glucose) on

Table 2. Lipase mediated synthesis of L-alanyl-D-glucose[a].

Amino acid (and amount as equiv.)	Enzyme (w/w of glucose used)	Solvent mixture (% by vol.)	Buffer salt	Conversion yield (%)
L-Alanine (2 eq)	RML (40%)	Chloroform+Hexane +DMF (45:45:10)	–	14.3
L-Alanine (2 eq)	RML (50%)	Chloroform+Hexane +DMF (45:45:10)	–	32.2
L-Alanine (1 eq)	RML (30%)	Chloroform+Hexane +DMF (45:45:10)	0.1 M, pH 4 0.1 ml	48
L-Alanine (3 eq)	RML (30%)	Chloroform+Hexane +DMF (45:45:10)	0.1 M, pH 4 0.1 ml	67.1
L-Alanine (4 eq)	RML (30%)	Chloroform+Hexane +DMF (45:45:10)	0.1 M, pH 4 0.1 ml	56.2
L-Alanine (2 eq)	PPL (30%)	Dichloromethane+DMF (90:10)	–	23.6
L-Alanine (2 eq)	PPL (40%)	Dichloromethane+DMF (90:10)	–	28
L-Alanine (2 eq)	PPL (50%)	Dichloromethane+DMF (90:10)	–	23.1
L-Alanine (2 eq)	PPL (40%)	Dichloromethane+DMF (90:10)	0.1 M, pH 5 0.1 ml	28.9
L-Alanine (2 eq)	PPL (40%)	Dichloromethane+DMF (90:10)	0.1 M, pH 6 0.1 ml	22.3
L-Alanine (2 eq)	PPL (40%)	Dichloromethane+DMF (90:10)	0.1 M, pH 7 0.1 ml	16

[a]Conversion yields from HPLC with respect to D-glucose. D-Glucose was used at 1 mmol throughout; temperature 40–60 °C; time 72 h.

the synthesis of L-leucyl-D-glucose ester was studied by increasing the L-leucine concentration from 1 to 5 molar eq (Table 1). The results showed that better conversions could be achieved with both the enzymes at 5 equiv of L-leucine.

Effect of buffer salts on esterification

Depending on the method of preparation, the enzyme always possesses a micro-aqueous layer around it. Buffer salts dissolve in such a micro aqueous layer and stabilize the enzyme structure against denaturation and subsequent loss of activity in non-polar solvents. A buffer of known pH and volume was added to the reaction mixture and their effect on imparting 'pH memory' to the enzyme was studied. Like some esterification reactions (Kiran *et al.* 2001), this reaction also gave better yields in presence of buffer salts.

Effect of buffer salts in terms of pH in the range 4 to 8 with RML at 40% (w/w) of D-glucose on the synthesis of L-leucyl-D-glucose ester at 1:2 molar equiv of D-glucose and L-leucine showed that 0.2 ml pH 5

buffer gave the highest yield of 63%. The yields were: 15.7% at pH 4, 16% at pH 6, 13.7% at pH 7 and 7.3% at pH 8.

The effect of various buffer volumes in the range 0.05 to 0.6 ml by using 0.1 M pH 7 phosphate buffer showed that the esterification yields increased with increase in the buffer volumes from 0.1 ml to 0.6 ml. The yields were: with 0.05 ml 5.2%, 0.1 ml 21.2%, 0.2 ml 29.2%, 0.4 ml 31.8% and 0.6 ml 85.2%. In general, the yields were better in the presence of buffer salts than those in their absence.

L-Alanyl-D-glucose ester

Esterification of D-glucose and L-alanine using RML and PPL was carried out. HPLC data showed that the total ester formation was in the range 14.3% to 67.1% (Table 2). The reactions were carried out both in presence and absence of buffer salts. In case of RML, the reaction gave the best conversion at 67% in presence of 0.1 ml 0.1 M acetate buffer, pH 4, with 3 equiv L-alanine (Table 2). In case of PPL, the reaction gave a

Table 3. Lipase mediated synthesis of L-phenylalanyl-D-glucose[a]

Amino acid (and amount as equiv.)	Enzyme (w/w of glucose used)	Solvent mixture (% by vol.)	Buffer salt	Conversion yield (%)
L-Phenylalanine (1 eq)	RML (10%)	Dichloromethane+DMF (90:10)	–	66.6
L-Phenylalanine (1 eq)	RML (40%)	Dichloromethane+DMF (90:10)	–	97.4
L-Phenylalanine (3 eq)	RML (30%)	Dichloromethane+DMF (90:10)	–	30.1
L-Phenylalanine (5 eq)	RML (30%)	Dichloromethane+DMF (90:10)	–	>99
L-Phenylalanine (1 eq)	RML (30%)	Chloroform+Hexane+DMF (45:45:10)	0.1 M, pH 4 0.2 ml	79.6
L-Phenylalanine (1 eq)	RML (30%)	Chloroform+Hexane+DMF (45:45:10)	0.1 M, pH 7 0.2 ml	76.2
L-Phenylalanine[b] (1 eq)	PPL (30%)	Dichloromethane+DMF (90:10)	–	58.3
L-Phenylalanine[b] (1 eq)	PPL (50%)	Dichloromethane+DMF (90:10)	–	69.7
L-Phenylalanine (1 eq)	PPL (30%)	Dichloromethane+DMF (90:10)	0.1 M, pH 4, 0.2 ml	57.5
L-Phenylalanine (1 eq)	PPL (30%)	Dichloromethane+DMF (90:10)	0.1 M, pH 6, 0.2 ml	87.4

[a]Conversion yields were from HPLC with respect to D-glucose. D-glucose was used at 1 mmol otherwise specified; [b]D-glucose and L-phenylalanine 2.5 mmol each; temperature 40–60 °C; incubation period 72 h.

yield of 29% in the presence of 0.1 ml 0.1 M acetate buffer, pH 5, with 2 equiv of L-alanine.

L-Phenylalanyl-D-glucose

Esterification of D-glucose with L-phenylalanine was carried out by using RML and PPL. HPLC data showed that the total ester formation of L-phenylalanyl-D-glucose was in the range 10.6 to >99% (Table 3).

RML at 40% (w/w) of glucose showed better esterification (97.4%) than 50% PPL (70%). In presence of buffer salts, 10% higher yields than the control were observed. 0.2 ml 0.1 M, pH 4, acetate buffer with RML at 30% (w/w) of glucose gave the highest yield of 80% and 0.2 ml 0.1 M phosphate buffer, pH 6, with PPL at 30% (w/w) of glucose gave the highest yield of 87%.

Discussion

HPLC data showed that the total ester formation in case of L-alanine, L-leucine and L-phenylalanine were 14% to 67%, 0.2 to 85% and 11 to >99%, respectively. Proton and ^{13}C signals from 2D HSQCT spectra, for C6 from *6-O-*, C3 from *3-O-* and C2 from *2-O-*monoesters and C2 and C6 from *2,6-di-O-* and C3 and C6 from *3,6-di-O-*esters clearly showed that as many as five esters were formed in this lipase catalyzed reaction in all the three above mentioned amino acids. Similar information could also be deduced from carbonyl and aromatic C1 signals of L-phenylalanyl-D-glucose ester. However, in case of L-alanyl-D-glucose and L-leucyl-D-glucose, the composition of monoesters and diesters were difficult to determine due to overlap of signals. In case of L-phenylalanyl-D-glucose, in an esterification yield of 81%, the composition of esters were found to be: *6-O-* 24.1% (62.9 ppm), *3-O-* 23.3% (60.9 ppm), *2-O-* 19.2% (62.8 ppm), *2,6-di-O-* 16.8% (63.2 ppm) and *3,6-di-O-* 16.6% (63 ppm). Although, the D-glucose employed showed an α:β anomeric composition of 40:60, the α-anomer was esterified to a greater extent than the β-anomer in L-leucine (26.1:14.8 in an esterification yield of 41%) and to almost equal extent in case of L-phenylalanine (32.25:32.25 in an esteri-

fication yield of 80%). The anomeric proton signals of the L-alanyl ester could not be clearly discerned due to overlap of the solvent HDO signal. Since very mild conditions were employed, the NMR data showed <3% peptide formation. The peptide formation was still very much less (<3%) in L-alanine and L-phenylalanine. All the three amino acids gave better yields when the reactions were carried out in the presence of buffer salts.

Even though free unprotected L-amino acids were employed in the present work, comparable yields to other researchers (whose reactions were carried out in shake flasks) were observed. This could be due to the experimental set up employed in the present work which facilitated excellent conversions even with PPL and RML which were reported to be less effective catalysts in converting N-protected and carboxyl activated L-amino acids in shake flasks. Even with protected and activated amino acids, earlier works, have yielded only a mixture of *6-O-, 4-O-, 3-O-* and *2-O-*amino acyl esters (Riva *et al.* 1988, Therisod & Klibanov 1986, Park *et al.* 1999, Boyer *et al.* 2001, Maruyama *et al.* 2002). Hence, the present work has shown the effectiveness of PPL and RML in the synthesis of *6-O-, 3-O-* and *2-O-*monoesters and *2,6-di-O-* and *3,6-di-O-*esters from unprotected amino acids in substantial yields.

Acknowledgements

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STRUCTURE, FUNCTION AND INTERACTIONS

Angiotensin converting enzyme inhibitory activity of amino acid esters of carbohydrates

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Abstract

L-Alanyl-D-glucose, L-valyl-D-glucose, L-phenylalanyl-D-glucose and L-phenylalanyl-lactose esters were synthesized enzymatically using two lipases viz., *Rhizomucor miehei* lipase (RML) and porcine pancreas lipase (PPL) and tested for their potential as inhibitors of angiotensin converting enzyme (ACE) in vitro. The esters exhibited concentration related ACE inhibitory activity. The potency of the various esters measured in terms of IC_{50} values were as follows: L-phenylalanyl-D-glucose, IC_{50}-0.121 mM (mixture of five diastereomeric esters: 6-*O*-24.1%; 3-*O*-23.3%; 2-*O*-19.2%; 2,6-di-*O*-16.6% and 3,6-di-*O*-16.8% from the total yield of 92.4%); L-phenylalanyl-lactose, IC_{50}-0.229 mM (mixture of three diastereomeric esters: 6-*O*-42.1%; 6'-*O*-30.9%; and 6,6'-di-*O*-27.0% from the total yield of 50.58%); alanyl-D-glucose, IC_{50}-0.23 mM (mixture of five diastereomeric esters: 6-*O*-46.7%; 3-*O*-11.5%; 2-*O*-19.9%; 2,6-di-*O*-6.6% and 3,6-di-*O*-15.3% from the total yield of 26.5%) and L-valyl-D-glucose, IC_{50}-0.396 mM (mixture of five diastereomeric esters: 6-*O*-32.4%; 3-*O*-26.5%; 2-*O*-26.4%; 2,6-di-*O*-8.8% and 3,6-di-*O*-5.9% from the total yield of 68.2%). These in vitro data suggest a potential therapeutic role for the aminoesters of carbohydrates as inhibitors of ACE.
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Keywords: Angiotensin converting enzyme inhibitor; L-Alanyl-D-glucose; L-Valyl-D-glucose; L-Phenylalanyl-D-glucose and L-phenylalanyl-lactose; Hypertension

1. Introduction

Hypertension is a leading cause of health concern all over the world. Renin angiotensin system (RAS) plays a major role in the development of hypertension and angiotensin converting enzyme (ACE; EC.3.4.15.1) constitutes a key component in this system. ACE raises blood pressure by converting angiotensin-I, released from angiotensinogen by renin, into the potent vasoconstrictor angiotensin-II. ACE also degrades vasodilative bradykinin in blood vessels and stimulates the release of aldosterone in the adrenal cortex. Increased ACE activity has been linked to narrowing of lumen of blood vessels, which results in increased blood pressure [1]. Inhibition of ACE is considered to be an important therapeutic approach for controlling hypertension. ACE inhibitors (viz., captopril, enalapril, fosinopril and ramepril) currently available in the market, exert antihypertensive effect by competitively binding to the active site of ACE [2]. However, some of the currently used ACE inhibitors have certain limitations like susceptibility to proteolytic degradation leading to side effects such as brochospasm and dry cough [3]. As a result, there have been renewed efforts in modifying available drugs or in developing new drugs with lesser side effects.

During the past decades, several fundamental studies have led to the search for ACE inhibitors from natural resources. Certain functional foods containing ACE inhibitory compounds have been found to act as alternatives for treatment of hypertension. Most of the food-derived ACE inhibitors are peptidic in nature [4]. Only a few non-peptidic compounds have been reported to possess the ACE inhibitory activity [5]. Park et al. [6] recently discussed the effectiveness of chitooligosaccharides for ACE inhibition while Huang et al. [7] have reported improved ACE inhibitory activity of chitooligosaccharides by carboxyl modification. Interestingly, the existence of carbohydrate-binding center on ACE has been proposed recently [8,9]. While many carbohydrate-peptide conjugates are reported to display a wide variety of potent biological activities of therapeutic value [10–12], use of amino acid esters of carbohydrates as inhibitors of ACE has not been reported so far. The main objective of

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the present investigation was to enzymatically synthesize amino acid esters of carbohydrates and to determine their potential as ACE inhibitors in vitro.

2. Experimental

2.1. General methods

Rhizomucor miehei lipase (RML) immobilized on a weak anion resin was purchased from Nova Nordisk Denmark, porcine pancreas lipase (PPL) type II, steapsin and *Candida rugosa* lipase (CRL) were purchased from Sigma Chemical Co., USA. Esterification activities for RML, PPL and CRL were found be 0.46, 0.06 and 0.03 μmol/min/mg of enzyme, respectively [13]. L-Amino acids were procured from Hi-Media (India), D-glucose was purchased from SD fine chemicals (India) and lactose was obtained from SISCO Research laboratories (India). Sephadex G-25 was purchased from Pharmacia, Sweden. All solvents used were of analytical grade and were procured from Qualigens Fine Chemicals (India).

2.2. Synthesis of amino acid esters of carbohydrates

Esterification was carried out as reported earlier [14] in presence of RML in case of L-alanyl-D-glucose and L-phenylalanyl-D-glucose, CRL in case of L-valyl-D-glucose and PPL in case of L-phenylalanyl-lactose (40% enzyme based on w/w of D–glucose/lactose) by refluxing with stirring 0.005 mol of amino acid (L-alanine, L-valine, L-phenylalanine) and 0.005 mol of D-glucose or 0.0025 mol of lactose in a 100 mL solvent mixture consisting of CH_2Cl_2:DMF (90:10 v/v) or hexane:$CHCl_3$:DMF (45:45:10 v/v) in two necked flat bottom flask for 3 days at 40 °C (boiling point of the dichloromethane). The enzyme was imparted with 'pH memory' by employing: 0.1 mL Na_2HPO_4 buffer (0.1 mM, pH 7.0) for L-valyl-D-glucose, 0.1 mL CH_3COONa buffer (0.1 mM, pH 4.0) for L-alanyl-D-glucose, 0.2 mL CH_3COONa buffer (0.2 mM, pH 4.0) for L-phenylalanyl-D-glucose and L-phenylalanyl-lactose. To facilitate complete removal of water from the reaction mixture, a very low water activity of $a_w = 0.0054$ was achieved by condensing azeotropic solvent vapor containing small amount of water of reaction into a desiccant which was then drained back into the reaction mixture [14–17]. The same reactions were carried out in the absence of lipase, which did not show any esterification. After completion of the reaction, the solvent was distilled off and then stirred with 20 mL of water and filtered to remove the lipase. The filtrate containing unreacted substrates and product esters was evaporated to dryness on a water bath and analyzed by HPLC employing a C-18 column or an aminopropyl column [14,17]. Conversion yields were determined from HPLC peak areas of the ester and L-amino acid and expressed as percentage esterification with respect to the L-amino acid concentration. L-Alanyl-D-glucose and L-phenylalanyl-D-glucose were separated from the reaction mixture by repeated HPLC injections and L-valyl-D-glucose and L-phenylalanyl-lactose separated by size exclusion chromatography using Sephadex G-25 with water as eluant. All the esters were characterized by UV, IR and two-dimensional Heteronuclear Single Quantum Coherence Transfer (2-D-HSQCT) NMR spectra. Two-dimensional HSQCT-NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer at 40 °C by dissolving 40 mg of the sample in DMSO-d_6.

2.3. ACE inhibitory assay

2.3.1. Preparation of ACE

ACE was prepared from porcine lung acetone powder by following the standard procedure [18]. Fresh pig lung was homogenized with 5–10 volumes of cold acetone in a waring blender. The homogenate was filtered and the residue was dried to remove the acetone. The dried lung acetone powder was stored at −20 °C and used for ACE extraction. ACE was extracted from the acetone powder as follows: the acetone powder was suspended in 10 volumes of Tris–HCl buffer (125 mM, pH 8.3) containing 1 M NaCl and homogenized. The homogenate was stirred overnight at 4 °C, and then centrifuged at 30,000 × g, for 20 min at 4 °C. The supernatant (the enzyme) was separated and was stored at −20 °C.

2.3.2. Determination of ACE activity

ACE activity was determined spectrophotometrically by a modification of the method of Cushman and Cheung [19] using Hippuryl-histidyl-leucine as the substrate. Twenty-five millimole HHL and appropriate quantity of ACE inhibitor were dissolved in Tris–HCl buffer (125 mM, pH 8.3) and incubated with the purified ACE for 60 min at 37 °C. The reaction was terminated by addition of 500 μl of 1 N HCl. The hippuric acid formed by the action of ACE was extracted into 1500 μl of ethyl acetate. After centrifugation at 800 × g for 10 min, 1000 μl of supernatant was transferred to a separate clean dry test tube and was evaporated to dryness at 80 °C. The residue was dissolved in 1.0 ml of distilled water and the absorbance was measured at 228 nm against distilled water. Concentration-dependent activities of inhibitors were studied and the concentration required to inhibit 50% of ACE activity was defined as the IC_{50} value.

3. Results and discussion

3.1. Chemistry

Two-dimensional heteronuclear single/multiple quantum coherence transfer (HMQCT/HSQCT) NMR data confirmed that three diasteriomeric mono esters (2-*O*-, 3-*O*- and 6-*O*-esters) and two diasteriomeric diesters (2,6-di-*O*- and 3,6-di-*O*-esters) were formed in various proportions in case of L-valyl and L-phenylalanyl esters of D-glucose and two diasteriomeric mono esters (6-*O*- and 6′-*O*-esters) and one diasteriomeric diester (6,6′-di-*O*-ester) were formed in case of L-phenylalanyl-lactose (Table 1). In case of L-alanyl-D-glucose only β-D-glucose was found to have reacted to give three mono and two di esters (Fig. 1). The composition of various esters were as follows: L-alanyl-D-glucose, mixture of five diastereomeric esters:-6-*O*-46.7%; 3-*O*-11.5%; 2-*O*-19.9%; 2,6-di-*O*-6.6% and 3,6-di-*O*-15.3% from the total yield of 26.5%; L-valyl-D-glucose, mixture of five diastereomeric

Table 1
Composition of mono and diesters of amino acyl-D-glucose esters and their average molecular weights[a]

Ester	6-O-Ester (%)	3-O-Ester (%)	2-O-Ester (%)	3,6-di-O-Ester (%)	2,6-di-O-Ester (%)	6'-O-Ester (%)	6,6'-di-O-Ester (%)	Average molecular weight
L-Alanyl-D-glucose[b]	46.7	11.5	19.9	15.3	6.6	–	–	266.5
L-Valyl-D-glucose[c]	32.4	26.5	26.4	8.8	5.9	–	–	293.5
L-Phenylalanyl–D-glucose[b]	24.1	23.3	19.2	16.8	16.6	–	–	376.3
L-Phenylalanyl-lactose[c]	42.1	–	–	–	–	30.9	27.0	528.7

[a] Percentage compositions of all five esters as determined from ^{13}C peaks of C-6 of esters.
[b] L-Alanyl-D-glucose and L-phenylalanyl-D-glucose were separated from the reaction mixture by repeated HPLC injections to obtain yields of 26.5% and 92.4%, respectively.
[c] L-Valyl-D-glucose and L-phenylalanyl-lactose were separated using sephadex G-25 to obtain yields of 68.2% and 50.58% respectively.

esters:-6-O-32.4%; 3-O-26.5%; 2-O-26.4%; 2,6-di-O-8.8% and 3,6-di-O-5.9% from the total yield of 68.2%; L-phenylalanyl-D-glucose mixture of five diastereomeric esters:-6-O-24.1%; 3-O-23.3%; 2-O-19.2%; 2,6-di-O-16.6% and 3,6-di-O-16.8% from the total yield of 92.4% and L-phenylalanyl-lactose mixture of three diastereomeric esters:-6-O-42.1%; 6'-O-30.9%; and 6,6'-di-O-27.0% from the total yield of 50.58%.

3.1.1. L-Alanyl-D-glucose esters

UV: 294.0 nm (log ε − 2.883), 227.0 nm (log ε − 3.061); IR: 3371 cm^{-1} (NH), 3410 cm^{-1} (OH), 2997 cm^{-1} (CH) and 1653 cm^{-1} (C=O); specific rotation-$[α]_D$ at 25 °C = −38.14°; CMC−2.25 mM (0.056%).

2-O-Ester: ^1H NMR $δ_{ppm}$: (500.13 MHz): 2.95 (αCH), 1.30 (βCH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6β); ^{13}C NMR $δ_{ppm}$ (125 MHz): 52.1(αCH), 15.7(βCH$_3$), 100.2 (C$_1$β), 82.6 (C$_2$β), 77.9 (C$_3$β), 68.8 (C$_4$β), 60.5 (C$_6$β). 3-O-Ester: ^1H NMR $δ_{ppm}$: 2.87(αCH), 3.93 (H-3β), 3.58 (H-4β), 3.82(H-6β); ^{13}C NMR $δ_{ppm}$: 51.4 (αCH), 83.3 (C$_3$β), 69.3 (C$_4$β), 63.5 (C$_3$β). 6-O-Ester: ^1H NMR $δ_{ppm}$ 2.95 (αCH), 1.30 (βCH$_3$), 3.86(H-2β), 3.4(H-4α), 3.76(H-5β), 3.36(H-6β); ^{13}C NMR $δ_{ppm}$: 50.2(αCH), 15.1(βCH$_3$), 171.4(CO), 101.8(C$_1$β), 65.4 (C$_6$β), 75.0 (C$_2$β), 70.1(C$_5$β), 57.3 (C$_6$β). 2,6-di-O-Ester: ^1H NMR $δ_{ppm}$: 3.36 (αCH), 1.30 (βCH$_3$), 3.78 (H-2β), 3.47 (H-6β), ^{13}C NMR $δ_{ppm}$: 49.5(αCH), 16.4 (βCH$_3$), 76.5 (C$_2$β), 62.7 (C$_6$β). 3,6-di-O-Ester: ^1H NMR $δ_{ppm}$: 1.30 (αCH), 3.78 (H-3β), 3.82 (H-6β) ^{13}C $δ_{ppm}$: 16.7 (αCH), 81.6 (C$_3$β), 63.1(C$_6$β).

3.1.2. L-Valyl-D-glucose esters

UV: 275.0 nm (log ε − 2.4), 227.0 nm (log ε − 2.65); IR: 3394 cm^{-1} (NH), 2985 cm^{-1} (OH), 2971 cm^{-1} (CH) and 1638 cm^{-1} (C=O); specific rotation-$[α]_D$ at 25 °C = +30.0°.

2-O-Ester: ^1H NMR $δ_{ppm}$ (500.13 MHz): 3.06 (αCH), 2.15 (βCH), 1.07 (γCH$_3$), 3.89 (H-2α), 3.75 (H-2β), 3.51 (H-6α); ^{13}C NMR $δ_{ppm}$ (125 MHz): 53.4 (αCH), 20.9(βCH), 9.51 (γCH$_3$), 76.1 (C$_2$α), 60.0 (C$_6$α). 3-O-Ester: ^1H NMR $δ_{ppm}$: 3.10 (αCH), 0.94 (γCH$_3$), 3.89 (H-3α), 4.01 (H-3β), 3.33 (H-6αβ); ^{13}C NMR $δ_{ppm}$: 52.4 (αCH), 9.39 (γCH$_3$), 82.9 (C$_3$α), 83.4 (C$_3$β), 60.3 (C$_6$αβ). 6-O-Ester: ^1H NMR $δ_{ppm}$: 3.20 (αCH), 2.01 (βCH), 0.90 (γ CH$_3$), 4.95 (H-1α), 4.22 (H-1β), 3.17 (H-4α), 3.0 (H-4β), 3.86 (H-6α); ^{13}C NMR $δ_{ppm}$: 51.9 (αCH), 21.0 (βCH), 8.94 (γ CH$_3$), 95.2 (C$_1$α), 104.5(C$_1$ β), 69.5 (C$_4$α), 69.8 (C$_4$β), 63.4 (C$_6$α). 2,6-di-O-Ester: ^1H NMR $δ_{ppm}$: 3.15 (αCH), 3.75 (H-2α), 3.64 (H-6β), ^{13}C NMR $δ_{ppm}$: 51.7 (αCH), 78.7 (C$_2$α), 61.6 (C$_6$α). 3,6-di-O-Ester: ^1H NMR $δ_{ppm}$: 3.21 (αCH), 1.55 (γ CH$_3$), 3.67 (H-3β), 3.15 (H-6αβ); ^{13}C NMR $δ_{ppm}$: 49.4 (αCH), 78.6 (C$_3$β), 61.3 (C$_6$αβ).

Fig. 1. Schematic representation for the lipase catalysed synthesis of L-amino acid esters of carbohydrates.

3.1.3. L-Phenylalanyl D-glucose esters

UV: 308.0 nm (log ε − 2.79), 257.5 nm (3.08) and 237.0 nm (log ε − 3.12); IR: 3642 cm^{-1} (NH), 3164 cm^{-1} (OH), 1722 cm^{-1} (C=O) and 1582 cm^{-1} (aromatic); specific rotation-[α]$_D$ at 25 °C = −24.2; CMC–3.25 mM (0.106%).

2-O-Ester: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.92(αCH), 2.51(βCH$_{2a}$), 4.6(H-1α), 3.67(H-2α), 3.4(H-6α); ^{13}C NMR δ_{ppm} (125 MHz): 52.0(αCH), 35.8(βCH$_2$), aromatic-136.5 (C$_1$), 96.3(C$_1\alpha$), 77.1(C$_2\alpha$), 62.0(C$_6\alpha$). 3-O-Ester: 1H NMR δ_{ppm}: 3.01(αCH), 3.11(βCH$_{2a}$), 2.96(βCH$_{2b}$), 4.4 (H-1α), 3.61 (H-2α), 3.66(H-2β), 3.82(H-3α), 3.91(H-3β), 3.40(H-6α); ^{13}C NMR δ_{ppm}: 53.0(αCH), 36.8(βCH$_2$), aromatic-136.4 (C$_1$), 97.3(C$_1\alpha$), 83.4(C$_3\alpha$), 83.9(C$_3\beta$), 61.9(C$_6\alpha$). 6-O-Ester: ^{1}H NMR δ_{ppm}: 3.07(αCH), 3.18(βCH$_{2a}$, J = 12.1); 3.06(βCH$_{2b}$, J = 12.1), aromatic-7.18 (H2,6, J = 3.5), 7.26(H3,5, J = 8.74), 7.16(H4), 3.16(H-5α), 3.78(H-6α), 3.66(H-6β); ^{13}C NMR δ_{ppm}: 54.2(αCH), 36.7(βCH$_2$) aromatic-136.3(C$_1$),128.9(C$_2$, C$_6$), 130.7(C$_3$, C$_5$), 130.3(C$_4$), 172.5(CO), 102.2(C$_1\alpha$), 70.5(C$_5\alpha$), 65.0(C$_6\alpha$, C$_6\beta$). 2,6-di-O-Ester: ^{1}H NMR δ_{ppm} 3.51(H-6α), 3.61(H-6β), 3.67(H-2α); ^{13}C NMR δ_{ppm} 77.0(C$_2\alpha$), 79.0(C$_2\beta$), 62.1(C$_6\beta$). 3,6-di-O-Ester: ^{1}H NMR δ_{ppm} 3.50(H-6α), 3.61(H-3α), 3.66(H-3β); ^{13}C NMR δ_{ppm} 82.3(C$_3\alpha$), 83.4(C$_3\beta$), 64.8(C$_6\alpha$).

3.1.4. L-Phenylalanyl-lactose

UV: 308.0 nm (log ε − 2.77), 258.0 nm (log ε − 3.00) and 237.5 nm (log ε − 3.48); IR: 3550 cm^{-1} (NH), 3383 cm^{-1} (OH), 1701 cm^{-1} (C=O) and 1556 cm^{-1} (aromatic).

6-O-Ester: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.67(αCH), 2.89(βCH$_{2a}$), aromatic-7.25 (H2,6), 7.28(H3,5), 7.2(H4), 4.32(H-1α), 4.23(H-1β), 3.36(H-2α), 3.49(H-2β), 3.32(H-3α), 3.80(H-3β), 3.56(H-4α), 3.72(H-4β), 3.90(H-5$\alpha\beta$), 3.47(H-6$\alpha\beta$), 4.16(H'-1β), 3.14(H'-2β), 3.42(H'-3β), 3.62(H'-4β), 3.39(H'-5β), 3.54(H'-6β); ^{13}C NMR δ_{ppm} (125 MHz): 51.9(αCH), 38.2(βCH$_2$) aromatic-138.0(C$_1$),128.3(C$_2$, C$_6$), 129.1(C$_3$, C$_5$), 126.1(C$_4$), 172.5(CO), 96.6(C$_1\alpha$), 101.2(C$_1\beta$), 70.5(C$_2\alpha$), 73.0(C$_2$ β), 74.0(C$_3\beta$), 82.5(C$_4\alpha$), 83.3(C$_4\beta$), 77.2(C$_5\alpha\beta$), 62.1(C$_6\alpha\beta$), 103.2(C$_1'\beta$), 69.9 (C$_2'$ β), 71.6(C$_3'\beta$), 68.2(C$_4'\beta$), 74.4(C$_5'\beta$), 60.5(C$_6'\beta$); 6'-O-Ester: ^{1}H NMR δ_{ppm}: 2.72(αCH), 3.00(βCH$_{2a}$), 5.16(H-1α), 4.32(H-1β), 3.16(H-2α), 3.45(H-2β), 3.43(H-3α), 3.50 (H-3β), 3.32(H-4α), 3.80(H-5$\alpha\beta$), 3.49(H-6$\alpha\beta$), 4.15(H'-1β), 3.36(H'-2β), 3.89(H'-4β), 3.89 (H'-5β), 3.45(H'-6β); ^{13}C NMR δ_{ppm}: 52.5(αCH), 37.1(βCH$_2$) aromatic-137.6(C$_1$), 92.0(C$_1\alpha$), 97.1(C$_1\beta$), 69.7(C$_2\alpha$), 72.7(C$_2\beta$), 72.1(C$_3\alpha$), 74.5(C$_3\beta$), 79.9(C$_4\alpha$), 80.7(C$_4\beta$), 76.0(C$_5\alpha\beta$), 61.0(C$_6\alpha\beta$), 104.0(C$_1'$ β), 69.3 (C$_2'$ β), 72.1(C$_3'$ β), 66.3(C$_4'$ β), 75.0(C$_5'$ β), 63.1(C$_6\beta$); 6,6'-di-O-Ester: ^{1}H NMR δ_{ppm}: 2.55(αCH), 2.89(βCH$_{2a}$), 4.32(H-1α), 3.23(H-2α), 3.68(H-2β), 3.33(H-3α), 4.02(H-4α), 4.01(H-5$\alpha\beta$), 3.45(H-6$\alpha\beta$), 4.21(H'-1β), 3.14(H'-2β), 3.58(H'-3β), 3.74(H'-4β), 3.45(H'-6β); ^{13}C NMR δ_{ppm}: 51.7(αCH), 37.9(βCH$_2$) aromatic-137.8(C$_1$), 95.6(C$_1\alpha$), 101.9(C$_1\beta$), 71.3(C$_2\alpha$), 73.2(C$_2$ β), 73.3(C$_3\alpha$), 74.7(C$_3\beta$), 82.2(C$_4\alpha$), 84.2(C$_4\beta$), 77.7(C$_5\alpha\beta$), 62.4(C$_6\alpha\beta$), 103.4(C$_1'$ β), 69.9 (C$_2'$ β), 72.4(C$_3'$ β), 67.4(C$_4'$ β), 74.7(C$_5'$ β), 62.7(C$_6\beta$).

