SELECTIVE DEBRANCHING OF GUAR GALACTOMANNAN BY STRUCTURALLY UNRELATED ENZYMES FOR IMPROVED FUNCTIONAL PROPERTIES

A thesis submitted to the department of

BIOTECHNOLOGY

of

University of Mysore

in fulfillment of the requirements for the degree of

Doctor of Philosophy

by

SHOBHA, M.S., M.Sc.

Under the supervision of

Dr. R.N.Tharanathan, M.Sc., Ph.D.

Department of Biochemistry and Nutrition

Central Food Technological Research Institute

Mysore - 570 020, India

April - 2009

.....Dedicated to my beloved grandmother

Acknowledgements

It is a feeling of justifiable pleasure that I wish to express my deep sense of gratitude and indebtedness to my research mentor **Dr. R.N. Tharanathan** for suggesting me the thesis topic and for his valuable, inspiring guidance, constant encouragement and concern throughout my research work. I will always cherish the academic freedom and friendly atmosphere that I enjoyed in his laboratory.

I wish to express my sincere thanks to Dr. V. Prakash, Director, CFTRI, for providing me an opportunity to work in this institute and permitting me to submit the results in the form of a thesis for the award of a Ph.D. degree. I am grateful to Dr. P.V. Salimath, Head, Department of Biochemistry and Nutrition, CFTRI, for providing the laboratory facilities and encouragement.

I am extremely thankful to Dr. G. Muralikrishna for his invaluable help and support. I am grateful to Dr. Lalitha R. Gowda for the guidance and help rendered during protein studies. Sincere thanks are due to Dr. A.S. Bawa, Director, DFRL, Mysore and DR. Anil Semwal, DFRL, Mysore, for DSC analysis. I also thank the Head, NMR Research Centre and Proteomic facility, IISc, Bangalore. I am extremely thankful to all the staff of our department for making my stay here fruitful and memorable. I appreciate the help and support of my seniors Dr. P.C. Srinivasa, Dr. K.V. Harish Prashanth and Dr. A.B. Vishu Kumar, labmates Mr. Puneeth Kumar, Mrs. Savitha, Mrs. Hemalatha, Mr. Revenappa and Mr. Madhu Kumar. I extend a warm gratitude to my friends Anitha, Smitha, Rajini, Chithra, Reena, Usha Prakash, Shubra, Sreenath, Sathish, Ayyappan and other well wishers in the department and in the institute.

My special thanks to dearest friends Shashi, Ashwini, Rashmi R and Rashmi N. Also I wish to thank Mrs Lalitha and Mr. Nagabushan and their family for constant support and help. My thanks to Mr. Ashwini Shankar and Mr. Raghava Simha for their technical support.

I am indebted to my family for sharing my dreams and being supportive all through. Sincere thanks are also due to my beloved Parents, Aunty, Sister and Brother

for the motivation and moral support. I would also like to thank my all other family members for their timely help and constant encouragement.

It is a privilege to thank all my esteemed teachers who were the source of inspiration during my school and college days.

Finally, I gratefully acknowledge the financial assistance in the form of Senior Research Fellowship (SRF) from the Council of Scientific and Industrial Research (CSIR), New Delhi.

Shobha, M.S.

DECLARATION

I hereby declare that the thesis entitled **"Selective debranching of guar galactomannan by structurally unrelated enzymes for improved functional properties"** submitted to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology,** is the result of research work carried out by me under the guidance of **Dr.R.N.Tharanathan**, Additional Director (Retd.), Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore - 570 020, India, during the period April 2006 - March 2009. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date: **(Shobha M.S.)**

Place: Mysore – 570 020

Head / B & N

Dr.R.N. Tharanathan, M.Sc., Ph.D.
Additional Director (Retd.)
Department of Biochemistry and Nutrition
CFTRI, Mysore - 570 020.
E-mail: tharanathan@yahoo.co.uk

Res.: 2941/1A, 'Sangeet'
S.N.S. Temple Street
Chamundipuram
Mysore - 570 004.

CERTIFICATE

I certify that the thesis entitled **"Selective debranching of guar galactomannan by structurally unrelated enzymes for improved functional properties"** submitted to the **University of Mysore** by **Ms. Shobha, M.S.,** for the award of **Doctor of Philosophy** in **Biotechnology** is the result of research work carried out by her in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore – 570 020, under my guidance during the period April 2006- March 2009.

Date: **(Dr.R.N.Tharanathan)**

Place: Mysore – 570 020 **Guide**

Contents

List of Illustrations

List of Tables

Abbreviations

^{o}C	Degree centrigrade
µL	Microlitre
µmole	Micro moles
A	Absorbance
Å	Angstrom
APS	Ammonium persulfate
cm	Centimetre
CrI	Crystallinity index
CZE	Capillary zone electrophoresis
CBB	Coomassie brilliant blue
DP	Degree of polymerization
Da	Daltons
EDAC	N- (3-methyl amino propyl) N'-ethylcarbodiimide
FT-IR	Fourier transform infra-red
GG	Guar galactomannan
g	Grams
GPC	Gel permeation chromatography
GME	Glycine methyl ester
HPLC	High performance liquid chromatography
hr	Hour
K_i	Inhibitory constant
kDa	kilo Dalton
LBG	Locust bean gum
M	Molar concentration
mg	Milligrams
min	Minutes
mL	Millilitre
Mw	Molecular weight
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of flight
nm	Nanometre
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein data bank
R_p	Reverse phase
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
TEMED	N,N,N'N'-Tetramethyl 1, 2-diaminoethane
TFA	Trifluoroacetic acid
TCA	Trichloroacetic acid
Vol.	Volume
w/v	Weight/volume
w/w	Weight/weight
v/v	Volume/volume
V_o	Void

Synopsis

Synopsis

Carbohydrates are the major representatives of naturally abundant bio-resources and undoubtedly they are the renewable raw materials of high (bio) – technological value. In the form of available (starch) and unavailable (dietary fiber) carbohydrates, they contribute a major portion of our diet, thereby providing a ready source of energy reserve and offer other beneficial physiological effects too. Galactomannans are one such complex carbohydrates which find importance due to their beneficial properties in food and non-food applications.

Galactomannans are reserve polysaccharides of plant origin (mostly *Leguminosae*) present in the endosperm portion of seeds. Constituting a group of commercially important hydrocolloids, Galactomannans, contain a 1,4-linked β-D-mannan backbone to which are attached D-galactopyranosyl residues α-1,6-linked as side chain branches. Fenugreek, guar gum, tara gum and locust bean gum are the classical galactomannan sources with varying galactose to mannose ratios of approximately 1:1, 1:2, 1:3, 1:4, respectively. Of these, guar galactomannan, the most sought-after hydrocolloid, exhibits high viscosity in aqueous solution, is non-ionic, and easily available in large quantities. It finds considerable use in various food and non-food industries like pharmaceutical, textile, printing, drilling, mining, explosives, etc.

In the food industry guar galactomannan is extensively used as a stabilizer in a variety of applications including ice cream, baked foods, soups, etc. It is also used as a thickener, syneresis control agent and as a formulation aid. The functional properties of guar galactomannan in controlling the release of drugs in the gastrointestinal tract for colon targeted drugs, anticancer drugs in the treatment of colorectal cancer, oral rehydration solutions in the

treatment of cholera and also as a visco-supplementation agent in osteoarthritis treatment are of pharmaceutical importance. As a source of water soluble fiber guar galactomannan serves as a fermentable substrate for the microflora normally present in GI tract. In addition, its other physiological properties are as hypocholesterolemic, hypolipidemic and hypoglycemic agent.

Although it has numerous applications, additional uses of guar galactomannan are limited due to its high branching, high viscosity and high molecular weight. Though expensive, locust bean gum (LBG) continues to enjoy a better and specific usage because of its less branching (with galactose), which facilitates synergistic interaction with xanthan and other hydrocolloid gums forming thermo-reversible gels of use in specific food products. Nevertheless, innumerable attempts have been made in several labs all over the world with little success to convert guar galactomannan to LBG-type, by debranching using specific enzymes (α-galactosidase) from various sources like germinating guar seeds, coffee bean, etc., which resulted in the removal of a few galactose residues. As the production of α-galactosidase in pure form involves laborious steps, which is non-economical, a search for alternative sources of enzymes which are easily available and inexpensive was made.

Our earlier studies have shown that several common enzymes such as pectinase, pepsin, papain, pronase, lysozyme, hemicellulase and lipase are involved (non-specifically) in the depolmerization of completely unrelated substrates, viz. chitosan. Sequel to this, a few of these enzymes were screened for any catalytic/debranching activities towards guar galactomannan. Infact, preliminary data indicated very encouraging results that prompted us to go further for detailed investigations. The main objective of the present study was to produce

modified guar galactomannan by selective enzymatic debranching, which mimics LBG in several of its rheological properties. Another objective of this study was to elucidate the mechanism for such non-specificity of pepsin in the removal of side chain galactose residues of guar galactomannan. Accordingly, the following work plan was envisaged to fulfill these two objectives.

a. Screening of enzymes such as pectinase, pullulanase, pepsin, pronase, lipase and cellulase for depolymerization and debranching activities on guar galactomannan.

b. To study the Gal-Man ratio, monomer sequence, viscosity and gelling characteristics of the modified guar galactomannan.

c. To study kinetic parameters and to elucidate the mechanism of action by pepsin in its catalytic activity towards guar galactomannan, and

d. To compare and contrast the rheological behaviour of modified guar galactomannan with those of LBG.

The results thus obtained have been consolidated in the form of a thesis entitled **"Selective debranching of guar galactomannan by structurally unrelated enzymes for improved functional properties"**, which has the following layout.

Introduction

It gives a brief introduction about the history, occurrence, structure, biosynthesis and application of native and modified galactomannans with a focus on their importance in various food and

non-food industries. The scope and objectives of the present investigation are also put forth in this chapter.

Chapter I: Screening of enzymes for (non-specific) activity towards GG

After a brief introduction about the non-specificity of some of the enzymes in catalyzing completely unrelated substrates, in comparison to their specific substrates, some information about the characterization of modified guar galactomannan (viz. molecular weight, GPC/HPSEC, viscosity and G-M ratio by GLC) is provided. Native guar galactomannan showed a molecular weight of ~240 kDa with a viscosity of 4800 cps and G-M ratio 1:2. Preliminary screening studies revealed pullulanase, pepsin and pectinase exhibiting considerable (non-specific) activity, particularly in selective debranching of side chain galactose residues of guar galactomannan.

Chapter II: Purification and kinetic studies of a few selected enzymes having debranching activity towards GG

It provides a detailed account on the purification and kinetic parameters of pullulanase, pepsin and pectinase, which showed considerable debranching activity towards guar galactomannan. The purity of these enzymes was established by PAGE (native and SDS), capillary zone electrophoresis and HPLC, in order to rule out the possibility of minor contaminants, if any, involved in the observed non-specificity. Native PAGE and SDS-PAGE of the purified fraction corresponded to the major band of crude enzymes. Studies on the effect of enzyme-substrate concentration obeyed Michaelis-Menton kinetics, showing an increase in the rate of catalysis to be directly proportional to enzyme concentration. The three enzymes showed

maximum debranching activity at pH 4.5, 5.5, 4.0 and 40^{o}C, 45^{o}C, 50^{o}C, respectively with K_m (substrate concentration at half maximal velocity) and V_{max} (maximum velocity) of 6.0, 5.0, 4.2 mg mL^{-1} and 1820± 8.8, 2790± 32.8, 3120± 37.11 nmoles min^{-1} mg^{-1} protein as calculated from Lineweaver – Burk (double reciprocal) plot.

Chapter III: Production of galactose-depleted guar galactomannan (GDGG) and characterization of resultant products

It gives a detailed account on the production and structural characterization of GDGG. GPC, viscometry and HPLC studies showed a decrease (~152 kDa) in the M_w of GDGG, which appeared as a single peak confirming its homogeneity. Its GLC analysis revealed a significant change in G-M ratio of 1:3.8, 1:3.6, 1:3.0, respectively, mimicking that of LBG. FTIR and solid-state CP- MASS ^{13}C-NMR analyses indicated subtle changes in the conformation of GDGG due to the removal of galactose residues. The nature of oligomers and monomers released as a result of enzyme catalysis was deduced by GPC and HPLC analyses. The G-M ratio and sequence analysis of the oligomers were deduced based on MALDI-TOF-MS data. Upon treatment with α-galactosidase and β-mannanase an increase in the reducing equivalents was observed. HPLC and ESI-MS profile of the oligosaccharides released upon hydrolysis of GDGG by β-mannanase provided additional structural features.

Chapter IV: Rheology of GDGG on co-gelation with other polysaccharides

The viscoelastic behaviour of GDGG on admixture with xanthan and κ-carrageenan was studied by rheometric analysis. A two fold increase in the magnitude of elasticity indicated a synergistic

interaction, with the formation of a three dimensional network. The blend of GDGG with xanthan (1% total biopolymer) and κ-carrageenan (0.8% total biopolymer) in 1:1 (w/w) ratio showed a maximum increase in magnitude with high yield stress. DSC analysis revealed maximum synergistic interaction with xanthan and κ-carrageenan at a temperature of 78^{o}C and 48^{o}C, respectively. Textural analysis indicated an improvement in its textural characteristics viz. hardness, cohesiveness, adhesiveness and gumminess, comparable to those of LBG.

Chapter V: Mode of action of pepsin in debranching of GG

This chapter provides information about the mode of action of pepsin on debranching of guar galactomannan. Zymogram analysis of the purified enzyme revealed the association of both proteolytic as well as debranching activities, confirming the dual catalytic activity of pepsin. Use of site-specific inhibitors and chemical modification agents showed the involvement of aspartic acid residue at the active site. Also, the peptide mapping of native and guar galactomannan-bound pepsin on proteolytic digestion with Glu-C V8 protease further confirmed the involvement of aspartic acid residue in the catalysis of guar galactomannan. Based on these results, a probable mechanism of action of pepsin has been proposed.

A brief account of **Summary** and **Conclusions** followed by a collective list of **References** (Bibliography) which forms the basis for interpretation of data obtained is given at the end.

Introduction

Utilization of plant products by mankind for their survival has been marked from the beginning of civilization. Accumulation of large amount of compounds which are mobilised during plant development and the resulting products formed are used for energy generation and production of vital ingredients (protein, nucleic acids, carbohydrates and lipids) for building cells and tissues (Mayer & Mayber, 1975). Carbohydrate polymers, also known as polysaccharides are one such compound stored by plants.

Polysaccharides, also known as Cinderella of biopolymers, are the most abundant organic polymers comprising about two-thirds of the dry weight of the total plant biomass. An awareness in using natural biopolymers for diversified applications in life science is increasing due to their diverse functionality (Tharanathan, 2002; Tharanathan, 2003). In recent years the application potential of polysaccharides has gained considerable interest academically and industrially due to their renewable, non-toxic, biodegradable features which make them natural fit for sustainable development (Srinivasa & Tharanathan, 2007).

Polysaccharides, formed by the condensation of monosaccharides via glycosidic bonds may be linear or branched in structure, and they perform two major biological functions (see Table 1) viz. as energy reservoirs and as structural elements. Structural polysaccharides namely cellulose, chitin, pectin and hemicelluloses, present in cell walls, seed coat, and intercellular spaces, are mainly responsible for structural integrity of plant tissues. Whereas, the reserve polysaccharides formed during intense photosynthetic activity enter into the energy generating metabolism involving rapid release of ATP and also producing carbon for the biosynthesis of other biomolecules in plants (Buckeridge *et al.*, 2000).

Table 1. Classification of polysaccharides based on function

		Function
Structural polysaccharides	Cellulose, Pectin, Hemicellulose, Chitin	Structural integrity
Storage polysaccharides	Starch, Fructans, Galactomannans	Energy reserve, Osmoprotectant

Among the various types of reserve polysaccharides in plants, starch, the most abundant and widely distributed from lower microalgae to higher plants, constitutes the major portion of our diet. Although starch is the principle and economically important polysaccharide, plants are not devoid of other multifunctional cell wall storage polysaccharides performing various functions like hardness, osmoprotectant and cell expansion (Tharanathan *et al.*, 1987). One such major multifunctional cell wall storage polysaccharide, best characterized with significant emphasis on their economic importance is galactomannans. Galactomannans usually do not co-exist with starch granules, but are present in the seeds rich in oligosaccharides of the raffinose family.

Galactomannans

Galactomannans are highly viscous water-soluble heteropolysaccharide mainly localized in the endospermic seeds of various plants belonging to the family *Leguminoseae* (Daniel *et al.*,

1994). These polysaccharides, also known as gums, were used as a substance for mummification during 3000 B.C. in ancient Egypt, hence, they are also known as pharaoh's polysaccharides (Rees, 1972). Galactomannans generally share a relatively simple structure consisting of a linear (1,4)-β-linked- D-mannopyranosyl backbone attached with single stubs of α-1,6-D-galactopyranosyl residues as side chain (see Fig.1). They differ in mannose (M) to galactose (G) ratio (ranging from 1:1 to 1:4) depending on their botanical origin (Meier & Reid, 1982) which is genetically controlled (Matheson, 1990) and also of taxonomical significance (Bailey, 1971). Aspinall (1959) defines galactomannans as mannans that contain more than 5% galactose.

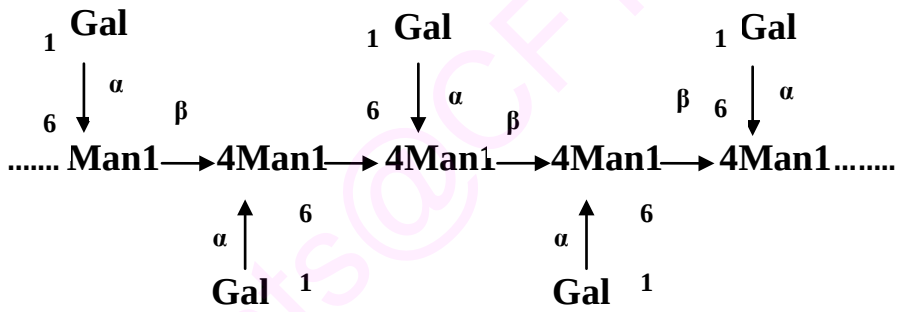


Fig. 1. General structure of galactomannan

Occurrence

Though the distribution of galactomannans is limited in the plant kingdom, the *Leguminosae* family serves as the rich source of galactomannans, particularly distributed in the endosperm of seeds. The distribution of galactomannan in *Leguminoseae* not only reflects the systematics but also the evolution pattern of the family (Buckeridge, 1995). Nearly 70 species of leguminous plants containing galactomannans have been reported (Dea & Morrison, 1975).Within

Leguminosae the plants of sub-family *Caesalpinioideae* contain a high level of galactomannans (15-38% of the dry weight of seed, Leschziner & Cerezo, 1970) followed by *Mimoseae, Crotalarieae, Trifoleae, Astragaleae* with 15-25% and *Glycineae, Loteae, Genisteae* having only 1-15% of polysaccharides (Dey, 1978). In relation to other two subfamilies *Caesalpinioideae* also represents the highest G/M ratio with the species *Ceratoniinae* showing high yield and relatively debranched galactomannans preserved during evolution. Table 2 presents the distribution of galactomannan with their G/M ratio in various species of *Leguminosae*.

Table 2. Leguminous plants containing galactomannan

Subfamily	*Species*	G/M ratio
Caesalpiniacae	*Cassia absus*	3 : 1
	Cassia fistula	3 : 1
	Cassia occidentalis	3 : 1
	Ceratonia siliqua	4 : 1
	Caesalpinia pulcherima	2.7 : 1
	Caesalpinia spinosa	3 : 1
	Delonix regia	4.2 : 1
	Parkinsonia aculeata	2.7 : 1
	Gleditsia amorphoides	2.5 : 1
	Gleditsia triacanthos	3.2 : 1
	Cyamopsis tetragonaloba	2 : 1
Mimosaceae	*Besmanthus illinoensis*	2.6 : 1
	Leucaena galauca	1.3 : 1

Apart from *Leguminosae* species the existence of galactomannans has been reported in members of *Annonaceae, Convolvulacea, Palmae, Ebenaceae* and *Loganiaceae.* Also an exception to the occurrence of galactomannan, other than in seeds, has been reported in glycine max, where it occurs in hull (Aspinall & Whyte, 1964), in *Gymnocladus diocia* - in the innerside of seed coat and in Mucuna – in the kernel (Larson & Smith, 1955). Table 3 reveals the list of various non-leguminous plants containing galactomannan.

Table 3. Non - leguminous plants containing galactomannan

Family	Species	G/M ratio
Annonaceae	*Annona muricata*	4.4 : 1
Convolvulaceae	*Convolvulus tricolor*	1.7 : 1
	Ipomoea muricata	1.8 : 1
Ebenaceae	*Diospyros virginiana*	4 : 1
Loganiaceae	*Strychnos nux-vomica*	2.7 : 1
Palmae	*Borassus flabellifer*	2.4 : 1
	Cocos mucifera	2.5 : 1
	Arenga saccharifera	2.2 : 1
	Phytelephas macrocarpa	2.5 : 1
	Hyphaene thebaica	3.2 : 1
	Phoenix dactylifera	2 : 1

Biosynthesis

Biosynthesis of galactomannan is a major metabolic activity followed during certain stages of seed development in the endospermic seeds of legumes. The mechanism and regulation of galactomannan biosynthesis in developing seed endosperm have been studied in comparison to the three leguminous plants, fenugreek (*Trigonella foenum-graecum*), guar (*Cyamopsis tetragonoloba*) and seenu (*Senna occidentalis*) (Edward *et al.*, 1992; Reid & Edwards, 1995). Campbell & Reid (1982) were the first to initiate studies on the enzymatic pathway of galactomannan biosynthesis by demonstrating a correlation between galactomannan deposition in fenugreek and the enzyme activity in the endosperm extract. Biosynthesis of galactomannan involves two Golgi membrane bound sugar nucleotide dependent glycosyltransferases namely mannansynthase and galactomannan galactosyltransferase which together catalyze the polymerization of galactomannan (Edward *et al.*, 1989).

Fig. 2 represents the outline of metabolic steps during the biosynthesis of galactomannans which accumulate in the cell wall of endosperm (Scheme taken from Naoumkina *et al.*, 2007). The accumulation of galactomannan in the cell wall of endosperm comprises up to 26 - 32% of seed dry weight (Kays *et al.*, 2006). Sucrose, a disaccharide acts as a building block for the galactomannan biosynthesis. The enzyme invertase (a hydrolase) brings about the catalysis of sucrose into glucose and fructose units whereas sucrose synthase, a glycosyl transferase in the presence of UDP accompanies the conversion of sucrose into UDP-glucose and fructose (Sturum & Tang, 1999).

The resulting glucose and fructose in the presence of hexokinase gets converted into glucose-6-phosphate and fructose-6-phosphate.

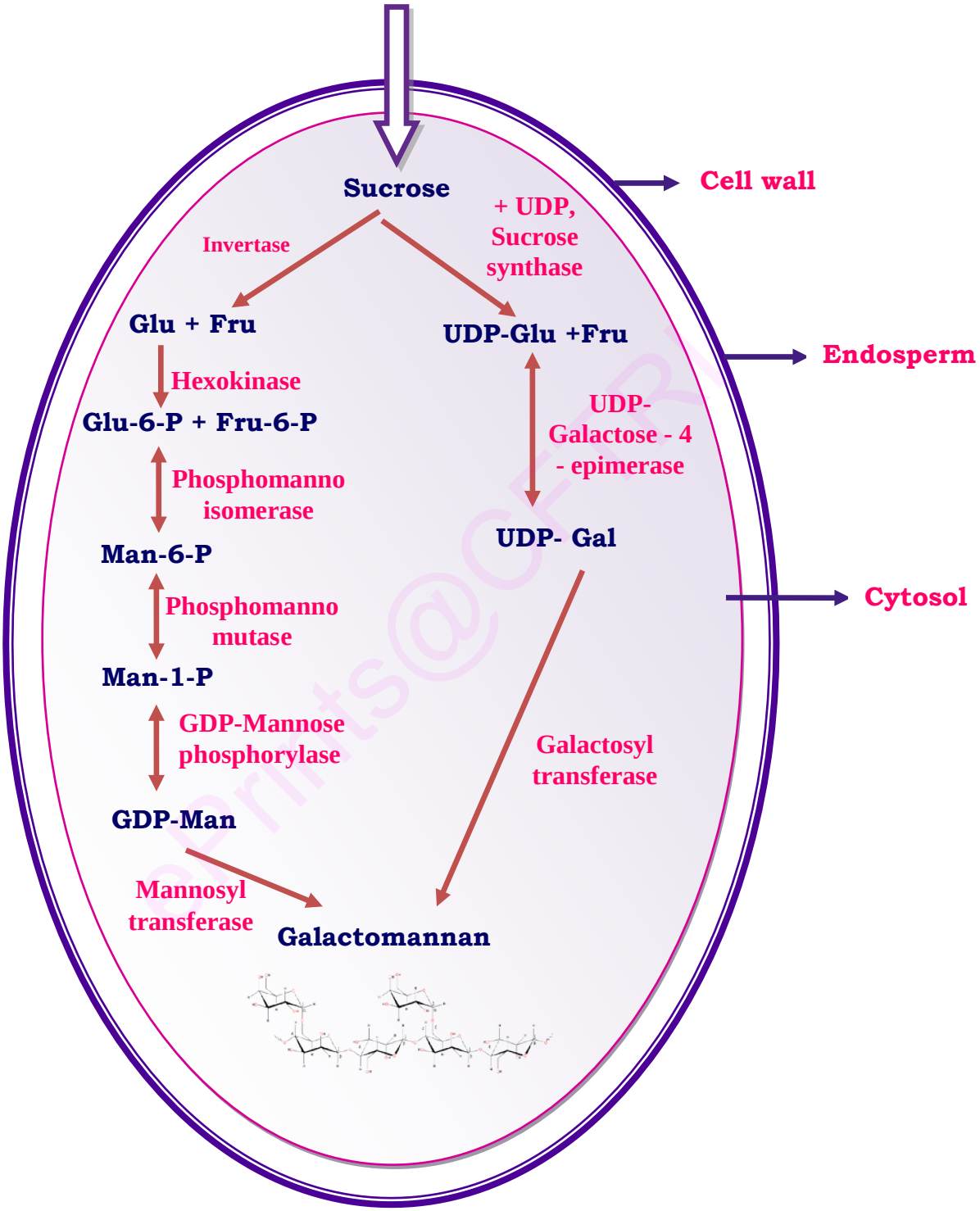


Fig. 2. Galactomannan biosynthesis in the endosperm (Scheme taken from Naoumkina *et al.*, 2007)

Further, the enzymes phosphomannoisomerase and phosphomannomutase convert fructose-6-phosphate into mannose - 6-phosphate and α-D-mannose 1-phosphate, respectively (Lee & Matheson, 1984). The precursors of galactomannan biosynthesis GDP-D-mannose and UDP-D-galactose are formed by the action of the enzymes GDP-mannose phosphorylase and UDP-galactose 4-epimerase. The *in vitro* experiments also report that the relative concentration of these precursors can affect the G/M ratio of the galactomannan (Edward *et al.*, 1992).

In the process of galactomannan biosynthesis the enzyme GDP - Man (and Mg^{2+}) - dependent (1, 4) β-D-mannosyltransferase or mannansynthase catalyse the sequential addition of mannosyl residues to the growing non-reducing end of mannose backbone followed by the attachment of galactosyl residues by UDP-Gal (and Mn^{2+}) - dependent (1, 6) α-D-galactosyltransferase or galactosyltransferase to galactosyl acceptor mannose residue on the mannan backbone (Reid *et al.,* 1992; Edwards *et al.,* 1999). The specificity of galactosyltranferase plays a key role in the regulation of galactose substitution in galactomannan biosynthesis and also the statistical distribution of galactosyl substituents along the mannan backbone (Edwards *et al.*, 2002). The activities of these two enzymes increase parallely such that the ratio of G/M remains constant in the polymer chain during the galactomannan biosynthesis.

Application Potential

Galactomannans, being functionally a seed cell wall storage polysaccharide, extend their biological role beyond as an energy reservoir by holding a role in water relationship during germination

and seed development. The hydrophilic nature of galactomannan allows the seed endosperm to imbibe an ample amount of water localized between the germinating embryo and external environment, thus protecting the developing embryo from desiccation during frost or drought, thereby acting as osmoprotectant (Reid & Bewley, 1979).

Galactomannans also form water dispersible hydrocolloids, which thicken when dissolved in water. This property is of commercial importance (see Table 4) and hence exploited in various industries as thickening and stabilizing agents (Cho & Prosky, 1999). The principal uses of galactomannans are in dairy products (in thickening desserts, and particularly in sorbets, ice creams, and low-energy fat substitutes), fruit-based water gels, powdered products (desserts and hot milk puddings), bakery goods (icings and cake mixes), dietary products, whiteners, milk formulations, seasonings, sauces and soups, tinned meats, and frozen and cured meat foods (Gidley & Reid, 2006).

Galactomannans exhibit a unique rheological behavior on co - gelation with other polysaccharides facilitating the formation of thermo-reversible gels, which find importance in various food and pharmaceutical applications. In the former their use imparts desirable texture and appearance to fabricated foods. Apart from these they also find application in pulp dispersing, sizing and finishing in paper industry; as flocculant, filterant and flotation of minerals in mining, cosmetics, drilling, and explosives and also in stabilizing fungicide and herbicide dips.

Table 4. Industrial application of galactomannans

Industry	Product	Galactomannan	Function
Food	Desserts Puddings Ice cream Dairy products Jellies, sauces	Guar gum and its derivatives, LBG, Tara gum	Thickening, stabilizer emulsifier
Pharmaceuticals	Tablet, Hydrogels, Encapsulated drugs	Guar gum and its derivatives	Water soluble preparations, dry binder, disintegrating agent
Paper and Textile	Hand towel, Tissues Sizing	Carboxymethyl guar, LBG	Paper strength Dyeing
Cosmetics	Hair decipher	*Gleditsia triacanthos* gum, guar gum	Moisturiser
Building	Plaster	Guar gum and LBG	Thickening agent
Fire fighting	Air drop	Guar gum	Provides viscosity stability
Oil well drilling and mining	Oil bores	Guar gum, LBG	Plugging leaks, Shock protection
Explosives	Gel	Guar gum	Improved resistance to water and ageing

Even though many leguminous plants posses galactomannan in their endosperm only few of them serve as important industrial hydrocolloids due to their abundance, easy availability, low cost and

increased functional properties. The latter depends on the substitution of galactose residues in the polymer chain. Fenugreek, guar gum, tara gum and LBG (Fig. 3) are the classical galactomannan sources with varying G/M ratios of approximately 1:1, 1:2, 1:3, 1:4, respectively (Picout *et al.*, 2002). Of these, locust bean gum and guar galactomannans are commercially produced in large quantities.

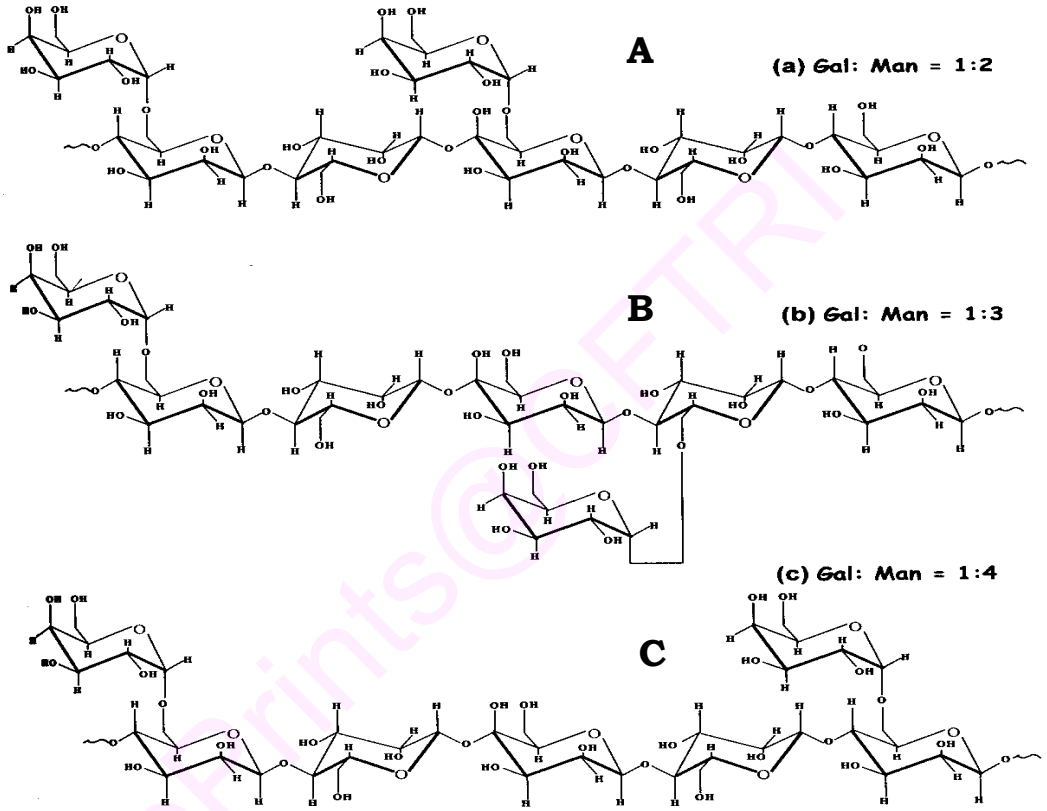


Fig. 3. Chemical structures of galactomannans **A:** Guar galactomannan, **B:** Tara gum, **C:** Locust bean gum

LBG, also known as carob galactomannan, obtained from the seeds of *Ceretonia siliqua,* is found growing in the Mediterranean region, and enjoys commercial importance due to its low viscosity and less branching, which facilitates synergistic interaction with other biocompatible polysaccharides forming thermo-reversible gels of use in specific food and non-food products (Rock, 1971; Fox, 1992).

Despite its positive aspects, LBG is known to undergo shortage due to its long growth period which takes nearly 8-10 years to reach maturity and produce pods. In addition, regional and climatic constraints with occasional sub-zero temperature and wet weather for long duration influence for decrease in seed size and gum content leading to shortage in supply of LBG which in turn has an impact on its price (Srivastava & Kapoor, 2005). The shortage in the supply of LBG in the mid eighties and nineties saw a huge price hike of thirty dollars compared to normal price of seven dollars per kilogram. These limitations in the production of LBG and also increasing demand of seed galactomannans, resulted in the search for an alternative hydrocolloid suitable to meet the ever increasing demands from various industries.

As a welcome alternative, guar galactomannan, an agro product from Indian subcontinent revealed to be an abundant and cheaper substitute for LBG in various applications. Over a period of time the production of guar galactomanann in India has considerably increased to meet the internal as well as global demands.

Guar galactomannan (GG)

The term guar is thought to be derived from Sanskrit word *go* or *gav*, meaning 'cow', indicating the use of this plant as cattle feed. It is believed that guar was originally domesticated in India as a rich protein food to feed cattle and also as green vegetable. In 1940s after the Second World War a major shortage in the supply of LBG adversely affected the textile and paper industries during which time GG got its big break and became the most suitable substitute for scarce LBG. In 1953, the extraction technology of GG was first commercialized in USA and a decade later in India. Since then guar

plant is being grown primarily as a commercial crop for gum production.

GG, derived from the endospermic seeds of *Cyamopsis tetragonolobo*, is an annual plant indigenous to Indian subcontinent. It is mainly grown in west and northwest parts of the country, particularly in the states of Haryana, Rajasthan, Gujarat and Punjab. Guar is also grown in Pakistan, United States and also in some parts of Arabian Peninsula, Africa and Australia. Guar plant is found growing in the semiarid conditions mainly in sandy soil with warm weather and moderate rainfall with plenty of sunlight. The plant grows to a size of 2 to 9 feet high with white flower buds, later changing to light pink and deep purple as the flower opens up followed by fleshy seed pods comprising nearly of 75-85% of galactomannan in its endosperm. The crop is usually sown after monsoon rain during mid July or early August and harvested during October - November with a short plantation period of 3 - 4 months.

Chemical structure and properties of GG

GG is a high molecular weight, polydisperse (PDI > 2) (Vijayendran & Bone, 1984) co-polymer mainly consisting of β-1,4-D-mannopyranose as backbone residues, having α-1,6-D-galactopyranose as a single residue side chain stubs on every alternative mannose units, and having G/M ratio of 1:2 (Crescenzi, 2004). The distribution of galactose residues on the mannan backbone plays a vital role in its functional property (McCleary *et al.*, 1985). The branches of galactose residues on the linear mannan backbone also prevents the aggregation of the molecule due to the intermolecular hydrogen bonding of cis-hydroxyl groups of mannose thereby preventing its easy dissolution in water (Pai & Khan, 2002).

GG is a non-ionic, salt tolerant, cold water-soluble hydrocolloid which hydrates rapidly to give highly viscous pseudo-plastic solutions of greater low-shear viscosity. The rate of dissolution and hydration of GG mainly depends on its average molecular weight, concentration and the temperature of dissolution (Wang *et al.*, 2003). A solution viscosity of ~5000 – 8000 cps at a concentration of ~1% depending on the conditions of extraction has been reported. Though GG lacks characteristic gelling property it shows good stability to freeze-thaw cycles. The water holding capacity of GG adds to its advantage in preventing ice crystal formation by slowing mass transfer across solid/liquid interface in frozen dessert products. It displays a unique ability of controlling rheolgy by water phase management. Also the presence of fee hydroxyl groups in the macromolecule facilitates reaction with various chemicals, thus leading to a wide array of derivatives for improvement in its functional properties viz. dispersion, viscosity and gelation.

Global market for GG

The global market for GG is estimated to be around 2-2.5 lakh tons/year. India dominates the production and trade of GG by contributing nearly 80% share in the global production. The other countries which are also involved in export of GG are Pakistan, USA, Italy, Spain, France, Greece, and Germany. The economic utility of guar seeds is largely influenced by the demand from overseas, mainly from the petroleum industry of USA and the oil fields in the Middle East as the derivatives of GG are particularly useful in the drilling process. Also the surging crude oil prices in the international market has super charged the oil exploration activities, which in turn influences the demand for guar gum. United States alone has a demand of over 40,000 tons of guar and its derivatives. Also, in rest of

the world, the trend of its consumption has increased significantly, that has lead to the cultivation of this crop in many countries. Export of Indian guar gum has increased from 83,000 tons to 205,000 tons in 2006-07. As per the trade estimates in the current year too, the export of guar is expected to remain almost steady in the range of 210,000 tons.

The production of GG is directly related to the agro-climatic conditions. Though the demand for guar seeds in the global market is almost constant in all the years the fluctuation in the rainfall influences the production of guar seeds with consequences of scarcity in the market. For example, 2002-03 was marked by a low production of 40,000 tons due to severe drought, whereas in 2003-04 the production increased to high levels of 16 lakh tons due to increased acreage and good rainfall.

Application of GG

GG, due to its easy availability in large quantities and low price is the most sought-after hydrocolloid of commerce, displaying wide application potential notably in food products, pharmaceuticals, cosmetics, textile, paper industry, mining, explosives, nutraceuticals, etc. Additionally, derivatization of GG by various chemical methods extends its range of application in various food and non-food industries.

Food products

The food industry constitutes the main platform for the utilization of GG and its derivatives due to their ability to improve the rheological and textural characteristics of food systems (see Table 5).

Often they are used as food additives for enhancing viscosity, creating gel-structures and lengthening the physical stability (Dziezak, 1991; Glicksman, 1991; Garti & Reichman, 1993; Dickinson, 2003; Hinrichs *et al.*, 2003). The high viscosifying, thickening and film forming property of GG prompts its use as a key functional food ingredient.

Table 5. Food applications of GG

Food products	Function
Frozen food products	Reduces crystal formation, acts as a binder & stabilizer to extend shelflife of ice-cream
Baked food products	Moisture retention of dough, retards fat penetration in baked foods
Dairy products	Improves texture, maintains uniform viscosity and colour
Sauces and salad preparations	Water binder, reduces water and oil separation
Confections	Controls viscosity, bloom, gel creation, glazing and moisture retention to produce the highest grade confectionary
Beverages	Outstanding viscosity control, reduces calorie value in low calorie beverages
Pet food	Gels, retains moisture, thickening, stabilizing and suspending agent for veterinary preparations

The high viscosity of GG helps to thicken and maintain the flow characteristics of sauces, gravy, dressings and other products. The ability of galactomannan to lockup water is useful in the preparation of mixture of oils, fats, proteins and water in salad dressings, yogurt,

and pudding (to reduce separation of water and oil, and to maintain their texture). Solubility of GG in cold water also enhances its usage in instant drinks as a cost-effective thickener and suspending agent. A report by Ward (1997) reveals the use of GG in bakery products as a component of fat mimetic system. Zambrano *et al.* (2004) reported the use of guar gum at low levels (0.1 - 0.5 %) to facilitate imbibition of water and to increase the viscosity in many bakery products. Maier *et al.* (1993) suggested that in bakery products such as bread, cakes and donuts addition of guar gum improves mixing and recipe tolerance with improvement in the shelflife of products by moisture retention. Incorporation of GG also allows preparation of low fat cakes with larger volumes and fine uniform cell structure similar to cakes with the original shortening levels (Glicksman, 1991).

