

POST HARVEST CHANGES IN THE CELL-WALLS OF MANGO FRUITS

by

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ABSTRACT

Comparative work on the structure of the cell walls of red kidney bean hypocotyls and mesocarp from unripe and ripe mango fruits showed significant differences. Cell-wall fractions from each were obtained using solvent extraction (water, alkali and acid) and enzymic (endopolygalacturonase) degradation procedures. The monosaccharide composition of each fraction was determined after TFA-hydrolysis by TLC-analysis. Greatest variation in monosaccharide compositions was observed in the water-soluble fractions which accounted for 18%, 48% and 11% of the cell walls of the bean, unripe and ripe mango, respectively.

Water-soluble carbohydrates present in the mango pulp were examined by TLC, gel-filtration and ion-exchange methods. Only sucrose, fructose, glucose and high-MW polysaccharides were detected. The ripe mango contained 8 times more soluble polysaccharide than the unripe. In the ripe fruit the approximate MW of the polysaccharide fraction, which was rich in uronic acid was 40,000. In the unripe fruit polysaccharides with MW's ranging from 40,000 to > 300,000 were detected. These polymers contained much smaller proportions of uronic acid than those from the ripe mesocarp.

A crude 3 M LiCl enzyme extract from the cell walls of the ripe mango was able to solubilize 14% of prepared cell walls from the unripe fruit. The mechanism of this process

was investigated. Endo-enzymes involved could not be identified.

The involvement of β -galactosidase in cell-wall degradation was examined. Using p-nitrophenyl- β -D-galactopyranoside as substrate, 3 wall-bound forms of the enzyme were found in the ripe fruit and 2 in the unripe. Four soluble forms which were different from the wall-bound forms were observed in the ripe fruit. Three of these were present in the unripe fruit. The action of the enzyme forms on mango pectin and cell walls was examined. There was no evidence that any were directly implicated in cell-wall degradation. Wall-bound exopolygalacturonase was detected for the first time in the mango fruit.

To Archna and Jaysen

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ABBREVIATIONS

PG	Endopolygalacturonase
MW	Molecular weight
TFA	Trifluoroacetic acid
Gal.	<u>D</u> -Galactose
Glu.	<u>D</u> -Glucose
Man.	<u>D</u> -Mannose
Ara.	<u>L</u> -Arabinose
Xyl.	<u>D</u> -Xylose
Rha.	<u>L</u> -Rhamnose
Gal. a.	<u>D</u> -Galacturonic acid
PNPG	<u>p</u> -Nitrophenyl- β - <u>D</u> -galactopyranoside
ONPG	O-Nitrophenyl- β - <u>D</u> -galactopyranoside
RKB	<u>Red Kidney Bean</u>
n/d	<i>Not determined</i>
u.ac.	<i>Uronic acid</i>

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INTRODUCTION

I. GENERAL

The softening of fruits during ripening is economically important as it affects their marketing quality and influences their shelf-life. The purpose of this work was to investigate changes occurring in the cell wall and the related enzymology during the ripening process of Ngowe mangoes Mangifera indica. The large number of varieties (approximately 1,000) of mango fruit have similar popularity in tropical countries as the apple does in more temperate regions, and possess a similar commercial potential as banana. The mango belongs to the dicotyledonous family Anacardiaceae which consists of 64 genera and are mostly trees and shrubs. The economic viability of the fruit crop may be realised by prolonging its shelf-life, which is of approximately 2 weeks duration for most varieties. The edible part of the fruit is the fleshy mesocarp region which is sweet in taste and has a distinct aroma.

The walls of the parenchyma cells in the pulp region of fruits are made up almost entirely of carbohydrate material. Mature unripe fruit cell walls generally have rigid well-defined structures, whereas ripe fruits have soft and diffused cell walls. It is thought that this change is caused mainly by the loss of carbohydrate material during ripening (1-5). Cell-wall breakdown is considered to be brought about by the co-ordinated action of hydrolytic enzymes and occur together with biochemical and physiological changes which convert the fruit from an inedible to an edible state.

II. COMPONENTS OF PRIMARY CELL WALL

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As fruit softening is accompanied by changes in the cell wall, the latter is discussed under two sections, namely components of the primary cell wall and structure of the primary cell wall. Physiological and biochemical changes during fruit development are discussed in separate sections.

The plant cell consists of cytoplasm surrounded by a cell membrane which in turn is surrounded by a cell wall. Each cell is connected to adjacent cells by a pectin-rich middle lamella. The cytoplasm of the cells are interconnected by a plasmadesmata which in the ripe fruit is thought to give a degree of cohesion. As long as a cell contrives to undergo growth, its wall remains relatively thin. At this stage of development it is called a primary cell wall and consists of roughly 90% polysaccharide and 10% protein. This is distinct from secondary walls which in some regions of the plant, such as the stem, is deposited after growth has stopped and is responsible for giving a woody texture. Cells present in fruit pulp are generally thought to contain primary cell walls.

The primary cell-wall constituents can be divided into fractions:

- A. Pectic polysaccharides (34%)
- B. Hemicellulose (24%)
- C. Cellulose (23%)
- D. Hydroxyproline-rich glycoprotein (19%)

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The ratio of these constituents vary with different cell walls. The percentage values given are for the walls of suspension-cultured sycamore cells (6). Structures have been proposed for various regions of each of the above fraction which have received varying degrees of acceptance and will be discussed in the following sections.

A. Pectic Polysaccharides

Pectic polysaccharides are generally considered to be portions of cell walls which can be extracted by a variety of mild methods such as hot water, ammonium oxalate solution, weak acids, chelating agents and PG. These reagents also extract varying amounts of other fractions from the cell wall which creates a certain amount of ambiguity. The quantity extractable varies from 20% to 30% of the walls of meristematic and parenchymatous tissues. It is accepted that pectin is essentially composed of galacturonic acid, rhamnose, arabinose and galactose, the proportions of these varying with the source. Other associated sugars are D-xylose, 2-O-methyl-L-fucose, D-apiose and D-glucuronic acid.

Fragments of pectic polysaccharide molecules have been isolated following removal from cell walls. Analysis of such materials suggests the presence of structurally different regions. These are referred to as rhamnogalacturonan I, rhamnogalacturonan II, homogalacturonan, arabinan, galactan and arabinogalactan.

1. Rhamnogalacturonan I

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Rhamnogalacturonan I accounts for approximately 7% of primary cell walls (7). These regions of pectin are large with estimated degrees of polymerisation of 2,000. The proposed structure for this fragment is shown in Fig. 1 which depicts a rhamnogalacturonan backbone to which side chains containing neutral sugars (arabinose and galactose) are linked via rhamnosyl residues. This has been demonstrated by Aspinall and co-workers (8-11) with work on pectin from rapeseed hull, soybean cotyledon, lucerne leaves, lucerne stems and lemon peel. Galacturonic acid residues in the backbone are attached to each other by α -1,4-glycosidic linkages. Analysis of rapeseed (11) and lucerne (8) pectin demonstrated that half of the rhamnosyl residues in the polymer are linked via their C-2 (kink; Fig. 1) and the rest via C-2 and C-4 (branch-point; Fig. 1). As no aldobouronic acid with a galacturonosyl residue attached to C-4 of a rhamnosyl residue has been identified, it has been assumed that this is the point of attachment of the side chains of arabinans, galactans or arabinogalactans. Rhamnose, galacturonic acid, arabinose and galactose were found to occur in the ratio 1:2:1.5:1.5 respectively (12). No information is available on whether the rhamnosidic bonds are in the α - or β -configuration.

2. Homogalacturonan

Homogalacturonan accounts for approximately 6% of the cell wall. Pectic polysaccharides contain regions of unbranched α -1,4-linked galacturonosyl residues in addition to high-MW rhamnogalacturonan I (13, 14). This is apparent from experiments which show that PG can successfully hydrolyse regions of pectin accounting for approximately

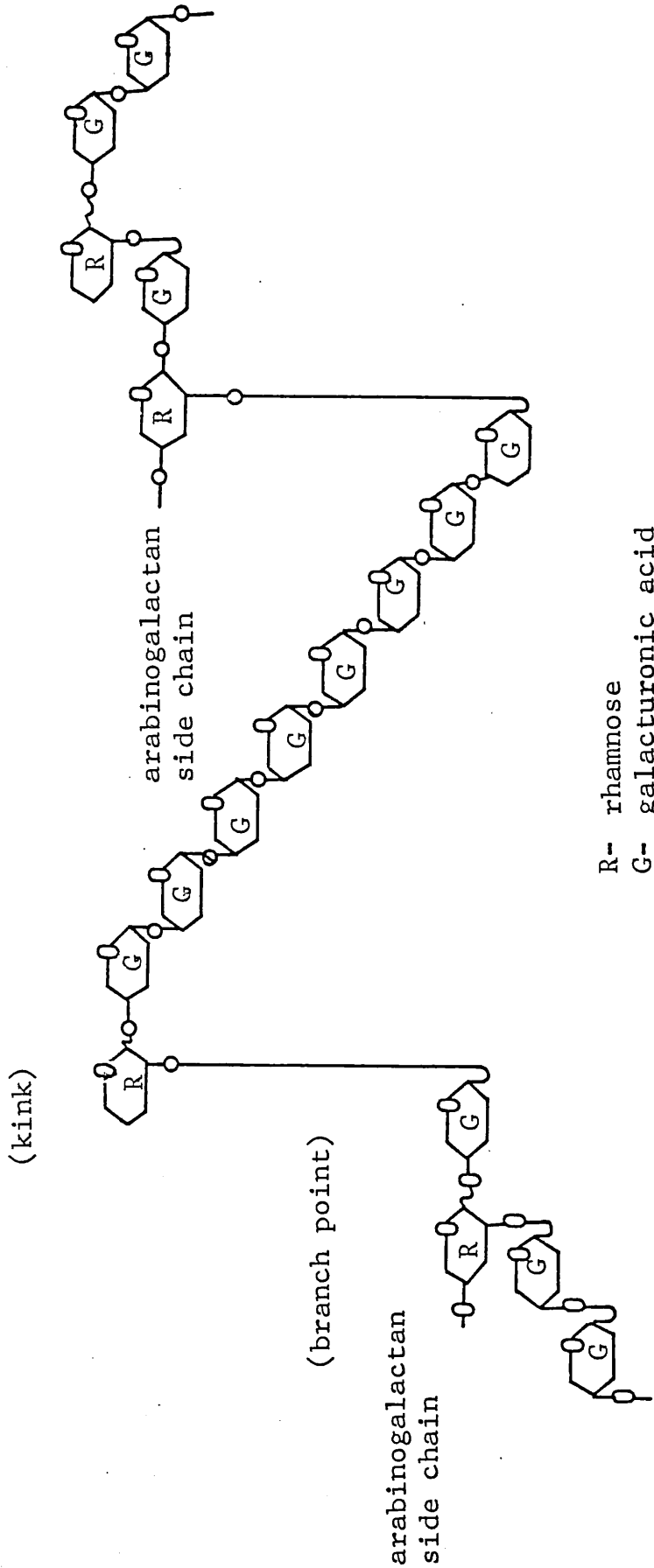


Fig. 1
Proposed structure for pectic rhamnogalacturonan I of primary cell walls of diotyledons, after Albersheim (13).

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15% of cell wall to give fragments of mono-, di- and tri-galacturonic acids. These products presumably arise from the hydrolysis of domains within large pectic polymers containing α -1,4-linked galacturonans. Also classified as homogalacturonans are regions of pectin which are stable to hydrolysis by PG due to esterification of the uronosyl carboxyl groups, and which do not contain side chains. Homogalacturonan fragments have been isolated from the walls of suspension-cultured sycamore cells (13), apple pectin (15) and sunflower seeds (16). The sycamore polymer had a degree of polymerisation greater than 25 (17).

3. Rhamnogalacturonan II

Rhamnogalacturonan II accounts for approximately 3% of the primary cell wall, and constitutes a small region of pectic polysaccharides. It contains 25-50 glycosyl residues (18) and monosaccharides that are rarely observed in other polymers, for example, 2-O-methyl-L-fucose, 2-O-methyl-D-xylose and D-apiose. A highly branched structure for this polymer is apparent from the wide variety of terminal glycosyl residues it possesses. The terminal residues detected are D-galacturonic acid, D-galactose, L-arabinose, L-rhamnose, 2-O-methyl L-fucose and also 2-O-methyl-D-xylose. Also present were 2-linked glucuronosyl, 3-linked apiosyl, 3-linked rhamnosyl, 2,4-linked galactosyl, 3,4-linked rhamnosyl and 3,4-linked fucosyl residues (18).

4. Arabinans

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Arabinans account for approximately 9% of the primary cell walls of dicotyledonous plants and a purified arabinan has been isolated from the walls of suspension-cultured sycamore cells (17). All the plant arabinans investigated from different sources appear to have similar structures (18-23). They are highly branched polymers containing L-arabinosyl residues that are mainly in the furanose ring form and α -anomeric configuration (18, 20). Regions of unbranched chains with 1,5-linked residues were shown to occur in sycamore pectic fraction (13), and arabinan from mustard cotyledon had branched structures (22). An arabinan from willow bark displayed a degree of polymerisation of 90 (21), whereas two such polymers found in Rosa glauca bark had degrees of polymerisation of 34 and 100 (20). Linkages that have been detected in plant arabinans include 5-linked, 3,5-linked, 2,5-linked and 2,3,5-linked residues (18, 20, 24, 25).

5. Galactans

Galactans together with arabinogalactans account for approximately 9% of the cell wall. Although galactans have been derived from citrus pectin (26), white willow (27) and beech (28), homogalactans are relatively rare. However, the well characterised galactans have been from Strychnurs nux vomica seeds (29) and potato tubers (30-32). Galactan preparations that have been obtained, generally contain β -1,4-linkages, however some 1,6-linkages have also been identified (31). Degrees of polymerisation have been found to range from 33 in white willow to 50 in sycamore (17). Several pectic polysaccharides have been shown to contain both 1,3- and 1,6-linked galactosyl residues (9,13). Many of the galactosyl residues found in pectic

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polysaccharides are probably not part of homogalactans (8, 9, 11, 13, 33-35). In rapeseed hull, the galactosyl residues attached to the uronic acid backbone have been shown to occur as β -1,4-linked dimers rather than as oligosaccharides or polymers (11). There is not sufficient data available at present to propose a structure for primary cell-wall galactan.

6. Arabinogalactans

It is uncertain whether arabinogalactans generally occur in the primary cell wall of dicotyledonous plants. Evidence supportive to their existence in sycamore cell wall came from the work of Albersheim's group (13). This involved analysis of a pectic fraction released by PG from the walls of suspension-cultured sycamore cells. The main arabinosyl- and galactosyl-containing components of these fractions appeared to come from a β -1,4-linked galactan and a highly-branched arabinan, but other linkages characteristic of arabinogalactans were also found. These included terminal, 3-, 6- and 3,6-galactosyl residues in addition to terminal, 3-, 5- and 2,5-linked arabinofuranosyl linkages which were all detected in substantial quantities. Earlier work on pectin has indicated the presence of arabinogalactans in apple (15) and sycamore suspension-cultured cells (36), however, these had not been rigorously characterised. There appears to be wide variations both in the composition and linkage structure of arabinogalactans. These polysaccharides isolated from rapeseed flour (37) contain 90% arabinosyl residues whereas those from larch contain 88% galactosyl residues (38). Of 7 arabinogalactans that have been studied, 3 were found to contain rhamnosyl residues

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(28). Arabinogalactans are generally characterised by the presence of significant amounts of 3,6-linked galactosyl residues and terminal α -L-arabinofuranosyl residues. Soybean arabinogalactan, however, has a linear β -1,4-linked galactosyl backbone with α -L-arabinosyl-(1,5)-O- α -arabinose residues linked to C-3 of some of the galactosyl residues (39-41). Other arabinogalactans also have a galactan backbone, but with larger arabinosyl side chains.

B. Hemicellulose

Hemicellulose accounts for 24% of the cell wall and is considered to be made up of xyloglucans and glucuronoarabinoxylans

1. Xyloglucans

Xyloglucans account for 19% of primary cell walls. Much more is known about the structure (Fig.2) of these polysaccharides compared to the relatively more complex pectic polymers. Xyloglucans from different sources share similar structural features (42-51). This includes a backbone of β -1,4-linked D-glucosyl residues with single D-xylosyl residues bonded via α -1,6-linkages to the backbone. Some of the xylosyl residues are substituted with β -1,2-linked D-galactosyl residues (42, 43, 52). Terminal fucosyl residues are linked to these galactosyl residues. This linkage is believed to be α -L-fucosyl attached to C-2 of the galactose. Attached to a few glucosyl residues of the backbone are arabinopyranose residues believed to be linked by 1,2-linkages; the anomeric nature of the linkages is not known. According to Darvill et al. (52) the present structure of xyloglucans

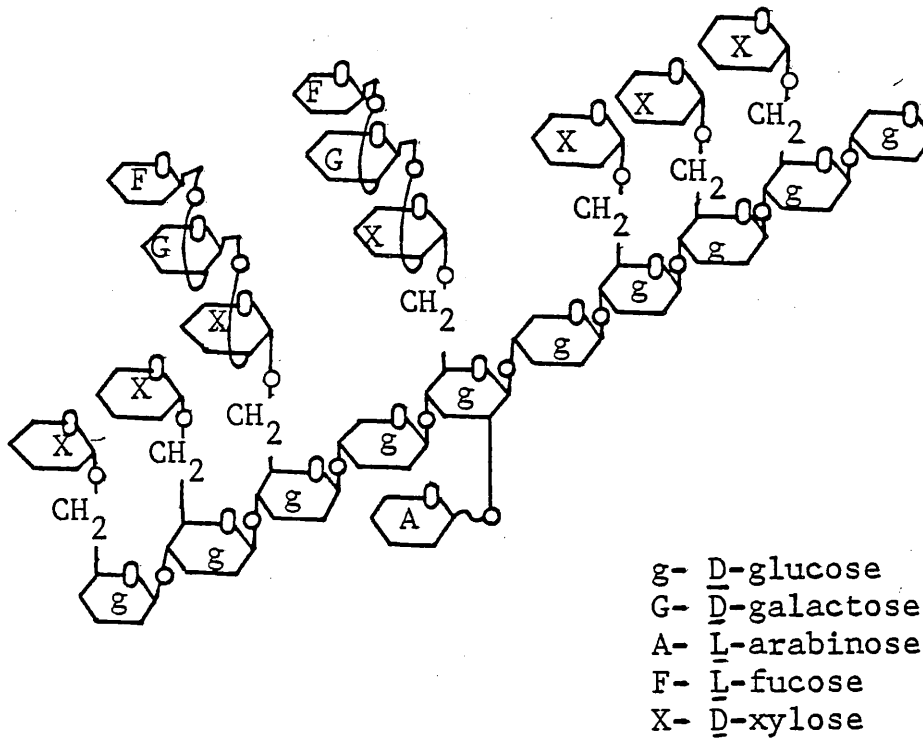


Fig. 2
Structural features of xyloglucan, after Albersheim et al. (12).

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suffer from the following shortcomings:

(a) The arabino-1,2-linkage is based solely on the finding that equimolar amounts of terminal arabinopyranosyl and 2,4,6-substituted glucosyl residues are present.

(b) Some glucosyl residues could be attached to C-6 of other glucosyl residues with an equivalent number of xylosyl residues attached to C-4 of glucosyl.

(c) The anomeric configuration of the glycosidic linkages in cell-wall xyloglucan have been assumed but not proved to be the same as those which occur in seed xyloglucans (45, 49, 53).

(d) Fucosyl linkages have been assumed to be in α -anomeric configuration because the linkage is hydrolysed by an enzyme mixture known to contain an α -1,2 fucosidase (54, 55).

2. Glucuronoarabinoxylan

Glucuronoarabinoxylan has only recently been identified in the primary cell wall of suspension-cultured sycamore cells (25) and accounts for 5% of it. It has been shown to contain terminal, 4-, 2,4- and 3,4-linked xylosyl residues, terminal and 2-linked arabinofuranosyl residues, terminal glucosyl residues and terminal 4-O-methyl-D-glucuronosyl residues. The overall structure is, however, not known.

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C. Cellulose

It is believed that the structure of cellulose which constitutes approximately 23% of the primary cell wall is similar in all plant sources (56, 57, 59). It is made up of long unbranched chains of β -1,4-linked glucose residues. The chains exist in parallel-sheet structures stabilised by interchain hydrogen-bonding to form cellulose fibres (56-60). Estimates from electron microscopic studies give values of 4.5 x 8.3 nm (56) for cross-sectional dimensions of these fibres which is estimated to contain 60-70 glucan chains. Aggregations of the glucan chains within a fibre are so ordered that they are crystalline (56-61) and can be subjected to X-ray diffraction studies which indicate that they may have parallel orientation with reducing ends facing the same direction (59-61). However, antiparallel orientation cannot be excluded (62). Purified cellulose invariably contains, in addition to a preponderance of glucosyl residues, minor amounts of other glycosyl residues. The possibility exists that these may be normal constituents of the glucan chains rather than non-covalently but tightly held impurities such as hemicellulose.

D. Glycoprotein

Proteins account for 5-10% of dicotyledonous primary cell walls and are generally present in glycosylated form (63-65). Proteins contain about 20% of hydroxyproline and relatively large amounts of alanine, serine and threonine. These are characteristic amino acids of structural protein of animals (52) which suggests that they may have a similar role in plant cell walls. Great difficulty is often

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encountered in extracting the protein from cell walls under non-degradative conditions (66). In effect, it has not been possible to isolate the wall glycoproteins without the use of drastic methods, which has made it difficult to assess their relationship with other cell-wall polymers in the intact wall, and to determine their full structures. Most of the detailed structural studies on glycoproteins have utilised cell walls isolated from plant suspension cultures (67, 69, 70) which have higher concentrations of hydroxyproline-rich proteins (53). Glycoproteins with low hydroxyproline content also exist (32, 67). The structure of hydroxyproline-rich glycoproteins (extensin) is characterised by the following features:

(a) L-Arabinofuranose oligosaccharides are O-glycosidically attached via most hydroxyl groups of hydroxyproline residues. Little or no unglycosylated hydroxyproline appear to be present (68, 69; see Fig. 3). The side chains vary in length from 1 to 4 residues (70-72).

(b) Single α -galactose residues are linked to the hydroxyl group of serine.

Analysis of cell-wall glycoproteins from a variety of sources show that hydroxyprolinyl-O-arabinosides and serinyl-O-galactosides are of universal occurrence (73). This conservation of structure, points to wall protein having a unique fundamental role in dicotyledonous plant cell wall.

E. Phenols

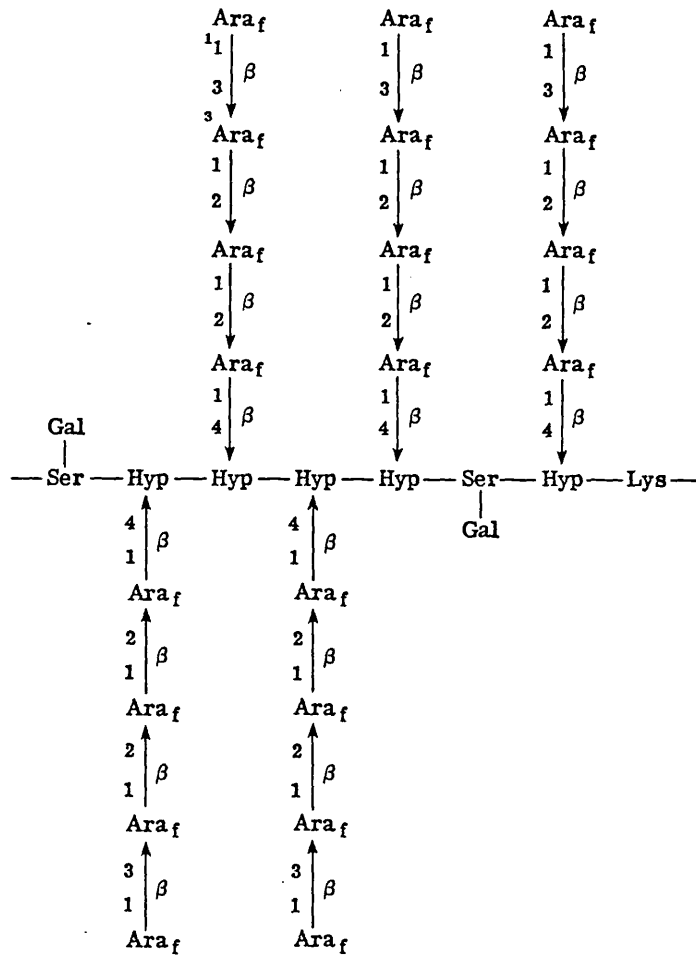


Fig. 3
Structure of hydroxyproline-rich glycoprotein, taken from Darvill et al. (52).

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The existence of phenolic materials in the primary cell wall of dicotyledonous plants has only recently attracted attention (74-76). Very little data is available from which general conclusions can be drawn. Ferulic acid, p-coumaric acid and other unidentified phenols were shown to be present in the cell-wall of spinach cells in suspension culture. Ferulic acids account for 0.5% of the cell wall and were suggested to be linked to the non-reducing termini of neutral arabinose and/or galactose containing regions of the pectic fraction. It was suggested that such residues can cross-link in vivo to form diferuolyl bridges (74-76) which would cause a lowering of the walls extensibility and could play a role in resistance to fungal pathogens. The significance of the presence of such a relatively small amount of material in plant cell wall is however open to speculation.

III. STRUCTURE OF PRIMARY CELL WALL

The way in which the various components of the cell wall are linked together is largely not known. This is due mainly to the limited amount of information currently available on their structures. Present evidence indicate that interconnections between some of the fractions exist and on this basis various models have been proposed. These interconnections will be described in the following sections:

A. Polymer Interconnections

1. Links Between Pectic Fractions

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It is believed that the various domains of pectic polysaccharides discussed in previous section are covalently linked together to form complex molecules (77, Fig. 4). All of these segments are known to be released from the primary cell wall by the action of polygalacturonase (13, 17, 18,). There is little doubt that arabinans and galactans are bonded to rhamnogalacturonan I as the whole complex can be isolated by ion-exchange column chromatography. Fragments containing these three characteristic components migrate as a single spot upon electrophoresis (15). Further, no arabinan or galactan has been extracted from primary cell walls that is free of galacturonic acids. An "egg-box" model of non-covalent bonding between pectic polysaccharides in the wall involving calcium ions (73, 78) has been proposed. This depicts the latter to be fitted between two or more chains of unesterified polygalacturonosyl molecules so that they are held by ionic attraction between the calcium ions and the oxygen atoms of 4 galacturonosyl residues. This arrangement results in greater rigidity and cross-linking of galacturonan chains (79). Other types of non-covalent associations may also exist but their relative importance in the arrangement of the pectic fraction in the cell wall is not known.

2. Links Between Xyloglucan and Cellulose

It has been suggested that xyloglucan which is the predominant hemicellulose in primary cell walls is strongly held to the surface of cellulose fibres by hydrogen-bonding. This is supported by the following evidence:

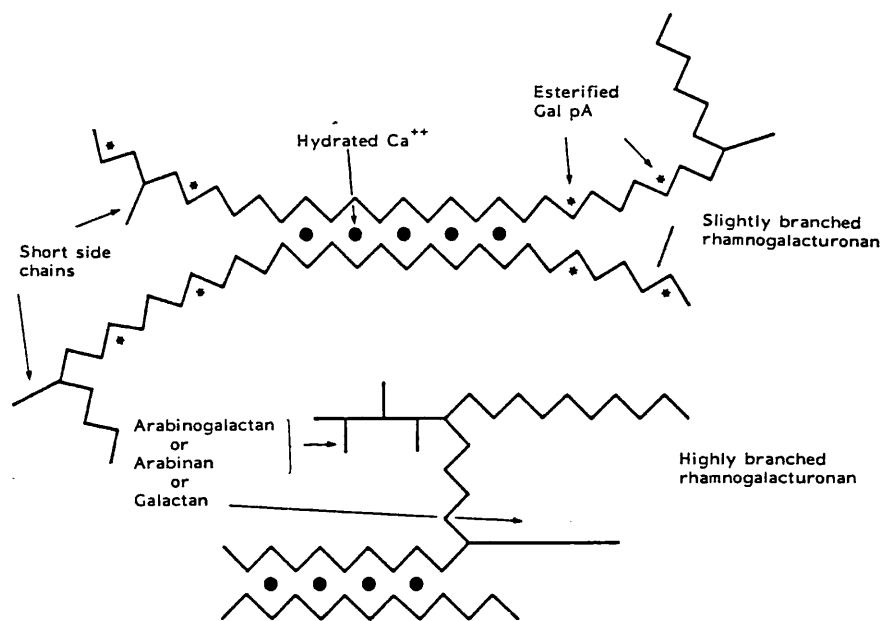


Fig. 4
Structural features of pectin, taken from Selvendran (77).

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(a) The amount of xyloglucan is quantitatively sufficient to form a monolayer coating of cellulose fibrils (43, 80).

(b) Space-filling models of xyloglucan show that it is capable of forming multiple hydrogen bonds to cellulose (43).

(c) Xyloglucan can be extracted from xyloglucan cellulose complexes in the cell wall by hydrogen-bond breaking reagents such as dilute base or 8 M urea (43).

(d) Xyloglucan strongly binds to isolated cellulose in the absence of enzymes or chemical catalysts (43, 81).

(e) The binding of xyloglucan to the cell wall and to isolated cellulose is reversible (43).

(f) Small fragments of xyloglucan can be extracted from cell walls and separated from cellulose fibrils by enzymes which degrade xyloglucan into fragments. It is assumed that these small polymers are not long enough to form stable hydrogen-bonded complexes with cellulose (43).

(g) Short, enzymically produced xyloglucan fragments can be induced to form complexes with cellulose by reducing the water activity of the solvent, thereby reducing the opportunity for the fragments to hydrogen-bond with the solvent (82).

Bonding of xyloglucans to cellulose has been suggested to prevent

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aggregation of cellulose fibres (52).

3. Links Between Xyloglucan and Pectin

Attempts at preparing large amounts of xyloglucan covalently attached to pectic polysaccharides have not been successful (83), though it has been suggested that these 2 fractions are covalently bonded together (13, 43, 80). The relative importance of this sort of interconnection within the cell wall is thus uncertain.

4. Glucuronoarabinoxylan

Glucuronoarabinoxylan is structurally related to arabinoxylans and xylans. The latter two are capable of hydrogen-bonding to cellulose (43, 84, 85). The observation that arabinoxylans aggregate in solution (86, 87; possibly forming a mixture of random coils and linear chains) has prompted the suggestion (52) that glucuronoarabinoxylan may do likewise, and may bind to themselves as well as to cellulose in cell walls. Such interaction would assist in the cross-linking of polymers in primary cell walls.

5. Role of Hydroxyproline-Rich Glycoproteins

As yet there is no evidence to indicate that hydroxyproline-rich glycoproteins are covalently attached to other fractions within primary cell walls. It is possible that linkages that exist are broken under the drastic conditions employed in the extraction of this polymer. In some tissues, alkali extraction of walls releases

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hydroxyproline-rich glycoproteins (88, 89) whereas in others, they are tenaciously held to the cell wall complex (90-92). Recently O'Neill et al. (72) released hydroxyproline-rich glycoproteins of "minimal modification" with chlorite / acid solution. They argued that such conditions are less effective against glycosidic or peptide linkages. They also proposed that glycoproteins could be held in the cell wall by phenolic cross-links which may be glycoprotein-protein or glycoprotein-polysaccharide in nature. No other evidence has been presented in support of this. However, Fry (93) has implicated a novel phenolic amino acid, isodityrosine, in providing inter-polypeptide cross-links in plant cell-wall glycoprotein (94-96). Such linkages contribute to glycoprotein insolubility (97).

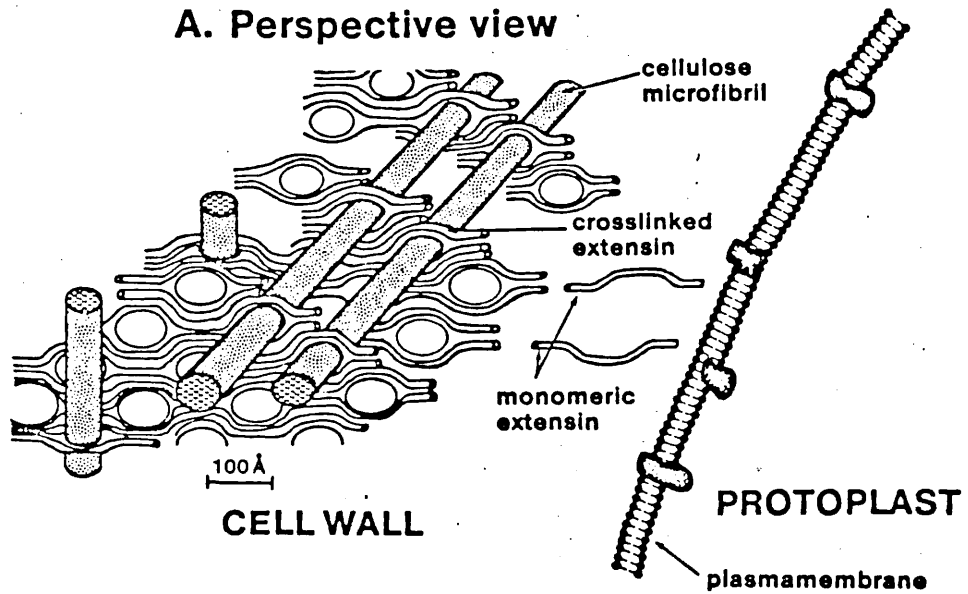
A completely new idea proposing non-covalent binding of hydroxyproline-rich glycoproteins to the primary cell wall has very recently been introduced by Lamport et al. (98). The structure they propose is shown in Fig. 5 and depicts a net of rod shaped hydroxyproline-rich glycoprotein molecules penetrated by cellulose microfibrils. Widespread cross-linking is proposed to occur between molecules of the former via isodityrosine cross-links.

B. Cell-Wall Model

Any model on primary cell-wall structure must be able to account for the following properties:

EXTENSIN-CELLULOSE NETWORK

A. Perspective view



B. Cross section view

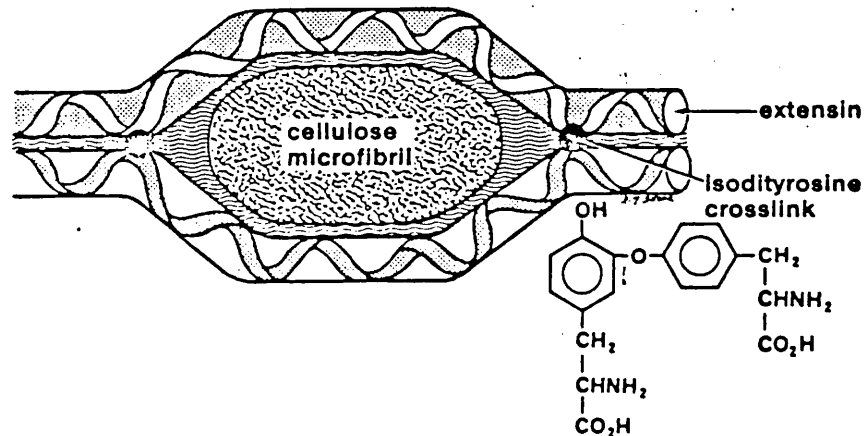


Fig. 5
Non-covalent bonding between cellulose microfibrils and extensin; after Lamport and Epstein (98).

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- (a) Their great strength in withstanding turgor pressure.
- (b) Their ability to grow without loss of strength.
- (c) Their behaviour under chemical and enzymic attack.

In addition, proposals on cell-wall structure should fit in with current observations on wall synthesis. Models have been proposed by Albersheim's group (80, 99, 100) and Monro et al. (101) which will be discussed. Other models will not be considered because they are in essence embodied by that of Albersheim's group. It should be borne in mind that the proposed models were based upon a limited number of plant studies and they should therefore be considered as only "theoretical models".

1. Model of Albersheim

Albersheim's most recent model on the primary cell wall (99) of dicotyledonous plants was put forward in 1978 and is shown in Fig. 6. This model is a modification of an earlier one presented in 1973 (80) and depicts a network of cellulose fibres each covered by monolayers of xyloglucan which are covalently cross-linked to each other by pectic polysaccharides via their neutral side chains.

