CEREAL STARCH DIGESTIBILITY BY PORCINE PANCREATIC α-AMYLASE ISOFORMS IN VITRO: EFFECT OF NON-STARCH POLYSACCHARIDES

A THESIS submitted to the DEPARTMENT OF BIOCHEMISTRY

Of

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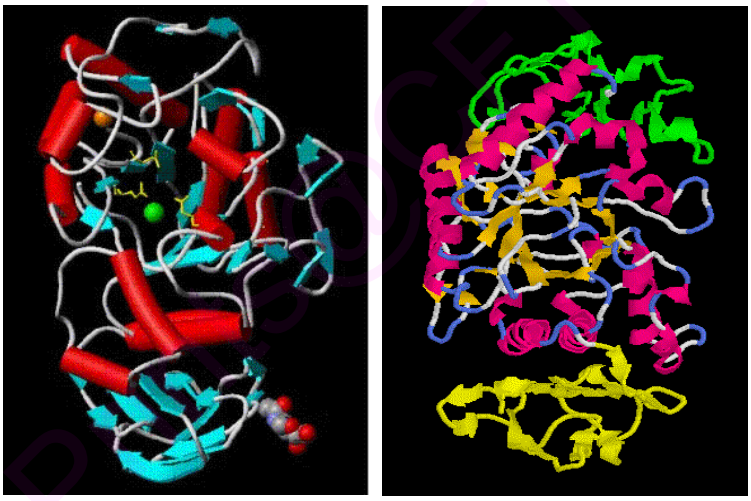

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DECLARATION

I declare that the thesis entitled, *"Cereal starch digestibility by porcine pancreatic α-amylase isoforms in vitro: effect of non-starch polysaccharides"* submitted to the University of Mysore, Mysore for the award of the degree of Doctor of Philosophy in Biochemistry is the result of the work carried out by me under the guidance of Dr. G. Muralikrishna, Scientist-F, Department of Biochemistry and Nutrition during the period of 2nd September 2004 to 1st September 2007. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

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CERTIFICATE

This is to certify that the thesis entitled, *"Cereal starch digestibility by porcine pancreatic α-amylase isoforms in vitro: effect of non-starch polysaccharides"* submitted by **Ms. Anitha Gopal. B.** for the award of the degree of **Doctor of Philosophy** in **Biochemistry** to the University of Mysore, Mysore is the result of research work carried out by her in the Department of Biochemistry and Nutrition, under my guidance during the period 2nd September 2004 to 1st September 2007. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

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To My Beloved Parents

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CONTENTS

Abbreviations

α	-	alpha
β	-	beta
λ_{max}	-	lamda max
μ	-	micro
μg	-	microgram
μ M	-	Micromolar
θ	-	theta
Ara	-	Arabinose
AAS	-	Atomic Absorption Spectrophotometer
BSA	-	Bovine Serum Albumin
BSS	-	British Standard Specifications
cm	-	Centimeter(s)
Da	-	dalton
°C	-	degree Celsius
DP	-	Degree of Polymerization
DEAE	-	Diethyl amino ethyl
DSC	-	Differential Scanning Calorimetry
DMSO	-	Dimethyl sulphoxide
DNS	-	Dinitro salicylic acid
ESI-MS	-	Electron spray ionization-mass spectrometry
ΔH	-	enthalpy of gelatinization
EC	-	Enzyme Commission
EDTA	-	Ethylene diamine tetra acetic acid
FUV-CD	-	Far- Ultra-Violet Circular Dichroism
Fig.	-	Figure
FID	-	Flame ionization detector
GLC	-	Gas Liquid Chromatography
GPC	-	Gel Permeation Chromatography

g	-	Gram
HPLC	-	High Performance Liquid Chromatography
HPSEC	-	High Performance Size Exclusion Chromatography
h	-	Hour(s)
kDa	-	kilo Dalton
Kv	-	Kilo Volts
L	-	Litre (s)
MS	-	Mass spectrometer
m/z	-	mass to-charge ratio
MRE	-	mean residue ellipticity
meq/g	-	milli equivalents /gram
mg	-	Milligram (s)
mL	-	Millilitre(s)
mm	-	Millimetre (s)
mM	-	Millimolar
min	-	Minute (s)
M	-	Molar
Mw	-	Molecular weight
nm	-	Nanometre
NSP	-	Non-starch polysaccharides
N	-	Normal
pI	-	Isoelectric point
ppm	-	Parts per million
PNGase F	-	Peptide-*N*-glycosidase F
PAS	-	Periodic acid schiff
pmol/µl	-	pico moles per microlitre
PAGE	-	Polyacrylamide Gel Electrophoresis
PPA	-	Porcine Pancreatic α-Amylase
RI	-	Refractive index
SEM	-	Scanning Electron Microscope
sec	-	Second (s)

SDS	-	Sodium Dodecyl Sulphate
Tc	-	conclusion temperature
T_o	-	onset temperature
T_p	-	peak temperature
TEMED	-	N, N, N', N'- Tetra methyl ethylene diamine
TFA	-	trifluoroacetic acid
UV	-	Ultra Violet
U/mg	-	Unit / milligram
U	-	Units
V	-	Volume
V_o	-	void volume
V_e	-	elution volume
v/v	-	Volume / volume
WSP	-	Water soluble polysaccharides
w/v	-	Weight/Volume

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Abstract

α-Amylases (EC 3.2.1.1) are a family of endo-amylases which catalyze the hydrolysis of α-D-(1,4) glycosidic linkages in starch and glycogen releasing malto-oligosaccharides. The amylose contents of ragi, rice, wheat and maize starches were 26.6, 25.1, 26.4 and 27.3% respectively. The gelatinization temperatures and the enthalpy of gelatinization of ragi, rice, wheat and maize starches were 64.9, 60.7, 62.2 & 66.5°C and 11.9, 8.9, 10.4 and 13.4 J/g respectively. X-ray crystallography revealed that all the starches showed typical A-type diffraction pattern. Pancreatic alpha amylase (PPA) was separated into two activity peaks i.e PPA-I and PPA-II on DEAE-cellulose chromatography. The molecular weights of PPA-I and PPA-II were found to be ~55 kDa, hence isoforms. The MS fragmentation pattern of the sugars released from starches by pancreatic α-amylase treatment indicated preponderantly maltose, maltotriose and maltotetraose. The pH and temperature optima of PPA, PPA-I and PPA-II were 6.9 and 45°C respectively. PPA was found to be stable in the pH range of 7.0-8.0 whereas PPA-I and PPA-II were stable in the pH range of 6.0-8.0. PPA, PPA-I and II were stable up to 45°C. K_m and V_{max} calculated from the Lineweaver Burk double reciprocal plot showed that maize starch was the least digestible among all the starches as indicated by its high K_m value. Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+} were found to have both activating and stabilizing effect whereas Al^{3+} and Hg^{2+} completely inactivated PPA and its isoforms. Citric acid and oxalic acid inhibited PPA and its isoforms in concentration dependent manner. The viscosity of water soluble non-starch polysaccharides (WSNSP) increased with increase in concentration (0.2 to 1.0%) and decreased with increase in temperature (20 to 70°C). Maize WSNSP reduced the activity of PPA and its isoforms to a greater extent compared to the WSNSP of ragi, wheat and rice. The glycoprotein nature of PPA and its isoforms were ascertained by colorimetric and periodic acid Schiff's (PAS) staining methods. The completeness of deglycosylation was observed after 24 h of PNGase F treatment. ESI-MS of the released oligosaccharide of these amylases showed the molecular fragmentation at 365 and 709 m/z. The deglycosylated enzymes were slightly less thermo-stable than the glycosylated forms. The excitation of the glycosylated enzymes were observed at 280 nm and that of deglycosylated samples were 286 nm indicating a red shift. The differences between the glycosylated and deglycosylated PPA and its isoforms with respect to the spectral characteristics in the far-UV region did not show any significant differences. The thesis ends with summary and conclusions along with a list of references.

SYNOPSIS

Synopsis of the thesis to be submitted for the award of Ph. D. degree (Biochemistry) of the University of Mysore, Mysore, India

Title of the thesis : **"Cereal starch digestibility by porcine pancreatic α-amylase isoforms in vitro: effect of non - starch polysaccharides"**

Candidate : B. Anitha Gopal

α-Amylases (α-1,4-glucan-4glucanohydrolases, EC 3.2.1.1) are a family of endo-amylases that catalyze the hydrolysis of α-D-(1,4) glycosidic linkages in starch and glycogen releasing malto-oligosaccharides in the α-anomeric form. α-Amylases are widely distributed in almost all the living organisms i.e. mammals, plants and several microorganisms. Crystallographic studies of α-amylases have shown that all α-amylases consist of three distinct domains, a central $(\beta/\alpha)_8$ barrel (domain A) forms the core of the molecule with three active site residues. Domains B and C are located roughly at opposite sides of this barrel. The B domain is firmly attached to the 'A' domain by disulphide bond. 'C' domain with a β-sheet structure is linked to the A domain by a simple polypeptide chain and may carry a carbohydrate chain depending on the origin of the enzyme.

Starch is mainly composed of linear amylose (α-1-4 linkage) and branched amylopectin {α-1-4 (~95%) & α-1-6 (~5%) linkage}. In mammals, starch digestion is initiated in the mouth by salivary α-amylase followed by digestion in the small intestine by the action of pancreatic α-amylase and the brush border enzyme complex (sucrase-isomaltase-α-glucosidase). Starch produces oligosaccharides of varying degree of polymerization (DP: 2-10) when digested by pancreatic α-amylase. Porcine pancreatic alpha amylase (PPA) has highest homology with

that of human pancreatic alpha amylase sequence, with a percentage identity of 87.1%. Most α-amylases are glycoproteins. PPA possesses three potential glycosylation sites. Enzymatic removal of the bound carbohydrate /oligosaccharides by PNGase F is often desirable to study the role of covalently linked carbohydrate on the structure and function of the enzyme. However, hardly very few studies were carried out till date in this direction.

Porcine pancreatic α-amylase inhibition is one of the ways to decrease the release of post-prandial glucose levels in diabetic patients. One of the important approaches which is not studied yet is the effect of individual water soluble polysaccharides isolated from cereals on the action of PPA and its isoforms. Water soluble polysaccharides (WSP) consists of arabinoxylans, (1→3)/ (1→4) β-glucans and phenolic acids that modulate the functional properties of foods. The WSP are viscous and are perhaps believed to inhibit the starch degrading enzymes, resulting in a slow release of post-prandial glucose. Soluble fibres vary in their molecular weight, arabinose/xylose ratio and the nature and content of covalently linked phenolic acids.

The present study is taken up with the following objectives:

1. Finger printing of the oligosaccharides produced from cereals/millet starches by the action of pancreatic α-amylase with respect to a) composition b) degree of polymerization and c) molecular weight.
2. Isolation of WSP from the cereals and millet and characterize the same with respect to their a) sugar composition b) molecular weight c) bound phenolic acids and d) associated proteins.
3. To study and understand the effect of water soluble polysaccharides on the activities of pancreatic α-amylase isoforms and study the mechanism if any.
4. To study the role of N-linked glycan portion of pancreatic α-amylase on its activity and stability.

The research work carried out towards achieving these objectives forms the subject matter of the thesis. The thesis is divided into 3 chapters followed by summary and conclusions.

Chapter I: ***Introduction***

The introduction begins with a general account on amylases followed by their classification, assay methods, structure, active site, and their catalytic mechanism. The chapter also deals with an introduction on the post translational modification of amylases with special emphasis on the N- linked glycosylation which is evident in porcine pancreatic alpha amylase. Also described in this chapter is about starch-the substrate for PPA, its characteristics and its hydrolysis *in vivo*. The nature of the non-starch polysaccharides is briefly described. This chapter also deals about the literature pertaining to the potential of phenolic-extracts with respect to their inhibitory activity on amylases. Also briefly described in this chapter is about acarbose, a powerful pseudo tetrasaccharide inhibitor of pancreatic α-amylase and α-glucosidase. The chapter concludes with the scope of the present investigation.

Chapter II: ***Material and Methods***

This chapter describes about the various materials, chemicals and instruments used in the present study. Various colorimetric estimation methods employed for the analysis of reducing sugar released and the amylase assay method are also described. Protocols followed for the purification of PPA i.e DEAE-cellulose anion exchange chromatography and Sephadex G-100 are mentioned along with the methods employed for the confirmation of the purity of PPA and its isoforms namely native PAGE and SDS-PAGE. The protocol followed for the enzymatic deglycosylation of PPA and its isoforms by PNGase F is also described. The methods pertaining to the physico-chemical characterization of starches and the composition of water soluble polysaccharides are also explained.

The GLC method adopted for the determination of sugar composition of the cereal NSP is described. HPLC and ESI-MS analysis methods are described which were performed to establish the nature and the molecular weights of the malto-oligosaccharides liberated from starches by the digestion by PPA and its isoforms respectively. UV, fluorescence and CD studies are also described which were carried out to study the effect of deglycosylation on the stability of PPA and its isoforms.

Chapter III: ***Results and Discussion***

Chapter-III
Section A.: ***Isolation and characterization of cereal and millet starches and non-starch polysaccharides***

In this section a comparative account of the characteristics of starches and non-starch polysaccharides isolated from ragi, rice, wheat and maize are presented.
The yield of starches from ragi, rice, wheat and maize were 63.5, 74.1, 53.9 and 64.4% respectively on the dry weight basis. Scanning electron photomicrographs (SEM) of the native starches from ragi, rice, wheat and maize revealed that the starch granules were regular and smooth. Wheat starch consisted of both large as well as small lenticular shaped granules. Rice starch granules were angular /pentagonal in shape and ragi starch granules were uneven with spherical, polygonal and rhombic shapes. The amylose contents of ragi, rice, wheat and maize starches were 26.6, 25.1, 26.4 and 27.3% respectively. Initial gelatinization temperatures for these starches ranged from 57.5 to 63.8°C and the final temperatures ranged from 66.4 to 84.5°C. The peak temperature (gelatinization temperature) and the enthalpy of gelatinization of ragi, rice, wheat and maize starches were 64.9, 60.7, 62.2 & 66.5°C and 11.9, 8.9, 10.4 & 13.4 J/g respectively. X-ray crystallography revealed that all the starches showed typical A-type diffraction pattern with strong reflection at 15° and 23° but the degree of

crystallinity of maize starch (41.4%) was significantly higher than that of ragi (37.3%), wheat (36.96%) and rice (35.8 %) starches.

The yield of WSP from the flours by aqueous extraction from ragi, rice, wheat and maize were 1.3, 0.8, 1.1 and 1.2% respectively, however these were contaminated by very small amount of starch (ragi-1.8%, rice-3.0%, wheat-2.6%, maize-1.1%) which was removed by glucoamylase digestion. The glucoamylase digested WSP consisted preponderantly carbohydrate (90%), very small amounts of protein and calcium. The neutral sugar composition of WSP indicated arabinose, xylose, glucose, rhamnose, mannose and galactose in varying ratios. Ferulic acid is the major bound phenolic acid in WSP followed by small amounts of coumaric acid. The viscosity of water soluble non-starch polysaccharides increased with increase in concentration (0.2 to 1.0%) and decreased with increase in temperature (20 to 70°C).

Chapter-III

Section B : ***Porcine pancreatic alpha amylase and its isoforms: purification, kinetic studies and effect of non-starch polysaccharides***

Pancreatic alpha amylase was separated into two activity peaks on DEAE-cellulose anion exchange chromatography at 0.25 M (PPA-I) and 0.34 M (PPA-II) of NaCl concentrations. PPA-I and PPA-II were eluted at the same elution volumes with symmetrical protein and activity peaks indicting their homogeneity as determined by gel filtration chromatography on Sephadex G-100. The apparent homogeneity of PPA-I and PPA-II were ascertained by both activity and protein staining procedures. The molecular weights of PPA-I and PPA-II were found to be identical i.e ~ 55 kDa as determined by GPC, SDS-PAGE and ESI-MS and therefore are iso-enzymes. The MS fragmentation pattern of the sugars released from starches by pancreatic α-amylase and its isoforms (PPA-I & PPA-II) indicated the m/z values of 365.3, 527.41 and 689.4 confirming them to

be maltose, maltotriose and maltotetraose however their quantity differed as determined by HPLC.

The pH and temperature optima of PPA, PPA-I and PPA-II were 6.9 and 45°C respectively. PPA was found to be stable in the pH range of 7.0-8.0 with 80% activity after 4 h of incubation whereas PPA-I and PPA-II were stable in the pH range of 6.0-8.0 retaining almost 80% activities after 4 h of incubation. PPA, PPA-I and PPA-II were stable up to 45°C. The reaction catalyzed by PPA and its isoforms was calculated from the Lineweaver Burk double reciprocal plot. The K_m values of PPA for ragi, rice, wheat and maize starches varied between 0.8-3.3% and the V_{max} was between 2841-6212 U/mg protein respectively. For PPA-I, K_m values for ragi, rice, wheat and maize ranged between 1.3-2.5% whereas V_{max} was found to be between 2740-4201 U/mg protein. K_m values of PPA-II, for ragi, rice, wheat and maize were in the range of 1.0-2.9% and the V_{max} values ranged between 2755-5102 U/mg protein**.** Maize starch was the least digestible among all the starches as indicated by its high K_m value.

Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+} were found to have both activating and stabilizing effects as indicated by the increased activities of PPA, PPA-I and PPA-II. Metals such as Al^{3+} and Hg^{2+} completely inactivated all the three amylases whereas Zn^{2+} and Cu^{2+} partially inactivated PPA and its isoforms. Organic acids such as citric acid inhibited PPA at 10 mM and PPA-I and PPA-II were inhibited at 7.0 mM concentrations. 10 mM Concentration of oxalic acid inhibited PPA whereas PPA-I and PPA-II were inhibited at 5 mM concentration. EDTA (125µm) completely inhibited the activities of PPA and its isoforms.

The effect of WSP from ragi, rice, wheat and maize were studied on the activities of PPA and its isoforms. The activities of all the three amylases were reduced in the presence of WSP. This reduction in the activity of α-amylases by maize polysaccharides was more followed by ragi, rice and wheat and this observation can probably be ascribed to the following reasons; (a) the arabinose to xylose

ratio (b) the phenolic acid present in the WSP samples and (c) the relative viscosity of the WSP.

Chapter-III

Section C : ***Deglycosylation & spectroscopic studies of porcine pancreatic alpha amylase & its isoforms***

The glycoprotein nature of PPA and its isoforms were ascertained by colorimetric and periodic acid Schiff's (PAS) staining methods. Native PAGE subjected to PAS staining developed pink bands against a white background indicating the glycoprotein nature of the enzyme while the deglycosylated samples did not develop the characteristic color indicating the complete removal of the carbohydrate moiety. The completeness of deglycosylation was observed after 24 h of PNGase F treatment. The carbohydrate content of PPA, PPA-I and PPA-II were 0.05, 0.006 and 0.004 mol of mannose per 1 mol of enzyme. SDS-PAGE of the deglycosylated PPA and its isoforms showed very little change in the relative mobility. The purified oligosaccharides were found to be mannose as identified by GLC. ESI-MS of the released oligosaccharide showed the ionization at 365 (mannose + mannose) and 709 m/z (mannose + mannose + mannose + *N*-acetyl-hexosamine). The deglycosylated enzymes were slightly less thermo-stable than the glycosylated forms. The absorbance of PPA, PPA-I and PPA-II indicated absorption maxima at 275 nm indicating the preponderance of aromatic amino acid residues such as tryptophan. PPA and its isoforms have an emission maximum at 340 nm. This is typical of an exposed tryptophan residue in protein. On deglycosylation a gradually increase in tryptophan fluorescence intensity of PPA, PPA-I and PPA-II was observed. The excitation of the glycosylated enzymes were observed at 280 nm and that of deglycosylated samples were 286 nm. In the far-UV region (260–200 nm), minima were observed at 231, 236 and 208 nm for PPA whereas for deglycosylated PPA the minima was

observed at 229 and 211 nm. PPA-I and PPA-II showed the minima at 231 and 208 nm and the deglycosylated isoforms showed the minima at 229 and 208 nm respectively.

Summary and conclusions

The thesis is concluded with the important findings of the Ph.D. work. Some of the major conclusions are as follows:

- Digestion of the rice, wheat, maize and ragi starches by PPA and its isoforms released qualitatively same sugars i.e maltose, maltotriose and maltotetraose. However, they differed quantitatively as determined by HPLC .
- The two activity peaks of PPA namely, PPA-I and PPA-II were found to be isoforms as determined by SDS-PAGE, Gel filtration and ESI-MS.
- Water soluble polysaccharides isolated from the flours of cereals showed nonspecific inhibition on the activities of PPA and its isoforms at 1% concentration.
- The glycoprotein nature of PPA and its isoforms were determined and the effect of glycosylation as determined by UV, fluorescence and CD studies revealed little effect on the activity and thermal stability.

The thesis ends with a list of references cited in alphabetical order.

CHAPTER - I

Introduction

I.1. Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are a family of endo-amylases (glycosyl hydrolase 13 family) that catalyse the hydrolysis of α-D-(1,4) glycosidic linkages in starch and its components, i.e. amylose and amylopectin and glycogen releasing malto-oligosaccharides in the α-anomeric form [McCarter, & Withers, 1994 ; Gottschalk, et al., 1998]. The term 'α' relates to the anomeric configuration of the free sugar group released. α-Amylases are widely distributed in almost all the living organisms, i.e. mammals, plants and several microorganisms [Machius, et al., 1998; Janecek, 1994; Muralikrishna, & Nirmala, 2005]. α-Amylases and related amylolytic enzymes are among the most important enzymes having great significance in several food industries and have a broad spectrum of technological applications. The first use of amylases in starch hydrolysis was reported in the 9th century AD for conversion of arrow root starch to sweetener using malt. The term 'diastase' was coined in 1833 for the mysterious material responsible for the reaction that precipitated malt extract. The French term 'diastase' meaning 'separation' became the generic term there after for amylases [Hebeda, & Teague, 1993]. Trivial enzyme names were later derived by adding the suffix 'ase' to the root of the word denoting the substrate or action of the particular enzyme. Fungal and bacterial α-amylases were developed and commercialized in the late 19th and early 20th centuries, respectively. The applications of these α-amylases in a variety of applications such as brewing, textiles, paper and corn syrup industries were initiated in the 1930's. α-Amylase from *Bacillus subtilis* and glucoamylase from *Aspergillus niger* were used to replace acid catalysis in the production of dextrose from starch.

α-Amylases are used for the liquefaction of cereal starch during the production of high fructose corn syrup and fuel ethanol. The paper and textile industry apply α-amylases in the sizing/desizing process. α-Amylases remove starch containing stains from clothes and dishes in household laundry and automatic dishwashing detergents. Besides these traditional applications, the spectrum of

amylase usage has widened in many other fields, such as clinical medicine and analytical chemistry. Genetic engineering tools developed in 1970's paved way for the production of cloned amylases (*Bacillus stearothermophillus*) in 1980's and 1990's for industrial applications [Brumm, et al., 1991]. Studies pertaining to the sequencing and X-ray crystallography of various amylases were also completed in 1980's [Lin, & Czuchajwska, 1998].

The Enzyme Commission (EC) classification of enzymes are based on the reaction catalysed. Each reaction is consequently assigned an EC number that uniquely identifies the reaction. For α-amylases this number is 3.2.1.1 and the reaction is described as the '*endohydrolysis of 1,4-α-glucosidic linkages in oligosaccharides and polysaccharides'* [Nielsen, & Borchert, 2000].

I.2.Types of starch degrading enzymes

Starch degrading enzymes can be classified into three main groups based on their mode of action: (1) endoamylases (2) exoamylases and (3) debranching enzymes.

I.2.a. ***Endoamylases***: Endoamylases, also known as 'liquefying' enzymes cleave the α-1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides such as glycogen but not the α-1,6 bonds in amylopectin. The products of hydrolysis are oligosaccharides of varying chain lengths, having α-configuration on the C_1 of the reducing glucose unit. Endoamylases hydrolyze the bonds located in the inner regions of the substrate resulting in rapid decrease of the viscosity as well as decrease in the iodine staining power of the gelatinized starch solution [Hill, & MacGregor, 1988]. Activity is defined in terms of the amount of enzyme required to breakdown starch under a given set of conditions.

I.2.b. ***Exoamylases***: Exoamylases, also known as 'saccharifying' enzymes act on the α-1,4 glycosidic bonds present in amylose, amylopectin and glycogen from

the non-reducing end and produce β-D-maltose/β-D glucose by the inversion reaction. Gelatinized starch when subjected to exoamylases, results in a slow decrease in the viscosity and iodine staining power of starch in contrary to endo amylases. Cereal and bacterial β-amylases and fungal glucoamylases come under this category [Banks, & Greenwood, 1975].

I.2.c. ***Debranching enzymes***: The branch points containing α-1,6 glycosidic linkages present in starch and glycogen are resistant to attack by α- and β-amylases resulting in α/β limit dextrins, respectively. Pullulanase, first discovered in 1961 attracted interest because of its specific action on pullulan, a linear D-glucose polymer with maltotriosyl units joined by α-1,6 bonds. Pullulanase is produced by mesophilic organisms such as *Klebisiella aerogenes* and *Aureobasidium pullulans* and are capable of specifically attacking α-1,6 linkages present in starch and glycogen [Abdulla, & French, 1970]. Glucoamylase can also attack α-1,6 linkages but the reaction proceeds at relatively slow speed compared to pullulanase action.

The different starch hydrolysing enzymes are given in table 1.

Table 1: Starch hydrolyzing enzymes

Enzyme	EC number	Glycosidic bond specificity	Mode of action	End product(s)
Phosphorylase (α-1,4-α-D-glucan orthophosphate α-D-glucosyl transferase)	2.4.1.1	α-(1-4)-glucosyl	exo	Glucose 1-phosphate
Alpha-amylase (α-1,4-α-D-glucan glucanohydrolase)	3.2.1.1	α-(1-4)-glucosyl	endo	Linear and branched Oligosacchar-ides
β-amylase (-1,4-α-D-glucan maltohydrolase)	3.2.1.2	α-(1-4)-glucosyl	exo	Maltose and limit dextrins
Amyloglucosidase (glucoamylase , exo-1,4-α-glucosidase)	3.2.1.3	α-(1-4)-glucosyl & α-(1-6)-glucosyl	exo/endo	Glucose
Isoamylase (glycogen 6-glucanohydrolase)	3.2.1.68	α-(1-6)-glucosyl	endo	Linear α-(1-4) glucan chains
Pullulanase (limit dextrinase; amylopectin 6-glucanohydrolase)	3.2.1.41	α-(1-6)-glucosyl	endo	Linear α-(1-4 malto triose units

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I.3. Assay methods for amylases

Amylase activity can be determined by a number of analytical methods. The quantitative procedures generally used involve the measurement of new reducing groups formed upon the amylolytic hydrolysis of starch. The amylase activity is represented as, the micromoles of products formed or substrate transformed per minute under defined conditions [Greenwood, & MacGregor, 1965]. The released reducing groups can be measured by colorimetric methods using the following reagents (a) alkaline copper [Nelson, 1944] (b) alkaline ferricyanide [Robyt, et al., 1972] and (c) alkaline 3, 5, dinitrosalicylate (DNS) [Bernfeld, 1955; Robyt, & Whelan, 1968]. Among these methods, DNS method is the most extensively used because of its reliability and simplicity. Copper and ferricyanide procedures give equimolar reducing values for equimolar reducing ends of maltooligosaccharides [Robyt, et al., 1972]. The decrease in loss of the ferricyanide ion concentration is measured by the ferricyanide reagent method. The other method is the Neocuprine method [Dygert, et al., 1965] which is used for the estimation of the reducing sugar released during amylase activity assay.

The measurement of decrease in the blue color produced by starch when complexed with iodine solution can be semi-quantitatively determined [vanDyk, & Caldwell, 1956]. This procedure reflects the endoclevage of starch and can be used routinely to assay α-amylases. In addition, the measurement of the decrease in the viscosity of the starch solution can also be taken as a measure of α-amylase activity [Greenwood, & MacGregor, 1965]. Since this procedure measures only endoactivity, it can be extensively used to detect α-amylase.

Another method to assay α-amylase activity is the use of chromogenic substrates which are useful for α-amylase assays of clinical samples. The dye is covalently linked to starch or one of its constituents (amylose or amylopectin) to give an insoluble azure derivative [Rinderkneet, et al., 1967]. When these substrates are acted on by α-amylase, fragments containing the dye is solubilized, the

remaining insoluble substrate is removed by centrifugation and the absorbance of the supernatant is taken as the measure of amylase activity.

α/β- amylase activities can also be detected by using the p-Nitrophenyl derivative of oligosaccharides [Ajandouz, & Marchis-Mouren, 1995; Kandra, et al., 1997]. The amount of p-nitrophenol released correlates with the amylase activity.

I.4. Structure of α-amylases

I.4.a. ***Primary structure***

α-Amylases are small proteins with a molecular weight ranging from 20–55 kDa. They are monomeric, calcium-containing enzymes. Nakajima, et al., [1986] clearly pointed out the existence of four highly conserved regions, especially in the catalytic and substrate binding regions in eleven different α-amylases. The three active site residues in porcine pancreatic amylase i.e Asp 212, Glu 248 and Asp 315 were identified.

I.4.b. ***Secondary structure***

X-ray studies of mammalian and bacterial α-amylases have shown that all α-amylases consist of three distinct domains i.e. domain 'A', 'B' and 'C'. A central $(\beta/\alpha)_8$ barrel (domain A) forms the core of the molecule with three active site residues. Domains 'B' and 'C' are located roughly at opposite sides of this barrel. The 'B' domain is firmly attached to the 'A' domain by disulphide bond. 'C' domain with a β-sheet structure is linked to the 'A' domain by a simple polypeptide chain and may carry a carbohydrate chain depending on the origin of the enzyme [Buisson, et al., 1987].

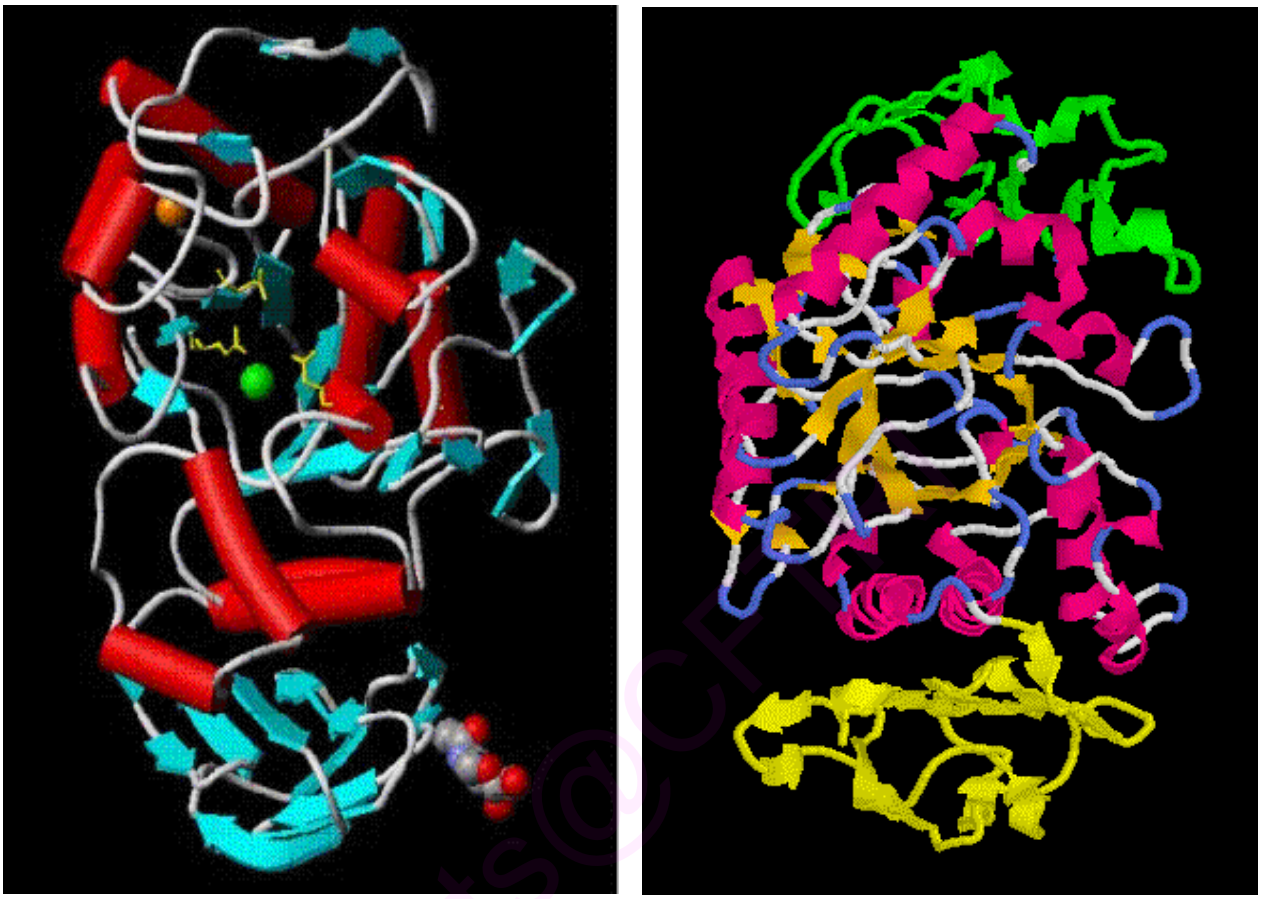

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Fig. 1: Ribbon diagram of pancreatic α-amylase

All known α-amylases contain a conserved calcium ion, which is located at the interface between domains 'A' and 'B' [Boel, et al., 1990 ; Machius, et al., 1995 Machius, et al., 1998; Vallee, et al., 1959]. The calcium ion is bound very tightly as shown by the dissociation constants for PPA and hog pancreatic amylase, 44 nM and 0.0050 nM respectively [Feller, et al., 1994]. The role of the conserved calcium ion is mainly structural since it is too far away from the active site to participate directly in catalysis [Machius, et al., 1998; Larson, et al., 1994; Buisson, et al., 1987]. The ribbon diagram of pancreatic α-amylase is presented in fig. 1.

I.4.c. *Tertiary structure*

X-ray crystallography was carried out to describe the crystal structure of amylases from barley malt [(isoform Amy-2 (68 Å x 53a x 36Å)], *Aspergillus oryzae* (Taka amylase 3.0 and 2.1 Å), *A. niger* (2.1 Å) and porcine pancreatic amylase (2.9 Å). The three-dimensional structures of porcine and human pancreatic α-amylases do not differ significantly at 2 Å resolution [Qian, et al., 1993; Brayer, et al., 1995].

I.5.a. Active site of α-amylase

The active site of the α-amylase is located in a long cleft of about 3 nm between the carboxyl end of the 'A' domain and the 'B' domain. A model for the organization of active site and subsites, each capable of binding glucose has been proposed [MacGregor, et al., 1994; MacGregor, 1988]. The active site of different α-amylases are there by made up of 5–11 subsites (A–K). The catalytic site is situated between subsites F and G. The reducing end of the α-glucose chain is located towards K subsite. Difference in the specificity of α-amylase, i.e. in the detailed way in which they hydrolyze a polysaccharide has been explained in terms of subsites at the active site. Each subsite interacts with one glucose unit of the substrate. The catalytic site of the enzyme is located between subsites F and G [fig. 2]. Interaction of the enzyme with the primary hydroxyl of a glucose ring unmodified at C-6 is an important requirement for binding at that subsite.

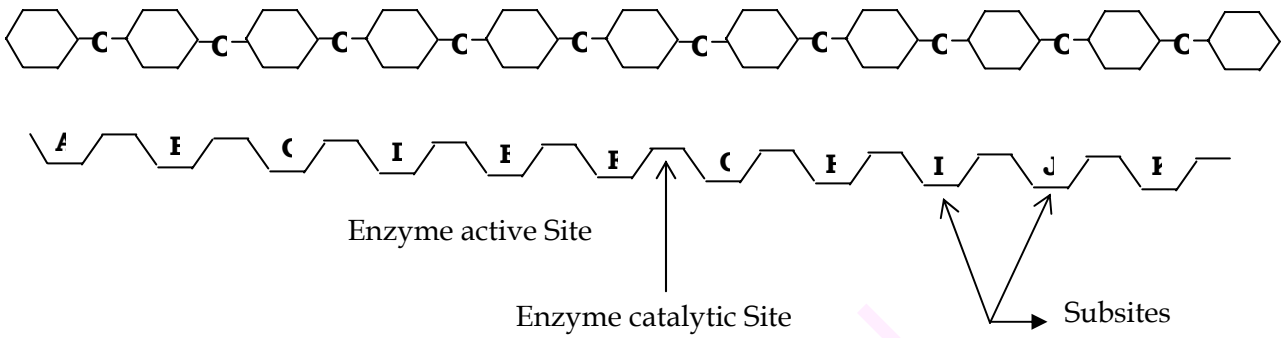

Fig. 2: Schematic representation of active site and sub-sites of α-amylases

I.5.b. α-Amylase catalytic mechanism

Catalytic mechanism of α-amylase was well characterized with respect to Taka amylase and found that active site has been localized in the cleft of the $(\beta/\alpha)_8$ barrel domain with Asp 206, Glu 230, Asp 297 residues playing the catalytic role whereas His 122 and His 296 might bind to glucosyl residues of substrate [Buisson, et al., 1987]. The active site is divided into two parts (a) the binding site made up of a number of subsites and (b) the catalytic site made up of 2–3 groups that are proton donors (electrophiles) and proton acceptors (nucleophiles) [fig. 3]. The number of subsites and their arrangement in conjunction with the catalytic groups determine the type of the products formed.

The retention of the configuration occurring during α-amylase action suggests a double displacement mechanism involving a covalent intermediate.

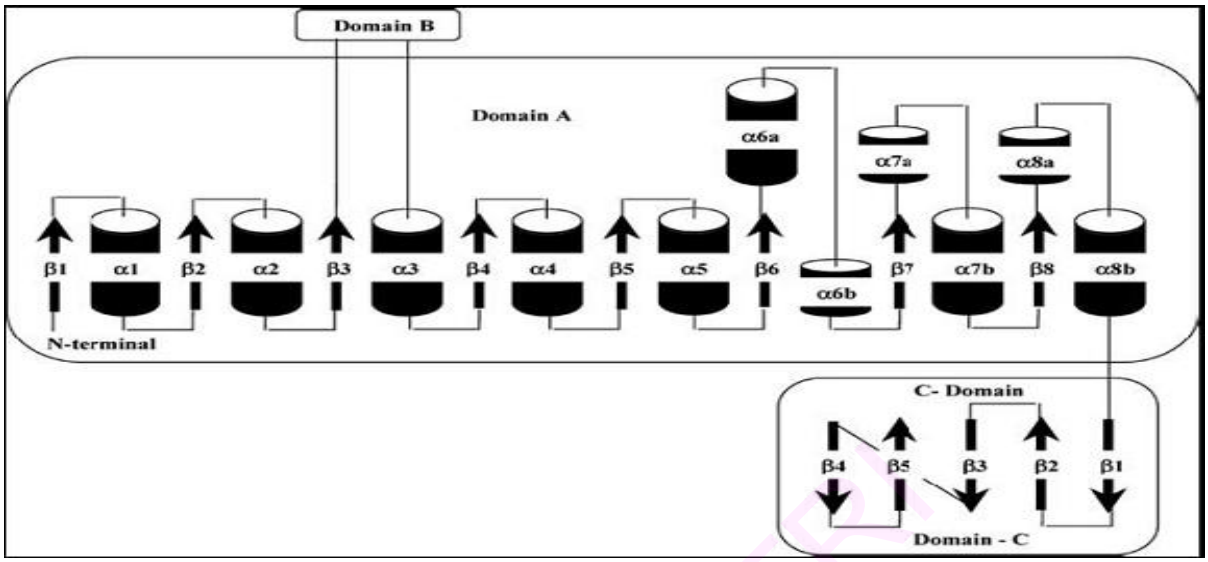

Fig. 3: A representation of domains and of the secondary structure of α-amylase. α-helices are shown as cylinders and β-strands as arrows.

It was proposed that transglycosylation which involves formation of both α-1,4 and α-1,6 bonds could be catalyzed by the same mechanism [Kuriki, & Imanaka, 1999].

I.6. Human α-amylases

Human α-amylases are divided into two groups by the site of their biosynthesis. One group is the pancreatic α-amylase and the other is the salivary α-amylase. Human α-amylases from both salivary and pancreatic origin (HSA and HPA) have been extensively studied with respect to their three dimensional structure [Qian, et al., 1993; Brayer, et al., 1995, Kandra, et al., 2000]. These groups are encoded by two loci Amy_1 (salivary) and Amy_2 (pancreatic) [Groot, et al., 1989]. Their amino acid sequences have been deduced from the nucleotide sequences of cDNAs [Nakamura, et al., 1984], and that of human salivary amylase was also determined from the cloned gene [Nishide, et al., 1986]. A 97% homology was observed with respect to the above two α-amylases.

Measuring α-amylase activity in serum, urine, saliva and other biological fluids is a useful diagnostic tool in evaluating diseases of the pancreas and salivary glands (e.g., acute pancreatitis, parotitis) [Greenberger, & Toskes, 1991; Kandra, et al., 1997].

I.6.a. Comparison of porcine pancreatic α-amylase amino acid (aa) sequences with other mammalian amylases

Structural comparisons were made between the PPA sequence and the established sequences of human [Nishide, et al., 1986], mouse [Tosi, et al., 1984] and rat [MacDonald, et al., 1980] α-amylases. The highest homology of PPA amino acid (aa) sequence was found with that of human pancreatic alpha amylase sequence with a percentage identity of 87.1%. The mouse and rat α-amylases share slightly lower identity with the porcine sequence (85.5%). As already known α-amylases contain five conserved sequence regions [Janecek, et al., 1993], four located at or around the β- strands of the $(\beta/\alpha)_8$-barrel (domain A) [Svensson, et al., 1994] and a short one near the 'C'-terminus of domain 'B' [Janecek, & Balaz, 1995]. The three-dimensional structures of porcine and human pancreatic α-amylases do not differ significantly at 2 Å resolution [Qian, et al., 1993; Brayer, et al., 1995]. However, according to Brayer, et al., (1995) two main regions of conformational differences exist near the active site, these include amino acid residues from 237-250 and 304-310. These above studies showed that the sequence of both porcine and human α-amylases are identical in the later region (aa 304-310). Together with the data obtained by X-ray analysis, the sequence conservation of the 304-310 region among mammalian α-amylases favours a common role of this loop in the enzymatic mechanism of α-amylases [Darnis, et al., 1999].