Syneresis is one of the most serious problems encountered in food industry which affects the quality and shelflife of food products. The water holding capacity of GG adds to its advantage in preventing syneresis and also ice crystal formation in frozen desserts (Morris, 1995) and baked products. Sidhu *et al.* (1997) indicated the improvement in consistency of tomato ketchup on addition of GG. Gujral *et al.* (2002) reported the addition of guar gum (0.5%) resulting in increased viscosity and reduced syneresis of tomato ketchups. The sensory properties and texture of frozen products are mainly influenced by the size and number of ice crystals formed during freezing. Addition of GG to ice cream mix and other frozen food products inhibited the elongated ice crystal and preventing the ice crystal size at low temperatures in abusive storage conditions with temperature fluctuations thereby protecting the food products from heat shock and dryness. Buyong & Fenema (1988) reported that GG at less than 2% (w/w) reduced the amount of ice crystals, while gelatin had no effect at all. Alexander (1999) described the importance

of GG in combination with LBG and carragennan as stabilizer in ice cream, filled pies and other frozen foods. Also the addition of GG to the cottage cheese fortified with food grade calcium lactate enhanced the flavor and sensory properties of cheese by masking bitterness of added calcium in comparison to other hydrocolloids (Puspitasari *et al.*, 1991; Kaup *et al.*, 1991).

Protein-polysaccharide conjugates have been proposed to be useful new functional biopolymer systems having excellent emulsifying properties and antimicrobial effect (Kato *et al.*, 1989; Nakamura *et al.*, 1991). Nakamura *et al.* (1992) report that lysozyme conjugated to GG, though controlled Maillard reaction, showed excellent emulsifying properties and significant bactericidal effect on Gram-negative bacteria. The conjugates prepared by non-enzymatic reactions without the use of chemicals can be used in formulated foods as a safe multifunctional food additive. The conjugation of GG with ovalbumin also enhanced the antioxidant effect thereby suppressing the rancidification of food products (Nakamura, 1992a).

GG also exhibits a unique property of synergistic interaction with other biocompatible polysaccharides, which imparts novel and improved texture and rheological characteristics to food products (Richardson *et al.,* 1999). The synergistic interaction of guar solution with xanthan resulted in enhanced viscosity rather than gelation. Whereas modified GG on admixture with xanthan showed improvement in gelation which is widely exploited by the food industry (Brooks *et al.*, 2000). Such differences in gelling behavior is attributed to distribution of galactose units along the mannan backbone (see details in Chapter IV). The interaction of GG with whey proteins also results in the enhancement of the performance of heat induced whey protein gels (Tavares & Lopes da Silva, 2003). Several authors have

reported the modification of gelatinization and retrogradation characteristics of starch-guar combination, which is useful in preparing a wide variety of foods for improvement in texture, mouthfeel and processing properties (Christianson *et al.,* 1981; Alloncle & Doublier, 1991).

The use of GG as a food additive is regulated by setting safety limits in various food products (Anderson, 1995). Table 6 denotes the desirable specified levels of GG in various food products as permitted by the U.S. code of federal regulations (Baird, 1992).

Table 6. U.S. code of federal regulation for usage of guar in various foods

Food	Maximum (%)
Baked foods and baking mix	0.35
Breakfast cereal	1.2
Cheese	0.8
Dairy products and analogs	1.0
Fats and oils	2.0
Sauce and gravies	1.2
Jams and jellies	1.0
Milk products	1.0
Processed vegetables and juices	2.0
Soups and soup mixes	0.8
Syrups and sweets	1.0
Other food products	0.5

Pharmaceuticals

GG and its derivatives are used in pharmaceutical industries for controlled delivery of drugs (Altaf *et al.*, 1998), which upon exposure to dissolution fluids gets hydrated and form viscous gel layer thereby slacking further seeping-in of dissolution fluid towards the core of the matrix tablet (Rama Prasad *et al.,* 1998). GG was effectively used as a release - retardant carrier in the design of three-layer matrix tablets for highly water-soluble drugs such as metoprolol tartrate (Krishnaiah *et al.*, 2002). Gilko-Kabir *et al.,* (1998; 2000) also report the potential of GG for colon specific drug targeting required in the treatment of ulcerative colitis, Crohn's disease, colon cancer and amebiasis. The matrix of GG is assumed to remain intact in the physiological environment of stomach and small intestine but once in the colon, they are acted upon by bacterial polysaccharases, thus releasing the drug (Chourasia & Jain, 2004). Recently, it has been demonstrated that GG can effectively enhance the oral delivery of therapeutic proteins and peptides due to the presence of favorable environment in colon in comparison to upper gastrointestinal tract. GG with compression coat of 0.61 to 0.91 mm was found sufficient to deliver the drug selectively to the colon (Krishnaiah *et al.*, 1999). Chourasia *et al.* (2006) evaluated the efficacy of GG by fabricating it into microspheres by emulsification technique using glutaraldehyde as a cross-linking agent for the improved delivery of anticancer drugs for the treatment of colorectal cancer. Use of GG in transdermal drug delivery systems (Murthy *et al.*, 2004) and as a visco supplementation agent in osteoarthritis treatment (Cunha *et al.*, 2005), is also known.

Nutraceuticals

GG is an important source of non-calorific functional dietary fiber, with 80% of it almost in the form of soluble fiber exhibiting interesting physiological functions (Fig. 4). As a viscous soluble dietary fiber GG has the potential to alter the rate of carbohydrate degradation during digestion thereby exhibiting beneficial effects on the regulation of postprandial blood sugar and insulin levels. It also plays key events in the prevention and treatment of obesity. The high viscosifying property of GG also decreases the protein efficacy (Poksay & Schneeman, 1983) and lipid utilization (Simons *et al.*, 1982) by interfering with the digestion and absorption of nutrients (Ikegami *et al.*, 1990).

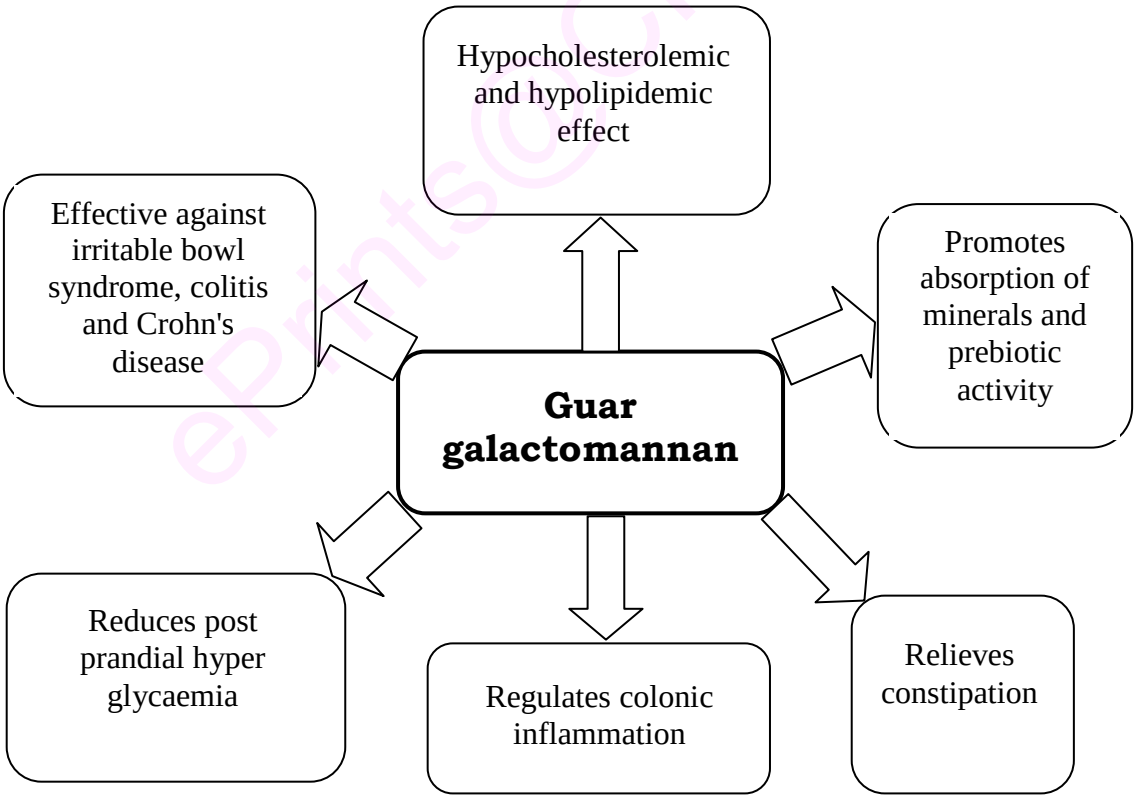


Fig. 4. Various physiological functions of GG

GG is claimed to be effective in promoting regular bowel movements, relieve constipation and chronic related functional bowel ailments such as diverticulosis, Crohn's disease, colitis and irritable bowel syndrome. The increased mass in the intestines stimulates the movement of waste and toxins from the system, which is particularly helpful for good colon health.

Several studies have found significant decrease in human serum cholesterol levels following guar gum ingestion. Jenkins *et al.* (1980) reported that consumption of 13 g of GG in the form of crisp bread over a period of 2-8 weeks produced 13% reduction in total serum cholesterol without changes in HDL cholesterol. About 44% reduction in LDL cholesterol and 22% decrease in triglycerides were observed in guinea pigs fed soluble fiber diet containing 2.5 g/100g of GG (Roy *et al.*, 2000). Intake of 15 g GG daily, elicited a significant reduction in serum concentrations of total and LDL cholesterol over 2 years of treatment (Salenius *et al.*, 1995). The clinical trials by Todd *et al.* (1990) and Uusitupa *et al.* (1984) have proved the efficiency of GG alone in reducing the serum total cholesterol by 10 to 15 %.

The addition of GG to glucose drinks or carbohydrate-rich meals reduces the post-prandial rise in blood glucose and plasma insulin levels in diabetic subjects (Jarjis *et al.*, 1984; Morgan *et al.*, 1985; Ellis *et al.*, 1988). The long-term improvements in carbohydrate and lipid metabolism of patients with insulin-dependent diabetes (IDDM) and non-insulin-dependent diabetes (NIDDM) have been achieved using either pharmaceutical preparations of guar gum or guar-containing foods (*Aro et al.*, 1981; Peterson *et al.*, 1987; Ebling *et al.*, 1988). The physiological action of guar gum is assumed to be mainly dependent on its capacity to hydrate rapidly thereby increasing the viscosity of digesta in the stomach and small intestine which slows

down the digestion and absorption of carbohydrate, which then leads to a reduction in post-prandial hyperglycaemia (Blackburn *et al.*, 1984; Edwards & Read, 1990). Takahashi *et al.* (1994) show that GG and its derivatives improve iron utilization in iron deficient rats.

Gamal-Eldeen *et al.* (2006) report the cancer chemopreventive, antioxidant and anti-inflammatory activities of chemically modified GG. The glycosylated and sulphated derivatives of GG enhanced the macrophage proliferation and phagocytosis and they also strongly inhibited the stimulated nitric oxide (NO) generation and tumor necrotic factor-α (TNF- α). The glycosylated guar derivative was a potent anti-initiator, wherein it inhibited not only the carcinogen activator enzyme, cytochrome P450 1A (CYP1A), but also induced carcinogen detoxification enzymes, viz. glutathione-*S*-transferases (GSTs). Whereas sulphated derivative of GG inhibited both CYP1A and GSTs and also was an effective radical scavenger against hydroxyl, peroxyl, and superoxide anion radicals. Sulphated GG could bring about a major disturbance in cell cycle by arresting both S - and G2/M-phases in Hep G2 cells associated with an induced cell death due to necrosis.

Oil field applications

GG and its derivatives have found a broad range of applications in oil well fracturing, oil well stimulation, mud drilling and also in preparations as a stabilizer, thickener, suspension, flocculation and turbulent friction reduction or mobility control agent. In the oil field industry, GG is used as a surfactant and deformer ideally suited for all rheological requirements of water-based and brine-based drilling fluids. High viscosity of GG is useful as drilling aid in oil well drilling, geological drilling and water drilling to maintain drilling mud

viscosities that enable drilling fluids to remove drill waste from deep holes.

Guar and its derivatives also reduce friction in the holes, and so minimizing power requirements. Some guar derivatives act to minimize water loss occurring in broken geological formations. The use of GG as a suspending agent for sand in drilling mud and in fracturing fluids led to the discovery that guar solutions have lower friction than water.

Miscellaneous applications

In mining process GG is widely used as a flocculant to effect liquid -solid separation in order to concentrate ores or tailings. In practice, slimes and tailings from ore beneficiation processes are concentrated so that the water can be reused and the solids are easily processed. GG also finds application as a depressant for talc (Rath *et al.*, 1997) or insoluble gangue mined along with the valuable minerals.

The application of GG in paper industry gives denser surface to the paper used for printing with improved erasive and writing properties, better bonding strength and increased hardness. Guar gum is used in the explosives industry as a waterproofing agent. Its ability to hydrate in saturated solutions of ammonium nitrate causes it to be widely used in this industry

GG was found to be an effective flocculant alternative to polyacyrlamide in water treatment. The use of GG also promoted the aggregation and growth of colloidal particles during potable water coagulation–flocculation treatment processes (Gupta & Ako, 2005). Sharma *et al.* (2006) also report the preparation of flocculant cum ion-

exchangers from GG by a general reaction using functionalized epoxides for treatment of industrial effluents. In addition to flocculation of suspended solids, these resins were capable of removing trace metal ions, dyes, surfactants, etc. from effluents.

In recent years efforts have been made to develop eco-friendly technologies to manage industrial waste resources into a value added products. Suthar (2007) reports the potential utilization of GG industrial waste for the production of vermifertilizer by using composting earthworm Perionyx sansibaricus. Recycling of guar industrial waste through vermicomposting also accelerates the mineralization of complex nutritents with relatively high content of humus-like compounds for sustainable plant production and land restoration practices.

The incorporation of GG derivatives prepared by acrylation in unsaturated polyester composites increased its mechanical properties as well as toluene and water resistance providing an option to mineral filler based composites thereby by rising environmental concern (D'Melo & Shenoy, 2008).

Selective debranching of GG

Inspite of its diversified applications and functionalities, GG is far less superior compared to LBG, due to its high branching, which causes less synergistic interaction with other polysaccharides. The fine structural difference in the distribution of galactose residue on mannan backbone contribute to significant variations in their properties. An earlier report on the primary structure of galactomannans, particularly LBG, reveals a random arrangement of galactosyl residues on the mannan backbone, thus forming a block

type sequence of unsubstituted mannose region, which favours gelling interaction with other polysaccharides. In contrast, the rather uniform distribution of galactose residues on every second mannose residue, resulting in a highly substituted mannose region hinders the synergistic behaviour of GG (Morris *et al.*, 1977). Thus, debranching by selective removal of galactose residues from the mannan backbone was felt necessary to transform GG for the diversified applications. Various attempts have been made in several labs all over the world to modify GG to LBG - type for improved functionalities.

Debranching, without significant damage to the mannan backbone of GG could be effectively achieved by enzymatic hydrolysis using α-galactosidase, which is specific for the removal of side chain galactose residues. McCleary (1986) reports studies on the treatment of GG with a highly purified α-galactosidase from germinated lucerne seed resulting in a series of galactose-depleted galactomannans. Also reports are available on the utilization of α-galactosidase extracted and purified from other sources such as plants (McCleary, 1983), bacterial and fungal sources (Ademark, 2001) in debranching GG to various extents.

Scope and objectives of the present investigation

Though successful usage of α–galactosidase in removing galactose residues from the side chain of GG is reported, the utility of this technology is not feasible because only the α–galactosidase isolated from germinated guar seeds is effective. Also the high cost involved for the isolation of this enzyme in a pure form and its laborious production steps are non-economical for bulk utilization. Hence, an alternative search for an economical and easily available source for the modification of GG was felt necessary and desirable.

Nevertheless, recent literature reveals the non-specificity of various enzymes belonging to hydrolases such as cellulase, lipase, hemicellulase, lysozyme, (Xia *et al.*, 2008; Lee *et al.*, 2008; Amano & Ito, 1978) and a few proteases like pepsin, papain, bromelain on chitosan (Liao *et al.*, 2000; Muzzarelli *et al.*, 2002) and other polysaccharides. Also earlier studies from our lab have shown the pectinase isozyme obtained from *A. niger* to exhibit chitosanolytic activity at acidic pH (Kittur *et al.*, 2003; 2003a). Vishu Kumar *et al.* (2007b) also report the preparation of low molecular weight chitosan with bactericidal activity towards *B.cereus* and *E.coli* by the action of pronase. At this juncture, it was felt interesting to try selective debranching of guar galactomannan using structurally unrelated enzymes such as carbohydrases, lipases and proteases to obtain modified GG with an altered G/M ratio which possibly mimic the properties of those of LBG. Possibly such an approach also adds to the cost effective replacement of LBG for various functional food applications. The economic feasibility for selective debranching of GG is of considerable commercial interest, as such modified galactomannans find multiple applications as functional food ingredients.

With these in mind, a detailed study on the non-specificity of a few enzymes in selective debranching of GG was initiated with the following two main objectives.

1. To prepare modified GG(s) having a G/M ratio akin to that of LBG, and which would exhibit useful rheological properties, and

2. To understand the mechanism of enzyme catalysis for their non-specificity in selective debranching.

In accordance with these objectives, the present investigation was focused mainly on the screening of various enzymes for their non-specific action towards guar galactomannan. Of these pectinase (*Aspergillus niger*), pullulanase (*Bacillus acidopullulyticus*) and pepsin (porcine stomach mucosa) showed better debranching activity towards GG. As the commercial enzyme preparations usually are admixed with inorganic substances, used as enzyme stabilizers, and also contain other contaminating proteinaceous matter, attempts were made to purify them before undertaking further detailed structural studies, in order to rule out the involvement of the contaminants in their non-specificity. The galactose-depleted GG was characterized for the conformational changes and improvement in rheological behaviour upon interaction with other polysaccharides.

Chapter I

Screening of enzymes for (non-specific) activity towards GG

Considering the drawbacks such as high viscosity and high branching, GG finds rather limited application in various food and non - food industries. Shortage in the supply of LBG due to its long maturation period, competition from other commercial crops and high cost, lead to an increased demand for GG (Bulpin *et al.*, 1990). Further, to enhance the usage of GG to suit to various purposes, removal of galactose residues from the main chain proved to be a significant step. Debranching of GG can efficiently be achieved by the enzymatic hydrolysis. α-Galactosidase obtained from various plants, bacterial and fungal sources have been effectively used in debranching of GG.

McCleary *et al.* (1981) and Bulpin *et al.* (1990) have reported the successful removal of galactose residues from GG by α-galactosidase obtained from germinating lucerne seeds and green coffee beans, with the modified guar galactomannan having improved functional properties. A report from Overbeeke *et al.* (1989) also describes the expression of α-galactosidase from guar plant by transformation using recombinant DNA technology. A thermostable α-galactosidase obtained from a highly thermophilic bacteria *Rhodothermus marinus* could facilitate the removal of galactose residues from GG (Anna *et al.*, 2000). Guar gum- induced α-galactosidase obtained from fungal culture *Penicillium ochrochloron* was employed by Prakash *et al.* (1993) for the depletion of galactose side chain to generate modified GG with less than 30 % galactose residues. Elina *et al.* (1998) report three different types of α-galactosidase isolated from *Penicillium simplicissimum* which effectively bring out the hydrolysis of galactose residues from GG.

Large scale usage of α-galactosidase in debranching of GG is restricted due to its high cost, probably because of its low yield due to

the presence of various other storage proteins and associated β-mannanase, which involves laborious, time consuming purification steps. In addition, the low quantity of specific protein production, its decreased activity, probably attributed to the localization of the enzyme and/or proteolysis, have also to be considered. In general α-galactosidase is more effective in hydrolyzing galactose residues from smaller substrates than polymeric substrates. α-Galactosidase obtained from *A. tamarii* (Civas *et al.,* 1984) and *B. stearothermophilus* (Talbot *et al.*, 1990) was found to show limited activity towards polymeric galactomannans probably due to the steric hindrance from the adjacent residues. Some α-galactosidases belonging to hydrolyase family 36 have been reported to have less activity or totally inactive towards polymeric substrates (Margolles *et al.*, 1996; Aslandis *et al.*, 1989). α-Galactosidase produced from recombinant DNA technology is very expensive and also involves the statutory approval for subsequent use of the modified GG.

Recent literature reveals the susceptibility of various substrates for the non-specific hydrolytic action of several unrelated enzymes belonging to the class - hydrolases. A non-specific action of β 1,4-glycanase on mannans and cellobiose has been reported (Macarron *et al.*, 1996). Effective hydrolysis of chitosan and its derivatives by various enzymes namely cellulase (Muraki *et al.*, 1993), lysozyme (Hirano *et al.,* 1989), papain (Muzzarelli *et al.*, 1994), wheat germ lipase (Muzzarelli *et al.*, 1995), hemicellulase (Qin *et al.*, 2003) and β-glucosidase (Zhang *et al.*, 2001) has been reported.

Preliminary screening of various enzymes by Qu-Ming (1999) has shown the action of lipases (from various sources), hemicellulase, papain, proteases (from various sources) and subtilisin on cationic GG. The majority of these enzymes could bring about the hydrolysis of

modified guar as manifested by reduction in viscosity of cationic guar solution at different pH conditions and 37°C. All these enzymes used were from commercial preparations which were not necessarily pure. Hence, the presence of minor contaminants that may be responsible for the reported hydrolytic activities could not be ruled out. But, so far no report is available on the physico-chemical characterization of the enzyme - treated GG.

Our continuing research on the non-specific activity of pectinase (Kittur *et al.,* 2005), pepsin (Vishu Kumar *et al.*, 2007), pronase (Vishu Kumar *et al.*, 2004a; Vishu Kumar *et al.*, 2005) and papain (Vishu Kumar *et al.*, 2004b) in hydrolysing chitosan to produce low molecular weight chitosan (LMWC), chitooligosaccharides and monomers in good yield has paved the way to look for a similar approach to obtain modified GG, which may be able to mimic the properties of LBG.

At this juncture, a variety of enzymes viz. pectinase from *A.niger* and *Rhizopus sps*, porcine pepsin, pullulanase from *Bacillus acidopullulyticus*, pronase, lipases (wheat germ, *Candida cylindreaceae*), amyloglucosidase, cellulase and hemicellulase were screened to study their action pattern towards GG. Some of these enzymes are relatively inexpensive and easily available. This chapter describes the screening of various commercially available enzymes for removal of galactose residues from GG with a view to improve its functional properties.

Materials

Guar gum was procured from Hindustan Gum Chemicals, Haryana, India. Enzymes – pectinase (*Aspergillus niger,* EC. 3.21.15),

pullulanase (*Bacillus acidopullulyticus,* EC.3.2.1.41), pepsin (porcine stomach mucosa, EC. 3.4.23.1), pronase (*Streptomyces griseus*, Protease Type XXV, EC. 3.4.23.18), lipase (wheat germ and *Candida cylindracea,* EC. 3.1.1.3), hemicellulase (*Aspergillus niger,* EC. 3.4.1.1), amyloglucosidase (*Rhizopus sps*, EC. 3.2.1.3) and Sepharose CL - 4B were from Sigma Chemical Co., St. Louis, MO, USA. All other reagents and chemicals were of highest purity.

Methods

Characterization of GG

The molecular mass (Mw) of GG was studied by three techniques, namely

a) Viscometry

The relative viscosity of GG dissolved in distilled water (0.1 - 0.5%) was measured using Ostwald viscometer at a constant temperature of 27±1^{o}C. The average molecular mass was calculated using Mark-Houwink's equation, $\eta = K \times (\text{molecular mass})^{\alpha}$, where η = intrinsic viscosity, K = 3.04x 10^{4} and α = 0.747 (Cheng *et al.*, 2002).

b) Gel permeation chromatography (GPC)

The GG solution (1 mL, 0.5% in water) was loaded onto Sepharose CL-4B column (bed volume 180 mL) pre-calibrated with T-series dextran standards of known Mw and eluted with water at a flow rate of 18 mL hr^{-1}. The Mw was determined from the standard graph obtained by plotting the log Mw of the standards versus Ve/Vo, (Ve-elution volume of the standards and Vo-Void volume).

c) High-performance size exclusion chromatography (HPSEC)

HPSEC was performed on Shimadzu LC 8A system connected to RI detector, using E-linear and E-1000 columns (Waters Associates, Millford, USA) connected in series with distilled water as the mobile phase at a flow rate of 0.6 mL min^{-1}. The column was pre-calibrated with dextran T-series of known Mw.

Viscosity measurement

Viscosity of GG (1%) dispersed in water was measured using Brookefield digital viscometer Model DV-II, version 2.0 using spindle No 5 at 50 rpm and 25^{o}C.

Galactose / mannose (G/M) content

G/M ratio of GG was determined after acid hydrolysis followed by reduction and acetylation. In brief, 10 mg of sample was dispersed in 0.3 mL of water and allowed to swell for about 10-12 hr at 4^{0}C followed by dropwise addition of 0.72 mL of concentrated sulphuric acid. Then the acid concentration was brought to 8% by the addition of distilled water and later keeping the mixture in boiling water bath for about 8-10 hr. The mixture was neutralized by adding solid barium carbonate followed by filtration. The filtrate was deionized with Amberlite IR-120 H^{+} and reduced with sodium borohydride. The resulting alditols were acetylated by heating with acetic anhydride and pyridine at 110^{o}C for 2 hr and the derivatized alditol acetates were analyzed by GC on OV-225 column (3% on Chromosorb W, 100-120 mesh) connected to Shimadzu 6A gas liquid chromatograph fitted with FID detector (Sawardekar *et al.*, 1965).

Phenol - Sulphuric acid assay

To a sample (0.5 mL) containing carbohydrate, phenol (0.3 mL, 5%) followed by concentrated sulphuric acid (H_2SO_4) were added. The absorbance was read at 480 nm after 20 min incubation at room temperature. Glucose (5-25 μg) was taken as reference sugar for the preparing the standard curve (Rao & Pattabiraman, 1989).

Reducing sugar assay

a. Dinitrosalicylic acid method

Dinitrosalicylic acid (1g) was dissolved in a solution containing sodium potassium tartrate (30 g) and 0.4 N NaOH (20 mL) and the contents were made up to 100 ml with distilled water. The reagent was filtered and stored at 4^0C.

To the sample (1 mL) in a test tube, DNS reagent (1 mL) was added, mixed well and incubated in a boiling water bath for 10 min. The contents were then cooled and diluted with double distilled water (2 mL) and the absorbance was read at 550 nm (Miller, 1959).

b. Potassium ferricyanide method

Potassium ferricyanide (1g) was dissolved in a solution containing sodium carbonate (5%) and the contents were made up to 100 mL with distilled water. The reagents were filtered and stored at 4^0C.

To the sample (1 mL) in a test tube, the reagent (2 mL) was added, mixed well and incubated in a boiling water bath for 15 min.

The contents were then cooled and the absorbance was read at 420 nm (Imoto & Yagishita, 1971).

Bradford assay

The reagent was prepared by dissolving Coomassie Brilliant Blue G-250 (100 mg) in 50 mL 95% ethanol. To this, 100 mL phosphoric acid (85%, w/v) was added and the volume was made up to 1 L with distilled water, filtered and stored at 4°C (protected from light).

To the enzyme samples and standard (bovine serum albumin, 0.2 mL, 2-10 μg), 0.8 mL of the reagent was added and after 10 min, the absorbance was measured at 595 nm (Zor & Selinger, 1996).

Determination of the non-specific activity towards GG

Non-specific activity of various enzymes towards GG was determined on co-incubation of GG solution (0.5 %) with the enzymes (100 μg, 1 mL) for about 1hr at 37°C. Then the reaction was terminated by heating the reaction mixture and adding of 3 volumes of ethanol followed by centrifugation. The supernatant was assayed for the released reducing sugar by the ferricyanide method. The enzyme activity (units) was expressed in terms of μmoles of reducing equivalents released min^{-1} mg^{-1} protein.

Determination of the specific activity of enzymes

The specific activity of the enzymes was determined using their respective substrates.

Pullulanase

Specific activity of pullulanase was determined on incubation of pullulan (1%) with the enzyme at pH 5.0 for about 1hr at 37°C and estimating the reducing sugar released by the DNS method. The specific activity was expressed as µm of reducing equivalents released min^{-1} mg^{-1} protein.

Pectinase

0.9 ml sodium pectate (0.25% of pectic acid in 0.1 M of citrate buffer, pH 3.0) was incubated with 0.1 mL of enzyme (60 µg) at 25°C for 30 min. One unit of activity is defined as the amount of enzyme required to liberate 1µmole reducing sugar per min at 25°C.

Proteolytic activity

Proteolytic activity of pepsin and pronase was determined by taking hemoglobin (2.5%) as a substrate followed by incubation with the enzyme at optimum conditions and estimating the TCA soluble peptides released at 280 nm. Specific activity expressed as one unit = absorbance at 280 nm / reaction time X mg protein in the reaction mixture (Anson, 1938).

Lipolytic activity

Lipase catalyzed hydrolysis was determined by using triacylglycerol as the substrate at pH 7.7 & 37°C and the activity was expressed as micro-equivalents of fatty acids released h^{-1}.

Results and Discussion

Characterization of GG

The reported average Mw of native GG was ~ 240 KDa which was in concordance with that derived from GPC and viscometric measurements. Appearance of a single peak upon GPC and HPSEC also confirmed its molecular homogeneity (Fig. 5). GG (1% w/v) showed a solution viscosity of ~ 4800 cps as determined by Brookfield viscometer. The galactomannan content of the guar was 90% with the galactose to mannose ratio of 1: 2 by GLC (Fig. 6).

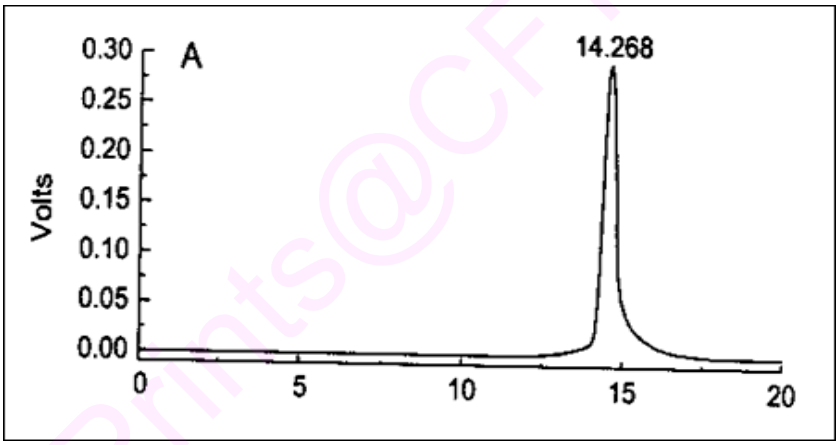


Fig. 5: HPSEC profile of native GG

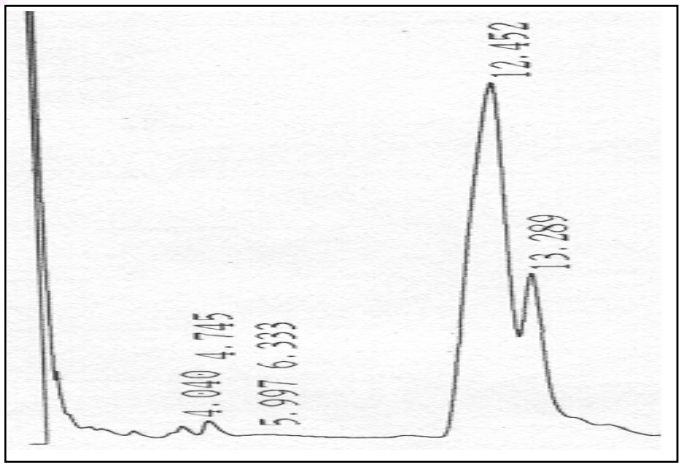


Fig. 6: GLC profile of native GG

The proximate composition of GG showed moisture content 9.1%, protein 5.3 %, ash 0.7% and dietary fiber 80.0%, which was in agreement with the literature data for the commercial food grade GG.

Non-specificity of enzymes towards guar galactomannan

A variety of commercially available enzymes, viz. carbohydrases (pectinase, pullulanase, hemicellulase and amyloglucosidase), proteases (pepsin and pronase) and lipases (wheat germ and *Candida cylindracea*) was initially screened for their catalytic activity on incubation with GG solution (Table 7).

Pectinase, pullulanase and pepsin showed a maximum catalytic activity towards GG followed by pronase. Whereas, both the lipases showed comparatively a very less activity, probably due to its esterase type of action rather than the hydrolase activity. Although hemicellulase, cellulase, amyloglucosidase belong to hydrolase class of enzymes, not much appreciable catalytic activity was observed, which might probably be due to varying inert filler ratios used in the commercial enzyme preparations. Pantaleone *et al.* (1992) reported higher specific activity values of some of these enzymes (pectinase, cellulase, lipase, papain, lactozyme and protease) to depolymerise chitosan and other aminoglycans. Yalpani *et al.* (1994) reported a more efficient chitosanolytic activity of pepsin and bromelain which could bring about 89 – 98% reduction in solution viscosity of chitosan, than commercial chitinase and lysozyme. A report by Zhang *et al.* (1999) also describes the higher hydrolytic rates of the mixture of enzymes (cellulase, amylase and a protease) towards chitosan.

Table 7. Activity of various enzymes towards GG and specific substrates

	Activity towards GG (Units)	Specific substrates	Units
Pectinase *(Aspergillus niger)*	3.01	Pectin	9.85
Pullulanase *(Bacillus acidopullulyticus)*	2.18	Pullulan	15.2
Porcine pepsin	1.58	Haemoglobin	530
Pronase	0.867	Casein	5.6
Wheat germ lipase	0.55	Triglycerides	22.5
Lipase *(Candida cylindracea)*	0.65	Triglycerides	20
Hemicellulase *(Aspergillus niger)*	0.92	LBG	8.5
Cellulase	0.752	Cellulose	5.0
Amyloglucosidase *(Rhizopus sps.)*	0.68	Starch	11.5

The decrease in solution viscosity of GG on co - incubation with enzymes is consistent with the reducing equivalents released, and indicated their non-specificity. A considerable change observed in the G/M ratio once again substantiated the non-specificity of these enzymes towards GG (Table 8). Kim *et al.* (2002) reported a similar time course of hydrolysis of GG by α - galactosidase obtained from cultured rice cells.

Table 8. Effect of enzymes on solution viscosity and galactomannan content of GG

	Viscosity (cps)	G/M
Pectinase *(Aspergillus niger)*	2500	1: 3.1
Pullulanase *(Bacillus acidopullulyticus)*	4850	1: 3.1
Porcine pepsin	4900	1: 2.5
Pronase	3800	1: 1.7
Wheat germ lipase	3900	1: 1.5
Lipase *(Candida cylindracea)*	3850	1: 1.7

The initial screening experiments however revealed a better catalytic efficiency of pectinase, pullulanase and pepsin towards GG. Hence, these three enzymes were selected for further in depth studies to understand their kinetic parameters and nature of products formed upon selective debranching of GG.

Cost effectiveness of non-specific modification of GG

The use of enzymes with non-specific activity (indicated by their activity data) for modification of GG is of commercial importance due to their inexpensive and easy availability. Although α-galactosidase showed a specific activity of 8 units towards guar galactomannan which is comparatively much higher than the other unrelated enzymes, it costs around Rs. 9,000 for 25 units. Nevertheless, pepsin showed an activity of only 1.8 units towards debranching of guar galactomannan. Inorder to achieve an activity similar to α-galactosidase the concentration of pepsin has to be increased by 6 folds. This increase in the enzyme concentration is compensated by its low cost which is around Rs.1855 (2500 units mg^{-1} protein) i.e. about 9 times lesser than the cost of α-galactosidase. Similarly pullulanase and pectinase, costing around Rs. 3,675≥(400 units mL^{-1}) and Rs. 2000 (5000 units) showed a non-specific activity of 2.18 units and 3.15 units, respectively, and to mimic the activity of α-galactosidase their concentration has to be increased by 4 and 3 folds, respectively, which is nevertheless compensated by their low cost. Such an approach in the modification of GG by inexpensive enzymes may result in cost effective replacement of LBG in various food and non-food applications.

Conclusion

The present study on screening several non-specific enzymes for their debranching action towards GG revealed an appreciable activity by pectinase, pullulanase and pepsin, which thus provided an alternative route for the production of modified GG. The unusual non-specific catalysis of these enzymes could be due to the catalytic site distinct from the usual one or due to the environment (change in pH) induced conformational changes in the enzyme. Nevertheless the

results are of interest since they open the prospects of commercial utilization of inexpensive enzymes for the modification of GG.

Though there are various reports on the possible usage of α-galactosidase in the production of modified GG, its application is rather limited due to its high cost and lengthy operational procedures. Instead, the non-specific activity exhibited by some of these unrelated enzymes may find great use in producing tailor made modified GG with enhanced functionalities, by suitably controlling the reaction conditions.

Chapter II

Purification and kinetic studies of a few selected enzymes having debranching activity towards GG

The Identification of various enzymes having multisubstrate specificities belonging to hydrolases rules out the concept of one enzyme to one substrate or group of related substrates. A report by Hashimoto *et al.* (1998) infers the action of β-glycosidases from *Bacillus* sp. *GLI to* cleave both α- as well as β- linkages in *p*-nitrophenyl-glycosides and positional isomers of β-1,4-glycopyranosyl linkages. Chitosanases from *Bacillus megaterium* have been reported to hydrolyse carboxymethylcellulose (Pelletier & Sygush, 1990). The β-1,3-/1,4-glucanase from *Bacillus circulans WL-12* has been reported to exhibit chitosanase activity (Mitsutomi *et al.*, 1998). The unique property exhibited by multifunctional enzymes may be a result of gene sharing, gene fusion and exon shuffling.

Based on the initial screening results of various enzymes for their non-specific mode of action towards GG, pectinase, pullulanase and pepsin were choosen for further detailed studies to understand their catalytic potential to cleave totally unrelated substrates. Since these enzymes were from the commercial sources it was felt desirable to purify them beyond ambiguity to eliminate the presence of other contaminants and inert fillers (Pantaleone & Yalpani, 1992).

This chapter mainly focuses on the purification of a few commercial enzymes, just to rule out the probability of contaminants in the enzyme preparation contributing for non-specificity. Also the kinetic parameters such as enzyme concentration, pH and temperature optima, K_m and V_{max} and stability studies for the maximum debranching of GG by the purified enzymes are also presented.

Materials

Sephadex G -100, Sephadex G -200, Coomassie brilliant blue R-250 and Trifluroacetic acid (TFA) were from Sigma Chemical Co., St. Louis, MO, USA. SDS-PAGE molecular weight markers was purchased from Bangalore Genie Pvt. Ltd., Bangalore, India. All other reagents and chemicals were of highest purity.

Methods

Purification of enzymes

Pullulanase

The crude enzyme solution (10 mg mL^{-1}) was loaded onto Sephadex G-200 column (100 cm length x 0.7 cm i.d.) equilibrated with 50 mM phosphate buffer of pH 7.4 and eluted at a flow rate of 12 mL hr^{-1} . The fractions (mL) were analyzed at 280 nm for protein and the one which showed catalytic activity towards both pullulan and GG was pooled, dialyzed and stored at 4°C for further assay.

Pepsin

The crude enzyme solution (10 mg mL^{-1}) was loaded onto Sephadex G-100 column (100 cm x 0.7 cm) and eluted with 50 mM acetate buffer of pH 5.6 at a flow rate of 12 mL hr^{-1}. The eluted fractions were analyzed at 280 nm for protein and the fraction which gave maximum proteolytic activity and also activity towards GG was pooled, dialyzed and stored at 4°C for further assay.

Homogeneity of the purified enzymes

Native & Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis (Native & SDS-PAGE)

The purity of the enzymes was ascertained by both Native and SDS-PAGE on vertical slab gel electrophoresis (Broviga mini electrophoretic unit, glass plate dimensions were 10.5 x 8.5 x 0.1 cm) at room temperature according to the method of Lamelli (1970).

Reagents:

(A) Monomer – 29.2 g of acrylamide + 0.8 g of bisacrylamide were dissolved in water (100 mL), filtered and stored in a brown bottle at 4°C.

(B) Running gel buffer – 18.15 g of Tris was dissolved in 100 mL water; pH was adjusted to 8.8 with 6N HCl and stored at 4°C.

(C) Stacking gel buffer – 3.06 g of Tris dissolved in 50 mL water, pH was adjusted to 6.8 with 6N HCl and stored at 4°C.

(D) Ammonium persulphate (15%) – 15 g in 100 mL water.

(E) Sodium dodecyl sulphate (SDS, 10%) – 10 g in 100 mL water.

(F) N, N, N', N'-Tetramethyl ethylene diamine (TEMED).

(G) Tracking dye – 2.5 mL stacking gel buffer + 4 mL distilled water + 2 ml glycerol + 1 mL 10% SDS containing 10% mercaptoethanol + 0.5 mL 1% (w/v) bromophenol blue (without SDS and mercaptoethanol for Native PAGE).

(H) Tank buffer – 0.3 g Tris + 1.44 g glycine dissolved in water and the volume was made up to 100 mL (pH 8.3).

Staining solution

Staining solution was prepared by dissolving 250 mg of Coomassie Brilliant Blue R-250 in 100 mL aqueous mixture of 45% methanol and 10% acetic acid, filtered and stored in a brown bottle.

Native-PAGE

Running gel (%T - 10) was prepared by mixing 2.0 mL solution [A] and 1.5 mL solution [B] with 2.4 mL distilled water. After degassing, 60 μL solution [D] and 15 μL [F] were added, mixed again and poured between the assembled glass plates having edges sealed with agar (1%). The gel was allowed to polymerize for 30 min.

For stacking gel (%T - 5), 0.35 mL solution [A] and 0.5 mL solution [C] with 1.23 mL distilled water was degassed. To this, 60 μL solution [D] and 15 μL [F] were added, mixed and poured onto the polymerized running gel. Immediately, comb was placed in the stacking gel (for well formation to load the protein samples) and allowed to polymerize for 30 min.