2. Model of Monro

This model was proposed by Monro and co-workers in 1976 (see Fig. 7) and is similar to that proposed by Albersheim in that it consists of

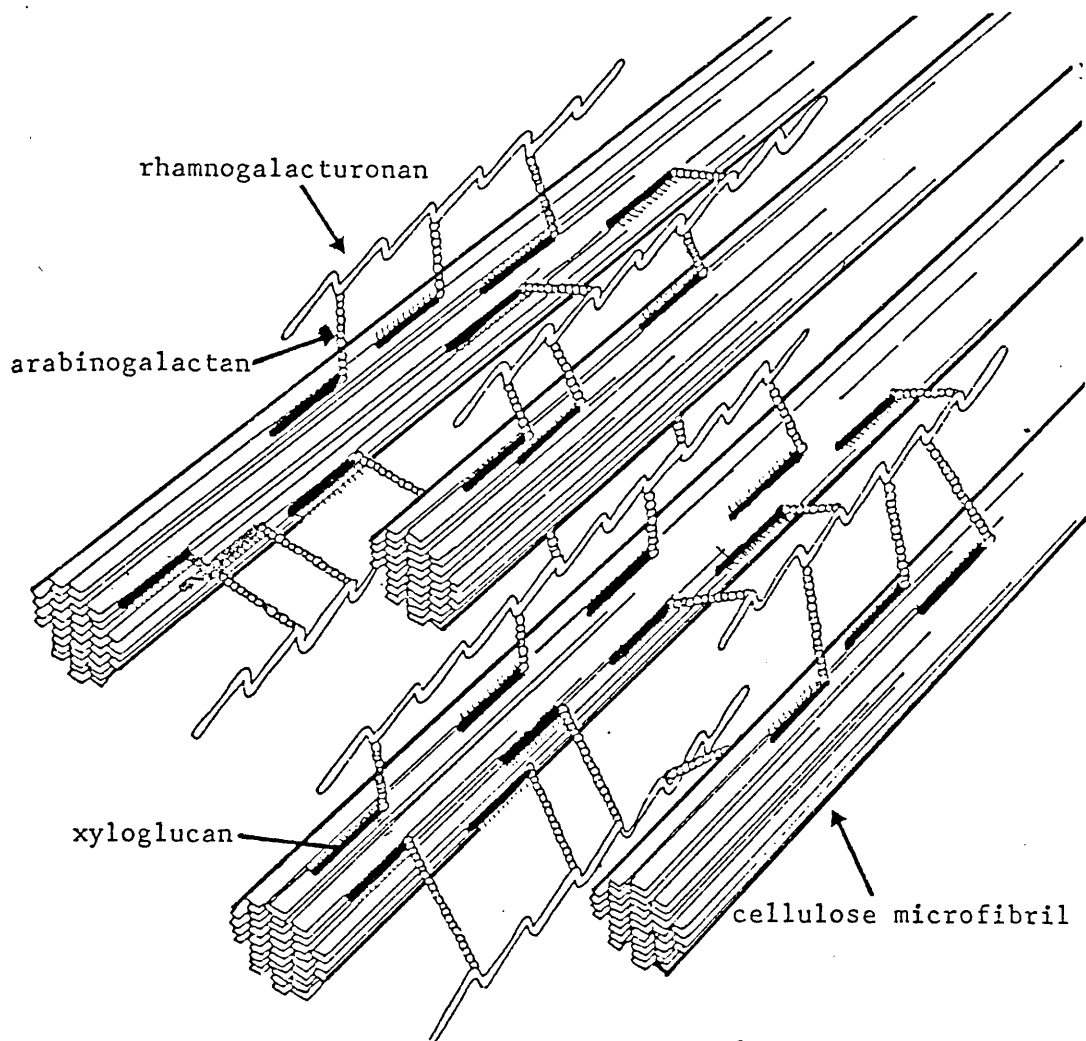
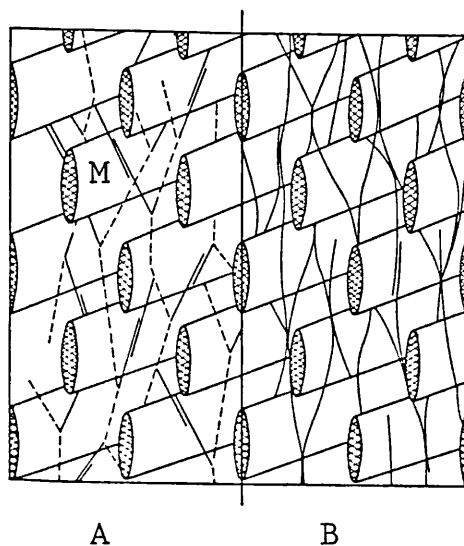


Fig. 6

Primary cell-wall model devised by Albersheim (99). Each cellulose microfibril is covered by a layer of xyloglucan. These are cross-linked by side chains of arabinogalactan.



- A- extensin-polysaccharide network
B- pectin network
M- cellulose microfibril
---- extensin
— polysaccharide
== junction zone

Fig. 7
Primary cell-wall model devised by Monro et al. (101).

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cellulose microfibrils interconnected by a network of polysaccharides. However, it differs from it in the following features:

(a) A polyuronide bridge is not used in the binding of hemicellulose to other components in the wall.

(b) Cellulose fibres are not necessarily covered by a layer of xyloglucan.

(c) A large proportion of the hemicellulose is proposed to be bound to the wall by alkali-labile covalent bonds.

(d) It depicts the interaction of wall protein and hemicellulose with cellulose microfibrils.

(e) Cellulose microfibrils are at right angles to the direction of elongation.

3. Appraisal of Albersheim's Model

(a) The current model of Albersheim (99, see Fig. 6) does not include hydroxyproline-rich glycoprotein which makes up a significant part of primary cell walls. This polymer was excluded from the model on the basis that it has not been shown to be covalently attached to cell-wall polysaccharides. The earlier model proposed in 1973 by Albersheim's group (69) depicted hydroxyproline-rich glycoproteins to be covalently bonded to the cell

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wall via arabinogalactan side chains of the pectic fraction. This linkage was proposed on the evidence that a protease was able to solubilize pectic fragments from suspension-cultured sycamore cell walls. However, since then evidence from other sources has suggested that there is no covalent linkage between cell-wall glycoprotein and cell-wall polysaccharides (101-104). Any model on cell wall structure has to account for this important component. The failure of Albersheim's model to do so is a serious reflection on its limitations.

(b) Although evidence for hydrogen-bonding between xyloglucans and cellulose fibres is convincing, there is very little to support the proposed covalent bonding between xyloglucans and neutral side chains of pectin. However, the observation that polygalacturonase catalysed removal of pectin facilitates the extraction of a small amount of xyloglucans from cell walls by 8 M urea (43) suggests that there is some degree of interaction (probably non-covalent) between the pectic and xyloglucan fractions.

(c) The model does not offer an explanation on how the fractions are distributed within the wall. For example, it does not distinguish between the locations of methylated and unmethylated region of pectin nor does it account for the presence of relatively greater amount of pectic polysaccharides in the middle lamella (56, 105). The latter has been confirmed by ferric hydroxamate staining and electron microscopy (106). It is of the utmost importance to know how the cellulose fibres within the wall are oriented as they play a major role in elongation during growth. It

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is accepted that in the primary cell walls of dividing, non-differentiated, higher plant cells, the orientation of microfibrils tend to be random. However, at the onset of elongation, the innermost newly formed microfibrils tend to become parallel in a direction transverse to the axis of growth (107).

(d) Much of the information used in developing the cell-wall model has been derived from analysis of suspension-cultured cells which cannot be totally compared with growing plant cells, for example, the presence or absence of a middle lamella in the former.

4. Appraisal of Monro's Model

(a) This model is based primarily on work on lupin hypocotyl cell walls which involved removal of fractions using varying concentrations of sodium hydroxide (102). Such conditions are expected to simultaneously result in transelimination of uronic acids (108, 109), hydrolysis of methyl galacturonates, β -elimination of serinyl glycosides (110), disruption of hydrogen-bonding and hydrolysis of glycosidic bonds. All these are non-specific reactions and make interpretation of data extremely difficult. Further, sodium hydroxide may cause conformational rearrangement of cell-wall components affecting their extractibility.

(b) No evidence has been provided to support the proposal that extensin is covalently bonded to cellulose microfibrils at one end and other polysaccharides at the other end.

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(c) No evidence has been provided for the suggested covalent linkage between pectic polysaccharides and cellulose microfibrils as depicted by the model.

C. Aspects of the Models

1. Growth

Cell elongation is highly sensitive to temperature (111, 112) and can be blocked by metabolic inhibitors such as potassium cyanide (111, 113), therefore it cannot be caused by simple mechanical stretching. Rapid and apparently normal cell elongation can be induced with a lag of less than 1 min. in Avena and corn coleoptiles by carbon dioxide (114-116), low pH (115, 116), indole acetic acid (IAA) and its ester (117, 118). It is believed that cell elongation occurs as a series of independent extension steps. This is apparent from the observation that although cells can elongate under normal conditions for up to 24 hrs. at a constant rate (119, 120) cell walls can only be induced to extend mechanically at a constantly diminishing rate (121). Each step of cell-wall extension probably involves a biochemical modification of the cell wall in addition to physical extension. Extension cannot result simply from cleavage of polysaccharide molecules interconnecting the cellulose fibres because walls which elongate manyfold would lose most of their strength. Elongated walls have essentially the same strength per unit length as walls which have not elongated. This means that, during elongation, polysaccharides interconnecting cellulose fibres must either be augmented by the insertion of new polysaccharides or existing

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cross-links must be broken and the freed ends re-joined to new partners (transglycosylation). It is doubtful, however, that wall expansion is due simply to an increase in the rate of wall synthesis.

Time-course studies by Ray and co-workers (122) have shown that auxin stimulation of wall synthesis is noticed only after a lag of nearly 1 hr., however as mentioned above, wall elongation can be observed after a lag of less than 1 min. More recently it has been shown that auxin induced elongation occurs in two biochemically distinct phases (123-128), an early rapid stage (probably stimulated by lowering of pH from 6.0 to 4.0), and a slower more steady phase (probably involving wall synthesis). In support of this, Vanderhoef and Dute (129) have demonstrated that soybean hypocotyl cell walls kept in a "loose" state at pH 4.0, undergo only the second stage of elongation when exposed to an exogenous source of auxin (the first stage having already been induced by pH). A scheme of events involved in cell-wall extension postulated by these authors is shown in Fig. 8, which proposes that auxin regulates and co-ordinates both wall loosening and the supply of wall material.

Two concepts on cell-wall extension have been considered (130, 131) which incombine biochemical and physical processes. Both assume that growth occurs as a continuous series of independent extension steps. However, they differ in one major aspect, namely whether the extension itself is irreversible or whether it must be rendered irreversible by a subsequent biochemical process. In this context it is important to mention that glycoside hydrolases have been demonstrated in the primary cell walls of plants (132-137). However

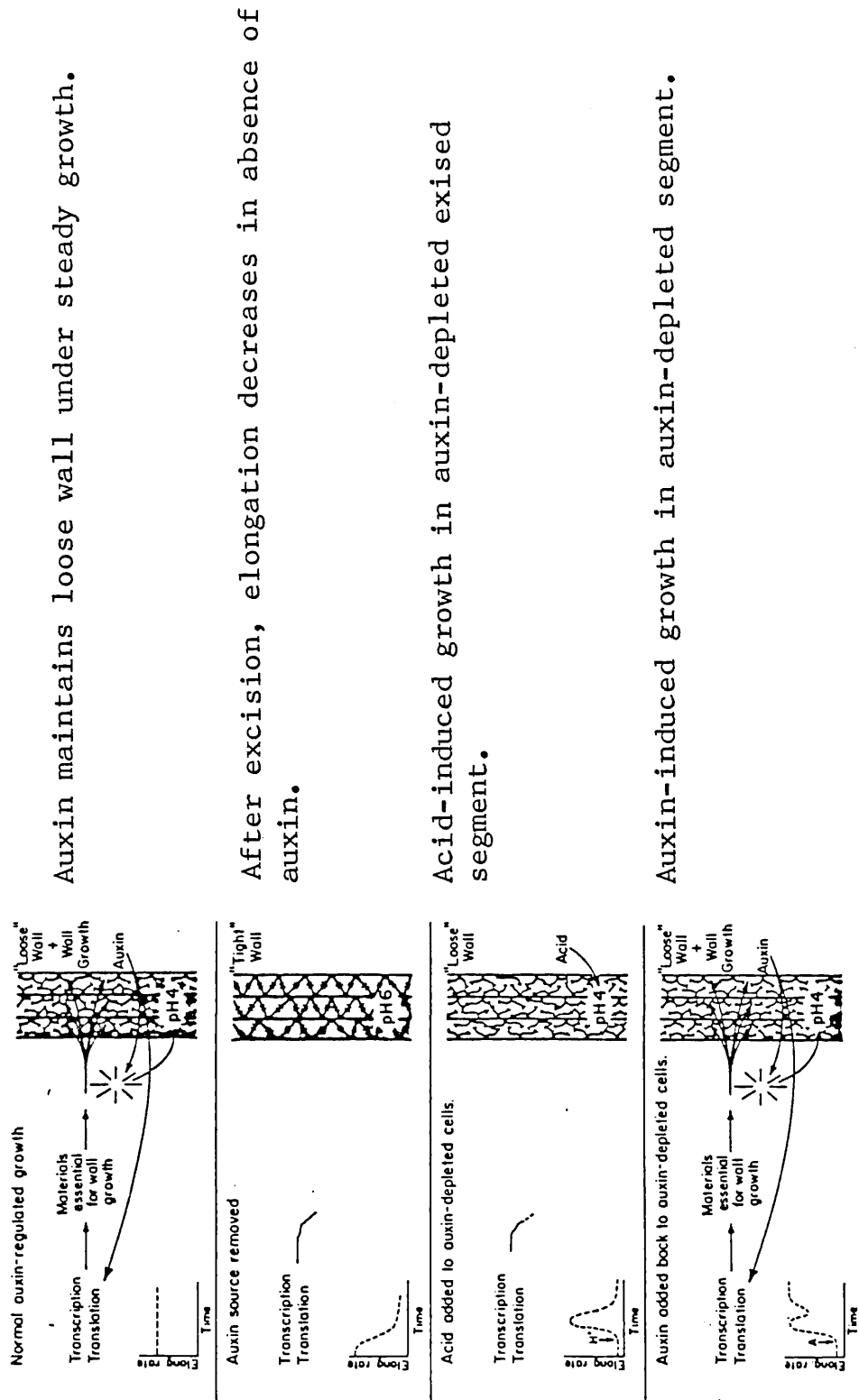


Fig. 8
 Events in cell elongation. Auxin is postulated to regulate and coordinate both wall loosening and synthesis, after Vanderhoef and Dute (129).

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it is not known whether any of these enzymes catalyse transglycosylation reactions in vivo.

Ray (138) has suggested that observed loosening of cell walls caused by auxins may be due to a shift from apposition (deposition of new wall only at the cell membrane) to intussuption (deposition throughout the wall). Intussuptioned polysaccharides would then cause loosening by forcing the cellulose microfibrils apart or by providing a "lubricant" to facilitate slippage. It has been shown (139) that in pea stem and Avena coleoptile tissues, wall synthesis was entirely by apposition in the absence of auxin, but that after treatment with auxin a sizeable amount of the deposition of hemicellulose, but not cellulose was found throughout the wall. However, evidence of this kind, based on correlation between wall synthesis and growth, cannot be very conclusive. It is difficult to be certain whether a positive correlation means a casual relationship between the two processes or whether they are affected in a parallel manner by some other agent.

Both models on cell-wall structure can be considered to be compatible with proposals on the mechanism of cell-wall extension. Initial extension could be caused by modification of interpolymer bonding which may or may not involve cleavage of polysaccharide molecules. This could be effected by a system that induces a change (in pH for example) in the whole or localised regions of the wall. Stimulation of enzyme activity (hydrolytic and transferase) may consequently occur, and/or a more direct influence may be brought on non-covalent polymer interaction. The thus modified wall would then be able to expand under turgor pressure. Albersheim and co-workers (80)

is an added problem in this context. In addition, some of the procedures used in the preparation of cell-wall material do not consider water-soluble polymers of the wall which leach out and are discarded. On the other hand procedures used may permit contamination of cell-wall preparations with intra-cellular water-soluble polymers. Chemical extraction procedures which have long been used to solubilise classic wall fractions may also cause diverse effects (153, 154; see p. 3, and 27)

3. PG Breakdown of Cell Walls

A significant amount of the information used by Albersheim's group in the construction of the cell-wall model has been derived from work using PG purified from Collitotrichum lindemuthianum (13, 42, 52, 80). This enzyme hydrolyses internal α -1,4-linked galacturonosyl bonds. Incubation of the enzyme with walls of suspension-cultured sycamore cells resulted in its solubilization (approximately 16%). This action resulted in the removal of 75% of the total galacturonic acid present in the walls. By comparison Knee (155) working with a similar enzyme from Sclerotinia fructigena was able to remove 50% of the total uronide content of apple cell walls. Roughly half of the released material from sycamore cell walls was made up of mono-, di- and tri-galacturonic acid, the rest consisting of polysaccharides containing both acidic (26%) and neutral (74%) sugars. The neutral sugars excluding rhamnose account for 5.5% of the cell wall, or 34.5% of carbohydrate solubilized by PG. Looking at the latest model of Albersheim (p. 23) hydrolysis of the rhamnogalacturonan backbone of the pectic fraction should not release polysaccharide molecules

containing arabinose or galactose, unless removal of part of the pectic fraction facilitated the dissociation of some non-covalently held xyloglucan from cellulose fibrils. It seems unlikely that this would occur under the mild conditions (50 mM acetate buffer pH 5.2) employed in the incubation of PG. In this regard, it is worth noting that 8 M urea was able to release less than 2% of cell walls pretreated with PG (43). These observations therefore conflict with the Albersheim model. The ratio of arabinose and galactose (presumably from the proposed arabinogalactan side chains of the model) to xylose and glucose (presumably from the xyloglucan fraction) in the PG released fraction is 8:1. The model predicts for each arabinogalactan chain solubilised from the cell wall one molecule of xyloglucan would also be removed. This is not consistent with the observation that pectic polysaccharides account for 35% of sycamore primary cell walls whereas xyloglucans account for 24%.

The 1973 model of Albersheim's group does not propose the linking of each arabinogalactan side chain to each xyloglucan polymer and therefore does not suffer from any of the above criticisms. However, it predicts the solubilisation of significant amounts of hydroxyproline-rich glycoproteins by PG which should be expected in conjunction with the removal of 75% of cell-wall uronic acid. Although PG removes some protein from plant cell walls (156-158) such solubilisation has not been shown to occur. Stevens et al. (159) did not observe any protein present in pectic fractions removed from cabbage cell walls which accounted for 45% of preparations.

4. Endoglucanase Breakdown of Cell Walls

Endoglucanase isolated from Trichoderma viride has been used to hydrolyse xyloglucans from the walls of apple cells (155) and cultured sycamore cells (43). It can only solubilise 1% of unmodified sycamore walls compared to 10-15% of walls pretreated with PG isolated from C. lindemuthianum. This suggests that the enzyme is unable to penetrate the outer matrix of the wall which is rich in pectin to reach its site of action further inside the wall. Klee (160) reported that endoglucanase from T. viride and S. fructigena released material of varying MW from apple mesocarp cell walls that had been pretreated with PG. Low-MW fragments accounted for approximately 44% of carbohydrate removed. These contained predominant amounts of galactose (39%) and glucose (33%). High-MW polymers which accounted for the other 56% of solubilized carbohydrate from the cell wall contained predominant amounts of galacturonic acid (34%) and arabinose (47%).

Looking at the 1978 model of Albersheim, hydrolysis of the cell wall by PG followed by endoglucanase (which presumably hydrolyses the xyloglucan fraction) should result in the release of small fragments of xyloglucan consisting essentially of glucose and xylose (unless, apple cell wall xyloglucan contains significant amounts of galactose).

High-MW polymers released should come from arabinogalactan side chains with attached pieces of rhamnogalacturonan and should contain greater proportions of arabinose galactose and galacturonic acid. The presence of large quantities of galactose in low-MW fractions removed by endoglucanase from apple cell walls is inconsistent with the model

proposed by Albersheim. Further, the small amount of galactose (9%) compared to arabinose(47%) present in the "high-MW peak" contradicts other data (155) which shows whole cell walls contain 1.4 times more galactose than arabinose.

5. Biosynthesis of Primary Cell Wall

Very little is known about synthesis of the primary cell wall (161-164). An overall hypothetical scheme for its assembly may however be projected. Sugar nucleotides synthesised from various pathways (see Fig. 9) serve as donors for the synthesis of polysaccharides, probably via glycolipid and glycoprotein intermediates (165). The polysaccharides except for cellulose, migrate outside the plasma membrane by a mechanism probably involving the Golgi system from where they are incorporated into the cell wall. Cellulose is probably synthesized in the membrane itself by an enzyme complex system that migrates from Golgi vesicles (164). This system can be transferred to the plasma membrane by fusion of the latter to it. Primary (and later secondary) cellulose-synthesizing complex appear to move freely within the lipid bilayer of the plasma membrane as fibril deposition proceeds and the pattern of deposition appears to be random (Fig.10). Cellulose and other polysaccharides can be bonded together covalently or non-covalently by mechanisms presently unknown. It is unclear how control of wall synthesis can be exerted beyond the plasma membrane. It is difficult to relate what is known about the synthesis of primary cell walls with models proposed.

D. A Suggested Modified Model

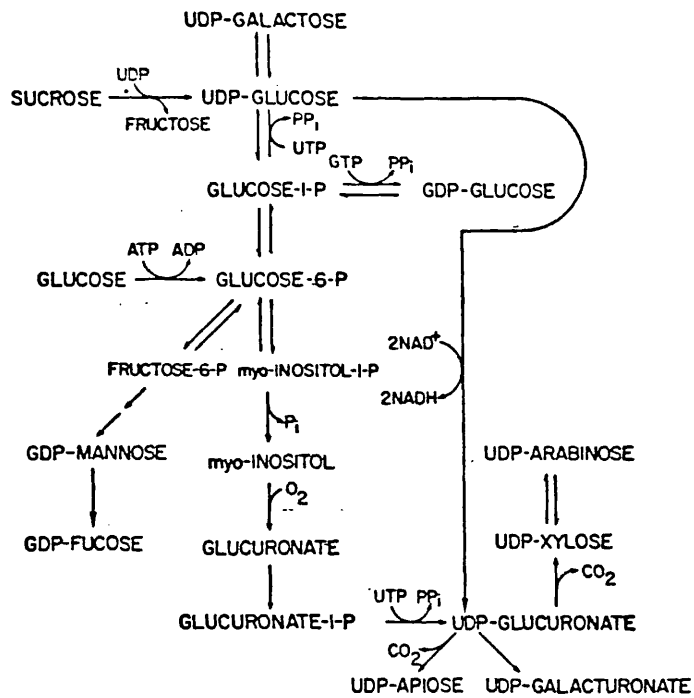


Fig. 9

Pathways for the synthesis and interconversions of nucleotide sugars in plants, taken from Delmer (165).

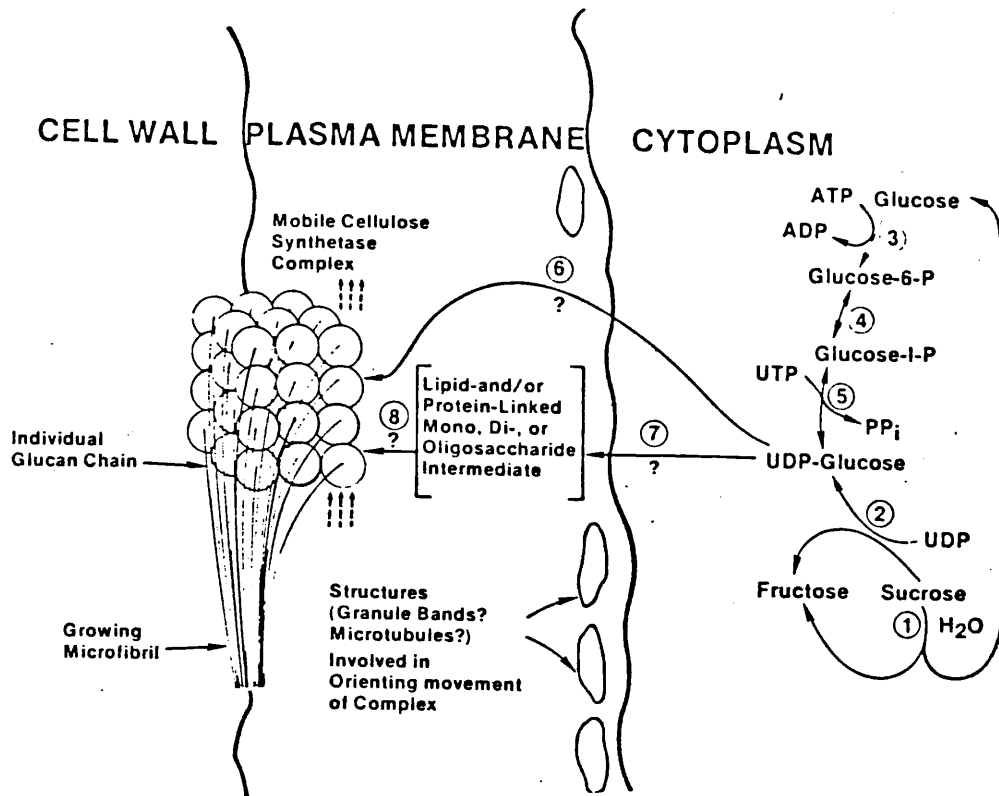


Fig. 10

Model for the biosynthesis of cellulose. Numbers refer to the reactions catalysed by the following enzymes: 1, invertase; 2, sucrose synthetase; 3, hexokinase; 4, phosphoglucomutase; 5, UDP-glucose pyrophosphorylase; 6, 7, and 8 hypothetical reactions, taken from Delmer (1965a).

In considering the various criticisms raised in the discussion of the cell-wall models, an attempt has been made to present a more up-dated version. This is shown in Fig. 11. The main features are:

(a) Several layers of pectin form an outer net around the whole of the cell wall constituting the middle lamella (cf. p. 26). The pectic molecules are interconnected by covalent bonds (cf. P. 15) and calcium ion bridges (cf. Fig. 4). A high proportion of the bridges are arranged parallel to the direction of elongation. No covalent connection between pectic and any other fraction is proposed, although some regions of the former fraction contain covalently bonded xylose and glucose.

(b) Inside the outer pectic layer are situated several layers of cellulose fibres which are non-covalently associated with xyloglucan and such assemblies are non-covalently attached to each other. Cross-linking of xyloglucan associated cellulose is also provided by hydroxyproline-rich glycoproteins in a novel non-covalent manner involving isodityrosine bridges similar to that suggested by Lamport (94, see Fig. 5). The region of the xyloglucan-associated cellulose layer nearest the cell membrane is arranged transverse to the direction of elongation, whereas the part nearest the pectic fraction is more randomly arranged (107).

1. Appraisal of the Model

(a) Growth

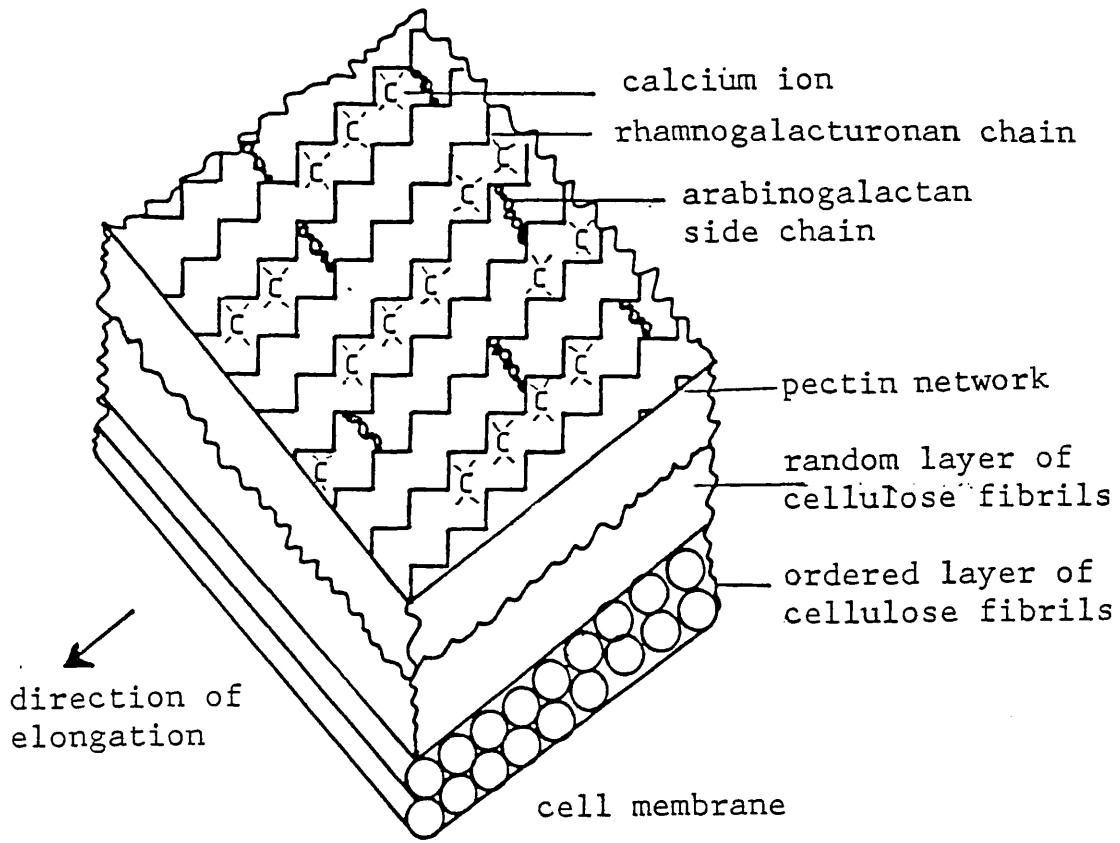


Fig. 11

A suggested modified model of the primary cell wall of dicotyledons. Some xylose and glucose is postulated to be part of the pectic fraction which is only cross-linked to itself. Cellulose microfibrils are cross-linked by hydroxyproline-rich glycoproteins.

Under normal conditions, the components of the cell wall are randomly turned over. This has been supported by both pulse labelling experiments (166, 167) and by gravimetric measurements (153, 168). Growth could therefore be considered to result from stimulation of synthesis or a slowing down of degradation. Wall extension induced by auxin has been suggested to result by the activation of two systems (169, 170): one involving proton pumping across the membrane into the cell wall and the other involving activation of wall synthesis. It is suggested that hydrogen ions directly weaken the cell wall, thus allowing the parallel pectic molecules to slide relative to each other under turgor pressure. This would correspond to the rapid early stage of auxin induced expansion (cf. P. 30). Loosening by low pH in cell walls has been previously proposed by Coil et al. (171) and Tagawa et al. (172). Evidence in support of this has recently been provided by Soil et al. (173) who found that proton loosening of cell walls could be mimicked by calcium chelating EDTA, potassium ions and sodium ions. In addition they found that externally applied calcium ions could increase rigidity of cell walls. It must be noted however, that Cleland (130) was unable to detect a redistribution of radioactive calcium between cell walls and the outer solution after auxin treatment. This could have been because the bonds involving calcium ions were not sufficiently weakened to allow appreciable exchange.

Wall loosening is not suggested to be caused by low pH stimulation of hydrolytic enzyme activity (cf. p. 32) within the wall as this action together with loosening of calcium bridges may cause the wall (as

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depicted by the model) to come apart under turgor pressure. In this regard a significant proportion of the arabinogalactan side chains of pectic polymers are needed to stabilise the loosened cell wall and also play a role in checking wall expansion. Thus the loosened wall is only able to expand up to a certain point after which the side chains have to be broken to permit further expansion. Soll et al. (173) were unable to find indications of acid-activated cell-wall loosening enzymes.

Once the pH of the wall is raised back to its physiological value, the calcium bridges become stronger and fix the pectic molecules firmly in place. The rapid early stage cell-wall elongation is therefore reduced, and insertion of new material continues beyond this point. The whole process can be sequentially repeated in response to further auxin stimulation. Elongation of the xyloglucan-associated cellulose layer is suggested to occur by direct insertion of newly synthesised material in a direction transverse to that of elongation. Such insertion would presumably be easier after pH-loosening of the wall. Extensive cross-linking of the xyloglucan-associated cellulose layer by hydroxyproline-rich glycoproteins could inhibit growth by preventing insertion of new material.

(b) Chemical and Enzymic Behaviour

As xyloglucans are *non*-covalently attached to the cell wall, the model predicts their removal by reagents which disrupt this type of bondings. Further, this process would be expected to be enhanced by degradation of the pectic net which covers the whole of the cell wall

(cf. p. 33). On the other hand pectic molecules would be removed by the breaking of covalent bonds. These predictions are consistent with observations discussed previously (sections C2, C3, and C4)

The difficulty encountered in solubilizing hydroxyproline-rich glycoproteins from cell walls can be explained by it being cross-linked to itself via isodityrosine (IDT) bridges (93, 98, 114, 174, 175), and its close association with cellulose. Partial solubilisation of this polymer with acidic sodium chlorite (62) can thus be explained by the hydrolysis of chlorite-labile isodityrosine linkages.

The suggested cross-linking of pectic molecules to each other (and not to xyloglucan) explains why it has not been possible to isolate significant quantities of xyloglucans covalently attached to pectic polymers (cf. p.19). The model also accounts for the ability of PG to solubilise neutral sugars as well as fragments of rhamnogalacturonans (cf. p.33)

IV. FRUIT DEVELOPMENT

From a physiological point of view, a fruit can be defined as the structural entity that results from the development of the tissue that supports the ovule of a plant. This definition encompasses dissimilar organs such as the floral axis of pineapple, receptacle of strawberry and apple, and syconium of fig in that an ovule is present in all these fruits. The function of a fruit is in principle to assist in the distribution of seeds. This is often achieved by

consumption of the ripe fruit and scattering of the residual seeds, or by passage of seeds through the digestive tract of the animal consuming them. On the plant, seeds develop along with the fruit, and are mature long before the fruit stops growing. Premature scattering of seeds is thought to be discouraged by the indigestibility of the unripe fruit which is attributable in part to the presence of phenolics and tannins.

A. Stages in Fruit Development

The life of a fruit starts with fertilisation followed by a phase of growth to maturity which in turn is followed by ripening and senescence.

1. Fruit Set

This is the early phase in the life of a fruit characterised by rapid growth of the ovary that usually follows pollination and fertilisation, and is accompanied by changes such as wilting of petals and stamens. It is considered that fruit growth begins in the floral primordium (176). The pericarp of the fruit develops from the ovary wall, and may differentiate into 3 distinct regions the exocarp, the mesocarp, and the endocarp. However fruit development is not restricted to the ovary and often involves noncarpellary parts of the flower (177). Initial development occurs mainly through cell multiplication.

2. Fruit Enlargement

This is the stage following fruit set where an increase in the size of fruits occur. It is marked by cell enlargement, although cell division also continues (177). In some fruits, such as the apple, expansion of intercellular spaces may be a contributing factor to enlargement. Generally, cell division predominates in the early stages of growth, whereas cell expansion predominates during the latter stages. There is, however, much varietal variation, and the cell division stage usually overlaps the cell enlargement stage. In one species of tomato Lycopersicon pimpinellifolium, some cell division continues even to maturity, whereas in L. esculentum, division ceases at anthesis. More complicated patterns of developments occur in other fruits in which cell division ceases at different times in different parts of the fruit. The period of fruit growth varies from 1 week to several years, although periods of several months are more usual.

3. Maturation

This stage is reached in the life of a fruit when full development (ripening and senescence) may be achieved independent of the parent plant. After maturation, there is no further increase in the size of fruits. Fruits are normally harvested at this stage, after which they live an independent life by utilising substrates accumulated during maturation (178).

4. Ripening

During ripening the fully mature fruit converts to a more palatable state, specific flavours are developed in conjunction with increased sweetness and decreased acid content. Softening of the fruit occurs and often is accompanied by a change in colouration. Chlorophyll in the chloroplasts of the outermost cells decreases while carotenoids and anthocyanins develop.

According to the respiratory behaviour late in their developmental sequence, fleshy fruits can be loosely classified as climacteric and non-climacteric (179-182). Climacteric fruits such as the mango, tomato and apple undergo an upsurge in respiration at the onset of ripening that is coincident with other activities resulting in changes characteristic to the ripening process. Known factors which influence the onset of the climactic in fruits are temperature (generally, lowering the temperature delays the onset of the climactic), oxygen and carbon dioxide tension (generally, lowering the oxygen tension below that of air or raising the carbon dioxide tension delays the climacteric) and the presence of ethylene. The pattern of climacteric (post-harvest) respiration in the mango is shown in Fig. 12. An upsurge in respiration occurs in fruits allowed to ripen whilst still attached to the plant, and in fruits detached after maturity has been reached. Non-climacteric fruits such as the strawberry, citrus fruits and pineapple however ripen gradually over a longer period of time and show no upsurge in respiration. The many changes that occur during the ripening process appear to be synchronized and are probably under genetic control. This contention is supported by the fact that the interval between anthesis and ripening under similar environmental

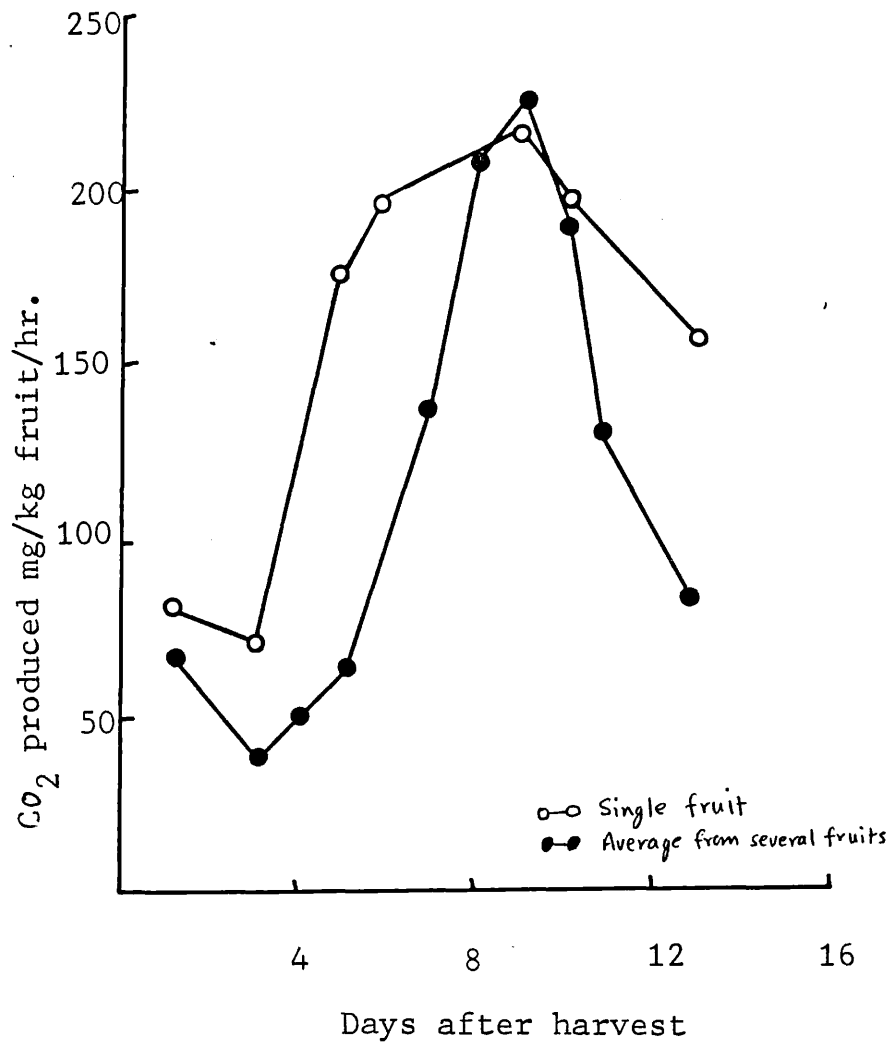


Fig. 12
Pattern of post-harvest respiration at 20 degrees C in mangoes, after
Krishnamurty and Subramanyam (218).

conditions is roughly constant for any given fruit. A summary of changes suggested by Biale and Young (183) that occur during the ripening process is presented in Table I. A strong demand for energy is placed upon the system for continuing these processes which include transcription, translation and synthesis of ethylene and flavour compounds. The energy is supplied by some of the degradative processes in particular the hydrolysis of starch. Glucose produced by this process is consequently utilized during the ripening process (184-187). The interrelation and mechanism whereby these changes are co-ordinated is presently unknown. One of the difficulties in determining this is in trying to discern causative factors from their effects. Ripening may be considered to occur as a chain of events which are dependent upon the completion of previous steps for its completion. However, Hobson (188) suggested that it should be considered as a number of key processes taking place simultaneously, each one having its own control mechanism which is loosely co-ordinated with that of the other processes.