Riva et al. [20] reported that three different mono esters of N-acetyl L-phenylalanyl-D-glucose were formed when reac-

Table 2
ACE inhibitory activities of the amino acid esters of carbohydrates

Ester	ACE inhibition IC$_{50}$, mM^a
L-Alanyl-D-Glucose	0.230 ± 0.02
L-Valyl-D-Glucose	0.396 ± 0.03
L-Phenylalanyl-D-Glucose	0.121 ± 0.01
L-Phenylalanyl-lactose	0.229 ± 0.01

^{a} Mean ± S.E. of three independent determinations. Respective L-alanine, L-valine, L-phenylalanine, D-glucose and lactose as controls showed no ACE inhibitory activity when tested at 0.2 mM.

tion was carried out in presence of subtilisin (2-O-, 3-O- and 6-O-mono esters). Tamura et al., [21] have reported synthesis of 2-O-and 3-O-mono esters of L-valyl-D-glucose chemically. Park et al. [22] have reported formation of three different esters of N-acetyl L-phenylalanyl-lactose (6-O-, 6'-O- and 6,6'-di-O-esters). The present work reports the synthesis of the 18 esters (mono and diesters) of which the following 10 esters have not been reported so far: 2-O-L-alanyl-D-glucose, 3-O-L-alanyl-D-glucose, 6-O-L-alanyl-D-glucose, 2,6-di-O-L-alanyl-D-glucose, 3,6-di-O-L-alanyl-D-glucose, 6-O-L-valyl-D-glucose, 2,6-di-O-L-valyl-D-glucose, 3,6-di-O-L-valyl-D-glucose, 2,6-di-O-L-phenylalanyl-D-glucose and 3,6-di-O-L-phenylalanyl-D-glucose. The average molecular weight of the esters in case of L-alanyl and L-phenylalanyl-D-glucose was evaluated from the percentage composition of all five esters determined from the C-6 peak area from ^{13}C spectra.

3.2. Biological evaluation

The synthesized amino acid esters of carbohydrates were evaluated for their ability to inhibit angiotensin-converting enzyme (ACE). All the amino acid esters (in the form of mixtures) showed moderate potency as inhibitors of ACE (Table 2, Fig. 2). While L-phenylalanyl-D-glucose showed highest ACE inhibitory activity with an IC$_{50}$ of 0.121 mM, L-valyl-D-glucose showed lowest ACE inhibitory activity with an IC$_{50}$ of 0.396 mM. The other two esters showed intermediate IC$_{50}$ value of 0.23 mM. Although D-glucose esters of L-alanine
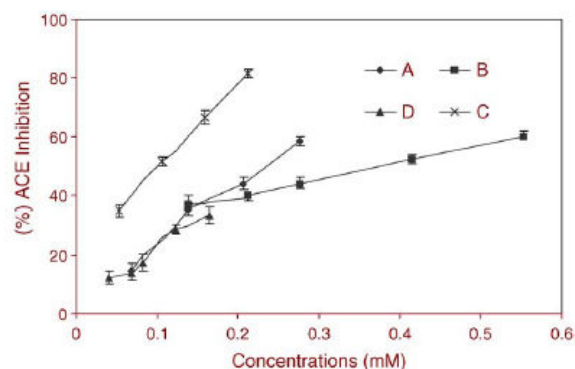

Fig. 2. ACE inhibitory patterns of L-amino acid esters of carbohydrates. Each value is a Mean ± S.E. of three determinations. ♦: L-alanyl-D-glucose; ■: L-valyl-D-glucose; ×: L-phenylalanyl-D-glucose; ▲: L-phenylalanyl-lactose.

Original article

Glycosides and amino acyl esters of carbohydrates as potent inhibitors of angiotensin converting enzyme

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Abstract

About 12 glycosides prepared through amyloglucosidase catalysis and 23 amino acyl esters of carbohydrates prepared through lipase catalysis in organic solvents showed angiotensin converting enzyme (ACE) inhibition activity. Both amino acyl esters of carbohydrates and glycosides exhibited IC_{50} values for ACE inhibition in the 0.5 mM to 15.7 mM range. Eugenyl-D-glucoside (IC_{50}: 0.5 ± 0.04 mM), L-isoleucyl-D-glucose (IC_{50}: 0.7 ± 0.067 mM), vanillyl-D-sorbitol (IC_{50}: 0.8 ± 0.09 mM), L-histidyl-D-fructose (IC_{50}: 0.9 ± 0.087 mM), L-tryptophanyl-D-fructose (IC_{50}: 0.9 ± 0.092 mM), octyl-D-glucoside (IC_{50}: 1.0 ± 0.093 mM), vanillyl-D-mannoside (IC_{50}: 1.0 ± 0.089 mM), L-valyl-D-mannitol (IC_{50}: 1.0 ± 0.092 mM) and L-phenylalanyl-D-glucose (IC_{50}: 1.0 ± 0.089 mM) were the compounds, which showed the best ACE inhibitory activities.
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Keywords: Glycosides; Amino acyl esters of carbohydrates; Enzymatic synthesis; ACE inhibition; IC_{50} values

1. Introduction

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc containing nonspecific dipeptidyl carboxypeptidase widely distributed in mammalian tissues [1]. Angiotensin-converting enzyme (ACE) regulates the blood pressure by modulating renin–angiotensin system [2]. This enzyme increases the blood pressure by converting the decapeptide angiotensin I into the potent vaso-constricting octapeptide, angiotensin II. Angiotensin II brings about several central effects, all leading to a further increase in blood pressure. ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin (blood pressure-lowering nanopeptide) and therefore inhibition of ACE results in an overall antihypertensive effect [1,3].

Several synthetic drugs and bio-molecules are available for ACE inhibition. Captopril is a successful synthetic anti-hypertensive drug and similar such molecules like enalapril, perindopril, ceranopril, ramipril, quinapril and fosinopril also show ACE inhibitory activities [4,5]. Some naturally occurring 'biologically active peptides' also act as ACE inhibitors [6–9].

Glycosides from the leaves of *Abeliophyllum distichum* like acteoside, isoacteoside, rutin, and hirsutrin moderately inhibited the Angiotensin I converting enzyme activity [10]. Glycosides like 3-*O*-methyl crenatoside from *Microtoena prainiana* also showed more than 30% ACE inhibitory activity [11]. Phenyl propanoid glycosides from *Clerodendron trichotomum* such as acteoside, leucosceptoside A, martynoside, aceteoside isomer and isomartynoside also showed ACE inhibitory effect [12].

Certain glycosides and amino acyl esters of carbohydrates are used widely in food and pharmaceutical applications as sweeteners, surfactants, microcapsules in pharmaceutical preparations, in delivery of biologically active agents such as antibiotics, nutraceuticals and antitumor agents [13–17]. However, they have not been shown to exhibit ACE inhibition activity so far. The present work deals with detection of ACE inhibition activities for some enzymatically synthesized glycosides using amyloglucosidase and amino acyl esters of carbohydrates using lipases in organic media. The results are presented here.

2. Results

A total of 35 compounds were tested for the inhibitory activities of ACE isolated from pig lung. General scheme for

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glycosylation and esterification are shown in Schemes 1 and 2. Enzymatic reactions were carried out under optimized conditions worked out for these reactions [18–21]. The enzymatic procedure employed unprotected and unactivated alcohols, phenols, amino acids and carbohydrates.
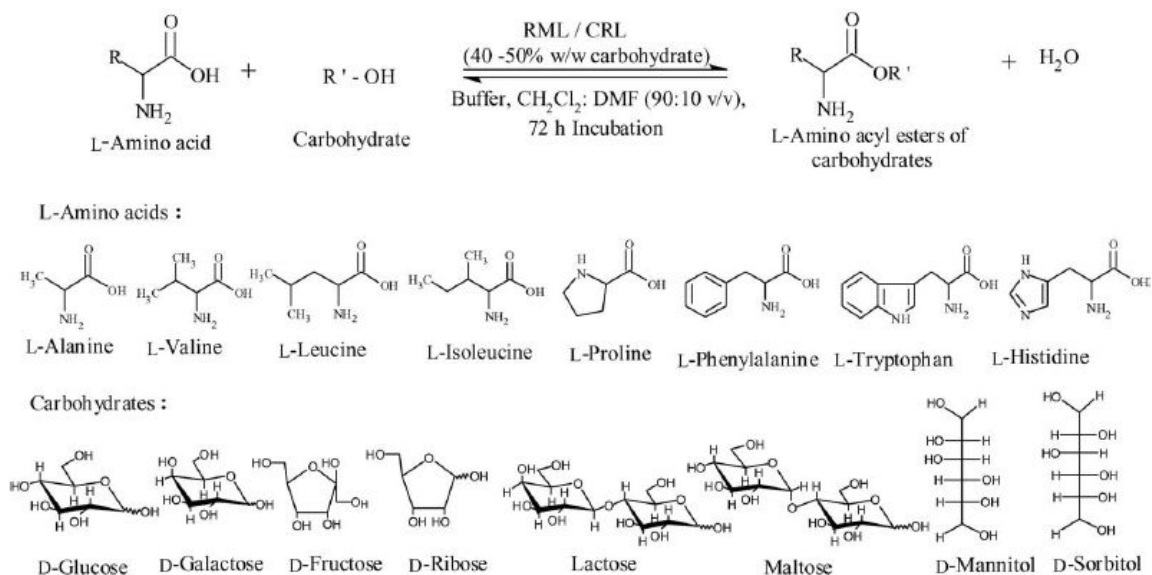
Typical ACE inhibition plots for eugenyl-D-glucoside, vanillyl-D-glucoside, L-isoleucyl-D-glucose and L-histidyl-D-fruc-

tose, are shown in Fig. 1. Tables 1 and 2, respectively, show the glycosides and amino acyl esters tested, their conversion yields from the respective enzymatic reactions, proportions of the products formed and ACE inhibitory activities for these compounds.

n-Octanol, guaiacol, eugenol, vanillin, curcumin and α-tocopherol glycosides of different carbohydrates (D-glucose, D-ga-
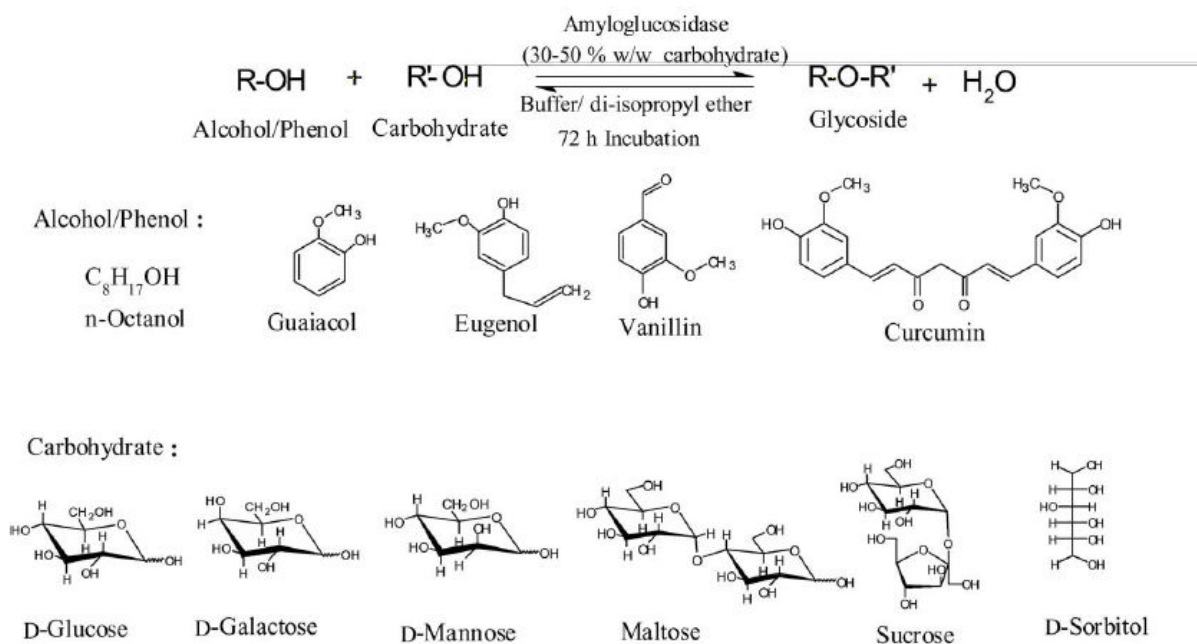
Scheme 1. Amyloglucosidase catalyzed synthesis of glycosides.

Scheme 2. Lipases catalyzed synthesis of L-amino acyl esters of carbohydrates.
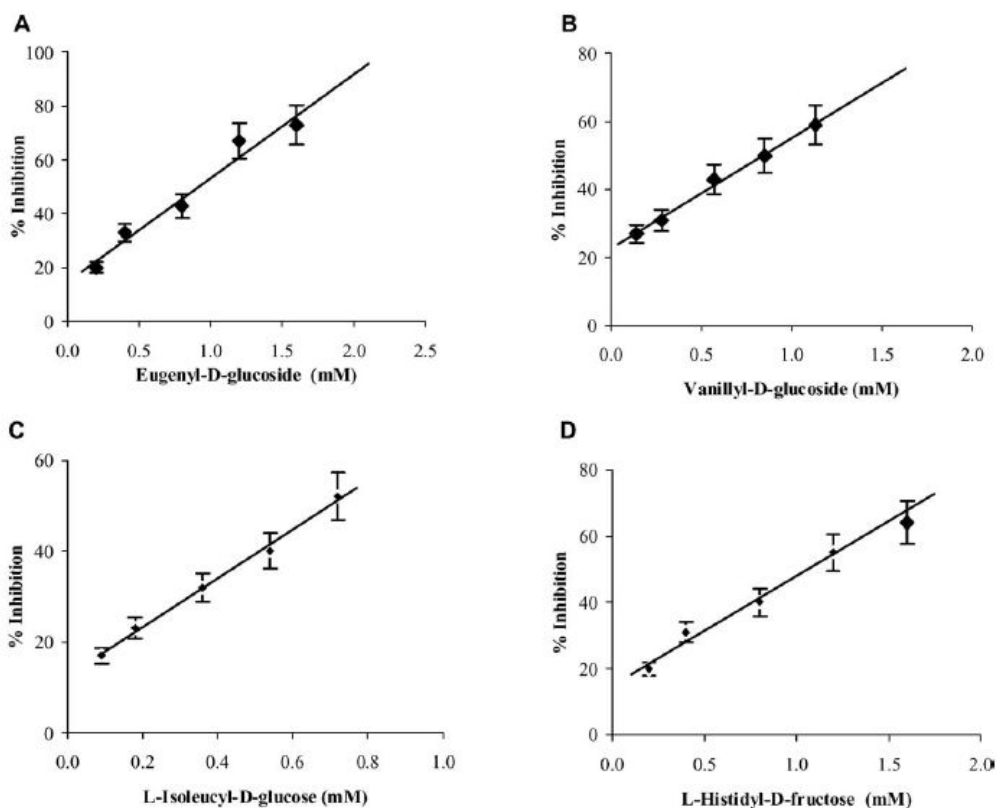
Fig. 1. **A.** Inhibition plot for eugenyl-D-glucoside, ACE: 0.1 ml (1.0 mg in 1.0 ml stock solution), Substrate: 0.1 ml hippuryl-histidyl-leucine (5 mM), Buffer: 100 mM phosphate buffer pH 8.3 contain 300 mM sodium chloride, Incubation period: 30 min, Temperature: 37 °C; **B.** Vanillyl-D-glucoside; **C.** L-Isoleucyl-D-glucose; **D.** L-Histidyl-D-fructose.

lactose, D-mannose, D-fructose, D-ribose, D-arabinose, maltose, sucrose, lactose, D-mannitol and D-sorbitol) were tested for ACE inhibition (Table 1). Among the carbohydrates employed, D-ribose, D-arabinose, D-fructose and lactose did not undergo any glycosylation.

Four amino acids containing alkyl side chains, three amino acids containing aromatic side chains and L-proline were employed for the preparation of esters. In case of aminoacyl esters, L-alanyl, L-valyl, L-leucyl, L-isoleucyl, L-prolyl, L-phenylalanyl, L-tryptophanyl and L-histidyl esters of D-glucose, D-galactose, D-fructose, D-ribose, lactose, maltose, D-sorbitol and D-mannitol were subjected to ACE inhibition activity studies (Table 2). ACE inhibition activity of the above-mentioned glycosides and amino acyl esters of carbohydrates were carried out by the Cushman and Cheung method [22]. Since hippuryl-L-histidyl-L-leucine (HHL) mimics the carboxyl dipeptide of angiotensin I, it has been routinely used as the substrate for screening ACE inhibitors.

Underivatized alcohols, phenols, L-amino acids and carbohydrates individually were also tested for ACE inhibition as controls and they did not show any ACE inhibitory activities. Only glycosides and esters showed activities. Isolated ACE inhibitor tested for lipase and protease activity (Table 3) showed

a small extent of protease activity (13.3%) compared to ACE activity but no lipase activity. In presence of glycosides and amino acids esters prepared, the isolated ACE showed 8.2% and 8.9% protease activity, respectively (Table 3) compared to the ACE activity. This confirmed that the ACE inhibition observed in the presence of glycosides and esters prepared is more due to ACE inhibition rather than protease inhibition.

The compounds were characterized by UV, IR, Mass and NMR (two-dimensional heteronuclear single quantum coherence transfer—2D-HSQCT) spectroscopic techniques. In case of glycoside synthesis, the major product was the glycosylated product and relatively lesser amounts of C6-O-alkylated or C6-O-arylated products were also detected. The reaction was between the alcohol or phenolic OH groups and the anomeric and/or primary C6-OH groups of the carbohydrates.

In case of esters, mono and di esters in different proportions were detected. Individual 1-O, 2-O, 3-O, 5-O, 6-O, and 6″-O monoesters and 1,6-di-O, 2,5-di-O, 2,6-di-O, 3,6-di-O, and 6,6″-di-O esters depending on the carbohydrate employed were found to be formed. It was not possible to separate the individual glycosides and esters from their reaction mixtures even by chromatographic separation on Sephadex G-10. Thus the activities described are for the mixture of these compounds.

Table 1
IC$_{50}$ values for ACE inhibition by glycosides [a]

Glycoside	Conversion Yield [b] (%)	Products formed (% proportions) [c]	IC$_{50}$ value (mM) [d]
n-Octyl-α/β-D-glucoside	46	*n*-Octyl-α-D-glucopyranoside (63)	1.0 ± 0.093
		n-Octyl-β-D-glucopyranoside (25)	
		C6-*O*-octyl-D-glucose (12)	
n-Octyl maltoside	22	*n*-Octyl maltoside	1.5 ± 0.13
n-Octyl sucrose	13	C1-*O*-octyl sucrose (44)	1.7 ± 0.15
		C6-*O*-octyl sucrose (56)	
Guaiacyl-α-D-glucoside	52	C1-*O*-guaiacyl-α-D-glucopyranoside (52)	3.7 ± 0.36
		C6-*O*-guaiacyl-α-D-glucose (48)	
Eugenyl-α-D-glucoside	32	C1-*O*-eugenyl-α-D-glucopyranoside (53)	0.5 ± 0.04
		C6-*O*-eugenyl-D-glucose (47)	
Vanillyl-α/β-D-glucoside	53	C1-*O*-vanillyl-α-D-glucopyranoside (52)	1.1 ± 0.10
		C1-*O*-vanillyl-β-D-glucopyranoside (17)	
		C6-*O*-vanillyl-D-glucose (31)	
Vanillyl-α-D-galactoside	18	C1-*O*-vanillyl-α-D-galactopyranoside	1.1 ± 0.13
Vanillyl-α-D-mannoside	13	C1-*O*-vanillyl-α-D-mannopyranoside	1.0 ± 0.089
Vanillyl maltoside	29	C1-*O*-vanillyl-α-maltopyranoside (42)	1.6 ± 0.14
		C6-*O*-vanillyl maltose (30)	
		C6″-*O*-vanillyl maltose (28)	
Vanillyl sucrose	23	C1-*O*-vanillyl sucrose (39)	15.7 ± 1.6
		C6″-*O*-vanillyl sucrose (61)	
Vanillyl-D-sorbitol	13	C1-*O*-vanillyl-D-sorbitol (13)	0.8 ± 0.09
		C6-*O*-vanillyl-D-sorbitol (25)	
		C1, C6 Di-*O*-vanillyl-D-sorbitol (62)	
Curcuminyl-bis-α-D-glucoside	48	C1-*O*-curcuminyl-bis-α-D-glucopyranoside (62)	1.5 ± 0.13
		C6-*O*-curcuminyl-bis-α-D-glucose (38)	

[a] Respective alcohols, phenols and carbohydrates as controls for ACE inhibition activity; non reducing sugar unit carbons of disaccharide are double primed;
[b] Conversion yields were from HPLC with errors in yield measurements ± 10%;
[c] Product proportions determined from ^{13}C 2D-HSQCT NMR C1/C6 peak areas or their cross peaks;
[d] IC$_{50}$ values compared to that of captopril 0.060 ± 0.005 mM determined by Cushman and Cheung method [22].

3. Discussion

Among the glycosides tested, eugenyl-D-glucoside (0.5 ± 0.04 mM), vanillyl-D-sorbitol (0.8 ± 0.09 mM), vanillyl-D-mannoside (1.0 ± 0.089 mM) and octyl-D-glucoside (1.0 ± 0.093 mM) exhibited the best ACE inhibitory activities (IC$_{50}$ ≤ 1.0 mM; Table 1). Among the carbohydrates employed glucosides showed the best ACE inhibitory activities. Alkyl glycosides showed better inhibitory activities than the phenolic glycosides. The glycosides also showed better ACE inhibitory activities than the amino acyl esters indicating higher potency of glycosides in ACE inhibition.

With increase in alkyl side chain branching, D-glucose esters of L-alanine (3.1 ± 0.30 mM), L-valine (6.0 ± 0.59 mM), L-leucine (2.8 ± 0.27 mM) and L-isoleucine (0.7 ± 0.067 mM) showed better inhibition (lesser IC$_{50}$ values), than the other esters, which could be directly correlated to increase in hydrophobicity (Table 2). IC$_{50}$ values ≤ 1.0 mM were detected for L-isoleucyl-D-glucose (0.7 ± 0.067 mM), L-histidyl-D-fructose (0.9 ± 0.087 mM), L-tryptophanyl-D-fructose (0.9 ± 0.092 mM), L-valyl-D-mannitol (1.0 ± 0.092 mM) and L-phenylalanyl-D-glucose (IC$_{50}$: 1.0 ± 0.089 mM). Among these, L-isoleucyl-D-glucose (0.7 ± 0.067 mM) was found to exhibit the best inhibitory activity. In general, aromatic amino acid esters showed comparatively higher IC$_{50}$ values than amino acids containing alkyl side chains, emphasizing that the latter were better inhibitors of ACE. Among the carbohydrates employed, D-mannitol esters showed better ACE inhibition (Ta-

ble 2) than the other carbohydrate esters. In case of L-prolyl esters, IC$_{50}$ values in the 1.6–4.4 mM range matched with those of alkyl side chain containing amino acid esters like L-alanine, L-valine, L-leucine and L-isoleucine (Table 2). Captopril is N-[(S)-3-mercapto-2-methylpropionyl]-L-proline containing prolyl unit as essential for ACE inhibition [4]. However, mere presence of a prolyl unit in the esters prepared does not give rise to a high level of ACE inhibition. Since, it was difficult to separate the individual esters, the actual potency of the individual esters could not be unequivocally established in the present work.

The present work for the first time has shown the ACE inhibitory potency of the above-mentioned glycosides and esters prepared enzymatically. Since milder reaction conditions were employed, the products formation did not suffer due to side reactions. However, the glycosides and esters tested in the present work, overall, clearly possess groups like alkyl side chains and phenolic groups which can be accommodated in the hydrophobic S$_1$ and S$_2$ subsites of angiotensin I converting enzyme [4,23]. The free amino group in the amino acid esters can also serve as good ligands for Zn^{2+} in the ACE active site. Carbohydrates in glycosides and esters could also bind to the hydrophobic and/or hydrophilic subsites of angiotensin I converting enzyme, as they possess both hydrophobic and hydrophilic groups in their structure. Although the ACE preparation of the present work from pig lung is ACE I [24], it showed a low protease inhibitory activity but no lipase activity indicating that the glycosides and esters inhibit ACE rather than the pro-

Table 2
IC_{50} values for ACE inhibition by L-aminoacyl esters of carbohydrates[a]

L-Amino acyl ester of carbohydrates	Conversion Yield (%)[b]	Products (% proportion)[c]	IC_{50} value (mM)[d]
L-Alanyl-β-D-glucose	30	2-O-L-alanyl-β-D-glucose (47)	3.1 ± 0.30
		3-O-L-alanyl-β-D-glucose (12)	
		6-O-L-alanyl-β-D-glucose (20)	
		2,6-di-O-L-alanyl-β-D-glucose (15)	
		3,6-di-O-L-alanyl-β-D-glucose (6)	
L-Alanyl-D-ribose	48	3-O-L-alanyl-D-ribose (16)	2.7 ± 0.25
		5-O-L-alanyl-D-ribose (32)	
		3,5-di-O-L-alanyl-D-ribose (52)	
L-Alanyl-lactose	20	6-O-L-alanyl-lactose (34)	2.0 ± 0.21
		6″-O-L-alanyl-lactose (34)	
		6,6″-di-O-L-alanyl-lactose (32)	
L-Valyl-D-glucose	84	2-O-L-valyl-D-glucose (26)	6.0 ± 0.59
		3-O-L-valyl-D-glucose (26)	
		6-O-L-valyl-D-glucose (33)	
		2,6-di-O-L-valyl-D-glucose (9)	
		3,6-di-O-L-valyl-D-glucose (6)	
L-Valyl-D-fructose	34	1-O-L-valyl-D-fructose	2.8 ± 0.29
L-Valyl-maltose	42	6-O-L-valyl-maltose (35)	3.1 ± 0.33
		6″-O-L-valyl-maltose (36)	
		6,6″-di-O-L-valyl-maltose (29)	
L-Valyl-D-mannitol	56	1-O-L-valyl-D-mannitol	1.0 ± 0.092
L-Leucyl-D-glucose	43	2-O-L-leucyl-D-glucose (17)	2.8 ± 0.27
		3-O-L-leucyl-D-glucose (20)	
		6-O-L-leucyl-D-glucose (48)	
		2,6-di-O-L-leucyl-D-glucose (8)	
		3,6-di-O-L-leucyl-D-glucose (7)	
L-Isoleucyl-D-glucose	47	3-O-L-isoleucyl-D-glucose (42)	0.7 ± 0.067
		6-O-L-isoleucyl-D-glucose (58)	
L-Prolyl-D-glucose	62	2-O-L-prolyl-D-glucose (26)	1.7 ± 0.19
		3-O-L-prolyl-D-glucose (26)	
		6-O-L-prolyl-D-glucose (48)	
L-Prolyl-D-fructose	61	1-O-L-prolyl-D-fructose (31)	4.4 ± 0.42
		6-O-L-prolyl-D-fructose (42)	
		1,6-di-O-L-prolyl-D-fructose (27)	
L-Prolyl-D-ribose	41	3-O-L-prolyl-D-ribose (35)	2.0 ± 0.18
		5-O-L-prolyl-D-ribose (65)	
L-Prolyl-lactose	68	6-O-L-prolyl-lactose (58)	1.6 ± 0.15
		6″-O-L-prolyl-lactose (42)	
L-Phenylalanyl-D-glucose	79	2-O-L-phenylalanyl-D-glucose (19)	1.0 ± 0.089
		3-O-L-phenylalanyl-D-glucose (23)	
		6-O-L-phenylalanyl-D-glucose (25)	
		2,6-di-O-L-phenylalanyl-D-glucose (17)	
		3,6-di-O-L-phenylalanyl-D-glucose (16)	
L-Phenylalanyl-D-galactose	45	2-O-L-phenylalanyl-D-galactose (32)	4.6 ± 0.45
		3-O-L-phenylalanyl-D-galactose (20)	
		6-O-L-phenylalanyl-D-galactose (19)	
		2,6-di-O-L-phenylalanyl-D-galactose (16)	
		3,6-di-O-L-phenylalanyl-D-galactose (13)	
L-Phenylalanyl-D-fructose	50	1-O-L-phenylalanyl-D-fructose (72)	13.6 ± 1.43
		6-O-L-phenylalanyl-D-fructose (28)	
L-Phenylalanyl-lactose	61	6-O-L-phenylalanyl-lactose (42)	7.8 ± 0.77
		6″-O-L-phenylalanyl-lactose (31)	
		6,6″-di-O-L-phenylalanyl-lactose (27)	
L-Phenylalanyl-D-mannitol	43	1-O-L-phenylalanyl-D-mannitol (62)	2.6 ± 0.25
		1,6-di-O-L-phenylalanyl-D-mannitol (38)	
L-Tryptophanyl-D-glucose	42	2-O-L-tryptophanyl-D-glucose (22)	7.4 ± 0.73
		3-O-L-tryptophanyl-D-glucose (21)	
		6-O-L-tryptophanyl-D-glucose (38)	
		2,6-di-O-L-tryptophanyl-D-glucose (10)	
		3,6-di-O-L-tryptophanyl-D-glucose (9)	
L-Tryptophanyl-D-fructose	18	1-O-L-tryptophanyl-D-fructose (45)	0.9 ± 0.092
		6-O-L-tryptophanyl-D-fructose (55)	

Table 2 *(continued)*

L-Amino acyl ester of carbohydrates	Conversion Yield (%)[b]	Products (% proportion)[c]	IC$_{50}$ value (mM)[d]
L-Histidyl-D-glucose	42	2-O-L-histidyl-D-glucose (25)	3.5 ± 0.34
		3-O-L-histidyl-D-glucose (24)	
		6-O-L-histidyl-D-glucose (28)	
		2,6-di-O-L-histidyl-D-glucose (12)	
		3,6-di-O-L-histidyl-D-glucose (11)	
L-Histidyl-D-fructose	58	6-O-L-histidyl-D-fructose	0.9 ± 0.087
L-Histidyl-D-mannitol	8	1-O-L-histidyl-D-mannitol	1.7 ± 0.16

[a] Respective amino acids and carbohydrates as controls for ACE inhibition activity;
[b] Conversion yields were from HPLC with errors in HPLC yield measurements ± 10%;
[c] Product proportions determined from ^{13}C 2D-HSQCT NMR C6 peak areas or their cross peaks, C5 cross peaks in case of ribose;
[d] IC$_{50}$ values compared to that of captopril 0.060 ± 0.006 mM determined by Cushman and Cheung method [22].