SDS-PAGE

While preparing both running and stacking gels, 60 μL 10% SDS was added prior to the addition of TEMED. Running gel (%T - 15) was prepared by mixing 3.0 mL solution [A] and 1.5 mL solution [B] with 1.5 mL distilled water, whereas for stacking gel, 1.17 mL distilled water taken instead of 1.23 mL as in case of native-PAGE. While performing SDS-PAGE, the tank buffer (100 mL) containing SDS (1 mL, 10%) was used.

Protein solutions (30 μg in 20 μL) mixed with 10 μL cocktail buffer without SDS for native and with SDS for SDS-PAGE were loaded into the well in stacking gel, subjected to electrophoresis at constant voltage (50 V) till the tracking dye just enters the lower tank buffer. After electrophoresis, protein bands were revealed by Coomassie Brilliant Blue staining. Excess dye was destained by keeping the gel in aqueous mixture of 5% methanol + 7% acetic acid. Protein markers were also run simultaneously to indicate Mw of the enzymes.

Capillary Zone Electrophoresis (CZC)

CZC was performed on Prince 550 system (Prince Technologies, Netherlands) with fused silica capillary column (80 cm l x 75 μm) connected to UV detector (280 nm) at 10 kV, 100 mbar, 26 ± 1^{o}C using Tris glycine buffer (pH 8.8). The enzyme solution (stock 10 mg mL^{-1}, ~10 nL) was injected under low - pressure hydrodynamic injection time of 2 sec. Data acquisition was performed using DAx software.

Reverse *phase High performance liquid chromatography (Rp - HPLC)*

Rp-HPLC was performed on Shimadzu LC-8A system connected with C -18 (ODS) column (Shimpak, 250 x 4.5 mm) precalibrated with mobile phase (solvent A - 0.1% trifluroacetic acid, TFA). The sample (20 μL) was injected into the column followed by gradient run consisting of 0% solvent B (70: 30 acetonitrile: water with 0.05 % TFA) traversing to 100% in 90 min at a flow rate of 1.0 ml min^{-1}. The elution was monitored spectrophotometrically at both 230 and 280 nm.

Kinetic studies

Effect of enzyme concentration

Optimum enzyme concentration towards debranching of GG was determined by the addition 1 mL of 10-200 µg of enzyme to 1mL of guar solution (0.5%) at 37°C and assaying the reaction products released after 60 min by the ferricyanide method.

Determination of temperature optima and stability

Optimum temperature

Effect of temperature on enzyme activity towards GG was studied on co-incubating the enzyme and GG at various temperatures (20° to 60°C) followed by assaying the reducing sugar released after hydrolysis. Temperature optimum was obtained by the plot of relative activity (%) versus temperature.

Temperature stability

Temperature stability was studied by pre-incubating the enzyme at different temperatures (20° to 60°C) for varying time intervals (0 - 540 min) prior to the enzyme assay. Different aliquots were removed at regular time intervals and assayed for the residual activity at optimum conditions.

Determination of pH optima and stability

pH optima

Enzyme activity as a function of pH was studied on co-incubation of enzyme and GG at various pHs (range 3.0 - 8.0) followed

by assaying the products released by reducing sugar assay. pH optimum was obtained by the plot of relative activity (%) versus pH.

pH stability

pH stability was studied by pre-incubation of the enzyme at different pHs for 0 - 540 min prior to the enzyme assay. Aliquots were removed at regular time intervals and assayed for the residual activity at optimum conditions.

K_m *and* V_{max}

By varying the substrate concentration between 1 and 10 mg ml^{-1}, the effect of substrate concentration on enzyme activity was determined, whereas the apparent K_m (substrate concentration at half the maximal velocity) and V_{max} (maximum velocity) were calculated from the double reciprocal plot (Lineweaver - Burk) of the initial velocity versus substrate concentration.

Results and Discussion

Criteria of enzyme purity

The native PAGE (Fig. 7) of pepsin (lane1) and pullulanase (lane 2) showed a major band contributing to ~80 % of protein, along with the presence of a few minor bands (~ 20 %), whereas pectinase (lane 3) showed the presence of three major bands. The appearance of minor bands in the former could be due to the minor contaminants in the commercial enzyme preparations or may be due to self degradation, since enzymes such as pepsin on long storage exhibits the phenomenon of auto-catalysis.

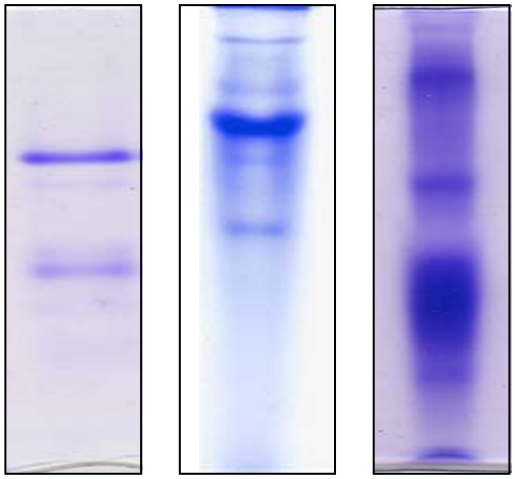


Fig. 7: Native PAGE of commercial enzymes: **Lane 1:** pepsin (%T - 12), **Lane 2:** pullulanase (% T-7.5) and **lane 3:** pectinase (% T- 10)

Thus, in order to rule out the possible contribution of these minor bands for their non-specific action towards guar galactomannan, the crude enzymes were subjected to partial purification by GPC. Fig. 8 represents the GPC profile of pepsin on Sephadex G-100, which revealed a single protein peak (see Table 9) having maximum debranching activity on GG.

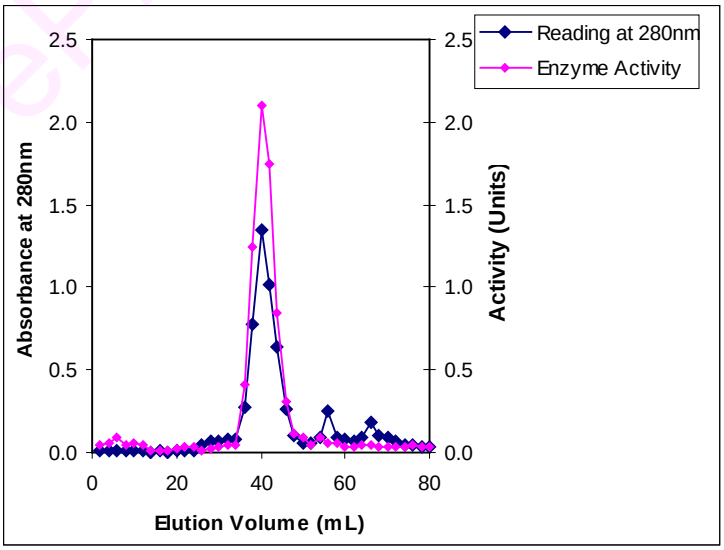


Fig. 8: GPC profile of pepsin

Similarly Fig. 9 represents the GPC profile of pullulanase, which showed one major peak (over 95 %) along with two very small peaks (Table 9). The major protein peak, nevertheless showed maximum debranching activity towards GG

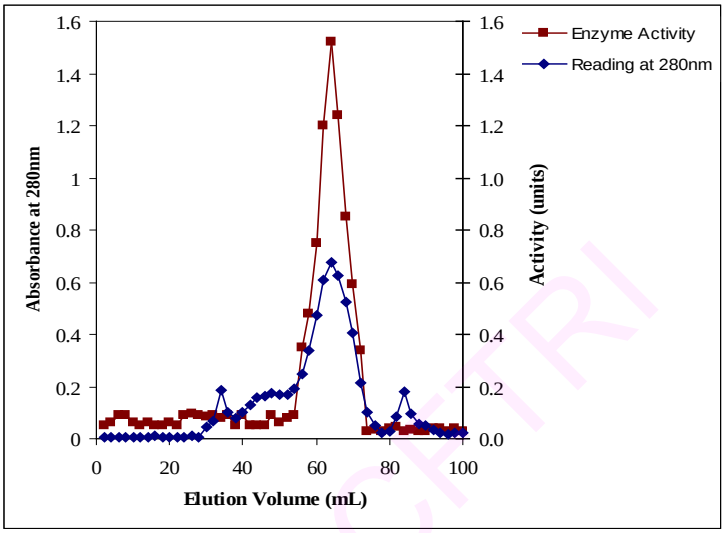


Fig. 9: GPC profile of pullulanase

The eluted fractions analyzed at 280 nm for the proteins and also the enzyme assay (Table 9) of the individual peaks revealed only the major peak to have activity for both the substrates.

Table 9: Enzyme activity of the pooled fractions

Enzyme	Fraction number	Specific substrate (units)	GG (units)
Pepsin	18-24	700	1.8
	28-29	-	-
	32-34	-	-
Pullulanase	20-24	-	-
	26-27	-	-
	30-36	9.9	2.1
	41-43	-	-

The GPC purified enzymes on both native PAGE as well as SDS - PAGE (Fig. 10A & B) showed the presence of a single band which corresponded to the earlier major band of crude enzymes, thus ruling out the involvement of contaminants in its non-specificity. This evidence proved beyond ambiguity that the major protein is responsible for the non-specific activity towards GG. The molecular weight of the purified enzymes, as calculated by comparison with the standard marker proteins (plotting log Mw of marker proteins versus relative mobility i.e. distance traversed by the dye / standard protein) were in concordance with their reported molecular weight.

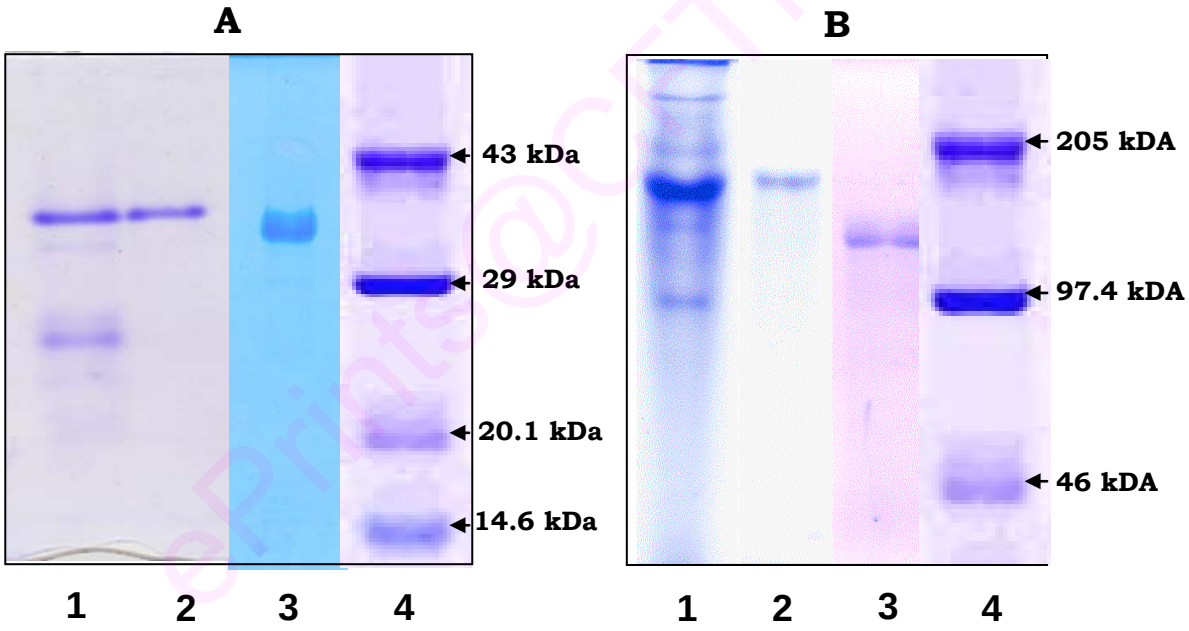


Fig. 10: A. Native PAGE of crude pepsin (Lane 1) and purified pepsin (Lane 2), SDS-PAGE of purified pepsin (Lane 3), protein markers (Lane 4)

B. Native PAGE of crude pullulanase (Lane 1) and purified pullulanase (Lane 2), SDS-PAGE of purified pullulanase (Lane 3), protein markers (Lane 4)

Purity of the enzymes was also confirmed by capillary zone electrophoresis and Rp-HPLC (Figs. 11 and 12). Appearance of a single peak also subscribed to the homogeneity of the purified enzymes.

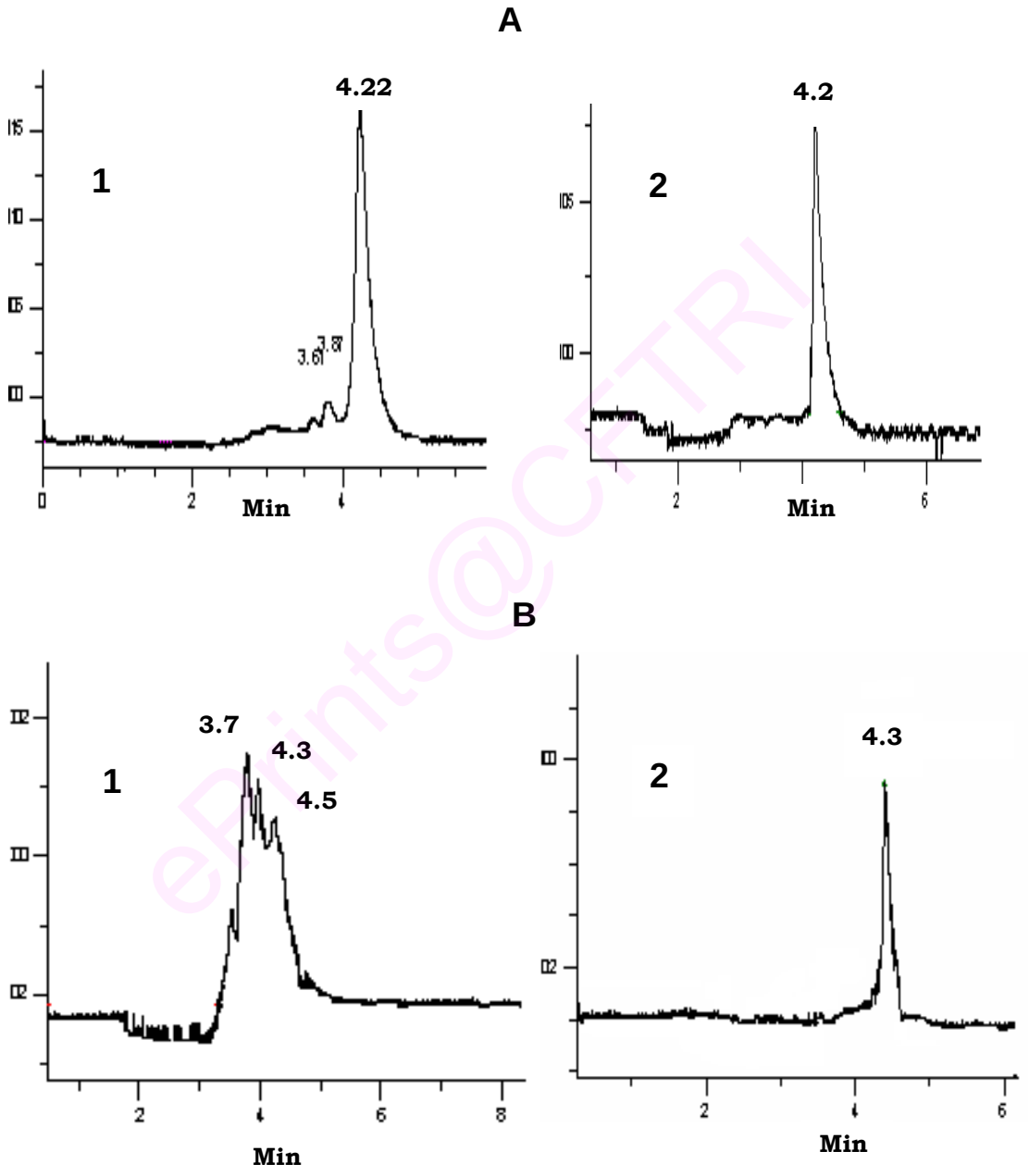


Fig. 11: Capillary zone electrophoresis of

A: 1. crude pepsin, 2. purified pepsin

B: 1. crude pullulanase, 2. purified pullulanase

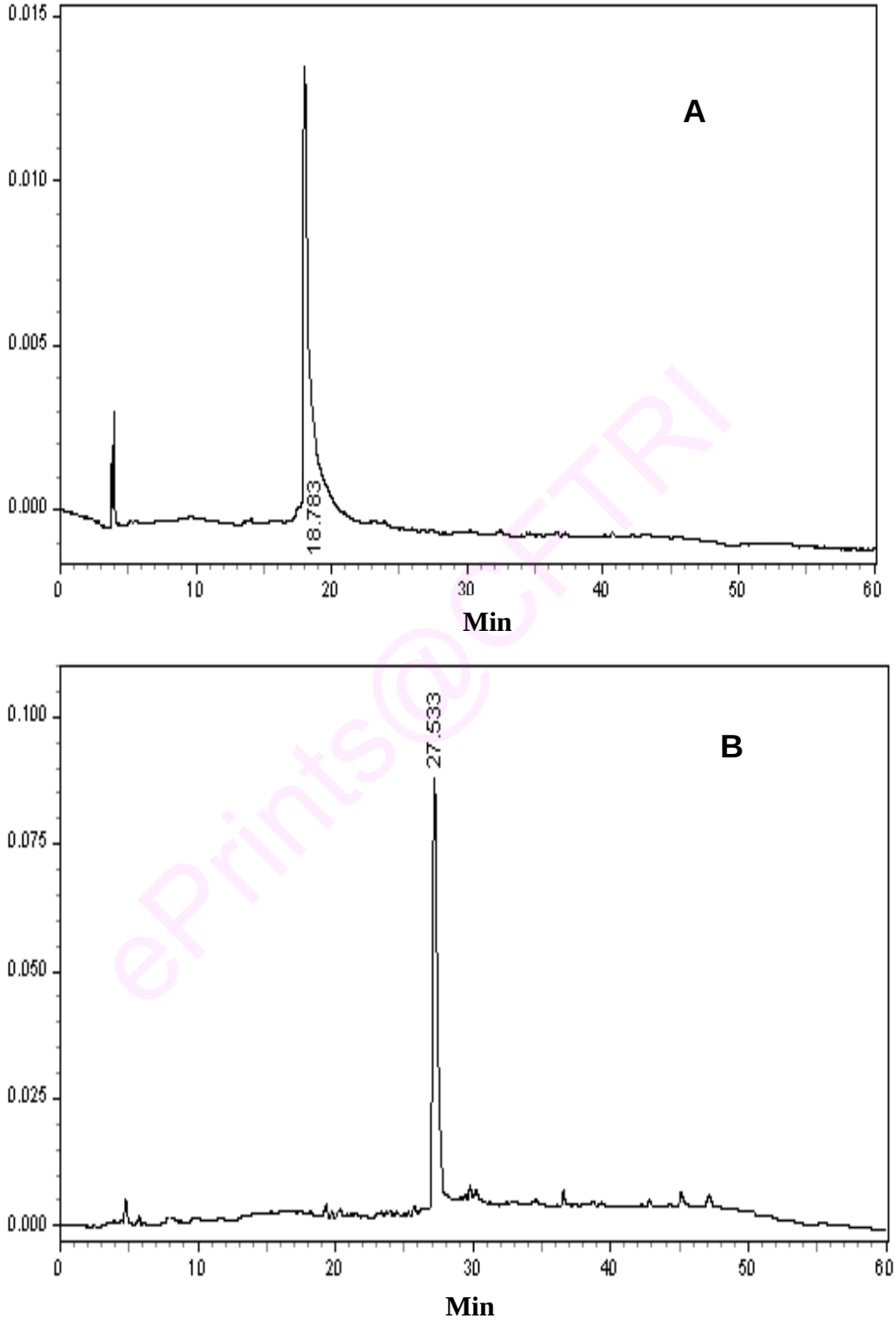


Fig. 12: Rp - HPLC profile of **A:** purified pepsin **B:** purified pullulanase

Whereas pectinase upon Native PAGE was resolved into four distinct bands whose zymogram analysis using the specific substrate (i.e. citrus pectin) showed association of pectinase activity with all the bands, indicating them to be isozymes (Fig. 13). Zymogram analysis using GG also revealed the association of all the isozymes in the catalytic activity. Therefore, without further purification pectinase was used to study its catalytic - kinetic parameters towards GG.

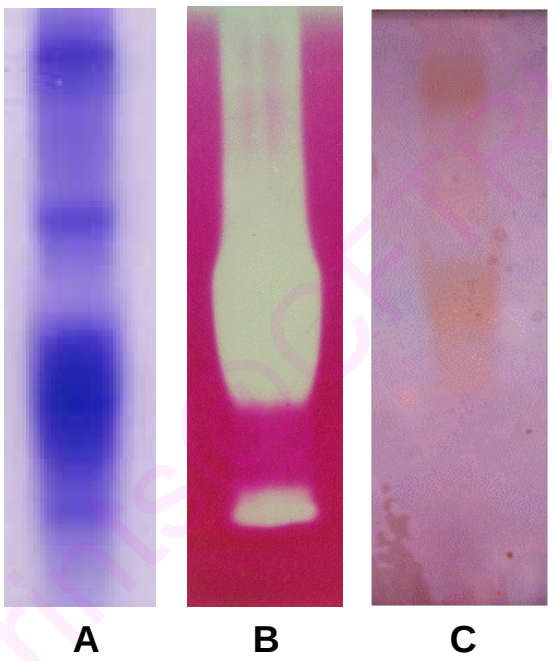


Fig. 13: Native PAGE of commercial pectinase (A) and its zymogram analyses on GG (B) and pectin (C) as substrates

Kinetic studies

Effect of enzyme concentration

Fig. 14 depicts the effect of enzyme concentration on debranching of GG. The rate of catalysis was directly proportional to the enzyme concentration (determined by the increase in reducing

equivalents released) with the conclusion that debranching of GG by these non - specific enzymes appears to follow Michaelis-Menton kinetics (Nordtveit *et al.*, 1994). Both pectinase and pepsin followed first order reaction kinetics upto a concentration of 100 µg, beyond which they were of second and zero orders and hence for further study, an enzyme : substrate ratio of 1: 100 was employed. Whereas pullulanase showed complete saturation at an enzyme: substrate concentration of 1:50, respectively.

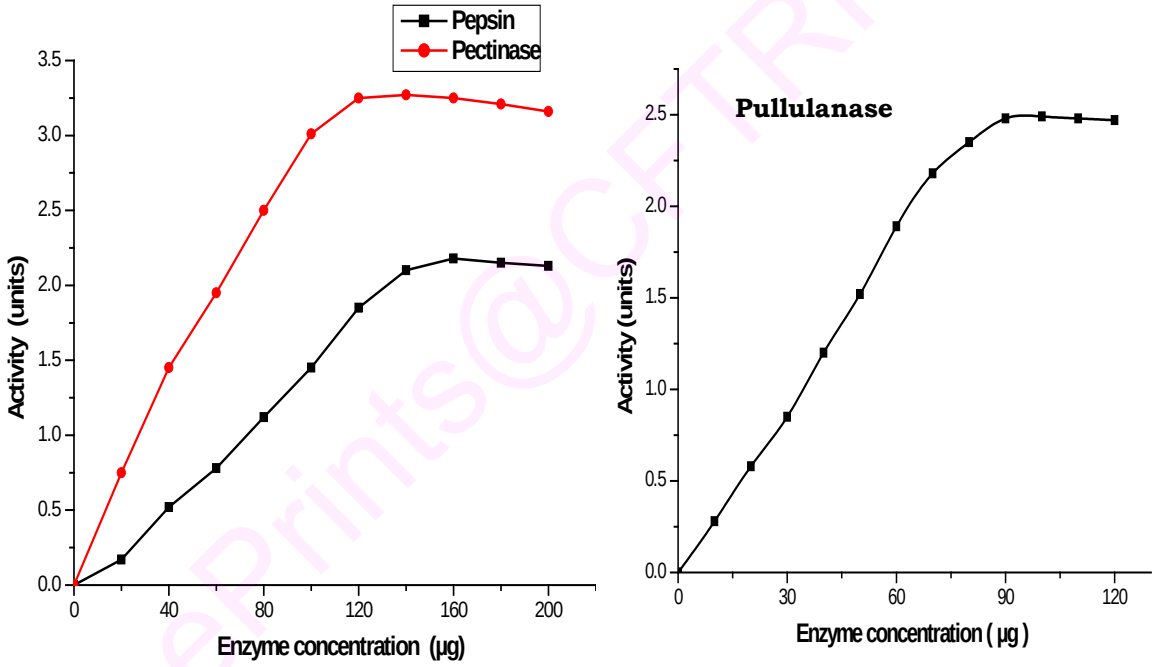


Fig. 14: Effect of enzyme concentration on debranching of GG

Effect of temperature and pH on enzyme activity

Fig. 15 reveals the non-specific activity of the enzymes towards GG as a function of temperature. The two carbohydrases namely pullulanase and pectinase showed maximum catalytic activity at a

temperature of 45°C and 50°C, respectively, whereas pepsin, a protease had a temperature optimum of 40°C.

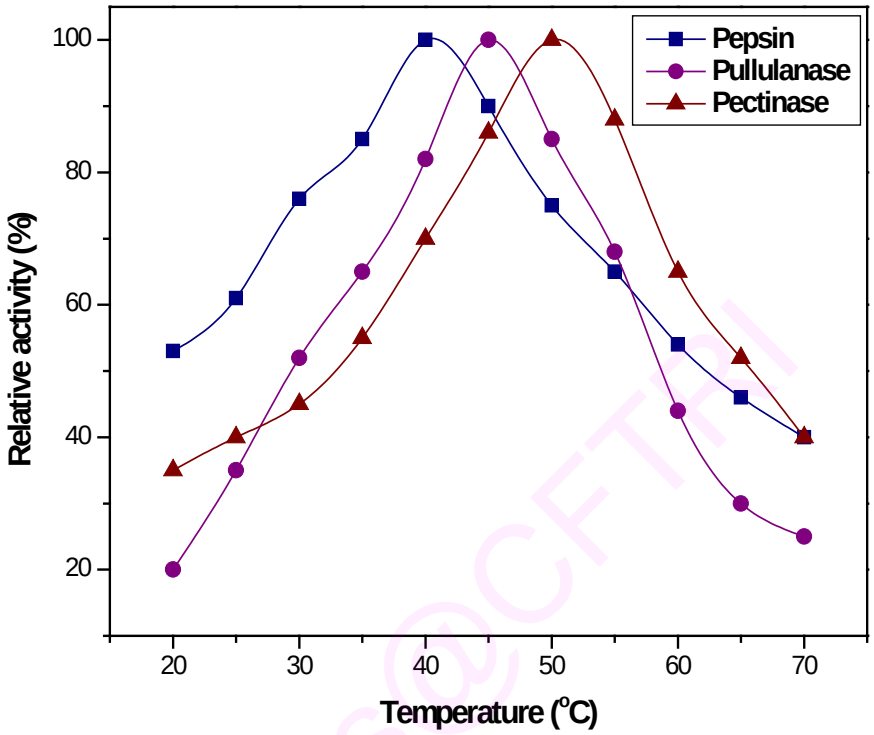


Fig. 15: Effect of temperature on enzyme activity towards GG

Fig.16 represents the pH profile of the enzymes with non-specific activity towards debranching of GG. Pullulanase and pectinase showed maximum activity at pH 4.5 and 5.0, respectively. The catalytic action of pepsin towards GG was exhibited at pH optimum of 5.5. Table 10 shows the differences in the pH optima of enzymes for their specific and non-specific activities. The shift in the pH optima of these enzymes could be attributed to the conformational changes that the enzymes undergo during its dual catalysis (Vishu Kumar & Tharanathan, 2004). Mill *et al.* (1961) reports two different pH optima for an endopolygalacturonase from *Aspergillus niger*.

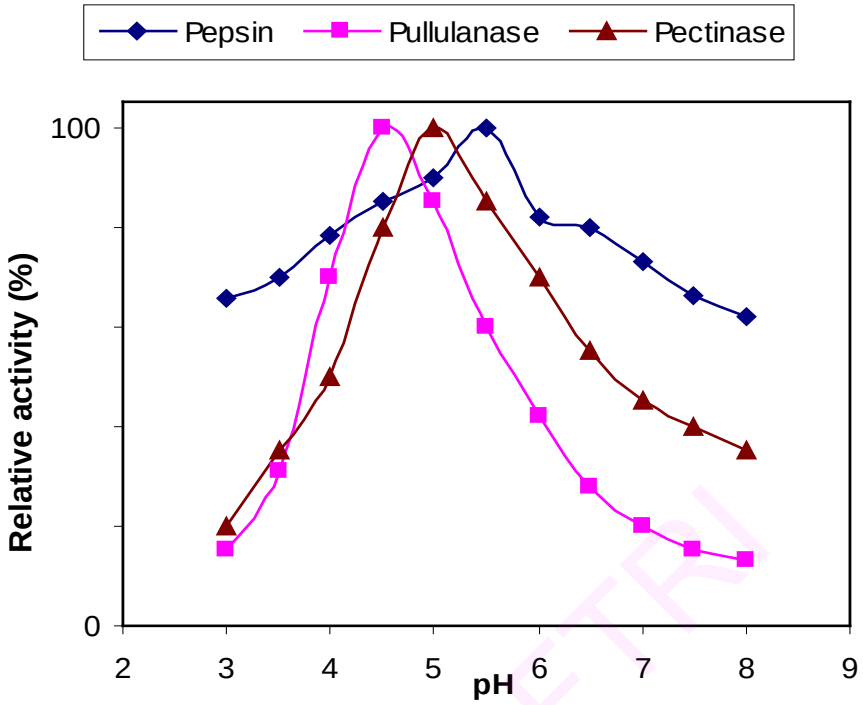


Fig. 16: Effect of pH on enzyme activity towards GG

Table 10: pH and temperature optima of enzymes towards specific and non-specific substrates

Enzyme	**pH optimum**		**Temperature optimum (°C)**
	Specific substrate	**GG**	**GG**
Pepsin	1.5 (Haemoglobin)	5.5	45
Pullulanase	5.0 (Pullulan)	4.5	40
Pectinase	4.0 (Pectin)	5.0	50

Campos & Sancho (2003) also describe the pH dependent conformational changes of pepsin between pH 4.5-6.0. The change in pH may slightly expand the protein allowing the compact active site to stretch, a common process under acidic conditions which probably occurs due to the non-specific long range interaction between different protanated groups (Goto *et al.*, 1989). Such conformational changes at acidic pH from a largely unfolded state to an intermediate conformational state have been reported for cytochrome C (Ohgushi *et al.*, 1983) and human interferon γ (Arakawa *et al.*, 1987) which appears to be a general property of many proteins.

Effect of substrate concentration

GG concentration of 10 mg mL^{-1} was taken for all these studies, as at higher concentration the viscosity of the guar solution became a limiting factor, as it restricted the penetration of enzyme resulting in reduced activity. Fig. 17 describes the influence of varying GG concentration on the activity of enzymes which obeyed Michaelis-Menton kinetics by displaying hyperbolic curve on plotting substrate concentration versus activity.

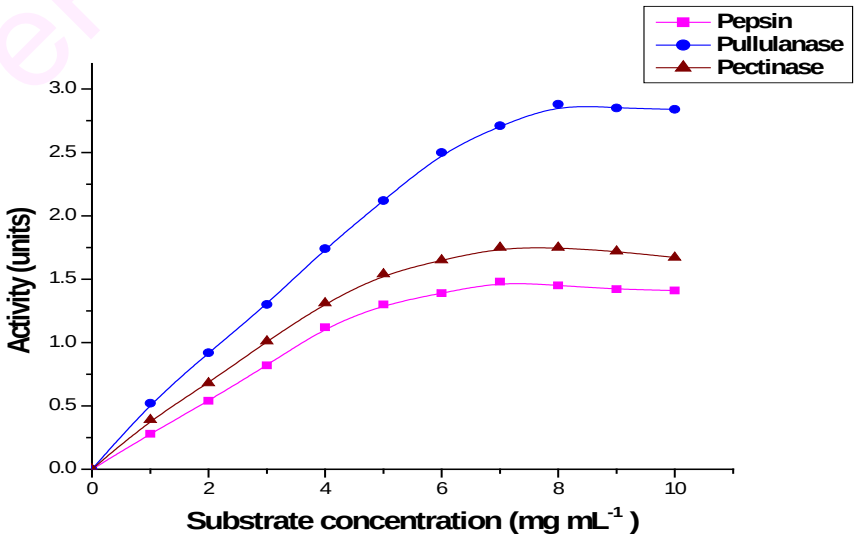


Fig. 17: Effect of GG concentration on enzyme activity

A decrease in the catalytic efficiency of pepsin, pullulanase and pectinase was observed for GG concentration beyond 6 mg mL^{-1}, which may be due to product or substrate inhibition. The former was ruled out by incubation of the enzymes with galactose and mannose (products of catalysis) for 30 min prior to the assay, where no appreciable change in the degree of hydrolysis was observed. This fact substantiated the susceptibility of enzymes to substrate inhibition. Several mechanisms have been proposed to explain substrate inhibition of various enzymes (Cleland, 1979). The phenomenon of substrate inhibition can be rationalized by assuming that a second substrate molecule binds non-productively to the active site thereby inhibiting the productively bound one (Sannchez - Ferres *et al.*, 1993).

K_m and V_{max} in enzymatic catalysis

The Michaelis constant K_m is the concentration of substrate at which half the active sites are filled thus providing a measure of substrate concentration required for the occurrence of significant catalysis. The K_m value of an enzyme also depends on the nature of substrate and environmental conditions such as pH, temperature and ionic strength. K_m also signifies the affinity of enzyme towards substrate suggesting that lower the K_m higher is the affinity thereby affecting the catalytic efficiency of the enzyme. V_{max}, another Michaelis parameter reveals the turnover number of an enzyme, denoting the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with the substrate.

Difficulties in the accurate measurement of maximum velocity (V_{max}) directly from the plot of substrate concentration (S) versus initial velocity (Vo) lead to an algebraically transformed graphical

representation by Lineweaver-Burk (double reciprocal) plot in which 1/[S] was plotted against 1/[Vo] that was useful in the practical determination of K_m and V_{max}. Of the two carbohydrases (Fig. 18 and Table 11) pullulanase showed K_m and V_{max} of 6.0 mg mL^{-1} and 1820±8.8 nmoles min^{-1} mg^{-1}, respectively,

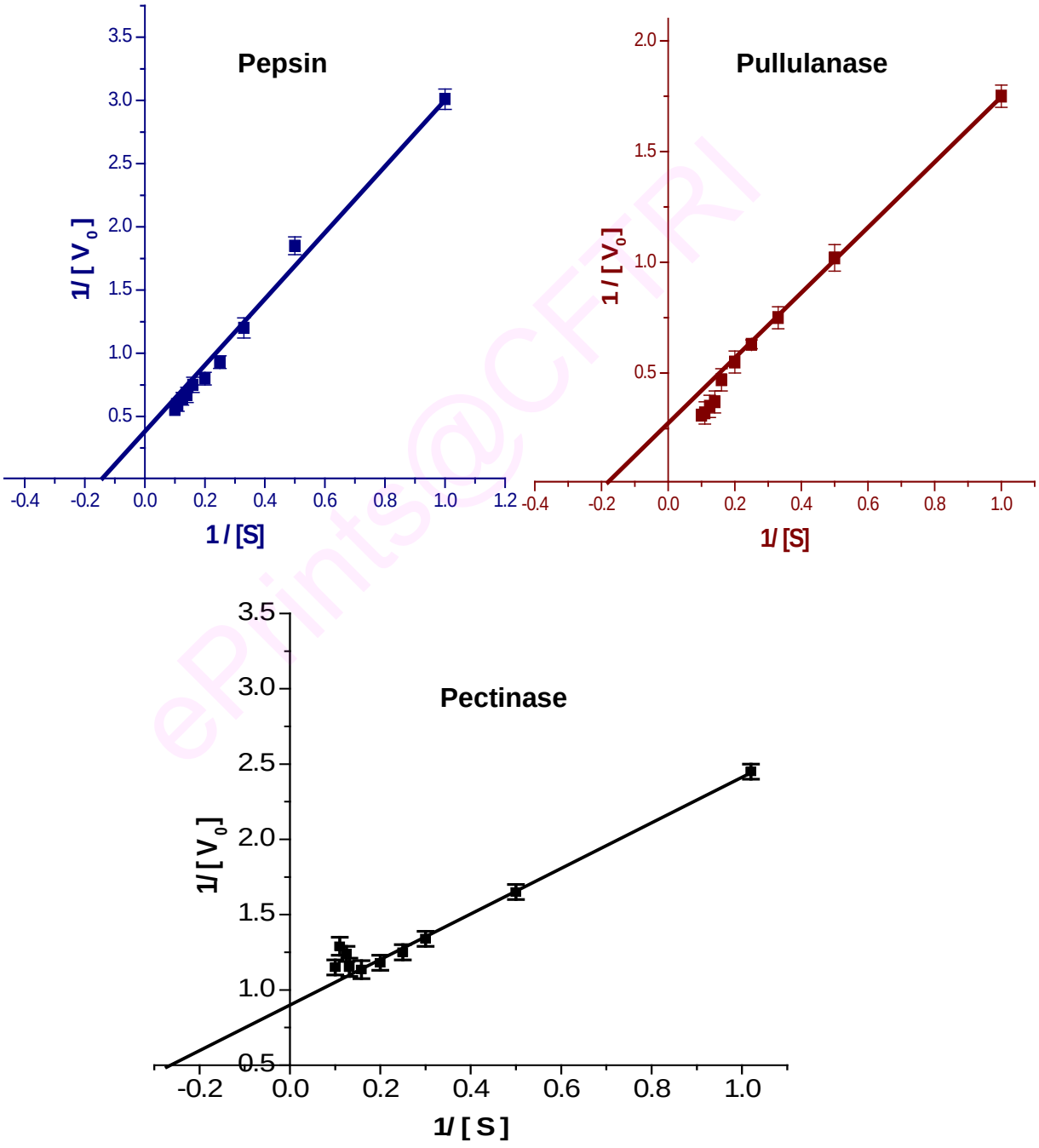


Fig. 18: Lineweaver-Burk (Double reciprocal) plot

whereas pectinase showed a K_m of 4.2 mg mL^{-1} and V_{max} of 3120±37.11 nmoles min^{-1} mg^{-1}, suggesting a higher affinity of this enzyme towards GG. The proteolytic enzyme pepsin displayed a K_m and V_{max} of 5.0 mg ml^{-1} and 2790±32.8 nmoles min^{-1} mg^{-1}, respectively.

Table 11: K_m and V_{max} of enzymes towards GG

Enzyme	**K_m (mg mL^{-1})**	**V_{max} (nmoles min^{-1} mg^{-1} protein)**
Pepsin	5.0	2790±32.8
Pullulanase	6.0	1820±8.8
Pectinase	4.2	3120±37.11

pH and temperature stability

Figs. 19 and 20 describe the pH and temperature stability of pepsin, pullulanase and pectinase. From Fig. 19 A & B it was inferred that both pepsin and pullulanase were stable upto 5 hr by retaining ~ 90-95% activity, beyond which there was a decline in the activity indicating the maximum stability of these enzymes at pH 5.5 and 4.5, respectively. Temperature stability studies (Fig. 20 A & B) of pepsin and pullulanase indicated maximum stability of the enzymes at 45°C and 40°C, respectively, for about 5 hr above which a decline in the activity was observed. Whereas preincubation of pectinase (Fig. 19 C) at different pH indicated that the enzyme is stable at pH 5.0 for about 2 hr beyond which their was a decrease in the activity. The

temperature stability (Fig. 20 C) study of pectinase also indicated that the enzyme was stable upto 50 °C for 2 hr after which there was 50% reduction in the activity.

