PECTIN HYDROLYSIS IN VIVO AND ITS ROLE IN FRUIT SOFTENING
DURING RIPENING IN MANGO
(MANGIFERA INDICA L)

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By

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October 2002

<u>DECLARATION</u>

I herby declare that the thesis entitled "PECTIN HYDROLYSIS IN VIVO AND ITS ROLE IN FRUIT SOFTENING DURING RIPENING IN MANGO (MANGIFERA INDICA L)" 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, Deputy Director, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore-570013, India, during the period 1998 -2002. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

(PRASANNA. V)

Place: Mysore.

Date: 28.10.2002

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Deputy Director,
Department of Biochemistry and Nutrition

CERTIFICATE

This is to certify that the thesis entitled "PECTIN HYDROLYSIS IN VIVO AND ITS ROLE IN FRUIT SOFTENING DURING RIPENING IN MANGO (MANGIFERA INDICA L)" submitted by Mr. Prasanna, V., for the award of DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY to the UNIVERSITY OF MYSORE is the result of research work carried out by him in the Department of Biochemistry and Nutrition, under my guidance during the period 1998-2002.

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Guide

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Date: 28.10.2002

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LIST OF ABBREVIATIONS

α	Alpha
A	Armstrong unit
Ac	Acetyl
ACC	α-Amino 2-cyclopropane carboxylic acid AG Arabinogalactan
AlP	Acetone insoluble powder AIR Alcohol insoluble residue Ara Arabinose
Amu (m/Z)	Atomic mass unit (mass/charge)
β	Beeta
DC	Degree centigrade
cDNA	Complementary Deoxyribonucleic acid
^{C}DTA	1 ,2-cyclohexaminediaminetetracetic acid

CE Capillary electrophoresis CM Carboxymethyl

CV.	Cultivar
Da	Daltons
DAc	Degree of acetylation OEAE Diethylaminoethyl
DM	Degree of methylation
EDTA	Ethylenediaminetetraacetic acid
EFE	Ethylene forming enzyme
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N', N' tetraacetic acid
eV	Electron Volts
eq	Equivalent
FID	Flame ionization detector
Fig.	Figure
FT -IR	Fourier Transform Infrared
Fuc	Fucose .
FW	Fresh weight g Gravity
g %	Gram percent (g / 100g)
GalA	Galacturonic acid
Gal	Galactose
Glc	Glucose
GC (GLC)	Gas (liquid) chromatography

GC-MS Gas chromatography-Mass spectrometry

GPC	Gel permeation chromatography
h	Hour(s)

HPSEC High performance size exclusion chromatography

lEC	Ion exchange chromatography
kDa	Kilo Daltons
K_m	Michealis-Menten constant

L	Litre(s)
M	Moles (molar concentration)
ma	Milli ampere
Me	Methyl
meq	Milli equivalents
mg %	Milligram percent (mg / 100g)
min	Minute
ml	Millilitre
MMT	Million metric tonnes
MSC	Methyl sulphinyl carbanion
μ	Micron
μI	Micro litre
μmol	Micro mole(s)
M_r	Relative molecular weight(s)
N	Newtons
N	Normality
nm	Nanometer
nmol	Nanomoles
NMR	Nuclear Magnetic Resonance
OD	Optical density (absorbance) .
p	Page number
PAGE	Polyacrylamide gel electrophoresis
PG	Polygalacturonase
PGA	Polygalacturonic acid (Pectic acid)
PME	Pectin methyl esterase
pp	Page to page
RG	Rhamnogalacturonan
Rha	Rhamnose
RID	Refractive index detector
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature s Second(s)
SAM	S-Adenosyl methionine
SDS	Sodium dodecyl sulphate
T_m	Thermal inactivation temperature at 50 % inacativation
TS	Total sugar
UA	Uronic acid
UV-Vis	Ultra violet- visible

V	Volt(s)
V_e	Elution volume
V_{max}	Maximum activity
Vo	Void volume
Vol.	Volume(s)
vs	Versus
v/v	Volume by volume
w/v	Weight by volume
%	Percent
>	Greater than
<	Lesser than
~	Approximately

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SYNOPSIS

Mango (Mangifera indica L), the 'King of fruits' is a commercially important fruit crop of tropical world. Despite India being the major producer of mango contributing 54% of the total world production, the fruit economy is far from satisfactory. One of the limiting factors that influence the economic value is the relative short ripening period and post-harvest life. In addition, excessive textural softening during ripening leads to adverse effects upon storage. Thus, delaying ripening-associated changes in this fruit will lead to greater 'commercial value addition' in fruit biotechnology.

Textural softening during ripening is of immense importance as it directly dictates the fruit shelf life and post-harvest physiology. Control or modification of fruit texture is the main objective of modern 'Fruit Biotechnology'. During textural softening from unripe to ripe stage (i.e., from 'stony hard' to 'soft' stage), carbohydrates undergo hydrolysis to various extent, resulting in depolymerization and decrease in molecular size of the polymers. It is generally partial hydrolysis of the large size carbohydrate polymers. Sometimes, there may be complete hydrolysis or nearly complete hydrolysis as in the case of starch in mango and banana.

In the area of fruit ripening, textural softening of tomato fruits has been fairly well studied. In this case, the expression of a-amino cyclopropane carboxylic acid synthase (ACC synthase) & ethylene forming enzyme (EFE) at ethylene level, and polygalacturonase (PG) & pectin methyl esterase (PME) at post ethylene level, were individually suppressed by antisense RNA. Interestingly, all these culminated in a desired end result. Ethylene suppression resulted in overall control of the ripening process, which was triggered by the exogenous ethylene-boost. Genetic manipulation at the textural level resulted specifically in "improved texture" in the transformed tomatoes, where PG and PME suppression yielded firmer fruits and higher solid content, respectively.

Since fruits differ in their biochemical make up, it is to be expected that the enzymic targets also differ from fruit to fruit. There may be other equally important hydrolases, which are crucial in fruit texture and textural softening.

This study in mango fruit was taken up to specifically identify the crucial and important hydrolases in connection with pectin metabolism, which was studied at both substrate (pectic polysaccharides) as well as enzyme level (pectin-hydrolyzing enzymes). Some new hydrolases other than PG/PME, hitherto unexplored, were observed in mango fruit, which could serve as important targets for pectin dissolution in vivo. It must be noted that the study here forms the basis for further investigation at the gene level. Identification of crucial substrates and their corresponding enzymes in connection with carbohydrate hydrolysis in vivo is important and useful for providing further insights.

The literature reports on mango fruit focus more on post harvest physiology, where most of them deal with biochemical changes during ripening, especially on the organic acid metabolism, fruit flavours, volatiles, overall composition and gross changes in total pectin during ripening. However, the precise nature of the pectic polysaccharides and the pectin- degrading enzymes; their quantitative and qualitative changes during ripening in relation to textural softening have not been studied. The present investigation in mango fruit (Mangifera indica cv. Alphonso) is novel and comprehensive.

The aim of this study is to understand the factors contributing to the textural changes in relation to pectin degradation during ripening in mango. The knowledge about the composition, structure and nature of the fruit substrates and enzymes during ripening provides a clear insight into the I physical, physiological and biochemical changes involved in the ripening I process. It is vital to understand (identify define) precisely the enzymes that are involved in textural softening of a fruit and the extent of their contribution towards the same. The profile of carbohydrate polymers of a fruit and the changes they undergo, in terms of abundance as well as molecular weight drop along with the activity profile of the related hydrolases during textural softening, and their action on the endogenous substrates give a direct clue to the involvement of specific enzymic targets in the process. This, information would clearly leads to identification of the gene targets, which may be different in different fruits.

The main objectives of this present investigation are

1) To study the pectin degradation in mango during ripening.
2) To characterize the major pectic polysaccharides from mango pulp.
3) To purify and to study the properties of a glycanase (PG) and a glycosidase (β galactosidase).
4) To study their involvement in pectin dissolution in vivo.

The thesis is divided into 5 chapters.

CHAPTER I: GENERAL INTRODUCTION

In this introductory chapter, various aspects of pectin regulation in fruits in the context of fruit ripening, with particular emphasis on textural softening has been reviewed in detail. A brief background on fruits, mango fruit, ripening associated changes and fruit cell wall is also presented. Current literature on important aspects of fruit ripening with particular emphasis on textural softening, pectins in relation to textural softening, pectin degradation during ripening in fruits, the enzymes involved in pectin metabolism, especially PG and β-galactosidase have been reviewed in detail. Also included here are the objectives and scope of the present investigation.

CHAPTER II: MATERIALS AND METHODS

Chapter II deals with the material procured, instruments used and various methodologies employed in this study. Fractionations, chromatographic techniques, spectrometric estimations, homogeneity criteria employed and structural studies carried out are described with standard literature references.

For clarity in understanding, the research component is divided into two chapters; III & IV, each having 3 sections. Each section starts with a summary, a brief introduction followed by results and discussion.

CHAPTER III: PECTIC POLYSACCHARIDES IN RIPENING MANGO

Section 1 deals with the degree of textural softening during ripening of mango fruit accompanied with microscopy and relevant biochemical data. Pectin dissolution during ripening correlated with textural softening. The total pectin decreased from 1.86 to 0.38 g% during ripening with a concomitant increase in soluble galacturonide, along with progressive textural softening as measured by texture analyzer. The microscopic data clearly showed the rigid and compact cell wall structure of the unripe fruit appeared more loosely structured and widened at the ripe stage. The microscopic data also showed the dissolution of pectin from middle lamella and primary cell wall.

In section 2, the entire range of pectic polymers present in mango pulp was studied both at unripe and ripe stages. About seven pectic fractions were resolved upon ion exchange chromatography (IEC) with differential gradient elution with weak and strong alkali gradients. Three of them were major, which got eluted out in neutral and lower strength gradients of $(NH4)2CO3$ (0.05 & 0.1 M). Quantitatively, there was a significant decrease from unripe to ripe stage in their abundance. The mg % drop in their levels for fractions I, II & III, from unripe to ripe stage was 60-7,89-13 & 74-2, respectively. There was an extensive drop in the molecular weight of all these pectic polymers, from unripe to ripe stage, as determined by high performance size exclusion

chromatography (HPSEC). The molecular weight drop was 250 to 70, 1300 to 21 and 473 to 298 kDa for the major peaks of fractions I, II & III, respectively. These indicated extensive depolymerization of pectic polymers in vivo. Gas liquid chromatography (GLC) analysis indicated that fraction I appeared to be arabinogalactan-type pectic polysaccharide, while fractions II and III were typical heterogalacturonans containing more than 62 % galacturonic acid, the rest being arabinose, galactose and rhamnose in different ratios. Loss of both acidic and neutral sugar residues from all the pectic fractions was evident.

Section 3 deals with structural characterization of the pectic fractions. The three major pectic polymers were further purified by gel permeation chromatography (GPC) and chosen for structural studies by using methods like optical rotation, Fourier Transformed Infrared Radiation spectroscopy (FT -IR), Gas chromatography-mass spectrometer (GC-MS) and Nuclear Magnetic Resonance (NMR). Structural studies revealed that fraction I, appears to be the arabinogalactan, with sugar compositions; galactose and arabinose in 3 : 1 ratio and were found to be 1-75 linked arabinan, linked to 1-74 linked galactan main chain through 1-73 linkages. Fractions II & III are found to be rhamnogalacturonans, having high amount of galacturonic acid content (69 and 62%, respectively) with different composition of neutral sugars; galactose, arabinose and rhamnose in the ratio, 14 : 15 : 2 and 23 : 10 : 4, respectively. They are a-(1-7 4) linked D-galacturonic acid interspersed with (1-72)-linked rhamnose, with galactan and arabinan side chains attached to rhamnose units.

CHAPTER IV: PECTIN-DEGRADING ENZYMES IN RIPENING MANGO

Section 1 deals with the enzymes related to pectin dissolution in vivo. The enzymes in this connection are PG, PME, galactanase, arabinanase and β-galactosidase. The activity profile of the pectic enzymes during ripening was obtained, where most of them showed an increased activity, with a characteristic climacteric peak, during ripening, except for PME, which showed a continuous decrease in activity. Further, when the total pectic fraction from mango was used as substrate for endogenous hydrolysis by the endogenous enzyme preparation (in vitro study), the loss of neutral sugars in the pectic fraction was found more prominent than the loss of GaiA residues. Further, there is a downward shift in molecular mass of pectic polysaccharides and loss of neutral sugar, which were more pronounced at pH 5.6, when compared to pH 3.6. These observations were comparable with the in vivo pectic changes from unripe to ripe stage.

In section 2, purification and properties of PG from mango pulp are presented. PG resolved into three distinct isoforms upon IEC on DEAE- cellulose column with a relative abundance of 68, 6 and 26%, respectively for isoforms I, II and III. They were subjected individually to GPC on Sephadex G-200. The apparent molecular weight for isoform I, II & III was 40, 51 & 45 kDa, respectively. Post GPC fractions were used for determining the enzymic properties. The pH optimum for isoforms I, II & III was between 3.2 to 3.9. The optimum temperature was around 40 °C for the three isoforms. Isoform III was more thermostable, comparatively. Their Km value for pectic acid

(PGA) was around 0.023%. The major metal ion inhibitors for mango PG were Cd++, Cu++ and Fe++. Galacturonic acid, galactose, fucose, rhamnose and arabinose stimulated the enzyme activity of PG-I particularly. The major endogenous substrates for mango PG were found to be the two rhamnogalacturonans.

Section 3 deals with purification and properties of β-galactosidase from mango. Three isoforms of p-galactosidase were identified from mango pulp, upon IEC on DEAE-cellulose. The % abundance of the three isoforms of β-galactosidase was 44, 38 & 18. They were further purified by GPC on Sephadex G-200. Their apparent molecular weight by GPC was 78, 58 & 91 kDa for isoforms I, II & III, respectively. The pH optima for activity and stability were in the range of 3.6 -4.3 and 3.6 -6.2, respectively. The optimum temperature for p-galactosidase activity was between 42 -47°C and the T m was in the range of 45 -51°C. The Km for paranitrophenyl-p-D- galactopyranoside (pNPG) was 0.98, 1.11 & 0.95 mM respectively for isoforms I, II & III. Hg++ showed a very powerful inhibition of all the three isoforms. Galacturonic acid, galactose, xylose, fucose and man nose slightly inhibited the activity of β-galactosidase isoforms. The ability of these isoforms to degrade the endogenous substrates (arabinogalactan) possibly suggests a role in pectin dissolution during tissue softening / fruit ripening.

CHAPTER V: HIGHLIGHTS OF THE RESEARCH

The thesis concludes with salient findings of this study. Pectin I regulation at the cell wall level during fruit ripening is important in the context of fruit texture and its changes. In mango fruit, a significant drop in the total pectin with a concomitant increase in soluble galacturonide correlated with progressive textural softening during ripening. The lEG profile of the pectic polymers of raw and ripe mango revealed seven distinct fractions all of which showed a drastic drop in their molecular weights as well as abundance at the end of ripening, clearly indicating controlled I depolymerization in vivo. The three major pectic polymers (further purified by GPC) were subjected to structural analysis using optical rotation, GCMS, FTIR & NMR. They were found to be an arabinogalactan and two rhamnogalacturonans with a difference in the sugar ratio and also in the linkage pattern.

Pectic hydrolases like PG, galactanase, arabinanase & β-galactosidase showed an increased activity with a climacteric peak during ripening. Initially PME prepares the pectin substrate, by demethylating it to pectic acid, thus making it amenable for the subsequent action by PG. PG (a glycanase) and (β-galactosidase (a glycosidase) were found to exist as three distinct isoforms upon purification and their enzymic properties studied. The two rhamnogalacturonans and the arabinogalactan were identified as the endogenous substrates for PG & (β-galactosidase respectively. These three major pectic polymers probably contribute to pectin dissolution in vivo at the cell wall level in mango. PG acts on the main homogalacturonan chains, while (β-galactosidase acts on the neutral side chains (galactans and arabinogalactans) of the ramified pectins, thus contributing to the dissolution of pectic polymers during fruit softening/ripening. Appearance of various cell wall-

degrading enzymes during ripening seems to be important in tissue softening during ripening. Enzymes like galactanase, arabinanase, (β- galactosidase act on side chains of pectins, while PME and PG act on homogalacturonan chains, finally resulting in the dissolution of pectic polysaccharides.

Finally, a collective bibliography is presented at the end of the thesis.

CHAPTER – I : GENERAL INTRODUCTION

Review of Literature

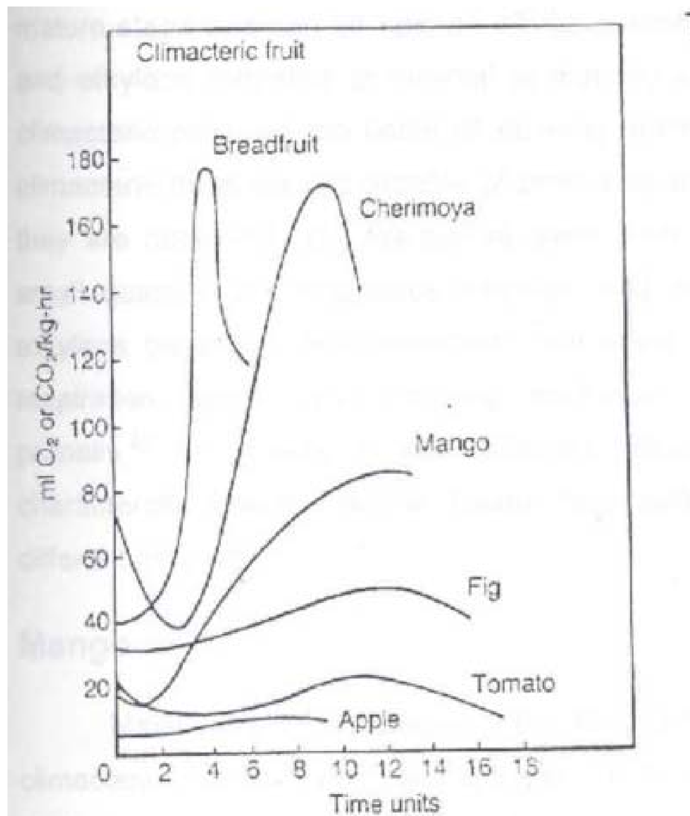
Fruit constitutes a commercially significant and nutritionally Indispensable food commodity. They are edible seed vessels or receptacles developed from a mature fertilized ovary. They are highly specialized organs in higher plants meant to brighten our lives, offering a great variety of aesthetic qualities with their complex / delicate aroma, pleasant taste, exotic colours, succulence, flavour and texture. They playa very important role in human nutrition, by supplying the necessary growth factors essential for maintaining the normal health. Nutritionally, they are known for their high energy, roughage value, minerals, vitamins (B-complex, C, K in some instances), β-carotene (pro-vitamin A) and phenolics (antioxidants).

Fruits are widely distributed in nature and depending upon their distribution, they are classified into tropical, subtropical and temperate fruit
[Table 1].

Table 1: Classification of fruits based on their distribution

Temperate	Sub-Tropical	Tropical
Apple	Avocado	Annona
Apricot	Limes	Banana
Cherry	Litchi	Guava
Grapes	Mandarin	Jackfruit
Kiwi fruit	Olive	Mango
Peach	Orange	Melon
Pear	Passion fruit	Papaya
Plum	Persimmon	Pineapple
Strawberry	Pomegranate	Sapota

Fruits are harvested at maturity. They are self -sufficient with their own catalytic machinery to maintain an independent life, even when detached from the parent plant. Based on the respiratory pattern and ethylene biosynthesis during ripening, harvested fruits can be divided into Climacteric and Non-climacteric [Fig. 1; Table 2]. Climacteric fruits are harvested at



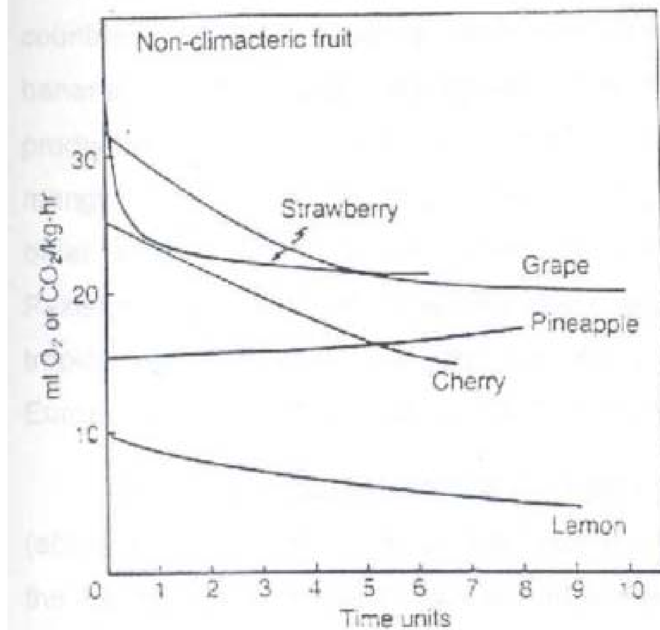


Table 2: Climacteric and Non-climacteric fruits

Climacteric

Apple
Apricot
Banana
Guava
Kiwifruit
Mango
Papaya
Passion fruit
Peach
Pear
Persimmon
Plum
Sapodilla
Tomato

Non-climacteric

Cherry
Cucumber
Grape
Grapefruit
Lemon
Lime
Litchi
Mandarin
Melon
Orange
Pineapple
Pomegranate
Raspberry
Strawberry

Fig.1: Respiration patters during ripening of climacteric and non-climacteric fruits[268]

mature stage and can be ripened off the parent plant. The respiration rate and ethylene formation is minimal at maturity and raise dramatically to aclimacteric peak, at the onset of ripening, after which it declines.[81]climacteric fruits are not capable of continuing their ripening process, once they are detached from the parent plant. Also, these fruits produce very small

quantity of endogenous ethylene, and do not respond to external ethylene treatment. Non-climacteric fruit show a gradual decline in their respiration pattern and ethylene production, throughout the ripening process.[81] Fruits vary in their inherent nature, composition, and their characteristic features: aroma, flavour, taste and texture, which make them different from others.

Mango

Mango (Mangifera indica L), the 'King of Fruits', is the most popular, climacteric, fleshy 'Drupe', belonging to the family Anacardiaceae. It is the choicest fruit crop of tropical world, grown commercially in more than 87 countries. Mango is currently rated in fifth position in total production after banana, citrus, grapes and apple.[71] The estimated world wide annual production of mango is around 24 MMT. Being the major producer of mango, India contributes around 54% of the total world production.[71] jor producers are China, Mexico, Thailand, the Philippines,Pakistan, etc. [Table 3]. Now, mangoes are widely available not only intropics and sub-tropics, but also year-round in North America, Japan and Europe, both in fresh as well as in processed forms.[164]

India is the richest source of quality mango varieties in the world (about 1000 varieties). The unique taste and flavour developed in some of the top mango varieties of India are unsurpassed. Mangoes have attractive fragrance, flavour, delicious taste, and high nutritional value, owing to high amount of vitamins A / C, β-carotene and minerals.[225,258] Thus, India has a great export potential for mango, which Can be increased through proper storage, packaging and marketing practices. In 1998-99, India exported around 45,000 tonnes of fresh mango and 455,000 tonnes of processed mango, worth Rs. 809 million.[247] The range of processed products of mango includes, raw mango powder (Amchur), pickles, mango sauce or chutney,green mango beverage (panna), frozen and canned slices, nectar, pulp, jam, squash, juice, ready-to-serve (RTS) beverages, mango puree, mango cereal flakes, mango leather, mango powder, mango toffee and mango fruit bars.[246] Alphonso (Badami) is the most wanted variety of Indian mangoes and is rated the best in the world. It is characterized by thin skin, soft flesh with low fibre content and sweet aroma.[132]

Table 3: Production status of mango in the world[71]

Countries	Mango Production Statistic (1000MT)			
	1976	1981	1991	1999
World	11,837	13,454	15,700	23,852
India	7,333	8,516	9,500	12,000
China	229	341	595	2,150
Mexico	388	561	800	1,538
Thailand	-	509	903	1,250
Philippines	241	367	348	950
Pakistan	594	547	760	917

Nigeria	-	-	-	731
Indonesia	374	444	640	605
Brazil	638	600	546	600
Egypt	88	123	208	231

Mango softens quickly and extensively during ripening. This high perishable nature of the fruit and the short harvest season limit utilization of mango, especially for commercial purposes. However, being a major fruit crop of great economic importance, mango has been extensively studied.[183]

Mango fruits are harvested at a physiologically mature-green stage,and are kept for normal ripening. In general, depending on the variety and environmental conditions, mangoes take 6-12 days for normal ripening under ambient conditions and become overripe and spoiled within 15 days after harvest.[136. 274] As a climacteric fruit, the period of ripening is characterized by a series of biochemical changes initiated by the Autocatalytic production of ethylene and increase in respiration.[217] Ripening results in the characteristic colour, taste and aroma with desirable softening.

Much work was expended to study the post-harvest physiology of mango fruit, where most of them deal with biochemical changes during ripening, [84, 108, 120, 136], [178] specifically the organic acid metabolism ,[191, 235,243] overall composition and gross changes in cell wall and total pectin during ripening.[33, 69,163,221,258,270] However, considerable differences exist between cultivars of same species.[235]

The post harvest life of mango can be divided into three phases; [179](a) Storage phase, which includes the transportation period from harvest,where the fruit remains raw (unripe). (b) Ripening phase, which includes the from harvest until the fruit attains the stage of maximum consumer acceptability, and (c) Shelf life phase, the period during which the period during which the fruit remais in an edible condition, after it attains full-ripe stage.

Mango fruit is vulnerable to post harvest losses due to its high perishable nature and are also susceptible to varieties of disorders during post harvest handling and storage.[288] Storage and ripening of mango are beset with number of problems. Various methods of post harvest techniques have been employed to extend the shelf life of mango fruit and reduce losses, through inhibition of respiration and ethylene production, which slows deterioration and senescence.[244] These can be classified as physical and chemical methods, which include refrigeration or cold storage, polyethylene film packaging, wax coating, sub-atmospheric pressure storage, controlled atmospheric

storage, modified atmospheric storage, irradiation, heat treatment and use of various chemicals. A combination of these can also be adopted to extend the shelf life of the fruit.[118]

The current storage techniques are expensive, inadequate and also not fully satisfactory.[17o] Further, a variety of disorders including development of off-flavour can result if fruits are exposed to O_2 / CO_2 concentrations below / above certain threshold values.[119] Therefore storage and ripening of mangoes continue to be a challenging problem and has received much attention. In recent years with molecular studies, 'Tomato Biotechnology' took a new turn of events.[86] Here fruit ripening was manipulated at gene level, which is considered to be a very promising approach. Control of ripening process has been very successfully proved to be possible where they have obtained firmer tomatoes with extended shelf life by individually suppressing ACC synthase,[172] ACC oxidase,[97] PG[252] and PME[267] expression by antisense RNA technology. One or more genes were identified and used in the 'sense' or 'antisense' orientation to extend the shelf life of commercially important fruits.[18] To control the post-harvest life of any fruit by molecular approach, a basic understanding of the events occurring during fruit ripening is essential.

Fruit ripening

Fruit ripening is a highly co-ordinated, genetically programmed and irreversible phenomenon involving a series of physiological, biochemical andorganoleptic changes that lead to the development of a soft and edible ripefruit with desirable quality. A spectrum of biochemical changes such asincreased respiration, chlorophyll degradation, biosynthesis of carotenoids,anthocyanins, essential oils and flavour and aroma components, increasedactivity of cell wall-degrading enzymes, and a transitory increase in ethyleneproduction are the major changes involved during fruit ripening.[32, 217]

The change in colour during fruit ripening is due to the unmasking of previously present pigments by degradation of chlorophyll and dismantlingof the photosynthetic apparatus, synthesis of different types of anthocyaninsand its accumulation in vacuoles,[269] and accumulation of carotenoids such as β-carotene, xanthophyll esters, xanthophylls and lycopene.[146. 269] The increase in flavour and aroma during fruit ripening is owing to the production of a complex mixture of volatile compounds like ocimene and myrcene,[146] and degradation of bitter principles, flavanoids, tannins and relatedcompounds.[269] The taste development is due to a general increase in sweetness, which is the result of increased gluconeogenesis, hydrolysis of polysaccharides, especially starch, decreased acidity and accumulation of sugars and organic acids with an excellent sugar/acid blend.[89, 235, 269] The metabolic changes during fruit ripening include increase in biosynthesis and evolution of the ripening hormone, ethylene,[289] increase in respiration mediated by mitochondrial enzymes, especially oxidases and de novo synthesis of enzymes catalyzing ripening specific changes.[269] Alteration of cell structure involves changes in cell wall thickness, permeability of plasmamembrane, hydration of cell wall, decrease in the structural integrity and increase in intracellular spaces.[21O, 269]

The major textural changes resulting in the softening of fruit are dueto enzyme-mediated alteration in the structure and composition of cell wall,partial or complete solubilization of cell wall polysaccharides, like pectinsand celluloses,[269] and hydrolysis of starch and other storage polysaccharides.[8o, 235] The changes in gene expression during ripening involves the appearance of new 'ripening- specific' mRNAs, tRNA, rRNA,poly A +RNA and proteins, and the disappearance of some mRNAs.[87, 88, 269] However, some mRNAs are found to remain constant throughout the ripening process.[84] These changes during fruit ripening are activated by plant hormones

Role of fruit ripening hormone

Ethylene, specifically a fruit ripening phytohormone, in minute amounts can trigger many facets of cell metabolism including initiation of ripening and senescence, particularly in a climacteric fruit. Ethylene, which is synthesized autocatalytically at levels as low as 0-0.01µl L^{-1} and 0.05-025µl L^{-1} triggered the ripening process in mango and banana,respectively.

[16]Number of reviews have been published on the role of ethylene in fruit ripening, particularly in mangoes as well as its biogenesis.[3],[122] Fruits treated with exo-polygalacturonase or other cell wall hydrolases or their products have been shown to elicit ethylene production.[17, 125] This response is not fruit specific.[17] In cultured pear cells it was shown that the pectic oligomers might also induce and regulate the ethylene biosynthesis.[37]

The pathway for ethylene biosynthesis has been elucidated in apple,[3] and other fruits such as avocado, banana and tomato.[122], [289] The first step is the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropanecarboxylic acid (ACC) by the enzyme ACC synthase (Fig. 2). At the onset of fruit ripening, expression of multiple ACC synthase genes are activated, resulting in increased production of ACC. In most cases, it is the ACC

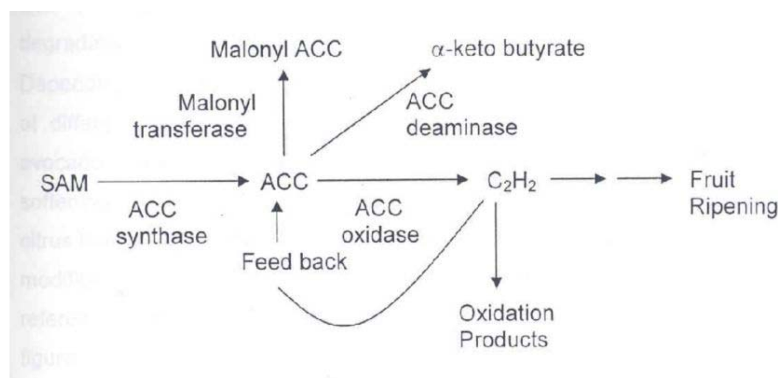


Fig. 2: Pathway for ethylene biosynthesis and metabolism

synthase activity, which determines the rate of ethylene biosynthesis. ACC is then oxidized to ethylene by ACC oxidase (Ethylene forming enzyme, EFE). Inhibition of ethylene biosynthesis by antisense RNA for ACC synthase 172 and ACC oxidase97 was demonstrated first in tomato fruit. Deamination of ACC to a.-keto butyrate by overexpressing ACC'deaminase enzyme also inhibited ethylene formation and fruit ripening[128] The resulted transgenic fruit did not overripe as normal controls, though some colour change occurred and a mere ethylene boost triggered back all the ripening related biochemical changes in a similar way as in normal fruit.[97 172] Recently the cDNA encoding for ACC oxidase enzyme has been isolated and characterized from mango.[293] The mango ACC synthase and ACC oxidase genes are now being used for transgenic works in mango, for extending the shelf life. Thus ethylene playing a crucial role in fruit ripening is clear.[265]

Textural softening during ripening

Fruit ripening is associated with textural alterations, which is dramatic in a climacteric fruits. Textural change is the major event in fruit softening, and is the integral part of ripening, which is the result of enzymatic degradation of structural as well as storage polysaccharides.[23. 89. 108. 269] Depending upon their inherent composition and nature, different fruits soften at different rates and to varying degrees.[269] Fruits like mango, papaya, avocado, sapota and banana undergo drastic and extensive textural softening from 'stone hard' stage to 'soft pulpy' stage. Fruits like apple and citrus fruit do not exhibit such drastic softening, though they undergo textural modifications during ripening. An overview of fruit ripening with special reference to textural softening has been diagrammatically represented in figure 3.

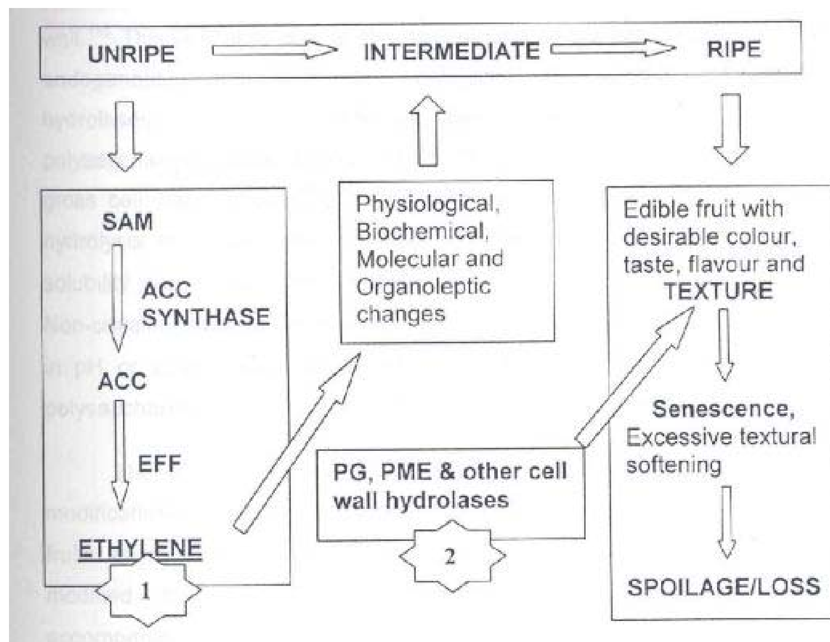


Fig. 3: An Overview of fruit ripening with particular emphasis on textural softening. Control points at ethylene (1) and post-ethylene (2) levels

Fruit texture is influenced by various factors like structural integrity of the primary cell wall and the middle lamella, accumulation of storage polysaccharides, and the turgour pressure generated within cells by osmosis.[112] Change in turgour pressure, and degradation of cell wall polysaccharides and starch determine the extent of fruit softening.[32, 89, 269] In citrus fruit, softening is mainly associated with change in turgour pressure, a process associated with the post harvest dehydration and / or loss of dry matter. Starch is the bulk polysaccharide present in some fruits like mango and banana, and its enzymatic hydrolysis results in pronounced loosening of cell structure and sweetness development, which is mainly due to sugar accumulation.269 However, textural changes during ripening of most of the fruits are largely due to changes in the physicochemical properties of the cell wall.[115] This is mainly due to the degradation of cell wall polysaccharides, Idogenously controlled and catalysed by various carbohydrate drolases.[76, 104, 269] Subtle structural changes of the constituent Ilysaccharides occur during fruit softening, without affecting much of the JSS cell wall composition.32, 76 Polysaccharides of the cell wall undergo hydrolysis or solubilization resulting in change in their molecular mass, solubility and the degree of substitution of the individual polysaccharides. Non-covalent changes in the cell wall are detected by the localized alteration pH or ionic concentration, whereas covalent modification of the wall polysaccharides generally results from the enzymatic processes.[39, 76. 78, 239]

The major classes of cell wall polysaccharides that undergo modifications during ripening are pectins, cellulose and hemicelluloses. In its, which are known for excessive softening, the cell walls are thoroughly modified by solubilization, de-esterification and depolymerization, companied by an extensive loss of neutral sugars and galacturonic acids, lowed by the solubilization of the remaining sugar residues and gosaccharides.[276]

The process of textural softening is of commercial importance as it directly dictates fruit shelf life and quality.[268] This should be considered to avoid mechanical damage during harvesting and transportation. The textural perties of fruits, in general play a very significant role in the consumer ceptability. The increased interest in controlling the textural qualities of it stimulated further research on the biochemistry of cell wall, with particular reference to cell wall polysaccharides and their degradation.[112 273] The textural qualities of fruits are attributed to its inherent composition, particularly the cell wall composition. Figure 4 shows the hematic representation of the levels of structure that contribute to fruit texture. The 'textural' characteristics is attributed to the mechanical perties of the final organ, which in turn depends on contributions and interactions of different levels of structure.[278] Attempt to understand the molecular mechanism of fruit softening have directly led to the investigation of cell wall polymers, their compositional changes and the related cell-wall degrading enzymes during ripening.[129]

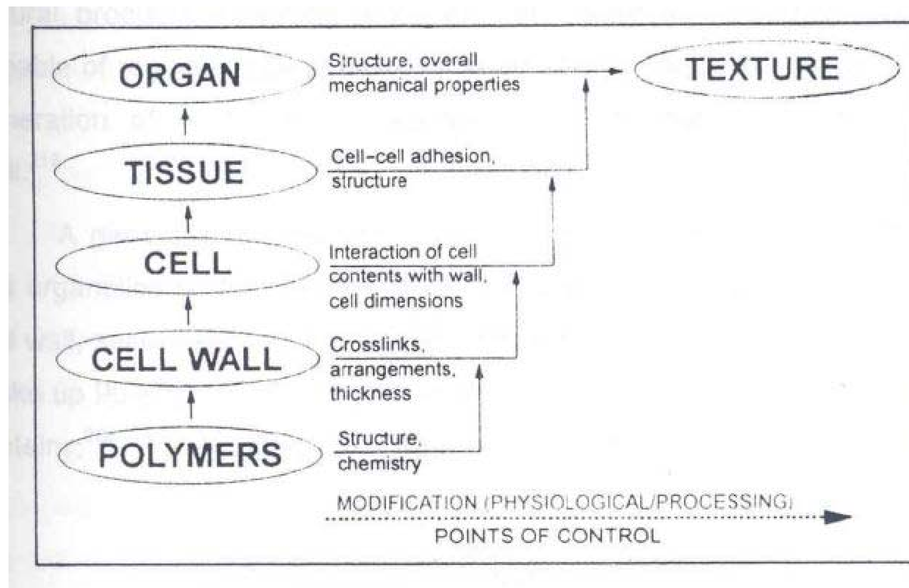


Fig. 4: Schematic representation of the levels of structure that contribute to the fruit texture[278]

Plant cell wall and its components

Plant polysaccharides, in general, are an extremely diverse set of biopolymers, which playa very important role as structural elements, such as pectins, celluloses, hemicelluloses; reserve polysaccharides like starch and galactomannans; gel formers such as gums and mucilages; and physiological information carriers like antigens. Fruit polysaccharides, upon their degradation, playa crucial role in textural softening during ripening. Polysaccharides from different sources vary in their chemical-biological, physico-chemical, and structural-functional characteristics.[263] Plant polysaccharides play a major role in storage, mobilization of energy and in maintaining cell and tissue integrity due to their structural and water binding capacity. Cell wall polysaccharides differ widely in their physical / nutritional properties and have the greatest potential for structural diversity.[11] They regulate the utilization of other dietary components in the food. Recently plant polysaccharides have emerged as important, bioactive, natural products exhibiting a number of biological properties. They are capable of regulating gene expression and host-defense mechanism by the generation of elicitor-active oligogalacturonide fragments from the cell wall.[218]

A generalized plant cell, showing plant cell wall, middle lamella and cell organelles is depicted in Fig. 50 The plant cell is composed of primary cell wall, secondary cell wall and middle lamella276 Carbohydrate polymers make up 90-95 % of the structural components of the wall, remaining being proteins.[268] The primary cell wall, a mechanically dynamic structure,[291]

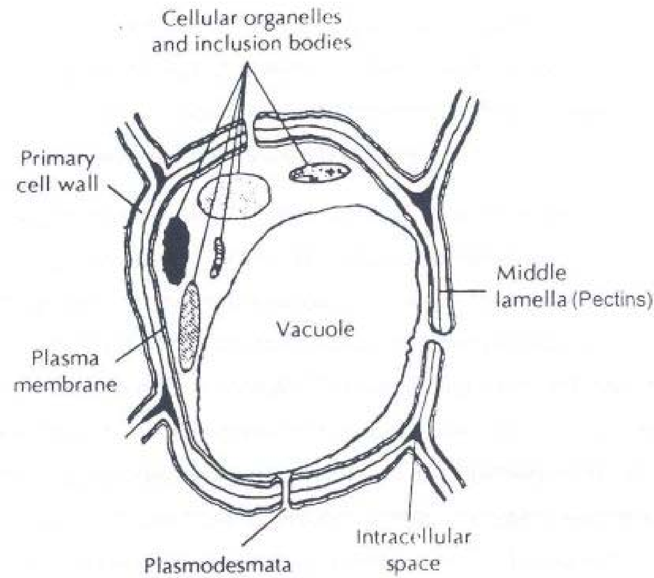


Fig. 5: Idealized plant cell[112]

constitutes the outer most part of the wall and is assembled when the cells are growing, expanding and dividing.[156] The secondary cell wall, a mechanically static structure,[291] consists of many layers, which are differentiated structurally by the orientation of the glucan chains, stabilized by the intra- and inter-specific hydrogen bonding. Secondary cell walls are deposited after the cessation of cell growth, internal to the primary cell wall and outside the plasmamembrane.[112] Tertiary cell wall, if present, forms a thin, amorphous, membranous layer around the plasma membrane, but inside the secondary cell wall.[11] The adjacent cells are separated by a non-cellular, amorphous, pectin-rich middle lamella, which holds the cells together. Middle lamella act much like adhesives (glue) between the cells, and bear some of the compressive or tensile stress and contribute to overall strength.[112]

Cell wall is an active organelle, vital to cell growth, metabolism, transport, attachment, shape, cell resistance and strength. The old notion of the cell wall being static, inert, and a mere load-bearing structure has changed to the newer concept of dynamic nature of the cell wall.[112] Carpita and Gibeaut[39] proposed a three-dimensional model for the primary cell wall on the basis of links between the various polymers.

According to this model (Fig. 6), hemicelluloses constitute the main chain-interlocking component. They are highly branched and the linear conformation favours orientation between cellulose microfibrils, to which they bind. Thus the hemicelluloses form a layer, coated around the cellulosic microfibrils to form a structural domain. The whole fibril is found embedded in a matrix of second domain consisting of pectin and pectic substances. The galactans and xyloglucans are involved in cross-linking the cellulose and pectin components. A third structural domain contains extensin and other structural protein units covalently cross-linked and oriented radially within the wall

matrix. Once cell growth completes, the extensin cross-linking is thought to be involved in locking the cell wall in a fixed geometric shape.[39]

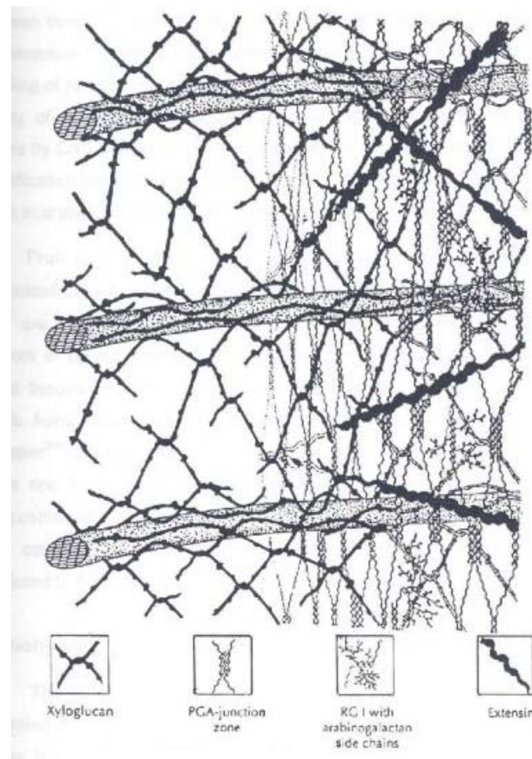


Fig. 6: A three dimensional model of primary cell wall[112]

The structure is held together by hydrogen bonding between the cellulose and hemicellulose chains and covalent linkages between the hemicellulose and neutral pectins and the neutral and acidic pectins.[269] Pectic polymers HG, RG-I and RG-II, which are solubilized by chelators, are not separated by gel permeation chromatography indicating covalent linkageIbetween them.[218] Non-covalent bonds are predominately involved in the wall structure.[263] Pectin cross-linking can also occur as a result of oxidative coupling of phenolic constituents, such as ferulic acid. More often, the cross-linking of the helical homogalacturonan chains of de-esterified pectins occurs by Ca^{++} bridging to form junction zones. However, not all sites of de-esterification become cross-linked. The side chains of rhamnogalacturonan-l, rich in arabinogalactans interrupt Ca++ bridge formation.[156]

Fruit pulp or the mesocarp is the edible part of the fruit, and is composed of thin-walled storage parenchymatous cells (50-500 µm). These cells are characterized by a prominent cell wall consisting of complex network of polysaccharides and proteins, which gives mechanical strength to the tissues. The primary cell wall contains 35 % pectin, 25 % cellulose, 20 % hemicellulose and 10 % structural, hydroxyproline-rich protein.[34] Whitake~84 reported the cell wall composition and percent pectin present in some ripe fruits, such as pears, tomato, apple and date. Neutral sugar i composition of fruit cell wall varies from fruit to fruit, and marked changes in their composition occur during ripening.91 Most of these changes are attributed to the action of cell wall (carbohydrate) hydrolases.