5. Senescence

This is the stage that begins somewhere during the ripening process and continues until the end of the life of the fruit. It is characterised by a general and increasing failure of many synthetic processes and susceptibility of the fruit to fungal attack.

B. Metabolic Aspects of Ripening

1. Protein Synthesis

Degradative	Synthetic
Destruction of chloroplast	Maintenance of mitochondrial structure
Breakdown of chlorophyll	Formation of carotenoids and of anthocyanins
Starch hydrolysis	Interconversion of sugars
Destruction of acids	Increased TCA cycle activity
Oxidation of substrate	Increased ATP generation
Inactivation by phenolic compounds	Synthesis of flavour volatiles
Solubilization of pectins	Increased amino acid incorporation
Activation of hydrolytic enzymes	Increased transcription and translation
Initiation of membrane leakage	Preservation of selective membranes
Ethylene induced cell wall softening	Formation of ethylene pathways

Table I
Suggested changes which occur during the ripening process, Biale and Young (183).

There is considerable evidence for the involvement of protein synthesis (de novo enzyme synthesis) at the climacteric stage during fruit ripening (189-193). Increases in the ratio of protein nitrogen to total nitrogen have been reported (184, 194, 195). It is not certain whether de novo synthesis of protein catalyse the climacteric rise. However recent reports of ripening related changes in the levels of different transfer-RNA's (196,197) and messenger-RNA's (198) in the tomato fruit support this contention. Further support is provided by the observation of ribosomal-RNA synthesis prior to the climacteric peak (189, 199, 200-202). Hobson (188) suggested that the synthesis of enzymes required for ripening may be at the expense of other proteins, as there is little evidence suggesting drastic alteration in total protein content during ripening.

2. Ethylene Production

It has long been recognised that ethylene acts as a hormone in plants, and can have profound effects on the ripening process particularly in climacteric fruits (203). Its true role in fruit ripening has not been determined. It is widely accepted that treatment of fruits with low concentrations of ethylene brings forward the time of onset of the climacteric upsurge without altering the shape of the cycle. It is only effective if applied before the climacteric phase has begun in the plant when it is not already influenced by endogenous ethylene production. There is no return to a pre-climacteric stage once an adequate exposure to this hormone has been achieved. This is in contrast to the effect of ethylene on non-climacteric fruits where a

response can be achieved throughout post-harvest life (204). Further, once the hormone is removed its effect on this type of fruit (non-climacteric) is reversed.

Detailed studies by Pratt and Goeschl (205, 206) showed that in the Honey Dew musk-melon, reduction in firmness and upsurge in respiration, appeared to be the only ripening parameters that were directly related to ethylene action. Other physiological changes, such as decrease in growth rate and increase in soluble solid content, preceded accelerated ethylene production. It was also observed that while ethylene was able to induce a respiratory climacteric in the immature Honey Dew musk-melon, this response did not result in the accumulation of sugars. From observations on apples, Rhodes and Reid (207) concluded that a factor other than a change in ethylene level determines the time of onset of ripening in the apple. Kosiyachinda and Young (208) came to the same conclusion while working on avocado and cherimoya fruits. The relationship between ethylene production and the respiratory rise in fruits appear to be varied. The onset of the rise precedes ethylene production in some fruits such as the avocado, banana, guava and Honey Dew musk-melon, whilst in fruits such as Cox's Orange Pippin apple, apricot and Cantaloup musk-melon it coincides with ethylene production, and in other fruits such as the Fuerte avocado, Chafley cherimoya, Haden mango and Rutgers tomato, it occurs after ethylene production (183). From such observation, Biale and Young (183) suggested that upsurge in ethylene synthesis is not the initial factor in inducing the respiratory climacteric although they questioned the reliability of some of these studies. McGlasson et al. (209) concluded that the onset of ripening

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in normal tomato is not controlled by endogenous ethylene, although increase in its production is probably an integral part of the ripening process. They found that treatment of 40-80% mature tomatoes with propylene stimulated respiration but did not bring about ethylene production. Normally treatment of climacteric fruits with ethylene stimulates an increase in its production (210, 211).

3. Starch Hydrolysis

Increased hydrolysis of stored reserves of starch often in the form of granules is closely related to the ripening process. In banana starch content of the pulp varies between 20-30% in the unripe fruit, and between 1-2% in the ripe fruit. The consequence of starch hydrolysis is the formation of sucrose, glucose and fructose. Young et al. (212) found that a sharp decrease in starch content in Valery bananas occurred when the respiratory rise was well on the way towards the peak. They suggested that hydrolytic enzymes involved were activated or synthesized de novo at the onset of ripening and reported the presence of 2 forms of α -amylase, 2 forms of β -amylase and 3 phosphorylases which were present at all stages of ripeness. Several inhibitors were found that prevented starch hydrolysis in extracts of pre-climacteric hormones.

4. Respiration

(a) Glycolysis

Fructose-1,6-diphosphate unlike glucose-6-phosphate and

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fructose-6-phosphate increases (20-fold) during ripening of bananas (212). Crossover plot analysis on the same fruit indicate that a regulatory site occurs at phosphofructokinase. Barker and Solomos (213) supported the contention that cellular fructose-1,6-diphosphate was a major controlling factor on the respiration rate, while Pearson et al. and Robertson (214) proposed that it was controlled by the ADP/ATP ratio.

(b) Phosphorylation

Millerd and co-workers (215) considered that the climacteric rise could be brought about by the uncoupling of phosphorylation. However, the enhancement of phosphorylative capacity with ripening does not support this proposal (183). Hobson (216) demonstrated that in tomatoes subjected to the action of uncoupling agents, production of enzymes necessary for the ripening process continued. He suggested the possibility that "loose" coupling of phosphorylation during the climacteric rise may result in a net increase in the synthesis of "energy rich" bonds at an early stage, leading to the formation of additional enzymes necessary for the furtherance of ripening. Biale and Young (183) indicated (from studies using isolated mitochondria and tissue discs) tighter coupling of oxidative phosphorylation in preparations from ripe avocados compared to unripe fruits.

(c) Mitochondrial Involvement

Using succinate as substrate, a rise in respiratory control together with an increase in ADP/O ratio (an index of degree of esterification

of ADP to ATP per oxygen atom consumed in substrate oxidation) is observed during ripening. Using malate, addition of ADP elicits a small response with mitochondria from preclimacteric avocados. Addition of thiaminepyrophosphate (TPP) is necessary to obtain respiratory control and maximal oxidation (in the presence of ADP-stage 3 oxidation). In mitochondria from the ripe fruit however, no TPP is needed to obtain the response. With α -ketoglutarate as substrate, the requirement for TPP is much more pronounced with mitochondria from unripe than ripe fruit. With pyruvate as substrate, a similar effect is obtained as for malate, except that differences in values for ripe and unripe fruits are more pronounced. The in vivo effect of TPP is not known.

C. Cell Wall Breakdown

1. Pectic Fraction

It is widely accepted that softening of fruit which accompanies ripening is essentially caused by the conversion of insoluble, wall-bound protopectin of high MW to water-soluble pectin. Dolendo et al. (217) reported that softening as indicated by pressure measurements coincided with increase in soluble pectins and decreases in protopectin. Solubilisation of pectin during ripening has been demonstrated in the mango (218-220), apple (221-224), tomato (3,225), avocado (226), peach (226, 227), pear (1, 228), dates (229), and strawberry (5). Doesburg (224) has proposed that movement of calcium in cell-walls may assist solubilization of pectin during ripening. Reported losses of pectin from cell-walls are consistent with observed

decreases in cell-wall galactose, galacturonic acid and in some cases arabinose content during the ripening process (1-5). Ahmed and Labavitch (1, 228) have shown that there is solubilisation of a high-MW branched arabinan from the cell-walls of ripening pears. This polysaccharide consists of a backbone of α -1,5-linked L-arabinosyl residues with α -linked L-arabinosyl side chains at C2 and/or C3 and has a similar structure to pectic polymers present in the primary cell-wall of cultured sycamore cells (17). There is also solubilisation of an acidic fraction of lower MW galacturonan free of arabinosyl residues.

Ultrastructural studies have shown that wall breakdown is accompanied by dissolution of the middle lamella region of parenchyma cells leading to cell separation in apple (230), tomato (231) pear (163), and strawberry (5). The mechanism whereby such drastic changes are brought about is not fully understood.

Enzymes capable of degrading pectin which have been identified in fruits are PG, pectinmethylesterase (PME), exopolygalacturonase, β -galactosidase and α -L-arabinosidase. The enzyme that is known to be directly involved in wall breakdown is PG (232, 233), although it seems certain that other enzymes are involved.

(a) Pectinmethylesterase

This enzyme catalyses the de-esterification of galacturonosyl residues (present in rhamnogalacturonan), in which the carboxyl group is methyl esterified. The optimum pH range is 5 to 8 and is affected by

cations. It does not hydrolyse any methyl esters in short chain galacturonans. Blocks of free carboxyl groups are produced following the action of PME (234), which suggests that de-esterification occurs in a linear manner. Lee and McMillan (235) have shown that the enzyme acts at both the reducing ends and interior regions of highly-esterified pectin chains. It has been suggested that hydrolysis catalysed by the enzyme only occurs adjacent to free carboxyl groups (236).

PME is present at the immature stage of most fruits and generally reaches maximal activity immediately preceding or early on in the climacteric rise. This enzyme has been found in peaches (237), pears (238), tomatoes (239-241), bananas (242), avocados (243) and mangoes (244). Correlation between the level of enzymic activity and cell-wall softening presently is not clear. For example, Hamson (245) reported that the level of PME activity is higher in firm than in soft tomatoes, whereas, Hobson (241) reported 40% greater activity at the ripe stage. Pressey (246) suggested that part of the confusion may be due to the complexity of the enzyme, which exists in multiple forms in tomato, the levels of which vary with degrees of ripeness and with variety. The level of PME activity in peaches also vary with variety, and show no particular trend with fruit softening (237). In banana however, the activity of the enzyme increases 10-fold during ripening (242). Three forms exist in this source, one of which increases continually during ripening. A role for PME in cell-wall softening is not apparent, although it has been considered that its action may be needed prior to the action of PG which has a preference for de-esterified pectate in some fruits (243-247).

(b) Endopolygalacturonase

PG catalyses random hydrolysis of the rhamnogalacturonan backbone of pectin. It is not present in all fruits. However, it has been identified in tomato (248), date (249), pear (250), cranberry (92), peach (251), cucumber (252, 253) and avocado (243). PG cleaves pectin randomly, first to oligogalacturonates and ultimately to galacturonic acid, but the rate of hydrolysis decreases with smaller polymers. The rate of hydrolysis of tetra-, tri- and di-galacturonate are 7%, 1.6% and 1% of the rate of a long chain polymer (254). Optimum pH for PG activity appears to vary between 4.5 and 5.5.

It is generally accepted that PG is primarily responsible for dissolution of the middle lamella during ripening in some fruits. Pressey and Avants (251) showed that the enzyme was effective in solubilizing pectin from washed peach cell-walls. McCready and McComb (226) reported that PG activity increases during ripening of avocados. The enzyme was shown to be capable of hydrolysing pectate to intermediate oligogalacturonates which were then slowly hydrolysed to galacturonic acid (255, 256). Ahmed et al. (1) found treatment of unripe pear cell-walls with purified PG solubilised an acidic unbranched arabinan with similar characteristics to a polymer solubilised during normal ripening. Wallner and Bloom (257) also found that tomato PG accomplished solubilisation of unripe tomato cell wall which appeared to be nearly as extensive as that which occurred during

in vivo ripening. However, the enzyme did not cause the 40-60% decrease in cell-wall galactose which occurs during the natural process. Wallner's group (3, 149, 257) has shown that a sharp increase in PG activity in the tomato is accompanied by an increase in the solubility of a rhamnogalacturonan fraction (which can be extracted from isolated cell walls by 4 hr. incubation with water at 30 degrees C). This fraction (average MW 20,000) was found to be almost free of neutral sugar residues, and could not be extracted from the cell-walls of unripe fruits. It was suggested that conversion of high-MW pectic polysaccharides within the cell-wall to water soluble rhamnogalacturonan of lower MW occurred during ripening. This was proposed to be accomplished by a 2 stage mechanism involving removal of neutral pectic side chains followed by PG catalysed hydrolysis of the exposed backbone.

Much attention has been focused on the involvement of PG in the ripening process particularly in tomato fruit. Its activity appears about 2 or more days after the onset of ethylene production, and increases sharply thereafter as ripening proceeds (258-260). Grierson and Tucker (261) showed that ethylene evolution occurs at least 20 hrs. before measurable PG activity. This was caused by de novo synthesis of the enzyme rather than by activation. It therefore seems that PG does not initiate fruit ripening or ethylene synthesis and that ethylene does trigger PG synthesis. Three forms of PG designated as PGI, PGIIA and PGIIB have been identified in tomato (251, 262, 263). Reports on the MW of PGI vary from 84,000 to 115,000 (251, 262, 263) and it has been suggested that it may be a dimer of PGIIA MW 43,000. However, Moshrefi and Luh (264) reported 199,000 in

Murrieta tomatoes. Further, they found PGI to yield 2 protein subunits under SDS electrophoresis in contrast to observations by Ali and Brady (263). PGIIB has MW 45,000 (263). During the early ripening process, PGI is the only form present (260, 262). As the tomato ripens PGII activity increases until it becomes the dominant form (260, 262). It is not certain whether this is due to the two forms of the enzyme being produced simultaneously, or to conversion of PGI to PGII. Evidence however suggest conversion may not cause an increase in enzymic activity as the two purified forms have similar specific activities. The roles of the various forms of the enzyme in cell-wall softening are still unclear.

(c) Exopolygalacturonase

Exopolygalacturonase catalyses the hydrolytic removal of small oligosaccharide fragments from the ends of pectin molecules. It has been found in pears (250), peaches (251), cucumbers (252) and apples (265). In the apple, it is capable of degrading cortical cell-wall preparations in vitro releasing low-MW uronic acid residues and polyuronide. Peach exopolygalacturonase showed optimum activity at pH 5.5 and required calcium ions for activity (251). It also displayed maximal activity against polymers containing 20 or more residues releasing galacturonic acid. Evidence indicated that cleavage occurred at the non-reducing end of pectate molecules which were progressively shortened. Exopolygalacturonase from cucumber also showed optimum activity at pH 5.5 (252) and displayed a similar mode of action to the enzyme from peach. It was also activated by calcium ions, but showed most activity against substrates containing 6 to 12

residues. There is very little evidence available at present to implicate exopolygalacturonase in cell-wall softening.

(d) β -Galactosidase

Increase in β -galactosidase activity with ripening has been reported in many fruits (51, 266-269). Its involvement in cell-wall breakdown has not been established although Bartley (269) has reported that it is capable of hydrolyzing a potato β -1,4-galactan similar to that found in apple cell-walls. Pressey (270) has recently reported the presence of multiple forms of β -galactosidase designated I, II and III, in tomato fruit. MWs calculated after isolation by gel-filtration chromatography were 144,000, 62,000 and 71,000 respectively. It was suggested that I may be a dimer of III. Only II was able to hydrolyse a galactose rich polysaccharide (58%) isolated from tomato cell walls releasing free galactose. During ripening, the levels of activity of I and III decreased whereas the activity of II increased. Knee and Bartley (271) have suggested that β -galactosidase may be involved in removing galactose from apple cell walls during ripening. Pressey (270) has suggested a similar role for the enzyme in tomato.

(e) Arabinosidase

As in the case of β -galactosidase, α -L-arabinosidase was reported in many fruits and its level of activity increases with ripening (265). Its involvement in cell-wall breakdown has not been established. Ahmed () was unable to generate reducing power from a purified arabinan using an enzyme preparation that contained α -L-arabinosidase

activity. It may be that glycosidase activity determined by incubation with p-nitrophenyl substrates give an inaccurate picture of in vivo enzyme specificity (272).

2. Hemicellulose

Loss of cell-wall hemicellulose during ripening has not been established as part of the process. The relative amounts of monomers characteristic of these polysaccharides such as xylose and glucose, have not been shown to decline in the cell wall during ripening of apples (2), strawberries (273), tomatoes (257) and pears (274). In addition, enzymes capable of degrading hemicellulose have not been identified in fruits. Pear (11) and tomato (149) lack xylanase activity although both contain β -D-xylosidase and β -D-glucosidase activities (11, 149). Tomato contains β -1,3-D-glucanase activity (149) but the likely natural substrates of this enzyme have not been shown to be present in the cell wall of fruits. β -1,4-glucanase activity has been found in strawberries, tomatoes and pears (275, 276). However, doubt that this enzyme has a primary role in fruit softening arises from the observation that its activity is normal in non-ripening mutants of tomatoes (277).

3. Cellulose

There is no evidence to suggest that cellulose is lost from cell walls during ripening, despite the presence in many fruits of β -1,4-glucanase activity which is often called cellulase (195, 244). Hydrolysis of cell-wall cellulose by this enzyme has not been

demonstrated.

D. Mango Ripening

Very little work has been done on the softening of mango cell wall during ripening. Attention has been directed primarily on other aspects such as effects of low temperature storage (279, 280) and metabolic enzymes (280-283). However, Mitzuta and Subramanyam (283) reported that there was an increase in cell wall content of water-soluble pectin during ripening whilst hemicellulose and cellulose content remained relatively constant. The enzymes bringing about these changes have not been studied. Decreases in molecular size and esterification of soluble protein in the pulp have been reported (284) as well as increases in uronic acid (285). A glucan isolated from mango has been partially characterised (286) and was found to contain repeating units of 1,3- and 1,4-linked glucose residues. In addition to these studies, Burg and Burg (287) have shown a pattern of ethylene evolution coinciding with the respiratory peak in Haden and Kent mangoes. Leley (288) has shown that starch content of the pulp increases from 1% to 13% throughout the period of growth of the mango.

V. SCOPE OF THE PROJECT

This project follows on from studies carried out by K. Brinson in the department. His main findings were:

- (a) PG is not present in the mango.

(b) D-Galactose and L-arabinose were lost from the cell wall during ripening.

→ (c) Using DNP-glycoside substrates, 6 exoglycosidases were shown to be present in the mango pulp. Five of these showed an increase in activity during ripening. The most pronounced increase occurred in the case of β -galactosidase.

The objectives of the present project were as follows:

(a) To compare the composition and structure of mango mesocarp cell wall with that of a typical plant primary cell wall. For the latter, red kidney bean hypocotyl cells were used.

(b) To study the compositional changes that occur in mango mesocarp cell walls during ripening.

(c) To investigate the roles played by wall-degrading enzymes in cell-wall softening.

It was considered that an overall understanding of the structure of the unripe and ripe mango cell walls and their component fractions, was needed to assist in the interpretation of later work which concentrated on carbohydrases possibly involved in cell-wall softening.

The necessity of a comparative investigation between the red kidney

bean hypocotyl cell wall and the unripe mango cell wall arose because of the very small amount of information available on fruit cell wall structure. Much of the current work on plant cell wall has been carried out on cells from other regions of plants and on cultured cells. Although present models of cell-wall structures are often employed in fruit research, there are few indicators to support their use. The walls of cells in the pulp region of fruits eventually undergo severe degradation during ripening, whereas cell walls from other regions of plant do not experience such a change. The red-kidney bean hypocotyl was chosen as the source for primary cell walls as its structure has already been compared to that of sycamore cells (42). Studies on the latter provided much of the information employed for constructing the cell-wall model by Albersheim and co-workers. Their conclusion from such work was that these two plant sources have very similar cell wall structures, and that a common structure probably exists for the primary cell wall of all dicotyledonous plants (99).

It was considered that an investigation of enzymic breakdown of the cell wall could be divided into two stages:

(a) An investigation of the ability of crude enzyme preparations from the ripe mango to degrade unripe mango cell walls, followed by the establishment of whether any in vitro changes that may consequently occur were similar to those that occur in vivo.

(b) Identification and characterisation of enzymes associated with such changes as mentioned above.

RESULTS AND DISCUSSION

PART 1

Cell-Wall Structure: Mango Mesocarp and Red-Kidney
Bean Hypocotyl

Cell Wall Structure: Mango Mesocarp and Red-Kidney Bean Hypocotyls

I. INTRODUCTION

The main purpose of the work carried out in this section was to investigate the level of similarity that exists between the walls of cells present in red-kidney bean hypocotyls and those in the pulp of mature unripe mangoes. It is generally assumed that the walls of cells present in the pulps of fruits are typical of other primary cell walls, although very little evidence exists to support this. At the same time some of the work on mango carried out by the previous worker (K. Brinson) was repeated, namely, examination of the changes that occur in the composition of cell walls from the mesocarp region of the fruit. K. Brinson (266) experienced difficulty in removing starch from mango cell-wall preparations. This was significant in view of the loss in cell-wall glucose which he suggested occurs during ripening.

II. RESULTS AND DISCUSSION

A. Preparation of Cell Walls

The hypocotyls of 4-6 day old red-kidney bean plants were chosen as the source of cell walls. Tests for lignin on such preparations by phloroglucin staining confirmed the absence of secondary wall thickening. Similar results were obtained with cell walls from the mesocarp region of unripe and ripe mangoes. However, phloroglucin staining showed up the presence of lignin in woody tissue from wild

plants.

Light microscope examination of homogenized tissues (Silverson blender) showed that whereas the cells of kidney bean hypocotyls and ripe mesocarp were disrupted, those of unripe mango were mostly intact. However, repeated sonication of the homogenized material brought about complete cell disruption. Macerated tissues thus obtained were passed through 5 layers of muslin in order to separate starch granules from cell walls. Repeated washing with 50 mM acetate buffer, pH 5.0 greatly enhanced this process. Cell-wall preparations obtained at this stage when incubated with salivary amylase showed no formation of gluco-oligosaccharides as tested by TLC analysis.

B. Cell-Wall Composition

The contents of protein, uronic acids and calcium were determined in the cell-wall preparations from red-kidney bean hypocotyl, unripe mango and ripe mango mesocarp cell walls. The results are presented in the Table II which shows that red-kidney bean hypocotyl cell walls contain 1.5 times more protein (11.9%) than unripe mango mesocarp cell walls (7.3%), while cell walls of ripe mango contained 21.1% protein. This suggests that very little protein is lost from cell walls during ripening of the fruit. Some protein may also be inserted into the cell wall. Protein synthesis is known to continue during the ripening process (184, 189-195).

Uronic acid content of cell-wall samples is shown in Table II. The content of this sugar at the ripe stage decreased by 28% in the mango.

Cell-wall sample	Content of cell wall (mg/100mg cell-wall preparation)		
	Protein	Uronic acid	Calcium
Red kidney bean	11.1	19.2	n/d
Unripe mango	7.3	25.0	0.55
Ripe mango	21.1	19.1	0.41

Table II

Protein, uronic acid and calcium content of typical cell-wall preparations from the red-kidney bean, unripe mango and ripe mango. Protein was estimated from the nitrogen content of samples and uronic acids by a modified version of the carbazole method (294). Assays for calcium were carried out by Elemental Micro Analysis, Devon.

If however, uronic acid content is calculated on the basis of total carbohydrate of cell-wall preparations (i.e. excluding the protein values), there appears to be very little difference between the two stages. Relative values become 27% for unripe mango and 24% for ripe mango. These figures suggest that uronic acids are not lost preferentially from cell walls during ripening compared to rest of the carbohydrate material. The content of calcium in unripe and ripe mango shows a loss of 25% at the ripe stage. However, this loss seems to be related to the loss of uronic acids (the ratio of uronic acid to calcium by weight is similar at both stages).

C. Fractionation of Cell Walls

1. Hot Water Fraction

The yield of hot water soluble fraction (100 degrees C for 6 hrs.) is shown in Table III. Hot water was able to remove almost 3 times more material (presumably pectic material) from the cell walls of the unripe mango (48%) than that from the red-kidney bean (18%). The former value is similar to 48% obtained for hot water fractions from cabbage (159). This indicates significant differences in the structure of the two samples which could be accounted for by the presence of larger amounts of pectin in the cell wall of the fruit and/or the presence of larger amounts of the insoluble calcium salt of pectic acid in the red-kidney bean cell wall. Another possibility is that hot water was able to remove greater quantities of hemicellulose (along with the pectic fraction) from the cell wall of the unripe mango as compared to the red-kidney bean. Water solubilised material

Cell-wall sample, mg/100mg cell-wall preparation			
Fraction	Red kidney bean	Unripe mango	Ripe mango
Hot water	18	48	11
4 M NaOH	18	22	24
2 M TFA	38	14	31
Residue	26	16	34

Table III

Fractionation of cell-walls prepared from the red-kidney bean, unripe mango and ripe mango. Hot water fractions were obtained by incubating samples (10 mg/ml) at 100 degrees Centigrade for 6 hrs. Remaining cell walls were treated with 4 M sodium hydroxide (10 mg/ml) for 4 hrs. at 25 degrees Centigrade to yield alkali-soluble fractions. TFA fractions were obtained by hydrolysing cell-wall residues at 100 degrees Centigrade for 6 hrs.

of ripe mango cell walls (11%) was very much less than that of the unripe fruit. This is probably a reflection of the loss of cell-wall carbohydrates during ripening in the former.

2. Alkali Fraction

Cell-wall residues left after extraction with hot water were extracted with 4 M sodium hydroxide for 4 hrs. at room temperature under nitrogen. Low temperature and the exclusion of oxygen are necessary to prevent extensive changes in the polysaccharides at high pH (289). Solubilized material (presumably hemicellulose) was precipitated with ethanol and dried in a stream of air. Alkali-soluble polysaccharides accounted for roughly the same amount of the total cell walls of all samples studied as shown in Table III. The residue left after hot water and alkali treatment accounted for 30% of the total unripe mango cell-wall sample compared to 65% for the ripe mango and 64% for the cell walls from red-kidney bean hypocotyls. The cellulose content of the red-kidney bean hypocotyl cell walls has been found to be 23% (6), thus ca. 41% kidney bean cell wall must comprise insolubilised forms of polysaccharides (eg. calcium pectate) and hydroxyproline-rich glycoproteins.

D. Analysis of Cell-Wall Fractions

1. Conditions of Hydrolysis

Conditions employed for the hydrolysis of polysaccharides are often a compromise between destruction of sugars and incomplete hydrolysis of

resistant glycosidic bonds. Complete hydrolysis of glycosidic linkages in many non-cellulosic or non-uronic acid containing polysaccharides can be achieved with dilute mineral acids. Those containing uronic acids however, undergo incomplete hydrolysis due to the stability of the glycosidic bond involving uronic acid residues. The carboxylic acid group exerts a stabilising effect on the uronosidic bond (290, 291). The resistance of aldobiouronic acids to acid hydrolysis is believed to result from steric factors which render difficult the formation of the partially planar carbonium ion intermediate in the hydrolysis reaction (292). Thermodynamic parameters such as energy of activation and entrophy of activation suggest that acidic disaccharides are hydrolysed by a different mechanism to neutral disaccharides (291) although the nature of the difference is not presently understood. The rate of hydrolysis of aldobiouronic acid is greatest with 1,4-linked disaccharides. This rate reduces by about 50% for the corresponding 1,2-linked compounds and is least for 1,6-linked compounds (291). Monosaccharides are also destroyed at different rates during acidic hydrolysis. Those in the furanoid form are more susceptible to destruction than those that are pyranoid (291).

For the whole of this investigation, the conditions of hydrolysis used were 2 M TFA at 100°C for 6 hrs.. Mankarios et al. (293) found that these conditions offered a good compromise between hydrolysis and monosaccharide destruction in the analysis of onion and potato pectin.

2. Uronic Acid Determination

During the initial stages of the work, uronic acid content of cell walls was determined by a modification of the method of Dische (294) and that of polysaccharides by the method of Blumenkrantz and Asboe-Hansen (295). Total carbohydrate was measured by a scaled down version of the phenol/sulphuric acid method of Dubois, et al. (296). Uronic acid composition was then expressed as a percent of total polysaccharide. However, polysaccharides that contained very high proportions of uronic acids gave values which were greater than 100% for their uronic acid composition. This was mainly because uronic acids give a less intense colour when assayed by the phenol/sulphuric acid method than glucose (which is normally used for preparing standard curves, see Fig. 13). To overcome this problem, neutral sugar content was determined by TFA hydrolysis followed by TLC analysis. These values were then added to uronic acid values determined by the carbazole or the Blumenkrantz method and the total taken to represent 100% of the polysaccharides. However there still remains 2 sources of error in this approach:

(a) Neutral monosaccharides which are destroyed during hydrolysis cannot be quantitated. The composition of polysaccharides in the literature is traditionally determined by acid hydrolysis and errors arising from monosaccharide destruction are never avoided. The errors may be minimised by estimating the quantity of each monosaccharide in the original polymer by linear extrapolation of hydrolysis time, but this method would be time consuming and it may not give more accurate results, for example, in the case of rhamnose estimation (293).

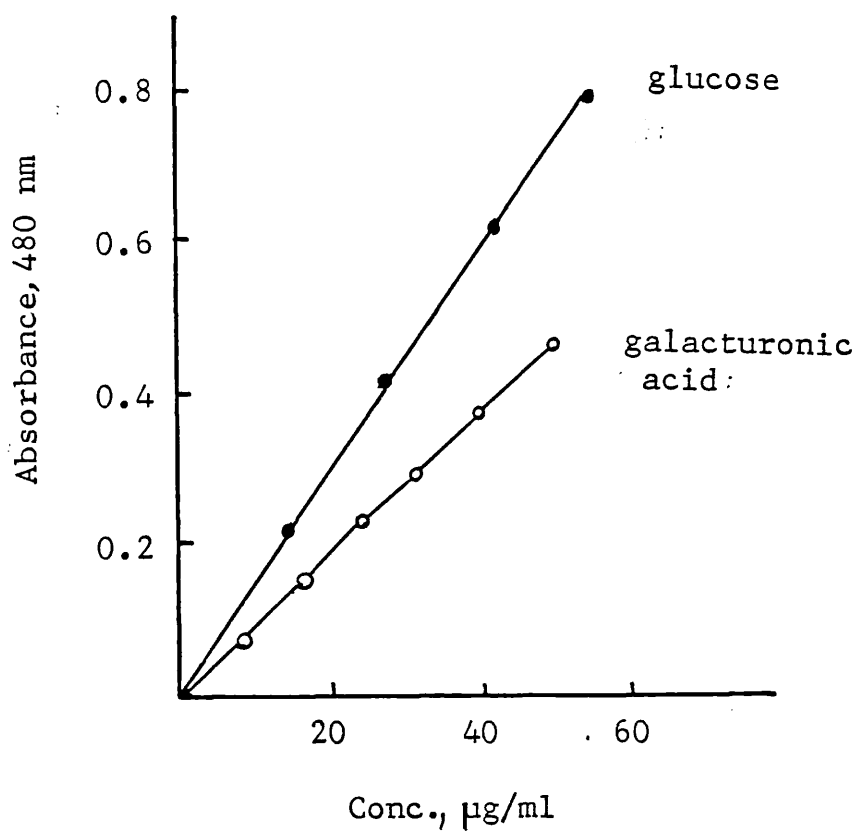


Fig. 13
Calibration curves for the phenol/sulphuric acid method (296) using galacturonic acid and glucose standards.

(b) Neutral sugars present in aldobiouronic acids would not be detected because of incomplete hydrolysis. It is unlikely that this would cause much error in the overall estimation of neutral sugars, as only a small proportion of such sugars are present in aldobiouronic acids (11, 13, 293, 297). For example, hydrolysis of sycamore rhamnogalacturonan (see Fig. 1) would only result in the formation of stable aldobiouronic acids containing rhamnose. However, decarboxylation of uronic acids prior to TFA hydrolysis would increase the yield of L-rhamnose by reducing the acid stability of the α -D-galacturonosyl-(1,2)-L-rhamnose glycosidic bond, but this method would be time consuming.

3. Hydrolysis of Hot-Water-Soluble Fraction

Samples were hydrolysed with 2 M TFA and the monosaccharide composition of the hydrolysate determined by TLC analysis by the method of Menzies and Mount (298). This method gives approximately 5% errors in the quantitation of neutral monosaccharide. Uronic acid composition of each sample was determined before hydrolysis.

Fig. 14 shows that the red-kidney bean hot-water fraction contained significantly larger amounts of uronic acids (29.1%), than that from unripe mango. The latter, however, contained larger quantities of arabinose and galactose. This suggests a greater amount of neutral side chain branching in unripe mango pectin. The presence of glucose and xylose in the hot-water fractions of the red-kidney bean and the unripe mango cell walls is consistent with the existence of these sugars in the pectic fraction (cf. p.40) or the solubilisation of some

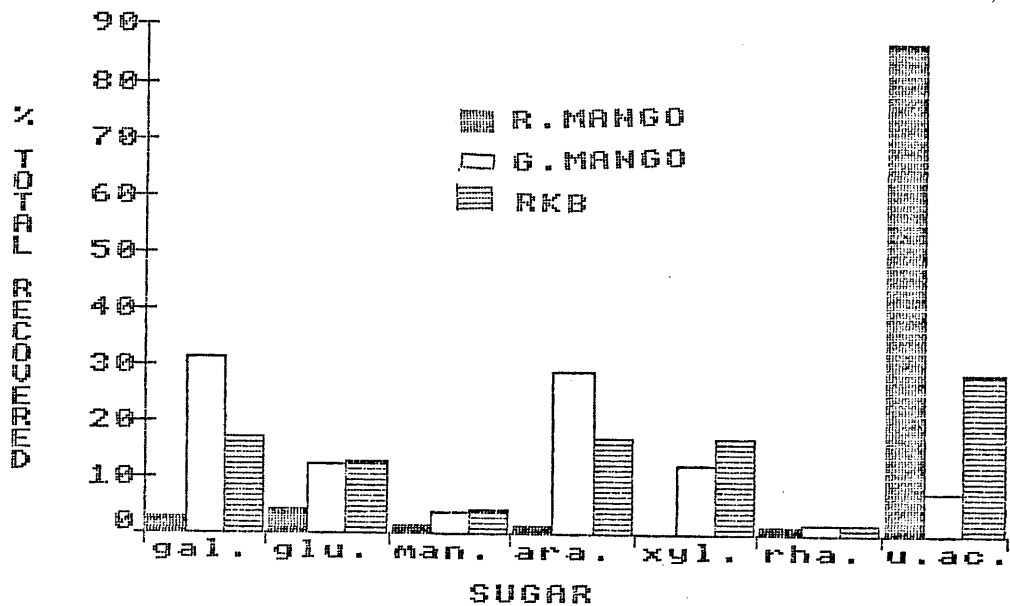


Fig. 14
 Monosaccharide compositions of the hot-water-soluble fractions of cell walls prepared from the red-kidney bean, unripe mango and ripe mango (see Table III). One ml samples containing 0.1 mg carbohydrate were heated at 100°C in 2M TFA for 6 hrs. prior to TLC analysis by the method of Menzies and Mount (298).

hemicellulose. This argues against earlier suggestions (section C1) that preferential removal of hemicellulose from unripe mango cell walls may have accounted for the greater yield of a hot water-soluble fraction.

The fraction obtained from the ripe mango contained 88% uronic acids which indicates almost complete loss of neutral sugars from this fraction during ripening.