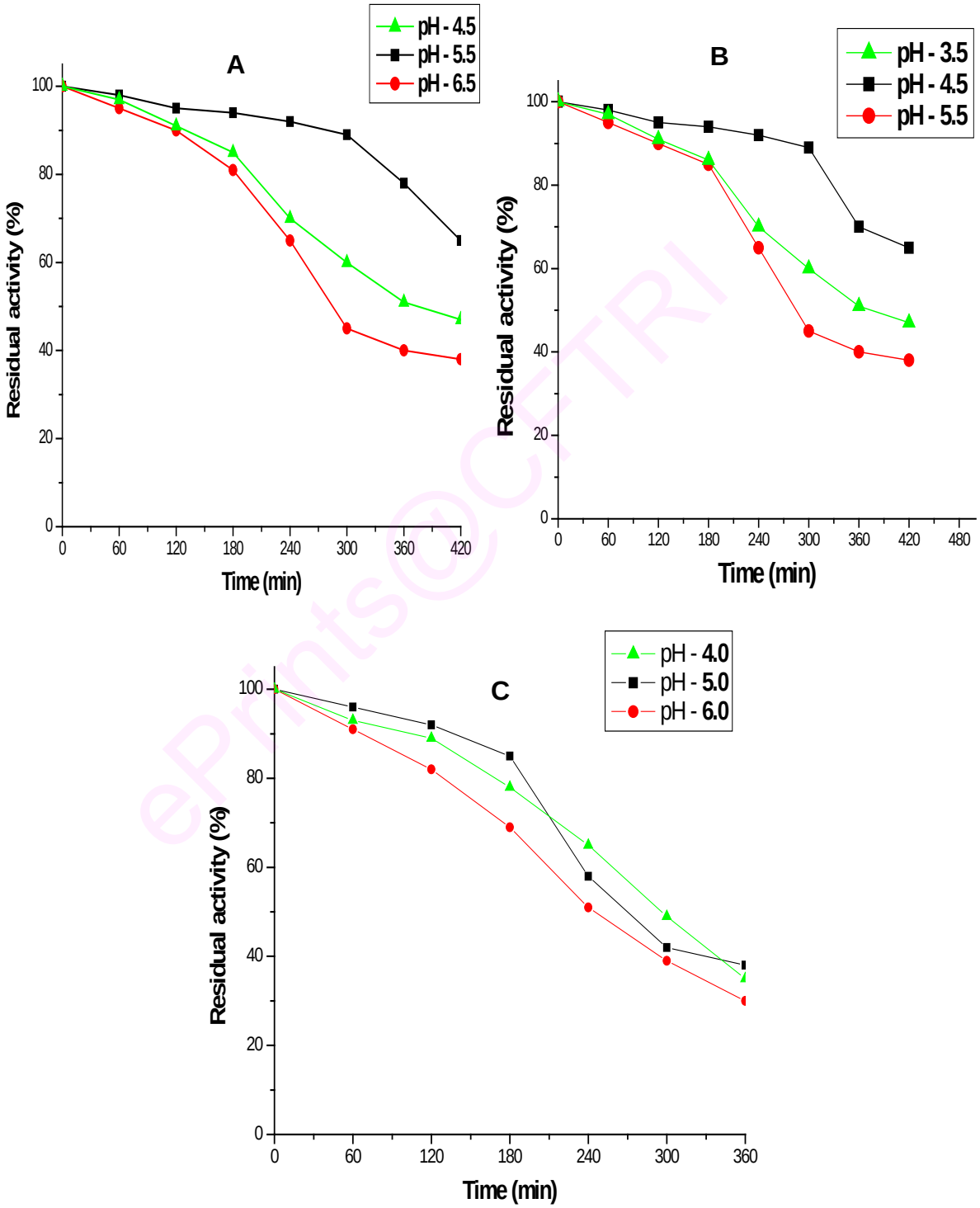


Fig. 19: pH stability of **A:** pepsin, **B:** pullulanase and **C:** pectinase

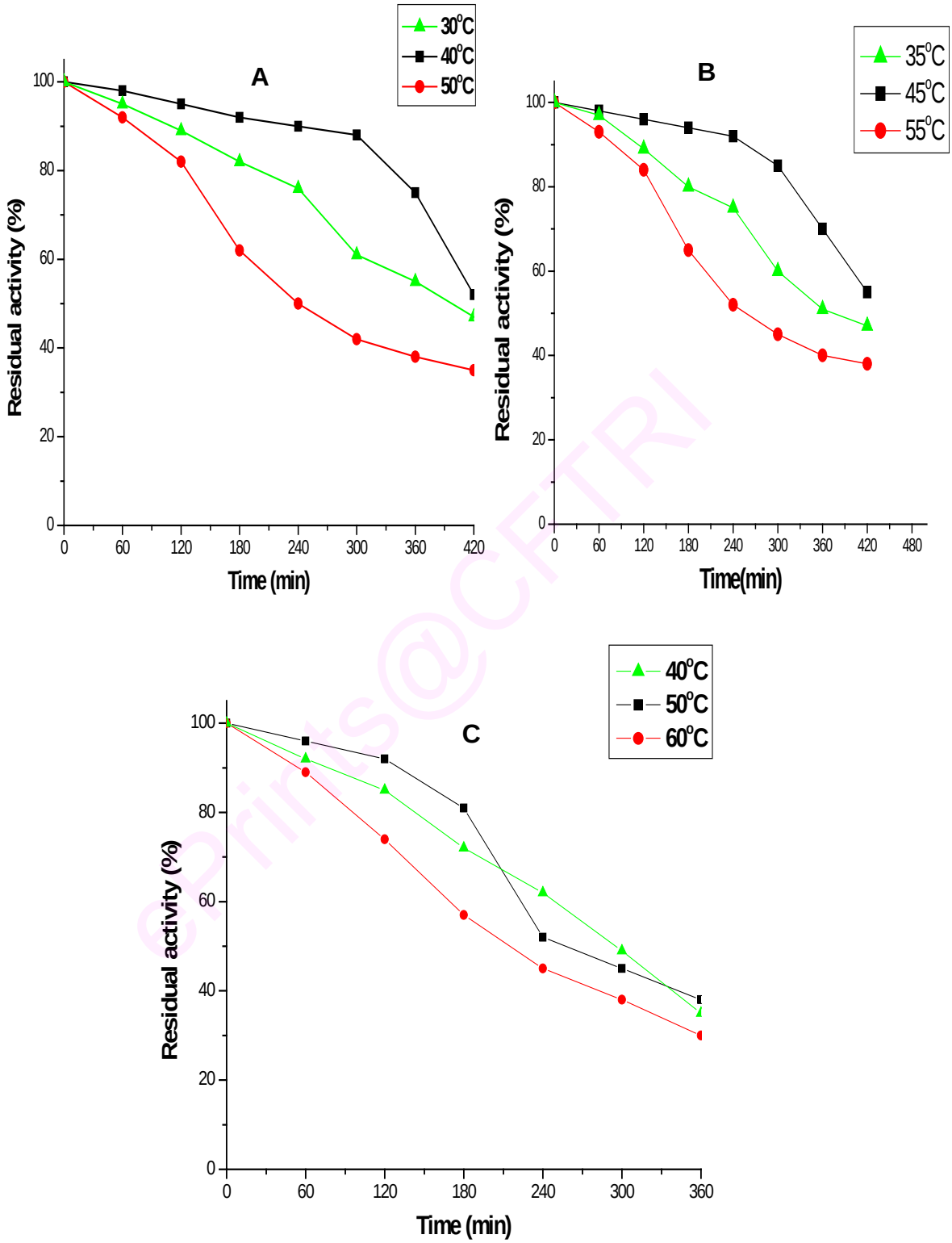


Fig. 20: Temperature stability of **A:** pepsin, **B:** pullulanase and **C:** pectinase

Conclusion

The present investigation involving the non-specific action of pullulanase, pepsin and pectinase towards debranching of GG establishes the affinity of a single protein towards both specific substrate and GG. The presence of contaminants in the enzyme preparation thus contributing for non-specific catalysis was ruled out by using GPC - purified enzyme preparations. The kinetic studies of purified enzymes also showed differences in their pH and temperature optima, which indicated the pH dependent conformational changes in the enzymes, thus estabilishing their dual role in catalysis. The effect of varying substrate concentration on initial velocity by enzymes obeyed Michaelis-Menton kinetics.

Chapter III

Production of galactose - depleted guar galactomannan (GDGG) and characterization of resultant products

GG derived from the seeds of endospermic leguminous plant *Cyamopsis tetragonalobo,* finds wide spread applications based on its ability to thicken and stabilize many food products, as functional food ingredients and also as a source of dietary fiber (Stephen & Churms, 1995; Greenberg & Salvin, 2003). In pharmaceutical sector its functional properties are of importance for controlling the release of drugs to gastrointestinal tract (Chourasia & Jain, 2004; Krishnaiah et al., 1998) and for transdermal drug delivery system (Murthy et al., 2004). Also GG is the material of choice in various other industries like textile (Kokol, 2002), oil recovery (Ebinger, 1989), mining, cosmetics (Glasser, 2000), etc. Inspite of various functionalities, its high viscosity due to high degree of substitution by galactose residues restricts its wide spread application potential. Therfore modification of GG by the selective removal of galactose residues is advantageous for its diversified application in various food and non-food industries.

The structural modification of GG using α-galactosidase enzyme has been widely studied for its extended range of industrial applications (Cheng & Prud'homme, 2000; Tayal et al., 1999). The α-galactosidase treated GG forms a hydrogel on complexation with helical polysaccharides like xanthan, which is of considerable interest in food and pharmaceutical applications (Chidwick et al., 1991). Yamatoya (1994) showed the enzyme-modified guar gum with low molecular weight exhibiting beneficial effect as water - soluble dietary fiber.

This chapter deals with the production and characterization of GDGG obtained by treatment with some selective (non-specific) enzymes. Also the characterization of the monomeric and oligomeric products released as a result of hydrolysis by enzyme treatment is described in this chapter.

Materials

β-Mannanase (From A.niger, EC. 3.2.1.25), β-Mannosidase (From Snail acetone powder, EC. 3.2.1.25), α-Galactosidase (From Coffee bean, EC. 3.2.1.22), Potassium Bromide (KBr) were from Sigma Chemical Co., St. Louis, MO, USA. BioGel - P2 (Biorad) and T series Dextran standards were procured from Pharmacia (Switerland). All other reagents and chemicals were of highest purity.

Methods

Isolation of galactose - depleted guar galactomannan (GDGG)

GG solution (0.5%) together with purified enzyme in the enzyme: substrate ratio of 1:100 (pepsin and pectinase) and 1:70 (pullulanase) (w/w) was incubated for different periods (1 to 10 hr) at optimum conditions followed by termination of the reaction by heating and adding 3 volumes of ethanol. After centrifugation (10,000 rpm, 15 min) the GDGG sediment was washed thoroughly with alcohol and freeze dried. The supernatant was concentrated and freeze dried.

Characterization of GDGG

The molecular mass (Mw) of GDGG was studied by two techniques, namely a) viscometric and b) GPC methods

Viscometric method

Viscosity of GDGG dissolved in water (0.5 %) was measured using Ostwald viscometer. The average molecular mass was calculated using Mark-Houwink's equation, $\eta = K \times (\text{molecular mass})^{\alpha}$, where η = intrinsic viscosity, K = 3.04 and $\alpha = 0.747$.

GPC

GDGG solution (1mL, 0.5%) was loaded onto Sepharose CL-4B (Bed volume, 120 mL) and eluted with water at a flow rate of 18 mL hr^{-1}. Each fraction was analyzed for total sugar by phenol-sulphuric method. The column was calibrated with T-series dextran standards.

Gas liquid chromatography

The galactomannan content of GDGG was evaluated, after acid hydrolysis followed by derivatization into alditol acetate, by GC on OV-225 (3% on Chromosorb W) column connected to Shimadzu 8A equipped with flame ionization detector**.**

Viscosity measurement

Viscosity of GDGG (1%) dissolved in water was measured using Brookefield viscometer model RV 11 at 20 rpm with spindle No. 5 at room temperature.

Infrared spectroscopy

IR spectral studies were done on Perkin-Elmer 2000 spectrometer under dry air at room temperature after pelletization with KBr. GDGG (3 mg) was mixed thoroughly with 200 mg of KBr of which 40 mg was pelletized for taking spectrum between 4000 and 200 cm^{-1}.

Solid-State CP- MASS ^{13}C-NMR

^{13}C-NMR spectra of galactomannans were recorded on a Bruker dsx_{300} spectrometer (Karlsruhe, Germany) with the utilization of cross polarization pulse sequence for all the samples. About 300 mg of dry

powder placed in a ceramic rotor was spun at the magic angle of 5 to 7.5 kHz, with the accumulation of more than 2000 scans at constant time of 2 ms and a pulse (repetition) time of 5 ms for each measurement.

Characterization of monomeric products

GPC

The oligomeric-monomeric mixture was subjected to GPC on Biogel P2 column (100 x 0.8 cm, bed volume, 80 mL, Bio Rad laboratories, CA) equilibrated with water. The column was pre-calibrated with standard oligomers and monomers.

HPLC

The supernatant from the enzyme digest was concentrated by flash treatment and studied by HPLC on a precalibrated (using galactose and mannose, 10 µL) µ-Bonda pack aminopropyl column (4.1 mm x 300 mm) using acetonitrile: water (75:25) mixture as mobile phase at a flow rate of 1 mL min^{-1} and RI Detector.

Matrix assisted laser desorption ionization – Time of flight – Mass spectrometry (MALDI-TOF-MS)

The oligomers were subjected to MS on a compact analytical SEQ MALDI-TOF-MS (Kratos, UK) with a nitrogen laser at 337 nm wavelength and 5 ns pulse width. The laser beam was focused onto the sample at an angle of 45^{o}. Ions were accelerated to an energy of 3 kV before entering the spectrometer. At the detector, ions were post accelerated to a maximum kinetic energy up to 30 kV for more efficient detection. The matrix was prepared by dissolving 2, 5-dihydroxybenzoic acid (1.0 g dL^{-1}) in 10% ethanol-water solution. The oligomers (0.1 g dL^{-1}) were prepared in triple distilled water and then

diluted with matrix solution (1: 10, v/v). NaCl solution (1μL, 0.1 M) was added to the sample-matrix mixture to increase the yield of cationized species. An aliquot of the resulting mixture (1 μL) was placed on a piece of silver plate; the solvent was removed by gentle steaming and the sample-matrix mixture was transferred into vacuum chamber of the mass spectrometer.

Results and Discussion

Characterization of GDGG

The decrease in Mw of GDGG as determined by viscometry, GPC and HPSEC was in good agreement with each other (Table 12). A steady decrease in Mw of GDGG was observed with the extended incubation time indicating an enhanced activity of the enzymes.

Table 12. Mw of GDGG

Enzyme	Reaction time (hr)	Mw, kDa		
		Viscometry	GPC	HPSEC
Native guar galactomannan		240	239	242
Pullulanase - GDGG	1	223	220	228
	3	184	184	185
	5	152	150	152
	7	110	113	112
Pepsin-GDGG	1	230	231	230
	3	200	205	210
	5	165	164	164
	7	130	129	131
Pectinase - GDGG	0.15	150	149	151
	0.30	110	105	110
	0.45	90	92	90
	1.0	70	71	71

Appearance of a single peak in GPC and HPSEC indicated the homogeneity of GDGG (Fig. 21). Pullulanase and pepsin showed a gradual decrease in Mw of GDGG as their action continued along with time from 1 to 7hr, whereas a sharp decrease in Mw occurred in the initial hours of reaction with pectinase indicating its endo-action (see Table 12).

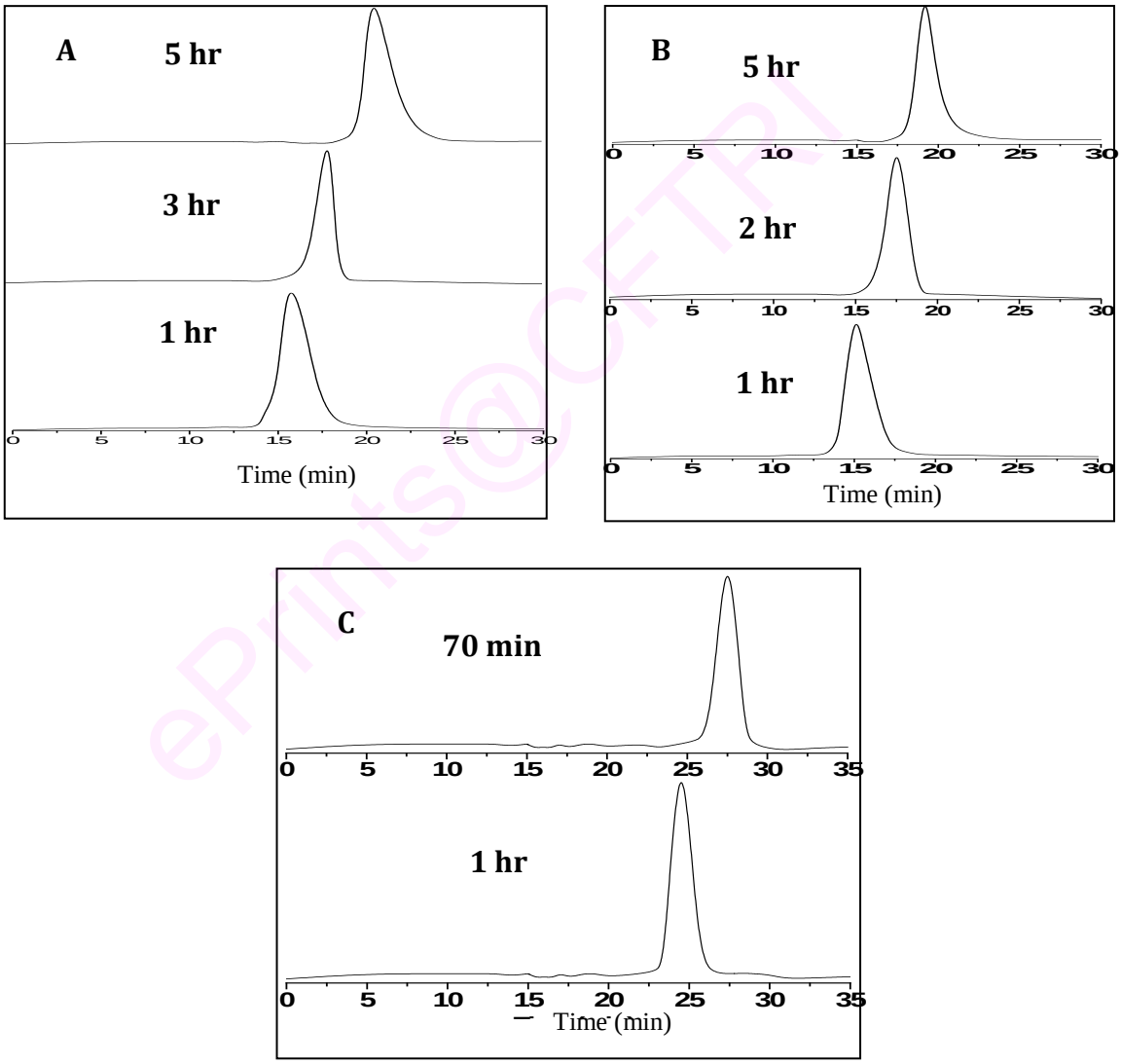


Fig. 21. HPSEC profiles of GDGG derived from **A.** pullulanase, **B.** pepsin, **C.** pectinase action

Galactomannan content (G/M ratio)

The changes in the G/M ratio (Fig. 22) of GDGG as determined by GC are given in Table 13. Extended action (1-8 hr) of pullulanase on GG could bring out a sequential removal of galactose residues with GDGG (5 hr) showing an increased G/M ratio of 1: 3.8, almost akin to LBG. Shibuya et al. (1999) reported the enzymic depletion of galactose from guar galactomannan by *Mortierella vinacea* α-galactosidase II resulting in an increased G/M ratio of 1:3.3. Subsequently, though the debranching process of GG continued the GDGG obtained after 7 hr was almost devoid of galactose residues, and it had a G/M ratio of 1: 5.1, which means that this residue may be essentially a β-D-mannan type polymer.

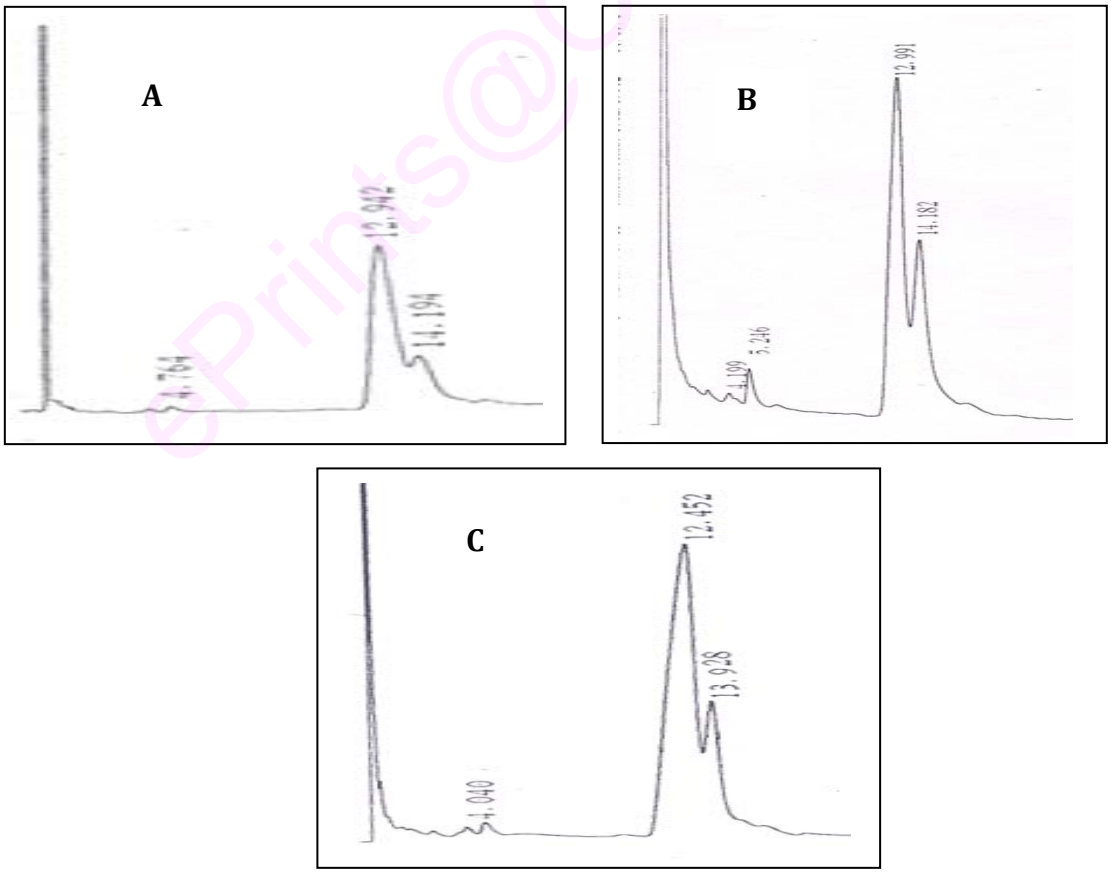


Fig. 22. GC profile of GDGG resulting from **A.** pullulanase, **B.** pepsin, **C.** pectinase action

Similarly, pepsin could also bring about reduction in the galactose content of GDGG, thus mimicking that of LBG, with G/M ratio of 1: 3.5. As the incubation time increased pepsin led to the formation of GDGG with completely altered G/M ratio of 1:3, indicating probably the scission of mannose residues in the backbone. Whereas, pectinase could bring about a gradual increase in the G/M ratio of 1:3.0 in about 1hr followed by depolymerization of the mannan backbone.

Table 13. GDGG obtained from pullulanase, pepsin and pectinase action

	Incubation time (hr)	Viscosity (cps)	G/M ratio
Native guar galactomannan	0	4800	1:2.0
Pullulanase -GDGG	1	4560	1: 2.4
	3	4080	1: 3.4
	5	3500	1: 3.8
	7	2600	1: 5.1
Pepsin -GDGG	1	4650	1: 2.2
	3	4180	1: 2.9
	5	3700	1: 3.6
	7	2900	1: 3.1
Pectinase -GDGG	0.15	1050	1: 2.1
	0.30	800	1: 2.5
	0.45	650	1: 2.8
	1.00	450	1: 3.0

Viscosity

Table 13 and Fig. 23 depict the viscosity profile of GDGG solution (1%) as determined by Brookfield viscometer. A gradual decrease in the viscosity of GG was observed upon co-incubation with pullulanase and pepsin, showing their ability of catalysis (Fig. 23A). Pullulanase and pepsin treatment showed a slight decrease in viscosity during the initial hours of reaction (1 to 5 hr) followed by a rapid decrease with the increased time. The former could be attributed to the exoaction of the enzymes leading to a selective stripping of galactose residues from the polymer, which is also evident from the changes in G/M ratio and M_W.

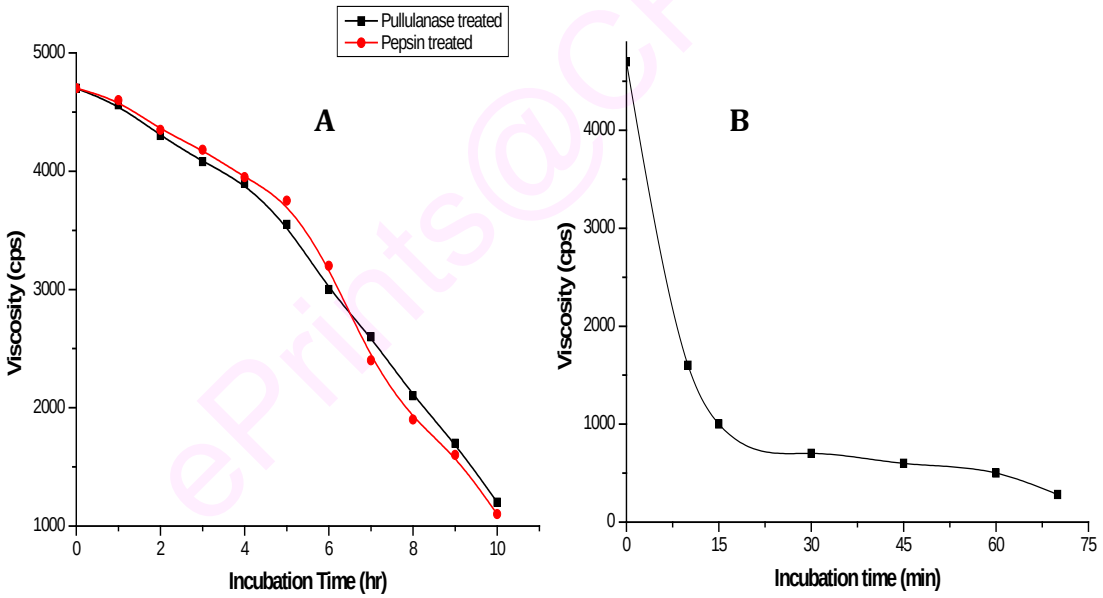


Fig. 23. Viscosity profile of GDGG resulting from **A.** pullulanase and pepsin, **B.** pectinase action

A report by Mahammad et al. (2007) states that the small decrease in viscosity upon hydrolysis by α-galactosidase can be attributed to slight changes in M_W by debranching of the galactomannan molecule. Pullulanase treatment for an extended time (>6 hr) resulted in a significant fall of the viscosity, due to complete

removal of galactose residues from the mannan backbone, leading finally to the formation of a mannan-type polymer. The GC data also substantiated the complete removal of galactose residues from the mannan backbone, thus resulting in an increased G/M ratio. McCleary *et al.* (1981) reports the hydrolysis of guar, carob and *L. leucocephala* galactomannans by α-galactosidase removing essentially all of the D-galactosyl residues and consequently bringing down the viscosity of the polymer solution.

Pepsin also showed a similar decrease in the viscosity on prolonged incubation (above 6 hr) which may also be due to the splitting of mannan backbone, as evident from the decrease in the G/M ratio, thus indicating an endo-action of the enzyme at the later stages. Action of β-mannanase on GG with the breakdown of mannan backbone and resulting in a drastic decrease of the solution viscosity has been reported by various authors (McCleary & Neukom, 1982).

Interestingly, pectinase action showed a drastic fall in viscosity (Fig. 23B), it decreased by over 75% (see Table 13) within 15 min of incubation. Continuing the enzyme action for 1 hr brought down the viscosity of galactomannan to ~450 cps. All these data suggest that the pectinase enzyme is depolymerising the galactomannan to oligomeric fragments and therefore bringing down the viscosity to such a low level. In accordance with this the G/M ratio of the final product (1 hr) got changed to 1:3.

IR - Spectra

FT-IR analysis of native guar galactomannan (Fig. 24A) showed a band in 1200–900 cm^{-1} region due to highly coupled C-C-O stretching modes of polymer backbone (Kacurakova *et al.,* 1998). The

band at about 1150 cm^{-1} contributing to the vibrations of C–O–C bonds of glycosidic bridges and that at 1077 cm^{-1} due to complex vibrations involving the stretching of the C6–O–C1 bonds linking the galactose residue to the main chain were also evident in the spectrum (Daniel et al., 2005) and the two bands at 871 and 813 cm^{-1} are also characteristic of guar gum (Jana *et al.*, 2001). The region between 3000-2800 cm^{-1} represents -C-H stretching modes and that at 3300 cm^{-1} was due to hydroxyl stretching vibration and water involved in hydrogen bonding (Fringant *et al.*, 1995). The absorption band around 1648 cm^{-1} was due to associated water and that around 1430 -1400 cm^{-1} was due to -CH_2 deformation (Kacurakova *et al.*, 1994).

The IR spectra of GDGG obtained at 5 hr of incubation with pullulanase (Fig. 24B) and pepsin (Fig. 24C) showed a gradual decrease in the 1077cm^{-1} band following enzyme treatment, suggesting a preferential debranching of GG. A decrease in the 1150 cm^{-1} stretching band of pepsin modified GG indicated the depolymerizing action of the enzyme on extended time. Similarly, a marked modification observed in the shape and intensity of –CH_2 (~2930 cm^{-1}) stretching band suggests decrease in the molecular size with structural changes in modified GG.

The IR spectra of pectinase - treated GG (Fig. 24D) showed a gradual appearance of a shoulder in the -CH stretching region (3000 - 2800 cm^{-1}), due to the galactosidase - type action of pectinase along with depolymerization as a result of exposure of the C6. A sharpening of the absorption band around 1648 cm^{-1} in the modified product was indicative of its increased association with water, which in turn explains its improved solubility compared to native GG.

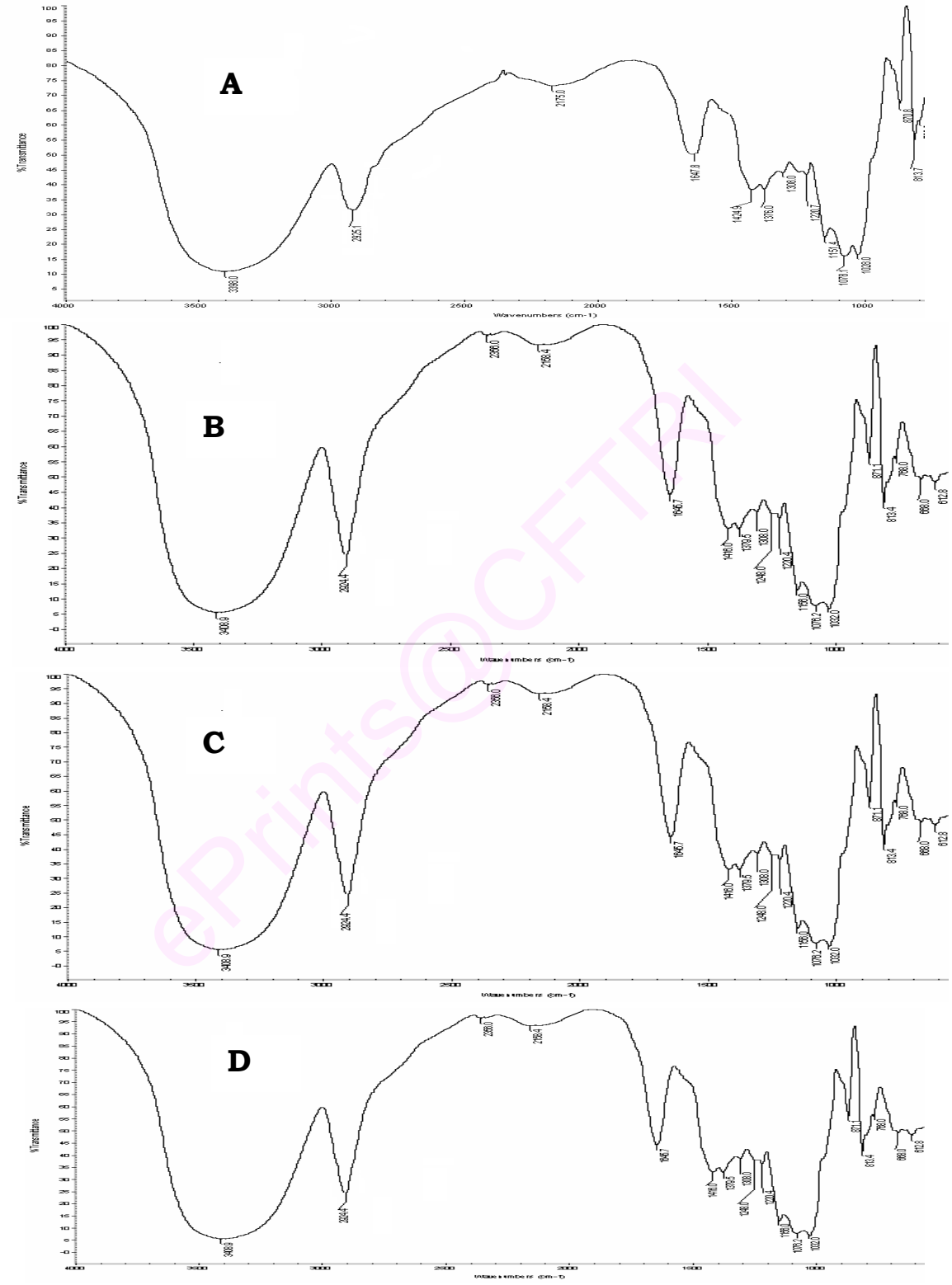


Fig. 24. FT-IR spectra of **A.** native GG and GDGG obtained from **B.** pullulanase, **C.** pepsin and **D.** pectinase action

Also the region between 700 and 500 cm^{-1} is thought to be sensitive to changes in crystallinity (Tul'Chinsky *et al.*, 1976) and the ratio of A_{1423} / A_{823} **cm-1** is indicative of crystallinity index (CrI) (Nelson & O'Connor, 1964). A change in this region was indicative of conformational changes and CrI calculated for native and enzyme modified GG was 0.24 and 0.32 (pectinase), 0.38 (pullulanase), 0.35 (pepsin), respectively, indicating more crystallinity of the products, which could be possible because of their smaller size.

CP- MASS ^{13}C-NMR

Selective debranching activity of these enzymes on GG was further confirmed by CP-MASS ^{13}C-NMR spectroscopy. Fig. 25A represents the spectrum of native GG with the anomeric signals around 103.810 ppm (C1-Man) and 101.589 ppm (C1-Gal) (Hans, 1980). Strong signals dominating around the region 63 ppm to 83 ppm corresponded to the various ring carbons of mannose main chain and galactose side chain. LBG (Fig. 25B) also showed a spectrum in the similar regions with a major difference in the intensity of mannosyl C1 signal around 102.3 ppm (upfield shift) and galactosyl C1 signal at 96.5 (Vieira, 2005). This may be attributed to changes in the G/M ratio and the structural environment. The broadening of peak observed for mannosyl group at C1 signal is due to the sensitivity of the carbon of non-reducing mannose units than the reducing units (McCleary, 1982).

Evidently the spectra of GDGG (Fig. 25 C&D) obtained on treatment with pullulanase and pepsin also revealed changes in the intensity of C1 region with an upfield shift of 102.3 (Man) and 96.85 (Gal) due to changes in the G/M ratio.

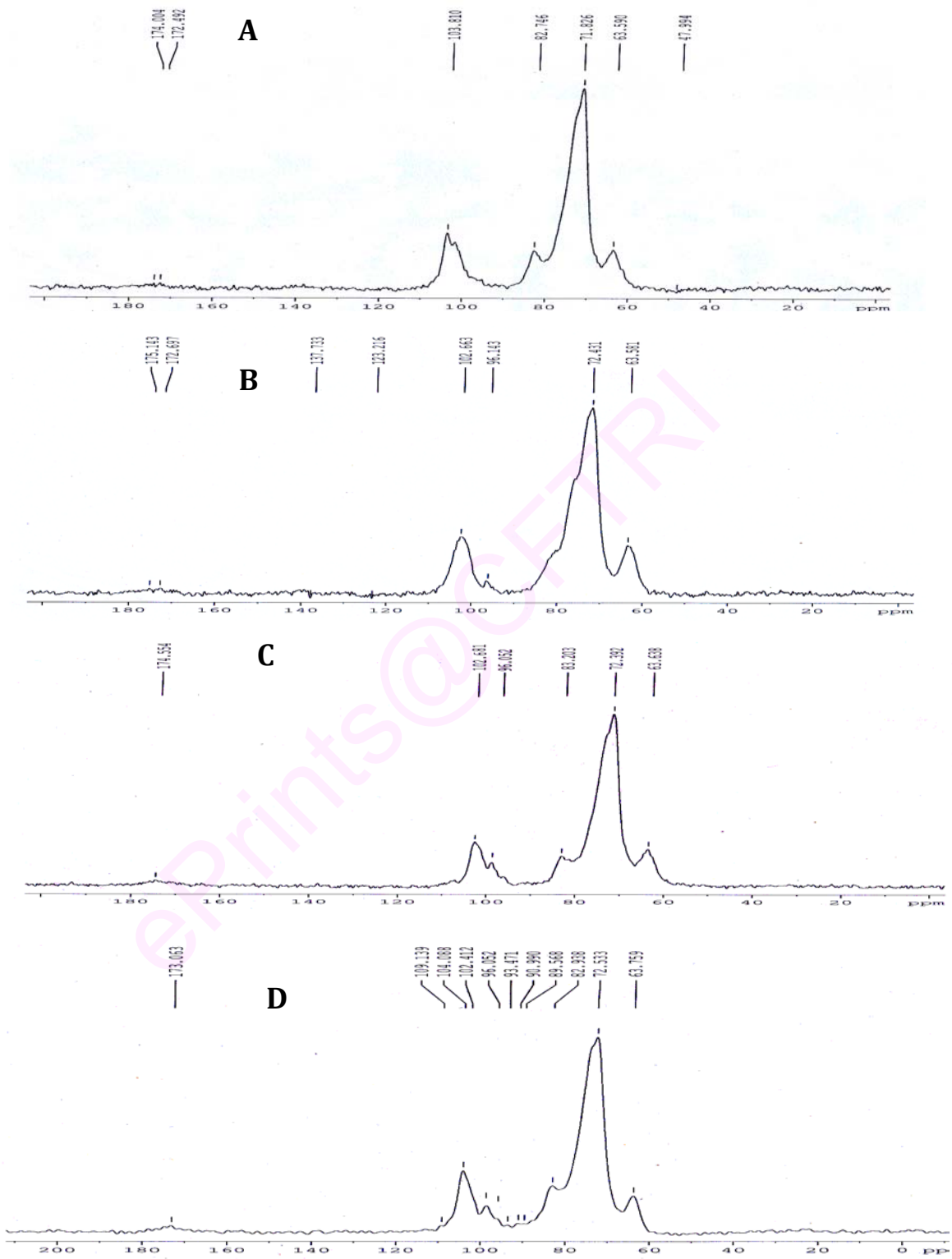


Fig. 25. CP - MASS ^{13}C-NMR spectra of **A.** native GG, **B.** LBG, and GDGG obtained from **C.** pullulanase and **D.** pepsin action

Characterization of monomeric products released

Figs. 26 and 27 represent the HPLC profiles of monomers and oligomers released on co-incubation of GG with pullulanase, pepsin and pectinase. The HPLC profile of the product released upon the action of pullulanase on GG (Fig. 26B) revealed the release of only galactose, indicating exoaction of the enzyme resulting in preferential debranching of GG. In contrast, pepsin and pectinase resulted in the release of oligomers along with galactose indicating a different action pattern (Fig. 27A & B).

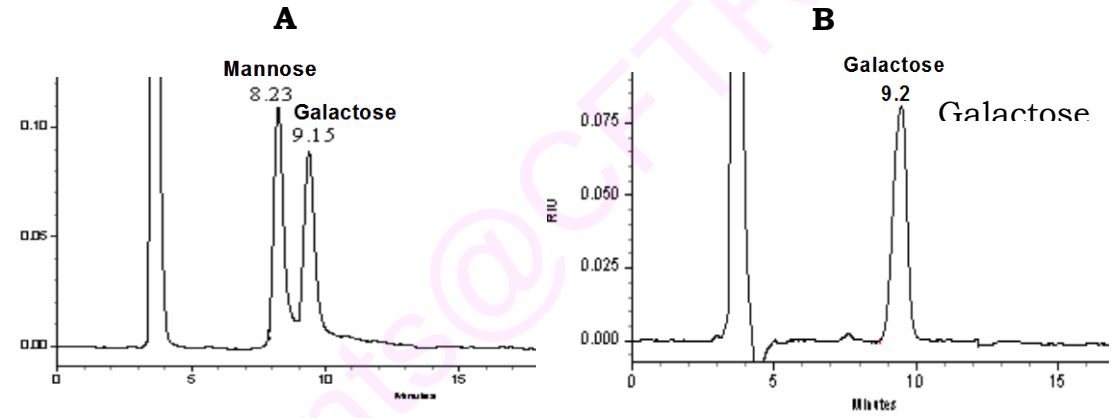


Fig. 26. HPLC profiles of monomer released from GG on treatment with **B:** pullulanase and **A**: Standard monomers

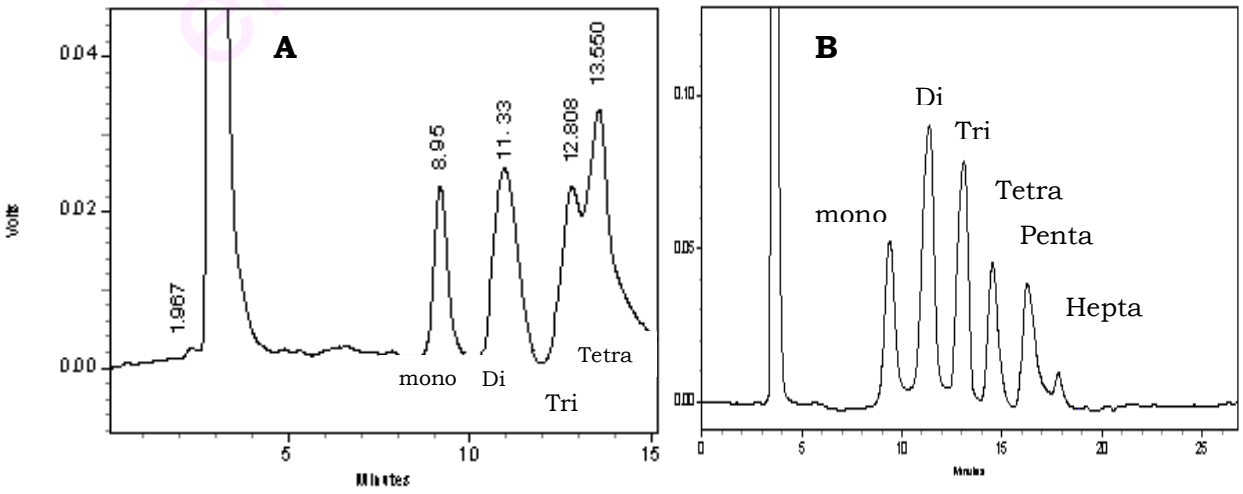


Fig. 27. HPLC profiles of products released from GG on treatment with **A**: pepsin and **B:** pectinase

Pepsin, during the early stages of reaction (1 to 3 hr) resulted in the release of galactose followed by oligomers as the incubation time extended. After 5 hr of incubation maximum debranching of galactose residues from the side chain was observed, resulting in GDGG with a G/M ratio similar to LBG. Though a trace quantity of dimer was observed along with galactose, its yield was negligible indicating the predominant exoaction of the enzyme. On continued incubation (above 6 hr), the enzyme released mannose, in addition to galactose and oligomers (ranging between DP 2 – 4) thus indicating the endoaction of the enzyme leading to the breakdown of mannan backbone (also reduction in viscosity as observed by viscometric measurements).