Carbohydrate hydrolases of fruits in relation to fruit softening

The changes in the cell wall composition, which accompany fruit softening during ripening, are due to the action of carbohydrate hydrolases. These hydrolases act on cell wall polymers, resulting in their degradation. Most of these enzymes are present in low levels and are constitutive throughout fruit development and ripening.[268] But during ripening, generally all the hydrolases increase in activity, particularly cell wall hydrolases, showing a peak activity at climacteric stage.

A wide range of cell wall hydrolases are identified in fruit tissues.[5, 76,78, 104, 281] The major hydrolases involved in polysaccharide dissolution in vivo can be broadly classified into 2 types of hydrolases; viz, glycanases and glycosidases. Glycanases (glycanohydrolases) by definition are a class of enzymes cleaving high molecular weight polymers (polysaccharides) into shorter chains, while glycosidases (glycohydrolases) generally act on shorter chain oligosaccharides, which may be homo- or heterooligomers or glycoproteins or glycolipids. They may be also involved in signal transduction by the way of deglycosylation.[205, 259] Recently, it has been reported that temperature plays a crucial role in the activities of these cell wall hydrolases.[208] The entire range of these enzymes has been systematically listed [Table 4].

Table 4: Different types of carbohydrate hydrolases in fruits

Glycanases	Glycosidases
Polygalacturonase*	∝-Mannosidase
Pectin methyl esterase*	∝-Galactosidase
Cellulase	β-Galactosidase*
Hemicellulase	∝-Glucosidase
Amylase	β-Glucosidase
Mannanase	∝-Hexosaminidase
Galactanase*	β-Hexosaminidase
Glucanase	∝-Xylosldase
Arabinosidase*	β-Xylosidase
Xylanase	∝-Arabinosidase
Rhamnogalacturonase	β-Arabinosidase

* Enzymes studied in the present investigation.

Hemicellulose / Cellulose degradation during ripening

Hemicelluloses are neutral sugar polysaccharides extracted by alkaline solutions from the cell wall residues after the extraction of pectic polysaccharides.[110] The inert, insoluble, crystalline cell wall

material remained after the hemicellulose extraction, which is mainly composed of β-glucose, is the cellulose.[273]

An apparent dissolution of the middle lamella and cell wall fibrillar network due to cellulolytic activity in ripening of avocado, pear and apple was demonstrated.[25. 130, 184] Ripening associated changes involving dramatic decrease in the molecular size of hemicellulose are reported in tomato,[103] strawberry,[102] pepper,[93] muskmelon,[153] kiwi,[212] melon[222] and mango.[161] The amount of hemicellulose decreased steadily during ripening of many fruits including mango.161 Decline or loss of substantial levels of characteristic monomers of hemicelluloses; glucose, xylose and mannose occur during ripening of fruits like strawberry,[165] tomato [92] and pear.[4]

Little is known about the enhancement of cellulase or hemicellulase in connection with fruit softening. Cellulases and hemicellulases Cellulase is a multienzyme system composed of several enzymes; endo-glucanase (EC 3.2.1.4), exo-glucanase (EC 3.2.1.91) and glucosidase (EC 3.2.1.21).[253] Endo-glucanase hydrolyses the β-1 ,4-link between adjacent glucose residues at random positions. Exo-glucanase breaks the bonds at non-reducing end of the chain, producing glucose or cellobiose (dimers of β-1 ,4-linked glucose), β-glucosidase split cellobiose into glucose molecules.

Cellulase activity increased during ripening in avocado,[184] papaya,[182] peach, strawberry and tomato.[104] Cellulase levels in unripe fruit are generally low and increase dramatically during ripening.[15] The loss of firmness, climacteric rise of respiration and ethylene evolution in ripening fruit was directly correlated with marked increase in cellulase activity.[184, 221, 2] Cellulase activity in normal and non-ripening mutants of tomato suggests that this enzyme has no primary role in fruit softening.[187] However, cellulase has been implicated in softening process in tomato.[100] Cellulase activity was reported in several Indian mango cultivars, which increased during ripening.[234] No cellulase activity was detected in pears.[5]

Xylanases (EC 3.2.1.8) catalyses the hydrolysis of β-1 ,4-xylan. β-1,4-D-endo-xylanase and β-1,4-D-exo-xylanase are reported as cell wall degrading enzymes from fruits including banana [189] and capsicum.[205] In papaya during ripening, a clear correlation between polygalacturonase and xylanase activities, climacteric rise in respiration and ethylene evolution and fruit softening were demonstrated.[182] Mannanase catalyses the hydrolysis of mannan polymer in capsicum[205] and mango.[190] Xylanase, arabinanase and mannanase are localized both in soluble and bound form, which increases during ripening. It was interesting to note that arabinanase, galactanase and mannanase were very prominent enzymes in mango fruit with activity peaks at climacteric stage of ripening.[27,190] Among glycosidase, the prominent enzymes found in ripening fruit were β-hexosaminidase, ∝-mannosidase and β-galactosidases.[205,2S9]

∝-Amylases (EC 3.2.1.1) and p-amylases (EC 3.2.1.2) are the two amylases in plant tissues capable of metabolizing starch, ∝-amylases hydrolyse the a-1,4-linkages of amylose at random to produce a mixture of glucose and maltose, whereas β-amylases attack only the penultimate linkage from the non-reducing end and thus releases only maltose. These enzymes are unable to degrade the ∝-(1→6) branch points of amylopectin, which are catalysed by debranching enzymes. Amylase activity increased to some extent during ripening of many fruits.[269, 80] Mango and Banana are the major starch containing fruits (-15 to 20 %, on fresh weight basis), where starch is almost completely hydrolysed to free sugars, thus contributing to loosening of cell structure and textural softening during ripening.[27]

As the present research investigation is specifically on pectin dissolution in vivo, pectic polymers and the related enzymes have been dealt here in detail.

Pectins and pectic substances

Pectins are the common components of the primary cell wall and middle lamella contributing to the fruit texture. Pectin content varies from fruit to fruit and pectins from fruits are used for commercial purposes. Eg.apple, guava and citrus [Table 5].[69,170,262,284]

Table 5: Pectin content of some fruits [69,170,262]

Fruits	Pectin content (%)
African Mango	0.72
Apple	0.5-1.6
Avocado	0.73
Banana	0.7-1.2
Cashew	1.28
Cherries	0.2-0.5
Guava	0.26-1.2
Lime	2.5-4.0
Litchi	0.42
Mango	0.66-1.5
Orange	1.35
Papaya	0.66-1.0
Passion fruit	0.5
Peach	0.1-0.9
Pineapple	0.04-0.13
Strawberry	0.44- 0.14
Tomato	0.2-0.6

The name 'Pectin' originated from the Greek word 'Pectos' meaning, 'gelled'. Native pectin plays an important role in the consistency of fruit and also in textural changes during ripening, storage, cooking or irradiation and other processing operations. Tissue softening is attributed to enzymatic degradation and solubilization of the protopectin.[226] Pectins are likely to be the key substances involved in the mechanical strength of the primary cell wall and are important to the physical structure of the plant.[248] Their degradation during ripening seems to be responsible for tissue softening, as reported for a number of fruits including tomato,[187, 242] kiwi,[211] apple[56] and bush butter.[159] The major changes in the cell wall structure are the dissolution of middle lamella and primary cell wall during ripening. Thus, elucidation of chemical structure of pectin is essential in understanding its role in plant growth / development and during ripening of fruits,[262]

Parenchymatous tissues are thought to consist principally of calcium salts of pectic substances, which are deposited in early stages of the cell growth, specifically when the area of cell wall is increasing,[276] Pectic substances are prominent structural constituents of primary cell wall and middle lamella and are the sole polysaccharides in middle lamella, along with some cellulose microfibrils, while they may be virtually absent in secondary walls.[273] Middle lamella are heat labile and their dissolution result in separation of plant cells. Ultrastructural studies in ripening fruits have also shown that cell wall breakdown was accompanied by dissolution of middle lamella and gradual dissolution of fibrillar network of primary cell wall.[25, 48,112,184] Deesterified pectins in the middle lamella are associated with calcium ions, and its removal also usually leads to cell separation.[11] The association involves binding of two or more polymeric chains, in the form of corrugated egg-box (Fig. 7), with interstices in which calcium ions are packed and coordinated, creating an "egg-box" system.[85] Specific binding of the divalent cations to pectins in an "Egg box model" leads to a firm cohesion between the chains,[85] Calcium treatment inhibited softening of fruits due to an

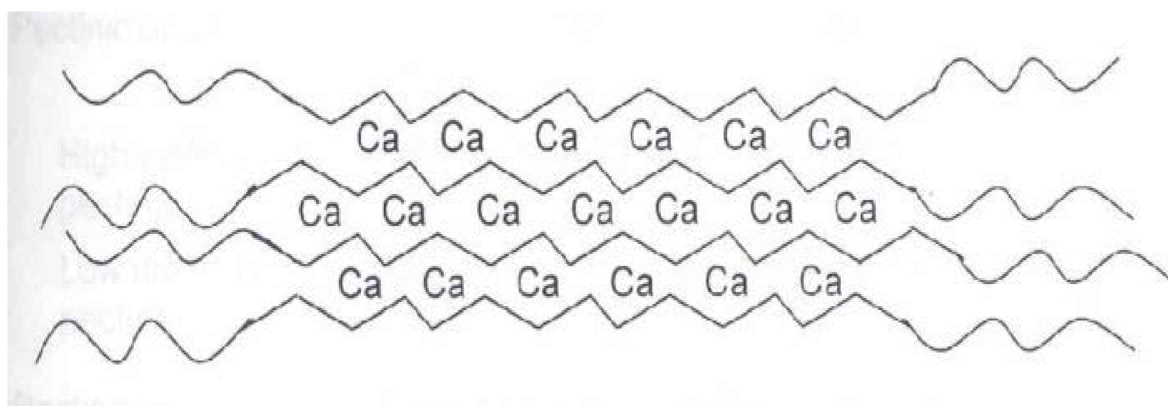


Fig. 7: Egg-Box model depicting association of pectins with Ca^{++} ions

Increase in cohesion of pectin network.[135] Separation of middle two layers, each remained attached to the primary cell wall was reported in strawberry during ripening,165 which was mimicked by EDTA chelation. Generally, pectins in the cell wall are cross-linked through ionic interaction.[148]

Due to this ability to form co-ordination complexes with Ca^{2+}, chelator soluble pectins are of special interest as they increase fruit firmness.[114]

Pectins are structural, acidic homo-/heteropolysaccharides obtained commercially from fruits but present universally in plant cell wall matrices.[13] They are structurally diverse, heteropolysaccharides containing partially methylated galacturonic acid residues; methyl esterified pectins, deesterified pectic acids and their salts; pectates [Table 6] and the neutral polysaccharides, which lack galacturonan backbone, i.e., arabinogalactans

Table 6: Description of pectic substances present in plant cell walls

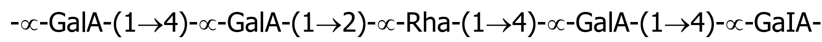
Pectic substances	Description
Pectic substances	Group of colloidal, complex polysaccharides of galacturonic acid linked in a chain fashion.
Protopectin	Water-insoluble parent pectic substances.
Pectic acids	Pectic substances free from methyl ester groups.
Pectates	Normal or acid salts of pectic acids.
Pectinic acids	Pectic substances partially esterified with methyl groups.
High methoxyl pectins	Highly esterified (>50% esterified) pectinic acids.
Low methoxyl pectins	Less esterified («50% esterified) pectinic acids.
Pectinates	Normal or acid salts of pectinic acids.

arabinans and galactans (Fig. 8).[13. 110] Neutral plant polysaccharides are also grouped under pectins mainly because of their association with acidic pectins as side chains to the main galacturonan backbone.[13, 65] They may also be present as free polymers,[34]

The pectin chain, ∝-D-galacturonans, i.e., ∝-D-galacturanoglycans or poly (∝-D-alactopyranosyluronic acid), consists largely of D-galacturonic acid linked by ∝. (1→4) linkages.[24] The carboxyl groups of pectin are partially esterified with methanol and the hydroxyl groups are partially acetylated with acetic acid.[185] They occur mainly in chair L-form and as both C-1 and C-4 hydroxyl groups are on the axial position, the polymer formed is a trans 1,4-polygalacturonan.[226]

Polygalacturonan

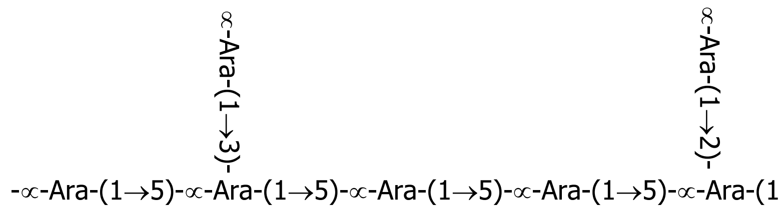
Rhamnogalacturonan



4

Side chains of
Galactan or
Arabinan or
Arabinogalactan-I or
Arabinogaiactan-II

Arabinan



Galactan

Fig. 8: Structure of pectic substances[250]

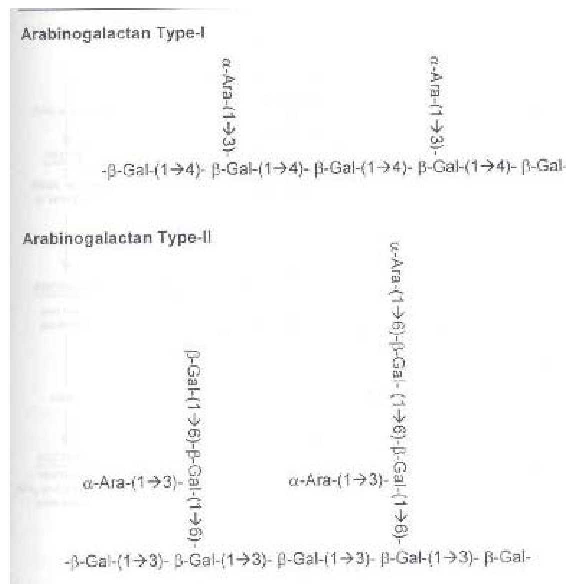


Fig. 8 (cont.) : Structure of pectic substances[250]

During ripening, softening of fruit is caused by the conversion of protopectin, the insoluble, high molecular weight parent pectin into soluble polyuronides.[115] This tightly bound protopectin is degraded into soluble pectins, which are found loosely bound to the cell walls. This phenomenon is

attributed to the textural softening during ripening.[67] Protopectin increases before physiological maturity, but decreases during mango fruit ripening.[250] Inter-relation between different pectic substances and their degradation is shown in Fig. 9.

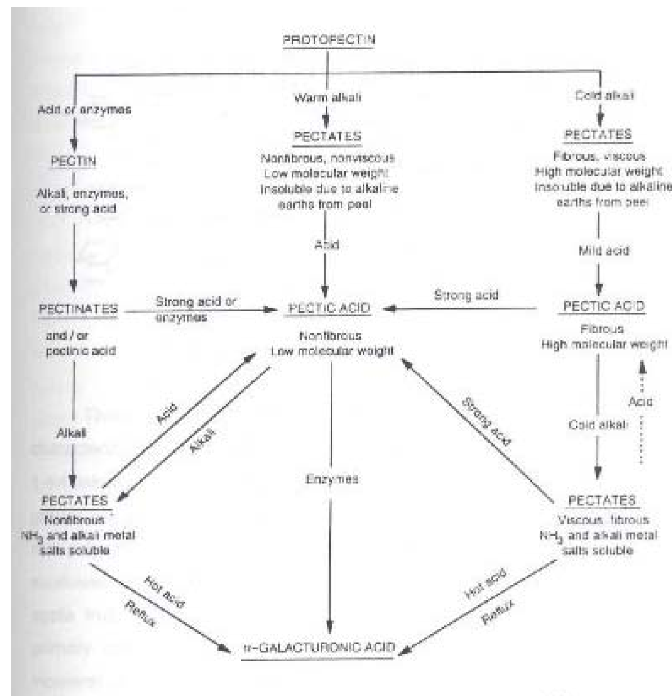


Fig. 9: Inter-relationship of pectic substances[226]

A detailed structure of pectin depicting various sugar units and their linkage is shown in figure 10. The degree of polymerization, degree of esterification, and the proportion of neutral sugar side chains are the principal factors contributing for heterogeneity of the pectic polysaccharides.[216] Pectins, like other polysaccharides, are both polydisperse and polymolecular, mainly due to their heterogeneous nature in both molecular weight and chemical structure.[23, 24, 134]

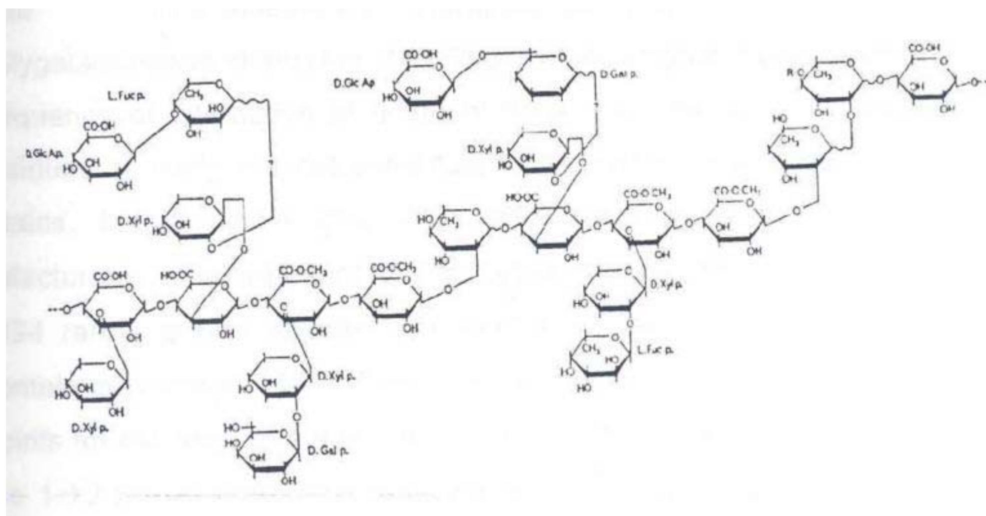


Fig. 10: A detailed structure of pectin[226]

Three types of pectic polysaccharides have been structurally characterized. Homogalacturonans (HG) consist solely of linear chain of 1→4 linked α-D-galacturonans (see Fig. 8), in which some of the carboxylic roups are methyl esterified. They are found to be 100 nm in length. It is a rare polysaccharide and has been isolated from only a few plant sources like sunflower heads and seeds, sisal, the bark of amabilis fir, Jack fruit and Ipple fruit.[19.185] It has been isolated from cell wall of rice endosperm, primary cell wall of Rosa, sycamore [156, 276] and recently from citrus.[294] However, it has been viewed that the homogalacturonan might be released rom the heterogeneous pectic substances by the conditions employed luring extraction.[276]

Rhamnogalacturonan-I (RG-I) is a peculiar type of pectic substance, and is the one, which is primarily responsible for the chemical and structural diversity of the pectins. It is the major component of the primary cell wall and middle lamella of dicotyledonous plants.[157] They consist mainly of the backbone of the repeating disaccharide units[24] →1)-α-D-GalA-(1→2)-α-L-Rha-(1→. The L-rhamnose residues are linked by α-1,2 linkage to the Jreceding galacturonic acid and β-1,4 linked to succeeding galacturonic acid.[141. 218] This insertion of rhamnose forms a 'T' shaped "kink" in the polygalacturonan chain (Fig. 11). This kinking of parent chain minimizes the frequency of interaction of adjacent polymeric chains.[85] Galacturonic acid residues typically are not substituted with mono- or oligosaccharides side chains, but a single glucuronic acid substitution on C-3 position of galacturonic acid was reported in sugar beet pectins.[215] Methyl esterified RG-I rarely occurs in plant cell wall. However, it has been reported to contain methylated RG-I in flax.[219] Rhamnose residues are found as branch points for the attachment of neutral sugar side chains.[148. 157] Almost 50% of the 1→2 linked rhamnose residues are branched at O-4 with side chains consisting of D-galactose and/or L-arabinose residues. Small amounts of fucose, glucuronic acid, 4-0-methyl f3-D-glucuronic acid units are also found linked to rhamnose units.[157, 176] RG-I was reported from a number of fruits including tomato,[241] grape,[169, 229] apple,[233] pear,[231] kiwi,[212] and raspberry,[255] although the nature and length of the neutral sugar side chain may vary.

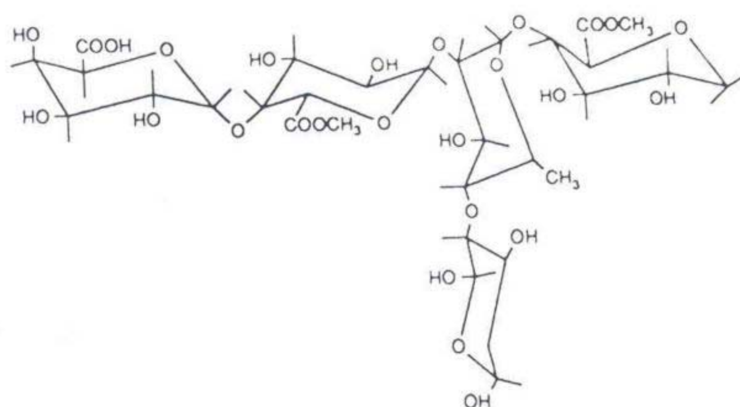


Fig. 11: T-shaped kinking of the pectin molecule

Rhamnogalacturonan-II (RG-II) is present as a minor component of the cell wall, and has extremely complex structures. It is not structurally related to RG-I, since it contains a high proportion of rhamnosyl residues, which occur as terminal (1→3) as well as branched (1→2, 3, 4,) units.[276] RG-II is a small complex polysaccharide containing a homogalacturonan lack bone composed of at least eight 1→4 linked α-D-galacturonic acid residues attached with side chain of 4 different complex oligoglycosyl residues.[256] The side chains are mainly composed of twelve glycosyl residues including several rare "diagnostic" monosaccharides such as piose, 2-0-methyl-α-L-fucose, 2-O-methyl-α-D-xylose, aceric acid, Kdo (2-keto-3-deoxy-D-manno-octulosonic acid) and Dha (3-deoxy-0-lyxo-heptulosaric acid).[275] Recently, it was shown that RG-II is present Iredominately as a dimer[175] These dimers are found cross-linked by borateliol esters, through apiosyl residues and play an important role in the tructure and function of pectins.[175] RG-II has been isolated from primary ell walls of tomato,[64] apple[64] and kiwi fruit.[213] High amounts of RG-II are Iresent in fruit juices.[64] RG-II binds heavy metals and has immunonodulating activities, which stimulated further research on structure of RG-II Ind its role in human nutrition and health.[218]

Substituted galacturonans are a diverse group of pectic olysaccharides that contain a backbone of linear 1,4-linked α-D-lalacturonic acid residues, substituted with other sugar residues.[215] (Xylogalacturonans, in which (β-D-xylose residues are attached to C-3 of the galacturonan backbone, are found in apple pectin.[232]

Regarding the neutral sugar side chains, considerable variations were found in the nature, type, length and structure of the side chains attached to rhamnosyl residues of rhamnogalacturonans (see Fig. 8).[13] Jsually, the ratio of rhamnose to galacturonic acid is 1 : 40, as reported for citrus pectin.[294] Side chains composed of neutral sugars such as D-galactose, L-arabinose occur most frequently, while O-xylose, D-glucose, D-mannose, D-apiose and L-fucose occur rarely in plant pectins.[50] These side chains are distributed discontinuously rather than continuously in pectins.[55] The branching occurs in the C-2177 or C-357 of galacturonic acid or through C-4[254] or C-3[50] of rhamnose. Arabinose and galactose form oligo- / polysaccharide substituting the hydroxyl groups of rhamnose units. The presence of galacturonans rich in xylose has also been reported in fruits like apple. [55, 57, 232] The proportion of branched rhamnose residues varies with fruits, It was in the range of 20-40% in grapes, tomato and kiwi fruit, while it was from 25-100% in apple.[276] RGs branched with several neutral polymers such as arabinans, galactans and arabinogalactans were reported for pectins.[55, 169. 173.229,233,257]

Arabinans are branched polysaccharide chains composed of α-(1→5) linked L-arabinose residues that contain single (terminal) L-arabinose side chains, linked to O-3 or O-2 position of the main chain (see Fig. 8).[284, 276] They resemble a "comb-like" structure. Arabinan associated pectins

have been isolated from fruit like apple[57] and has been recently characterized from sugar beet pulp.[173]

Galactans are linear chains of β-(1→4) linked D-galactose residues (see Fig. 8).[13] They occur as oligosaccharide chains attached to the rhamnose residues of the RG backbone[157]. Tomato pectin rich in galactose side chains were structurally characterized using NMR.[203]

Arabinogalactans (AG) are heteropolymers of D-galactose and L-arabinose residues (see Fig. 8). Two structurally different forms of arabinogalactans are found in plants.[13, 250, 284] AG-I is a simple polysaccharide composed of chains of β-(1→4) linked D-galactose residues with single L-arabinose residues linked to O-3 of the galactose residues.[250,284] They have been isolated from different fruits including apple,[57] kiwi,[213] tomato[241] and pineapple.[251] AG-II are complex and branched polysaccharides, consisting of chains of β-(1→3) linked D-galactose residues linked to chains of f3-(1→6) linked D-galactose residues at the O-6 position of the main chain. The O-3 and O-6 positions of the side chains are in turn linked to terminal L-arabinose residues.[250, 284] Plant arabinogalactans are known for their multifaceted physiological and functional characteristics.[50] They possess freeze-inhibition, water holding and adhesive properties. Due to their specific carbohydrate binding properties, they may possibly affect cell-cell interaction.[50] The pectic polymers of primary cell wall have a relatively higher proportion of neutral oligosaccharide chains on their backbone (i.e., highly substituted pectins) and these side chains are much longer than those of the pectins of middle lamella.[131, 225, 235] The side chains are not distributed regularly but are concentrated in some regions called "hairy regions". Highly esterified and slightly branched rhamnogalacturonan, the "smooth regions", are present in middle lamella whereas highly branched rhamnogalacturonan, the "hairy regions" are present in primary cell wall (Fig. 12).[235] In plant cell wall, the side chains of the pectin molecules link to protein, hemicellulose and cellulose.

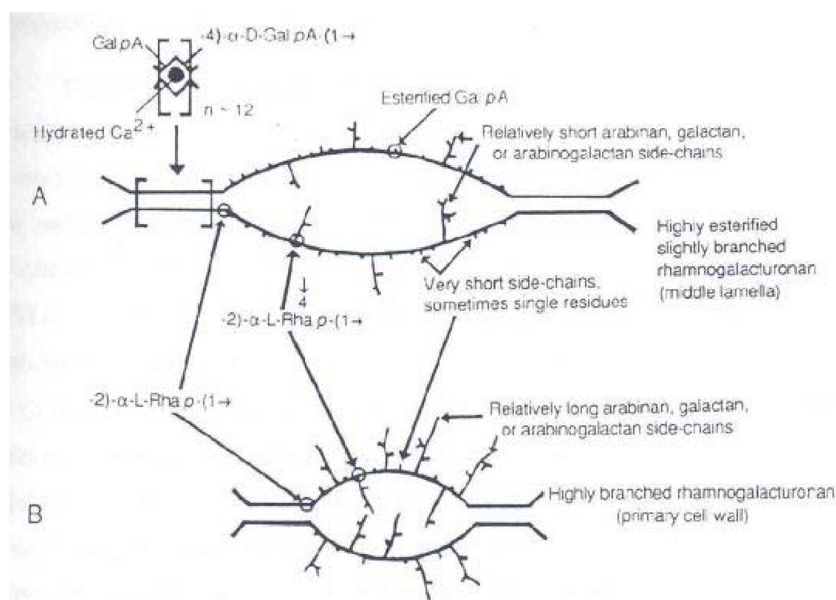


Fig. 12: Schematic representation of some structural aspects of pectins from middle lamella (A) and primary cell wall (B) [236]

The acidic and neutral pectins carry non-sugar substituents, essentially methanol, acetic acid, phenolic acids and amide groups, and contribute further for the structural diversity of pectins.[148] The esterification of galacturonic acid with methanol or acetic acid is a very important structural characteristic of pectins. The degree of methylation (DM) is defined as the percentage of carboxylic groups esterified with methanol. Degree of acetylation (DAc) is defined as the percentage of galacturonic acid residues esterified with one acetyl group.[276] Chelator-soluble pectins have high DM and DAc than those extracted with alkali, which is mainly due to the liberation of methyl ester and acetyl groups by alkali.[266] Phenolic acids, especially ferulic acid and p-coumaric acid are found esterified to the non-reducing ends of the neutral arabinose / galactose residues. These non-sugar substituents, especially ferulic acid facilitate oxidative cross-linking between pectins or with other polysaccharides in the cell walls, by formation of diferuloyl bridges, which would limit wall extensibility[34] and plays a significant role in growth regulation and defense mechanism.

Pectins are extracted from plant material using a wide variety of extracting media.[291] Some pectins are found soluble in water indicating little or no binding to the other cell wall components.[79] It is assumed that pectins are held together by calcium bridges. This forms the basis for the wide use of chelating agents such as; oxalates, hexametaphosphate, EOTA, COTA, EGTA, etc., for extracting pectins. Chelating treatment is often combined with heating, and this treatment does not give a real proof for the presence of Ca-bridges, as heating cleaves pectic backbone irrespective of pH.[79] At cold condition and at neutral pH, CDTA removes all the Ca-bridges from the pectins, rendering its solubilization. These types of pectins are found abundantly in fruit, moderately in leafy vegetables and in low levels in cultured tissues[79] and originate from the middle lamella.[266] These pectins are found complexed with calcium ions.[266] Pectins extracted with HCl (pH 1.5), had a wider molecular weight range with a peak molecular weight slightly lower than the similar type of pectins extracted with 0.5% EDTA or 0.25% ammonium oxalate. This suggests that acid might hydrolyse pectins during extraction and EDTA or ammonium oxalate may be preferred for pectin extraction.[181] Cold sodium carbonate (containing sodium borohydride) treatment would cause hydrolysis of inter polymeric ester bonds with negligible β-elimination degradation.[266] It solubilizes the CDTA-insoluble pectins and suggests that inter polymeric ester bonds help to hold pectins in the cell wall.[79] A simple model consisting of five types of basic pectin interactions (S-, A-, B- C- and P-) was proposed based on their extraction behaviour.[42]

Pectins form gels under certain conditions and this property has made them as useful additive in jams, jellies and marmalades, as well as in confectionery industries as stabilizers for acid milk products.[276] They are used in number of foods as thickeners, texturizers, emulsifiers, etc.[262] In recent

years, pectin has been used as a fat or sugar replacer in low-calorie foods.[262] They have a wide application in pharmaceutical industries, mainly because of their activities like antidiarrhea, detoxicant, regulation and protection of gastrointestinal tract, lowering blood cholesterol level, and glucose metabolism.[16 .262, 276] Also, it is the major constituent in fruit cell wall that undergoes drastic degradation by the carbohydrate hydrolases, during ripening, leading to fruit softening.

Changes in pectic polysaccharides during ripening

During ripening fruits loose firmness, and unless the fruit is dehydrated, osmotic properties of the cell and the turgour pressure usually remain constant during ripening. While in plant tissues, it is assumed that turgour pressure alone is not contributing for the loss of firmness, instead it is the result of changes in the cell wall polysaccharides.[273] Much work done to relate chemical changes in cell walls to fruit softening has been focused towards the characterization of changes in pectic substances.[135] Pectins are the lone cell wall polysaccharides that are easily soluble in water and due to this property they can be deesterified and depolymerized mostly by enzymatic reactions. Also, retardation of textural softening by the addition of Ca^{++} ions to fruit is related to the ability of divalent cations to form calcium bridges between the pectic polysaccharide chains.[135] Limited degradation of the pectic polymers might be due to the methylation of galacturonic acid groups or their accessibility for depolymerization.[276]

Loss of firmness during heat treatment of acid fruit has been attributed to acid hydrolysis of glycosidic bonds in cell wall polysaccharides.[63] It was suggested earlier that hydrolysis of neutral sugar glycosidic bonds was involved in softening process.[63] Arabinofuranosyl linkages are most labile in pectins than glycosidic linkages between neutral sugars and between neutral sugar and galacturonans. Glycosidic linkages between galacturonans are most stable.[276] However in acidic pH (pH 2.5-4.5), hydrolysis of galacturonans occurs faster than neutral sugars, as uronic acid was lost from the cell wall, while the neutral sugars were found associated with the pectic substances.[249] Thus, the possible mechanism involved in softening during ripening at acidic condition is the hydrolysis of pectin.[135]

Changes in the proportion and characteristics of pectic substances are reported in many fruits.[123] During ripening, the progressive loss of firmness is the result of a gradual solubilization of protopectin in the cell walls to form pectin and other products.[89, 115.226] Solubilization followed by depolymerization and deesterification of pectic polysaccharides is the most apparent change occurring during ripening of many fruits like pear,[26] apple,[56] tomato,[242] muskmelon,[153, 207] persimmon,[54] Spanish pear,[150] bell pepper,[93, 205] strawberry,[102, 168] kiwifruit,[211, 167] bush butter,[160] apricot,[73] melon,[222] peach,[99] and olive fruit.[114] Pectins from ripe fruit exhibited a lower degree of esterification, a lower average molecular weight and decreased neutral sugar content compared to pectins from unripe fruits.[105]

Among cell wall hydrolases, pectin-degrading enzymes are mostly implicated in fruit softening. Increased solubilization of the pectic substances, progressive loss of tissue firmness and rapid rise in the PG activity accompany normal ripening in many fruits.[32, 76, 104, 195, 268] Since the pectic polymers begin to acquire solubility only after PG has become active, it is believed that this enzyme is involved in the breakdown of the insoluble complex polysaccharides by reducing the length of the chains cross-linked by calcium.[88] A correlation between appearance of PG and initiation of softening is shown in number of fruits like guava,[69] papaya[182] and mango.[221] PG and PME activity increased remarkably in peach, tomato and pear.[269] In apple and strawberry fruit the mechanism of solubilization of polyuronide is thought to be different from peach, pear and tomato, due to the absence of the endo-PG, although exo-PG is present.[276] PG activity was not detected in plum fruit.[29]

In ripening fruits, much attention was focused on the depolymerization of acidic pectins by polygalacturonase. However, experiments with transgenic tomatoes have shown that even though PG is important for the degradation of pectins, it is not the sole determinant of tissue softening during ripening.[86] PG antisense constructs for various tomato lines have little effect on the fruit characteristics, viz, reduced susceptibility to cracking, and decay and other damages at the later stages of ripening.[86] Now the focus is on hydrolysis of neutral sugar side chains, which may weaken the complex network of cell wall polymers thus, contributing to textural softening.[92, 251] The variation in pectins from different sources is mainly attributed to arrangement of these neutral sugars side chains resulting in configurational rearrangements.[134]

The loss of neutral sugar side chains from the pectin is one of the most important features occurring during ripening. Substantial variation in the cell wall composition among fruits and fruit tissues exists. Further, their metabolism in relation to softening also varies from fruit to fruit.[91] Out of [17] types of economically important fruits,[14] types showed a net loss of neutral sugars; galactose and arabinose, from the cell wall during ripening.[91] No such loss of neutral sugars occurs in ripening plum and cucumber fruits.[91] A net loss of neutral sugars during ripening of pear,[4] apple[56] and tomato[92] was reported earlier. The mutant tomato fruit ('rin') containing little or no PG activity showed substantial loss of galactose from the cell wall suggesting that this loss is not due to the action of PG.[92] These evidences suggest that other cell wall hydrolases, especially glycosidases play an important role in textural softening during ripening.[86]

One novel approach to elucidate the role of enzymes in cell wall degradation and softening is to employ antisense RNA technology. This technology was one of the first molecular approaches used for delaying fruit ripening.[18] It has been possible to obtain firmer tomatoes with longer shelf-life by specific suppression of PG gene expression with antisense RNA.[252] Pectin methyl esterase (PME)

suppression resulted in increased solid content in tomato.[267] The genes coding for PG, PME and other enzymes have been cloned in tomato[86] and other fruits.[18]

Enzymes related to pectin dissolution in vivo

Pectolytic enzymes are wide spread in plants, fungi and bacteria. They constitute a unique group of enzymes that are responsible for the degradation of pectin and pectic substances in plant cell walls [Table 7]. They act on plant tissues, especially on the main polyuronide chains of pectins and eventually cause cell lysis. The other enzymes act on the side chains of the galacturonide backbone, eventually degrading the entire pectic substance. These enzymes are arabinanase, galactanase and β-galactosidase.

Pectic enzymes have been used for the clarification of wines since the beginning of 19th century. They are industrially useful enzymes for

Table 7: Classification of pectin-degrading enzymes [10,276,283]

Enzymes	Substrate	Products	Mechanism
Pectin Methyl Esterase	Pectin	Pectic acid + Methanol	Hydrolysis
Polygalacturonases			
Protopectinase	Protopectin	Pectin	Hydrolysis
Endo-PG	Pectic acid	Oligogalacturonates	Hydrolysis
Exo-PG	Pectic acid	Monogalacturonates	Hydrolysis
Oligogalacturonate hydrolase acid	Trigalacturonic	Monogalacturonates	Hydrolysis
△4:5 unsaturated Oligogalacturonate hydrolase	△4:5	Unsaturated	Hydrolysis
Endopolymethyl galacturonase	Pectin	Methyl-Hydrolysis oligogalacturonates	Hydrolysis
Rhamnogalacturonase	Pectin	α-(1 ,2) linked L-Rha, α-(1,4) linked D-Gal	Hydrolysis
Pectin acetyl esterase	Pectins (Smooth region)	Unsaturated oligogalacturonates	Hydrolysis
Lyases			
Endopectate lyases	Pectic acid	Unsaturated oligogalacturonates	Trans elimination
Exopectate lyases	Pectic acid	Unsaturated digalacturonates	Trans elimination

Oligogalacturonate lyases	Unsaturated digalacturonate	Unsaturated onogalacturonates Trans elimination	
Endopectin lyases	Pectin	Unsaturated oligogalacturonates	Trans elimination
Arabinanase			
α-L-Arabino- furanosidase	Arabinans	α-L-Arabinose	Hydrolysis
Endoarabinanase	(1,5)-α-Arabinans	Arabinose and higher oligosaccharides	Hydrolysis
Galactanase			
β-D-Galactanase	Galactans	β-D-Galactose	Hydrolysis

extraction, clarification and liquefaction of fruit juices and wines.[43] They are also used in fabric industry to soak plant fibers and in paper making industry to solve the retention problems by de-clogging the pulps.[226] They hydrolyse the pectic substances and aid in the flocculation of suspended particles and clarification of wines and juices.[43] Recently, immobilized pectic enzymes are gaining importance in this area.[10] PG from fungal source is commercially utilized in fruit juice industries. One of the technically important differences between PG from tomato and fungal source is the inhibition of the latter by some vegetable extracts, which may render them useless in the preparation of vegetable macerates for baby foods. Thus, fruit PGs are gaining importance.

Pectin-degrading enzymes are classified, based on their mode of action on pectin and pectic substances into PG, PME, pectate lyase and pectin lyase (Fig. 13).[10,286,226]

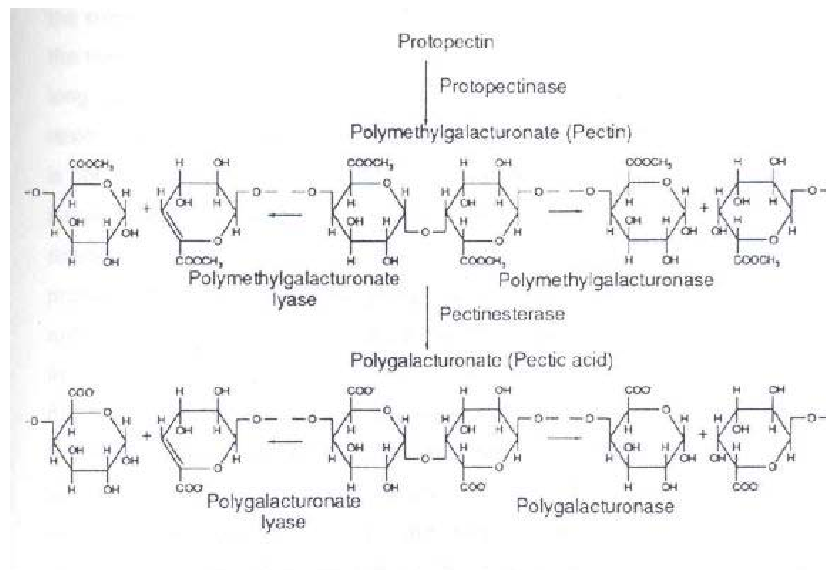


Figure 13: Action of pectic enzymes

Polygalacturonase (PG)

PG, an important pectolytic glycanase, is the primary enzyme playing a significant role in pectin dissolution in vivo. [187] PG is a hydrolytic enzyme, which acts on pectic acid (polygalacturonic acid (PGA)). It hydrolyses the α-1,4-glycosidic bonds between the galacturonic acid residues in galacturonans.

Based on their mode of action, PGs are classified into exo-PG (exopoly (1,4 α-D-galacturonide) galacturonohydrolase, EC 3.2.1.67) and endo-PG (endo-poly (1,4 a-D-galacturonide) glycanohydrolase, EC 3.2.1.15). Exo-PG catalyses the hydrolysis of the glycosidic bonds between the de-esterified galacturonans from the non-reducing end, which results in the release of galacturonic acid as the major reaction product. The rate of hydrolysis depends on degree of polymerization and it increases with increase in molecular size of the substrate.[199] The branching that occurs in the substrate interrupts hydrolysis. Exo-PG action causes a large increase in the formation of reducing groups and a slow decrease in viscosity. From the long polygalacturonan chain mere removal of terminal galacturonic acid residue does not show much effect on pectin solubility.[197] Thus, this enzyme is not involved in ripening, as pectate degradation does not occur. However, some evidences suggest a possible implication of this enzyme in fruit ripening.[115] Recently, exo-PG in tomato was found to elicit ethylene production, which in turn triggers the ripening process.[17] On the other hand, endo-PG depolymerizes pectic acid randomly, resulting in a rapid decrease in viscosity and only endo-PG is involved in ripening process. The rate of hydrolysis decreases with the decrease in the length of the chain. Some fruits like apple, Freestone peach and persimmon posses only exo-PG, while other fruit such as apple, avocado, Clingstone peach, lemon, mango, musk melon, raspberry, kiwi, and tomato contain only endo-PG.[140] , Cucumber, papaya, passion fruit, peach, pear and strawberry contain both endo- and exo-PGs.[140] The marked difference in the textural characteristics two types of peaches (Clingstone and Freestone) is attributed to the difference in PG.[195, 197] The extent and rate of textural softening during ripening is directly related to PG composition, i.e., extensive softening occur if endo- or both endo- and exo-PG are present and limited softening occur if exo-PG is present.[21,102] PG is the major enzyme in tomato, avocado and peach.[94, 195] It is generally accepted that PG is primarily responsible for dissolution of the middle lamella during fruit ripening.[112, 115] There is a clear correlation between the appearance of PG and the onset of dissolution of middle lamella and primary cell wall during ripening.48 PG alone is sufficient to dissolve middle lamella in apple, but both PG and cellulase are required in pear for the same.[25] PG activity was detected in a number of fruits including avocado, peach, pear, pineapple, tomato 185 and even in mango.[221] The presence of PG in fruit during ripening resulted in softening of fruit.[195, 197] One of the most characteristic changes during fruit ripening is decrease in firmness. This has been shown to be associated with the increased activity of the pectic enzymes, particularly PG.[48, 282] An increase in the total PG activity prior to respiratory climacteric stage of tomato suggested that this enzyme might playa role in initiating the ripening process.[187] However, no detectable endo-PG, an enzyme thought to play a role in tomato softening, was found in pre-climacteric tomato and appearance of endo-PG in tomatoes after the onset of

climacteric ethylene was reported. [17,87] The absence of PG in unripe fruits and appearance near the onset of ripening with increased activity during ripening, along with concomitant pectin degradation suggest that this enzyme is implicated in pectin solubilization.[280] The appearance of soluble pectin was the result of the increased activity of PG during ripening.[269] This suggests that fruit softening is regulated by the accumulation of PG and the rate of splitting of pectin. PG acts on the de-esterified portion of the galacturonan chains, particularly on those glycosidic bonds, which have the carboxylic groups adjacent to the glycosidic linkage, and free from esterification.[145]

PG was first found in ripe tomato fruit, and still it remains the richest plant source of the enzyme.[19S, 286] Increased activities of PG during ripening have also been demonstrated in fruits.[5, 15. 58, 195, 204] Recently, increase in PG activity with a peak at climacteric stage in mango,[190], capsicum[205] and banana[189] was reported from our lab. Increase in PG activity in seven Indian mango cultivars during ripening was also reported[234] but have not been purified. In climacteric fruits, whose texture alters considerably during ripening, maximum loss of firmness was directly correlated with rapid increase in PG.[2, 193, 221] Apart from fruits, other plant parts like roots, stem, leaf explants and seedlings are also reported to contain PG.[195] But the PGs may vary as the biochemical/physiological aspects in other tissues or organs differ from those of fruit.