I.6.b. **Pancreatic alpha amylase and its isoform**s

Porcine pancreatic α-amylase (PPA) was purified to homogeneity from tissue extracts [Granger, et al., 1975] as two forms i.e. PPA-I and PPA-II [Alkazaz, et al., 1996]. They differ in their pI values of 7.5 (PPA-I) and 6.4 (PPA-II) [Ajandouz, & Marchis-Mouren, 1995]. The crystal structures of α-amylase from *Aspergillus oryzae* (TAA) [Matsuura, et al., 1984] and α-amylase from porcine pancreas (PPA) [Buisson, et al., 1987] have revealed that the binding sites are similar in both the enzymes. Porcine pancreatic α-amylase contains 2 sulfhydryl groups [Schramm, 1964; Granger, et al., 1975].

The entire amino acid sequence of PPA-I was established [Kluh, 1981; Pasero, et al., 1986], while only partial unordered and non-overlapping peptides have been determined for PPA-II [Meloun, et al., 1980]. However, the three dimensional structure of both PPA-I and PPA-II are known and were found to be identical at 2.1 Å resolution [Qian, et al., 1993; Gilles, et al., 1996]. Moreover, the three-dimensional structure of mixed PPA-I and PPA-II crystals has been reported at the same resolution [Larson, et al., 1994].

According to the multiple attack hypothesis porcine pancreatic α-amylase (PPA), PPA (E) remains bound after the first endoglycosidic attack to the new reducing end of the glycoside half chain and switches to the exoglucosidase mode of action, releasing several maltose molecules before the ES complex is dissociated [Robyt, & French, 1970; Matsumoto, et al., 1997]. This highly unusual mechanism has not been properly elucidated yet nor have the functions of several structural features of PPA been explained [Koukiekolo, et al., 2001]. The active site of PPA is a long deep polysaccharide binding cleft, crosses the C-terminal end of the β/α-barrel [Larson, et al., 1994]. An additional surface carbohydrate-binding site has been detected in the C-terminal domain segment between β_9 and β_{10} [Buisson, et al., 1987]. Porcine pancreatic amylase has two metal ion binding sites, one selective for calcium and the other to which zinc or copper may bind [Agarwal, & Henkin, 1987].

I.7. Post-translational modification of amylases

Glycosylation is the most important post-translational modification in newly synthesized proteins. Addition of oligosaccharides (glycans) to protein structures is a common occurrence in higher organisms [Lis, & Sharon, 1993; Varki, 1993]. The structural complexity of glycans far greater than that of proteins and nucleic acids allows them to encode information for specific molecular recognition and to determine protein folding, stability and pharmacokinetics. The interest in glycosylation is also due to the implication of carbohydrates in many pathological states such as cancer, atherosclerosis and rheumatoid arthritis.

There are two main type of protein glycosylation: *N*-glycosylation, in which the glycan is attached to an Asn residue present in the tripeptide consensus sequon Asn-X-Ser/Thr (where X can be any amino acid except Proline), and *O*-glycosylation, in which the glycan is attached to a Ser or Thr residue [fig 4]. In rare cases other amino acid residues, e.g., cysteine or lysine, may also be glycosylated. N-linked glycans contain N-acetylglucosamine (GlcNAc) linked via an amide bond to asparagine residues. The complexity of N-linked oligosaccharide structures are reduced by the existence of their core region but enhanced through the variations in the antennal region. *N*-glycosylation is a co-translational event where prefabricated oligosaccharide units are transferred from the lipid carrier, i.e. dolichol diphosphate to *Asn* residues as soon as the growing polypeptide chain enters the lumen of the endoplasmic reticulam [Kobota, 1992; Woods, 1994].

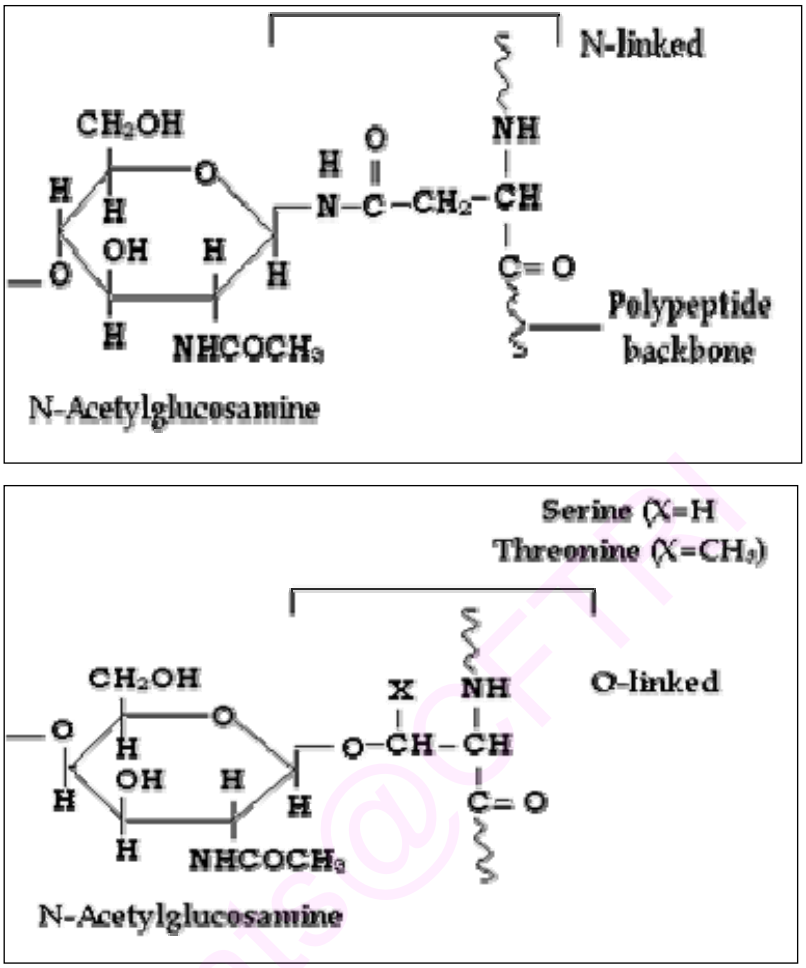

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Fig. 4: N-linked and O-linked oligosaccharides of glycoproteins

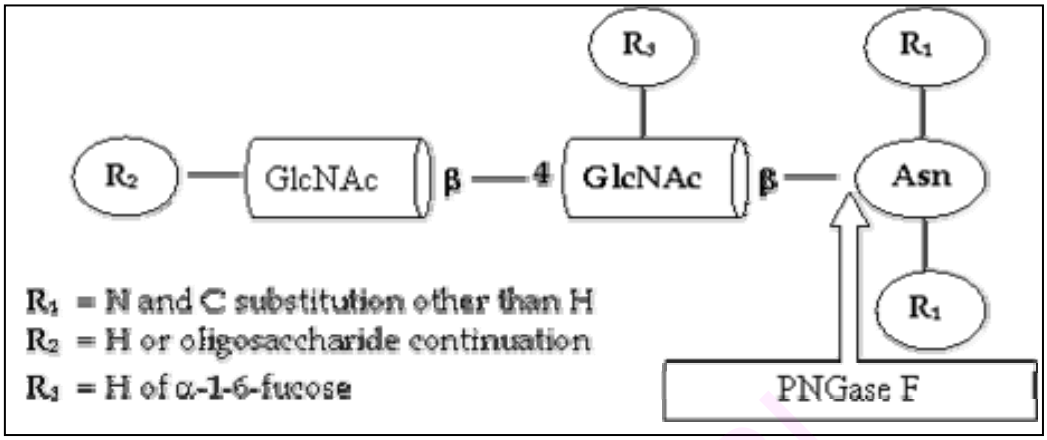
Complex oligosaccharides attached to an asparagine residue in glycoprotein termed *N*-linked oligosaccharides have a common pentasaccharide core structure and one or more antennae attached to each of the mannose residues at the non reducing terminus. Glycosylation is observed in all eukaryotes and has also been found in prokaryotes [Moens, & Vanderleyden, 1997]. Porcine pancreatic α- amylase was found to be a glycoprotein [Beaupoil-Abadiee, et al., 1973]. In mammalian systems, these antennae consist either of mannose (Man) chains (termed "high-mannose" sugars) or chains containing N-acetyl-2-amino-2-

deoxyglucose (GlcNAc) with or without galactose (Gal) [Harvey, et al., 1997]. The biological significance of the carbohydrate moiety of a glycoprotein has been a matter of much speculation. Carbohydrates in the form of asparagine-linked (*N*-linked) or serine/threonine-linked (*O*-linked) are major structural components of many eukaryotic proteins.

The diversity of oligosaccharides structure often results in heterogeneity in the mass and charge of glycoproteins. These glycosylation modifications are not merely decorations. The carbohydrate perform critical biological functions/ processes such as embryonic development, inter and intracellular activities [Helenius, & Aebi, 2001], coordination of immune functions [Rudd, 2001], cell division processes and protein regulations and interactions.

To study the structure and function of a glycoprotein it is often desirable to remove either only one or all selected class of oligosaccharides. This approach allows the assignment of specific biological functions to particular components of the glycoprotein. For the glycomic analysis glycans are often released from protein back-bones. Asparagine (Asn)-linked glycans can be cleaved enzymatically by peptide *N*-glycosidase F (PNGase F, EC 3.5.1.52) and chemically by hydrazinolysis.

The enzymatic methods are more preferably employed since they yield intact oligosaccharides regardless of size or structure of the substrate/carbohydrate moiety resulting in a slightly modified protein in which Asn residues at the site of de-N-glycosylation are converted to aspartate (Asp) [fig. 5]. Chemical deglycosylation by hydrazinolysis causes chemical modification including N-deacetylation of sialic acids and N-acetyl-D-hexosamines such as GlcNAc and GalNAc residues, as well as extensive cleavage of polypeptide backbones [Harvey, 2000; Morelle, & Michalski, 2005].



Source: www.QA-Bio.com QA-Bio E-PNG05 Product Specifications - Protocol

Fig.5: Schematic representation of the action of PNGase F on glycoproteins

The reasons for deglycosylation experiments are manifold: i) for simplifying amino acid sequence determination of glycoproteins ii) to remove heterogeneity in glycoproteins for X-ray crystallographic analysis iii) to remove carbohydrate epitopes from antigens iv) to enhance or reduce blood clearance rates of glycoprotein therapeutics v) to investigate the role of carbohydrates in enzyme activity and solubility vi) to investigate ligand binding vii) for quality control of glycoprotein as pharmaceuticals.

Even though all animal α-amylases include glycosylation sequons (Asn-Xaa-Thr/Ser) in their sequences, amylases purified from natural sources are not quantitatively glycosylated. These glycosylation sequons suggest that the protein is a potential target of the glycosylation machinery. There are one or two of these sequons per amylase depending on the species and they are always located at the C-terminus of the protein in the C-domain. The two rat glycosylation sequons are located at positions 410 (Asn^{410}-Gly-Ser-Asn) and 459 (Asn^{459}-Cys-Thr- Gly) [MacDonald, et al., 1980]. According to studies on the influence of the Xaa

residues in the glycosylation sequon on the efficiency of glycosylation, the glycine and the asparagine of the rat Asn^{410} sequon favour N-glycosylation [Mellquist, et al., 1998]. However, it is remarkable that all glycosylation sequons of animal α-amylases are located in the C-domain, a domain of unknown function. One of the roles proposed for this domain is to help stabilize the $(\beta/\alpha)_8$ barrel of the A-domain [Strobl, et al., 1998].

The N-glycosylation site of PPA is located between the amino acid residues from position 235 to 293 and for taka-amylase it is located between the amino acid residues 234 to 290. These regions are located on the enzyme surface close to their active-site cleft in their three-dimensional structures. Such a carbohydrate chain might interact with the substrate entering into the active-site cleft. The presence of the carbohydrate chain near the active-site cleft might improve its efficiency since α-amylase is known to hydrolyze the soluble starch several times by single/multiple attack mechanisms [Robyt, & French, 1967; Kondo, et al., 1978].

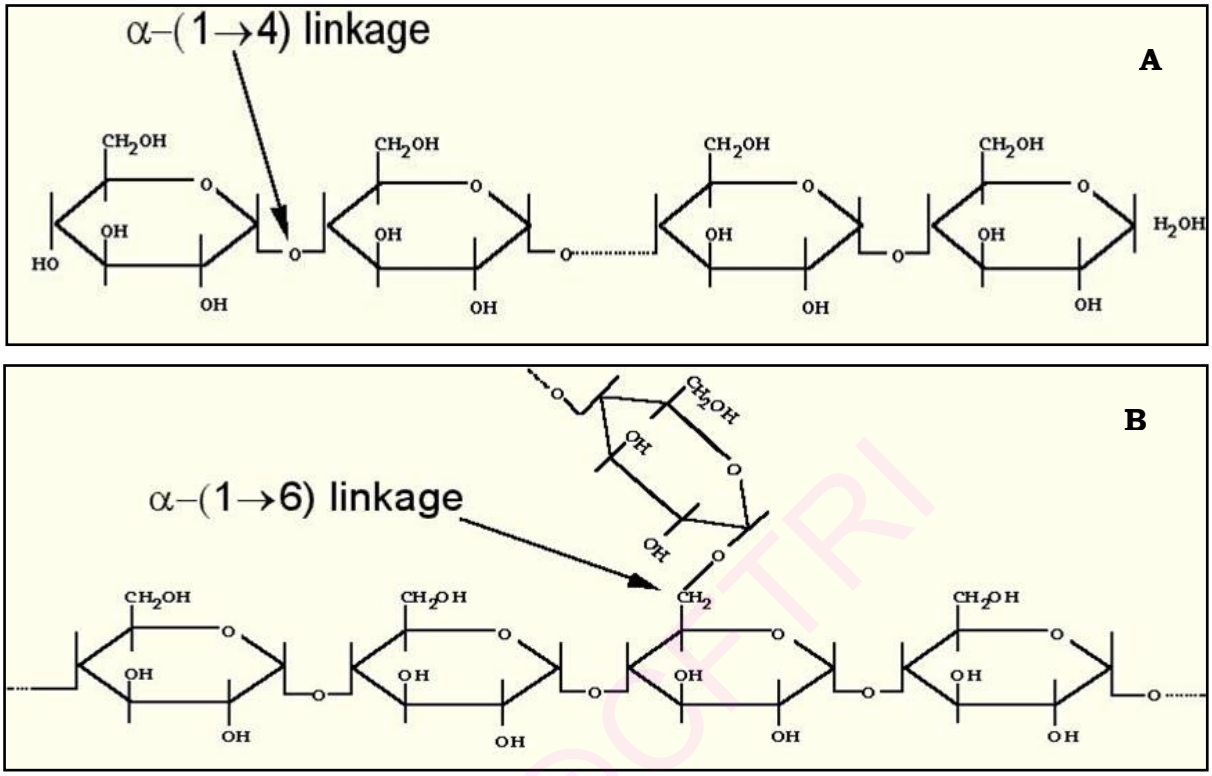
Effects of carbohydrate chains on thermostability were reported for glucoamylase [Takegawa, et al., 1988]. These results suggest that the presence of the carbohydrate chain is important for stabilization of the three-dimensional structure. It is important to consider the position of the N-linked carbohydrate chain on the enzyme to elucidate the effects of the carbohydrate chain on the enzyme kinetics.

Structural identification of these oligosaccharides requires determination of the constituent monosaccharide, their sequence, the branching pattern and the hydroxyl groups involved in the linkage of one residue with another. Composition and linkage are best determined by gas chromatograph and MS following hydrolysis, whereas sequence and branching information can be deduced from fragment ions produced by fast atom bombardment (FAB) [Harvey, et al., 1997].

I.8.a. Starch - the substrate

Starch is the major digestible carbohydrate in the human diet and contributes a substantial amount of calories. Starch granules are composed of two types of α-glucans, amylose and amylopectin which represent approximately 98-99% of the weight. Amylose, a linear polymer of α-D-glucopyranosyl units linked by the α-1,4 bonds and amylopectin is a branched polymer of α-D-glucopyranosyl units linked by α-1,4 and α-1,6 linkages (4.0–5.5% branching) [fig. 6] [Hizukuri, 1996; Englyst, et al., 1992]. Amylopectin has a cluster-like organization and forms crystalline regions developed from double helices of linear branched chains. This results in the formation of amorphous and crystalline lamellae arranged in an overall semi crystalline structure.

Starch is acted upon by the salivary and pancreatic α-amylases and the brush border enzymes to produce glucose which is utilized by the human body for various metabolic activities. Undigested or resistant starch is fermented by bacteria in the large intestine thereby producing short chain fatty acids such as acetate, propionate and butyrate which are utilized for further metabolic activities.



Source: http://images.google.co.in/images

Fig. 6: Structure of (A) Amylose and (B) Amylopectin

The susceptibility of starch granules to degradation by α-amylase depends on their botanical origin and the α-amylase source [Planchot, et al., 1995; Leach, & Schoch, 1961]. An important parameter correlated to the extent of amylolysis is the variation in starch crystallinity (A or B) [Katz, 1934]. Starch granules from various botanical sources have one of the three X-ray diffraction patterns. Most cereal starches, normal maize, rice, wheat and oats exhibit the type A pattern, alternatively called the cereal type which are more readily hydrolyzed by α-amylase. Potato, lily, canna and tulip starches show B-type diffraction pattern called the tuber type. Some of the rhizome and bean starches exhibit the C-type pattern which is a mixture of A and B types in varying proportions.

Porcine pancreatic α-amylase degrades rice or wheat starches (A-type) over 6 times faster than banana starch and over 20 times faster than potato starch (B-type) [Williamson, et al., 1992]. The final rates after extensive hydrolysis of the banana starch by common α-amylases from plants or animals are in the range of 5 to 20% [Ring, et al., 1988]. The lower susceptibility of potato starch has been attributed to a higher granular size than the normal cereal starches [Ring, et al., 1988] and to a greater crystalline structure [Gallant, et al., 1992]. Studying reference crystalline materials is a way of understanding the differences observed in the susceptibility of native starch granules towards amylolysis [Planchot, et al., 1997].

The processed i.e. cooked starch is easily digested in the human body as its crystalline structure is destroyed during cooking. Digestion of starch is effected by hydrolysing enzymes in a complex process which depends on many factors, these include the botanical origin of starch, whether the starch is amorphous or crystalline, the source of enzymes, substrate to enzyme concentration, temperature and time as well as the presence of other substances in the multi component matrix in which starch occurs naturally, e.g. cereal grains [table 2]. Native starch is digested slowly compared to processed (gelatinised) starch whose crystallinity has been lost and wherein the accessibility of substrate to enzyme is greater and not restricted by α-glucan associations such as double helices (especially in crystallites) or amylose-lipid complexes (cereal starches). The restriction of starch digestion in the human digestive system due to forms which are resistant to hydrolysis has led to the concept of dietary 'resistant-starch' [Tester, et al., 2004].

Table*. 2: Characteristics of native starch granules from common sources

Starch	Size (μm)	Shape	Distribution
Barley	2-5 (B-granules) 15-25	Spherical Lenticular	Bimodal
Maize (waxy and normal)	2-30	Spherical/polyhedral	Unimodal
Amylomaize	2-30	Irregular	Unimodal
Millet	4-12	Polyhedral	Unimodal
Oat	3-10 (single) 80 (compound)	Polyhedral	Unimodal
Pea	5-10	Rentiform	Unimodal
Potato	5-100	Lenticular	Unimodal
Rice	3-8 (single) 150 (compound)	Polyhedral	Unimodal
Rye	5-10 (B-granules) 10-40 (A-granules)	Spherical Lenticular	Bimodal
Sorghum	5-20	Spherical	Unimodal
Tapioca	5-35	Spherical/ Lenticular	Unimodal
Triticale	1-30	Spherical	Unimodal
Wheat	2-10 (B-granules) 15-35 Lenticular (A-granules)	Spherical	Bimodal

*Reprinted with permission (Tester, et al., 2004)

I.8.b. Starch digestion *in vivo*

Animals and humans produce a range of digestive enzymes which hydrolyse starch. Starch digestion and absorption essentially consists of three phases: the intraluminal phase, the brush border phase and the phase of glucose absorption. The digestion of starch is initiated in the oral cavity by salivary α-amylase secreted from the parotid glands. Chewing disintegrates the food, thus increasing the surface area to volume ratio in the solid phase and hence enzyme accessibility. The starch which is partially digested by salivary α-amylase is further acted upon by pancreatic α-amylase and cleaves α-(1- 4) bonds and thus produce glucose, maltose, oligosaccharides and dextrins in the duodenum of the small intestine.

Glucose can be absorbed from the small intestine (especially terminal end of the duodenum and jejunum) whereas maltose and malto-dextrins generated from starch hydrolysis cannot be absorbed. Absorptive epithelial cells which line the intestinal villi produce a number of other 'brush border' enzymes that allow digestion and subsequent absorption of disaccharides [Ao, et al., 2007; Eliasson, 1996].

The α-limit dextrins and small linear oligomers are not absorbable into the bloodstream and are converted to glucose in the human small intestine by the combined action of mucosal maltase-glucoamylase (MGAM, E.C. 3.2.1.20 & 3.2.1.3) and sucrase-isomaltase (SIM, E.C. 3.2.1.48 & 3.2.1.10). These enzymes are not released into the lumen of the small intestine but are bound to the membrane of microvilli. Consequently, some carbohydrate hydrolysis occurs at the surface of epithelial cells in the small intestine rather than the lumen of the gut. Brush border enzymes include sucrase which converts sucrose to glucose and fructose and lactase converting lactose to glucose and galactose, but these are not relevant to starch catabolism. However, maltase (converting maltose generated from α-amylase activity to glucose) and isomaltase (α-dextrinase) which hydrolyses

α-(1-6) bonds of isomaltose and α-dextrins continue the digestive process and thus convert the available starch to glucose.

I.9. Non-starch polysaccharides

Cereals, the staple food for millions of people across the world, are the chief source of both soluble and insoluble dietary fibre (DF) [Plaami, 1997]. The nature of carbohydrates with a potential to help prevent diseases is a growing field of interest. Non-glycemic carbohydrates, i.e. those carbohydrates or their components that are not absorbed in the small intestine, transit down to become fermented in the colon. In fact, food carbohydrates can be broadly classified on the basis of their *in vivo* digestibility into digestible and non-digestible carbohydrates [Asp, 1996; Englyst, et al., 1992]. Non-digestible carbohydrates have been collectively referred to as 'dietary fibre' [Hipsley, 1953]. Some of these carbohydrates are of particular interest to the food industry for the purpose of developing 'functional foods', i.e., foods that are able to exert positive health effects. Non-digestible oligo/polysaccharides are considered as prebiotics, which stimulate the growth of bifidobacteria in the colon.

Arabinoxylans, along with 1,3/1,4 β-D-glucans, are the major components of soluble DF [Rao, & Muralikrishna, 2004]. Cereals form the quantitatively most important source of non-starch-polysaccharides (NSP). Consuming cereals and cereal based products are known to have beneficial roles in human nutrition and health and have been linked to their phytochemical profiles [Adom, & Liu, 2002; Adom, et al., 2003; Charalampopoulos, et al., 2002; Mori, et al., 1999]. The common diseases such as cardiovascular diseases, diverticulosis, diabetes and colon cancer are linked to the deficiency of complex carbohydrates/dietary fibre in the food. Prevention of constipation and regulation of transit time are mainly caused by the bulking effect of DF. It is also partly fermented in large intestine by a mixed flora of anaerobic bacteria and most of the physiological effects of DF are thought to be based on this property [Scheeman, 1998]. After more than 30 years

of research into many and varied claims for its benefits, it is now clear that fibre has uniquely significant physical/physiological effects. A daily intake of approximately 30g is encouraged to promote health benefits associated with fibre. Because of the increased nutritional awareness, the food industry is facing the challenge of developing new food products with special health enhancing characteristics [Charalampopoulos, et al., 2002]. To meet this challenge it must identify new sources of neutraceuticals and other natural and nutritional materials with the desirable functional characteristics [Izydorczyk, et al., 2001]. In view of the therapeutic potential of DF more fibre incorporated food products are being developed all over the world. However, consumer acceptability of these functional foods depends not only on the nutritional property, but also on the functional and sensory qualities. These factors are considered while developing functional foods.

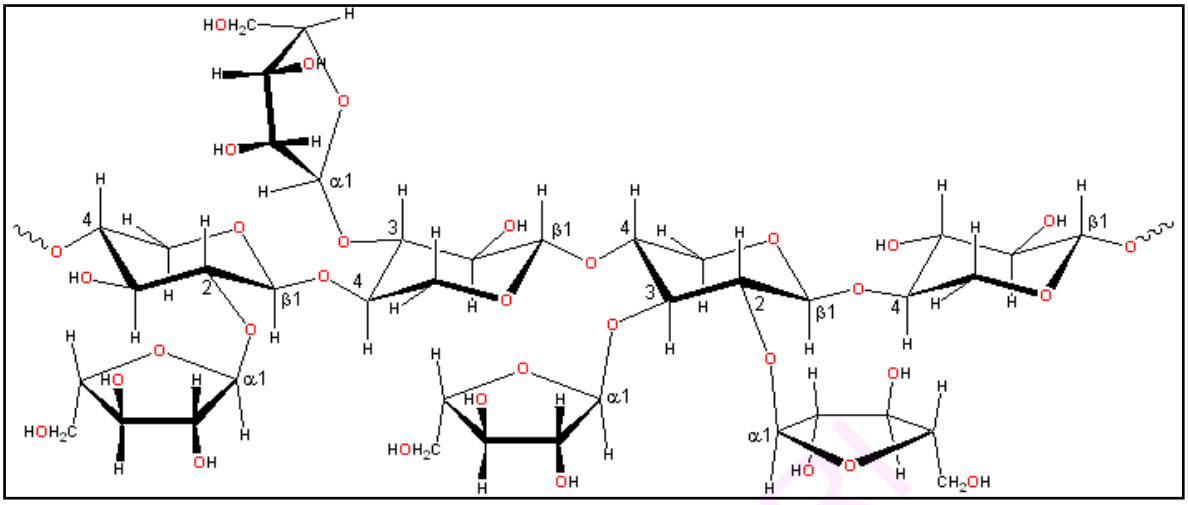
Polysaccharides may be extracted with various extractants such as water (for water soluble polysaccharides and mixed glucans), polar non-aqueous solvents (for starch and glycogen) [Leach, & Schoch, 1962], chelating agents (for pectins) [Selvendran, 1985], N-methyl morpholine-N-oxide (MMNO, for cellulose) [Chanzy, et al., 1979] and alkali (for hemicellulose A and B) [Wilkie, 1979]. Water extraction at different temperatures can be carried out to obtain gums and mucilages.

Polysaccharides are highly complex and diverse unlike proteins. They are heterogeneous in their chemical characteristics like molecular weight and composition which in turn affects their physical properties. The heterogeneity arises because of their biosynthesis which is controlled indirectly by glycosyltransferase genes. Glycosyltransferases, the enzymes with individual specificities are responsible for the transfer of sugar residues from particular glycosyl donor to the growing polysaccharide chain. Variations in polysaccharide structures may result from (a) departure from absolute specificity of the transferases (b) incomplete formation of segments/side chains and (c) post

polymerization changes. If these variations are continuous with respect to parameters such as molecular size, proportions of sugar constituents and linkage type, separation into discrete molecular species will be impossible and the polysaccharide sample would be called 'polydisperse'. If the heterogeneity lies in their molecular size, but not in their chemical composition, they are called 'polymolecular' [Aspinall, 1983].

In cereal grains, arabinoxylans (AX), 1,3/1,4 β-D-glucans are non starch polysaccharides from cell walls [Fincher, et al., 1975]**.** Arabinoxylans from endosperm are partly water-soluble and result in highly viscous aqueous solutions. This high viscosity of cereal grain water extract has a positive effect in some technological processes (bread-making) [Biliaderis, et al., 1995]. AX are constituted of a linear backbone of β-(1,4)- linked D-xylopyranosyl units to which α-arabinofuranosyl substituents are attached through O-2 and/or O-3, some ferulic acid moieties may esterify arabinofuranoyl residues at O-5 [fig. 7]. On an average, an arabinose to xylose ratio of 0.6 is usually found in wheat water-soluble AX [Izydorczyk, et al., 1991, Hoffmann, et al., 1991] but high natural variations are observed [Cleemput, et al., 1995; Izydorczyk & Biliaderis, 1995].

Andrewartha, et al., [1979] have ascribed the high viscosifying effect of AX to an extended rod-like conformation. Furthermore, it was generally assumed that variations in Ara : Xyl ratio affect the behavior of AX, the increase in arabinose content leading to a more 'stiffened' and extended conformation [Andrewartha, et al., 1979 ; Dea, & Rees, 1973].



Source: http://images.google.co.in/images

Fig. 7: Structure of arabinoxylan

However, ferulic ester bridges have been detected in these fractions which indicate that the behaviour of the polysaccharide in solution not only depend on the degree of polymerization (DP) of the xylan backbone and on its degree of substitution but also on the number of crosslinks.

I.10. Amylase inhibitors

α-Amylase inhibitors are diverse as they can be synthetic, microbial and of plant origin. α-Amylase and α-glucosidase have been targeted as potential avenues for modulation of postprandial hyperglycemia through mild inhibition of the enzymatic breakdown of complex carbohydrates to decrease meal-derived glucose absorption. Hyperglycemia is a condition characterized by an abnormal presence of sugar in the blood. Elevated postprandial blood glucose levels are widely recognized as one of the earliest disease markers in the prediction of subsequent microvascular and macrovascular complications that can progress to full symptomatic type 2 diabetes (T2DM) [Zimmerman, 2001; Fonseca, 2003]. Majority of available synthetic antidiabetic drugs target the dual metabolic

defects that characterize T2DM, impaired insulin secretion and insulin resistance, while some of these drugs (e.g. metformin) can have negative side effects at high doses [Mudaliar, & Henry, 2001; Carroll, et al., 2003; Fonseca, 2003]. Thus, a major goal of antidiabetic research is the discovery of anti-hyperglycemic agents that are safe and that lack any negative side effects. Dietary α-glucosidase and α-amylase inhibitors that act in the gut by inhibiting the enzymatic breakdown of starch/soluble carbohydrates have been identified as a potentially natural and safe approach for controlling hyperglycemia through modulation (i.e. decrease) of meal-derived glucose absorption.

The potential of phenolic-optimized aqueous extracts of selected foods in a typical American and Asian diets for anti-amylase and anti-glucosidase activities (antidiabetic potential) have been investigated [Patrick, et al., 2005]. Tannins are natural polyphenolic compounds of high molecular weight which form insoluble complexes with proteins. The hydroxyl groups of these molecules are partially or totally esterified with phenolic compounds such as gallic acid (gallotannins) or ellagic acid (ellagitannins). The tannins have general protein complexing properties which can cause variable enzyme inhibition. Inhibition of salivary α-amylase by tea polyphenols was documented [Zhang, & Kashket, 1998]. Recently, soft fruit extracts (rich in ellagitannins and anthocyanins) were found to be effective α-amylase inhibitors [McDougall, et al., 2005]. The exact structure of active tea polyphenols and kinetic properties of tea and fruit extracts have not yet been investigated**.** It is a well known from the published literature that the inclusion of polyphenols in the diet can lead to perturbation of mineral absorption from the intestinal canal, decrease the bodyweight gain, retard growth and inhibit the digestive enzymes [Bennick, 2002; Baxter, et al., 1997].

Hydroxycinnamic acids, especially ferulic acid are the most important phenolic compounds to form cross-links in plant cell wall. Ferulic acid and p-coumaric acid are ester-linked to arabinoxylans in cereals and other grasses and ferulic acid is linked to also pectins in some dicots [Ishii, 1997]. Similarly, sinapic acid is

thought to be bound to polysaccharides via ester-linkages [Bunzel, et al., 2002]. These polysaccharides can potentially be developed into useful therapies for treatment of some diseases like diabetes, obesity, hyperlipoproteinemia, cancer and HIV [Mehta, et al., 1998]. The search for glucosidase inhibitors has yielded a number of chemically distinct inhibitors from microbes and plants [Atsumi, et al., 1990; Fischer, et al., 1995; Kwon, et al., 2001]. Among them acarbose, voglibose and miglitol have been in clinical use. Most of them were isolated from microbial origins and so microorganisms are an important source for screening α-glucosidase and α-amylase inhibitors.

Acarbose is a powerful pseudotetrasaccharidic inhibitor of amylase and α-glucosidase [Schmitt, et al., 1977; Talamond, et al., 2002]. The half-chair conformation of the cyclitol unit mimics the distortion expected in the transition state prior to hydrolysis. The enzyme activity is lost upon binding to the active site [Gilles, et al., 1996]. Acarbose may also bind to a secondary carbohydrate binding site to make another abortive complex [Al Kazaz, et al., 1996].

The structure of PPA complexed with acarbose a pseudotetrasaccharide [Gilles, et al., 1996] was determined by Fourier difference analysis and the interactions occurring at the active site were identified [Koukiekolo, et al., 1999]. An additional surface carbohydrate binding site has been detected in the $A_{\alpha 7}A_{\alpha 8}$ region facing the C-terminal domain segment between β9 and β10 in the 4,4'-dithio-α-maltotrioside-PPA complex [Buisson, et al., 1987; Qian, et al., 1997]. Two additional carbohydrate-binding sites have also been observed in the maltopentaose-PPA complex [Qian, et al., 1995]. PPA is much more sensitive to subtilisin attack in the inhibitor-enzyme complex which suggests that the conformation of PPA changes when complexed. A change in the three-dimensional structure has in fact been detected by Fourier difference analysis [Gilles, 1996].

Scope of the present investigation

Pancreatic α-amylase is the most important enzyme present in mammals which is mainly responsible for the *in vivo* degradation of starch. Sequence similarities among PPA and human pancreatic amylase revealed a percentage identity of 87.1%. Literature pertaining to the studies with respect to the purification and kinetic studies of porcine pancreatic α-amylase are available. A comparative study of the digestibility of cereal and millet starches by PPA and its isoforms is lacking. Porcine pancreatic α-amylase inhibition is one of the ways to decrease the release of post-prandial glucose levels in diabetic patients. One of the important approaches that did not receive due attention is pertaining to the effect of individual water soluble polysaccharides isolated from cereals on the activity of PPA and its isoforms. Water soluble polysaccharides (WSP) consists of arabinoxylans, (1→3)/ (1→4) β-glucans and phenolic acids that modulate the functional properties of foods. The WSP are viscous and are perhaps believed to reduce the activities of starch degrading enzymes, resulting in a slow release of post-prandial glucose. Soluble fibres vary in their molecular weight, arabinose/xylose ratio and the nature and content of covalently linked phenolic acids. Most α-amylases are glycoproteins. PPA possess three potential glycosylation sites. Enzymatic removal of the bound carbohydrate/ oligosaccharide/s by PNGase F is often desirable to study the role of covalently linked carbohydrate on the function of the pancreatic α-amylase which has not been carried out till date.

The modulation of postprandial hyperglycemia can be brought about by the inhibition of the PPA by complex carbohydrates to decrease post prandial (meal-derived) glucose absorption. The significance of food-grade, plant-based amylase inhibitors for modulation of carbohydrate breakdown and control of glycemic index of foods in the context of preventing hyperglycemia and diabetes mellitus complications is hypothesized in the literature. PPA is a glycoprotein with a carbohydrate moiety anchored to the protein/enzyme molecule. Studying the

differences in the glycosylated and deglycosylated forms of the glycoprotein (PPA, PPA-I and PPA-II) are very useful to monitor the effect of the bound carbohydrate on temperature stability and changes in spectral and circular dichroism (CD) spectra. In the present study an attempt is made to see the effect of WSP derived from the flours of ragi, rice, wheat and maize on the activities of PPA and its isoforms.

Keeping the above mentioned in proper perspective, the present investigation is envisaged with the following objectives:

- Finger printing of the oligosaccharides produced from cereals/millet starches by the action of pancreatic α-amylase with respect to a) composition b) degree of polymerization and c) molecular weight.
- Isolation of WSP from the cereals and millet and characterize the same with respect to their a) sugar composition b) bound phenolic acids and c) associated proteins.
- To study and understand the effect of water soluble polysaccharides on the activities of pancreatic α-amylase isoforms and study the mechanism if any.
- To study the role of N-linked glycan portion of pancreatic α-amylase on its activity and stability.

CHAPTER - II

Materials & Methods

II.1. General

- All the results are average values of minimum of three experiments.
- Enzyme purification was carried out at 4°C unless and otherwise mentioned.
- Starch gelatinization was carried out at ~ 95°C in a boiling water bath for 15 min.
- Extractions and reagents were prepared using double glass-distilled water.
- Room temperature was ~ 25°C.
- Boiling water bath temperature was ~ 95°C, unless otherwise mentioned.
- Dialysis, against double distilled water or buffer, was carried out at ~ 4°C by using dialysis bags with a cut off range of ~ 12,000 Da
- Concentration of samples was carried out by using Buchi Rotavapor (RE 111) with a water bath temperature ranging from 30-40°C.
- Colorimetric and spectrophotometric readings of test solutions with appropriate blanks were taken by using Shimadzu double beam Spectrophotometer (UV – 160A), Japan.
- Lyophilization was carried out using Virtis Freeze Mobile (12 SL), Germany.
- Centrifugation was carried out either in Sigma (202 C), HermLe (Z 320 K), Kobata and Remi (RC 8) centrifuges.
- Gel permeation fractions were collected using Gilson FC 203 B and LKB Bromma 2211 fraction collector.

II.2. Materials

Finger millet, ragi (*Eleusine coracana,* variety Indaf-15), rice (*Oryza sativa,* variety Jaya) and maize (*Zea mays,* variety NAC-6002) were procured from Zonal Agricultural Research Centre, Mandya of UAS, Bangalore, Karnataka, India. Wheat (*Triticum aestivum,* variety DPW-225) was procured from the local market.

II.3. Chemicals

II.3.a. Sigma Chemical Company, St.Louis, USA.

Enzymes: Porcine pancreatic α-amylase (E.C. 3.2.1.1), glucoamylase (E.C. 3.2.1.3) from *Rhizopus* and PNGase F from *Chyseobacterium (Flavobacterium) meningseptium* (E.C. 3.5.1.52).

Other chemicals: Blue dextran, coomassie brilliant blue G-250 coomassie brilliant blue R-250, amonium per sulphate (APS), protein molecular weight standards (GPC) [β-amylase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14 kDa), cytochrome C (12.4 kDa)], carbazole, dinitrosalicylic acid (DNS), sodium azide, sodium borohydride, Tris (Hydroxy methyl) methyl amine [2-Amino-2-(hydroxy methyl) propane-1,3-diol], TEMED (N,N,N′,N′-Tetramethyl ethylene diamine).

Standard malto-oligosaccharides: maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.

Phenolic acids: Ferulic, coumaric, caffeic, gentisic, protocatechuic, vanillic, syringic, gallic and cinnamic acids

Gel matrices and ion exchange resins: Sepharose CL-2B (2%, cross-linked) (fractionation range for dextrans 30,000-5,000,000 Da), DEAE-cellulose (0.99 meq/g), Amberlite IR-120-P (8% cross-linked, 16-50 mesh).

II.3.b. Other sources

Protein molecular weight markers (SDS) [Lactoglobulin (18.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase (97.4 kDa)] obtained from Genei, Bangalore, India.

T-series dextran standards (T-10, T-20, T-40, T-70, T-150, T-500, T-2000) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Sugar standards (rhamnose, xylose, arabinose, glucose, galactose, mannose, maltose, inositol) were from ICN Pharmaceuticals Inc., Life Sciences group, Cleveland, USA.

Biogel P-2 (100-200 mesh, fractionation range 100 to 1800 Da) was from Bio Rad Laboratories, Richmond, California, USA.

Termamyl was obtained from Novozymes, Denmark.

Dimethyl sulphoxide [DMSO] (UV spectroscopic grade), Folin phenol reagent (2 N) and bovine serum albumin (BSA) were from Sisco Research Laboratories, Mumbai (Bombay), India.

II.4. Distillation/purification of solvents

Phenol, acetic anhydride, pyridine, diethyl ether and DMSO were purified by distilling in glass distillation apparatus following standard distillation protocols.

II.5. Columns

II.5.a. GLC

OV-225 (ϕ 1/8″ x 8 ft), 3% on Chromosorb W (80 – 100 mesh) was obtained from Pierce Chemical Company, Rockford, Illinois, USA.

II.5.b. HPLC

μ-Bondapak-NH_2 carbohydrate column (ϕ 4.1 mm × 30 cm)

E-linear (ϕ 7.8 mm x 300 mm) and E-1000 (ϕ 3.9 mm x 300 mm) gel permeation columns were from Waters Associates, Milford, USA.

Shimpack C_{18} column (ϕ 4.6 mm x 250 mm) was obtained from Shimadzu Corporation, Tokyo, Japan.

II.6. Instruments

The following instruments were used for various experiments:

- GC-15A, equipped with FID Shimadzu, Shimadzu Corporation, Tokyo, Japan.
- HPLC-LC 10A, equipped with RI and UV-photo diode array detectors, from Shimadzu, Japan.

- Scanning Electron Microscope, LEO-435VP from Leo Electron Microscopy Ltd., UK.
- LC-MS / MS from Waters, UK.
- UV-visible Spectrophotometer (UVH 60A) from Shimadzu, Japan.

II.7. Colorimetric estimations

II.7.a. Total Carbohydrate

To the sample (0.5 mL) in a test tube (ϕ 12 x 150 mm), phenol (0.3 mL 5%) and concentrated H_2SO_4 (1.8 mL, specific gravity: 1.84) were added successively and mixed thoroughly. After incubation at room temperature for 20 min, absorbance was read at 480 nm [McKelvy, & Lee, 1969; Dubois, et al., 1956].