Pectinase during the initial stages of incubation resulted in the release of galactose, a few oligomers and also mannose (see Fig. 27B). This dual action of pectinase (i.e. debranching as well as depolymerizing) on GG was further confirmed by TLC analysis of the supernatant (profile not shown) which also showed the presence of galactose, mannose and oligomers.

MALDI -TOF- MS

For the identification of the monomeric sequence of the oligomers separated by HPLC, they were subjected to MALDI-TOF-MS. Fig. 28 represents the mass spectra of the oligomers obtained by the non-specific catalysis of pepsin. The major peaks were identified as oligomers of DP - 2 (m/z, 342+23.13), DP - 3 (m/z, 504+23.21), DP – 4 (m/z, 665+23.92) and a trace of DP- 5 (m/z, 826+23) as minor peak. The increase in the m/Z value of the oligomers is assigned to the existence of sodiated form of the individual oligosaccharides.

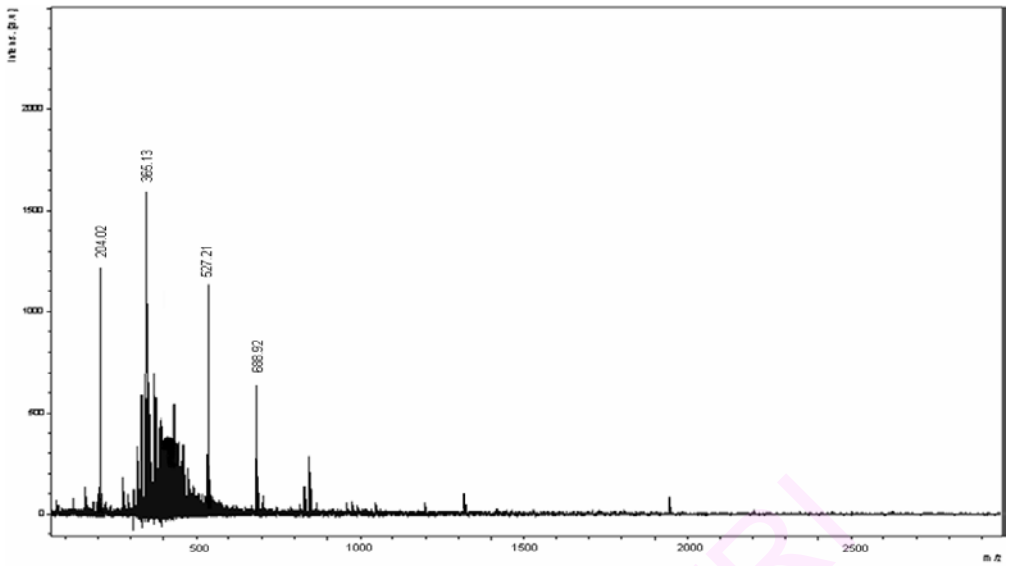


Fig. 28. MALDI -TOF- MS of oligomeric mixture obtained by using pepsin

The catalytic action of pectinase (Fig. 29) on guar galactomannan gave rise to oligomers of DP - 2 (m/z, 365) which was predominant, along with DP - 3 (m/z, 527), DP - 4(m/z, 688), DP - 5 (m/z, 849), DP - 6 (m/z, 1010). Also the intensities of the peaks in MALDI spectrum correlated very well with the peak areas in the HPLC pattern.

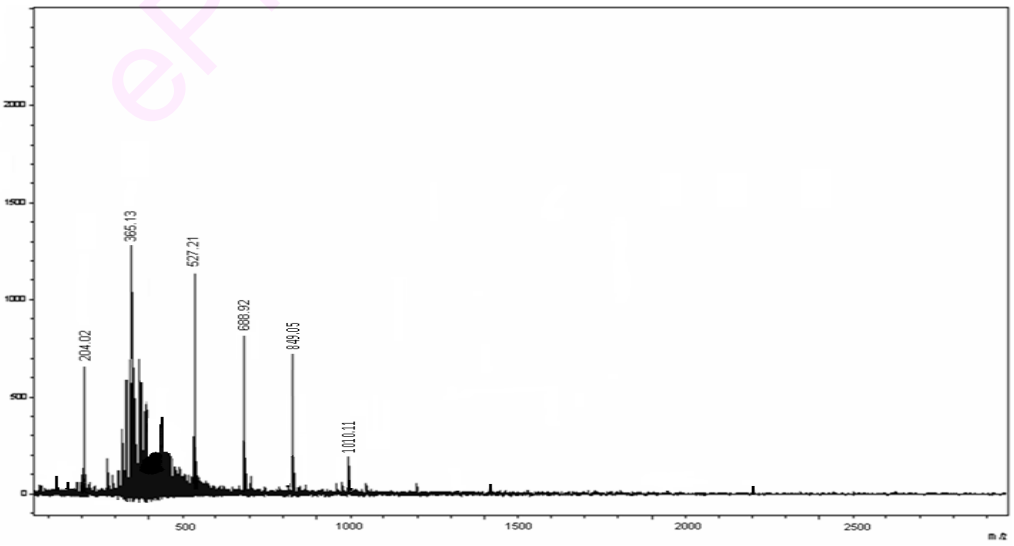


Fig. 29. MALDI-TOF-MS of oligomeric mixture obtained by using pectinase

Further, the individual oligomers, separated on Biogel - P2 column were treated with β-mannanase (an enzyme which hydrolyses the mannose residues in the backbone), β-mannosidase (which hydrolyses non-reducing, terminal mannose residues) and α-galactosidase (which releases galactose residues from the side chain) in order to know the mode of action and the possible alignment of monomers in the oligomeric sequence. Addition of β-mannanase and β-mannosidase to the individual oligomers released by both pepsin and pectinase resulted in the increased reducing equivalents suggesting the action of both the enzymes on mannan backbone. The pepsin derived dimer and trimer on treatment with β- mannosidase showed a 2 and 3 fold increase in the reducing equivalents, whereas α-galactosidase did not alter their reducing equivalents thus supporting the absence of galactose residue(s) in them. These results established the products to be $(Man)_2$ and $(Man)_3$, respectively. Whereas addition of α-galactosidase to the oligomers DP- 4 to DP-5 showed a 2 and 3 fold increase respectively, in the reducing equivalents, thus indicating the presence of galactose in the side chain of these oligomers. On the other hand, addition of β-mannanase and β-mannosidase to the oligomers derived from pectinase resulted in the release of more reducing equivalents from trimer to heptamer indicating the splitting of mannose backbone. A two fold increase in the reducing equivalents of pentamer to heptamer and one fold increase in tetramer respectively on treatment with α- galactosidase confirmed the presence of galactose residue in the side chain.

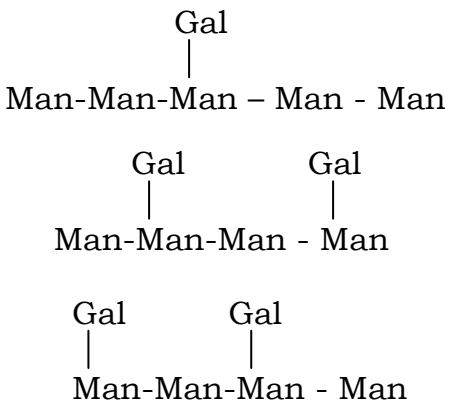
Based on the m/Z ratios obtained by MALDI-TOF-MS and changes in the reducing equivalents upon enzyme treatment the possible monomeric sequence of these various oligomers is given in Tables 14 and 15.

Table 14. Possible sequence of the monomers in the oligomeric mixture obtained after hydrolysis by pepsin

Oligomer	m/Z ratio	Monomeric sequence
Monomer	204.02	Gal, Man
Dimer	365.13	Man - Man
Trimer	527.21	$(Man)_2$ - Man
Tetramer	688.92	Gal | $(Man)_2$ - Man Gal | Man - Man - Man

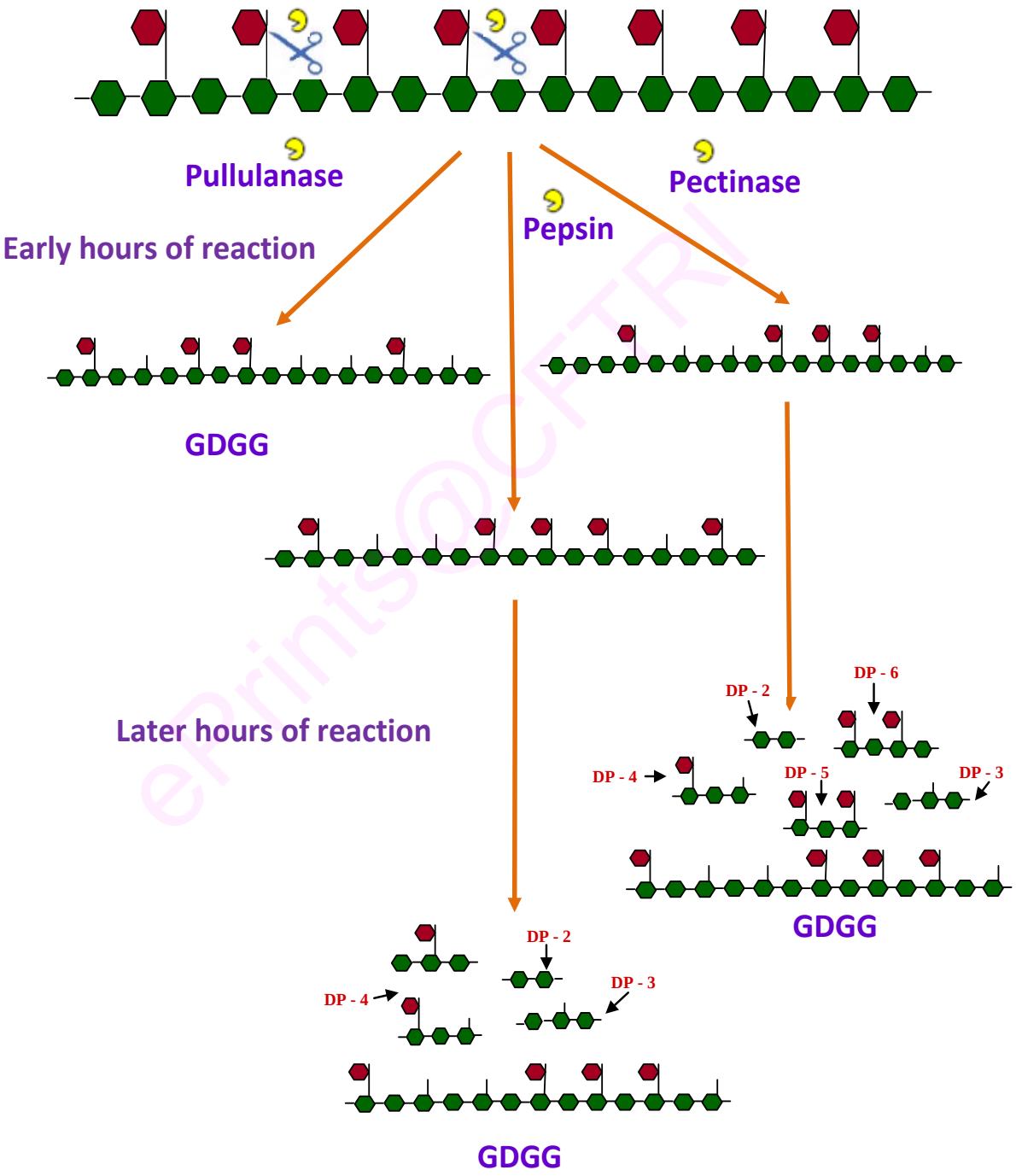
Table 15. Possible sequence of the monomers in the oligomeric mixture obtained after hydrolysis by pectinase

Oligomer	m/Z ratio	Monomeric sequence
Monomer	204.02	Gal, Man
Dimer	365.13	Man - Man
Trimer	527.21	Man-Man - Man
Tetramer	688.92	Gal | Man-Man-Man Gal | Man-Man-Man
Pentamer	849.25	Gal Gal Gal | | | Man-Man-Man - Man Man-Man-Man Gal | Man-Man-Man - Man

Hexamer	1010.56	

Differences in the type of products formed after treatment with pullulanase, pepsin and pectinase was a clear indication of their different action patterns on GG. Stripping of only galactose residues from the side chain by pullulanase, as evident by HPLC pattern and also decrease in galactose content with increase in G/M ratio was indicative of the exoaction of the enzyme. Similarly the action of pepsin on GG with the release of galactose residues during the early hours of reaction along with sight decrease in viscosity and increase in G/M ratio explains the exo-action of enzyme. In addition, the release of oligomers on extending the incubation period (over 5 hr) resulted in a sudden drop of the solution viscosity and also decrease in the G/M ratio, which indicated an endo-action of pepsin leading to the scission of mannan backbone. A sudden decrease in the viscosity with the release of mono- and oligomers was indicative of simultaneous endo- and exo-actions by pectinase. The initial release of galactose by the exo-action probably provides more unsubstituted regions in the polymeric molecules, thereby minimizes the steric hinderence by galactose and ultimately increasing the susceptibility of mannan backbone for enhanced enzymic hydrolysis.

Based on the above results, the mode of action of these unrelated (non-specific) enzymes towards GG can be represented as follows.



Possible structure of GDGG

GG with an uniform distribution of galactose residues on mannan backbone in a ratio of 1: 2 (i.e. one galactosyl residue on every second mannosyl residue) would yield the trisaccharide 6^1-α-D-galactosyl-(1,4) -β-D-mannobiose as the major product (Baker & Whistler, 1975). Whereas, LBG having a block type distribution of galactosyl residues on the mannan backbone in a ratio of 1: 4 would yield β-D-mannobiose and β-D-mannotriose with higher DP oligosaccharides containing D-galactose residues, suggesting a higher proportion of unsubstituted mannose regions (McCleary et al., 1984) (see figs. 30 & 31).

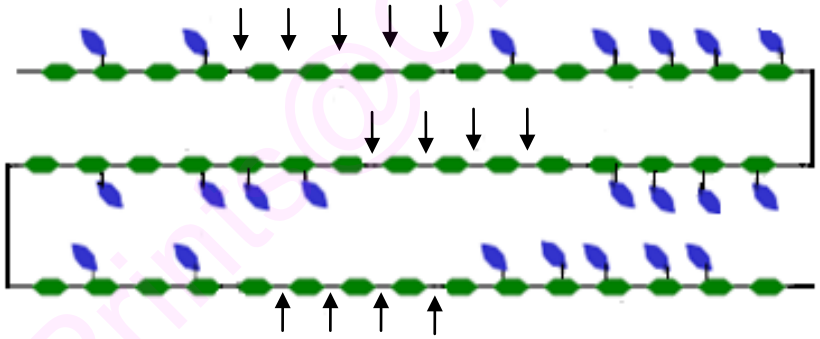


Fig. 30. Schematic representation of block-wise pattern of LBG

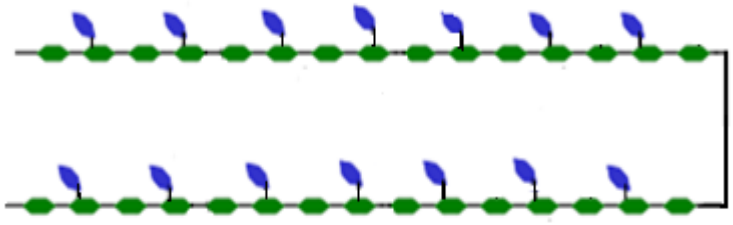


Fig. 31. Schematic representation of uniform sequence of GG (↑ indicates the site of enzyme action)

Further, information on the distribution of galactosyl residues on mannan backbone of GDGG was obtained by treating with highly purified β-mannanase. The HPLC and ESI-MS profile (Fig. 32) of the resultant oligosaccharides revealed the co-existence of both lower and higher DP oligosaccharides. The individual oligosaccharides fractionated on Biogel-P2 were characterized by enzymic analysis using β-mannosidase, α-galactosidase and β-mannanase which revealed the co-existence of β-D-mannobiose and β-D-mannotriose along with the significant 6^1-α-D-galactosyl (1,4) β-D-mannotriose and decreased level of higher DP oligomer with galactose residue. The decrease in galactose content of GDGG results in the increased degree of hydrolysis with lower DP oligosaccharides (McCleary *et al.,* 1981). Table 16 reveals the pattern of oligosaccharides released on hydrolysis of GDGG by β-mannanase.

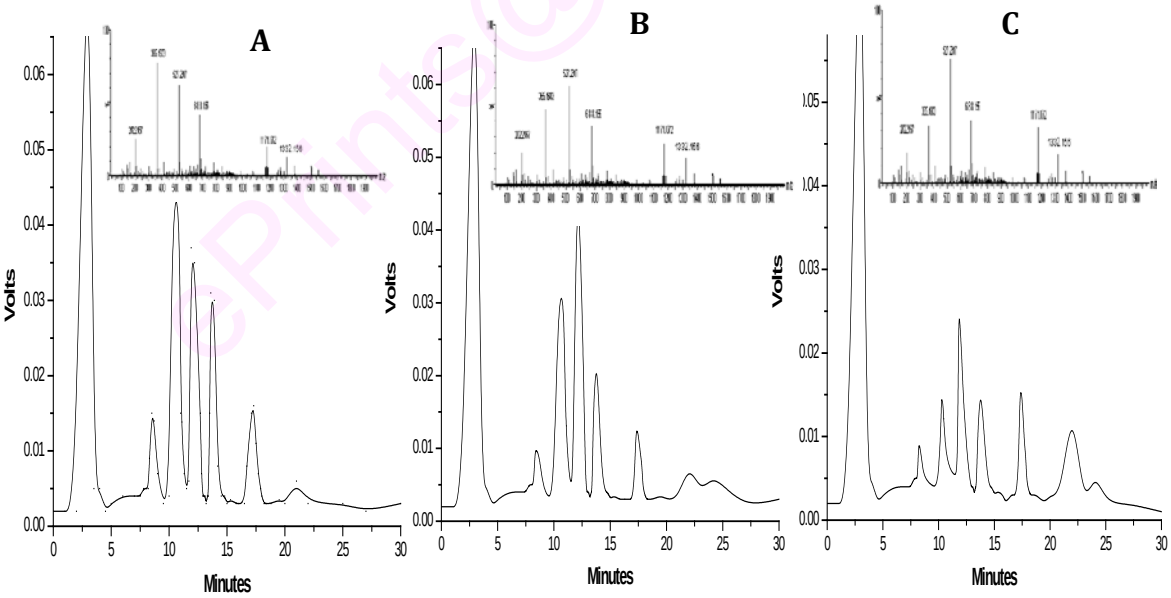


Fig. 32. HPLC profiles of the oligosaccharides released on hydrolysis of GDGG (**A:** by pullulanase, **B:** by pepsin and **C:** by pectinase) by β-mannanase **Inset:** ESI-MS profile of oligosaccharides

Though GDGG showed a G/M ratio similar to LBG, the array of oligosaccharides released upon the action of β–mannanase indicates rather a different distribution of galactose residues on modified GG. Differences in the type of oligosaccharides formed suggests random release of galactose residues by pullulanase, pepsin and pectinase action. McCleary & Nurthen (1983) and McCleary & Matheson (1983) report that the modified GG (formed by α-galactosidase II from guar seeds) to have galactose content similar to LBG, which upon continued hydrolysis with β-mannanase resulting in a different oligosaccharide pattern.

Table 16. Monomeric sequence of the oligosaccharides released on hydrolysis of GDGG by β-mannanase

Oligomer	m/Z ratio	Probable sequence
Monomer	204.02	Gal, Man
Dimer	365.13	Man - Man
Trimer	527.21	$(Man)_2$ - Man
Trimer	527.92	Gal | Man-Man
Tetramer	688.15	Gal | Man-Man-Man
Heptamer	1171.7	Gal Gal | | Man-Man-Man-Man-Man

Conclusion

The non-specific action of pullulanase, pepsin and pectinase on GG resulted in selective removal of galactose residues, leading to the formation of GDGG which showed a decrease in its Mw and appeared as a single peak in HPLC and GPC, justifying its homogeneity. Its GC analysis revealed a significant change in galactose - mannose content of 1:3.1, respectively, and also reduced viscosity, thus mimicking that of LBG. FT-IR and solid-state CP-MASS ^{13}C-NMR analyses indicated subtle changes in the conformation of modified galactomannan.

Characterization of the products released (galactose residues) as a result of hydrolysis showed the exoaction of these enzymes. In addition, pepsin and pectinase upon extended incubation time also exhibited the endo-action with the release of oligomers, whose monomeric sequence was established. Both pepsin and pectinase shared a similar mode of action pattern compared to pullulanase.

Chapter IV

Rheology of GDGG on co-gelation with other polysaccharides

Galactomannans, the most sought-after hydrocolloids of commerce, find extensive application in various food and non-food industries due to their ability to modulate rheological properties. The union of galactomannan with other (biocompatible) polysaccharides such as xanthan and carrageenan brings about synergistic interaction by binding with their helices forming gelation of the two components which are individually non-gelling, and results in the improvement of product quality and reduction in production cost, which are the main contributing factors for their industrial application. Though the mechanism of gelation exhibited by these polysaccharides is still a matter of debate, different types of models have been proposed by various authors to elucidate the mechanism underlying the process of gelation.

A widely accepted model proposed by Dea *et al.* (1977) for gel formation between galactomannan and xanthan involves the interaction between xanthan backbone in the ordered, rod-like conformation and galactose-free regions of the galactomannan backbone. Taka *et al.* (1984) report the interaction of the xanthan side chain with mannan backbone, but still retaining the ordered conformation of xanthan backbone. Based on the X-ray fibre diffraction studies on stretched gels Cairns *et al.* (1986) have proposed a different model for the gelation where a mixed junction zone is formed with xanthan side chains staggered on either side of a sandwiched galactomannan. In addition, Chandrasekaran & Radha (1997) projected a model for the interaction of xanthan with galactomannan suggesting a different molecular arrangement from two chain ordered complex to hybrid-helix model. Miles *et al.* (1984) also suggest that the synergistic interaction between carrageenan and galactomannan leads to highly flexible gels due to the formation of a

three dimensional network involving bonds between double helix and a part of galactose free galactomannan chain. Viebke *et al.* (1994) explain the gelation of k-carrageenan with galactomannan preceeding by the coil–helix transition occurring as a consequence of the aggregation of double helices.

Specifically, the blends of galactomannans such as guar and LBG are used in combination with xanthan and carrageenan in a range of applications that include coatings, drug delivery, oil/gas production and food additives (Fox, 1992). Admixture of LBG with xanthan and carrageenan induces a strong synergistic gelling interaction, whose rheological behaviour is well established. On the contrary, the addition of GG to xanthan and carrageenans only increases the viscosity of the solution without gelation (Tako & Nakamura, 1985). The latter is mainly attributed to the greater proportion of sidechain galactose residues on the mannan backbone which unables to bind with the helices of xanthan or carrageenan.

Contemporary investigations by various groups have demonstrated that the fine structure of GG can be altered by employing highly specific enzymes for the removal of galactose residues which subsequently enhance its synergistic interaction with other biocompatible polysaccharides (Chidwick, 1991; Kloek, 1996). As mentioned earlier, the high cost involved for the isolation of these enzymes and their laborious production steps are non-economical, and have paved the way to look for alternative approach such as use of other unrelated enzymes which are able to selectively debranch (non-specifically) the GG without depolymerising the core mannan backbone.

Xanthan gum, an extracellular polysaccharide secreted by *Xanthomonas compestris,* is mainly made up of pentasaccharide repeating units with β 1,4- linked cellulosic backbone, attached with charged trisaccharide side chain stubs on every second glucose residue. The degree of acetylation at O(6) of mannose residue and substitution of pyruvate group at the terminal mannose residue varies depending on the fermentation conditions (Kennedy & Bradshaw, 1984). Exploitation of xanthan gum in various food industries is mainly due to its non-Newtonian behaviour of forming strong shear thinning solutions at low concentration (Launay *et al.*, 1984).

Carrageenans are another family of polymeric sulfated galactans extracted from various species of red seaweed (i.e. *Eucheuma cottoni*, *Eucheuma spinosum*, *Gigartina acicularis*) which exhibit strong synergy on interaction with galactomannans. The gel-forming members of carrageenans have linear backbones of a repeating disaccharide sequence of 1,3-linked-β-D-galactopyranose and 1,4-linked 3,6-anhydro-D-galactopyranose residues**.**

Materials:

Xanthan, Locust Bean Gum, κ-Carrageenan were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other reagents and chemicals were of highest purity.

Methods

Preparation of galactomannan solution

GG solution (1%, w/v) was prepared by slow addition of the gum

powder to deionised water at room temperature with continuous stirring for 12 hr at 80 rpm to enhance complete hydration of the gum and to attain maximum viscosity.

LBG solution (1%, w/v) was prepared by adding the gum into hot water and further heating to 80°C for about 30 min with continuous stirring to ensure complete solubility and to obtain a clear homogenous solution. Sodium azide (0.1% solution) was added to prevent bacterial contamination.

Preparation of xanthan solution

Xanthan solution (1%, w/v) was prepared by dispersion of gum in deionised water with stirring at room temperature for about 2 hr. The solution was then centrifuged at 5000 rpm for 15 min to remove the air bubbles trapped during stirring.

Preparation of κ-carrageenan solution

κ-Carrageenan (1%, w/v) was prepared by dispersion of gum in deionised water with stirring at room temperature for about 2 hr. The solution was then centrifuged at 5000 rpm for 15 min to remove the entrapped air bubbles.

Preparation of xanthan / galactomannan blends

Xanthan / galactomannan blends were prepared by mixing the two gum (total biopolymer concentration 1%, w/w) solutions in 1:1 ratio followed by homogenization of the mixture for about 15 min at the desired temperature. The mixture was centrifuged at 5000 rpm for 15 min to remove the entrapped air bubbles and poured into a gel jars and allowed to stand for 24 hr at room temperature.

Preparation of κ-carrageenan / galactomannan blends

κ-Carrageenan / galactomannan mixed gels (0.8%, total biopolymer) were prepared in a ratio of 1:1 (w/w) by dispersing in deionised water under constant stirring for about 30 min. Then the mixture was heated to 60^{o} C for 15 min and centrifuged at 5000 rpm for 15 min to remove the entrapped air bubbles. The solution was poured into a gel jar and allowed to set at room temperature for 24 hr.

Rheological measurements

Rheological measurements were performed on RV Rheometer-SR-5 fitted with a probe of diameter of 25 mm using parallel plate geometry. Elastic (G') and viscous moduli (G") were measured in the linear domain by dynamic frequency test with the frequency range of 0.1-100 rads sec^{-1}. Dynamic stress sweep test was performed in the range of 10-1000 dynes/cm^{2}. All the experiments were carried out at 25^{o}C.

Texture profile analysis

Textural parameters, viz. hardness and cohesiveness of mixed gels (45 mm diameter x 35 mm height) were measured by Instron Texture analyzer equipped with a load cell of 50 N, 35 mm probe, speed 20 mm min^{-1} followed by double compression (50%). The samples were cooled to 20^{o}C before taking measurement.

Differential scanning calorimetry

Differential scanning calorimetric studies were performed on Micro DSC III (Waters) supported by Dac software. The temperature in the DSC cell was controlled at a programmed rate using proportional

integral derivative control. The DSC cell was purged with liquid nitrogen at a rate of 40 ml min^{-1} and each run was carried out at a scan rate of 5°C min^{-1} from 10 to 100°C. About 15 - 20 mg of sample were weighed into an aluminium crucible and hermetically sealed with an aluminium lid using an encapsulating press.

Results and Discussion

Rheology of galactomannan solutions

Native GG solution (Fig. 33A) at 1% concentration exhibited a typical macromolecular biopolymer behaviour with the viscous modulus (G'') dominating the elastic modulus (G') in the low frequency range followed by the domination of elastic modulus with a cross point at high frequency range. GDGG solution (Fig. 33B) with depleted galactose residues also showed a similar property, with the elastic modulus dominating the viscous modulus with a cross point in high frequency range. That means the removal of galactose residues did not alter the gross rheological behaviour of galactomannan.

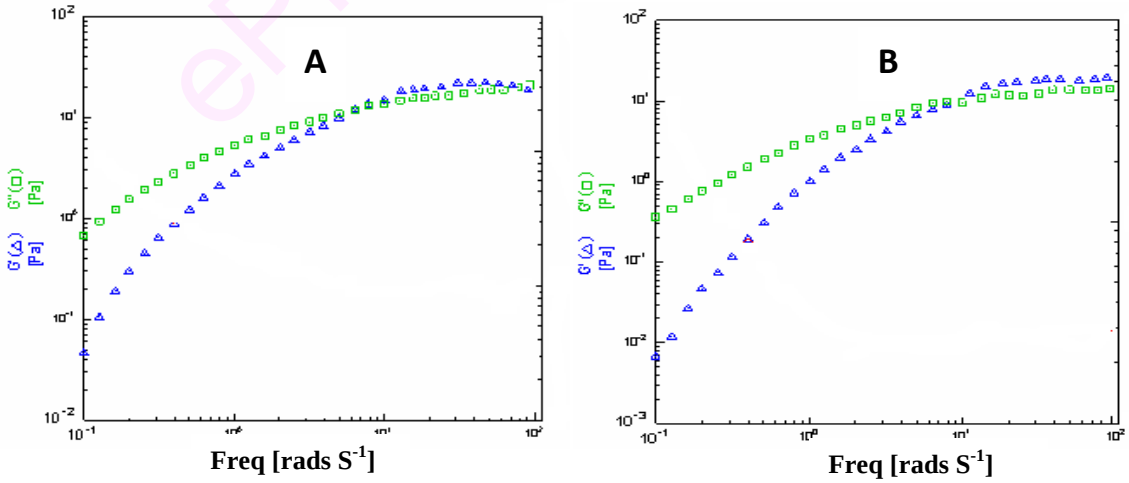


Fig. 33. Rheological behaviour (1%, w/v) of **A:** Native GG **B:** GDGG at frequency range 0.1-100 rads sec^{-1}

Fig. 34A depicts the rheology of LBG solution showing the viscous modulus (G'') dominating the elastic modulus (G') without a cross point which is frequency dependent, thus suggesting the behaviour of a dilute polymer solution. In comparison, xanthan solution (Fig. 34B) showed the elastic modulus greater then the viscous modulus over the entire frequency range indicating the behaviour of a weak gel with strong network. Though κ-carrageenan solution (Fig. 34C) exhibited a trend similar to xanthan, it showed a significant increase in the magnitude of G' indicating the formation of a firm gel.

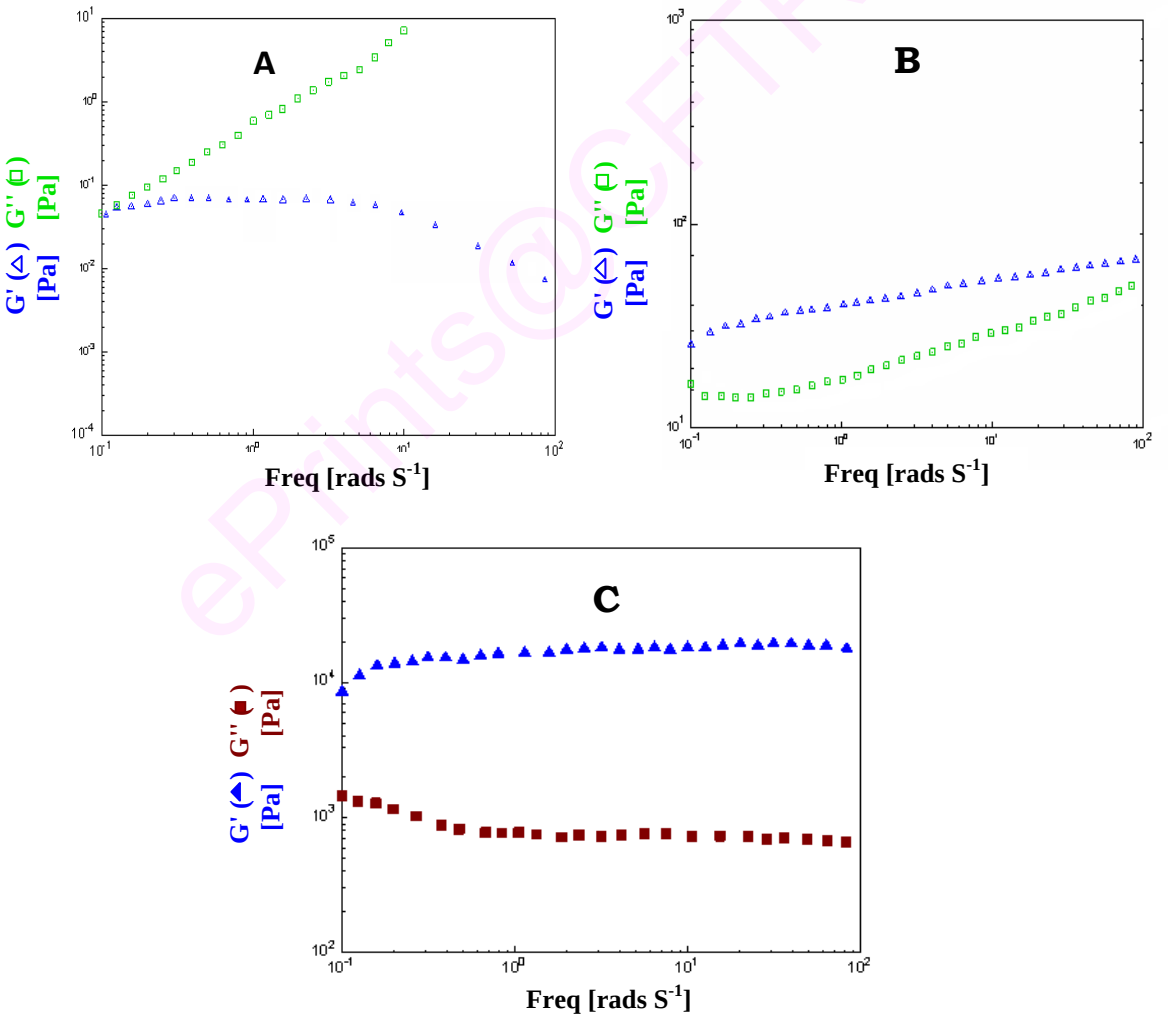


Fig. 34. Viscoelastic behaviour (1%, w/v) of **A:** LBG, **B:** xanthan, **C:** κ-carrageenan solutions

Galactomannan / xanthan blends

Viscoelastic behaviour (Fig. 35A) of LBG mixed (1:1) with xanthan at a concentration of 1%, (w/w, total biopolymer concentration) showed a significantly high elastic behaviour with elastic modulus greater than viscous modulus throughout the experiment with both the moduli as frequency independent indicating a three dimensional network of the polymer mixture. It also showed a two-fold increase in the magnitude of the elastic modulus when compared to xanthan solution, which clearly depicted the synergistic interaction of both the polysaccharides (Cuvelier & Launay, 1986).

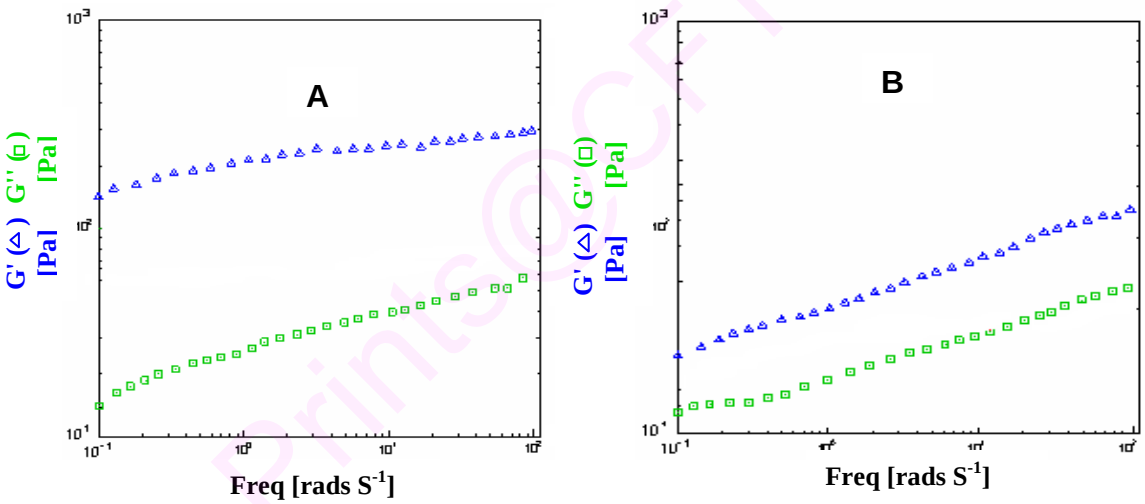


Fig. 35. Viscoelastic behaviour of blends **A:** LBG/ xanthan, **B:** GG/ xanthan

GG and xanthan mixture (Fig. 35B) exhibited gel like behaviour with elastic modulus greater than viscous modulus with the magnitude of the elastic modulus close to that of xanthan solution, showing the contribution of xanthan rather than a synergistic interaction. Doublier & Llamas (1991) suggest that xanthan at a very low level induces a transition from a macromolecular solution to a structured system.

Galactomannan / κ-carrageenan blends

Fig. 36A depicts the viscoelastic behaviour of LBG/κ-carrageenan blend (1%, w/w, total biopolymer concentration). It showed a significant increase in the elastic (G') and viscous modului (G'') independent of frequency as compared to carrageenan alone. A two-fold increase in elasticity (G' > G'') over the entire frequency range corresponded to the characteristic feature of a true gel, indicating a synergistic interaction (Clark & Ross-Murphy, 1987). Cairns *et al.*, (1987) interpret such an interaction as due to the entrapment of LBG within the κ-carrageenan network.

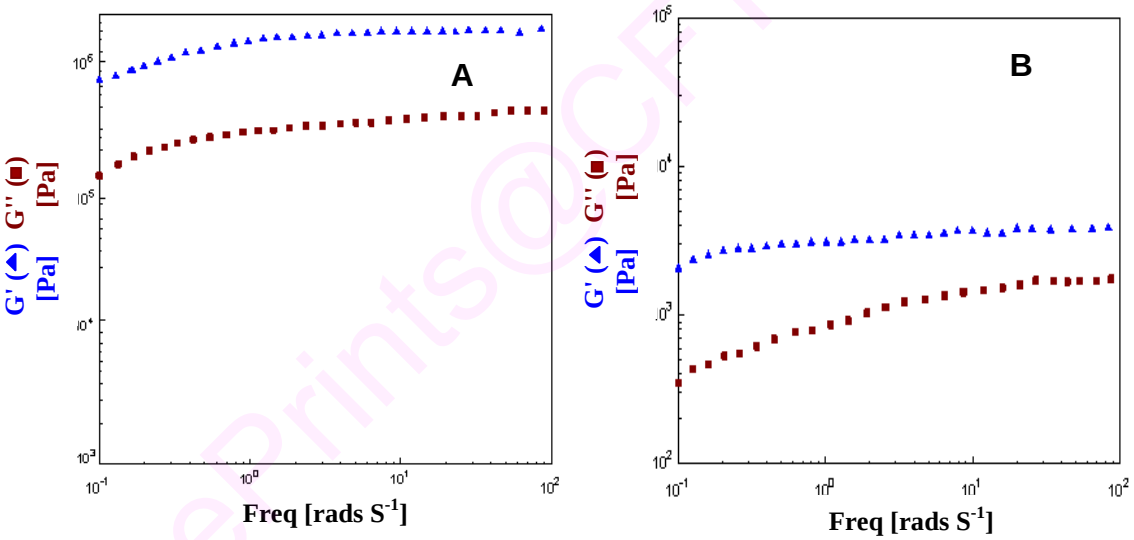


Fig. 36. Viscoelastic behaviour of blends **A:** LBG/ κ-carrageenan **B:** GG/ κ-carrageenan

In comparison, GG/κ-carrageenan mixture showed a different viscoelastic behavior (Fig. 36B), which resulted in the elastic modulus (G') dominating viscous modulus (G' > G''), with an increase in viscous modulus (G'') over the entire frequency range. The magnitude of G' was similar to that of native κ-carrageenan solution, suggesting the role of the latter for increase in elasticity. Lopes *et al.* (1992) and

Shatwell *et al.* (1991) report a similar increase of viscosity in blends of GG and xanthan due to molecular interaction.