Recently, three isoforms of PG were reported from banana[180] and strawberry.[168] Multiple forms of PG have also been reported for pear[198] and peach.[200] In tomato, PG exists in two forms and both are endo-acting.[201] Both the PGs found in tomato fruit, split glycosidic bonds randomly releasing oligogalacturonides.8 Both enzymes have pH optima in the acidic range and analysis using SDS-PAGE seems to suggest that PG1 is a dimer of PG2.[272] Later studies suggest that PG1 is produced by the combination of both PG2 and a β-subunit (converter).[193, 195] Both PGs are glycosylated. Two PG2 isoenzymes (PG2A and PG2B) have been characterized and are the product of post-translational modification or glycosylation. It was shown that the two PG2 isoenzymes have similar polypeptides, but have differences in the degree of glycosylation.[52] DellaPenna and coworkers[53] demostrated that all the PG isozymes arise by differential processing of a single gene product. The physiologically active form of PG in tomato is PG1, which is enough to carryout both solubilization and depolymerization.[53]

Multiple forms appear due to genetic variants (allelic), genetically independent proteins, or heteropolypeptide chains that are bound non-covalently. However conformational differences, covalent alteration or conjugation may also cause multiplicity of enzymes.[59] The significance of these multiple forms may be related to the complex nature of the pectic substrates and their modification during ripening.[202]

PG gene was the first to be cloned from tomato for studying textural regulation in ripening fruit and the transformed tomato with PG antisense gene resulted in improved fruit with firmer

texture and extended shelf- life[35.252] This gave remarkable clues regarding the role of PG in fruit cell wall metabolism. However, despite similar catalytic properties, PGs differ from fruit to fruit, thus reducing the percent homology of the PG genes. Thus it is necessary to study this enzyme individually in the fruit of choice.

Methods for quantification assay for PG have been well documented.[195] PG activity is generally measured by the increase in reducing equivalents. The usual spectrophotometric methods for quantification of reducing equivalent are arseno-molybdate method,[166] dinitrosalicylic acid method,[158] potassium ferricyanide method,[109] 2,2-bicinchoninate method154 and 2-cyanoacetamide method.[101]

Measurement of viscosity changes using an Oswald viscometer is less convenient for routine measurement but is useful in distinguishing between endo- and exo-splitting PGs. This is by comparing the rate of decrease in viscosity with rate of hydrolysis, as measured by increase in reducing equivalents. An endo-splitting enzyme causes around 50 % reduction in viscosity when only 3-5 % of the glycosidic bonds are cleaved, while an exo-splitting enzyme causes similar reduction in viscosity with as much as 10-15 % of the glycosidic bond cleavage. Other difference between these enzymes is in the nature of product formed, at the beginning of the reaction. The endo-splitting enzyme does not produce low molecular weight products at the beginning of the reaction, whereas, the a splitting enzyme results in low molecular weight products.

Due to the presence of rhamnose in almost all fruit pectins, PG alone is not liable for pectin degradation. It seems that other glycanases, such as rhamnogalacturonase, are also responsible for the degradation of rhamnogalacturonan backbone.

Rhamnogalacturonase (RGase)

Rhamnogalacturonase is an enzyme that catalyses the hydrolysis of glycosidic bonds between galacturonic acid and rhamnose units in RG backbone, the "hairy regions" of many fruit pectins.[47,231] The products are oligomers with alternating galacturonic acid and rhamnose units, rhamnose forming the non-reducing end.[231] RGase activity enhances strongly when the ester groups are de-esterified and the side chains are removed.[231] RGase are hindered by o-acetyl group. Thus, they act along with rhamnogalacturonan acetylesterase, which splits off acetyl groups from the 'hairy regions' of pectin.[276] Recently, the probable presence of RGase was also reported for bush butter fruit.[160]

These cell wall glycanases (PG and RGase) appear to be more active on de-esterified pectins than esterified pectins.[242] Therefore, deesterification is most important and is catalysed by a unique group of enzymes, the pectin methyl esterase.

Pectin methyl esterase (PME)

PME (Pectin pectylhydrolase, EC 3.1.1.11) catalyses the hydrolysis of methyl ester groups, resulting in deesterification, PME is specific for galacturonide esters and its action is to remove methoxyl groups from methylated pectin by nucleophilic attack. This results in the formation of an acyl enzyme intermediate with the release of methanol, followed by deacylation (hydrolysis) to generate the enzyme and a carboxylic acid. PMEs of plant origin exhibit an action pattern that results in the formation carboxylate groups along the pectin chain.[286]

De-esterification of pectin by PME appears to proceed linearly along the chain of the molecule resulting in blocks of free carboxyl groups.[216] It appears that PME preferentially attacks methyl ester bonds of a galacturonate unit next to non-esterified galacturonate unit.[185] Thus, they de-esterify the esterified pectic substances, making them vulnerable for PG action.[145] Its action may be a prerequisite for the action of PG during ripening.

PME activity was detected in fruits like apple, banana, cherry, citrus, grape, papaya, peach, pear, tomato and strawberry.[185] The activity of PME increases as mature green tomatoes pass through different colour stages to become full red. Unripe fruits are rich in PME, while ripe fruits are rich in hydrolase enzymes. Activity of PME was shown to decrease[2, 15,69, 190,221] or increase[7, 234] or remained constant[5], [14] during fruit ripening. PME has been purified and characterized in few ripening fruits[70, 202, 271] Several PME isoenzymes have been identified in tomato.[271] The slow ripening of 'Abu-Samaka' in spite of high PG activity, suggests a key role to PME in controlling fruit softening.[2]

PME acts on commercial methylated pectin (citrus) to liberate carboxyl group and methanol. The activity may be assayed by estimating the released methanol chromatographically.[287] A new continuous spectrophotometric assay has been developed based on the reaction of PME on pectins in the presence of a pH indicator, bromothymol blue. The carboxylic groups produced by hydrolysis of ester groups lower the pH, causing indicator dye to change the colour.[95]

By genetic engineering, it has been shown that PME may not be the sole determinant of softening, and other enzymes may be involved in textural softening. But increase in total soluble solid was a very important and significant finding in ripening tomato as demonstrated from PME suppression by antisense construct.[86. 267]

Lyases

The lyases or trans eliminases cleave the glycosidic bond by trans β-elimination mechanism, i.e., elimination of hydrogen from the C-4 and C-5 position of the aglycone portion of the substrate.[284] It is known that in alkaline medium, pectin undergoes deesterification, accompanied by degradation by β-elimination reaction. Similar splitting of glycosidic bonds also occurs in neutral pH at elevated temperature. These enzymes are absent in fruit but are present only in microorganisms.

Pectate lyases (PL) catalyses the cleavage of de-esterified or esterified galacturonate units by a trans β-elimination of hydrogen from the C-4 and C-5 positions of galacturonic acid. Exo-PL (exo-poly 1,4 α-galacturonide) lyase, EC 4.2.2.9) acts from non-reducing end, whereas endo-PL (endo-poly 1,4 α-D galacturonide) lyase, EC 4.2.2.2) acts randomly on de-esterified galacturonans. Pectin lyase (PNL) (EC 4.2.2.10) catalyzes the cleavage of esterified galacturonate units by trans β-elimination. All PNLs studied so far are endo-enzymes, acting randomly.[286]

Arabinanase and galactanase

Arabinanase are of two types; arabinofuranosidase (EC 3.2.1.55) and endo-arabinanase (EC 3.2.1.99). They are among the enzymes, which reduce the degree of branching and increase the polymer-polymer association.[284] Endo-arabinase hydrolyses linear arabinan in a random fashion producing oligomers of shorter lengths. Arabinofuranosidase degrades branched arabinan to a linear chain by splitting of terminal α-1,3-linked arabinofuranosyl side chains and sequentially breaks the α-1,5 links at the non-reducing end of linear arabinan.

This enzyme hydrolyses the terminal non-reducing arabinofuranosyl groups from various range of arabinose-containing polysaccharides such as α-L-arabinofuranoside, arabinogalactans, arabinans and arabinoxylans.

The substrates most widely used for the assay of arabinofuranosidase are p-nitrophenyl-a-L-arabinofuranoside, phenyl-α-L-arabinofuranoside and β-L-arabinan. The release of L-arabinose is quantitated either by reducing group estimation or by HPLC.

Galactanases are of two types; endo-galactanase (EC 3.2.1.89), which catalyses the random cleavage of the β-1,4 linkages of galactan chains and galactanases (EC 3.2.1.90), which also randomly hydrolyses the β-1 ,3 and β-1,6 linkages of galactans, present as side chains in pectins. β-1,4-Galactanase from Bacillus subtilis degraded the structural components of dicotyledonous primary cell wall.[137] Increase in the activity of arabinanase and galactanase in mango, banana and capsicum was reported.[190, 27, 189, 205] Recently, exo-(1-4)-β-galactanase was purified and characterized from tomato.[38]

β-Galactosidases

It is very well understood by molecular evidence that PG activity alone is not responsible for the degradation of the pectins to the extent that occurs during fruit ripening.[82] Initial softening was not correlated with the increase of PG activity in ripening apples. Further, in ripening inhibitor mutant 'rin' tomato, little or no PG activity detected, but a substantial amount of galactose was lost indicating the involvement of other enzymes.[92] The apparent absence of PG in some fruits that soften normally has implied other alternative mechanisms of cell wall dissolution.[93, 207] These evidences stimulated further research on this glycosidase. This enzyme is also implicated in pectin dissolution by way of eglycosylating the galactan, which is generally present in pectin-type of polymers. Thus, loss of neutral sugars has become a general feature of fruit ripening.[91] This loss of neutral sugar residues is separate and independent of polyuronide solubilization during ripening,[92] and independent of PG activity.[40]These suggest the involvement of β-galactosidase/galactanase, which have been associated with many ripening fruits.[38. 222]

β-Galactosidase (EC 3.2.1.23), a glycosidase, acts on short chain oligomers of galactose units present either as glycoprotein, glycolipid or hetero-/homopolysaccharides. This enzyme partially degrades the pectic and hemicellulosic components of the cell wall and is possibly related to breakdown of polysaccharides at over-ripening. β-Galactosidase was detected in a wide variety of fruit systems. [59] Increase in β-galactosidase activity during ripening was reported in many fruits.[22. 115.279] It was reported that this enzyme also increases during development of fruits like mango.[206] This enzyme has been purified from a number of fruits including tomato,[196] apple,[62. 224] orange,[36] muskmelon,[207] avocado,[54] coffee berry,[83] kiwi,[223] sweet cherry,[12] sapota[66] and 'Harumanis' mango.[9] This enzyme is incapable of degrading native galactans in citrus fruit.[36] However, in some fruits like tomato,[196] muskmelon,[207] apple[224] and kiwifruit,[223] they attack native galactan polymers. In most studies of fruit β-galactosidase, the synthetic substrate, para-nitrophenyl-β-D-galactopyranoside was widely used. The other substrates used for assaying the activity were phenyl-β-D-galactopyranoside, arabinogalactans, galactomannan and lactose.

Scope of the Investigation

Textural softening during ripening is of immense importance as it directly dictates the fruit shelf life and post harvest physiology. Control or modification of fruit texture is the main objective of modern 'Fruit Biotechnology'. During textural softening from unripe to ripe stage (i.e., from 'stony hard' to 'soft spongy' stage), carbohydrates undergo hydrolysis to various extent, resulting in depolymerization and decrease in molecular size of the polymers. It is generally a partial hydrolysis of the high molecular weight carbohydrate polymers. Sometimes, there may be complete hydrolysis or nearly complete hydrolysis as in the case of starch in mango and banana fruit.

In the recent research trends in the area of fruit ripening, it could be pointed out that "Tomato Biotechnology" is fairly advanced where the expression of ACC synthase & EFE at ethylene level, and PG & PME at post ethylene level, were individually suppressed by antisense RNA. Interestingly, all these culminated in a desired end result. Ethylene suppression resulted in overall control of the ripening process, which was triggered by the exogenous ethylene-boost. Genetic manipulation at the textural level resulted specifically in "improved texture" in the transformed tomatoes, where PG and PME suppression yielded firmer fruits and higher solid content, respectively. There may be other equally important carbohydrate hydrolases, which are crucial in fruit texture and textural softening. Since fruits differ in their biochemical make up, it is to be expected that the enzymic targets may also differ from fruit to fruit. Thus, to control the post-harvest life of any fruit by molecular approach a basic understanding of the events occurring during fruit ripening is essential.

This study was taken up to specifically identify the crucial and important hydrolases involved in pectin metabolism, which was studied at both substrate (pectic polysaccharides) as well as enzyme level (pectin-hydrolyzing enzymes). It must be noted that the study here forms the basis for any further investigation at the gene level. Identification of crucial substrates and their corresponding enzymes implied with carbohydrate hydrolysis in vivo is important and useful for providing further leads and scope. The fruit of choice was the commercially important Indian mango, Mangifera indica L. cv. Alphonso.

The aim of this study was to understand the factors contributing to the textural changes in relation to pectin degradation during ripening in mango. The knowledge about the composition, structure and nature of the fruit substrates and enzymes during ripening provides a clear insight into the physical, physiological and biochemical changes involved in the ripening process. This study on events that occur during ripening are essential for investigating systems in which the post-harvest as well as pre-harvest physiology of the fruits can be controlled. It was vital to understand (identify/define) precisely the enzymes that were involved in textural softening of a fruit and the extent of their contribution towards the same. So far, the literature reports on mango fruit focus more on post harvest physiology, where most of them deal with overall biochemical changes during ripening. However, the precise nature of the pectic polysaccharides and the pectic enzymes; their quantitative and qualitative changes during ripening in relation to textural softening have not been studied well especially in Indian mango cv.

The main objectives of the present investigation were as follows:

1). To study the pectin degradation during ripening.

2). To characterize the major pectic polysaccharides from mango pulp.

3). To purify and to study the properties of a glycanase (PG) and a glycosidase (β-galactosidase), and

4). To study their involvement in pectin dissolution in vivo.

From the results it was obvious that the profile of carbohydrate polymers of mango and the changes they underwent, in terms of abundance as well as molecular weight drop along with the activity profile of the related hydrolases during textural softening, and their action on the endogenous substrates gave a direct clue to the involvement of specific enzymic targets in the softening process.

CHAPTER – II
MATERIALS AND METHODS

Instruments and accessories

Analytical balances, Sartorius, Germany; Mettler H20, Zurich, Switzerland.
Beckmann Microzone Electrophoretic Cell, model R-1 01, Beckmann, USA.
Capillary electrophoresis (CE) unit, CE-56 model, Prince Technologies, The Netherlands.
Centrifuges, HermIe Z 320K; Rem; RC8.
Lyophilizer, Virtis Freeze Mobile, model 12 SL.
Flash evaporator, Buchi Rotavapor, model RE111, Switzerland.
Fraction collector, LKB, Bromma, Sweden.
FT -IR spectrometer, Perkin-Elmer Spectrum 2000, USA.
FT -IR pellet maker, Delta press, Tetragon Scientific, USA.
Gas Chromatograph, GC-15A, Shimadzu, Kyoto, Japan., fitted with a flame ionization detector (FID).
GC column, OV-225 (3 % on Chromosorb W (100-120 mesh) packed column (SS column, 5'x 1/8"), Pierce Chemical Co., Rockford, IL, USA.
GC-MS, Shimadzu Mass Spectrometer QP-5000, combined with GC-17 A. Kyoto, Japan.
GC-MS column, SP-2330 capillary column, 30 m x 0.32 mm i.d, 0.02 μ film coating, Supelco, USA.
HPSEC column, E-linear and E-1000 μ-Bondagel, Waters Associates, Milford, USA.
HPSEC unit, Shimadzu HIC-6A equipped with Shimadzu RID-6A refractive index detector, SCL-6A systems controller and C-R 4A Chromatopac integrator units.
Homogeniser, Sorvall Omni-mixer, USA; Johnson mixie, India.
Light Microscope, Leitz Labovert, 520573, Leitz Wetzlar, Germany.
Magnetic Stirrer, Remi, India.
Millipore membrane filter, Millipore Corporation, USA.
NMR, Brucker 400MHz., Germany.
Pharmacia Mini Slab Gel Electrophoresis, model SE250-10A-.75, Hoefer
Pharmacia Biotech Inc., CA, USA.
pH meter, Control Dynamics, Bangalore, India.
Polarimeter, Perkin Elmer, model 243, USA
Sep-pack C18 cartridges, Waters Associates, Milford, USA.
Texture analyzer, Llyod Universal Texture Measurement Instrument, LR5K, Fareham, Hampshire, UK.
UV-Vis double beam Spectrophotometer, Shimadzu, UV-160A, Kyoto, Japan.
Water bath with temperature control, Tempo, Instruments and equipments
(I) Pvt. Ltd., Bombay.

Double distilled water was used throughout the study. Degassed double distilled water was used for chromatographic analyses. Degassed triple distilled water was filtered through Millipore membranes (Type HA, 0.45 jlm) and used for HPSEC, Capillary electrophoresis (CE) and PAGE.

Chemicals

All the chemicals used were of analytical reagent (AR) grade.

Acrylamide, ammonium persulphate, arabinogalactan, bis-acrylamide, Blue dextran (20,00,000 Da), bromophenol blue, bromothymol blue, BSA (bovine serum albumin, 66,000 Da), Citrus pectin, CMC-carbodiimide p-toluenesulphonate), CM-cellulose, cytochrome C (12,300 Da), Coomassie Brilliant Blue (CBB) G-250, CBB R-250, DEAE-cellulose (0.86 meq./ml), deuterium oxide, Dialysis tubing (MWCO 12,000 Da), dinitrosalicylic acid (DNS), galactan, galactomannan, galacturonic acid (GaiA), iodomethane (methyl iodide), Microcrystalline cellulose, metahydroxydiphenyl, molecular weight markers (14-94 kDa), ovalbumin (45,000 Da), phenyl methyl sulfonyl fluoride (PMSF), pNP-substrates, polygalacturonic acid (PGA), polyvinyl pyrrolidone (PVP), polyvinyl polypyrrolidone (PVPP), potassium ferricyanide, ruthenium red, Sepharose CL-4B (fractionation range, 30,000 to 50,00,000 Da), sodium azide, sodium borodeuteride, sodium borohydride, starch, TEMED (N, N, N1, NI-tetramethyl ethylene diamine), triflouroacetic acid (TFA), triphenyl methane and Tris (hydroxymethyl) amino methane were procured from Sigma Chemical Company, St. Louis, MO, USA.

SDS-PAGE Mr markers, Sephadex G-200 and standard dextrans (T-10, T-20, T-40, T-70, T-150, T-500 and T-2000) were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. Sugar standards arabinose, fucose, galactose, glucose, mannose, meso-inositol, rhamnose and xylose were purchased from ICN Pharmaceuticals Inc. Life Science Group, Cleveland, USA. L (+) Cystenium chloride and potassium bromide (spectroscopic grade) were obtained from E-merck, Darmstadt, Germany. Ammonium carbonate, ammonium thiosulphate, boric acid, calcium chloride, calcium hydride, disodium EDT A, potassium hydroxide, sodium acetate, sodium carbonate, sodium chloride, sodium citrate, sodium diphosphate, sodium hydride, sodium hydroxide, sodium monophosphate, silver nitrate, sodium sulfite, sodium tetraborate and sucrose were obtained from Qualigens Fine Chemicals, Mumbai, India; S. D. Fine-chem Limited, Mumbai, India; E-merck (India) Limited, Mumbai, India; Ranbaxy Laboratories Limited, S.A.S. Nagar, Punjab, India; or British Drug House
(India) Pvt. Limited, Bombay, India.

Acetic anhydride, acetonitrile, aniline, chloroform, methanol, phenol, pyridine and toluene (procured from Qualigens, SO Fine chemicals or E-merck) were distilled in an all glass apparatus. Anhydrous ether was distilled over calcium chloride and stored in brown bottles with metallic sodium

wires. Anhydrous dimethyl sulphoxide (DMSO, from Sisco Research Laboratories, Mumbai, India) was distilled over fresh calcium hydride, under reduced pressure. The constant boiling fraction (BP 63°C) was collected and stored with molecular sieves (4A). Acetone was obtained from Qualigens. Absolute alcohol (99%) was procured from Hayman Limited, Witham, England. Acids like acetic acid, formic acid, hydrochloric acid, perchloric acid, and sulphuric acid were from Qualigens or Ranbaxy. Formaldehyde was from Qualigens.

Cellulose acetate membranes were from Beckman Instruments International, SA, Geneva, Switzerland. Crimper, decapitator and vials (used for methylation analysis) were from Pierce Chemical Company, Rockford, Illinois, USA. Enzyme 'Termamyl' was procured from Nova, Denmark.

Plant Material

Mangoes (Mangifera indica L. cv. Alphonso) were freshly harvested from a local farm (around Mysore), washed with tap water and rinsed with double distilled water, wiped and were stored at ambient temperature for normal ripening. Experiments with mango fruit pulp was conducted at various stages of ripening from unripe to ripe stage. Freshly harvested mango fruits were taken immediately to denote unripe stage, while the subsequent stages of ripening were followed from the fruit kept for normal ripening. The 4 stages of ripening (Fig. 14) chosen were as follows (determined subjectively by firmness measurements) –

Stage-I. Mature unripe (raw) -hard, dark green, 0th day.

Stage-II. Pre-climacteric -intermediate, light green, 4th day.

Stage-III. Post-climacteric -intermediate, yellowish green, 8th day.

Stage-IV Ripe- yellow, soft, 12th day

Fig. 14: Mangoes at different stages of ripening (stages I, II, III & IV)

Texture measurements

Texture measurements were conducted on fruits at all the 4 stages of ripening to know the exact differences between the ripening stages. Textural properties were evaluated by three parameters, namely penetration, piercing and compression using a computer interfaced universal

texture analyzer (Llyod Universal Texture Measurement Instrument) equipped with a 100 kg load cell. The loss in firmness was expressed as force (in Newtons) required by the probe for penetration, piercing and compression.[6, 20, 183]

Penetration

A cylindrical 8 mm probe was used to penetrate one side (cheek) of the mango fruit (with or without peel) to a depth of 10 mm with a constant speed of 10 mm / min. The maximum force required by the probe to penetrate the tissue was recorded and expressed in Newtons. One measurement was made per fruit and 6 fruits were tested per treatment and the average values were taken to represent the textural value.

Piercing

A 5 mm probe was used to pierce one side (cheek) of the mango fruit (with peel) to a depth of 10 mm with a constant speed of 10 mm/min. The maximum force required by the probe was recorded and expressed in Newtons. One measurement was made per fruit and 6 fruits were tested per treatment and the average values were taken to represent the textural value.

Compression

Tissue blocks (15 mm x 15 mm x 15 mm) from each cheek of the mango fruit were compressed using a 50 mm circular flat plate with a stroke speed of 100 mm/min. The maximum force required to compress the block by 50% was recorded and expressed in Newtons representing the firmness. Two measurements were made per fruit (blocks from each cheek) and 3 fruits were tested per treatment and the average values were taken to represent the textural value.

Microscopy

Small blocks of fresh unripe and ripe mango fruits were sectioned using a sharp blade into double distilled water and mounted on a slide. The sections were viewed using a light microscope, Leitz Labovert, Germany. Some sections were stained using ruthenium red (0.02% in double distilled water) to observe the dissolution of pectin-rich regions in the cell wall.[251]

I. CARBOHYDRATE ANALYSES

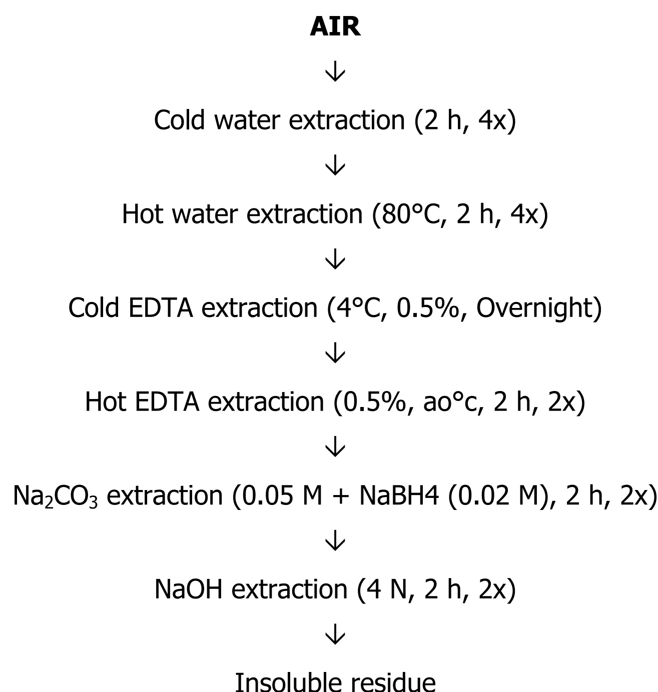
Preparation of alcohol-insoluble residue (AIR)

Unripe (I) and ripe (IV) fruits were selected for separation and purification of pectic polymers. The fruits were peeled and the endocarp (seed) was removed. The pulp tissue was sliced immediately

into 3 volumes of 95% ethanol to arrest the endogenous enzymatic hydrolysis and homogenized using a mixie. The resulting slurry was kept at 60°C for 40 minutes to arrest endogenous enzyme activity and to facilitate protein coagulation.[42, 222] The slurry was kept at RT for cooling and then filtered through two layers of cotton cloth. The residue was subsequently washed with 80 % ethanol (until the filtrate is sugar-free), methanol/chloroform (1:1,v/v) , and finally with petroleum ether. The left over insoluble material was air-dried overnight at RT to obtain alcohol insoluble residue (AIR). The yields obtained were recorded and this AIR was used for carbohydrate extraction. The ethanol extracts (filtrate) were combined, concentrated and finally washed with petroleum ether. The concentrated extracts were used for estimating soluble total sugar and uronic acid contents.

Fractionation of AIR for extracting pectins

The AIR from unripe (I) and ripe (IV) mango was fractionated based on their differential solubility into 8 different fractions. The fractionation procedure[222, 227] adapted here was slightly modified. Extraction of pectins from AIR of unripe and ripe fruits was carried out separately. The complete fractionation step is illustrated in flow chart 1.



Flow chart 1 : Flow chart for the sequential extraction of carbohydrates based on differential solubility

AIR was suspended in distilled water separately, stirred vigorously using a magnetic stirrer, for 2 h at room temperature. The slurry was filtered through 4 layers of nylon cloth. The residue was resuspended in distilled water and repeated the extraction (thrice). The filtrates of cold water solubles (CWS) were combined together, dialysed against distilled water and kept for alcohol precipitation or

for lyophilization. Fresh dialysis tubings were washed accordingly to the instruction manual and used for dialysis. Cold water insoluble residue (CWIR) was suspended in hot water, stirred vigorously for 2 h at 80°C on a water bath and filtered through 4 layers of nylon cloth. Termamyl was used during extraction, to solubilize starch. The above extraction with hot water was repeated (thrice) and the filtrates of hot water solubles (HWS) were pooled together, dialysed against distilled water and kept for alcohol precipitation or lyophilization.

The leftover hot water-insoluble residue (HWIR) was used for chelator-soluble pectin extraction. The residue was suspended in 0.5% Na2-EDTA (pH 4.8) and stirred vigorously at 4°C, overnight (8 h) and the resulting slurry was filtered through 4 layers of nylon cloth. The residue (CEIR) was resuspended in 0.5% Na2-EDTA (pH 4.8) and stirred vigorously at 80°C for 2 h and filtered as above. The residue was re-extracted again with same solution for 2 h at 80°C, and filtered. Aliquots from the filtrates from cold and hot Na2-EDT A extractions were taken separately for estimations and combined thereafter as EDT A solubles, exhaustively dialysed against running tap water for 2 days and against distilled water at 4°C for 2 days with several changes, and kept for precipitation or for lyophilization.[162] The hot EDT A-insoluble residue (HEIR) was suspended in 50 mM Na_2CO_3 containing 20 mM $NaBH_4$, stirred vigorously for 2 h at 4°C to extract alkali soluble pectins. The slurry was filtered as earlier, and the extraction of the residue was repeated again for 2 h. The filtrates were combined, dialysed extensively against distilled water at 4°C for 2 days with several changes, and kept for precipitation or for lyophilization. The left over residue was used for extraction of hemicelluloses (Hemicelluloses A and B) with 4 N NaOH, for 2 h (twice) under N2 atmosphere and filtered through nylon cloth. The filtrate was kept cold and dilute (50%) acetic acid was added to adjust the pH to 4.5, and the precipitated hemicellulose A was centrifuged out. To the clear supernatant, ethanol was added (3 vol.) and the precipitate (hemicellulose B) was collected by centrifugation. The alkali insoluble residue was washed with water/alcohol and dried by solvent exchange method.

Ethanol precipitation of extracted polysaccharides

To each of the above dialyzed extracts of polysaccharides, 3 volumes of cold ethanol was added with vigorous stirring to incipient turbidity (80%). The solution was kept overnight at 4°C to facilitate aggregation of the precipitate. It was centrifuged, washed with 80% ethanol and then filtered through nylon cloth, solubilized in small volume of distilled water and lyophilized. Fractionation of pectic polysaccharides on DEAE-cellulose (lEG) DEAE-cellulose (anion exchanger, fine mesh, exchange capacity 0.86 meq./g, Sigma) was washed with distilled water to remove fines and regenerated with 0.5 N HCl and 0.5 N NaOH with successive washings with distilled water. The column was then equilibrated by washing with 0.5 M $(NH_4)_2CO_3$ solution (3 bed vol.) and excess carbonate was removed with distilled water and equilibrated with buffer (0.02 M acetate buffer, pH

4.8). The aqueous solution (0.5 g/ 40 ml of 0.02 M acetate buffer, pH 4.8) of the combined pectic fractions (cold and hot EDTA soluble) was loaded on top of the DEAE-cellulose (3.2 x 10 cm, 80 ml) column bed and eluted with 0.02M acetate buffer (pH 4.8) and successively with stepwise increasing gradients of $(NH_4)2CO3$ (0.05 M, 0.1 M, 0.15 M, 0.3 M & 0.45 M) and NaOH (0.15 M, 0.3 M & 0.45 M).245 The flow rate was maintained at 50 ml/h. Fractions (8 ml) were collected using fraction collector and monitored both for total sugar and galacturonic acid contents by phenol-sulphuric acid and metahydroxydiphenyl methods, respectively. The peak fractions were pooled appropriately, dialyzed exhaustively against distilled water, concentrated by flash evaporation and lyophilized.

Preparative Gel Permeation Chromatography (GPC)

Sepharose CL-4B was swollen in distilled water overnight, washed with double distilled water (thrice) to remove any fine particles. The slurry was then packed into a column (1.4 x 100 cm, 154 ml) at a flow rate of 20 ml/h. The column was equilibrated by washing with 0.05 M acetate buffer (pH 4.8). Pectic polysaccharides (25-30 mg in 4 ml of 0.05 M acetate buffer, pH 4.8) were loaded on top of the column bed and eluted with 0.05 M acetate buffer (pH 4.8) at a flow rate of 15 ml/h. Fractions (4 ml) were collected and monitored for total sugar and galacturonic acid. The peak fractions were pooled appropriately, dialyzed and lyophilized.

Analytical GPC

To determine the relative molecular weight (M_r) of the crude and GPC purified pectic polysaccharide fractions, Sepharose CL-4B column (1.4 x 100 cm, 154 ml) was calibrated with standard dextrans of known molecular weight (T-10, T-40, T-70, T-150, T-500 and T-2000). The void volume (Va) was determined using pre-dialysed Blue dextran (20,00,000 Da). Pectic polysaccharides (5 mg in 1 ml of 0.05 M acetate buffer, pH 4.8) were loaded on top of the column and eluted with 0.05 M acetate buffer (pH 4.8) at a flow rate of 15 ml/h.222, 240 Fractions (2 ml) were collected and the elution volume (V_e) was determined by monitoring the total sugar content. The relative molecular weight of the fraction was obtained from the standard plot of V_e/V_o vs log molecular weight.

High Performance Size Exclusion Chromatography (HPSEC)

Molecular weights of all the pectic fractions were also determined by HPSEC,[77] using the columns E-linear and E-1000. The flow rate was 1aintained at 0.6 ml / min and temperature at 40°C. 5-1 0 μl of 0.2 % queous solution of purified pectic fractions were injected. The eluate was automatically monitored by RID and the M_r of the sample was determined standard dextran markers (T -10, T -20, T -40, T-70, T-500 and T-2000). The relative molecular weight of the fractions was obtained from the standard plot of retention time vs log molecular weight of dextran markers.

ANALYTICAL METHODS

Acid hydrolysis

Crude and purified pectic polymers (1-3 mg) were dispersed in 72% sulphuric acid (0.5 ml) in an ice-water bath and left for 3 h at 20°C for hydrolysis.[238] The hydrolysate was diluted to 3 ml with distilled water, and refluxed for 10-12 h to effect complete hydrolysis, and filtered through Millipore membrane filter and known aliquots taken for both total sugar and uronic acid estimations.

Total Sugar (TS)

Total sugar estimation was done using phenol-sulphuric acid method[68] with slight modification. To 0.5 ml of the sample (5-50 I1g), 0.3 ml of 5% aqueous phenol was added, and mixed by vortexing. Added 1.8 ml of conc. H_2SO_4 directly to the reaction mixture and mixed. The tubes were cooled to RT and absorbance at 480 nm was read against reagent blank. A calibration curve was prepared using glucose or galacturonic acid as standard, in the concentration range 5-50 µg.

Galacturonic acid

Galacturonic acid (GalA) was estimated using metahydroxydiphenyl method[28] as described by Kintner and Van Buren.[126] The sample (0.2 ml) was allowed to cool in an ice bath for 10 min. To this, 1.2 ml of conc. H_2SO_4 containing 0.0125 M $Na_2B_4O_7$, was added and vortexed vigorously. The tubes were heated in a boiling water bath precisely for 5 min, and cooled immediately in an ice-cooled water bath. 20 µl of 0.15 % metahydroxydiphenyl (prepared in 0.5 % NaOH) was added, vortexed vigorously and left at RT for full colour development. After 20 min, the absorbance was read at 520 nm using reagent blank. A calibration curve using galacturonic acid (0-1 00 µg / ml) as standard was prepared similarly (Fig.15).

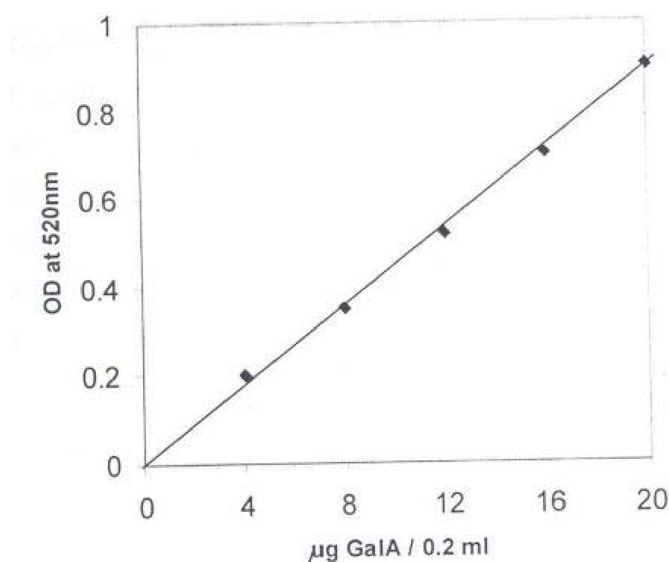


Fig. 15: Standard curve for galacturonic acid estimation using metahydroxydiphenyl

Neutral sugar composition by GC

The individual neutral sugar composition of all the fractions was determined by TFA hydrolysis of the pectic fraction,[238] followed by derivatization and analyzed as their alditol acetates as described by Sawardeker and coworkers.[230, 291] 1-2 mg of pectic sample was dispersed in 0.5 ml of double distilled water in a test tube. 0.5 ml of 4 N TFA was added to the tube, sealed and kept at 121°C for 4 h. TFA was removed by flash evaporation, washed with double distilled water (2 ml x 5) to remove completely any traces of TFA (as indicated by pH paper) and dissolved in 1 ml of water. $NaBH_4$ (10 mg) or $NaBD_4$ (sodium borodeuteride, in case of methylated samples) was added, tubes were stoppered and left overnight (8-10 h) at RT for reduction. The excess borohydride was destroyed by adding dilute acetic acid (2 M) dropwise till the effervescence of hydrogen stopped. The boric acid formed was removed by co-distillation with methanol (2 ml x 5) and desiccated. To the dried residue, 0.5 ml each of distilled acetic anhydride and pyridine was added, tubes sealed, and kept at 100°C for 2 h for acetylation. The excess acetylating reagent was removed by flash evaporation, and traces were removed by washing with both water and toluene (2 ml x 4). The alditol acetates were extracted with chloroform, filtered through glass wool and dried by flushing N_2 gas. The derivatives were taken in known volume of chloroform and injected to GC for qualitative and quantitative analyses.

A Gas chromatograph GC-15A, 8himadzu, fitted with a flame ionization detector (FID) and an OV-225 (3 % on Chromosorb W (100-120 mesh) packed column (88 column, 5'x 1/8") was used to analyse and quantify the alditol acetates. The column was maintained at an isothermic temperature of 185°C, with injection and detection port temperatures, 250°C. Nitrogen was used as carrier gas at

a flow rate of 40 mil min. 1-2 µl of the extracted alditol acetates were injected and run for 60 min. The retention time of alditol acetates was compared with that of standards, and the neutral sugars were identified (Fig. 16).

CRITERIA OF PURITY

Cellulose acetate membrane electrophoresis

Cellulose acetate membrane was moistened with the electrophoretic running buffer; ammonium carbonate: NaCl buffer (0.05 M, pH 9.3). Excess buffer was removed by blotting between the blotter papers, and the membrane was inserted into the Beckmann Microzone Electrophoretic Cell. Pectic sample solution (1 %) was spotted on the membrane with the help of

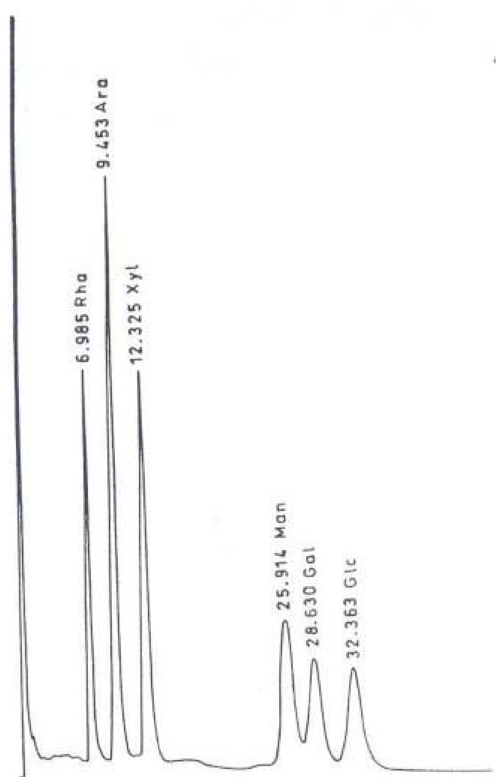


Fig. 16: GC profile of standard alditol acetates

a Beckmann microzone sample applicator. Electrophoresis was performed 3t 150 V and 15 mA current for 45 min. The air dried electrophoresed nembrane was dipped in an aqueous solution of ruthenium red (0.1 %), and Nashed successively with water and dilute acetic acid, until the background Nas clear. Pectic polysaccharides appeared as pink coloured bands.[124]

Capillary electrophoresis

Capillary electrophoresis (CE) was performed using capillary ectrophoresis unit, CE-56 fitted with a silica column (75 μ id x 100 cm ength). The column was regenerated with 0.1 M NaOH and equilibrated Nith 0.02 M borate buffer (pH 9.3) before running the sample. Sample solution (0.5 ml, 1-2 % pectic fractions) was taken in a sample holder and run using 0.02 M borate buffer (pH 9.3) at 8 mA current for 5-10 min. PGA (Sigma) was used as standard to compare the migration time of the purified sample.[228]

STRUCTURAL STUDIES

Specific (Optical) rotation

Specific rotation of the pectin samples (0.6 ml, 0.25% in distilled water) was determined using a Perkin Elmer, Model 243 polarimeter, using sodium D line lamp at a wavelength of 589 nm.[18S,186] The optical rotation was measured by the shift in plane polarized light, and specific rotation $[\propto]_D$ at 20°C (c, 0.25 in water) was calculated using the formula,

$$[\propto]_D = \theta \times 100 / c \times l$$

where, θ = angle of rotation of plane polarized light

c = concentration of the sample (in %)

l = path length (in dm)

Carboxyl-reduction of pectic fraction (Fr. II)

The reduction of the galacturonic acid of the purified pectin was done as described by Taylor and Conrad[261] 25-30 mg of pectic sample was dissolved in 6 ml distilled water, and pH adjusted to 4.75 using 0.1 N HCl. 250 mg CMC-carbodiimide (Sigma) was slowly added over a period of 4h, maintaining the pH at 4.75 by the addition of 0.1 N HCl. The reduction was carried out by dropwise addition of NaBH4 solution (250 mg in 3 ml), over a period of 2 h, during which the pH was maintained at 7.0, using 4 N HCl. A drop of 1-octanol was added as an anti-foaming agent to prevent foaming during the additions. After reduction, the pH was reduced to 4.5, and dialysed extensively, lyophilized and stored for further experimentation. This reduction was repeated 4 times to obtain a sample containing <6% GalA.

Methylation analysis

Pectic fractions (Frs. I & II) were methylated using the method of Hakomori[96, 113] Briefly, the methyl sulfinyl carbanion (MSC) was prepated by treating dry DMSO (5 ml) with dry sodium hydrides (500 mg, which was washed with dry ether (x3) and dried using N_2 gas) at 60°C for 4 h. The hydrogen liberated was removed intermittently and the resulting greenish yellow solution was tested for its characteristic blood red colour with triphenyl methane. [291]

The prepared MSC was added dropwise (using a syringe) to pectic fractions (3-5 mg) dissolved in dry DMSO (1 ml), in a sealed reaction vial and kept stirring for 4 h at RT. To this was added, methyl iodide (1 ml) in ice-cold condition and left stirring for 7 h. The reaction product was diluted with water (1:1) and applied to activated Sep-pack C 18 cartridges. [298] Activaation was carried out by flushing the cartridges with 40 ml ethanol, 2 ml acetonitrile followed by 4 ml water, 15% (3: 17). And 20% (1:4) aqueous acetonitrile, dry acetonitrile, methanol and finally with ethanol, at a flow rate of 1-2 drops / s. The presence of methylated polysaccharides in fraction was tested on TLC strips by charring with 5% sulphuric acid in methanol (v/v). The fractions eluted in dry acetonitrile and methanol showed positive for this test. These fractions were plled, dried and washed with water (2 ml x 4), flash evaporated to dryness and desiccated. To the desiccated material, 2 ml of aqueous formic acid (90%) was added and hydrolysed at 100°C for 2 h. Further, after removing formic acid by washing with methanol (2 ml x 4), the dried residue was subjected to 2 N TFA hydrolysis, at 100°C for 4 h in a sealed tube. After evaporation of excess TFA, traces of TFA ws removed by washing with methanol (2 ml x 4), the dried residue was subjected to 2 N TFA hydrolysis, at 100°C for 4 h in a sealed tube. After evaporation of excess TFA, traces of TFA was removed by washing with methanol (2 ml x 4) and D_2O (0.5 ml) and flash evaporated to dryness. To the washed residue, again D_2O (0.5ml) was added and reduced with 10 mg $NaBD_4$, overnight and converted to their corresponding alditol acetates as described earlier (p61).

The permethylated alditol (^2H) acetates were analysed by GC and combined GC-Ms.[291] GC was run as described above. GC-MS was performed on a high performance quadrupole Shimadzu mass spectrometer QP-5000 combined with GC-17A, fitted with a SP-2330 capillary column (30 m x 0.32 mm i.d, 0.02 ~ film coating, Supelco, USA) at a programmed column temperature of 180-200°C, with a 4°C raise per min. The mass range was between 40-400 amu (mil) for the analysis, operating at an ionization potential of 70 eV. Solvent used was chloroform and the carrier gas was He (helium).[113]

FT -IR study

The polysaccharide samples (6mg each) were incorporated into crystalline KBr (spectroscopic grade), by grinding and pressed into a 1 mm pellet, using a Delta press.46 The pellet was mounted on the FT -IR window and the spectra were recorded in the absorbance mode at a resolution of $4cm^{-1}$ with the wave number range between 400-4000 cm^{-1}, using a Perkin-Elmer Spectrum 2000 FT -IR spectrometer. Fourier transform infrared spectroscopy (FT -IR) was performed for all the major purified pectic fractions.

^{13}C NMR study

^{13}C NMR spectrum was recorded with a Brucker AMX-400 spectrometer, operating in the FT mode. Purified pectic fraction (Fr. II, 25 mg) was dissolved in 0.6 ml 020 and was injected into 5 mm tube (probe) and the spectra were obtained at a probe temperature of 60°C.[203] The ^{13}C resonance was used as a field frequency lock and the shifts were referenced to external TMS. 100 MHz 13C NMR spectra were recorded. The acquisition time was 0.68 s with a 2 s delay between each scan and more than 10,000 scans were accumulated for a total acquisition time of 4-5 h.

II. ENZYME ANALYSES

Acetone insoluble powder (AlP) preparation from mango pulp

Mango, at 4 different stages of ripening as described earlier (p. 53 were peeled and grated with 0.2 M sodium phosphate buffer (pH 7.0) usin a stainless steel grater. While grating, the pulp tissue was adjusted to pH 7. by drop wise addition of 6 N NaOH. The pulp tissue was then poured immediately into homogenizer containing chilled acetone (-18°C) and blended for 1 min. The homogenate was filtered through cotton cloth and the residue was extracted similarly (twice), air-dried and stored in freezer for further use.[41]

Enzyme extraction

AIP of mango pulp, at different stages I, II, III and IV, was used to extract various pectic enzymes, using different buffer systems. Extraction of PG was performed using 0.5M NaCl buffer (pH 6.0). PME was extracted using 1.5M NaCI buffer (pH 7.5). Galactanase/arabinanase were extracted using the buffer system, 0.1 M sodium acetate (pH 5.0), while β-galactosidase was extracted with 0.25 M phosphate buffer (pH 6.6).[205] The ratio of buffer to AI P was 1: 1 O. The extraction was performed at 4°C for 12 h with intermittent stirring. After extraction, the resultant slurry was filtered through nylon cloth and clarified by centrifugation at 3500 g for 15 min. The supernatant was termed as crude enzyme extract and were dialysed against 1 L of 10 mM sodium acetate buffer (pH 4.8) at 4°C with three-four changes over a period of 24 h and used for assaying enzyme activities.