Table 3
Protease inhibition assay for eugenyl-D-glucosides and L-isoleucyl-D-glucose[a]

System	Protease activity Unit min^{-1}mg^{-1} enzyme protein[b]	Percentage of protease activity with respect to ACE activity[c]
Control ACE- 0.5 ml + 0.5 ml of 0.6% hemoglobin + 0.5 ml Buffer	0.0436	13.3
Eugenyl-D-glucoside: 0.5 ml glycoside + ACE - 0.5 ml + 0.5 ml of 0.6% hemoglobin	0.0292	8.2
L-Isoleucyl-D-glucose: 0.5 ml ester + ACE -0.5 ml + 0.5 ml of 0.6% hemoglobin	0.0267	8.9

[a] Conditions: ACE: 0.5 ml (0.5 mg), All the solutions were prepared in 0.1 M pH 7.5 Tris–HCl, incubation period: 30 min, temperature: 37 °C, 0.5 ml of 10% trichloroacetic acid added to arrest the reaction; Blank performed without enzyme and glycoside or ester; Absorbance measured at 440 nm; Eugenyl-D-glucoside and isoleucyl-D-glucose: 0.5 ml of 0.8 mM;
[b] Average absorbance values from three individual experiments;
[c] Percentage protease activity with respect to an ACE activity of 0.327 µmol min^{-1} mg^{-1} protein.

tease activity as protease inhibition activity can be construed to be ACE inhibition. The results indicate that both the glycosides and esters bind to these enzymes and hence hold promise as potential inhibitors for the ACE.

4. Experimental section

4.1. Enzymes

Lipozyme IM20 *Rhizomucor miehei* (RML) immobilized on weak anion exchange resin from Novo Nordisk, Denmark, *Candida rugosa* lipase (CRL) from Sigma Chemical Co., USA, for the esterification reactions and amyloglucosidase from *Rhizopus* sp. purchased from M/S Sigma Chemical. Co., MO, USA, for the glycosylation reactions, were used in the present work. RML and CRL employed showed esterification activities of 0.46 and 0.03 µmol min^{-1} mg^{-1} enzyme preparation, respectively [25]. Amyloglucosidase activity determined by the Sumner and Sisler method [26] was found to be 4.7 µmol min^{-1} mg^{-1} protein.

ACE was extracted from pig lung using the method described by Andujar-Sanchez et al. [24]. A 100 g of pig lung was minced and homogenized using a blender with 10 mM, pH 7.0, HEPES buffer containing 0.4 M NaCl at a volume ratio of 5:1 (v/w of pig lung) at 4 °C throughout the procedure. The homogenate was centrifuged at 9000 × g for 60 min. The supernatant was discarded and the precipitate was washed twice with 200 ml of 10 mM, pH 7.0, HEPES buffer containing 0.4 M NaCl. The final precipitate was resuspended in 200 ml of 10 mM, pH 7.0, HEPES buffer containing, 0.4 M NaCl, 10 µM ZnCl$_2$, 0.5% (w/v) Triton X100 and stirred over

night. The solution was centrifuged to remove the pellets. The supernatant was dialyzed against water and later lyophilized. The protein content of ACE determined by Lowry's method [27] was found to be 8.3%. The remaining is constituted by 17% carbohydrate, lipid and other solid tissue [5,28].

4.2. Chemicals and reagents

L-Amino acids, guaiacol, D-galactose, D-fructose, gum acacia and Tris–HCl from HiMedia (Ind.) Ltd.; D-glucose, sucrose, ZnCl$_2$, trichloroacetic acid, NaOH and NaCl from SD fine chemicals (Ind.) Ltd.; maltose, hippuryl-L-histidyl-L-leucine, bovine hemoglobin and tributyrin from Sigma Chemical Co. USA; D-mannose, D-arabinose, D-ribose, D-mannitol, eugenol, sodium dodecyl sulfate, sodium benzoate, Triton X-100 and hippuric acid from LOBA Chemie Pvt. Ltd. India; curcumin (purity > 95%) from Flavors and Essences Pvt. Ltd. India; HEPES buffer, lactose, *n*-octanol and vanillin (purity > 98%) from Sisco Research Laboratories Pvt. Ltd. India and D-sorbitol from Rolex Laboratory Reagent India Ltd., were employed as such. Sephadex G-10 from Sigma Chemical Co., USA was used. Solvents–CH$_2$Cl$_2$, dimethylformamide, di-isopropyl ether, petroleum ether, ethyl acetate and acetonitrile from SD Fine Chemicals (Ind.) Ltd. were employed after distilling once.

4.3. Glycosylation procedure

A general procedure for the glycosylation involved reaction in a 150 ml two necked flat bottom flask by refluxing phenols (1.0 mmol) and carbohydrate (1.0 mmol) in 100 ml di-isopropyl ether in presence of buffer and appropriate quantity of amyloglucosidase (30–50% w/w carbohydrate) for 72 h. In

case of *n*-octanol and guaiacol glycosides, carbohydrate and *n*-octanol/guaiacol were taken in 1: 50 molar ratio. Buffers employed were 0.4 ml to 1.0 ml of sodium acetate for pH 4.0 and 5.0, sodium phosphate for pH 6.0 and 7.0 and sodium borate for pH 8.0. After incubation, the reaction mixture was held in a boiling water bath for 5–10 min to denature the enzyme and 15–20 ml of water was added to dissolve the unreacted carbohydrate and the product glycoside. The unreacted alcohol was separated in a separating funnel with petroleum ether or *n*-hexane and phenols were separated by extracting with chloroform. The aqueous layer was evaporated to dryness to get the unreacted carbohydrate and the product glycoside. The reaction mixtures were analyzed by HPLC using an amino-propyl column (3.9 × 300 mm length) and acetonitrile: water in 80:20 ratio (v/v) as the mobile phase at a flow rate of 1 ml/min with refractive index detector. Conversion yields were determined from HPLC peak areas of the glycoside and free carbohydrate with respect to the free carbohydrate employed. Error in HPLC measurements will be ± 10%. The glycosides formed were separated through size exclusion chromatography using Sephadex G-10 eluting with water.

The isolated glycosides and esters were characterized by UV, IR, MS, 2D-NMR (HSQCT) and optical rotation, which provided good information on the nature and proportions of the products formed. A Shimadzu UV–1601 spectrophotometer was used for recording UV spectra of the isolated glycosides and esters in aqueous solutions at 0.2–1.0 mM concentration. A Nicolet 5700 FTIR instrument was used for recording the IR spectra with 1.0–3.0 mg of glycosides and ester samples as KBr pellet. Specific rotations of the isolated glycosides and esters were measured at 25 °C using Perkin-Elmer 243 polarimeter with 0.5% aqueous solution of the esters. Mass spectra of the isolated glycosides and esters were recorded using a Q-TOF Waters Ultima instrument (No. Q-Tof GAA 082, Waters corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz ^{13}C). Proton and carbon 90° pulse widths were 10.5 and 12.25 μs, respectively. About 40 mg of the sample dissolved in DMSO-d_6 and D_2O was used for recording the spectra at 35 °C. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard to within ± 0.01 ppm. 2D-HSQCT spectra were recorded for the glycosides and esters. In the NMR data, only resolvable signals are shown. Some assignments are interchangeable. *n*-Octanol/phenol signals are primed and signals from non reducing end units in maltose, lactose and sucrose are double primed. Since, the compounds are surfactant molecules, they appear to aggregate in the solvent and usually give broad signals, thus, making it difficult to resolve the coupling constant values accurately.

4.3.1. Glycosides

4.3.1.1. n-Octyl-α/β-D-glucoside.
Solid; UV (H$_2$O, λ_{max}): 206 nm (σ→σ*, ε_{206}–1230 M^{-1}), 278 nm (n→π*,

ε_{278}–72 M^{-1}). IR (KBr): 1053 cm^{-1} (glycosidic C-O-C symmetrical), 3605 cm^{-1} (OH). Optical rotation (*c* 1, H$_2$O) [α]$_D$ at 25 °C = +45.8°. MS (*m/z*) 294 [M + 2]$^+$, 317 [M + 2 + Na]$^+$. 2D-HSQCT (DMSO-d_6) *n-Octyl-α-D-glucopyranoside:* ^{1}H NMR δ$_{ppm}$: (500.13 MHz) 4.62 (H-1α), 3.18 (H-2α), 3.42 (H-3α), 3.74 (H-4α), 3.18 (H-5α), 3.42 (H-6α), 3.1 (CH$_2$-1′), 1.51 (CH$_2$-2′), 1.23 (CH$_2$-3′-7′), 0.85 (CH$_2$-8′); ^{13}C NMR δ$_{ppm}$: (125 MHz) 98.5 (C1α), 72.0 (C2α), 72.4 (C3α), 70.2 (C4α), 72.1 (C5α), 60.8 (C6 α), 14.0 (C8′), 23.0 (C7′), 31.2 (C6′), 29.8 (C3′), 30.0 (C5′), 70.2 (C1′). *n-Octyl-β-D-glucopyranoside:* ^{1}H NMR δ$_{ppm}$: 4.17 (H-1β), 2.88 (H-2β), 3.12 (H-5β), 3.60 (H-6β), 3.1 (CH$_2$-1′), 1.51 (CH$_2$-2′), 1.23 (CH$_2$-3′-7′), 0.85 (CH$_2$-8′); ^{13}C NMR δ$_{ppm}$: 103.2 (C1β), 74.7 (C2β), 77.1 (C3β), 71.0 (C4β), 77.1 (C5β), 61.5 (C6 β), 14.0 (C8′), 23.0 (C7′), 31.2 (C6′), 30.0 (C5′), 70.5 (C1′). *C6-O-octyl-D-glucose:* ^{1}H NMR δ$_{ppm}$: 4.90 (H-1α), 3.20 (H-2α), 3.10 (H-5α), 3.64 (H-6α), 3.1 (CH$_2$-1′), 1.51 (CH$_2$-2′), 1.21 (CH$_2$-3′-7′), 0.85 (CH$_2$-8′); ^{13}C NMR δ$_{ppm}$: 92.2 (C1α), 72.5 (C2α), 72.1 (C5α), 67.2 (C6 α), 14.0 (C8′), 29.5 (C3′), 30.0 (C5′), 70.5 (C1′).

4.3.1.2. n-Octyl maltoside.
Solid; UV (H$_2$O, λ_{max}): 194 nm (σ→σ*, ε_{194}–1479 M^{-1}), 278.5 nm (n→π*, $\varepsilon_{278.5}$–95 M^{-1}). IR (KBr): 1033 cm^{-1} (glycosidic C-O-C symmetrical), 1255 cm^{-1} (glycosidic C-O-C asymmetrical), 3415 cm^{-1} (OH). Optical rotation (c 1, H$_2$O), [α]$_D$ at 25 °C = +91.1°. MS (*m/z*) 455 [M + 1]$^+$. 2D-HSQCT (DMSO-d_6) ^{1}H NMR δ$_{ppm}$: (500.13 MHz) 4.63 (H-1α), 3.30 (H-5α), 3.66 (H-6α), 4.99 (H-1″α), 3.46 (H-2″), 3.20 (H-3″), 3.08 (H-4″), 3.44 (H-6″), 2.9 (CH$_2$-1′), 1.11–1.25 (CH$_2$-2′-7′), 0.85 (CH$_2$-8′); ^{13}C NMR δ$_{ppm}$: (125 MHz) 98.8 (C1α), 75.0 (C5α), 60.5 (C6α), 100.8 (C1″α), 71.8 (C2″), 72.1 (C3″), 70.2 (C4″), 61.0 (C6″), 14.1 (C8′), 23.0 (C7′), 31.5 (C6′), 29.8 (C3′), 29.0 (C2′), 70.3 (C1′).

4.3.1.3. n-Octyl sucrose.
Solid; UV (H$_2$O, λ_{max}): 205 nm (σ→σ*, ε_{205}–2570 M^{-1}), 276 nm (n→π*, ε_{276}–257 M^{-1}). IR (KBr): 1054 cm^{-1} (glycosidic C-O-C symmetrical), 1259 cm^{-1} (glycosidic C-O-C asymmetrical), 3357 cm^{-1} (OH). Optical rotation (*c* 1, H$_2$O), [α]$_D$ at 25 °C = +13.3°. MS (*m/z*) 455 [M + 1]$^+$. 2D-HSQCT (DMSO-d_6) *C1-O-octyl sucrose*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.76 (H-1), 3.81 (H-4), 3.79 (H-5), 3.40 (H-6), 5.18 (H-1″α), 3.10 (H-3″), 3.03 (H-4″), 3.54 (H-5″), 3.62 (H-6″), 3.01 (H-1′), 1.01–1.23 (H-2-7′), 0.84 (H-8′); ^{13}C NMR δ$_{ppm}$ (125 MHz): 62.8 (C1), 104.0 (C2), 75.4 (C4), 83.0 (C5), 62.0 (C6), 91.5 (C1″α), 72.2 (C3″), 70.5 (C4″), 72.0 (C5″), 61.0 (C6″), 14.4 (C8′), 23.2 (C7′), 31.5 (C6′), 29.2 (C5′), 29.6 (C2′), 70.2 (C1′). *C6-O-octyl sucrose*: ^{1}H NMR δ$_{ppm}$: 3.54 (H-1), 3.87 (H-3), 3.72 (H-4), 3.72 (H-5), 3.25 (H-6), 4.90 (H-1″α), 3.17 (H-3″), 3.11 (H-4″), 3.44 (H-5″), 3.48 (H-6″), 3.01 (H-1′), 1.0–1.25 (H-2-7′), 0.85 (H-8′); ^{13}C NMR δ$_{ppm}$: 61.8 (C1), 104.04 (C2), 77.4 (C3), 76.0 (C4), 82.0 (C5), 63.0 (C6), 92.1 (C1″α), 72.2 (C3″), 70.2 (C4″), 72.0 (C5″), 61.2 (C6″), 14.6 (C8′), 23.3 (C7′), 31.3 (C6′), 29.6 (C4′), 29.7 (C2′), 70.0 (C1′).

4.3.1.4. Guaiacyl-α-D-glucoside.
Solid; UV (H$_2$O, λ_{max}): 210 nm (σ→σ*, ε_{210}–398 M^{-1}), 270 nm (π→π*,

ε_{270}–69 M^{-1}). IR (KBr): 1030 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 1236 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = +92.3°. MS (m/z) 286 [M]$^+$. *C1-O-guaiacyl-α-D-glucopyranoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.65 (H-1α), 3.3 (H-2α), 3.72 (H-3α), 3.75 (H-4α), 3.10 (H-5α), 3.43 (H-6α), 7.08 (H-6′), 6.98 (H-5′), 6.94 (H-4′), 7.09 (H-3′); ^{13}C NMR δ_{ppm} (125 MHz): 98.7 (C1α), 74.9 (C2α), 72.0 (C3α), 70.1 (C4α), 74.9 (C5α), 60.9 (C6α), 113.4 (C6′), 118.5 (C5′), 121.9 (C4′), 110.5 (C3′). *C6-O-guaiacyl-α-D-glucose*: ^{1}H NMR δ_{ppm}: 4.97 (H-1α), 3.06 (H-2α), 3.16 (H-3α), 3.63 (H-4α), 3.14 (H-5α), 3.60 (H-6α), 6.80 (H-5′), 6.84 (H-4′), 6.88 (H-3′); ^{13}C NMR δ_{ppm} 100.0 (C1α), 74.9 (C2α), 74.0 (C3α), 70.2 (C4α), 74.0 (C5α), 67.0 (C6α), 119.0 (C5′), 121.7 (C4′), 110.0 (C3′).

4.3.1.5. Eugenyl-α-D-glucoside. Solid; UV (H$_2$O, λ_{max}): 205 nm ($\sigma{\rightarrow}\sigma^*$, ε_{205}–1792 M^{-1}), 279 nm ($\pi{\rightarrow}\pi^*$, ε_{279}–396 M^{-1}). IR (KBr): 1653 cm^{-1} (allylic C=C), 1033 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 1268 cm^{-1}, (glycosidic aryl alkyl C-O-C asymmetrical), 3330 cm^{-1} (OH). Optical rotation (c 1, H$_2$O) at 25 °C = +59.6°. MS (m/z) 347 [M-2+Na]$^+$. *C1-O-eugenyl-α-D-glucopyranoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.65 (H-1α), 3.55 (H-2α), 3.28 (H-3α), 3.73 (H-4α), 3.42 (H-6α). 6.86 (1H, s, H-3′), 6.70 (1H, d, H-5′), 6.85 (1H, d, H-6′), 3.28 (H-7′), 5.92 (1H, m, H-8′), 5.10 (H-9′), 3.40 (OCH$_3$); ^{13}C NMR δ_{ppm} (125 MHz): 97.0 (C1α), 72.5 (C2α), 74.0 (C3α), 69.0 (C4α), 61.0 (C6α), 52.0 (OCH$_3$), 113 (C3′), 120 (C5′), 115.3 (C6′), 39.0 (C7′), 137.0 (C8′), 116.0 (C9′). *C6-O-eugenyl-D-glucose*: ^{1}H NMR δ_{ppm}: 3.6 (H-2α), 3.50 and 3.68 (H-6α a & b), 6.90 (1H, s, H-3′), 6.78 (1H, d, H-5′); ^{13}C NMR δ_{ppm}: 69.0 (C2α), 66.0 (C6α), 52.0 (OCH$_3$), 112 (C3′), 119 (C5′).

4.3.1.6. Vanillyl-α/β-D-glucoside. Solid; UV (H$_2$O, λ_{max}): 195.5 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{195.5}$–2241 M^{-1}), 279.5 nm ($\pi{\rightarrow}\pi^*$, ε_{279}.–5291 M^{-1}). IR (KBr): 3358 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1030 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1408 cm^{-1} (C=C), 1636 cm^{-1} (CO), 2933 cm^{-1} (CH). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = +62.8. MS (m/z) 316 [M + 2]$^+$. 2D-HSQCT (DMSO-d_6) *C1-O-vanillyl-α-D-glucopyranoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.65 (H-1α, 5.5 Hz), 3.23 (H-2α), 3.42 (H-3α), 3.78 (H-4α), 3.15 (H-5α), 3.60 (H-6α), 6.59 (H-2′), 6.20 (H-5′), 3.73 (OCH$_3$); ^{13}C NMR δ_{ppm} (125 MHz): 99.2 (C1α), 72.3 (C2α), 73.5 (C3α), 70.2 (C4α), 72.5 (C5α), 60.5 (C6α), 111.4 (C2′), 114.5 (C5′). *C1-O-vanillyl-β-D-glucopyranoside*: ^{1}H NMR δ_{ppm}: 4.94 (H-1β, 5.3 Hz), 2.98 (H-2β), 3.22 (H-3β), 3.68 (H-6β); ^{13}C NMR δ_{ppm}: 101.5 (C1β), 74.6 (C2β), 76.1 (C3β), 60.8 (C6β). *C6-O-vanillyl D-glucose*: ^{1}H NMR δ_{ppm}: 4.91 (H-1α), 3.23 (H-2α), 3.20 (H-3α), 3.62 (H-4α), 3.23 (H-5α), 3.55 (H-6α); ^{13}C NMR δ_{ppm}: 92.7 (C1α), 72.3 (C2α), 72.6 (C3α), 70.2 (C4α), 75.2 (C5α), 68.0 (C6α).

4.3.1.7. Vanillyl-α-D-galactoside. Solid; UV (H$_2$O, λ_{max}): 198.0 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{198.0}$–2909 M^{-1}), 281.0 nm ($\pi{\rightarrow}\pi^*$, $\varepsilon_{281.0}$–519 M^{-1}). IR (KBr): 3271 cm^{-1} (OH), 1261 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1031 cm^{-1} (glycosidic

aryl alkyl C–O–C symmetric), 1406 cm^{-1} (C=C), 1664 cm^{-1} (CO). Optical rotation (c 1, H$_2$O), [α]$_D$ at 25 °C = +8.82. MS (m/z) 314 [M]$^+$. 2D-HSQCT (DMSO-d_6): *C1-O-vanillyl-α-D-galactopyranoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.22 (H-1α), 3.69 (H-2α), 3.52 (H-3α), 3.48 (H-4α), 3.43 (H-5α), 3.35 (H-6α), 7.38 (H-2′), 6.90 (H-5′), 7.33 (H-6′), 3.86 (OCH$_3$), 9.74 (CHO); ^{13}C NMR δ_{ppm} (125 MHz): 95.8 (C1α), 69.4 (C2α), 69.9 (C3α), 70.8 (C4α), 71.1 (C5α), 62.0 (C6α), 129.2 (C1′), 111.3 (C2′), 148.6 (C3′), 153.5 (C4′), 115.9 (C5′), 126.4 (C6′), 56.1 (OCH$_3$), 191.4 (CHO).

4.3.1.8. Vanillyl-α-D-mannoside. Solid; UV (H$_2$O, λ_{max}): 198.5 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{198.5}$–3401 M^{-1}), 278.0 nm ($\pi{\rightarrow}\pi^*$, $\varepsilon_{278.0}$–284 M^{-1}). IR (KBr): 3365 cm^{-1} (OH), 1249 cm^{-1} (glycosidic aryl alkyl C–O– C asymmetric), 1030 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1406 cm^{-1} (C=C), 1651 cm^{-1} (CO), 2940 cm^{-1} (CH). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = –3.6. MS (m/z) 314 [M]$^+$. *C1-O-vanillyl-α-D-mannopyranoside*: ^{13}C NMR (DMSO-d_6) δ_{ppm} (125 MHz): 100.8 (C1α), 70.5 (C2α), 71.3 (C3α), 67.1 (C4α), 73.8 (C5α), 61.3 (C6α), 109.4 (C2′), 114.72 (C5′), 121.9 (C6′).

4.3.1.9. Vanillyl maltoside. Solid; UV (H$_2$O, λ_{max}): 194.5 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{194.5}$–4782 M^{-1}), 278.5 nm ($\pi{\rightarrow}\pi^*$, $\varepsilon_{278.5}$–328 M^{-1}). IR (KBr): 3361 cm^{-1} (OH), 1265 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1024 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1412 cm^{-1} (C=C), 1651 cm^{-1} (CO), 2930 cm^{-1} (CH), 1205 cm^{-1} (OCH$_3$). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = +92.0. MS (m/z) 478 [M + 2]$^+$. 2D-HSQCT (DMSO-d_6): *C1-O-vanillyl-α-maltoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.68 (H-1α), 3.10 (H-2α), 3.20 (H-3α), 3.30 (H-4α), 3.72 (H-5α), 3.48 (H-6α), 4.94 (H-1″α, 5.7 Hz), 3.25 (H-2″), 2.88 (H-3″), 3.65 (H-4″), 3.60 (H-6″), 6.26 (H-2′), 6.62 (H-5′), 7.18 (H-6′), 3.73 (OCH$_3$); ^{13}C NMR δ_{ppm} (125 MHz): 98.2 (C1α), 70.1 (C2α), 75.1 (C3α), 79.1 (C4α), 69.8 (C5α), 60.8 (C6α), 100.3 (C1″α), 73.8 (C2″), 74.5 (C3″), 70.0 (C4″), 60.8 (C6″), 130.0 (C1′), 109.5 (C2′), 114.2 (C5′), 126.8 (C6′). *C6-O-vanillyl maltose*: ^{1}H NMR δ_{ppm}: 4.88 (H-1α), 3.54 (H-6α), 4.94 (H-1″α); ^{13}C NMR δ_{ppm}: 92.4 (C1α), 67.2 (C6α), 100.3 (C1″α). *C6″-O-vanillyl maltose*: ^{1}H NMR δ_{ppm}: 4.88 (H-1α), 4.94 (H-1″α), 3.69 (H-6″), ^{13}C NMR δ_{ppm}: 92.4 (C1α), 100.3 (C1″α), 66.1 (C6″).

4.3.1.10. Vanillyl sucrose. Solid; UV (H$_2$O, λ_{max}): 194.0 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{194.0}$–6820 M^{-1}), 278.5 nm ($\pi{\rightarrow}\pi^*$, $\varepsilon_{278.5}$–423 M^{-1}). IR (KBr): 3374 cm^{-1} (OH), 1254 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1026 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1412 cm^{-1} (C=C), 1650 cm^{-1} (CO), 2936 cm^{-1} (CH), 1211 cm^{-1} (OCH$_3$). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = +48.6. MS (m/z) 476 [M]$^+$. 2D-HSQCT (DMSO-d_6): *C1-O-vanillyl sucrose*: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.49 (H-1), 3.88 (H-3), 3.89 (H-4), 3.86 (H-5), 3.4 (H-6), 4.72 (H-1″α), 3.68 (H-2″), 3.46 (H-3″), 3.62 (H-4″), 3.65 (H-5″), 3.59 (H-6″), 7.22 (H-2′), 6.60 (H-5′), 8.35 (H-6′); ^{13}C NMR δ_{ppm} (125 MHz): 66.0 (C1), 76.8 (C3), 80.9 (C4), 81.5 (C5), 62.2 (C6), 98.5 (C1″α), 71.0 (C2″), 72.2 (C3″), 69.8 (C4″), 72.1 (C5″), 60.5 (C6″), 112.8 (C5′), 126.3 (C6′). *C6″-O-vanillyl*

sucrose: ^{1}H NMR δ_{ppm}: 3.48 (H-1), 3.67 (H-3), 3.57 (H-5), 3.46 (H-6), 4.63 (H-1″α), 3.08(H-2″), 3.42 (H-3″), 3.15 (H-4″), 3.19 (H-5″), 3.72 (H-6″); ^{13}C NMR δ_{ppm}: 62.3 (C1), 76.5 (C3), 82.2 (C5), 60.5 (C6), 98.6 (C1″α), 69.9 (C2″), 72.3 (C3″), 69.8 (C4″), 71.5 (C5″), 66.1 (C6″).

4.3.1.11. Vanillyl-D-sorbitol.

Solid; UV (H$_2$O, λ_{max}): 193.5 nm ($\sigma{\rightarrow}\sigma^*$, $\varepsilon_{193.5}$–2940 M^{-1}), 273.0 nm ($\pi{\rightarrow}\pi^*$, $\varepsilon_{273.0}$–290 M^{-1}). IR (KBr): 3386 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1038 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1409 cm^{-1} (C=C), 2943 cm^{-1} (CH). Optical rotation (c 1, H$_2$O): [α]$_D$ at 25 °C = +13.9. MS (m/z) mono-arylated 316 [M]$^+$, di-arylated 451 [M + 1]$^+$. 2D-HSQCT (DMSO-d_6): *C1-O-vanillyl-D-sorbitol*: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.65 (H-1), 3.37 (H-2), 3.48 (H-3), 3.57 (H-4), 3.54 (H-5), 3.58 (H-6), 7.40 (H-2′), 7.20 (H-5′), 7.58 (H-6′), 3.81 (OCH$_3$), 9.75 (CHO); ^{13}C NMR δ_{ppm} (125 MHz): 67.2 (C1), 70.5 (C2), 74.1 (C3), 71.2 (C4), 69.0 (C5), 62.9 (C6), 130.5 (C1′), 111.2 (C2′), 153.8 (C4′), 111.1 (C5′), 124.5 (C6′), 55.9 (OCH$_3$), 191.5 (CHO). *C6-O-vanillyl-D-sorbitol*: ^{1}H NMR δ_{ppm}: 3.55 (H-1), 3.54 (H-2), 3.44 (H-3), 3.68 (H-4), 3.46 (H-5), 3.58 (H-6), 6.88 (H-2′), 6.85 (H-5′), 7.39 (H-6′); ^{13}C NMR δ_{ppm}: 63.2 (C1), 70.8 (C2), 72.5 (C3), 73.1 (C4), 68.2 (C5), 66.2 (C6), 129.1 (C1′), 110.8 (C2′), 153.8 (C4′), 111.4 (C5′), 126.1 (C6′). *C1, C6 Di-O-vanillyl-D-sorbitol*: ^{1}H NMR δ_{ppm}: 3.46 (H-1), 3.35 (H-2), 3.36 (H-3), 3.54 (H-4), 3.65 (H-6), 6.65, 6.68 (H-5′), 6.69, 6.84 (H-6′); ^{13}C NMR δ_{ppm}: 66.5 (C1), 67.0 (C2), 73.5 (C3), 76.1 (C4), 65.5 (C6), 129.1, 130.5 (C1′), 111.3,111.7 (C2′), 153.5, 153.5 (C4′), 115.3, 115.9 (C5′), 119.8, 120.3 (C6′).