Sugar content was determined by referring to the standard graph, prepared by using either D-glucose or maltose (5-50 µg/0.5 mL).

II.7.b. Reducing sugar by dinitro salicylic acid

Preparation of DNS reagent: Dinitro salicylic acid (1g) was dissolved in a solution containing sodium potassium tartrate (30 g) and 2.0M NaOH (20 mL) and the contents were made up to 100 mL with water. The reagent was filtered using Whatman No. 1 filter paper. Stored at 4°C and used whenever required.

To the sample (1 mL) in a test tube (ϕ 12 x 150 mm), DNS reagent (1 mL) was added and incubated in boiling water bath for 10 min. Contents were then cooled and diluted with equal volume (2 mL) of distilled water. Absorbance was read at 550 nm [Cleemput, et al., 1997; Miller, 1959].

Reducing sugar content was determined by referring to the standard graph prepared by using maltose (0.1-1.0 mg/mL).

II.7.c. Uronic acid

To the sample (0.5 mL) in a test tube (ϕ 12 x 150 mm), concentrated H_2SO_4 (3 mL) was added keeping in an ice-cold water bath and mixed well. After

incubation in boiling water bath for 20 min, carbazole* solution (0.1 mL, 0.1% in ethanol) was added and kept in dark for 2 h. Absorbance was read at 530 nm [Knutson, & Jeanes, 1968].

Uronic acid content was determined by referring to the standard graph prepared by using either D-glucuronic or D-galacturonic acid (10-50 µg/0.5 mL).

* Carbazole was re-crystallized in benzene to remove impurities.

II.7.d. Protein estimation

i) Bradford method

Preparation of Bradford reagent: Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5 mL, 95%) and phosphoric acid (10 mL, 85%) was added. The solution was made up to 100 mL with water and filtered through Whatman No. 1 filter paper and stored at 4°C in a brown bottle.

To the sample (0.2 mL) Bradford reagent (0.8 mL) was added and mixed well. Absorbance was read at 595 nm [Bradford, 1976; Zor & Sclinger, 1996]. Protein content was determined by referring to the standard graph prepared by using BSA (2 – 10 µg/0.2 mL).

ii) Extinction coefficient method

Protein concentration of porcine pancreatic amylase was determined using a pre-determined value of specific extinction co-efficient $E^{1\%}_{280}$ =26 [Caldwell, et al., 1952].

iii) Spectrophotometric method

The presence of protein/peptide bonds in column fractions were monitored by taking absorbance at 280/220 nm respectively [Peterson, 1983].

II.7.e. Amylose by iodometry

Preparation of iodine reagent: Iodine (8.2 mg) was dissolved in water (50 mL) containing KI (216 mg) and the final volume was made up to 100 mL with water. The reagent was stored at room temperature in a brown bottle.

Quantification of amylose: Total and soluble amylose contents were determined and the λ_{max} of the starch-iodine complex was recorded in a Shimadzu UV-Visible spectro-photometer [McCready, & Hassid, 1943; Sowbhagya, & Bhattacharya, 1979; McGrance, et al., 1998]. Calibration curve was prepared using corn amylose (40-200 µg).

II.8. Enzyme assays

II.8.a. Amylase (E.C. 3.2.1.1)

Gelatinized soluble starch (1%, 1 mL) in phosphate buffer (0.05 M, pH 6.9) was incubated with enzyme (10 µL) at 37°C for 60 min. The reaction was stopped by adding 1 mL of DNS reagent. The enzyme activity was expressed as the amount of reducing sugar liberated (estimated by DNS method) /min/g flour under the assay conditions [Bernfeld, 1955].

One unit of the enzyme activity was defined as µmol maltose equivalent released per minute under the assay condition and the specific activity was calculated as activity / mg protein.

II.8.b. Glucoamylase digestion of starch

Sample (10 g) was dissolved in sodium acetate buffer (100 mL, 0.1 molar, pH 4.8) and incubated with termamyl (1 mL, ~1000 units) at 95°C for 1 h. After cooling glucoamylase (100 mg, ~7000 units) was added and incubated at 37°C for 48 h. Glucose released from the starch hydrolysis was quantified by glucose oxidase method

II.8.c.Glucose oxidase method

Preparation of glucose oxidase reagent: Glucose oxidase (125 mg, ~6250 units) was dissolved in tris buffer (0.1 molar, pH 7.0) and peroxidase [0.5 mL (10

mg/mL in water), ~1250 units], O-dianisidine (0.5 mL, 10 mg/mL in 95% ethanol) and triton X-100 (1 mL, 10 mL detergent in 40 mL ethanol) were added. The solution was made up to 100 mL with tris buffer.

Quantification of glucose: To the sample (0.5 mL) glucose oxidase reagent (3 mL) was added and incubated at 37°C for 1 h. Absorbance was read at 420 nm [Dahlqvist, 1964].

Glucose content was determined by referring to the standard graph prepared by using glucose (10 – 50 μg/0.5 mL).

II.9. Isolation and characterization of starches

II.9.a. Isolation of starches

Starches were isolated as per the standard protocol reported earlier [Muralikrishna, et al., 1982; Madhusudhan, & Tharanathan, 1996]. Starches from the flours (ragi, rice, wheat and maize) were isolated by steeping in water in the presence of $HgCl_2$ (0.01 M) and passing through 60μ and 240μ mesh sieve (BSS). The filtrate containing crude starch was purified by suspending in dilute NaOH (pH 9.0) for 10 mins followed by repeated washing with NaCl-toluene (10:1 v/v). The floating scum layer of starch-protein-toluene complex was removed by centrifugation (3000 x g, ~10 min) and pooled together. The bottom dense, firm sediment was the starch isolate. This was taken in excess water (~ 8 L), stirred and kept overnight and again centrifuged (3000 x g, ~10 min). The starch isolate was washed with water, ethanol (x 4), acetone (x 2), finally with ether and dried. The purity was ensured by microscopic examination.

II.9.b. Gel permeation chromatography

Sepharose CL-2B was packed into a glass column (ϕ 1.7 x 90 cm) and equilibrated with the running eluent overnight. The samples (starches) dispersed in 85% DMSO (2 mL) was applied to the column bed and eluted with water containing 0.02% sodium azide at a constant flow rate (18 mL /h)

[Billiaderis, et al., 1979]. Fractions (1.5 mL) were collected and an aliquot (0.2 mL) of the fraction was analyzed for total sugar [Dubois, et al., 1956] as well as for starch iodine blue color at 630 nm.

II.9.c. Molecular weight determination

The approximate molecular weight (MW) of the starches were determined from a calibration curve prepared for standard dextrans (T-10, T-20, T-40, T-70, T-500 and T-2000) of known molecular weight on the same GPC column. The void volume (V_o) was determined by using a predialyzed blue dextran (5 mg/0.5 mL water). The molecular weight values were computed from the standard plot of log M_w vs $V_e/V_{o.}$, where V_e was the elution volume of the respective fractions [Wankhede, et al., 1979; Praznik, et al., 1998].

II.9.d. High Performance Size Exclusion Chromatography (HPSEC)

HPSEC was carried out using Shimadzu HPLC system (LC-8A model) equipped with refractive index (RI) detector and CR 4A recorder. E-Linear (7.8 mm × 30 cm) and E-1000 (3.9 mm × 30 cm) columns connected in series were used for the analysis. The fractions (10 mg/mL) were dissolved in water and centrifuged at 3000 x g, for 5 min to remove any undissolved /particulate materials prior to the analysis. Samples and standards (10 mg/mL each) were individually loaded (10 µl) and eluted with water at a flow rate of 0.6 mL/min and the elution was monitored by using refractive index detector set at 8 x 10^{-6} RIU.

II.9.e. Scanning electron microscopy (SEM)

Starch samples were mounted on metal stubs with the aid of double sided Scotch ® adhesive tape. The samples were gold coated (about 100 Å, 10 nm thickness) in a KSE 24M sputter coater and viewed in a scanning electron microscope (LEO 435 VP) and the selected regions depicting distinct morphological features were photographed and scanned at a constant voltage of 20 kV [Mohan, et al., 2005].

II.9.f. Differential scanning calorimetry (DSC)

Gelatinization: onset (T_o), peak (T_p) and conclusion (T_c) temperatures and enthalpy of gelatinization were measured on a TA Instruments DSC 2010 (New Castle, DE) temperature programme controlled calorimeter. To sample (10 mg) taken in an aluminium pan, water (40 μL) was added, the pans were sealed, equilibrated for 1 h and scanned in a differential scanning colorimeter. The instrument was programmed to rise 10°C/min. Heat flow and temperature calibration were performed using pure indium with a heat fusion of 28.4 J and onset melting temperature of 156.6°C. Values of T_o, T_p, T_c and ΔH were calculated from the output obtained from 3 runs for each starch samples [Stevens, & Elton, 1971; Rosa, et al., 1989]. The enthalpy of gelatinization (ΔH) was estimated by integrating the area between the thermogram and was expressed in terms of Joules per gram of dry starch.

II.9.g. X-ray diffraction

Isolated starches were examined for their crystallinity using EG-7G solid state germanium liquid nitrogen cooled detector, Scintag XDS-2000 (Sunnyvale, CA, USA) instrument equipped with a θ-θ goniometer at 25 mA and 30 kV. The samples were exposed for 5 h to Cok α filtered radiation (λ 1.54184 nm). Diffractograms were scanned from 2 to 40°, which covers all the significant diffraction peaks of starch crystallites at a diffraction angle of 2θ. The degree of crystallinity was quantitatively estimated [Norman, et al., 1998; Nara, & Komiya, 1983]. The moisture contents of all starch samples were adjusted to ~19% by keeping in a desiccator over saturated $BaCl_2$ solution (25 °C) for one week [Barron, et al., 2000].

II.10. Purification of Porcine Pancreatic Amylase (PPA)

*II.10.a Chromatographic method*s

II.10.a.1. Ion exchange chromatography

Ion exchange chromatography was carried out on DEAE-cellulose column.

DEAE-cellulose regeneration

Anion exchanger was washed with water to remove the fines and then treated successively with five volumes of HCl (0.5 M) and NaOH (0.5 M) for 60 min each. After each treatment it was thoroughly washed with water to bring the pH to neutrality. The regenerated anion exchanger was suspended in five volumes of tris-HCl buffer (20mm, pH 8), and stored at 4°C.

Fractionation of PPA

Regenerated and degassed ion exchanger (50 mL) was packed in a column (ϕ 3 x 25 cm) and equilibrated with tris-HCl buffer (500mL, 20 mM, pH 8.0) at a flow rate of 18 mL/h. PPA was dialyzed against equilibrating buffer and loaded on the column. The unbound components were eluted with the loading buffer. A linear NaCl gradient (0-0.6) in equilibrating buffer was used to elute the bound components. Fractions (3 mL) were collected and monitored for protein as well as amylase activity.

II.10.a.2. Gel Permeation Chromatography on Sephadex G-100

Sephadex G-100 superfine (10 g) was kept in sodium phosphate buffer (50 mM, pH 6.9) for 24 h at room temperature for swelling. The fines were removed by repeated washing with buffer and degassed under vacuum and packed in a glass column (ϕ 0.8 x 80 cm) and equilibrated with the above buffer at a flow rate of 6 mL/h. Fractions (1 mL) were collected, monitored for protein and amylase activity.

II.10.a.3. Molecular weight by GPC

The molecular weights of the amylases were determined by GPC on Sephadex G-100. The experimental conditions were as described earlier. 100 mM NaCl was added to the elution buffer in order to reduce the interaction between the protein and the column matrix. The column was calibrated using Sigma molecular weight marker proteins such as β-amylase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14 kDa) and cytochrome C (12.4 kDa). A plot of V_e/V_o versus log molecular weight was used for calculating the molecular weight of the amylases, where V_o is the void volume (as described in II.9.C) and V_e is the elution volume of the standard proteins/amylase [Whitaker, 1963].

II.10.b. Electrophoresis methods

II.10.b.i. SDS Polyacrylamide Gel Electrophoresis (PAGE)

Vertical slab gel electrophoresis was carried out at room temperature according to Walker, 1996.

Reagents:

a) *Monomer:* Acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved in water (50 mL) and the volume was made up to 100 mL, filtered and stored in a brown bottle at 4°C.

b) *Separating gel buffer:* Tris (18.15 g) was dissolved in water (50 mL) and the pH of the solution was adjusted to 8.8 with 6 N HCl. The volume of the solution was made up to 100 mL with water and stored at 4°C.

c) *Stacking gel buffer:* Tris (3 g) was dissolved in water (25 mL) and the pH of the solution was adjusted to 6.8 with 6 N HCl. The solution was made up to 50 mL, filtered and stored at 4°C.

d) Ammonium persulphate: 200 mg was dissolved in 2 mL water.

e) *Tank buffer:* Tris (0.3 g) and glycine (1.44 g) and SDS (0.1 g) were dissolved in water and made up to 100 mL.

f) *Cocktail buffer:* Solution 'C' (25 mL), glycerol (2 mL) and bromophenol blue (0.01%, 0.5 mL) were mixed and stored at 4°C.

II.10.b.ii. Staining reagents

Coomassie staining

Staining reagent: Coomassie brilliant blue R-250 (100 mg) was dissolved in methanol: acetic acid: water (100 mL, 12.5: 7.5: 30 v/v). The reagent was filtered and stored at room temperature.

Destaining solution: Methanol: acetic acid: water (12.5: 7.5: 30 v/v).

II.10.b.iii. Preparation of vertical slab gel

Running gel (7.5%, 0.7 mm): It was prepared by mixing solutions - A (1.7 mL), B (1.1 mL) and water (1. 3 mL). The resulted solution was degassed and to it TEMED (30 µl), solution - D (60 µl) was added. The solution was mixed, poured between the assembled glass plates with edges sealed with 2% agar. Gels were allowed to polymerize at room temperature for 2 h.

Stacking gel (5%): It was prepared by mixing solution - A (0.67 mL) and C (0.9 mL) with water (2.1 mL) and degassed. To this mixture was added TEMED (30 µl) and solution - D (60 µl) and mixed well. The resulting solution was poured above the polymerized separating gel and the polymerization was facilitated at room temperature for 2 hrs.

II.10.b.iv. Electrophoresis run

The protein samples containing protein (5 µg) were dissolved in solution-F (50 µl), heated in a boiling water bath for 5 min and loaded (25 µl) on the wells immersed in solution 'E'. The electrophoresis was carried out at a constant voltage of 50 V for 3 h or until the tracking dye was just above the lower end of the gel.

II.10.b.v. Gel Staining

After electrophoresis the gels were stained for proteins.

Coomassie blue staining

The gels after electrophoresis were kept in solution *(a.1)* for 2 h and destained by solution *(a.2).*

II.10.b.vi. Polyacrylamide Gel Electrophoresis (PAGE)

PAGE under native conditions was carried out to evaluate the purity of amylases. The electrophoresis was carried out as described above without SDS [Walker, 1996]. The sample containing ~5µg of protein was dissolved in 50 µL of sample buffer devoid of SDS and β-mercaptoethanol. 25 µL of this sample was loaded on the wells and were run at a constant voltage of 50 V for 3 h and the gel was stained for protein as well as for amylase activity.

II.10.b.vii. Amylase Activity staining

After electrophoresis the gel was incubated in gelatinized soluble starch (2%) prepared in sodium acetate buffer (50 mM, pH 5.0) at 45°C for 30 min. Subsequently the gel was stained with iodine reagent (3%) and the amylase bands were visualized as transparent clear zones against a dark blue background [Acevedo, & Cardemil, 1997].

II.10.b.viii. Glycoprotein staining

Reagents:

a) *Solution A:* Trichloroacetic acid (100 X) 12.5 mL madeup to 100 mL in distilled water.
b) *Solution B:* 1g of periodic acid dissolved in 100 mL of water containing glacial acetic acid (3%, v/v).
c) *Solution C:* Schiff's reagent

8 g of potassium metabisulphite + 10.5 mL of concentrated HCl + 4 g of fuschin, dissolved in 1 lt water. The mixture then clarified by adding 1g of activated charcoal, filtered and stored in brown bottle at 4°C.

d) *Solution D:* Aqueous glacial acetic acid (7%).

Initially the gel after electrophoresis was fixed by keeping in solution 'a' for 30 min. Gels were then immersed in solution 'b' for 50 min at room temperature. The gel was repeatedly washed with distilled water to remove traces of periodic acid and then immerse in solution 'c' for 60 min at 4°C in dark followed by destaining in solution 'd' [Zacharius, et al., 1969]. Ovalbumin and BSA were taken as positive and negative controls.

Observation: Glycoprotein stains pink against white background.

II.11. Kinetic studies

II.11.a. Effect of temperature

Amylase activity was determined at a temperature range of 30-70°C (with an interval of 5°C) with 1% soluble starch as substrate in 50 mM sodium phosphate buffer (pH 6.9). The maximum activity was taken as 100% and relative activities were plotted against different temperatures [Nirmala, & Muralikrishna, 2003a].

II.11.b. Effect of pH

Amylase activity was determined at different pH values using different buffers such as sodium acetate & sodium succinate (pH 4.0 - 6.0), sodium phosphate (pH 6.0 - 7.0), Tris-HCl (pH 7.0 - 9.0) at 50 mM concentration and 37°C. The maximum activity was taken as 100% and relative activities were plotted against different pH values [Nirmala, & Muralikrishna, 2003a].

II.11.c. Thermal stability

Amylases in sodium phosphate buffer (50 mM, pH 6.9) were incubated at a temperature range of 30-70°C for 15 min. The residual activity was estimated taking original activity as control (100%) and relative activity was plotted against different temperatures [Nirmala, & Muralikrishna, 2003b].

II.11.d. pH stability

Stability of amylases was carried out using different buffer, pH ranging from 2.0-9.0. The following buffers such as phathalate-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), sodium phosphate (pH 7.0-8.0) and Tris-HCl (pH 8.0-9.0) were used at 50 mM concentration. The amylases were incubated at 37°C and the residual activities were measured at different time intervals. The pH stability at extreme pH's were also cross checked by using buffers such as glycine-HCl (pH 2.0-3.0) and borate (pH 8.0 -9.0). The original activity was taken as 100% and relative activity was plotted against different time intervals [Nirmala, & Muralikrishna, 2003b].

II.11.e. Effect of metal ions

Amylases were incubated with 5mM solutions of chloride salts of metal ions (Ca^{2+}, Ba^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Al^{3+}, Hg^{2+}) at 37°C for 15 min and the residual activities were measured. The enzyme activities without any metal ion were taken as control (100%) and relative activities were calculated [Nirmala, & Muralikrishna, 2003b].

II.11.f. Effect of inhibitors

Inhibitors such as EDTA (25-125 µM), citric acid (2.5 - 12.5 mM) and oxalic acid (2.5 - 12.5 mM) were incubated with amylases at 37°C for 15 min and residual activity was measured. The enzyme activity without inhibitor was taken as 100% and relative activity was plotted against different inhibitor concentrations [Nirmala, & Muralikrishna, 2003a].

II.11.g. Effect of substrate concentration

Different concentrations of gelatinized cereal and millet starches from ragi, rice, wheat and maize (0.25 -1.25%) in sodium phosphate buffer (50 mM, pH 6.9) were incubated with purified amylases for 120 min at 37°C and the activities were measured at every 15 min. Initial velocities (V_o) were calculated for all the substrate concentrations and the K_m and V_{max} values were calculated from the Lineweaver double reciprocal plot [Lineweaver, & Burk, 1934].

II.12.a. Deglycosylation

Pancreatic α-amylase (PPA), PPA-I and PPA-II (500µg) were dissolved in phosphate buffer (500 µL, pH 6.9, 20 mM) and PNGase F was (0.1 U) added and incubated in water bath at 37°C for 72 h [Lee, & Park, 2002]. The completeness of deglycosylation was assayed by SDS-PAGE followed by PAS staining and quantification of the released oligosaccharides.

One unit of PNGase F activity is defined as the amount of enzyme required to catalyze the release of *N*-linked oligosaccharides from 1 micromole of denatured Ribonuclease B (RNase B) in 1 minute at 37°C and pH 7.5.

II.12.b.Quantification of the oligosaccharides

PNGase F digests were desalted on Bio-Gel P-2 column (0.9 x 40 cm) using water as eluent [Packer, et al., 1998]. Quantification of the oligosaccharides gave a check to the completeness of deglycosylation. The oligosaccharides released were concentrated and the composition was established by GLC. The mass of the oligosaccharides were studied by ESI-MS positive mode by taking in 50% methanol.

II.13. Spectral studies

II.13.a. Absorption spectra

The U.V. absorption spectra of glycosylated and deglycosylated derivatives of PPA and its isoforms dissolved in 0.02 M sodium phosphate buffer, pH 6.9,

were recorded at room temperature using Shimadzu UV-160 spectro-photometer in a wavelength range of 200-400 nm [Peterson, 1983; Ken, et al., 2004].

II.13.b. Fluorescence Spectroscopy

Intrinsic fluorescence of glycosylated and deglycosylated PPA and its isoforms were measured using a Shimadzu RF-5000 Fluorospectrometer. The emission spectra were recorded after excitation of the samples [Suelter, 1985]. The relative fluorescence intensities of the enzymes were recorded. An excitation wavelength of 280 nm was applied, whereas emission spectra were recorded in the range between 300-450 nm. All fluorescence spectra were corrected for background scattering as measured with pure buffer.

II.13.c. Far UV-CD measurements of glycosylated and deglycosylated PPA and its isoforms

The structural changes accompanying deglycosylation were evaluated by far-UV-CD spectroscopy. FUV-CD studies was performed from 200-260 nm using a Jasco J 810 automatic recording spectropolarimeter at 30 ± 0.5°C with the slits programmed to give 1 nm bandwidth. The CD instrument was calibrated prior to use using d-10-camphorsulfonic acid (ammonium salt). The samples were scanned with a protein concentration of 1.0 mg/mL in a 2 mm cell at a wavelength range of 200-260 nm with 0.1 nm increment. Spectra were collected at 0.1 nm intervals, with a scan speed of 20 nm/min and a response time of 1 s. Each spectrum was the average of three scans. A mean amino acid residue weight of 115 was used to calculate mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ [Wiegand, et. al., 1995].

MRW is defined as:

$$MRW = (\theta)\,obs \frac{(mdeg)}{10} \times n\,l\,C_p$$

where, (θ) obs is the CD in millidegree, n is the number of amino acid residues, *l* is the path length of the cell and Cp is the mole fraction [Khodarahmi, & Yazdanparast, 2005]. The helical content can be calculated from the MRW value at 222 nm using the following equation: Percent helix = [(MRW_{222} – 2340) / 30300] x 100 [Chen, & Yang, 1972; Muzammil, et al., 2000]. The secondary structure analysis can be done according to Yang, et al. [1986].

II.14. In vitro digestibility of cereal starches and flours

Cereal starches and flours (20 mg/2 mL) suspended in sodium phosphate buffer (50 mM, pH 6.9) were gelatinized, cooled to 37°C and incubated with PPA, PPA-I and PPA-II for different time intervals (15, 30, 60 & 120 min) and the reducing sugar released was estimated by DNS method. The reducing sugar value was taken as % hydrolysis.

II.15. Separation and identification of oligosaccharides by HPLC

HPLC was carried out to determine the degree of polymerization (DP) of the products. Amylase digested starches (60 min) were precipitated with 3 volumes of ethanol and kept overnight at 4°C and the undigested residual starch was separated by centrifugation at 3000 x g. The supernatants were concentrated by vacuum evaporation and taken in ultra pure water, filtered through millipore filter and analyzed by HPLC on µ-Bonda pak amino column using acetonitrile water solvent system (70:30) as eluent keeping the flow rate at 1 mL/min [Nirmala, & Muralikrishna, 2003c]. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose served as the standards. The relative retention time of glucose, maltose and malto-oligosaccharides (G3-G7) and area under each peak was taken to identify and quantify the released malto-oligosaccharides.

Purified oligosaccharides were subjected to HPLC analysis. Oligosaccharide samples (2 mg) were dissolved in water (0.2 mL) and 20 µl of it was injected to the µ-Bondapak-NH_2 carbohydrate column and eluted at a flow rate of 1

mL/min and detected using refractive index detector [Nikolov, et al., 1984]. The HPLC profile of standard malto-oligosaccharides are presented in fig. 8.

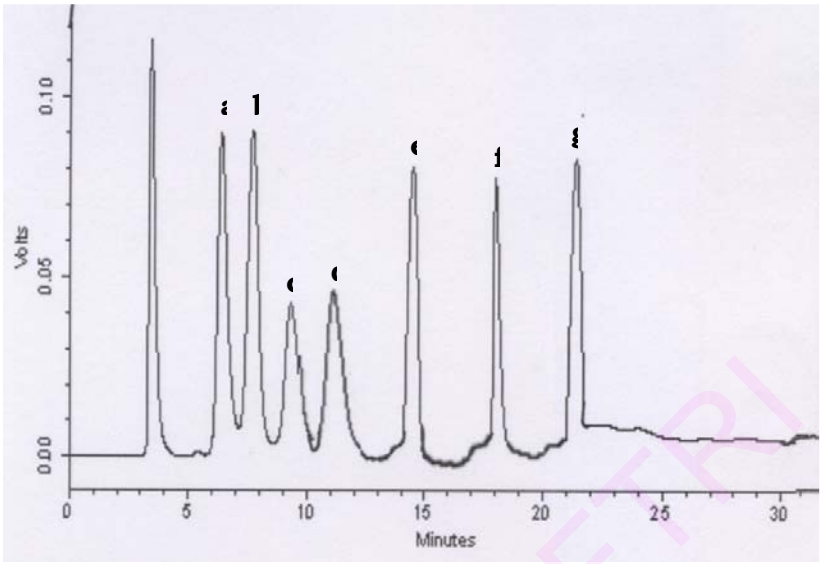


Fig.8: HPLC profile of standard oligosaccharides on µ-Bondapak amino column (a) Glucose-6.2′ (b) maltose-7.3′ (c) maltotriose-9.2′ (d) maltotetraose-11.0′ (e) maltopentaose-14.4′ (f) maltohexaose-18.0′ & (g) maltoheptaose-21.2′.

II.16. ESI-MS

MS analysis was performed on a Alliance, Waters 2695 mass spectrometer operated at a resolution of >10000 full-width at half maximum peak height (Waters; MicromassMS Technologies, Manchester, U.K.) [Rabin, et. al., 2001; Careri, et al., 2002].

II.16.a. Proteins

External mass calibration was obtained over the m/z range 50– 2000 using a solution of NaI (2 mg/mL) in 2-propanol/water (1:1, v/v). Intact proteins in ultrapure water or dilute volatile buffer solutions were diluted to 1–5 pmol/µl in acetonitrile/water (1:1, v/v) and introduced into the mass spectrometer by infusion (syringe pump, 300–500 nl/min) through a nano-Z spray source (source voltage, approx. 3000 V; cone voltage, 30-35 V).

II. 16.b. Maltooligosaccharides

Mass spectra of the oligosaccharides obtained by pancreatic α-amylase digestion of the starches were measured by using positive mode electrospray ionization [Fernandez, et al., 2004; Levigne, et al., 2004]. Capillary voltage was 3.5 kV, core voltage 100 V, source 80°C, desolvation temperature 150°C, core gas (Argon) 35 lt h^{-1} and desolvation gas (Nitrogen) 500 lt h^{-1}. Data were typically acquired over the m/z range 500–2000.

II.17. Isolation and **characterization of water soluble polysaccharides (WSP)**

II.17.a. Isolation

Flour (100 g) was extracted with water (200 mL x 4 at 25°C) for 2 h each and the supernatant obtained after centrifugation (3000 x g for 20 min) was precipitated with 3 volumes of ethanol. Precipitate was separated out, dialyzed and lyophilized to obtain water soluble/extractable polysaccharides (WSP/WEP) [Rao, & Muralikrishna, 2004]. The residue obtained after centrifugation was digested with glucoamylase to remove the associated starch.

II.17.b. Determination of neutral sugar composition by GLC

II.17.b.i. ***Hydrolysis of polysaccharide with sulfuric acid***

Polysaccharide sample (10 mg) was dissolved in water (0.2 mL) and ice-cold sulfuric acid (0.6 mL) and water (6.4 mL) were added to obtain a final acid concentration of 8% and hydrolyzed by keeping in boiling water bath for 12 h. Contents were neutralized with barium carbonate, filtered, de-ionized with Amberlite IR 120-H^+ resin and concentrated [Selvendran, & O'Neil, 1987].

II.17.b.ii. ***Hydrolysis of polysaccharide with trifluroacetic acid***

PPA (2-3 µg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 400 µ1) at 100°C for 5-6 h [Miguel, et al., 2003].

II.17.b.iii. ***Preparation of alditol acetate derivatives***

To the polysaccharide hydrolysate inositol (1 mg) was added as an internal standard and sodium carbonate was added (0.1 mL, 2 M) to saponify any glucuronolactones formed. Reduction was done by adding either sodium borohydride (10 mg) and kept at room temperature for 6 h. Excess borohydride was destroyed by adding acetic acid (2 N) drop wise till the hydrogen-effervescence stopped. Boric acid formed was removed by co-distillation with methanol (2 mL x 5). To the resultant glycitol, acetic anhydride (1 mL) and pyridine (1 mL) were added and kept in boiling water bath for 2 h. After acetylation excess reagents were removed by co-distillation with water (2 mL x 3) and toluene (2 mL x 3). Alditol acetates were extracted with chloroform, filtered though glass wool and dried using nitrogen stream [Sawardekar, et al., 1965]. They were further dissolved in a known amount of chloroform and analyzed by GLC. The GLC profile of the standard sugars are presented in fig. 9.

Identification of component sugars was done by comparing the retention times of the sample with those of standard sugars (rhamose, arabinose, xylose, mannose, galactose, glucose and inositol used as internal standard).

II.17.b.iv. ***GLC analysis***

Alditol acetates were analyzed on Shimadzu system (GC - 15 A) GLC fitted with an OV-225 (3%) stainless steel column using flame ionization detector (FID). Column, injector and detector port temperatures were maintained at 200°C, 250°C and 250°C respectively. Nitrogen was used as the carrier gas (40 mL/min).

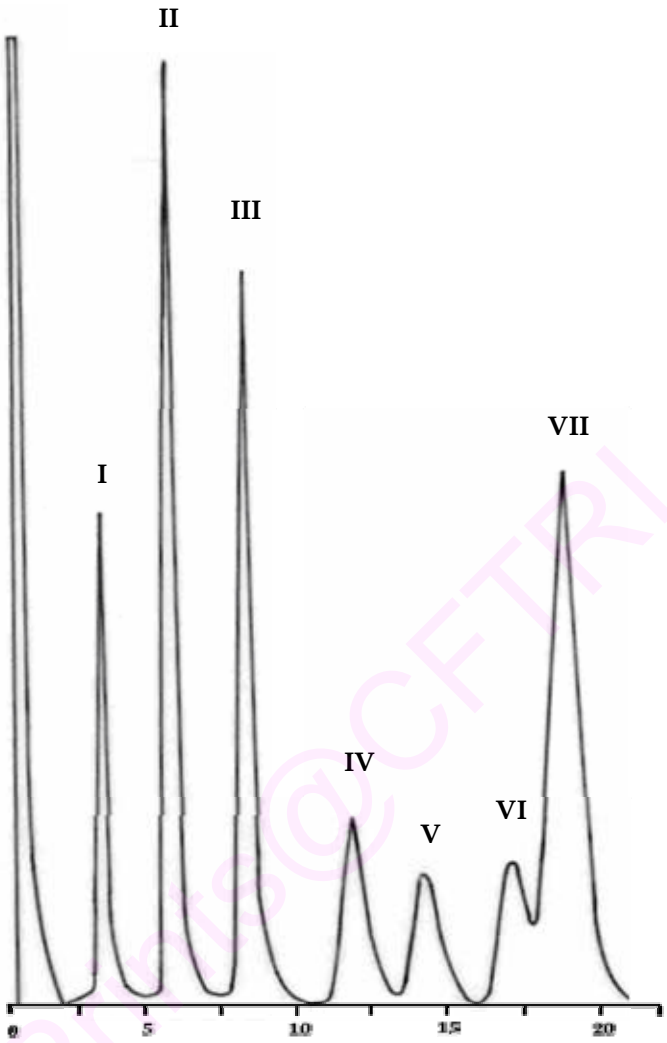


Fig. 9: GLC profile of standard sugars on OV-225 column.
(I) rhamnose-2.8 min, (II) arabinose-5.5 min, (III) xylose-7. 5 min, (IV) mannose-11.3 min (V) galactose-13.7 min, (VI) glucose-16.9 min (VII) inositol-18.2 min

II.18. Isolation and characterization of bound phenolic acids

Native and malted flours (2 g each) were first extracted with ethanol (70%, 4 x 50 mL, 1 h each) and hexane (4 × 50 mL, 1h each) to remove free phenolics, sugars and fat. The dried samples were extracted with NaOH (1M, 2 × 100 mL, 2 h each) containing sodium borohydride (0.5%) under nitrogen. The clear supernatants obtained after centrifugation were pooled, acidified with HCl (4 M) till the pH became 1.5 and the released phenolic acids were extracted, characterized and quantified as before [Nordkvist, et al., 1984; Ayumi, et al., 1999]. The HPLC profile of the standard phenolic acids is presented in fig. 10.

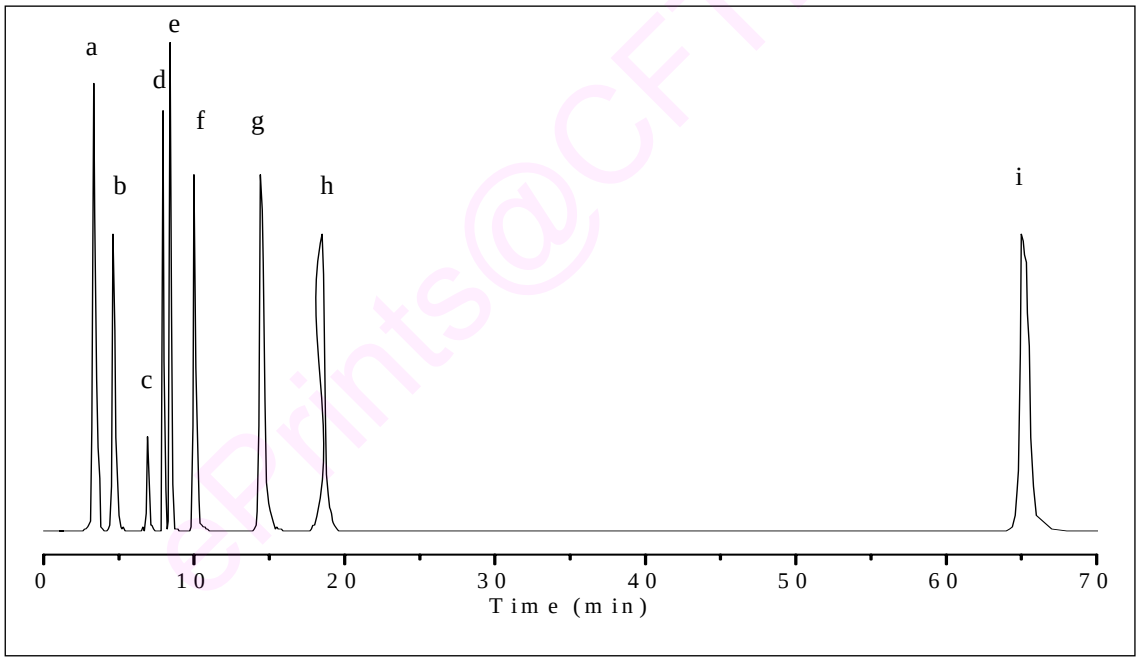


Fig. 10: HPLC profile of standard phenolic acids on C-18 column.
(a) Gallic acid (3.2 min); (b) Protocatechuic acid (4.5 min)
(c) Gentisic acid (6.9 min); (d) Vanillic acid (7.9 min);
(e) Caffeic acid (8.3 min); (f) Syringic acid (9.6 min);
(g) Coumaric acid (14.4 min); (h) Ferulic acid (18.4 min);
(i) Cinnamic acid (65.9 min)

II.19. Estimation of calcium

II.19.a. Atomic Absorption Spectrophotometric (AAS) method

Water soluble polysaccharides (1 g) was taken in a tared silica dish heated by using a soft flame to volatilize organic matter followed by placing it in a muffle furnace maintained at 300°C, subsequently the furnace temperature was increased to 450°C till it became carbon free ash. Any remains of the carbon was removed by adding 1 to 2 mL of concentrated HNO_3 to the ash, evaporated to dryness and again heated at 450°C in muffle furnace. To the carbon free ash concentrated HCl (4 to 5 mL) was added and kept for heating in a steam bath for 2-3 min. This was filtered through a Whatman No. 41 filter paper into a volumetric flask (25 mL) and the volume of the solution made up to the mark [AOAC, 1975]. The ash solutions were diluted suitably and the content of calcium present in the WSP were determined by AAS (Perkin Elmer, PE 3110, USA) by reading against a standard curve.

II.19.b. ***AAS conditions***

Wave length: 422.7 nm, flame: air-acetylene, lamp current: 10 mA, slit width: 0.7 nm, range: linear.

The standard curve was prepared by reading the absorbance of a series of concentrations of calcium (2 and 4 ppm) and plotting the absorbance against concentration of the metal. The instrument was set as per the guidelines given in the instrument instruction manual.

Working standard was prepared by diluting the calcium stock solution (1000 ppm, BDH, Poole, England) to 2 and 4 ppm working range.

II.20. Viscosity

The relative viscosities (with respect to water) of samples (WSP) were determined using Ostwald viscometer as a function of their concentration (0.1- 1.0%) and temperature (10-70°C) [Muralikrishna, et al., 1987].

II.21. Effect of WSP on enzyme activity

Starches (1%) from ragi, rice, wheat and maize were gelatinized in buffer (sodium phosphate, pH 6.9, 50 mM). The gelatinized starch solutions were incubated along with their respective water soluble polysaccharides at different concentrations (0.1-1.0%) with appropriate amounts of PPA and its isoforms (0.3 U) at 37°C for 1 h. The reducing sugars released were assayed by DNS method.

CHAPTER - III

Section A

Isolation and characterization of cereal and millet starches and non-starch polysaccharides

III.A.1 Introduction

Starch is the principal reserve polysaccharide of plants and forms a high proportion of world's food intake and it is readily assimilated in the human gut. The common sources of starch are maize, potato, wheat and rice. Starch occurs as discrete granules whose size and shape depend upon the source. The variations in size and shape of the different starch granules may be due to their biological origin [Wang, et al., 2006]. Starch from different sources are usually isolated by aqueous extraction and purified by defined protocols, i.e. by alkaline treatment followed by NaCl: toluene treatments [Muralikrishna, et al., 1982; Madhusudhan, & Tharanathan, 1996]. Starch is normally consumed after processing. Different products containing starch elicit different glycemic index. The digestion of starch is initiated in the oral cavity by salivary α-amylase. Chewing the food facilitates an increase in surface area to volume ratio in the solid phase and hence enzyme accessibility. The degradation products from starch with maltose dominating diffuse from the lumen to the brush border of the small intestinal mucosa where the final digestion to glucose take place through the action of di- and oligosaccharidases (sucrase-isomaltase complex) [Andersson, et. al. 2002; Eliasson, 1996].

Non-starch polysaccharides from cereals and millets form the quantitatively most important source of both soluble and insoluble dietary fibers [Bunzel, et al., 2001]. Water soluble polysaccharides (WSP) consists of arabinoxylans, (1→3)/ (1→4) β-glucans and phenolic acids that modulate the functional properties of foods. They are also known to reduce the risk of diseases such as diabetes, atherosclerosis and colon cancer [Karppinen, et al., 2000; Plaami, 1997]. Phenolic acids such as coumaric and ferulic acids mainly bound to arabinoxylans further influence these properties in addition to their strong antioxidant properties. [Dervilly-Pinel, et al., 2001; Subba Rao, & Muralikrishna, 2002].

In this chapter a comparative account of the characteristics of starches and non-starch polysaccharides isolated from ragi, rice, wheat and maize are presented.

III.A.2.i. Isolation and purification of starches

The yield of starches isolated from ragi, rice, wheat and maize were 63.5, 74.1, 53.9 and 64.4% respectively on the dry weight basis. The low yield of the starches may be perhaps due to (a) loss of the incompletely biosynthesized water soluble starch granules which is removed upon repeated washings and (b) the loss of the protein bound starch granules upon NaCl-toluene treatment. Small starch granules tend to associate with the protein fraction and may be lost during the isolation procedure [McDonald, & Stark, 1988] and these losses during cereal starch isolation has been reported earlier [Oscarsson, et al., 1997].

III.A.2.ii. Scanning electron microscopy (SEM)

Scanning electron photomicrographs of the native starches from ragi, rice, wheat and maize are depicted in fig. 11. The surface of the starch granules seemed to be regular and smooth. The morphological features of the native starches differed considerably with respect to their granule size. Wheat starch consisted of both large as well as small lenticular shaped granules. Rice starch granules were angular/pentagonal in shape and ragi starch granules were uneven with spherical, polygonal and rhombic shapes. Some of the rice starch granules showed some cavities at one side of the granule surface. Maize starch was circular to pentagonal in shape.