Assay for different pectic enzymes

PG activity was assayed by measuring the formation of reducing sugar by DNS method. The reaction mixture contained 250 µl of 0.1 M acetate buffer (pH 3.8), 125 µl of 0.25% polygalacturonic (PGA, sigma) adjusted to pH 3.8 with 1 N NaOH and 125 µl of suitably diluted enzyme. The reaction was initiated by adding the enzyme and incubated at 37°C for 2 h. The reaction was terminated by immersing the reaction tubes in boiling water for 3 min and estimated the reducing sugar by DNS method, as mentioned below. One enzyme unit (U) is defined as Ilmol galacturonic acid equivalent released in 1 h under the conditions of the enzyme assay.

Activities of galactanase and arabinanase were assayed similarly as above using respective substrates; galactan and arabinogalactan, by measuring the amount of reducing group liberated by DNS method. One enzyme unit is defined as µmol reducing group released in 1 h.

PME activity was measured by decrease in pH upon demethylation of citrus pectin, as determined by the decrease in absorbance at 620 nm.95 To 2 ml of 0.5% citrus pectin, 150 III of bromothymol blue (8T8) and 850 III of distilled water were added, mixed well and the pH was adjusted to 7.5, such that the absorbance of this solution at 620 nm was approximately equal to 0.28. The reaction was started by adding appropriately diluted enzyme (100 µl), and the rate of decrease in absorbance at 620 nm was recorded, every minute. One unit was defined as µmol of acid released in 1 min.

β-Galactosidase activity was assayed by following the release of paranitrophenol (pNP) from pNP-β-D-galactopyranoside (pNPG, Sigma). The reaction mixture consisted of 0.1 M acetate buffer (pH 3.8, 100 Ill), 13 mM pNPG (50µ) and suitably diluted enzyme extract (50 µl). After 15 min at 37°C, the reaction was terminated by adding 1 ml of 0.25 M Na_2CO_3, and the liberated paranitrophenol was measured at 420 nm. One enzyme unit is defined as Ilmol pNP released in 15 min.

Determination of reducing sugar using DNS method

For estimating the reducing group released from PGA, galactan or arabinogalactan, 3,5-dinitrosalicylic acid (DNS) method158 was employed. The DNS reagent was prepared by dissolving 1 g of DNS and 30 g of sodium potassium tartrate in 0.4 N NaOH (100 ml). DNS reagent was stored in brown bottles.

To 1 ml of standard sugar solution (100-1000 µg / ml), 1 ml of DNS reagent was added and heated in a boiling water bath for 20 min. Reducing sugar was determined by taking absorbance at 540 nm, after cooling to RT.[158] A standard graph was plotted and reducing sugar concentration of samples was estimated using the standard slope value. Galacturonic acid or galactose was used as standard.

Determination of reducing group using Potassium ferricyanide method

For estimating the reducing group released from the chromatographic and the purified fractions, a more sensitive alkaline potassium ferricyanide method[109] was used, as McFeeters[154] reported the occurrence of variations in the results obtained by Nelson-Somogyi methods. The

reagent was prepared by dissolving 500 mg of potassium ferricyanide in 1 L of 0.5 M Na_2CO_3, filtered and stored in brown bottle.

To 750 µl of galacturonic acid solution (0-100 µg/ml), 1 ml reagent was added and heated in boiling water bath for 15 min in a test tube stoppered by aluminium foil. After cooling to RT, the reducing sugar was measured at 420 nm. A standard graph was plotted and galacturonic acid equivalent reducing sugar concentration of samples was estimated using the standard slope value (Fig. 17).

Determination of paranitrophenol (ρNP)

To 100 µl of standard pNP solution (0-1 µg / ml), 1 ml of 0.25 M Na2CO3 was added and the yellow colour was measured at 420 nm, using a reagent blank containing water and 0.25 M Na_2CO_3. A standard graph was plotted and the released paranitrophenol of the assay mixture was estimated using the standard slope value.

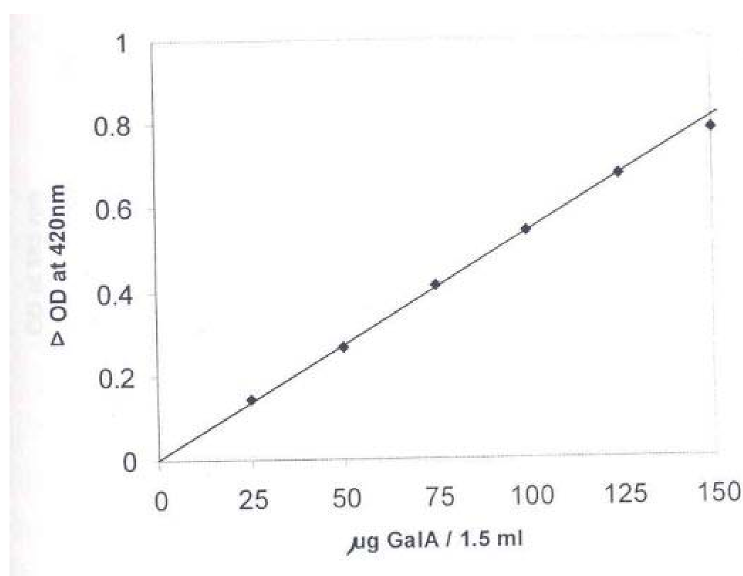


Fig. 17: Standard curve of reducing group estimation using potassium ferricyanide (△OD = Difference OD)

Determination of protein

Protein was estimated by modified Bradford's method[31] as described recently by lor and Selinger[95] using BSA as standard. The reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol. To this, 100 ml of 85% (w/v) phosphoric acid was added and

diluted to 1 L with double distilled water, and stored in brown bottle at 4°C, protected from light. 200 μl of standard protein solution (0-20 μg BSA/ml) or suitably diluted sample was mixed with 800 μl of the above reagent and the absorbance was read at 595 nm in a Shimadzu UV-Vis spectrophotometer against a reagent blank containing double distilled water, after 10 min. A standard graph was plotted and protein concentration of samples was estimated using the standard slope value (Fig. 18).

For chromatographic fractions, absorbance at 280 nm in the UV range was used to estimate the protein concentration.

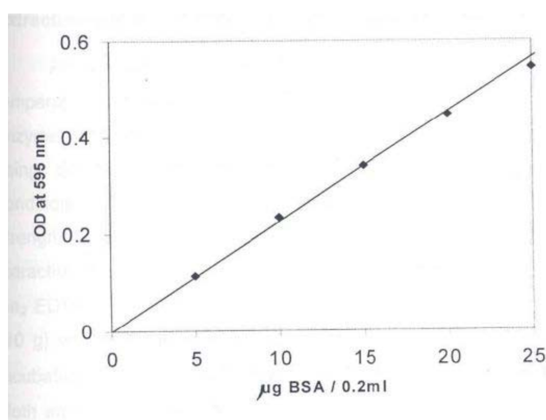


Fig. 18: Standard curve of protein estimation using modified Bradford's method

GPC of enzyme treated EDT A-soluble pectic fraction (crude) from unripe mango

EDTA-soluble pectic polysaccharides (5 mg each) were dissolved in 1 ml of 0.05M acetate buffer of pH 3.6/ pH 5.6. To this, added 500 μl crude enzyme extract (obtained from stage-III mango AlP, using citrate: NaCl buffer (see below) and were incubated at 42°C for 24 h. Aliquots were drawn to estimate reducing sugar released. After 24h of incubation, the tubes were heated at 100°C for 5 min, to arrest the reaction. Each enzyme-treated pectic fraction (at pH 3.6 and pH 5.6) was directly loaded on top of the column bed and eluted with 0.05 M acetate buffer (pH 4.8) at a flow rate of 15 ml/h. Fractions (2.4 ml) were collected and monitored for total sugar and galacturonic acid contents.

Extraction and purification of PG and β-galactosidase from mango

All extraction and purification steps were performed at 10 :emperature not exceeding 5°C. Mango, at stage-III of ripening was used for enzyme purification. Enzyme extracts were prepared from AlP. Experiments using different extraction buffers were done to optimize extraction conditions. Sodium citrate buffer (0.1 M; pH 4.9) containing high ionic strength salt (1.3 M NaCl) was found to be the best and used for enzyme extraction. The extraction buffer (citrate: NaCl buffer) also contained 13 mM Na_2 EOTA, 1% PVPP, 0.1% PMSF and 0.1% cystenium hydrochloride. AIP (10 g) was

homogenized with the extraction buffer (1:10) in a mixer and incubated overnight at 4°C. The resultant slurry was filtered through nylor cloth and clarified by centrifugation at 3500 g for 15 min. The supernatant was termed as crude enzyme extract and used for further purification.

Concentration of enzyme extract

Experiments with different concentration methods were tried and sucrose concentration was found to be most effective in concentrating PG and β-galactosidase enzyme extract, with little loss in activity.

The dialysis bags were kept immersed in saturated sucrose solution, to get a concentrated enzyme extract.[180] The bags were then dialysed against 1 L of 10 mM sodium acetate buffer (pH 4.8) at 4°C with three-four changes over a period of 24 h, to remove sucrose. Suitably concentrated enzyme extract was used for purification.

Ion exchange chromatography

IEC was performed using DEAE-cellulose. The ion exchanger was soaked overnight at RT in 10 mM sodium phosphate buffer (pH 5.8). The swollen gel was packed into a glass column (3.2 x 12.5 cm, 100 ml) at a flow rate of 1 ml / min and washed with double distilled water.

Suitably concentrated enzyme extract was loaded on to the column and eluted with 20 mM sodium acetate buffer (pH 4.8, 200 ml) and with buffer containing linear gradient of NaCI (0-1 M, 400 ml). Fractions (4 ml) were collected using fraction collector at a flow rate of 50 ml/h. Protein concentration was monitored in the fractions by reading absorbance (00) at 280 nm. Enzyme activity of the fractions was assayed and active fractions were pooled appropriately. The activity of pooled fractions was assayed to determine the fold purification and recovery. The pooled fractions were designated as I, II and III according to their order of elution from the IEC column. The fractions were dialysed extensively against 10 mM sodium acetate buffer (pH 4.8), with three changes over a period of 12 h and concentrated using sucrose. Fraction I was further purified using CM-cellulose (3.2 x 7 cm, 56 ml) and eluted with 20 mM sodium acetate buffer (pH 4.8), and with increasing gradients of NaCl. Fractions II and III were rechromatographed on OEAE-cellulose before GPC.

Gel permeation chromatography

GPC was performed using Sephadex G-200. The gel was swollen in 50 mM sodium acetate buffer (pH 4.8) and was packed into a narrow glass column (1.2 x 85 cm, 96 ml) at a flow rate of 12 mil/h. The void volume was determined using pre-dialysed blue dextran, (20,00,000 Da, sigma), and calibrated with protein Mr markers; BSA (66,000 Da), ovalbumin (45,000 Da) cytochrome C (12,300

Da) and purified tomato a-O-mannosidase-11 (75,000 Da).[259] The standard curve was obtained by plotting VeNa VS log molecular weight (V_e= elution volume of protein; V_a= void volume).

Suitably concentrated post IEC fraction was applied to the column, separately and eluted with 50 mM sodium acetate buffer containing 50 mM NaCI (pH 4.8). Fractions (2.5 ml) were collected using fraction collector. The protein and enzyme activity of each fraction was determined as described arlier. The Mr of the purified enzyme was obtained from the standard curve.

The active fractions were pooled, concentrated and estimated the protein and enzyme activity to determine the fold purification and recovery. The remaining purified enzymes were dispensed (500 µl) in eppendorfs and stored in freezer for further studies.

Electrophoresis

SDS-PAGE was carried out using discontinuous buffer system as described by Laemmli.[139] The polyacrylamide gel (10% T) containing 0.1 % SDS was casted in 1.5 mm slab gel apparatus. The electrophoresis was carried out at 100 V in 0.025 M Tris : 0.3 M glycine buffer, pH 8.3, containing 0.1 % SDS as electrode buffer. The protein was mixed with the sample buffer, pH 6.8 containing 10% (v/v) glycerol, 2% (w/v) SOS and 0.1% bromophenol blue. Samples were heated in a boiling water bath for 5 min and subjected to electrophoresis. The SOS-PAGE molecular weight markers were also treated similarly and electrophoresed. After electrophoresis, the gels were stained using silver nitrate method, as described below.

Silver staining [188]

All the steps were carried out with mild agitation (40 rpm). The gel was transferred to fixer (30% methanol and 11 % acetic acid) and allowed to stand for 1 h/overnight. The gel was then washed with 50% methanol for exactly 10 min, with 3 changes. The methanol was drained and sodium thiosulphate solution (40 mg / 100 ml) was added immediately and allowed to stand for exactly 1 min, after which the solution was again drained. The gel was thoroughly rinsed with triple distilled water, precisely for 5 min with three intermittent changes. Freshly prepared silver nitrate solution (100 mg AgNO3 in 50 ml water containing 25 III formaldehyde) was added immediately and allowed to stand for 15 min, with mild agitation. The gel was rinsed with water for 10 min, with three changes intermittently. After complete draining, developer (3 g sodium carbonate, 20 µl formaldehyde and 200 µl sodium thiosulphate solution (prepared as above) in 50 ml water) was added, and hand-shaken till bands appeared. The gel was immediately washed and transferred to fixer to stop the reaction and were stored in 5-7% acetic acid until photographed.

PG and β-Galactosidase assay

Suitably diluted chromatographic or purified enzyme solution (125 µl) was assayed for PG as described earlier (p. 67), and estimated the reducing groups by alkaline potassium ferricyanide method (p. 69). Suitably diluted chromatographic or purified enzyme solution (50 µl) was assayed for β-galactosidase activity as described earlier (p. 68).

Enzyme properties

Effect of pH on activity and stability

The effect of pH on the activity of purified enzymes (isoforms) was examined over a wide range of pH in 100 mM of each buffer (KCI: HCI buffer, 1.2 -2.0; Glycine: HCI buffer, 2.2 -3.4; acetate buffer, 3.6 -5.4; phosphate buffer, 5.8 -8.0; Glycine: NaOH, 8.6-10.0). Suitably diluted purified enzyme was added to the reaction mixture consisting of buffer at defined pH and the enzyme activity at 37°C was determined. The effect of pH on the stability of enzyme isoforms was determined by incubating the enzyme in 100 mM of buffer at different indicated pH (as mentioned above) for 24 h at 4°C. The enzyme activity was determined after adjusting to optimal pH.

Effect of temperature on activity and stability

The effect of temperature on the hydrolytic activity of purified enzyme isoforms at optimal pH was examined at different temperatures between 27-77°C. The enzyme was incubated with 100 mM acetate buffer containing substrate, after which the product formed was estimated. The stability of isoforms at different temperatures was determined by incubating purified enzyme with 100 mM acetate buffer (optimum pH) for 15 min at different temperatures in the range 27-77°C. After the incubation time, the enzyme was immediately cooled and the remaining activity was assayed. The temperature at which 50% of the activity is retained (T_m) was calculated.

K_m and V_{max}

The kinetic parameters (K^m and V^{max}) were determined by incubating suitably diluted purified enzyme with 100 mM acetate buffer (at optimum pH) containing different substrate concentration. The Michaelis –Menten constants: K_m and V_{max} for substrate hydrolysis were calculated for the isoforms by double reciprocal Lineweaver -Burk plot.

PGA, at concentration ranging from 0.001- 0.1 % was used for PG isoforms, whereas concentration of ρNPG, ranging from 0.6 -6.7 mM was used for β-galactosidase isoforms.

Effect of metal ions and EDT A on activity

The effect of metal ions (Ca^{++}, Mg^{++}, Fe^{++}, Mn^{++}, Zn^{++}, Cc^{++}, Cd^{++}, Hg^{++}) EDTA on the hydrolytic activity of isoforms was determined by assaying the remaining activity, after incubating the enzymes with acetate buffer (optimum pH) containing metal ions (1 / 0.1 mM concentration) for 20 min at 4°C.

Effect of product analogues on activity

The effect of product analogues on the hydrolytic activity of isoforms was determined by incubating the enzymes with acetate buffer (optimum pH) containing 2 mM product analogues, such as galacturonic acid, galactose, glucose, man nose, fucose, rhamnose, arabinose and xylose for 20 min at 4°C. The remaining activity was assayed immediately.

Substrate (synthetic) specificity

Substrate (synthetic) specificity of isoforms was determined by incubating the enzyme with various pNP-glycosides at 13 mM concentration. The pNP released was estimated after incubation at 47°C for 15 min.

Activity of isoforms on natural substrates

The action of purified enzyme (isoforms) on natural substrates like, PGA (0.06%), pectin (0.06%), galactomannan (0.45%) and microcrystalline-cellulose (0.45%) was examined by incubating these substrates with the purified enzymes. The enzyme activity was assayed after incubation.

Activity of isoforms on endogenous substrates

Activity of purified enzyme isoforms towards the endogenous substrates was tested. Each of the three purified substrates (0.5-1 mg) was incubated with the purified enzyme at optimum temperature for 7 h. Controls containing substrate without enzyme were also incubated simultaneously. The reaction was stopped by adding potassium ferricyanide reagent (1 ml), heated in a boiling water bath for 15 min and estimated the reducing sugar released.

Note: (unless otherwise specified).

- For PG isoforms, PGA (0.25%) was the substrate and incubation was for 2 h at 37° (for isoforms I and III) and at 42°C (for isoform II).

- For β-galactosidase isoforms, pNPG (13 mM) was the substrate and incubation was for 15 min at 47°C (for isoforms I and II) and at 42°C (for isoform III).
- All the values given here represent the mean of three replicate.

CHAPTER – III
PECTIC POLYSACCHARIDES IN RIPENING MANGO

1. Textural Softening: Cell Wall and Pectic Changes During Ripening

Summary

The total pectin in mango fruit decreased from 1.86 to 0.38 g % during ripening with a concomitant increase in soluble galacturonide, along with progressive textural softening as measured by texture analyzer. The rigid and compact cell wall structure of the unripe fruit appeared more loosely structured and widened at the ripe stage as seen by light microscopy. Microscopic data also revealed the loss of cell wall integrity, cell wall swelling, increase in the intracellular spaces, loosening of cells and disappearance of starch. Pectin dissolution in quantitative terms correlated with the microscopic observations on cell structure as well as organoleptic changes in fruit texture.

Introduction

Texture is one of the most important organoleptic characteristics of fruits that is altered during ripening. Fruit texture is of prime importance as it directly dictates fruit shelf life. The cell wall chemistry determines the fruit texture, which in turn influences the overall quality and consumer acceptability.[112, 220, 273] The complex nature of cell wall makes it difficult to pinpoint the crucial factors specifically contributing to textural softening.[278] The increased interest in controlling the textural qualities of fruit stimulated research in cell wall biochemistry, with particular reference to cell wall polysaccharides and their degradation. At cell wall level, the extent of pectin dissolution and solubilization is generally related to the degree of textural softening and wall changes during ripening.[104, 278]

Textural properties of fruits were evaluated by firmness measurement, which is an index of fruit ripeness. The textural qualities of fruit are attributed to its inherent composition, particularly the cell wall composition. Three classes of instrumental or objective methods of texture evaluation, viz; fundamental, empirical and imitative are usually employed for firmness measurement.[20] Empirical tests, which are simple and rapid, include penetration, piercing, compression, puncture, extrusion, shear, and so on, which are now widely used for determining textural properties of fruit. Textural softening and changes in cell structure during ripening were studied iin a number of fruits, with the help of texture analyzer and microscopy.[1, 6, 69, 112,114,133,152,163,169,183,210,222]

Microscopic observation of many fruit tissues revealed changes in the cell wall structure during ripening. Apparent change in the cell wall thickness, i.e., swelling of cell wall, as observed by

light microscope, was detected during ripening of many fruits such as kiwifruit.[210] However, no such cell wall swelling was noticed in grapes.[169] Textural softening is accompanied with the disruption of cell wall and middle lamella, appearing as electron-translucent area in electron micrographs of ripening fruits. These wall changes were correlated with textural softening in tomato,[48, 112] avocado [184] and pear.[25] Softening of many fruits including kiwifruit is accompanied by a marked swelling of cell wall and this is strongly correlated with pectin solubilization.[210]

Pectins or pectic polysaccharides are diverse group of acidic polysaccharides abundant in fruits and contribute in a substantial way to their texture.[34,276] Loss of pectin and increase in soluble galacturonide during ripening were shown in fruits like tomato,[242] apple,[56] kiWi,[211] plum,[29] peach,[99] melon,[222] banana,[277] persimmon,[49] muskmelon,[153] bell pepper, [93, 205] apricot,[73] cherry, 75 olive fruit, [114] bush butter fruit [160] and mango[33, 69,163,221]

The major changes in mango fruits during ripening are reduction in fruit weight, firmness, acid content, starch, vitamin C with concomitant increase in total soluble solids, pH, sugar/acid ratio and carotenoids.[67, 235] 'he objective here was to see the extent of correlation with textural oftening in mango fruit in relation to cell structure and pectic changes during ripening.

Results and Discussion

The texture analysis on mango fruit based on penetration (with and without peel), piercing (with peel) and compression (on tissue blocks), indicated progressive decrease in the force requirements at different stages during ripening (Fig,19a). The firmness data are expressed in Newtons [N].61 The force required for the penetration of 8 mm probe into the fruit, through the peel, decreased from 240 N, at stage-I to 32 N at stage-IV, with drastic decrease occurring between stage-III to IV (153 to 32 N). Similar results were obtained with compression analysis on tissue blocks, where the force requirement decreased from 274 to 169 N (from stage I-III), which further dropped to 30 N, at stage-IV. Similarly, the force required by the 8 mm probe to penetrate the tissue without peel decreased from 138 to 6 N, showing a dramatic decrease between stage-III to stage-IV. Same was noticed in the force requirement for 'piercing' with peel. Thus, the energy requirement sharply decreased in later stages of ripening (i.e., from stage-III to IV) than the earlier stages (stage-I and II), showing a more pronounced degree of softening at the end of ripening. Similar trend of tissue softening was reported for fruits including melon,[222] banana[27] and pepino.[171] This loss of firmness is due to the change in the ordered and compact arrangement of cell wall and middle lamella polysaccharides.[61]

The high energy requirement for penetration of mango with peel than without peel, indicated that the peel tissue is much stronger than the pulp, and also the degradation of peel constituents is

much slower when compared to the pulp tissue in mango. This also suggests that the softening process is from inside to outside, as reported for other mango varieties.[143,]

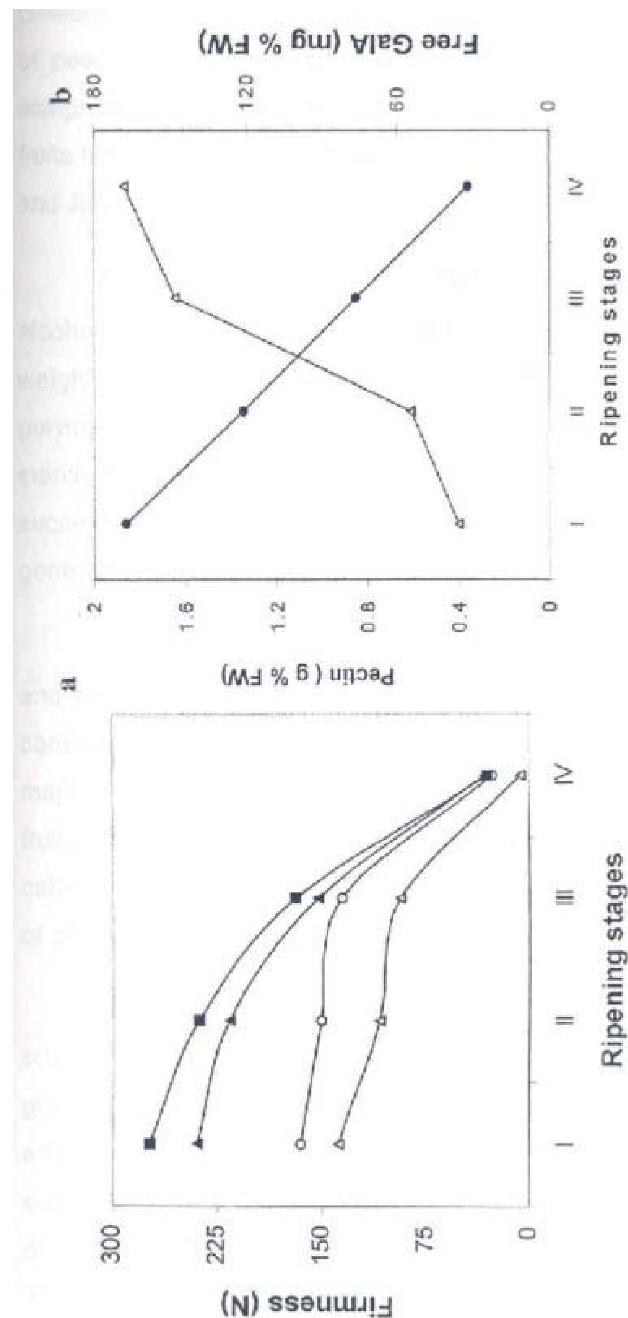


Fig. 19: (a): Changes in fruit firmness (in N) at various stages during ripening in mango. Penetration, with peel (-▲); without peel (-△-); Piercing with peel (-o-); Compression of tissue blocks (-■-).

(b) Pectic changes during ripening: Pectin (g % FW -•-); Free GaiA (mg% FW -△-).

I, II, III & IV denote ripening stages.

Further, it has been reported that the pulp constituents decrease and peel constituents increase during ripening,[67] which may contribute to the firmness of peel. Texture analysis parameters such as penetration, piercing and compression were widely used for assessing textural attributes of many fruits including apple,[1] avocado,[183] cherry,[75] mango,[161] papaya,[133] tomato[112] and Japanese pear.[248]

AIR was prepared from unripe and ripe mango pulp using aqueous alcohol (~80 %) at 60°C.[42] Its yield decreased from 12.9 to 2.4 g % fresh weight (FW) from unripe to ripe stage, indicating that large alcohol-insoluble polymers are degraded to alcohol-soluble polymers during ripening[69] and starch to soluble sugars.[27, 221, 234] Extraction of fruit tissue in hot ethanol successfully inactivates cell-wall enzymes and prevents the autocatalytic generation of reducing groups.[40, 222]

The carbohydrate and uronic acid (UA) contents of AIR from unripe and ripe fruits were 97 and 58 %, and 8 and 32 %, respectively. Starch constitutes the bulk of the AIR of unripe fruits, while protein constitutes the major part of AIR of ripe fruits. As fruit ripens, protein content in AIR of ripe fruit increases as a consequence of generation of more number of free carboxylic groups (by deesterification of pectins), which bind more amounts of proteins.[211]

During ripening, decline in AIR and concomitant increase in total soluble sugar was reported for mango[33. 221, 234] and other fruits like papaya,[41] guava, date[69] and strawberry.[102] Decrease in AIR as well as fruit firmness are the general physicochemical features accompanying the ripening of seven Indian mango varieties[234] and other fruits.[25] Apparent increase in AIR during development and decrease during ripening was reported in 'Oashehari' mango.[260] Increase in AIR during ripening was reported for bush butter fruit, which is mainly attributed to dehydration.[159]

EDTA solubilized -14% of the AIR from both unripe and ripe mango. The other components of AIR were water-soluble pectins, alkali-soluble pectins, hemicelluloses, celluloses, proteins and probably non-sugar cell wall constituents such as polyphenols and lignins. [237. 266] EDTA was used as extractant as it extracts more chelator-solubles than other chelators.[214] A steady decline in the EDTA-solubles was observed during ripening. The drop in EDT A-soluble pectins from unripe to ripe stage was 1.86 to 0.38 g % FW (w/w) , which was more than four-fold decrease (Fig. 19b). The carbohydrate content of EDTA-solubles was only 16 -21 %, remaining being other constituents, which may be components of pectins (methanol and acetic acids) or proteins (cell wall proteins or co-precipitated intracellular proteins).[266] However, the major constituent was EDTA itself, which found associated with pectins even after prolonged dialysis.[162] Similar low sugar content in the EDTA-soluble pectins and persistence of high amount of EDT A in spite of prolonged dialysis was reported for sugar beet pectins.[72] Banana chelator-soluble pectins decreased from 1.1 to 0.8 % during ripening.[27]

Concomitantly, the soluble galacturonide content increased from 36 to 168 mg % FW (>four-fold increase) during ripening. The increase was more between stage-II and III, which was in accordance with decreased fruit firmness and increased activity of pectin degrading enzymes [Chapter-IV; Section 1]. The low level of soluble galacturonide (free GaiA) is due to the fact that the galacturonic acid content was only 8 % (- 39 % of the total carbohydrate content) of the total EDT A-solubles of unripe fruit. The solubilization of chelator-solubles was not due to heating while AIR preparation, since Huber and Lee 105 reported that the solubility, molecular size or neutral sugar composition of pectins solubilized from AIR is not affected by heating. Further, there exists a clear correlation between the loss of polyuronide and increase in soluble galacturonides.185 Infact, degradation of pectic polysaccharides resulted in the accumulation of soluble galacturonide in ethanol-soluble fraction. Thus, loss in chelator-soluble pectin content in mango pulp during ripening was clear.

The chelator-soluble pectins decreased during ripening in this mango cv. (Alphonso), unlike other mango varieties. Similar decrease in chelator-soluble pectins have been reported in other fruits such as cherry,[75] peach,[98] strawberry,[168] kiwi,[211] nectarine,[147] and bell pepper,[205] while increase of the same was reported in pear,[26] Spanish pear,[150] apple,[56] avocado,[106] apricot,[73] banana,[189] 01ive,[107] 'Keitt' and 'Tommy Atkins' varieties of mango.[161, 163, 221] In tomato there was no change in the chelator-soluble pectin during ripening.[90] Increase in soluble galacturonide during ripening was also reported for pear,[26] tomato,[103] muskmelon,[153] and kiwi.[211] Generally, fruit softening is accompanied by the decrease in the amount of insoluble pectic substances and a concomitant increase in soluble polyuronides.[63, 123] The results showed that EDTA-soluble pectins are the major polysaccharides that undergo drastic degradation during ripening. This suggests that pectic polysaccharides are implicated in tissue softening during ripening in mango fruit.

Microscopic study showed loss of middle lamella (i.e., pectin) resulting in loosening of cell wall structure (Figs. 20 & 21). The more compact and rigid cell wall at the mature unripe stage appeared loosely structured and broader at the end of ripening (Fig. 20). The loosening of cell wall in ripe stage makes the cells larger in size than the unripe cells. Dissolution of cell wall and middle lamella is apparent from the micrographs showing ruthenium red stained cell wall (Fig. 21). The geometry and the cell wall shape also changed during ripening. Loss of cell wall integrity, increase in the intracellular spaces and loosening of cells during ripening are clearly observed. Cell wall stained with ruthenium red was reported for pineapple fruit.[251] Microscopic studies on mango fruit with specific staining for xylem and tannins has been reported.[174] In kiwi fruit, swelling of cell wall and a

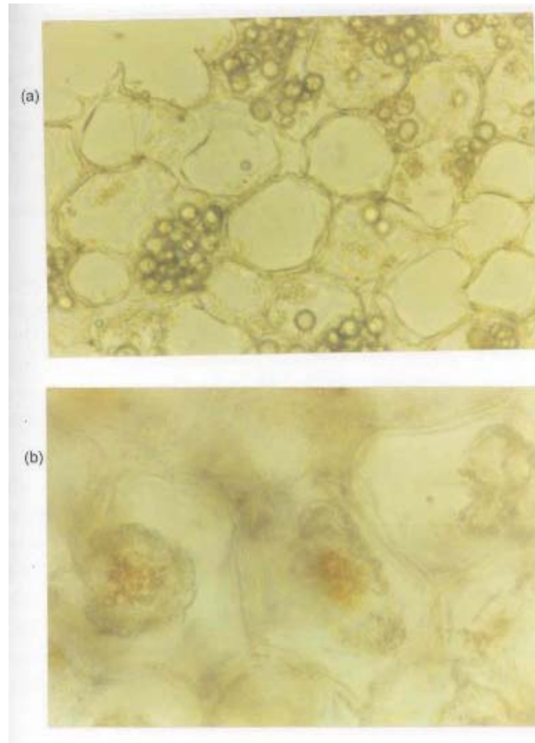


Fig.20: Micrographs (LM) of mango fruit cell wall at unripe (a) and ripe (b) stages (100x)

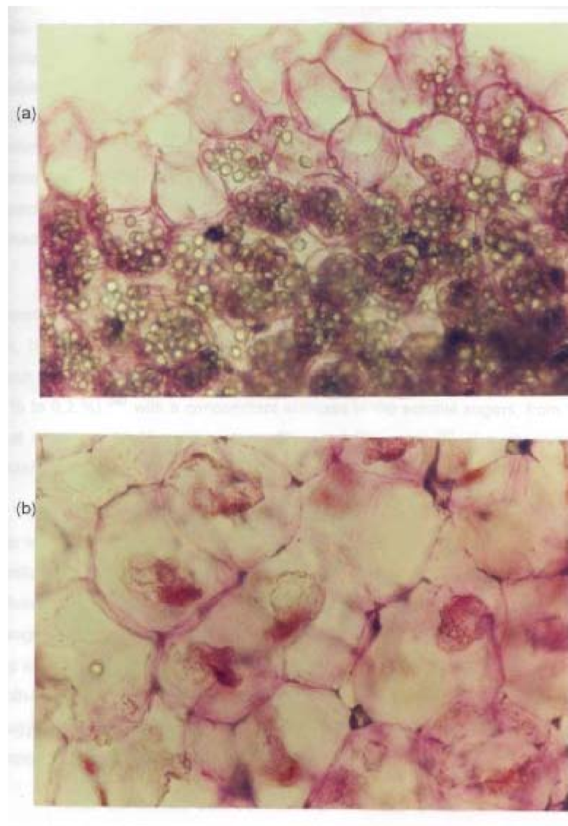


Fig.21: Micrögaphs (LM) of ruthenium red stained mango fruit cell wall at unripe (a) and ripe (b) stages

three- to four-fold increase in cell wall thickness during ripening has been reported.[210] This marked swelling is strongly correlated with pectin solubilization during ripening.[210] This swelling has resulted from the water moving to the voids left by the pectin solubilization.[167] However, no such swelling was observed in grapes during ripening.[169] As the physical interconnection between adjacent cells occur primarily through interlocking of pectins, the change in the degree of interconnection affects the rigidity of interaction between the polymers,[152] and eventually the tissue firmness.

Apart from cell wall dissolution, microscopic observation also showed almost complete disappearance of starch granules at the ripe stage (Fig. 20a, b). This is in consistence with the decrease in starch content. The starch is one of the major polysaccharides in mango, which decreased (from 18 % to 0.2 %),[290] with a concomitant increase in the soluble sugars, from 1 % at unripe to 18 % at ripe stage. Roe and Breumme[221] also observed similar correlation between starch and soluble sugars.

In mango, the loss of cellulose (2.0 to 0.9 %) during ripening was also noticed. In ripening tomato fruit, cellulose was not considered to be significant for textural loss.[269] In mango, it appears that apart from pectin, cellulose (to some extent) may also be a component contributing to the changes in cell structure at the cell wall level. The disappearance of starch may also be an important factor contributing to textural softening. Banana is another climacteric fruit, which is rich in starch (18 %) and Prabha and Bhagyalakshmi[189] reported that this starch was almost completely disappeared during ripening.

A clear correlation between textural loss and pectin content was reported in Japanese pear, where chelator-soluble pectin was found to affect the fruit texture.[248] The texture of sweet cherry fruit was reported to be related to equilibrium between the relative pectic fractions.[75] Loss of pectic polysaccharide in relation to textural softening was reported in olive[149] and tomato.[112] A positive correlation between chelator-soluble pectins and loss of tissue firmness was reported.[42,292] Chelator-soluble pectins exist as pectic acids that bind calcium and form cross-links, which is responsible for tissue firmness.[292, 165] In apple, papaya, and capsicum, a clear correlation between textural loss and cell wall degradation, especially the breakdown of pectin, cellulose and hemicellulose was reported by many authors.[1. 27. 133, 190. 205]

Thus, there is a clear correlation between textural softening, microscopic observation, biochemical changes (change in pectin, soluble galacturonide, starch and total sugar content) and also a climacteric rise in pectin-solubilizing enzymes during ripening [Chapter-IV; Section 1]. As the compactness of the cell wall structure was modified during ripening, polymers and enzymes were able to come in contact with each other resulting in degradation of wall polymers during ripening. Pectic polymers, which are the hydrophilic polymers in the cell wall, are the first to be solubilized during

ripening.[211] The breakdown of tightly bound insoluble protopectin to soluble polyuronides, which are loosely bound to the cell wall, appears to influence the fruit / textural softening during ripening.[67] The pectin solubilization results in the loosening of middle lamella and primary cell wall by lowering the degree of cross-linking.[167] There exist a clear correlation between the cell wall swelling and the degree of pectin solubilization, suggesting that the wall swelling is the consequence of the changes occuring in the viscoelastic properties of the cell wall during pectin solubilization.[210] The highly branched pectic polysaccharides in the primary cell wall are accessible for hydration and degradation, and as a result their solubilization leads to marked decrease in neutral sugar side chains and eventually textural softening.

Mango, being a climacteric fruit and also rich in starch, is known to undergo extensive textural softening during ripening. At the cell wall level, pectin may be an important component contributing to loosening of cell wall structure, as the loss of pectin-rich middle lamella during ripening is quite significant. Though various factors influence textural softening during ripening, in mango there seems to be interplay of pectin and cellulose at cell wall level and starch changes at cellular level. Thus, it is difficult to pinpoint the factors contributing to textural softening in precise qualitative and quantitative terms. This has also been pointed out by Waldron and coworkers.[278] However, pectins are the key substances responsible for the mechanical strength of the primary cell wall.[248] Their degradation seems to be responsible for tissue softening during ripening, as reported for number of fruits including tomato,[241] kiwi,[211] apple[56] and bush butter.[159, 160]

2. Changes in the Profile of pectic Polysaccharides: Their Implication in Fruit Softening

Summary

When the EDTA soluble total pectic fraction was fractionated by ion exchange chromatography (IEG) on DEAE-cellulose followed by differential elution with weak and strong alkali gradients, it resolved into seven distinct polymeric peaks, three major and four minor peaks. The three major peaks were found in neutral (buffer), 0.05M and 0.10M ammonium carbonate eluates. All the seven polymeric fractions showed a drastic drop in their abundance as well as their molecular weight at the end of ripening process, indicating significant depolymerization in vivo. The mg % drop in their levels for fractions I, II and III, from unripe to ripe stage was 60 to 7,89 to 13 and 74 to 2, respectively. The molecular weight drop in kDa (for post HPSEG fractions) from unripe to ripe stage was 250 to 70, 1300 to 21 and 473 to 298 kDa for the fractions I, II and III. Based on gas liquid chromatographic composition, fraction I appeared to be arabinogalactan-type pectic polysaccharide, while fractions II and III were typical rhamnogalacturonans containing> 62 % galacturonic acid, the rest being arabinose, galactose and rhamnose in different ratios. During ripening, loss of both acidic

and neutral sugar residues from all the pectic fractions was evident. The results are discussed in the light of fruit softening during ripening.

Introduction

Much work in the area of fruit ripening has been focused on ripening-associated changes with specific reference to textural softening which is mainly due to changes in cell wall structure and composition.[32. 34.100.239,248,270,278] Pectin at the cell wall level is one of the major polysaccharides contributing to the above phenomenon.[248]

These complex cell wall polysaccharides can be extracted by hot water, weak acids, weak base and chelating agents. The chelators such as ammonium oxalate, hexametaphosphate, EOTA, EGTA and COTA are frequently employed to extract chelator-soluble pectins.[214, 250] After extraction, fractionation of pectic polysaccharides was normally accomplished by a combination of various fractionation methods; precipitation with alcohol, ion exchange chromatography (IEC) and/or gel permeation chromatography (GPC). High performance size exclusion chromatography (HPSEC) is the most versatile technique used for separating molecules based on their molecular size differences as well as to determine the relative molecular weight of pectic polysaccharides. Once the sample is fractionated, composition analysis of monosaccharides, in qualitative and quantitative terms, was performed by GLC, after converting the sugars into volatile derivatives.[238, 291]

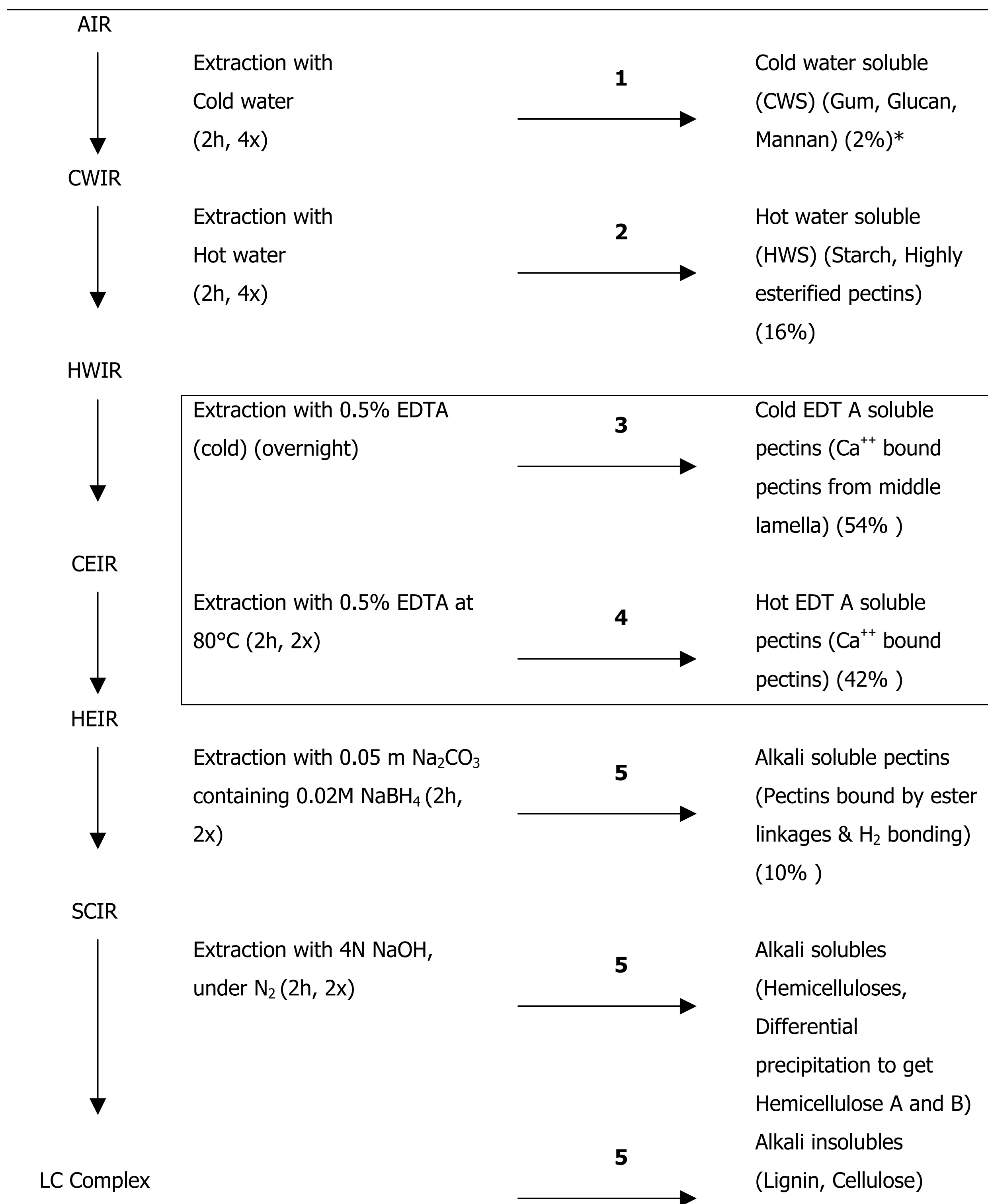
Changes in the pectic fraction of the cell wall, especially increased solubility, depolymerization, deesterification, and loss of neutral sugar side chains are described in many fruits.[26, 49,56,73,98,99,147,150, 153, 160, 168,205,207,209 211 222] However, a detailed investigation to show the entire spectrum of pectic polymers present and their qualitative and quantitative changes during ripening is not well documented in fruits.

Very little is known about the cell wall structure and pectin composition of mango fruit. Only gross changes in the mango cell wall pectins with a net loss of sugar residues in total pectin during ripening have been reported.[33, 69,161,163,221] One of the approaches to deduce the nature of the physicochemical and enzymological process involved in pectin hydrolysis in vivo is to characterize the changes occuring in the polyuronide structure and composition during ripening. This section specifically deals with pectin dissolution in vivo, giving a complete profile of pectic polymers in unripe and ripe mango, their qualitative and quantitative changes, their drop in the molecular weight in vivo. The profile of carbohydrate polymers and the changes they undergo during ripening give a direct clue to identify the enzyme targets in a fruit. Such a comprehensive study is lacking, especially so, in mango fruit.

Results and Discussion

AIR from unripe (stage-I) and ripe (stage-IV) mango pulp was extracted sequentially based on their differential solubility into 8 different fractions (Fig. 22). The carbohydrate polymers, extracted with cold and hot EDTA (fractions 3 and 4) showed the highest galacturonic acid content [Fig.22; Table 8]. They were combined and designated as 'chelator-soluble pectins. Two extreme stages of ripening were taken by many authors to study carbohydrate changes during ripening. [105,107,129]

It should be noted here that the galacturonic acid to neutral sugar ratio was higher in the ripe stage than unripe stage [Table 8], indicative of a more pronounced dissolution of neutral sugar residues than galacturonic acid in the pectic fractions upon ripening. Further, the pectin extractability (as galacturonic acid) at ripe stage was much higher in cold (59 %) than in hot condition (41 %) and is vice versa in unripe stage, lower in cold (33 %) and higher in hot condition (67 %) [Table 9]. Huber and Lee 105 also reported that EDTA solubilized more pectins from ripe fruit than from unripe tomato. These observations suggest that higher dissolution of pectin-rich middle lamella occur during ripening. Further, as ripening progress more of the pectic polymers get solubilized, favouring its extraction even in mild condition. Redgwell and coworkers[211] also reported that pectic polymers, which were solubilized in harsh conditions, were found solubilized in mild condition at the later stages of ripening.