4.3.1.12. Curcuminyl-bis-α-D-glucoside.

Solid; UV (H$_2$O, λ_{max}): 210 nm ($\sigma{\rightarrow}\sigma^*$, ε_{210}–1242 M^{-1}), 252 nm ($\pi{\rightarrow}\pi^*$, ε_{252}–1537 M^{-1}), 430 nm ($\pi{\rightarrow}\pi^*$extended conjugation, ε_{430} – 42 M^{-1}). IR (KBr): 1664 cm^{-1} (CO), 1027 cm^{-1} (aryl alkyl C-O-C symmetrical), 1254 cm^{-1} (aryl alkyl C-O-C asymmetrical). Optical rotation (C 1, H$_2$O): [α]$_D$ at 25 °C = +30.3°. MS (m/z) 691 [M-1]$^+$. *C1-O-curcuminyl-bis-α-D-glucopyranoside*: ^{1}H NMR δ_{ppm} (500.13 MHz): 4.66 (H-1α), 3.15 (H-2α), 3.73 (H-3α), 3.75 (H-4α), 3.53 (H-6α). 3.85 (6H, s, 2-OCH$_3$), 6.06 (1H, s, H$_1$), 6.71 (2H, d, J = 15.8Hz, H$_{3, 3'}$), 7.51(2H, d, J = 15.8, H$_{4, 4'}$), 7.25 (2H, s, H$_{6, 6'}$), 6.81 (2H, d, J = 8.2Hz, H$_{9, 9'}$), 7.11 (2 H, dd, J = 1.45 Hz, H$_{10, 10'}$); ^{13}C NMR δ_{ppm} (125 MHz): 99.0 (C1α), 72.2 (C2α), 73.6(C3α), 70.6 (C4α), 61.3 (C6α), 56.1 (OCH$_3$), 101.0 (C$_1$), 183.5 (C$_2$·C$_{2'}$), 121.5 (C$_3$,C$_{3'}$), 141.0 (C$_4$,C$_{4'}$), 126.8 (C$_5$,C$_{5'}$), 111.6 (C$_6$,C$_{6'}$), 148.4 (C$_7$,C$_{7'}$), 150.0 (C$_8$,C$_{8'}$), 116.1 (C$_9$,C$_{9'}$), 123.4 (C$_{10}$,C$_{10'}$). *C6-O-curcuminyl-bis-α-D-glucose*: ^{1}H NMR δ_{ppm}: 3.25 (H-2α), 3.65 (H-4α), 3.52, 3.70 (H-6α a & b), 6.81 (H$_{3, 3'}$), 7.10 (H$_{9, 9'}$); ^{13}C NMR δ_{ppm}: 75.0 (C2α), 70.5 (C4α), 66.5 (C6α), 123.0 (C$_3$, C$_{3'}$), 116 (C$_9$,C$_{9'}$).

4.4. Esterification procedure

A general procedure employed for the esterification reaction involved reacting 0.001–0.008 mol unprotected L-amino acid (L-alanine, L-valine, L-leucine and L-isoleucine, L-proline, L-phenylalanine, L-tryptophan and L-histidine) and 0.001–0.002 mol of carbohydrate (D-glucose, D-galactose, D-fructose, D-ribose, lactose, maltose, D-sorbitol and D-mannitol) along with 100 ml CH$_2$Cl$_2$/DMF (90:10 v/v, 40 °C) or hexane/CHCl$_3$/DMF (45:45:10 v/v, 61 °C) in presence of 0.60–0.180 g of lipases (40–50% w/w carbohydrate employed) under reflux for a period of three days in a flat bottom two necked flask. *Rhizomucor miehei* lipase (RML) in presence of 0.1 mM (0.1 ml), pH 4.0, acetate buffer (L-alanyl carbohydrate esters), *Candida rugosa* lipase (CRL) in presence of 0.1 mM (0.1 ml), pH 7.0, phosphate buffer (L-valyl, L-leucyl, and L-isoleucyl esters of carbohydrates) and CRL in presence of 0.2 mM (0.2 ml), pH 4.0, acetate buffer (L-prolyl, L-phenylalanyl, L-tryptophanyl and L-histidyl esters of carbohydrates) were employed. The condensed vapor of solvents which formed an azeotrope with water was passed through a desiccant before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction [18] and also maintaining a very low water activity of a_w = 0.0054 throughout the reaction. After completion of the reaction, the solvent was distilled off, 20–30 ml of warm water was added, stirred and filtered to remove the lipase. The filtrate was evaporated to get a mixture of the unreacted carbohydrate, unreacted L-amino acids and the product esters, which were then analyzed by HPLC. A Shimadzu LC10AT HPLC connected to LiChrosorb RP-18 column (5 μm particle size, 4.6 × 150 mm length) with acetonitrile/water (v/v 20:80) as a mobile phase at a flow rate of 1 ml min^{-1} was employed using an UV detector at 210 nm in case of L-alanyl, L-valyl, L-leucyl, L-isoleucyl and L-prolyl esters and at 254 nm in case of L-phenylalanyl, L-tryptophanyl and L-histidyl esters of carbohydrates. The conversion yields were determined with respect to peak areas of L-amino acids and those of the esters. The esters formed were separated by size exclusion chromatography using Sephadex G-10.

4.4.1. Amino acyl esters of carbohydrates

4.4.1.1. L-Alanyl-β-D-glucose.

Solid; UV (H$_2$O, λ_{max}): 227.0 nm ($\sigma{\rightarrow}\sigma^*$ $\varepsilon_{227.0}$–1151 M^{-1}), 294.0 nm ($n{\rightarrow}\pi^*$ $\varepsilon_{294.0}$–764 M^{-1}). IR (KBr): 3371 cm^{-1} (NH), 3410 cm^{-1} (OH), 2297 cm^{-1} (CH), 1653 cm^{-1} (CO). Optical rotation (c 0.5, H$_2$O): [α]$_D$ at 25 °C = -38.1°. MS (m/z) 274 [M + Na]$^+$. 2D-HSQCT (DMSO-d_6) *2-O-ester*: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.95 (αCH), 1.07 (βCH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 52.1 (αCH), 15.7 (βCH$_3$), 102.8 (C1β), 82.6 (C2β), 77.9 (C3β), 68.8 (C4β), 60.5 (C6β). *3-O-ester*: ^{1}H NMR δ_{ppm}: 2.87 (αCH), 3.93 (H-3β), 3.58 (H-4β), 3.36 (H-6β); ^{13}C NMR δ_{ppm}: 51.4 (αCH), 83.3 (C3β), 69.3 (C4β), 57.3 (C6β). *6-O- ester*: ^{1}H NMR δ_{ppm}: 2.95 (αCH), 1.30 (βCH$_3$), 3.86 (H-2β), 3.76 (H-5β), 3.82 (H-6β); ^{13}C NMR δ_{ppm}: 50.2 (αCH), 15.1 (βCH$_3$), 171.4 (CO), 101.8 (C1β), 75.0 (C2β), 70.1 (C5β), 63.5 (C6β). *2,6-di-O-ester*: ^{1}H NMR δ_{ppm}: 3.36 (αCH), 1.30 (βCH$_3$), 3.78 (H-2β), 3.47 (H-6β); ^{13}C NMR δ_{ppm}: 49.5 (αCH), 16.4 (βCH$_3$), 100.8 (C1β), 76.5 (C2β), 62.7 (C6β). *3,6-di-O- ester*: ^{1}H NMR δ_{ppm}:

1.30 (βCH₃), 3.78 (H-3β), 3.82 (H-6β); ¹³C NMR δ$_{ppm}$: 51.4 (αCH), 16.7 (βCH₃), 81.6 (C3β), 63.1 (C6β).

4.4.1.2. ʟ-Alanyl-ᴅ-ribose. Solid; UV (H₂O, λ$_{max}$): 224.0 nm (σ→σ* ε$_{224.0}$–3802 M⁻¹), 294.0 nm (n→π* ε$_{294.0}$–1288 M⁻¹). IR (KBr): 3402 cm⁻¹ (NH), 3242 cm⁻¹ (OH), 2887 cm⁻¹ (CH), 1625 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = +22.0°. MS (m/z) 221 [M]⁺; 2D-HSQCT (DMSO-d₆) *2-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 1.25 (αCH), 3.12 (βCH₃), 3.67 (H-2α), 3.50 (H-3α), 3.63 (H-4α), 3.64 (H-5α); ¹³C NMR δ$_{ppm}$ (125 MHz): 48.2 (αCH), 15.9 (βCH₃), 75.7 (C2α), 67.2 (C3α), 68.1 (C4α), 60.6 (C5α). *5-O- ester:* ¹H NMR δ$_{ppm}$: 3.39 (αCH), 1.25 (βCH₃), 4.95 (H-1α), 4.20 (H-1β), 3.27 (H-3α), 3.88 (H-4α), 3.61(H-5α); ¹³C NMR δ$_{ppm}$: 53.0 (αCH), 18.5 (βCH₃), 173.5 (CO), 101.6 (C1α), 103.9 (C1β), 75.0 (C3α), 71.0 (C4α), 63.4 (C5α). *2,5-di-O-ester:* ¹H NMR δ$_{ppm}$: 1.20 (βCH₃), 3.45 (H-2α), 3.79 (H-4α), 3.52 (H-5α); ¹³C NMR δ$_{ppm}$: 18.5 (βCH₃), 74.9 (C2α), 71.0 (C3α), 63.4 (C5α).

4.4.1.3. ʟ-Alanyl lactose. Solid; UV (H₂O, λ$_{max}$): 220.0 nm (σ→σ* ε$_{220.0}$–436 M⁻¹), 294.0 nm (n→π* ε$_{294.0}$–240 M⁻¹). IR (KBr): 3378 cm⁻¹ (NH), 3378 cm⁻¹ (OH), 2946 cm⁻¹ (CH), 1624 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = + 7.4°. MS (m/z) 436 [M + Na]⁺. 2D-HSQCT (DMSO-d₆) *6-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.55 (αCH), 1.25 (βCH₃), 4.78 (H-1α), 4.82 (H-1β), 2.95 (H-2α), 3.25 (H-2β), 2.95 (H-3α), 4.05 (H-4α,β), 3.15 (H-5α), 3.35 (H-5β), 3.80 (H-6α,β), 4.90 (H-1″β), 3.90 (H-2″), 2.85 (H-3″), 3.70 (H-4″), 3.60 (H-5″), 3.40 (H-6″); ¹³C NMR δ$_{ppm}$ (125 MHz): 51.0 (αCH), 15.5 (βCH₃), 173.0 (CO), 98.0 (C1α), 100.2 (C1β), 70.3 (C2α), 72.4 (C2β), 74.3 (C3α), 81.0 (C4α,β), 73.3 (C5α), 73.4 (C5β), 61.2 (C6α,β), 100.2 (C1″β), 76.5 (C2″), 75.1 (C3″), 68.5 (C4″), 78.5 (C5″), 60.6 (C6″). *6″-O-ester:* ¹H NMR δ$_{ppm}$: 3.35 (αCH), 3.85 (H-4α), 3.70 (H-6″); ¹³C NMR δ$_{ppm}$: 53.5 (αCH), 81.5 (C4α), 64.0 (C6″). *6,6″-di-O- ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.85 (H-6″), ¹³C NMR δ$_{ppm}$ (125 MHz): 67.5 (C6″).

4.4.1.4. ʟ-Valyl-ᴅ-glucose. Solid; UV (H₂O, λ$_{max}$): 200.0 nm (σ→σ* ε$_{200.0}$–2630 M⁻¹), 295.0 nm (n→π* ε$_{295.0}$–2089 M⁻¹). IR (KBr): cm⁻¹ 2889 (NH), 3407 cm⁻¹ (OH), 2950 cm⁻¹ (CH), 1622 cm⁻¹(CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = +8.8°. MS (m/z) 302 [M + Na]⁺. 2D-HSQCT (DMSO-d₆) *2-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.30 (αCH), 1.90 (βCH), 0.98 (γCH₃), 0.98 (δCH₃), 3.83 (H-2α), 3.65 (H-2β); ¹³C NMR δ$_{ppm}$ (125 MHz): 53.2 (αCH), 29.2 (βCH), 18.1 (γCH₃), 18.1 (δCH₃), 76.1 (C2α), 60.0 (C6α). *3-O- ester:* ¹H NMR δ$_{ppm}$: 3.10 (αCH), 0.94 (γCH₃), 3.89 (H-3α), 4.01 (H-3β), 3.33 (H-6α,β); ¹³C NMR δ$_{ppm}$: 52.4 (αCH), 9.39 (γCH₃), 82.9 (C3α), 83.4 (C3β), 60.3 (C6α,β). *6-O-ester:* ¹H NMR δ$_{ppm}$: 3.20 (αCH), 2.01 (βCH), 0.90 (γ CH₃), 4.95 (H-1α), 4.22 (H-1β), 3.17 (H-4α), 3.0 (H-4β), 3.86 (H-6α); ¹³C NMR δ$_{ppm}$: 51.9 (αCH), 21.0 (βCH), 8.94 (γ CH₃), 95.2 (C1α), 104.5(C1β), 69.5 (C4α), 69.8 (C4β), 63.4 (C6α). *2,6-di-O-ester:* ¹H NMR δ$_{ppm}$: 3.15 (αCH), 3.75 (H-2α), 3.64 (H-6α); ¹³C NMR δ$_{ppm}$: 51.7 (αCH), 78.7 (C2α), 61.6 (C6α). *3,6-di-O-ester:* ¹H NMR

δ$_{ppm}$: 3.21 (αCH), 1.55 (γ CH₃), 3.67 (H-3β), 3.15 (H-6α,β); ¹³C NMR δ$_{ppm}$: 49.4 (αCH), 78.6 (C3β), 61.3 (C6α,β).

4.4.1.5. .ʟ-Valyl-ᴅ-fructose. Solid; UV (H₂O, λ$_{max}$): 223.0 nm (σ→σ* ε$_{223.0}$–53 M⁻¹), 288.0 nm (n→π* ε$_{288.0}$–23 M⁻¹). IR (KBr): 3352 cm⁻¹ (NH), 3290 cm⁻¹ (OH), 2946 cm⁻¹ (CH), 1623 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = -6.7°. MS (m/z) 304 [M + 2 + Na]⁺. 2D-HSQCT (DMSO-d₆) *1-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.30 (αCH), 1.90 (βCH), 0.98 (γCH₃), 0.98 (δCH₃), 3.80 (H-1α), 4.85 (H-2β), 3.85 (H-3α), 3.30 (H-6α); ¹³C NMR δ$_{ppm}$ (125 MHz): 3.30 (αCH), 1.90 (βCH), 0.98 (γCH₃), 0.98 (δCH₃), 175.0 (CO), 63.5 (C1α), 102.4 (C2β), 70.8 (C3α), 62.8 (C6α).

4.4.1.6. ʟ-Valyl-maltose. Solid; UV (H₂O, λ$_{max}$): 221.0 nm (σ→σ* ε$_{221.0}$–1622 M⁻¹), 291.0 nm (n→π* ε$_{291.0}$–776 M⁻¹). IR (stretching frequency): 3419 cm⁻¹ (NH), 3267 cm⁻¹ (OH), 2936 cm⁻¹ (CH), 1634 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = +341.7°. MS (m/z) 464 [M + Na]⁺. 2D-HSQCT (DMSO-d₆) *6-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.05 (αCH), 2.25 (βCH), 0.99 (γ,δCH₃), 4.80 (H-1α), 4.20 (H-1β), 4.05 (H-2α,β), 3.30 (H-3α), 3.85 (H-4α,β), 3.65 (H-5α,β), 3.94 (H-6α,β), 4.90 (H-1″α), 2.95 (H-2″), 3.10 (H-3″), 3.50 (H-4″), 3.60 (H-5″), 3.60 (H-6″); ¹³C NMR δ$_{ppm}$ (125 MHz): 51.0 (αCH), 28.9 (βCH), 18.9 (γ,δCH₃), 175.5 (CO), 97.0 (C1α), 100.5 (C1β), 79.7 (C2α,β), 76.4 (C3α), 81.5 (C4α,β), 77.2 (C5α,β), 67.2 (C6α,β), 100.7 (C1″α), 70.3 (C2″), 71.8 (C3″), 69.9 (C4″), 72.4 (C5″), 60.6 (C6″); *6″-O-ester:* ¹H NMR δ$_{ppm}$: 2.99 (αCH), 3.81 (H-6″); ¹³C NMR δ$_{ppm}$ (125 MHz): 52.5 (αCH), 68.0 (C6″). *6,6″-di-O- ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.81 (H-6″); ¹³C NMR δ$_{ppm}$ (125 MHz): 64.0 (C6″).

4.4.1.7. ʟ-Valyl-ᴅ-mannitol. Solid; UV (H₂O, λ$_{max}$): 225.0 nm (σ→σ* ε$_{225.0}$–372 M⁻¹), 270.0 nm (n→π* ε$_{270.0}$–178 M⁻¹). IR (KBr): 3294 cm⁻¹ (OH), 2957 cm⁻¹ (CH), 1630 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = +6.6°. MS (m/z) 464 [M + Na]⁺. 2D-HSQCT (DMSO-d₆): *1-O-ester:* ¹H NMR δ$_{ppm}$ (500.13 MHz): 3.42 (αCH), 2.05 (βCH), 0.85 (γCH₃), 3.52 (H-1), 3.46(H-2), 3.53 (H-3), 3.56 (H-4), 3.41 (H-5), 3.46(H-6); ¹³C NMR δ$_{ppm}$ (125 MHz): 55.8(αCH), 29.8(βCH₂), 19.8 (γCH₃), 60.6(C1), 69.6(C2), 68.2(C3), 71.3 (C4), 71.2(C5) 63.6(C6).

4.4.1.8. .ʟ-Leucyl-ᴅ-glucose. Solid; UV (H₂O, λ$_{max}$): 221.0 nm (σ→σ* ε$_{221.0}$–1622 M⁻¹), 291.0 nm (n→π* ε$_{291.0}$–776.2 M⁻¹). IR (KBr): 3419 cm⁻¹ (NH), 3267 cm⁻¹ (OH), 2936 cm⁻¹ (CH), 1634 cm⁻¹ (CO). Optical rotation (c 0.5, H₂O): [α]$_D$ at 25 °C = +34.7°, MS (m/z) 464 [M + Na]⁺. 2D-HSQCT (DMSO-d₆): *2-O-ester:* ¹H δ$_{ppm}$ (500.13 MHz): 2.8 (αCH), 0.75 (δ, ε CH₃), 3.79 (H-2α); ¹³C δ$_{ppm}$ (125 MHz): 46.1 (αCH), 23.4 (δCH₃), 23.9 (εCH₃), 76.9 (C2α). *3-O-ester:* ¹H δ$_{ppm}$: 3.15 (αCH), 1.5 (γCH), 0.77 (δ,εCH₃), 3.86 (H-2β), 3.97 (H-3α), 3.88 (H-3β); ¹³C δ$_{ppm}$: 50.0 (αCH), 25.0 (γCH), 23.3 (δCH3), 23.1 (εCH3), 84.0 (C3α), 83.2 (C3β). *6-O-ester:* ¹H δ$_{ppm}$: 3.08 (αCH), 2.51 (βCH₂), 1.57 (γCH), 0.81 (δ, ε CH₃), 3.86 (H-6α); ¹³C δ$_{ppm}$:

53.5 (αCH), 36.0 (βCH$_2$), 25.3 (γCH), 22.5 (δ-CH$_3$), 23.0 (εCH$_3$), 173.6 (CO), 102.5 (C1α), 65.0 (C6α). *2,6-di-O-ester*: ^{1}H δ$_{ppm}$: 3.45 (H-6α), 3.44 (H-6α); ^{13}C δ$_{ppm}$: 75.5 (C2α), 62.8 (C6α). *3,6-di-O-ester*: ^{1}H δ$_{ppm}$: 3.68 (H- 3α), 3.45 (H-6α); ^{13}C δ$_{ppm}$: 82.5 (C3α), 63.1 (C6α).

4.4.1.9. ʟ-Isoleucyl-ᴅ-glucose. Solid; UV (H$_2$O, λ$_{max}$): 230.0 nm (σ→σ* ε$_{230.0}$–724 M^{-1}), 297.0 nm (n→π* ε$_{297.0}$–363 M^{-1}). IR (KBr): 3383 cm^{-1} (NH), 3360 cm^{-1} (OH), 2240 cm^{-1} (CH), 1657 cm^{-1} (CO). Optical rotation (c 0.5, H$_2$O): [α]$_D$ at 25 °C = –3.1°. MS (m/z) 316 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *3-O-ester*: ^{1}H δ$_{ppm}$: 3.13 (αCH), 1.61 (βCH), 1.08 (γCH$_2$), 0.59 (δ CH$_3$), 0.61 (ε CH$_3$), 5.0 (H-1α), 4.4 (H-1β), 4.01 (H-3α), 3.88 (H-3β), 3.46 (H-4α), 3.58(H-6α,β); ^{13}C δ$_{ppm}$: 51.0 (αCH), 35.4 (βCH), 25.6 (γCH$_2$), 11.2 (δ CH$_3$), 14.0 (ε CH$_3$), 171.4 (CO), 91.8 (C1α), 95.8 (C1β), 82.0 (C3α), 81.9 (C3β), 67.5 (C4α), 63.0 (C6α,β). *6-O-ester*: ^{1}H δ$_{ppm}$: 3.11 (αCH), 1.61 (βCH), 1.08 (γCH$_2$), 0.59 (δ CH$_3$), 0.61 (ε CH$_3$), 3.59 (H-2α), 3.48 (H-3α), 3.64 (H-4α), 3.63 (H-5α), 3.82 (H-6α,β); ^{13}C δ$_{ppm}$: 53.1 (αCH), 35.3 (βCH), 25.2 (γCH$_2$), 11.2 (δ CH$_3$), 14.0 (ε CH$_3$), 70.0 (C2α), 72.2 (C3α), 69.0 (C4α), 69.2 (C5α), 63.6 (C6α,β).

4.4.1.10. ʟ-Prolyl-ᴅ-glucose. Solid; UV(H$_2$O, λ$_{max}$): 200 nm (σ→σ* ε$_{200}$–1862 M^{-1}), 285 nm (n→π* ε$_{285}$–512 M^{-1}). IR (KBr): 3261 cm^{-1} (OH), 1631 cm^{-1} (CO), 1384 cm^{-1} (CN). Optical rotation (c 0.6, H$_2$O): [α]$_D$ at 25 °C = 19.6°. MS (m/z) 302 [M + 2 + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *2-O-ester*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.85 (αCH), 2.84 (βCH$_2$), 3.75 (H-2α), 3.63 (H-2β), 3.55 (H-6α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): 58.0 (αCH), 32.0 (βCH$_2$), 75.0 (C2α), 80.0 (C2β), 61.5 (C6α,β). *3-O-ester*: ^{1}H NMR δ$_{ppm}$: 3.46 (αCH), 2.84 (βCH$_2$), 2.20 (δCH$_2$), 3.84 (H-3α), 3.93 (H-3β), 3.46 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 53.0 (αCH), 32.0 (β-CH$_2$), 28.0 (δ-CH$_2$), 98.5 (C1α), 82.4 (C3α), 84.0 (C3β), 61.0 (C6α,β). *6-O-ester*: ^{1}H NMR δ$_{ppm}$: 3.75 (αCH), 2.85 (βCH$_2$), 1.94 (γCH$_2$), 3.15 (δCH$_2$), 4.38 (H-1α), 4.20 (H-1β), 3.38 (H-3α), 4.20 (H-4α), 3.90 (H-4β), 3.82 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 56.0 (αCH), 35.0 (βCH$_2$), 28.0 (γCH$_2$), 46.0 (δCH$_2$), 171.6 (CO), 95.2 (C1α), 101.4 (C1β), 72.0 (C3α), 71.0 (C4αβ), 63.6 (C6α,β).

4.4.1.11. ʟ-Prolyl-ᴅ-fructose:. Solid; UV (H$_2$O, λ$_{max}$): 213 nm (σ→σ* ε$_{213}$–1479 M^{-1}), 280 nm (n→π* ε$_{280}$–145 M^{-1}). IR (KBr): 3070 cm^{-1} (OH), 1604 cm^{-1} (CO), 1402 cm^{-1} (CN). Optical rotation (c 0.5, H$_2$O): [α]$_D$ at 25 °C = –44.0°. MS (m/z) 300 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *1-O-ester*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.15 (αCH), 2.72 (βCH$_2$), 2.08 (γCH$_2$), 3.12 (δCH$_2$), 4.13 (H-1α), 3.13 (H-3α), 3.40 (H-3β), 3.80 (H-4α), 3.62 (H-4β), 3.30 (H-5α), 3.93 (H-5β), 3.23 (H-6α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): 60.0 (αCH), 31.0 (βCH$_2$), 24.0 (γCH$_2$), 48.2 (δCH$_2$), 170.8 (CO), 66.1 (C1αβ), 104.2 (C1α), 71.4 (C3α), 82.1 (C3β), 69.9 (C4α), 78.2 (C4β), 74.0 (C5α), 82.9 (C5β), 63.7 (C6α,β). *6-O- ester*: ^{1}H NMR δ$_{ppm}$: 3.32 (αCH), 3.33 (δCH$_2$), 3.78 (H-1α), 3.78 (H-3α), 3.29 (H-3β), 3.38 (H-5α), 4.12 (H-5β), 4.02 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 59.2 (αCH), 49.3 (δCH$_2$), 64.4 (C1α), 99.1 (C2α), 71.9 (C3α), 70.7 (C4α), 75.2 (C5α), 81.9 (C5β), 65.8 (C6α,β). *1,6-di-O-ester*: ^{1}H NMR δ$_{ppm}$: 2.91 (αCH), 4.37 (H-1α), 3.42 (H-3α), 3.54 (H-5α), 4.28 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 60.0 (αCH), 66.5 (C1αβ), 102.0 (C2α), 70.1 (C3α), 75.6 (C5α), 66.2 (C6α,β).

4.4.1.12. ʟ-Prolyl-ᴅ-ribose. Solid; UV (H$_2$O, λ$_{max}$): 210 nm (σ→σ* ε$_{210}$–1820 M^{-1}) and 280 nm (n→π* ε$_{280}$–575 M^{-1}). IR (KBr): 3307 cm^{-1} (OH), 1621 cm^{-1} (CO), 1403 cm^{-1} (CN). Optical rotation (c 0.5, H$_2$O): [α]$_D$ at 25 °C = –45.1°. MS (m/z) 272 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *2-O-ester*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.52 (αCH), 2.53 (βCH$_2$), 1.78 (γCH$_2$), 3.30 (δCH$_2$), 4.20 (H-1α), 3.55 (H-2α), 3.45 (H-4α), 3.45 (H-5α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): 61.0 (αCH), 34.5 (βCH$_2$), 23.7 (γCH$_2$), 54.0 (δCH$_2$), 170.9 (CO), 103.8 (C1α), 71.0 (C2α), 73.0 (C4β), 63.0 (C5α,β). *5-O- ester*: ^{1}H NMR δ$_{ppm}$: 3.62 (αCH), 2.88 (βCH$_{2a}$), 1.90 (γCH$_2$), 3.08 (δCH$_2$), 3.32 (H-2α), 3.42 (H-3α), 3.45 (H-4α), 3.20 (H-5α,β); ^{13}C NMR δ$_{ppm}$: 61.2 (αCH), 35.8 (βCH$_2$), 172.0 (CO), 97.0 (C1α), 73.2 (C2α), 66.5 (C3α), 75.0 (C4α), 65.8 (C5α,β).

4.4.1.13. ʟ-Prolyl-lactose. Solid; UV (H$_2$O, λ$_{max}$): 201 nm (σ→σ* ε$_{201}$–2239 M^{-1}) and 278 nm (n→π* ε$_{278}$–447 M^{-1}). IR (KBr): 3084 cm^{-1} (OH), 1609 cm^{-1} (CO), 1419 cm^{-1} (CN). Optical rotation (c 0.5, H$_2$O): [α]$_D$ at 25 °C = –11.4°. MS (m/z) 462 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *6-O-ester*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.06 (αCH), 2.72 (βCH$_2$), 1.73 (γCH$_2$), 3.01 (δCH$_2$), 4.12 (H-1β), 3.29 (H-2α), 3.36 (H-3α), 4.10 (H-4α), 3.84 (H-5α), 3.76 (H-5β), 3.85 (H-6α,β), 4.01 (H-1″β), 3.24 (H-2″), 3.33 (H-3″), 3.41 (H-4″), 3.36 (H-5″), 3.44 (H-6″); ^{13}C NMR δ$_{ppm}$ (125 MHz): 59.6 (αCH), 30.4 (βCH$_2$), 23.8 (γCH$_2$), 44.5 (δCH$_2$), 101.4 (C1β), 70.6 (C2α), 73.8 (C3α), 81.6 (C4α), 77.1 (C5α,β), 64.8 (C6α,β), 102.9 (C1″β), 70.0 (C2″), 71.6 (C3″), 68.2 (C4″), 74.4 (C5″), 60.5 (C6″). *6″-O- ester*: ^{1}H NMR δ$_{ppm}$: 2.86 (αCH), 2.48 (βCH$_2$), 3.99 (H-4α), 3.78 (H-5α), 3.75 (H-6α,β), 3.27 (H-2″), 3.58 (H-4″), 3.38 (H-5″), 3.45 (H-6″); ^{13}C NMR δ$_{ppm}$ (125 MHz): 60.0 (αCH), 33.2 (βCH$_2$), 82.4 (C4α), 77.3 (C5α,β), 60.8 (C6α,β), 70.2 (C2″), 68.9 (C4″), 75.0 (C5″), 62.8 (C6″).

4.4.1.14. ʟ-Phenylalanyl-ᴅ-glucose. Solid; UV (H$_2$O, λ$_{max}$): 237.0 nm (σ→σ* ε$_{237.0}$–1318 M^{-1}), 257.0 nm (π→π* ε$_{257.0}$ 1259 M^{-1}) and 308.0 nm (n→π* ε$_{308.0}$ – 616 M^{-1}). IR (KBr): 3186 cm^{-1} (OH), 1722 cm^{-1} (CO), 1582 cm^{-1} (aromatic – C=C–). Optical rotation (c 0.6, H$_2$O): [α]$_D$ at 25 °C = –24.2°. MS (m/z) 350 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *2-O-ester*: ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 2.92 (αCH), 2.51 (βCH$_{2a}$), 4.6 (H-1α), 3.79 (H-2α), 3.80 (H-2β), 3.4 (H-6α); ^{13}C NMR δ$_{ppm}$ (125 MHz): 52.0 (αCH), 35.8 (βCH$_2$), aromatic- 136.5 (C1), 96.3 (C1α), 75.1 (C2α), 77.3 (C2β), 62.0 (C6α). *3-O- ester*: ^{1}H NMR δ$_{ppm}$: 3.01 (αCH), 3.11 (βCH$_{2a}$), 2.96 (βCH$_{2b}$), 4.4 (H-1α), 3.61 (H-2α), 3.66 (H-2β), 3.82 (H-3α), 3.91 (H-3β), 3.40 (H-6α); ^{13}C NMR δ$_{ppm}$: 53.0 (αCH), 36.8 (βCH$_2$), aromatic-136.4 (C1), 97.3 (C1α), 83.4 (C3α), 83.9 (C3β), 61.9 (C6α). *6-O-ester*: ^{1}H NMR δ$_{ppm}$: 3.07 (αCH), 3.18 (βCH$_{2a}$), 3.06 (βCH$_{2b}$), aromatic- 7.18 (H2, H6), 7.26 (H3, H5), 7.16 (H4), 3.16 (H-5α), 3.78 (H-6α), 3.66 (H-6β); ^{13}C NMR δ$_{ppm}$: 54.2 (αCH), 36.7 (βCH$_2$), aromatic- 136.3 (C1), 128.9 (C2, C6), 130.7 (C3, C5), 130.3 (C4), 172.5 (CO), 102.2 (C1α),

70.5 (C5α), 65.0 (C6αβ). *2,6-di-O-ester:* ^{1}H NMR δ_{ppm}: 3.51 (H-6α), 3.61 (H-6β), 3.67 (H-2β); ^{13}C NMR δ_{ppm}: 77.0 (C2α), 79.0 (C2β), 62.1 (C6β). *3,6-di-O-ester:* ^{1}H NMR δ_{ppm}: 3.61 (H-3α), 3.66 (H-3β), 3.50 (H-6α); ^{13}C NMR δ_{ppm}: 82.3 (C3α), 83.4 (C3β), 64.8 (C6α).