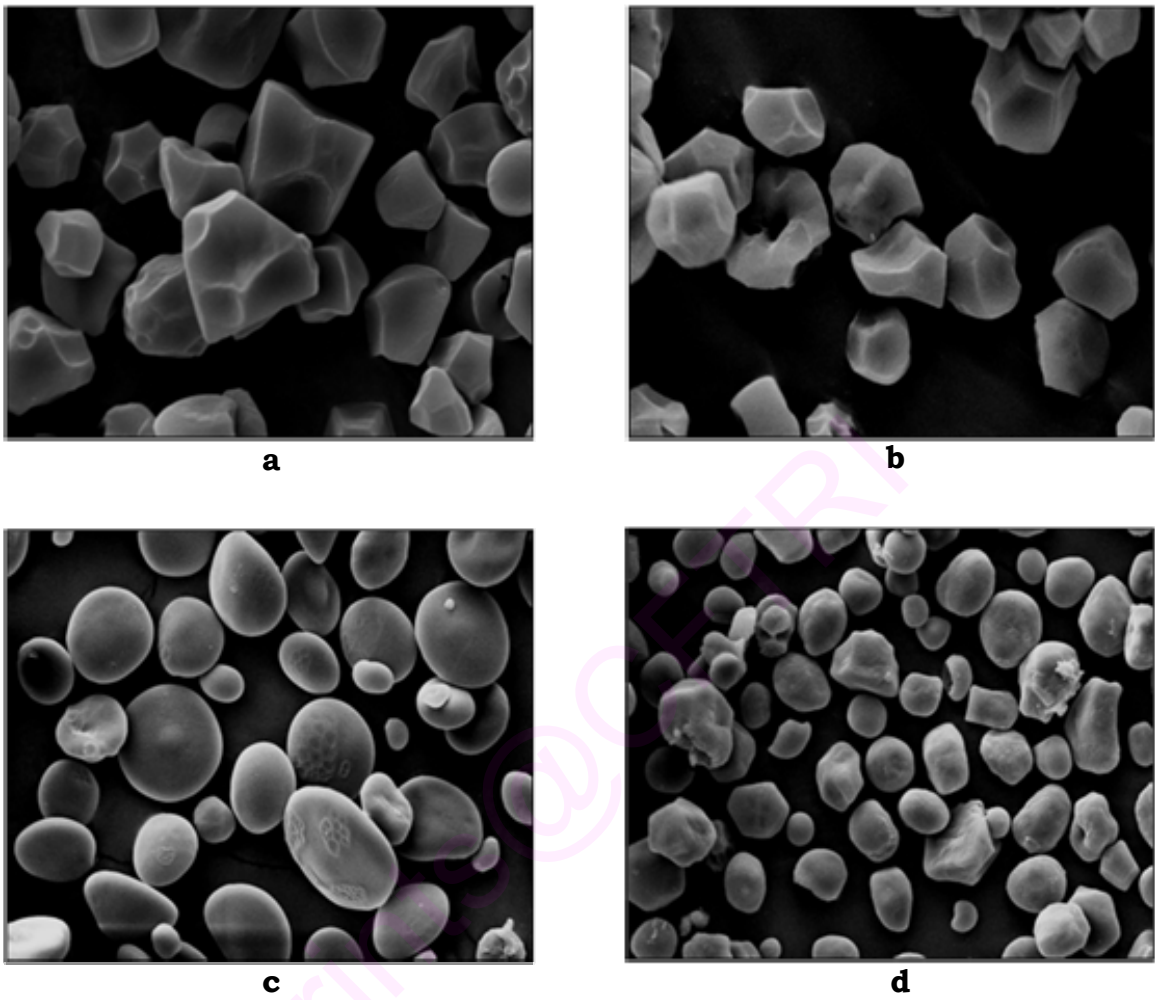


Fig. 11: SEM of millet and cereal starches a) ragi b) rice c) wheat d) maize

III.A.2.iii. Amylose content

The apparent amylose content of ragi, rice, wheat and maize starches were 26.6, 25.1, 26.4 and 27.3% respectively. In general the amylose content in cereals varies between 16-38% [Tharanathan, et al., 1987]. The λ_{max} of the iodine blue color complex was in the range of 603-616 nm. Branched amylopectin molecules contribute to the values obtained by iodine binding method [Yamade, et al., 1978] which may result in high amylose content in some varieties.

The total amylose content determined by iodine method was similar which was in the range of 25-27% in wheat, rye and barley starches [Fredriksson, et al., 1998].

III.A.2.iv. a) Gel permeation chromatography

The starches solubilized in DMSO were fractionated into two main components on Sepharose CL-2B gel (fig. 12). The high molecular weight (M_w) component of the starch was eluted first in the void volume (fraction 1) and the smaller M_W (fraction 2) was eluted later. In general, fraction I is considered as high molecular weight, i.e. branched chain amylopectin and fraction 2, low molecular weight compound, i.e. straight chain amylose.

The GPC of normal and high-amylose barley starches resulted in almost identical cumulative curves [Fredriksson, et al., 1998].

III.A.2.iv.b) Molecular weight determination

The molecular weight of amylopectin could not be determined as it got eluted from the column before T-2000 (2×10^6) standard, the highest molecular weight reference standard available in our laboratory. The exact M_w determination was difficult but the approximate M_w for maize amylose was high i.e. 375,800 followed by ragi, wheat and rice i.e 272,230, 201,800 and 139,600 ± 10,000 amyloses respectively (fig. 13). These values were within the range reported in the literature for amylose from cereal and millet starches [Wankhede, et al., 1979; Praznik, et al, 1998; Billiaderis, et al., 1979; Mohan, & Malleshi, 2006].

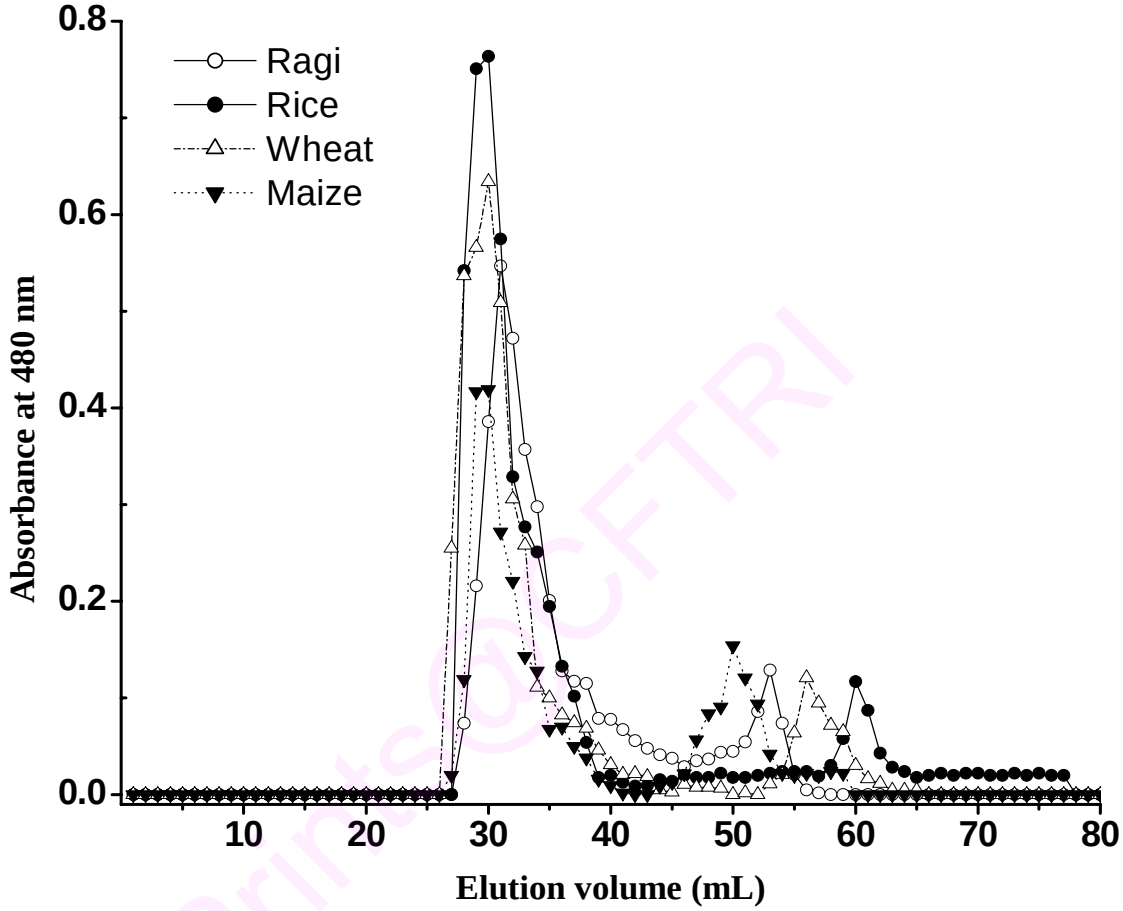


Fig. 12: Elution profile of starches on Sepharose CL-2B

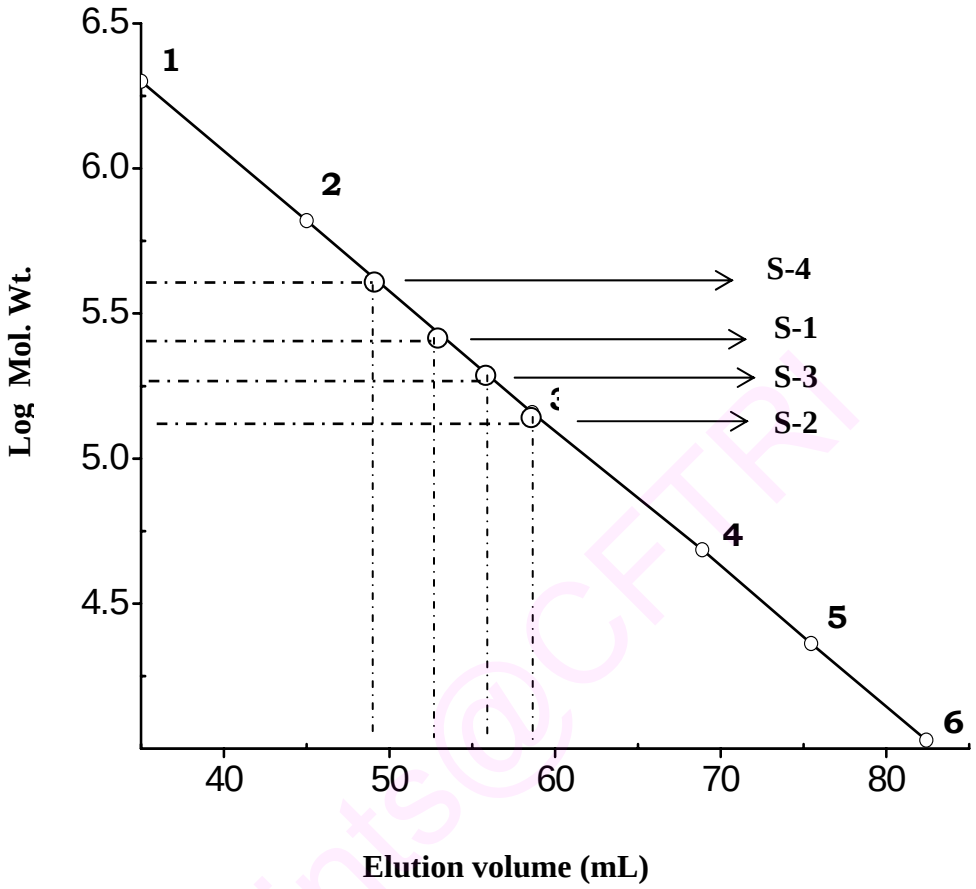


Fig. 13: Calibration curve for the determination of molecular weight of native starch constituents

1) T-2000	2) T-500	3) T-150
4) T-40	5) T-20	6) T-10

S-1: Ragi starch **S-2**: Rice Starch
S-3: Wheat Starch **S-4**: Maize Starch

III.A.2.v. Differential scanning calorimetry

Gelatinization: onset (T_o), peak (T_p) and conclusion (T_c) temperatures and enthalpy of gelatinization (ΔH) of the starches isolated from ragi, rice, wheat and maize starches were studied using DSC. Initial gelatinization temperatures for these starches ranged from 57.5 to 63.8°C and the final temperature from 66.4 to 84.5°C which was in agreement with the values reported earlier [Wankhede, et al., 1979; Mohan, et al., 2005; Mohan, & Malleshi, 2006]. The peak temperatures (gelatinization temperatures) of ragi, rice, wheat and maize starches were 64.9, 60.7, 62.2 & 66.5°C respectively and the enthalpy of gelatinization were 11.9, 8.9, 10.4 and 13.4 J/g respectively (table 3).

Table 3. DSC thermogram data of millet and cereal starches

	Endothermic transition (°C)			Enthalpy of
Sample	**T_o**	**T_p**	**T_e**	**gelatinization ΔH (J/g)**
Ragi	60.1	64.9	76.2	11.9
Rice	57.5	60.7	66.4	8.9
Wheat	58.3	62.2	68.1	10.4
Maize	63.8	66.5	84.5	13.4

Average of three values obtained from three independent experiments

The higher energy needed to gelatinize maize starch denotes a stronger crystalline nature compared to ragi, wheat and rice starches. The endothermic transition or the glass transition temperature was maximum for maize starch followed by ragi, wheat and rice starches.

When starch is heated in the presence of enough water its crystalline organization decomposes to form amorphous regions [Atwell, et al., 1988]. This molecular disordering is called gelatinization and is a frequently observed

endothermic phenomenon using DSC [Kruger, et al., 1987]. The endothermic peak associated with gelatinization originates from amylopectin [Russell, 1987]. DSC is a valuable tool in the study of starch characteristics [Stevens, & Elton, 1971] as it provides a quantitative measurement of the enthalpy (ΔH), the energy transformation that occurs during melting of crystallites in the starch granule and it provides a precise measurement of the temperature range over which these transformations occur [Longston, & LeGys, 1981].

Amylose interrupts the packing of the amylopectin double helices in the crystalline regions. An increase in amylose content will therefore decrease the crystallinity in the starch granules, thereby decreasing the enthalpy change [Jenkins, & Donald, 1995]. The differences in gelatinization temperatures may be attributed to the difference in amylose content, shape, size and distribution of starch granules and also to the internal arrangement of starch fraction within the granules [Wang, et al., 2006].

III.A.2.vi. X-ray diffraction

The X-ray diffractograms of ragi, rice, wheat and maize starches are presented in fig. 14. All the starches showed typical A-type diffraction pattern with strong reflection at 15° and 23° [Imberty, et al., 1988] but the degree of crystallinity of maize starch (41.4%) was significantly higher than ragi (37.3%), wheat (36.9%) and rice (35.8%) starches. While the scattering angle at which the diffraction intensities observed was 2θ, the 'd' spacing was used to discriminate the planes of different sites. The X-ray diffraction patterns were comparable to those reported earlier for cereal starches [Zobel, 1988; Wu, & Sarko, 1978].

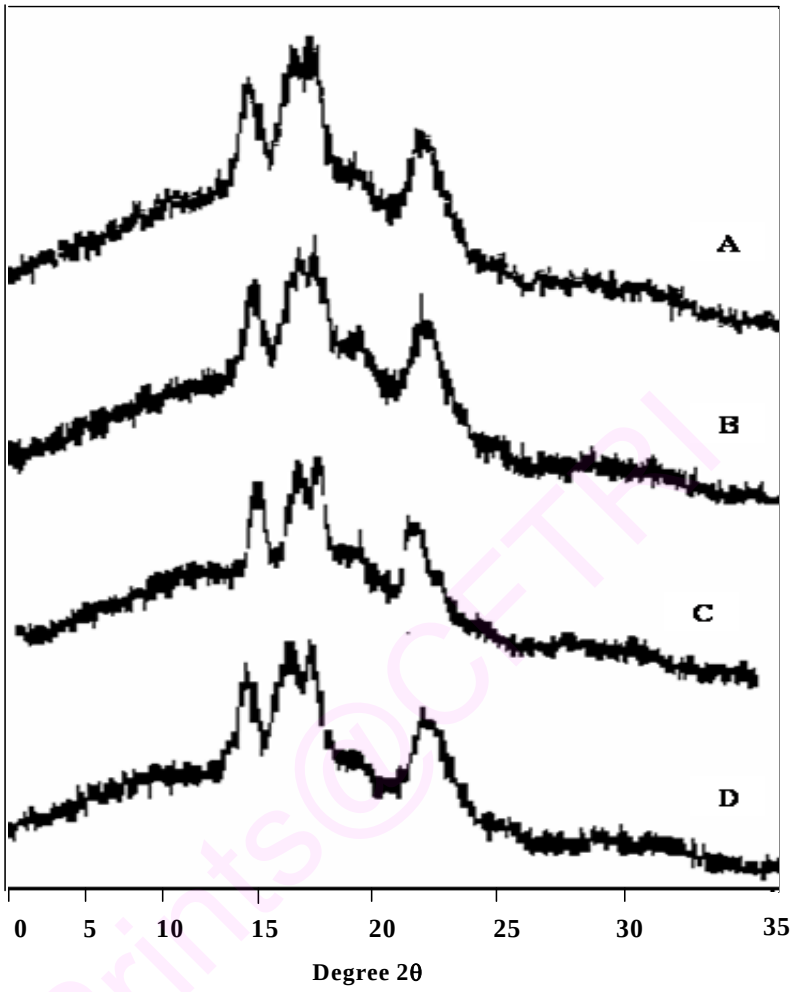


Fig. 14: X-ray diffractograms of cereal and millet starches
A- ragi, B-rice, C-wheat, D-maize

X-ray powder diffraction can monitor crystal structure and relative amounts of crystalline and amorphous phases in starch. The ratio of the upper area to the total diffraction area was calculated as the relative crystallinity. 'A' and 'B' type starches are based on parallel stranded double helices in which the helices are closely packed in the 'A' type starch, but loosely packed in the 'B' type starch. They differ in content of intrahelical water (B > A). Earlier reports on X-ray

investigation of maize starches with amylose content ranging from 0-84% showed that there is a transition of crystalline types from A through C to B in these starches corresponding crystallinity decrease and an increase in average chain length in amylopectin [Cheetam, & Tao, 1997]. The degree of crystallinity of the granule is inversely related to the amylose content confirming that crystallinity is a feature of amylopectin rather than amylose. Generally differences in relative crystallinity between starches could be attributed to the (1) crystal size (2) amount of crystalline regions (influenced by amylopectin content and amylopectin chain length) (3) orientation of the double helices within the crystalline domain and (4) extent of interaction between double helices [Hoover, & Ratnayake, 2002; Zhou, et. al, 2004].

III.A.2.vii. Scanning electron microscopy (SEM)

Scanning electron photomicrographs of the pancreatic alpha amylase digested starches from ragi, rice, wheat and maize are depicted in fig. 15 & 16.

When starch granules were subjected to enzymatic treatment the granules are damaged or eroded, they crack open and exposes as pronounced layer like structures**.** Cereal and millet starches were subjected to digestion for 2 h with PPA and the regions depicting the erosions caused by the enzyme action were photographed. Among the enzyme digested starches it is observed that rice and wheat starch granules were more disrupted compared to ragi and maize starches.

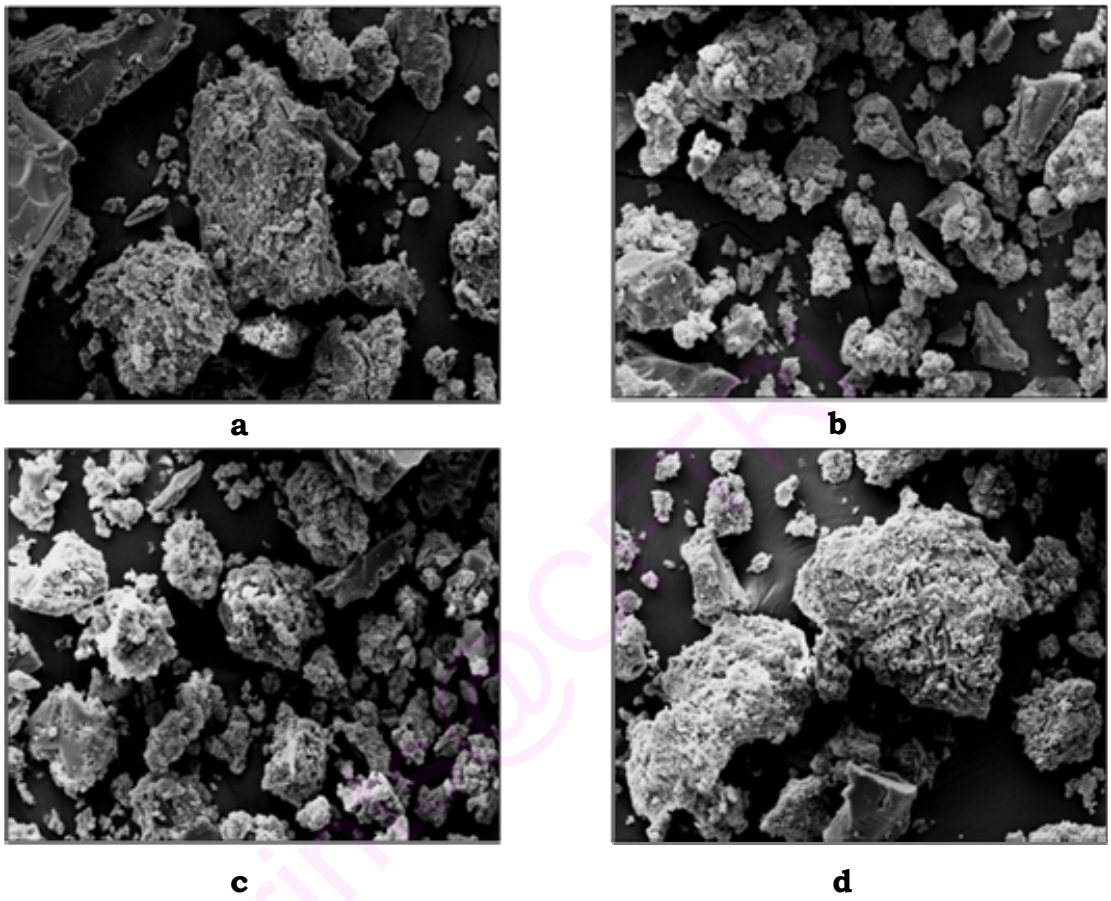


Fig. 15: SEM of enzyme digested (1hr) millet and cereal starches
a) ragi b) rice c) wheat d) maize

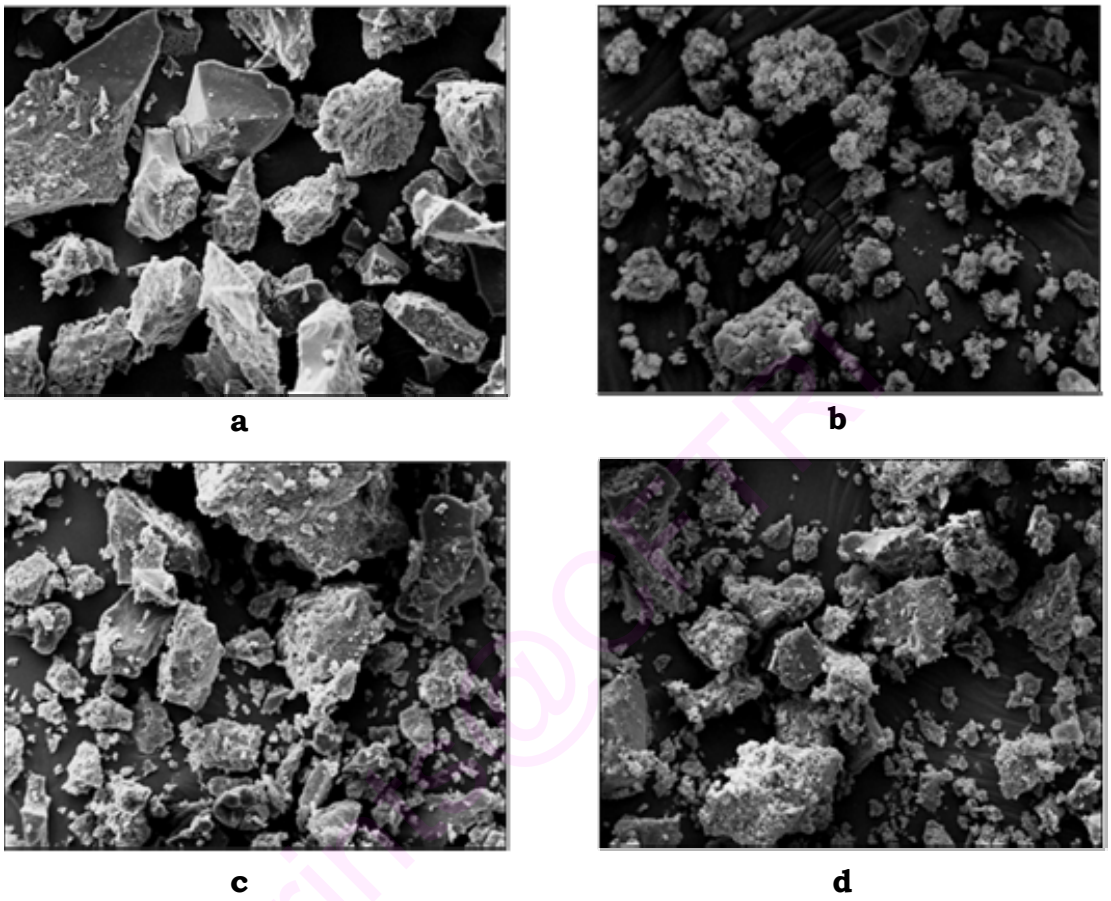


Fig. 16: SEM of enzyme digested (2 hr) millet and cereal starches
a) ragi b) rice c) wheat d) maize

Different starches are attacked in a pattern correlated to the surface and internal structure of the starch granules [French, 1981]. The degradation of the starch granules by amylase was found to be in the exo fashion degrading the entire granule surface in an orderly manner [Mohan, et al., 2005; Tharanathan & Ramadas Bhat, 1988].

Scanning of several hundred granules over several frames revealed that differences in appearance of granules among the samples subjected to the same period of hydrolysis were barely observable, although differences in the degree of hydrolysis were found. However, signs of degradation were more obvious as the period of hydrolysis was extended. After 1 h of hydrolysis the surface had small shallow holes and/or stretch marks on part of the granule surface. More damaged granules, greater areas of eroded surface, and large numbers of small particles deposited on the surface were observed with longer periods of hydrolysis. The small fragments of the granules were believed to be pieces of starch granules which were the products of hydrolysis. Most of the granules were degraded by exocorrosion.

SEM of the A-type crystalline starches from maize, barley, sorghum and wheat starches showed erosion of specific areas resulting in circular pits/pinholes [Demirkan, et al., 2005; Kunamneni, & Singh, 2005; Stevnebø, et al., 2006; Svihus, et al., 2005].

III.A.3.i. Isolation of water soluble polysaccharides (WSP)

The yields of the WSP's from ragi, rice, wheat and maize were 1.3, 0.8, 1.1 and 1.2% respectively. Water soluble polysaccharides from the flours were extracted as mentioned in the scheme below (fig 17).

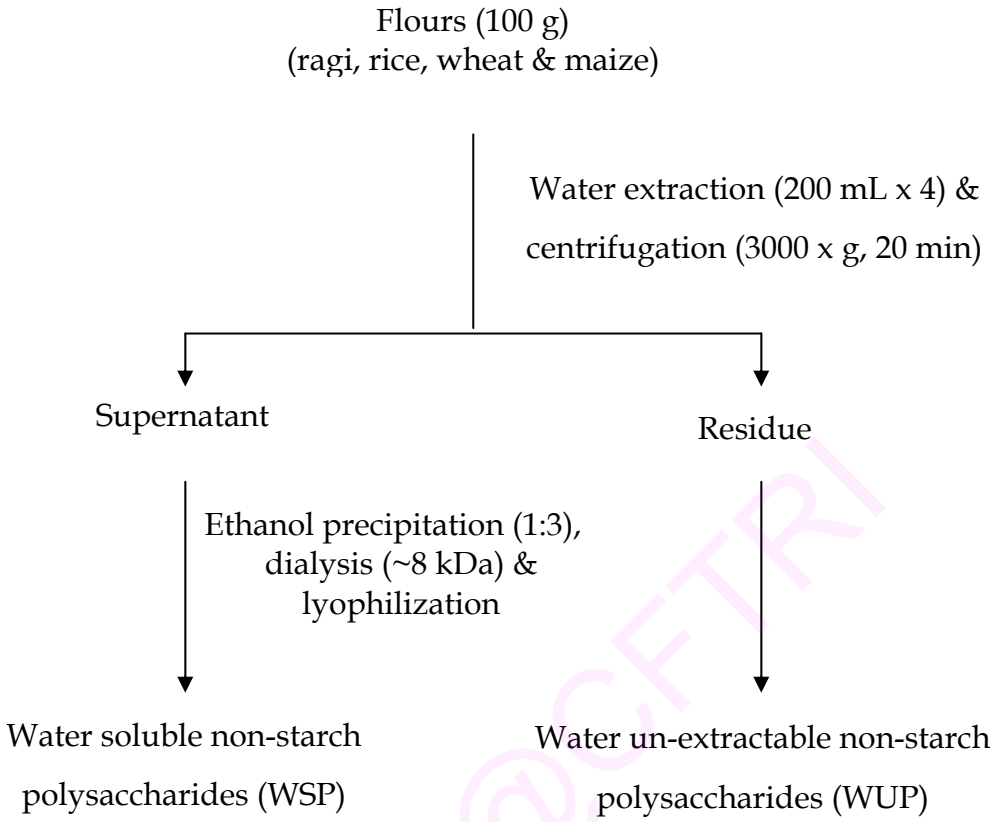


Fig. 17: Scheme for obtaining WSP from cereals and millet flours

III.A.3.ii. Characterization of WSP

WSP from ragi, rice, wheat and maize contained small amounts of starch (ragi - 1.8%, rice - 3.0%; wheat - 2.6%, maize - 1.1%). WSP also contained small amounts of proteins (table 4). The starch and protein might be soluble in water and hence co-extracted with cold water. WSP contained about 90% sugars and small amounts of uronic acid and calcium (table 4). The calcium in the polysaccharides might be in the bound form that was extracted in water.

Table. 4: Total sugar (%), uronic acid (%), protein (%) and calcium (ppm) contents of WSP from ragi, rice, wheat and maize.

Samples	Total sugar (%)	Uronic acid (%)	Protein (%)	Calcium (ppm)
Ragi	92	5.4	1.0	177.0
Rice	94	2.3	1.1	8.66
Wheat	94	5.2	2.1	5.55
Maize	91	6.4	1.8	3.82

* uronic acid gives partial positive answer for total sugar.

III.A.3.iii. Neutral sugar composition of WSP

WSP from ragi, rice, wheat and maize consisted of arabinose, xylose and glucose with small amounts of rhamnose, mannose and galactose (table 5), which are in comparison with the values reported earlier for cereals [Rao, & Muralikrishna, 2004].

Table. 5: Neutral sugar composition (%) of WSP from ragi, rice, wheat and maize

Samples	Rha	Ara	Xyl	Man	Gal	Glc	Ara / Xyl	P/H
Ragi	1.7	30.4	22.5	6.9	1.9	36.6	1:4	1:2
Rice	8.1	20.3	16.5	7.7	0.5	46.9	1:2	0:8
Wheat	2.5	24.8	19.3	6.8	1.1	45.4	1:3	0:9
Maize	1.8	34.7	21.5	8.8	1.6	31.6	1:6	1:4

Rha - Rhamnose; Ara - Arabinose; Xyl - Xylose; Man - Mannose; Gal - Galactose; Glc - Glucose; Ara : Xyl - Arabinose:Xylose; P : H - Pentose:Hexose

The ratio of arabinose to xylose and pentose to hexose ratio was more in maize followed by ragi, wheat and rice polysaccharides.

III.A.3.iv. Glucoamylase digestion of WSP

The neutral sugar composition of the glucoamylase digested, destarched WSP samples had small amounts of glucose as determined by GLC analysis indicating complete removal of associated starch (table 6). The glucose remaining in the WSP after glucoamylase digestion must have resulted from β-linked 1,3/ 1,4 glucans which are usually present in the NSP of cereals and millet along with arabinoxylans (Muralikrishna & Rao, 2007).

Table. 6: Neutral sugar composition (%) of WSP from ragi, rice, wheat and maize after glucoamylase treatment

Samples	Rha	Ara	Xyl	Man	Gal	Glc	Ara / Xyl	P/H
Ragi	7.9	41.9	22.7	8.5	9.2	9.1	1.8	2.7
Rice	9.4	33.3	25.1	8.3	13.2	10.6	1.3	2.1
Wheat	9.6	33.5	24.9	10.1	10.5	11.1	1.3	2.1
Maize	2.2	44.4	26.4	8.5	11.3	6.4	1.7	2.8

Rha - Rhamnose; Ara - Arabinose; Xyl - Xylose; Man - Mannose; Gal - Galactose; Glc - Glucose; Ara : Xyl - Arabinose:Xylose; P : H - Pentose:Hexose

III.A.3.v. Bound phenolic acids from WSP

Ferulic acid was the major bound phenolic acid identified in WSP's of ragi, rice, wheat and maize (table 7) which are in accordance with earlier reports on cereals [Durkee, & Thivierge, 1977; Hahn, et al., 1983; Harukaze, et al., 1999; Salomonsson, et al., 1978; Shibuya, 1984; Subba Rao, & Muralikrishna, 2001]. Ferulic acid content was higher in all the WSP fractions. Apart from ferulic acid, small amounts of coumaric acid was also identified in the WSP samples.

Table. 7: Bound phenolic acids (µg/g) of WSP from ragi, rice, wheat and maize

Samples	Ferulic acid	Coumaric acid
Ragi	221.0	4.62
Rice	116.0	6.65
Wheat	102.4	0.19
Maize	139.0	19.64

Phenolic acids and their derivatives are known to be distributed widely in plants. Ferulic acid is the major bound phenolic acid identified in graminaceous plants [Smith, & Hartley, 1983]. The predominance of phenolic acids is in bran portion with little amount present in the cell walls [Nordkvist, et al., 1984]. However, there is no report on the distribution of phenolic acids based on the solubility of non-starch polysaccharides. Earlier investigation showed that the major amounts of phenolic acids are bound to WSP [Rao, & Muralikrishna, 2004].

III.A.3.vi. Viscosity

III.A.3.vi.a) Effect of concentration on viscosity

The viscosity of WSP increased with increase in concentration (0.2 to 1.0%). At 0.2% WSP concentration the relative viscosity of ragi, rice, wheat and maize were 0.7, 0.6, 0.6 and 0.8 respectively. The increased values of viscosities observed at 1% concentration for ragi, rice, wheat and maize WSP's were 1.0, 0.8, 0.8 and 1.2 respectively. This may be perhaps due to the greater chain interactions of the polysaccharide molecules.

It is observed that the relative viscosity of maize WSP was maximum followed by ragi, wheat and rice WSP at 1% concentration (fig. 18). Above 1% concentration, the WSP samples could not be dispersed uniformly and hence viscosity was not measured.

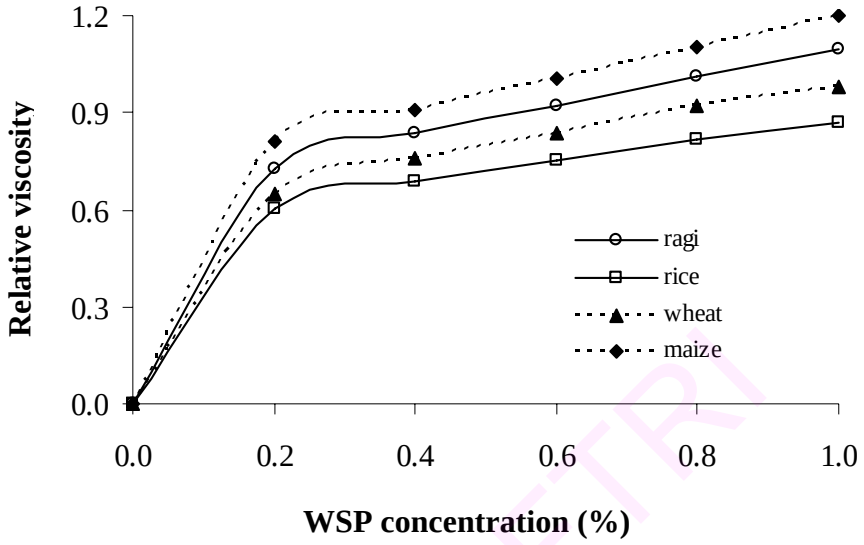


Fig. 18: Effect of concentration on the viscosity (η_r) of WSP

III.A.3.vi.b) Effect of temperature on viscosity

The viscosity of WSP decreased with increase in temperature (20 to 70°C) (fig 19). At 10°C, the viscosities of ragi, rice, wheat and maize WSP's were 1.0, 0.9, 0.9 and 1.3 respectively and at higher temperature (70°C), the viscosities of the WSP's from ragi, rice, wheat and maize reduced to 0.6, 0.5, 0.5 and 0.7 respectively. This may be due to increased thermal mobility of polysaccharide molecules [Whistler, 1973].

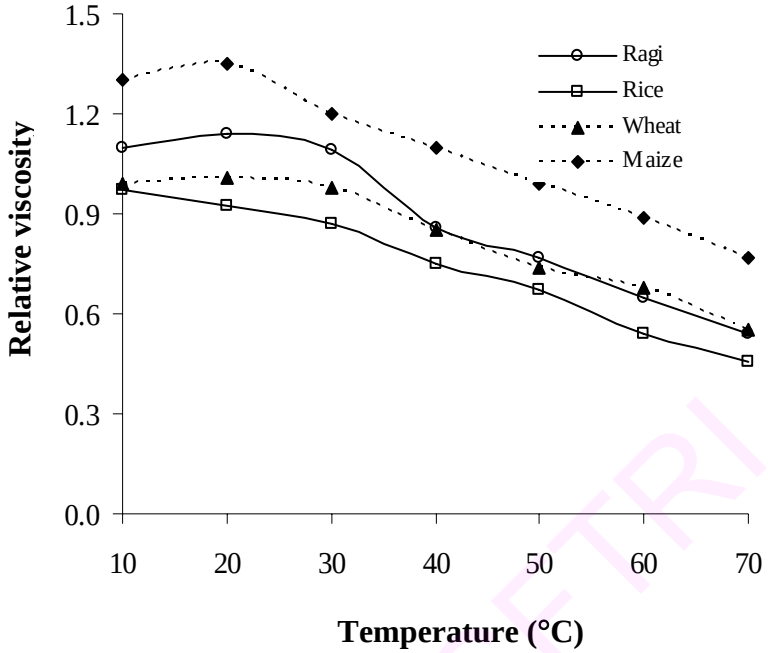


Fig. 19: Effect of temperature on the viscosity (η_r) of WSP

The viscosity of any polysaccharide solution is governed not only by its overall conformation but also by the specific arrangement of substituent residues along the polysaccharide backbone [Izydorczyk, & Biliaderis, 1995].

CHAPTER - III

Section B

Porcine pancreatic alpha amylase and its isoforms: purification, kinetic studies, and effect of non-starch polysaccharides

III.B.1. Introduction

Pancreatic α-amylase is mainly responsible for *in vivo* degradation of starch [Andersson, et al., 2002]. In humans starch digestion is initiated by the salivary α-amylase in the mouth wherein starch is degraded into oligomers which are further degraded by pancreatic α-amylase into maltose, maltotriose and low molecular weight maltooligosaccharides (DP 3-6) in the small intestine. These oligosaccharides are further acted upon by the brush border enzymes (sucrase-isomaltase-α-glucosidase complex) which converts the oligosaccharides to glucose [Marchel, et al., 1999]. Existence of PPA in multiple forms was reported way back four decades ago [Marchis-Mouren, & Pasero, 1967].

Porcine pancreatic α-amylase inhibition is one of the ways to decrease the release of post-prandial glucose levels in diabetic patients. One of the important approaches which is not studied is the effect of individual water soluble polysaccharides isolated from cereals on the action of PPA and its isoforms. The WSP are viscous and are perhaps believed to inhibit the starch degrading enzymes resulting in a slow release of post-prandial glucose. Soluble fibres vary in their molecular weight, arabinose/xylose ratio and the nature and content of covalently linked phenolic acids. However, comparative information on different non-starch polysaccharides from cereals and millet, their bound phenolic acids and their effect on porcine pancreatic alpha amylase and its isoforms are not reported in the literature.

In the present section focus is on (a) purification of PPA by ion-exchange chromatography (b) determination of the molecular weights of isoforms by gel permeation chromatography on Sephadex-G-100, SDS-PAGE and ESI-MS (c) determination of kinetic parameters, i.e. K_m and V_{max}, pH optima and stability, temperature optima and stability (d) studying the effect of metal ions e) studying the effect of organic acids and EDTA on the activities of PPA and its isoforms and (f) studying the effect of WSP on the activities of PPA and its isoforms.

III.B.2.i. Ion exchange chromatography

PPA (5.4 mg) was dialyzed against the equilibrating buffer (Tris pH 8.0, 20 mM) and loaded onto the DEAE-cellulose column (ϕ 3.0 x 25 cm) which was pre-equilibrated with the above mentioned buffer (500 mL) at a flow rate of 30 mL/h. The unbound proteins were removed by the equilibrating buffer and the bound enzymes were eluted with a linear NaCl gradient (0-0.6 M) in the aforementioned buffer (fig. 20). Pancreatic alpha amylase was separated into two activity peaks and eluted at 0.25 M (PPA-I) and 0.34 M (PPA-II) NaCl concentrations. The peaks were designated as PPA-I and PPA-II in the order of their elution.