Fiig. 22: Flow chart for the sequential extraction of carbohydrates based on differential solubility. AIR-Alcohol insoluble residue; CWIR- Cold water insoluble residue; HWIR-Hot water insoluble residue; CEIR-Cold EDTA insoluble residue; HEIR-Hot EDTA insoluble residue; SCIR-Sodium carbonate insoluble residue. LC- Lignin/cellulose

* % GaiA of Total Sugars (shown for unripe stage to indicate extractability)

Table 8: GaiA and neutral sugar contents in EDT A soluble pectic extract (mg % FW)

EDTA-Solubles	Stages	Total Sugar* (mg)	Uro.nic Acid# (mg)	Neutra! Sugar+ (mg)	Uronic Acid (%)	UA : NS ratio
Cold EDTA	Unripe	204	109	95	54	1.1:1.0
	Ripe	34	23	11	68	2.1 : 1.0
HotEDTA	Unripe	534	220	314	42	0.7:1.0
	Ripe	23	16	7	70	2.3:1.0
Total EDTA	Unripe	738	329	409	45	0.8 : 1.0
(cold + hot)	Ripe	57	39	18	68	2.2: 1.0

* As monitored by Phenol-sulphuric acid

As monitored by metahydroxybiphenyl method

+ By difference (arbitrary).

The presence of high galacturonic acid content along with relatively high galactose and arabinose residues indicates that these fractions are pectic in nature [Table 9]. Chelator-solubles are usually pectic polysaccharides.[251] Pectins are generally associated with Ca^{++} ions. EDTA by chelating with Ca^{++} ions favours pectin solubilization. EDTA extracts pectins from the middle lamella, while Na_2CO_3 extracts covalently bound pectins, essentially from the primary cell wall.[213] The amount of galacturonic acid was much higher in EDTA-solubles than in Na_2CO_3 solubles and other fractions [Fig. 22; Table 9], indicating that the pectins in the cell wall of mango pulp are essentially ionic in nature and are concentrated in the middle lamella region. High amount of pectins in chelator-solubles were reported for other fruits.[159,212] The amount of polysaccharides solubilized by Na_2CO_3 was higher than that solubilized by EDTA [Table 9], which was as reported for kiwi fruit.[211] However, the galacturonic acid content was low in Na_2CO_3-soluble fraction when compared to chelator soluble fraction. Similar low level of galacturonic acid in Na_2CO_3-solubles was reported for olive fruits,[114] while high level was reported for nectarines.147 Generally, arabinose and rhamnose residues were higher and galacturonic acid was lower in Na_2CO_3-solubles than chelator-solubles pectins.[99] A high amount of man nose was also observed in Na_2CO_3-solubles. The amount of EDTA- and Na_2CO_3-solubles decreased during ripening, as in many ripening fruits. However increase in chelator-soluble in the expense of Na_2CO_3-soluble was reported during ripening in avocado.[73]

Table 9: Relative composition of sugars in EDTA - and Na_2CO_3-soluble pectins

Yield* (mg% FW)		Relative ratio of the sugar composition						
		GalA	Rha	Ara	Xyl	Man	Gal	Glc
EDTA solubles								
Unripe	396	39	5	19	2	6	20	9
Ripe	57	55	6	15	2	5	7	10
Na_2CO_3 solubles								
Unripe	530	10	9	28	4	20	20	9
Ripe	105	17	10	26	4	17	16	10

* Yield is as determined by phenol-sulphuric acid total sugar estimation.

In order to allow more specific characterization, the chelator-soluble pectins were subjected to ion exchange chromatography. Figure 23 gives the qualitative and quantitative profile of the entire range of pectic polymers in unripe and ripe mango pulp as fractionated on DEAE-cellulose column. Fractions were eluted stepwise from the column by sodium acetate buffer (O.02M, pH 4.8) and with increasing molarities of ammonium carbonate and sodium hydroxide. Pectic polysaccharide from unripe and ripe mango pulp resolved into seven distinct polymeric peaks and were designated as fractions I -VII, respectively, based on their order of elution. Three of them

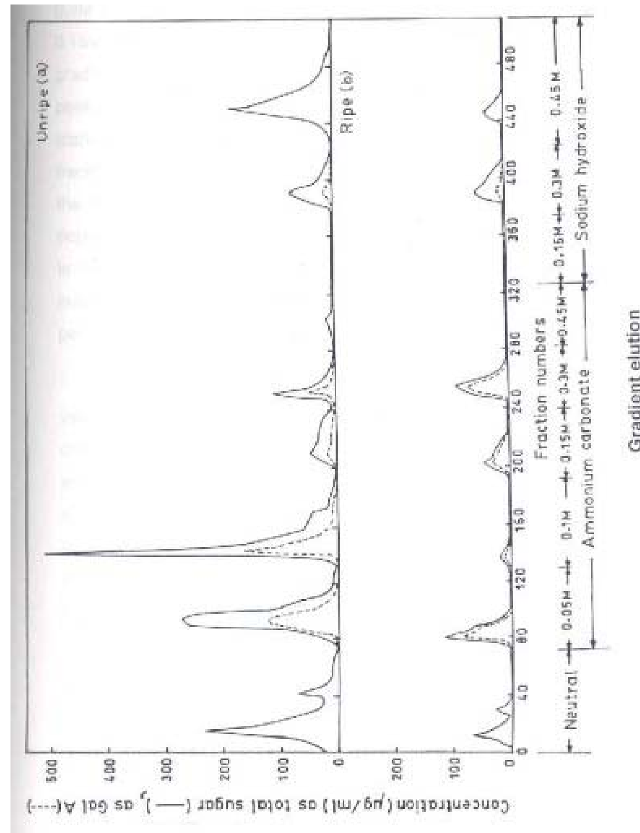


Fig.23 DEAE-cellulose profile of chelator-soluble pectic polymers from unripe (a) and ripe (b) mango

were major fractions, which distinctly resolved in neutral (acetate buffer), 0.O5M and 0.10M $(NH_4)2CO3$ elution. At higher strength gradients (0.15 and 0.3M $(NH_4)_2CO_3$), there were minor peaks while 0.45M $(NH_4)_2CO_3$) and 0.15M NaOH eluates showed no carbohydrate peaks. Stronger alkali gradients (0.3 and 0.45M NaOH) also showed some distinct polymeric Jeaks but with lower levels of uronic acid « 5 %). Loss in qualitative levels carbohydrate content) from unripe to ripe stage was observed in all the factions. A slight increase in fraction V (0.3M $(NH_4)^2CO_3$) may indicated that he degradation of both 0.05M and/or 0.1 M $(NH4)^2CO3$ generated pectic)opulations of low degrees of methylation, which was retained and eluted n higher gradients (0.3M $(NH_4)_2CO_3$). Similar observation was shown in bush butter fruit (Missang, et al., 2001 b). Changes in the lEG profiles of pectins from unripe and ripe tomatoes were also reported.[105]

Ion exchange chromatography has been successively applied by various Investigators to fractionate pectins.[51, 72 254] Based on their net charge and degree of esterification, pectic fractions were eluted from the anion exchange column.[72] However, molecular weight and covalent linkages of neutral sugar also influence the elution pattern.[55] Ammonium carbonate (a weak alkali) and NaOH (a strong alkali) were used for fractionating pectins on DEAE-cellulose column.[245] Ammonium ions are found useful for quantitative elution of pectic fractions from anion exchanger.[44]

Unbound fraction was devoid of galacturonic acid, while the retained fractions were rich in galacturonic acid and are poor in neutral sugar residues as reported for sugar beet pectins.[72] From lEG column, highly esterified homogalacturonans eluting earlier than highly branched heterogalacturonans was reported for kiwi, peach, pear and bush butter fruit pectins.[51, 99,150.212]

The relative abundance and their sugar composition, as well as the changes from unripe to ripe stage of all the pectic fractions are clearly detailed in Table 10. A significant decrease in their levels from unripe to ripe stage is evident in all the fractions, except fraction V. The mg % drop in their levels for the major fractions I, II and III, from unripe to ripe stage was 60 to 7, 89 to 13 and 74 to 2, respectively. All the other fractions showed abundance at -20 mg % FW and declined at the ripe stage, while fraction V increased slightly. Nevertheless, there was no complete disappearance of any of these fractions at the end of ripening indicating controlled depolymerization and partial hydrolysis of pectic polymers. This suggests that the high molecular weight polymers underwent degradation leading to formation of alcohol soluble polymers, and thus are not accumulated as a peak, except fraction V (Fig. 23).

Fraction I was completely devoid of galacturonic acid, while all other fractions showed the presence of galacturonic acid [Table 10]. Further, all the fractions showed decrease in the quantitative levels from raw to ripe stage [Table 10]. Fractions II and III showed high galacturonic acid content (> 60%) compared to other fractions. Fractions IV and V contained high galacturonic acid content (- 58%), but their yield were in low quantities. Further, Fraction VI contained low level

of galacturonic acid « 6%), while fraction VIII was devoid of galacturonic acid and contained glucose as the major neutral sugar. Hence these latter fractions were not considered for further studies.

All the fractions showed the presence of neutral sugars such as galactose, arabinose and rhamnose, which are the characteristic neutral sugars of pectic-type polymers. Based on the composition obtained, fraction I appeared to be an arabinogalactan-type polymer, probably present as arabinogalactans covalently linked to pectic polymers 16() or strongly linked to cellulose.209 Fractions II and III were heterogalacturonans containing relatively high amount of galacturonic acid, 62 and 61 %, respectively, with different ratios of neutral sugars; arabinose, galactose and rhamnose [Table 10]. In the major fractions (II and III), the in vivo hydrolysis of the neutral

Table 10: Changes in abundance and composition of pectic polymers from unripe and ripe mango pulp (mg/FW) ,

Fr.	Eluate	Pectic	Abundance*		Sugar composition							
No.		Frs.	Unripe	Ripe	GalA**		Ara		Gal		Rha	
					Unripe	Ripe	Unripe	Ripe	Unripe	Ripe	Unripe	Ripe
I	Buffer	Neutral	60.0	7.0	nil		14.4	2.2	38.2	0.7	2.8	0.2
II	$(NH4)_2CO_3$	0.05M	89.0	13.0	55.0	10	17.1	1.5	8.2	0.3	4.4	0.6
III		0.1M	74.0	2.0	45.0	1.3	11.9	0.2	9.2	0.1	4.4	0.1
IV		0.15M	20.0	6.0	12.0	5.0	3.4	0.9	2.0	0.1	1.2	0.1
V		0.3M	18.0	23.0	10.0	20.0	7.0	0.9	nil		Tr	1.3
VI	NaOH	0.3M	+17.0	7.0	1.0	1.0	0.5	0.8	0.2	0.5	nil	
VII		0.45M	+34.0	4.0	nil	3.1	0.8	0.7	0.0	2.0	0.8	

* As Total sugar by phenol-sulphuric acid method.

** As GaiA by metahydroxydiphenyt-sulphuric acid method.

+ Majar part is Glc.

→ Indicates change in levels. :

Tr- Trace.

sugar chain seemed to be more pronounced than the galacturonic acid chain as shown before. The presence of both galacturonic acid and neutral sugar in these fractions may suggest that they contained branched pectins as reported for chelator-solubles from apple and sugar beet pectins.[214] Further, the presence of rhamnose indicates that these heterogalacturonans are rhamnogalacturonan-type polymer. The presence of rhamnose may serve as branch point for the attachment of neutral sugar side chains.[157] Dick and Labavitch[61] also reported that 'Bartlett' pears contained heterogalacturonans having different proportions of neutral sugars arabinose, galactose and rhamnose.

Loss of galacturonic acid, galactose, arabinose and rhamnose residues in all the pectic fractions were clearly observed during ripening in mango [Table 10]. Almost complete loss (>95%) of neutral sugar residues from ripe mango pulp was very evident from the compositional analysis, while that of galacturonic acid was in the range 60-90%. Similar loss of acidic and neutral sugars was observed in total pectic fraction of ripening fruits such as persimmon,[49] 'Ngowe' mango[33] and tomato[90] Gross and Sams[91] reported a net loss of galactose/arabinose from the cell wall of 14 types of fruits out of 17 tested. A dramatic loss of pectin associated monomers, rhamnose, arabinose, galactose and galacturonic acid was reported for kiwi fruit, after six days of ethylene treatment.[167] Loss of galactose from the cell wall during ripening was reported in fruits like apples,[56], [130] kiwi,[211], [209] melon,[222] muskmelon,[153] tomato[90] and pineapple,[251] while loss of arabinose was reported in pear[4] and nectarines.[147] Significant decrease of only galactose was reported for 'Sensation' variety of mango.[270] Loss of both galactose and arabinose was observed in apples,[130] peach,[99] hot pepper,[93] 01ive,[114] 'Keitt', and 'Tommy Atkins' varieties of mango.[161] Loss of arabinogalactan, the major component of the side chain of pectic polysaccharide, has been observed in a number of ripening fruits.[91, 103, 169] Loss of galactose is mainly due to the degradation of arabinogalactan polymer, which is abundant in plant cell walls.[209] However, in ripening plum and cucumber, no change occurred in the composition of neutral sugars associated with pectins.[29.91]

Loss of galacturonic acid was also reported in other varieties of mango[33,161] However, polyuronide synthesis during ripening was reported for plum and cherry fruits[29, 75] Increase in the levels of neutral sugars of chelator-soluble pectins during ripening was also reported for olive fruit.[107] Thus, the cell wall neutral sugar composition varies among fruits and the metabolism of pectins in relation to textural softening differs from fruit to fruit, as mentioned by Gross and Sams.[91] In peach, softening is associated with change in the sugar composition of pectin and hemicellulose as well as their change in molecular mass.[98, 99]

Only a few reports have been published in concern with molecular weight changes of pectic fractions during ripening.[114] Mort and coworkers [162] and Fishman and coworkers[77] concluded that pectin-aggregation makes gel permeation chromatography a poor technique for the determination of molecular weight, and thus HPSEC was used to determine the molecular weight. Figure 24 and Table 11 give the molecular weight changes from unripe to ripe stage of all the pectic fractions, as determined by HPSEC. Molecular weight determination by HPSEC was reported by many authors.[107, 114, 160, 173, 174] The molecular weight difference from unripe to ripe stage was 250 to 70, 1300 to 21 and 473 to 298 kDa, for the major peaks of fractions I, II and III, respectively and this drop in molecular weight from unripe to ripe stage was obvious in all the pectic fractions [Fig. 24a, b; Table 11]. The profiles showed a peak in the molecular weight range for unripe (-14 -16 min) and ripe stages (-16 -21 min). Similar profiles for unripe and ripe fruits were reported for olive 107 and bush butler fruit pectins.160 Molecular weight of pectic polysaccharides ranging from -250 -40 kDa, with

broadening of the peak was also shown in olive fruits during ripening.[114] Broad distribution of molecular weight of water soluble pectins, eluting in -13-14 min and -14-17 min was reported in mango.[174]

The drop in molecular weight not only reflected the quantitative loss but also reflected the dissolution of a large polymer into a number of smaller ragments at the end of ripening [Table 11]. This is especially so in the most abundant pectic polymer (fraction II), where 1300 kDa at unripe stage 'resulted in low molecular weight peaks of 21, 10, 5 and 1 kDa. Low nolecular weight (1.1 kDa) homogalacturonan was reported in alcohol-insoluble polymers from soft bush butter fruit.[159] Drop in molecular weight of Jolyuronides, from 1000 kDa to 50 kDa, was also reported for kiwi fruit.[211] A high molecular weight of 1300 kDa was reported for sugar beet pulp pectins rich in neutral sugars.[173] A possible explanation for such a high molecular weight is the presence of diferulate cross-links connecting several rhamnogalacturonans (RG).[173] Extensive degradation of this polymer during ripening suggests that diferulate cross-linkings are probably present between these highly branched heterogalacturonans.

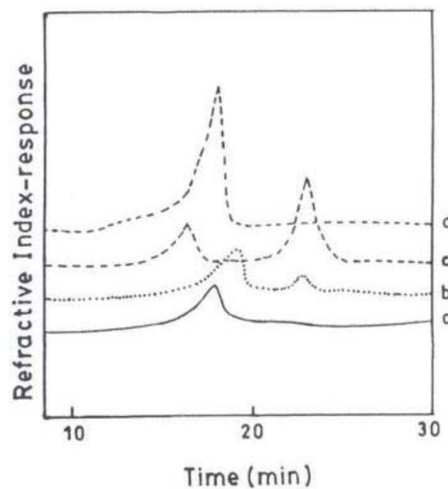


Fig. 24a: HPSEC profiles of (a) neutral unripe; (b) neutral ripe; (c) 0.15M $(NH_4)_2CO_3$)unripe; (d) 0.15M $(NH_4)_2CO_3$)ripe

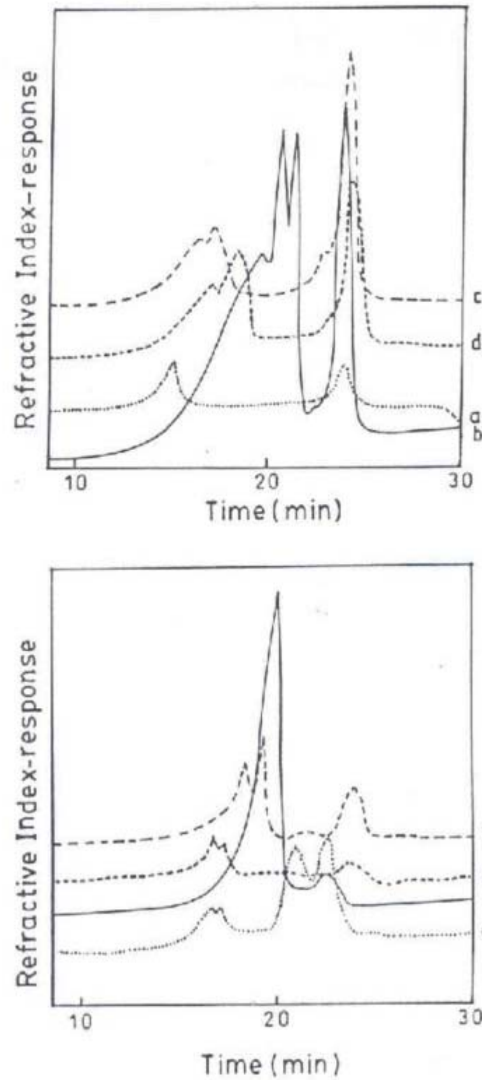


Fig.24b: HPSEC profiles of (a) 0.05M $(NH_4)_2CO_3$) unripe; (b) 0.05M $(NH_4)_2CO_3$)ripe; (c) 0.1M $(NH_4)_2CO_3$) unripe (d) 0.15M $(NH_4)_2CO_3$) ripe; (e) 0.3 N NaOH unripe; (f) 0.3 N NaOH ripe; (g) 0.45 N NaOH unripe; (h) 0.45 N NaOH ripe

Table 11: Changes in molecular weights of post HPSEC pectic fractions in unripe and ripe mango

Pectic fractions	Molecular weight (kDa)	
	unripe	Ripe
I	250	70
II	1,300	21
		10
		5
		1
III	473	298
	298	100

IV	298	100
V	nd	nd
VI	335	15
	224	
VII	376	70
	237	33

* as in Table 10; nd = not determined

Such a drop in molecular weight was also shown for pectic fraction of ripening tomato,[242] kiwi,[211] 0live,[107, 114] nectarines[147] and bush butter fruit.[159, 160] Preliminary observation for total pectin in ripening mango fruit also indicated the change in molecular weight[163, 270] as well as loss of sugar residues during ripening.[33] No such drop in molecular weight of chelatorsoluble pectins was reported for banana[277] and strawberry. [102]

The results gave a clear picture of the number and type of pectic polymers present in mango, their qualitative and quantitative changes during ripening, which may be taken as factors contributing to loss of middle lamella, which in turn are implied in change in texture of a fruit during ripening. This apparent pectic changes; increase in pectin dissolution, decrease in molecular weight and loss of both acidic and neutral sugars were also reported for ripening fruits.[90 163. 242] Further, in mango fruit, the major part of the textural softening was mainly due to degradation of chelator-soluble pectin, particularly, neutral (fraction I), 0.05M and 0.1 M eluates (fractions II and III). Pectin hydrolysis can also be influenced by temperature, pH and presence of divalent cations, especially calcium.[135, 249] However, pectin hydrolysis during ripening is mainly attributed the presence of pectin-hydrolyzing enzymes [Chapter IV].

In mango, it could be concluded that fractions I (arabinogalactan), II and III (both rhamnogalacturonans) contribute more to pectin hydrolysis in vivo. It must be noted here that the two rhamnogalacturonans are also rich in galactose and arabinose residues, apart from containing more than 60% galacturonic acid.

The presence of specific substrates in qualitative and quantitative terms and their changes during ripening give a direct evidence to speculate the corresponding enzyme targets. However, only gross information about the changes in the substrates can be obtained by compositional data and molecular weight analysis. Thus, a clear understanding of the pectin structure is necessary to determine the type of enzymes involved in the degradation of these substrates during softening of mango fruit.

3. Structural Studies of the Major Pectic Polysaccharides

Summary

A detailed separation of the entire range of pectic polymers of unripe mango pulp revealed seven distinct fractions clearly resolved upon lEG [Chapter-III; Section 2]. The striking drop in their abundance and molecular weight at the end of ripening denoted depolymerization in situ. The three major fractions were purified by GPC for further structural studies. These three fractions were identified as arabinogalactan (Fr. I) and two rhamnogalacturonans (Frs. II and III). Optical rotation, FT -IR, GC-MS and NMR data revealed that fraction I to be a 1$\rightarrow$ 4 linked galactose residues, which were occasionally involved in side chain branches consisting of single residues of galactose and/or arabinose or oligomeric 1 $\rightarrow$5 linked arabinofuranose residues, linked through 1$\rightarrow$3 linkages. Fraction was identified to be a rhamnogalacturonan with 1,4-linked galactopyranosyluronic acid residues in the main backbone chain, which are interspersed with (1$\rightarrow$2)-linked L-rhamnopyranosyl residue, which are further involved in branching.

Introduction

Pectins are composed of linear chain of D-galactopyranosyluronic acid, which are methylated and/or acetylated to various degrees with methanol and acetic acid, respectively.[24] They fall into three classes, namely homogalacturonan (HGA), rhamnogalacturonan-1 (RG-I) and rhamnogaicturonan-II (RG-II). The HGA consists of 1,4-linked α-D-galacturonic acid backbone carrying a very few side chains. RG-I is made up of alternating 1,4-linked α-D-galacturonic acid and 1,2-linked L-rhamnopyranosyl units and is interspersed with long stretches of neutral sugar side chains composed mainly of galactose and arabinose. These side chains are terminated occasionally by rare sugars.[156] RG-II is present in low level, and is composed of homogalacturonan backbone with side chains that are extremely complex in nature.[275]

Despite an in-depth study on tomato fruit at gene level, studies at substrate level are lacking and information regarding the precise substrates (carbohydrates) involved in textural regulation in fruits is scanty. Though gross changes in the pectin content during ripening were reported for a number of fruits, the structural aspects on pectic polymers have been reported only for a few fruits like apple,[47. 233] grapes,[169, 229, 275] kiwi,[212] raspberry[255] and tomato[203], [241] Most of these studies on pectins are based on methylation followed by GCMS, FT -IR and 13C NMR. These physical methods have been usually employed for structural analysis of pectic polysaccharides.[65] However, structural investigations of pectins are difficult due to their high molecular weight, the lack of homogeneity, and the absence of repeating units.[276]

A convenient and complete methylation of all the accessible functional groups can be accomplished in one step using a methylation method developed by Hakomori.[96] Subsequent acid hydrolysis, reduction and acetylation yields partially methylated alditol acetates, which separate on GC-MS based on ions having difference in mass: charge (mil) ratio, which are differentially deflected by the magnetic and electrostatic fields. This gives structural information regarding the carbohydrate composition and glycosidic linkages of the polysaccharides. Methylation analysis was employed for determination of linkage analysis of most of the fruit pectins.[47,169,212,229,241,275]

Molecules containing asymmetric carbon atom have the ability to rotate plane polarized light, which is the basis of Polarimeter. This gives information regarding the configuration of the sugar residues of polymers.

Carbohydrates contain functional groups that absorb infrared radiation due to vibration or rotation. Analytical instruments (FT -IR) based on infrared absorbance are thus suitable for structural analysis. FT -IR was used to detect functional groups, configuration and substitution patterns of pectic polysaccharides of plant origin.[45,46,74,117,255] 13C NMR is a valuable technique in the structural elucidation of polysaccharides, which allows spectra of the polysaccharides to be obtained using only their naturally abundant 13C atoms. It gives detail information on composition, sequence and conformation of polysaccharides. The better signal separation is mainly due to the wide range of chemical shifts involved. This technique is widely used for elucidating structure of pectic polysaccharides from fruits.[47, 203, 211, 229, 241] Mango is almost untouched for such detailed studies on pectic polymers. In the previous section, the entire spectrum of pectic polymers, their qualitative and quantitative changes and their extent of contribution towards textural softening were well documented. This chapter specifically deals with the structural aspects of the three major post IEC pectic polymers of unripe mango. These fractions were characterized using optical rotation, methylation analysis (GCMS), FT -IR and NMR. The drop in their molecular weight and abundance at the end of ripening denoting in situ depolymerization are also discussed.

Results and Discussion

The three major pectic polymeric fractions of unripe mango, designated as I, II and III, which were found eluted in neutral, 0.05M and 0.10M $(NH_4)_2CO_3$ eluates, respectively, were chosen for structural studies (Fig. 25). Due to very low recovery, minor fractions and pectic polymers from ripe mango pulp were not taken for structural studies. The striking drop in their abundance and molecular weight at the end of ripening is depicted in figure [25], which also denoted in vivo depolymerization of pectic fractions. They are the major pectic polysaccharides, which undergo drastic degradation during ripening in mango.

These three pectic fractions were individually subjected to GPC on Sepharose CL-4B (Fig. 26). They were eluted as a single broad peak, due to their high polydispersity. The molecular weights were in the range between 500-150,2000-1000, -500 kDa for fractions I, II and III respectively. Similar results were observed for pectic polymers from other fruits like tomato,[242] kiwi,[211] melon,[222] nectarine[147] and bush butter.[160] However, in pear, the IEG fraction resolved into 2-3 peaks upon GPC, suggesting the presence of unique pectic molecular species.[61] The molecular weights of the fractions I, II and III, as determined by HPSEC (Fig. 27) were 250, 1300 and [475] kDa, respectively. High molecular weight RG, reported in sugar beet PLUP also had a molecular weight of 1300 kDa,173 which is similar to that of fraction II. Molecular weights ranging from 1000 to 50 kDa were reported for pectins from 'Kiett' mango[163,174] and other fruits.[211, 222, 226] Kokini and Chou 134 reported that tomato pectins also exhibit high polydispersity, with the majority having the molecular weight in the range of 3 to 5.5 x 10^6.

High molecular weight (1300 kDa) was reported for sugar beet pulp pectins rich in neutral sugars, which may be due to the presence of diferulate cross-links connecting several rhamnogalacturonans.[173] The extensive degradation of this polymer during ripening [Chapter-III; Section 2] possibly suggests a probable diferulate cross-linking between rhamnogalacturonan molecules. Covalent linkages between rhamnogalacturonan and homogalacturonan through borate ester cross-links in sugar beet pulp pectins, as reported recently[111, 175, 294] may also contribute to such high molecular weights of pectic polymers.

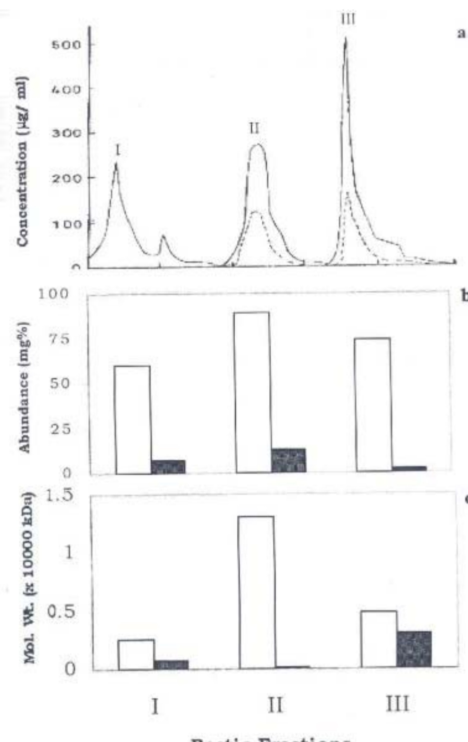


Fig.25(a) IEC profile of the three major pectic fractions of unripe mango pulp: monitored as Total sugar (_____); GalA (-----) (b & c) Abundance and molecular weight changes of the major pectic fractions from unripe (□) and ripe (■) mango

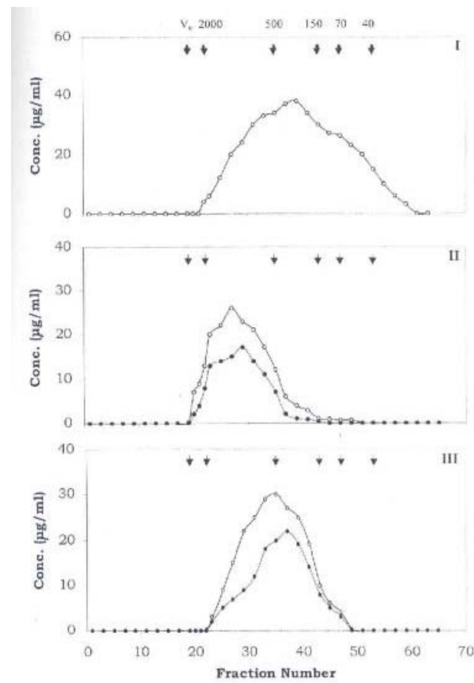


Fig. 26: GPC profile of the pectic fraqctions I, II & III on Sepharose CL-4B column; monitored as Total sugar (-O-); GalA (-●-) ↓ indicates void (V0) and elution volume (Ve) of standard molecular weight markers as indicated

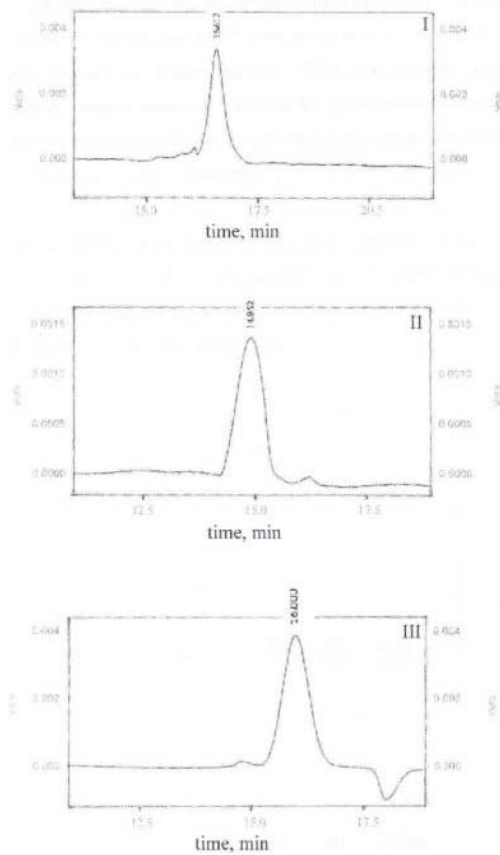


Fig.27: HPSEC profiles of the major pectic fractions I, II & III

Homogeneity of pectic fractions

The cellulose acetate electrophoresis profile (Fig. 28) showed single band for fractions II and III, which corresponds to the standard PGA (Sigma). Fraction I, being devoid of charged groups (i. e., GaiA), remained at the origin. Based on their charges, PGA was clearly separated on cellulose acetate membranes, as reported for plant pectic substances.[124] In capillary electrophoresis (CE), a single migration peak at -4.8 min, was observed for the acidic pectic fractions (Fr. II and III) and was similar to that of the standard pectic acid (PGA) (Fig.29). Further, during cellulose acetate electrophoresis, fraction I remained at the origin, but moved under capillary electrophoresis (peak at -4.0 min), owing to electroosmotic flow.[228] During capillary electrophoresis, the applied voltage creates a net flow of ions migrating towards electrode, which sweep even neutral molecules towards

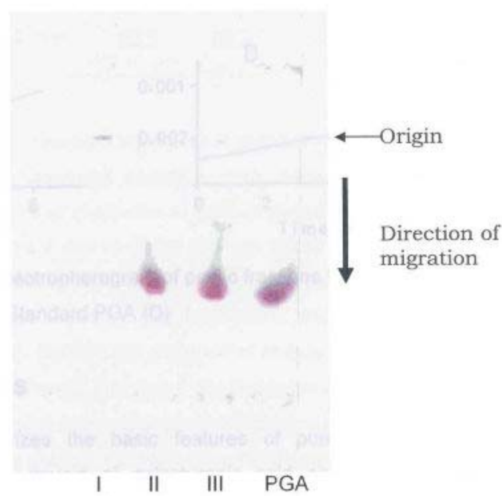


Fig.28: Cellulose-acetate electrophoresis of the pectic po ys. fractions I, II, III and standard PGA (Sigma)

the electrode, but at different rates.[228] Based on the result obtained, all these major pectic fractions, purified by GPC, appeared homogeneous.

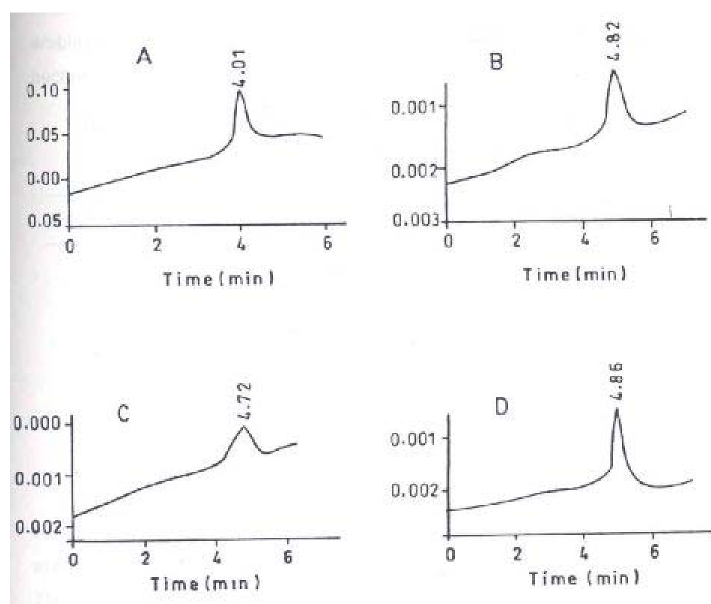


Fig. 29: Capillary electropherogram of pectic fractions I (A), II (B),III (C) and Standard PGA (0)

STRUCTURAL STUDIES

Table 12 summarizes the basic features of purified polymeric fractions. Fraction I was devoid of galacturonic acid and was rich in galactose and arabinose (Fig.3D). It may be an arabinogalactan-type polysaccharide, with a relative ratio of galactose and arabinose, 3 : 1, with small amount of rhamnose and traces of other neutral sugars. The presence of rhamnose in fraction I indicate that these arabinogalactans may be linked to galacturonic acid main chain through rhamnose residues. Similar observation for arabinan from sugar beet was reported.[173] Rhamnogalacturonans branched with several neutral polymers such as arabinans, galactans and arabinogalactans were reported for plant pectins.[169, 173,229,233,257]

Table 12: Physico-chemical characteristics of the three major pectic polymers of mango pulp

Fr. No	Polymeric Frs.	Specific rotation	Composition (Relative %)			
			GalA	Gal	Ara	Rha
I	Neutral	+202°	nil	69.4	23.3	3.3
II	0.05M $(NH_4)_2CO_3$	+287°	69.4	13.7	15.2	1.7
III	0.10M $(NH_4)_2CO_3$	+173°	62.3	23.2	10.3	4.2

Fractions II and III contained a high level of galacturonic acid, 69 and 62%, respectively. They contained neutral sugars, such as galactose, arabinose and small amount of rhamnose in

various proportions (Fig. 30). The composition of fractions II and III [Table 12] was found to be typical of acidic pectic polysaccharide containing more than 62% galacturonic acid. The presence of rhamnose indicates that fractions II and III are typical rhamnogalacturonans (RG). Further, the presence of neutral sugars such as galactose and arabinose showed that these rhamnogalacturonans contain side chains made up of arabinose or galactose or arabinose and galactose oligomers, and presumably present as "hairy region". Rhamnogalacturonans containing side chains composed of arabinose and galactose were reported for other fruits like tomato,241 grape,229 kiwi212 and apple55. 57 The presence of high galacturonic acid content (69 and 62%) compared to neutral sugars in mango pectins probably suggests that large stretches of HGA were

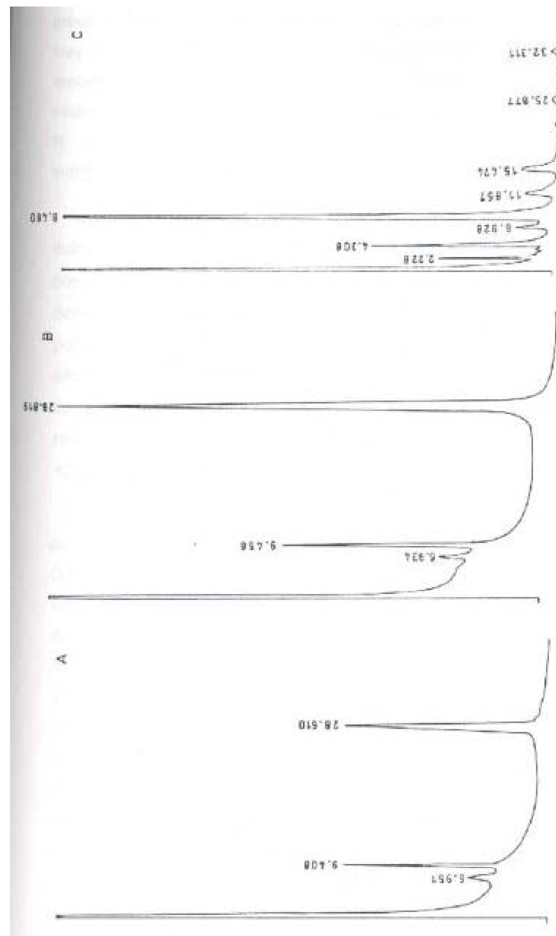


Fig.30: GC profile of sugars (as alditol acetates derivatized from fraction_I (A); fraction – II (carboxyl-reduced) (B) and premethylated fraction – I (C)

present between RG molecules, as reported recently.[294] Covalent linkages may also exist between RG and HGA through borate ester cross-links, as reported recently for sugar beet pectins.[111] Discontinuous distribution of neutral sugar side chains was also reported for apple pectins.[55] Compared to apple pectins, uronic acid content was lower in tomato pectins, suggesting more neutral sugar side chains.[134]

The high positive specific rotation for these fractions [Table 12] indicated that the anomeric configuration of the main chain is 0,- type. High positive specific rotation (+230°) was also reported for citrus pectin.123. 185 Specific rotation was found to be increased upon purification of pectins. High positive rotation, +300°, +277° and +308° was reported, for purified apple, citrus and sunflower pectates, respectively.[185] Recently, a high positive specific rotation (+175°) for pectic polysaccharides from tansy (which is related to sugar beet pectins) and its enzyme treated fractions (+185.8° and +228.4°) was reported.[186]

In order to assess the glycosidic linkage patterns of fractions I and II, permethylation analysis was performed. The derivatives were analyzed by GC-MS on SP2330 capillary column and identified by their relative retention times with respect to 2,3,4,6-tetra-0-methyl glucose and confirmed by their diagnostic mass fragments (mil). Data [Fig. 30, 31 & 32; Table 13] indicated that the major sugars in fraction I led to a 1,4-linked galactose backbone. Thus fraction I was found to be an arabinogalactan having a backbone made up of 1-74 linked galactose residues, which were occasionally involved in side chain branches at 0-6, 0-3,6 and 0-2,3,6 positions, and consisting of single residues of galactose andlor arabinose or oligomeric 1-75 linked arabinofuranose residues, linked through 1 ,3-linkages to the main galactan chain. Arabinose was essentially found in the furanosidic form. Rhamnose was also found as chain linked residues (identification of 3, 4-Me2-Rha) possibly may be through side chain appendages.

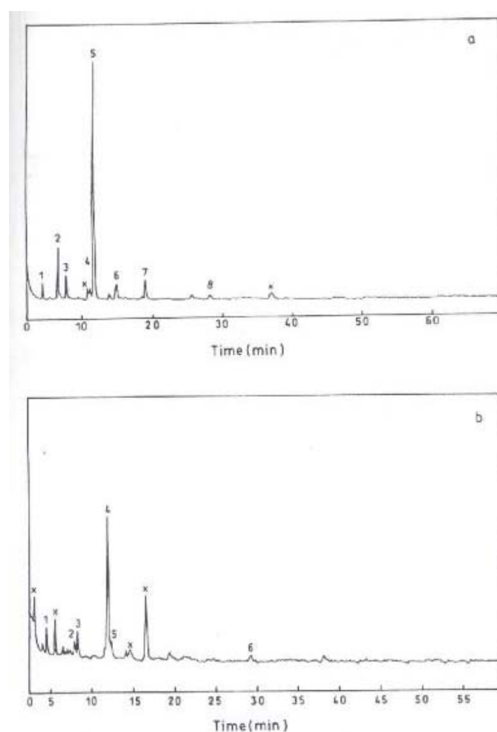


Fig.31: GC-MS profile of permethylated pectic fractions (a) Fraction I (b) Fraction II (carboxyl-reduced). X-non-sugar peak; Peaks 1-8 (6), permethylated alditol acetates [see Table 13]

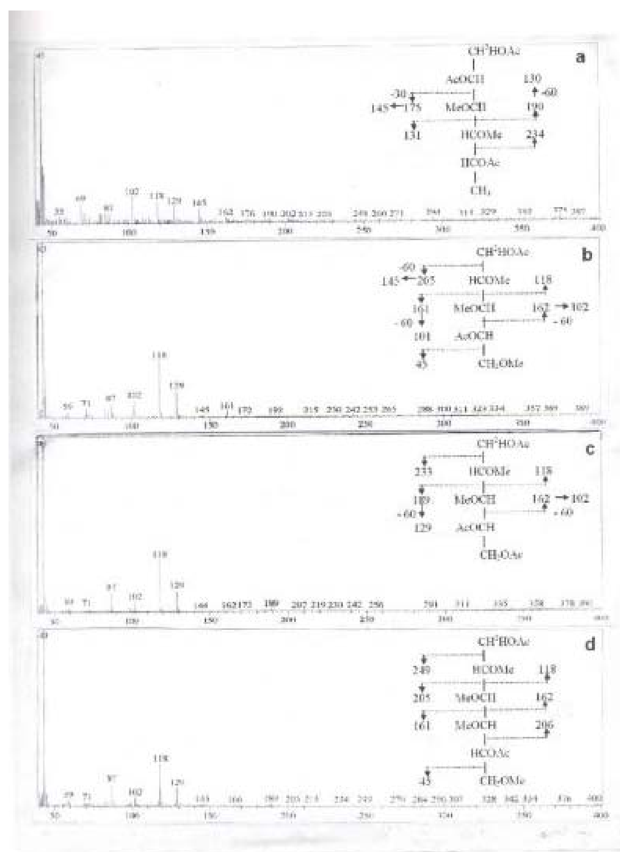


Fig.32: Mass spectra and fragmentation pattern of alditol acetates (a) 3,4-Rha; (b) 2, 3, 5-Araf, (c) 2, 3-Araf, (d) 2, 3, 4, 6-Gal

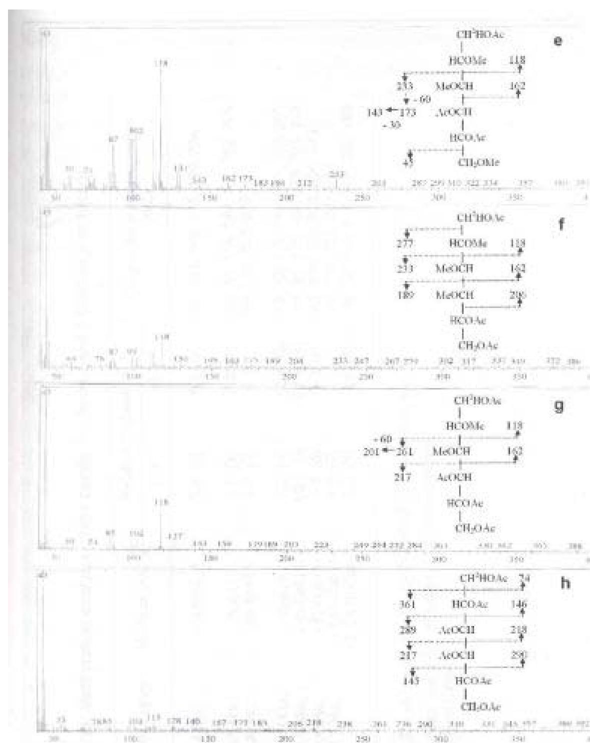


Fig.32: cont..): Mass spectra and fragmentation pattern of alditol acetates (e) 2, 3, 6-Gal; (f) 2, 3, 4-Gal; (g) 2, 3-Gal & (h) Gal

Table 13: Methylation analysis of the pectic fractions I and II (carboxyl-reduced)

Monosaccharide	O-methyl ether	Mode of linkage	Relatice% (peak no*)		Diagnostic mass fragments (m/Z)
			Fr.I	Fr.II**	
Rha	3,4-Me_2	-2)-Rha-(1-	9.4 (2)	5.2 (2)	43, 131, 160, 175, 190,234
Ara	2,3,5-Me_3	Ara-(1-	2.7 (1)	7.5 (1)	43,45,118,145,161,162,205
	2,3-Me_3	-5)-Ara-(1-	3.9 (3)	-	43,118,203,233
Gal	2,3,4,6-Me_4	Gal-(1	3.2(4)	10.3 (3)	43,45,118,145,161,162,205
	2,3,6-Me_3	-4)-Gal-(1-	66.1 (5)	65.9 (4)	43,45,118,162,173,201,233
	2,3,4-Me_3	-6)-Gal-(1-	4.8 (6)	6.3 (5)	43, 118, 162,201,206,233
	2,3-Me2	-4,6)-Gal-(1-	7.6 (7)	-	43,118,162,201,261
	--	-2,3,4,6)-Gal-(1-	2.3 98)	4.9 (6)	43,45,146,217,218,289,290

3,4- Me_2-Rha = 1,2,5 -tri-O-acetyl-3,4-di-O-methyl -L-rhamnitol, etc.