4.4.1.15. L-Phenylalanyl-D-galactose. UV (H$_2$O, λ_{max}): 222.0 nm (σ→σ* $\varepsilon_{222.0}$–871 M^{-1}), 257.5 nm (π→π* $\varepsilon_{257.5}$ 437 M^{-1}) and 299.0 nm (n→π* $\varepsilon_{299.0}$ – 331 M^{-1}). IR (KBr): 3379 cm^{-1} (OH), 1761 cm^{-1} (C=O), 1603 cm^{-1} (aromatic, –C=C–). Optical rotation (*c* 0.5, H$_2$O): [α]$_D$ at 25 °C = +31.1°. MS (*m/z*) diester – 512[M + K]$^+$; ^{13}C NMR DMSO-d$_6$ δ_{ppm} (125 MHz,): *2-O-ester:* 55.7 (αCH), 36.7 (βCH$_2$), aromatic- 136.5 (C1), 127.4 (C2, C6), 129.6 (C3, C5), 129.1 (C4), 171.4 (CO), 97.2 (C1α) 76.3 (C2α), 75.1 (C2β), 60.8 (C6α). *3-O-ester:* ^{13}C NMR δ_{ppm}: 97.2 (C1α), 82.4 (C3α), 81.5 (C3β), 61.0 (C6α). *6-O-ester:* ^{13}C NMR δ_{ppm}: 97.2 (C1α), 70.6 (C2α), 72.2 (C3α), 73.1 (C4α), 63.0 (C6α,β). *2,6-di-O-ester:* ^{13}C NMR δ_{ppm}: 77.2 (C2αβ), 63.1 (C6β). *3,6-di-O-ester:* ^{13}C NMR δ_{ppm}: 81.8 (C3α), 62.7 (C6α,β).

4.4.1.16. L-Phenylalanyl-D-fructose. Solid; UV (H$_2$O, λ_{max}): 198.0 nm (σ→σ* $\varepsilon_{198.0}$–4467 M^{-1}) and 257.5 nm (π→π* $\varepsilon_{257.5}$ 776 M^{-1}). IR: 3380 cm^{-1} (OH), 1630 cm^{-1} (CO), 1598 cm^{-1} (aromatic, –C=C–). Optical rotation (*c* 0.4, H$_2$O): [α]$_D$ at 25 °C = –14.3°. MS (*m/z*). 365 [M – 1 + K]$^+$. ^{13}C NMR DMSO-d$_6$ δ_{ppm} (125 MHz): *1-O-ester:* 55.1 (αCH), aromatic- 138.4 (C1), 64.9 (C1α,β), 102.4 (C2β), 76.2 (C4β), 69.6 (C5β), 62.3 (C6α,β). *6-O-ester:* ^{13}C NMR δ_{ppm}: 55.0 (αCH), 63.4 (C1α,β), 98.1 (C2β), 75.8 (C4β), 81.4 (C5β), 64.2 (C6α,β).

4.4.1.17. L-Phenylalanyl-lactose. Solid; UV (H$_2$O, λ_{max}): 214.0 nm (σ→σ* $\varepsilon_{214.0}$–6026 M^{-1}), 257.5 nm (π→π* $\varepsilon_{257.5}$ 562 M^{-1}) and 290.0 nm (n→π* $\varepsilon_{290.0}$ – 302 M^{-1}). IR (KBr): 3378 cm^{-1} (OH), 1632 cm^{-1} (CO), 1556 cm^{-1} (aromatic, –C=C–). Optical rotation (*c* 0.2, H$_2$O): [α]$_D$ at 25 °C = +31.3°. MS (*m/z*) 512 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *6-O-ester:* ^{1}H NMR δ_{ppm} (500.13 MHz): 2.67 (αCH), 2.89 (βCH$_{2a}$), aromatic- 7.25 (H-2,H-6), 7.28 (H-3,H-5), 7.20 (H-4), 4.32 (H-1α), 4.23 (H-1β), 3.36 (H-2α), 3.49 (H-2β), 3.32 (H-3α), 3.80 (H-3β), 3.56 (H-4α), 3.72 (H-4β), 3.90 (H-5α,β), 3.47 (H-6α,β), 4.16 (H-1″β), 3.14 (H-2″), 3.42 (H-3″), 3.62 (H-4″), 3.39 (H-5″), 3.54 (H-6″); ^{13}C NMR δ_{ppm} (125 MHz): 51.9 (αCH), 38.2 (βCH$_2$) aromatic- 138.0 (C1), 128.3 (C2, C6), 129.1 (C3, C5), 126.1 (C4), 172.5 (CO), 96.6 (C1α), 101.2 (C1β), 70.5 (C2α), 73.0 (C2β), 74.0 (C3β), 82.5 (C4α), 83.3 (C4β), 77.2 (C5α,β), 62.1 (C6αβ), 103.2 (C1″β), 69.9 (C2″), 71.6 (C3″), 68.2 (C4″), 74.4 (C5″), 60.5 (C6″). *6″-O-ester:* ^{1}H NMR δ_{ppm}: 2.72 (αCH), 3.00 (βCH$_{2a}$), 5.16 (H-1α), 4.32 (H-1β), 3.16 (H-2α), 3.45 (H-2β), 3.43 (H-3α), 3.50 (H-3β), 3.32 (H-4α), 3.80 (H-5α,β), 3.49 (H-6α,β), 4.15 (H-1″β), 3.36 (H-2″), 3.89 (H-4″), 3.89 (H-5″), 3.45 (H-6″); ^{13}C NMR δ_{ppm}: 52.5 (αCH), 37.1 (βCH$_2$) Aromatic- 137.6 (C1), 92.0 (C1α), 97.1 (C1β), 69.7 (C2α), 72.7 (C2β), 72.1 (C3α), 74.5 (C3β), 79.9 (C4α), 80.7 (C4β), 76.0 (C5α,β), 61.0 (C6α,β), 104.0 (C1″β), 69.3 (C2″), 72.1 (C3″), 66.3 (C4″), 75.0 (C5″), 63.1

(C6″). *6,6″-di-O-ester:* ^{1}H NMR δ_{ppm}: 2.55 (αCH), 2.89 (βCH$_{2a}$), 4.32 (H-1α), 3.23 (H-2α), 3.68 (H-2β), 3.33 (H-3α), 4.02 (H-4α), 4.01 (H-5α,β), 3.45 (H-6α,β), 4.21 (H-1″β), 3.14 (H-2″), 3.58 (H-3″), 3.74 (H-4″), 3.46 (H-6″); ^{13}C NMR δ_{ppm}: 51.7 (αCH), 37.9 (βCH$_2$), aromatic- 137.8 (C1), 95.6 (C1α), 101.9 (C1β), 71.3 (C2α), 73.2 (C2β), 73.3 (C3α), 74.7 (C3β), 82.2 (C4α), 84.2 (C4β), 77.7 (C5α,β), 62.4 (C6α,β), 103.4 (C1″β), 69.9 (C2″), 72.4 (C3″), 67.4 (C4″), 74.7 (C5″), 62.7 (C6″).

4.4.1.18. L-Phenylalanyl-D-mannitol. Solid; UV (H$_2$O, λ_{max}): 215.0 nm (σ→σ* $\varepsilon_{215.0}$–631 M^{-1}), 257.5 nm (π→π* $\varepsilon_{257.5}$ 170 M^{-1}). IR (KBr): 3290 cm^{-1} (OH), 1637 cm^{-1} (CO), 1532 cm^{-1} (aromatic, –C=C–). Optical rotation (*c* 0.6, H$_2$O): [α]$_D$ at 25 °C = +1.6°. MS (*m/z*) 352 [M + Na]$^+$. 2D-HSQCT (DMSO-d$_6$): *1-O-ester:* ^{1}H NMR δ_{ppm} (500.13 MHz): 3.48 (αCH), 2.88 (βCH$_{2a}$), aromatic- 7.26 (H-2, H-6), 7.26 (H-3, H-5), 7.18 (H-4), 3.38 (H-1), 3.46 (H-2), 3.70 (H-3), 3.84 (H-4), 3.52 (H-5), 3.36 (H-6); ^{13}C NMR δ_{ppm} (125 MHz): 55.8 (αCH), 37.1 (βCH$_2$), aromatic- 137.6 (C$_1$), 131.2 (C$_2$, C$_6$), 126.7 (C$_4$), 171.0 (CO), 66.0 (C1, C6), 75.0 (C2), 70.0 (C3), 70.2 (C4), 77.8 (C5). *1,6-di-O-ester:* ^{1}H NMR δ_{ppm}: 3.39 (αCH), 3.46 (H-1), 3.45 (H-2, H-6), 3.11 (H-3, H-4), 3.46 (H-6); ^{13}C NMR δ_{ppm}: 55.3 (αCH), 66.8 (C1), 77.0 (C2, C5), 70.4 (C3, C4), 66.8 (C$_6$).

4.4.1.19. L-Tryptophanyl-D-glucose. Solid; UV (H$_2$O, λ_{max}): 213.0 nm (σ→σ* $\varepsilon_{213.0}$–1479 M^{-1}), 276 nm (π→π* ε_{276} 389 M^{-1}), 315 nm (n→π* ε_{315} – 118 M^{-1}). IR (KBr): 3523 cm^{-1} (NH), 3336 cm^{-1} (OH), 1633 cm^{-1} (C=O), 1524 cm^{-1} (aromatic –C=C–). Optical rotation (*c* 0.6, H$_2$O): [α]$_D$ at 25 °C = –21.7°. MS (*m/z*) 366.1 [M]$^+$. 2D-HSQCT (DMSO-d$_6$): *2-O-ester:* ^{1}H NMR δ_{ppm} (500.13 MHz): 2.92 (αCH), 3.06 (βCH$_{2a}$), Aromatic - 6.96–7.59, 4.6 (H-1α), 3.69 (H-2α), 3.72 (H-2β), 3.68 (H-3α), 3.54 (H-6α,β); ^{13}C NMR δ_{ppm} (125 MHz): 53.2 (αCH), 35.0 (βCH$_2$), aromatic-109.4 (C$_1$), 124.2 (C$_2$), 136.0 (C$_4$), 114.6 (C$_6$), 120.8 (C$_7$), 121.1 (C$_8$), 100.8 (C1α), 75.9 (C2α), 76.2 (C2β) 72.9 (C3α), 62.8 (C6α,β). *3-O-ester:* 1H NMR δ_{ppm}: 2.87 (αCH), 2.84 (βCH$_2$), 3.82 (H-2α), 3.92 (H-3α), 3.59 (H-3β), 3.58 (H-6α,β); ^{13}C NMR δ_{ppm}: 52.3 (αCH), 35.9 (βCH$_2$), aromatic-109.3 (C$_1$), 124.3 (C$_2$C$_3$), 136.3 (C$_4$), 121.0 (C$_5$), 113.0 (C$_6$), 116.5 (C$_7$), 120.0 (C$_8$), 100.1 (C1α), 101.8 (C1β), 74.2 (C2α), 71.2 (C2β), 82.0 (C3α,β), 62.5 (C6α,β). *6-O-ester:* ^{1}H NMR δ_{ppm}: 2.81 (αCH), 2.70 (βCH$_2$), 3.41 (H-2α), 3.51 (H-2β), 3.68 (H-3α), 3.52 (H-4α), 3.64 (H-5α), 3.70 (H-5β), 3.65 (H-6α,β); ^{13}C NMR δ_{ppm}: 51.5 (αCH), 31.0 (βCH$_2$), aromatic- 109.2 (C$_1$),124.4 (C$_2$,C$_3$), 136.4 (C$_4$), 121.0 (C$_5$), 111.5 (C$_6$), 118.2 (C$_7$), 118.5 (C$_8$), 172.0 (CO), 96.9 (C1α), 101.9 (C1β), 71.8 (C2α), 69.5 (C2β), 72.6 (C3α), 69.6 (C4α), 68.9 (C5α), 70.2 (C5β), 63.0 (C6α,β). *2,6-di-O-ester:* ^{1}H NMR δppm: 2.78 (βCH$_2$), 3.92 (H-2α), 3.82 (H-2β), 3.54 (H-6α,β). ^{13}C NMR δ_{ppm}: 31.0 (βCH$_2$), 77.0 (C2α), 77.3 (C2β), 63.2 (C6α,β). *3,6-di-O-ester:* ^{1}H NMR δ_{ppm} 2.82 (αCH), 2.61 (βCH$_2$), 3.74 (H-3α), 3.44 (H-6α,β); ^{13}C NMR δ_{ppm}: 51.8 (αCH), 31.0 (βCH$_2$), 81.2 (C3α), 63.9 (C6α,β).

4.4.1.20. L-Tryptophanyl-D-fructose. Solid; UV (H$_2$O, λ$_{max}$): 212.0 nm (σ→σ* ε$_{212.0}$–5495 M^{-1}), 265.0 nm (π→π* ε$_{265.0}$ 1862 M^{-1}), 308 nm (n→π* ε$_{308}$ – 1175 M^{-1}). IR (KBr): 3284 cm^{-1} (OH), 1631 cm^{-1} (C=O), 1364 cm^{-1} (CN) and 1492 cm^{-1} (aromatic, –C=C–). Optical rotation (c 0.7, H$_2$O): [α]$_D$ at 25 °C = –10.8°. MS (m/z) 165 [M – 1]$^+$. 2D-HSQCT (DMSO-d$_6$): *1-O-ester:* ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.44 (αCH), 2.59 (βCH$_{2a}$), aromatic - 7.1 (H-2), 8.18 (H-3), 7.32 (H-7), 7.74 (H-8), 4.32 (H-1α), 3.78 (H-3α), 3.48 (H-4α), 3.42 (H-6α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): 58.2 (αCH), 30.2 (βCH$_2$), aromatic-109.5 (C$_1$), 124.0 (C$_2$), 122.0 (C$_3$), 111.5 (C$_6$), 120.6 (C$_7$), 119.0 (C$_8$), 66.4 (C1α), 104.2 (C2α), 71.6 (C3α), 70.6 (C4α), 63.1 (C6α,β). *6-O-ester:* ^{1}H NMR δ$_{ppm}$: 3.32 (αCH), 2.50 (βCH$_2$), 3.27 (H-1α), 3.78 (H-4α), 4.38 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 59.0 (αCH), 29.8 (βCH$_2$), 63.9 (C1α), 102.0 (C2α), 72.5 (C4α,β), 66.4 (C6α,β).

4.4.1.21. L-Histidyl-D-glucose. Solid; UV (H$_2$O, λ$_{max}$): 210.0 nm (σ→σ* ε$_{210.0}$–1072 M^{-1}), 264.0 nm (π→π* ε$_{264.0}$ 933 M^{-1}). IR (KBr): 3126 cm^{-1} (OH), 1720 cm^{-1} (C=O), 1343 cm^{-1} (NH), 1588 cm^{-1} (aromatic, –C=C–). Optical rotation (c 1.0, H$_2$O): [α]$_D$ at 25 °C = –33.3°. MS (m/z) 318 [M + 1]$^+$. 2D-HSQCT (DMSO-d$_6$): *2-O-ester:* ^{1}H NMR δ$_{ppm}$ (500.13 MHz): 3.08 (αCH), 2.70 (βCH$_2$), Aromatic - 6.93 (H2), 7.69 (H3), 4.78 (H-1α), 3.86 (H-2α), 3.76 (H-2β), 3.19 (H-3α), 3.12 (H-3β), 3.62 (H-4α), 3.58 (H-6α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): 52.0 (αCH), 30.8 (βCH$_2$), aromatic-115.6 (C1), 134.2 (C2), 134.8 (C3), 171.5 (CO), 96.2 (C1α), 75.0 (C2α), 76.7 (C2β), 70.0 (C3α), 80.0 (C3β), 69.3 (C4α), 62.2 (C6α,β). *3-O-ester:* ^{1}H NMR δ$_{ppm}$: 3.00 (αCH), 2.82 (βCH$_2$), aromatic- 6.93 (H2), 7.71 (H3), 4.75 (H-1α), 3.60 (H-2α), 3.32 (H-2β), 3.77 (H-3α), 3.93 (H-3β), 3.67 (H-4α), 3.42 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 52.8 (αCH), 27.7 (βCH$_2$), aromatic-116.2 (C1), 134.4 (C2), 134.8 (C3), 171.0 (CO), 95.5 (C1α), 72.2 (C2α), 74.9 (C2β), 81.7 (C3α), 83.2 (C3β), 68.9 (C4α), 61.3 (C6α,β). *6-O-ester:* ^{1}H NMR δ$_{ppm}$: 2.84 (αCH), 3.06 (βCH$_2$), aromatic – 6.93 (H2), 7.72 (H3), 4.72 (H1-α), 3.59 (H2-α), 3.12 (H3-α), 3.80 (H-4α), 2.90 (H-5α), 3.80 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 51.2 (αCH), 26.8 (βCH$_2$), aromatic-115.6 (C1), 134.4 (C2), 134.9 (C3), 170.3 (CO), 95.0 (C1α), 100.2 (C1β), 72.5 (C2α), 73.1 (C3α), 70.6 (C4α), 75.0 (C5α), 63.6 (C6α,β). *2,6 -di-O-ester:* ^{1}H NMR δppm 2.90 (αCH), 2.78 (βCH$_2$), 3.73 (H-2α), 3.75 (H-2β), 3.47 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 52.5 (αCH), 30.8 (βCH$_2$), 102.1 (C1β), 76.7 (C2α), 78.0 (C2β), 70.5 (C4α), 62.7 (C6α,β). *3,6-di-O-ester:* ^{1}H NMR δ$_{ppm}$: 2.80 (βCH$_2$), 3.73 (H-3α), 3.60 (H-3β), 3.26 (H-6α,β); ^{13}C NMR δ$_{ppm}$: 30.0 (βCH$_2$), 81.7 (C3α), 82.3 (C3β), 70.2 (C4α), 62.4 (C6α,β).

4.4.1.22. L-Histidyl-D-fructose. Solid; UV (H$_2$O, λ$_{max}$): 210.0 nm (σ→σ* ε$_{210.0}$–617 M^{-1}), 267.0 nm (π→π* ε$_{267.0}$ 240 M^{-1}), 321.0 nm (n→π* ε$_{321.0}$–170 M^{-1}). IR (KBr): 3136 cm^{-1} (OH), 1605 cm^{-1} (C=O), 1393 cm^{-1} (CN), 1592 cm^{-1} (aromatic, –C=C–). Optical rotation (c 0.6, H$_2$O): [α]$_D$ at 25 °C = –20.0°. MS (m/z) 340 [M + Na]$^+$. 2D-HSQCT NMR: *6-O-ester:* ^{1}H NMR δ$_{ppm}$(500.13 MHz): 3.45 (αCH), 3.21 (βCH$_2$), aromatic – 6.85 (H2), 7.42 (H3), 3.67 (H-3α), 3.52 (H-4α), 3.24 (H-6α,β); ^{13}C NMR δ$_{ppm}$ (125 MHz): aromatic-116.5 (C1), 134.2 (C2), 124.5 (C3), 170.5 (CO), 62.8 (C1α), 102.1 (C2α), 69.2 (C3α), 70.0 (C4α) 70.6 (C4α), 64.2 (C6α,β).

4.4.1.23. L-Histidyl-D-mannitol. Solid; UV (H$_2$O, λ$_{max}$): 210.0 nm (σ→σ* ε$_{210.0}$–3802 M^{-1}), 267.0 nm (π→π* ε$_{267.0}$ 1349 M^{-1}), 324.0 nm (n→π* ε$_{324.0}$–776 M^{-1}). IR (KBr): 3344 cm^{-1} (OH), 1631 cm^{-1} (C=O), 1319 cm^{-1} (CN), 1511 cm^{-1} (aromatic). Optical rotation (c 0.2, H$_2$O): [α]$_D$ at 25 °C = +17.4°. 2D-HSQCT NMR: *1-O- ester:* ^{1}H NMR δ$_{ppm}$(500.13 MHz): 3.46 (αCH), 3.32 (βCH$_2$), aromatic 7.38 (H3), 3.44 (H-1), 3.39 (H-2), 3.47 (H-3), 3.54 (H-4), 3.57 (H-5), 3.38 (H-6); ^{13}C NMR δ$_{ppm}$ (125 MHz): 54.3 (αCH), aromatic- 128.2 (C2), 123.4(C3), 63.8 (C1, C6), 71.4 (C2, C5), 69.8 (C3, C4).

4.5. Angiotensin converting enzyme (ACE) inhibition assay

ACE inhibition assay for the esters prepared were performed by the Cushman and Cheung method [23]. Aliquots of glycoside or ester solutions in the concentration range 0.12 to 1.60 mM (0.1–0.8 ml of 2.0 mM stock solution) were taken and to this 0.1 ml of ACE solution (0.1% in 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl) was added. To this solution, 0.1 ml of 5.0 mM hippuryl-L-histidyl-L-leucine (HHL) was also added and the total volume made up to 1.25 ml by adding phosphate buffer (0.95–0.25 ml of 0.1 M pH 8.3 containing 300 mM NaCl). The solution was incubated on a Heto-Holten shaking water bath for 30 min at 37 °C. Blanks were performed without the enzyme by taking only the glycoside or ester solutions (0.1 to 0.8 ml) along with 0.1 ml of 5.0 mM HHL. The total volume was made up to 1.25 ml by adding same buffer (1.05–0.35 ml). The reaction was terminated by adding 0.25 ml of 1 M HCl. Hippuric acid formed in the reaction was extracted with 1.5 ml of ethyl acetate. One ml of the ethyl acetate layer was evaporated to dryness and treated with equal amount of distilled water and absorbance was measured at 228 nm for hippuric acid. The hippuric acid formed in 1.5 ml of ethyl acetate was determined from a calibration plot prepared by using a standard hippuric acid in 1 ml of distilled water in the concentration range 0–400 nmol and measuring its absorbance at 228 nm.

Specific activity was expressed as hippuric acid formed (mM) per min, per mg of enzyme protein.

$$\text{Specific activity} = \frac{A_{ts} - A_{blank}}{T \times S \times E}$$

A_{ts} = absorbance of test solution, A_{blank} = absorbance of blank solution, T = incubation period in min, S = slope value of the calibration plot (1.00·6 × 10^{-2} Abs units/nmol of hippuric acid), E = amount of the enzyme in mg protein. The specific activity value for each glycoside and ester is an average from two independent measurements. Percentage inhibition was expressed as the ratio of the specific activity of ACE in the pre-

sence of the inhibitor to that in the absence of the inhibitor, the latter being considered as 100%. IC_{50} value was expressed as the concentration of the inhibitor required for 50% reduction in ACE specific activity. Molecular weights of the glycosides and esters employed in the calculations are weighted averages of molecular weights of glycosides and esters detected by NMR and Mass spectroscopy. In case of samples where NMR spectra were not recorded, molecular weights of the mono glycosides and mono esters were considered for the calculations.

4.6. Protease and lipase assay

Protease activity for the ACE inhibitor was determined by the method described by Dubey and Jagannadham [29] and lipase activity by the tributyrin method [30] in presence of eugenyl-D-glucoside (0.8 mM in 0.1 M, pH 7.5, Tris–HCl buffer) and L-isoleucyl-D-glucose (0.8 mM in the same buffer) individually. Specific protease activity was expressed as the increase in absorbance at 440 nm min^{-1} mg^{-1} of the protein employed. Similarly specific lipase activity was determined as µmol of butyric acid formed per min per mg of the protein employed.

Acknowledgements

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Lipase catalyzed synthesis of L-alanyl esters of carbohydrates

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Abstract

Synthesis of L-alanyl esters of carbohydrates like aldohexoses (D-glucose, D-galactose and D-mannose), ketohexose (D-fructose), pentoses (D-arabinose and D-ribose) and disaccharides (lactose, maltose and sucrose) were carried out using *Rhizomucor miehei* lipase (RML) in organic solvents with conversion yields in the range 8–56%. Enzymatic esterification between L-alanine and D-glucose using RML and porcine pancreas lipase (PPL) was investigated in terms of incubation period, enzyme concentration, substrate concentration, buffer salts (pH and concentration) and enzyme reusability. RML has shown a maximum conversion yield of 28% (1.12–0.88 mmol monoesters and 0.24 mmol diesters) at 1 mmol D-glucose and 4 mmol L-alanine in presence of 30% RML (w/w, D-glucose) and 0.1 mM (0.1 ml) pH 4.0 acetate buffer in 72 h. PPL showed a maximum yield of 18% (0.36–0.28 mmol monoesters and 0.08 mmol diesters) at 1 mmol D-glucose and 2 mmol L-alanine in presence of 40% lipase (w/w, D-glucose) and 0.1 mM (0.1 ml) pH 5.0 acetate buffer in 72 h. In presence of buffer salts, RML showed 22% higher ester yield than in its absence under the same experimental conditions. However, buffer salts did not enhance esterification with PPL. Two-dimensional HSQCT NMR confirmed formation of 1-O-, 2-O-, 3-O-, 4-O-, 5-O-, 6-O- and 6'-O-monoesters and 1,6-di-O-, 2,5-di-O-, 2,6-di-O-, 3,5-di-O-, 3,6-di-O-, 4,6-di-O and 6,6'-di-O-diesters to varying extents depending on the carbohydrate employed.
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Keywords: L-Alanyl esters of carbohydrates; Diesters; Enzymatic esterification; Monoesters; Porcine pancreas lipase; *Rhizomucor miehei* lipase

1. Introduction

Amino acyl esters of carbohydrates are used as sweetening agents, surfactants, microcapsules in pharmaceutical preparations, active nucleoside amino acid esters, antibiotics and in the delivery of biological active agents [1–5]. Chemical acylation of carbohydrates regio-selectively is complex due to the presence of multiple hydroxyl groups, which require protection and deprotection [2,6]. When enzymes are used in organic media, they exhibit specificity [7], thermostability [8,9], molecular memory [10,11] and capacity to catalyze reverse hydrolytic reactions [12,13].

Hitherto, very few references are available on the lipase catalyzed esterification of amino acyl esters of sugars. Most of the earlier workers used proteases and N-protected and carboxyl group activated amino acids for synthesizing aminoacyl esters of carbohydrates [14–17]. Therisod and Klibanov [18] used subtilisin to acylate carbohydrates with activated carboxylic acids in anhydrous organic solvents. Riva et al. [14] carried out subtilisin catalyzed synthesis of N-acetyl-L-alanyl-methyl-β-D-galactopyranoside in anhydrous DMF, with an yield of 70% (6-O- 84% and 4-O- 8%) and N-acetyl-D-alanyl-methyl-β-D-galactopyranoside with an yield of 35% (6-O- 68%, 4-O- 10%, 3-O- 10% and 2-O- 10%). Suzuki et al. synthesized L-alanyl-D-glucose by using D-glucose, methyl-L-alaninate hydrochloride and intact cells of *Rhodotorula lactosa* [19]. Park et al. reported that lipase from porcine pancreas and Lipozyme IM20 gave very low yields (<2%), compared to proteases which gave conversion yields ranging from 15% to 98% when N-protected and carboxyl group activated amino acid was used for the acylation of D-glucose in pyridine [16]. All these reactions were conducted in shake flasks using lesser quantity of substrates and larger quantity of enzymes.

We have earlier carried out some preliminary investigation [20] on the lipase catalyzed synthesis of L-alanyl-D-glucose, L-phenylalanyl-D-glucose and L-leucyl-D-glucose. However, in the present work, a comprehensive investigation has been carried out on the lipase catalyzed synthesis of L-alanyl esters of nine carbohydrate molecules. In the present investigation, lipases from *Rhizomucor miehei* (RML) and porcine pancreas (PPL) were employed to synthesize L-alanyl esters of D-glucose. Besides, RML was also employed to synthesize L-alanyl esters of aldo-

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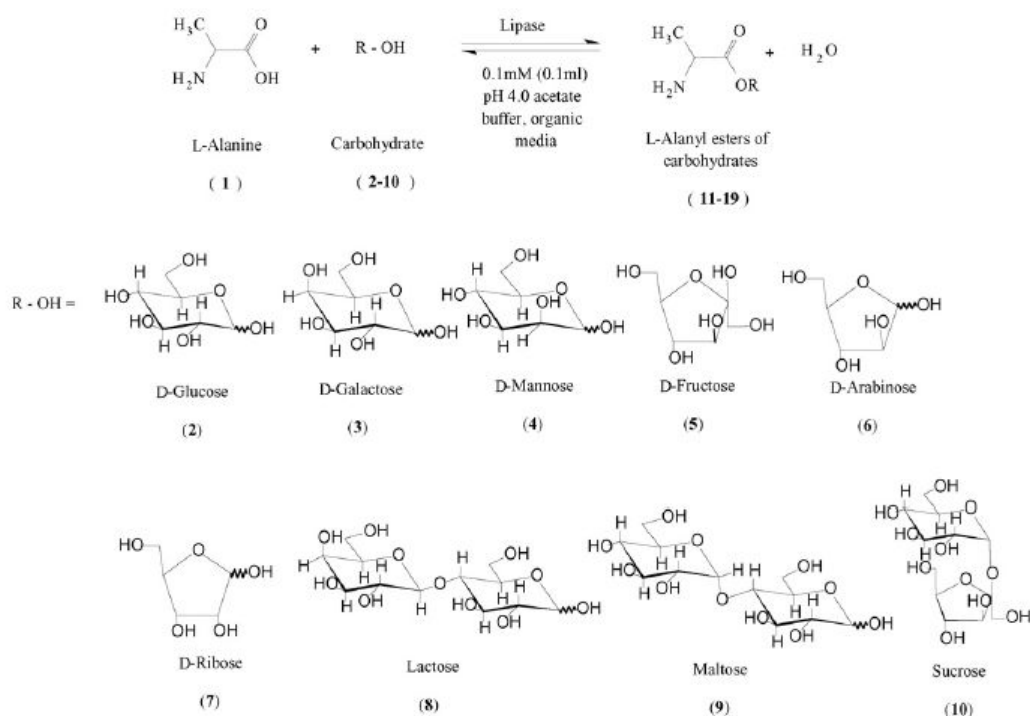
Scheme 1. Lipases catalyzed synthesis of L-alanyl esters of carbohydrates.

hexoses (D-glucose 2, D-galactose 3 and D-mannose 4), ketohexose (D-fructose 5), pentoses (D-arabinose 6 and D-ribose 7) and disaccharides (lactose 8, maltose 9 and sucrose 10). Attempts to synthesize the same using N-acetyl-L-alanine resulted in very little conversion with the above-mentioned enzymes. Hence, unprotected and unactivated L-alanine (1) and carbohydrates (2–10) were employed (Scheme 1). The results are presented below.