4. Hydrolysis of Alkali Fractions

Fig. 15 shows that the alkali-soluble fraction from the cell wall of the red-kidney bean and the unripe mango are very similar in composition. Nearly 60% of the monosaccharides obtained in each case was accounted for by glucose, xylose and mannose which were present in roughly double the amount than was obtained from the hot-water-soluble fraction. This is presumably due to a greater removal of hemicellulose by sodium hydroxide. Very little uronic acid is present in this fraction and most of the remaining monosaccharides are accounted for by galactose and arabinose. This suggests that a significant amount of pectin, rich in neutral sugars, is still left in the cell wall after hot-water treatment. The composition of the alkali-soluble fraction from the ripe mango is not dissimilar to that obtained from the unripe mango. The reason for the lower glucose content in the ripe mango fraction especially when there is little difference in the xylose content, is not clear. The higher proportion of uronic acids in the ripe mango fraction may have arisen because the ripening process modified the cell wall in such a manner that

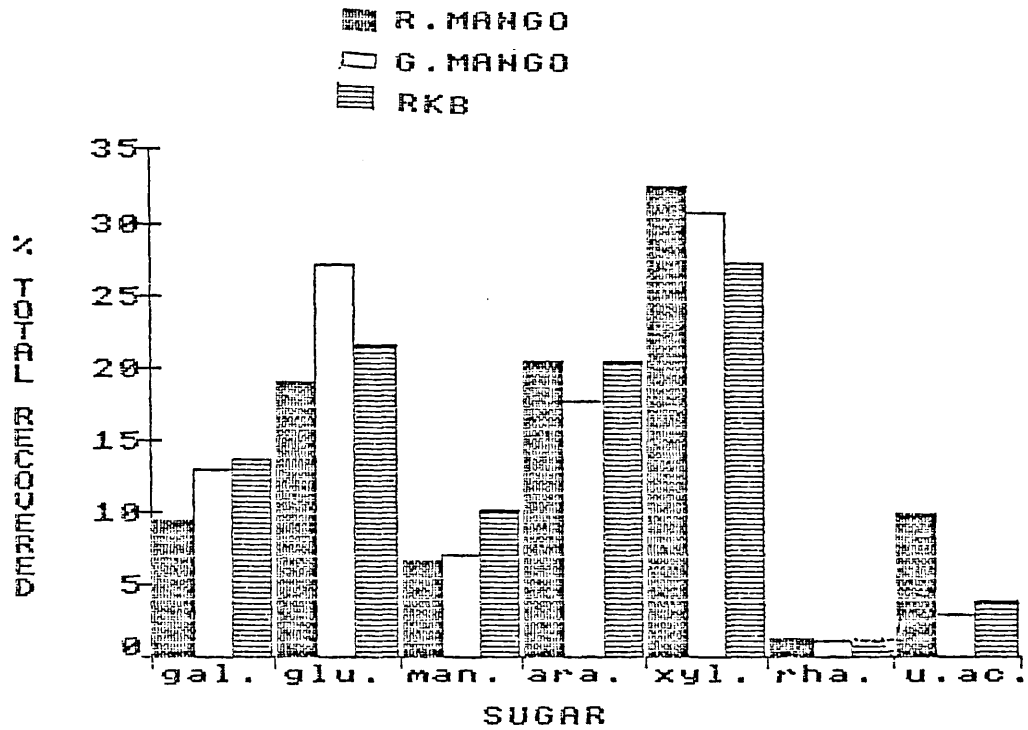


Fig. 15
 Monosaccharide composition of the 4 M sodium hydroxide soluble fraction of cell-walls prepared from the red-kidney bean, unripe mango and ripe mango (see Table III). One ml. samples containing 0.1 mg carbohydrate were heated at 100° C in 2M TFA for 6 hrs. prior to TLC analysis by the method of Menzies and Mount (298).

extraction of uronic acid containing polymers by 4 M sodium hydroxide was enhanced.

5. Hydrolysis of Residues

Acid hydrolysis was carried out on the cell-wall residues remaining after hot water and alkali extractions. It can be deduced from Table III that 60% of the remaining cell-wall material after the above extractions can be solubilised by TFA treatment in the case of the red-kidney bean. The respective values for the unripe and ripe mango cell walls are 47% and 48%. Presumably most of the residual material is cellulose and hydroxyproline-rich proteins.

TFA hydrolysis of cell-wall residues released galactose and arabinose in significant quantities (see Fig. 16) from all samples. Although a small amount of these monosaccharides may have been released by hydrolysis of the glucan chains of hydroxyproline-rich glycoproteins (total protein of cell walls are shown in Table II), most of the monosaccharides probably originated from pectic molecules which were not removed by previous extractions. The presence of high amounts of xylose and glucose in TFA hydrolysates suggests that 4 M sodium hydroxide was unable to remove all of the hemicellulose fractions from cell-wall samples. This may be because some of this polymer are trapped between cellulose fibres. The variation in xylose content between red-kidney bean and unripe mango samples supports earlier suggestions of fundamental differences between the structure of these 2 types of cell walls.

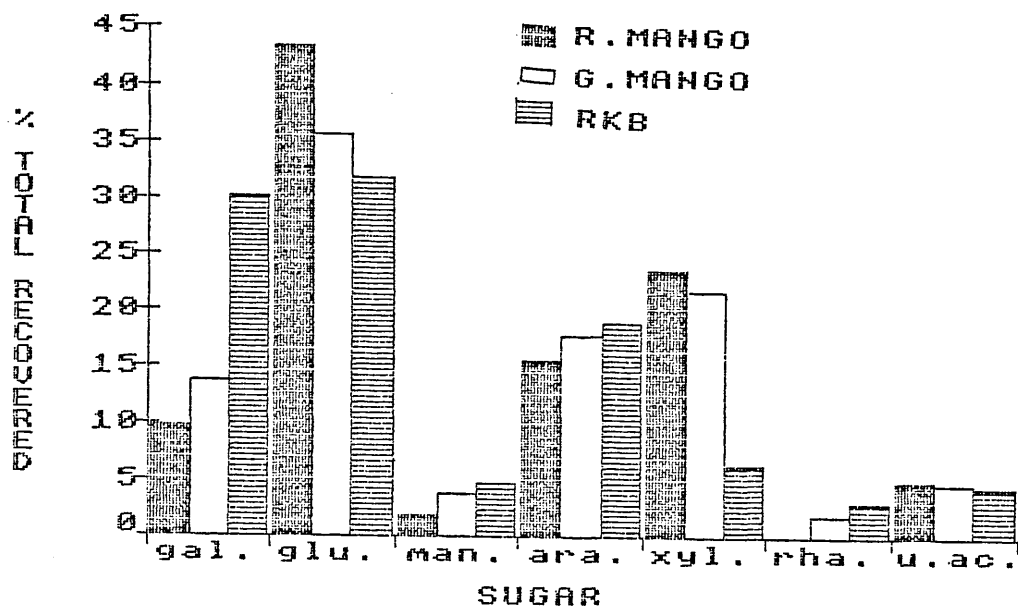


Fig. 16

Monosaccharide composition of the 2 M TFA soluble fraction of cell walls prepared from the red-kidney bean, unripe mango and ripe mango (see Table III). Analysis was carried out by TLC using the method of Menzies and Mount (298).

E. Action of PG on Cell Walls

1. PG-Soluble Fraction.

The main differences between red-kidney bean hypocotyl and unripe mango cell walls is the presence of greater amounts of pectic material in the latter. It was therefore decided to further examine the similarity/dissimilarity of the pectic fractions in these samples. Thus soluble and insoluble fractions obtained after PG treatment of cell walls were examined. PG released 8.1% of red-kidney bean hypocotyl cell wall. This figure is low compared to 16% obtained by Talmadge et al. for walls of sycamore suspension-cultured cells. The enzyme released 6.6% of unripe mango mesocarp cell wall and 0.2% of the ripe mango mesocarp cell wall. The low yield from ripe mango cell wall is probably due to prior solubilisation of pectin during ripening. Bio-Gel P-2 elution profiles of solubilised carbohydrates (see Fig.17) show that roughly 40% of solubilized carbohydrates from red-kidney bean and unripe mango cell walls are excluded in the void volume (peak P1). As the exclusion limit of Bio-Gel P-2 is 2,000 this fraction probably contains species of this molecular size or of higher values. Other peaks (P2 and P3), thus contain oligosaccharides.

2. Composition of Bio-Gel P-2 Fractions

TLC analysis following TFA-hydrolysis show that the composition of the polysaccharides present in peak P1 of the unripe mango and the red-kidney bean samples are very different (see Fig. 18). The most striking differences are in their uronic acid and glucose contents

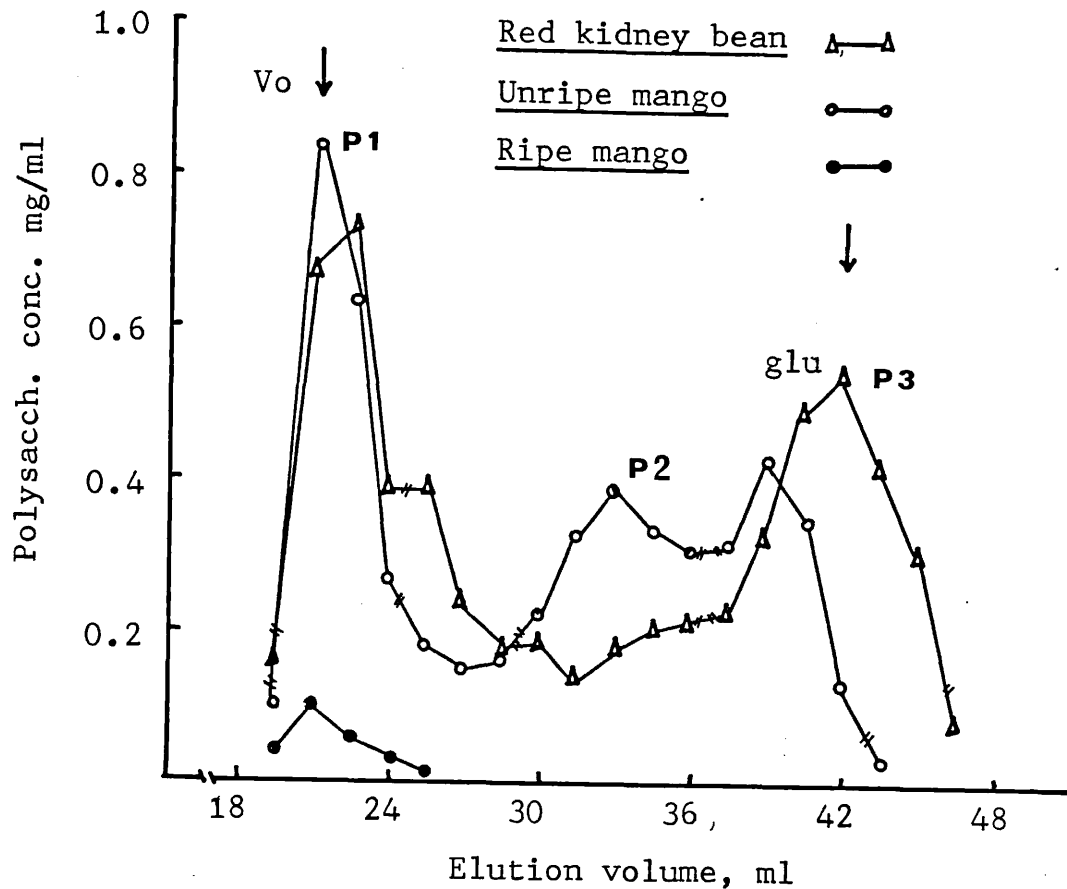


Fig. 17
 Bio-Gel P-2 gel-filtration chromatography of carbohydrate material solubilized by PG from cell walls prepared from the red-kidney bean, unripe mango and ripe mango. After freeze-drying, soluble products, were redissolved in 1 ml 50 mM acetate buffer pH 5.0 and applied to a column (74 x 1.4 cm). Elution was carried out with the same buffer at a rate of 0.1 ml/min. Fractions of 1.5 ml were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296).

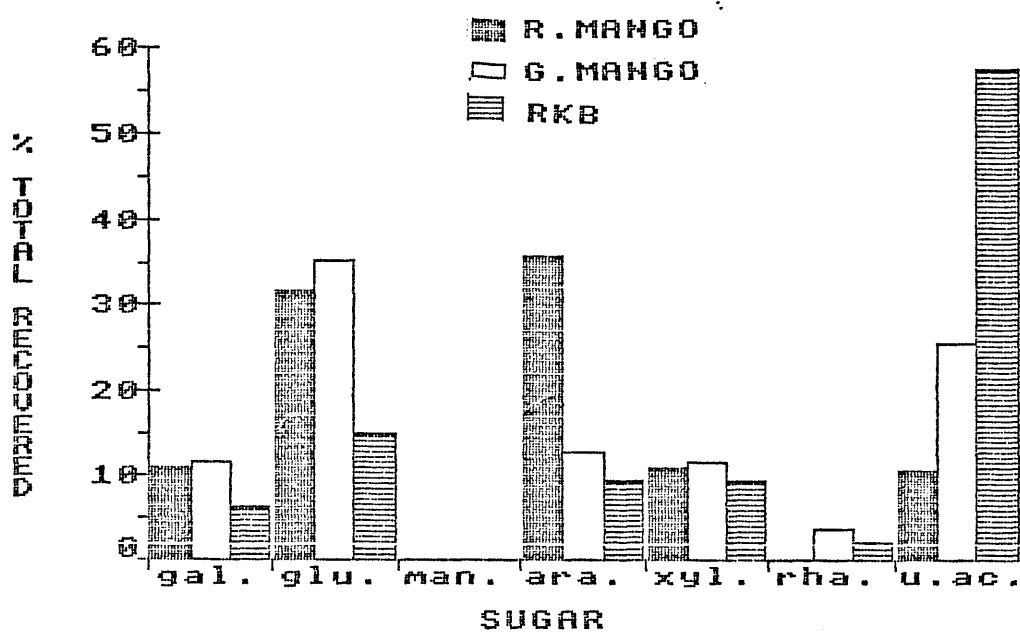


Fig. 18

Monosaccharide composition of the void peak P1 obtained after Bio-Gel P-2 separation of PG solubilized material from cell walls prepared from the red-kidney bean, unripe mango and ripe mango (see Fig. 17). Fractions were pooled, freeze-dried and analysed as described in Fig. 14.

which are 58.1% and 14.9% respectively for the red-kidney bean, and 25.6% and 35.2% respectively for the unripe mango. High proportions of glucose also occur in peak P1 of the ripe mango. On the other hand peak P2 from both sources show similar compositions, uronic acid being the main constituent (see Fig. 19). This suggests regions of similarity in the pectic fractions of the 2 sources.

Without knowing whether other hydrolytic enzymes were present in the PG preparation, it is difficult to explain the presence of large amount of glucose and xylose in peak P1. However, no other carbohydrase activity was reported by English et al. (14) who employed the same purification procedure for the enzyme. Xylose and glucose are characteristic sugars of xyloglucans in the hemicellulose fraction of the primary cell wall. If the unripe mango and red-kidney bean cell wall possess the same type of structure as that of the generalised model proposed by Albersheim, it seems unlikely that PG could remove xyloglucans strongly hydrogen-bonded to cellulose fibres. However the above results are consistent with the cell-wall model suggested on p. 36 which depicts covalent association of xylose and glucose with some regions of the pectic fraction. Knee et al. (160) have also shown the presence of xylose and glucose in similar cell-wall fractions.

3. Urea Solubilised Fraction

The residue obtained after PG digestion of cell-wall sample was treated with 8 M urea and the resulting solubilised fraction was further analysed. Urea was used to solubilise (without degrading) hydrogen-bonded xyloglucan from PG treated cell walls. The amount of

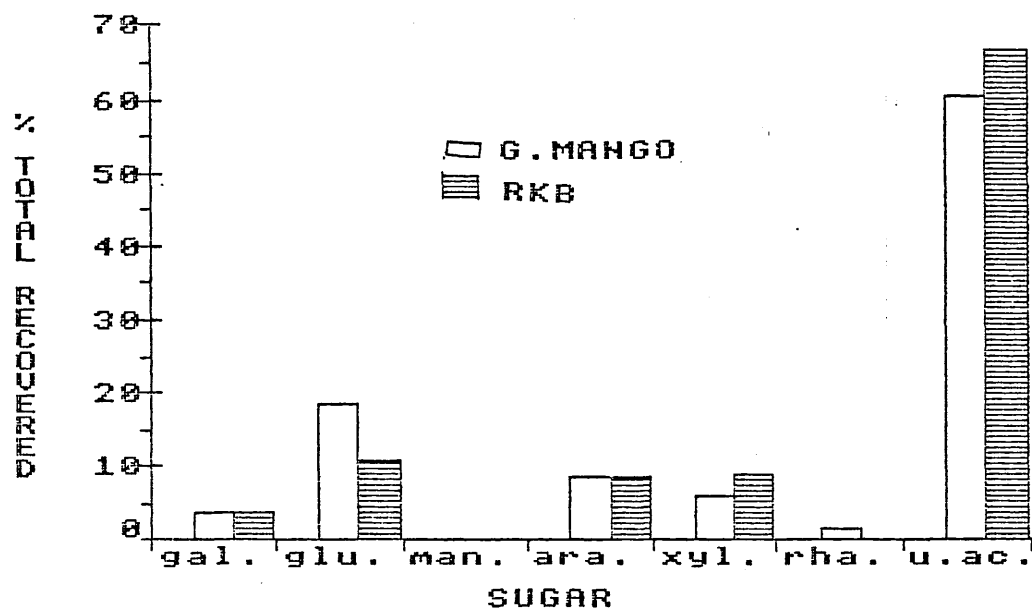


Fig. 19

Monosaccharide composition of the included peak P2 (fractions 31-36) obtained after Bio-Gel P-2 separation of PG solubilised material from cell walls prepared from the red-kidney bean and unripe mango (see Fig. 17). Fractions were pooled, freeze-dried and analysed as described in Fig. 14.

carbohydrate released was not quantitated. However, Talmadge et al. (13) found that less than 2% was removed from PG-treated suspension-cultured sycamore cell walls under similar conditions. Prior to analysis this fraction was dialysed to remove urea and the soluble material precipitated with ethanol. The monosaccharide composition of urea-solubilised fractions are presented in Fig. 20. Only trace amounts of uronic acids and rhamnose were present in all samples. Sugars present were those characteristic of arabinogalactan side chains of pectic molecules and of xyloglucan. These observations are compatible with solubilisation of hydrogen-bonded xyloglucan and residual fragments of pectic material essentially free of uronic acids. It is unlikely that urea would enhance the solubilisation of calcium pectate fragments which partially explains the absence of uronic acids. Significant amounts of mannose were present in the solubilised material from unripe and ripe mango cell walls, but not from the red-kidney bean cell walls. This further suggests differences in the structure of red-kidney bean hypocotyl and mango pulp primary cell walls.

III. CONCLUSION

Mango pulp cell wall is not typical of other dicotyledonous primary cell wall. The largest difference is in the pectic fraction which is present in much greater quantities in the cell walls of mango and which contains higher proportions of neutral sugars (arabinose and galactose). There is severe degradation of the cell wall during ripening caused by solubilisation of the pectic fraction from which galactose and arabinose are preferentially lost. There appears to be little loss of protein.

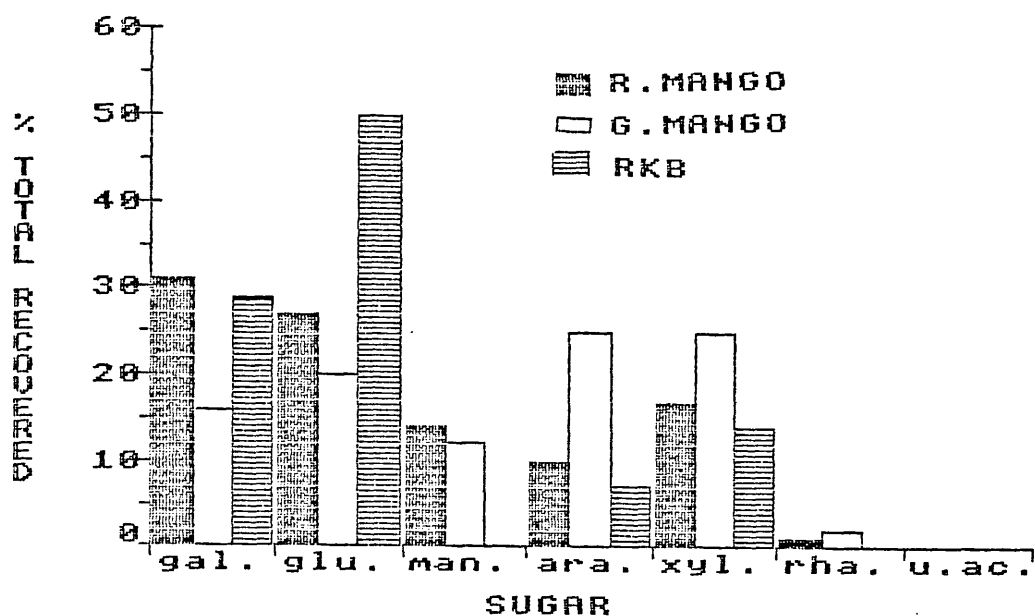


Fig. 20

Monosaccharide composition of urea solubilised fractions of PG-treated cell-wall samples of the red-kidney bean, unripe mango and ripe mango. After dialysis, solubilised material was freeze-dried and analysed as described in Fig. 14.

PART 2

Soluble Carbohydrates

I. INTRODUCTION

In the last chapter the composition of the mango cell wall was examined. Very little information is presently available in the literature on the soluble carbohydrates present in the mesocarp tissue of mango. In the ripe fruit, most of the soluble polysaccharides have presumably been solubilised by enzymic action on the cell wall during ripening. The purpose of the work presented in this chapter was to investigate the composition of soluble carbohydrates of unripe and ripe mango which, together with the work carried out in the last chapter, would provide an overall view of the carbohydrate composition of the pulp. In addition, autolytic activity of carbohydrases present in soluble fractions from the ripe and unripe fruits was investigated. It was hoped that this work would provide information that would be of value in assessing the nature of enzymes involved in mango ripening.

II. RESULTS AND DISCUSSION

A. Composition of Mango Mesocarp Tissue

Fig. 21 shows the general composition of mango pulp from unripe and ripe fruits. As determined by freeze-drying, both tissues contained approximately 84% water. Of the solid materials, 2.3% constituted a soluble fraction (50 mM acetate buffer pH 5.0) in the case of the unripe mango and 31.9% in the ripe fruit. Of this soluble fraction, polysaccharide content increased by 8-fold at the ripe stage, whilst smaller sugars increased by 15-fold. These results are similar to a 14-15-fold increase in soluble carbohydrate which occurs in

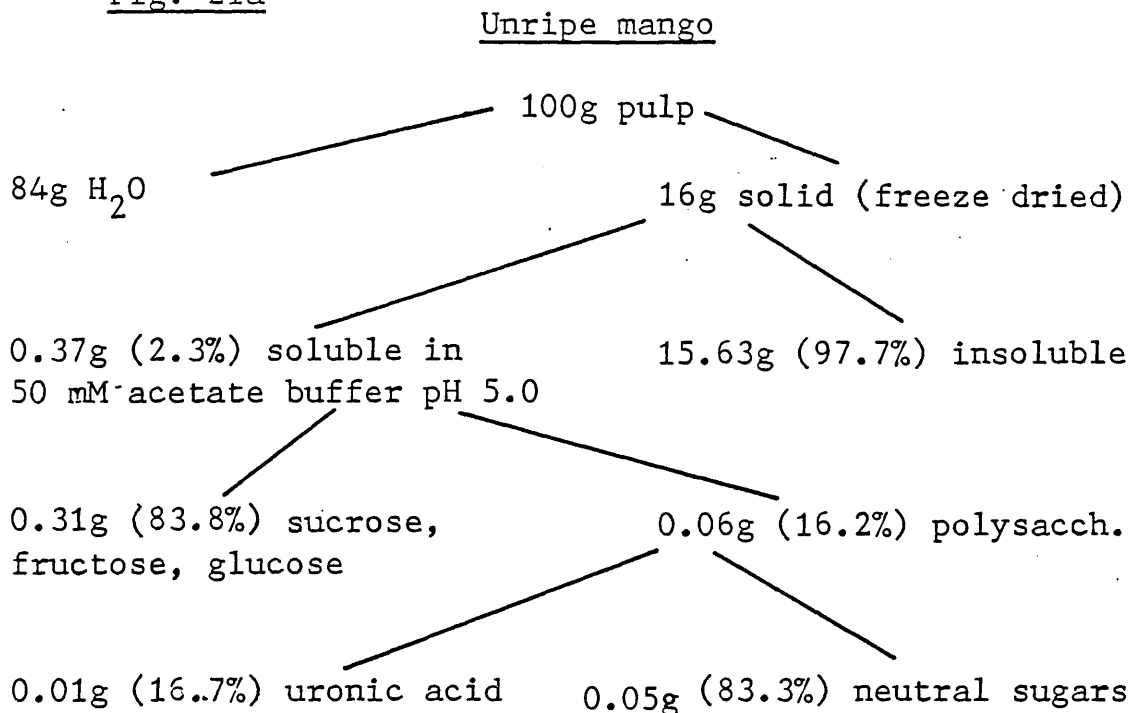
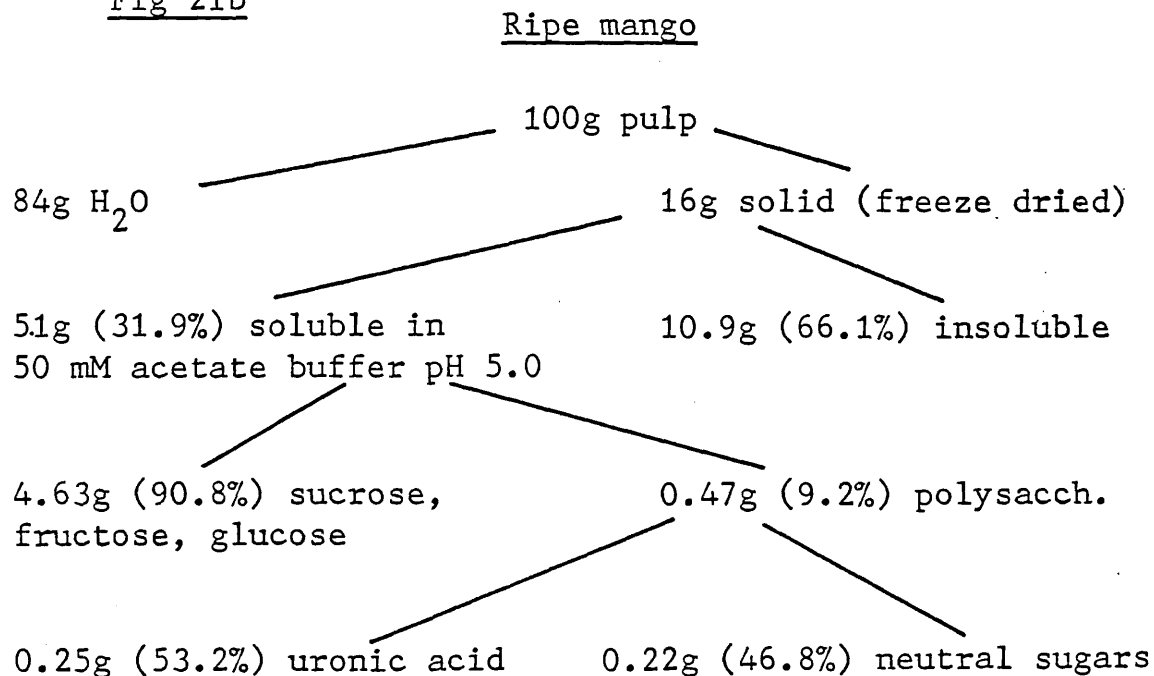
Fig. 21aFig 21b

Fig. 21

Composition of the pulp of unripe (Fig. 21a) and ripe mango (Fig. 21b). Soluble polysaccharide and smaller sugar composition were determined by the phenol/sulphuric acid method (296) after separation of soluble fractions by Bio-Gel P-150 gel-filtration chromatography. Uronic acid composition was determined by the method of Blumenkrantz (295). Solid content of pulp is that portion left after freeze-drying.

banana (196). Analysis of the buffer soluble fraction by TLC and gel-filtration showed the presence of glucose, fructose, sucrose and high-MW polysaccharides. Sucrose, fructose and glucose make up 84% of soluble carbohydrates in the unripe mango and 91% in the ripe fruit. No oligosaccharides were detected in either unripe or ripe mango fractions. This tends to support previous findings that PG is absent from mango fruit (266); the presence of this enzyme would probably have resulted in the detection of oligosaccharides in the ripe mango.

Increases in sucrose, fructose and glucose are probably the result of ripening related mobilisation of reserves of starch. Light microscopy after iodine staining showed the presence of large numbers of starch granules in cells of the unripe fruit which were absent from ripe fruit. In banana, starch content was noted to drop from 10-20% of the total carbohydrate to 1-2% on ripening.(183).

B. Gel-Filtration of Soluble Polysaccharides

1. Elution Profiles of Soluble Polysaccharides

Marked differences in molecular size were noted between soluble polysaccharides of the unripe and ripe mango (see Fig. 22a and Fig. 22b). Peaks containing fructose glucose and sucrose are not shown. The ripe fruit contained polysaccharides of average MW 45,000 which eluted as a single peak from a Bio-Gel P150 column. In contrast the soluble polysaccharides of unripe mango eluted as a main peak (58%) in the void volume (MW > 150,000) with a distinct indication of a second peak

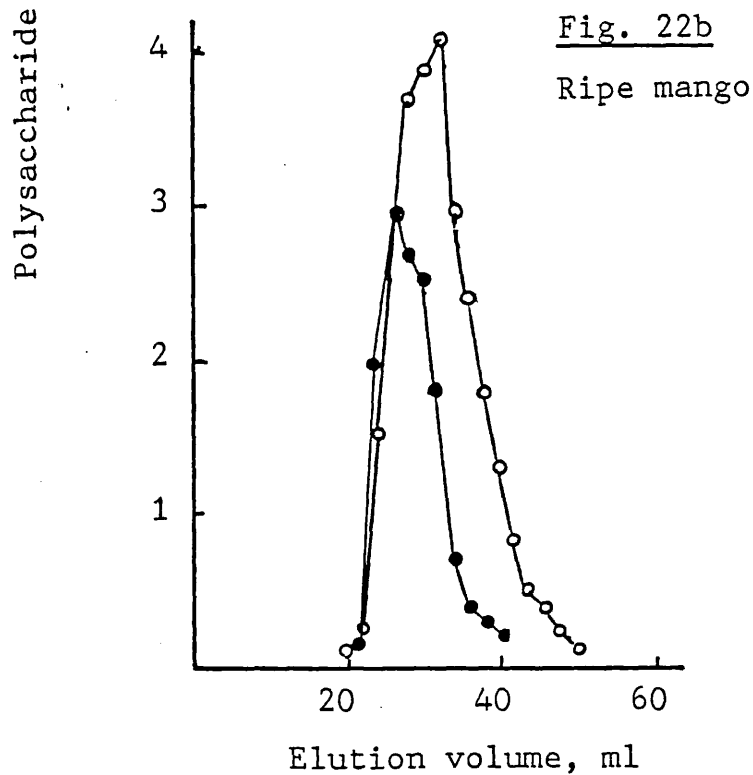
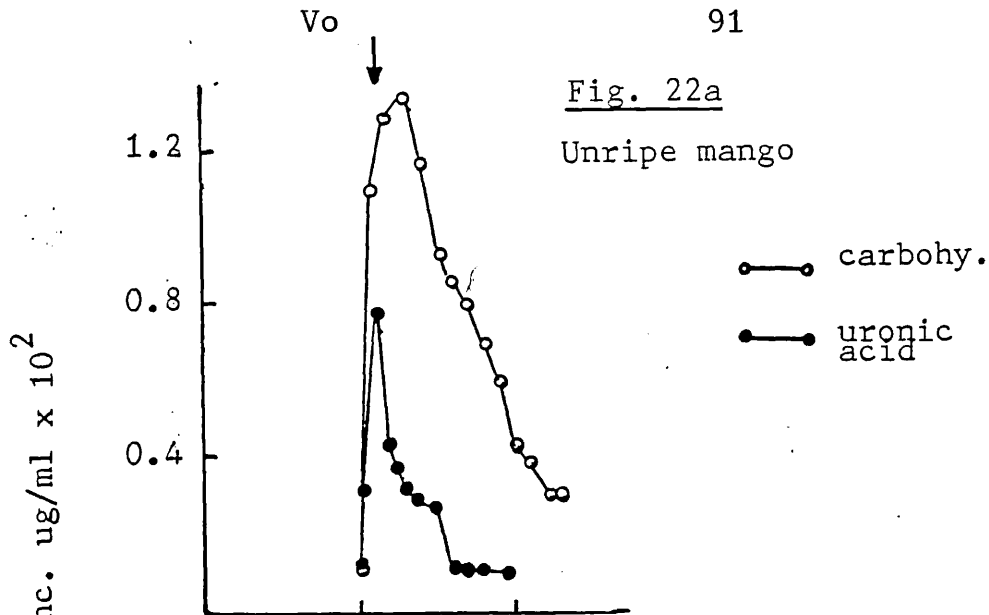


Fig. 22

Bio-Gel P-150 gel-filtration of soluble polysaccharides (50 mM acetate buffer, pH 5.0) present in the pulp of unripe (Fig. 22a) and ripe (Fig. 22b), mango. One ml samples were applied to a column (76 x 0.8 cm) equilibrated in 50 mM acetate buffer, pH 5.0. Elution was carried out with the same buffer and 2 ml fractions were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296) and uronic acids by the method of Blumenkrantz (295).

(42%) as a shoulder; MW 45,000). These observations suggest that polysaccharides in the soluble fraction of the ripe fruit are in a more degraded state than those in the unripe fruit.

2. Autolytic Changes of Soluble Polysaccharides

Upon incubation at 25 degrees C (in the presence of thymol to prevent microbial growth) the elution profile of the unripe mango soluble fraction polysaccharides displayed a distinct change (Fig. 23a) the shoulder (cf. Fig. 22a) becoming more pronounced with a concomitant decrease in the size of the void peak. This change is probably brought about by endogenous enzymes as it is inhibited by the presence of 10 mM mercury chloride in the incubation medium (Fig. 23b). No mono- or oligosaccharides were detected after the incubation, indicating that the enzymes involved were endo-acting enzymes.

Such incubation showed no change in the elution profile of the soluble polysaccharides of ripe mango. This is probably because polysaccharides present at this stage have already been hydrolysed.

C. Ion-Exchange Separation of Soluble Polysaccharides

1. Elution profiles

Soluble polysaccharide fractions from mango fruits at the unripe and ripe stages were subjected to ion-exchange chromatography on DEAE Sephadex A 25 after removal of sucrose, fructose and glucose by dialysis. Approximately 20% of the soluble unripe mango fraction

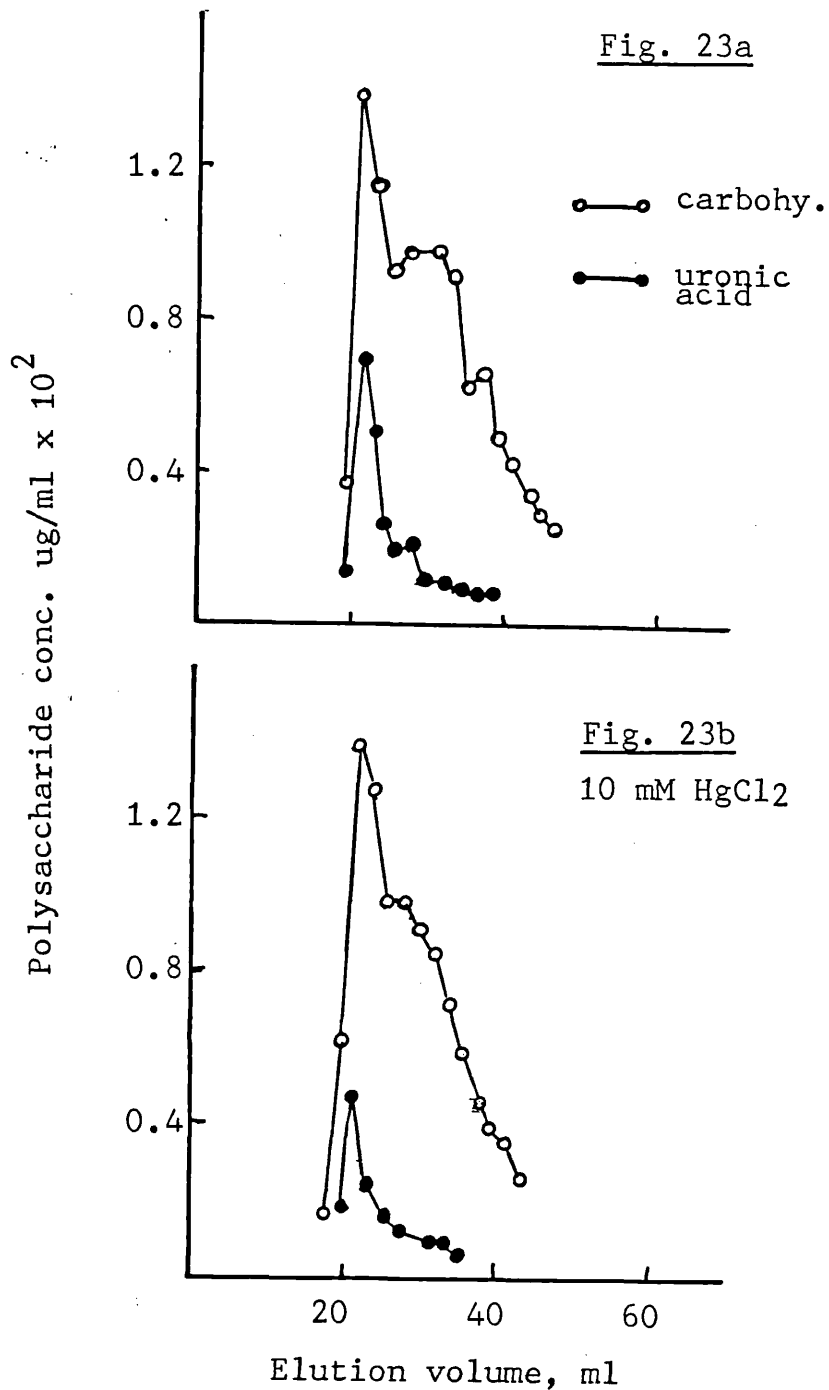


Fig. 23

Bio-Gel P-150 gel-filtration of soluble polysaccharides (50 mM acetate buffer, pH 5.0) present in the pulp of unripe mango after autolysis at 25 degrees C for 24 hrs. (Fig. 23a) Fig. 23b shows the affect of the presence of 10 mM mercury chloride in the incubation medium. Details of experimental procedure are as in Fig. 22.

and about 50% from ripe mango eluted through the column unretarded. Presumably these "Neutral" fractions have polysaccharides with lower proportions of negatively charged groups. Thus they probably contain esterified uronic acid groups, and/or very little charged uronic acid residues. Material which was held by the column was eluted with a sodium chloride gradient after it was washed with equilibrating buffer.

The elution profile of the unripe mango preparation that bound to DEAE Sephadex is presented in Fig. 24a. Two distinct polysaccharide peaks were obtained; the first peak containing the major proportion of the polysaccharides was eluted at 0.07 M sodium chloride and the second and smaller peak at 0.34 M sodium chloride. The elution profile for the ripe mango is shown in Fig. 24b, the first peak being eluted at 0.07 M whereas the second peak at 0.25 M sodium chloride. Soluble polysaccharides present in the pulp of the ripe mango thus appear to be less acidic than those in the unripe fruit.