Table. 8: Summary of the purification of PPA by ion-exchange chromatography

Samples	**Total activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Fold purification**	**% recovery**
PPA	2746.9	5.4	513.5	1	100
PPA-I	1867.8	2.1	876.9	1.7	67.9
PPA-II	1799.8	2.0	908.9	1.8	65.5

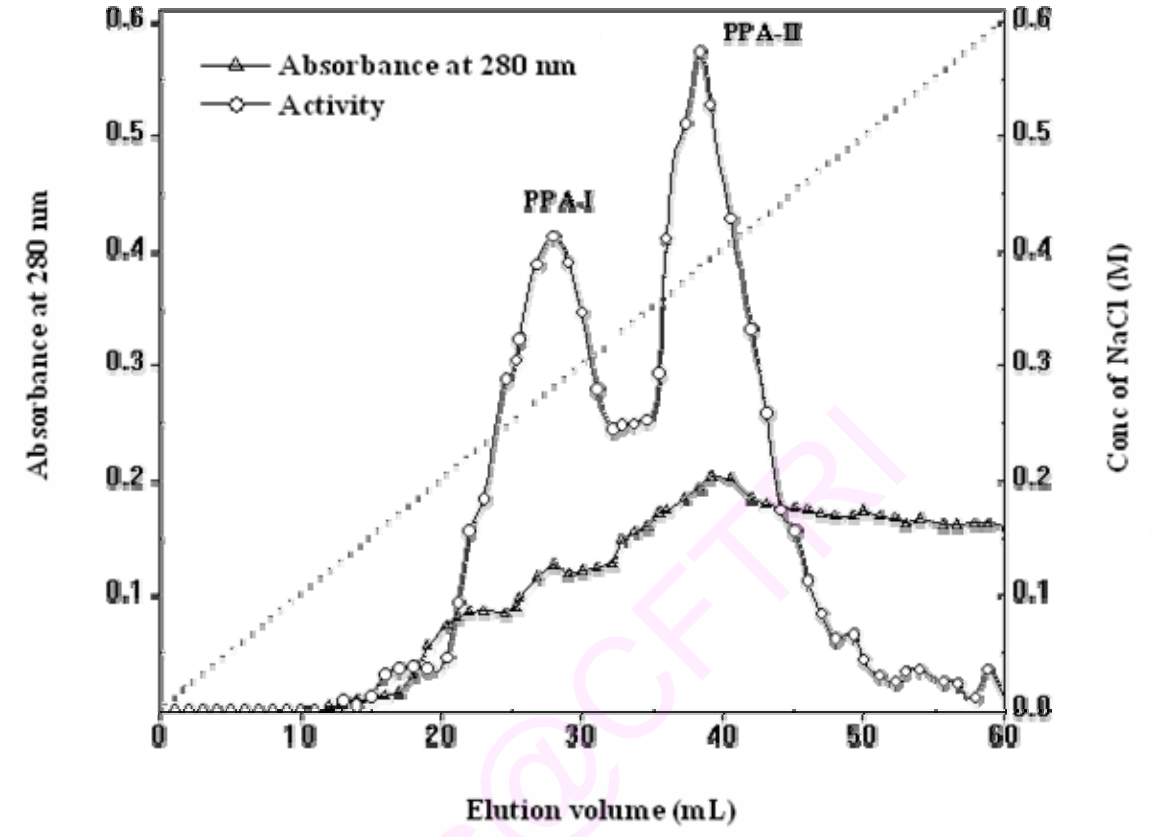


Fig. 20: Elution profile of porcine pancreatic alpha amylase on DEAE-Cellulose

The % recovery of PPA-I and PPA-II were found to be 1.7 and 1.8 with a fold purification of 67.9 and 65.5% respectively (table 8).

The resolution of PPA-I and PPA-II components were obtained by DEAE-cellulose under slightly modified conditions compared to the results reported by earlier investigators [Marchis-Mouren & Pasero, 1967; Robyt, et al., 1971; Cozzone, et al., 1970].

Elution of proteins from the DEAE-cellulose column can be brought about by changes in either the salt concentration or pH. As the concentration of salt (NaCl) increases, protein is displaced from DEAE-cellulose column by the anion (Cl^-). If

the pH is altered over a relatively narrow working range proteins are eluted as their isoelectric point is reached. Ion exchange chromatography has been used in the purification of soluble enzymes. PPA-I and PPA-II were purified from porcine pancreas by quaternary methylamine 50 nm (Waters) replacing DEAE-cellulose in the chromatographic separation [Ajandouz, & Marchis-Mouren 1995; Alkazaz et al., 1996]. These results suggest that PPA-I and PPA-II are isoforms of the same enzyme [Ajandouz, & Marchis-Mouren 1995; Alkazaz, et al., 1996].

Pancreatic α-amylase isoenzyme from hog pancreas was purified by chromatography on DEAE-cellulose [Kluh, 1981]. Parotid and mandibulary saliva amylase from red kangroos were separated by horizontal electrophoresis on either cellulose acetate or on starch gels [Beal, 1998].

The existence of carbohydrases in camel pancreas compared to some other ruminants was carried out on DEAE-Sepharose and gel filtration chromatography on Sepharose 6B [Saleh, et al., 2005]. The existence of disaccharidases (maltase, cellobiase, lactase, trehalase and sucrase), α-amylase, and glucoamylase in the pancreas of four ruminants (sheep, cow, buffalo and camel) were ascertained. The levels of these enzymes were almost equal in cow, buffalo and camel whereas it was very low in sheep compared to the other ruminants. The highest activity levels were detected for α-amylase (660–3480 units/g pancreas), followed by moderate levels of glucoamylase (2.1–61.4 units/g pancreas) and maltase (2.1–19.3 units/g pancreas). Very low activity levels were detected for the other disaccharidases: cellobiase (0.15–1.0 units/g pancreas), lactase (0.54–0.78 units/g pancreas), trehalase (0.15–1.1 units/g pancreas) and sucrase (0.15–0.78 units/g pancreas) [Saleh, et al., 2005].

Bacillus amyloliquefaciens α-amylase was purified to homogeneity from the culture filtrate by a combination of ion-exchange and gel filteration chromatography. α-amylase from post-harvest *Pachyrhizus erosus* tuber was purified using DEAE cellulose and CM cellulose chromatography technique [Noman, et al., 2006].

III.B.2.ii. Gel permeation chromatography

The purity and the molecular weight of the individual components (PPA-I & PPA-II) were ascertained by gel permeation chromatography on Sephadex-G-100 column (fig. 21).

Both PPA-I and PPA-II have the same elution volumes with symmetrical protein peaks indicting their homogeneity on the basis of molecular weight (fig. 22).

Porcine pancreatic α-amylase was thought to be a homogeneous protein until 1954 [Caldwell, et al., 1954]. Later investigations indicated the presence of two isoenzymes by DEAE-cellulose chromatography and gel electrophoresis [Marchis-Mouren, & Pasero, 1967; Rowe, et al., 1968].

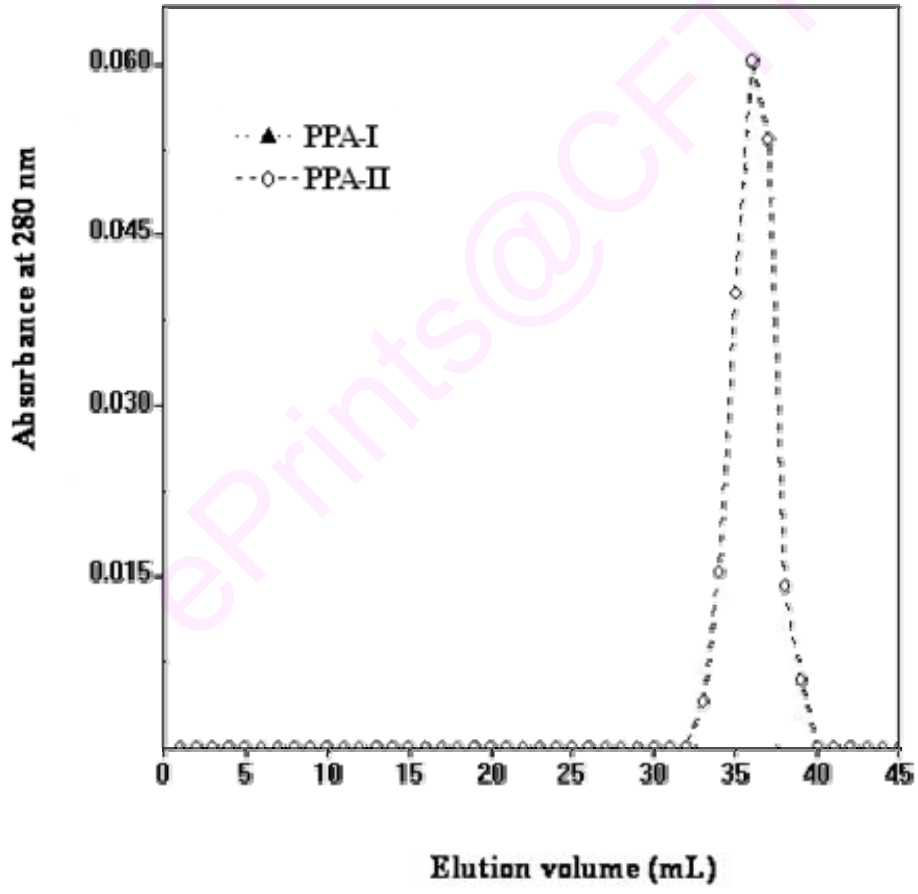


Fig. 21: Elution profile of PPA-I and PPA-II on Sephadex G-100

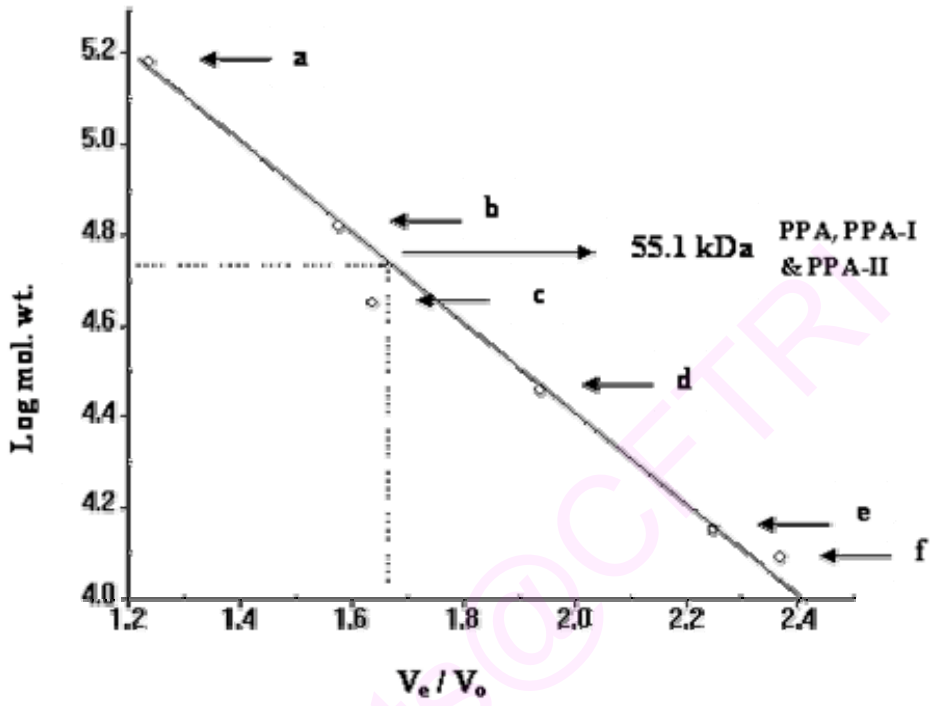


Fig: 22: Calibration curve for the determination of molecular weight of PPA-I and PPA-II on Sephadex- G-100

a : β-amylase (150 kDa) b : Bovine Serum Albumin (66 kDa)
c : Ovalbumin (45 kDa) d : Carbonic anhydrase (29 kDa)
e : Lysozyme (14 kDa) f : Cytochrome C (12.4 kDa)

III.B.3 Electrophoresis

III.B.3.i. Native PAGE and activity staining

Native page was carried out to check the purity of amylases with respect to the nature of protein and activity. PPA showed a broad band with two main amylase activity bands (fig. 23).

The α-amylase isoenzymes present in *Araucaria aracuana* was resolved by native PAGE followed by amylase activity staining using KI-I_2 solution [Acevedo, & Cardemil, 1997].

III.B.3.ii. SDS PAGE

The apparent homogeneity of the protein was ascertained by SDS-PAGE. The two PPA fractions, PPA-I and PPA-II have the same mobility on SDS-PAGE indicating them to isoenzymes (fig. 24). Upon purification of PPA by DEAE-cellulose and gel filteration, the two amylases were separated from one another and other contaminating proteins.

A) PPA **B)** PPA-I **C)** PPA-II

Fig. 23: PAGE of PPA, PPA-I and PPA-II - activity staining

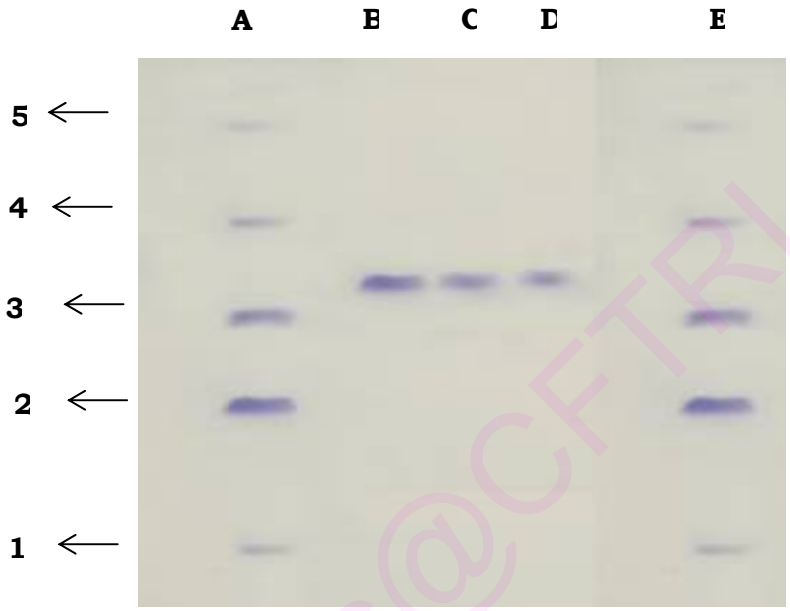


Fig. 24: SDS-PAGE of PPA, PPA-I and PPA-II - protein staining

A) & E) Molecular weight markers:* **B)** PPA **C)** PPA-I **D)** PPA-II

* **1**. *Lactoglobulin* (18.4kDa) **2.** *Carbonic anhydrase* (29 kDa)
3. *Ovalbumin* (43 kDa) **4**. *Bovine serum albumin* (66 kDa)
5. P*hosphorylase* (97.4 kDa)

III.B.4. Molecular weight

The molecular weights of PPA-I and PPA-II were found to be 55.1 kDa both by GPC on Sephadex G-100 and SDS-PAGE.

The molecular weight of PPA and its isoforms were further confirmed by ESI-MS (fig. 25). The mass analyzed by ESI-MS of PPA, PPA-I and PPA-II were 55.6, 55.0 and 54.9 kDa respectively.

Molecular weights of amylases fall in the range of 15.6-139.3 kDa [Manning, & Campbell, 1961; De Pinto, & Campbell, 1968] however, most of them are monomeric and their molecular weight are in the range of 45-60 kDa.

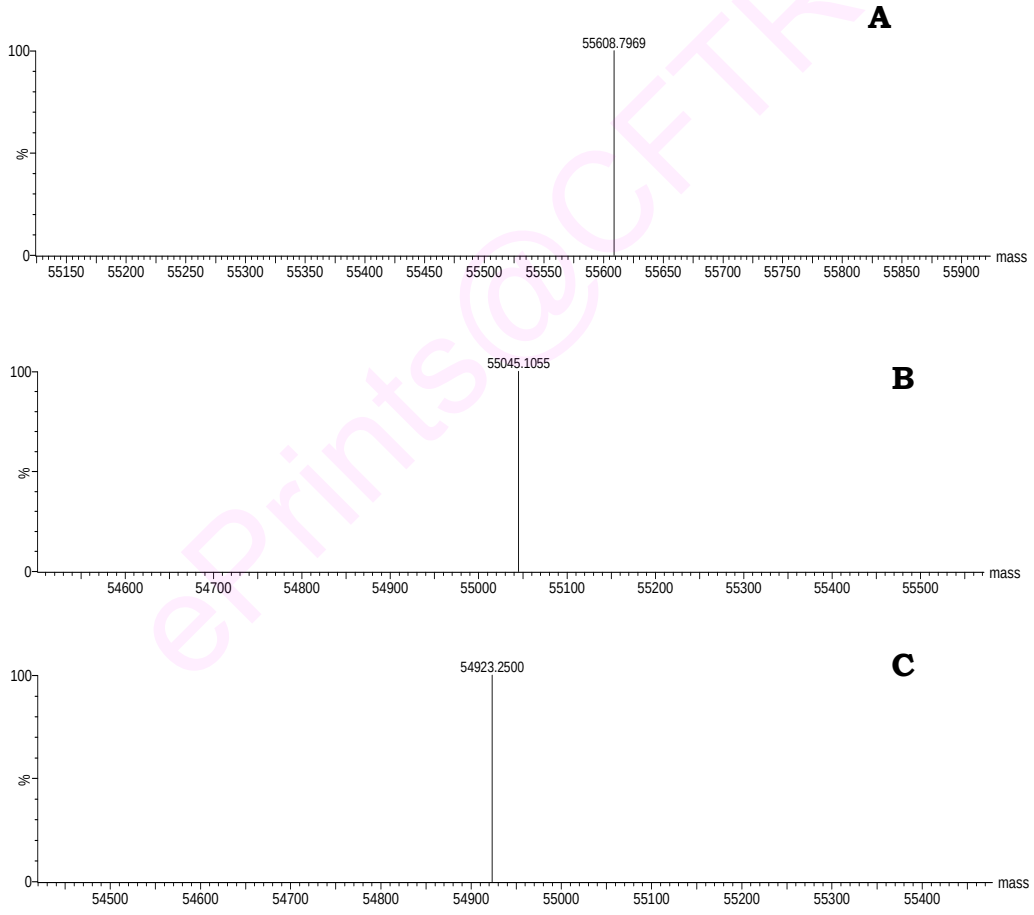


Fig. 25: Molecuar weight of (A) PPA (B) PPA-I and (C) PPA-II as determined by ESI-MS

III.B.5. Nature of amylase

The nature of the amylases were determined by carrying out the digestion of the isolated starches by PPA, PPA-I and PPA-II and analyzing the resultant products by HPLC and ESI-MS.

III.B.5.i. HPLC

The results of HPLC and ESI-MS of the starches digested by PPA and its isoforms indicate the abundance of oligosaccharides varying in DP 2 to 4 which is the pattern characteristic of the hitherto known alpha amylases.

The oligosaccharides obtained from cereal/millet starches differ with respect to the amount of maltose, maltotriose and maltotetraose. Glucose was absent indicating the absence of any contaminating α-glucosidase activity. Though the end products have the similar pattern, quantitatively there exists variation with respect to the oligosaccharide composition amongst the above starches by pancreatic α-amylolysis. The amount of oligosaccharides released by PPA and its isoforms are presented in table 9. Very small amount (2.4 -12.7%) of higher oligosaccharides (DP 4) are present in the hydrolysate in addition to maltose, maltotriose and maltotetraose put together (87.3-97.6%). The amount of higher oligosaccharides (G>4) liberated from maize starch substantiated its slower digestion compared to ragi, wheat and rice starches.

Temperature was found to have a distinct influence on the oligosaccharide composition during the hydrolysis of amylopectin from potato starch with three different *Bacillus* α-amylases. A higher temperature led to a less heterogeneous molecular weight distribution during the initial phase of the hydrolysis. The way in which temperature effects the saccharide composition will hold good for most starches although differences in molecular structure will affect the exact composition of the hydrolysate [Marchel, et al., 1999].

Table. 9: Oligosaccharides composition (%) obtained by the hydrolysis of starches by PPA, PPA-I and PPA-II after 60 min incubation (as determined by HPLC)

Sample	PPA				PPA-I				PPA-II			
	G-2	**G-3**	**G-4**	**HO**	**G-2**	**G-3**	**G-4**	**HO**	**G-2**	**G-3**	**G-4**	**HO**
Ragi	36.6	38.1	19.6	5.7	33.9	36.8	21.4	7.9	35.5	46.0	13.1	2.4
Rice	37.4	38.8	19.5	4.3	41.8	41.4	14.4	2.4	40.8	41.8	15.3	2.0
Wheat	34.9	37.7	22.2	5.1	36.8	41.7	18.4	3.0	40.3	41.5	15.1	3.1
Maize	31.1	34.5	21.8	12.7	32.4	37.3	20.0	10.3	33.5	43.7	14.6	8.1

G - 2 - maltose: G - 3 - maltotriose: G - 4 – maltotetraose:
HO - Higher oligosaccharides (G > 4)

III.B.5.ii. Molecular weight determination of maltooligosaccharides by ESI-MS

The fragmentation patterns of the malto-oligosaccharides liberated from the starches by pancreatic α-amylase and its isoforms were confirmed by ESI-MS (fig. 26, 27 & 28). PPA digested starches of ragi showed the MS spectrum of the ion at 365.3 m/z (maltose: fragmentation, 180 x 2 = 360-18 = 342+23 = 365), 527.4 m/z (maltotriose: fragmentation, 180 x 3 = 540-36 = 504+23 = 527) and 689.4 m/z (maltotetraose: fragmentation, 180 x 4 = 720-54=666+23= 689) and trace of maltohexaose at 1013 m/z (fragmentation, 180 x 6 = 1080-90 = 990+23 = 1013). PPA digested starches of rice showed the MS spectrum of the ion at 365.3, 527.4 and 689.4 m/z and small amount of maltopentaose at 851 m/z (fragmentation, 180 x 5 = 900 – 72 = 828+23 = 851). 23 is the sodium adducts $[M+ Na]^+$ that is detected in all the samples.

With respect to PPA digestion of wheat and maize starches, MS spectrum of the ions were observed at 365.3, 527.41 and 689.4 m/z which correspond to maltose, maltotriose and maltotetraose. Digestion of starches by PPA-I and PPA-II showed similar patterns of fragmentation at 527.4 (maltotriose: fragmentation,

180 x 3 = 540-36 = 504+23 = 527) and 689.4 m/z (maltotetraose: fragmentation, 180 x 4 = 720-54 = 666+23 = 689).

The variety of oligosaccharides as the products from α-amylase digestion indicates that all the glycosidic bonds were not equally susceptible to amylase attack. The digestion pattern of PPA-I and PPA-II are similar to that of PPA. Maize starch with higher amount of G>4 oligosaccharides is the least digestible followed by ragi, wheat and rice starches.

Studies with starch, amylose, maltooligosaccharides and their nitrophenylated derivatives show that full amylase activity is obtained only when the five sub-sites are occupied [Ajandouz, & Marchis-Mouren, 1995].

Thoma, et al. [1971] showed that porcine pancreatic α-amylase gives 100% retention of the α-configuration during catalysis. The mechanism for α-amylase glycosidic bond hydrolysis was a double-displacement process involving a covalent carbohydrate-enzyme intermediate that gives the required retention of configuration at the reducing-end of the products [Yoon, et al., 2007].

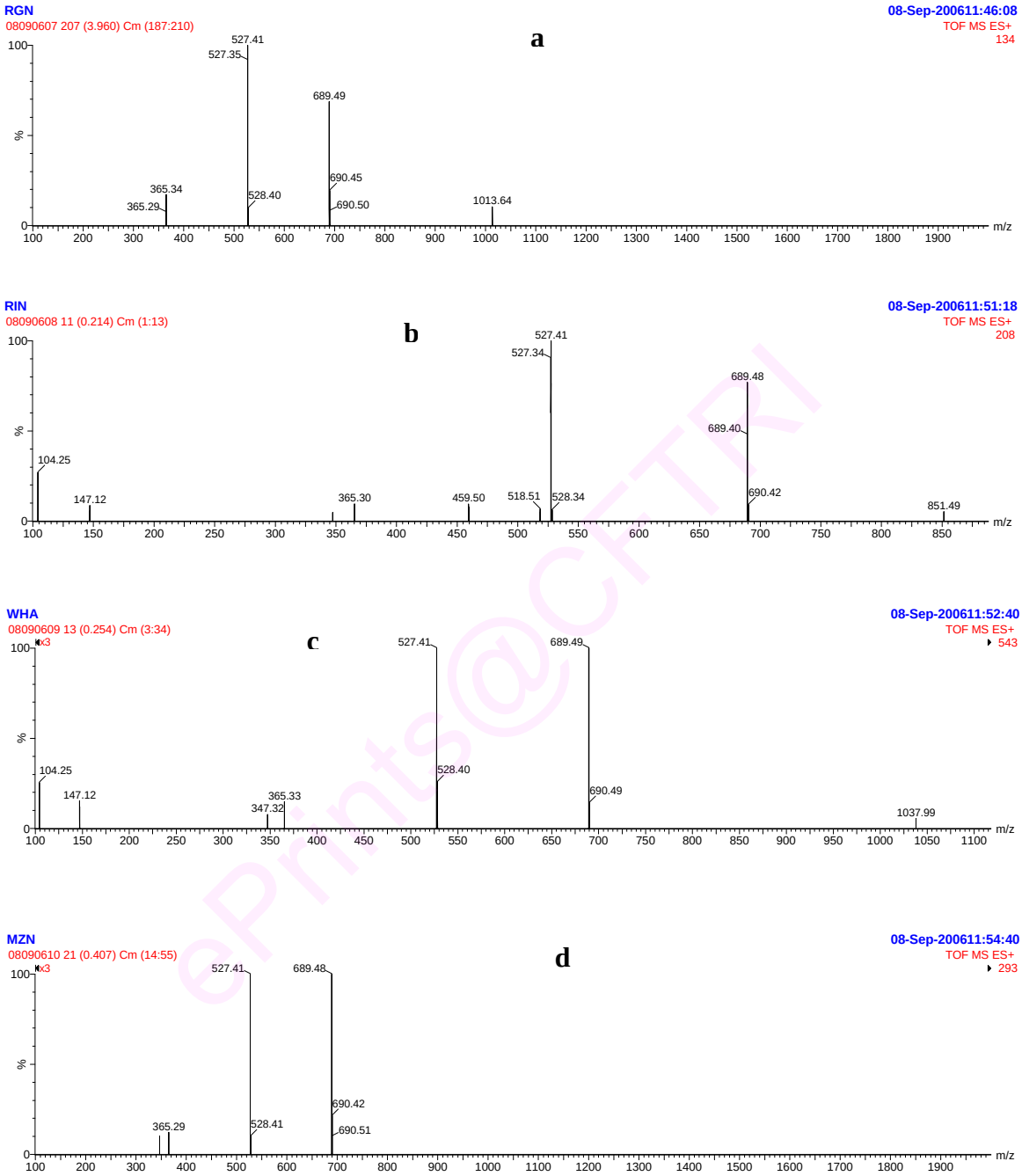


Fig. 26: ESI-MS spectra of maltooligosacharides obtained by PPA digestion of starches a) ragi b) rice c) wheat d) maize

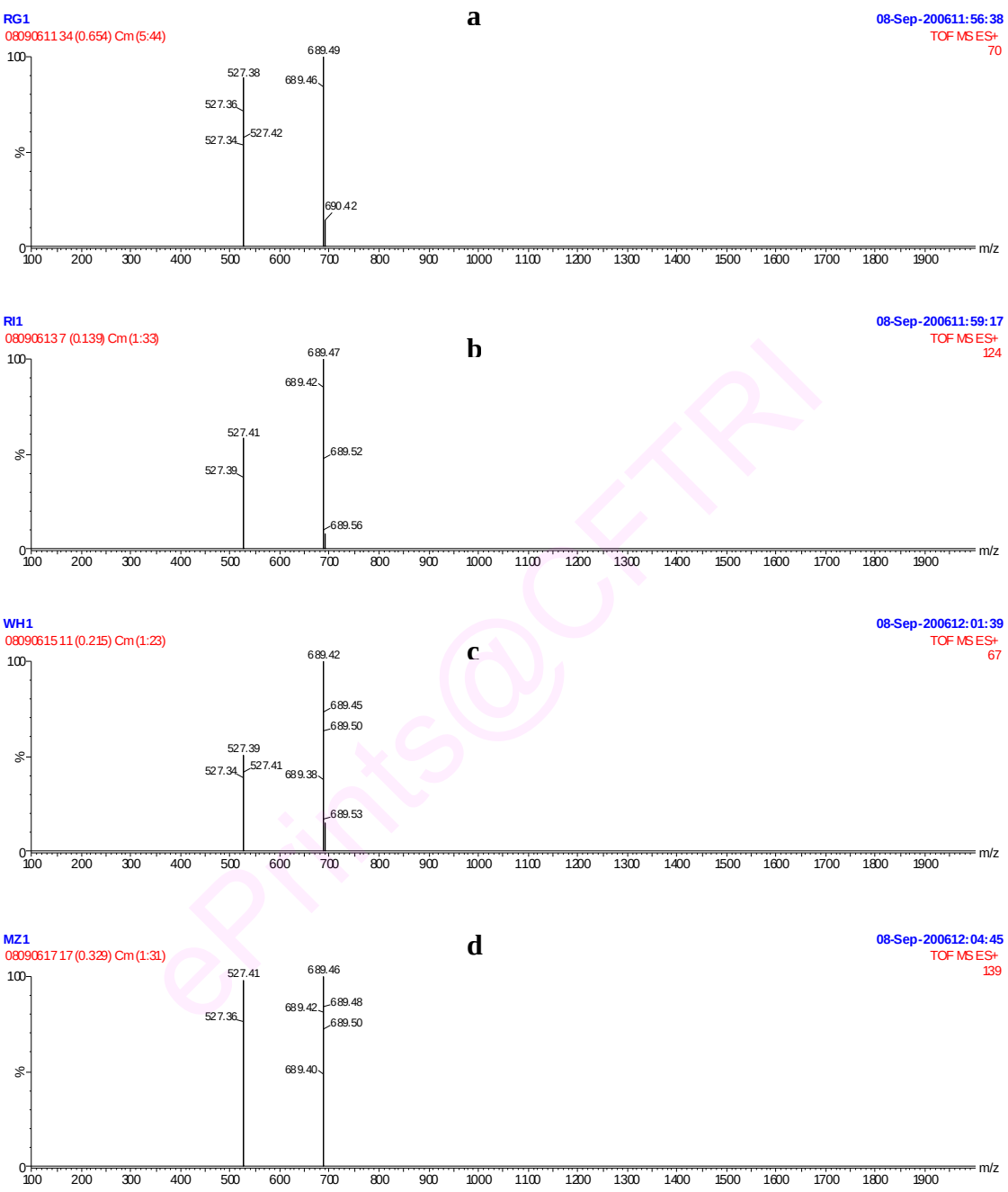


Fig. 27: ESI-MS spectra of maltooligosacharides obtained by PPA-I digestion of starches a) ragi b) rice c) wheat d) maize

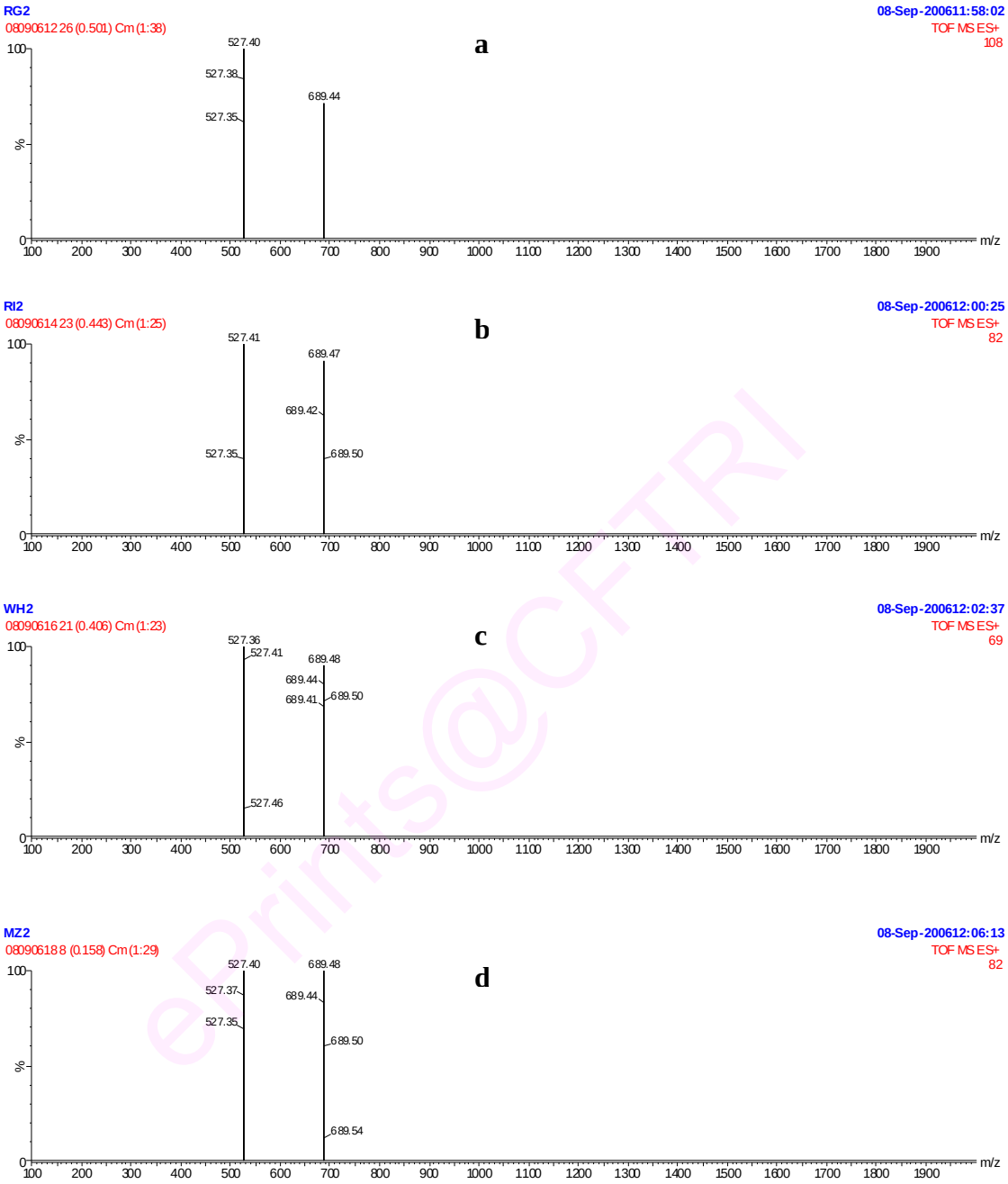


Fig. 28: ESI-MS spectra of maltooligosacharides obtained by PPA-II digestion of starches a) ragi b) rice c) wheat d) maize

The application of homologous oligomeric substrates is an effective way to explore the nature of the binding site and the process of catalysis for depolymerizing enzymes. Although the overall structure and the tertiary folding of the polypeptide chain of porcine pancreatic alpha amylase (PPA) have been determined, less is known about the differences in the action of PPA on the homologous maltooligosaccharide series. β-maltooligosaccharide glycosides series were envisaged as good substrates for studying the action pattern of PPA [Kandra, 1997] compared to other substrate series so far reported, for example, maltooligosaccharides [Robyt, & French, 1970] or α-NP-maltooligosaccharides [Ajandouz, & Marchis-Mouren, 1995]. In high amylose starches, the amylose fraction has been suggested to have synergetic effects on the amylopectin degradation [Russell, 1987].

III.B.6.i. pH optima

To determine the pH optima of the amylases, the activities were determined using different buffers such as sodium acetate and succinate (pH 4.0-6.0), sodium phosphate pH 6.0-8.0) and Tris-HCl (pH 8.0-9.0) at 50 mM concentration. Effect of pH on the activities of amylases are influenced by the kind of substrate and the assay conditions [Witt, & Slauger, 1996]. In the present study soluble starch was taken as substrate at 1% concentration. pH optima of PPA, PPA-I and PPA-II were 6.9. The activities of PPA and its isoforms were more in neutral range of pH compared to the activities on either side of pH's (fig. 29 & 30). The activity of PPA in sodium acetate and sodium succinate buffers were around 80% at pH 5.0 and at higher pH the activity decreased. A similar observation was made with respect to PPA-I and PPA-II where the activities at pH 5.0 were around 85 and 65% respectively. Sodium phosphate buffer showed the maximum activity at pH 6.9 for PPA and its isoforms. Though PPA was stable in sodium phosphate buffer (pH 6.0-7.0) with an activity of around 80% at pH 8.0, PPA-I showed decrease in activity (60%) at pH 8.0 and with respect to PPA-II the activity reduced to around

40% at pH 7.5. Tris-HCl buffer had a stabilizing effect on the activities of PPA, PPA-I as well as PPA-II. PPA retained about 80% activity at pH 8.5 and at pH 9.0 the activity retained was around 60%. PPA-I and PPA-II did not show a similar trend. Though the activities were retained the relative activity percentage was comparatively less. PPA-I showed a maximum of 80% activity at pH 8.0 and at pH 9.0 the activity reduced to around 35%. PPA-II showed a maximum activity of 60% at pH 8.0 and the activity was reduced to 30% at pH 9.0. The optimum pH for the hydrolytic activity of PPA under physiological conditions has long been believed to be around neutrality on the basis of the study on soluble starch [Wakim, et al., 1969]. PPA-II acts in a narrow range of pH when compared to PPA and PPA-I. The activity loss is more in the case of PPA-II in both acidic as well as alkaline pH.

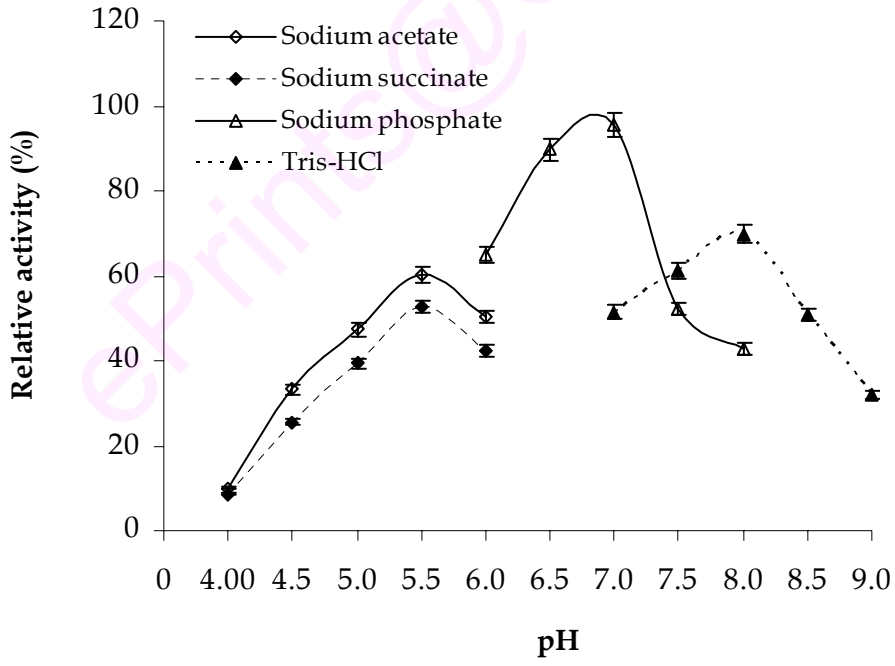


Fig. 29: Effect of pH on the activity of PPA

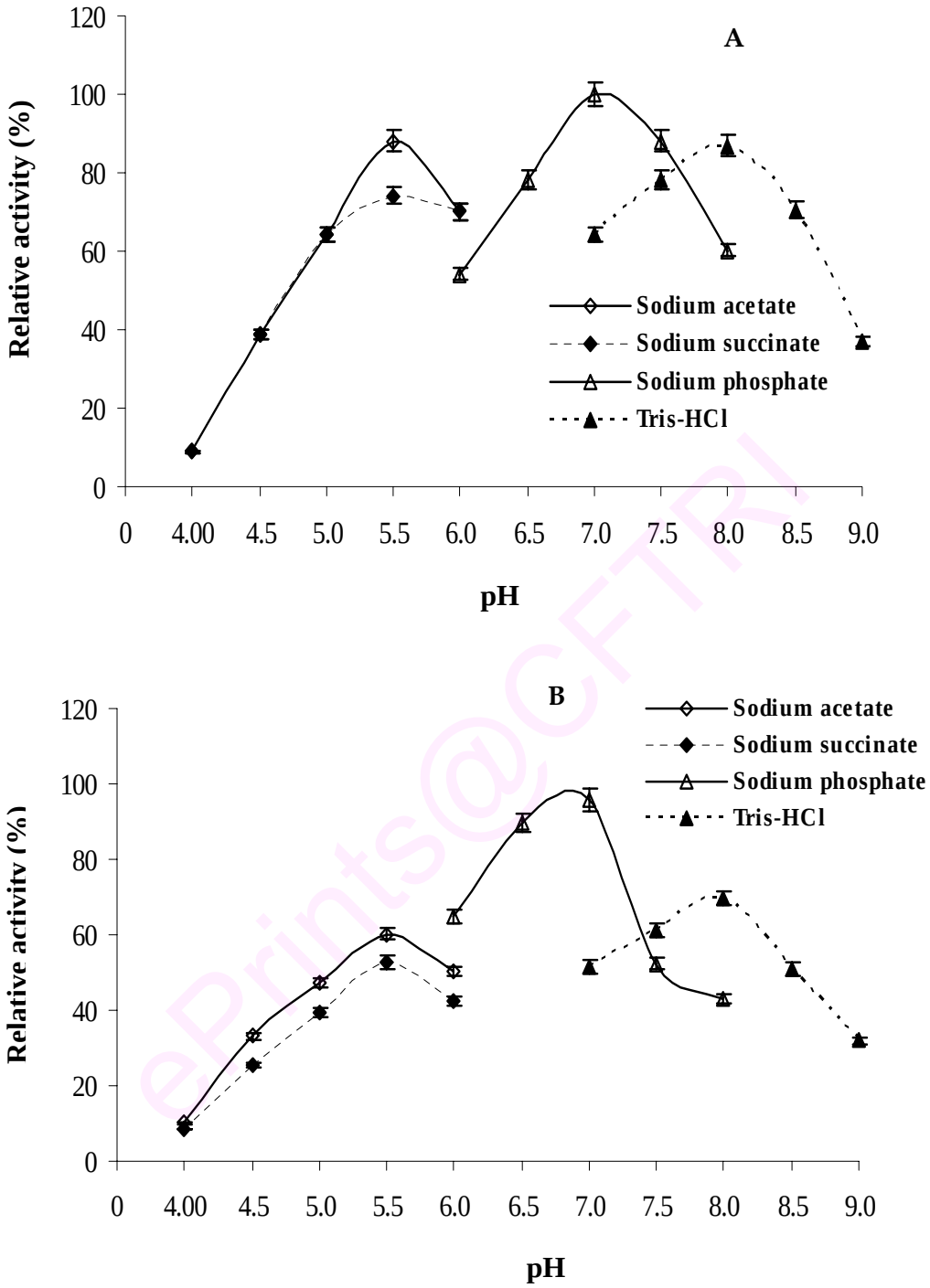


Fig. 30: Effect of pH on the activities of (A) PPA-I & (B) PPA-II

α-amylase, including porcine pancreatic alpha amylase [Levitzki, & Steer, 1974], and bacterial amylase have been found to require chloride to show full catalytic activity. The binding of chloride ion to mammalian α-amylase leads to an increase in the activities and shift in the optimum pH from acidic values to neutrality [Levitzki, & Steer, 1974; Wakim, et al., 1969]. The shift in the pH-optimum may be attributed to the repulsion between the anion (chloride ion) and the residue Glu233.