2. Materials and methods

2.1. Enzymes

Lipozyme IM20 (*Rhizomucor miehei* lipase, RML) immobilized on weak anion exchange resin, from Novo Nordisk, Denmark and Porcine pancreas lipase (PPL), Type II, crude Steapsin purchased from Sigma Chemical Co., USA, were used in the present work. RML and PPL employed showed esterification activities of 0.46 μmol/min/mg enzyme preparation and 0.06 μmol/min/mg enzyme preparation, respectively, [21].

2.2. Chemicals and reagents

L-Alanine, D-galactose and D-fructose from HiMedia Ltd., India; D-glucose, sucrose and Coomassie Brilliant Blue-G 250 from SD fine chemicals Ltd., India; maltose from Sigma Chemical Co., USA; D-mannose, D-arabinose, D-ribose and D-mannitol from LOBA Chemie Ltd., India; lactose from SISCO Research Laboratories Ltd., India; D-sorbitol from Rolex Laboratory Ltd., India and Karl Fischer Reagent from Qualigens Fine Chemicals Ltd., India, were employed as such. Sephadex G-10 from Sigma Chemical Co., USA, and Bio Gel P-2 from Bio-Rad Laboratories were used as such. Solvents: CH_2Cl_2, $CHCl_3$, n-hexane, HPLC grade acetonitrile and DMF from SD fine Chemicals (Ind.) Ltd., were employed after distilling once.

2.3. Esterification procedure

Esterification was carried out in a flat bottomed two necked flask by reacting unprotected L-alanine 1 (0.001–0.008 mol) and 001–0.008 mol of carbohydrate (2–10) along with 100 ml of CH_2Cl_2:DMF (v/v, 90:10, 40 °C) or hexane:$CHCl_3$:DMF (v/v/v, 45:45:10, 60 °C) in presence of 0.018–0.25 g of lipases (10–50% by weight of 2–10) under reflux for a period of three days. The enzymes were imparted with 'pH memory' in some experiments by adding known volumes of 0.1 M buffer solutions of specified pH value to 100 ml (solvent) of the reaction mixture [22,23]. The buffer salts thus employed were CH_3COONa for pH 4.0 and 5.0, Na_2HPO_4 for pH 6.0 and 7.0 and $Na_2B_4O_7·10H_2O$ for pH 8.0 buffers. The condensed vapours of solvent which formed an azeotrope with water during reflux was passed through a desiccant before being returned into the reaction mixture, thereby facilitating complete removal of water of reaction [24]. This set-up maintained a very low water activity of $a_w = 0.0054$ throughout the reaction period which was determined by Karl Fischer titration of the reaction mixture using Karl Fischer reagent by examining aliquots for the water content during the course of the reaction. After completion of the reaction, the solvent was distilled off, 20–30 ml of warm water was added, stirred and filtered to remove the lipase. The filtrate was evaporated to get a mixture of the unreacted carbohydrate, unreacted L-alanine and the product esters 11–19 which were then analyzed by HPLC. The esters formed were separated by size exclusion chromatography using Sephadex G-10 and Bio Gel P-2 as column materials and eluted with water and subjected to spectral characterization. The isolated esters were also tested for Angiotensin Converting Enzyme (ACE) inhibition activity according to the method of Cushman and Cheung [25]. Critical micellar concentration was determined for L-alanyl-β-D-glucose by using Coomassie Brilliant Blue-G 250 reagent at 470 nm [26] and it was found to be 2.25 mM (0.056%).

2.4. HPLC

The reaction mixture was monitored by employing a Shimadzu LC10AT high-performance liquid chromatography instrument connected to a μ-Bondapack aminopropyl column (10 μm particle size, 3.9 mm × 300 mm length)

with acetonitrile:water (v/v, 80:20) as a mobile phase at a flow rate of 1 ml/min and refractive index detector. Also, a LiChrosorb RP-18 column (5 μm particle size, 4.6 mm × 150 mm length) with acetonitrile:water (v/v, 20:80) as a mobile phase at a flow rate of 1 ml/min and UV detector at 210 nm was employed. Since different equivalents of L-alanine were employed, the conversion yields were determined based on the peak areas of L-alanine and L-alanyl ester of carbohydrate and were expressed relative to the L-alanine concentration employed. The error in HPLC yields were ±10–15%. In μ-Bondapack aminopropyl column, retention times of D-glucose, L-alanine and L-alanyl-D-glucose are found to be 5.2, 9.7 and 12.3 min, respectively. In LiChrosorb RP-18 column, retention times are: L-alanine, 2.6 min; L-alanyl-D-glucose, 3.4 min; L-alanyl-D-galactose, 3.4 min; L-alanyl-D-mannose, 3.4 min; L-alanyl-D-fructose, 3.5 min; L-alanyl-D-arabinose, 3.2 min; L-alanyl-D-ribose, 3.2 min; L-alanyl-lactose, 3.3 min; L-alanyl-maltose, 3.3 min; L-alanyl-sucrose, 3.3 min. Respective carbohydrates were not detected at 210 nm.

2.5. Spectral characterization

A Shimadzu UV-1601 spectrophotometer was used for recording UV spectra of the isolated esters in aqueous solutions at 0.2–1.0 mM concentration. A Nicolet 5700 FTIR instrument was used for recording the IR spectra with 1.0–2.0 mg of ester sample as KBr pellet. Specific rotations of the isolated esters were measured at 25 °C using Perkin-Elmer 243 polarimeter with a 0.5% aqueous solution of the esters. Mass spectra of the isolated esters were recorded using a Q-TOF Waters Ultima instrument (No. Q-Tof GAA 082, Waters corporation, Manchester, UK) fitted with an electron spray ionization (ESI) source.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz for ^{13}C). Proton and carbon 90° pulse widths were 10.5 and 12.25 μs, respectively. About 40 mg of the sample dissolved in DMSO-d_6 and D_2O was used for recording the spectra at 35 °C. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard to within ±0.01 ppm. Two-dimensional Heteronuclear Single Quantum Coherence Transfer spectra (2D HSQCT) were recorded [21] for the esters. In the NMR data, only resolvable signals are shown. Some assignments are interchangeable. Non-reducing end carbohydrate signals are primed. Since, the esters are surfactant molecules, they appear to aggregate in the solvent and usually give broad signals, thus, making it difficult to resolve the coupling constant values accurately. Mass data for the monoesters are shown. However, although NMR data clearly indicated the presence of di-O-esters, they were not detected in the mass spectra which could be due to instantaneous decomposition.

2.5.1. L-Alanine 1

UV (H_2O, λ_{max}): 190.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{190.0}$, 111.9 M^{-1}), IR (stretching frequency): 3415 cm^{-1} (OH), 2945 cm^{-1} (CH), 1715 cm^{-1} (CO); optical rotation (c 1.0, H_2O): $[\alpha]_D$ at 25 °C = +3.33°; 2D HSQCT (DMSO-d_6): ^{1}H NMR δ_{ppm} (500.13 MHz): 3.35 (α-CH), 1.25 (β-CH$_3$); ^{13}C NMR δ_{ppm} (125 MHz): 53.0 (α-CH), 15.5 (β-CH$_3$), 170.5 (CO).

2.5.2. L-Alanyl-β-D-glucose 11

UV (H_2O, λ_{max}): 227.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{227.0}$, 1150.8 M^{-1}), 294.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{294.0}$, 763.8 M^{-1}); IR (stretching frequency): 3371 cm^{-1} (NH), 3410 cm^{-1} (OH), 2297 cm^{-1} (CH), 1653 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = −38.1°; mass, 274 [M + Na]$^+$; ACE activity: IC$_{50}$ value = 3.1 ± 0.3 mM; 2D HSQCT (DMSO-d_6) 2-O-ester **11a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.95 (α-CH), 1.07 (β-CH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 52.1 (α-CH), 15.7 (β-CH$_3$), 102.8 (C-1β), 82.6 (C-2β), 77.9 (C-3β), 68.8 (C-4β), 60.5 (C-6β); 3-O-ester **11b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.87 (α-CH), 3.93 (H-3β), 3.58 (H-4β), 3.36 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 51.4 (α-CH), 83.3 (C-3β), 69.3 (C-4β), 57.3 (C-6β); 6-O-ester **11c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.95 (α-CH), 1.30 (β-CH$_3$), 3.86 (H-2β), 3.76 (H-5β), 3.82 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 50.2 (α-CH), 15.1 (β-CH$_3$), 171.4 (CO), 101.8 (C-1β), 75.0 (C-2β), 70.1 (C-5β), 63.5 (C-6β); 2,6-di-O-ester **11d**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.36 (α-CH), 1.30 (β-CH$_3$), 3.78 (H-2β), 3.47 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 49.5 (α-CH), 16.4 (β-CH$_3$), 100.8 (C-1β), 76.5 (C-2β), 62.7 (C-6β); 3,6-di-O-ester **11e**: ^{1}H NMR δ_{ppm} (500.13 MHz): 1.30 (β-CH$_3$), 3.78 (H-3β), 3.82 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 51.4 (α-CH), 16.7 (β-CH$_3$), 81.6 (C-3β), 63.1 (C-6β).

2.5.3. L-Alanyl-D-galactose 12

UV (H_2O, λ_{max}): 200.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{200.0}$, 2630.3 M^{-1}), 295.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{295.0}$, 2089.3 M^{-1}); IR (stretching frequency): 2889 cm^{-1} (NH), 3407 cm^{-1} (OH), 2950 cm^{-1} (CH), 1622 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = +8.8°; mass, 274 [M + Na]$^+$; 2D HSQCT (DMSO-d_6) 2-O-ester **12a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.95 (α-CH), 3.38 (H-2α), 3.36 (H-2β), 3.55 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 51.5 (α-CH), 76.4 (C-2α), 76.5 (C-2β), 60.7 (C-6β); 3-O-ester **12b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.75 (H-3α), 3.60 (H-3β), 3.35 (H-6α); ^{13}C NMR δ_{ppm} (125 MHz): 81.6 (C-3α), 82.6 (C-3β), 60.7 (C-6α); 6-O-ester **12c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.05 (α-CH), 4.90 (H-1α), 4.85 (H-1β), 3.85 (H-3α), 3.30 (H-6α); ^{13}C NMR δ_{ppm} (125 MHz): 52.0 (α-CH), 175.0 (CO), 95.4 (C-1α), 101.8 (C-1β), 70.8 (C-3α), 63.1 (C-6α).

2.5.4. L-Alanyl-D-mannose 13

UV (H_2O, λ_{max}): 194.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{194.0}$, 2630.3 M^{-1}), 295.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{295.0}$, 1047.1 M^{-1}); IR (stretching frequency): 2887 cm^{-1} (NH), 3387 cm^{-1} (OH), 2816 cm^{-1} (CH), 1626 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = −20.0°; mass, 274 [M + Na]$^+$; 2D HSQCT (DMSO-d_6) 3-O-ester **13a**: ^{13}C NMR δ_{ppm} (125 MHz): 51.5 (α-CH), 15.2 (β-CH$_3$), 74.0 (C-2α), 89.2 (C-3α), 60.1 (C-6α); 4-O-ester **13b**: ^{13}C NMR δ_{ppm} (125 MHz): 49.7 (α-CH), 14.7 (β-CH$_3$), 75.32 (C-4β), 60.1 (C-6α); 6-O-ester **13c**: ^{13}C NMR (DMSO-d_6) δ_{ppm} (125 MHz): 52.1 (α-CH), 15.9 (β-CH$_3$), 172.0 (CO), 95.5 (C-1α), 101.5 (C-1β), 69.7 (C-2α), 69.0 (C-3α), 68.4 (C-4α), 74.9 (C-5α), 63.1 (C-6α); 3,6-di-O-ester **13d**: ^{13}C NMR δ_{ppm} (125 MHz): 82.0 (C-3α), 82.9 (C-3β), 62.8 (C-6α); 4,6-di-O-ester **13e**: ^{13}C NMR δ_{ppm} (125 MHz): 77.2 (C-4α), 62.5 (C-6α).

2.5.5. L-Alanyl-D-fructose 14

UV (H_2O, λ_{max}): 200.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{200.0}$, 2630.3 M^{-1}), 295.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{295.0}$, 2089.3 M^{-1}); IR (stretching frequency): 2889 cm^{-1} (NH), 3407 cm^{-1} (OH), 2950 cm^{-1} (CH), 1622 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = +8.8°; mass, 274 [M + Na]$^+$; 2D HSQCT (DMSO-d_6) 1-O-ester **14a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.05 (α-CH), 3.40 (H-1α), 4.85 (H-2β), 3.85 (H-3α), 3.30 (H-6α); ^{13}C NMR δ_{ppm} (125 MHz): 52.0 (α-CH), 175.0 (CO), 63.5 (C-1α), 102.4 (C-2β), 70.8 (C-3α), 62.8 (C-6α); 6-O-ester **14b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.75 (H-3α), 3.60 (H-3β), 3.30 (H-6α); ^{13}C NMR δ_{ppm} (125 MHz): 81.6 (C-3α), 82.6 (C-3β), 63.6 (C-6α); 1,6-di-O-ester **14c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 2.95 (α-CH), 3.12 (H-1α), 3.38 (H-2α), 3.36 (H-2β), 3.55 (H-6β); ^{13}C NMR δ_{ppm} (125 MHz): 51.5 (α-CH), 63.6 (C-1α), 76.4 (C-2α), 76.5 (C-2β).

2.5.6. L-Alanyl-D-arabinose 15

UV (H_2O, λ_{max}): 226.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{200.0}$, 1584.9 M^{-1}), 276.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{295.0}$, 933.3 M^{-1}); IR (stretching frequency): 3397 cm^{-1} (NH), 3443 cm^{-1} (OH), 2940 cm^{-1} (CH), 1629 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = +34.0°; mass, 219 [M − 2]$^+$ and 293 [M + 1]$^+$; 2D HSQCT (DMSO-d_6) 2-O-ester **15a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.15 (α-CH), 1.22 (β-CH$_3$), 5.0 (H-1α), 4.93 (H-1β), 3.60 (H-2α), 3.15 (H-3α), 3.35 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 48.2 (α-CH), 17.2 (β-CH$_3$), 172.5 (CO), 96.0 (C-1α), 102.0 (C-1β), 77.8 (C-2α), 72.0 (C-3α), 63.5 (C-5α); 5-O-ester **15b**: ^{1}H NMR δ_{ppm} (500.13 MHz) 3.92 (α-CH), 1.20 (β-CH$_3$), 4.30 (H-1α), 4.18 (H-1β), 3.35 (H-2α), 3.25 (H-3α), 3.60 (H-4α), 3.60 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 48.2 (α-CH), 16.0 (β-CH$_3$), 97.1 (C-1α), 104.0 (C-1β), 72.9 (C-2α), 72.0 (C-3α), 67.8 (C-4α), 65.1 (C-5α); 2,5-di-O-ester **15c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 1.30 (β-CH$_3$), 3.45 (H-2α), 3.30 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 17.2 (β-CH$_3$), 76.9 (C-2α), 65.1 (C-5α).

2.5.7. L-Alanyl-D-ribose 16

UV (H_2O, λ_{max}): 224.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{224.0}$, 3801.9 M^{-1}), 294.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{294.0}$, 1288.2 M^{-1}); IR (stretching frequency): 3402 cm^{-1} (NH), 3242 cm^{-1} (OH), 2887 cm^{-1} (CH), 1625 cm^{-1} (CO); optical rotation (c 0.5, H_2O): $[\alpha]_D$ at 25 °C = +22.0°; mass, 221 [M]$^+$; ACE activity: IC$_{50}$ value = 2.7 ± 0.3 mM; 2D HSQCT (DMSO-d_6) 3-O-ester **16a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 1.25 (α-CH), 3.12 (β-CH$_3$), 3.50 (H-2α), 3.67 (H-3α), 3.63 (H-4α), 3.64 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 48.2 (α-CH), 15.9 (β-CH$_3$), 67.2 (C-2α), 75.7 (C-3α), 68.1 (C-4α), 60.6 (C-5α); 5-O-ester **16b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.39 (α-CH), 1.25 (β-CH$_3$), 4.95 (H-1α), 4.20 (H-1β), 3.27 (H-3α), 3.88 (H-4α), 3.61 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 53.0 (α-CH), 18.5 (β-CH$_3$), 173.5 (CO),

Table 1
Effect of lipase concentration on the synthesis of L-alanyl-D-glucose[a]

Lipase concentration (%, w/w D-glucose)	Yield (%) (mmol)		
	Rhizomucor miehei lipase (RML)[b]	Porcine pancreas lipase (PPL)[c]	*Rhizomucor miehei* lipase (RML)[d]
10	2 (0.04)	14 (0.27)	7 (0.14)
20	1 (0.02)	15 (0.29)	10 (0.21)
30	3 (0.05)	17 (0.35)	26 (0.53)
40	9 (0.17)	18 (0.36)	30 (0.60)
50	18 (0.37)	15 (0.29)	22 (0.44)

[a] Conversion yields from HPLC determined with respect to L-alanine. Error in yield measurements will be ±10–15%. This applies to all the yields given in the subsequent tables also. D-Glucose, 1 mmol and L-alanine, 2 mmol.

[b] Solvent—CHCl₃:hexane:DMF (v/v/v, 45:45:10) at 60 °C.

[c] Solvent—CH₂Cl₂:DMF (v/v, 90:10) at 40 °C.

[d] Carried out in presence of buffer with 100 ml of solvent system b containing 0.1 mM (0.1 ml) acetate buffer pH 4.0.

101.6 (C-1α), 103.9 (C-1β), 75.0 (C-3α), 71.0 (C-4α), 63.4 (C-5α); 3,5-di-*O*-ester **16c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 1.20 (β-CH₃), 3.45 (H-3α), 3.79 (H-4α), 3.52 (H-5α); ^{13}C NMR δ_{ppm} (125 MHz): 18.5 (β-CH₃), 74.9 (C-3α), 63.4 (C-5α).

2.5.8. L-Alanyl-lactose 17

UV (H₂O, λ_{max}): 220.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{220.0}$, 436.5 M^{-1}), 294.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{294.0}$, 239.9 M^{-1}); IR (stretching frequency): 3378 cm^{-1} (NH), 3378 cm^{-1} (OH), 2946 cm^{-1} (CH), 1624 cm^{-1} (CO); optical rotation (c 0.5, H₂O): $[\alpha]_D$ at 25 °C = +7.4°; mass, 436 $[M + Na]^+$; ACE activity: IC$_{50}$ value = 2.0 ± 0.2 mM; 2D HSQCT (DMSO-d₆) 6-*O*-ester **17a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.55 (α-CH), 1.25 (β-CH₃), 4.78 (H-1α), 4.82 (H-1β), 2.95 (H-2α), 2.95 (H-3α), 4.05 (H-4α,β), 3.15 (H-5α), 3.35 (H-5β), 3.80 (H-6α,β), 4.90 (H'-1β), 3.90 (H'-2), 2.85 (H'-3), 3.70 (H'-4), 3.60 (H'-5), 3.40 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 51.0 (α-CH), 15.5 (β-CH₃), 173.0 (CO), 98.0 (C-1α), 100.2 (C-1β), 70.3 (C-2α), 72.4 (C-2β), 74.3 (C-3α), 81.0 (C-4α,β), 73.3 (C-5α), 73.4 (C-5β), 61.2 (C-6α,β), 100.2 (C'-1β), 76.5 (C'2), 75.1 (C'3), 68.5 (C'4), 78.5 (C'5), 60.6 (C'6). 6'-*O*-ester **17b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.35 (α-CH), 3.85 (H-4α), 3.70 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 53.5 (α-CH), 81.5 (C-4α), 64.0 (C'6). 6,6'-di-*O*-ester **17c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.85 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 67.5 (C'6).

2.5.9. L-Alanyl-maltose 18

UV (H₂O, λ_{max}): 228.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{228.0}$, 114.8 M^{-1}), 294.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{294.0}$, 56.2 M^{-1}); IR (stretching frequency): 3283 cm^{-1} (NH), 3380 cm^{-1} (OH), 2937 cm^{-1} (CH), 1626 cm^{-1} (CO); optical rotation (c 0.5, H₂O): $[\alpha]_D$ at 25 °C = +84.0°; mass, 436 $[M + Na]^+$; 2D HSQCT (DMSO-d₆) 6-*O*-ester **18a**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.55 (α-CH), 1.25 (β-CH₃), 4.80 (H-1α), 4.20 (H-1β), 4.05 (H-2α,β), 3.30 (H-3α), 3.85 (H-4α,β), 3.65 (H-5α,β), 3.50 (H-6α,β), 4.90 (H'-1α), 2.95 (H'-2), 3.10 (H'-3), 3.50 (H'-4), 3.60 (H'-5), 3.60 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 50.0 (α-CH), 175.5 (CO), 92.0 (C-1α), 96.7 (C-1β), 79.7 (C-2α,β), 76.4 (C-3α), 81.5 (C-4α,β), 77.2 (C-5α,β), 67.5 (C-6α,β), 100.7 (C'-1α), 70.3 (C'2), 71.8 (C'3), 69.9 (C'4), 72.4 (C'5), 60.6 (C'6); 6'-*O*-ester **18b**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.35 (α-CH), 3.95 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 53.0 (αCH), 67.0 (C'-6α); 6,6'-di-*O*-ester **18c**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.75 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 63.0 (C'6).

2.5.10. L-Alanyl-sucrose 19

UV (H₂O, λ_{max}): 224.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{224.0}$, 2344.2 M^{-1}), 294.0 nm ($n \rightarrow \pi^*$ $\varepsilon_{294.0}$, 1288.2 M^{-1}); IR (stretching frequency): 3100 cm^{-1} (NH), 3319 cm^{-1} (OH), 2958 cm^{-1} (CH), 1625 cm^{-1} (CO); optical rotation (c 0.5, H₂O): $[\alpha]_D$ at 25 °C = −17.4°; mass, 436 $[M + Na]^+$; 2D HSQCT (DMSO-d₆) 6-*O*-ester **19**: ^{1}H NMR δ_{ppm} (500.13 MHz): 3.59 (α-CH), 1.30 (β-CH₃), 3.41 (H-1β), 3.79 (H-3β), 3.54 (H-4β), 3.47 (H-5β), 3.67 (H-6β), 4.35 (H'-1α), 3.31 (H'-2), 3.45 (H'-3), 3.31 (H'-4), 3.58 (H'-5), 3.54 (H'-6); ^{13}C NMR δ_{ppm} (125 MHz): 49.5 (α-CH), 18.0 (β-CH₃), 172.0 (CO), 64.0 (C-1β), 104.2 (C-2β), 77.33 (C-3β), 82.71 (C-4β), 74.5 (C-5β), 67.0 (C-6β), 96.0 (C'-1α), 71.0 (C'2), 75.5 (C'3), 71.0 (C'4), 74.0 (C'5), 60.71 (C'6).

3. Results

3.1. L-Alanyl-D-glucose

The esterification reaction between unprotected and unactivated L-alanine and D-glucose was studied in detail using RML and PPL.

3.1.1. Reaction profile

In presence of RML, the conversion yields showed an increase in esterification from 15% at 24 h to 26% at 72 h and thereafter remained constant up to 120 h at 26%. From the initial slope value of the plot, the rate of esterification was found to be 0.004 mmol h^{-1}.

3.1.2. Effect of lipase concentration

In case of RML (Table 1), maximum esterification (18%) was achieved at 50% (w/w) D-glucose (1 mmol D-glucose and 2 mmol L-alanine for incubation up to 72 h). In case of PPL (Table 1), maximum yield of 18% was achieved at 40% (w/w) D-glucose (1 mmol D-glucose and 2 mmol L-alanine for a incubation period of 72 h). In presence of 0.1 mM (0.1 ml) pH 4.0 acetate buffer, RML at 40% (w/w) D-glucose showed (Table 1) a maximum esterification of 30% (for 1 mmol D-glucose and 2 mmol L-alanine for incubation up to 72 h). While lesser amounts of enzymes (≤20% RML and PPL employed) could be inhibited by L-alanine (2 mmol) and D-glucose (1 mmol), >30% RML and PPL could favour better conversion as the enzyme/substrate ratio is enhanced at >30% enzyme concentration for the L-alanine and D-glucose employed.

3.1.3. Effect of buffer salts

Carrying out this esterification reaction in presence of buffers of certain pH not only imparted 'pH memory' to the enzyme, but also provided the optimum water activity necessary for better performance of the enzyme. Besides, addition of buffer salts of certain concentration also affected ionic activities of especially the microaqueous layer around the enzyme, where the buffer salts are concentrated during the course of the reaction. All these have been found to be operative in these esterification reactions.

Table 2
Effect of buffer salts (pH and buffer concentration) on the synthesis of L-alanyl-D-glucose[a]

RML				PPL			
pH[b]	Yield (%) (mmol)	pH 4.0[c] concentration (Mm)	Yield (%) (mmol)	pH[d]	Yield (%) (mmol)	pH 5.0[e] concentration (mM)	Yield (%) (mmol)
4.0	26 (0.53)	0.05	25 (0.49)	4.0	9 (0.18)	0.05	13 (0.25)
5.0	7 (0.13)	0.1	26 (0.53)	5.0	17 (0.33)	0.1	17 (0.33)
6.0	20 (0.39)	0.2	8 (0.16)	6.0	15 (0.31)	0.2	11 (0.23)
7.0	8 (0.16)	0.3	7 (0.15)	7.0	11 (0.23)	0.3	10 (0.21)
8.0	10 (0.19)	0.4	17 (0.34)	8.0	No yield	0.4	12 (0.24)
–	–	0.5	18 (0.35)	–	–	0.5	17 (0.34)

[a] D-Glucose, 1 mmol and L-alanine, 2 mmol; incubation period, 72 h; RML, 30% (w/w of D-glucose); PPL, 40% (w/w of D-glucose). One hundred millilitres of the solvent containing specified volumes, concentration and pH of the buffer.
[b] Solvent—100 ml CHCl$_3$:hexane:DMF (v/v/v, 45:45:10) at 60 °C. Buffer, 0.1 mM (0.1 ml) of 0.1 M appropriate pH buffer.
[c] Solvent—100 ml CH$_2$Cl$_2$:DMF (v/v, 90:10) at 40 °C. Buffer, 0.05–0.5 ml of 0.1 M acetate pH 4.0.
[d] Same solvent system as c. Buffer, 0.1 mM (0.1 ml) of 0.1 M appropriate pH.
[e] Solvent—same solvent system as c. 0.05–0.5 ml of 0.1 M acetate pH 5.0.

In presence of buffer salts, conversion yield increased in case of RML. In presence of 0.1 mM (0.1 ml) pH 4.0 buffer, RML showed maximum esterification of 26% (at 1 mmol D-glucose and 2 mmol L-alanine for a period of 72 h, Table 2). However, PPL, in presence of 0.1 mM (0.1 ml) pH 5.0 buffer showed maximum esterification of 17% only (Table 2), similar to the conversion yield obtained in the absence of buffer salts.

The effect of buffer salt concentration was studied by using different concentrations (0.05–0.5 mM) of pH 4.0 buffer in case of RML and pH 5.0 buffer in case of PPL. In case of RML, the maximum conversion yield of 26% (at 1 mmol D-glucose and 2 mmol L-alanine for 72 h) was obtained when 0.1 mM (0.1 ml) pH 4.0 buffer was employed (Table 2). In case of PPL, 0.5 mM (0.5 ml) pH 5.0 buffer showed the maximum conversion yield (Table 2) of 17% (at 1 mmol D-glucose and 2 mmol L-alanine for 72 h incubation).

By imparting 'pH memory', the catalytic activity of the lyophilized substilisin Carlsberg in the pH range 5–11, in organic solvents like acetonitrile and 3-pentanone was reported to be enhanced [22]. The enzymatic activity of substilisin cross-linked crystals in anhydrous 3-pentanone was accelerated by the addition of organic soluble (a mixture of a suitable acid and sodium salt) buffers [22]. The enantioselectivity of *Candida antarctica* lipase B in organic media was increased by 'pH tuning' of the enzyme by the addition of certain buffer salts which altered the protonation state of the enzyme and selectively tuned enantioselectivity and catalytic activity [23]. Similarly, the present work also showed enhanced activity of RML in presence of buffer salts. However, buffer salts did not enhance esterification with PPL.

3.1.4. Effect of substrate concentration

When L-alanine was varied from 1 to 5 mmol at a constant 1 mmol D-glucose, there was in general, an increase in esterification (Fig. 1) from 0.68 mmol (68% with respect to 1 mmol L-alanine—0.54 mmol monoesters and 0.14 mmol diesters) to 1.12 mmol (28% with respect to 4 mmol L-alanine—0.88 mmol monoesters and 0.24 mmol diesters) in presence of 0.1 mM
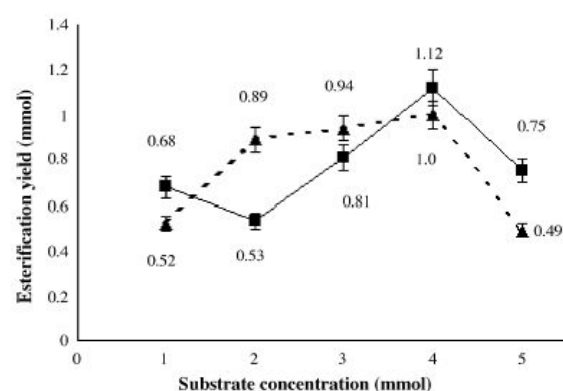
Fig. 1. Effect of substrate concentration on esterification. RML, 30% (w/w D-glucose); solvent, CH$_2$Cl$_2$:DMF (v/v, 90:10) at 40 °C; buffer, 0.1 mM (0.1 ml) pH 4.0 acetate buffer. L-Alanine (■) 1–5 mmol at 1 mmol D-glucose; D-glucose (▲) 1–5 mmol at 1 mmol L-alanine and a constant enzyme concentration of 54 mg.