2. Autolytic changes

The DEAE bound component of both unripe and ripe mango soluble fractions appear to become more acidic upon incubation at 25 degrees C, pH 5.0 for 24 hrs. This is reflected in Fig. 25a and 25b in which greater proportions of polysaccharides are eluted at relatively higher salt concentrations. This change is more pronounced in the ripe fruit. Such change in activity may be caused by enzymic de-esterification of the polysaccharides; pectinesterase has been found in the mango (224).

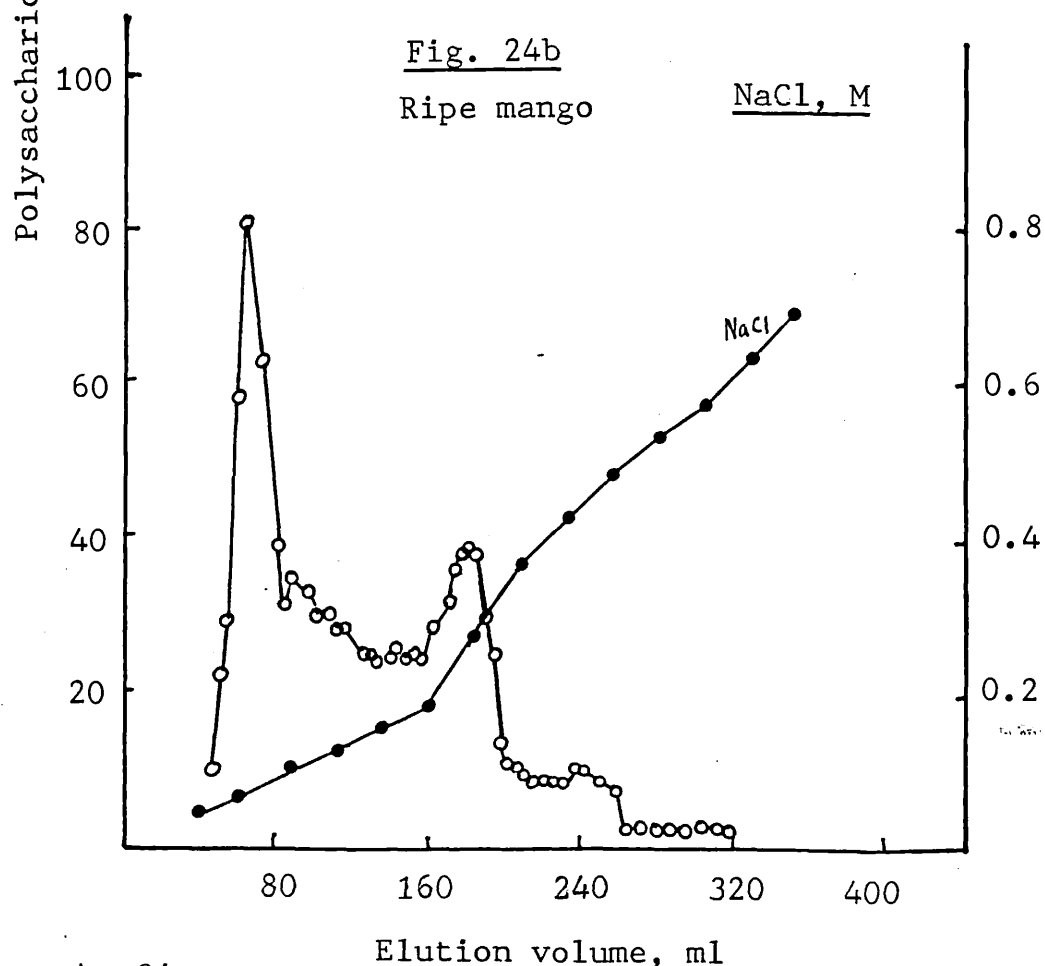
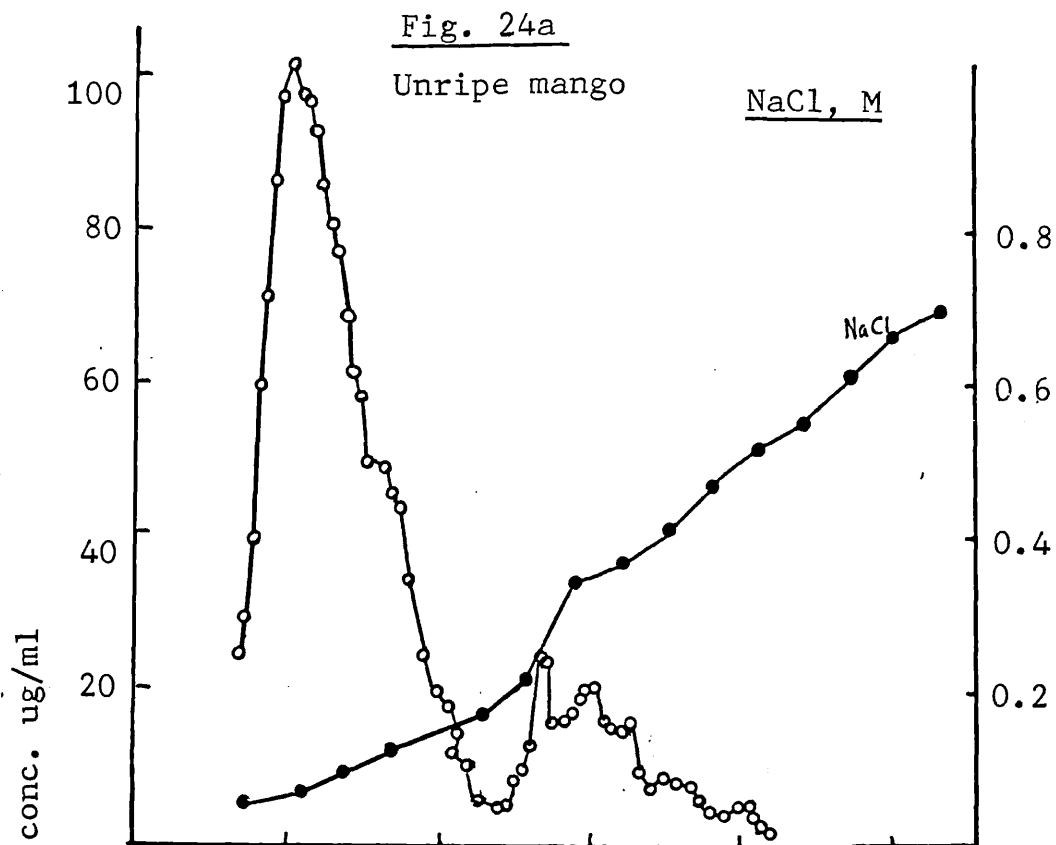


Fig. 24

DEAE Sephadex A 25 separation of soluble polysaccharides (50 mM acetate buffer, pH 5.0) present in the pulp of the unripe mango (Fig. 24a) and ripe mango (Fig. 24b). After application of samples (4ml) to a column (11.2 x 2.5 cm) equilibrated at pH 5.0 and washing, bound material was eluted by a sodium chloride gradient. Four ml fractions were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296).

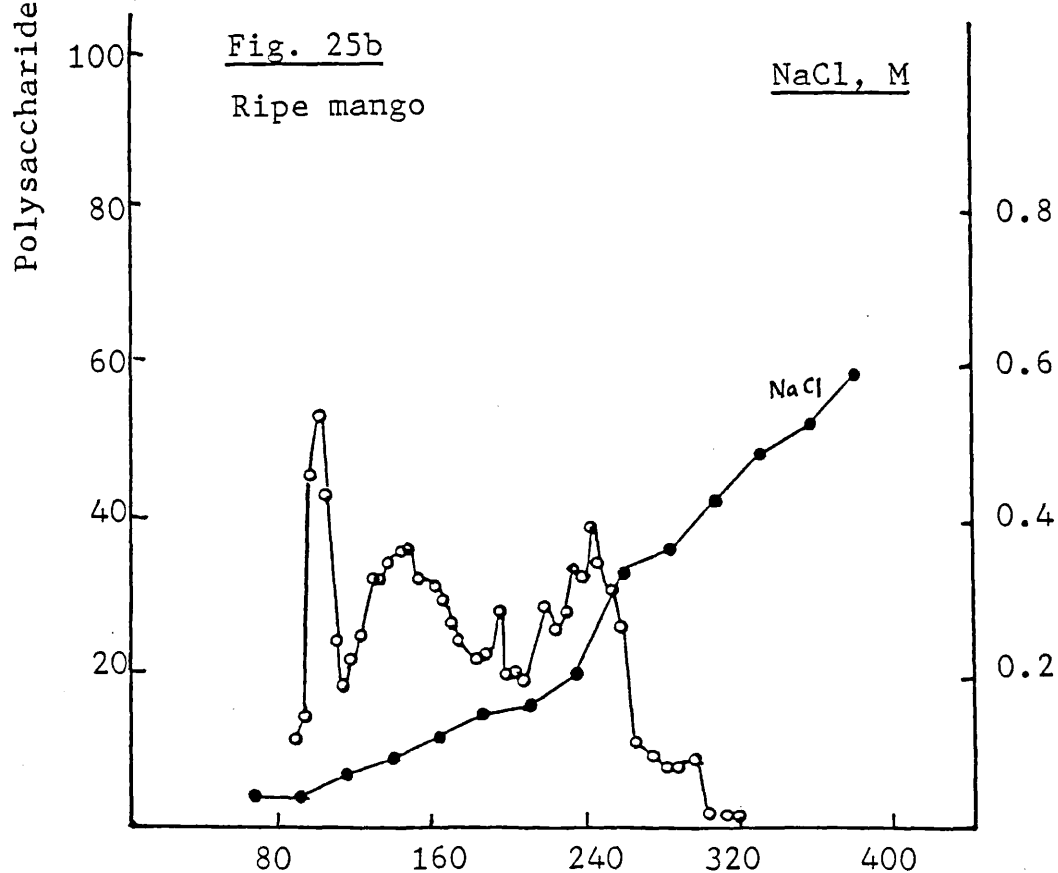
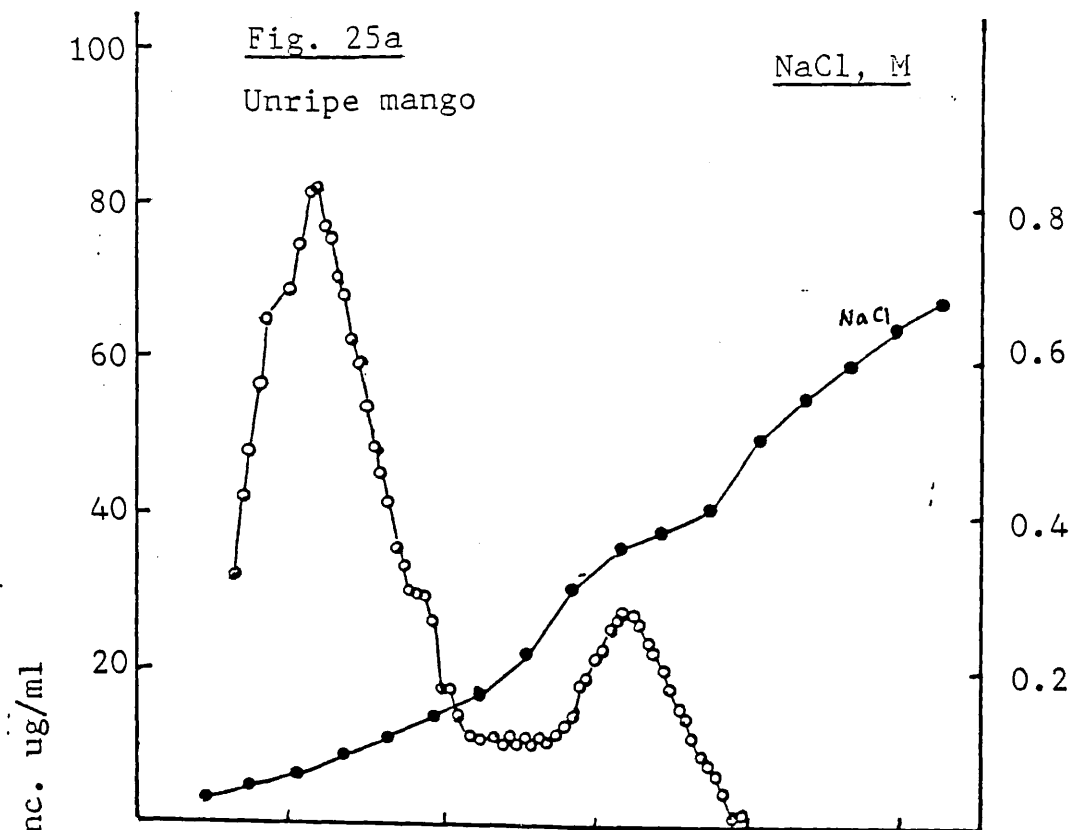


Fig. 25 Elution volume, ml
 DEAE Sephadex A 25 separation of soluble polysaccharides (50 mM acetate buffer, pH 5.0) present in the pulp of the unripe mango (Fig. 25a) and ripe mango (Fig. 25b) after incubation at 25 degrees Centigrade for 24 hrs. Details of experimental procedure are as in Fig. 24.

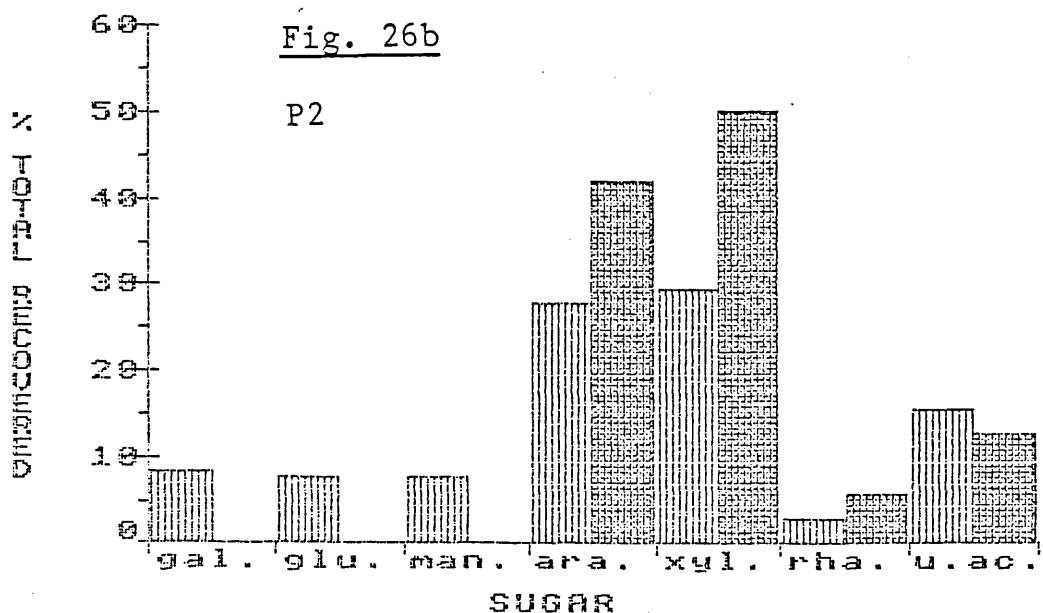
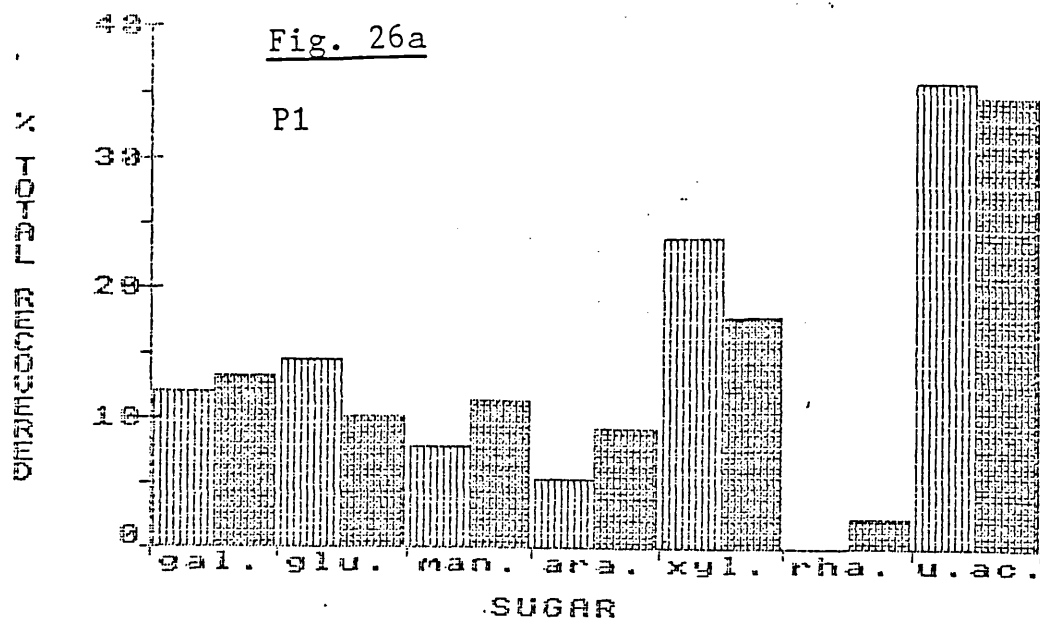
D. Monosaccharide Composition of Soluble Polysaccharides of the Unripe Mango

1. Bio-Gel P150 Fractions

Pooled fractions obtained after Bio-Gel P-150 gel-filtration of unripe mango soluble polysaccharides (see Fig. 22; fractions 11-14 designated as P1 and 15-20 as P2) were freeze-dried and analysed for their monosaccharide composition. P1 and P2 differed markedly in their composition. P1 was made up of 6 different monosaccharides (see Fig. 26a, 0 hrs.) while P2 consisted essentially of arabinose (42%) and xylose (50%, Fig. 26b, 0 hrs.). Uronic acids accounted for 35% of the polysaccharides of P1 of which it was the major component. It is difficult to compare the composition of P1 and P2 in relation to cell-wall softening, as it is uncertain whether the polysaccharides have resulted from in vivo hydrolysis of the cell wall, or have arisen from other cellular source as a result of de novo synthesis.

After autolysis of the soluble preparation of the unripe mango the composition of P1 showed little change (Fig. 26a 24 hrs.). On the other hand, there were increases in P2 of galactose, glucose, mannose and uronic acid while a general decrease in the level of arabinose, xylose and rhamnose occurred (Fig. 26b 24 hrs.). Some of the products found in P2 probably originated from P1 as a result of the action of endo-acting enzymes during incubation process.

2. DEAE Sephadex Fractions





 0 hrs.
 24 hrs.

Fig. 26
 Monosaccharide composition of polysaccharides present in pooled fractions 11-14 (P1, Fig. 26a) and 15-20 (P2, Fig. 26b) obtained after Bio-Gel P-150 separation of unripe mango soluble fractions before and after incubation at 25 degrees C for 24 hrs. After freeze-drying samples were hydrolysed in 2 M TFA (0.1 mg/ml) at 100 degrees C for 6 hrs. and then subjected to TLC analysis by the method of Menzies and Mount (298). Uronic acid content was determined by the method of Blumenkrantz ^{and Asbee-Hansen} (295) on unhydrolysed samples.

Pooled fractions were dialysed against distilled water to remove salt prior to analysis. The monosaccharide compositions of the "Neutral" unripe mango polysaccharide fraction that passed through the DEAE Sephadex column and the large acidic peak were determined. The composition of the smaller, more acidic peak was not determined. The results are presented in Fig. 27a and Fig. 27b and show that the two fractions are fairly similar in composition. Both contain significant amounts of uronic acids, pronounced quantities of arabinose, xylose and galactose, and smaller amounts of glucose, mannose and rhamnose. The results also show that binding of polysaccharides to the ion-exchange column is possible with fairly low uronic acid content (12.6%). The presence of significant amounts of xylose (21.9%) in the bound fraction suggests covalent linking of glycans rich in this sugar with the rhamnogalacturonan backbone (cf. suggested model p 36). Similarly as in the previous section, loss of xylose cannot be linked to cell-wall softening.

E. Monosaccharide Composition of Soluble Polysaccharides of the Ripe Mango.

1. Bio-Gel P-150 Fraction

The Bio-Gel P-150 fractions derived from the ripe mango (12-14, 15-17 and 18-20 see Fig. 22b) were pooled, freeze-dried and analysed for monosaccharide composition. Although these fractions were eluted as one peak various sections of the peak showed the presence of polysaccharide species with different monosaccharide compositions (see

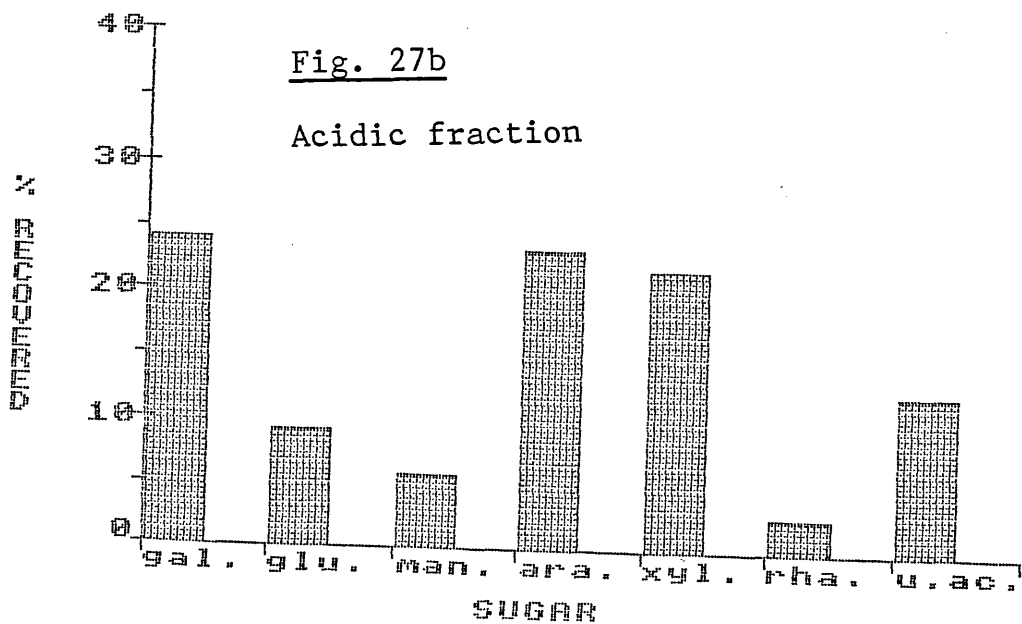
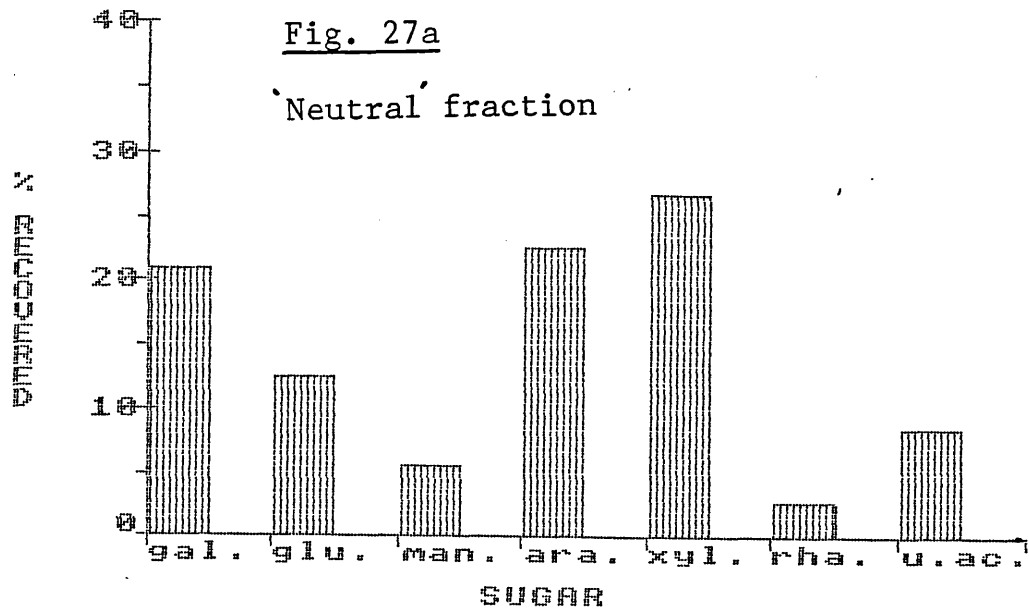


Fig. 27
 Monosaccharide composition of polysaccharides present in pooled "Neutral" (Fig. 27a) and acidic (15-35, Fig. 27b) fractions of a soluble unripe mango preparation after DEAE Sephadex ion-exchange chromatography (see Fig. 24a). Details of analysis are as described in Fig. 26 except that the acidic fraction was dialysed before freeze-drying.

Fig. 28).

The polysaccharides eluting in the early fractions (12-14) contain very high proportions of uronic acids (93.2%). The content of this sugar decreases markedly in the later fractions and therefore with polymer size. Values of 46% and 16.5% for fractions 15-17 and 18-20 respectively were obtained. The latter two fractions contained predominant amounts of arabinose and xylose. It has been shown in part 1 that galactose is lost from the cell wall together with arabinose during ripening. However the present data do not show the presence of high proportions of galactose in any of the pooled fractions. This could be because this monosaccharide is preferentially metabolised following release from the cell wall, probably to provide energy to support various processes during ripening. Arabinose on the other hand is present in high proportion in the soluble polysaccharides (Fig. 28).

The presence of significant amounts of xylose and glucose in fractions 15-17 and 18-20 (Fig. 28b and 28c) may be an indication of some solubilisation of xyloglucan during ripening or of pectic polysaccharides with these sugars covalently attached (cf. model R36). However xylose was present in greater amounts in soluble polysaccharides at the unripe stage (see Fig. 27a and 27b).

2. DEAE Sephadex Fraction

The data obtained from the analysis of "Neutral" and acidic fractions of the mango polysaccharides separated by DEAE Sephadex is presented

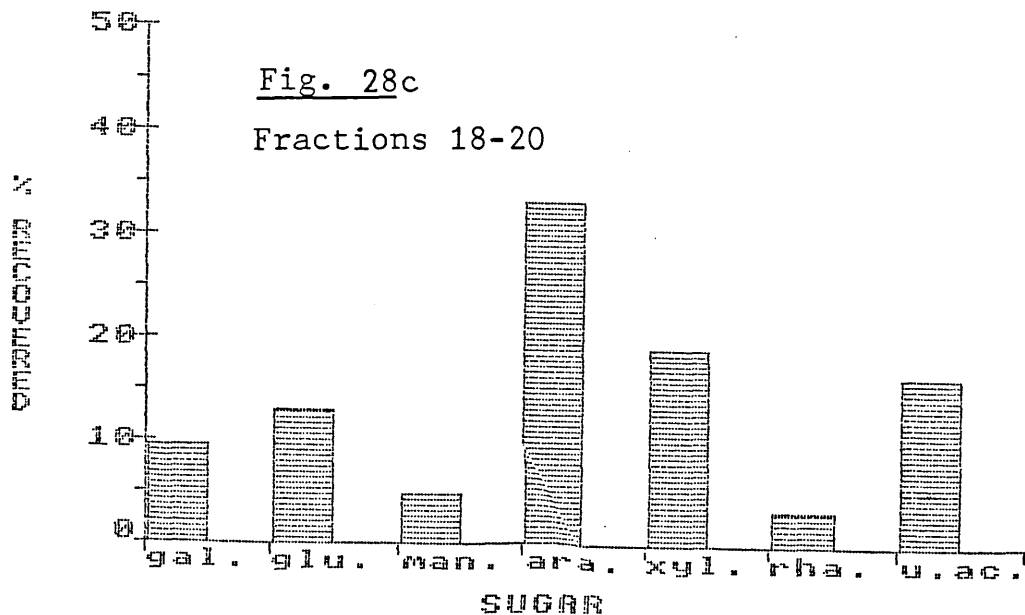
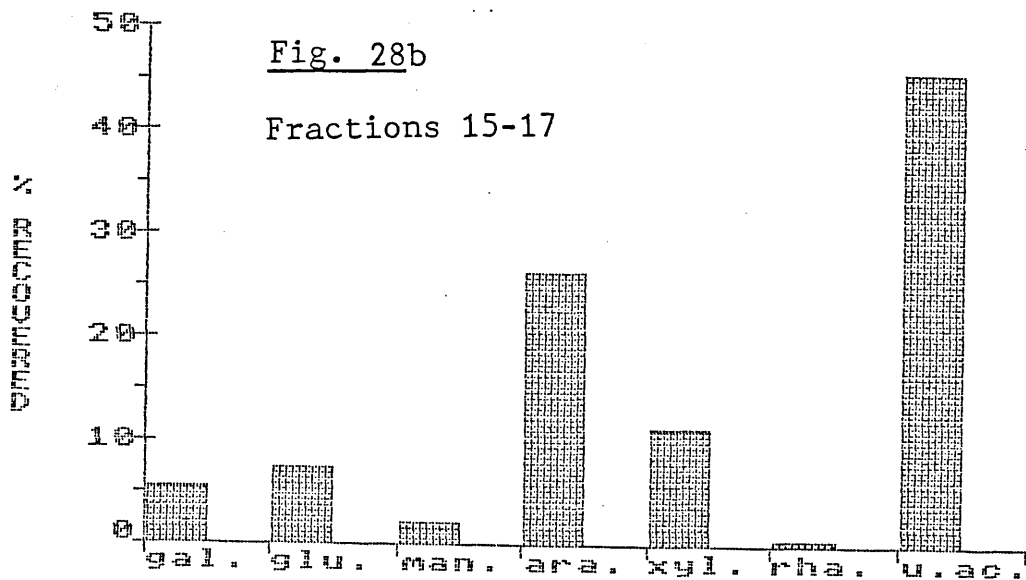
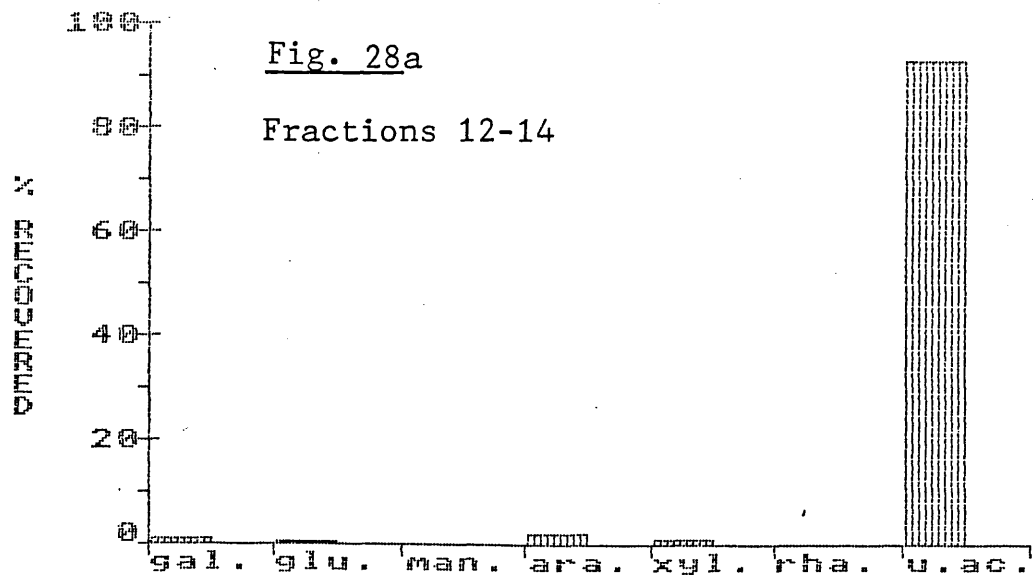


Fig. 28
Monosaccharide composition of polysaccharides present in pooled fractions 12-14 (Fig. 28a), 15-17 (Fig. 28b) and 18-20 (Fig. 28c) of a soluble ripe mango preparation after separation by Bio-Gel P-150 gel-filtration chromatography (see Fig. 22b). Experimental details are as described in Fig. 26.

RESULTS AND DISCUSSION

PART 2

in Fig. 29a and 29b. It is interesting to note that although the neutral fraction accounts for 50% of the soluble polysaccharides it has uronic acid content of only 4.7%. This may be an indication of the action of an endo-enzyme releasing fragments rich in neutral sugars from the cell wall, especially when considered in conjunction with previous data showing loss of neutral sugars compared to uronic acids from cell walls during ripening.

The Bio-Gel P-150 elution profile of the soluble polysaccharides (Fig. 22b ripe mango) has shown that the larger molecules have a greater uronic acid content. This implies that the "Neutral" fraction contains material of lower MW. There is some degree of similarity between the composition of the "Neutral" fractions obtained from the unripe and ripe mangoes, however, this fraction increases 20-fold in the ripe fruit. The acidic fractions however differ greatly in composition. The first acidic peak (fractions 15-35 Fig. 24) for example, from the ripe mango contain high amounts of uronic acids, 75.8% compared to only 12.6% for the equivalent peak from the unripe mango. It is surprising however that these two peaks of such varying uronic acid content should elute at similar positions on ion-exchange chromatography. It may be due to the presence of large amounts of esterified uronic acid residues in the ripe mango fraction.

III. Conclusion

High-MW polysaccharides are removed from the cell wall of the mango during ripening. Endo-enzyme(s) exist in the soluble fraction of the unripe fruit which is (are) capable of hydrolysing high-MW

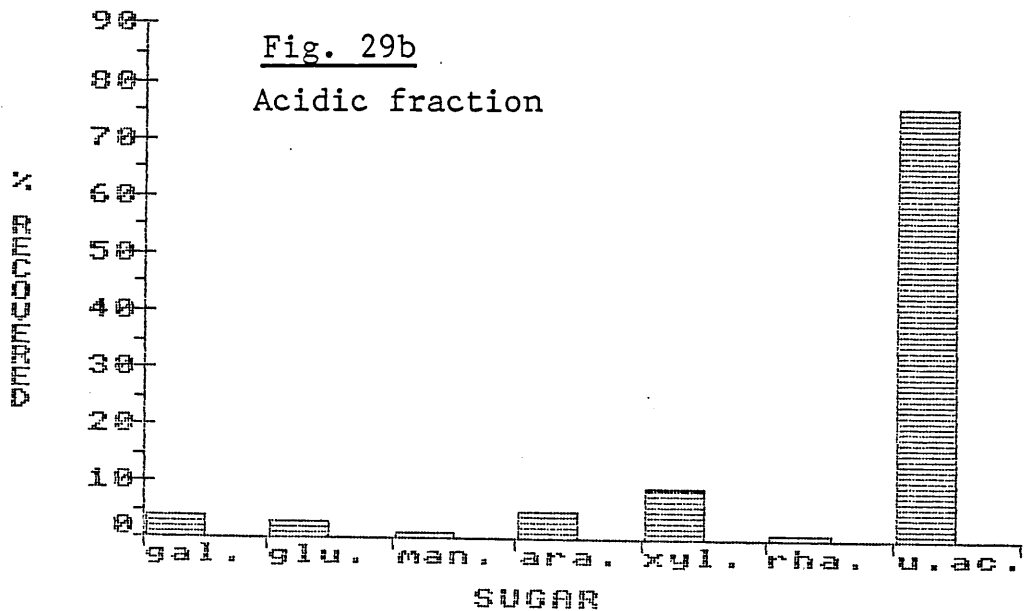
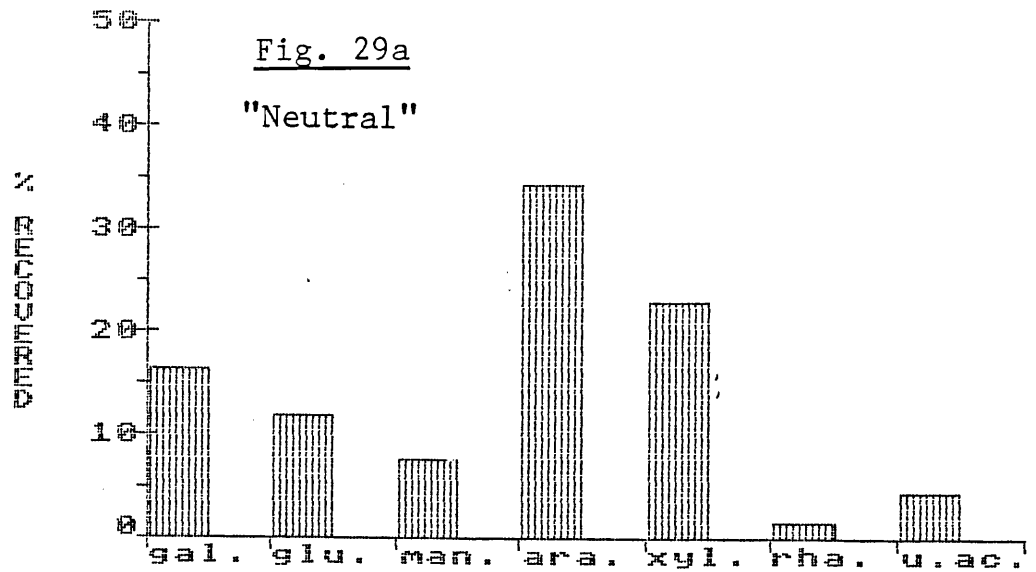


Fig. 29
Monosaccharide composition of polysaccharides present in pooled "Neutral" (Fig. 29a), and acidic fractions (15-35; Fig. 29b) of a soluble ripe mango preparation after separation by DEAE Sephadex ion-exchange chromatography (see Fig. 24b). Experimental details are as described in Fig. 26 except that acidic fractions were dialysed before freeze-drying.

polysaccharides. No oligosaccharides are present in the mango at either the ripe or unripe stages. These observations strongly suggest that cell-wall softening during ripening is brought about by endo-enzymes which solubilise large fragments.