III.B.6.ii. pH stability

The pH stability of PPA, PPA-I and PPA-II were tested after their incubation at different pH's for varying time intervals followed by determining the residual activities. Buffers such as phthalate buffer (pH 2.0-3.0), sodium acetate (pH 4.0-6.0), sodium phosphate (pH 6.0-7.0) and Tris-HCl (pH 8.0-9.0) at 50 mM were used to maintain the buffering capacity and to check the effect of buffer components on the stabilities of PPA, PPA-I and PPA-II.

PPA was found to be stable in the pH range of 7.0-8.0 and retained almost 80% activity after 4h of incubation. PPA-I and PPA-II were stable in the pH range of 6.0-8.0 and retained almost 80% activities after 4h of incubation. However, the activities of these amylases decreased drastically at pH 2.0-3.0 after pre-incubation for 30 min. Buffers such as glycine-HCl (pH 2.0-3.0) and borate (pH 8.0-9.0) were used and the percent activity retained after different time intervals were depicted in the fig. 31, 32 & 33. Though the enzymes lost their activities at pH 3.0, they retained almost 40% (PPA), 35% (PPA-I) and 30% (PPA-II) activities after incubation for 60 min. This indicated the stabilizing effect of glycine at lower pH. This may be perhaps due to the protection of the active site residues from the surrounding environment through electrostatic interaction. Borate buffer, alkaline pH retained 50% (PPA) and 40% (PPA-I & PPA-II) activities after 4 h of incubation.

α-amylases are generally stable in the pH range of 5.5-8.0 but exceptions exists on both sides of the pH scale amongst the amylases. An acidophilic α-amylase from a *Bacillus* sp. has a pH optima of 2.0 [Uchino, 1982] and an alkalophilic α-amylase from another *Bacillus* sp. has pH optima of 10.5 [Horikosi, 1971]. This broad range of optimum pH values for activities of individual α-amylases indicates their evolutionary adaptability to environmental circumstances [Toda, et al, 1982]. At extreme pH, the tertiary structure of the enzyme may be disrupted and the enzymes denatured and even at moderate pH, where the tertiary structure is not disrupted, enzyme activity depend upon the degree of ionization of the amino acid side chains [Palmer, 2001].

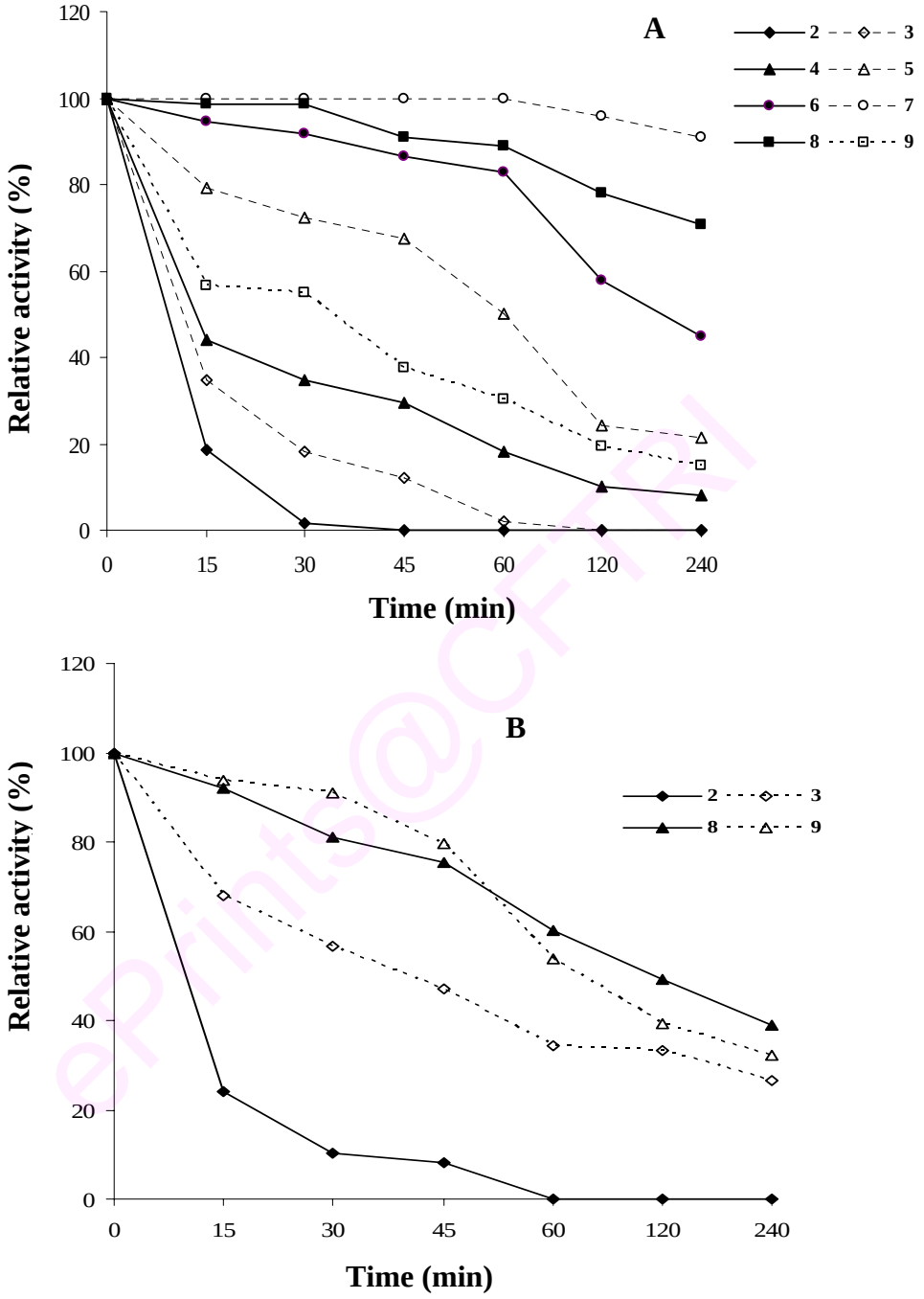


Fig. 31: Effect of pH on the stability of PPA

A. pH 2.0-3.0: Phathalate-HCl; pH 4.0-6.0: Sodium acetate; pH 7.0-8.0: Sodium phosphate; pH 8.0-9.0: Tris-HCl

B. pH 2.0-3.0: Glycine HCl; pH8.0-9.0: Borate

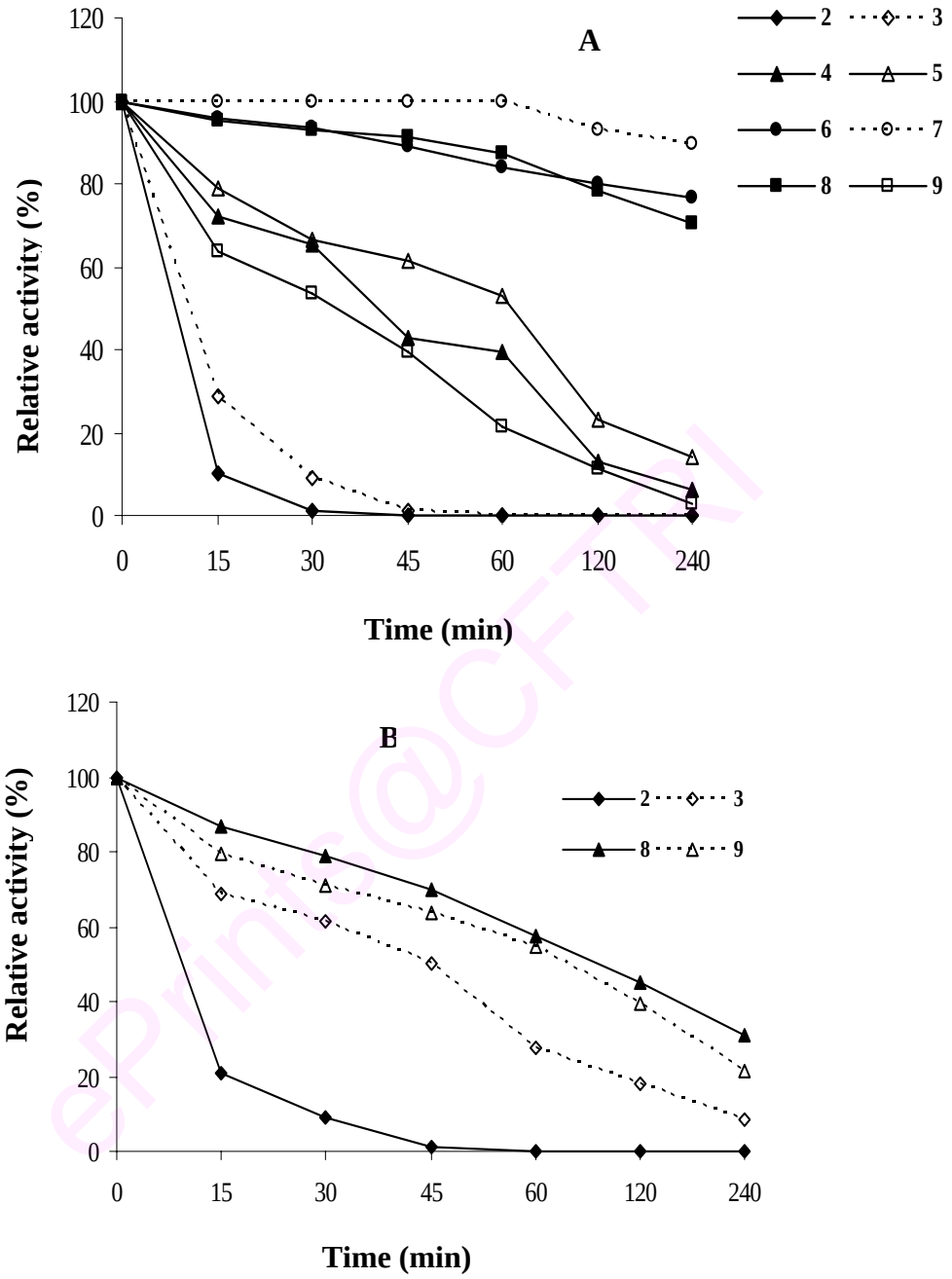


Fig. 32: Effect of pH on the stability of PPA-I

A. pH 2.0-3.0: Phathalate-HCl; pH 4.0-6.0: Sodium acetate; pH 7.0-8.0: Sodium phosphate; pH 8.0-9.0: Tris-HCl
B. pH 2.0-3.0: Glycine HCl; pH8.0-9.0: Borate

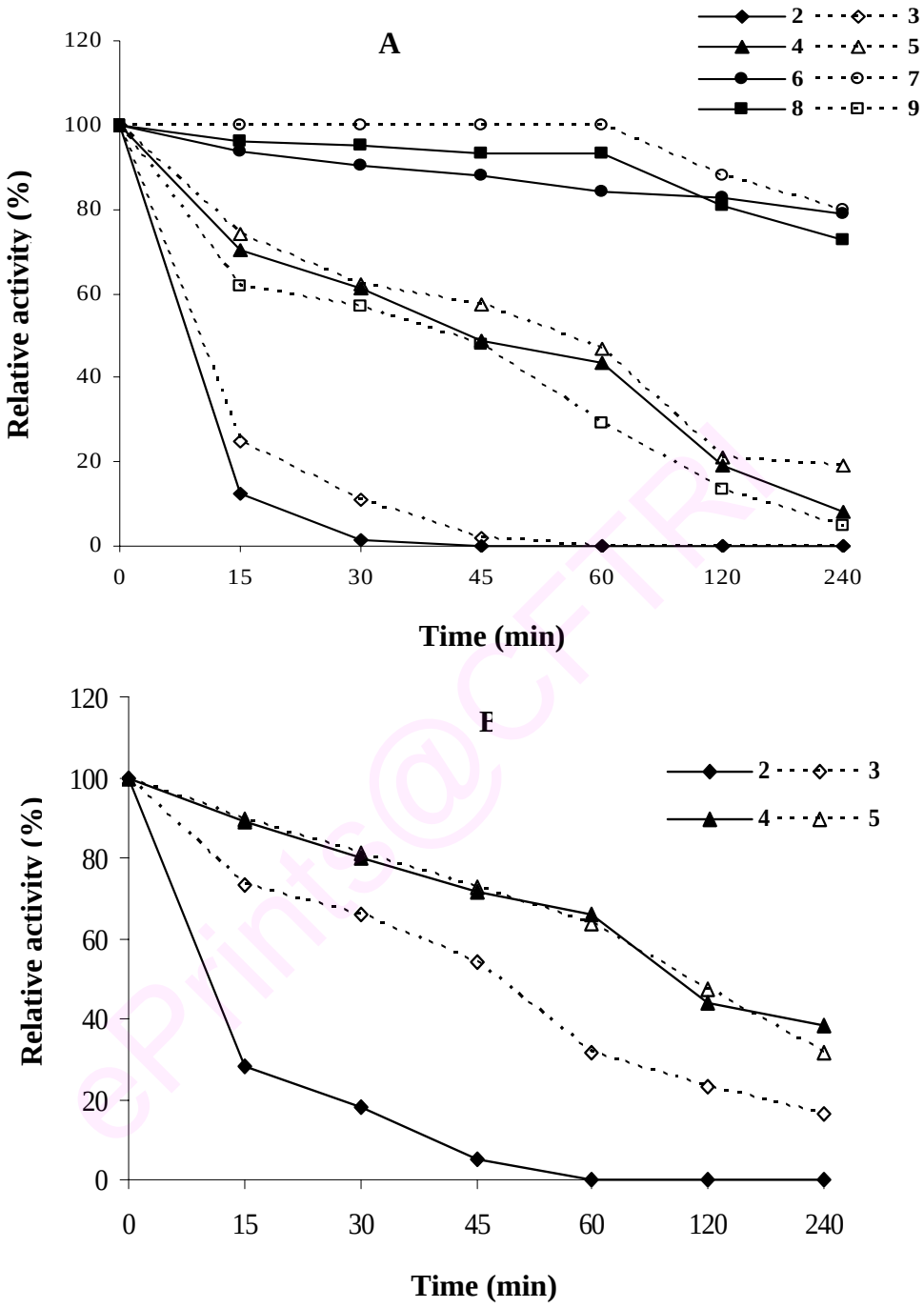


Fig. 33: Effect of pH on the stability of PPA-II

A. pH 2.0-3.0: Phathalate-HCl; pH 4.0-6.0: Sodium acetate; pH 7.0-8.0: Sodium phosphate; pH 8.0-9.0: Tris-HCl;
B. pH 2.0-3.0: Glycine HCl; pH8.0-9.0: Borate

III.B.6.iii. Temperature optima

PPA, PPA-I and PPA-II were found to have temperature optima of 45°C (fig. 34 & 35).

The activities increased sharply with gradual increase in temperature up to 45°C and there after showed decrease in activities with further increase in temperature indicating the loss in the active conformation of the enzyme. At higher temperature (70°C) PPA, PPA-I and PPA-II showed less than 20% activities.

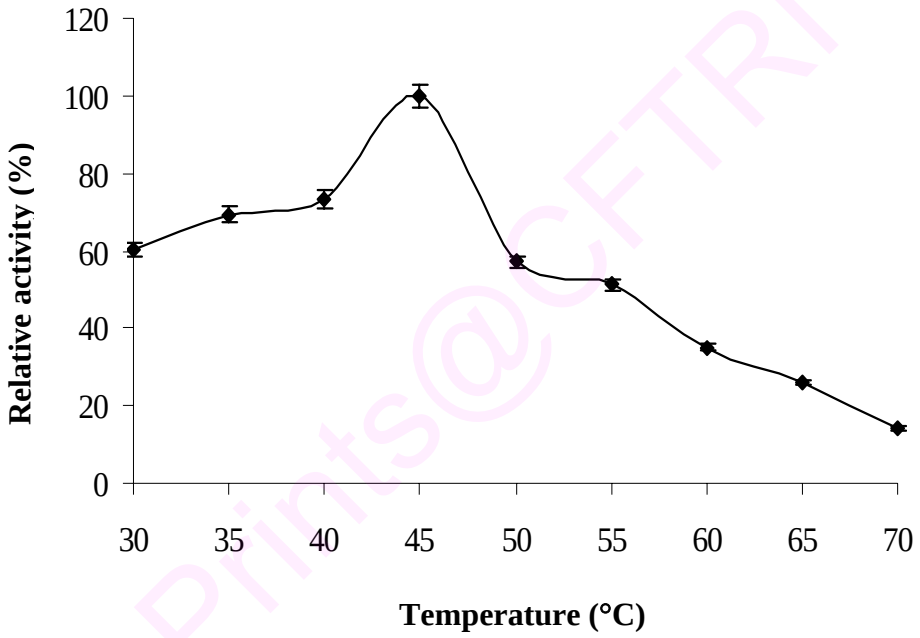


Fig. 34: Effect of temperature on the activity of PPA

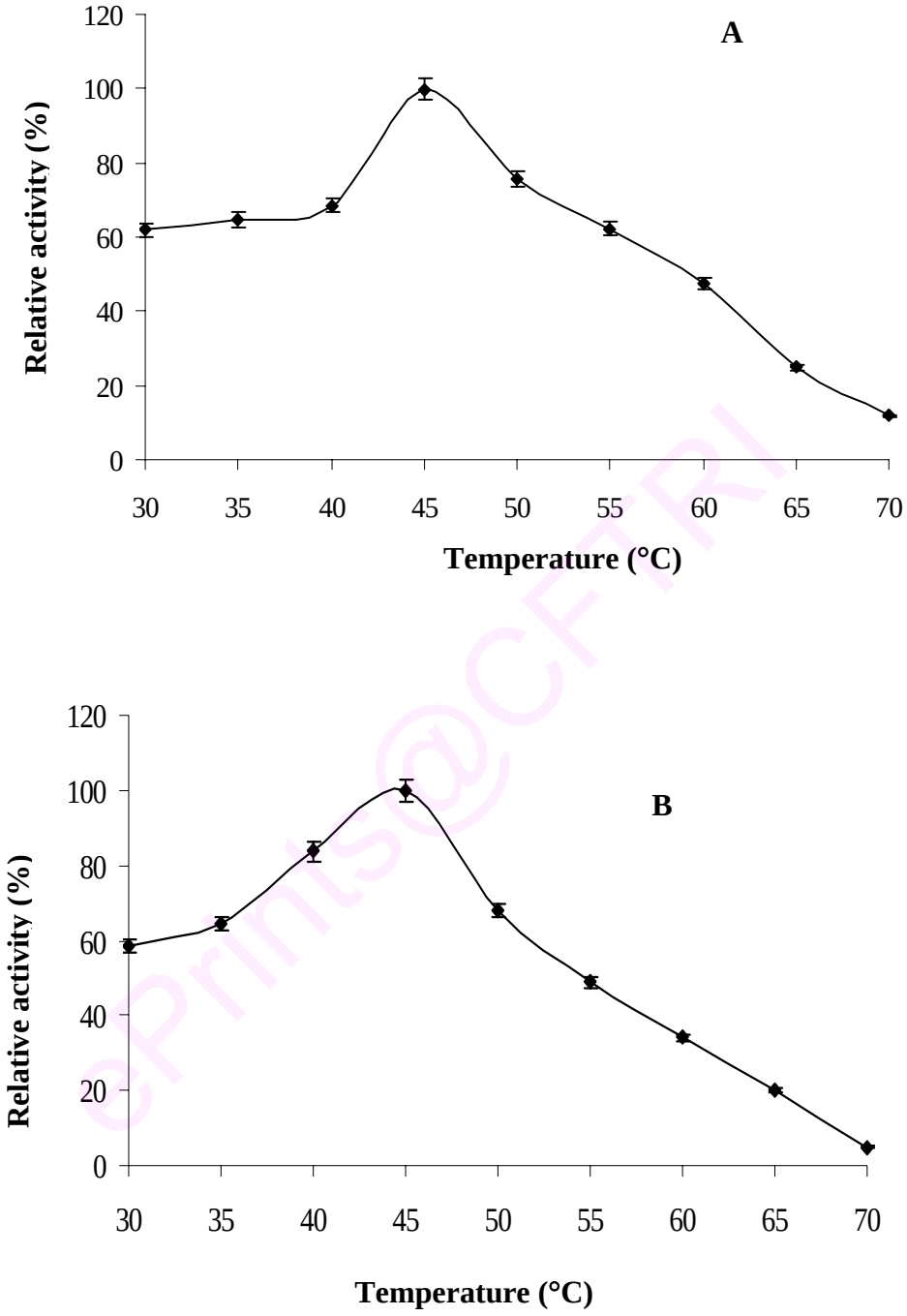


Fig. 35: Effect of temperature on the activities of (A) PPA-I and (B) PPA-II

The lowest temperature optimum for amylase has been reported to be 25°C for *Fusarium oxysporum* α-amylase [Janecek, & Balaz, 1992; Chary, & Reddy, 1985] and the highest for *Bacillus licheformis* alpha amylase (90°C) [Krishanan, & Chandra, 1983]. Most of the temperature optima of α-amylases are between 35 and 60°C. The extra thermostability of thermophilic *Bacillus licheniformis* α-amylase has been found to be mainly due the additional salt bridges involving a few specific lysine residues [Tomazic, & Klibanov, 1988]. *Bacillus stearothermophilus* α-amylase [Brumm, & Teague, 1989] as well as a mutant α-amylase from *Bacillus amyloliquefaciens* [Suzuki, et al., 1989] have been suggested to be stable against thermal denaturation through ionic interaction.

III.B.6.iv. Thermal stability

The thermal stability of PPA and its isoforms were observed to be upto 45°C as indicated by the residual activities (fig. 36 & 37). At 50°C the activities of PPA, PPA-I and PPA-II dropped to 50, 63 and 65% respectively. However at 70°C, PPA and its isoforms were almost completely inactivated (< 10% activity).

The residual enzyme activities decreased as the temperature increased from 50 to 70°C. Investigation on the effect of temperature on the enzymatic hydrolysis of corn, rice and wheat starches by three commercial α-amylases from *Bacillus* sp, *Aspergillus oryzae* and *Bacillus licheniformis* showed that temperature and incubation time are involved in the inactivation of α-amylases. The inactivation time and mechanism of each α-amylase studied at various temperatures were different and were specific to the enzyme [Daniel, et al., 1996; Apar, & Özbek, 2004].

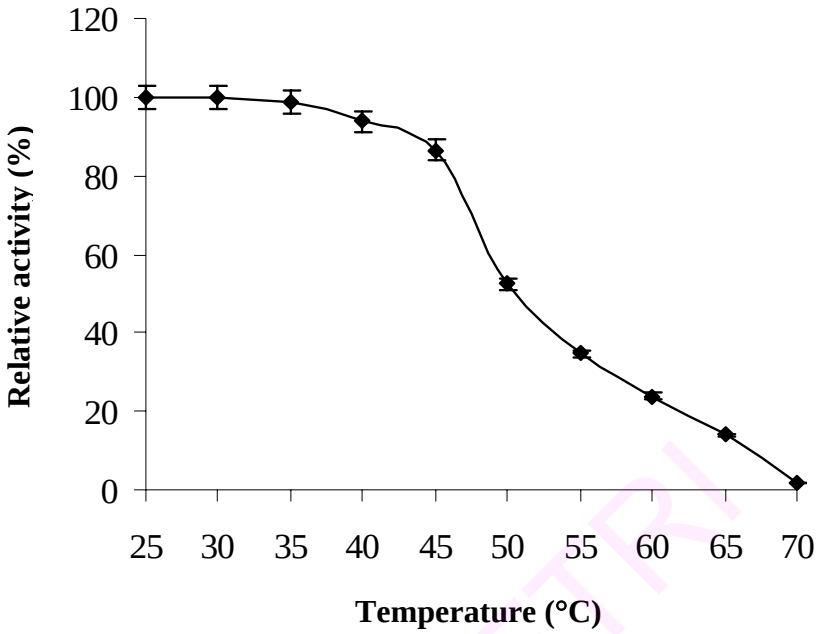


Fig.36: Effect of temperature on the stability of PPA

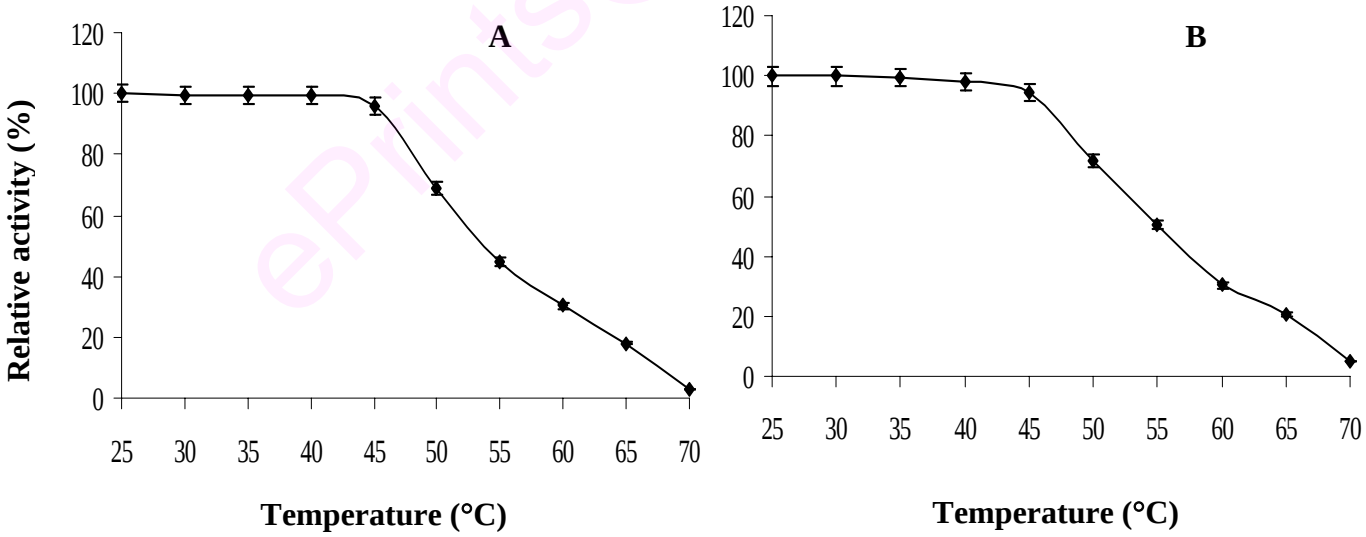


Fig. 37: Effect of temperature on the stabilities of (A) PPA-I and (B) PPA-II

III.B.7. Effect of substrate concentration

The kinetic constants (K_m & V_{max}) of PPA and its isoforms were calculated using the Lineweaver double-reciprocal plots (LB plot) [Lineweaver, & Burk, 1934]. The K_m value of PPA for ragi, rice, wheat and maize starches varied between 0.8 - 3.3% and V_{max} was found to be between 2841 - 6212 U/mg respectively. For PPA-I, K_m value for ragi, rice, wheat and maize starches varied between 1.3 - 2.5% and V_{max} was found to be between 2740 - 4201 U/mg respectively and for PPA-II, the K_m value for ragi, rice, wheat and maize starches varied between 1.0 - 2.9% and V_{max} was found to be between 2755 - 5102 U/mg respectively (figs. 38, 39, 40 & 41).

Higher the K_m, lower the digestibility. The observed K_m values for the starches digested by PPA, PPA-I and PPA-II revealed that maize starch with high K_m values was least digestible followed by ragi, wheat and rice starches (table 10).

Table.10: Kinetic constants of PPA, PPA-I and PPA-II for cereal and millet starches (K_m and V_{max})

Starches	**PPA**		**PPA-I**		**PPA-II**	
	K_m	**V_{max}**	**K_m**	**V_{max}**	**K_m**	**V_{max}**
Ragi	2.3	4149	2.5	4201	1.4	4149
Rice	0.8	2841	1.3	2740	1.0	2755
Wheat	1.0	3571	1.8	3125	1.5	3571
Maize	3.3	6212	2.5	3571	2.9	5102

K_m (%) V_{max} (U/mg protein)

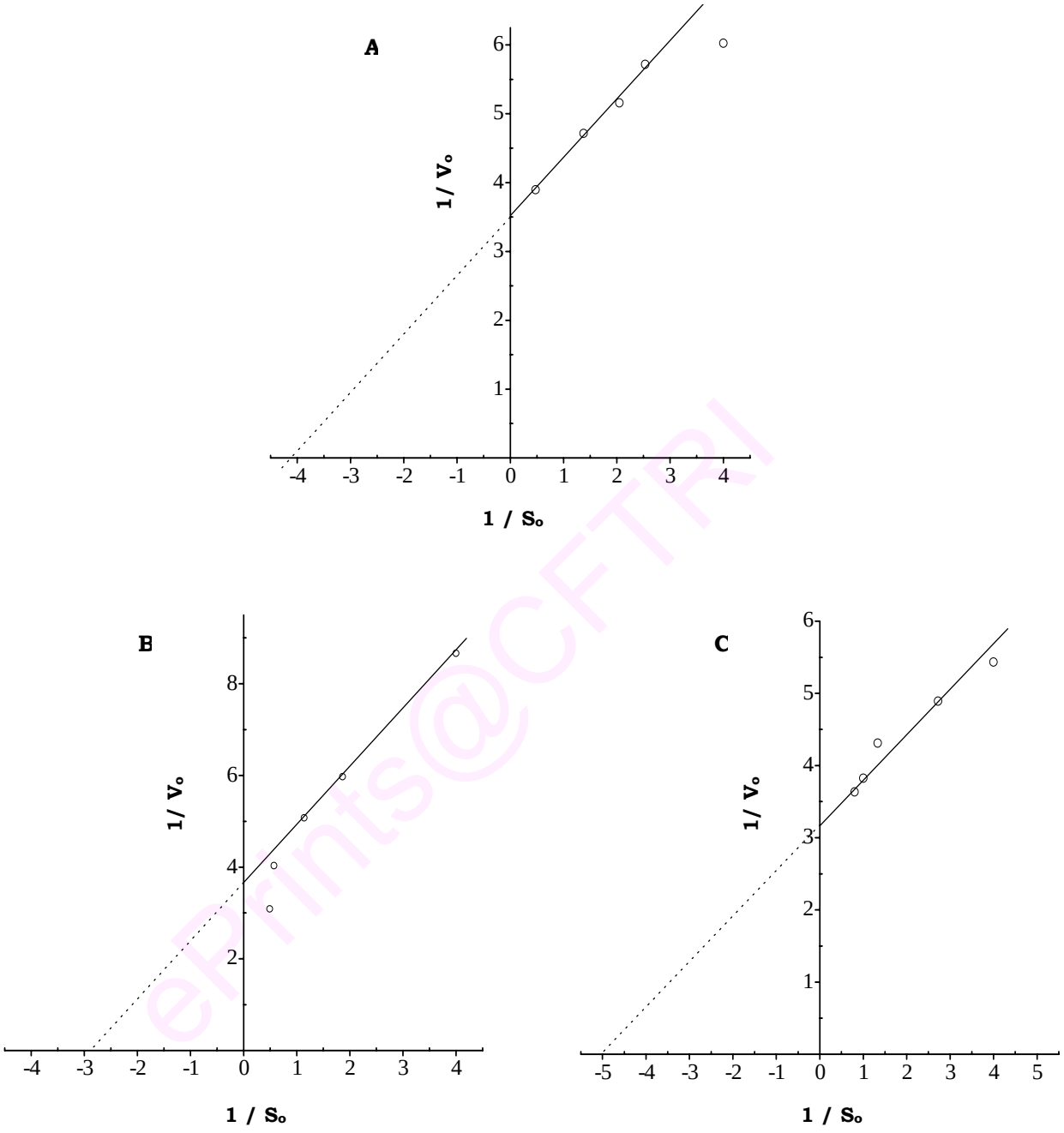


Fig. 38: Lineweaver-Burk plot for the hydrolysis of gelatinized ragi starch by (A) PPA (B) PPA-I and (C) PPA-II

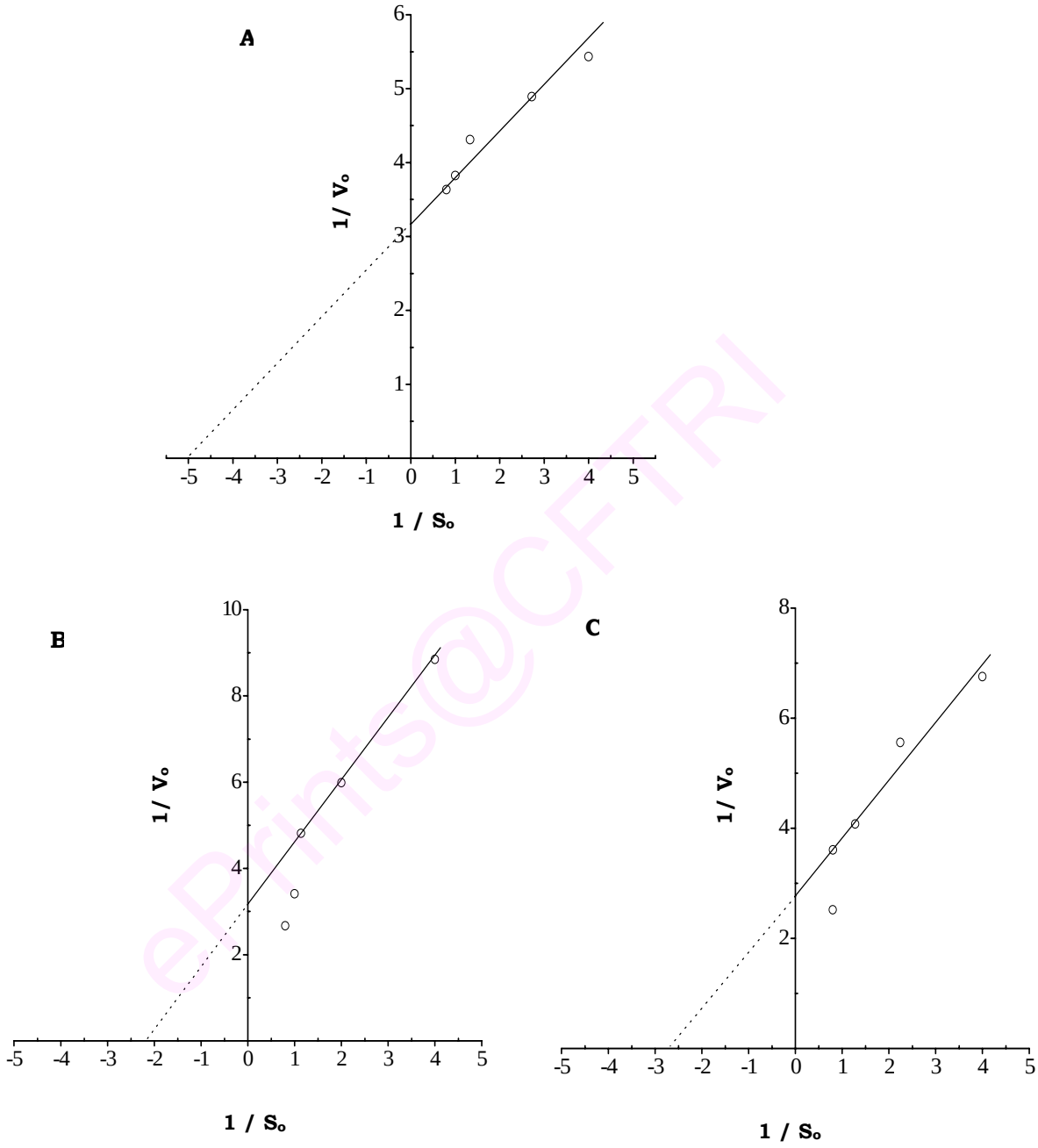


Fig. 39: Lineweaver-Burk plot for the hydrolysis of gelatinized rice starch by (A) PPA, (B) PPA-I and (C) PPA-II

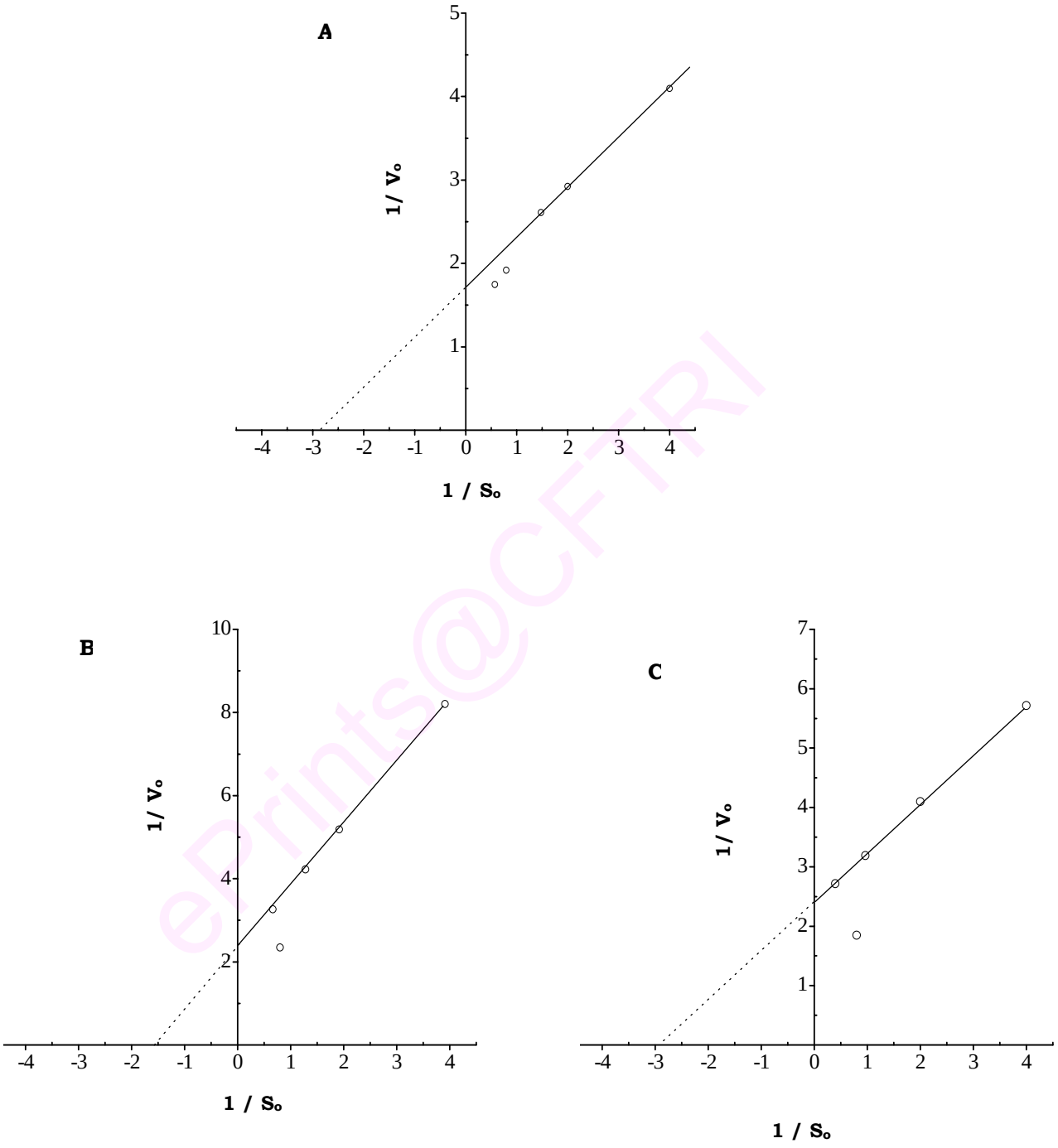


Fig. 40: Lineweaver-Burk plot for the hydrolysis of gelatinized wheat starch by (A) PPA (B) PPA-I and (C) PPA-II

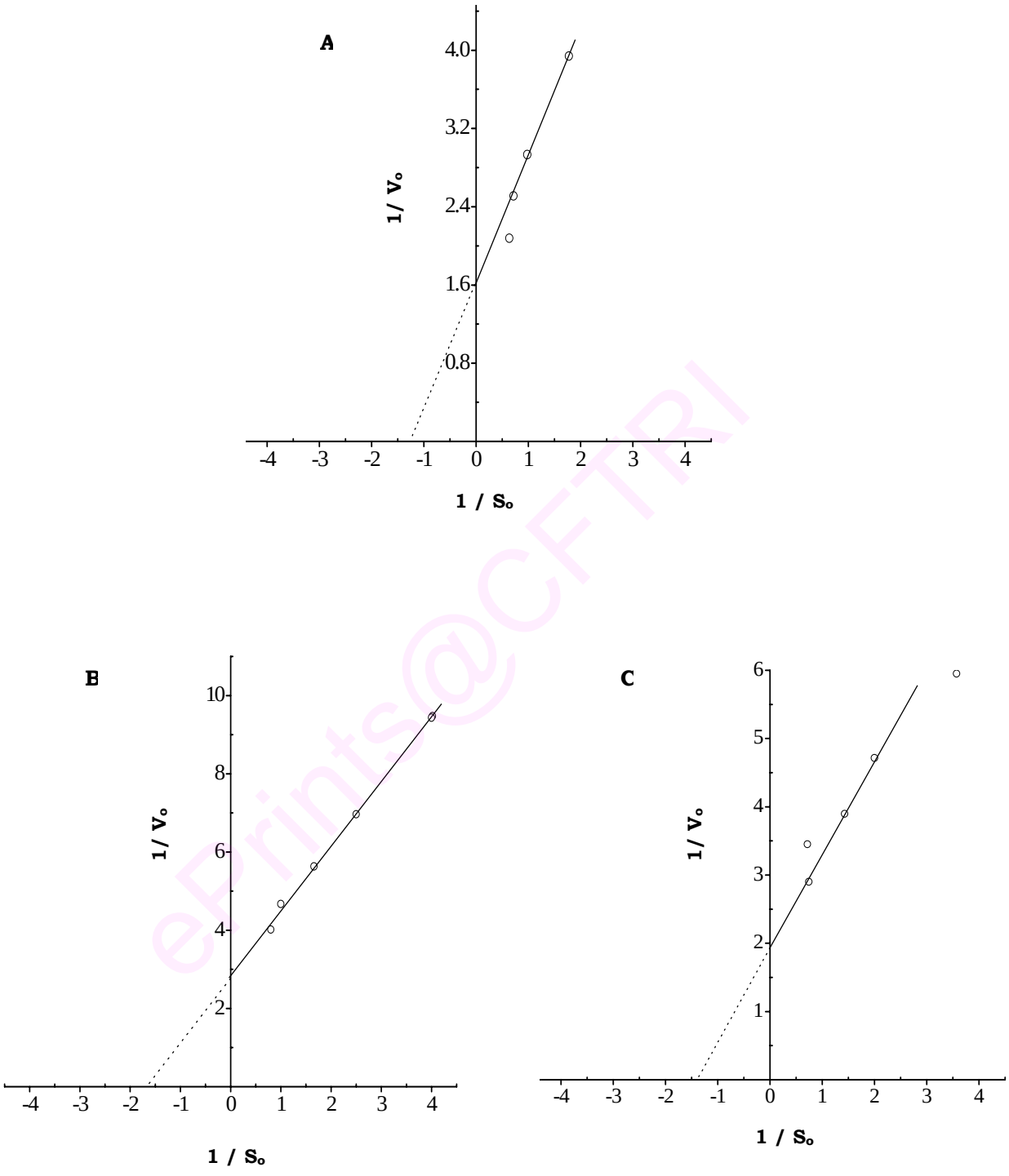


Fig. 41: Lineweaver-Burk plot for the hydrolysis of gelatinized maize starch by (A) PPA, (B) PPA-I and (C) PPA-II.