* Relative peak numbers of figure 31 in parentheses

** Carboxyl reduced

Arabinogalactan having these types of linkages has been reported.[250] The presence of high amount of 1,4-linked galactose residues in the neutral fraction is the characteristic feature of type-I arabinogalactan. Arabinogalactan-1 polymer from the chelator-extracted pectins was reported for pineapple fruit cell wall.[251]

When fraction II was directly methylated, without carboxyl reduction, it resulted in poor methylation, as it was rich in galacturonic acid content and was not easily soluble in DMSO. Therefore, prior to methylation, fraction II was carboxyl-reduced using CMC carbodiimide and sodium borohydride. After 4 treatments, the galacturonic acid content was reduced from 69 to 6%, thus resulting in >85% reduction of galacturonic acid. Similar carbodiimide activated carboxyl-reduction was performed for grape and apple pectins.[229, 233]

The major sugars in fraction II were found to be 1,4-linked galactose followed by 1,2-linked rhamnose and terminal arabinoses [see Fig. 31 & 32; Table 13]. Eventually all the galacturonic acid residues were involved in linear 1,4-linkages in the backbone, as evidenced by the quantitative presence of 2,3,6-Me_3-Gal. Some amount of branching was evident by the identification of small amount of free galactose, which were involved in branching off with probably single residues of galactose and arabinose. Identification of 3,4-Me_3-Rha indicates its possible involvement as a 'Kinking' residue in the main chain, and which is further involved in side chain appendages. Thus the carboxyl-reduced fraction II was identified to be a rhamnogalacturonan with 1,4-linked galactopyranosyluronic acid residues in the main backbone chain, which are interspersed with (1-72)-linked L-rhamnopyranosyl residues, which are further involved in branching with galactose. Similar type of linkages was reported for fruit pectins.[47, 169,212,]

The FT -IR spectra of these fractions (Fig. 33) in the frequency range of 400-4000 cm^{-1} characteristic of pectic

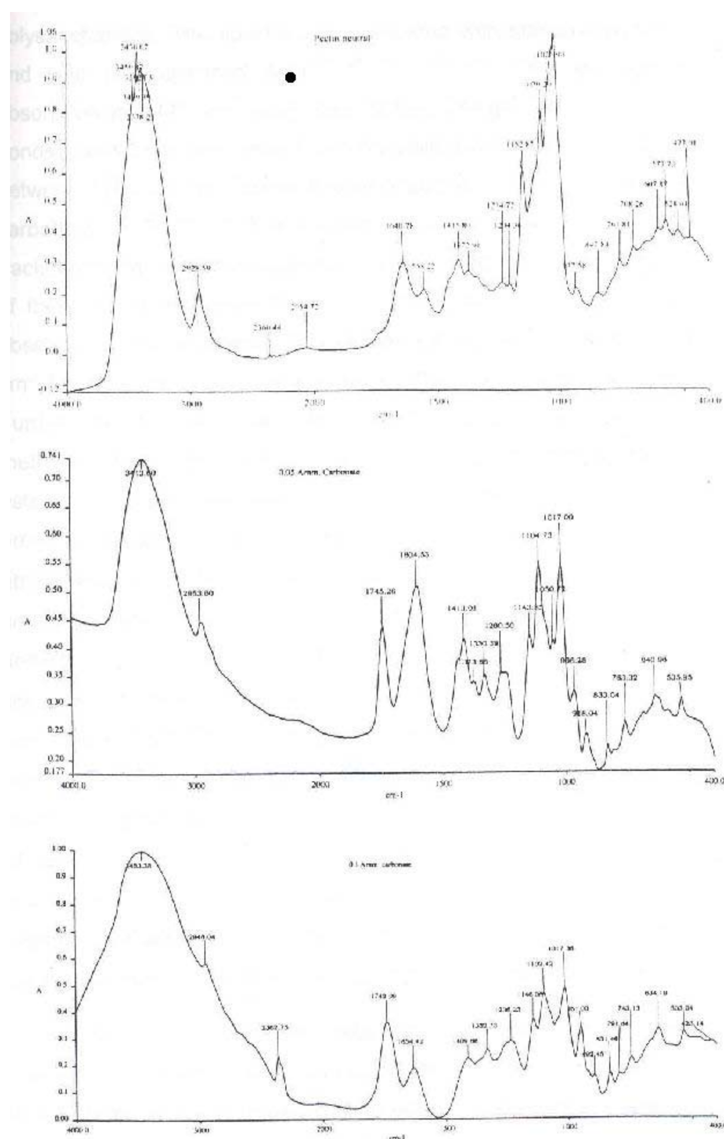


Fig.33: FT-IR spectra of the major pectic fractions

polysaccharides. The spectra was compared with standard pectin (Sigma) and with the published data.45, 46, 117, 151, 255, The spectrum showed absorption at 3440 cm-1 (indicative of free OH groups), 1750 cm-1 (ester bonds), and 1630 cm-1 (due to carboxylate functional group). The region between 1200-850 cm-1 showed several absorption peaks, characteristic of carbohydrates.45, 46,117 The intense peaks at 1104 and 1017 cm-1, for fractions II and III corresponded to galacturonic acid residues. Total absence of these peaks in fraction I indicates that galacturonic acid is completely absent, as shown by metahydroxydiphenyl analysis. The absorption at 1745 cm-1 for fractions II and III, is indicative of the presence of ester bond (C=O). Further, the absorbance at -1420 cm-1, indicative of the presence of pectin ., methyl ester group (O-CH3), probably suggests that these

fractions are esterified. The absorbance at -1260 cm-1 for fraction II is an indication of the presence of acetyl group, as reported by Mathlouthi and Koenig.[151] The absorbance at -830 cm-1 was an indicative of a-configuration, which also correlated with high positive specific rotation for fractions II and III. A minor absorption peak at 892 cm-1 for fraction III may be due to (3-glycosidic linkages[117] between the sugar residues in the side chain galactan, which coincides with its higher relative ratio of galactose. The absorbance at –945 cm^{-1} in fractions I and III is also indicative of a high content of galactose.46 Further, fraction I showed intense peak at 1079 cm-1, which is an indication of galactose residue, as well as (3-(1-76) and (3-(1-73) linked galactan.[117] Intense peak at 1026 cm-1 may correspond to arabinan side chain as , reported by Kacurakova and coworkers.[117] The high positive specific rotation for this fraction is rather surprising, as this fraction is rich in (3-linkages.

The 13C NMR spectrum (obtained only for fraction II) showed signals characteristic of pectic-type polysaccharides (Fig. 34). The spectrum quality is very poor, which is mainly due to viscous nature of the sample, at the concentration required for NMR analysis. Furthermore, the galacturonic acid main chain occurring as backbone of the polysaccharide is too large and

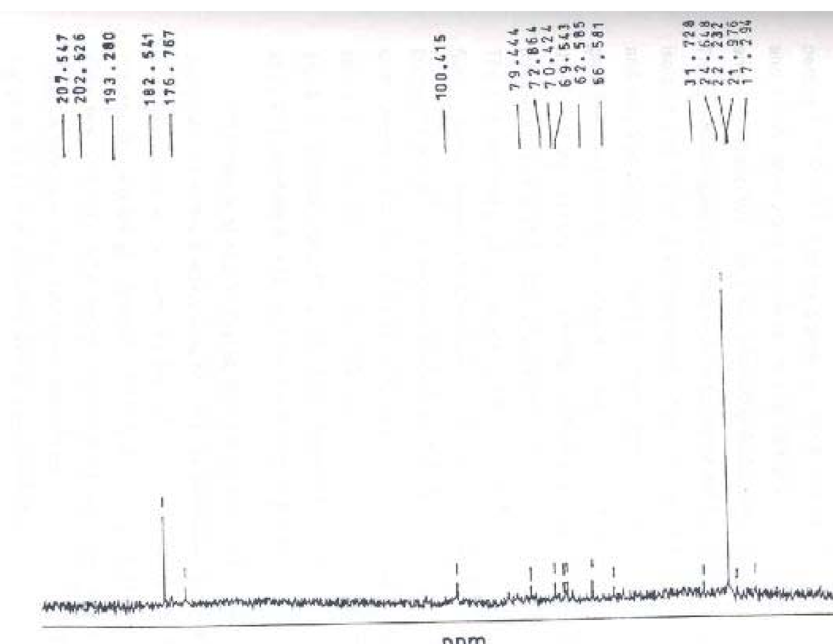


Fig.34: 13C NMR spectrum of the major pectic fractions (Fr.II)

rigid to yield detectable signals, as strong signals are the result of highly flexible chains.[203]

Signal assignments were based on published spectra of several plant pectins.[47, 51, 121,241] The six signals at 100.41, 69.54, 70.42, 79.44, 72.86 and 176.76 ppm, corresponds to the ring carbons, C-1, C-2, C-3, C-4, C-5 and C-6, respectively of 1,4-a-linked-D-galactopyranosyluronic acid units. The assigned signals showed a close similarity with those of a-galacturonan isolated from mung bean hypocotyls,[51] flax, [51] sugar beet,[121] tomato fruit[241] and kiwi fruit pectins.[167] The six signals assigned to

the six carbons of a-galacturonan from apple,[47] grape,[229] and tansy pectins [186] slightly vary from those of mango pectins. The variation in the signals may be attributed to the differences in the molecular organization of pectins from different origins. The C-1, anomeric carbon, signal provides useful information since other five signals occur at chemical shift, which coincides with other polysaccharides.[167] A signal at 17.29 is attributed to C-6 of a-rhamnose units, whereas the one at 62.58 is assigned to C-6 of β-galactose residue linked to a-rhamnose unit by 1→4 linkage. Relatively small intensity of signal at 17.29 indicates the presence of low levels of rhamnose. The signal at 22.23 is possibly due to the presence of methyl groups.

The signal at 182.54 is probably due to the -C=O of acetyl groups, suggesting that this fraction is acetylated. Furthermore, a signal at 22.23 ppm suggests that the pectic fraction (Fr. II) is methylated too. The FT –IR spectra showed an absorbance centered at around 1420 cm-1, which is indicative of methyl ester group. But no signal was observed at 53 ppm, which is indicative of pectin methyl ester bond (-OCH3).47 The absence of signal at 53 ppm is probably due to poor solubility of this pectic fraction. Thus, fraction II is both acetylated and methylated, as reported for other plant pectic polymers.[121, 160,233] Acetylated pectins are reported from other fruits like raspberry.[255]

The data establish that the pectic fraction (Fr. II) is a branched a-1 ,4-linked D-galacturonan. The α-linkage is in good agreement with high positive specific rotation and IR absorption at 820-830cm^{-1} for this fraction (Fr. II). Furthermore, the galacturonan is interspersed with L-rhamnose units, which are further involved in branching.

CHAPTER – IV
PECTIN-DEGRADING ENZYMES IN RIPENING MANGO

I. 1. Enzymes Related to Pectin Hydrolysis in vivo

Summary

The enzymes implied in pectin dissolution are polygalacturonase (PG), pectin methyl esterase (PME), galactanase, arabinanase and β-galactosidase. Generally, all the enzymes showed a climacteric peak in activity during ripening, except for PME, which showed a continuous decreased activity, after an initial increase. A very prominent activity of galactanase and arabinanase in mango pulp coincided with a significant loss of galactose and arabinose residues. When the total pectic fraction was used as substrate for endogenous hydrolysis by the endogenous enzyme preparation (in vitro) the loss of neutral sugars in the pectic fraction was found more prominent than the loss of galacturonic acid residues. A down ward shift in the molecular weight of pectins was observed. Further, this was more pronounced at pH 5.6, when compared to pH 3.6. These observations were comparable with the in vivo study on pectic changes from unripe to ripe stage.

Introduction

The changes in the cell wall composition, which accompany fruit softening during ripening, are due to the action of carbohydrate hydrolases. These hydrolases act on cell wall/cellular polymers, resulting in their degradation. Most of these enzymes increase in activity during ripening, showing an activity peak at climacteric stage. A wide range of cell wall hydrolases was identified in fruit tissues.s, 32, 76, 78, 104,269 Most of them are known to catalyze cell wall disassembly by acting on pectin. The best characterized pectin degrading enzymes are polygalacturonase (PG), pectate lyase, pectin methyl esterase (PM E) and r3-galactosidase. PME activity has been reported for a number of ripening fruits.7. 69. 70.189.202.205.221. 234 Activity of mango PME was shown to decrease69. 190.221 or increase7. 234 or remained constant14 during ripening. PG and r3-galactosidase/ galactanase were reported more extensively in ripening fruits.[36, 38, 54, 62, 140, 168, 180, 198, 199, 200, 201, 202, 207, 223, 224, 285] However, glycanases like galactanase and arabinanase have not been well documented, especially in fruits. Interestingly, these two enzymes were found to be very prominent in mango fruit, which also correlated with loss of galactose and arabinose from the pectic fraction, at the end of ripening.

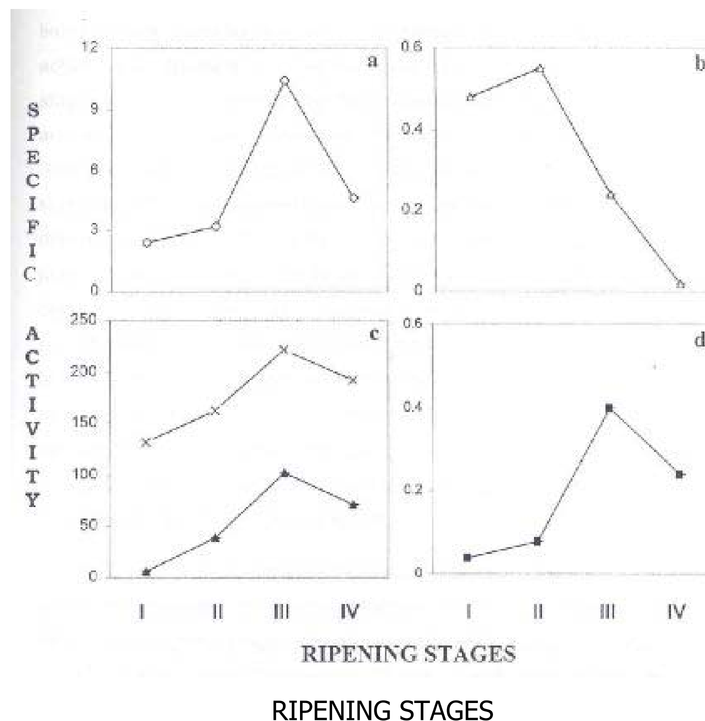
Downward shift in the molecular weights of pectins during ripening of many fruits occur due to the action of PG and other cell-wall degrading enzymes. In in vitro condition, PG was found to degrade the cell-wall polymers from tomato and many other fruits, thus decreasing the molecular weight considerably.[264, 280] Water-soluble polyuronides of strawberry released galacturonic acid and small oligomers, when treated with exo-PG.[168] Thus shift in molecular weight is mainly due to enzymatic cleavage during ripening.

Among the pectin-hydrolyzing glycanases, PG, PME, galactanase and arabinanase were chosen here for following their activity profiles during ripening. The choice glycosidase was β-galactosidase as it was involved in pectin hydrolysis in vivo. In addition, an attempt was made to mimic the in vivo hydrolysis of pectic polysaccharides using endogenous substrates and enzymes.

Results and Discussion

Activity profiles of pectic enzymes during ripening

Fruit softening during ripening is believed to be the result of enzyme-mediated modification of cell wall polysaccharides, particularly the pectin modification. The climacteric peak of mango fruit is reached around 8 days after harvest and ready-to-eat ripe stage by 12th day of harvest, which is in accordance with published report.[136] The enzyme activity of five pectic hydrolases of mango followed at different stages of ripening is depicted in Fig. 35. The enzyme activities were followed at different, defined stages of ripening (see materials and methods). Generally, all the hydrolases showed a climacteric peak in activity during ripening, while PME, an esterase,



RIPENING STAGES

Fig. 35: Activity profile of pectin-hydrolyzing enzymes at different stages (I, II, III & IV) during ripening.

a (-○-) Polygalacturonase (µmol GaiA eq./ h / mg protein)

b (-△-) Pectin methyl esterase (µmol Acid / min / mg protein)

c (-▲-) Galactanase and

(-×-) Arabinanase (µmol reducing group / h / mg protein)

d (-■-) β-Galactosidase (µmol pNP / min / mg protein)

showed a steady decrease in activity. In general, during ripening the rise in enzyme activity results in increased charge density by diminishing the degree of esterification and / or decreasing the molecular weight.114 In mango, the increase in pH (from pH 3.4 to 6.0) during ripening 143 may be a factor influencing the enzyme activities.

Total PG showed an increased activity reaching a maximum (4-5 times the initial activity) at stage-III of ripening. Thereafter, a decrease in PG activity was observed reaching two-times of the initial activity at the final stage (stage-IV) of ripening (Fig. 35a). Similar observation on increasing PG activity upto half-ripe stage and declining thereafter was reported for 'Alphonso' mango.[234] PG activity is barely detected in unripe fruits,[100] but slight activity was observed here. Similar increase in PG activity during ripening was reported in fruits including tomato,[48] banana,[180] papaya,[41] pear,[26] peach,[204] kiwi,[285] nectarine,[147] mango[234] and African mango.[7] In climacteric fruits, the rapid synthesis of PG activity coincides with considerable textural alteration (loss of firmness) during ripening.[194, 221] Part of this increase may probably be due to the de novo synthesis of PG.[143] The rise in the activity of PG coincides with the conversion of pectic polysaccharides to water-soluble galacturonides, observed earlier [Chapter-III, Section 1 & 2]. Thus, PG plays a key role during ripening/tissue softeninG, as suggested by Ahmed and Labavitch.[5]

Activity of PME increased initially, reaching a maximum at stage-II, but dropped steadily as the fruit softened (Fig. 35b). Decreased activity of PME during ripening was also shown in lime,[70] orange,[70] tomato,[202, 282] kiwi,[285] guava,[69] date,[69] strawberry,[69] capsicum[205] and African mango.[7] Similar observation was also reported for other varieties of mango.[69, 221] Recently, increase in PME activity during ripening was reported for banana fruit (six-fold) [189] and papaya.[182]

There was an inverse correlation between PG and PME activities. When PG activity was highest, PME was lowest and vice versa (Fig. 35a & b). PME makes the pectin substrate amenable for PG action. The high activity of PME in the initial stage of ripening is perhaps required for preparing the pectins (i.e., demethylating pectins to pectic acid) for the subsequent attack by PG. This suggests that during ripening deesterification occurs first followed by depolymerization of pectins. Thus there may be a synergistic action between PG and PME in pectin degradation during ripening.[15,160]

A 17 -fold increase in galactanase activity was observed at stage-III of ripening, which decreased to 12-fold at the ripe stage (Fig. 35c). Arabinanase also increased its activity, which actually doubled its initial activity at stage-III, and decreased thereafter. But the initial activity was very high compared to other hydrolases (Fig. 35c). Thus a very high activity of galactan- and arabinan-degrading enzymes, viz; galactanase and arabinanase was noted (Fig. 35c) in mango pulp, which also correlated with a significant loss of the corresponding sugar residues (galactose and

arabinose) from the polymeric fractions of ripe mango (Chapter-III; Section 2). High galactanase activity was also reported during ripening of 'Harumanis' mango.[9]

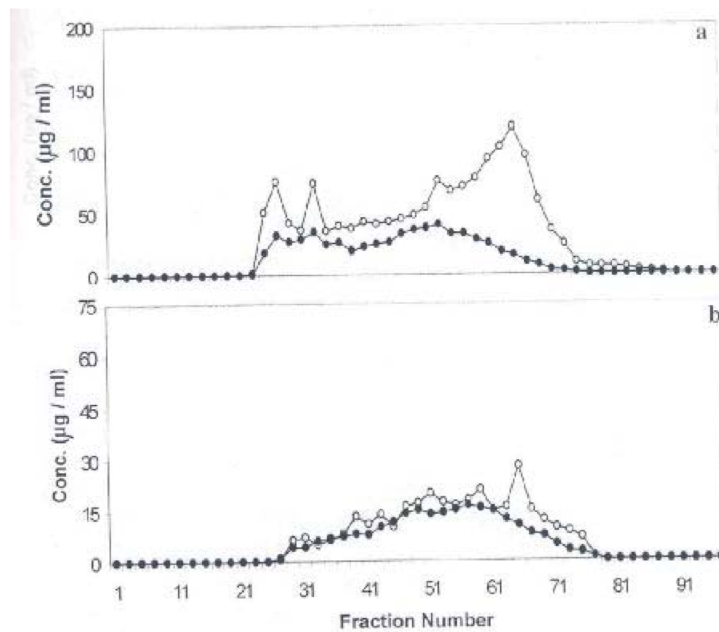
β-Galactosidase activity also increased during ripening, showing a maximum activity (10-fold of the initial) at stage-III of ripening, which then fell to 6-fold in the ripe fruit (Fig. 35d). Increase in activity of β-galactosidase during ripening and over-ripening was reported in fruits like apple,[279] apricot,[30] plum,[59] peach,[59] pear, [59] hot pepper,[93] muskmelon,[207] and tomato.[282,196] However, the low activity of β-galactosidase remained unchanged during ripening in banana [189] and kiwi fruits.[285] It was also found to be the second major glycosidase at stage-III of ripening mango fruit. In avocado mesocarp, despite low PG activity, the in vivo pectin degradation was high which was attributed to β-galactosidase activity. [54] In persimmon fruit, PG activity was almost absent, while the pectin solubilization was high which was attributed to certain other enzymes like galactosidase/ galactanase.[49] Loss of galactose residues during ripening could occur independently of PG activity, which might involve other classes of enzymes like β-galactosidase and galactanase.[9, 38, 40] In mango, PG activity is quite low compared to tomato fruit [142, 143] [Chapter-IV; Section 2], while glycanases like galactanase and arabinanase activities are very prominent as reported recently from our lab.[190, 290] Cellulase was also reported to be an equally important enzyme in mango.[234]

In mango, it was found that all these pectic enzymes; PG, PME, galactanase, arabinanase and β-galactosidase are related to softening of fruit pulp during ripening. Since rhamnogalacturonans are the major pectic polysaccharides of mango [Chapter-IV; Section 2 & 3], the enzyme rhamnogalacturonase (RGase), which degrades these polymers,[233] may be present in mango. The presence of RGase was also shown in other fruits such as bush butter.[160] PG may solubilize the backbone of rhamnogalacturonan-l, while the RGase depolymerizes RG-I into 0ligogalacturonides.[160] It may be concluded that no one enzyme is responsible for the complete degradation of pectic polysaccharides. A variety of cell-wall degrading enzymes may be involved directly in the ultimate modification and dissolution of cell wall.1Oo Thus apart from PG, enzymes such as pectinesterase, cellulase and galactosidase have also been implicated in softening during fruit ripening.[15,93,196]

In vivo and In vitro studies on total pectin and its degradation

The total EDTA-soluble pectic fractions from unripe and ripe mango were subjected to GPC individually (Fig. 36), which may be considered as the in vivo hydrolysis profile.[211] Interestingly, the loss of neutral sugar from the pectic fraction of the ripe fruit was significantly more prominent than the loss of galacturonic acid (Fig. 36). Disappearance of high molecular weight peak was observed indicating a clear downward shift in the molecular weight. Decrease in the proportion of large molecular weight with no change in the proportion of low molecular weight polymers was reported for

persimmon49 and peach fruit chelator-soluble pectins.98 The total pectins from unripe mango fruit are polydisperse in nature and so are not expected to give distinct separations on GPC. However, GPC revealed extensive depolymerization of pectic polymers during ripening, as reported for tomato pectins.[105] Polyuronides from tomato and avocado also eluted throughout the fractionation range of the gel, owing to high molecular weight and polydispersity.[106] However, tomato polyuronides exhibit less rapid and less

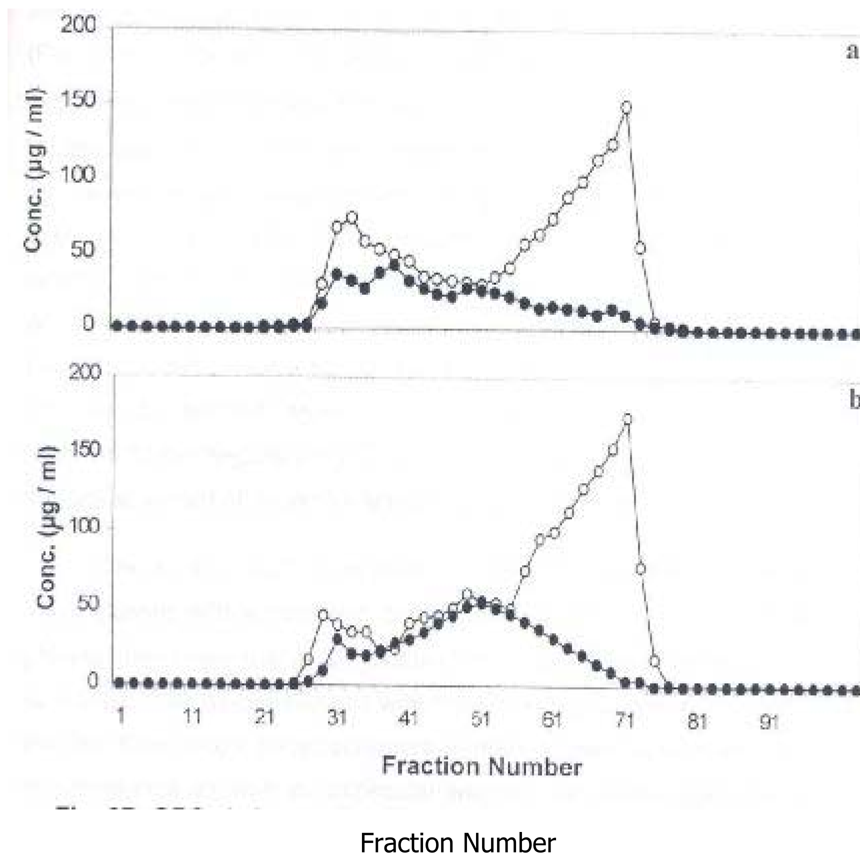


Fraction Number

Fig. 36: GPC elution profile of total chelator-soluble pectic polymers from unripe (a) and ripe mango (b) on Sepharose CL-4B; monitored as Total sugar (-○-); GalA (-●-)

extensive downward shift than those of avocado,[106] indicating that this shift is a general phenomenon during fruit ripening and varies betwee.n species. Similar observation was reported for total chelator-soluble pectins during ripening of kiwi,[211] melon[222] and bush butter fruits.[159]

The observation in vivo could be mimicked in the in vitro (enzyme-treated) experiments (Fig. 37). The in vitro study on total pectin hydrolysis was followed by incubating the substrate (total chelator-soluble pectic fraction from AIR of unripe fruit) with the endogenous enzyme (enzyme preparation from the stage-III). In vitro degradations were performed using acetate buffer at different pHs (pH 3.6 and 5.6). PG and β-galactosidase



Fraction Number

Fig. 37: GPC elution profile of in vitro enzyme-treated total chelator-soluble pectic polymers from unripe mango on Sepharose

CL-4B (a at pH 3.6; (b) at pH 5.6; Total su ar(-○-); GalA (-●-)

were more active at pH ~3.6, while galactanase and arabinanase were more active at higher pH value. The GPC for enzyme- treated pectins were done directly (without dialyzing) to see the presence of liberated monomers or dimers, especially of galacturonic acid, by exo-acting enzymes.

A clear-cut downward shift, i.e., a shift from high molecular weight range to low molecular weight range, of total pectins of unripe mango, when treated with enzymes at pH 5.6, was observed. Further, a more pronounced loss of neutral sugars was observed at pH 5.6, rather than at pH 3.6. As PG is active at pH -3.6, there may be other enzymes, which are active at pH 5.6 and eventually causing dissolution. Further, a large neutral sugar peak in the low molecular weight range, which was absent in in vivo experiment (Fig. 36b), indicates the presence of exo-acting cell wall hydrolases especially β-galactosidase. Enzyme extracts from ripe tomatoes are shown to degrade tomato cell wall material in vitro.280 Degradation experiments on water-soluble polyuronides of strawberry indicated release of galacturonic acid and small oligomers, while large molecular weight polymers remain undegraded mainly due to the presence of exo-acting PG, which removes only terminal galacturonic acid residues.168 The strawberry polyuronide was found to be degraded rapidly to low molecular weight polymers by purified tomato PG.1O2 Tomato cell wall polymers were also reported

to be degraded by PG.[264, 280] However, increase in the apparent molecular weight of pectic fractions was reported in peaches.[98]

The loss of neutral sugars; i.e., galactose and arabinose residues accompanied with a very high activity of galactanase, arabinanase and β-galactosidase unambiguously establish their role in pectin dissolution in vivo in mango. This also correlates with the findings [Chapter-III; Section 2 & 3] that the three major pectic polymers of mango undergoing drastic decrease in abundance as well as molecular weights are arabinogalactan and two rhamnogalacturonans, rich in arabinose and galactose residues.

2. Purification and Properties of Polygalacturonase (a Glycanase)

Summary

Polygalacturonase (PG) from mango pulp revealed three distinct isoforms, which were resolved upon IEC on DEAE-cellulose column with a relative abundance of 68, 6 and 26%, respectively for isoforms I, II and III. They were further purified by GPC using Sephadex G-200 column. The pH optimum for isoforms I, II and III was found to be 3.2, 3.6 and 3.9, respectively. Isoform I was stable over a wide pH range between (4 -7.5), unlike isoforms II and III, which were stable at pH 4 and 5, respectively. The optimum temperature was around 40°C for the three isoforms. Isoform III was more thermostable, comparatively. Their Km value for pectic acid (PGA) was ~0.023%. The Vmax for isoforms I, II and III was 5.7,3.6 and 4.4 µmol galacturonic acid released / h, respectively. The major metal ion inhibitors for mango PG were Cd^{++}, Cu^{++} and Fe^{++}. EDTA completely inhibited the activity of PG-III of mango. Galacturonic acid, galactose, fucose, rhamnose and arabinose stimulated the enzyme activity of PG-I particularly. The major endogenous substrates for mango PG were found to be the two rhamnogalacturonans, having more than 60% galacturonic acid with a difference in the relative ratio of galactose, arabinose and rhamnose. The apparent molecular weight for isoforms I, II and III was 40, 51 and 45 kDa, respectively.

Introduction

PG acts on polygalacturonic acid (PGA) and splits the glycosidic bonds between galacturonic acid units to release oligogalacturonides or free galacturonic acid units. Increase in levels of total PG during ripening has been demonstrated in ripening fruits.[58, 195] PG gene was the first to be cloned from tomato for studying the textural regulation in ripening tomato.[35] The transformed tomato with PG antisense gene resulted in improved fruit with fermer texture and extended shelf life.[35,94] This gave remarkable clues regarding the role of PG in fruit cell wall metabolism.

PG has been purified and studied only from very few fruits, such as, tomato,[8,48,201] pear,[198] peach,[200] cucumber,[155,199] papaya,[41] strawberry[168] recently banana,180 where the existence of PG isoforms was also Nn. Classical separation techniques like ion exchange chromatography IEC), gel permeation chromatography (GPC) and electrophoresis in various combinations are usually employed to purify PG isoforms from different fruits [8, 168, 180]

Despite similar catalytic properties, PGs differ from fruit to fruit, thus reducing the percent homology of the PG genes. Thus, it is necessary to study this enzyme individually in the fruit of choice. Also, PG has not been Idied in mango fruit. PG activity was observed in "Keitt', 'Tommy Atkins' d 'Harumanis' mango [143,161,221] cultivars but was not detected in 'Ngowe' mango.[33] Lazan and

Ali[142] noticed a very low level of PG in mango fruit when compared to tomato. PG activity in ripe mango was found to be 300-fold lower than those of ripe tomato.[163] This low level posed problems in studying PG from mango.

This section describes the method for purification of PG, which was resolved into three distinct isoforms. Their enzymic properties were compared. In addition, their action on the purified endogenous substrates [Chapter-lll; Section 2 & 3] was studied.

Despite the fact that mango fruit showed significantly higher activities of galactanase and arabinanase, PG was the choice enzyme here for purification and further study, mainly because the major pectic polymers of ,mango are rhamnogalacturonans containing high amount of galacturonic acid (>60%). They were found to be the most susceptible substrates for PG isoforms. This study on purification of PG from mango fruit is novel.

Results and Discussion

Total PG activity, when monitored at different stages of ripening showed an increased activity reaching a maximum at stage-III of ripening, and then declined at the final stage of ripening [Chapter-IV; Section 1]. The activity of PG enzyme is generally very low in mango compared to other fruits such as tomato, banana and papaya [Table 14]. Acetone powders prepared from the climacteric stage were extracted and assayed for PG activity. Tomato fruit exhibited -17 times higher PG activity than mango, while banana and papaya showed 4 and 2 times higher activity than mango, respectively. Thus, mango fruit has lowest PG activity when compared to tomato, banana and papaya. A very low level of PG in ripe mango when compared to ripe tomato was also reported. [142] Furthermore very low level (260-fold decreased level) of PG in 'Kiett' and 'Tommy Atkins' varities than 'Harumanis' variety was reported. [143] 'Mangoa' mango had lower PG activity than 'Harumanis' mango but softened more extensively than the 'at' Similar low level (300-fold decreased level) of PG activity was reportE ripe kiwi Fruit when compared to tomato. However, no PG activity was

Table 14: Comparison of PG activity in some important climacteric fruits

Fruits	PG activity (µmol GaIA eq./h)
Tomato	35.44
Banana	9.19
Papaya	4.06
Mango	2.17

detected in ripening fruits such as hot pepper,93 muskmelon 153 and 'Ngowe' mango.33 Thus considerable differences exist between species and even between cultivars of the same species.

Purification of PG from mango fruit pulp

Initially, different buffers were tried for PG extraction, based on literature reports.[2, 8, 41,180,194.198,205] Citrate buffer (0.1 M) containing 1.3 NaCl (pH 4.9) was chosen for further extraction and purification as this buffer system, when used showed highest specific activity for PG, though the total activity was higher in 0.5 M NaCI, pH 6.0 [Table 15],

Table 15: Extraction of mango PG using different buffer systems

No.	Buffer system	Units	Protein (mg/g AIP)	Sp. Act.
1	0.5 M NaCI (pH, 6.0)	13.26	1.93	6.9
2	1.3 M NaCI: citrate (pH, 4.9)	10.38	0.74	14.0
3	1.0 M NaCI (pH, 5.8)	9.66	1.47	6.6
4	1.2 M NaCI (pH, 6.2)	5.97	1.75	3.4
5	1.0 M NaCI: acetate (pH, 4.5)	5.93	1.03	5.8

Units=μmol GalA equivalent / h / g acetone insoluble powder (AIP)

For concentrating the enzyme during purification, sucrose was preferred as it retained -90% of the initial enzyme activity [Table 16]. Sucrose was used for concentrating enzyme extract of banana PG.[180] Generally PGs are heat labile and sucrose present in the enzyme extracts protects PG from the heat inactivation.[123] In addition, during $(NH_4)_2SO_4$ precipitation, considerable loss of tomato PG activity was reported.[194]

Table 16: Different ways of concentrating PG extract

Concentration	PG activity (μmol/h/1 0 9 AIP)
Crude	98.8
Sucrose	89.96
Salt ppt.(40-80%)	69.60
Lyophilization	49.02

The PG activity in mango fruit pulp was clearly resolved into three distinct isoforms separable upon IEG with DEAE-cellulose (Fig. 38). Based on the order of their elution profile from the column, they were designated as PG-I, II and III, with relative abundance of 68, 6 and 26 % (on the basis of activity peak area), respectively. PG-I (the most abundant isoform) was found eluted in the buffer wash, before gradient elution. The unadsorbed PG-I was not retained even on GM-cellulose. The column retained PG activity was further resolved into two different peaks upon elution with linear gradients of NaGI (0 -1M). PG-II and III were found eluted between 0.1-0.2 M and between 0.2-0.35 M NaGl, respectively (Fig. 38). Different molecular forms of enzymes show different elution pattern on ion exchange column chromatography.[168, 180, 198, 200, 201] Further, since the protease inhibitor (PMSF)

was used during extraction, these isoforms do not represent any artifact caused by the action of protease enzyme.

Three isoforms of PG were reported for banana[180] and strawberry[168] upon IEC, at the climacteric stage of ripening, while pear,[198] peach[200] and tomato[201] showed only two isoforms. In tomato, IEG was used to separate the exo-PG.[192] Differential expression of tomato PG isoforms at different stages of ripening was demonstrated.[48, 193, 195] The significance of the occurrence of multiple forms may be related to the complex nature of pectic polymers and the variety of modification they undergo during ripening.[202]

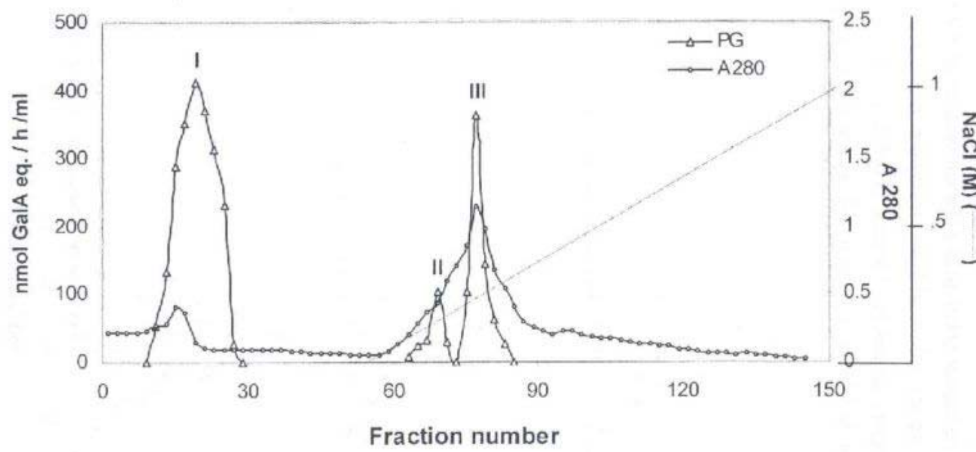


Fig.38: IEC profile fo PG from mango on DEAE-cellulose

All the three isoforms were individually subjected to GPC, based on which they showed molecular weights in the range of Mr 40-51 kDa (Fig. 39). Isoforms I, II and III showed a molecular weight of 40, 51 and 45 kDa respectively. Generally, the molecular weight of mango PG isoforms

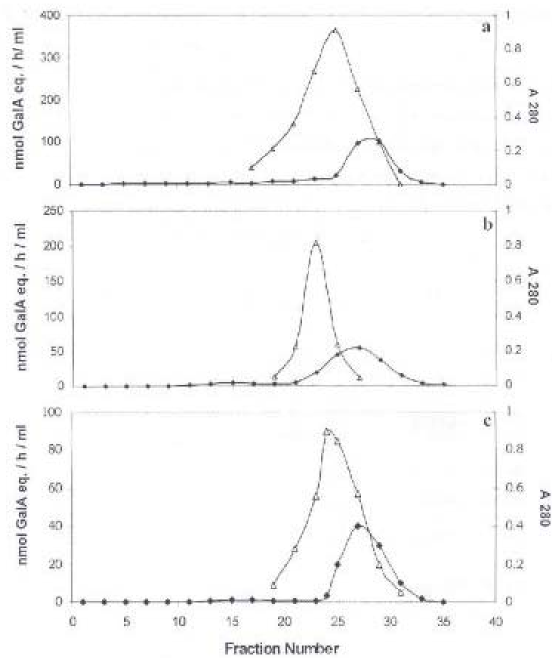


Fig. 39: GPC profiles of PG isoforms on Sephadex G-200

(a) PG-I, (b) PG-II & (c) PG-III

A 280 (-◆-); Activity (-△-)

was slightly lower when compared to other fruit PG isoforms, which were in the range of 50-59 kDa.[168, 180, 199] However, Bartley and Knee23 reported molecular weight in the range 160 -41 kDa for fruit PGs. In banana PG-I and III showed a molecular weight of 23.2 and 130 kDa, respectively.[180] The molecular weight of mango PG-I and PG-III was close to one of the tomato PGs.[8, 48, 201] Molecular weight of PG-II of mango is almost similar to that of PG2 of strawberry.[168] Difference in molecular weights is one of the characteristics of isoforms.

Figure 40 shows the PAGE profiles for the enzyme fractions, which revealed a faint band around 33 kDa for the lEG fraction. Fraction II additionally showed a few protein bands in the higher molecular weight range, while fraction III showed protein bands both at high and low molecular weight range. Post GPG fractions could not be subjected to PAGE (native and SDS) due to their low yield.

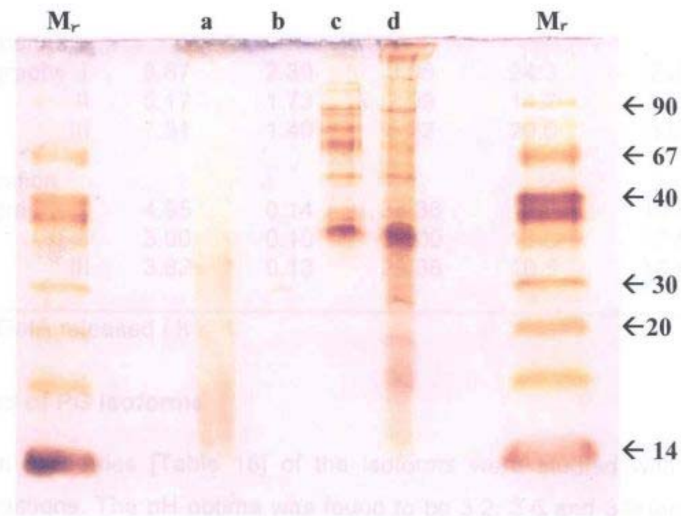


Fig. 40: SDS-PAGE of IEC fractions. Mr: reference marker proteins in the range 14-94 kD. A) crude; b) unadsorbed (PG-I); (c) 0.1-0.2 M NaCl eluate (PG-II); d) 0.2 – 0.35 M NaCl eluate (PG-III)

Table 17 summarizes the purification of PG from mango pulp. The specific activity for PG-I, II and III was 3.9, 3.0 and 5.2, respectively upon 'lEG, while it was 34, 30 and 29, upon GPG. The fold purification of isoforms I, II and III were 2.2, 1.7 and 3.0, respectively, upon lEG, while it was 19.4, 17.0 and 16.6 upon GPG, with a final recovery (post GPG) of 13.6, 11.0 and 10.5 % of the initial enzyme activity. It must be noted that PG in general was not found to be a stable enzyme.[123] Low recoveries of the initial enzyme activity were also reported for other fruits like cucumber,[155] papaya,[41] tomato,[8] strawberry [168] and banana.[180]

Table 17: Summary of purification of PG from mango

FractIons	Activity (U)	Protein (mg)	Sp. Activity	Yield (%)	Fold Purification
Crude	36.47	20.62	1.77	100	1.00
Anion Exchange					
Chromatography I	8.87	2.30	3.86	24.3	2.2
II	5.17	1.73	2.99	14.2	1.7
III	7.31	1.40	5.22	20.0	3.0
Gel permeation					
chromatography I	4.95	0.14	34.36	13.6	19.4
II	3.00	0.10	30.00	11.0	17.0
III	3.82	0.13	29.38	10.5	16.6

U= μmol GalA released / h

Properties of PG isoforms

The properties [Table 18] of the isoforms were studied with GPC purified fractions. The pH optima was found to be 3.2, 3.6 and 3.9 for PG-I, II and III, respectively. PG-I showed pH stability over a wide range of pH, 4 to 7.5, with high stability at pH 5.0. PG-II and III showed pH stability at pH 4 and 5, respectively (Fig. 41). The PG isoforms from mango resembled those of banana fruit180 in their pH optima, which was ~3.5, while that of pear,[198] cucumber,[199], peach,[201] and strawberry168 showed a higher pH optima in the range of 4-5.5. The pH optimum of ~4.8 for crude mango PG was reported. [138,161]

Table 18: Properties of PG isoforms of mango

Properties	Polygalacturonase		
	I	II	III
% Abundance (IEC)	68	6	26
Optimum pH	3.2	3.6	3.9
PH stability	3.9-7.5	3.9	5.1
Optimum Temp. (°C)	37	42	37
Thermal stability (°C) (50% activity retained)	54	41	69
K_m (%PGA)	0.025%	0.023%	0.022%
V_{max} (µmol GalA / h)	5.7	3.6	4.4
M_r (kDa)	40	51	45

The three isoforms of PG showed differences in their temperature optima and thermal tability. The temperature optima were found to be 37, 42 and 37°C, respectively for PG-I, II and III. Labib and coworkers[138] reported a temperature of 30-35°C, with maximum activity at 30°C for crude mango PG. The temperature stability (i.e., T_m= Temperature at which 50% activity is retained) was 54, 41 and 69°C for PG-I, II and III, respectively. Comparatively, PG-III appeared to be more themostable than PG-I and II (Fig. 41), which is similar to PG3 of banana.[180] Thermostable isoform (PG-I) was also reported for tomato.[201] PG-I and II showed closer thermal stability to that of tomato[201] PG-II and strawberry[168] PG2 isoforms

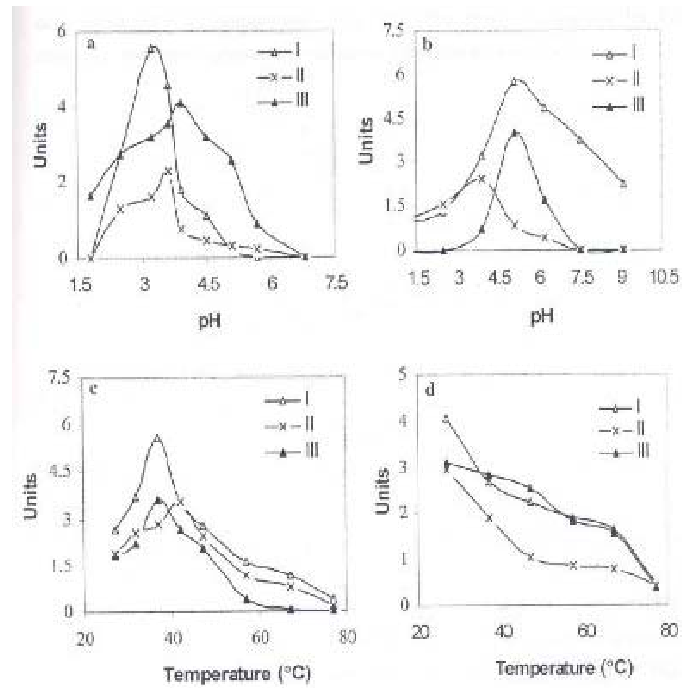


Fig. 41: Effect of pH on activity (a) and stability (b) and effect of Temperature on activity (c) and stability (d) of PG isoforms

Units / 20µl = Amount of enzyme required to release 1 µmol GalA / h

respectively. Differences in the thermostability of the isoforms of PG were also reported in tomato[201] and banana.[180]

The apparent K_m values for PG-I, II and III were 0.025, 0.023 and 0.022% for PGA, the V_{max} values being 5.7, 3.6 and 4.4 µmol galacturonic acid released / h, respectively (Fig. 42). The lower Km values for PG isoforms indicated higher affinity towards polygalacturonic acid.