PART 3

In Vitro Breakdown of Cell Walls

I. INTRODUCTION

Work thus far has indicated that polysaccharides essentially pectic in nature, are solubilized from the cell wall during ripening. Little is known about the enzymes responsible for bringing about this change although endo-enzyme involvement is necessary. Brinson (266) was unable to detect PG in the mango and could not implicate other carbohydrases with the above change. The purpose of work presented in this section was to identify and study enzymes that may be involved in cell-wall breakdown. The enzymes that were considered likely to be involved are endogalactanases and endoarabanases although these have not been identified in fruits. These enzymes could hydrolyse the arabinogalactan side chains of pectic molecules (see Fig. 1 and 11) thus causing solubilisation. Brinson (266) obtained a crude enzyme preparation from ripe mangoes that caused the release of free galactose from cell walls of the unripe fruit. However, he did not show whether large polysaccharide molecules were released as occurs during the ripening process. There was a high level of carbohydrate impurities present in the enzyme preparation he used, and these were difficult to remove. It was therefore difficult to study the composition of enzymically released material.

II. RESULTS AND DISCUSSION

A. Enzyme Preparation

1. Removal of Carbohydrates

A soluble enzyme fraction was obtained by homogenising ripe mango pulp in 50 mM acetate buffer pH 5.0 (1g/ml.) followed by centrifugation. The polysaccharides present in this fraction co-precipitated with proteins when treated with acetone or ammonium sulphate. Application of gel-filtration chromatography using Bio-Gel P-150 and Bio-Gel A.5m was also unsuccessful in separating protein from carbohydrate.

2. Isolation at High Salt Concentration

In view of the above difficulties and in consideration of the lack of available evidence to support the presence of endogalactanases and endoarabanases in the soluble fractions of the ripe mango, attention was shifted towards investigating the presence of these enzymes in cell-wall preparations. LiCl was used for isolating enzymes bound to cell-wall preparations from ripe mango. It was hoped that this would solubilise wall-degrading enzymes with reduced carbohydrate contamination. The possibility existed however, that relevant enzymes present in the soluble fraction would be discarded. Salt extraction has been used with success by Huber and Nevins (299) working with Zea seedlings and by Wallner and Walker (257) working with tomato fruit. The latter workers used 0.5 M citrate buffer at pH 4.6 to obtain extracts containing PG from the cell wall of ripe tomatoes. From such work it was demonstrated that this was the only enzyme present in the preparation which was responsible for degrading cell walls of unripe tomato with the release of polysaccharides and smaller amounts of neutral sugars. Huber and Nevins (299) found that 3 M LiCl enzyme preparations were able to cause extensive degradation of isolated cell walls. They also found that enzyme samples prepared using LiCl above 3

M were less effective in causing these changes. Further, the use of 3 M LiCl gave better extraction of enzyme activity than with 3 M sodium chloride. Although such preparations contained polysaccharide impurities, they were capable of releasing easily measurable quantities of carbohydrate from Zea cell walls.

The effect of LiCl concentration on the isolation of enzymic activity responsible for solubilizing carbohydrates from cell walls prepared from unripe mango is shown in Fig. 30. It is evident that greater amounts of activity were isolated at higher salt concentrations. The preparation obtained using 3 M LiCl caused release of 14.3 mg of carbohydrate per 100 mg cell walls in an incubation period of 48 hours. A control incubation using a boiled enzyme preparation caused very little increase in soluble carbohydrate (see Fig. 30). Huber and Nevins (299) reported the release of 10 % of carbohydrate from cell walls of Zea seedlings during a similar incubation period. The effect of LiCl concentration on the extractibility of β -galactosidase activity and protein was also examined. Fig. 31 and Fig. 32 show that both increased with higher salt concentrations. However, the specific activity of the enzyme decreased (Fig. 33) reflecting greater dissociation of other proteins from the cell walls with increasing salt concentration.

B. Properties

The enzyme preparation was quite stable and lost only 15-25% of activity (tested using mango cell walls) on storage for 2 weeks at 4 degrees C. The pH profile shown in Fig. 34 demonstrates highest

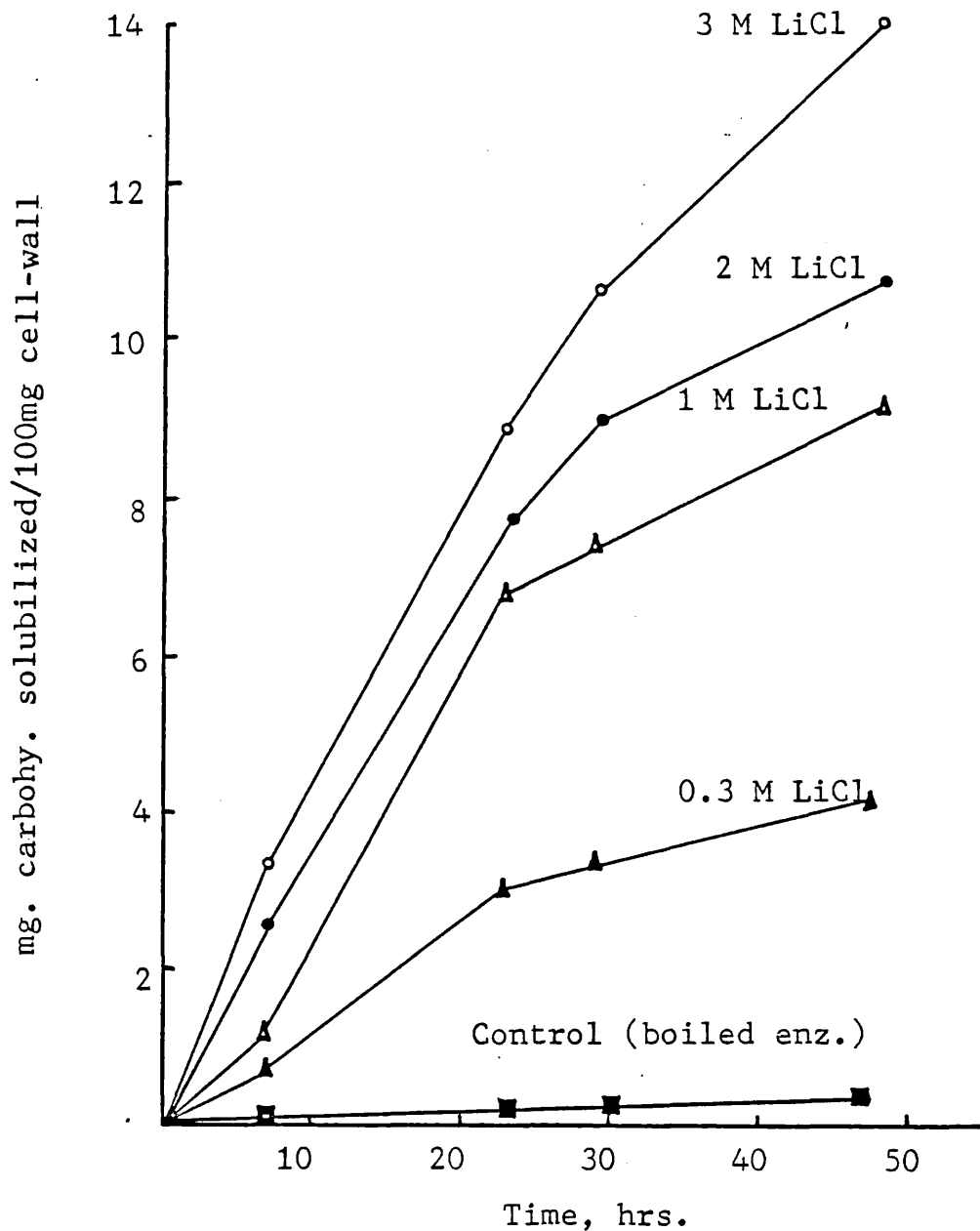


Fig. 30

Release of carbohydrate from unripe mango cell-wall substrate by crude LiCl enzyme extracts from ripe mango cell-wall preparations. Incubations were carried out in 50 mM acetate buffer, pH 5.0 at 30 degrees C for 48 hrs. Carbohydrate was determined by the phenol/sulphuric acid method (296).

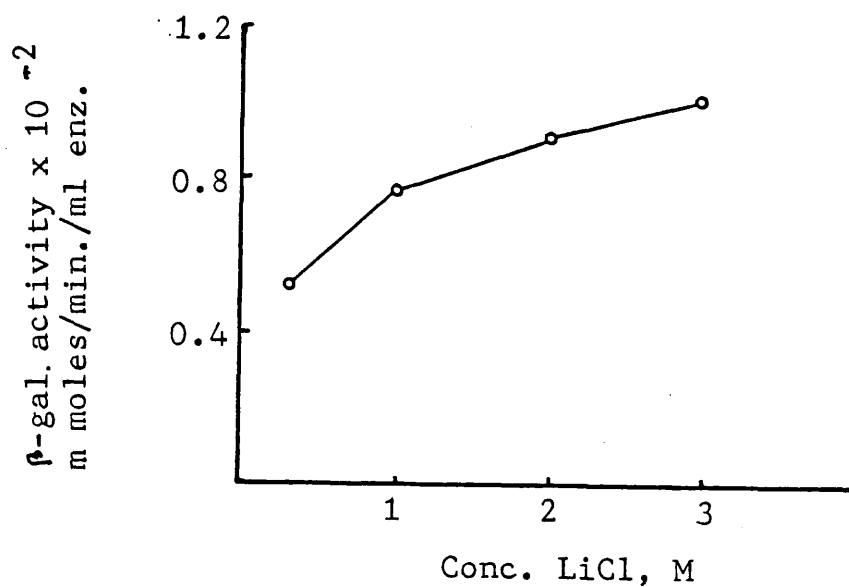


Fig. 31

β-Galactosidase activity (PNPG substrate) of crude LiCl enzyme extract from ripe mango cell-wall preparations. Aliquots of substrate (50 μl, 10mg/ml in water) were incubated with suitably diluted preparations and buffer (50 mM acetate, pH 5.0) to give final volumes of 0.43 ml. After 15-20 mins. reactions were terminated with 1 ml 0.3 M sodium carbonate solution and absorption values at 405 nm were determined.

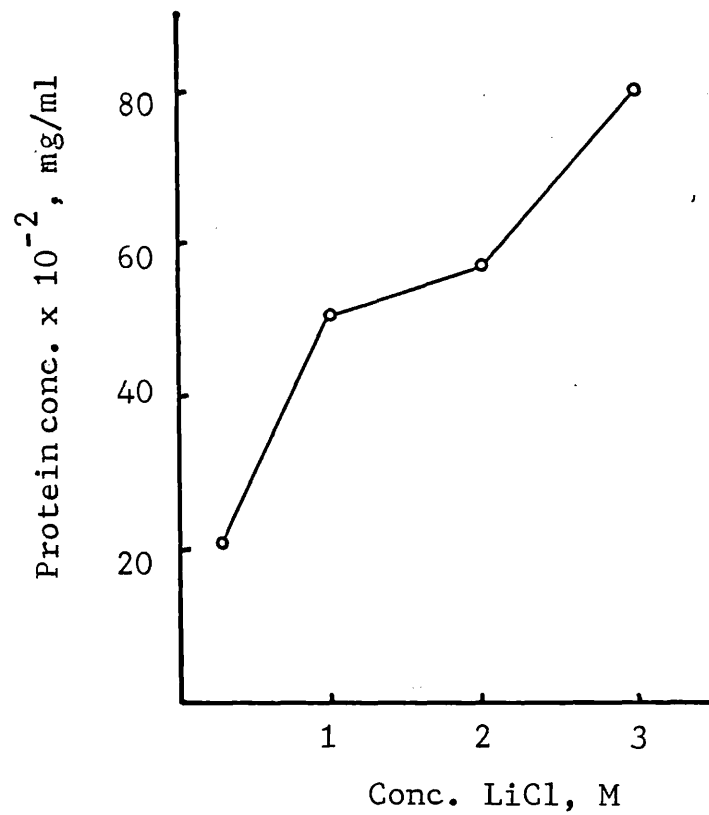


Fig. 32
Concentration of protein in crude LiCl enzyme extracts from unripe mango cell-wall preparations. Estimations were made using the Bradford method (300).

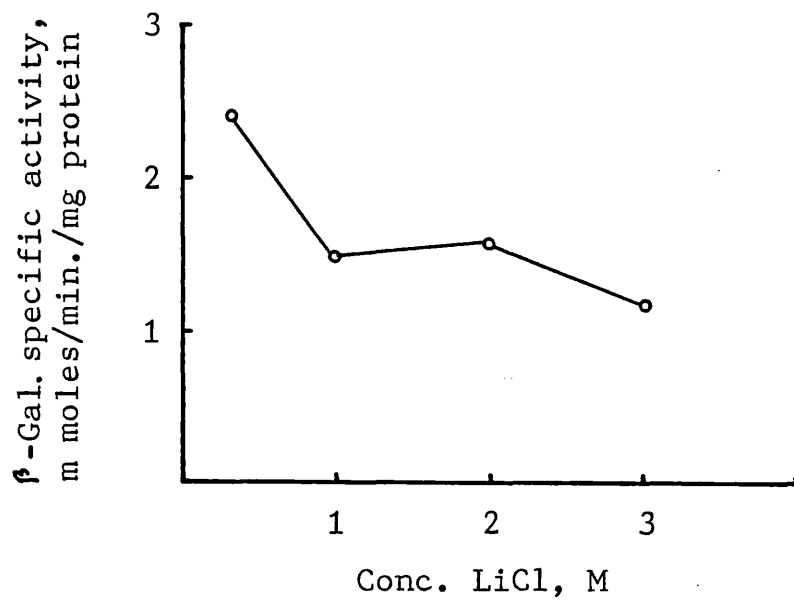


Fig. 33
Specific β -galactosidase activity (PNPG substrate) of crude LiCl enzyme extracts from ripe mango cell-wall preparation. Protein was determined by the Bradford method (300). Experimental details for the determination of β -galactosidase activity are as in Fig. 31..

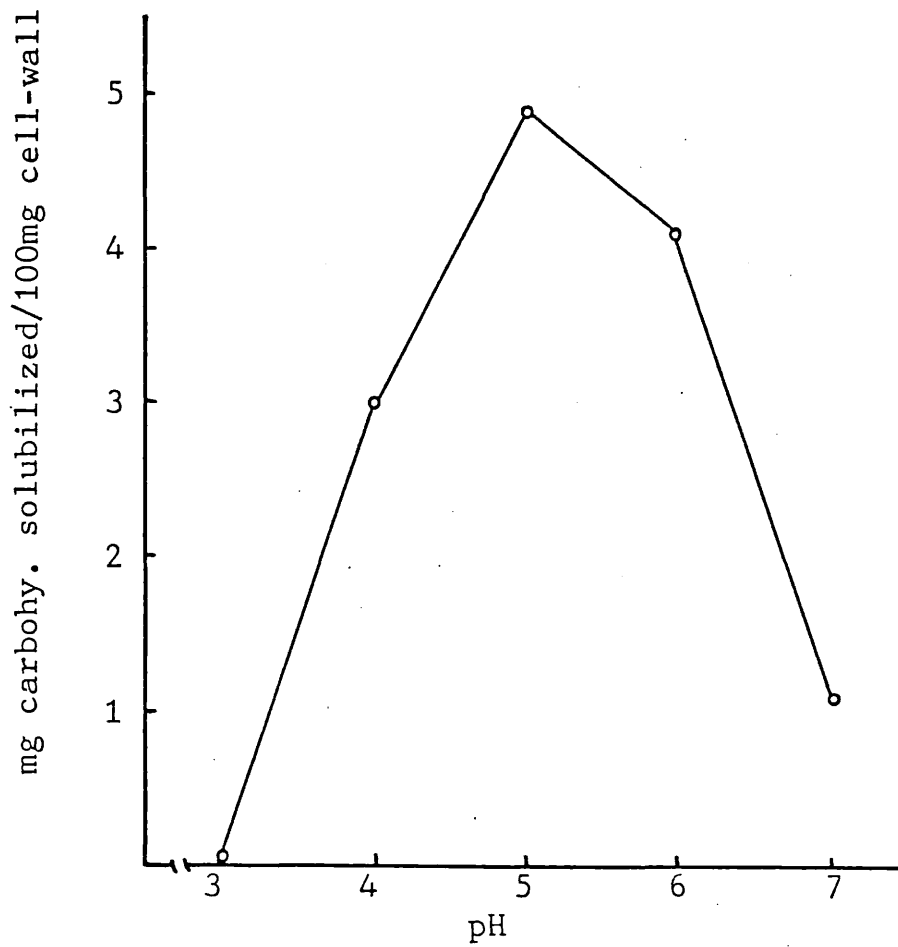


Fig. 34

Variation of the activity of crude 3 M LiCl enzyme extracts from ripe mango cell-wall preparations with pH (unripe mango cell-wall substrate). Experimental details are as outlined in Fig. 30.

activity at pH 5.0. There was no detectable activity at pH 3.0 whilst 22% of the maximal activity was noted at pH 7.0. The effect of calcium ions on the activity of the enzyme preparation was examined. The latter was dialysed against 10 mM EDTA followed by de-ionised water and then incubated with cell walls in a buffered medium. Table IV shows that solubilisation of cell-wall material was inhibited by 30.4% and 33.9% in the presence of 10 mM calcium nitrate and calcium chloride respectively. This is consistent with previous suggestions that calcium has a rigidifying effect on cell walls which in this case might have retarded hydrolysis by the enzyme preparation. However, direct inhibition by calcium cannot be excluded. In this context it is important to point out that the calcium content of mango cell walls decreases by 25% during ripening (Table II). Such loss may result in the enhancement of cell-wall softening.

Mercury chloride (10 mM) was found to completely inhibit solubilisation of unripe mango cell walls by the enzyme preparation.

C. Release of Carbohydrate from cell wall

1. Monosaccharides

Examination of solubilised carbohydrates by TLC showed that 23% of the material could be accounted for by monosaccharides. This liberation did not occur when boiled enzyme was incubated with cell walls or when the enzyme preparation was incubated by itself. Table V shows the liberation of galacturonic acid, galactose, glucose, arabinose and xylose. The most abundant monosaccharide was galacturonic acid; (1.22

Incubation	Solubilised carbohy. mg/100mg cell-wall
Enzyme + cell wall	6.72
Enzyme + cell wall + calcium nitrate	4.68
Enzyme + cell wall + calcium chloride	4.44

Table IV

Effect of calcium (10 mM) on the activity of the enzyme preparation at 30 degrees Centigrade for 40 hrs.. The enzyme preparation was previously dialysed against 10 mM EDTA and de-ionised water.

Monosaccharide	Amount released mg/100mg cell wall
Galactose	0.72
Glucose	0.56
Arabinose	0.35
Xylose	0.36
Mannose	0.00
Rhamnose	0.00
Galacturonic acid	1.22

Table V

Release of monosaccharides from unripe mango cell-wall substrate by the crude 3.0 M LiCl enzyme extract from ripe mango cell-wall preparation. Incubations were carried out as outlined in Fig. 30. Values were determined by TLC analysis by the method of Menzies and Mount (298).

mg/100 mg cell wall or 8.72% of solubilised material). The release of monosaccharide with time was examined. The results are presented in Fig. 35 which show that release occurred in a similar manner to that of total carbohydrate presented in Fig.30. The liberation of galactose with time was not quantitated, although in a separate experiment (see Table V) after 50 hrs. it was greater than that of glucose. These results indicate that glycosidases are present in the enzyme preparation which liberate the corresponding monosaccharides. Earlier work on enzyme preparations from ripe mango mesocarp demonstrated the presence of glycosidases when nitrophenyl glycosides were used as substrates (266).

No oligosaccharides could be detected by TLC analysis, or gel-filtration chromatography on Bio-Gel P-2 at any of the incubation times shown in Fig. 35. This indicates that the other 77% of the solubilised carbohydrates is made up of polysaccharides.

3. Polysaccharides

Polysaccharides released by the enzyme preparation could not be separated by gel-filtration (Bio-Gel P-150) from the carbohydrates already present in the enzyme preparation. The elution profiles of carbohydrates present at various times of incubation with the enzyme are shown in Fig 36. A small peak made up of monosaccharides was noticeable after 24 hours and became more pronounced with time. The soluble polysaccharide peak also became larger.

D. Changes in the Composition of Incubated Cell Walls

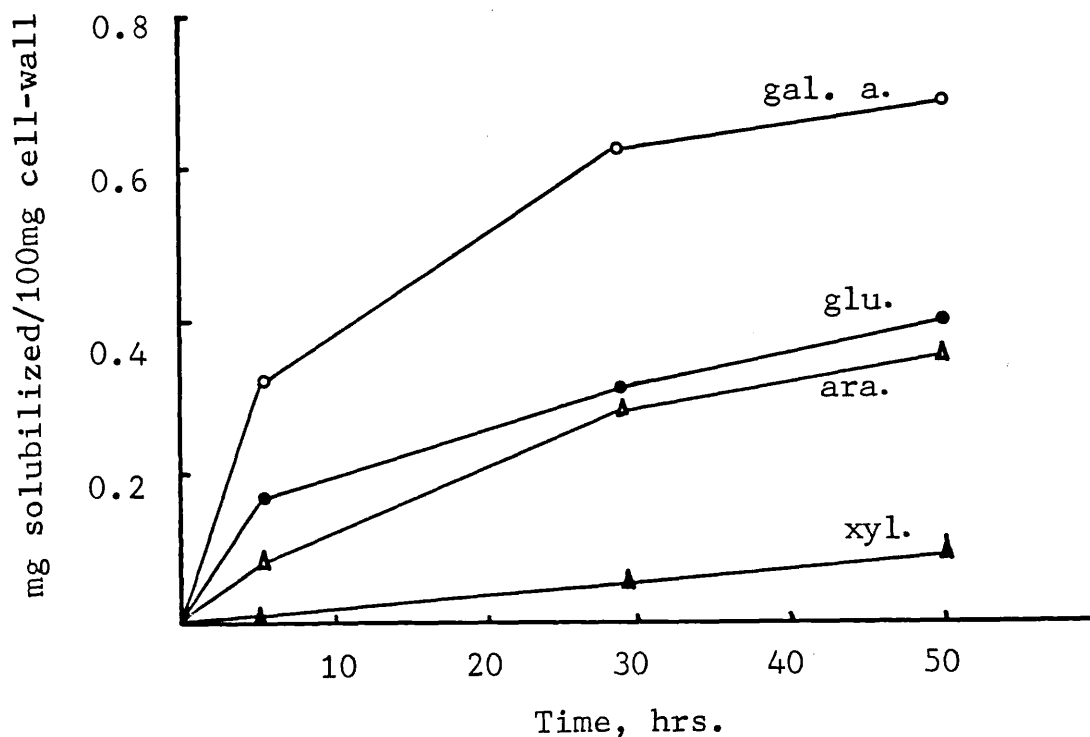


Fig. 35

Release of monosaccharides from unripe mango cell-wall substrate by crude 3 M LiCl enzyme extract from unripe mango cell-wall preparations. Incubations were carried out in 50 mM acetate buffer pH 5.0 at 30 degrees Centigrade. Monosaccharides were quantitated by TLC analysis by the method of Menzies and Mount (298) after removal of soluble polysaccharides by ethanol precipitation. The release of galactose was not quantitated.

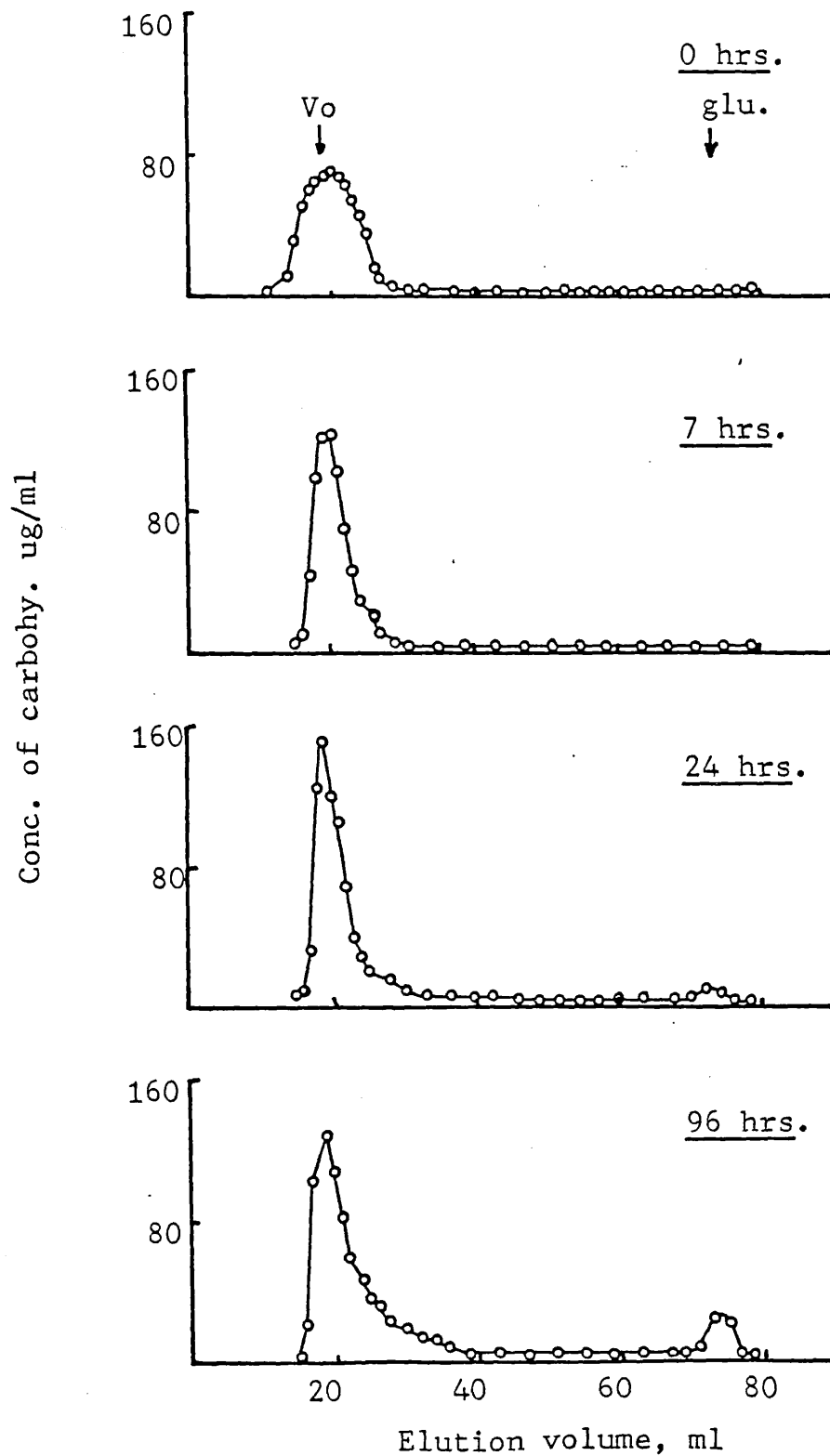


Fig. 36
Bio-Gel P-150 separation of soluble carbohydrate material during the incubation of the 3 M LiCl enzyme preparation with unripe mango cell walls.

The residual cell walls left after incubation with the enzyme preparation were assayed for uronic acid by a modified carbazole method. The values obtained are presented in Table VI which show that uronic acid content of cell walls that had been incubated with enzymes extracted with higher salt concentrations were smaller than those incubated with enzyme prepared with lower salt concentrations. There was a decrease of 35.1% in the uronic acid content of cell walls incubated with the 3M LiCl extracted enzyme. As free galacturonic acid accounted only for 8.7% of solubilized material, polysaccharides released must have contained high proportions of this sugar. Monosaccharide composition of the solubilised polysaccharides could not be determined because of carbohydrate contamination of the enzyme preparation. The results however suggest that pectin was solubilised during incubation. This is supported by data on acid hydrolysis of the residual cell wall presented in Table VII. There were reduced levels of some of the sugars characteristic of pectin (galactose and arabinose) after incubation. Furthermore, the difference was larger in the cell walls that underwent greater degrees of degradation. The percentage of xylose in the hydrolysate showed a small decrease which suggests that this sugar is also being removed from the cell wall during incubation. As there was no corresponding decrease in glucose, xyloglucans are probably not solubilised during incubation. Thus some xylose may constitute a part of the pectic fraction. In this regard, it is worth noting that significant amounts of xylose was found in soluble acidic polysaccharides of the unripe and ripe mango (Fig. 27 and 29).

E. Search For Endo-Acting Carbohydrases

LiCl conc., M	0.3	1.0	2.0	3.0	3.0*
Uronic acid content of residue {mg/100mg} cell wall	25.7	24.3	24.3	19.2	28.0

* Control using a boiled enzyme preparation

Table VI

Uronic acid content of unripe mango cell-wall residues after degradation by crude LiCl enzyme extracts from ripe mango cell-wall preparations (see Fig. 30). Values were determined by a modified version of the carbazole method (294).

Amount released by 2 M TFA mg/100mg cell wall					
Monosaccharide	LiCl conc. used for extraction, M				
	0.3	1.0	2.0	3.0	3.0*
Galactose	8.6	7.0	7.0	5.9	9.0
Glucose	13.0	14.8	14.2	14.9	13.6
Arabinose	13.2	11.4	11.4	10.3	15.1
Xylose	7.5	7.5	6.9	6.4	8.3
Rhamnose	1.9	1.8	1.2	1.2	2.1
Total	44.2	42.5	40.7	38.7	48.1

* Control using a boiled enzyme preparation

Table VII

Neutral sugars released by 2 M TFA hydrolyses (6 hrs. at 100 degrees C) of unripe mango cell-wall preparations (see Fig. 30). Values were determined by TLC analysis by the method of Menzies and Mount (298).

The ability of the enzyme preparation to bring about changes in the viscosity of various polymeric substrates (hot water extracts of unripe mango cell walls, 4 M alkali extracts of unripe mango cell walls, and citrus pectin) was examined. No changes in viscosity occurred as compared to control experiments with any of the substrates. This however, may have been due to the unsuitability of any of the substrates rather than the absence of endo-acting enzymes. Thus availability of suitable substrates is a limiting factor in carrying out further work on endo-enzymes.

III. CONCLUSION

The ripe mango cell walls are associated with enzymes (not including PG) that possess the capacity to degrade unripe mango cell walls. The changes brought about bear similarity to those that occur during natural ripening. The presence of the following exoglycosidases are suggested in the mango: β -D-galactosidases, α -L-arabinosidase, β -D-xylosidase and exopolygalacturonase. However it is not clear whether the roles of these enzymes if any, is to act on polysaccharides after they are released from the cell wall, or to act directly on the cell wall itself.

PART 4

Involvement of β -Galactosidase

I. INTRODUCTION

The release of both polysaccharides and monosaccharides from unripe mango cell wall by enzyme preparations extracted from ripe mango cell-walls using 3 M LiCl, has demonstrated the presence of both endo- and exo-acting enzymes in ripe mango cell-wall preparations. Whilst the action of endoglycanases can be envisaged as being necessary to cleave inter-polymer bonds to bring about solubilization of carbohydrate material, the requirement for exoglycosidases is not so apparent. Pressey (270) has identified 3 forms of β -galactosidase in tomato and has reported that 1 of these is capable of hydrolysing a β -1,4- galactan prepared from the cell-wall of the fruit. Bartley (269,301) and Knee and Bartley (271) have argued that in apple the enzyme may be responsible for catalysing the hydrolysis of a cell-wall galactan. In support of this contention, Bartley showed that β -galactosidase was capable of attacking a potato β -1,4-galactan similar to that found in apple cell walls and of releasing galactose residues from apple cell-wall preparations.

Exoglycosidases can be envisaged as being involved in any of the following ways in fruit ripening:

(a) Direct action on cell walls before cleavage of polysaccharide molecules by endo-enzymes.

(b) Action on cell walls after cleavage by endo-enzymes.

I. INTRODUCTION

The release of both polysaccharides and monosaccharides from unripe mango cell wall by enzyme preparations extracted from ripe mango cell-walls using 3 M LiCl, has demonstrated the presence of both endo- and exo-acting enzymes in ripe mango cell-wall preparations. Whilst the action of endoglycanases can be envisaged as being necessary to cleave inter-polymer bonds to bring about solubilization of carbohydrate material, the requirement for exoglycosidases is not so apparent. Pressey (270) has identified 3 forms of β -galactosidase in tomato and has reported that 1 of these is capable of hydrolysing a β -1,4- galactan prepared from the cell-wall of the fruit. Bartley (269,301) and Knee and Bartley (271) have argued that in apple the enzyme may be responsible for catalysing the hydrolysis of a cell-wall galactan. In support of this contention, Bartley showed that β -galactosidase was capable of attacking a potato β -1,4-galactan similar to that found in apple cell walls and of releasing galactose residues from apple cell-wall preparations.

Exoglycosidases can be envisaged as being involved in any of the following ways in fruit ripening:

(a) Direct action on cell walls before cleavage of polysaccharide molecules by endo-enzymes.

(b) Action on cell walls after cleavage by endo-enzymes.

(c) Action on polysaccharide fragments after their release from cell-walls by endo-enzymes.

(d) By any combination of (a), (b) and (c).

If (c) is the only mode of exoglycosidase action, the enzymes involved may not be considered to be directly involved in cell-wall softening. It was therefore important to investigate the release of carbohydrate material from inactive cell-walls by the action of purified exoglycosidases.

The work presented in this section is of a study on the role played by β -galactosidase in cell-wall softening during ripening. This enzyme undergoes a 13-fold increase in activity (against PNPg substrate) during ripening in the mango (266).

II. RESULTS AND DISCUSSION

A. Levels of β -Galactosidase Activity

Crude 3 M LiCl enzyme extracts were obtained from unripe and ripe mango cell walls. These were assayed for β -galactosidase activity using PNPg as the substrate and the results expressed with respect to the amount of tissue used. Higher levels of activity were detected in the unripe (5.1 nmoles/min/g pulp) than the ripe fruit (3.9 nmoles/min/g pulp). These results are in striking contrast to the values obtained from buffer soluble fractions (50 mM acetate pH 5.0) of the tissue (ie.

excluding cell walls); activities obtained were 12.9 nm/min/g pulp of unripe fruit and 133.5 nm/min/g pulp of ripe fruit (Table VIII). This 10-fold increase in buffer soluble β -galactosidase activity during ripening is in agreement with a 13 fold increase reported by Brinson (266). The factors responsible for this are not clear, however they may involve any of the following:

- (a) Activation of existing enzymes.
- (b) Removal of inhibitors of the enzymes.
- (c) Solubilisation of enzymes from the cell-wall.
- (d) Synthesis of enzymes.

There appears to be a fall in cell-wall associated β -galactosidase activity at the ripe stage when results are expressed in terms of wet pulp weight, however the difference is negligible if results are expressed in terms of dry weight of cell-walls. As starch was not exhaustively removed from unripe mango cell-wall preparations, the value of 32.6 nm/min/g for β -galactosidase activity is likely to be lower than the true value. Further, not all of the enzyme activity present in cell-wall preparations may have been extracted, particularly from unripe mango. Results presented in Fig. 37 showing β -galactosidase activity remaining in ripe mango cell-wall preparations after extraction with LiCl support this suggestion. Similar determinations for unripe mango cell-walls were not made.

Table VIII

Levels of β -galactosidase activity using PNPG substrate in buffer soluble (50 mM sodium acetate pH 5.0) and 3 M LiCl enzyme extracts from cell-wall preparations of unripe and ripe mango.

Activity, n moles/min/g wet wt. pulp		
Fraction	Unripe mango	Ripe mango
Buffer fraction	12.9	133.5
3 M LiCl fraction	5.1 (32.6)*	3.9 (35.8)*

* Values in brackets show activity expressed as n moles/min/g dry wt. cell-wall preparation.

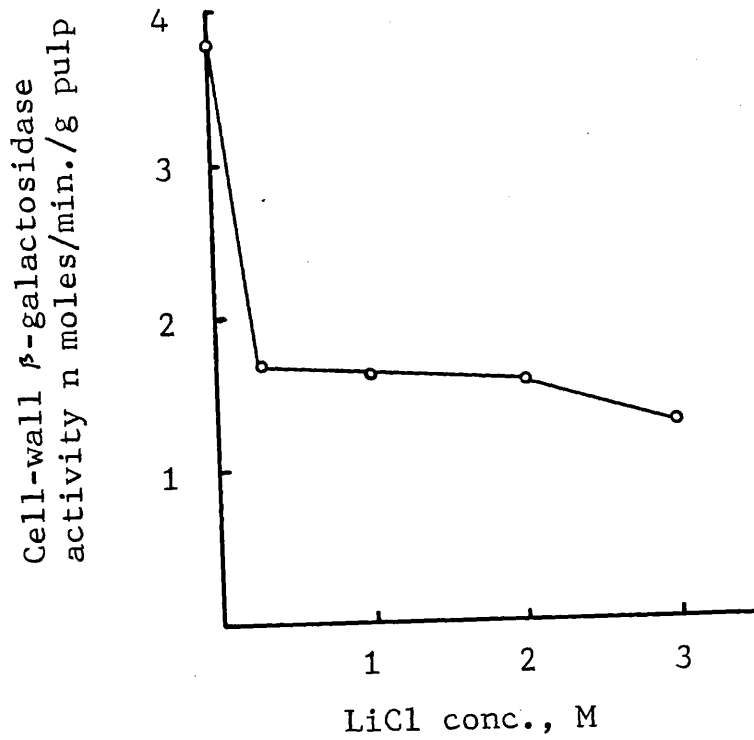


Fig. 37
 β -galactosidase activity of ripe mango cell-wall preparations (against PNPG) after extraction with 3 M LiCl (see Fig. 31). Samples were incubated with substrate in 0.1 M acetate buffer (0.3 g wet weight/ml) at 30 degrees C for 30 mins. After centrifugation, 0.2 ml of supernatants were removed and added to 1.0 ml of 0.1 M carbonate; absorption values at 410 nm were determined.