III.B.8.i. Effect of metal ions

Various metal ions such as Ca^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Fe^{2+} and Hg^{2+} at 5 mM concentrations were tested for amylase activation/inhibition effect. Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+} were found to have both activating and stabilizing effects as indicated by the increased activities of PPA, PPA-I and PPA-II. Metals such as Al^{3+} and Hg^{2+} completely inactivated all the three amylases whereas Zn^{2+} and Cu^{2+} partially inactivated PPA and its isoforms (table 11).

The inactivation by these metal ions may be due to their binding to either catalytic residues or replacing the Ca^{2+} from the substrate-binding site of the enzymes.

Table. 11: Effect of metal ions on PPA, PPA-I and PPA-II (represented as % activity)

Salts (5mM)	PPA	PPA-I	PPA-II
Control	100	100	100
$CaCl_2$	114.9	58.6	48.9
$BaCl_2$	108.4	14.1	0.8
$CoCl_2$	105.9	21.2	2.6
$MgCl_2$	111.6	18.8	25.5
$ZnCl_2$	15.5	12.6	5.22
$Cucl_2$	0.7	0.3	0.4
$AlCl_3$	0	0	0
$HgCl_2$	0	0	0

Enhancement of amylase activity by Ca^{2+} ions is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which results in stabilization as well as maintenance of enzyme confirmation. Calcium is known to have a role in substrate binding, optimum activity and

stability of the enzyme [Bush, et al., 1989]. Alpha-amylases have long been known to require at least one Ca^{2+} ion per enzyme molecule for maintaining their tertiary structure and catalytic activity [Vallee, et al., 1959]. This represents a new type of Ca^{2+} interaction site in a protein. One way of explaining the 'essential' role of Ca^{2+} ion in α-amylases is suggested by the observation that its ligands belong to domains A and B, and that the active site cleft is located between these two domains. The essential Ca^{2+} ion appears to stabilize the active site cleft by inducing an ionic bridge between domains A and B [Buisson,et al., 1987].

A minimum of 1 gram-atom of calcium per mole of protein is present in all purified amylases. α-amylase binds calcium very tightly with a binding constant of 2×10^{11} [Steer, & Levitzki, 1973]. The maximum number of cations that amylases can bind is much larger. If a crystalline sample of *A. oryzae* amylase is exposed to calcium ions and then dialyzed thoroughly against distilled water the protein will bind as many as 9 or 10 equivalents of calcium. Most of this extrinsically [Vallee, 1959] and weakly bound calcium can be removed easily during purification and recrystallization, whereas the other nonspecifically bound ions are removed whenever there is a change in pH or ionic strength.

Divalent cations, Ca^{2+} and Mg^{2+} can form six co-ordinate bonds to produce octahedral complexes [Palmer, 2001]. Calcium in metallo-enzymes perform dual functions, it forms a tight metal-chelate structure with the protein molecule maintaining the protein in the proper configuration for biological activity and on the other hand, it stabilizes the secondary and tertiary structure thus conferring on the amylase molecule a complete resistance to proteolytic degradation [Vallee, 1959]. Emission spectroscopic analysis of crystalline amylases from human saliva, hog pancreas, *Bacillus subtilis* and *Aspergillus oryzae* demonstrated that all these enzymes contain at least 1 gram-atom of very firmly bound calcium per mole of enzyme.

III.B.8.ii. Effect of citric and oxalic acids

Citric acid inhibited PPA at 10 mM concentration and PPA-I and II were inhibited at 7.0 mM concentration (fig. 42A).

Oxalic acid inhibited PPA at 10 mM concentration whereas PPA-I and II were inhibited at 5 mM concentration (fig. 42B).

Carboxylic acids such as oxalic acid and citric acids inhibit α-amylases by selective removal of Ca^{2+} present in the enzyme. The differential inhibition effect of citric/oxalic acid on the activities of PPA, PPA-I and PPA-II may be attributed to their relative binding (strong/ weak) of Ca^{2+} to the enzymes.

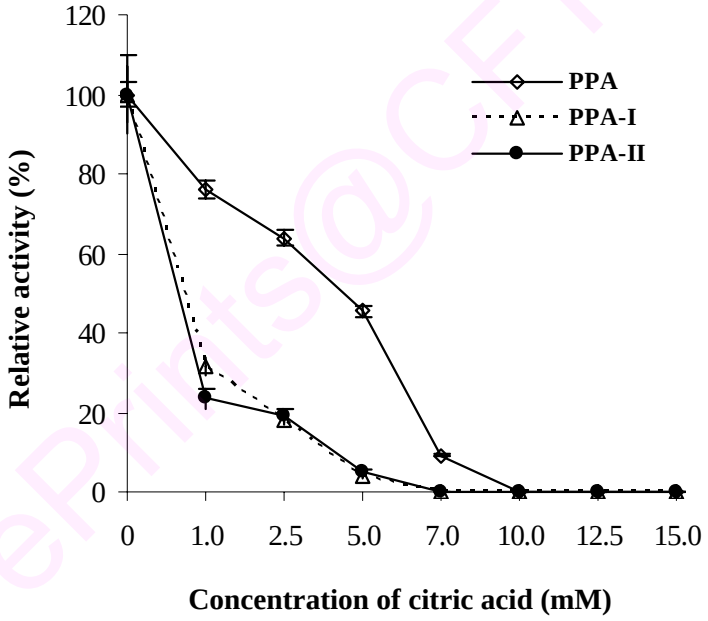


Fig. 42 A. Effect of citric acid on the activities of PPA, PPA-I and PPA-II

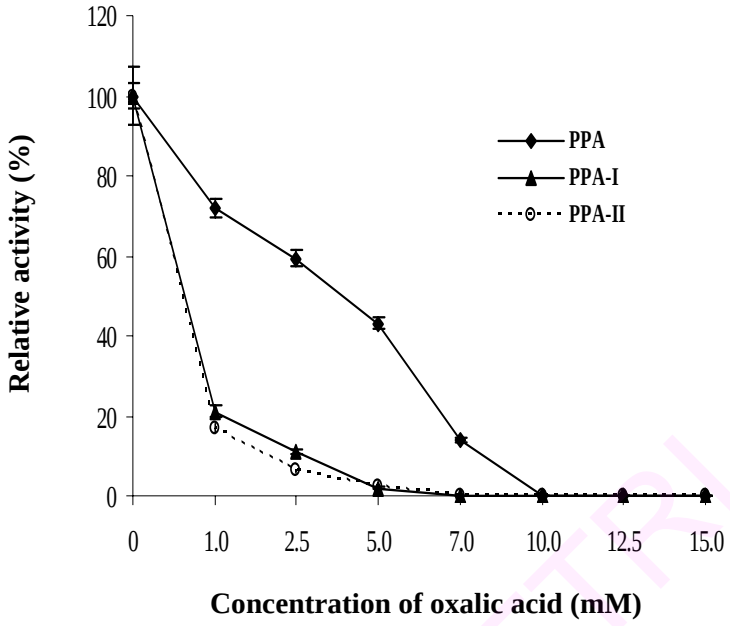


Fig.42 B. Effect of oxalic acid on the activities of PPA, PPA-I and PPA-II

III.B.8.iii. Effect of EDTA

The effect of various concentrations (0-150µm) of EDTA on the activities of PPA and its isoforms were tested in the presence of substrate at 37°C and the results are depicted in fig. 43. EDTA at 125µm concentration completely inhibited the activities of PPA and its isoforms.

The inhibition by EDTA may be due to the chelating of bound calcium present in the enzymes [Boyer, & Ingle, 1972]. Calcium ions appear to stabilize the active site cleft by inducing an ionic bridge between the domains. However EDTA inhibited isoforms PPA-I and PPA-II much more strongly than PPA. This may be perhaps due to the presence of more amount of bound calcium in the native enzyme than its isoforms which may require higher concentrations of EDTA to chelate the bound calcium present in the native enzyme. The inhibition may be due to the preferential and rapid formation of enzyme-inhibitor complex over substrate-enzyme complex indicating this to be a competitive type of inhibition.

Once substrate-enzyme complex is formed, the bound Ca^{2+} is no more accessible to EDTA hence, resulting in very poor inhibition.

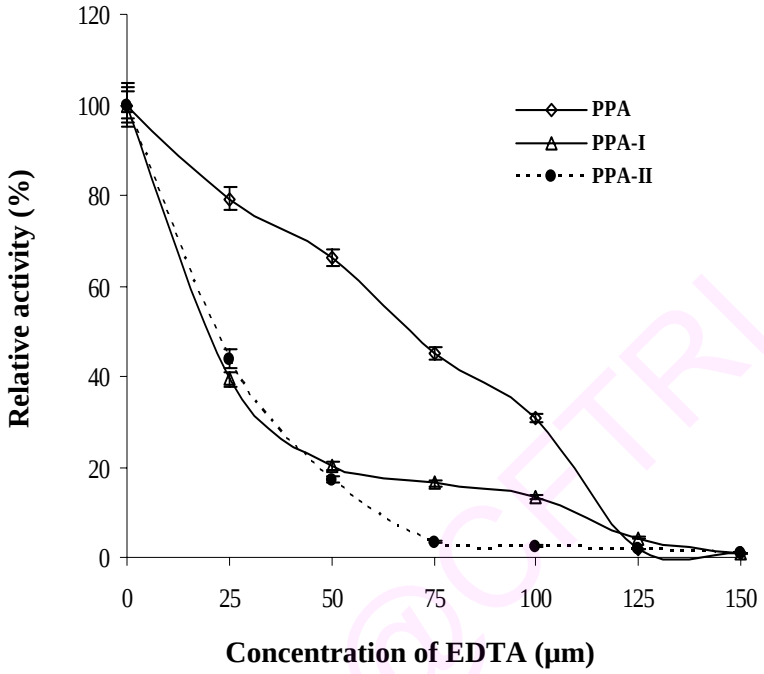


Fig. 43: Effect of EDTA on the activities of PPA, PPA-I and PPA-II

Amylases from molds were found to be more stable towards inhibition by EDTA followed by bacterial amylases and hog pancreatic amylase [Stein, & Fischer, 1958]. Inhibition of the enzyme activity by EDTA indicates the enzyme to be a metallo-protein containing Ca^{2+} ion. EDTA binds most divalent metal ions. On removal of the Ca^{2+} from the enzyme, the two sulfhydryl groups become exposed and the enzyme loses its activity [Steer, & Levitzki, 1973].

III.B.9. *In vitro* digestibility of cereal and millet flours

In the present study the digestibility of the flours and starches were restricted to 120 min since the digestibilities of the starches were almost complete.

a) Cereal and millet flours

The digestibility of rice flour was higher when compared to wheat, ragi and maize flours. The % hydrolysis of ragi, rice, wheat and maize flours by PPA, PPA-I and PPA-II are represented in fig 44. The hydrolysis percentage was more for rice flour (PPA-97.8%, PPA-I- 98.5% & PPA-II-98.3%) and least hydrolysis was observed for the maize flour (PPA-83.5%, PPA-I- 88.1% & PPA-II-91.1%). The variation in the hydrolysis percentage may possibly be due to the presence of more viscogenic polysaccharides and polyphenols which limit the digestibility of flours of ragi and maize.

b) Cereal starches

The hydrolysis rate of the starches by PPA and its isoforms was almost similar. The percentage hydrolysis of rice starch was more followed by wheat, ragi and maize starches (fig. 45).

The percentage hydrolysis of rice starch by PPA, PPA-I and PPA-II were 99.0, 98.5 and 99.1% respectively. Cereal starches were digested more easily compared to the flour samples.

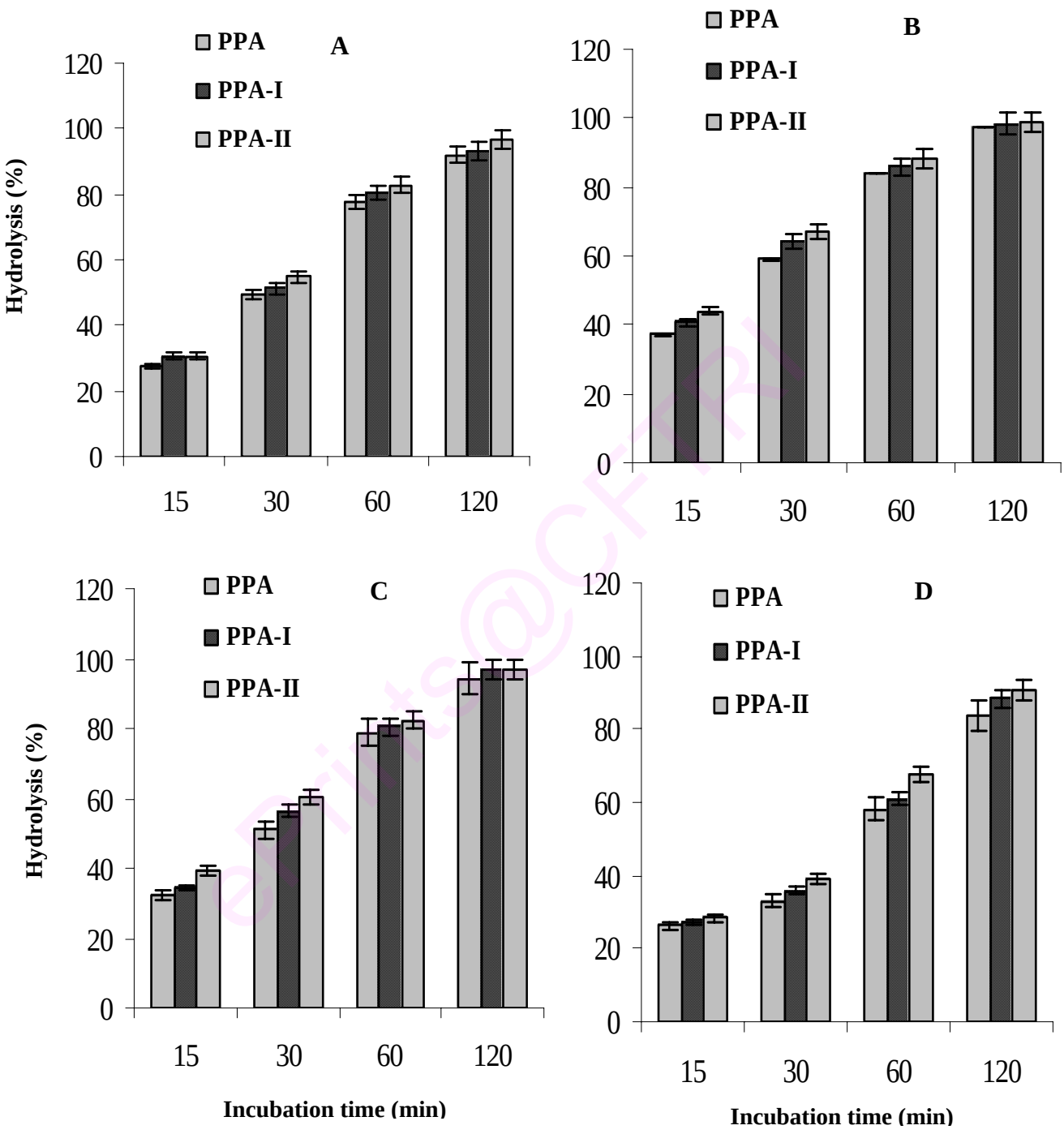


Fig. 44: Hydrolysis (%) of millet and cereal flours by PPA and its isoforms (A) ragi (B) rice (C) wheat and (D) maize

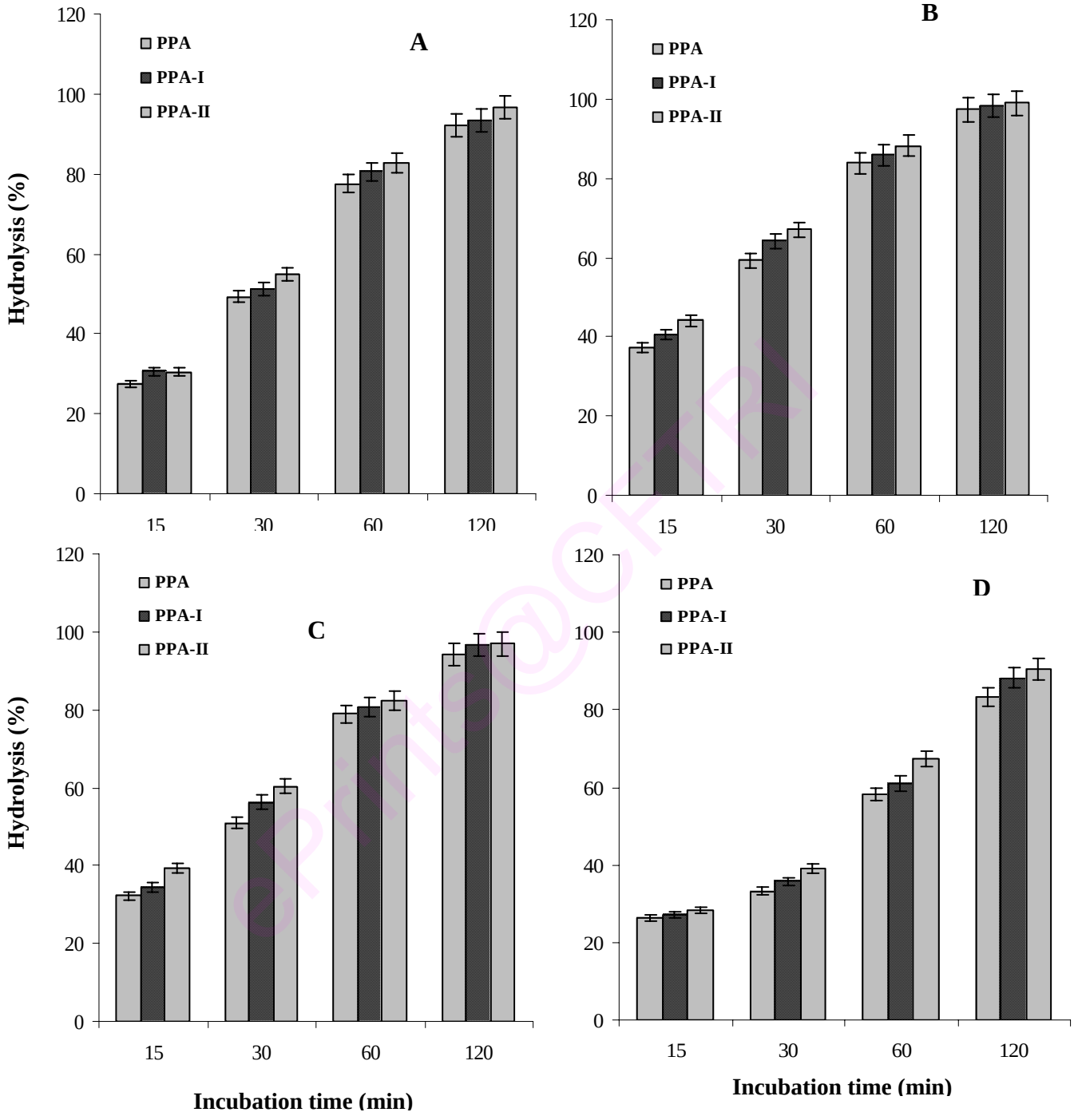


Fig. 45: Hydrolysis (%) of millet and cereal starches by PPA and its isoforms (A) ragi (B) rice (C) wheat and (D) maize

Many studies on starch hydrolysis by α-amylases have been carried out to obtain information on its structure and activity. Often α-amylases from bacteria have been used. In the present study we have used pancreatic α-amylases from porcine source since PPA shows highest homology (87.1%) with human pancreatic α-amylase sequence [Darnis, et al., 1999]. In general starch hydrolysis is carried out using gelatinized starches since the native starch granules were hydrolyzed very slowly compared to the gelatinized starches.

The rate of hydrolysis varies with the source of the enzyme as well as the substrate. The susceptibility of isolated rice starch to PPA and its isoforms was more when compared to the starches isolated from ragi, wheat and maize. Research in this direction to study the kinetics of α-amylolysis of the starches followed by SEM observations have proven that the enzyme attack was by exo-corriosion, i.e. attack from the exterior portion of the granule inwards. Further the amylolysis proceeded most rapidly in the amorphous or the less crystalline regions in the molecule. The rate and extent of amylolysis was essentially a cumulative action of several factors such as (1) the presence of contaminating enzymes in the amylase (2) the stability of the enzyme preparation over a period of time (3) the enzyme to substrate ratio (4) its debranching ability to hydrolyze large and small substrate (5) the availability of non-reducing ends on its surface (6) the overall composition of the starch per se, particularly the amylose content and its chain length (DP) and (7) shape-size variation in the starch population as also on the interactions between granules of different sizes. Small spherical granules were reported to be less digestible. Higher the amylose content, slower the amylolysis [Tharanathan, et al., 1987]. Enzymatic hydrolysis of starch granules is affected by granule structure, crystal type, granule size, amylose/amylopectin ratio, average molecular weight, presence of lipids and proteins, reaction conditions and the enzyme specificity [Yook, & Robyt, 2002; Kong, et al., 2003].

III.B.10. Effect of WSP from ragi, rice, wheat and maize on the activities of PPA and its isoforms

Ragi WSP at 0.5% concentration showed reduction (non specific inhibition) in activity i.e 5% in the case of PPA and < 5% reduced activity of PPA-I and PPA-II. It is observed that the activity decreased with increase in the amount of polysaccharide addition (up to 1%). At 1% concentration of ragi WSP the reduction in activity of PPA, PPA-I and PPA-II were 21.8, 17.2 and 18.6% respectively. An increase in concentration above 1% was not taken due to the lower solubility of WSP in buffer/water.

Percentage reduction in activity by rice WSP (1%) was 19.6% for PPA and 14.7 and 17.6% for PPA-I and PPA-II respectively. Wheat WSP at 1% concentration showed decrease in activity of 17.3, 11.8 and 15.9% for PPA, PPA-I and PPA-II respectively. The reduction in activity was high by maize polysaccharides with a percent reduction of 30.4, 21.7 and 24.6% for PPA, PPA-I and PPA-II respectively (fig. 46). The loss of activity of PPA was more followed by PPA-II and PPA-I. The reduction in activity by maize polysaccharides was higher followed by ragi, rice and wheat WSP's. The following may be the probable reasons for the reduced activity (non specific inhibition) observed by these WSP samples; (a) the arabinose to xylose ratio (maize-1:8, ragi-1:4, wheat and rice-1:3), (b) the percentage of phenolic acids present in the WSP samples (ferulic and coumaric acids) or (c) the relative viscosity of WSP. The viscosity of the substrate do play a significant role with respect to its hydrolysis by the enzyme. The enzymes can not penetrate the viscous substrate as easily compared with that of a less viscous substrate and hence there is a likely reduction in enzyme-substrate interaction resulting in a slow release of the products. the probable mechanism of reduction in activity of PPA and its isoforms may be attributed to the effect of viscosity of the WSP.

α-Amylase and α-glucosidase have been targeted as potential avenues for modulation of postprandial hyperglycemia through mild inhibition of these

enzymes by non-starch polysaccharides to decrease meal-derived glucose absorption. The significance of food-grade, plant-based amylase inhibitors for modulation of carbohydrate breakdown and control of glycemic index of foods in the context of preventing hyperglycemia and diabetes mellitus complications were hypothesized. Porcine pancreatic α-amylase (PPA) react with WSP and the enzyme-WSP-substrate mixture obtained were assayed for the amylase activity. Reduction of activity of enzymes may be brought about by a single factor or by the combination of many factors as described above.

Protenacious inhibitors of pancreatic α-amylase are known [Tanizaki, et al., 1985; Tanizaki, & Lajolo, 1985; Frels, & Rupnow, 1984]. The inhibition is high in aspartic acid, glutamic acid, serine, threonine and valine and low in cysteine/cystine. These protenacious inhibitors have inhibitory activity against porcine pancreatic α-amylase and human salivary α-amylase but were inactive against *Bacillus subtilis* α-amylase, *Aspergillus oryzae* α-amylase, barley α-amylase and red kidney bean α-amylase. The rate of complexation with porcine pancreatic α-amylase was very slow which required almost 12-24 h for maximum inhibition at 30°C [Frels, & Rupnow, 1984]. They exhibited specificity to human salivary and porcine pancreatic α-amylases but had no inhibitory activity on *Bacillus subtillis*, *Aspergillus oryzae* and endogenous triticale α-amylases [Ida, et al., 1994]. Inhibitory activities in eight plant seed extracts were determined against ten different pancreatic amylases where in protenacious inhibitors from maize had no inhibitory activity [Ambalath, & Pattabiraman, 1986]. The phenolic acids are known to inhibit the activities of the amylases [Patrick, & Kalidas, 2004; Patrick, et al., 2005]. Phenolic substances interact with and/or inhibit proteins/enzymes [Arts, 2002; Griffiths, 1986; Watanabe, et al., 1997]. Phenolic substances that are able to form quinones (i e caffeic acid, chlorogenic acid, gallic acid, etc) are more reactive than those phenolics that cannot form quinones and suggested that semi-quinones formed may react with

amino-acid side chains and free thiol groups of the enzyme and inhibit the enzyme activity [Rohn, et al. 2002].

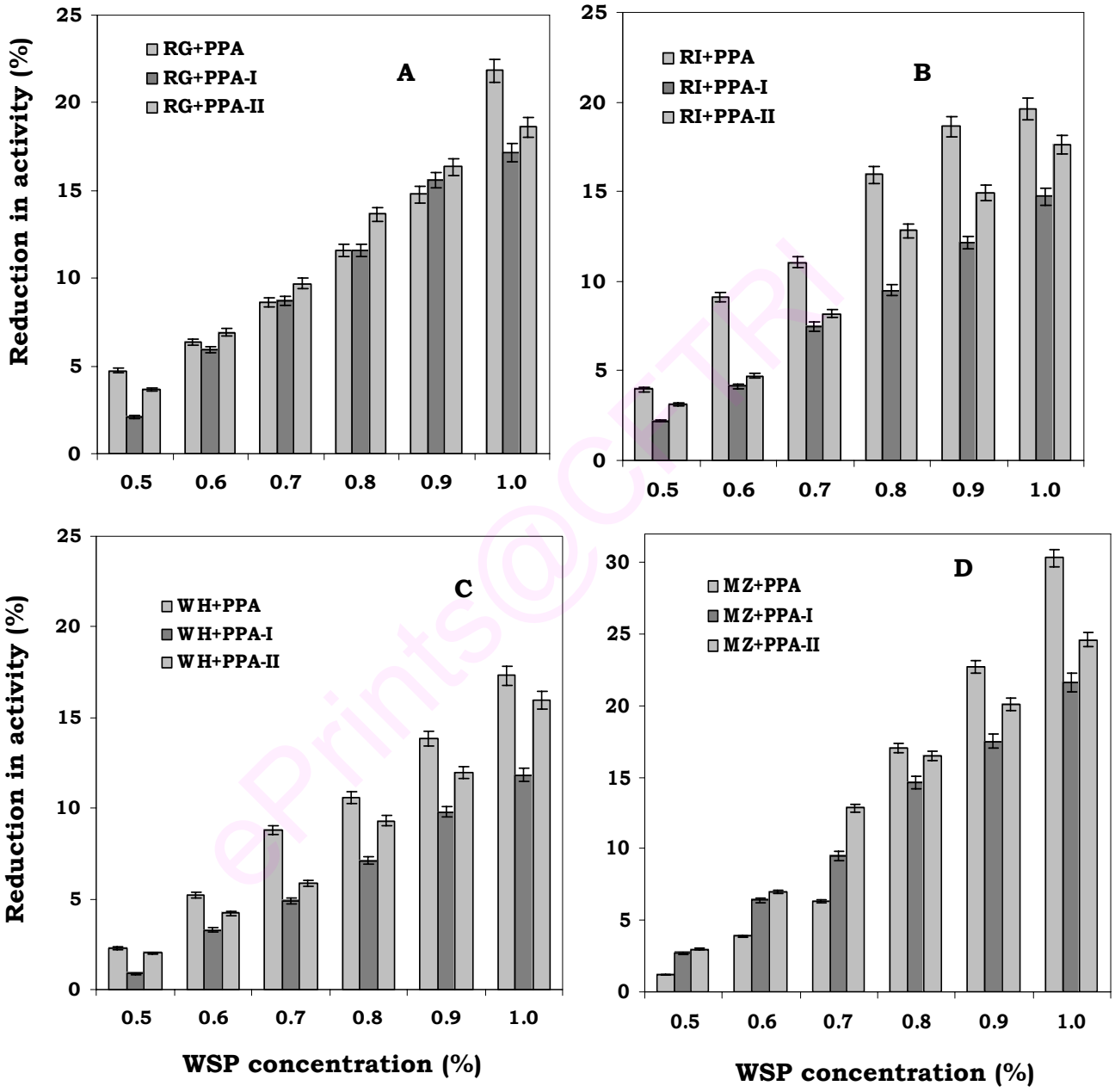


Fig. 46: Effect of WSP on the activities of PPA and its isoforms
(A) ragi (B) rice (C) wheat (D) maize

Acarbose, a pseudotetrasaccharide, is a powerful inhibitor of α-glucosidases [Schmidt, et al., 1977]. Kinetics of the acarbose-inhibited hydrolysis of long (amylose) and short (reduced maltodextrin) substrates were discussed in literature. Acarbose can also interact with PPA [Muller, et al., 1980]. One molecule of acarbose per amylase molecule binds either directly to free enzyme at the active site or to the enzyme-substrate complex at a secondary carbohydrate-binding site which becomes functional after the substrate has bound to the enzyme molecule at the active site [Al Kazaz, et al., 1998]. The three-dimensional structure of the PPA-I-acarbose and PPA-II-acarbose complexes has been determined [Gilles, et al., 1996]. Two acarbose molecules react giving five-sugar and six-sugar ring structures respectively localized in the active center [Alkazaz, et al., 1996].

Two inhibitors the pseudotetrasaccharide inhibitor acarbose and the protein inhibitor of *Phaseolus vulgaris* α-A-II, inhibit PPA catalyzed hydrolysis of amylose and short maltodextrin by blocking the active centres of PPA-I and PPA-II [Alkazaz, 1996; Alkazaz, 1998; Berre-Anton, et al., 1997].

CHAPTER - III

Section C

Deglycosylation & spectroscopic studies of porcine pancreatic alpha amylase & its isoforms

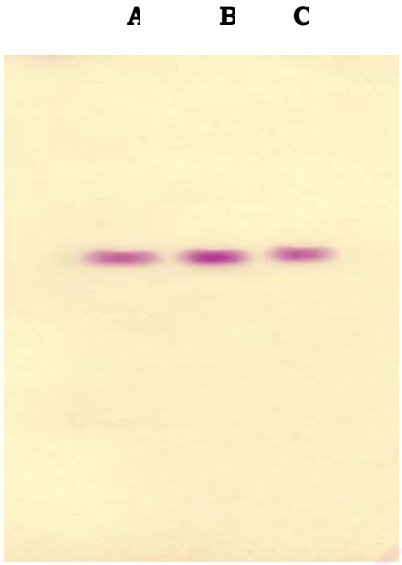
III.C.1. Introduction

One of the most important post-translational modifications in newly synthesized proteins is glycosylation and is common in several higher organisms [Lis, & Sharon, 1993; Varki, 1993]. Most plant lectins and α-amylases are glycoproteins. Porcine pancreatic α-amylase possess three potential glycosylation sites [Alkazaz, 1998]. The carbohydrate moiety plays a major role in secretion, targeting and modulation mechanism of the enzymes *in vivo* [Matsushita, 2002]. Removal of the oligosaccharide/s using endoglycosidase enzyme is often desirable to study the structure and function of an enzyme with out the covalently anchored carbohydrate moiety. The N-glycosylation (NG) sites of PPA are positioned in the junction between the A- and C-domains of the enzyme [Qian, et al., 1993; Kadziola, et al., 1994]. Peptide N-glycosidase F (PNGase F) is widely used for enzymatic deglycosylation of the glycoproteins. PNGase F cleaves the intact glycans as glycosylamine which are converted to glycans. The mechanism of action involves the hydrolysis of the amide bond between the sugar and the side chain of the asparagines which forms aspartic acid.

The present investigation is aimed at studying the differences in the glycosylated and deglycosylated forms of the glycoproteins (PPA, PPA-I and PPA-II) using different parameters such as (i) temperature stability (ii) quantification of oligosaccharides (iii) change in absorption and fluorescence spectra and (iv) circular dichroism (CD) studies.

III.C.2. Carbohydrate composition

PPA and its isoforms were tested positive for carbohydrates by phenol-sulphuric acid method as well as by periodate Schiff's staining of PAGE gel (fig. 47).



A) PPA **B) PPA-I** **C) PPA-II**

Fig. 47: PAGE of PPA, PPA-I and PPA-II-glycoprotein staining

The carbohydrate content of PPA, PPA-I and PPA-II were 0.05, 0.006 and 0.004 mol per 1 mol of enzymes respectively. The completeness of deglycosylation by PNGase F treatment was observed after 24 h of incubation at 37°(fig. 48). PNGase F treatment of PPA, PPA-I and PPA-II liberated oligosaccharides.

Carbohydrate analysis by Matsushita, et al., [2002] indicated that PPA contained 0.04 mol each of D-mannose and *N*-acetyl-D-glucosamine per 1 mol of PPA. The carbohydrate content reported in the present investigation are in agreement with the values reported by earlier investigators [Matsushita, et al., 2002; Beaupoil-Abadie, et al., 1973]. L-Fucose, *N*-acetyl-D-galactosamine and D-xylose were not detected according to the literature reports. PPA has three potential glycosylation sites but most parts of PPA are unglycosylated and only a very small amount (< 1% of potential sites) is *N*-glycosylated.

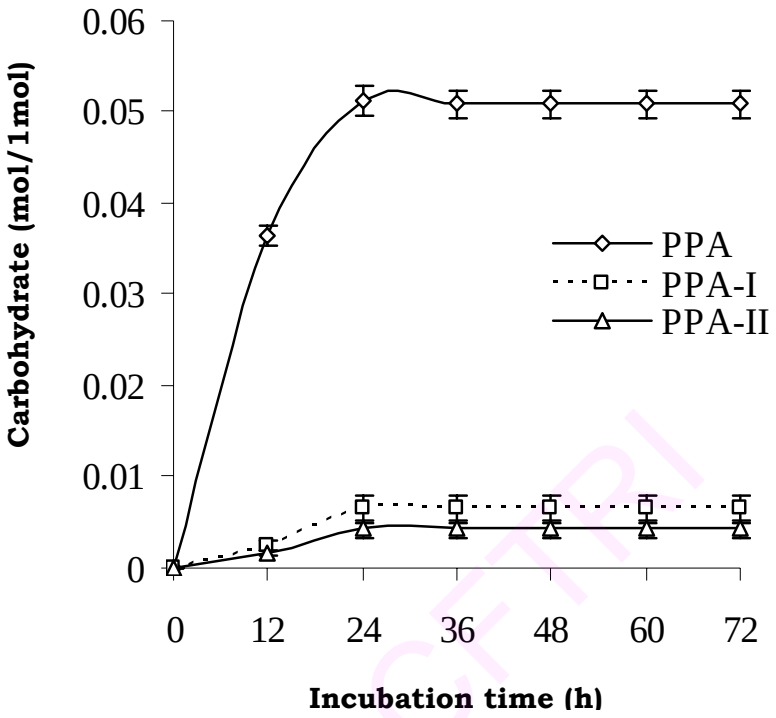


Fig. 48: Carbohydrate (moles/1 mol) released from PPA, PPA-I and PPA-II upon PNGase F treatment

Enzymatic deglycosylation of glucose oxidase (from *Aspergillus niger,* Type X-S), and fetuin (from fetal calf serum) were investigated using peptide-*N*-glycosidase F [Lee, & Park, 2002]. Glucose oxidase had 8 potential *N*-linked glycosylation sites and only a few sites that were occupied by oligosaccharides. The attached oligosaccharides were of high-mannose type and corresponds to 11-20% of the molecular weight [Frederick, et al., 1990]. Fetuin had 3 potential *N*-linked glycosylation sites and on an average 2.4 of them were glycosylated [Takasaki, et al., 1986].

Although the presence of oligosaccharide is critical for the biological activity of glycoprotein, it was reported that the nature and structure of the oligosaccharide was not an important factor [Patel, et al., 1993].

III.C.3. Electrophoresis

III.C.3.i. Periodic Acid-Schiff (PAS) Staining

Native PAGE of PPA, PPA-I and PPA-II subjected to PAS staining developed magenta/pink bands against a white background indicating the glycoprotein nature of the enzyme. PNGase F digested samples of PPA and its isoforms did not develop the characteristic color indicating the complete removal of the carbohydrate moiety (fig. 49).

When treated with periodic acid the glycols are oxidized to aldehydes. The reaction with Schiff's reagent (a mixture of pararosaniline and sodium metabisulfite) a pararosaniline adduct is released that stains the glycol-containing components and the reaction produces a pink color.

III.C.3.ii. SDS-PAGE

Deglycosylated PPA and its isoforms showed very little change in their relative mobility (fig. 50) compared to their glycosylated forms on SDS-PAGE. This indicated that molecular weights of the deglycosylated PPA, PPA-I and PPA-II were close to the molecular weights of glycosylated PPA, PPA-I and PPA-II. The relatively less mobility of the deglycosylated PPA and its isoforms indicated a small change in the molecular weight upon deglycosylation.

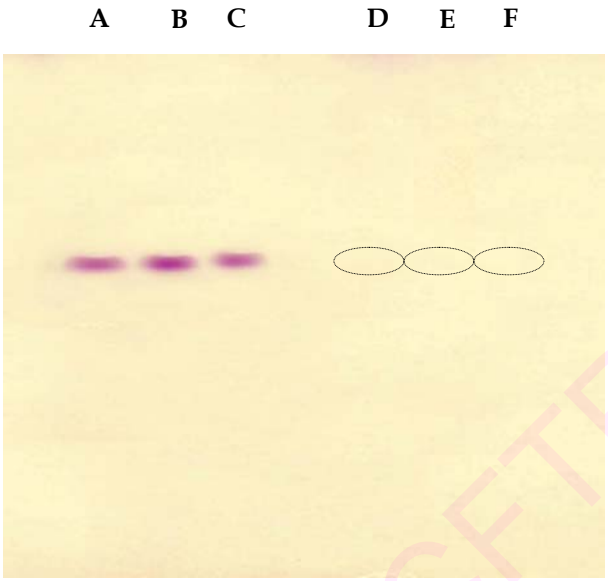


Fig. 49: PAGE of glycosylated and deglycosylated PPA, PPA-I and PPA-II - glycoprotein staining

Lanes: A-PPA; B-PPA-I; C-PPA-II; D-PPA,deglycosylated; E-PPA-I, deglycosylated; F-PPA-II, deglycosylated

Deglycosylated PPA and its isoforms - negative to PAS staining

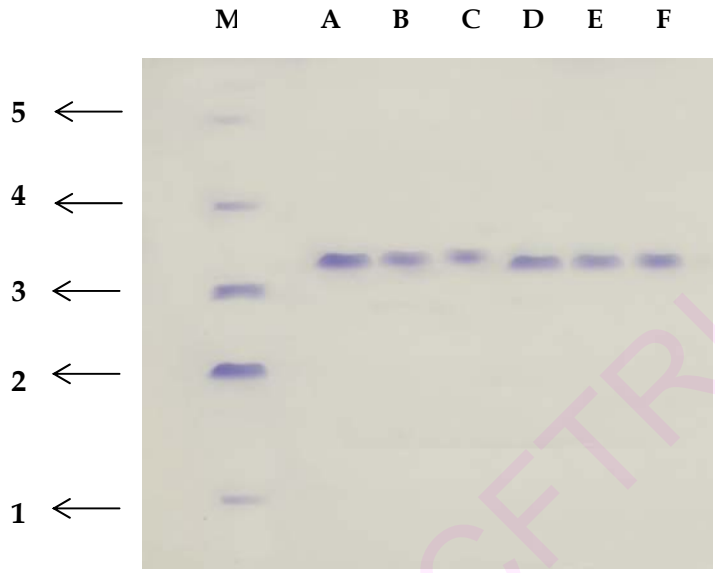


Fig. 50: SDS-PAGE of glycosylated and deglycosylated PPA, PPA-I and PPA-II - protein staining

Lanes M- Molecular weight marker:

1. *Lactoglobulin (18.4kDa)*
2. *Carbonic anhydrase (29 kDa)*
3. *Ovalbumin (43 kDa)*
4. *Bovine serum albumin (66 kDa)*
5. *Phosphorylase (97.4 kDa)*

Lanes: A-PPA; B-PPA-I; C-PPA-II; D-PPA,deglycosylated; E-PPA-I, deglycosylated; F-PPA-II, deglycosylated

III.C.4. Effect of temperature

The deglycosylated PPA and its isoforms were slightly less thermo-stable than their glycosylated forms (fig. 51 & 52). This is represented based on the calculation of the relative activity taking control as 100% activity.