Interaction of GDGG with xanthan and κ-carrageenan

Figs. 37 A&B show the viscoelastic behaviour of blends of GDGG with xanthan and κ-carrageenan, respectively (1%, w/w total biopolymer, 1:1 ratio), which shows elastic modulus (G') dominating the viscous modulus (G'') over the entire frequency range, indicating synergistic interactions. As the galactose content of GG decreased, an increase in the synergistic behaviour was observed (Shobha & Tharanathan, 2009). A two fold increase in the magnitude of G' in the plateau region of GDGG with decrease in tan δ suggest a gain in elasticity, a characteristic feature for forming more strong and elastic gels (Schorsch *et al.*, 1997). Burchard & Ross-Murphy (1990) describe the appearance of plateau in the elastic moduli region over the entire frequency range with the magnitude of more than one order for true gels which are self standing.

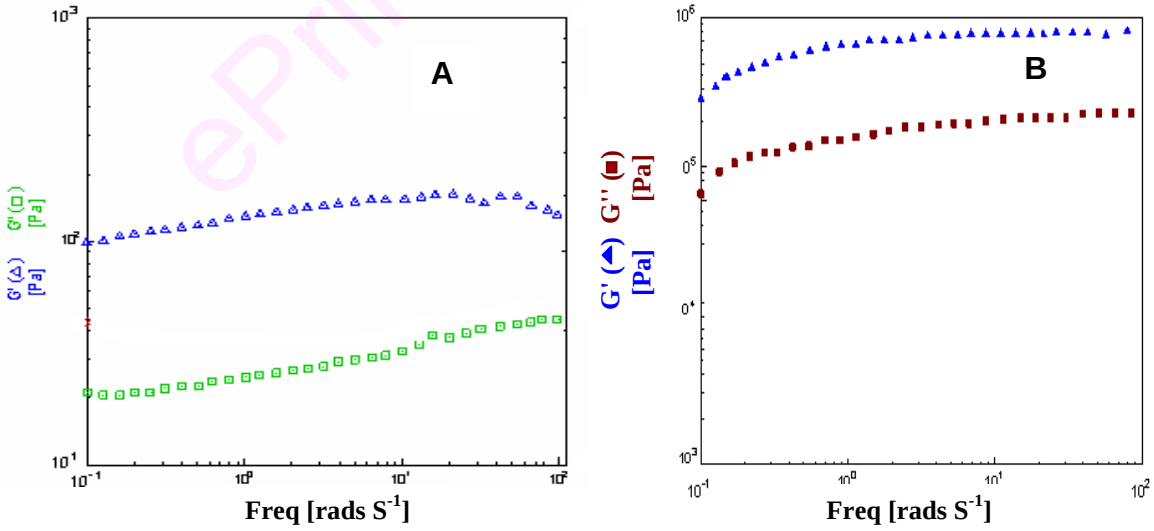


Fig. 37. Viscoelasticity of blends, **A:** GDGG/ xanthan **B:** GDGG/ κ-carrageenan

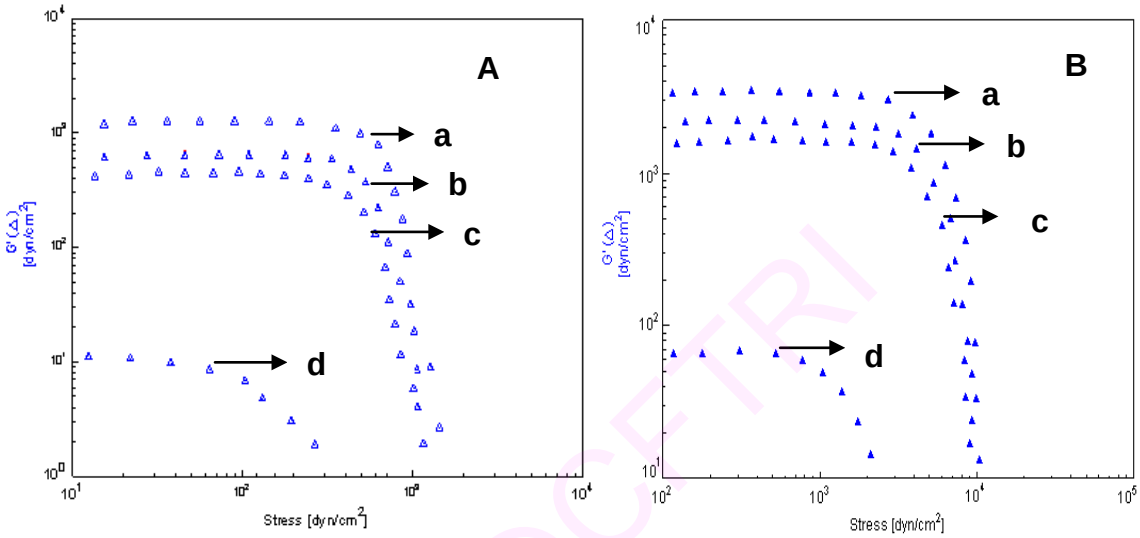
Measurement of yield stress is one of the parameters for describing the textural qualities of gelling materials for commercial exploitation. Table 17 describes the yield stress as measured by the dynamic stress-sweep method. The GG blends with xanthan and κ-carrageenan showed a very low yield stress compared to LBG blends. A sharp decrease in the G' region was observed at lower range, which may be due to rupture in the gel network, suggesting a weak association of GG. Whereas LBG blends showed highest yield stress with the rupture point at a very high range showing its strong interaction.

Table 17. Yield stress of galactomannan / xanthan blends

Blend	Yield stress (Pa)
Xanthan / native GG	110
Xanthan / LBG	825
Xanthan / pullulanase treated GG	800
Xanthan / pepsin treated GG	790
κ-Carrageenan / native GG	450
κ-Carrageenan / LBG	1450
κ-Carrageenan / pullulanase treated GG	1430
κ-Carrageenan / pepsin treated GG	1410

Figs. 38 A&B show the yield stress pattern exhibited by the native and modified polysaccharide blends. The latter showed the capacity to withstand the stress of higher range, thus mimicking LBG blends (Luyten *et al.*, 1994). An increase in the strength of GDGG

blend is attributed to the enzymatic removal of galactose residues from guar molecules with the exposure of unsubstituted mannan backbone to interact with xanthan and κ-carrageenan forming junction zones, thus giving rise to strong gels.



a. LBG / xanthan
b. Pullulanase treated GG/ xanthan
c. Pepsin treated GG/ xanthan
d. Native GG/ xanthan

a. LBG / κ-carrageenan
b. Pullulanase treated GG/ κ-carrageenan
c. Pepsin treated GG/ κ-carrageenan
d. Native GG/ κ-carrageenan

Fig. 38. Yield stress pattern

Effect of xanthan concentration

The concentration of xanthan plays a crucial role in determining the synergistic interaction of these biopolymers. Fig. 39A shows the effect of varying xanthan concentration on the rheological behaviour of GG/ xanthan blends. Varying the concentration of xanthan (0 to 100%, w/w) showed the magnitude of G' dominating G''. Elasticity of the polysaccharide blends increased as the concentration of xanthan increased without affecting the viscous modulus. A report from Mannion *et al.* (1992) suggests increase in elasticity as due to the

cross linking between the two biopolymers. Xanthan at very low as well as high concentrations, though formed elastic gels the magnitude of the elasticity was very low indicating the contribution of xanthan alone. Whereas, the concentration in the range of 40-60% displayed a constant increase in the magnitude of elasticity. Xanthan/GDGG blend mixed at 50% xanthan concentration in a ratio of 1:1 (w/w) showed a maximum increase in magnitude with high yield stress (Fig. 39B). The above data is consistent with those reported by Pai & Khan (2002) and Schorsch *et al.* (1997).

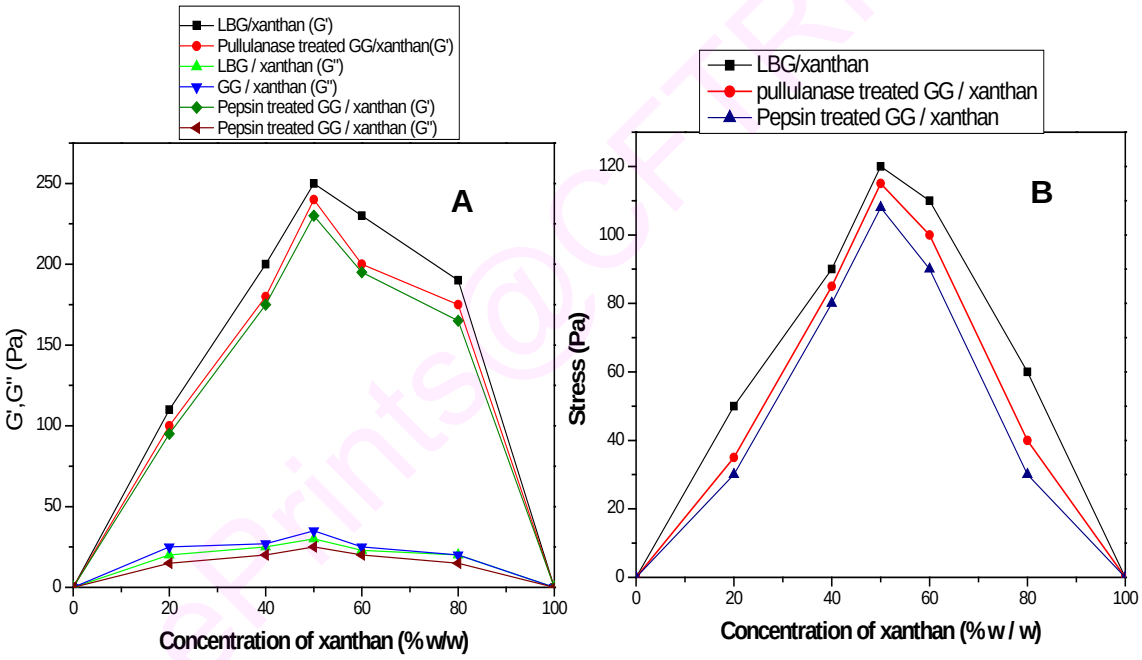


Fig. 39. Effect of xanthan concentration on gelation with galactomannans at 1 rad sec^{-1} **A:** Dynamic frequency test, **B:** Dynamic stress - sweep test

Effect of κ-carrageenan concentration

Fig. 40A shows the effect of varying κ-carrageenan concentration on the rheological behaviour of galactomannan blends.

Various concentrations of κ-carrageenan (0 to 100%, w/w) on both GDGG and LBG blends showed increase in the magnitude of G'. Though an increase in the elasticity was observed, κ-carrageenan at a concentration of 50% in a ratio of 1:1 (w/w, 0.8% total biopolymer concentration) showed a maximum increase in the magnitude of elasticity (G'). A report from Fernandes *et al.* (1991) also suggests similar synergistic interaction as the concentration of κ-carrageenan increases, displaying maximum G' (elasticity) at 80:20 ratio. The κ-carrageenan/modified GG blend mixed at 50% κ-carrageenan concentration in a ratio of 1:1 (w/w, 0.8% total biopolymer concentration) showed a maximum increase in magnitude with high yield stress (Fig. 40B).

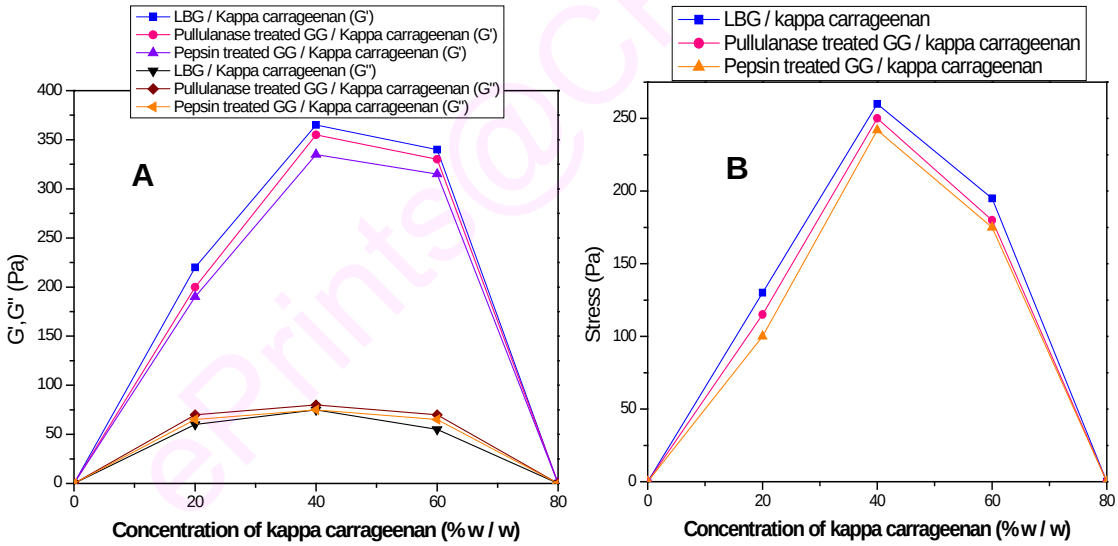


Fig. 40. Effect of κ-carrageenan concentration on gelation with galactomannans at 1 rad sec^{-1} **A:** Dynamic frequency test, **B:** Dynamic stress - sweep test

Effect of mixing temperature

Xanthan exhibits ordered conformation at lower temperature followed by disordered conformation with increase in temperature.

Fig. 41 depicts the studies on effect of temperature (20-60°C) on the interaction of galactomannan / xanthan blends. An increase in the elastic modulus compared to the viscous modulus was observed as the temperature increased. Maximum synergistic behaviour was shown at 60°C, which was attributed to the interaction of galactose depleted region with disordered xanthan molecules forming junction zone (Tako, 1991). A report by Norton *et al.* (1984) also states that the increase in temperature leads to the transition of ordered conformation of xanthan molecules to disordered conformation, favoring better interaction and giving rise to strong gels.

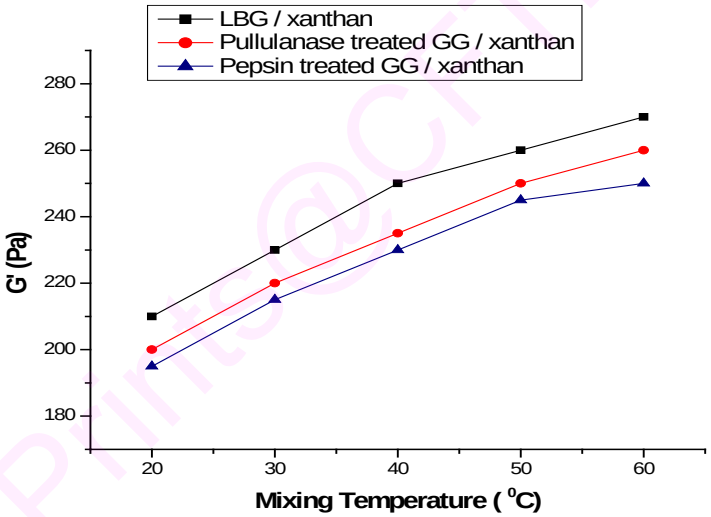


Fig. 41: Effect of mixing temperature on elastic modulus (G') of galactomannan / xanthan blends

The conformational transition temperature (T_m) of xanthan in water as measured by DSC was 52°C, which was in concordance with the value reported by William *et al.* (1991). Table 18 summarises the T_m and transition enthalpy (Δ H) values of these blends. Addition of LBG to xanthan showed an increase in T_m to 80°C with Δ H of 31.25 kJ. GDGG/xanthan blend also showed an increase in the value of T_m

almost similar to LBG/xanthan blend indicating improvement in its synergistic interaction compared to native GG. An earlier report by Goycoolea *et al.* (2001) suggests that increase in the proportion of the galactomannan component leads to a decrease in ΔH, along with an increase in the T_m, due to the stabilization of the helical conformation.

Table 18. T_m and Δ H of galactomannan / xanthan blends

Blend	T_m (^{0} C)	Δ H (J/g)
Xanthan	53	35.38
Xanthan / Native GG	54	32.12
Xanthan / LBG	80	31.25
Xanthan / pullulanase treated GG	78	30.56
Xanthan / pepsin treated GG	77	31.75

Fig. 42 shows the effect of variation of temperature on the synergistic interaction of galactomannan/κ-carrageenan blends. The elastic modulus of κ-carrageenan/LBG blend was higher then viscous modulus indicating gelling characteristics. The mixture showed a synergistic interaction at 30^0C which was slightly higher than that of κ-carrageenan alone. GDGG on admixture with κ-carrageenan also displayed a similar increase in the elasticity as the temperature increased to 29^0C with maximum yield stress. Whereas the addition of GG though showed elastic modulus greater than viscous modulus the

magnitude of elasticity was less than of κ-carrageenan/LBG blend, indicating the formation of a weak gel. The above results are consistent with that reported by Fernandes *et al.* (1991) suggesting a similar gelation mechanism.

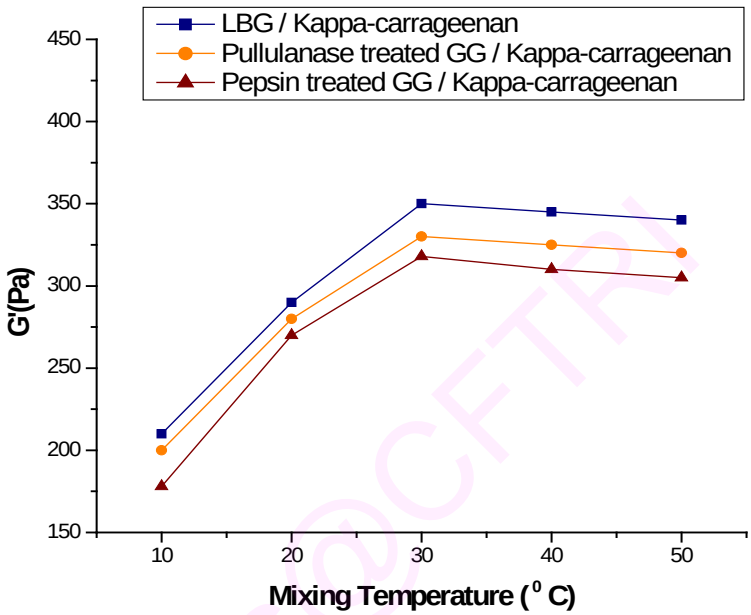


Fig. 42: Effect of mixing temperature on elastic modulus (G') of galactomannan / κ-carrageenan blends

The T_m of κ-carrageenan (0.8%) as measured by DSC was 33^{0}C (Table 19) in accordance with the previous report (Fernandes *et al.*, 1992). Upon heating, the hydrogen bonds are broken and double helices change their conformation giving rise to fusion of aggregates with the break up of network. The difference between melting temperature and gelling temperature is known as "thermal hysteresis" which is associated with the aggregation of double helices leading to gel formation (Mangione *et al.*, 2003). The κ-carrageenan/LBG mixture had a T_m of 48^{0}C. Similarly GDGG/κ-carrageenan blend also showed increase in T_m similar to LBG/κ-carrageenan blend. This increase is

mainly attributed to the increase in the gel rigidity which is induced by the addition of galactomannan.

Table 19. T_m and Δ H of galactomannan / κ-carrageenan blends

Blend	T_m (^{0} C)	Δ H (J/g)
κ - Carrageenan	33	25.65
κ - Carrageenan / native GG	36	25.16
κ - Carrageenan / LBG	48	22.89
κ - Carrageenan / pullulanase treated GG	48	20.65
κ - Carrageenan / pepsin treated GG	47	22.95

Texture profile analysis of polysaccharide blends

The synergistic behaviour exhibited by GDGG was studied by texture profile analysis (Table 20) on cogelling with xanthan and κ-carrageenan. The native GG blends did not show any significant gelling, whereas the LBG blends showed synergistic interaction of the polysaccharides by strong gelation, thereby regulating the textural parameters viz. hardness, cohesiveness, adhesiveness, gumminess, etc. GDGG with varying galactose content also showed a good gelling synergy with xanthan and κ-carrageenan, and having texture parameters akin to those of LBG blends.

Table 20. Texture profile analysis of polysaccharide blends

	Hardness [N]	Cohesiveness	Springeness (mm)	Chewiness (N, mm)	Adhesiveness (N)	Gumminess (N)
LBG / κ-carrageenan	10.5	0.067	9.42	6.672	0.043	3.39
GG/ κ-carrageenan	NG	NG	NG	NG	NG	NG
Pullulanase treated GG/ κ-carrageenan	9.1	0.054	9.10	5.94	0.063	3.68
Pepsin treated GG / xanthan	8.9	0.049	8.85	5.21	0.057	2.95
LBG/xanthan	8.5	0.052	7.25	5.25	0.035	2.65
GG/ xanthan	NG	NG	NG	NG	NG	NG
Pullulanase treated GG/ xanthan	7.8	0.048	6.83	4.91	0.029	2.12
Pepsin treated GG/ xanthan	6.9	0.039	6.25	4.31	0.025	2.09

NG – No gel formation

α-Galactosidase-treated guar galactomannan blends have been shown to give strong gels (Brooke *et al.*, 2000). The enhanced synergistic interaction of GDGG is attributed to the intermolecular interaction of galactose-depleted region of galactomannan backbone with the double helices of carrrageenan forming secondary network (Fernandes *et al.*, 1991).

Conclusion

Although catalysis by unrelated enzymes on GG is unusual and non-specific, it enables an alternative way to produce modified guar galactomannan with significant reduction in galactose content. GDGG blend with xanthan and κ-carrageenan exhibited a rheological behaviour similar to LBG blends. Also a two fold increase in the magnitude of elasticity compared to xanthan and κ-carrageenan alone suggested a synergistic interaction with the formation of three dimensional networks. GDGG with G/M ratio almost akin to LBG, showed a better interaction. Dynamic stress-sweep study of polysaccharide blends also showed increase in the yield stress indicating the synergistic behaviour. Modification of guar galactomannan by (non-specific) unrelated enzymes is an alternative route to produce galactose-depleted guar galactomannans with enhanced rheological characteristics and as a cost effective replacement to LBG. Such an approach may assume greater significance in view of product development requirements of the food and other allied industries.

Chapter V

Mode of action of pepsin in debranching of GG

Pepsin, an endopeptidase with maximum catalytic activity at acidic pH is a well known aspartic protease which has been extensively studied eversince Northrop crystallized it in 1929. Though secreted as pepsinogen (the zymogen of pepsin) by the chief cells lining the stomach, the hydrochloric acid released by the parietal cells of gastric lumen activates the enzyme which facilitates the removal of 42 amino acid residues from the N-terminal, thereby leading to the breakdown of dietary proteins into peptides and aminoacids.

Pepsin is a monomeric β-protein (M_r, 34.6 kDa) with two domains containing high percentage of acidic residues (43 out of 327 are aspartic and glutamic acids, pK_a – 1.5), of which two aspartates (Asp 32 and Asp 215) are mainly responsible for the catalysis. It acts exclusively on the peptide bonds containing aromatic amino acids (phenylalanine, tyrosine and tryptophan), leucine and acidic residues at the N-terminal. During catalysis, of the two aspartate residues, one will be ionized and the other unionized (Antonov *et al.*, 1978). It is a very well known fact in classical biochemistry that enzymes are highly substrate specific, more so in the case of carbohydrate substrates whose anomeric configuration (α/β – pyranosyl or furanosyl forms) as well as linkage pattern play a crucial role. **Such being the case, why and how a proteolytic enzyme could bring about catalysis of structurally unrelated carbohydrate substrates????**

Nevertheless, a few earlier reports have claimed the multiple specificity of several enzymes on completely unrelated substrates, for example, though many workers have demonstrated chitosanolysis by several commercial enzymes including pepsin, purity of these enzymes was in doubt (Pantaleone *et al.*, 1992; Yalpani & Pantaleone, 1994). Liao *et al.* (2000) partially purified chitosanolytic enzymes from commercial crude porcine pepsin preparation and recently Fu *et al.*

(2003) could isolate and characterize three chitosanase isozymes from the same. They showed that 95% of the N-terminal amino acid residues of chitosanase isozyme PSC-III (i.e., 14 residues out of first 15) were identical with those of pepsin B. Also earlier research from our lab reports the non-specificity of various enzymes in the depolymerisation of chitosan and debranching of GG (Shobha *et al.*, 2005). Vishu Kumar *et al.* (2006) report effective depolymerization of chitosan with the aid of purified procine pepsin for the production of low molecular weight chitosan (LMWC). Though pepsin is a proteolytic enzyme, functionally its non-specificity towards glyco-substrates may be due to its structural features mimicking several carbohydrate degrading enzymes (hydrolases), which is characterized by the presence of two domains with barrel-like structures having one end of the barrel wider than the other. At the wider end is an elongated cleft, which could be narrower/wider depending on the size of the substrate (Jedrzejas, 2000). Further, the multiple sequence alignment of pepsin and α-galactosidase (from guar) showed 25% sequence homology (Fig. 43) involving aspartic acid residues at the active site. Keeping all these perplexing views in mind, an attempt has been made in this investigation to answer the question raised before, by solving the controversies related to the purity of pepsin and also its dual catalysis towards different substrates. The use of site specific inhibitors, chemical modification agents and peptide mapping of native and GG-bound pepsin on proteolytic digestion with Glu-C V8 protease were carried out to know the binding site residue and also to elucidate the mode of action of pepsin in debranching of GG.

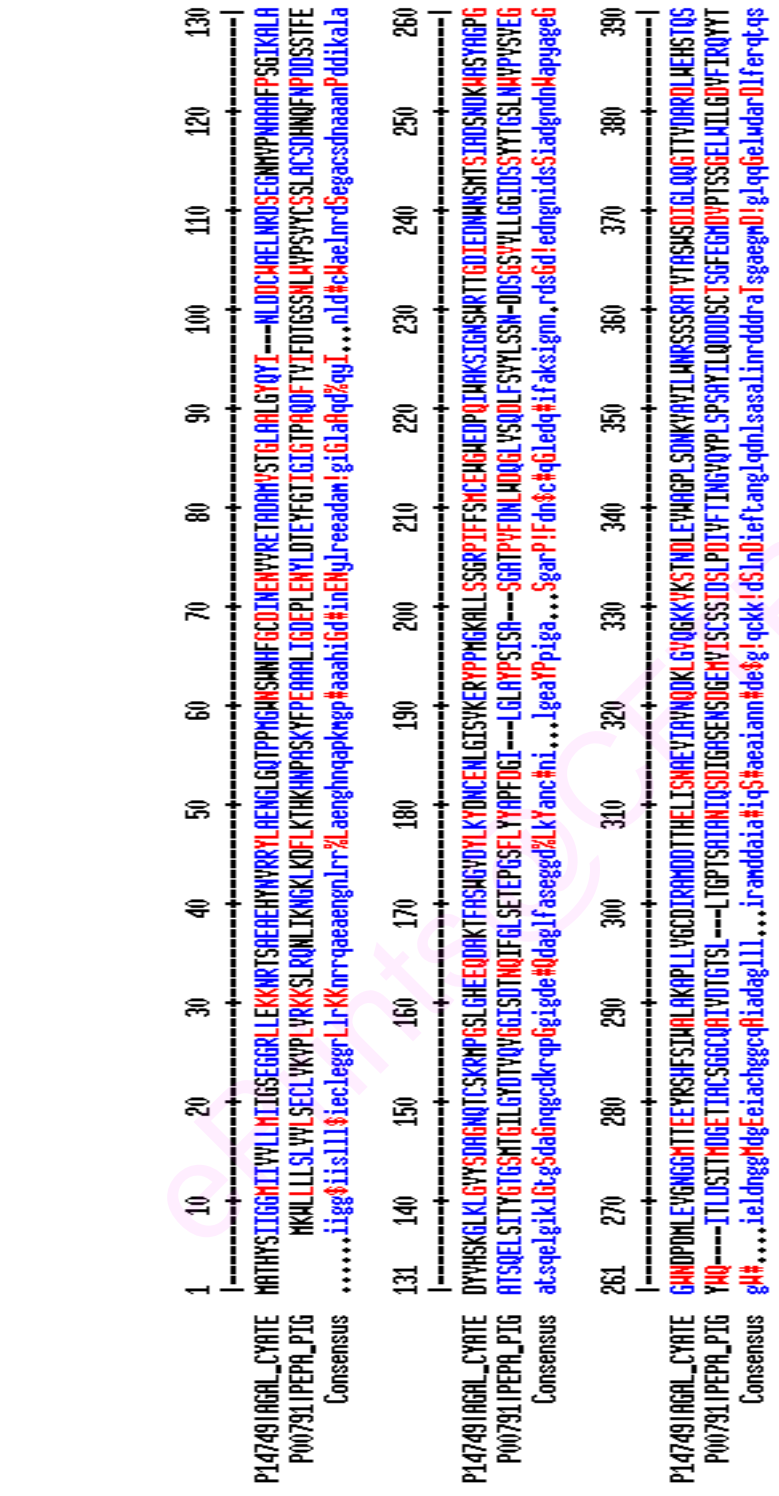


Fig. 43: Multiple sequence alignment of porcine pepsin (P00791lPEPA_PIG) and α-galactosidase (P1474911AGAL_CYATE from guar)

Materials

Haemoglobin (Bovine), Pepstatin, Phenylmethylsulfonylketone (PMSF), N-tosyl-L-lysine-chloromethylketone (TLCK) hydrochloride, iodoacetate, N-(3-methyl amino propyl) N'-ethylcarbodiimide hydrochloride (EDAC), Glycine Methyl Ester (GME) and Endoproteinase Glu-C (*Staphylococcus aureus* strain V8, E.C. 3.4.21.19) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other reagents and chemicals were of highest purity.

Methods

GPC

The commercial preparation of porcine pepsin was purified on Sephadex G-100 column (see Chapter II) in order to free the enzyme from the proteinaceous contaminants present if any, and also to remove stabilizers and inorganic fillers, which may contribute for the non-specificity of the enzyme.

Homogeneity of the purified enzyme

The homogeneity of the purified enzyme was ascertained by PAGE (Native and SDS), Rp-HPLC, and capillary zone electrophoretic and other techniques (see Chapter II)

MALDI-TOF-MS

The purified enzyme was subjected to MS on a Compact analytical SEQ MALDI-TOF-MS (Kratos, UK) in a reflective positive ion mode. The matrix used was α-cyano-4-hydroxycinnamic acid prepared in CH_3CN:H_2O:TFA (80:20:0.1). The purified sample was mixed with equal volume of matrix, dried at 25^0C under atmospheric

pressure and transferred into vacuum chamber of the mass spectrometer.

Automated gas-phase protein sequencing

The N-terminal sequencing of purified procine pepsin was carried out on Applied Biosystems 491A automated gas phase protein sequencer (Procise 491A, USA) by Edman degradation supplying gaseous reagents for the coupling and cleavage reactions. Fig. 44 shows the flow diagram for the reaction sequence that occurs during sequencing of proteins.

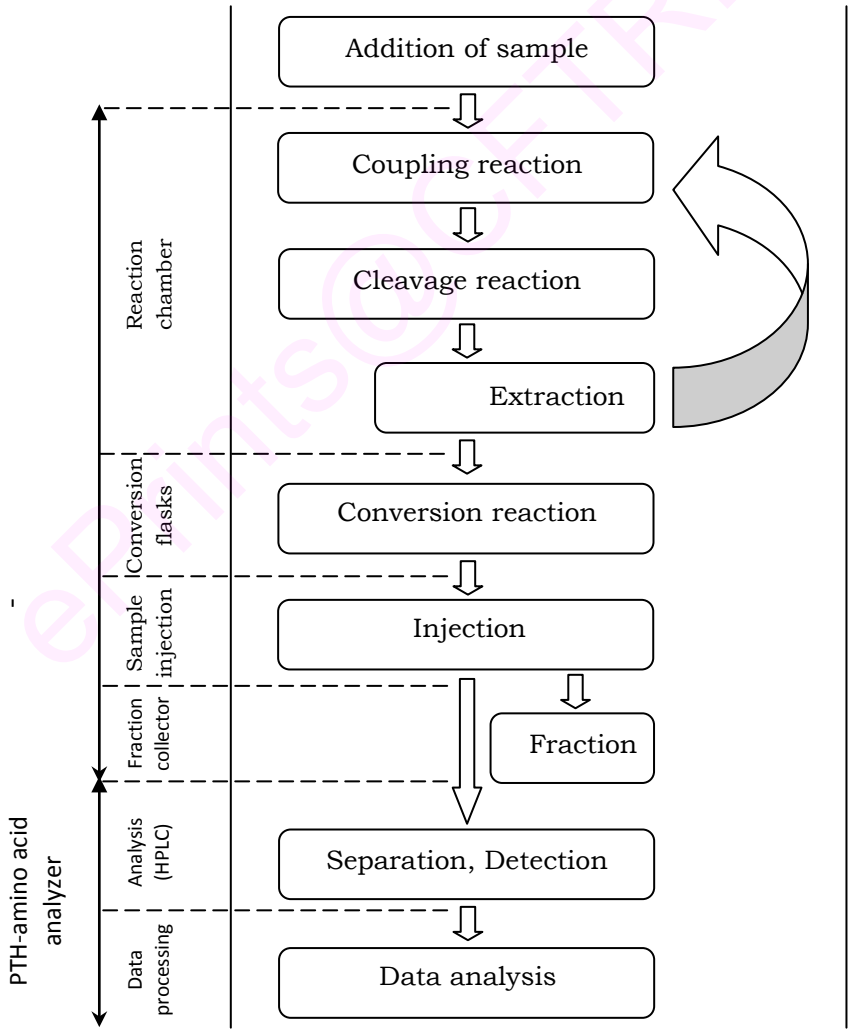


Fig. 44: Flow diagram for the reaction sequence that occurs during sequencing of proteins

In brief, the purified protein was subjected to SDS-PAGE followed by equilibrating the gel in Tris-glycine (192 and 24 mM, respectively) buffer containing 20% methanol and 0.1% SDS after which the protein (on the gel) was subjected to blotting on to PVDF membrane (for 3 hr). The latter was stained with CBB to locate the protein band, which was cut, thoroughly washed with water to remove excess glycine adsorbed on the membrane surface, followed by destaining with methanol. Thus processed membrane was directly placed on a glass fiber disc. The coupling reaction was carried out with phenyl isothiocyanate in the presence of gaseous trimethylamine. Excess reagents and by-products were washed with *n*-heptane and ethyl acetate. The cleavage reaction was carried out with gaseous TFA to form an anilino thiazolinone (ATZ) derivative. Both the coupling and cleavage reactions were performed in a temperature controlled reaction chamber. The free ATZ-amino acid was extracted into the conversion flask by *n*-butyl chloride and converted to more stable PTH-amino acid by reaction with 25% TFA, which was dissolved in acetonitrile and automatically injected into the HPLC. The residual PTH-amino acid was collected in a sample tube by a fraction collector. Separation of PTH-amino acid by HPLC was performed using an isocratic elution and the former in each cycle was identified using PROCISE software version 2.0.

Zymogram analysis

Proteolytic activity

The zymogram analysis of pepsin towards specific substrate was done according to Lopez *et al.* (1998). After electrophoresis (acid PAGE), the gel was soaked in 0.1 M HCl to reduce the pH to 2.0, for the enzyme to become active. After 15 min, the gel was resoaked for

30 min in a solution containing 0.25% haemoglobin in 0.1 M glycine-HCl, pH 2.0 at 4°C, then for 90 min in a fresh haemoglobin solution at 37°C. The gel was washed with distilled water and fixed for 15 min in 12% TCA, after which, stained with 0.1% CBB in methanol-glacial acetic acid-water (5:2:5). Destaining was carried out using an aqueous solution of 30% methanol-10% acetic acid.

Activity towards GG

After electrophoresis the gel was incubated with 0.5% GG at 40^0 C for 8 hr in acetate buffer of pH 5.5 and later stained with 0.1% Congo red (1 hr), excess stain was removed using 1 M potassium chloride.

Fluorescence studies

Fluorescence measurements were recorded on a Shimadzu RF 5000 spectrofluorimetre using 5 nm path length, at 27°C. Fluorescence emission spectra were recorded in a range of wavelength 300 to 450 nm with the excitation wavelength of 280 nm. Appropriate blanks were used for baseline correction of fluorescence intensity.

For fluorimetric titration, 3.0 mL of solution with appropriate concentration of pepsin (100 µg/mL) was titrated in acetate buffer of pH 5.5 with successive addition of GG solution (0-0.5 %).

Effect of inhibitors

Pepstatin, phenylmethylsulfonylketone (PMSF), N-tosyl-L-lysine-chloromethylketone (TLCK) hydrochloride and iodoacetate were used

to study the inhibition of both proteolytic and debranching activities of pepsin.

Each inhibitor (1 mg) was dissolved in 1 mL of solvent (i.e., pepstatin in 95:5 ethanol:acetic acid mixture, PMSF in absolute alcohol, TLCK and iodoacetate in water). Pepsin (1 mg mL^{-1} in 1 mM acetate buffer, 0.1 mL) was pre-incubated with different concentrations of inhibitors (2-10 mM) at 45°C for 1 hr followed by adding 1 mL of 0.5% GG solution, (pH 5.5) and assaying the products released. Simultaneously, proteolytic activity was studied using haemoglobin (0.25%) as the substrate at pH 2.0 using HCl-KCl buffer (1 mM).

Chemical modification of carboxyl residues

The importance of chemical modification of carboxyl groups in the activity of pepsin was investigated on reaction with N-(3-methyl amino propyl) N'-ethylcarbodiimide hydrochloride (EDAC) according to the method of Hoare & Koshland (1967). Pepsin was conjugated to glycine methyl ester (GME) using EDAC. To 5 mL (1mg/mL) of pepsin solution, 350 mg of GME and 85 mg of the carbodiimide were added. The pH was adjusted to 4.75 and the reaction was allowed to proceed for 10 min. The mixture was desalted and concentrated using a 10 kDa cut off centrifugal filter device.

Kinetics of carboxyl residue modification

Carboxyl residue modifications were performed at 25°C with pepsin in 0.05 M sodium acetate (pH 4.8) containing 0.275 M GME and 0-0.3 M EDAC. The latter were dissolved in water immediately before use and inactivation was initiated by the addition of EDAC.

A control experiment of enzymes and the nucleophile GME in buffer was run simultaneously which corresponds to 100% activity of pepsin.

Aliquots at 10 min intervals were removed for determination of residual activity and the pseudo first order rate constants for the inactivation were determined by the plot of log% residual activity against time. The inactivation kinetics was fitted to the equation: log (% residual activity) = $-k_i$ t, where k_i is the pseudo first-order inactivation rate constant for a given concentration of EDAC and t is time of inactivation. The inactivation order (n) was calculated from the equation: log K_i = n log [inactivator] + K_i, where log K_i is the second-order inactivation constant. The stoichiometry of the inactivation reaction was determined by a plot of log K_i versus log (EDAC). The slope of the curve represented the stoichiometry.

Peptide mapping of GG binding site of pepsin

Native and GG-bound pepsin were incubated with endoproteinase Glu-C from *Staphylococcus aureus* strain V8 (100:3, w/w) in 0.1 M phosphate buffer of pH 7.8 for 18 hr at 37°C. The proteolytic digestion was arrested by heating the reaction mixture for 20 min in a boiling water bath. The digest was concentrated to dryness and re-dissolved in 0.5 mL of 0.1 % TFA (Matsudaira, 1989).

The peptides released were then separated on R_p-HPLC using C_{18} column (4.6 mm × 150 mm; 5 μm) on a Shimadzu LC-10 system equipped with a binary pump at 230 nm at a flow rate of 0.7 mL min^{-1} using solvent A (0.1 % TFA in water) and solvent B (70 % CH_3CN containing 0.05 % TFA) (Mahoney & Hermodson, 1980; Hermodson & Mahoney, 1983). The solvent strength was increased linearly from 0 to 100 % solvent B in 85 min. The peptide profile of GG-bound pepsin

was compared and analyzed for the different peaks from the peptide profile of native pepsin. The distinct peptide was collected over several runs. The amino acid sequence of this peptide was determined by automated Edman degradation in an Applied Biosystems 491A automated gas phase protein sequencer.

Sequence alignment

The amino acid sequence of pepsin was obtained from Swiss-Port/TrEMBL (Acc.No.P00791). The sequence alignment of peptides with pepsin (Residue 139-148) was generated using CLUSTAL X (Higgins *et al.*, 1996) with subsequent gap adjustments.

Docking of GG with pepsin

The model for porcine pepsin was derived from the co-ordinates of the structure labeled 5pep in the RCSB Protein Data Bank, which represents pepsin at 2.3 Å resolution (Cooper *et al.*, 1999). The ligand GG (with 6 residues) was constructed and submitted to the PRODRG site (Schuettelkopf & van Aalten, 2004). The initial geometry and topologies were retrieved. The ligand GG was docked against porcine using Arguslab (Thompson, Planaria software, USA) with a grid spacing of 0.4 Å. Further calculations and graphical manipulations were performed using pyMol.

Results and Discussion

The commercial preparation of porcine pepsin on Native PAGE showed the presence of a few minor bands as contaminants along with a major band (over ~95%) and was purified by GPC (discussed in Chapter II).

Criteria of homogeneity

From GPC, the major protein band which showed maximum catalytic activity towards both protein as well as GG was pooled for further studies. The GPC-purified pepsin on Native PAGE showed a single band which corresponded to the earlier major band of crude pepsin (Fig. 45). Capillary zone electrophoresis and Rp-HPLC profile also showed the existence of a single peak (Figs. 46 & 47). The N-terminal sequence of the purified protein by Edman degradation revealed Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Thr-Glu-Tyr-Phe which was identical to the sequence of porcine pepsin reported by Sepulveda *et al.* (1973). All these results confirmed beyond ambiguity, the homogeneity the purified pepsin.