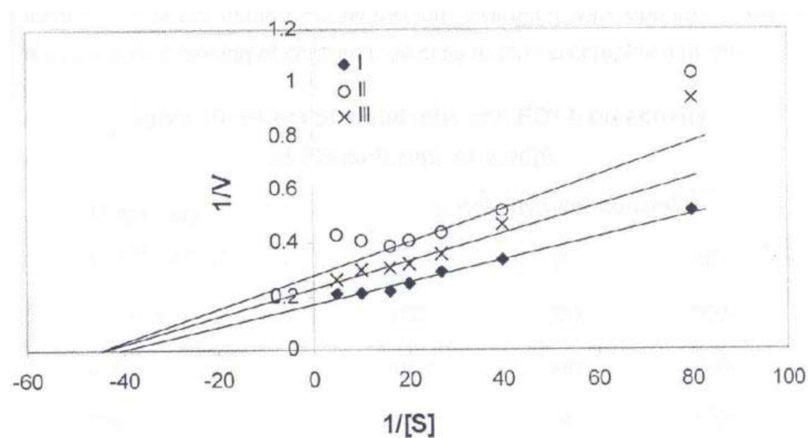


Fig. 42: Double reciprocal Lineweaver-Burk plot for PG isoforms I, II & III

As for the effect of metal ions and EDTA, at 1 mM level [Table 19], Cd^{++} showed a percent inhibition of 76, 57 and 100, respectively for PG-I, II and III. Fe^{++} and Cu^{++} inhibited only PG-III to

the extent of 55%. EDTA showed 100% inhibition for PG-III, while it did not show such a significant inhibition for PG-I and II. Ca^{++} and Zn^{++} stimulated the activity of PG-II significantly, while there was only a slight activation of PG-I and III. Mg^{++} also has the stimulatory effect on PG-I isoform. Ions like Mn^{++} and Hg^{++} were not followed as they interfere with the ferricyanide method of reducing group estimation. Similar inhibition was reported for banana PG.[180] Additionally, banana PG180 was also inhibited by Mg^{++} unlike in mango. Infact, Mg^{++} showed stimulation of PG-I activity in mango. Similar inhibition of strawberry PG2 by metal ions was also reported.[168] The inhibition of mango PG by EDTA was similar to that of strawberry[168] and cucumber PGS.[199] Interestingly, EDTA activated PG-II of banana fruit.[180] Activation of PG-II of mango by Ca^{++} was also similar to Ca^{++} activation of PG1 of banana 180 andPG2 of strawberry.168 Tomato exo-PG was also found to be activated by Ca^{++}, as 0.4 mM calcium was required for maximum activity.[192] Recently, inhibition of crude mango PG by calcium infiltration was reported,[208] which may be due to binding of calcium to pectins to form a complex network.

Table 19: Effect of metal ions and EDTA on activity of PG isoforms of mango

% Activity over control

Metal ions (1mM conc.)	% Activity over control		
	I	II	III
Control	100	100	100
Ca^{++}	100	156	108
Mg^{++}	124	94	100
Fe^{++}	95	63	46
Zn^{++}	119	169	108
Cu^{++}	105	63	46
Cd^{++}	24	43	0
EDTA	86	81	0

The effect of product analogues (monosaccharides) on PG isoforms is shown in Table 20. All the sugars tested, except fucose, showed significant inhibitory action on PG-II. Mannose and xylose inhibited PG-I and III considerably, while glucose inhibited PG-I, apart from PG-II. Strikingly, PG-I was stimulated by sugars like fucose, rhamnose, arabinose, galactose and galacturonic acid quite significantly, while PG-III was activated only by galacturonic acid and fucose, and by glucose to some extent. It is worth noticing here that PG-II was not activated by any sugars tested, intact it was inhibited by most of the sugars.

Table 20: Effect of product analogues on the activity of PG isoforms of mango

Product analogues (2mM conc.)	% Activity over control

	I	II	III
Control	100	100	100
GaiA	114	75	140
Gal	133	18	60
Glc	29	36	116
Man	10	4	40
Fuc	276	100	216
Rha	295	71	96
Ara	176	18	36
Xyl	19	23	12

Table 21 shows the substrate specificity of PG isoforms. There was considerable hydrolytic activity of all the three PGs on galactomannan, which may be due to a-linkage of galactose units, similar to that of galacturonic acid units in pectic polymers. The activity of purified PGs on purified endogenous substrates when tested showed some hydrolytic activity of PG-I and II towards arabinogalactan, while PG-III showed no activity on that substrate. Both the rhamnogalacturonans were hydrolysed by all the three PG isoforms [Table 21]. PG activity was much higher towards rhamnogalacturonan-1 than rhamnogalacturonan-2 [Table 21], which may be due to higher ratio of galacturonic acid to neutral sugars and their branching, which interrupts hydrolysis. Purified tomato PG was also found to degrade the tomato cell wall material in vitro.[264] However, PG from tomato and pears showed no activity towards rhamnogalacturonans.[198, 201]

Table 21: Activity of PG isoforms of mango on natural and endogenous substrates

Substrates	% Activity over controi		
	I	II	III
Control (0.06% PGA)	100	100	100
Natural substrates			
Pectin (0.06%)	0	0	0
Galactomannan (0.045%)	30	29	20
Microcrystalline-Cellulose (0.045%)	0	0	0
Endogenous substrates			
Arabinogalactan	8	9	0
Rhamnogalacturonan-1 (0.05M (NH4)2CO3 eluate)	57	73	75
Rhamnogalacturonan-2 (0.10M (NH4)2CO3 eluate)	43	55	61

Though, total PG activity in mango fruit is much less when compared to tomato, banana and papaya, mango PG was also expressed as three distinct isoforms at the stage-III, as in the case of banana fruit PG.[180] When the endogenously hydrolysable substrates and the in vivo hydrolysis were assessed in mango pulp, the two major pectic polymers, i.e., rhamnogalacturonans, were found to be the susceptible substrates for PG isoforms. The two rhamnogalacturonans were composed of galacturonic acid, arabinose, galactose and rhamnose in the relative ratio of 69 : 15 : 13 : 2 and 62 : 10 : 23 : 4, respectively [Chapter-III; Section 2 & 3]. Mostly, they are the endogenous substrates hydrolysed by PG in mango, probably contributing to the loosening of cell structure at the middle lamella of the cell wall. Both the rhamnogalacturonan contained more than 60% galacturonic acid and thus formed ideal endogenous substrates for PG, an enzyme that is specific for galacturonic acid chains. The other major residues such as galactose and arabinose found in the heterogalacturonans could be hydrolysed by galactanase/ galactosidase and arabinanase, which are very active in mango [Chapter-IV; Section 1]. These may be responsible for total pectin solubilization. The decrease in the molecular weights of solubilized pectins during ripening suggests a key role for PG in pectin solubilization, and eventually in textural softening.[200] In vitro hydrolysis of cell walls by purified PG also suggests a possible role for this enzyme in cell wall degradation and fruit softening process.[264] The product of PG action on cell wall pectins, viz, galacturonic acid and oligomers are found to regulate, induce or to elicit ethylene biosynthesis.[37,125] Thus this enzyme playing a role in ripening process is very clear.

To summarize, the results indicated that mango PG is responsible for the degradation of pectins in mango during ripening. Inhibition of this enzyme is of commercial importance for prolonging the post harvest shelf-life of mango.35 However, as evident by structural studies, mango pectins being rhamnogalacturonans interspersed with long stretches of homogalacturonans, both PG and rhamnogalacturonase are implicated in tissue softening and inhibition of anyone of these enzymes will not be sufficient for delaying the softening process during ripening. This might be the reason why inhibition of PG in tomato did not result in delayed softening.

3. Purification and Properties of β-Galactosidase (a Glycosidase)

Summary

Mango contains three isoforms of β-galactosidase, which are distinctly separable on IEG. The isoforms were designated as I, II and III, based on their order of elution from the IEG column. They were further purified individually by GPC. The overall specific activity increased from 20 to 727 upon purification, resulting in -36 fold purification with a recovery of 28%. The pH optima for activity and stability were in the range of 3.6-4.3 and 3.6-6.2, respectively. The optimum temperature for β-galactosidase activity was between 42-47°C and the T m was in the range of 45-51 °C. The Km for pNPG was 0.98, 1.11 and 0.95 mM respectively for isoforms I, II and III, similarly their Vmax was 0.56, 0.53 and 0.35 /lmol pNP / min. Hg^{++} showed a very powerful inhibition of all the three isoforms. Galacturonic acid, galactose, xylose, fucose and mannose slightly inhibited the activity of β-galactosidase isoforms. Their apparent molecular weight by GPC was 78, 58 and 91 kDa for isoforms I, II and III, respectively. The ability of these isoforms to degrade the endogenous substrates (arabinogalactan) possibly suggests a role in pectin dissolution during tissue softening / fruit ripening.

Introduction

Tissue softening during ripening of many fruits is the end result of enzymatic degradation of cell wall and cellular polysaccharides. The recent model of cell wall structure suggests that galactans and xyloglucans are instrumental in cross-linking cellulose and pectin components.[34] Arabinan, galactan and arabinogalactan250 are the structural components of pectic polysaccharides, apart from rhamnogalacturonans.

It is very well understood by molecular evidence that PG activity alone is not responsible for the degradation of the pectins to the extent that occurs during fruit ripening.[82] These evidences stimulated further research on other cell wall hydrolases, especially glycosidases. β-Galactosidase, a glycosidase, acts on short chain oligomers of galactose residues present either as homo- / heteropolysaccharides or glycoproteins or glycolipids. Loss of galactose from the branched pectins during ripening is mainly attributed to the action of this enzyme[129]

β-Galactosidase has been detected in a wide variety of fruit systems,[59] but purification and study of enzyme properties were reported only for a few fruits like tomato,[196] apple,[62, 224] orange,[36] muskmelon,[207] avocado,[54] coffee berry,[83] kiwi,[127,223] sweet cherry[12] and sapota.[66] Recently the enzyme was purified from the 'Harumanis' mango cv. (Indonesian CV.)[132] and some preliminary properties were reported, where they showed the existence of isoforms.[9] The endogenous substrates for this enzyme and the molecular weight for these isoforms have not been reported. This enzyme is also

implied in pectin dissolution by way of deglycosylating the galactan, which is generally present in pectin-type of polymers. Here, β-galactosidase was purified and studied in 'Alphonso' mango fruit. A more detailed study of the properties for all the three isoforms is presented here. In addition, we have shown the presumable endogenous substrates of pectic-type polysaccharide identified for this enzyme from the same fruit. The significance of this enzyme in the context of fruit ripening / softening is discussed.

Results and Discussion

β-Galactosidase activity increased during ripening with an activity peak at stage-III of ripening, and decreased thereafter as softening progressed [Chapter-IV; Section 1]. This increased activity of β-galactosidase was well correlated with loss of galactose during ripening [Chapter-III; Section 2]. Similar observation was reported for apple and 'Harumanis' mango, which accompanied fruit softening.[9, 279] The activity of β-galactosidase also increased during development of mango fruit.[206] Furthermore, the low level of polygalacturonase in mango also accelerated the study on these glycosidases. Purification of this enzyme was performed with the enzyme collected at stage-III of ripening.

Purification of β-galactosidase from mango fruit pulp

β-Galactosidase from mango pulp, when subjected to purification upon lEG with DEAE-cellulose was resolved into three distinct activity peaks, which were designated as isoforms I, II and III, based on their order of elution from the column (Fig. 43). Different isoforms having difference in their elution profile on DEAE-cellulose have been reported for many fruits including muskmelon.[207] Their relative % abundance based on activity peak area was 44, 38 and 18 for isoforms I, II and III, respectively. β-Galactosidase isoform-1 was found in the initial buffer wash, while isoforms II and III were eluted in the range 0.02-0.2 M and 0.2-0.27 M NaCl, in the linear gradient of NaCI (0-1 M). Surprisingly, the enzyme activity of isoform was lost when subjected to CM-cellulose chromatography. They were individually subjected to GPC purification on pre-calibrated Sephadex-G-200 (Fig. 44). Based on the elution volume, their molecular weights were found to be 78, 58 and 91 kDa, respectively for isoforms I, II and III. Multiple forms of β-galactosidase have been already reported in ripening fruits.9. 196. 207 The occurrence of multiple forms is attributed to the complex nature of the side chains of pectins and their breakdown during ripening. The molecular weight for the three isoforms of mango was similar to those of tomato isoforms 196 and differed from that of sapota66 and apple.62 The molecular weight of Isoform-11 was similar to β-galactosidases of sweet cherry,12 kiwi,223 and avocado.54 Various other plant β-galactosidases in the molecular weight ranging from 46 -300 kDa have been reported.[23.59]

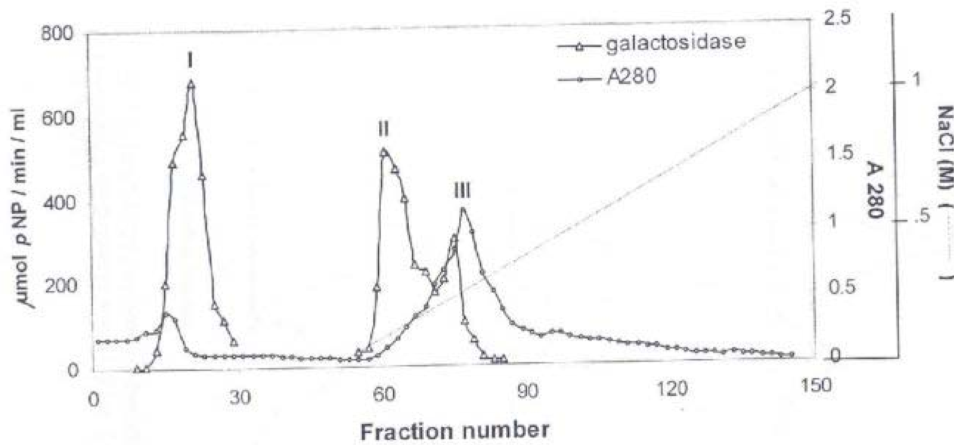


Fig.43: IEC profile of β-Galactosidase from mango on DEAE-cellulose

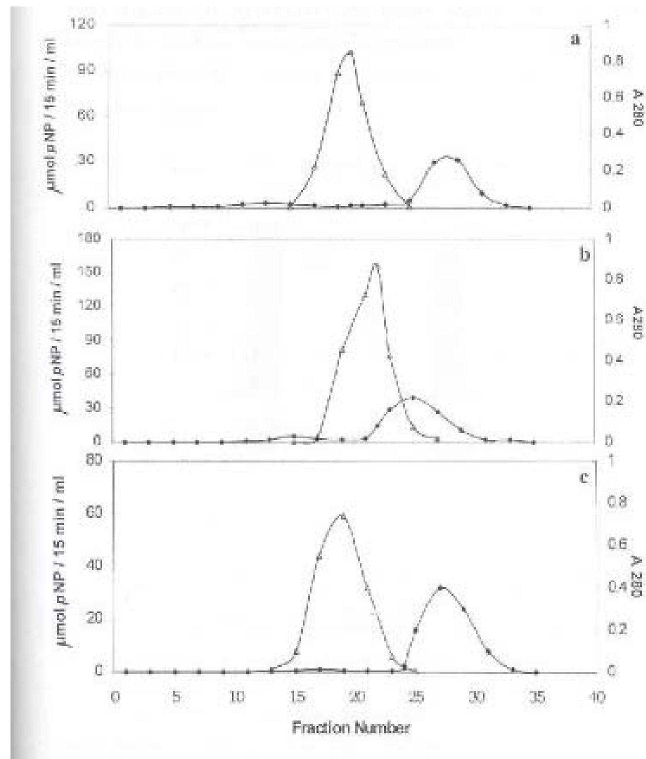


Fig.44: GPC profiles of β-galactosidase isoforms on Sephadex G-200
(a) β-galactosidase-I, (b) β-galactosidase-II (c) β-galactosidase-III.
A_{280} (-◆-); Activity (-△-)

bands suggests that the purified β-galactosidase is almost homogeneous. With SDS and marcaptoethanol, the relative mobility changed to a molecular weight of ~ 26 kOa. Thus, Isoform-11 appeared to be a dimer. For Isoforms I Ind III, PAGE profile could not be obtained for post GPC fraction due to very low protein yields.

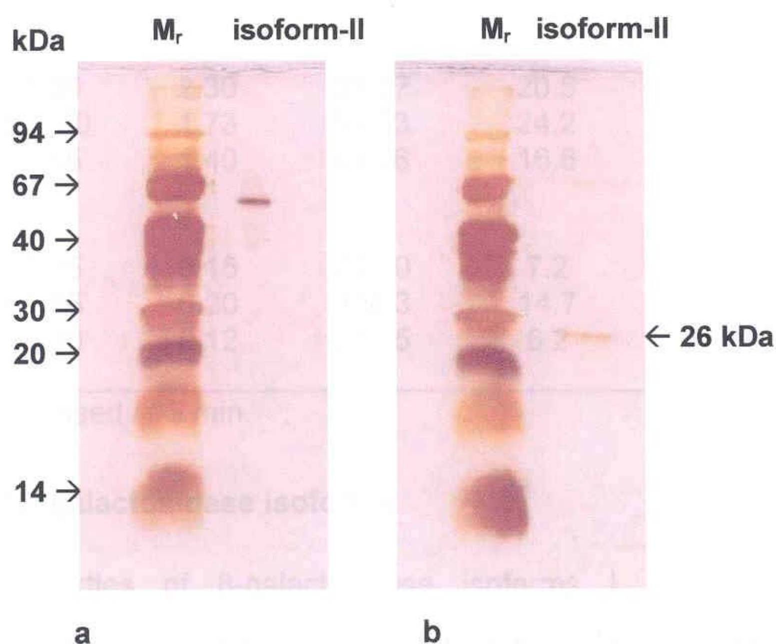


Fig. 45: SDS-PAGE of post GPC fraction of β-galactosidase isoform-II. Left lanes: reference Mr marker.
a) without marcaptoethanol; b) with marcaptoethanol

The entire purification profile of β-galactosidase of mango is summarized in Table 22. The specific activity of 20 increased to 37, 59 and 50 upon IEG, which further increased to 201, 308 and 218 upon GPC for isoforms I, II and III respectively. The total activity finally resulted in 7- and 36-fold purification upon IEG and GPC, respectively. Individually it was 10, 15.2 and 10.7 -fold purification, with a recovery of 7, 15 and 6%, respectively for isoforms I, II and III, with an overall recovery of 28%.

Table 22: Summary of purification of β-galactosidase from mango

Fractions		Activity (U)	Protein (mg)	Sp. Activity	Recovery (%)	Fold Purification
Crude		418.50	20.62	20.29	100	1.00
IEC						
	I	85.95	2.30	37.37	20.5	1.8
	II	101.20	1.73	58.53	24.2	2.9
	III	69.65	1.40	49.76	16.6	2.5
GPC						
	I	30.15	0.15	201.0	7.2	10.0
	II	61.65	0.20	308.3	14.7	15.2
	III	26.10	0.12	217.5	6.2	10.7

U=μmol pNP released / 15 min

Properties of β-galactosidase isoforms The properties of β-galactosidase isoforms I, II and III are consolidated in Table 23. The pH optima for β-galactosidase-l, II and III were 3.6,4.3 and 3.6 respectively. Isoforms II was stable over a range of pH 3.6-6.2, while Isoform I and III were stable at a narrow pH range, with maximum at 6.2 and 5.1, respectively (Fig. 46 a & b). Similar pH optima were reported for tomato,[196] apple,[62] coffee berry,[83] and 'Harumanis' mango[9] while it was lower than that of sapota β-galactosidase.[66] The pH stability of mango β-galactosidases was generally similar to that of tomato β-galactosidase isoforms.[196] The optimum temperature for β-galactosidase activity was 47°C for isoforms I and II, and 42°C for isoform-III (Fig. 46 c & d). The thermal stability (T_m = temperature at which 50% activity retained) was 51, 48 and 45 °C for isoforms I, II and III, respectively.

The Km for pNP-β-galactopyranoside was 0.98, 1.11 and 0.95 mM respectively for isoforms I, II and III, similarly their V_{max} was 0.56, 0.53 and 0.36 μmol *p*NP / min (Fig. 46e). The K_m values for all the three isoforms of

Table 23: Properties of β-galactosidase isoforms of mango

Properties	β-Galactosidase		
	I	II	III
% Abundance	44	38	18
Optimum pH	3.6	4.3	3.6
pH stability	6.2	3.6-6.2	5.1
Optimum Temp. (°C)	47	47	42
Thermal stability (T_m) (°C)	51	48	45
K_m (mM *p*NPG)	0.98	1.11	0.95
V_{max} (μmol *p*NP/ min)	0.56	0.53	0.35
M_r (kDa)	78	58	91

'Alphonso' mango, were significantly lower than those of 'Harumanis' mango[9] indicating high affinity of these isoforms to *p*NP-β-galactopyranoside. Low K_m values were also reported for β-galactosidase isoforms of tomato and coffee berry.[83,196]

Table 24 shows the effect of divalent metal ions and EDTA on β-galactosidase activity of these isoforms. Among the metal ions tested, Hg^{++} showed a very powerful inhibition of all the three isoforms, even at 0.1 mM level, which was 100, 91 and 91 % inhibition for isoforms I, II and III, respectively. Powerful inhibition by Hg^{++} was also shown for tomato and muskmelon β-galactosidase

isoforms.[196, 207] Generally, isoform-1 was inhibited more by various metal ions, especially by Cu^{++}, followed by Mg^{++},Zn^{++}, Fe^{++}, Mn^{++}, Ca^{++}, and Cd^{++}. Mn^{++} showed significantly high activation of isoform-lll, while Fe^{++} and Zn^{++} showed slight stimulation of activity. Only K^{+} inhibition, and Ca^{++} activation was shown in "Harumanis" mango, [9]

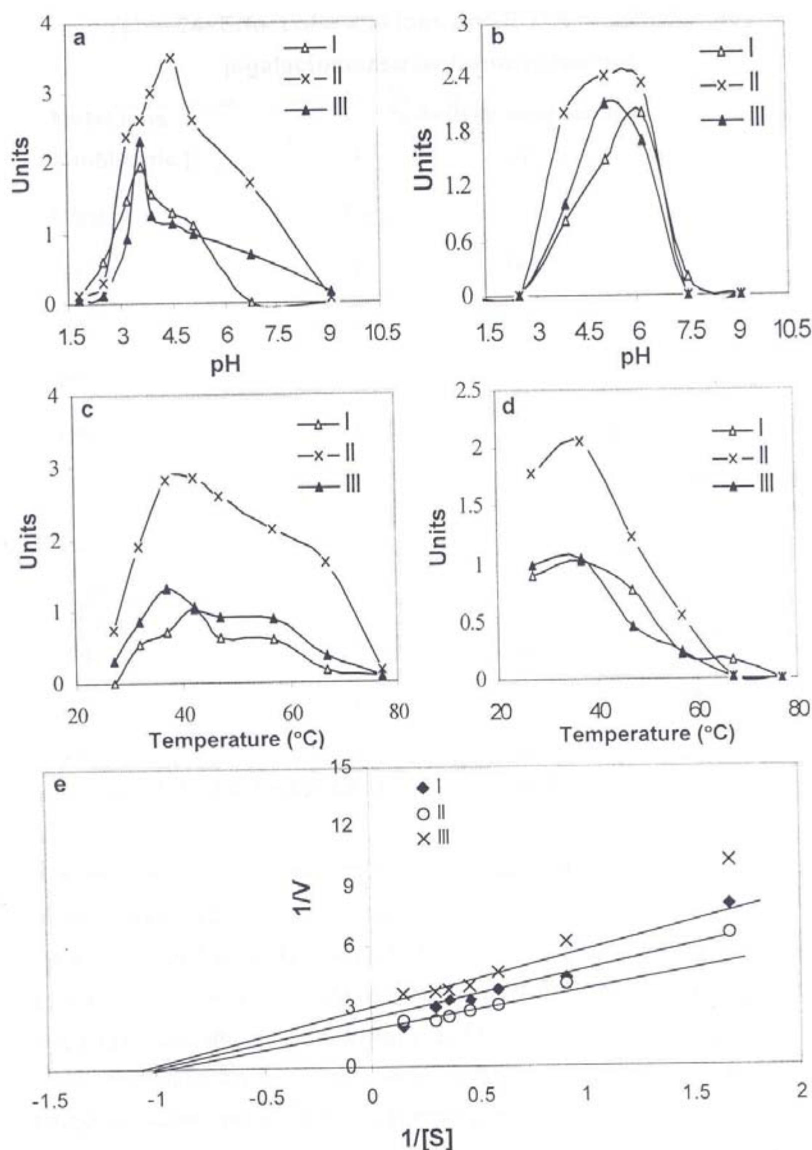


Fig. 46: Effect of pH on activity (a) and stability (b) and effect of temperature on activity (c) and stability (d) of β-galactosidase isoforms. (e) Double reciprocal Lineweaver-Burk plot for isoforms I, II and III Units / 20μl = Amont of enzyme required 1μmol pNP/min

Table 24: Effect of metal ions and EDT A on activity of β-galactosidase isoforms of mango

Metal ions (1mM conc.)	% Activity over control		
	I	II	III
Control	100	100	100
Ca^{++}	69	102	103
Mg^{++}	30	94	82

Fe^{++}	45	82	132
Mn^{++}	60	89	272
Zn^{++}	37	83	106
Cu^{++}	3	66	66
Cd^{++}	70	67	96
Hg^{++}	0 [0]	3 [9]	4 [9]
EDTA	64	98	91
EDTA+ Ca^{++}	30	87	70

Values in parenthesis indicate % activity at 0.1 mM level

whereas deleterious effect of several metal ions on the activity of β-galactosidase isoforms was observed. Ca^{++} activation was not found in our case. The most powerful metal ion inhibition was found with Hg^{++}, followed by Cu^{++}, Mg^{++} and Fe^{++} (except for isoform-III). The inhibition by Hg^{++}, suggests that sulfhydril groups may play an essential role in enzyme activity. The inhibition by Cu^{++} may be due to oxidation of sulfhydril groups.[12] These inhibitions were not shown for 'Harumanis' mango.[9]

The effect of product analogues (simple sugars) on the β-galactosidase activity is shown in Table 25. Rhamnose appeared to stimulate the activity of all the isoforms, especially that of isoforms I and III. Generally, all the other sugars at 2 mM level showed some inhibition of β-galactosidase activity. Galactose inhibition of β-galactosidase was also shown for tomato and coffee berry.[83, 196]

Table 25: Effect of product analogues on the activity of β-galactosidase isoforms of mango

Product Analogues (2mM conc.)	% Activity over control		
	I	II	III
Control	100	100	100
GalA	59	47	74
Gal	65	51	65
Glc	94	68	79
Man	77	57	66
Fuc	88	54	58
Rha	141	105	174
Ara	108	70	88
Xyl	65	57	74

Various glycosides as *p*NP-substrates were tested to see the activity of isoforms I, II and III [Table 26]. All isoforms showed some hydrolytic activity with pNP-α-galactopyranoside. Isoform-I showed some hydrolytic activity with pNP-α-glucopyranoside and pNP-N-acetyl-β-D-glucosaminide while

isoform-III also hydrolysed pNP-N-acetyl-β-D-glucosaminide and pNP-α-mannopyranoside. Isoform-II showed little activity towards *p*NP-α-mannopyranoside. All these activities were in the range of 10-20% of the control. Purified β-galactosidase isoforms having other glycosidase activities like α-mannosidase, β-fucosidase and α-arabinosidase have been reported for mung bean seedlings.[144]

Table 26: Activity of β-galactosidase isoforms of mango on synthetic substrates

Synthetic Substrates (13 mM)	% Activity over control		
	I	II	III
Control(pNP-β-Gal)	100	100	100
pNP-α-Gal	20	14	10
pNP-β-Glc	0	0	4
pNP-α-Glc	11	0	0
pNP-β-GlcNAc	17	0	12
pNP-α-Man	0	10	20
pNP-β-Xyl	0	0	0
pNP-α-Fuc	0	0	0

β-Galactosidase in mango showed little activity towards the natural substrates like PGA and pectin, whereas, it showed a slightly higher activity towards galactomannan [Table 27]. Galactanase activity was also found for f3-galactosidase of apple,22 tomato 196 and coffee berry.83 Galactanase activity of β-galactosidase observed in this cv. (Alphonso) was similar to that of 'Harumanis' mango.[9] As for the endogenous substrates for β-galactosidase of mango, only arabinogalactan was hydrolysed by all the 3 isoforms. Rhamnogalacturonans were not the endogenous substrates for

Table 27: Activity of β-galactosidase isoforms of mango on natural and endogenous substrates

Subtrates	Activity (μmol/h)		
	I	II	III
Natural substrates			
PGA (0.06%)	0.046	0.077	0.034
Pectin (0.06%)	0.017	0.017	0.020
Galactomannan (0.045%)	0.080	0.097	0.067
Endogenous substrate			
Arabinogalactan	0.214	0.264	0.288
Rhamnogalacturonan-1 (0.05M $(NH_4)_2CO_3$ eluate)	-	-	-
Rhamnogalacturonan-2	-	-	-

(0.1 M $(NH_4)_2CO_3$ eluate)

this enzyme despite the presence of high galactose residues in the two rhamnogalacturonans identified in mango [Chapter-III; Section 2 and 3]. It may be due to the concentration of these galactan side chains in 'hairy regions' of mango pectin, which make them resistant towards the action of these enzymes. The ability of β-galactosidase to degrade pectins has been detected in tomato,[196] muskmelon,[207] kiwi,[223] apple[224] and avocado,[54] but not in orange.[36] β-Galactosidase acting on galactans was reported in fruits such as apple[224] and Japanese pear.[127] The kiwi β-galactosidase was 10-times more active towards kiwi galactan and 20-times more active towards apple galactan than the apple β-galactosidase.[223, 224] Hemicelluloses were found degraded by β-galactosidase from mung bean seedlings.[144]

The precise role of β-galactosidase is not well understood.59 The present knowledge of the structure of mango pectin and other fruit pectins suggests the presence of rhamnogalacturonans profusely branched with side chains consisting of arabinan, galactan or arabinogalactans. Arabinogalactan, having high amount of galactose was found to be a possible susceptible endogenous substrate in mango for β-galactosidase enzymes. These enzymes did not act on the two rhamnogalacturonans.

Redgwell and coworkers[211] stated that potential substrates for β-galactosidase are present in unripe fruit but because of their localization in the cell wall, they are inaccessible for the action. In the cell wall, pectins are connected to the cellulose by galactan side chains and the β-galactosidase, having also the galactanase activity, may act on these galactans probably resulting in pectin solubilization. The ability of these isoforms to degrade the endogenous substrates (arabinogalactan) possibly suggests a role in pectin dissolution during tissue softening / fruit ripening.[9] Further, β-galactosidase also acts on glycolipids of membrane, changing the membrane integrity, which may bring other enzymes in contact with cell wall and eventually result in further cell wall degradation. However, solubilization of pectic polymers by PG also renders galactan type-polymers susceptible to enzyme action.[211]

Gross and Sams[91] reported that the hydrolysis of neutral sugar polymers may weaken the strong bonding between the pectins and cellulose, and thus contribute to loss of fruit firmness. This modification of pectic polysaccharide may influence the action of other enzymes like PG towards these polymers.[92] PG acts on those pectins, which contain less neutral sugar residues.92 Thus limited β-alactosidase activity on the side chains of the pectin backbone could have major implications for the matrix of the cell wall rendering substrates susceptible for hydrolysis by other cell-wall degrading enzymes.224 Removal of neutral sugar side chain paves the way to attack the exposed galacturonan

chain by PG, thus contributing for the overall pectin degradation and eventually tissue softening during ripening. Limited in vivo β-galactosidase activity on the pectins could have a significant effect on pectin solubility, through decreasing the ability of pectin molecules to aggregate.[54] Thus both PG and β-galactosidase enzymes play synergistically to solubilize cell wall during ripening.

This knowledge about the probable role of β-galactosidase in the modification of cell wall polysaccharides provides information that this enzyme may serve as a useful target at cell wall level apart from PG and probably rhamnogalacturonase (RGase) to extend shelf life and improve quality of mango by genetic engineering. The expression of these enzymes may be suppressed by molecular approach to give desired end result for controlled fruit firmness.

CHAPTER – V
HIGHLIGHTS OF THE RESEARCH

An Overview

Pectin regulation at the cell wall level during fruit ripening is important in the context of fruit texture and its changes. Loosening of cell wall structure and some loss of pectin is a common feature noticed during textural softening, especially in climacteric fruits.

Fruits may differ in their target enzymes depending upon their inherent nature and composition. The present investigation on mango fruit is comprehensive. The aim was to pinpoint the major pectic hydrolases of mango, which were studied extensively at both substrate and enzyme levels. Thus, the crucial pectic polymers as substrates for the corresponding hydrolases were identified and characterized. One of the related glycanases (PG) and a glycosidase (β-galactosidase) were purified and found to exist as three isoforms. Their properties and action on endogenous substrates were also studied. These findings are novel and hitherto not reported in mango (Mangifera indica cv. Alphonso) fruit.

In mango pulp, the total pectin content was reduced from 1.8 g % to 0.38 g % from extreme unripe to ripe stage, accompanied by progressive textural softening, loss of middle lamella and loosening of cell wall. The related enzymes of pectin dissolution, viz: PG, arabinanase, galactanase and β-galactosidase (hydrolases), showed increased activity with a climacteric peak during ripening, except for PME (esterase), which decreased during ripening. Initially PME prepares the pectin substrate, by demethylating it to pectic acid, thus making it amenable for the subsequent action by PG.

Out of the seven pectic polymers present in mango pulp, all of them showed a drop in their quantitative levels as well as their molecular weights. The major pectic polymers identified in mango were arabinogalactan (fraction I, with arabinose : galactose, 1 : 3 ratio) and two rhamnogalacturonans (fractions II & III). They differed in their relative ratio of sugars, viz; galacturonic acid: galactose: arabinose: rhamnose; 9: 14: 15:2 and 62: 23: 10: 4 for fractions II and III, respectively, and there was difference also in the nature of the side chains and branching. The abundance of the major pectic polymers of mango was 60, 89 and 74 mg % FW, respectively for fractions I, II and III, which dropped to 7, 13 and 2 mg % at the end of ripening. Similarly, the molecular weight drop for the major polymers from unripe to ripe stages was 250 to 70, 1300 to 21, 473 to 298 kD, respectively for fractions I, II & III.

It is interesting to note that arabinanase and galactanase activities were very powerful in mango fruit in comparison with other fruits. Infact PG activity is quite low in mango when compared to fruits like

tomato, papaya and banana. It is quite possible that arabinanase, galactanase and galactosidase have an important significance in pectin hydrolysis in vivo in mango apart from PG. In addition, the major pectic polysaccharide substrates identified in this fruit, i.e., the two rhamnogalacturonans and an arabinogalactan, which are rich in galactose and arabinose residues correlate with the observation that mango fruit is particularly abundant in tc respective glycanases. These pectic polymers may form the endogenous substrates for the high activity of arabinanase, galactanase and β-galactosidase. β-Galactosidase from mango exists as three isoforms and the arabinogalactan, purified and characterized from mango was found to be an endogenous substrate for p-galactosidase. Similarly, three isoforms of PG were purified and characterized from mango and the two rhamnogalacturonans, which had >60% galacturonic acid units, were found to be the endogenous substrates for the PG isoforms. Thus, in mango, enzymes like arabinanase, galactanase and p-galactosidase may play an equally crucial role than PG, in pectin dissolution in vivo at cell wall level. Activitywise, these enzymes are very powerful in mango when compared to PG. On the other hand, in tomato fruit, PG has been shown to playa significant role in textural changes during ripening.[32, 269] In apple, a different type of pectic polysaccharides, i.e., highly ramified (hairy region) rhamnogalacturonans, containing substituted rhamnose and galacturonic acid as alternating residues, were reported to be hydrolysed by a novel fungal enzyme rhamnogalacturonase (RG).[231, 233]

Cellular compartmentalization, which also undergoes changes during ripening is another important parameter, influencing the fruit softening phenomenon. Loosening of cell structure during ripening, which has been reported in some fruits, might bring a better substrate-enzyme contact. What exactly is the situation in the natural milieu (in situ) cannot be explained fully In precise terms. Nevertheless, at substrate level, the abundance and nature of the polymers, their extent of loss and drop in molecular weight and degree of depolymerization during ripening give the best clue for guessing the enzymes involved. Especially so, if they are supported and correlated by enzyme activity, profile of polymers and their action on the endogenous substrates. Here, all the seven pectic polymers of mango pulp showed a significant drop in their abundance and molecular weight and none of them showed total disappearance during ripening. This in vivo hydrolysis is due to combined action of both glycanases and glycosidases. Thus, depolymerization results in smaller size polymers, oligomers, or glycoproteins, which may have a role in signal transduction, thereby controlling fruit ripening. It has been suggested that some pectic oligomers might act like signal molecules in inducing and regulating ethylene biosynthesis, which in turn triggers fruit ripening.[37, 125]

CONCLUSION

This study identifies the crucial carbohydrate polymers and the key ezymes contributing to the textural softening in mango fruit.

1. The degree of loss in tissue firmness, loss of cellular integrity, extensive hydrolysis of pectic polysaccharides and increased activity of pectic enzymes, decreased activity of PME during ripening show a clear correlation between each other.
2. Pectins in mango are essentially ionic in nature. These fractions are normally affected during mango fruit ripening process.
3. In general, pectin degradation is accompanied by release of small molecular weight polymers, loss of neutral sugars and concomitant increase in the level of total sugar / galacturonic acid in alcohol-soluble fraction.
4. Changes in the apparent molecular weight during ripening were quantified.
5. By methylation analysis (GC-MS), FTIR and NMR, the structure of the major pectic polysaccharide of mango was established and found to be similar to that of most of the fruits.
6. Three isoforms of PG and β-galactosidase are present in the pulp of mango fruit at the climacteric stage.
7. The isoforms were resolved by ion exchange chromatography and partially purified by gel permeation chromatography.
8. Each isoform differs slightly in its physico-chemical properties and in molecular weight.
9. Further research is needed to clarify specific role of these enzymes in the context of fruit softening.

BIBLIOGRAPHY

1. Abbott, J. A., Watada, A. E. and Massie, D. R. (1984). Sensory and instrument measurement of apple texture. J. Amer. Soc. Hort. Sci. 109: 221-228.
2. Abu-Sarra, A. F. and Abu-Goukh, A. A. (1992). Changes in pectinesterase, polygalacturonase and cellulase activity during mango fruit ripening. J. Hort. Sci. 67: 561-568.
3. Adams, D. O. and Yang, S. F. (1979). Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. USA 76: 170-174.
4. Ahmed, A. E. R. and Labavitch, J. M. (1980). Cell wall metabolism in ripening fruit. I. Cell wall changes in ripening' Bartlett' pears. Plant Physiol. 65: 1009-1013.
5. Ahmed, A. E. R. and Labavitch, J. M. (1980). Cell wall metabolism in ripening fruit. II. Changes in carbohydrate-degrading enzymes in ripening 'Bartlett' pears. PlantPhysiol. 65: 1014-1016.
6. Ahmed, E. M. and Dennison, R. A. (1971). Texture profile of irradiated mangoes and peaches. J. Texture Studies 2: 489-496.
7. Aina, J. O. and Oladunjoye, O. O. (1993). Respiration, pectolytic activity and textural changes in ripening African mango (Irvingia gabonensis) fruits. J. Sci. Food Agric. 63: 451-454.
8. Ali, Z. M. and Brady, C. J. (1982). Purification and characterization of the polygalacturonases of tomato fruits. Aust. J. Plant Physiol. 9: 155-169.
9. Ali, Z. M., Armugam, S. and Lazan, H. (1995). p-Galactosidase and its significance in ripening mango fruit. Phytochem. 38: 1109- 1114.
10. Alkorta, I., Garbisu, C., Llama, M. J. and Serra, J. L. (1998). Industrial applications of pectic enzymes: a review. Proc. Biochem. 33: 21-28.
11. Aman, P. and Westerlund, E. (1996). Cell wall polysaccharides: Structural, chemical, and analytical aspects. In: Eliasson, A-C (Ed.). Carbohydrates in Food. Marcel Dekker, Inc. New York. pp. 191-226.
12. Andrews, P. K. and Li, S., (1994). Partial purification and characterization of p-D-galactosidase from sweet cherry, a non-climacteric fruit. J. Agric. Food Chern. 42: 2177-2182.
13. Aspinall, G. O. (1980). Chemistry of cell wall polysaccharides. In: Preiss, J. (Ed.) The Biochemistry of Plants. Academic Press, Inc. New York. Vol. 3. pp.473-500.
14. Ashraf, M., Khan, N., Ahmed, M. and Elahi, M. (1981). Studies on the pectinesterase activity and some chemical constituents of some Pakistani mango varieties during storage-ripening. J. Agric. Food Chern. 29: 526-528.
15. Awad, M. and Yang, R. E. (1979). Postharvest variation in cellulase, polygalacturonase and pectinmethylesterase in avocado (Persea Americana mill, cv, Fuerte) fruits in relation to respiration and ethylene production Plant Physiol. 64: 306-308.

16. Baker, R. A. (1997). Reassessment of some fruit and vegetable pectin levels. J. Food Sci. 62: 225-229.
17. Baldwin, E. A. and Pressey, R. (1990). Exopolygalacturonase elicits ethylene production in tomato. HortSci. 25: 779-780.
18. Bansal, K. C. (2000). Biotechnology for improving product quality in horticultural crops. In: Chadha, K. L, Ravindran. P. Nand Leela Sahijram (Eds.) Biotechnology in Horticultural and Plantation Crops. Malhotra Publishing House, New Delhi, India. pp. 201-218.
19. Barrett, A. J. and Northcote, D. H. (1965). Apple fruit pectic substances, Biochem. J. 94: 617-627.
20. Barrett, D. M., Garcia, E. and Wayne, J. E. (1998). Textural modification of processing tomatoes. Crit. Rev. Food Sci. Nutr. 38: 173-258.
21. Bartley, I. M. (1978). Exo-polygalacturonase of apple. Phytochem. 17: 213-216.
22. Bartley, I. M. (1974). [3-Galactosidase activity in ripening apples. Phytochem. 13: 2107-2111.
23. Bartley, I. M. and Knee, M. (1982). The chemistry of textural changes in fruit during storage. Food Chern. 9: 47-58.
24. BeMiller, J. N. (1986). An introduction to pectins: Structure and properties. In: Fishman, M. Land Jen, J. J. (Eds.) Chemistry and Function of Pectins. Amer. Chem. Soc., Washington D.C., pp. 2-12.
25. Ben-Arie, R., Kislev, N. and Frenkel, C. (1979). Ultrastructural changes in the cell walls of ripening apple and pear fruit. Plant Physiol. 64: 197-202.
26. Ben-Arie, R., Sonego, L. and Frenkel, C. (1979). Changes in pectic substances in ripening pears. J. Amer. Soc. Hort. Sci. 104: 500-505.
27. Bhagylakshmi, N., Prabha, T. N., Yashoda, H. M., Prasanna, V., Jagadeesh, B. Hand Tharanathan, R. N (2002). Biochemical studies related to textural regulation during ripening of banana and mango fruit. Acta Hort. 575: 717-724.
28. Blumenkrantz, N. and Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484-489.
29. Boothby, D. (1983). Pectic substances in developing and ripening plum fruits. J. Sci. Food Agric. 34: 1117-1122.
30. * Bouranis, D. L. and Niavis, C. A. (1992). Cell wall metabolism in growing and ripening stone fruits. Plant Cell Physiol. 33: 999-1008.
31. Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
32. Brady, C. J. (1987). Fruit ripening. Ann. Rev. PlantPhysiol. 38: 155-178.
33. Brinson, K., Dey, P. M., John, M. A. and Pridham, J. B. (1988). Post-harvest changes in Mangifera indica mesocarp cell walls and cytoplasmic polysaccharides. Phytochem. 27: 719-723.