(0.1 ml) pH 4.0 buffer for 72 h incubation. The yields were determined with respect to L-alanine concentrations which were usually greater than that of D-glucose (1 mmol). In terms of D-glucose, one D-glucose molecule forms mono as well as diesters with L-alanine. Hence, in presence of higher equivalents of L-alanine, yields >1 mmol (D-glucose concentration) are reported. Similarly, when D-glucose was varied from 1 to 5 mmol, at a constant 1 mmol L-alanine, there was a steep increase in esterification from 52% (0.52–0.41 mmol monoesters and 0.11 mmol diesters) at 1 mmol D-glucose to >99% (>0.99–0.78 mmol monoesters and 0.21 mmol diesters) at 4 mmol D-glucose. In both the cases, esterification decreased after 4 equiv. which could be due to inhibition at higher concentrations of L-alanine and D-glucose.

3.1.5. Reusability of lipases

Reusability of both RML and PPL employed were studied at optimized conditions. After completion of each reaction

Table 3
Reusability of lipase in presence and absence of buffer salts[a]

Number of reactions (cycles)	RML		PPL	
	Yield (%) (total enzyme activity, μmol/min)	Yield[b] (%) (total enzyme activity, μmol/min)	Yield (%) (total enzyme activity, μmol/min)	Yield[c] (%) (total enzyme activity, μmol/min)
1	24 (99.4)	17 (99.4)	19 (15)	11 (15)
2	17 (66.4)	11 (66.9)	12 (5.6)	6 (4.1)
3	10 (43.1)	8 (42.2)	10 (3.3)	–
4	8 (23.5)	5 (24.6)	3 (0.7)	–

[a] D-glucose, 4 mmol and L-alanine, 8 mmol; incubation period, 72 h; Solvent—100 ml CH$_2$Cl$_2$:DMF (v/v, 90:10) at 40 °C. Total enzyme activity of the recovered enzyme employed for the reaction. Percentage yields are an average from two independent experiments.

[b] In presence of 0.2 mM (0.2 ml) of 0.1 M acetate buffer pH 4.0 in each cycle.

[c] In presence of 0.2 mM (0.2 ml) of 0.1 M acetate buffer pH 5.0 in each cycle.

(cycle, 72 h), the enzyme was separated from the reaction mixture by filtration, air dried and reused in the next reaction. After each cycle, total esterification activity (μmol/min) of the enzyme was determined. In case of RML, there was a steady loss of 15–22% of enzyme concentration after each cycle both in presence as well as absence of 0.2 mM (0.2 ml) buffer pH 4.0 (Table 3). In the absence of buffer salts, the esterification activity decreased from 24% (1st cycle: total enzyme activity, 99.4 μmol/min) to 8% (4th cycle: total enzyme activity, 23.5 μmol/min). The yields in 2nd and 3rd cycles were 17% (total enzyme activity, 66.4 μmol/min) and 10% (total enzyme activity, 43.1 μmol/min), respectively. In presence of buffer salts (pH 4.0), the esterification activity decreased from 17% (1st cycle: total enzyme activity, 99.4 μmol/min) to 5% (4th cycle: enzyme activity of 23 μmol/min). The yields in 2nd and 3rd cycles were 11% (total enzyme activity, 66.9 μmol/min) and 8% (total enzyme activity, 42.2 μmol/min), respectively. However, in case of PPL, there was a drastic loss of enzyme concentration from 20% to 60% after each cycle, as PPL was partially soluble in water and the reaction was stopped after the 2nd cycle due to reduction in enzyme (Table 3). In the absence of buffer salts, esterification activity of the PPL decreased from 19% (1st cycle: total enzyme activity, 15 μmol/min) to 3% (4th cycle: total enzyme activity, 0.7 μmol/min). The 2nd and 3rd cycle yields were 12% (total enzyme activity, 5.6 μmol/min) and 10% (total enzyme activity, 3.3 μmol/min), respectively. However, in presence of buffer salts (pH 5.0), the esterification activity decreased slightly from 11% (1st cycle: total enzyme activity, 15 μmol/min) to 6% (2nd cycle: total enzyme activity, 4.1 μmol/min).

3.2. L-Alanyl esters of carbohydrates

L-Alanyl esters of different carbohydrates like aldohexoses (D-glucose 2, D-galactose 3 and D-mannose 4), ketohexose (D-fructose 5), pentoses (D-arabinose 6 and D-ribose 7) and disaccharides (lactose 8, maltose 9 and sucrose 10) were synthesized by refluxing the reaction mixture consisting of 2 mmol L-alanine (1), 1 mmol carbohydrate at 40% RML (w/w of carbohydrate 2–10) in presence of 0.1 mM (0.1 ml) pH 4.0 buffer in CH$_2$Cl$_2$:DMF (v/v, 90:10) for a period of 72 h. The esters were isolated and characterised as described in Section 2.3.

3.3. Spectral characterization of L-alanyl esters of carbohydrates

The isolated esters were characterized by UV, IR, MS and 2D NMR spectroscopic studies (data in Section 2.5). Infra red spectral data showed that the ester carbonyl stretching frequency for the prepared esters were in the range 1606–1653 cm^{-1} compared to 1715 cm^{-1} observed for free L-alanine indicating that L-alanine carboxylic group had been converted into its corresponding carbohydrate ester.

Two-dimensional HSQCT NMR spectroscopy of the L-alanyl esters of carbohydrates prepared by using RML gave good information on the nature and proportion of esters formed (Table 4). In case of L-alanyl-D-glucose (11) three monoesters (2-O-, 3-O- and 6-O-) and two diesters (2,6-di-O- and 3,6-di-O-) were found to be formed with only β-anomer of D-glucose, the D-glucose employed being a 40:60 mixture of α- and β-anomers, respectively. The down field chemical shift values for C1 at 102.6 (2-O-ester), 101.8 (6-O-ester) and 100.8 (2,6-di-O-ester) clearly indicated that only β-D-glucose was esterified. The chemical shift values for C-2β at 82.6 ppm and the corresponding H-2β cross-peak at 3.62 ppm indicated the formation of 2-O-ester, C-3β at 83.3 ppm (H-3β at 3.93 ppm) indicated the formation of 3-O-ester, C-6β at 63.5 ppm (H-6β at 3.82 ppm) indicated the formation of 6-O-ester, C-6β at 62.7 ppm (H-6β at 3.47 ppm) and C-2β at 76.5 ppm (H-2β at 3.78 ppm) indicated the formation of 2,6-di-O-ester and the chemical shift values for C-6β at 63.1 ppm (H-6β at 3.82 ppm) and C-3β at 81.6 ppm (H-3β at 3.78 ppm) indicated the formation of 3,6-di-O-ester. In case of L-alanyl-D-galactose (12), three diastereomeric monoesters (2-O-, 3-O- and 6-O-) only were found to be formed. The down field chemical shift values for C-2α at 76.4 ppm (H-2α at 3.38 ppm) and C-2β at 76.5 ppm (H-2β at 3.36 ppm) indicated the formation of 2-O-ester, C-3α at 81.6 ppm (H-3α at 3.75 ppm) and C-3β at 82.6 ppm (H-3β at 3.60 ppm) indicated the formation of 3-O-ester and C-6α at 63.1 ppm (H-6α at 3.30 ppm) indicated the formation of 6-O-ester. In case of L-alanyl-D-mannose (13), five diastereomeric esters (three monoesters—3-O-, 4-O- and 6-O- and two diesters—3,6-di-O- and 4,6-di-O-) were found to be formed. The chemical shift values for C-3α at 89.2 ppm indicated the formation of 3-O-ester, C-4β at 75.32 ppm indicated the formation of 4-O-ester, C-6α at 63.1 ppm indicated the for-

Table 4
Synthesis of L-alanyl esters of aldohexoses, ketohexose, pentoses and disaccharides[a]
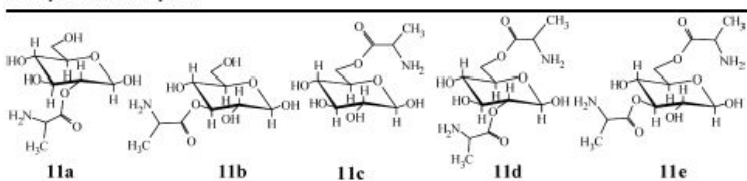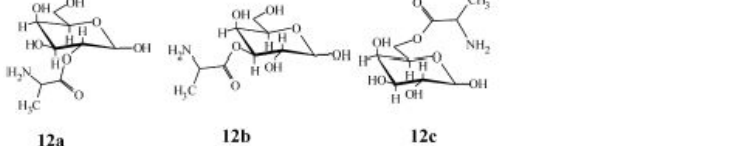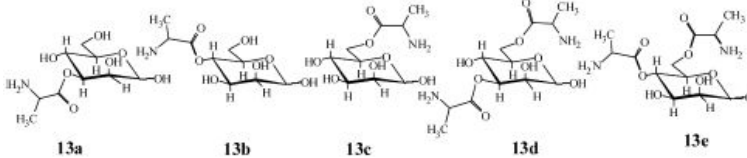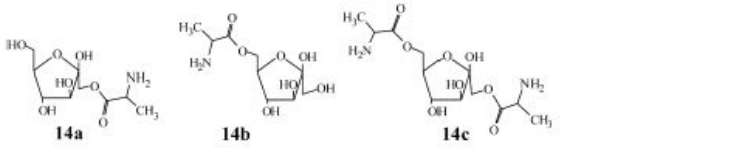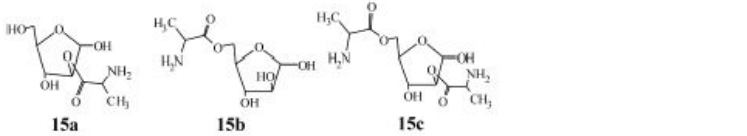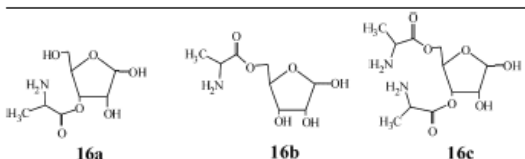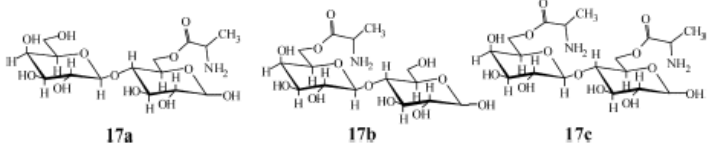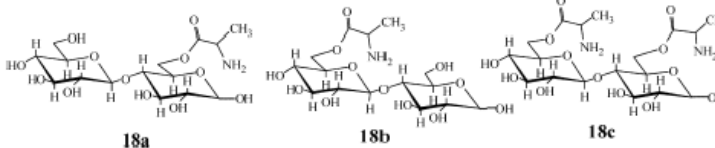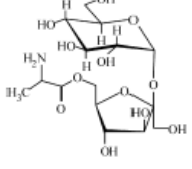
L-Alanyl esters of carbohydrates	Esterification yield (%)	Esters (% proportions[b])
11a **11b** **11c** **11d** **11e**	30 (monoesters, 2, diesters, 6)	11a: 2-O-L-alanyl-β-D-glucose (47) 11b: 3-O-L-alanyl-β-D-glucose (12) 11c: 6-O-L-alanyl-β-D-glucose (20) 11d: 2,6-di-O-L-alanyl-β-D-glucose (15) 11e: 3,6-di-O-L-alanyl-β-D-glucose (6)
12a **12b** **12c**	21 (only monoesters)	12a: 2-O-L-alanyl-D-galactose (33) 12b: 3-O-L-alanyl-D-galactose (32) 12c: 6-O-L-alanyl-D-galactose (35)
13a **13b** **13c** **13d** **13e**	49 (monoesters, 39, diesters, 10)	13a: 3-O-L-alanyl-D-mannose (25) 13b: 4-O-L-alanyl-D-mannose (25) 13c: 6-O-L-alanyl-D-mannose (30) 13d: 3,6-di-O-L-alanyl-D-mannose (9) 13e: 4,6-di-O-L-alanyl-D-mannose (11)
14a **14b** **14c**	52 (monoesters, 35, diester, 17)	14a: 1-O-L-alanyl-D-fructose (34) 14b: 6-O-L-alanyl-D-fructose (34) 14c: 1,6-di-O-L-alanyl-D-fructose (32)
15a **15b** **15c**	9 (monoesters, 6, diester, 3)	15a: 2-O-L-alanyl-D-arabinose (33) 15b: 5-O-L-alanyl-D-arabinose (34) 15c: 2,5-di-O-L-alanyl-D-arabinose (33)

Table 4 (*Continued*)

L-Alanyl esters of carbohydrates	Esterification yield (%)	Esters (% proportions[b])
16a　**16b**　**16c**	48 (monoesters, 23, diester, 25)	16a: 3-*O*-L-alanyl-D-ribose (16) 16b: 5-*O*-L-alanyl-D-ribose (32) 16c: 3,5-di-*O*-L-alanyl-D-ribose (52)
17a　**17b**　**17c**	20 (monoesters, 14, diester, 6)	17a: 6-*O*-L-alanyl-lactose (34) 17b: 6′-*O*-L-alanyl-lactose (34) 17c: 6,6′-di-*O*-L-alanyl-lactose (32)
18a　**18b**　**18c**	56 (monoesters, 38, diester, 18)	18a: 6-*O*-L-alanyl-maltose (34) 18b: 6′-*O*-L-alanyl-maltose (34) 18c: 6,6′-di-*O*-L-alanyl-maltose (32)
19	8 (only monoester)	19: 6-*O*-L-alanyl-sucrose

[a] L-Alanine, 2 mmol; carbohydrates, 1 mmol; RML, 40% (w/w based on carbohydrate); buffer, 0.1 mM (0.1 ml) 0.1 M pH 4.0 acetate buffer; CH$_2$Cl$_2$:DMF (v/v, 90:10) at 40 °C; incubation period, 72 h. Conversion yields were from HPLC with respect to L-alanine concentration.

[b] Percentage proportions of individual esters determined from the peak areas of the ^{13}C C6, C5 (in case of pentoses) signals or from cross peaks of the 2D HSQCT spectrum.

mation of 6-*O*-ester, and C-6α at 62.8 ppm, C-3α at 82.9 ppm indicated the formation of 3,6-di-*O*-ester and the chemical shift values for C-6α at 62.5 ppm and C-4α at 77.2 ppm indicated the formation of 4,6-di-*O*-ester. In case of L-alanyl-D-fructose (**14**), two diastereomeric monoesters (1-*O*- and 6-*O*-) and one diastereomeric diester (1,6-di-*O*-) were found to be formed. The down field chemical shift values for C-1α at 63.1 ppm (H-1α at 3.40 ppm) indicated the formation of 1-*O*-ester, C-6α at 63.6 ppm (H-6α at 3.30 ppm) indicated the formation of 6-*O*-ester and C-1, 6α centered at 63.6 ppm (H-1, 6α centered at 3.12 ppm) indicated the formation of 1,6-di-*O*-ester. In case of L-alanyl-D-arabinose (**15**), two diastereomeric monoesters (2-*O*- and 6-*O*-) and one diastereomeric diester (2,6-di-*O*-) were found to be formed. The chemical shift values for C-2α at 77.8 ppm (H-2α at 3.60 ppm) indicated the formation of 2-*O*-ester, C-5α at 65.1 ppm (H-5α at 3.60 ppm) indicated the formation of 5-*O*-ester and C-2α at 76.9 ppm (H-2α at 3.45 ppm) and C-5α at 65.1 ppm (H-5α at 3.30 ppm) indicated the formation of 2,5-di-*O*-ester. Both lactose and maltose showed the formation of two diastereomeric monoesters (6-*O*- and 6′-*O*-) and one diester (6,6′-di-*O*-). In case of L-alanyl-D-ribose (**16**), two diastereomeric monoesters (3-*O*- and 6-*O*-) and one diastereomeric diester (3,6-di-*O*-) were found to be formed. The chemical shift values for C-3α at 75.7 ppm (H-3α at 3.67 ppm) indicated the formation of 3-*O*-ester, C-5α at 63.4 ppm (H-5α at 3.61 ppm) indicated the formation of 5-*O*-ester and C-3α at 74.9 ppm (H-3α at 3.45 ppm) and C-5α at 63.4 ppm (H-5α at 3.52 ppm) indicated the formation of 3,5-di-*O*-ester. In case of L-alanyl-lactose (**17**), the down field chemical shift values for C-6α,β at 61.2 ppm (H-6α,β at 3.80 ppm) of glucose moiety indicated the formation of 6-*O*-ester, C-6′ at 64.0 ppm (H-6′ at 3.70 ppm) of galactose moiety indicated the formation of 6′-*O*-ester and C-6,6′ centered at 67.5 ppm (H-6,6′ centered at 3.85 ppm) of both galactose and glucose moieties indicated the formation of 6,6′-di-*O*-ester. In case of L-alanyl-maltose (**18**), the chemical shift values for C-6α,β at 67.5 ppm (H-6α,β at 3.50 ppm) of reducing end glucose moiety indicated the formation of 6-*O*-ester, C-6′ at 67.0 ppm (H-6′ at 3.95 ppm) of the non-reducing end glucose moiety indicated the formation of 6′-*O*-ester and C-6,6′ centered at 63.0 ppm (H-6,6′ centered at 3.75 ppm) of both the reducing and non-reducing end glucose moieties indicated the formation of 6,6′-di-*O*-ester. In case of L-alanyl-sucrose (**19**), only one monoester (6-*O*-) was formed. This was confirmed by the chemical shift values for C-6β at 67.0 ppm (H-6β at 3.67 ppm) of the fructose moiety. In all these esters, shifts in L-alanyl signals, α-CH and β-CH₃, also indicated esterification of L-alanine clearly.

4. Discussion

The optimum conditions determined for this esterification reaction by studying the effect of variables like incubation period, enzyme and substrate concentration, pH and buffer concentration clearly explain the behaviour of the lipases. Most of the effects show that esterification increases upto a certain point, and thereafter they remain as such or decrease a little. This complex esterification reaction is not controlled by kinetic factors or thermodynamic factors or water activity alone.

Use of lower enzyme concentrations did not result in thermodynamic yields. The thermodynamic binding equilibria regulates the concentrations of the unbound substrates at different enzyme and substrate concentrations and thereby conversion as the reaction proceeds with time. At lesser enzyme concentrations, for a given amount of substrates (enzyme/substrate ratio low), rapid exchange between bound and unbound forms of both the substrates with the enzyme (on a weighted average based on binding constant values of both the substrates) leaves substantial number of unbound substrate molecules at the start of the reaction and they decrease progressively as conversion takes place [27,28]. This becomes more so, if one of them binds more firmly to the enzyme than the other (higher binding constant value) as the respective enzyme/substrate ratios keep changing (during the course of the reaction) unevenly till the conversion stops due to total predominant binding (inhibition). At intermediatory enzyme concentrations, such a competitive binding results in a favourable proportions of bound and unbound substrates to effect quite a good conversion. At higher enzyme concentrations, most of the substrates would be in the bound form leading to inhibition and lesser conversion (higher enzyme/substrate ratios). Also, the esterification reaction requires larger amount of enzyme compared to hydrolysis. While this leads to lesser selectivity, they also give rise to varying bound and unbound substrate concentrations till the conversion ends. For a given amount of enzyme and substrates there is no increase in conversion beyond 72–120 h. Longer incubation periods of especially lesser enzyme concentrations could also result in partial enzyme inactivation as shown in Table 3. However, not all the enzyme are inactivated before the end of the reaction.

Besides imparting 'pH memory', added water is essential for the integrity of the three-dimensional structure of the enzyme molecule and therefore its activity [1]. Zaks and Klibanov [29] reported that at low water activities, lower the solvent polarity, the higher the enzyme activity. Beyond the critical water concentration, esterification decreases because the size of the water layer formed around the enzyme retards the transfer of acyl donor to the active site of the enzyme [30,31] and also the water layer surrounding the enzymes makes enzyme to be more flexible by forming multiple H-bonds and interacting with organic solvent causing denaturation [32]. Increase in buffer volume affected this esterification reaction significantly. It could increase the water activity of the system in the initial stages by increasing the thickness of the microaqueous layer around the enzyme. Higher volumes of the buffer in the microaqueous layer could also cause slight inactivation of the enzyme due to increase in salt concentration beyond a critical point. Partridge et al. reported that when an enzyme is suspended in a low-water organic solvent, the counter ions are in closer contact with the opposite charges on the enzyme because of the lower dielectric constant of the medium [33]. Thus, protonation of the ionizable groups on the enzyme could be controlled by the type and availability of these ions as well as hydrogen ions resulting in a 'pH memory'. The third factor is the increase in ionic strength which could play a favourable role in esterification. Optimum pH for this reaction (4.0 for RML and 5.0 for PPL) clearly indicates a slight unfavourable conformational change in the enzyme at

about pH 4.0–5.0 leading to lesser conversion beyond pH 5.0 for both the enzymes.

The experimental set-up employed in the present work is such that it maintained a low water activity ($a_w = 0.0054$) due to azeotropic distillation and recycling the solvent back into the reaction system after passing through a bed of desiccant. Even the water of reaction formed could also be used to constitute the microaqueous layer around the enzyme and the excess water could be removed by azeotropic distillation. The same could occur even with the addition of added enzyme (with little water content) and buffer volume. The added carbohydrate molecule could also reduce the water content of the reaction mixture. Adachi and Koyayashi [34] have reported that the hexose which is more hydrated decreased the water activity in the system and shifts the equilibrium towards synthesis. All these factors lead to maintenance of an equilibrium concentration of water around the enzyme all the time. Hence, thermodynamic binding equilibria interplayed by inactivation and inhibition along with maintenance of an optimum water activity could be governing this reaction as reflected by the extent of conversion under different reaction conditions of added buffer, enzyme and substrate concentrations.

As monosaccharides contain five hydroxyl groups, 31 diastereomeric esters (mono, di, tri, tetra and penta) are possible for both the anomers. In case of L-alanyl-D-glucose (**11**) only 6-*O*- was the major ester produced (47%). While most of the carbohydrates showed 6-*O*- and 5-*O*-esters as major products, D-ribose formed 3,5-di-*O*-ester as the major product. Hence, primary hydroxyl groups invariably formed esters in major proportions. Very low conversions (<10%) were detected for D-arabinose and sucrose. However, D-ribose exhibited 48% conversion. Among the secondary hydroxyl groups of carbohydrates, 2-*O*- and 3-*O*-esters were found to be prominent than 1-*O*- or 4-*O*-esters. Among the secondary hydroxyl groups, 4-*O*-ester was formed only in case of D-mannose. Out of eleven carbohydrates employed, carbohydrate alcohols such as D-sorbitol and D-mannitol were not esterified.

Commercial crude PPL preparations contain variety of estero-/lipo-lytic enzymes with low PPL concentrations [35,36] which could also perform facile esterification. Hence, a small amount of esters formed from esterases along with those of lipases in the present reaction cannot be ruled out. Since the reactions were carried out at a low temperature of 40–60 °C, the formation of peptide was less than 3%, even though unprotected L-alanine was used for the reaction. NMR data clearly indicated that no Maillard reaction occurred. Under these reaction conditions, formation of Maillard reaction products are quite likely. For instance, Maillard and Pictet-Spengler phenolic condensation products were reported in the reaction between phenolic amino acids and D-glucose in phosphate buffer at different pH from 5.0 to 9.0 at 90 °C [37]. Similarly Maillard products from the reaction between D-glucose and N^α-*t*-Boc-L-lysine incubated with aminoguanidine in pH 7.4 phosphate buffer at 70 °C was also reported [38]. No such Maillard reaction type products were detected by mass as well as NMR in the present investigation. RML and PPL showed significant esterification (up to 68%) when unprotected L-alanine was used. When *N*-acetyl-L-

alanine was used in the present work, both RML and PPL gave <5% yield. Riva et al. [14] have reported two monoesters (6-*O*- and 4-*O*-esters) and no diester for L-alanine, using subtilisin, a protease. Our present study has shown that comparable esterification yields to others could be achieved by employing PPL and RML instead of protease. Thus, our study clearly indicates that unprotected L-alanine could be used for esterification of carbohydrates.

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Inhibition of *Rhizomucor miehei* and *Candida rugosa* Lipases by D-Glucose in Esterification between L-Alanine and D-Glucose

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A detailed kinetic study of the esterification of D-glucose with L-alanine catalyzed by lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) showed that both lipases follow the Ping-Pong Bi-Bi mechanism, in which L-alanine and D-glucose bind in subsequent steps releasing water and L-alanyl-D-glucose, with competitive substrate inhibition by D-glucose at higher concentrations leading to the formation of dead-end lipase·D-glucose complexes. An attempt to obtain the best fit of this kinetic model through curve fitting yielded good approximates of the apparent values of four important kinetic parameters: for RML-k_{cat}=0.29±0.028×10^{-3} M h^{-1} mg^{-1}, $K_{m\,L\text{-alanine}}$= 4.9±0.51×10^{-3} M, $K_{m\,D\text{-glucose}}$=0.21±0.018×10^{-3} M, and $K_{i\,D\text{-glucose}}$=1.76±0.19×10^{-3} M; for CRL-k_{cat}= 0.75±0.08×10^{-3} M h^{-1} mg^{-1}, $K_{m\,L\text{-alanine}}$=56.2±5.7×10^{-3} M, $K_{m\,D\text{-glucose}}$=16.2±1.8×10^{-3} M, and $K_{i\,D\text{-glucose}}$ =21.0±1.9×10^{-3} M.

[Key words: L-alanyl-D-glucose, *Candida rugosa* lipase, *Rhizomucor miehei* lipase, Ping-Pong Bi-Bi mechanism, competitive D-glucose inhibition, dead-end lipase·D-glucose complex, hydrogen-bonding interactions]

Lipases (EC 3.1.1.3) are enzymes, that cleave ester linkages of triacylglycerides releasing glycerol and fatty acids at the water/lipid interface. Lipase is a single-domain molecule that belongs to the family of α/β-hydrolase proteins (1, 2). Most of the lipases reported contain Ser-His-Asp/Glu catalytic triads in their active site (2) with the exception of esterases from *Streptomyces scabies*, which contain only Ser-144 and His-283 (3).

Kinetic studies of esterification (4–8), racemization (9) and hydrolysis (10) using lipases have been performed. In some esterifications, lipases follow the Ping-Pong Bi-Bi mechanism (5, 11–13). This mechanism involves binding acid with alcohol in successive steps, which is followed by the release of water and the ester products in succession. The kinetic behavior of *Candida rugosa* lipase (CRL) in the esterification of long-chain fatty acids with alcohols (12) and tetrahydrofurfuryl alcohol with butyric acid (14) follows the Ping-Pong Bi-Bi mechanism, in which the binding of an acid leads to an acyl enzyme complex followed by the release of water molecules. The subsequent binding of an alcohol leads to the transfer of the acyl group to the alcohol, which results in ester formation. Thereafter, an ester product is released. *n*-Octanol is inhibitory to *Rhizomucor miehei* lipase (RML) and CRL in the transesterification between vinyl acetate and *n*-octanol (4) following the Ternary Complex Bi-Bi mechanism, in which *n*-octanol binds twice, once to bind to lipase to yield a dead-end lipase·*n*-octanol complex and a second molecule of *n*-octanol again binds to the above lipase to give another dead-end lipase·*n*-octanol complex.

For citronellyl laurate synthesis, RML follows the ordered Bi-Bi mechanism wherein β-citronellol binds to the enzyme to yield the β-citronellol·enzyme complex, which again binds to lauric acid to form the ternary enzyme·β-citronellol·lauric acid complex. Finally, it decomposes to give β-citronellyl laurate and water as products in this process (13). A series of dead-end RML·lauric acid complexes were also reported in this process.

In our previous investigation, we have reported an enzymatic synthesis of L-alanyl-D-glucose and other carbohydrate esters of L-alanine (15). In this work, the kinetics of the esterification between L-alanine and D-glucose to form L-alanyl-D-glucose with RML and CRL is carried out in which both lipases follow the Ping-Pong Bi-Bi mechanism with L-alanine and D-glucose binding. In subsequent steps, water is released followed by L-alanyl-D-glucose. A competitive substrate is inhibited by D-glucose at higher concentrations that leads to the formation of dead-end lipase·D-glucose complexes.

For the first time, we have shown that D-glucose could be inhibitory to both lipases at higher concentrations. The apparent values of the important kinetic parameters k_{cat}, $K_{m\,L\text{-alanine}}$, $K_{m\,D\text{-glucose}}$ and $K_{i\,D\text{-glucose}}$ are evaluated and compared between both lipases by graphical and curve-fitting procedures.

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MATERIALS AND METHODS

Enzyme and chemicals   The lipozyme IM20 (RML, immobilized on weak anion-exchange resin) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark), and CRL from Sigma Chemical (St. Louis, MO, USA). The esterification activities of RML and CRL were found to be 0.5 and 0.03 μmol min^{-1} (mg enzyme preparation)$^{-1}$, respectively, using 0.13 M butyric acid and 0.33 M n-butanol as substrates in heptane [16].

L-Alanine was purchased from Himedia Laboratories (Mumbai, India), and D-glucose from SD Fine Chemicals (Mumbai, India). HPLC-grade acetonitrile, dichloromethane and dimethylformamide were obtained from Qualigens Fine Chemicals (Mumbai, India). The other solvents employed were distilled once before use.

Kinetic experiments   Kinetic experiments were conducted by refluxing L-alanine and D-glucose along with 90 mg of RML or CRL in 100 ml of dichloromethane and dimethylformamide (v/v, 90:10) solvent mixture [15, 17, 18] containing 0.1 ml of 0.1 M sodium acetate buffer (pH 4.0) for RML or 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.0) for CRL. Unprotected and inactivated molecules of L-alanine and D-glucose were employed as substrates (Fig. 1). The temperature of the reaction mixture was maintained at the reflux temperature of dichloromethane (40°C). Experiments with RML were conducted by maintaining the concentration of one of the substrates constant in the range of 0.005–0.05 M and varying the concentration of the other in the same concentration range. With CRL, the concentration range employed was 0.005–0.1 M. Product workout involved distilling off the solvent, heating to denature the enzyme, stirring and filtering to remove the lipase. The filtrate was then evaporated to obtain a residue containing L-alanine, D-glucose and the ester. The residue was subjected to high performance liquid chromatography (HPLC) on a Shimadzu LC 10A (Shimadzu, Kyoto) using a C-18 column (LiChrosorb 100 Å, 5 μm, 25 cm×4.6 mm) with water/acetonitrile (80:20, v/v), as the mobile phase at a flow rate of 1 ml/min and monitored by a UV detector at 210 nm for L-alanyl-D-glucose. The retention times of L-alanine and L-alanyl-D-glucose were found to be 2.6 min and 3.4 min, respectively. No D-glucose was detected at 210 nm. The molar concentrations of the ester products formed were determined from the L-alanyl-D-glucose peak area with reference to that of free unreacted L-alanine in the reaction mixture. The error in yield measurement was within ±10%. The esters formed were separated by size exclusion chromatography using a Sephadex G-10 column, eluted with water, and subjected to spectral characterization by ultraviolet (UV), infrared (IR) and 2D heteronuclear single quantum coherence transfer-nuclear magnetic resonance (HSQCT-NMR) spectroscopies.