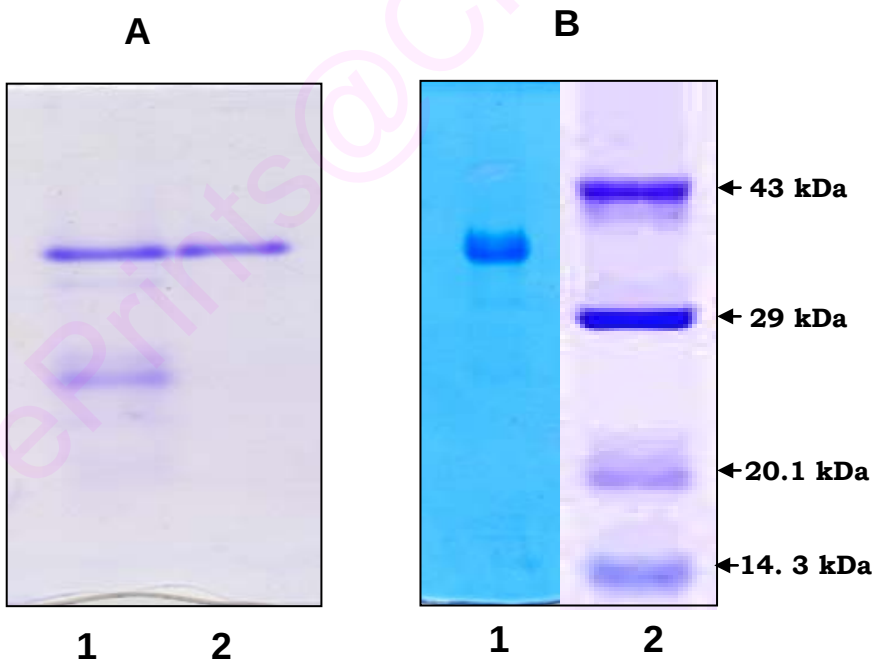


Fig. 45: Native PAGE of crude pepsin (Lane 1) and purified pepsin (Lane 2); SDS-PAGE of purified pepsin (Lane 3) and protein markers (Lane 4)

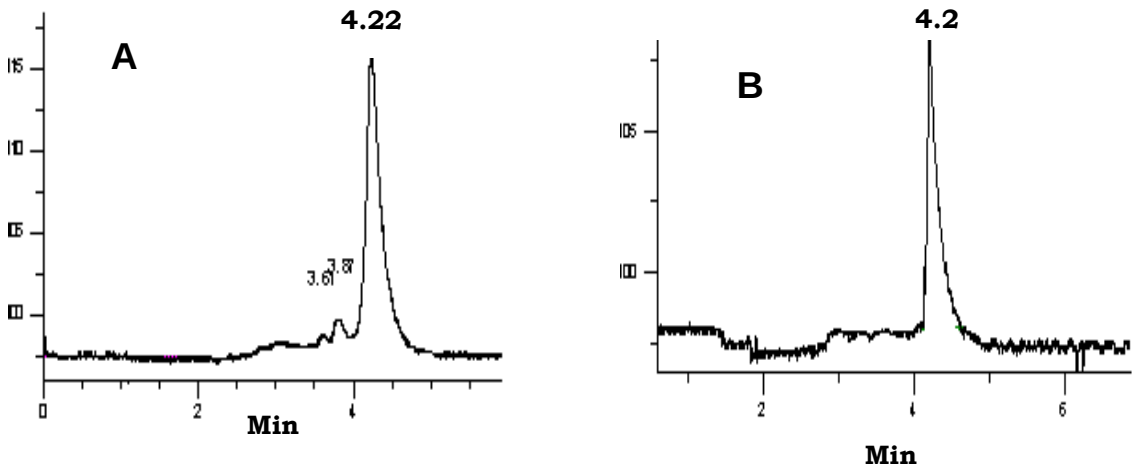


Fig. 46: Capillary zone electrophoresis of **A.** crude pepsin, **B.** purified pepsin

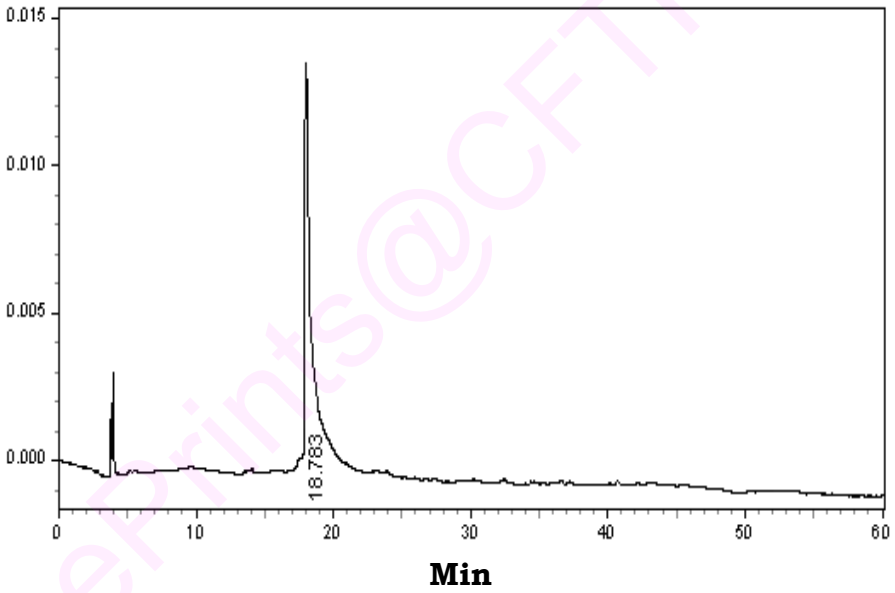


Fig. 47: Rp - HPLC profile of purified pepsin

The purified pepsin (with molecular markers ovalbumin - 43,000 Da, carbonic anhydrase - 29,000 Da, Soyabean trypsin inhibitor - 20,100 and Lysozyme - 14,300 Da) on SDS-PAGE showed a single band with Mw of ~ 34,900 ± 1200 Da. MALDI-TOF-MS also revealed a single peak with a Mw 34,511 Da (Fig. 48), which was in concordance with the reported molecular weight of porcine pepsin.

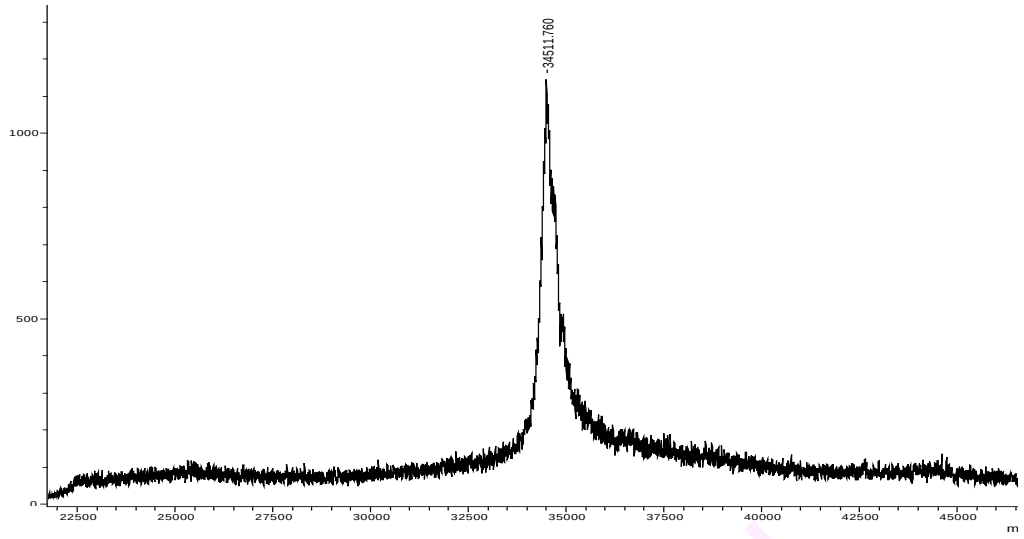


Fig. 48: MALDI- TOF- MS of purified pepsin

The zymogram analysis of the purified pepsin on acid-PAGE for proteolytic activity and activity towards GG showed a single clear zone (Fig. 49) confirming the role of pepsin in both proteolysis and debranching of GG. These results clearly established the involvement of pepsin in dual catalysis.

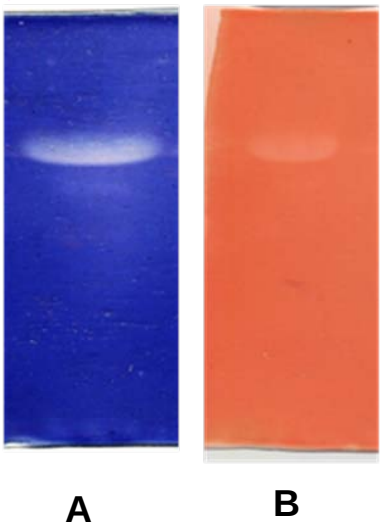


Fig. 49: Zymogram analysis of pepsin, **A.** Proteolysis, **B:** Activity on GG

Fluorescence studies on binding of pepsin with GG

Fig. 50 reveals the fluorescence spectra of native and GG-bound pepsin. The titration of GG with pepsin resulted in an increased quenching as the concentration of GG increased. The changes in the fluorescence intensity of the protein upon addition of GG also indicated the conformational changes favoring the hydrolytic process.

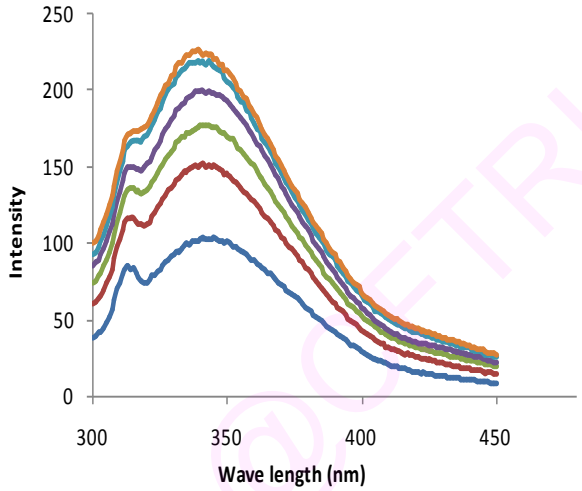


Fig. 50: Fluorescence spectra of pepsin on titration with GG

Effect of inhibitors on dual activity of pepsin

Table 21 shows the effect of various inhibitors on the activity of pepsin towards both haemoglobin and GG. Pepstatin (Isovaleryl-Val-Val-Sta-Ala-Sta, where Sta = statin (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid), an inhibitor of acid proteases such as pepsin, rennin, cathepsin D, etc. could bring about 100% inhibition of proteolytic activity of pepsin without any inhibition for its activity towards GG (Fig. 51). Whereas the addition of PMSF and TLCK (inhibitors of serine – cysteine and histidine – cysteine, respectively) did not bring about the inhibition of both proteolytic as well as activity

towards GG, thereby ruling out the contribution of serine, cysteine and histidine during catalysis of GG by pepsin.

Table 21. Effect of inhibitors on activity of pepsin towards haemoglobin and GG

Inhibitor	Inhibition (%)	
	Proteolysis	GG
Pepstatin		
2 mM	90	0
4 mM	100	0
10 mM	100	0
PMSF		
5 mM	0	0
10 mM	0	0
15 mM	0	0
TLCK		
5 mM	0	0
10 mM	0	0
15 mM	0	0

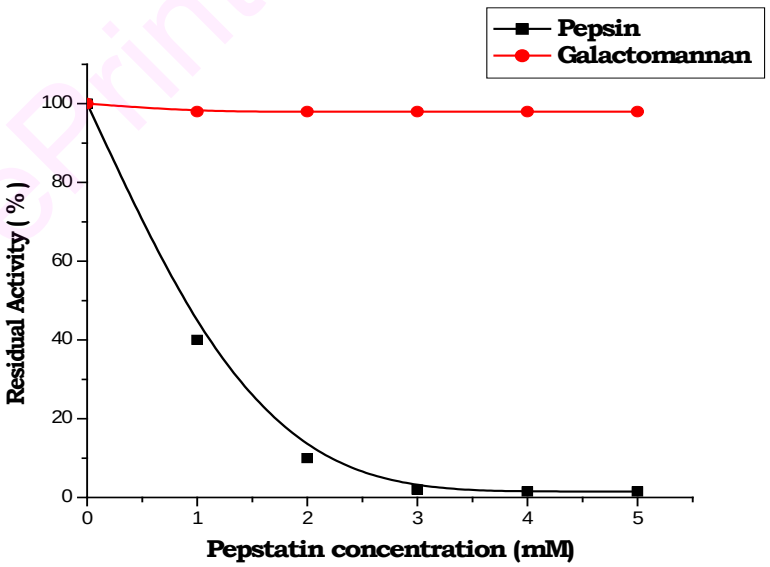


Fig. 51: Effect of pepstatin on the activity of pepsin towards haemoglobin and GG

Chemical modification of carboxylic groups

The inefficiency of pepstatin in inhibiting the activity of pepsin towards GG indicated the role of different amino acid(s) for catalysis. The confirmation of the absence of serine, cysteine and histidine and also shift in the pH optima of pepsin indicated the role of (different) carboxyl groups during catalysis of GG by pepsin. An examination of α-galactosidase (for debranching of GG) sequence indicated Asp residues in the catalysis. Hence, EDAC, which is specific for the modification of carboxylic groups was attempted to know the involvement of a carboxylic residue at the active site of pepsin. Fig. 52 shows the kinetic studies on the inactivation of pepsin with various concentrations of EDAC.

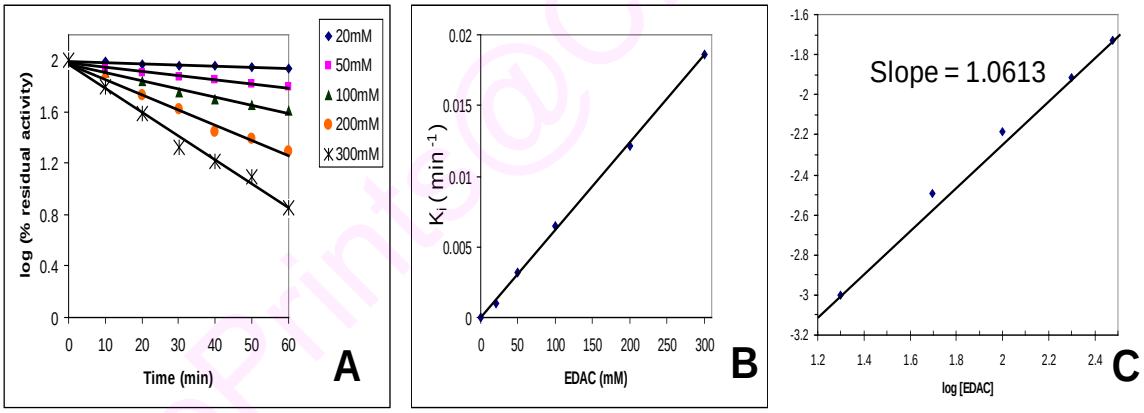


Fig. 52: Inactivation of pepsin by EDAC and GME **A:** Plot of log (% residual activity) versus time, **B:** Plot of pseudo first order inactivation rate constant as a function of EDAC concentration, **C:** Double logarithimic plot of pseudo first order inactivation rate constant as a function of EDAC concentration

Incubation of pepsin individually with GME and EDAC resulted in the lack of inhibition of the enzyme activity, thus supporting the inactivation due to direct involvement of carboxylic groups and not due to the cross linking of other amino acids. The requirement of EDAC concentration, for the inactivation reaction was relatively high which could be due to the high content of carboxylic groups in pepsin. A similar requirement of high concentration of EDAC for the modification of glutathione transferase (100 mM, Xia *et al.*, 1993), oxalate oxidase (150 mM, Kotsira & clonis, 1998), α-1,4-glucan lyase (200 mM, Nyvall *et al.*, 2000), field bean PPO and sweet potato ibCO (300 mM, Gowda & Paul, 2002; Klabunde *et al.*, 1998) has been reported.

The semi-logarithmic plot of residual activity at various EDAC concentrations versus time was linear for pepsin indicating the inactivation following pseudo first-order kinetics. A plot of first-order inactivation rate constant (k_i) against EDAC concentration was also linear. A plot of log K_i versus log [EDAC] yielded a slope of 1.0613. Since the stoichiometry of the inactivation reaction was near to 1.0 with respect to EDAC, it suggested the involvement of a single carboxylate group in the catalytic activity of pepsin towards GG.

Identification of GG binding site of pepsin

Analysis of the primary structure of protein followed by peptide mapping is essential in the studies of ligand-protein interaction (Lundell & Schreitmuller, 1999). Thus, inorder to identify the GG binding site the pepsin, conjugated with and without GG was subjected to proteolysis with Glu-C V8 protease and analysed by Rp-HPLC (Fig. 53 A & B).

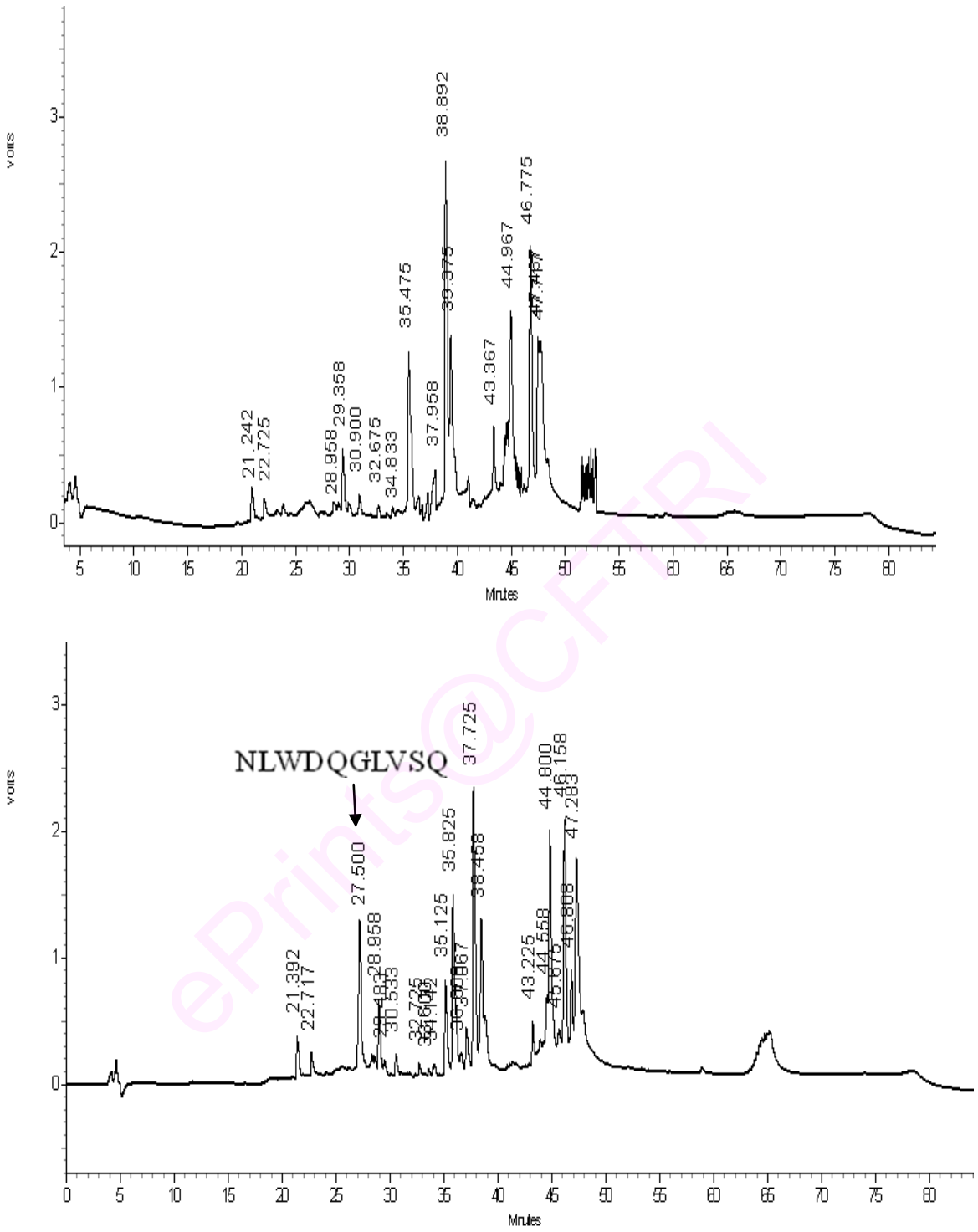


Fig. 53: Rp-HPLC profile of the proteolytic digests of pepsin in the **A:** absence, **B:** presence of GG

The cleavage of pepsin at the carboxyl side of glutamic acid residues did not show any distinct peak in camparison to the native profile of the peptides, thus ruling out the contribution of glutamic acid residues in the binding site of pepsin. Whereas the cleavage at the carboxyl side of aspartic acid resulted in a distinct peak (at 27.5 min) which was absent in the peptide map of pepsin unbound to GG. This peak was collected and subjected to Edman degradation, which showed the sequence NLWDQGLVSQ corresponding to the residues 139-148 of porcine pepsin. In other words, binding of GG protected the Asp residue in pepsin from proteolysis, which also substantiated the loss of enzyme activity upon chemical modification of carboxylic groups.

Docking of GG with pepsin

The docking simulations use computational methodologies to mimic the biochemical process of a ligand approaching the active site of the receptor. The prediction of ligand binding site is an essential part of the protein-ligand interaction studies. Fig. 54 shows the computational simulation of the interaction of GG with pepsin, which showed Asp^{138} of pepsin to be involved in catalyzing the removal of galactose residues. The perfect ligand binding pattern of GG-pepsin complex showed a mean binding energy of -6.5 kcal mol^{-1} with Asp - 138 residue at a distance of 6.4 Å from the glycosidic oxygen of galactose residue. Also, the proline and phenylalanine residues of pepsin indicate a hydrophobic interaction with the ring oxygen of galactose and mannose moieties thereby facilitating effective binding. Liu *et al.* (2007) report the hydrophobic interaction of aromatic side chain of Phenylalanine and proline with the glucose ring of β-cyclodextrin influencing the surrounding residues in the binding of ligand. The contribution of Asp^{142} (identified from GG-binding peptide

sequence) in debranching of GG is also evident from computational model of ligand complex.

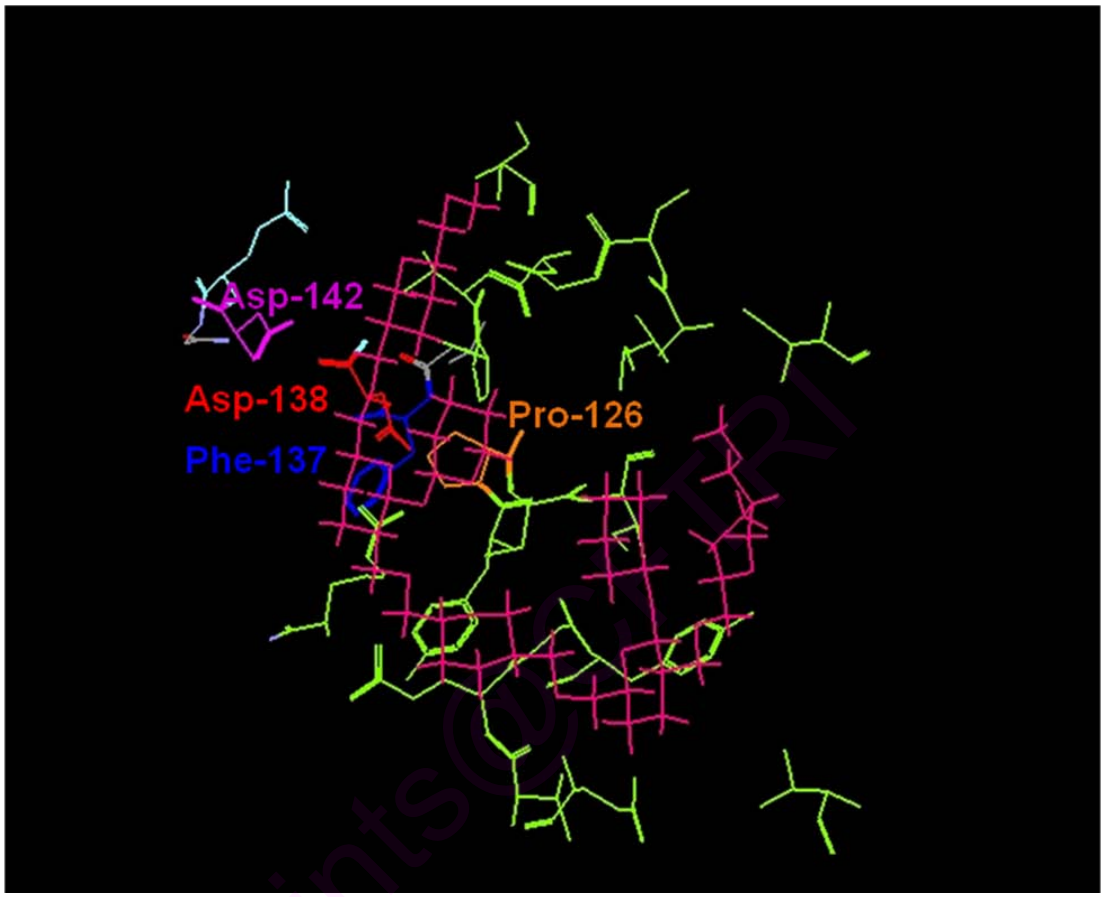


Fig. 54: Docking of GG with pepsin

Plausible mechanism of action of pepsin on GG

The computational modeling studies also revealed a single carboxylate group (Asp^{138}) of pepsin to be involved in the catalysis of GG. This result is in concordance with the chemical modification of carboxylate group which showed a stoichiometry of the inactivation reaction near 1.0. Generally acid catalysis by hydrolases follows double displacement reaction which requires two acidic residues in the active site leading to the hydrolysis of the substrate. Nevertheless,

reports by various authors also reveal an alternative mechanism with a single active site carboxylate residue involved in the catalytic process. Weaver *et al.* (1995) discount the need of a second carboxylate group (Asp^{52}) for catalysis in goose lysozyme thereby ruling out the double displacement mechanism for goose egg white lysozyme (GEWL). Perrakis *et al.* (1994) and Terwisscha van Scheltinga *et al.* (1994) report the X-ray crystal structure of two chitinases (family 18) with no second acidic residue in the active site capable of stabilizing the oxocarbenium ion. Based on the above results, a probable mechanism of action of pepsin towards GG has been proposed (Fig. 55).

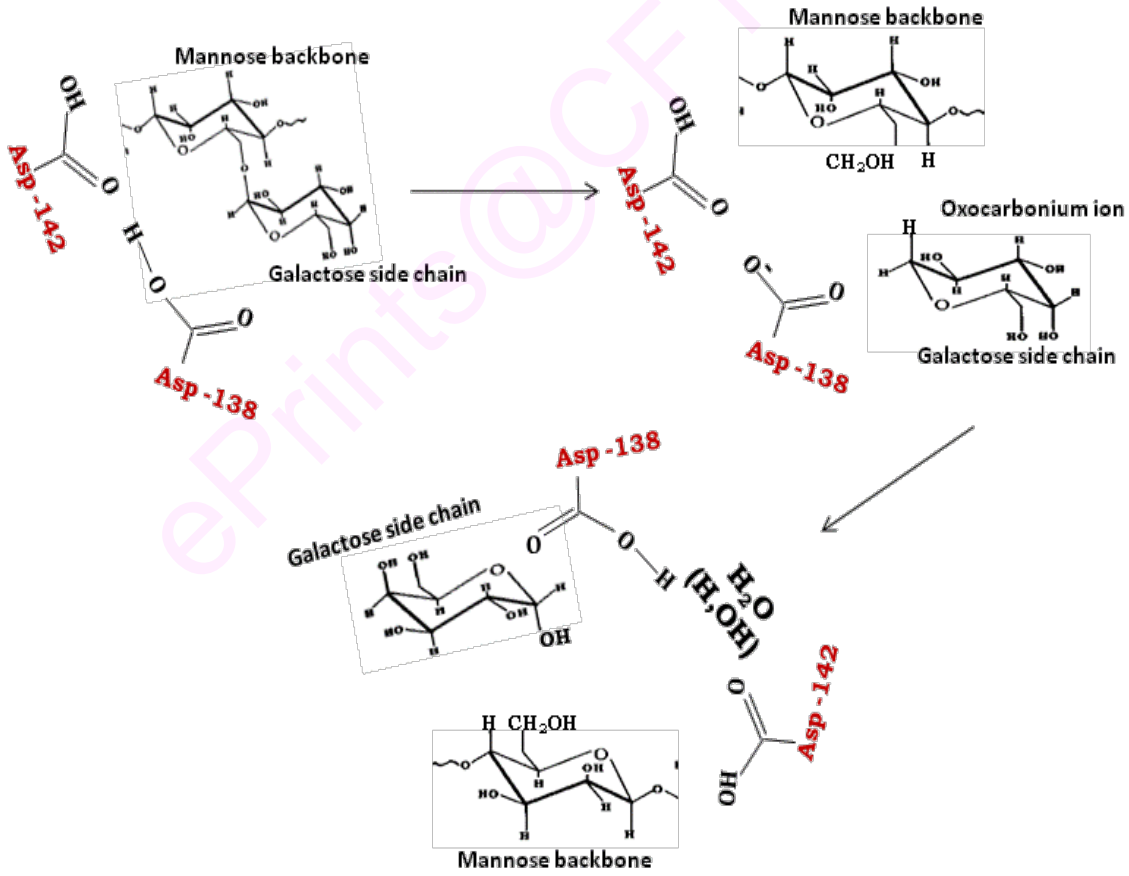


Fig. 55: Plausible mechanism of action of pepsin towards GG

Asp^{138} of porcine pepsin in a nonpolar milie contributed by Trp^{141}, Val^{136}, Phe^{137} and Leu^{140} is protonated. Therefore upon galactomannan binding Asp^{138} donates a H^+ ion to the glycosidic oxygen between the C1 of galactose and C6 of mannose residue (α-1,6 linkage). Thus, the transfer of proton cleaves the glycosidic bond thereby creating a positive charge on the C1 of the galactose ring by forming a transient oxocarbonium ion, which is stabilized by neighbouring Asp^{142} which is in a polar environment. The resulting oxocarbonium ion intermediate reacts with OH^- of the water thereby completing the reaction and also reprotonating the Asp^{138} by H^+ ion which becomes available for subsequent catalysis. Bjelic & Aquist (2004) report a favoured mechanism involving the direct participation of single catalytic aspartate performing both acid-base functions, with the neighbouring histidine residue providing required stabilization for the reaction. The docking simulations of GG to pepsin showed distance of 6.4 Å between the Asp-138 and glycosidic oxygen and 10.4 Å between Asp-142 and oxocarbonium ion, which is greater than the normally required 3-4 Å. This increased distance may possibly explain the lower activity towards guar galactomannan when compared to its proteolytic activity. It is also evident that the increased distance contributes to the slow down of hydrolysis (Brameld & Goddard III, 1998).

Conclusion

The present investigation shows that a single protein is involved in the catalysis of both specific and other (non-specific) substrates. The commercial porcine pepsin showed a few minor bands as contaminants with the major band which was purified to homogeneity. The Mw of the purified pepsin was in concordance with that reported. Zymogram analysis revealed the association of both

proteolytic as well as debranching activities, confirming the dual catalytic activity of purified pepsin. Use of site-specific inhibitors and chemical modification agents showed the involvement of aspartic acid residue at the active site. Docking simulations of GG-pepsin complex further confirmed the involvement of Asp-138 residue in the catalysis of GG.

Summary and Conclusions

Guar galactomannan (GG), is a high molecular weight co-polymer consisting of β-1,4-D-mannopyranose as backbone residues and α-1,6-D-galactopyranose as a single residue side chain stub on every alternative mannose unit, with a G/M ratio of 1:2. Compared to the native GG, debranched (by enzymatic hydrolysis with α-galactosidase) GG showed enhanced functional properties. But the utility of this technology is not feasible because only the α-galactosidase isolated from germinated guar seeds is effective. Also, the high cost involved for the isolation of this enzyme and laborious production steps are non-economical for bulk use.

Screening of several enzymes for their (non-specific) activity towards GG revealed appreciable debranching activity by pectinase, pullulanase and pepsin. Purification of these enzymes by GPC followed by activity measurements established the affinity of a single protein towards both specific substrate and GG. The kinetic studies of purified enzymes showed differences in pH and temperature optima, due to pH dependent conformational changes in the enzymes. Studies on the effect of enzyme-substrate concentration obeyed Michaelis-Menton kinetics, showing an increase in the rate of catalysis to be directly proportional to the enzyme concentration.

The resulting galactose-depleted GG (GDGG) showed a decrease in Mw and appeared as a single peak in HPLC and GPC. Its GLC analysis revealed a significant change in G/M ratio with reduced viscosity, thus mimicking that of LBG. FT-IR and NMR analyses indicated subtle changes in its conformation. Characterization of the products (galactose residues) released as a result of hydrolysis showed the exoaction of these enzymes. In addition, pepsin and pectinase upon

extended incubation time exhibited an endoaction with the release of oligomers containing both galactose and mannose.

The blend of GDGG with xanthan and κ-carrageenan exhibited a rheological behaviour having elastic modulus (G') greater than viscous modulus (G'') with a decrease in tanδ value similar to LBG/xanthan blend. Also a two fold increase in the magnitude of elasticity compared to xanthan and κ-carrageenan suggested synergistic interaction with the formation of a three dimensional network.The blend also showed a maximum increase in magnitude with high yield stress. DSC analysis revealed maximum synergistic interaction.

Zymogram analysis, use of site-specific inhibitors, chemical modification of carboxyl residues, peptide mapping of native and GG-bound pepsin on proteolytic digestion with Glu-C V8 protease and molecular docking studies revealed the involvement of Asp 138 in the catalysis of GG by pepsin.

Although catalysis by unrelated enzymes on GG is unusual and non-specific, it enables an alternative way to produce GDGG with enhanced rheological characteristics and as a cost-effective replacement for LBG. Such an approach may assume greater significance in view of product development requirements of the food and other allied industries.

Ademark, P., Larsson, M., Tjerneld, F. and Stalbrand, H. Multiple alpha-galactosidase from *Aspergillus niger*: Purification, characterization and substrate specificities. Enzyme and Microbial Technology, 2001, 29, 441-448.

Alexander, R.J. Hydrocolloid gums. Part I. Natural products. Cereal Foods World, 1999, 44, 684-686.

Alloncle, M. and Doublier, J. L. Viscoelastic properties of maize starch/hydrocolloid pastes and gel. Food Hydrocolloids, 1991, 5, 455-467.

Altaf, S.A., Yu, K., Parasrampuria, J. and Friend, D.R. Guar gum-based sustained release diltiazem. Pharmcological Research, 1998, 15, 1196–1201.

Amano, K. and Ito, E. Action of lysozyme on partially deacetylated chitin. European Journal of Biochemistry, 1978, 85, 97-104.

Anderson, D.M.W. Regulatory aspects. In Stephen, A.M. (ed.), Food Polysaccharides and their Applications, Academic press, New York, 1995, 607-632.

Anna, B., Eva, N.K. and Olle, H. Substrate-dependent production and some properties of a thermostable α-galactosidase from *Rhodothermus marinus*. Biotechnology Letters, 2000, 22, 663-669.

Anson, M. Estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. Journal of General Physiology. 1938, 22, 79-85.

Antonov, V.K., Ginodman, L.M., Kapitannikov, Y.V., Barshevskaya, T.N., Gurova, A.G. and Rumsh, L.D. Mechanism of pepsin catalysis: general base catalysis by the active site carboxylate ion. FEBS Letters, 1978, 88, 87-90.

Arakawa, T., Hsu, Y. R. and Yphantis, D. A. Acid unfolding and self-association of recombinant *Escherichia coli* derived human interferon gamma. Biochemistry, 1987, 26, 5428-5432.

Aro, A., Uusitupa, M., Voutilainen, E., Hersio, K., Korhonen, T. and Siitonen, O. Improved diabetic control and hypocholesterolaemic effect

induced by long-term dietary supplementation with guar gum in type 2 (insulin independent) diabetes. Diabetologia, 1981, 21, 29-33.

Aslandis, C., Schmid, K. and Schmitt, R. Nucleotide sequence and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. Journal of Bacteriology, 1989, 171, 6753-6763.

Aspinall, G.O. and Whyte, J.N.C. Polysaccharides of soy-beans. Part-I. galactomannans from the hulls. Journal of Chemical Society, 1964, 5058-5068.

Aspinall, G.O. Structural chemistry of the hemicelluloses. Wolfrom, M.L. (ed.), Advances in Carbohydrate Chemistry, Academic press, New York, 1959, 14, 429-468.

Bailey, R.W. In Chemotaxonomy of the leguminosae. In Harborne, J.B., Boulter, D. and Turner, B.L. (eds.), Academic Press, London, 1971, 503-541.

Baird, J.K. Analysis of gums in foods. In Whistler, R.L. and BeMiller, J.N. (eds.), Industrial polysaccharides and their derivatives (3rd ed.), Academic Press, San Diego, 1992, 605-618.

Baker, C.W. and Whistler, R.L. Distribution of D-galactosyl groups in guaran and locust bean gum. Carbohydrate Research, 1975, 45, 237-243.

Bjelic, S. and Aqvist, J. Computational prediction of structure, substrate binding mode, mechanism, and rate for a malaria protease with a novel type of active site. Biochemistry, 2004, 43, 14521-14528.

Blackburn, N. A., Redfern, J. S., Jarjis, H., Holgate, A**.** M., Hanning, I., Scarpello, J. H., Johnson, I. T. and Read, N. W. The mechanism of action of guar gum in improving glucose tolerance in man. Clinical Science, 1984, 66, 329-336.

Brameld, K.A. and Goddard III, W.A. Substrate distortion to a boat conformation at subsite -1 is critical in the mechanism of family 18 chitinases. Journal of American Chemical Society, 1998, 120, 3571-3580.

Brooks, M., Philip, K., Cooney, G. and Horgan, L. Designing galactomannans for the food industry. In Williams, P.A. and Phillips, G.O. (eds.), Gums and stabilizers for the food industry, RSC, Cambridge, UK, 2000, 10, 421-428.

Buchard, W. and Ross-Murphy, S.B. Physical gels from synthetic and biological macromolecules. In Burchard, W. and Ross-Murphy, S.B. (eds.), In Physical networks: Polymers and gels, Elsevier, Oxford, 1990, 1-14.

Buckeridge, M.S., Panegassi, V.R., Rocha, D.C. and Dietrich, S.M.C. Seed galactomannan in the classification and evolution of leguminoseae. Phytochemistry, 1995, 38, 871-875.

Buckeridge, M.S., Santos, H.P and Tine, M.A.S. Mobilization of storage cell wall polysaccharides in seeds. Plant Physiology and Biochemistry, 2000, 38, 141-156.

Bulpin, P.V., Gidley, M.J., Jeffcoat, R. and Underwood, D.R. Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase. Carbohydrate Polymers, 1990, 12, 155-168.

Buyong, N. and Fenema, O. Amount and size of ice crystals in frozen samples as influenced by hydrocolloids. Journal of Dairy Science, 1988, 71, 2630-2639.

Cairns, P., Miles, M.J. and Morris, V.J. Intermolecular binding of xanthan gum and carob gum. Nature, 1986, 322, 89-90.

Cairns, P., Miles, M.J., Morris, V.J. and Brownsey, G.J. X-ray fiber diffraction studies of synergistic, binary polysaccharides gels, Carbohydrate Research, 1987, 160, 411-423.

Campbell, J. McA. and Reid, J.S.G. Galactomannan formation and guanosine-5-diphosphate–mannose:galactomannan mannosyltransferase in developing seeds of fenugreek (*Trigonella foenum – graecum*, L., *Leguminosae*). Planta, 1982, 155,105-111.

Campos, L. A. and Sancho, J. The active site of pepsin is formed in the intermediate conformation dominant at mildly acidic pH. FEBS Letters, 2003, 538, 89-95.

Chandrasekaran, R. and Radha, A. Molecular modeling of xanthan: galactomannan interactions. Carbohydrate Polymers, 1997, 32, 201-208.

Cheng, Y. and Prud'homme, R. K. Enzymic degradation of guar and substituted guar galactomannans. Biomacromolecules, 2000, 1, 782-788.

Cheng, Y., Brown, K.M. and Prudhome, R.K. Preparation and characterization of molecular weight fraction of guar galactomannan using acid and enzymatic hydrolysis. International Journal of Biological Macromolecules, 2002, 31, 29-35.

Chidwick, H., Dey, P., Hart, R., McKenzie, A. and Pridham, J. B. Cogelation of xanthan gum with modified guar galactomannan. Biochemical Society Transactions, 1991, 19, 269S.

Cho, S.S. and Prosky, L. Application of complex carbohydrates to food product - fat mimetics. In Complex carbohydrates in foods, Marcel Dekker, Ltd., New York, 1999, 411-429.

Chourasia, M.K. and Jain, S.K. Polysaccharide for colon targeted drug delivery. Drug Delivery, 2004, 11, 129–148.

Chourasiya, M., Chourasiya, M.K., Jain, N.K., Jain, A., Soni,V., Gupta, Y. and Jain, S.K. Crosslinked guar gum microspheres: A viable approach for improved delivery of anticancer drugs for the treatment of colorectal cancer. AAPS PharmSciTech, 2006, 7, 74-78.

Christianson, D.D., Hodge, J.E., Osborne, D. and Detroy, R.W. Gelatinization of wheat starch as modified by xanthan gum, guar gum and cellulose gum. Cereal Chemistry, 1981, 58, 513-517.

Civas, A., Eberhard, R., Le Dizet, P. and Petek, F. Glycosidases induced in *Aspergillus tamarii.* Mycelial α-galactosidases. Biochemical Journal, 1984, 219, 849-855.