PPA, PPA-I and PPA-II retained almost 100% activities up to 40°C and the activities decreased with increase in temperature. This indicated that the bound carbohydrates have a role in the thermal stability of the enzymes.

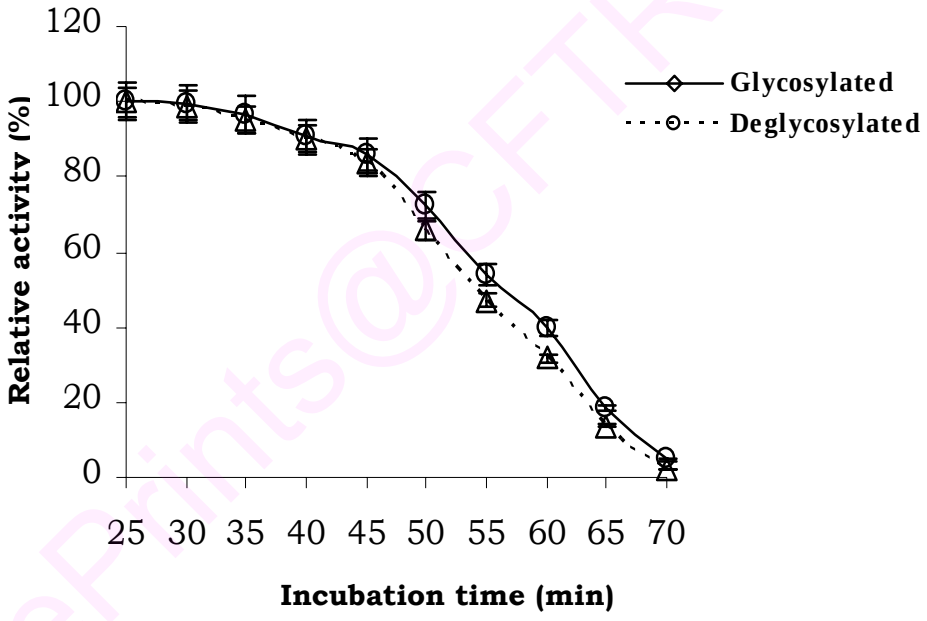


Fig.51: Effect of temperature on the activities of glycosylated and deglycosylated PPA

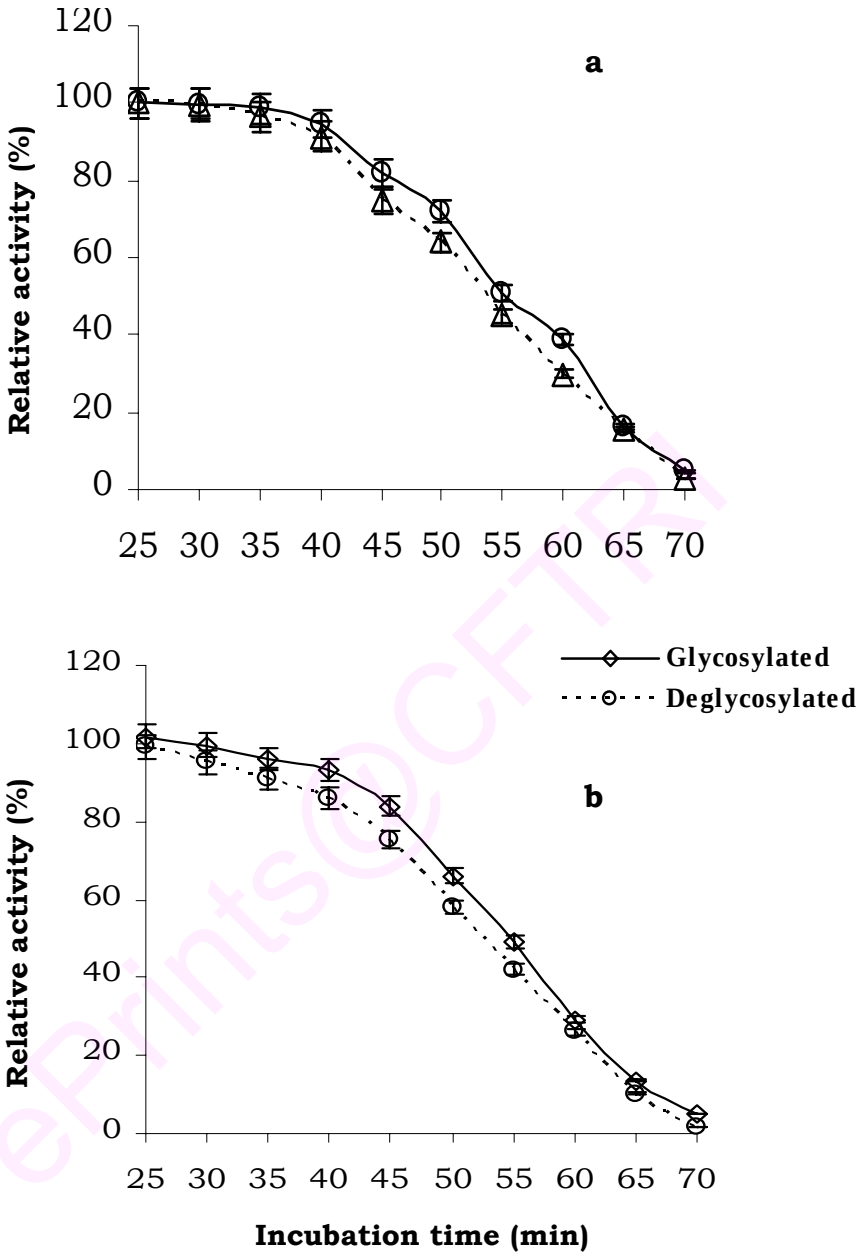


Fig. 52: Effect of temperature on the activities of glycosylated and deglycosylated a) PPA-I & b) PPA-II

Literature pertaining to the effect of deglycosylation show that the carbohydrate moiety in different enzymes offers different functionality. In some of the enzymes they are positively contributing to the stability and and in a few others their role is negligible. Chicken egg white ovatransferrin and chicken egg white avidin are thermally stable glycoprotein. Although they are less glycosylated, their resistance to glycosidase action is apparently higher than that of yeast external invertase and bovine serum fetuin. This is probably due to the inaccessibility of the glycosidic bonds which might be masked and buried under the protein surface [Wang, et al., 1996].

The loss of α-amylase activity during prolonged exposure at 60°C was identical for deglycosylated and control samples of α-amylase from *Aspergillus oryzae* [Eriksen, 1998]. The removal of the three N-glycosylation recognition sites (*NGRS*) significantly reduced the thermostability of recombinant peroxidase from peanut. A *NGRS* is a three amino acid sequence of asparagine to which the glycan chains are attached. The removal of one of the three recognition sites significantly reduced the thermostability and removal of the other two recognition sites also decreased the activity levels [Lige, et al. 2001]. The same observation was made with respect to rice α-amylase where removal of the single *NGRS* significantly decreased thermostability [Terashima, et al. 1994]. Yeast glucanases were glycosylated and enzymatic deglycosylation reduced their thermostability [Meldgaard, & Svendsen, 1994]. Deglycosylation of the recombinant phytase showed decrease in the thermostability of the enzyme [Han, & Lei, 1999].

Effects of carbohydrate chains on thermostability were also reported for glucoamylase which indicates the importance of the carbohydrate moiety for stabilization of the three-dimensional structure [Takegawa, et al., 1988].

The removal of *N*-linked carbohydrate from the preparations of Taka-amylase A and species of *Aspergillus* did not affect the α-amylase activity [Takahashi, et al.,1982; Chiba, et. al., 1993].

N-glycosylation occurs in the early stage and plays a central role in protein folding [Wang, 1996]. One possible way for the attached carbohydrate moiety to stabilize protein conformation is to form hydrogen bonds with the polypeptide backbone. Hecht, et al., [1993] in studying the crystal structure of glucose oxidase, have shown that the N-linked mannose residues form strong hydrogen bonds with the backbone nitrogen and the carbonyl oxygen of glutamic acid.

III.C.5. Purification of the released oligosaccharides

The oligosaccharides released from PPA and its isoforms by PNGase F were purified on Bio-Gel P-2. The sugar composition and molecular weights were established by GLC and ESI-MS respectively.

III.C.5.i. Gas Liquid Chromatography (GLC)

GLC analysis of the oligosaccharides released after PNGase F treatment of PPA and its isoforms indicated preponderantly mannose.

Plant glycoproteins usually have fucose residues linked to the proximal GlcNAc residues, while this is not a common feature in animal glycoproteins. The amount of fucose was relatively high in plant peroxidases [Sun, et al., 1997; Kim, & Kim, 1995; Tams, & Welinder, 1995].

III.C.5.ii. Electron spray ionization-mass spectrometry (ESI-MS)

ESI-MS of the oligosaccharide released from PPA and its isoforms showed the ionization at 365 and 709 m/z (fig. 53). Based on the GLC analysis and the mass determination by ESI-MS the nature of the oligosaccharides may be probably ManMan *(m/z 365)* and Man_3 Hex NAc *(m/z 709)* [Harvey, et al., 1997].

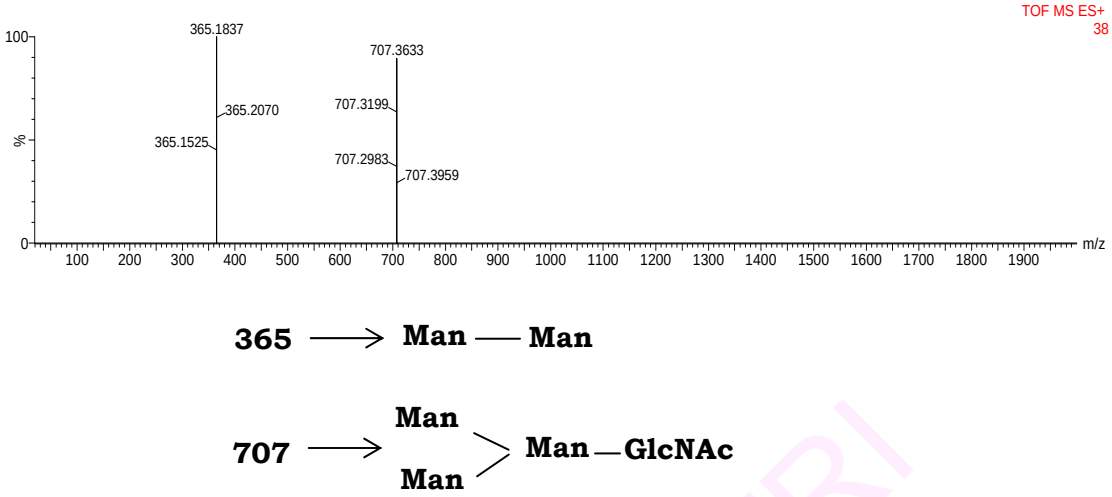


Fig. 53: ESI-MS spectra of the oligosaccharides released on deglycosylation by PNGase F

Among the modern spectroscopic techniques, electrospray ionization mass spectrometry (ESI-MS) has emerged as a powerful tool for the analysis of large and non-volatile molecules [Morelle, & Michalski, 2005].

The positive ion fragmentation spectra of the singly- and doubly-charged sodiated adducts from high-mannose, hybrid and complex N-linked glycans has been deduced [Harvey, 2000]. An acetyl group identified in the terminal peptide of porcine pancreatic amylase proves that the enzyme is an acetyl-protein [Cozzone, & Marchius-Mouren, 1970].

III.C.6. Spectral studies

III.C.6.i. Absorbance spectra

The absorption spectra of PPA and its isoforms are represented in fig. 54 & 55. The absorbance of PPA, PPA-I and PPA-II indicated an absorption maximum at 275 nm indicating the preponderance of aromatic amino acid residues such as tryptophan. The absorbance was slightly more in deglycosylated PPA and its

isoforms when compared to the glycosylated enzymes having taken the same concentration of the protein solution in buffer.

These absorption spectra are also sensitive to the local protein environment, but much less than the fluorescence spectra [Ken, et al., 2004].

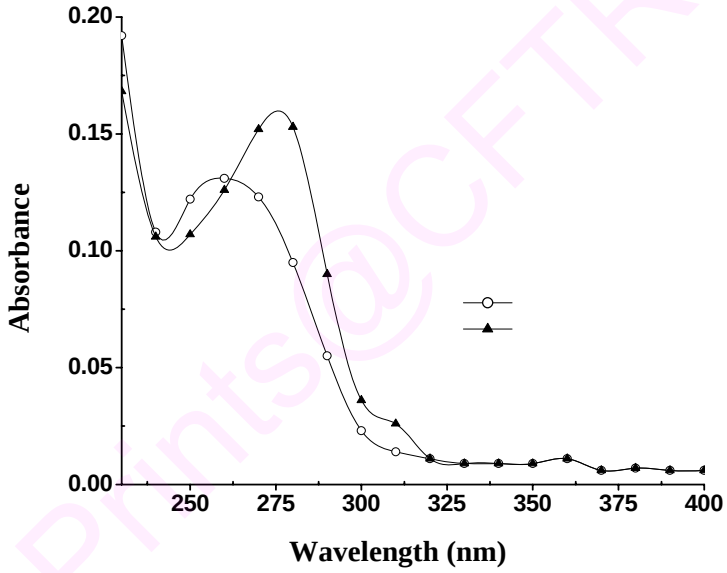


Fig. 54: Absorption spectra of glycosylated and deglycosylated PPA

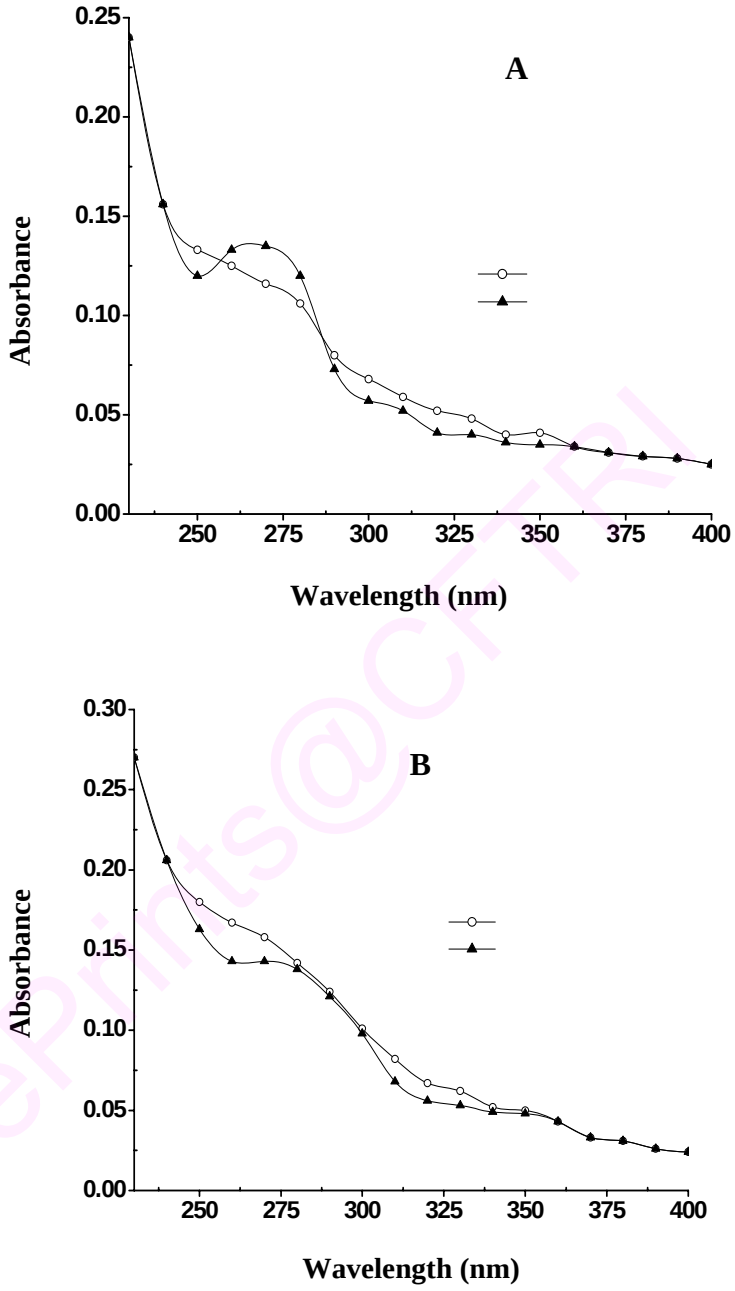
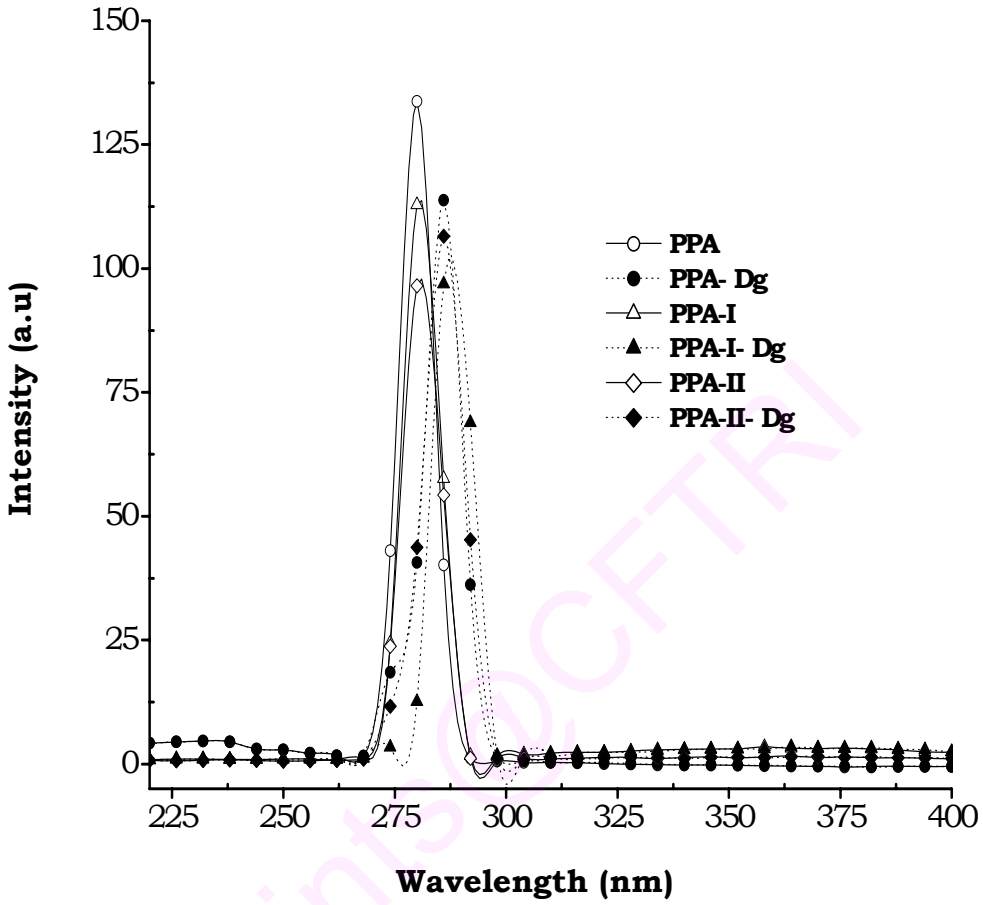


Fig. 55: Absorption spectra of glycosylated and deglycosylated (A) PPA-I and (B) PPA-II

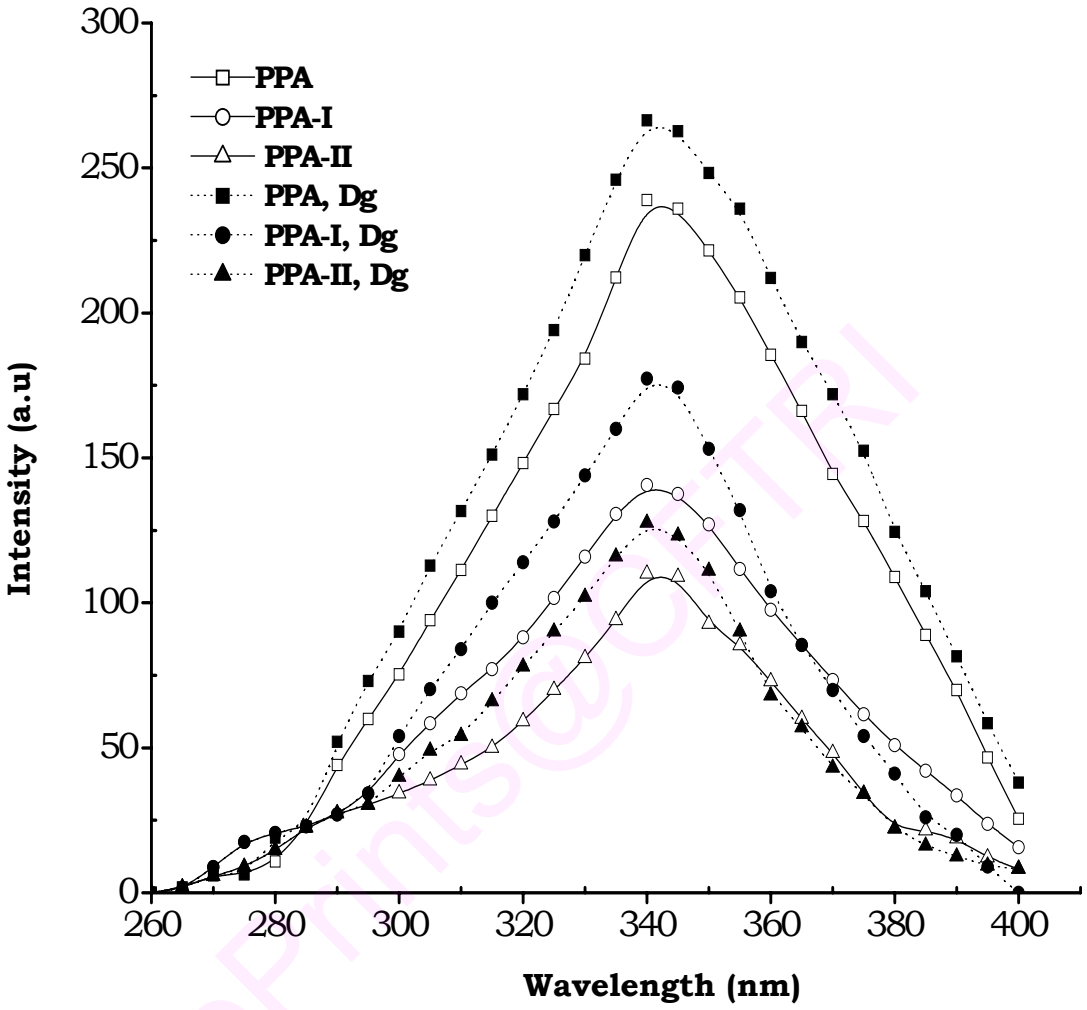
III.C.6.ii. Fluorescence spectra

The fluorescence excitation and emission spectra of glycosylated and deglycosylated PPA and its isoforms are presented in fig. 56 & 57. PPA and its isoforms had an emission maximum at 340 nm. This is typical of an exposed tryptophan residue in protein. Upon deglycosylation the tryptophan fluorescence of PPA, PPA-I and PPA-II gradually increased. The excitations of the glycosylated enzymes were observed at 280 nm and that of deglycosylated PPA and its isoforms were 286 nm. The excitation intensity of the deglycosylated PPA and its isoforms were higher than that of glycosylated enzymes. A slight shift in the excitation observed indicates that the Trp residues are in a fairly hydrophilic environment. The fluorescence spectra showed an increase in the fluorescence intensity. A small change in the relative fluorescence intensities for the glycosylated and deglycosylated enzymes were observed (+6 nm), indicating a slight change in tertiary structure of PPA and its isoforms.



Dg-deglycosylated

Fig. 56: Excitation spectra of glycosylated and deglycosylated PPA, PPA-I and PPA-II



Dg-deglycosylated

Fig. 57: Emission spectra of glycosylated and deglycosylated PPA, PPA-I and PPA-II

The emission maximum at 343 nm is an indication of the partial exposed nature of tryptophan in the native state. The excitation maximum of PPA and its isoforms after deglycosylation shifted by +6 nm from 280 to 286 nm (red shift). Red shift in λ_{max} may be due to deglycosylation.

The fluorescence of proteins originates from aromatic residues (phenylalanine, tyrosine and tryptophan). Porcine pancreatic α-amylase a 55 kDa protein with 19 tryptophan residues is a single polypeptide chain (496 residues) with five disulfide bonds [Tarentino, & Plummer, 1994]. All α-amylase structure bear multiple tryptophan residues more or less equally distributed over the whole structure. The several tryptophan residues in PPA are located at the surface and have more or less direct contact with the solvent when compared to other α-amylases where the tryptophan residues are buried in the interior of the protein [Tarentino, & Plummer, 1994]. The Trp-residues of α-amylase dominate the fluorescence emission at 340-345 nm [Duy, & Fitter, 2006]. As a consequence the observed emission spectra of individual proteins (and of their corresponding conformational states) do not vary with respect to λ_{max} but show different spectral shapes and widths [Burstein, et al., 2001; Reshetnyak, & Burstein, 2001]. It is well-known that for multi-tryptophan proteins the fluorescence emission spectra depend on the excitation wavelength and therefore may differ slightly. This generally occurs because the individual tryptophan residues are located in different local environments in the native state. The emission spectra measured with excitation wavelengths of 275 and 280 nm show a significantly larger intensity in the region below 330 nm, which most probably is caused by tyrosine fluorescence. Tyrosine fluorescence emission (λ_{max} 305 nm) is not observed in protein fluorescence because the spatial vicinity of tyrosine and tryptophan residues in the protein structure give rise to a distinct energy transfer from tyrosine to tryptophan [Fitter, & Haber-Pohlmeier, 2004]. A small change in the relative fluorescence intensities observed in the emission and excitation spectra

indicates a slight change in the tertiary structure of PPA and its isoforms.

Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today. Although fluorescence measurements do not provide detailed structural information, the technique has become quite popular because of its acute sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes [Royer, 1995]. The fluorescence parameters of tryptophan residues are sensitive to the micro-environment of fluorophore in protein structure [Burstein, et al., 2001]. Tryptophan fluorescence is widely used to study the location, physical and dynamic properties of micro-environment and the structural features of the protein molecules. The study of λ_{max} values of tryptophan fluorescence emission are meaningful parameter to characterize properties of the unfolded state of proteins [Duy, & Fitter, 2006]. The fluorescence properties of intrinsic tryptophan residues in proteins invariably change on denaturation of the protein. Proteins with heme prosthetic groups typically exhibit an increase in intensity on unfolding due to the loss of the heme group which quenches the tryptophan emission in the native state by Forster energy transfer [Grinvald, & Steinberg, 1976].

The average energy of the emission of the tryptophan residues usually shifts to red on unfolding because of the solvent exposure. The magnitude of the shift in the energy depends on the extent to which the proteins are buried in the native state. In the unfolded state most tryptophan residues in proteins have spectra similar to that of N-acetyl tryptophanamide in water with a maximum of ~355 nm [Royer, 1995].

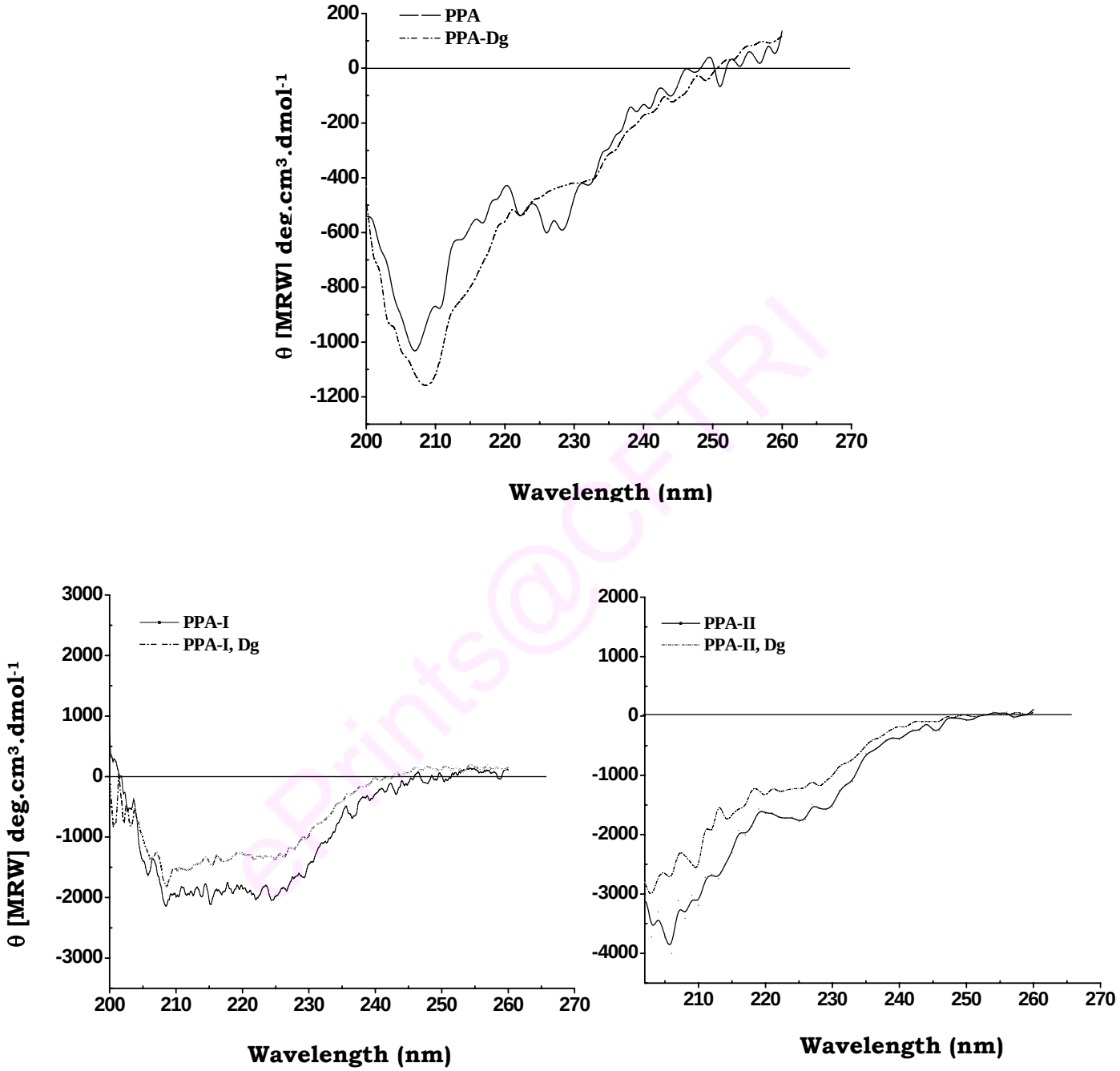
III.C.6.iii. CD spectra of PPA, PPA-I and PPA-II: Effect of deglycosylation

CD spectra of glycosylated and deglycosylated PPA and its isoforms were obtained in the far UV region (fig. 58). In the far-UV region (260–200 nm), minima were observed at 231, 225 and 208 nm for PPA whereas for

deglycosylated PPA the minima were observed at 229 and 211 nm. PPA-I and PPA-II showed the minima at 225 and 208 nm and the deglycosylated forms had lower ellipticity values. PPA-II had mostly random structure.

The influence of carbohydrate depletion on the secondary structure of glycoprotein were measured by CD spectra in the amide region at 25°C. Yeast external invertase and bovine serum fetuin showed the minimum at 218 and 208 nm respectively. The essential absence of the 222 nm transition in the spectra suggests that the protein contain a low amount of α-helical structure [Chang, et al., 1978]. Glucoamylase from *Aspergillus niger* had two minima at 209 and 220 nm. The thermal stability of a recombinant α-amylase from *Bacillus halmapalus* α-amylase (BHA) has been investigated using circular dichroism spectroscopy (CD) [Nielsen, et al., 2003].

Enzymatic deglycosylation of yeast invertase, bovine serum fetuin and glucoamylase from *Aspergillus niger* did not show any significant changes in secondary structure [Wang, et al., 1996]. Results from CD studies strongly suggest that although the carbohydrate moiety did not influence the conformation of the polypeptide backbone, its presence considerably enhanced protein conformational stability. This was probably due to hydrogen bonding with the polypeptide backbone [Wang, et al., 1996].



Dg-deglycosylated

Fig. 58: Far UV-CD spectra of glycosylated and deglycosylated PPA, PPA-I and PPA-II

Summary & Conclusions

Summary & conclusions

The main findings of the present investigation are summarized as follows:

- The morphological features of the native starches differed considerably with respect to their granule size as observed by SEM. The amylose content of ragi, rice, wheat and maize starches were 26.6, 25.1, 26.4 and 27.3% respectively. The gelatinization temperatures of ragi, rice, wheat and maize starches were 64.9, 60.7, 62.2 & 66.5°C respectively and the enthalpy of gelatinization were 11.9, 8.9, 10.4 and 13.4 J/g respectively for ragi, rice, wheat and maize starches as determined by DSC. **(Chapter-III, Section A: page no.'s: 57-63)**

- X-ray crystallography data of the starches showed typical A-type diffraction pattern with strong reflection at 15° and 23°. The degree of crystallinity of maize starch (41.4%) was maximum followed by ragi (37.3%), wheat (36.9%) and rice (35.8 %) starches. **(Chapter-III, Section A: page no.'s: 63 -65)**

- Neutral sugar composition of WSP indicated a higher ratio of arabinose to xylose in maize WSP followed by ragi, wheat and rice polysaccharides. Ferulic acid was the major bound phenolic acid identified in all the WSP along with small amounts of coumaric acid. **(Chapter-III, Section A: page no.'s: 68- 72)**

- The viscosities of water soluble NSP increased with increase in concentration (0.2 to 1.0%) and decreased with increase in temperature (20 to 70°C). **(Chapter-III, Section A: page no.'s: 72-74)**

- Pancreatic alpha amylase (PPA) was separated into two activity peaks i.e. PPA-I and PPA-II eluted at 0.25 M and 0.34 M NaCl concentrations respectively by DEAE-cellulose anion exchange chromatography. The % recovery of PPA-I and PPA-II were found to be 67.9 and 65.5% with a fold purification of 1.7 and 1.8 respectively. The molecular weight of PPA-I and

PPA-II were found to be 55.1 kDa as determined by SDS-PAGE. The molecular weights of PPA, PPA-I and PPA-II as analyzed by ESI-MS were 55.6, 55.0 and 54.9 kDa respectively. **(Chapter-III, Section B: page no.'s: 76- 84)**

- The oligosaccharides obtained from cereal/millet starches by the digestion of PPA and its isoforms differed quantitatively with respect to the amount of maltose, maltotriose and maltotetraose upon digestion by PPA and its isoforms. **(Chapter-III, Section B: page no.'s: 85 -90)**

- pH and temperature optima of PPA, PPA-I and PPA-II were 6.9 and 45°C respectively. PPA was found to be stable in the pH range of 7.0-8.0 where as PPA-I and PPA-II were stable in the pH range of 6.0-8.0 retaining 80% of their respective activities after 4h of incubation. All of them were completely stable upto 45°C. **(Chapter-III, Section B: page no.'s: 91- 102)**

- Higher K_m values for maize starch as determined by the Lineweaver double reciprocal plot substantiated its lowest digestibility among all the starches digested by PPA and its isoforms. **(Chapter-III, Section B: page no.'s: 103 - 107)**

- Ca^{2+}, Ba^{2+}, Co^{2+} and Mg^{2+} were found to have both activating and stabilizing effects on PPA and its isoforms. Al^{3+} and Hg^{2+} completely inactivated PPA and its isoforms. Carboxylic acids such as citric acid and oxalic acid inhibited PPA and its isoforms in concentration dependent manner. EDTA (125 μm) completely inhibited the activities of PPA and its isoforms. **(Chapter-III, Section B: page no.'s: 108- 112)**

- The WSP from ragi, rice, wheat and maize reduced the activities (non specific inhibition) of PPA and its isoforms. The reduction in activity was maximum by maize polysaccharides followed by ragi, rice and wheat WSP's. This may

be attributed to the higher viscosity of the maize polysaccharides. **(Chapter-III, Section B: page no.'s: 117 - 119)**

- PPA and its isoforms were confirmed to be glycoproteins by colorimetric assay and PAS staining method and the carbohydrate content of PPA, PPA-I and PPA-II were found to be 0.05, 0.006 and 0.004 (mol per 1 mol of enzyme) respectively. **(Chapter-III, Section C: page no.'s : 121 - 123)**

- The complete deglycosylation of PPA and its isoforms was observed after 24 h of treatment with PNGase F. The complete removal of carbohydrate was confirmed by PAS staining. SDS-PAGE of deglycosylated PPA and its isoforms showed very little change in their relative mobility. The thermal stabilities of deglycosylated PPA and its isoforms were slightly less than their glycosylated counter parts. **(Chapter-III, Section C: page no.'s : 124 -130)**

- The oligosaccharide released by PNGase F digestion of PPA and its isoforms were found to be high mannose type. ESI-MS of the oligosaccharides released by PPA and its isoforms after PNGase F treatment showed the ionization at 365 and 709 m/z and the nature of the oligosaccharides may be probably ManMan *(m/z 365)* and Man_3 HexNAc *(m/z 709)* types. **(Chapter-III, Section C: page no.'s : 130&131)**

- The absorbance of PPA, PPA-I and PPA-II indicated absorption maxima at 275 nm indicating the preponderance of aromatic amino acid residues such as tryptophan. PPA and its isoforms had an emission maximum at 340 nm and the excitation of the glycosylated and deglycosylated enzymes were observed at 280 nm and 286 nm respectively. The fluorescence spectra showed an increase in the fluorescence intensity as observed in the emission spectra for the deglycosylated enzymes and showed a red shift in the excitation spectra. **(Chapter-III, Section C: page no.'s : 131 - 138)**

- CD studies showed that in the far-UV region (260–200 nm) minima were observed at 231, 236 and 208 nm for PPA whereas for deglycosylated PPA the minima were observed at 229 and 211 nm. PPA-I and PPA-II showed the minima at 225 and 208 nm and the deglycosylated forms had lower ellipticity values. PPA-II had mostly random structure. **(Chapter-III, Section C: page no.'s : 138 -140)**

Conclusions

- Digestion of ragi, rice, wheat and maize starches by PPA and its isoforms released qualitatively same sugars, i.e maltose, maltotriose and maltotetraose. However, they differed quantitatively as indicated by HPLC analysis.

- The two activity peaks of PPA namely, PPA-I and PPA-II were found to be isoforms as determined by SDS-PAGE, gel filtration and ESI-MS.

- Water soluble polysaccharides isolated from the flours of cereals showed nonspecific inhibition of PPA and its isoforms (visible reduction in enzyme activity) at 1% concentration.

- The glycoprotein nature of PPA and its isoforms was determined and the effect of deglycosylation as determined by UV, fluorescence and CD studies revealed little effect on their activity and thermal stability.

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PUBLICATIONS

List of Publications and Posters Presented In National/International Symposia

Manuscripts from the Ph.D thesis

1) Anitha Gopal. B. & Muralikrishna Gudipati. "*Porcine pancreatic α-amylase and its isoforms: purification and kinetic studies*" **- accepted for publication in *International Journal of Food Properties*. (In press).**

2) Anitha Gopal. B. & Muralikrishna Gudipati. "*Physico-chemical characteristics of native and pancreatic alpha amylase digested cereal and millet starches*" **accepted for publication in *Journal of Food Science and Technology*. (In press).**

3) Anitha Gopal. B., Sridevi A. Singh & G. Muralikrishna. "*Porcine pancreatic alpha amylase and its isoforms - effect of deglycosylation by peptide-N-glycosidase F*"**- published online: April-2008 in *International Journal of Biological Macromolecules* (In press)**.

Posters presented in National/ International Symposia

1) Anitha Gopal. B. & G Muralikrishna. "*A comparative study of cereal/millet starch digestibility using pancreatic α-amylase*". Presented in colloquium on Novel Proteins in Nutrition and Health organized by the Solae Company and Central Food Technological Research Institute (C.F.T.R.I.), Mysore, held at C.F.T.R.I., Mysore, **22nd March 2005**.

2) Anitha Gopal. B. & G Muralikrishna. "*Cereal starch digestibility using porcine pancreatic α-amylase and its isoforms*"- Presented in CARBO-XXI, held at Delhi University, New Delhi, **26-29th November 2006**.

3) Anitha Gopal. B., Sridevi A. Singh & G. Muralikrishna. "*Fluorimetric and circular dichroism studies of porcine pancreatic alpha amylase and its isoforms*". Presented in 77th annual session and symposium of the National Academy of Sciences India (NASI), held at C.F.T.R.I., Mysore, **6-8th December 2007**.