B. Purification of β -galactosidase

1. Precipitation

Attempts at precipitation of β -galactosidase activity from LiCl extracts of cell-wall preparations and buffer soluble fractions by pH variation with 1 M citric acid within the range of pH 5.0-3.0 were unsuccessful; no precipitate was formed and below pH 3.0 activity was irreversibly lost. Precipitation by adding ammonium sulphate resulted in co-precipitation of carbohydrate component. Similar results were obtained using ethanol except that there was also sharp inactivation of β -galactosidase.

2. Ion-Exchange Chromatography

Both β -Galactosidase and carbohydrate components present in the LiCl extract of cell-walls and soluble β -galactosidase preparations of tissue did not bind to DEAE Sephadex equilibrated with buffer in the pH range 5-9. However, a high proportion of the enzyme attached to Bio-Rex 70, a weak cationic exchange gel at pH 4.4. At this pH carbohydrates present in the enzyme preparations did not bind to the column and were completely removed by washing with equilibrating buffer (50 mM acetate, pH 4.4). Results presented in Table IX show that 60% of the total activity applied in the case of the unripe mango stayed as compared to 84 % of that of the ripe fruit. No greater binding was achieved for either of these enzyme preparations by lowering the pH of the equilibrating buffer.

Table IX

Binding and recovery values of β -galactosidase activity present in 3 M LiCl enzyme extracts from cell-wall preparations of the unripe and ripe mango after Bio-Rex 70 ion-exchange chromatography. Total number of units eluted by pH gradient was calculated by adding up the activity of each fraction.

Fruit sample	Units *	Bound units	Units eluted	Recovery **
Unripe	978	590	445	85%
Ripe	916	767	493	70%

* One unit of β -galactosidase activity is the amount which hydrolyses 1 μ mole of PNPG in 1 min.

** Recovery = $\frac{\text{Total no. of units eluted} + \text{No. of units that passed through}}{\text{No. of units applied}} \times \frac{100}{1}$

The inability of all the β -galactosidase enzyme to bind may have been due to its presence in association with other polymers (most likely polysaccharides). In support of this, it was noticed that approximately half of the β -galactosidase present in unbound fractions (see Table IX) attached to a second column. The amount of protein applied to the column was well below its maximum capacity. However, the possibility that this activity was caused by other forms of the enzyme that do not bind to Bio-Rex 70 under the conditions of the experiment cannot be ruled out.

C. Multiple Forms of β -Galactosidase

1. Cell-Wall Bound Enzymes

Elution of β -galactosidase bound to Bio-Rex 70 was carried out by pH gradient (pH 4.5 to 5.5, see Fig. 38). Between these pH values, 75% of total column-bound activity in the case of the unripe mango and 64 % in the case of the ripe mango were recovered. No further elution of enzyme activity was obtained by raising the pH of the column to 7.0. Carbohydrate was not present in any of the fractions eluted. Three main peaks containing β -galactosidase activity were obtained from cell-wall solubilised preparations of the ripe fruit (RW1, RW2 and RW3 eluting at pH 5.16, 5.36 and 5.41 respectively) and 2 main peaks from the unripe fruit (UW1 and UW2) eluting at pH similar to RW1 and RW2 respectively, (see Fig. 38). The presence of multiple forms of β -galactosidase has been previously reported in tomato fruit (270). The role of β -galactosidase in fruits may be in bringing about effective hydrolysis of cell-wall galactans which contain appreciable

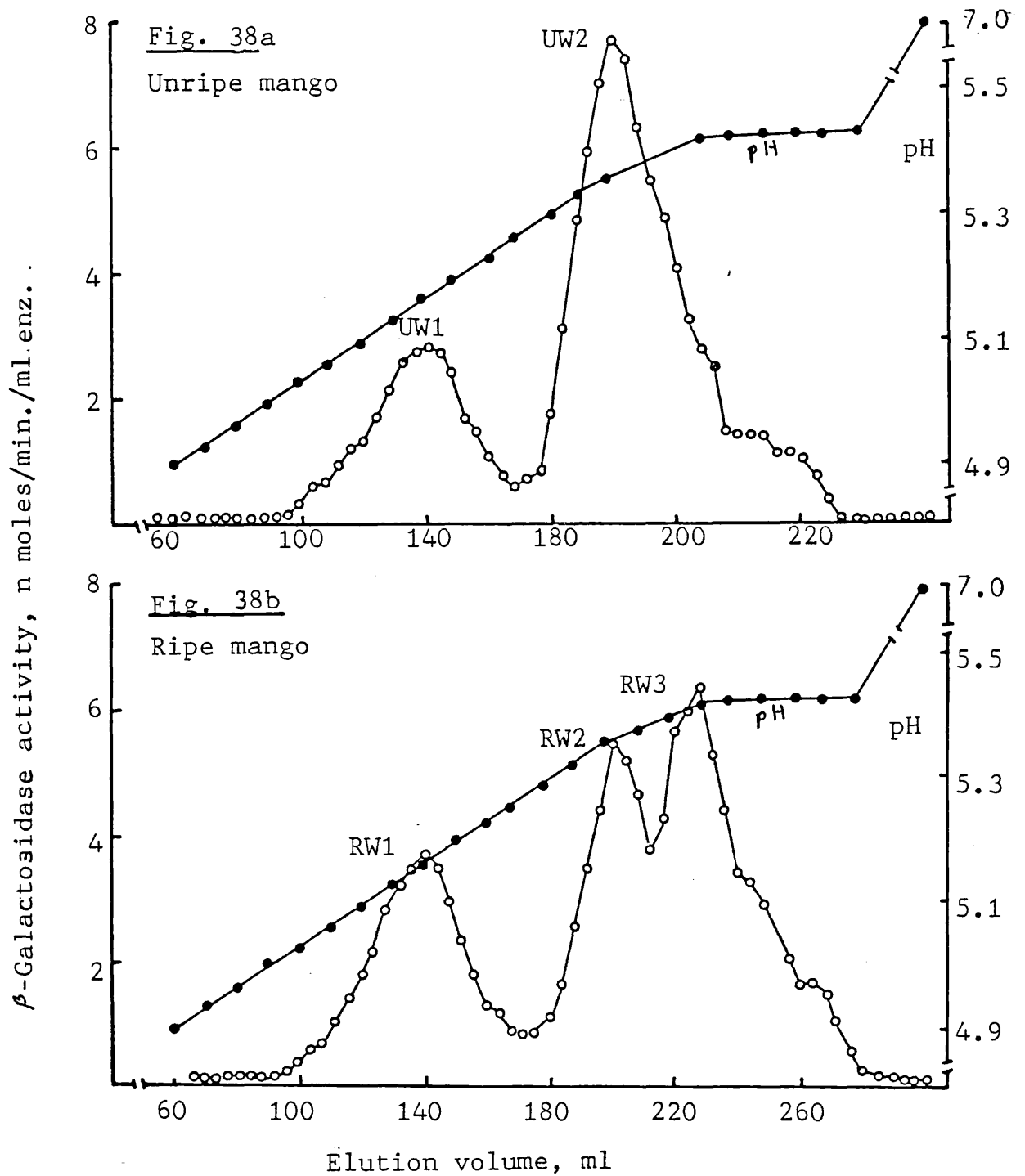


Fig. 38
Separation of forms of β -galactosidase present in 3 M LiCl enzyme extracts from unripe (Fig. 38a) and ripe (Fig. 38b) mango cell-wall preparations by Bio-Rex 70 ion-exchange chromatography. Details of pH gradient elution are given on p. 165.

quantities of 3-, 4-, 6-, 3,6- and 2,6-linked galactosyl residues (13,83).

Further purification of the resolved β -galactosidase peaks was attempted by using affinity chromatography. An affinity column was prepared by attaching a p-aminophenyl- β -D-thiogalactopyranoside ligand to ω -aminohexyl Sepharose using succinic anhydride and carbodiimide by the method of Cuatrecasas (302). A similar column has been successfully used by this author to purify bacterial β -galactosidase. However, no binding of any of the mango forms occurred.

Fractions constituting peaks shown in Fig. 38 were pooled and concentrated by dialysis against a solution of polyethylene glycol. Attempts at volume reduction by freeze-drying and ethanol precipitation gave poor recoveries. Concentrated fractions were subjected to gel-filtration chromatography on Sephacryl S-200 (Fig.39); elution profile showed 1 major peak for each sample. MW estimations from the elution volumes are presented in Table X.

Kinetic data (K_m and V_{max}) derived from Lineweaver-Burk plots of β -galactosidase activity against PNPG and ONPG substrates (Fig. 40 and 41) show that the properties of the 2 peaks of unripe mango (UW1, UW2) are similar to that of the ripe mango (RW1, RW2; see Table X). The molecular weights of the enzymes are also comparable. All the forms of β -galactosidase showed a preference for PNPG compared to ONPG which is apparent from the lower K_m and higher V_{max} values for the

Fig. 39

Sephacryl S-200 gel-filtration of β -galactosidases obtained after Bio-Rex 70 separation of 3 M LiCl enzyme extracts from mango cell-wall preparations (see Fig. 38). Two mls were applied to a column (2.4 x 90 cm) equilibrated with 100 mM acetate buffer, pH 5.0 containing 100 mM NaCl. Elution was carried out with the same buffer at a rate of 1.8 ml/min.. Fractions of 3 ml were collected and assayed for β -galactosidase activity using PNPG substrate.

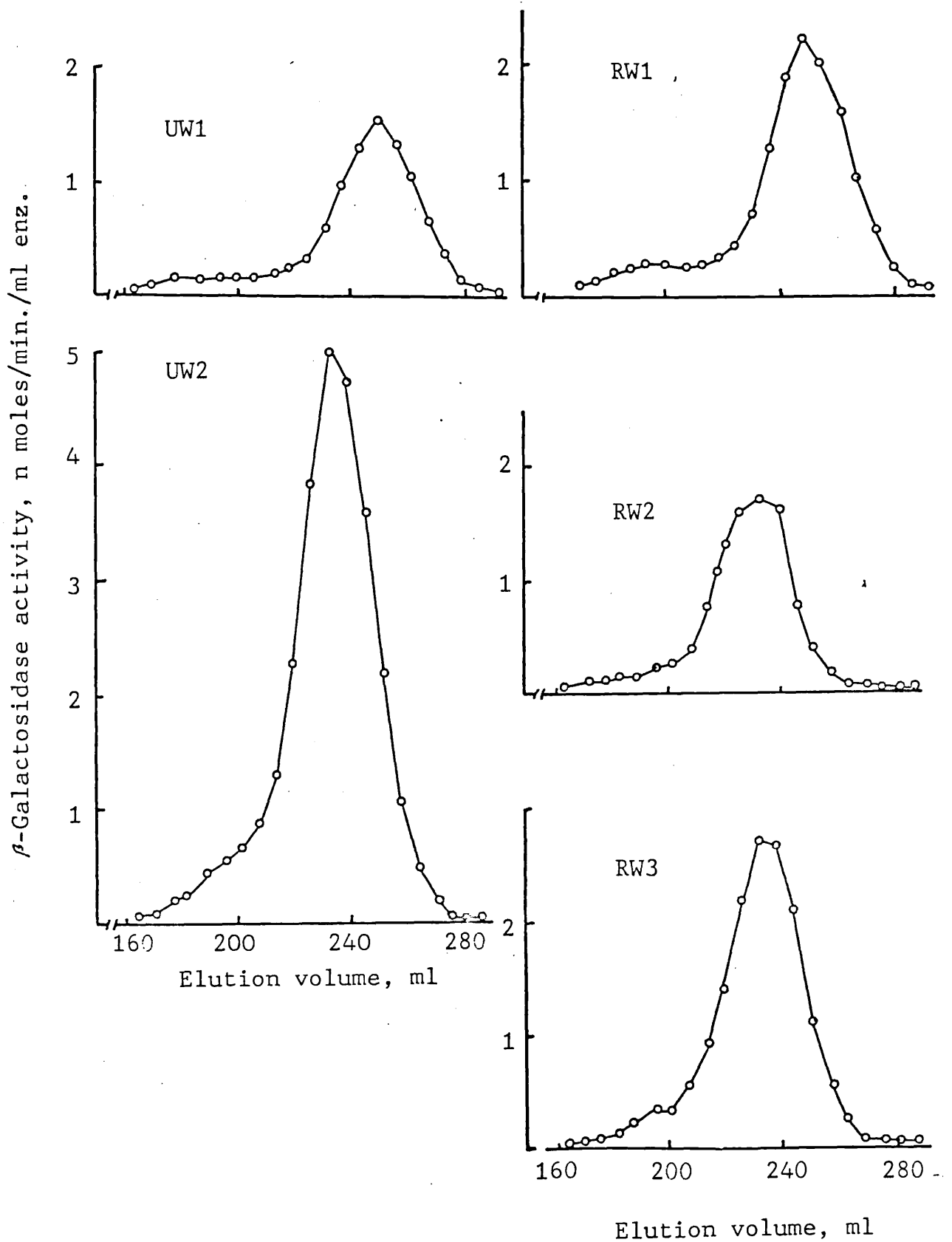


Table Xa

		<u>Unripe</u>					
Enz. form	pH*	PNPG**		ONPG**		MW***	pH optimum
		Km	Vmax	Km	Vmax		
UW1	5.16	1.35	0.37	4.55	0.14	79,000	4.3
UW2	5.36	0.94	0.57	1.18	0.13	108,000	4.5 & 6.2

Table Xb

		<u>Ripe</u>					
Enz. form	pH*	PNPG**		ONPG**		MW***	pH optimum
		Km	Vmax	Km	Vmax		
RW1	5.16	1.35	0.42	4.55	0.20	77,000	4.3
RW2	5.36	0.94	0.59	n/d		113,000	4.5 & 6.2
RW3	5.4	1.03	0.53	2.33	0.13	110,000	4.6 & 6.1

* Elution pH using a Bio-Rex 70 column

** Vmax = μ moles substrate hydrolysed/min./mg protein
Km = mM

*** Determined by using a Sephacryl S-200 column

Table X

Summary of data on forms of β -galactosidase present in 3 M LiCl enzyme extracts from unripe (Table Xa) and ripe (Table Xb) mango cell-wall preparations.

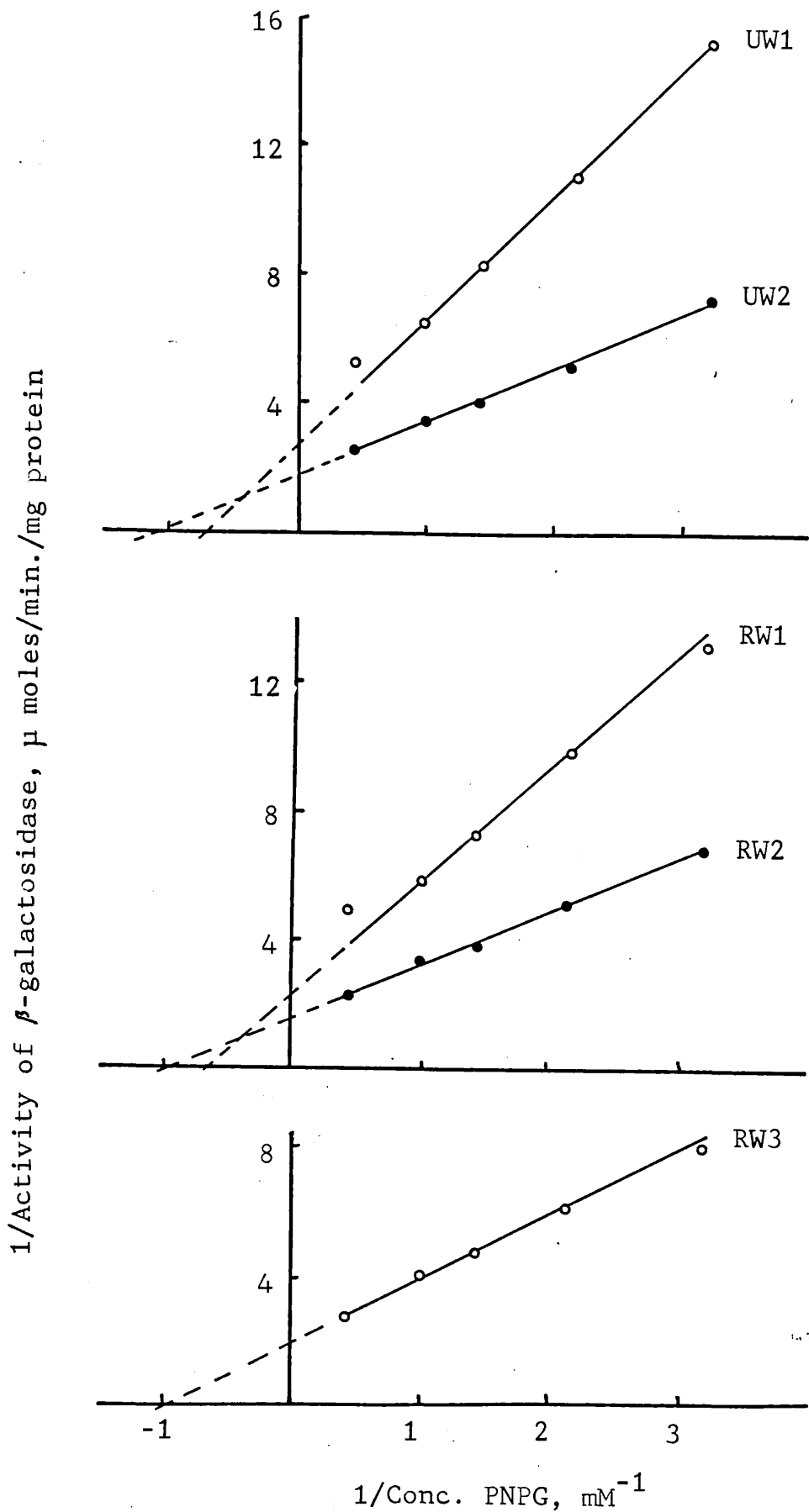
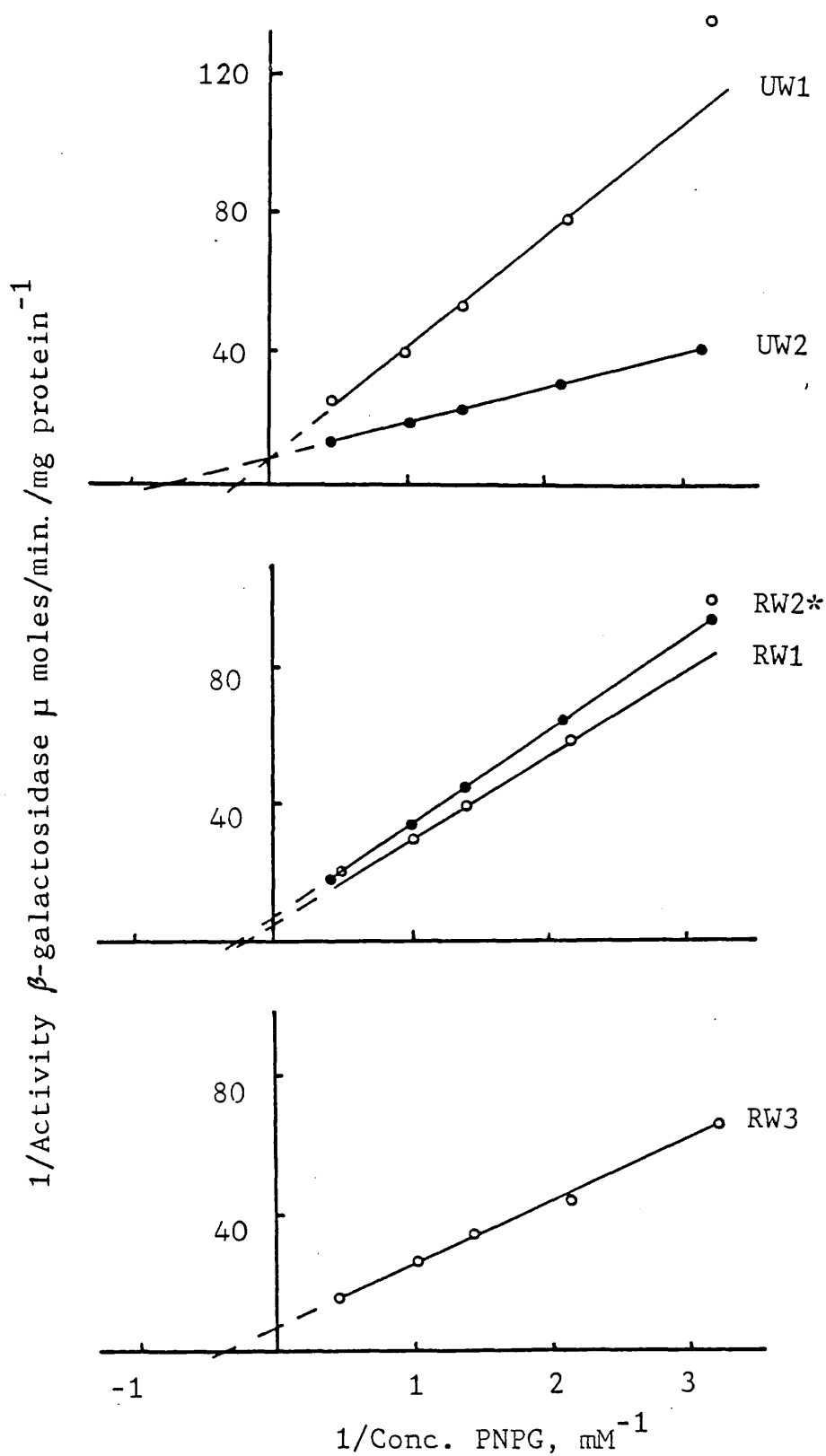


Fig. 40
 Lineweaver-Burk plots for forms of β -galactosidase (using PNPG substrate) present in 3 M LiCl enzyme extracts of cell-wall preparations from unripe and ripe mango after purification with Bio-Rex 70 (Fig. 38) and Sephacryl S-200 chromatography (Fig. 39).



* Values for RW2 have been multiplied by 10^{-1}

Fig. 41
Lineweaver-Burk plots for forms of β -galactosidase (using ONPG substrate) present in 3 M LiCl enzyme extracts of cell-wall preparations from unripe and ripe mango after purification by Bio-Rex 70 (Fig. 38) and Sephacryl S-200 gel-filtration chromatography (Fig. 39).

former. This is in contrast to *E. Coli* β -galactosidase which shows 7-fold more activity against ONPG than PNPG (303). K_m values for all the enzyme forms using PNPG substrate lie in the range 0.94-1.35 mM which is roughly twice as high as those reported for the β -galactosidase forms present in tomato (270). The K_m values for the mango enzymes using ONPG lie in the range 1.18-4.55 mM which is comparable to a value of 2.7 mM reported for sugar cane β -galactosidase (304).

Fig. 42 shows activity variation with pH for the 3 enzyme forms (RW1, RW2, RW3) using PNPG as substrate. RW1 has a single optimum at pH 4.3 which is near a value of 4.0 reported for apple β -galactosidase (269). RW1 and RW2 both show double optima, at pH 4.6 and 6.2. The 2 peaks of the unripe mango (UW1 and UW2) show similar pH profile to RW1 and RW2, respectively.

The presence of an extra peak (RW3) in ripe mango cell-wall enzyme preparation in much larger amounts than in unripe cell-walls is very striking. The following explanations may be given for this difference:

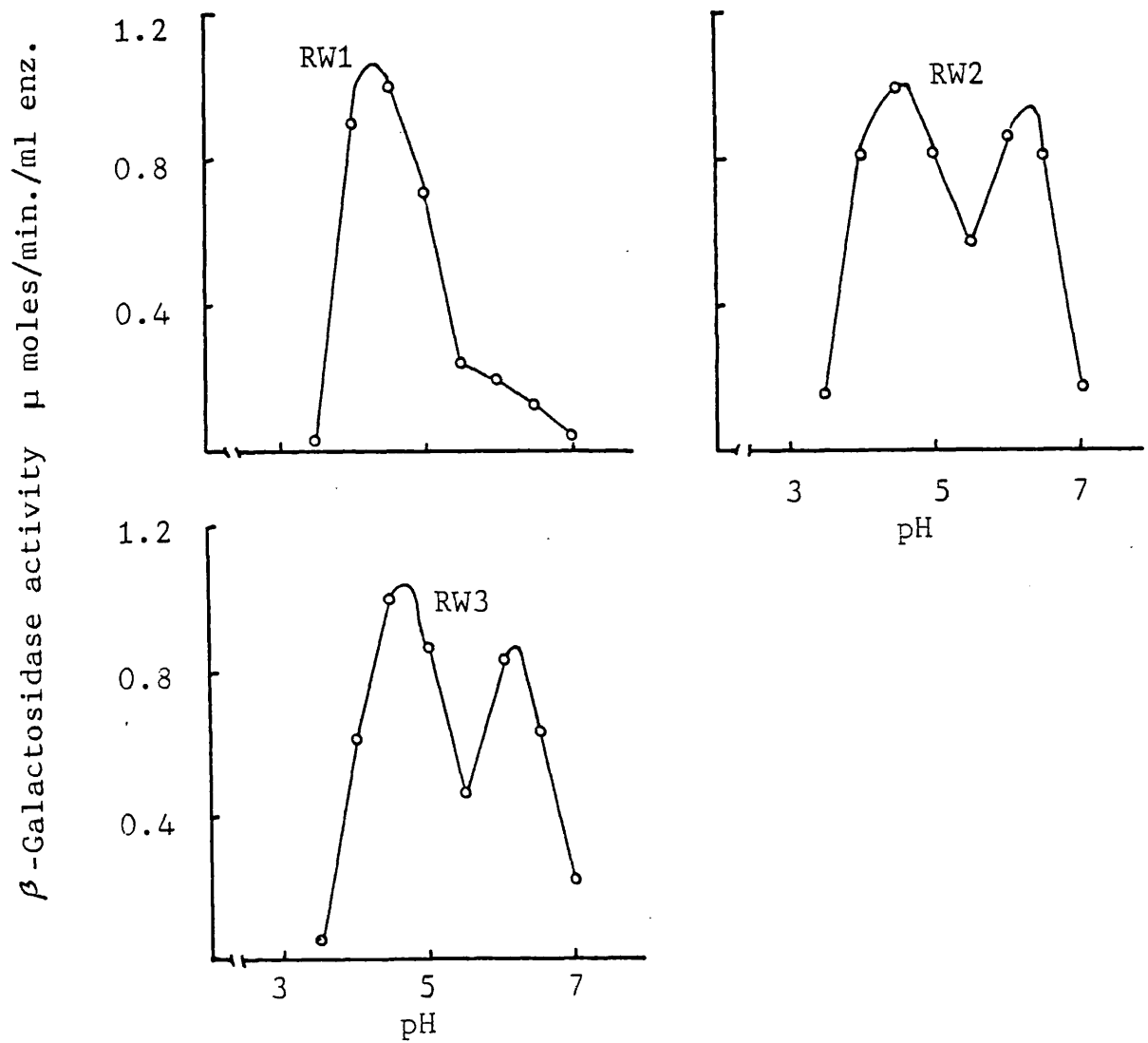
(a) Existence of smaller quantities of this form in unripe mango cell wall.

(b) Inability of 3 M LiCl to extract the enzyme from unripe mango cell wall.

(c) Presence of this form in an inactive state in the unripe mango cell wall.

Fig. 42

Activity /pH profiles of forms of β -galactosidase (using PNPG substrate present in 3 M LiCl enzyme extracts of cell-wall preparations from ripe mango after purification with Bio-Rex 70 (Fig. 38b) and Sephacryl S-200 chromatography, see Fig. 39.



If (a) is true, then the enzyme has to be inserted into the cell-wall at some stage during the ripening process.

2. Soluble Fraction

Table XI shows that 87% and 88% of soluble (50 mM acetate buffer pH 5.0) β -galactosidase activity from unripe and ripe mango tissue, respectively were bound to a Bio-Rex 70 column. The enzyme activities were eluted by pH gradient and total recoveries of 51% and 86% were achieved for the unripe and ripe preparations, respectively. The reason for the low recovery for the unripe sample is not clear. However it could have been caused by deactivation of one specific form of the enzyme which consequently did not appear in eluted fractions, or by a more distributed loss of activity of forms which were observed to bind to the column (Fig. 43). Four peaks of activity (RS1; at pH 4.87, RS2; at pH 5.09, RS3 at pH 5.23, RS4; at pH 5.40) were obtained from ripe samples and 3 from unripe (US1; at pH 5.09, US2; at pH 5.18, US3 at pH 5.40) as shown in Fig.43. Most of the activity from the ripe fruit is accounted for by RS1 which is absent from the elution profile of the unripe mango preparation. Sephacryl S-200 gel-filtration resolved both RS2 and RS3 into 2 peaks, RS2a, RS2b and RS3a and RS3b, respectively. RS1 and RS4 gave single peaks (Fig.44). On the other hand, all the peaks (US1, US2, US3) from unripe mango preparation gave single peaks on gel-filtration (Fig. 45). The molecular weights of the enzyme forms from both ripe and unripe mango preparations were determined by calibrating the gel-filtration column. These results together with the kinetic properties of the enzyme forms present in soluble fraction are presented in Table XII.

Fruit sample	Units* applied	Bound units	Units eluted	Recovery**
Unripe	757	655	283	52%
Ripe	1,815	592	1,326	85%

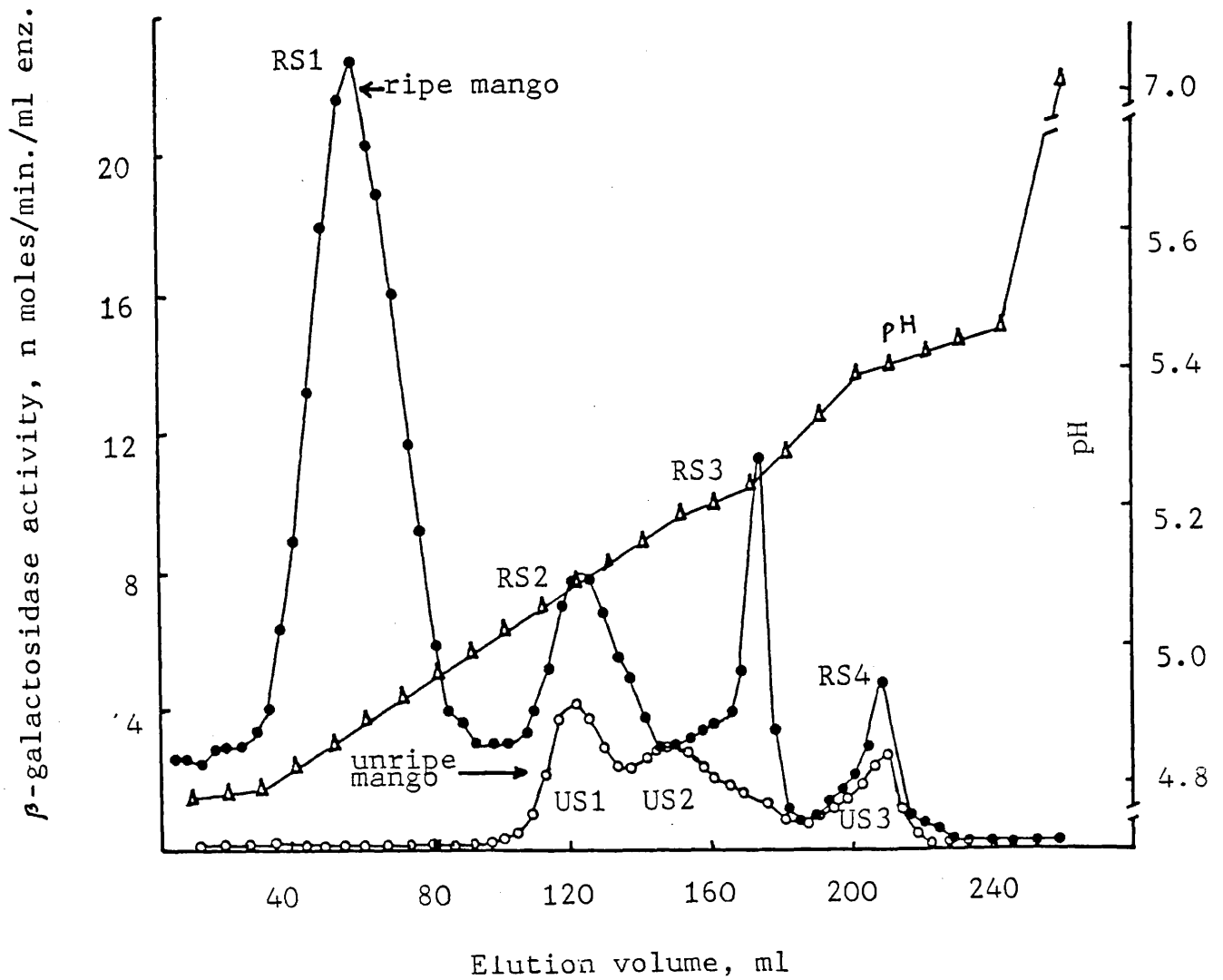
* One unit of β -galactosidase activity is the amount which hydrolyses 1 μ mole of PNPG in 1 min.

$$** \text{ Recovery} = \frac{\text{Total no. of units eluted} + \text{No. of unbound units}}{\text{No. of units applied}} \times \frac{100}{1}$$

Table XI

Binding and recovery values of β -galactosidase activity present in soluble fractions (50 mM acetate buffer) of the unripe and ripe mango tissues after Bio-Rex 70 ion-exchange chromatography. Total number of units eluted by pH gradient was calculated by adding up the activity of each fraction. Details of experimental procedures are given on P. 165.

Fig. 43
 Separation of β -galactosidases present in soluble fractions (50 mM acetate buffer pH 5.0) of unripe \circ - \circ and ripe \bullet - \bullet mango tissue by Bio-Rex 70 ion-exchange chromatography. Details of pH gradient elution are given on p. 165.



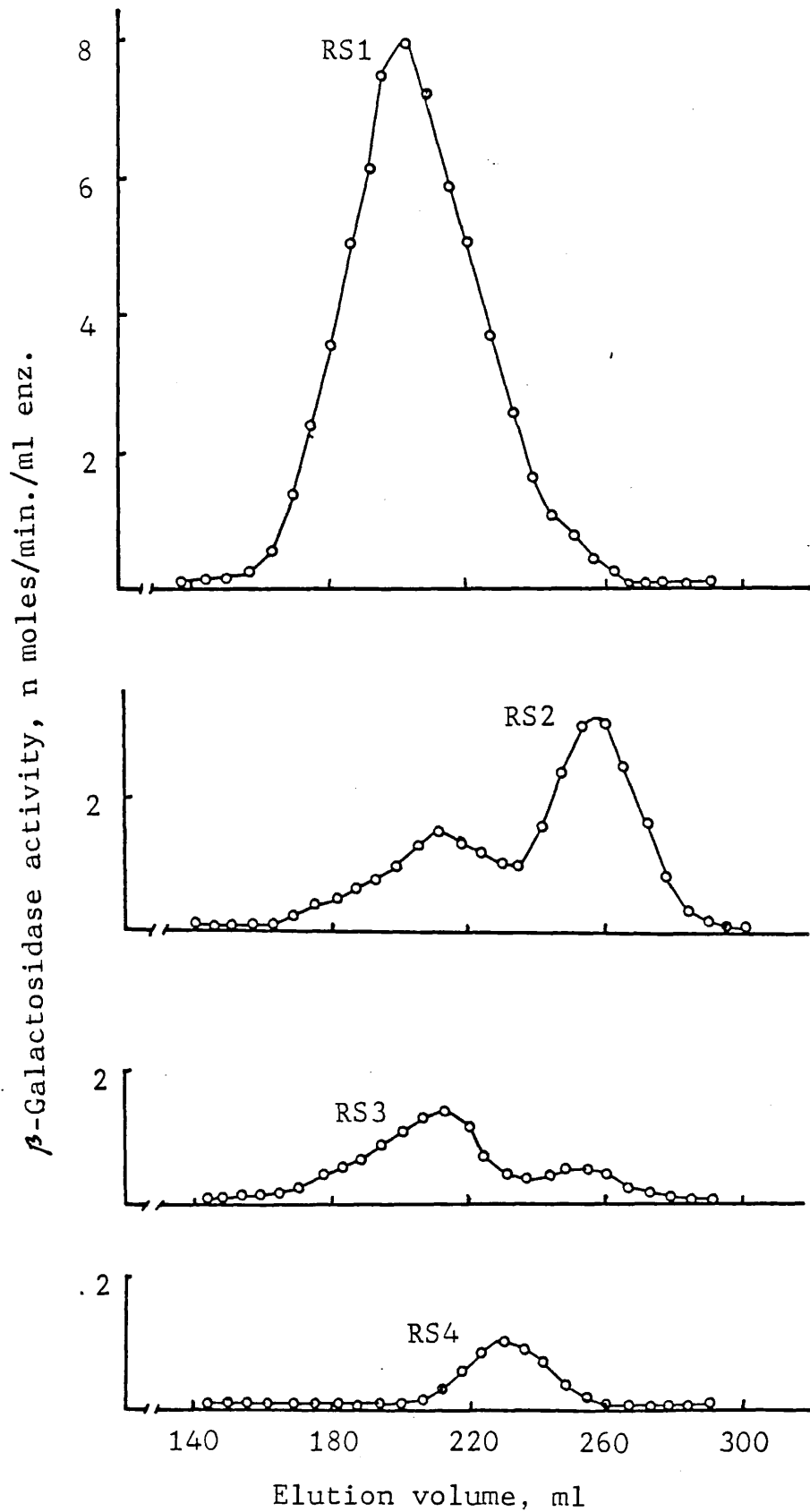


Fig. 44
Sephacryl S-200 gel-filtration of β -galactosidases obtained after Bio-Rex 70 separation of soluble fractions from ripe mango tissue (Fig. 43). Experimental details are as presented in Fig. 39.