Clark, A.H. and Ross-Murphy, S.B. Structural and mechanical properties of biopolymers gels. Advances in Polymer Science, 1987, 83, 55-192.

Cleland, W.W. Substrate inhibition. Methods in Enzymology, 1979, 63, 500-513.

Cooper, J.B., Khan, G., Taylor, G., Tickle, I.J. and Blundell, T.L. X-ray analyses of aspartic proteases. II. Three dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 A resolution. Journal of Molecular Biology, 1990, 214, 199-222.

Crescenzi, V., Dentini, M., Risica, D., Spadoni, S., Skjak-Braek, G., Capitani, D., Mannina, L. and Viel, S. C(6)-oxidation followed by C(5) epimerization of guar gum studied by high field NMR. Biomacromolecules 2004, 5, 537–546.

Cunha, P.L.R., Castro, R.R., Rocha, F.A.C., de Paula, R.C.M. and Feitosa, J.P.A. Low viscosity hydrogel of guar gum: Preparation and physicochemical characterization. International Journal of Biological Macromolecules, 37, 2005, 99–104.

Cuvelier, G. and Launay, B. Viscoelastic properties of xanthan / carob mixed gels. In Phillips, G.O., Williams, P. A. and Wedlock, D.J. (eds.). In Gums and stabilizers for the food industry, Pergamon Press, Oxford, 1986, 3, 147-158.

D'Melo, D.J. and Shenoy, M.A. Evaluation of mechanical properties of acrylated guar gum - unsaturated polyester composites. Polymer Bulletin, 2008, 61, 235–246.

Daniel, J.R., Whistler, R.L., Voragen, A.G.J. and Pilnik, W. Starch and other polysaccharides. Ullmann's encyclopedia of Industrial Chemistry, Verlagsgesellschaft MBH Weinheim. Germany, 1994, A25.

Daniel, R., Mariella, D. and Vittorio, C. Guar gum methyl ethers. Part I. Synthesis and macromolecular characterization. Polymer, 2005, 46, 12247–12255.

Dea, I.C.M. and Morrison, A. Chemistry and interaction of seed galactomannans. In Tipson, R.S. and Horton, D. (eds.), Advances in Carbohydrate Chemistry and Biochemistry, Academic press, New York, 1975, 31, 241-312.

Dea, I.C.W., Morris, E.R., Rees, D.A., Welsh, E.J., Barnes, H.A. and Price, J. Associations of like and unlike polysaccharides: Mechanism and specificity in galactomannans, interacting bacterial

polysaccharides, and related systems. Carbohydrate Research, 1977, 57, 249-272.

Dey, P.M. Biochemistry of plant galactomannans. In Tipson, R.S. and Horton, D. (eds.), Advances in Carbohydrate Chemistry and Biochemistry, Academic press, New York, 1978, 35, 341-376.

Dickinson, E. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. Food Hydrocolloids, 2003, 17, 25-39.

Doublier, J.L. and Llamas, G. Flow and viscoelastic properties of mixed xanthan gum and galactomannan systems. Dickinson, E. (ed.). In Food polymers, gels and colloids, RSC, Cambridge, UK, 1991, 349 – 356.

Dziezak. J.D. A focus on gums. Food Technology, 1991, 45, 116-132.

Ebinger, C. D. and Hunt, E. Keys to good fracturing - 6. New fluids help increase effectiveness of hydraulic fracturing. Oil & Gas Journal, 1989, 87, 52-55.

Ebling, P., Hannele, Y. J., Aro, A., Helve, E., Sinisalo, M. and Koivisto, V. A. Glucose and lipid metabolism and insulin sensitivity in type 1 diabetes: the effect of guar gum. American Journal of Clinical Nutrition, 1988, 48, 93-103.

Edwards, C. A. and Read, N. W. Fibre and small intestinal function. In Leeds, A. R. (ed.), In Dietary Fibre Perspectives 2, John Libbey, London, 1990, 52-75.

Edwards, M., Bulpin, P.V., Dea, I.C.M. and Reid, J.S.G. Biosynthesis of legume seed galactomannans *in vitro*. Planta, 1989, 178, 41-51.

Edwards, M., Dickson, C.A., Chengappa, S., Sidebottom, C., Gidley, M.J. and Reid J.S.G. Molecular characterization of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. Plant Journal, 1999, 19, 691-697.

Edwards, M., Marshall, E., Gidley, M.J. and Reid, J.S.G. Transfer specificity of detergent solubilized fenugreek galactomannan galactosyl transferase. Plant Physiology, 2002, 129, 1391-1397.

Edwards, M., Scott, C., Gidley, M.J. and Reid, J.S.G. Control of mannose/galactose ratio during galactomannan formation in developing legume seeds. Planta, 1992, 187, 67-74.

Elina, L., Maija, T. and Liisa, V. Substrate specificities of *Penicillium simplicissimum* α-galactosidase. Enzyme and Microbial Technology, 1998, 22, 192-198.

Ellis, P. R., Kamalanathan, T., Dawoud, F. M., Strange, R. N. and Coultate, T. P. Evaluation of guar biscuits for use in the management of diabetes: tests of physiological effects and palatability in non-diabetic volunteers. European Journal of Clinical Nutrition, 1988, 42, 425-435.

Fernandes, P.B., Goncalves, M.P. and Doublier, J.L. A rheological characterization of kappa-carrageenan/galactomannan mixed gels: A comparison of locust bean gum samples. Cabohydrate Polymers, 1991, 16, 253 – 274.

Fernandes, P.B., Goncalves, M.P. and Doublier, J.L. Effect of galactomannan addition on the thermal behaviour of κ-carrageenan gels. Carbohydrate Polymers, 1992, 19, 261-269.

Fernandes, P.B., Goncalves, M.P. and Doublier, J.L. Phase diagrams in kappa-carrageenan / locust bean gum systems. Food Hydrocolloids, 1991, 5, 71-73.

Fox, J. E. In Thickening and Gelling Agents for Food. In Imeson, A., (ed.) Blackie Academic and Professional, London, 1992, 153-171.

Fringant, C., Tvaroska, I., Mazeau, K., Rinaudo, M. and Desbrieres, J. Hydration of α-maltose and amylase; Molecular modeling and thermodynamic study. Carbohydrate Research, 1995, 278, 27-41.

Fu, J.Y., Wu, S.M., Chang, C.T. and Sung, H.Y. Characterization of three chitosanase isozymes isolated from a commercial crude porcine pepsin preparation. Journal of Agricultural and Food Chemistry, 2003, 51, 1042-1048.

Gamal-Eldeen, A. M., Amer, H. and Helmy, W. A. Cancer chemopreventive and anti-inflammatory activities of chemically modified guar gum. Chemico-Biological Interactions, 2006, 161, 229-240.

Garti, N. and Reichman, D. Hydrocolloids as food emulsifiers and stabilizers. Food Structure, 1993, 12, 411-426.

Gidley, M.J. and Reid, J.S. Galactomannans and other cell wall storage polysaccharides in seeds. In Stephen, A.M., Phillips, G.O. and Williams, P.A. (eds.), Food Polysaccharides and their Applications, 2nd edition, Academic press, New York, 2006, 181-185.

Glasser, W. G. Polysaccharide applications - cosmetics and pharmaceuticals. Wood and Fiber Science, 2000, 32, 387-388.

Glicksman, M. Hydrocolloids and search for oily grail. Food Technology, 1991, 45, 96-103.

Gliko-Kabir, I., Yagen, B., Penhasi, A. and Rubinstein, A. Low swelling, cross-linked guar and its potential use as colon-specific drug carrier. Pharmacological Research, 1998, 15, 1019–1025.

Gliko-Kabir, I., Yagen, B., Penhasi, A. and Rubinstein, A. Phosphated cross-linked guar for colon-specific drug delivery. I. Preparation and physicochemical characterization. Journal of Controlled Release, 2000, 63, 121–127.

Goto, Y. and Fink, A. L. Conformational states of β - lactomase: Molten-globule states at acidic and alkaline pH with high salt. Biochemistry, 1989, 28, 945-952.

Gowda, L.R. and Paul, B. Diphenol activation of the monophenolase and diphenolase activities of field bean (*Dolichos lablab*) polyphenol oxidase. Journal of Agricultural and Food Chemistry, 2002, 50, 1608-1614.

Goycoolea, F.M., Milas, M. and Rinaudo, M. Associative phenomena in galactomannan-deacetylated xanthan systems. International Journal of Biological Macromolecules, 2001, 29, 181–192.

Greenberg, N.A. and Slavin, J.L. Partially hydrolysed guar gum: Clinical nutritional uses, Nutrition, 2003, 19, 549-552.

Gujral, H.S., Sharma, A. and Singh, N. Effect of hydrocolloids, storage temperature and duration on the consistency of tomato ketchup. International Journal of Food Properties, 2002, 5, 179-191.

Gupta, B.S. and Ako, J.E. Application of guar gum as a flocculant aid in food processing and potable water treatment. European Food Research and Technology, 2005, 221, 746–751.

Hans, G. and Terence, P. NMR studies of composition and sequence in legume bean gum galactomannan. Carbohydrate Research, 1980, 81, 59-66.

Hashimoto, W., Miki, H., Wanakai, H., Sato, N., Kawai, S. and Murata, K. Molecular cloning of two genes from β-D-glucosidase in *Bacillus sp. GL1* and identification of one as a gellan-degrading enzyme. Achieves of Biochemistry and Biophysics, 1998, 360, 1-9.

Hermodson, M., and Mahoney, W. C. Separation of peptides by reversed-phase high-performance liquid chromatography, Methods in Enzymology, 1983, 91, 352–359.

Higgins, J. D. G., Thompson, D., and Gidson, T. J. Using CLUSTAL for multiple sequence alignments, Methods in Enzymology, 1996, 266, 383-402.

Hinrichs, R., Gotz, J. and Weisser, H. Water holding capacity and structure of hydrocolloid gels, WPC-gels and yogurts characterized by means of NMR. Food Chemistry, 2003, 82, 155-160.

Hirano, S. and Nagao, N. Effect of chitosan, pectic acid, lysozyme and chitinase on the growth of several phytopathogens. Agricultural and Biological Chemistry, 1989, 53, 3065-3066.

Hoare, D.G. and Koshland, JR. D.E. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. The Journal of Biological Chemistry, 1967, 2447-2453.

Ikegami, S., Tsuchihashi, F., Harada, H., Tsuchihashi, N., Nishide, E. and Innami, S. Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. Journal of Nutrition, 1990,120, 353–360.

Imoto, T. and Yagishita, K. A simple activity measurement of lysozyme. Agricultural and Biological Chemistry, 1971, 33, 1154-1157.

Jana, C., Andriy, S., Marcela, C., Jitka, K. and Miroslava, N. Application of FTIR spectroscopy in detection of food hydrocolloids in confectionery jellies and food supplements. Czech Journal of Food Science, 2001, 19, 51-56.

Jarjis, H. A., Blackburn, N. A., Redfern, J. S. and Read, N. W. The effect of ispaghula (Fybogel and Metamucil) and guar gum on glucose tolerance in man. British Journal of Nutrition, 1984, 51, 371-378.

Jedrzejas, M.J. Structural and functional comparison of polysaccharide-degrading enzymes. Critical Reviews in Biochemistry and Molecular Biology, 2000, 35, 221-251.

Jenkins, D. J., Reynolds, D., Slavin, B., Leeds, A. R., Jenkins, A. L and Jepson, E. M. Dietary fiber and blood lipids: treatment of hypercholesterolemia with guar crisp bread. American Journal of Clinical Nutrition, 1980, 33, 575-581.

Kacurakova, M., Belton, P.S., Wilson, R., Hirsch, J. and Ebringerova, A. Hydration properties of xylan-type structure on FTIR study of xylooligosaccharides. Journal of the Science of Food and Agriculture, 1998, 77, 38-45.

Kacurakova, M., Ebringerova, A., Hirsch, J. and Hromadkova, Z. Infrared study of arabinoxylans. Journal of the Science of Food and Agriculture, 1994, 66, 423-427.

Kato, A., Sato, T. and Kobayashi, K. Emulsifying properties of protein-polysaccharide complexes and hybrids. Agricultural and Biological Chemistry, 1989, 53, 2147-2152.

Kaup, S., Greger, J.L. and Lee, K. Nutritional evaluation with an animal model of cottage cheese fortified with calcium and guar gum. Journal of Food Science, 1991, 56, 692-695.

Kays, S.E., Morris, J.B. and Kim, Y. Total and soluble dietary fiber variation in *Cyamopsis tetragonoloba* (L.) Taub. (guar) genotypes. Journal of Food Quality, 2006, 29, 383-391.

Kennedy, J.F. and Bradshaw, I.J., Production, properties and application of xanthan. Progress in Industrial Microbiology, 1984, 19, 319-371.

Kim, W., Kobayashi, O., Kaneko, S., Sakakibara, Y., Park, G., Kusakabe, I., Tanaka, H. and Kobayashi, H. α-Galactosidase from culture rice (*Oryza sativa* L. var. Nipponbare) cells. Phytochemistry, 2002, 61, 621-630.

Kittur, F.S., Vishu Kumar, A.B., and Tharanathan, R.N. Low molecular weight chitosans – preparation by depolymerization with *Aspergillus niger* pectinase and characterization. Carbohydrate Research, 2003, 338, 1283 – 1290.

Kittur, F.S., Vishu Kumar, A.B., Gowda, L.R. and Tharanathan, R.N. Chitosanolysis by a pectinase isozyme of *Aspergillus niger* – A non-specific activity. Carbohydrate Polymers, 2003a, 53, 191-196.

Kittur, F.S., Vishu Kumar, A.B., Varadaraj, M.C. and Tharanathan, R.N. Chitooligosaccharides – preparation with the aid of pectinase isozyme from *Aspergillus niger* and their antibacterial activity. Carbohydrate Research, 2005, 340, 1239 – 1245.

Klabunde, T., Eicken, C., Sacchettini, J.C. and Krebs, B. Crystal structure of plant catechol oxidase containing a dicopper center. Nature Structural Biology, 1998, 5, 1084-1090.

Kloek, W., Luyten, H. and van Vliet, T. Small and large deformation behaviour of mixtures of xanthan and enzyme modified galactomannan. Food Hydrocolloids, 1996, 10, 123-129.

Kokol, V. Interactions between polysaccharide polymer thickener and bifunctional bireactive dye in the presence of nonionic surfactants. Part 1: surface tension and rheological behavior of different polysaccharide solutions. Carbohydrate Polymers, 2002, 50, 227-236.

Kotsira, V.P. and Clonis, Y.D. Chemical modification of barley root oxalate oxidase shows the presence of a lysine, a carboxylate, and disulfides essential for enzyme activity. Archives of Biochemistry and Biophysics, 1998, 10, 117-126.

Krishnaiah, Y.S.R., Karthikeyan, R.S. and Satyanarayana, V. A three-layer guar gum matrix tablet for oral controlled delivery of highly soluble metoprolol tartrate. International Journal of Pharmaceutics, 2002, 241, 353–366.

Krishnaiah, Y.S.R., Satyanarayana, S., Rama Prasad, Y.V. and Narasimha Rao, S. Evaluation of guar gum as a compression coat for drug targeting to colon. International Journal of Pharmacology, 1998, 171, 137–146.

Krishnaiah, Y.S.R., Satyanaryana, S. and Rama Prasad, Y.V. Studies of guar gum compression-coated 5-aminosalicylic acid tablets for colon-specific drug delivery. Drug Development and Industrial Pharmacy, 1999, 25, 651–657.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 1970, 227, 680-685.

Larson, E.B. and Smith, F. The constitution of the galactomannan of the Kentucky coffee bean (*Gymnocladus dioica*). Journal of American Chemical Society, 1955, 77, 429-432.

Launay, B., Cuvelier, G., and Martinez-Reyes, S. Xanthan gum in various solvent conditions: intrinsic viscosity and flow properties. In Phillips, G.O., Wedlock, D. J. and Williams, P. A. (eds.). In Gums and stabilisers for the food industry, Pergamon Press, Oxford, 1984, 2, 79-98.

Lee, B.T. and Matheson, N.K. Phosphomannoisomerase and phosphoglucoisomerase in seeds of *Cassia coluteoides* and some other legumes that synthesize galactomannan. Phytochemistry, 1984, 23, 983-987.

Lee, D. X., Xia, W.S. and Zhang, J.L. Enzymatic preparation of chitooligosaccharides by commercial lipase. Food Chemistry, 2008, 111, 291-295.

Leschziner, C. and Cerezo, A.S. The structure of a galactomannan from the seed of *Gleditsia triacanthos*. Carbohydrate Research, 1970, 15, 291-299.

Liao, Y.M., Su, J.L., Chiang, C.L. and Chang, C.T. Studies on the degradation of chitosan by crude proteolytic enzymes from hog stomach mucosa and *Bacillus subtilis*. Taiwanese Journal of Agricultural Chemistry and Food Science, 2000, 38, 239-247.

Liu, Y.N., Lai, Y.T., Chou, W.I., Chang, M.D. and Lyu, P.C. Solution structure of family 21 carbohydrate-binding module from *Rhizopus oryzae* glucoamylase. Biochemical Journal, 2007, 403, 21-30

Lopes, L., Andrade, C.T., Milas, M. and Rinaudo, M. Role of conformation and acetylation of xanthan on xanthan-guar interaction. Carbohydrate Polymers, 1992, 17, 121 – 126.

Lopez, M.D., Lopez, F.J., Lopez, A.J., Carreno, F.L. and Toro, M.A.N. Characterization of fish acid proteases by substrate-gel electrophoresis. Comparitive Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology, 1998,121, 369-377.

Lundell, N. and Schreitmuller, T. Sample preparation for peptide mapping. A pharmaceutical quality control perspective, Analytical Biochemistry, 1999, 266, 31-47.

Luyten, H., Kloek, W., and van Vliet, T. Yielding behaviour of mixtures of xanthan and enzyme-modified galactomannans. Food Hydrocolloids, 1994, 8, 431-440.

Macarron, R., Acebal, C., Castillon, M.P. and Claeysens, M. Mannanase activity of endoglucanase III from *Trichoderma reesei* QM 9414. Biotechnology Letters. 1996, 18, 559-602.

Mahammad, S., Comfort, D.A, Kelly, R.M. and Khan, S.A. Rheological properties of guar galactomannan solution during hydrolysis with galactomannanase and alpha-galactosidase enzyme mixtures. Biomacromolecules, 2007, 8, 949-956.

Mahoney, W. C. and Hermodson, M. A. Separation of large denatured peptides by reverse phase high performance liquid chromatography: Trifluoroacetic acid as a peptide solvent. The Journal of Biological Chemistry, 1980, 255, 11,199-203.

Maier, H., Anderson, M., Karl, C. and Magnuson, K. Guar, locust bean, tara and fenugreek gums. In Whistler, R.L. and BeMiller, J.N. (eds.). Industrial polysaccharides and their derivatives (3rd ed.), Academic Press, New York, 1993, 182-205.

Mangione, M. R., Giacomazza, D., Bulone, D., Martorana, V., and San Biagio, P. L. Thermoreversible gelation of κ-carrageenan: relation

between conformational transition and aggregation. Biophysical Chemistry, 2003, 104, 95–105.

Mannion, R.O., Melia, C.D., Launay, B., Cuvelier, G., Hill, S.E., Harding, S.E. and Mitchell, J.R. Xanthan / locust bean gum interaction at room temperature. Carbohydrate Polymers, 1992, 19, 91-97.

Margolles-Clark, E., Tenkanen, M., Luonteri, E. and Penttila, M. Three α-galactosidase genes of *Trichoderma reesei* cloned by expression in yeast. European Journal of Biochemistry, 1996, 240, 104-111.

Matheson, N.K. In Carbohydrates. Dey, P.M. and Harbourne, J.B. (eds.), Academic Press, London, 1990, 372-413.

Matsudaira, P. T. A Practical guide to peptide and protein purification for microsequencing. Matsudaira, P.T. (ed.), Academic Press, New York, 1989, 53–58.

Mayer, A.M. and Poljaloff-Mayber, A. The germination of seeds, Pergamon press, New York, 1975, 192-209.

McCleary, B. V. and Neukom, H. Effect of enzymic modification on the solution and interaction properties of galactomannans. Progress in Food and Nutrition Science, 1982*, 6*, 109-118.

McCleary, B.V. and Matheson, N.K. Action pattern and substrate binding requirements of β-D-mannanase with mannosaccharides and mannan-type polysaccharides. Carbohydrate Research, 1983, 119, 191-219.

McCleary, B.V. and Nurthen, E. Characterization of the oligosaccharides produced on hydrolysis of galactomannan with β-D-mannanase. Carbohydrate Research, 1983, 118, 91-109.

McCleary, B.V. Enzymatic interaction in the hydrolysis of galactomannan in germinating guar: The role of exo-β-mannanase. Phytochemistry, 1983, 22, 649-65.

McCleary, B.V. Enzymatic modification of plant polysaccharides. International Journal of Biological Macromolecules, 1986, 8, 349-354.

McCleary, B.V. Preparative scale isolation and characterization of 6^1 - α-D galactosyl (1, 4)-β-mannobiose and 6^2-α-D galactosyl-(1, 4) - β-D mannobiose. Carbohydrate Research. 1982, 104, 285-297.

McCleary, B.V., Clark, A.H., Dea, I.C.M. and Rees, D.A. The fine structure of carob and guar galactomannans. Carbohydrate Research, 1985, 139, 237-260.

McCleary, B.V., Dea, I.C.M., Windust. J. and Cooke, D. Interaction properties of D-galactose-depleted guar galactomannan samples. Carbohydrate Polymers, 1984, 4, 253-270.

McCleary, B.V., Renato, A., Robert, W. and Hans, N. Effect of galactose content on the solution and interaction properties of guar and carob galactomannans. Carbohydrate Research, 1981, 92, 269-285.

Meier, H. and Reid, J.S.G. Reserve polysaccharides other than starch in higher plants. In Loewus, F. A. and Tanner, W. (eds.), Encyclopedia of plant physiology, New series, 13A: Plant carbohydrates I. Springer, Berlin, 1982, 418-471.

Miles, M.J., Morris, V.J. and Carroll, V. Carob gum/κ-carrageenan mixed gels: Mechanical properties and X-ray fiber diffraction studies. Macromolecules, 1984, 17, 2443 – 2445.

Mill, P. J. and Tuttobello, R. The pectic enzymes of *Aspergillus niger*. 2. Endopolygalacturonase. Biochemical Journal, 1961, 79, 57- 64.

Miller, G.L. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Analytical Chemistry, 1959, 31, 426-432.

Mitsutomi, M., Isono, M., Uchiyama, A., Nikaidou, N., Ikegami, T. and Watanabe, T. Chitosanase activity of the enzyme previously reported as β-1,3/1,4-glucanase from *Bacillus circulans WL-12.* Bioscience, Biotechnology and Biochemistry, 1998, 62, 2107-2114.

Morgan, L. M., Tredger, J. A., Madden, A., Kwasowski, P. and Marks, V. The effect of guar gum on carbohydrate, fat, and protein-stimulated gut hormone secretion: modification of post-prandial gastric inhibitory polypeptide and gastrin responses. British Journal of Nutrition, 1985, 53, 467-475.

Morris, E.R., Rees, D.A., Young, G., Walkinshaw, M.D. and Darke, A. Order-disorder transition for a bacterial polysaccharide in solution. Journal of Molecular Biology, 1977, 110, 1-16.

Muraki, E., Yaku, F. and Kojima, H. Preparation and characterization of D-glucosamine oligosaccharides with DP 6-8. Carbohydrate Research, 1993, 239, 227-237.

Murthy, S.N., Hiremath, S.R.R. and Paranjothy, K.L.K. Evaluation of carboxymethyl guar films for the formulation of transdermal therapeutic systems. International Journal of Pharmaceutics, 272, 2004, 11-18.

Muzzarelli, R.A.A., Terbojevich, M., Muzzarelli, C. and Francescangeli, O. Chitosan depolymerization with the aid of papain and stabilized as glycosamines. Carbohydrate Polymers, 2002, 50, 69-78.

Muzzarelli, R.A.A., Tomasetti, M. and Ilari, P. Depolymerization of chitosan with the aid of papain. Enzyme and Microbial Technology, 1994, 16,110-114.

Muzzarelli, R.A.A., Xia, W., Tomasetti, M. and Ilari, P. Depolymerization of chitosan and substituted chitosans with the aid of wheat germ lipase preparation. Enzyme and Microbial Technology, 1995, 17, 541-545.

Nakamura, S., Kato, A. and Kobayashi, K. Bifunctional lysozyme - galactomannan conjugate having excellent emulsifying properties and bactericidal effect. Journal of Agricultural and Food Chemistry, 1992, 40, 735-739.

Nakamura, S., Kato, A. and Kobayashi, K. Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. Journal of Agricultural and Food Chemistry, 1992a, 40, 2033-2037.

Nakamura, S., Kato, A. and Kobayashi, K. New antimicrobial characteristics of lysozyme-dextran conjugate. Journal of Agricultural and Food Chemistry, 1991, 39, 647-650.

Naoumkina, M., Torres-Jerez, I. Allen, S., Ji, H., Zhao, P.X., Dixon, R. A. and May, G.D. Analysis of cDNA libraries from developing seeds of guar (*Cyamopsis tetragonoloba* (L.) Taub). BMC Plant Biology, 2007, 7, 62-73.

Nelson, M.L. and O'Connor, R.T. Relation of certain infrared bands to cellulose crystallinity and crystal lattice type. Part I. Spectra of lattice types I, II, III and amorphous cellulose. The Journal of Applied Polymer Science, 1964, 8, 1311-1318.

Nordtveit, R., Varum, K.M. and Smidsrod, O. Degradation of fully water soluble, partially N-acetylated chitosans with lysozyme. Carbohydrate Polymers, 1994, 23, 253-260.

Norton, I.T., Goodall, D.M., Frangou, S.A., Morris, E.R. and Rees, D.A. Mechanism and dynamics of conformational ordering in xanthan polysaccharide. Journal of Molecular Biology, 1984, 175, 371-394.

Nyvall, P., Pedersen, M., Kenne, L. and Gacesa, P. Enzyme kinetics and chemical modification of α-1,4-glucan lyase from *Gracilariopsis sp*. Phytochemistry, 2000, 54, 139-145.

Ohgushi, M. and Wada, A. Molten-globule state: a compact form of globular proteins with mobile side-chains. FEBS Letters, 1983, 164, 21-24.

Overbeeke, N., Fellinger, A.J., Toonen, M, Y., Wassenaar, D.V. and Verrips, C.T. Cloning and nucleotide sequence of the α-galactosidase cDNA from *Cyamopsis tetragonoloba* (guar). Plant Molecular Biology, 1989, 13, 541-550.

Pai, V.B. and Khan, S.A. Gelation and rheology of xanthan/modified guar blends. Carbohydrate Polymers, 2002, 49, 207-216.

Pantaleon, D., Yalpani, M. and Scollar, M. Unusual susceptibility of chitosan to enzymic hydrolysis. Carbohydrate Research, 1992, 237, 325-332.

Pelletier, A. and Sygush, J. Purification and characterization of three chitosanase activities from *Bacillus megaterium* P1. Applied Environmental Microbiology, 1990, 56, 844-848.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., Wilson, K.S. and Vorgias, C.E. Crystal structure of a bacterial chitinase at 2.3 Å resolution. Structure, 1994, 12,1169-1180.

Peterson, D. B., Ellis, P. R., Baylis, J. M., Fielden, P., Ajodhia, J., Leeds, A. R. and Jepson, E. M. Low dose guar in a novel food product: improved metabolic control in non-insulin-dependent diabetes. Diabetic Medicine, 1987, 4,111-115.

Picout, D.R., Ross-Murphy, S.B., Jumel, K. and Harding, S.E. Pressure cell assisted solution characterization of polysaccharides. 2. Locust bean gum and Tara gum. Biomacromolecules, 2002, 3, 761-767.

Poksay, K.S. and Schneeman, B.O. Pancreatic and intestinal response to dietary guar gum in rats. Journal of Nutrition, 1983,113, 1544–1549.

Prakash, M.D., Smitha, P. and Michael, D.B. Induction of α-galactosidase in *Penicillium ochrochloron* by guar (*Cyamopsis tetragonoloba*) gum. Biotechnology and Applied Biochemistry, 1993, 17, 361-371.

Puspitasari, N.L., Lee, K. and Greger, J.L. Calcium fortification of cottage cheese with hydrocolloid control for bitter flavor defects. Journal of Dairy Science, 1991, 74, 1-7.

Qin, C., Du, Y., Zong, L., Zeng, F., Liu, Y. and Zhou, B. Effect of hemicellulase on the molecular weight and structure of chitosan. Polymer Degradation and Stabilization, 2003, 80, 435-441.

Qu-Ming, G. Enzyme-mediated reactions of oligosaccharides and polysaccharides. Journal of Environmental Polymer Degradation, 1999, 7, 1-7.

Rama Prasad, Y.V., Krishnaiah, Y.S.R. and Satyanarayana, S. *In vitro* evaluation of guar gum as a carrier for colon specific drug delivery. Journal of Controlled Release. 1998, 51, 281–287.

Rao, P. and Pattabiraman, T.N. Reevaluation of the phenol-suphuric acid reaction for the estimation of hexoses and pentoses. Analytical Biochemistry, 1989, 181, 18-22.

Rath, R. K., Subramanian, S. and Laskowski, J. S. Adsorption of dextrin and guar gum onto Talc. A Comparative Study. Langmuir, 1997, 13, 6260-6266.

Rees, D.A. Shapely polysaccharides. Biochemical Journal, 1972, 126, 257-273.

Reid, J.S.G, Edwards, M., Gidley, M.J. and Clark, A.H. Mechanism and regulation of galactomannan biosynthesis in developing leguminous seeds. Biochemical Society Transactions, 1992, 20, 23-26.

Reid, J.S.G. and Bewley, J.D. A dual role for the endosperm and its galactomannan reserves in the germinative physiology of fenugreek (*Trigonella foenum-graecum* L.), an endospermic leguminous seed, Planta, 1979, 147, 145-151.

Reid, J.S.G. and Edwards, M. Galactomannans and other cell wall storage polysaccharides in seeds. In Stephen, A.M., (ed.), Food polysaccharides and their applications, Marcel Dekker, New York, 1995, 155-186.

Richardson, P. H., Clark, A. H., Russell, A. L., Aymard, P. and Norton, I. T. Galactomannan gelation: A thermal and rheological investigation analyzed using the cascade model. Macromolecules, 1999, 32, 1519-1527.

Rocks, J.K. Xanthan gum. Food Technology, 1971, 25, 22-31.

Roy, S., Vaga, L. S. and Fernandez, M. L. Gender and hormonal status affect the hypolipidemic mechanisms of dietary soluble fiber in guinea pigs. Journal of Nutrition, 2000,130, 600–607.

Salenius, J. P., Harju, E., Jokela, H., Riekkinen, H. and Silvasti, M. Long term effects of guar gum on lipid metabolism after carotid endarterectomy. British Medical Journal, 1995, 310, 95–96.

Sanchez-Ferres, A., Franscisco, L. and Franscisco, G.C. Partial purification of soluble potato polyphenol oxidase by partitioning in an aqueous two-phase system. Journal of Agricultural and Food Chemistry, 1993, 41, 1219-1224.

Sawardekar, J.S., Sloneker, L.S. and Jeanes, A. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Analytical Chemistry, 1965, 37, 1602-1604.

Schorsch, C., Garnier, C. and Doublier, J.L. Viscoelastic properties of xanthan / galactomannan mixtures: comparison of guar gum with locust bean gum. Carbohydrate Polymers, 1997, 34, 165-175.

Schuettelkopf, A. W. and van Aalten, D. M. F. PRODRG-a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallographica, 2004, 60, 1355-1363.

Sepulveda, T.P., Marciniszyn, J., Chen, K.C.S., Huang, W.Y., Tao, N. and Liu, P. Amino acid sequence of porcine pepsin. Proceedings of the National Academy of Sciences, USA, 1973, 70, 3437-3439.

Sharma, B. R., Dhuldhoya, N. C. and Merchant, U. C. Flocculants - an ecofriendly approach. Journal of Polymers and the Environment, 2006, 14, 195–202.

Shatwell, K.P., Sutherland, I.W., Ross-Murphy, S.B. and Dea, I.C.M. Influence of acetyl substituent on the interaction of xanthan with plant polysaccharides-III. Xanthan – konjac mannan system. Carbohydrate Polymers, 1991, 14, 131 – 147.

Shibuya, H., Kobayashi, H. and Kusakabe, I. Galactose depletion by *Mortierella vinacea* α-galactosidase II increases the synergistic interaction between guar gum and xanthan gum. Food Science and Technology Research, 1999, 5, 271-272.

Shobha, M.S. and Tharanathan, R.N. Non specific activity of *Bacillus acidopullulyticus* pullulanase on debranching of guar galactomannan. Journal of Agricultural and Food Chemistry, 2008, 56, 10858-10864.
Shobha, M.S. and Tharanathan, R.N. Rheological behavior of pullulanase-treated guar galactomannan on co-gelation with xanthan. Food Hydrocolloids, 2009, 23, 749-754.

Shobha, M.S., Vishu Kumar, A.B., Rathna, K., AnilKumar, G. and Tharanathan, R.N. Modification of guar galactomannan with the aid of *Aspergillus niger* pectinase, Carbohydrate Polymers, 2005, 62, 267-273.

Sidhu, J.S., Bawa, A.S. and Singh, N. Studies on the effect of hydrocolloids on the consistency of tomato ketchup. Journal of Food Science and Technology, 1997, 34, 423-429.

Simons, L.A., Gayst, S., Balasubramaniam, S. and Ruys, J. Long-term treatment of hypercholesterolaemia with a new palatable formulation of guar gum. Atherosclerosis, 1982, 45, 101–108.

Srinivasa, P.C. and Tharanathan, R.N. Chitin/chitosan – safe, ecofriendly packaging material with multiple potential uses. Food Reviews International, 2007, 23, 53-72.

Srivastava, M. and Kapoor, V.P. Seed galactomannans - an overview. Chemistry & Biodiversity, 2005, 2, 295-317.

Stephen, A.M. and Churms, S.C. In Stephen, A.M. (ed.), Food polysaccharides and their applications, Marcel Dekker, New York, 1995, 1-18.

Sturum, A. and Tang, G.Q. The sucrose cleaving enzymes of plant are crucial for development, growth and carbon partitioning. Trends in Plant Science, 1999, 4, 401-407.

Suthar, S. Production of vermifertilizer from guar gum industrial wastes by using composting earthworm *Perionyx sansibaricus* (Perrier). Environmentalist, 2007 27, 329–335.

Taka, M. and Nakamura, S. Rheological properties of deacetylated xanthan in aqueous solution. Agricultural and Biological Chemistry, 1984, 48, 2987-2991.

Takahashi, H., Yang, S.L., Ueda, Y., Kim, M. and Yamamoto, T. Influence of intact and partially hydrolysed guar gum on iron utilization in rats fed on iron-deficient diets. Comparative Biochemistry and Physiology. 1994, 109, 75-82.

Tako, M. and Nakamura, S. Synergistic interaction between xanthan and guar gum. Carbohydrate Research, 1985, 138, 207-213.

Tako, M. Synergistic interaction between deacetylated xanthan and galactomannan. Journal of Carbohydrate Chemistry, 1991, 10, 619-633.

Talbot, G. and Sygusch, J. Purification and characterization of thermostable β-mannanase and α-galactosidase from *Bacillus*

stearothermophilus. Applied and Environmental Microbiology.1990, 56, 3505-3510.

Tavares, C. and Lopes da Silva, J. A. Rheology of galactomannan-whey protein mixed systems. International Dairy Journal**,** 2003, 13, 699-706.

Tayal, A., Kelly, R. M. and Khan, S. A. Rheology and molecular weight changes during enzymic degradation of a water-soluble polymer. Macromolecules, 1999, 32, 294-300.

Terwisscha van Scheltinga, A.C., Lalk, K.H., Beintema, J.J. and Dijkstra, B.W. Crystal structure of hevamine, a plant defense protein with chitinase and lysozyme activity and its complex with an inhibitor. Structure, 1994, 12, 1181-1189.

Tharanathan, R.N. Biodegradable films and composite coatings: past, present and future. Trends in Food Science and Technology, 2003, 14, 71-78.

Tharanathan, R.N. Food derived carbohydrates – structural complexity and functional diversity. Critical Reviews in Biotechnology, 2002, 22, 65-84.

Tharanathan, R.N., Muralikrishna, G., Salimath, P.V. and Raghavendra Rao, M.R. Plant carbohydrates - an overview. Proceedings of Indian Academy of Sciences, 1987, 97, 81-155.

Todd, P. A., Benfield, P. and Goa, K. L. Guar gum: A review of its pharmacological properties and use as a dietary adjunct in hypercholesterolemia. Drugs, 1990, 39, 917–928.

Tul'chinsky, V.M., Zurabyan, S.E., Asankozhoev, K.A., Kogan, G.A. and Ya, A. A study of the infrared spectra of oligosaccharides in the region 1000-400 cm^{-1}. Carbohydrate Research, 1976, 51, 1-8.

Uusitupa, M., Tuomilehto, J., Karttunen, P. and Wolf, E. Long term effects of guar gum on metabolic control, serum cholesterol and blood pressure levels in type 2 (non-insulin dependent) diabetic patients with high blood pressure. Annals of Clinical Research, 1984, 43, 126–131.

Viebke, C., Piculell, L. and Nilsson, S. On the mechanism of gelation of helix-forming biopolymers. Macromolecules, 1994, 27, 4160-4166.

Vieira, M.C. and Gil, A.M. A solid state NMR study of locust bean gum galactomannan and konjac glucomannan gels. Carbohydrate Polymers, 2005, 60, 439-448.

Vijayendran, B.R. and Bone, T. Absolute molecular weight and molecular weight distribution of guar by size exclusion chromatography and low angle laser light scattering. Carbohydrate Polymers, 1984, 4, 299-313.

Vishu Kumar, A.B. and Tharanathan, R.N. A comparative study on depolymerization of chitosan by proteolytic enzymes. Carbohydrate Polymers, 2004, 58, 275-283.

Vishu Kumar, A.B., Lalitha, R.G. and Tharanathan, R.N. Non-specific depolymerization of chitosan by pronase and characterization of the resultant products. European Journal of Biochemistry, 2004a, 271, 713-723.

Vishu Kumar, A.B., Varadaraj, M.C., Lalitha, R.G. and Tharanathan, R.N. Low molecular weight chitosan: preparation with the aid of papain and characterization. Biochimica et Biophysica Acta, 2004b, 1670, 137-146.

Vishu Kumar, A.B., Varadaraj, M.C. and Tharanathan, R.N. Low molecular weight chitosan - preparation with the aid of pepsin, characterization, and Its bactericidal activity. Biomacromolecules, 2007, 8, 566-572.

Vishu Kumar, A.B., Varadaraj, M.C., Gowda, L.R. and Tharanathan R. N. Low molecular weight chitosans - preparation with the aid of pronase, characterization and their bactericidal activity towards *Bacillus cereus* and *Escherichia coli*. Biochimica et Biophysica Acta, 2007b, 1770, 495-505.

Vishu Kumar, A.B., Varadaraj, M.C., Lalitha, R.G. and Tharanathan, R.N. Characterization of chito-oligosaccharides prepared by chitosanolysis with the aid of papain and pronase, and their bactericidal action against *Bacillus cereus* and *Escherichia coli*. Biochemical Journal, 2005, 391,167-175.

Wang, Q., Ellis, P.R. and Ross-Murphy, S.B. Dissolution kinetics of guar gum powders II. Effects of concentration and molecular weight. Carbohydrate Polymers, 2003, 53, 75-83.

Ward, F.M. Hydrocolloids system as fat mimetics. Cereal Foods World, 1997, 42, 386-395.

Weaver L.H. Grutter, M.G. and Matthews, B.W. The refined structure of goose lysozyme and its complex with a bound trisaccharide shows that the goose type lysozymes lack a catalytic aspartate residue. Journal of Molecular Biology, 10, 245, 1995, 54-68.

Williams, P. A., Day, D. H., Landon, M. J., Phillips, G. O. and Nishinari, K. Synergistic interaction of xanthan gum, glucomannans and galactomannans. Food Hydrocolloids, 1991, 4, 489–493.

Xia, C., Meyer, D.J, Chen, H., Reinemer, P., Huber, R. and Ketterer, B. Chemical modification of GSH tranferase P1-1 confirms the presence of Arg-13, Lys-44 and one carboxylate group in the GSH-binding domain of the active site. Biochemical Journal, 1993, 293, 357-362.

Xia, W., Liu, P. and Liu, J. Advances in chitosan hydrolysis by non-specific cellulases. Bioresource Technology, 2008, 99, 6751-6762.

Yalpani, M. and Pantaleon, D. An examination of the unusual susceptibility of aminoglycans to enzymatic hydrolysis. Carbohydrate Research, 1994, 265, 159-175.

Yamatoya, K. Hydrolyzed guar gum. A new generation water-soluble dietary fiber.International Food Ingredients, 1994, 20,15-19.

Zambrano, F., Despinoy, P., Ormenese, R. C. S. C. and Faria, E. V. The use of guar and xanthan gums in the production of 'light' low fat cakes. International Journal of Food Science and Technology, 2004, 39, 959-966.

Zhang, H. and Neau, S.H. *In vitro* degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of acetylation. Biomaterials, 2001, 22, 1653-1658.

Zhang, H., Du, Y., Yu, X., Mitsutomi, M. and Aiba, S. Preparation of chitooligosaccharides from chitosan by a complex enzyme. Carbohydrate Research, 1999, 320, 257-260.

Zor, T. and Selinger, Z. Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. Analytical Chemistry, 1996, 236, 302-308.