For the concentrations of D-glucose and L-alanine, individual experiments in duplicate (30×2 lipases) were performed for incubation periods of 3 h, 6 h, 12 h, 24 h and 36 h. Initial rate (specific reaction rate, v) was determined from the initial slope of the plot of the amount of esters formed (M) versus incubation period (h) and expressed as M h^{-1} (mg protein)$^{-1}$. R^2 obtained from least-squares analysis for the initial rate in each case was found to be within 0.88–0.95. Each plot shown in this work was constructed from all experimentally determined values; a few initial rates were obtained by curve-fitting.

Spectral characterization   A Shimadzu UV–1601 spectrophotometer was used for recording the UV spectra of isolated L-alanyl-D-glucose esters in aqueous solutions at 0.5 mM. A Nicolet 5700 FTIR instrument (Madison, WI, USA) was used for recording the IR spectra. The specific rotation of the isolated esters was measured at 25°C using a Perkin-Elmer 243 polarimeter (Überlingen, Germany) with a 0.5% aqueous solution of the esters. The mass spectra of the isolated esters were recorded using a Q-TOF Waters Ultima Instrument (Manchester, UK) fitted with an electron spray ionization (ESI) source. ^{1}H and ^{13}C NMR spectra (500.13 MHz for ^{1}H and 125 MHz for ^{13}C) were recorded on a Brüker DRX-500 MHz spectrophotometer (Fallanden, Switzerland). The proton and carbon 90° pulse widths were 10.5 and 12.25 μs, respectively. About 40 mg of the sample dissolved in DMSO-d_6 and D$_2$O was used for recording the spectra at 35°C. Chemical shift value was expressed in ppm relative to an internal tetramethylsilane standard within ±0.01 ppm.

In the NMR data, only resolvable signals are shown. Some assignments are interchangeable. Based on ^{13}C NMR data, the proportions of mono- and diesters produced, detected by measuring the peak areas of the C2, C3 and C6 signals, were found to be 2-O-ester, 20%; 3-O-ester, 12%; 6-O-ester, 47%; 2,6-di-O-ester, 15%; and 3,6-di-O-ester, 6% [15].

Since the polarities of the five esters (2-O-, 3-O-, 6-O-, 2,6-di-O- and 3,6-di-O-esters) formed were identical, all the esters eluted as a single peak at 3.4 min on HPLC. Attempts to further separate these esters through HPLC and other chromatographic procedures were unsuccessful. Only ^{13}C NMR gave a clear indication of the type and proportion of the esters. The down-field chemical shift values for C1 at 102.8 ppm (2-O-ester), 101.8 ppm (6-O-ester) and 100.8 ppm (2,6-di-O-ester) clearly indicate that only β-D-glucose was esterified.

L-Alanyl-β-D-glucose UV (H$_2$O, λ_{max}): 227.0 nm ($\sigma \rightarrow \sigma^*$ $\varepsilon_{227.0}$-1151 M^{-1}), 294.0 nm (n$\rightarrow \pi^*$ $\varepsilon_{294.0}$-764 M^{-1}); IR (KBr, stretching frequency): 3371 cm^{-1} (NH), 3410 cm^{-1} (OH), 2297 cm^{-1} (CH), 1653 cm^{-1} (CO); optical rotation: $[\alpha]_D = -38.1°$ (c 0.5, H$_2$O, 25°C); MS: m/z 274 [M+Na]$^+$; 2D HSQCT (DMSO-d_6)-2-O-ester: ^{1}H-NMR δppm: 2.95 (αCH), 1.07 (βCH$_3$), 3.62 (H-2β), 3.83 (H-3β), 3.67 (H-4β), 3.44 (H-6β), ^{13}C-NMR δppm: 52.1 (αCH), 15.7 (βCH$_3$), 102.8 (C1β), 82.6 (C2β), 77.9 (C3β), 68.8 (C4β), 60.5 (C6β); 3-O-ester: ^{1}H-NMR δppm: 2.87 (αCH), 3.93 (H-3β), 3.58 (H-4β), 3.36 (H-6β), ^{13}C-NMR δppm: 51.4 (αCH), 83.3 (C3β), 69.3 (C4β), 57.3 (C6β); 6-O-ester: ^{1}H-NMR δppm: 2.95 (αCH), 1.30 (βCH$_3$), 3.86 (H-2β), 3.76 (H-5β), 3.82 (H-6β), ^{13}C-NMR δppm: 50.2 (αCH), 15.1 (βCH$_3$), 171.4 (CO), 101.8 (C1β), 75.0 (C2β), 70.1 (C5β), 63.5 (C6β); 2,6-di-O-ester: ^{1}H-NMR δppm: 3.36 (αCH), 1.30 (βCH$_3$), 3.78 (H-2β), 3.47 (H-6β), ^{13}C-NMR δppm: 49.5 (αCH), 16.4 (βCH$_3$), 100.8 (C1β), 76.5 (C2β), 62.7 (C6β); 3,6-di-O-ester: ^{1}H-NMR δppm: 1.30 (βCH$_3$), 3.78 (H-3β), 3.82 (H-6β), ^{13}C-NMR δppm: 51.4 (αCH), 16.7 (βCH$_3$), 81.6 (C3β), 63.1 (C6β).

FIG. 1. Lipases-catalyzed synthesis of L-alanyl-β-D-glucose esters. 2-O-Ester: R$_2$=L-CH$_3$CH(NH$_2$)COO, R$_1$=R$_3$=OH; 3-O-ester: R$_3$=L-CH$_3$CH(NH$_2$)COO, R$_1$=R$_2$=OH; 6-O-ester: R$_1$=L-CH$_3$CH(NH$_2$)COO, R$_2$=R$_3$=OH; 2,6-di-O-ester: R$_2$=R$_1$=L-CH$_3$CH(NH$_2$)COO, R$_3$=OH; 3,6-di-O-ester: R$_3$=R$_1$=L-CH$_3$CH(NH$_2$)COO, R$_2$=OH.

RESULTS AND DISCUSSION

Because both RML and PPL showed good esterification potencies in L-alanyl-D-glucose synthesis, they were employed in this kinetic study to evaluate the salient features of the kinetic behavior of both these lipases in esterification.

To graphically evaluate the apparent values of the kinetic parameters, initial rate (specific reaction rate) was determined from the concentration of L-alanyl-D-glucose at different incubation periods, and typical time courses of RML and CRL-catalyzed reactions are shown in Fig. 3.

The initial rates (v) for RML were found to be in the range of 15–$176 \times 10^{-6}\,\mathrm{M\,h^{-1}}$ (mg protein)$^{-1}$. CRL experiments showed the initial rates to be in the range of 20–$460 \times 10^{-6}\,\mathrm{M\,h^{-1}}$ (mg protein)$^{-1}$. At the initial periods of incubation, the reaction is relatively fast owing to the shift in equilibrium towards esterification. The process slows down at incubation periods longer than 24 h, resulting in a stable steady-state equilibrium. The effects of external mass transfer phenomena-internal and external diffusions ([14], 19), if any, on the RML and CRL enzymes employed were not tested in this work.

Using initial rates, double reciprocal plots were constructed to graphically evaluate the apparent values of k_{cat}, $K_{m\,L\text{-alanine}}$, $K_{m\,D\text{-glucose}}$ and K_i: RML, Fig. 4A ($1/v$ versus $1/[\text{D-glucose}]$) and 4B ($1/v$ versus $1/[\text{L-alanine}]$); CRL, Fig. 5A ($1/v$ versus $1/[\text{D-glucose}]$) and 5B ($1/v$ versus $1/[\text{L-alanine}]$). Figure 6A shows a replot of the slopes from Fig. 4B (RML), and Fig. 6B shows a replot of the slopes from Fig. 5B (CRL). Figure 4A from RML reactions and Fig. 5A from CRL reactions show a series of curves obtained for different fixed concentrations of L-alanine for varying D-glucose concentration, in which slight increase in initial rates are observed at lower D-glucose concentrations. At higher D-glucose concentrations, the rates markedly decrease. Also, increasing L-alanine concentration increases the initial rates at all D-glucose concentrations. Figure 4B from RML reactions and Fig. 5B from CRL reactions show a series of parallel lines for different fixed low D-glucose concentrations at varying L-alanine concentration. The slopes of these lines change at higher D-glucose concentrations.

The plots in Figs. 4A, 4B, 5A and 5B show that the kinetics could be best described by the Ping-Pong Bi-Bi model, in which L-alanine and D-glucose bind in subsequent steps that release water and L-alanyl-D-glucose. This also happens in subsequent steps (Fig. 2) with competitive substrate inhi-
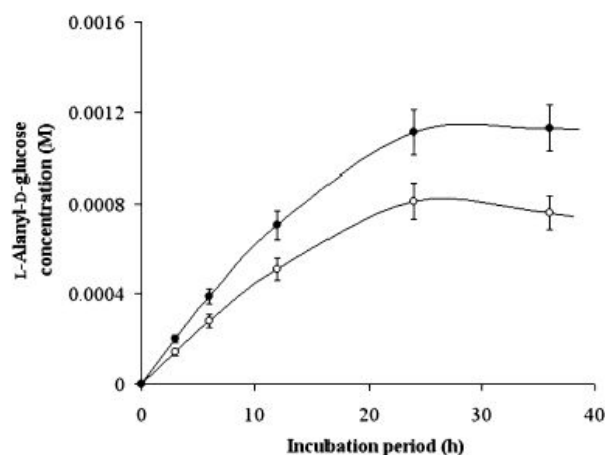

FIG. 3. Time courses of esterification reactions–concentration of L-alanyl-D-glucose versus incubation period (open circles, RML and closed circles, CRL). The RML enzyme (90 mg) or the CRL enzyme (90 mg) was reacted with 0.005 M L-alanine and 0.020 M D-glucose in 100 ml of mixture of dichloromethane/dimethylformamide (90:10, v/v) mixed with 0.1 ml of 0.1 M sodium acetate buffer (pH 4.0) for RML or 0.1 ml of 0.1 M sodium phosphate buffer (pH 7.0) for CRL.

bition that leads to dead-end inhibition ([20]). Both RML and CRL were found to be inhibited by D-glucose. This model could be described by the rate equation

$$\frac{V}{V_{max}} = \frac{[A][B]}{K_{mA}[B](1+[B]/K_i)+K_{mB}[A]+[A][B]} \quad (1)$$

where v is initial rate, V_{max} is maximum velocity, $[A]$ is L-alanine concentration, $[B]$ is D-glucose concentration, K_{mA} is Michaelis–Menten constant for the lipase·L-alanine complex, K_i is dissociation constant for the lipase·inhibitor (D-glucose) complex and K_{mB} is Michaelis–Menten constant for the lipase·D-glucose complex. Because the initial rates are in M h^{-1} (mg protein)$^{-1}$, V_{max} is expressed as $k_{cat}=V_{max}/$enzyme concentration.

The apparent values of the four important kinetic parameters $K_{i\,D\text{-glucose}}$, $K_{m\,L\text{-alanine}}$, $K_{m\,D\text{-glucose}}$ and k_{cat} were graphically evaluated. The intercepts of the positive slopes of the curves in Figs. 4A and 5A on the Y-axis, particularly, at the highest L-alanine concentration (0.05 M/0.1 M) employed, gave $1/k_{cat}$ for RML and CRL (Table 1). Figure 6A (RML) and 6B (CRL) shows a replot of the slopes from Figs. 4B and 5B versus D-glucose, respectively, for which slope $= K_{m\,L\text{-alanine}}/(k_{cat}K_i)$, Y intercept $= K_{m\,L\text{-alanine}}/k_{cat}$ and X intercept $= -K_i$, where K_i represents the dissociation constant for the lipase·D-glucose complex. $K_{m\,D\text{-glucose}}$ was obtained using Eq. 2 derived by rearranging Eq. 1.

$$K_{mB}=k_{cat}[B]/v - K_{mA}[B]/[A] - K_{mA}[B]^2/[A]K_i \quad (2)$$

Here, K_{mB} is Michaelis–Menten constant for the lipase·D-glucose complex.

To confirm that the kinetics of the RML- and CRL-catalyzed syntheses of L-alanyl-D-glucose follow the above-mentioned model, the apparent values of the four important kinetic parameters k_{cat}, K_i, K_{mA} and K_{mB} were also estimated through curve fitting using Eq. 1.

The range of values tested for these parameters and the
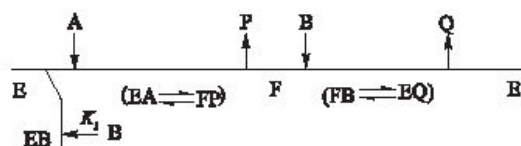
FIG. 2. Ping-Pong Bi-Bi mechanism of RML and CRL-catalyzed syntheses of L-alanyl-D-glucose. A, L-Alanine; P, H$_2$O; B, D-glucose; E, lipase-*Rhizomucor miehei* lipase/*Candida rugosa* lipase; F, lipase·L-alanyl complex; EA, lipase·L-alanine complex; FP, lipase·L-alanyl·water complex; EB, lipase·D-glucose complex; K_i, dissociation constant of lipase·D-glucose complex; FB, lipase·L-alanyl·D-glucose complex; EQ, lipase·L-alanyl-D-glucose complex and Q, L-alanyl-D-glucose.

FIG. 4. Double reciprocal plots for RML-catalyzed synthesis of L-alanyl-D-glucose. (A) $1/v$ versus $1/[$D-glucose$]$ plots; a series of curves show the effect of varying D-glucose concentration at different fixed L-alanine concentrations in the range of 0.005–0.05 M. The inset shows plots obtained by computer simulation for 0.0003 M and 0.0006 M L-alanine concentrations (open trapeziums, 0.0003 M; closed trapeziums, 0.0006 M; open circles, 0.005 M; closed circlse, 0.01 M; open triangles, 0.015 M; closed triangles, 0.02 M; open squares, 0.025 M; closed squares, 0.05 M). (B) $1/v$ versus $1/[$L-alanine$]$ plots, a series of lines show the effects of varying L-alanine concentration at different fixed D-glucose concentrations in the range of 0.005–0.025 M; plots shown for 0.0003 and 0.0006 M D-glucose concentrations are from computer simulation (open circles, 0.0003 M; closed circles, 0.0006 M; open triangles, 0.005 M; closed triangles, 0.01 M; open squares, 0.015 M; closed squares, 0.02 M; open trapeziums, 0.025 M).

constraints employed for the iteration procedure are: $k_{cat} < 1\ \mathrm{M\,h^{-1}\,mg^{-1}}$, $K_{\mathrm{iD\text{-}glucose}} > K_{\mathrm{mD\text{-}glucose}}$, $K_{\mathrm{mD\text{-}glucose}} < K_{\mathrm{mL\text{-}alanine}}$, and $K_{\mathrm{mL\text{-}alanine}} < 10\ \mathrm{M}$.

The iteration procedure for the curve fitting involved nonlinear optimization by minimizing the sum of squares of deviations between v_{exptl} and v_{pred}, such that the values of the four kinetic parameters mentioned above correspond to the best fit achieved.

Table 1 lists the graphical and curve fitted values for comparison. Table 2 shows a comparison between the experimental and predictive initial rates obtained under different reaction conditions. Although the computer-simulated v_{pred} values showed R^2 values of 0.84 for RML and 0.86 for

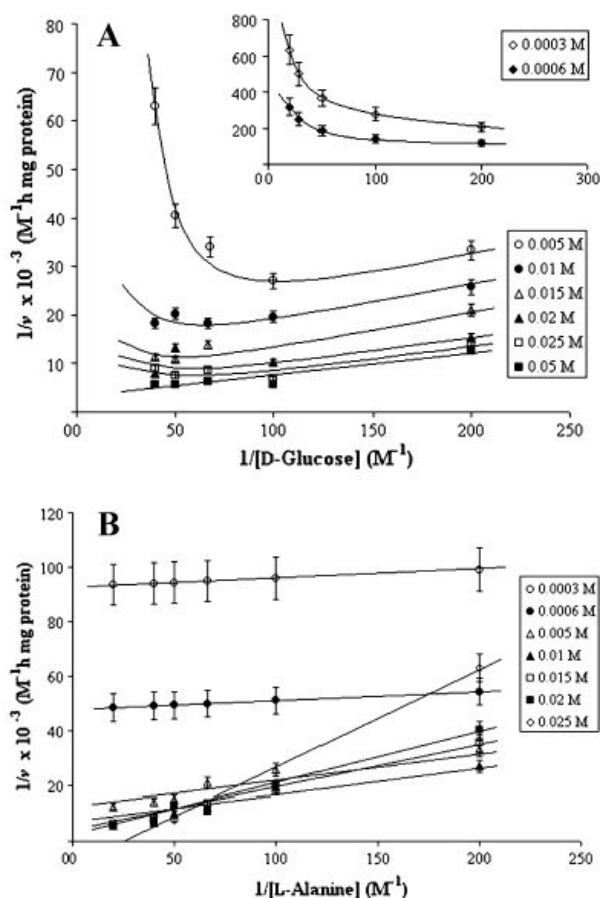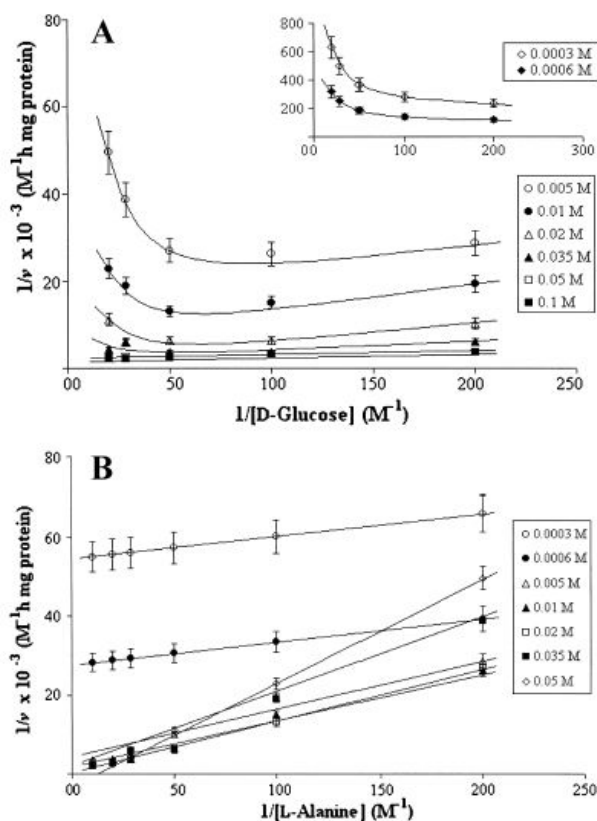FIG. 5. Double reciprocal plots for CRL-catalyzed synthesis of L-alanyl-D-glucose. (A) $1/v$ versus $1/[$D-glucose$]$ plots; a series of plots show the effect of varying D-glucose concentration at different fixed L-alanine concentrations in the range of 0.005–0.1 M; the plots shown for 0.0003 and 0.0006 M L-alanine concentrations are from computer simulation (open trapeziums, 0.0003 M; closed trapeziums, 0.0006 M; open circles, 0.005 M; closed circles, 0.01 M; open triangles, 0.02 M; closed triangles, 0.035 M; open squares, 0.05 M; closed squares, 0.1 M). (B) $1/v$ versus $1/[$L-alanine$]$ plots, a series of plots show the effect of varying L-alanine concentrations at different fixed D-glucose concentrations in the range of 0.005–0.05 M. The plots shown for 0.0003 and 0.0006 M D-glucose concentrations are from computer simulation (open circles, 0.0003 M; closed circles, 0.0006 M; open triangles, 0.005 M; closed triangles, 0.01 M; open squares, 0.02 M; closed squares, 0.035 M; open trapeziums, 0.05 M).

CRL, the discrepancy between v_{exptl} and v_{pred} appeared to be significant at several substrate concentrations. This could be due to (i) the constraints employed in the iteration (curve fitting), which limits the flexibility required to examine a real system in solution, (ii) the error in the experimental

TABLE 1. Apparent values of kinetic parameters for RML and CRL-catalyzed syntheses of L-alanyl-D-glucose

Lipase		$k_{cat} \times 10^3$ (M h^{-1} mg^{-1})	$K_{mA} \times 10^3$ (M)	$K_{mB} \times 10^3$ (M)	$K_i \times 10^3$ (M)
RML	a	0.29±0.028	4.9±0.51	0.21±0.018	1.76±0.19
	b	0.4±0.038	11.2±1.23	10.0±0.96	5.5±0.59
CRL	a	0.75±0.08	56.2±5.7	16.2±1.8	21.0±1.9
	b	1.0±0.11	56.2±5.4	16.1±1.5	21.0±2.3

A, L-Alanine; B, D-glucose. a, Graphical method; b, curve-fitted values.

FIG. 6. Replot of slopes obtained from Fig. 4B versus [D-glucose] (RML) (A) and Fig. 5B versus [D-glucose] (CRL) (B).

graphical methods based on HPLC measurements, which itself involves errors on the order of ±10% and (iii) the heterogeneous experimental conditions employed involving un-dissolved carbohydrates and enzymes, on the one hand, and, partly due to dissolved amino acids, on the other, in the mixture of dichloromethane and dimethylformamide.
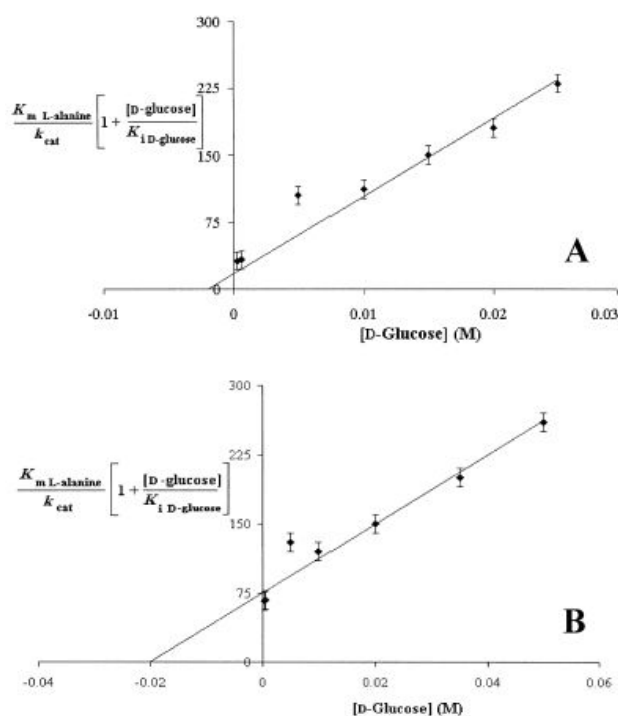
With increasing L-alanine concentration (Fig. 4A for RML and Fig. 5A for CRL), the initial rate increases with decreasing D-glucose concentration. With increasing D-glucose concentration up to the minimum $1/v$, the initial rate decreases, and the plots tend to become closer to the $1/v$ axis (Y-axis).

Figures 4B (RML) and 5B (CRL) also show the same behavior, in which at low D-glucose concentrations, the plots appear parallel probably as long as $K_i > K_{mB}$ concerned. However, at high fixed D-glucose concentration, the slopes of the plots drastically vary. Thus, in these reactions, the kinetic data clearly shows the inhibitory nature of D-glucose. The competition between L-alanine and D-glucose for the active site (binding site) of lipases (RML/CRL) could result in a predominant binding of D-glucose at high concentrations, displacing L-alanine, and thus leading to the formation of the dead-end lipase·D-glucose complex.

For the RML reaction, K_{mA} ($4.9 \pm 0.51 \times 10^{-3}$ M) is always higher than K_{mB} ($0.21 \pm 0.018 \times 10^{-3}$ M, Table 1), which shows that L-alanine is bound to RML less firmly than D-glucose ($K_{mA}/K_{mB} = 23.3$). A similar behavior is also observed with CRL (Table 1) K_{mA} ($56.2 \pm 5.7 \times 10^{-3}$ M), K_{mB} ($16.2 \pm 1.8 \times 10^{-3}$ M), $K_{mA}/K_{mB} = 3.5$. However, the respective values are very much higher for CRL than for RML, indicating that CRL can yield better conversions than RML. Between RML and CRL, the K_i for D-glucose is lower for RML ($5.5 \pm 0.59 \times 10^{-3}$

TABLE 2. Experimental and predicted initial rates for synthesis of L-alanyl-D-glucose by RML and CRL

L-Alanine (M)	D-Glucose (M)	$v_{experimental}{}^{a} \times 10^{6}$ (M h^{-1} mg^{-1})	$v_{predictive}{}^{b} \times 10^{6}$ (M h^{-1} mg^{-1})
RML			
0.005	0.005	30	51
0.005	0.01	37	45
0.005	0.015	28	37
0.005	0.02	25	31
0.005	0.025	15	27
0.01	0.005	38	73
0.01	0.01	51	72
0.01	0.015	55	64
0.01	0.02	49	56
0.01	0.025	55	49
0.015	0.005	48	84
0.015	0.01	46	91
0.015	0.015	72	84
0.015	0.02	91	75
0.015	0.025	88	67
0.02	0.005	65	92
0.02	0.01	97	104
0.02	0.015	41	99
0.02	0.02	76	91
0.02	0.025	127	83
0.025	0.005	71	97
0.025	0.01	146	114
0.025	0.015	116	112
0.025	0.02	132	104
0.025	0.025	111	96
0.05	0.005	79	109
0.05	0.01	176	142
0.05	0.015	158	149
0.05	0.02	176	147
0.05	0.025	176	141
CRL			
0.005	0.005	35	55
0.005	0.01	38	52
0.005	0.02	37	42
0.005	0.035	26	32
0.005	0.05	20	25
0.01	0.005	52	89
0.01	0.01	66	92
0.01	0.02	76	78
0.01	0.035	53	61
0.01	0.05	44	49
0.02	0.005	99	130
0.02	0.01	157	148
0.02	0.02	158	137
0.02	0.035	162	112
0.02	0.05	90	92
0.035	0.005	164	161
0.035	0.01	275	201
0.035	0.02	267	202
0.035	0.035	168	174
0.035	0.05	228	148
0.05	0.005	269	178
0.05	0.01	314	234
0.05	0.02	347	250
0.05	0.035	370	224
0.05	0.05	404	195
0.1	0.005	269	203
0.1	0.01	320	291
0.1	0.02	362	345
0.1	0.035	449	338
0.1	0.05	460	310

[a] Graphical method.
[b] Curve-fitted values.

M) than for CRL ($21.0 \pm 2.3 \times 10^{-3}$ M), indicating that the RML is inhibited by D-glucose far more efficiently than CRL. This could also explain the better conversion observed with CRL than with RML.

Both RML and CRL contain amino acids in their active sites capable of forming hydrogen bonds with suitable donor molecules. The catalytic triad in RML consists of Ser-144, His-257 and Asp-203 (21). CRL contains Ser-209, Glu-341 and His-449 (2, 22). Brzozwski *et al.* (23) showed in an atomic model of the inhibitor *n*-hexylchlorophosphonate ethyl ester–RML complex that in the oxyanion hole, which is directly responsible for the substrate binding, a direct covalent bond formation between the nucleophilic O$_\gamma$ of Ser-144 and the phosphorous atom of *n*-hexylchlorophosphonate ethyl ester is possible. In CRL, the oxyanion hole O$_\gamma$ (Ser-209) is formed by the amide backbones of Gly-123, Gly-124 and Ala-210 through the hydrogen bonding between the amide-CO-NH- and the hydroxyl of Ser-209, which is stabilized by the helix dipole (23). D-Glucose possesses five hydroxyl groups and L-alanine possesses carboxyl and amino groups capable of forming hydrogen bonds with polar side chains of amino acids. Ser-144 hydroxyl and Asp-203 carboxyl groups of RML and Ser-209 and Glu-341 of CRL (2) residues are very good candidate molecules for exhibiting hydrogen-bonding interactions.

Between D-glucose and L-alanine, the former possesses more hydrogen-bonding functional groups than the amino or carboxyl groups of L-alanine. Ser-144 in RML and Ser-209 in CRL can form hydrogen bonds with the amino N atom of L-alanine as well as the O atom of D-glucose. Because the $K_{\text{m L-alanine}}$ values are higher than the $K_{\text{m D-glucose}}$ values for both enzymes, D-glucose could strongly bind to these enzymes than L-alanine.

Zaidi *et al.* (12) reported that the interaction between nylon-immobilized CRL and alcohol through hydrogen-bonding could block the nucleophilic site of the enzyme engaged in acylation, leading to inhibition. A similar behavior can also be envisaged between D-glucose hydroxyl groups and the above-mentioned oxygen of serine and the carboxylate groups of glutamic acids. Hence, this kinetic study could clearly explain the inhibition of both RML and CRL by D-glucose.

This is the first report in which D-glucose has been unequivocally shown to inhibit both RML and CRL.

ACKNOWLEDGMENTS

NOMENCLATURE

2D-HSQCT: two-dimensional heteronuclear single quantum coherence transfer
[α]   : optical rotation
A   : reactant A, L-alanine
B   : reactant B, D-glucose

CRL   : *Candida rugosa* lipase
DMSO   : dimethyl sulfoxide
E   : lipase: *Rhizomucor miehei* lipase/*Candida rugosa* lipase
EA   : lipase·L-alanine complex
EB   : lipase·D-glucose complex
EQ   : lipase·L-alanyl-D-glucose complex
F   : lipase·L-alanyl complex
FB   : lipase·L-alanyl·D-glucose complex
FP   : lipase·L-alanyl·water complex
HPLC   : high-performance liquid chromatography
IR   : infrared
k_{cat} : catalytic efficiency of enzyme
$K_{\text{i D-glucose}}$: dissociation constant for lipase·inhibitor (D-glucose) complex
K_{i} : dissociation constant for lipase·inhibitor (D-glucose) complex
K_{mA} : Michaelis–Menten constant for lipase·L-alanine complex
K_{mB} : Michaelis–Menten constant for lipase·D-glucose complex
$K_{\text{m D-glucose}}$: Michaelis–Menten constant for lipase·D-glucose complex
$K_{\text{m L-alanine}}$: Michaelis–Menten constant for lipase·L-alanine complex
MS   : mass spectrometry
NMR   : nuclear magnetic resonance
P   : H_2O
Q   : L-alanyl-D-glucose
R   : regression coefficient
RML   : *Rhizomucor miehei* lipase
UV   : ultraviolet
v : initial rate
V_{max} : maximum velocity

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