34. Brownleader, M. D., Jackson, P., Mobasheri, A., Pantelides, A. T., Sumar, S., Trevan, M. and Dey, P. M. (1999). Molecular aspects of cell wall modifications during fruit ripening. Grit. Rev. Food Sci. Nutr. 39: 149-164.

35. Bryant, J. A. and Cuming, A. C. (1999). Molecular control of development. In: Lea, P. J and Leegood, R. C. (Eds.) Plant Biochemistry and Molecular Biology. II edition. John Willey & Sons. New York, Brisbane, Singapore. pp. 287-333.

36. Burns, J. K. (1990). a- and p-galactosidase activities in juice vesicles of stored Valencia oranges. Phytochem. 29: 2425-2429.

37. Campbell, A. D. and Labavitch, J. M. (1991). Induction and regulation of ethylene biosynthesis by pectic oligomers in cultured pear cells. Plant Physiol. 97: 699-705.

38. Carey, A. T., Holt, K., Picard, S., Wilde, R., Tucker, G. A., Bird, C. R., Schuch, W. and Seymour, G. B. (1995). Tomato exo-(1-4)-p-D-galactanase. Plant Physiol. 108: 1099-1107.

39. * Carpita, N. C. and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3: 1-30.

40. Carrington, C. M. S., Greve, C. L. and Labavitch, J. M. (1993). Cell wall metabolism in ripening fruit VI. Plant Physiol. 103: 429-434.

41. Chan, H. T. Jr. and Tam, S. Y. T. (1982). Partial separation and characterization of papaya endo- and exo- polygalacturonase. J. Food Sci. 47: 1478-1483.

42. Chang, C. Y., Tsai, Y. R. and Chang, W. H. (1993). Models for the interactions between pectin molecules and other cell-wall constituents in vegetable tissues. Food Chern. 48: 145-157.

43. Chauhan, S. K., Tyagi, S. M. and Singh. D. (2001). Pectinolytic liquefaction of apricot, plum and mango pulps for juice extraction. Int. J. Food Properties 4: 103-109.

44. Cheng, L. and Kindel, P. L. (1995). Effect of cations on the elution of pectic polysaccharides from anion-exchange resins. Anal. Biochem. 228: 109-114.

45. Coimbra, M. A., Barros, A., Rutledge, D. N. and Delgadillo, I. (1999). FTIR Spectroscopy as a tool for the analysis of olive pulp cell-wall polysaccharide extracts. Carbohydr. Res. 317: 145-154.

46. Coimbra, M. A., Barros, A., Barros, M., Rutledge, D. N. and Delgadillo, I. (1998). Multivariate analysis of uronic acid and neutral sugars in whole pectic samples by FT-IR spectroscopy. Carbohydr. Polym. 37: 241-248.

47. Colquhoun, I. J., de Ruiter, G. A., Schols, H. A. and Voragen, A. G. J. (1990). Identification by n. m. r. spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectin with rhamnogalacturonase. Carbohydr. Res. 206: 131-144.

48. Crookes, P. R. and Grierson, D. (1983). Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. Plant Physiol. 72: 1088-1093.

49. * Cutilias-iturralde, A., Zarra, I. and Lorences, E. P. (1993). Metabolism of cell wall polysaccharides from persimmon fruit: Pectin solubilization during fruit ripening occurs in the apparent absence of polygalacturonase activity. Physiol Plant. 89: 369-375.
50. Darvill, A. G., McNeil, M. and Albersheim, P. (1978). Structure of plant cell walls VIII. A new pectic polysaccharide. Plant Physiol. 62: 418-422.
51. Davis, E. A., Derouet, C. and Du Penhoat, C. H. (1990). Isolation and N.M.R. study of pectins from flax (Linum usitatissimum L.). Carbohydr. Res. 197: 205-215.
52. DellaPenna, D. and Bennett, A. B. (1988). In vitro synthesis and processing of tomato fruit polygalacturonase. Plant Physiol. 86: 1057-1063.
53. DellaPenna, D, Lashbrook, C. C., Toenjes, K., Giovannoni, J. J., Fischer, R. L. and Bennett, A. B. (1990). Polygalacturonase isoenzymes and pectin depolymerization in transgenic rin tomato fruit. Plant Physiol. 94: 1882-1886.
54. * De Veau, E. J. I. (1993). Degradation and solubilition of pectin by -galactosidases purified from avocado mesocarp. Physiol Plant. 87: 279-285.
55. De Vries, J. A., Voragen, A. G. J., Rombouts, F. M. and PilniK, W. (1986). Structural studies of apple pectins with pectolytic enzymes. In: Fishman, M. Land Jen, J. J. (Eds.) Chemistry and Function of Pectins. Amer. Chem. Soc., Washington D.C. pp. 38- 48.
56. De Vries, J. A., Voragen, A. G. J., Rombouts, F. M. and PilniK, W. (1984). Changes in the structure of apple pectic substances during ripening and storage. Carbohydr. Polym. 4: 3-14.
57. De Vries J. A., den Uijl, C. H., Voragen, A.G.J., Rombouts, F. M. and PilniK, W. (1983). Structural features of the neutral side chains of apple pectic substances. Carbohydr. Polym. 3: 193-205.
58. Dey, P. M. and Brinson, K. (1984). Plant cell-walls. Adv. Carbohydr. Chern. Biochem. 42: 265-382.
59. Dey, P. M. and Del Campillo, E. (1984). Biochemistry of the multiple forms of glycosidases in plants. Adv. Enzymol. 56: 141-249.
60. * Dey, P. M. and Dixon, R. A. (1985). Biochemistry of Storage Carbohydrates in Green Plants. Academic Press Inc., London.
61. Dick, A. J. and Labavitch, J. M. (1989). Cell wall metabolism in ripening fruit. IV. Characterization of the pectic polysaccharides solubilized during softening of 'Bartlett' pear fruit. Plant Physiol. 89: 1394-1400.
62. * Dick, A. J., Opuku-Gyanifua, A. and DeMacro, A. C. (1990). Glycosidases of apple fruit: A multifunctional (3-galactosidase. Physiol. Plant. 80: 250-256.
63. Doesburg, J. J. (1965). Pectic Substances in Fresh and Processed Fruits and Vegetables. I.B.V.T. communication, Wageningen, The Netherlands.
64. Doco, T., Williams, P., Vidal, S. and Pellerin, P. (1997). Rhamnogalactur-onan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables. Carbohydr. Res. 297: 181-186.

65. Doner, L. W. (1986). Analytical methods for determining pectin composition. In: Fishman, M. Land Jen, J. J. (Eds.) Chemistry and Function of Pectins. Amer. Chem. Soc., Washington D.C., pp. 13-21.

66. Dore Raju, M. R. and Karuna Kumar, M. (1996). Purification and characterization of β-D-galactosidase from the pulp of sapota (Achrus sapota) fruit. J. Food Sci. Technol. 33: 36-40.

67. Doreyappa Gowda, I. N. and Huddar, A. G. (2001). Studies on ripening changes in mango (Mangifera indica L.) fruits. J. Food Sci. Technol. 38: 135-137.

68. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chern. 28: 350-356.

69. El-Zoghbi, M. (1994). Biochemical changes in some tropical fruits during ripening. Food Chern. 49: 33-37.

70. Evans, R. and McHale, D. (1978). Multiple forms of pectinesterase in limes and oranges. Phytochem. 17: 1073-1075.

71. FAO, Production Yearbook (1983, 1993, 1999). Food and Agricultural organization of the United Nations. Rome. Italy.

72. Fares, K., Renard, C. M. G. C., R'zina, O. and Thibault, J-F. (2001). Extraction and composition of pectins and hemicelluloses of cell walls of sugar beet roots grown in Morocco. Int. J. Food Sci. Technol. 36: 35-46.

73. Femenia, A., Sanchez, E. S., Simal, S. and Rosello, C. (1998). Developmental and ripening-related effects on the cell wall of apricots (Prunus armeniaca) fruit. J. Sci Food Agric. 77: 487-493.

74. Filippov, M. P. (1992). Practical infrared spectroscopy of pectic substances. Food Hydrocolloids 6: 115-142.

75. Fils-Lycaon, B. and Buret, M. (1990). Loss of firmness and changes in pectic fractions during ripening and overripening of sweet cherry. HortSci. 25: 777-778.

76. Fisher, R. L. and Bennett, A. B. (1991). Role of cell wall hydrolases in fruit ripening. Ann. Rev. Plant Physiol. Plant Mol. Bioi. 42: 675-703. 77. Fishman, M. L., Pepper, L., Damert, W. C., Phillips, J. G. and Barford, R. A (1986). A critical reexamination of molecular weight and dimensions for citrus pectins. In: Fishman, M. Land Jen, J. J. (Eds.) Chemistry and Function of Pectins. Amer. Chem. Soc., Washington D.C. pp. 22-37.

78. Fry, S. C. (1995). Polysaccharide-modifying enzymes in the plant cell wall. Ann. Rev. Plant Physiol. Plant Mol. BioI. 46: 497-520.

79. Fry, S. C. (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann. Rev. Plant Physiol. 37: 165-186.

80. Fuchs, Y., Pesis, E. and Zauberman, G. (1980). Changes in amylase activity, starch and sugar contents in mango fruit pulp. Sci. Hort. 13: 155-160.

81. Gamage, T. V. and Rehman, M. S. (1999). Post harvest handling of foods of plant origin. In: Rehman, M. S. (Ed.) Handbook of Food Preservation. Marcel Dekker Inc. New York. pp. 11-46.

82. Giovannoni, J. J., DellaPenne, D., Bennett, A. B. and Fischer, R. L. (1989). Expression of a chimeric polygalacturonase gene in transgenic rin "(ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. Plant Cell 1: 53-63.

83. Golden, K. D., John, M. A. and Kean, E. A. (1993). p-Galactosidase from Correa arabica and its role in fruit ripening. Phytochem. 34: 355-360.

84. Gomez-Um, M. A. (1997). Postharvest physiology. In: Litz, R. E. (Ed.) The Mango; Botany, Production and Uses. CAB International, Florida. pp. 425-445.

85. Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C. and Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: The Egg-Box model. FEBS Letters 32: 195-198.

86. Gray, J., Picton, S., Shabbeer, J., Schuch, W. and Grierson, D. (1992). Molecular biology of fruit ripening and its manipulation with antisense genes. Plant Mol. BioI. 19: 69-87.

87. Grierson, D., Slater, A., Spiers, J. and Tucker, G. A. (1985). The appearance of polygalacturonase mRNA in tomatoes; one of a series of changes in gene expression during development and ripening. Planta 163:263-271.

88. Grierson, D. and Tucker, G. A. (1983). Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. Planta 157: 174-179.

89. Grierson, D., Tucker, G. A. and Robertson, N. G. (1981). The molecular biology of ripening. In: Friend, J., Rhodes, M. J. C., (Eds.). Recent Advances in the Biochemistry of Fruits and Vegetables. Academic Press, London, pp. 149-160.

90. Gross, K. C. (1984). Fractionation and partial characterization of cell walls from normal and non-ripening mutant tomato fruit. Physiol. Plant. 62: 25-32.

91. Gross, K. C. and Sams, C. E. (1984). Changes in cell wall neutral sugar composition during fruit ripening: A Species survey. Phytochem. 23: 2457-2461.

92. Gross, K. C. and Wallner, S. J. (1979). Degradation of cell wall polysaccharides during tomato fruit ripening. Plant Physiol. 63: 117-120.

93. Gross, K. C., Watada, A. E., Kang, M. S., Kim, S. D., Kim, K. S. and Lee, S. W. (1986). Biochemical changes associated with the ripening of hot pepper fruit. Physiol. Plant. 66: 31-36.

94. Hadfield, K. A. and Bennett, A. B. (1998). Polygalacturonases: Many genes in search of a function. Plant Physiol. 117: 337-343.

95. Hagerman, A. E. and Austin, P. J. (1986). Continuous spectrophotometric assay for plant pectin methyl esterase. J. Agric. Food Chem. 34: 440-444.

96. Hakomori, S. I. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. (Tokyo) 55: 205-208.

97. Hamilton, A. J., Lycett, G. W. and Grierson, D. (1990). Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature 346: 284-287.
98. Hegde, S. and Maness, N. O. (1998). Changes in apparent molecular mass of pectin and hemicellulose extracts during peach softening. J. Amer. Soc. Hart. Sci. 123: 445-456.
99. Hegde, S. and Maness, N. O. (1996). Sugar composition of pectin and hemicellulose extracts of peach fruit during softening over two harvest seasons. J. Amer. Soc. Hart. Sci. 121: 1162-1167.
100. Hobson, G. E. (1981). Enzymes and texture changes during ripening. In: Friend, J., Rhodes, M. J. C., (Eds.). Recent Advances in the Biochemistry of Fruits and Vegetables. Academic Press, London, pp. 123-132.
101. Honda, S., Nishimura, Y., Takahashi, C. and Kakehi, K. (1982). A manual method for the spectrophotometric determination of reducing carbohydrates with 2-cyanoacetamide. Anal. Biochem. 119: 194-199.
102. Huber, D. J. (1984). Strawberry fruit softening: The potential roles of polyuronides and hemicelluloses. J. Food Sci. 49: 1310-1315.
103. Huber, D. J. (1983). Polyuronide degradation and hemicellulose modifications in ripening tomato fruit. J. Amer. Soc. Hart. Sci. 108: 405-409.
104. * Huber, D. J. (1983). The role of cell wall hydrolases in fruit softening. Hart. Rev. 5: 169-219.
105. Huber, D. J. and Lee, J. H. (1986). Comparative analysis of pectins from pericarp and locular gel in developing tomato fruit. In: Fishman, M and Jen, J. (Eds.) Chemistry and Functions of Pectins. Amer. Chem. Soc., Washington D.C., USA. pp. 141-156.
106. Huber, D. J. and O'Donoghue, E. M. (1993). Polyuronides in avocado (Persea amen'cana) and tomato (Lycopersicon esculentum) fruits exhibit markedly different patterns of molecular weight downshifts during ripening. Plant Physiol. 102: 473-480.
107. Huisman, M. M. H., Schols, H. A. and Voragen, A. G. J. (1996). Changes in cell wall polysaccharides from ripening olive fruits. Carbohydr. Polym. 31: 123-133.
108. Hulme, A. C. (1971). The mango. In: Hulme, A. C. (Ed.). The Biochemistry of Fruits and their Products. Vol. 2. Academic Press, London & New York. pp. 233-254.
109. Imoto, T. and Yagishita, K. (1971). A simple activity measurement of lysozyme. Agn'c. BioI. Chern. 35: 1154 -1156.
110. Isherwood, F. A. (1970). Hexosans, pentosans and Gums. In: Hulme, A. C. (Ed.) The Biochemistry of Fruits and their Products. Vol. I. Academic Press, London & New York. pp.33-52.
111. Ishii, T. and Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. Phytochem. 57: 969-974.
112. Jackman, R. L. and Stanley, D. W. (1995). Perspectives in the textural evaluation of plant foods. Trends Food Sci. Technol. 6: 187-194.

113. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Longren, J. (1976). A practical guide to the methylation analysis of carbohydrates. Univ. Stockholm Chern. Commun. 8: 1-25.

114. Jimenez, A., Rodriguez, R., Fernandez-caro, I., Guillen, R., Fernandez- Bolanos, J. and Heredia, A. (2001). Olive fruit cells wall: Degradation of Pectic polysaccharides during ripening. J. Agric. Food Chern. 49: 409-415.

115. John, M. A. and Dey, P. M. (1986). Postharvest changes in fruit cell wall. Adv. Food Res. 30: 139-193.

116. Johnson, G. I., Sharp, J. L., Milne, D. L. and Oosthuyse, S. A. (1997). Postharvest technology and quarantine treatments. In: Litz, R. E. (Ed.) The Mango,' Botany, Production and Uses. CAB International, Florida. pp 447-507.

117. Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N. and Ebringerova, A. (2000). FT -IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. Carbohydr. Polym. 43: 195-203.

118. Kader, A. A. (1980). Prevention of ripening in fruits by use of controlled atmospheres. Food Technol. 34(3): 51-54.

119. Kader, A. A., Zagory, D. and Kerbel, E. L. (1989). Modified atmosphere packaging of fruits and vegetables. Crit. Rev. Food Sci. Nutr. 28: 1-30.

120. Kalra, S. K., Tandon, D. K. and Singh, B. P. (1995). Mango. In: Salunkhe, D. K., Kadam, S. S. (Eds.) Handbook of Fruit Science and Technology.

106. Huber, D. J. and O'Donoghue, E. M. (1993). Polyuronides in avocado (Persea amen'cana) and tomato (Lycopersicon esculentum) fruits exhibit markedly different patterns of molecular weight downshifts during ripening. Plant Physiol. 102: 473-480.

107. Huisman, M. M. H., Schols, H. A. and Voragen, A. G. J. (1996). Changes in cell wall polysaccharides from ripening olive fruits. Carbohydr. Polym. 31: 123-133.

108. Hulme, A. C. (1971). The mango. In: Hulme, A. C. (Ed.). The Biochemistry of Fruits and their Products. Vol. 2. Academic Press, London & New York. pp. 233-254.

109. Imoto, T. and Yagishita, K. (1971). A simple activity measurement of lysozyme. Agn'c. BioI. Chern. 35: 1154 -1156.

110. Isherwood, F. A. (1970). Hexosans, pentosans and Gums. In: Hulme, A. C. (Ed.) The Biochemistry of Fruits and their Products. Vol. I. Academic Press, London & New York. pp.33-52.

111. Ishii, T. and Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. Phytochem. 57: 969-974.

112. Jackman, R. L. and Stanley, D. W. (1995). Perspectives in the textural evaluation of plant foods. Trends Food Sci. Technol. 6: 187-194.

113. Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Longren, J. (1976). A practical guide to the methylation analysis of carbohydrates. Univ. Stockholm Chern. Commun. 8: 1-25.

114. Jimenez, A., Rodriguez, R., Fernandez-caro, I., Guillen, R., Fernandez- Bolanos, J. and Heredia, A. (2001). Olive fruit cells wall: Degradation of Pectic polysaccharides during ripening. J. Agric. Food Chern. 49: 409-415.

115. John, M. A. and Dey, P. M. (1986). Postharvest changes in fruit cell wall. Adv. Food Res. 30: 139-193.

116. Johnson, G. I., Sharp, J. L., Milne, D. L. and Oosthuyse, S. A. (1997). Postharvest technology and quarantine treatments. In: Litz, R. E. (Ed.) The Mango, Botany, Production and Uses. CAB International, Florida. pp 447-507.

117. Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N. and Ebringerova, A. (2000). FT -IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses. Carbohydr. Polym. 43: 195-203.

118. Kader, A. A. (1980). Prevention of ripening in fruits by use of controlled atmospheres. Food Technol. 34(3): 51-54.

119. Kader, A. A., Zagory, D. and Kerbel, E. L. (1989). Modified atmosphere packaging of fruits and vegetables. Crit. Rev. Food Sci. Nutr. 28: 1-30.

120. Kalra, S. K., Tandon, D. K. and Singh, B. P. (1995). Mango. In: Salunkhe, D. K., Kadam, S. S. (Eds.) Handbook of Fruit Science and Technology.

136. Krishnamurty, S., Patwardhan, M. V. and Subramanyam, H. (1971). Biochemical changes during ripening of the mango fruit. Phytochem. 10: 2577-2581.

137. Labavitch, J. M., Freeman, L. E. and Albersheim, P. (1976). Structure of plant cell walls. Purification and characterization of a β-1 ,4-galactanase which degrades a structural component of the primary cell walls of dicots. J. BioI. Chern. 251: 5904-5910.

138. Labib, A. A. S., EI-Ashwah, F. A., Omran, H. T. and Askar, A. (1995). Heat-inactivation of mango pectinesterase and polygalacturonase. Food Chern. 53: 137-142.

139. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature 227: 680-685.

140. Lang, C. and Dornenburg, H. (2000). Perspectives in the biological function and the technogical application of polygalacturonases. Appl. Microbiol. Biotechnol. 53: 366-375.

141. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1985). Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. Carbohydr. Res. 137: 111-125.

142. Lazan, H. and Ali, Z. M. (1993). Cell wall hydrolases and their potential in the manipulation of ripening of tropical fruits. ASEAN Food J. 8: 47-53.

143. Lazan, H., Ali, Z. M., Wah, L. K., Voon, J. and Chaplin, G. R. (1986). The potential role of polygalacturonase in pectin degradation and softening of mango fruit. ASEAN Food J. 2: 93-98.

144. Li, S-H., Han, J-W., Chen, K-C. and Chen, C-S. (2001). Purification and characterization of isoforms of ~-galactosidases in mung bean seedlings. Phytochem. 57, 349-359.

145. Lineweaver, H. and Jansen, E. F. (1951). Pectic enzymes. Adv. Enzymol. 11: 267-295.
146. Lizada. C. (1993). Mango. In: Seymour, G. B., Taylor, J. E., Tucker, G. A. (Eds.) Biochemistry of Fruit Ripening. Chapman and Hall, London. pp. 255-271.
147. Lurie, S., Levin, A., Greve, C. and Labavitch, J. M. (1994). Pectic polymer changes in nectarines during normal and abnormal ripening. Phytochem. 36: 11-17.
148. Mac Dougall, A. J., Brett, G. M., Morris, V. J., Rigby. N. M., Ridout. M. J. and Ring, S. G. (2001). The effect of peptide-pectin interactions on the gelation behaviour of a plant cell wall pectin. Carbohydr. Res. 335: 115-126.
149. Marsilio, V., Lanza, B., Campestre, C. and De Angelis, M. (2000). Oven-dried table olives: textural properties as related to pectic composition. J. Sci. Food Agric. 80: 1271-1276.
150. Martin-Cabrejas, M. A., Waldron, K. W., Selvendran, R. R., Parker, M. L. and Moates, G. K. (1994). Ripening-related changes in the cell walls of Spanish pear (Pyrus communis). Physiol. Plant. 91: 671-679.
151. Mathlouthi, M. and Koenig, J. L. (1986). Vibrational spectra of carbohydrates. Adv. Carbohydr. Chern. Biochem. 44: 7-89.
152. McCann, M.C., Wells, B. and Roberts, K. (1990). Direct visualization of cross-links in the primary plant cell wall. J. Cell. Sci. 96: 323-334.
153. McCollum, T. G., Huber, D. J. and Cantliffe, D. J. (1989). Modification polyuronides and hemicellulose during muskmelon fruit softening. Physiol. Plant. 76: 303-308.
154. McFeeters, R. F. (1980). A manual method for reducing sugar determinations with 2, 2'-bicinchoninate reagent. Anal. Biochem. 103: 302-306.
155. McFeeters, R. F., Bell, T. A. and Fleming, H. P. (1980). An endo-polygalacturonase in cucumber fruit. J. Food Biochem. 4: 1-16.
156. McNeil, M., Darvill, A. G., Fry, S. and Albersheim, P. (1984). Structure and function of the primary cell walls of plants. Ann. Rev. Biochem. 53: 625-663.
157. McNeil, M., Darvill, A. G. and Albersheim, P. (1980). The structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiol. 66: 1128-1134.
158. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chern. 31: 426-428.
159. Missang, C. E., Renard, C. M. G. C., Baron, A. and Drilleau, J-F. (2001a). Cell wall polysaccharides of bush butter (Dacryodes edulis (G Don) HJ Lam) fruit pulp and their evolution during ripening. J. Sci. Food Agric. 81:773- 780.
160. Missang, C. E., Renard, C. M. G. C., Baron, A. and Drilleau, J-F. (2001b). Changes in the pectic fraction of bush butter (Dacryodes edulis (G Don) HJ Lam) fruit pulp during ripening. J. Sci. Food Agric. 81: 781-789.
161. Mitcham, E. J. and McDonald, R. E. (1992). Cell wall modification during ripening of 'Keitt' and 'Tommy Atkins' mango fruit. J. Amer. Soc. Hort. Sci. 117: 919-924.

162. Mort, A. J., Moerschbacher, B. M., Pierce, M. L. and Maness, N. O. (1991). Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them. Carbohydr. Res. 215: 219-227.

163. Muda, P., Seymour, G. B., Errington, N. and Tucker, G. A. (1995). (Compositional changes in cell wall polymers during mango fruit ripening. Carbohydr. Polym. 26: 255-260.

164. Mukherjee, S. K. (1997). Introduction: Botany and Importance. In: Litz, R. E. (Ed.) The Mango,. Botany, Production and Uses. CAB International, Florida. pp. 1-19.

165. Neal, G. E. (1965). Changes occurring in the cell walls of strawberries during ripening. J. Sci. Food Agric. 16: 604-611.

166. Nelson. N. (1944). A photometric adaptation of the somogyi method for the determination of glucose. J. BioI. Chern. 153: 375-380.

167. Newman, R. H. and Redgwell, R. J. (2002). Cell wall changes in ripening kiwifruit: 13C solid state NMR characterization of relatively rigid cell wall polymers. Carbohydr. Polyrn. 49: 121-129.

168. Nogata, Y., Ohta, H. and Voragen, A. G. J. (1993). Polygalacturonase in strawberry fruit. Phytochern. 34: 617- 620.

169. Nunan, K. J., Sims, I. M., Bacic, A., Robinson, S. P. and Fincher, G. B. (1998). Changes in cell wall composition during ripening of grape berries. Plant Physiol. 118: 783-792.

170. Nwanekezi, E. C., Alawuba, O. C. G. and Mkpolulu, C. C. M. (1994). Characterization of pectic substances from selected tropical fruits. J. Food Sci. Technol. 31: 159-161.

171. O'Donoghue, E. M., Somerfield, S. D., De Vre, L. A. and Heyes, J. A. (1997). Developmental and ripening-related effects on the cell wall of Pepino (Solanum rnuricaturn) fruit. J. Sci. Food Agric. 73: 455-463.

172. Geller, P. W., Min-Wong, L., Taylor, L. P., Pike, D. A. and Theologis, A. (1991). Reversible inhibition of tomato fruit senescence by antisense RNA. Science 254: 437-439.

173. Oesterveld, A., Beldman, G., Schols, H, A. and Voragen, A. G. J. (2000). Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp. Carbohydr. Res. 328: 185-197.

174. Olle, D., Lozano, Y. and Brillouet, J-M. (1996). Isolation and characterization of soluble polysaccharides and insoluble cell wall material of the pulp from four mango (Mangifera indica. L) cultivars. J. Agric. Food Chern. 44: 2658-2662.

175. O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G. and Albersheim, P. (1996). Rhamnogalacturonan-ll, a pectic polysaccharides in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. in vitro conditions for the formation and hydrolysis of the dimer. J. BioI. Chern. 271: 22923-22930.

176. O'Neill, M. A., Albersheim, P. and Darvill, A. G. (1990). The pectic polysaccharides of primary cell walls. In: Dey, D. M. (Ed.) Methods in Plant Biochemistry, Vol. 2. Academic Press, London, pp. 415-441.

177. Ovodov, Y. S., Ovodova, R. G., Bondarenko, O. D. and Krasikova, I. N. (1971). The pectic substances ofzosteraceae. Carbohydr. Res. 18: 311-318.

178. Padmini. S. and Prabha, T. N. (1997). Biochemical changes during acetylene-induced ripening in mangoes (var. Alphonso). Trop. Agric. 74: 265-271.

179. * Pantastico, E. B., Lam, L. R., Ketsa, S., Yuniarti, Y. and Kosittrakul. S. (1984). Mango -Fruit development, post harvest physiology and marketing in ASEAN. In: Mendoza, D. B. Jr. and Wills, R. B. H. (Eds.). ASEAN Food Handling Bureau, Kuala Lumpur, pp. 41-43.

180. Pathak, N. and Sanwal, G. G. (1998). Multiple forms of polygalacturonase from banana fruits. Phytochem. 48: 249-255.

181. Pathak, L., Chang, K. C. and Brown, G. (1988). Isolation and characterization of pectin in sugar beet pulp. J. Food Sci. 53: 830-833.

182. Paull, R. E. and Chen, N. J. (1983). Postharvest variation in cell wall degrading enzymes of papaya (Carica papaya. L.) during fruit ripening. Plant Physiol. 72: 382-385.

183. Peleg, M. (1979). Evaluation by instrumental methods of the textural properties of some tropical fruits. J. Texture Studies 10: 45-65.

184. Pesis, E., Fuchs, Y. and Zauberman, G. (1978). Cellulase activity and fruit softening in avocado. Plant Physiol. 61: 416-419.

185. Pilnik. W. and Voragen, A. G. J. (1970). Pectic substances and other uronides. In: Hulme, A. C. (Ed.) The Biochemistry of Fruits and their Products. Vol. I. Academic Press, London & New York. pp.53-87.

186. Polle, A. Y., Ovodova, R. G., Shashkov, A. S. and Ovodov, Y. S. (2002). Some structural features of pectic polysaccharide from tansy, Tanacetum vulgare. L. Carbohydr. Polym. 49: 337-344.

187. Poovaiah, B. W. and Nukaya, A. (1979). Polygalacturonase and cellulase enzymes in the normal Rutgers and mutant rin tomato fruits and their relationship to the respiratory climacteric. Plant Physiol. 64: 534-537.

188. Porro, M., Viti, S., Antoni, G. and Saletti, M. (1987). Ultrasensitive silver-stain method for the detection of proteins on polyacrylamide gels and immunoprecipitates on agarose gels. Anal. Biochem. 127: 316-321.

189. Prabha, T. N. and Bhagyalakshmi, N. (1998). Carbohydrate metabolism in ripening banana fruit. Phytochem. 48: 915-919.

190. Prabha, T. N., Yashoda, H. M., Prasanna, V., Jagadeesh, B. H. and Bimba Jain, M. V., (2000). Carbohydrate metabolism in relation to textural softening during fruit ripening. Trends Carbohydr. Chern. 6: 89-95.

191. Prabha, T. N., Veena, B. A. and Patwardhan, M. V., (1987). Metabolism of organic acids in modified atmosphere stored ripening mangoes (Mangifera indica). J. FoodSci. Technol. 24: 111-115.

192. Pressey, R. (1987). Exopolygalacturonase in tomato fruit. Phytochem. 26: 1867-1870.

193. Pressey, R. (1986). Changes in polygalacturonase isoenzymes and converter in tomatoes during ripening. HortSci. 21: 1183-1185.

194. Pressey, R. (1986). Extraction and assay of tomato polygalacturonases. HortSci. 21: 490-492.

195. Pressey, R. (1986). Polygalacturonases in higher plants. In: Fishman, M. L and Jen, J. J. (Eds.) Chemistry and Function of Pectins. Amer. Chem. Soc., Washington D.C. pp. 157-174.

196. Pressey, R. (1983). p-Galactosidase in ripening tomatoes. Plant Physiol. 71: 132-135.

197. Pressey, R. and Avants, J. K. (1978). Difference in polygalacturonase composition of Clingstone and Freestone peaches. J. Food Sci. 43: 1415-1417,1423.

198. Pressey, R. and Avants, J. K. (1976). Pear polygalacturonases. Phytochem. 15: 1349-1351.

199. Pressey, R. and Avants, J. K. (1975). Cucumber polygalacturonase. J. Food Sci. 40: 937-939.

200. Pressey, R. and Avants, J. K. (1973). Separation and characterization of endopolygalacturonase and exopolygalacturonase from peaches. Plant Physiol. 52: 252-256.

201. Pressey, R. and Avants, J. K. (1973). Two forms of polygalacturonase in tomatoes. Biochem. Biophys. Acta. 309: 363-369.

202. Pressey, R. and Avants, J. K. (1972). Multiple forms of pectinesterase in tomatoes. Phytochem. 11: 3139-3142.

203. Pressey, R. and Himmelsbach, D. S. (1984). 13C-N.m.r. spectrum of a D-galactose-rich polysaccharide from tomato fruit. Carbohydr. Res 127: 356-359.

204. Pressey, R., Hinton, D. M. and Avants, J. K. (1971). Development of polygalacturonase activity and solubilization of pectin in peaches during ripening. J. Food Sci. 36: 1070-1073.

205. Priya sethu, K. M., Prabha, T. N. and Tharanathan, R. N. (1996). Post-harvest biochemical changes associated with the softening phenomenon in Capsicum annuum fruits. Phytochem. 42: 961-966.

206. Rahman, M. M., Akhter, T. and Absar, N. (2000). Activities of glucosidase and galactosidase enzymes during fruit development of ten mango varieties. Ind. J. Plant Physiol. 5: 374-376.

207. Ranwala, A. P., Suematsu, C. and Masuda, H. (1992). The role of β-galactosidases in the modification of cell wall components during muskmelon fruit ripening. Plant Physiol. 100: 1318-1325.

208. Reddy, Y. V. and Srivastava, G. C. (1999). Regulation of cell wall softening enzymes during ripening of mango fruits. Ind. J. Plant Physiol. 4: 194-196. polysaccharides. Planta 189: 499-506.

224. Ross, G. S., Wegrzyn, T., MacRae, E. A. and Redgwell, R. J. (1994). Apple β-galactosidase. Activity against cell wall polysaccharides and characterization of a related cDNA clone. Plant Physiol. 106: 521-528.

225. Sagar, V. R., Khurdiya, D. S. and Balakrishnan, K. A (1999). Quality of dehydrated ripe mango slices as affected by packaging material and mode of packaging. J. Food Sci. Technol. 36: 67-70.

226. Sakai, T., Sakamoto, T., Hallaert, J. and Vandamme, E. J. (1993). Pectin, pectinase and protopectinase: Production, properties and application. Adv. Appl. Microbiol. 39: 213-294.
227. Salimath, P. V. and Tharanathan, R. N. (1982). Carbohydrates offield bean (Dolichos lablab). Cereal Chern. 59: 430-435.
228. Sanchez, A. and Smith, A. J. (1997). Capillary electrophoresis. Methods Enzyrnol. 289: 469-478.
229. Saulnier, L., Brillouet, J. M. and Joseleau, J. P. (1988). Structural studies of pectic substances from the pulp of grape berries. Carbohydr. Res. 182: 63-78.
230. Sawardeker, J. S., Sioneker, J. H. and Jeanes, A. (1965). Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chern. 37: 1602-1604.
231. Schols, H. A. and Voragen, A. G. J. (1994). Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. Carbohydr. Res. 256: 83-95.
232. Schols, H. A., Bakx, E.J., Schipper, D. and Voragen, A. G. J. (1995). A xylogalacturonan subunit present in the modified hairy regions of apple pectin. Carbohydr. Res. 279: 265-279.
233. Schols, H. A., Posthumus, M. A. and Voragen, A. G. J. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. Carbohydr. Res. 206: 117-129.
234. Selvaraj, Y. and Kumar, R. (1989). Studies on fruit softening enzymes and polyphenol oxidase activity in ripening mango (Mangifera indica L.) fruit. J. Food Sci. Technol. 26: 218-222.
235. Selvaraj, Y., Kumar, R. and Pal, D. K. (1989). Changes in sugars, organic acids, amino acids, lipid constituents and aroma characteristics of ripening mango (Mangifera indica L.) fruit. J. Food Sci. Technol. 26: 308-313.
236. Selvendran, R. R. (1985). Developments in the chemistry and biochemistry of pectic and hemicellulosic polymers. J. Cell Sci. (Suppl.) 2: 51-88.
237. Selvendran, R. R. (1975). Analysis of cell wall material from plant tissues: Extraction and purification. Phytochern. 14: 1011-1017.
238. Selvendran, R. R., March, J. F. and Ring, S. G. (1979). Determination of aldoses and uronic acid content of vegetable fiber. Anal. Biochem. 96: 282-292.
239. * Seymour, G. B. and Gross, K. C. (1996). Cell wall disassembly and fruit softening. Postharvest News and Information 7: 45N-52N.
240. Seymour, G. B. and Harding, S. E. (1987). Analysis of the molecular size of tomato (Lycopersicon esculentum Mill.) fruit polyuronides by gel filtration and low speed sedimentation equilibrium. Biochem. J. 245: 463-466.
241. Seymour, G. B., Colquhoun, I. J., Dupont, M. S., Parsley, K. R. and Selvendran, R. R. (1990). Composition and structural features of cell wall polysaccharides from tomato fruits. Phytochem. 29: 725-731.

242. Seymour, G. B., Harding, S. E., Taylor, A. J., Hobson, G. E. and Tucker, G. A. (1987). Polyuronide solubilization during ripening of normal and mutant tomato fruit. Phytochem. 26: 1871-1875.

243. Shasirekha, M. S. and Patwardhan, M. V. (1976). Changes in aminoacids, sugars and non-volatile organic acids in a ripening mango fruit (Mangifera indica Badami variety) Lebensm. Wissens. Technol. 9: 369-370.

244. Shewfelt, R. L. (1986). Postharvest treatment for extending the shelf life of fruits and vegetables. Food Technol. 40(5): 70-80, 89.

245. Siddiqui, I. R. and Wood, P. J. (1971). DEAE-Cellulose carbonate form. A useful medium for fractionating polysaccharides. Carbohydr. Res. 16: 452-454.

246. Singh, R. N. (1990). Mango. Indian Council Agricultural Research, New Delhi, India.

247. * Singhal, V. (2001). Handbook of Export Statistics. Indian Economic Data Research Centre, New Delhi.

248. Sirisomboon, P., Tanaka, M., Fujitha, S. and Kojima, T. (2000). Relationship between the texture and pectin constituents of Japanese pear. J. Texture Studies 31: 679-690.

249. * Smidsrod, 0., Haug, A. and Larsen, B. (1966). The influence of pH on the rate of hydrolysis of acidic polysaccharides. Acta Chern. Scand. 20: 1026-1034.

250. Smith, C. J. (1999). Carbohydrate biochemistry. In: Lea, P. J and Leegood, R. C. (Eds.) Plant Biochemistry and Molecular Biology. II edition. John Willey & Sons. New York. pp. 81-118.

251. Smith, B. G. and Harris, P. J. (1995). Polysaccharide composition of unlignified cell walls of pineapple (Ananas comosus L. Merr.) fruit. Plant Physiol. 107: 1399-1409.

252. Smith, C. J. S., Watson, C. F., Ray, J., Bird, C. R., Morris, P. C., Schuch, W. and Grierson, D. (1988). Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. Nature 334: 724-726.

253. Sobotka, F. E. and Stelzig, D. A. (1974). An apparent cellulase complex in tomato (Lycopersicon esculentum) fruit. Plant Physiol. 53: 759-763.

254. Stevens, B. J. H. and Selvendran, R. R. (1984). Structural features of cell-wall polymers of the apple. Carbohydr. Res. 135: 155-166.

255. Stewart, D., lannetta, P. P. M. and Davies, H. V. (2001). Ripening-related changes in raspberry cell wall composition and structure. Phytochem. 56: 423-428.

256. Strasser, G. R. and Amado, R. (2002). Pectic substances from red beet (Beta vulgaris conditiva) Part-II. Structural characterization of rhamnogalacturonan II. Carbohydr. Polym. 48: 263-269.

257. Strasser, G. R. and Amado, R. (2001). Pectic substances from red beet (Beta vulgaris L. var. conditiva) Part-I. Structural analysis of rhamnogalacturonan I using enzymic degradation and methylation analysis. Carbohydr. Polym. 44: 63-70.

258. Subramanyam, H., Krishnamurthy, S. and Parpia., H. A. B. (1975). Physiology and biochemistry of mango fruit. Adv. Food Res. 21: 223-305.

259. Suvarnalatha, G. and Prabha, T. N. (1999). a-D- Mannosidase from Lycopersicon esculentum II. Phytochem. 50: 1111-1115.

260. Tandon, D. K. and Kalra, S. K. (1984). Pectin changes during the development of mango fruit cv Dashehari. J. Hort. Sci. 59: 283-286.

261. Taylor, R. L. and Conrad, H. E. (1972). Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxylic groups. Biochem. 11: 1383-1388.

262. Thakur, B. R. Singh, R. K. and Handa, A. K. (1997). Chemistry and uses of pectin- A review. Grit. Rev. Food Sci. Nutr. 37: 47-73.

263. Tharanathan, R. N., Muralikrishna, G., Salimath, P. V and Raghavendra Rao, M. R. (1987). Plant carbohydrates- an overview. Proc. Ind. Acad. Sci. (Plant Sci.) 97: 81-155.

264. Themmen, A. P. N., Tucker, G. A. and Grierson, D. (1982). Degradation of isolated tomato cell walls by purified polygalacturonase in vitro. Plant Physiol. 69: 122-124.

265. Theologis, A., Zarembinski, T. I., Geller, P. W., Liang, X. and Abel, S. (1992). Modification of fruit ripening by suppressing gene expression. Plant Physiol. 100: 549-551.

266. Thomas, M. and Thibault, J-F. (2002). Cell wall polysaccharides in the fruits of Japanese quince (Chaenomeles japonica): extraction and preliminary characterization. Carbohydr. Polym. 49: 345-355.

267. Tieman, D. M., Harriman, R. W., Ramamohan, G. and Handa, A. K. (1992). An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. Plant Cell 4: 667-679.

268. Tucker, G. A. (1993). Introduction. In: Seymour, G. B., Taylor, J. E., Tucker, G. A. (Eds.). Biochemistry of Fruit Ripening. Chapman and Hall, London. pp. 1-51.

269. Tucker, G. A. and Grierson, D. (1987). Fruit ripening. In: Davies, D. (Ed.). The Biochemistry of Plants. Vol. 12. Academic Press Inc., New York, pp. 265-319.

270. Tucker, G. A. and Seymour, G. (1991). Cell wall degradation during mango fruit ripening. Acta Hort. 291: 454-460.

271. Tucker, G. A., Robertson, N. G. and Grierson, D. (1982). Purification and changes in activities of tomato pectinesterase isoenzymes. J. Sci. Food Agric. 3: 396-400.

272. Tucker, G. A., Robertson, N. G. and Grierson, D. (1980). Changes in polygalacturonase isoenzymes during the "ripening" of normal and mutant tomato fruit. Eur. J. Biochem. 112: 119-124.

273. Van Buren, J. P. (1979). The chemistry of texture in fruits and vegetables. J. Texture Studies 10: 1-23.

274. Vazquez-Salinas, C. and Lakshminarayana, S. (1985). Compositional changes in mango fruits during ripening at different storage temperatures. J. FoodSci. 50: 1646-1648.

275. Vidal, S., Williams, P., O'Neill, M. A. and Pellerin, P. (2001). Polysaccharides from grape berry cell walls. Part I: tissue distribution and structural characterization of the pectic polysaccharides. Carbohydr. Polym. 45: 315-323.

276. Voragen, A. G. J., Pilnik, W., Thibault, J-F, Axelos, M. A. V. and Renard, C. M. C. G. (1995). Pectins. In: Stephen, A M (Ed.) Food Polysaccharides and their Applications. Marcel Dekker, Inc. New York. pp. 287-339.

277. Wade, N. L., Kavanagh, E. E., Hokley, D. G. and Brady, C. J. (1992). Relationship between softening and the polyuronides in ripening banana fruit. J. Sci. Food Agric. 60: 61- 68.

278. Waldron, K. W., Smith, A. C., Parr, A. J., Ng. A. and Parker, M. L. (1997). New approaches to understanding and controlling cell separation in relation to fruit and vegetable texture. Trends Food Sci. Technol. 8: 213-221.

279. Wallner, S. J. (1978). Apple fruit β-galactosidase and softening in storage. J. Amer. Soc. Hort. Sci. 103: 364-366.

280. Wallner, S. J. and Bloom, H. L. (1977). Characteristics of tomato cell wall degradation in vitro. Implications for the study of fruit-softening enzymes. Plant Physiol. 60: 207-210.

281. Wallner, S. J. and Walker, J. E. (1975). Glycosidases in cell wall-degrading extracts of ripening tomato fruits. Plant Physiol. 55: 94-98.

282. Watkins, C. B., Haki, J. M. and Frenkel, C. (1988). Activities of polygalacturonase, ∝-D-mannosidase, and ∝-D- and β-D-galactosidase in ripening tomato. HortSci. 23: 192-194.

283. * Whitaker, J. R. (1994). Pectic enzymes. In: Principles of Enzymology for the Food Sciences. (II ed.) Marcel-Dekker Inc., New York.

284. Whitaker, J. R. (1984). Pectic substances, pectic enzymes and haze formation in fruit juices. Enzym. Microb. Technol. 6: 341-349.

285. Wegrzyn, T. F. and MacRae, E.A. (1992). Pectinesterase, polygalacturonase and p-galactosidase during softening of ethylene-treated kiwifruit. HortSci. 27: 900-902.

286. Wong, D. W. S. (1995). Pectic enzymes. In: Food Enzymes. Structure and Mechanisms. Chapman and Hall, New York. pp. 212-236.

287. Wood, P. J. and Siddiqui, I. R. (1971). Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. Anal. Biochem. 39: 418-428.

288. * Yahia, E. (1998) Modified and controlled atmospheres for tropical fruits. Hort. Rev. 22: 123-183.

289. Yang, S. F. and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. Ann. Rev. Plant Physiol. 35: 155-189.

290. Yashoda. H. M., Prasanna, V., Jagadeesh, B. H. and Prabha, T. N. (2001). In situ depolymerization of carbohydrate polymers with reference to fruit ripening: Its implication. Trends Carbohydr. Chern. 7: 97-102.

291. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P. (1985). Isolation and characterization of plant cell walls and cell wall components. Methods Enzymol. 118: 3-40.

292. Yu. L., Reitmeier. C. A. and Love. M. H. (1996). Strawberry texture and pectin content as affected by electron beam irradiation. J. Food Sci. 61: 844-846.

293. * Zainal, Z., Tucker, G. A. and Lycett, G.W. (1999). Isolation and characterization of cDNA encoding 1-ACC oxidase from mango (Mangifera indica. L). Asia Pacific J. Mol. BioI. Biotechnol. 7: 53-59.

294. Zhan, D., Janssen, P. and Mort, A. J. (1998). Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. Carbohydr. Res. 308: 373-380.

295. Zor, T. and Selinger, Z. (1996). Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. Anal. Biochem. 236: 302-308.

* Original reprints were not referred to.