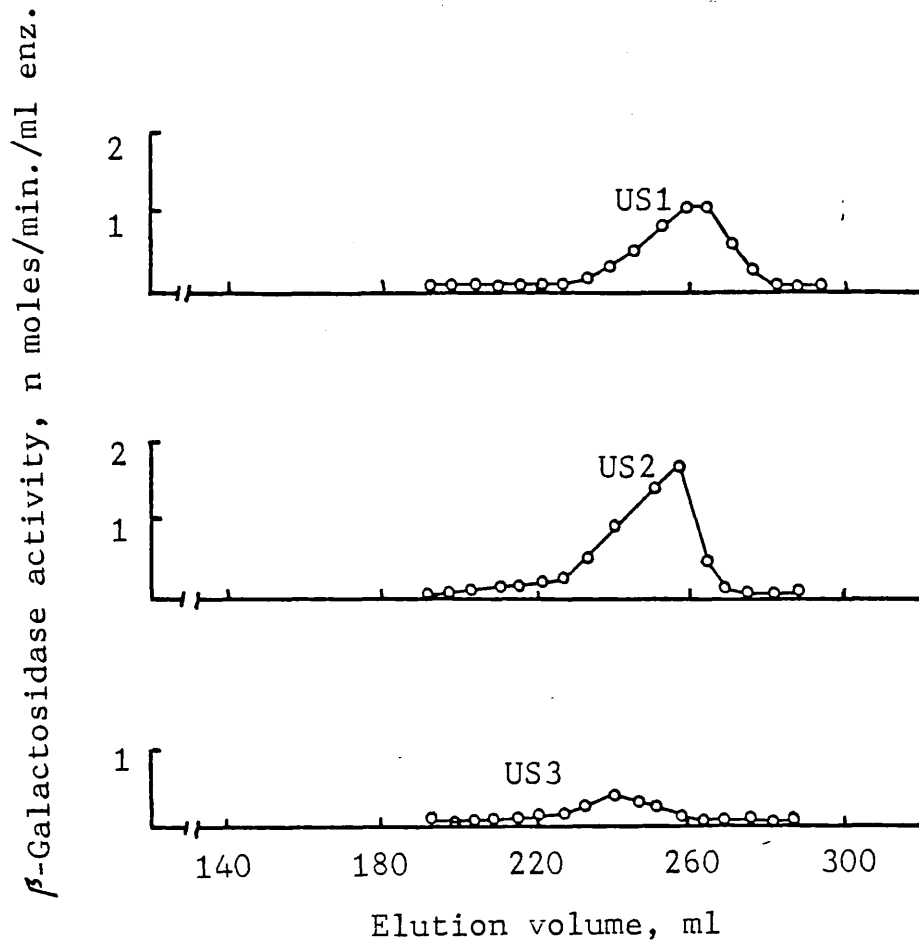


Fig. 45
Sephacryl S-200 gel-filtration of β -galactosidases obtained after Bio-Rex 70 separation of soluble fractions from unripe mango tissue (Fig. 43). Experimental details are as presented in Fig. 39.

Table XIIa		Unripe					
Enz. form	pH*	PNPG**		ONPG**		MW***	pH optimum
		Km	Vmax	Km	Vmax		
US1	5.09	0.85	0.17	1.02	0.13	60,000	4.3
US2	5.18	7.14	1.33	2.94	0.31	64,000	4.3
US3	5.4	n/d		n/d		94,000	4.3

Table XIIb		Ripe					
Enz. form	pH*	PNPG**		ONPG**		MW***	pH optimum
		Km	Vmax	Km	Vmax		
RS1	4.87	0.31	5.37	1.26	2.37	275,000	4.3
RS2a	5.09	0.64	2.68	0.67	0.39	229,000	4.3
RS2b	5.09	0.93	5.12	1.17	0.85	62,000	4.3
RS3a	5.23	0.58	1.95	0.37	0.19	229,000	4.3
RS3b	5.23	3.14	1.22	0.44	0.10	72,000	4.3
RS4	5.4	n/d		n/d		n/d	n/d

* Elution pH using a Bio-Rex 70 column

** Vmax = μ moles substrate hydrolysed/min./mg protein
Km = mM

*** Determined by using a Sephacryl S-200 column

Table XII

Summary of data on forms of β -galactosidase present in 3 M LiCl enzyme extracts from unripe (Table XIIa) and ripe (Table XIIb) mango soluble preparations.

All forms displayed single optima at pH 4.3 (cf. Table X). It is interesting to note that the molecular weight and K_m of RS2b are similar to those of the single MW form of US1 (Fig. 45 and Table XII). It seems that none of the enzymes solubilised with 3 M LiCl from the cell-wall preparations are present in appreciable amounts in the soluble preparations (compare Tables X and XII). Contrary to these findings, Pressey (282) found that tomato β -galactosidases extractable with 1.0 M NaCl were similar to those of water extractable enzymes. The possibility exists that some of the forms of β -galactosidase observed in this investigation may be artifacts of isolation and preparation. Causes of this are not clear and may not be easily determined (cf. α -galactosidase from Vicia fabia, 305). Probable factors are interactions of mono- or oligomeric forms of enzymes (association/dissociation), modification of carbohydrate regions of proteins by carbohydrases, interactions with phenolic compounds and modifications by proteases.

D. Action Against Natural Substrates.

1. β -Galactosidase From Cell-Wall Preparations

The ability of the forms of β -galactosidase isolated from cell-wall preparations to degrade cell-wall and pectic materials isolated from unripe mango tissue was investigated. RW1 and RW3 did not release measurable amounts of carbohydrate from cell-wall samples at pH 5.0. However RW2 did solubilize small quantities (0.5mg/100mg cell-wall in 36 hrs.)¹. The activity of RW2 was not enhanced in the presence of RW1 or RW3. These observations support the contention that β -galactosidases

may not act on cell-walls prior to the action of endoglycanases. All forms of the enzyme, released reducing sugars when incubated with hot water extracted pectin from unripe mango cell-walls. This is apparent from Fig. 46 which shows an increase in reducing sugar with time during incubation. Attempts at identifying released product by TLC analysis were hindered by an unknown compound (arrow in Fig. 47) present in the pectin substrate (it appears to increase during incubation) which caused a streaking effect on TLC plates (see Fig. 46). However, there was a band in the position of the plate where galactose would be found, the intensity of which also appeared to increase with incubation time. Various attempts including use of a mixed deioniser Zerolitz at removing the streak were unsuccessful. Galactose release was therefore not firmly established.

Galacturonic acid was released from unripe mango cell-wall pectin by RW2 during incubation. This was established by TLC analysis of the incubation mixtures (see Fig. 47). No galacturonic acid band was detected when the reaction mixture was treated with a mixed ion-exchange resin prior to TLC analysis. These observations suggest the presence of exopolygalacturonase activity in the enzyme preparation. It was not however investigated whether this activity and β -galactosidase activity were displayed by the same protein. Although exo-polygalacturonase has been found in several fruits (250,252,253,265,) its presence in mango has not been previously shown. This enzyme was not found in the soluble extract (50 mM acetate buffer, pH 5.0) prepared from ripe mango tissue. Thus it must be firmly attached to the cell-wall. This may be a reason why Brinson (266) who used low sodium chloride concentrations instead of 3 M

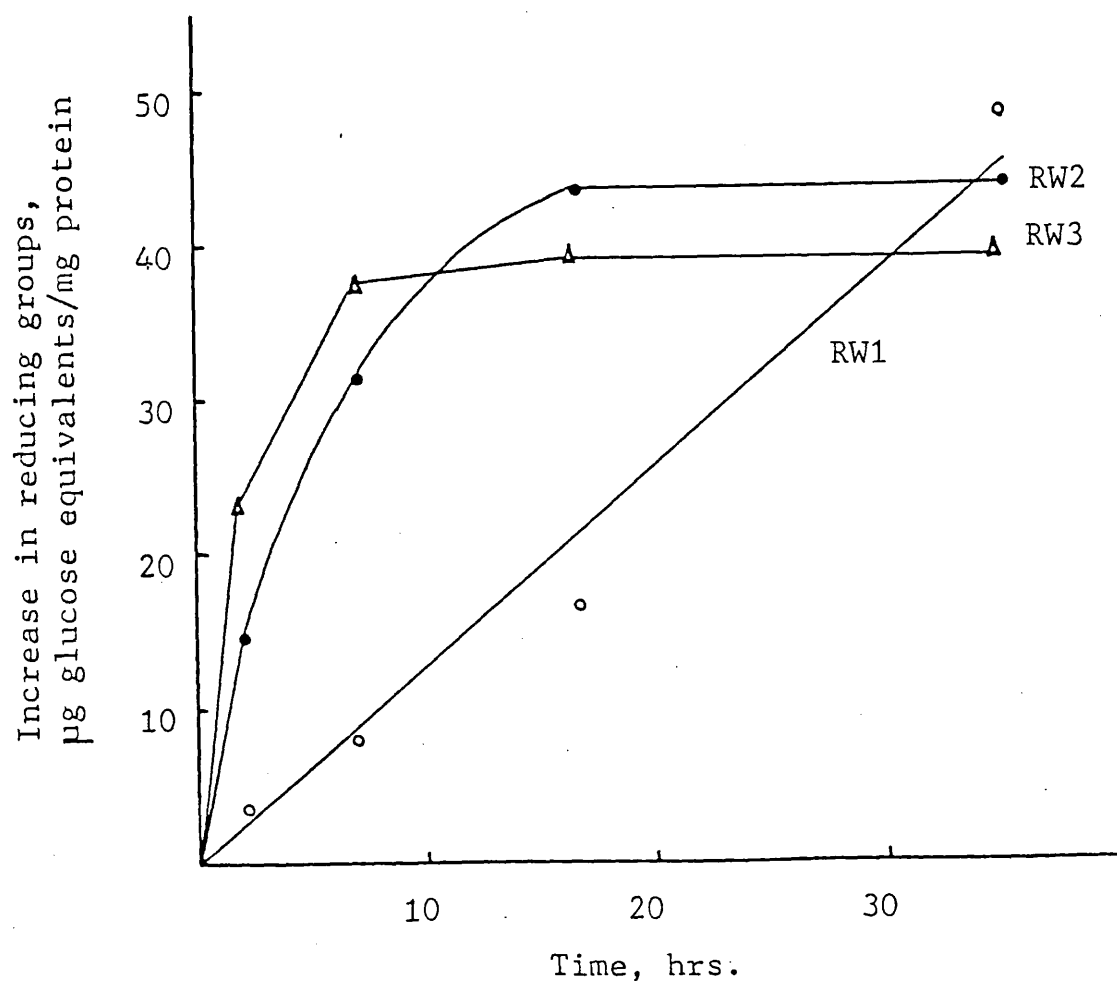


Fig. 46
Hydrolysis of pectin derived from unripe mango cell-walls by different forms of β -galactosidase isolated from ripe mango cell-walls preparations by 3 M LiCl (Fig. 38). At various time intervals aliquots of incubating samples were removed and assayed for reducing sugar content by the Park-Johnson ferricyanide method (306).

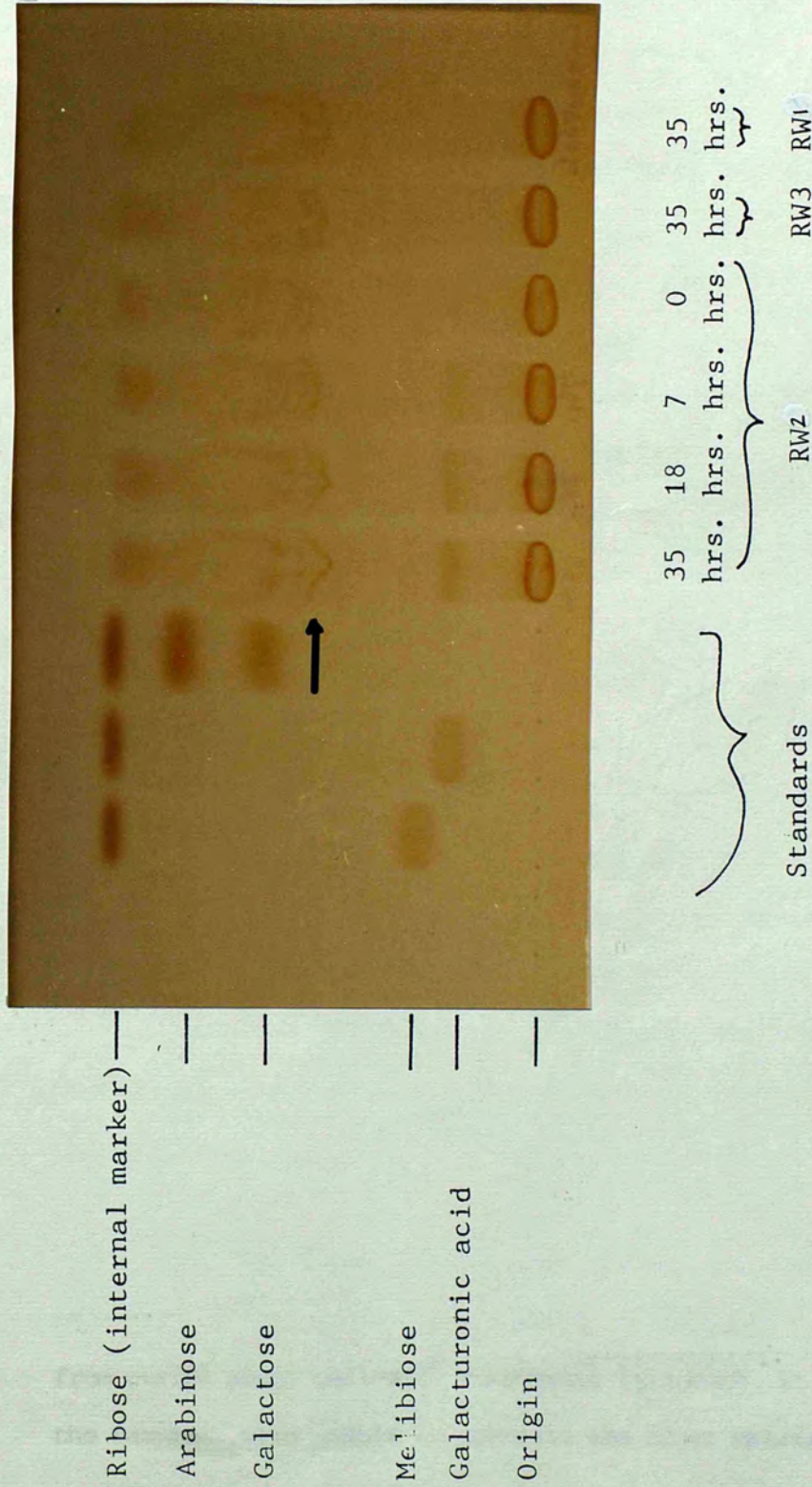


Fig. 47
 Release of galacturonic acid with time by RW2 from pectin derived from unripe mango cell-walls. At varying time intervals, aliquots were removed from incubating samples and applied to TLC plates. Separation of samples after incubation (35 hrs.) of RW1 and RW3 with the same substrate is also shown. Streaking is present in incubated samples and appears to increase with incubation time.

LiCl for enzyme extraction, failed to detect this activity.

2. β -Galactosidase From Soluble Preparations

None of the forms of β -galactosidase present in the soluble fractions displayed measurable activity against unripe mango cell-wall substrate. The ability of the forms to generate reducing sugars against pectin prepared from unripe mango cell wall was not quantitated. However activity against the same substrate was monitored by TLC analysis. As in the case of wall-associated β -galactosidases, streaking of samples on TLC plates prevented definite establishment of galactose release after incubation.

III. CONCLUSION

The ability of the different forms of β -galactosidase obtained from cell-wall preparations from the ripe mango to degrade pectin derived from cell-walls of the unripe fruit show they have the capacity to partake in in vivo wall breakdown. From the results obtained so far it is suggested that β -galactosidase acts on cell-walls after cleavage of pectic side chains by endo-enzymes during ripening. This contention is supported by following observations:

(a) The forms of β -galactosidase isolated from cell-wall preparations were unable to solubilise appreciable amounts of material from unripe mango cell-wall substrates (although it is possible that the enzymes were unable to permeate the outer matrix of the latter to

reach their sites of action).

(b) Wall associated forms of β -galactosidase are absent from the soluble pool of enzymes. This suggests that their site of action is in the wall itself where they are situated.

CONCLUDING REMARKS

A. Structure of Unripe Mango Pulp Cell Wall

1. Comparison With Red-Kidney Bean Hypocotyl Cell Wall

From the results discussed earlier (pp. 66-87), it seems that the cell wall of the unripe mango pulp is very different from that of the red-kidney bean hypocotyl. The water-soluble fraction of the former accounts for nearly 50 % of the cell wall compared to 18 % of the latter. Analysis has shown this fraction is essentially pectin in nature in both (pp. 75-77). The physiological significance of this is not apparent, but the following points warrant consideration:

(a) The high level of pectin material in mango may provide a reserve supply of carbohydrate in addition to starch for the generation of energy required for the ripening process, and/or the making of low molecular

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weight sugars (sucrose, D-glucose D-fructose) to give the mango its sweetness. It has been shown (pp. 88-89) that these sugars increase 15-fold during ripening. As the fate of the red-kidney bean hypocotyl is very different to that of the mango (it eventually undergoes secondary thickening), its wall may be considered to be more suitable for this purpose.

(b) Assuming softness is a desirable quality in the mango which helps in its appeal as a source of food and thus in distribution of the seed in nature, it is important that the wall be degraded more easily and this may be possible if it is rich in pectin material, rather than if it is similar to the wall of the red-kidney bean hypocotyl, which has low levels of this type of polymer. The fruit may be best equipped for enzymic breakdown of pectin molecules.

In support of (a), it has been noticed that the level of D-galactose was very low in both the cell wall (see p. 76) and soluble polysaccharides of the ripe mango (see pp. 101-102). Presumably this sugar has been metabolised

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following its liberation. Further, carbohydrate from the water-soluble fraction of the unripe fruit cell wall contained twice as much D-galactose as that found in a similar fraction of red-kidney bean hypocotyl cell wall (see pp. 75-76). D-Galactose can be readily metabolised in the cell following its conversion to D-glucose or its derivative.

Once the pectin fraction is removed, the alkali-soluble fraction from the cell walls of both samples seem very similar in composition (see p. 78).

2. Comparison With Cell-Wall Models

Considering the suggested modified model (see p. 40) the difference between the cell walls of the unripe fruit and red-kidney bean hypocotyl could be explained by the presence of a thicker layer of pectin network in the former. The greater proportion of neutral sugars in unripe mango pectin as compared to that of red-kidney bean (see p. 75) could be due to the presence of longer neutral side chains, or a greater number of smaller side chains in the former. All of the three models discussed

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(pp. 20-43) are compatible with these two possibilities, although greater numbers of side chains would mean greater cross-linking which could restrict growth. In this regard, it is worth noting that the red-kidney bean hypocotyl cells may still have been growing, but those in the mango pulp were fully developed. The concept of a localised rich region of pectin on the outside of the cell compared to a more scattered distribution throughout the wall is more appealing when considered in the context of efficient degradation by enzymes within the structure.

The presence of D-xylose and D-glucose in the acidic buffer-soluble fraction in the unripe mango mesocarp (see p. 99) is consistent with the model which proposes that these two monosaccharides occur in the pectin fraction. They may exist in arabinogalactan side chains or in side chains of their own. The other models however, suggest that D-xylose and D-glucose are found in xyloglucans which are proposed to be covalently linked to arabinogalacturonan side chains of pectin and hydrogen-bonded to cellulose fibres.

From an evolutionary point of view, basic features of plant primary cell wall which are required for essential

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features such as growth and strength would be preserved between species (and in different regions of the same plant), but variations would be expected to occur depending on the more specific function of the cell. Thus any attempt at protraction of a model to fit all dicotyledonous species must be restricted within the context of general structural features. The fact that much of the structural features of the various types of polysaccharides that make up the cell wall, have been deduced from work on tissue-cultured cells indicates possible inherent weaknesses in current concepts of cell-wall structure. Whether tissue-cultured cells have the same basic structural polysaccharides and 3-dimensional features in their walls as normal cells is still to be established.

3. Comparison With Ripe Mango Cell Wall

Comparison between the unripe and ripe mango cell wall show loss of the water-soluble pectin fraction during ripening. Table XII shows the yield of various fractions extracted from cell walls in terms of fresh weight of pulp. The figures were obtained by recalculating values

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previously presented in Table III which were expressed in terms of dry weight of cell-wall preparations.

Cell-wall sample, g/ 100 g fresh wt. of pulp		
Fraction	Unripe mango	Ripe mango
Hot water	4.42 ± 0.31	0.36 ± 0.03
4 M NaOH	2.02 ± 0.07	0.79 ± 0.03
2 M TFA	1.29 ± 0.08	1.02 ± 0.04
Residue	1.47 ± 0.15	1.12 ± 0.13
Total	9.20	3.29

Table XIII

Fractionation of cell walls prepared from the unripe and ripe mango. Details of experimental procedure are the same as those mentioned in Table III (p. 70).

The data show an apparent decrease of 92 % in the water-soluble pectin fraction at the ripe stage. Solubilisation of pectic polysaccharides during ripening has already been demonstrated in mango (218,266) and other fruits such as apple, tomato, avocado, peach, pear, date and strawberry (3, 221-229). In the present study, the value of 92 % seems very high. This may be because of the comparatively greater loss of cell-wall material during the preparation process using ripe mango pulp. This was because more of the wall material from the ripe fruit might have passed through the muslin sieve used for filtration as a result of the relatively degraded nature of the wall. This error could have been avoided if walls from the ripe fruit were prepared in a centrifuge tube.

Analysis of cell-wall fractions from the unripe and ripe fruit (see pp. 75-79) suggested preferential loss of D-galactose and L-arabinose which is in agreement with solubilisation of the pectin fraction during ripening.

B. Mechanism of Cell-Wall Softening

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Solubilisation of carbohydrate materials from the cell wall to the extent which occurs during ripening can only be brought about by the enzymic cleavage of internal bonds of polysaccharide molecules. In fruits such as tomato and pear, PG plays a major role in this process (1, 3, 149, 255-262). No other enzyme has been directly implicated, although it is unlikely that this is the only enzyme involved in the process. The cell-wall model proposed by Albersheim's group which proposes the binding of each neutral side chain to a xyloglucan molecule, predicts that other enzyme(s) capable of hydrolysing neutral sugars (eg. endo-L-arabanase and endo-D-galactanase) would also be required to explain reported losses of D-galactose and L-arabinose (see Fig. 48a). If no other enzyme than PG is involved then only fragments containing D-galacturonic acid and L-rhamnose would be released. By contrast the suggested modified model predicts that action of PG would result in the solubilisation of both acidic and neutral sugars (see Fig. 48b). This is substantiated by observations made by Ahmed *et. al.* (1) who found that treatment of unripe pear cell walls with purified PG solubilised an acidic fraction containing an unbranched arabinan; this had

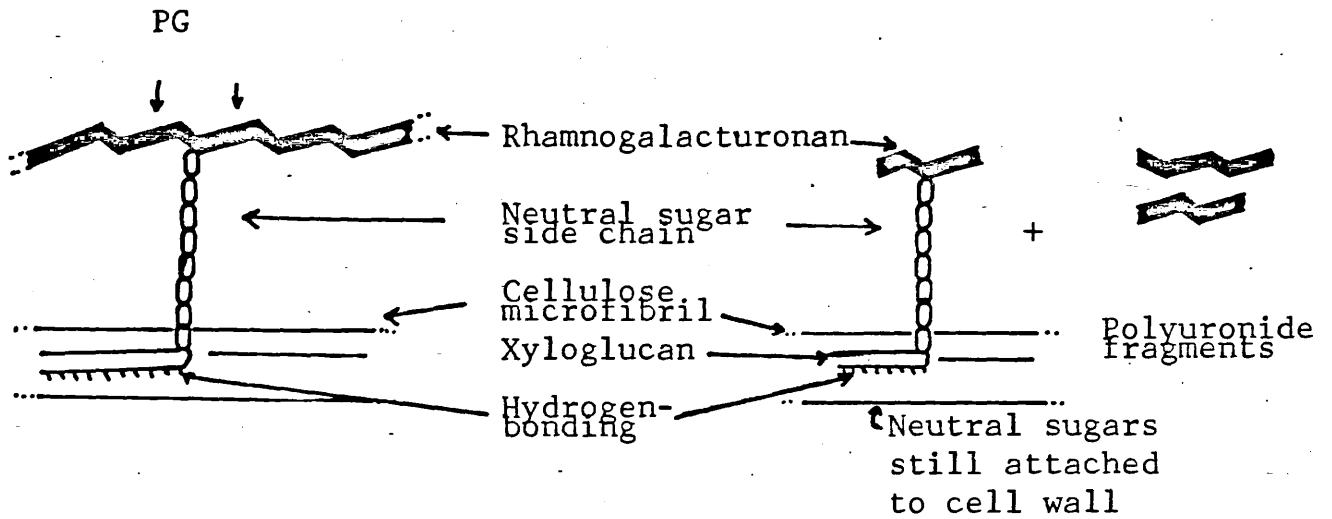


Fig. 48a

Action of PG on the Albersheim model.

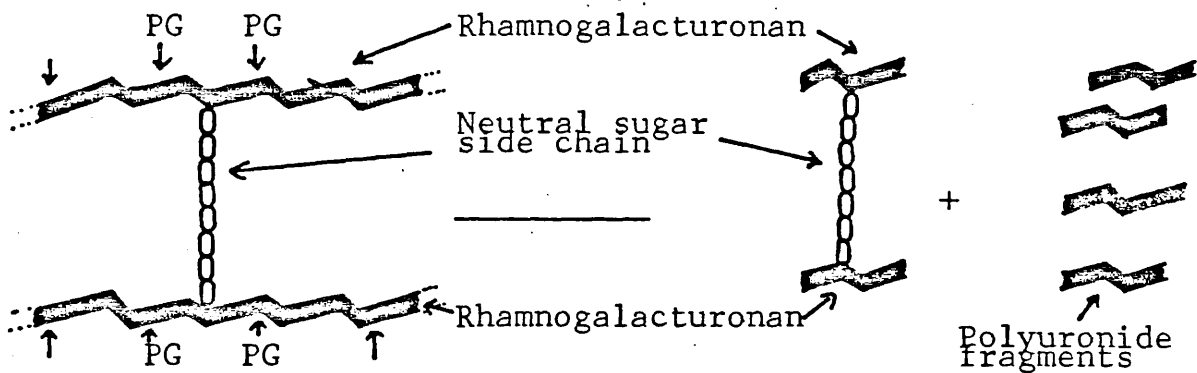


Fig. 48b

Action of PG on modified model

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similar characteristics to a polymer solubilised during in vivo ripening. Further support was obtained from work on the mango. It was found that a purified preparation of PG solubilised polysaccharide molecules containing appreciable amounts of neutral sugars (D-glucose, L-arabinose, D-xylose and D-galactose) from the cell wall of the unripe mango, the ripe mango and red-kidney bean hypocotyl (see pp. 81-85). In addition to this, smaller fragments (molecular weight less than 2,000) containing mainly uronic acids and reduced amounts of neutral sugars were also released.

In fruits such as the mango and apple, where PG is absent, cell-wall softening must occur by a different mechanism to those which possess this enzyme. It is difficult to imagine extensive cell-wall degradation (using any current model) in fruits that do not have PG without the action of endo-enzymes capable of hydrolysing neutral side chains of pectin. Thus the crude 3 M LiCl enzyme extract from the ripe mango cell-wall which was able to remove substantial amounts (14%) of carbohydrate material from unripe mango cell wall substrates (see Fig. 30 p. 109) must have contained this type of activity. Large polysaccharide molecules and a variety of

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monosaccharides were liberated during incubation (see pp. 117-119). TFA hydrolysis of the cell wall left after degradation by this crude enzyme preparation showed that the most pronounced changes they caused were decreases in D-galactose, L-arabinose and D-galacturonic acid content (compared to undegraded cell walls from control experiments; see pp. 120-122). These observations are consistent with the solubilisation of pectin material.

The significance of the presence of exopolygalacturonase (see pp. 132, 147-148) seemingly attached to ripe mango cell walls and absent from the soluble fraction is not clear. Although this enzyme has been identified in pears (250), peaches (251), cucumber (256) and apples (265), its role in fruit cell-wall softening has not been established. It is capable of hydrolysing apple cell wall to a certain extent (265) and has now been shown to be able to do the same to mango cell walls (see pp. 147-148). It is possible that in the absence of PG, this enzyme plays a vital role in cell-wall softening; namely, cleaving off uronic acid residues to allow more space for other endo-enzymes to hydrolyse the neutral side chains.

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The role of β -galactosidase appears to be of secondary importance in cell-wall softening. Its function seems to be in hydrolysing D-galactose residues from neutral side chains of pectin after initial hydrolysis by endo-enzymes has been completed. Although many soluble and apparently wall-bound forms of this enzyme were observed in the mango (see p. 135 and 145 for summary of forms of β -galactosidase identified) they showed none or negligible activity against undegraded cell-wall substrate (pp. 146-147). Bartley (269) and Pressey (270) have shown that this enzyme from the apple and tomato respectively is capable of hydrolysing β -1,4-galactans, but its action on cell walls was very small. Another role for β -galactosidase could be to assist in normal cell-wall turnover. In fruits that contain PG, it may be needed to assist in the cleaving of neutral side chains to leave an exposed rhamnogalacturonan backbone in order to enhance the action of this enzyme.

C. Future Work

The study of polysaccharide and cell-wall structure is very complex and labour intensive. However, progress on

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the mechanism of enzymic breakdown of covalent linkages leading to softening is dependant on a sound knowledge of fruit cell-wall structure. This area of research has been almost completely neglected and requires more attention. Purified fungal enzymes have been used with increasing effect and could also be utilised in further studies on the mango. Certainly more information is needed about the nature of the side chains of pectin, and how these molecules interact with the hemicellulose fraction. As more data of this kind is gained, modifications can be made to current cell-wall models which in turn may serve to inspire future advancement.

Perhaps the greater scope for further progress in the study of post harvest changes in mango cell walls lies in identification and purification of enzyme(s) present in crude 3 M LiCl extracts responsible for in vitro degradation of cell walls. Once achieved this could be followed by experiments aimed at monitoring levels of the enzyme(s) in the fruit at different stages of ripening. Experience has shown that identification of enzymes is likely to be hindered by two factors:

- (a) Availability of suitable substrates for

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viscometric assay. A rapid reduction in the viscosity of a solution is strong indication of endo-enzyme activity. By contrast, the generation of reducing sugars during incubation can be caused by either endo- or exo-acting enzymes. It may be possible to produce a suitable substrate by using purified PG to obtain intact side chains from the unripe mango cell wall. Fragments obtained could be partially purified by ion-exchange chromatography on DEAE Sephadex and/or gel-filtration on Bio-Gel P 150. Preliminary investigations in this direction has shown that a crude preparation of the enzyme from tomato was very active on this substrate.

(b) The presence of large amounts of contaminating polysaccharides in the enzyme preparation. Due to the relatively higher negative charge density of pectin it may be possible to remove significant amounts of these from proteins by ion-exchange chromatography using a weak anion exchange gel such as DEAE Sephadex. This process could be enhanced by mild de-esterification of the pectin with dilute alkali in the cold (without denaturing proteins).

After purification of the pertinent enzymes, antibodies

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could be raised and work similar to that of Tucker et. al. (262) on tomato PG could be carried out. This would involve an investigation of total enzyme activity and quantity to see if they increase in a parallel manner using radioimmunoassay techniques. This could provide indications of whether increase in activity is caused by de novo synthesis or activation. If the former possibility seems likely, further work can then centre on investigations into whether increase is due to translation of already existing mRNA or whether new mRNA is synthesized.

In the long term work at the genetic level could greatly assist in control of the ripening process, and may eventually lead to the creation of new species of the mango which have a longer shelf-life.

MATERIALS AND METHODS

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MATERIALS

General laboratory chemicals and Zerolit DM-F were obtained from BDH Ltd., Poole, Dorset and were of 'AnalaR' grade where possible. P-Nitrophenyl- β -D-galactopyranoside (PNPG), o-Nitrophenyl- β -D-galactopyranoside (ONPG) p-hydroxydiphenyl and monosaccharide standards used for TLC analysis were purchased from Koch-Light, Colnbrook, Bucks. Starch, citrus pectin, larchwood arabinogalactan, ω -amino-hexylsepharose p-aminophenyl-thio- β -D-galactopyranoside and protein standards used to calibrate the Sephacryl S-200 superfine column were obtained from Sigma (London) Chemical Co. Kingston-Upon-Thames, Surrey. Plastic backed Fl50 silica-gel plates were obtained from Schleicher and Schull, Dassel, Germany.

Polysaccharide standards used to calibrate the Bio-Gel P-150 column, DEAE Sephadex A 25 and Sephacryl S-200 superfine were obtained from Pharmacia (S.B) Ltd., London. Bio-Rex 70, Bio-Gel P-150 and Bio-Gel P-2 were obtained from Bio-Rad Laboratories, Watford. All buffers used, were prepared by the procedures described in *Methods in Enzymology* (307).

METHODS

A. Cell-wall Analysis

1. Preparation of Cell-walls

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All operations were carried out at 4 degrees C. Equal amounts of tissue (20g) were taken from the pulp of 5 mangoes and homogenised in 50mM sodium acetate buffer pH 5.0 (1g/ml). In the case of red kidney bean, hypocotyls of 4-6 day old plants were used. After sonication (4 half minute bursts with rest intervals of 2 minutes), samples were filtered through 5 layers of muslin. The cell-wall residue was washed generously with cold distilled water and resuspended in buffer (1g/ml). The sonication, filtration and washing process was repeated until virtually all starch granules (as seen under the microscope after iodine staining) were removed. Cell-wall residues were stirred continuously for 3 hrs. This was filtered through a sintered glass funnel and the residue was washed thoroughly with a 50% v/v chloroform/methanol mixture. The walls were finally dried in a stream of air and stored in a desiccator.

2. Test for Lignin

Drops of concentrated hydrochloric acid were added to cell-walls previously soaked in phloroglucin solution (0.1g/ml in 90% ethanol) for 30 min. The presence of lignin is apparent by the formation of a violet colour.

3. Test for starch

Cell-wall preparations were incubated overnight with

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salivary amylase (1g dry weight /ml). After filtration in a microcentrifuge, samples were concentrated by freeze-drying and redissolved in minimum amounts of water. These were then subjected to TLC analysis to identify gluco-oligosaccharides.

4. Protein Content

Analysis for nitrogen content of cell-walls was carried out by Elemental Micro-Analysis Limited, Devon. Percent values were multiplied by 6.25 to obtain approximations of protein content. Average values were obtained using 3 estimates for each cell-wall sample.

5. Uronic Acid Content

Uronic acid content of cell-walls was determined after the carbazole method (294). Dry cell-wall samples were suspended in water (1 mg in 2 ml). Carbazole solution (0.4 ml; 25 mg/ml in 80% ethanol) and 10 ml sodium tetraborate solution (25 mM in concentrated sulphuric acid) were added to this. After cooling, absorption values at 525 nm were determined. A calibration curve was done using galacturonic acid. For test samples average values were determined after 5 assays.

6. Calcium Content

Analysis of calcium content was carried out by Elemental Micro-Analysis Limited, Devon. Average values were obtained from 3 estimates for each cell-wall sample.

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B. Fractionation of Cell Walls

1. Water Fraction

Cell-wall preparations in water (10 mg/ml) were heated in screw cap bottles at 100 degrees C for 6 hrs. in a water bath. Solubilised material was recovered by filtration. Residues were washed with water and filtrates and washings were pooled and freeze-dried. Average water-soluble yield values were calculated from 5 separate incubations.

2. Alkali Fraction

Cell-wall residues obtained after hot water treatment were incubated for 4 hrs. at 25 degrees C on a shaker with 4M NaOH (10 mg/ml). Solubilised material was recovered by filtration and the residues were washed. Ethanol (80% v/v) was added to the combined filtrate and washings (3:1 v/v). This was left at -20 degrees C overnight for precipitation. Precipitates were separated by filtration and were washed with 80% ethanol at -20 degrees C and finally freeze-dried. Alkali-soluble yield values were calculated from 5 separate incubations.

3. TFA Fraction

Cell-wall residues left after water and alkali treatment were hydrolysed in screw top bottles with 2 M TFA at 100 degrees C for 6 hrs. (2 mg/ml). Acid was removed by drying under vacuum over potassium hydroxide and samples were

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redissolved in water prior to TLC analysis by the method of Menzies and Mount (298).

4. PG Fraction

PG was isolated from C. Lindemuthianum and incubated with cell-wall preparations by the method of English et al. (14). Samples were filtered and washed with water. Filtrates and washings were pooled and freeze-dried. After redissolving in water (1mg/ml) a 2 ml sample was applied to a Bio-Gel P-2 column (98 x 1.3 cm) equilibrated in 50 mM sodium acetate buffer pH 5.2. Elution was carried out with the same buffer at the rate of 0.1 ml/min. Fractions of 1.5 ml were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296).

5. Urea Fraction

Cell-wall residues obtained after PG treatment were resuspended (5 mg/ml) in 8 M urea solution (made up in 50 mM sodium acetate pH 5.0) and incubated at 25 degrees C for 24 hrs. with continuous stirring. Samples were next filtered and washed with water. Filtrates and washing were pooled, dialysed exhaustively against water and freeze-dried.

C. Analysis of Fractions

1. Total Carbohydrate

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Total Carbohydrate was measured by a scaled down version of the phenol/sulphuric acid method of Dubois (296). Samples were suitably diluted with water to give a final volume of 1.0 ml. Phenol (25 μ l of an 80% solution in water) was added to this followed by 2.5 ml concentrated sulphuric acid. Upon cooling, absorption values were read at 480 nm. Standard curves were prepared using glucose. Carbohydrate content of each sample was determined routinely using the average of 3 to 5 assays.

2. Uronic Acid

Uronic acid content of soluble polysaccharides was measured by the method of Blamenkrantz ^{Hansen} and ~~Asbr~~ (295). Samples 20 μ l containing uronic acid were added to 1.2 ml ice-cold 12.5 mM sodium tetraborate in concentrated sulphuric acid. These were heated at 100 degrees C for 5 minutes and then cooled in a water-ice bath. Meta-hydroxydiphenyl (20 μ l of a 0.15% solution in 12.5 mM sodium hydroxide solution) was added to each sample and absorbance values at 520 nm determined after 5 mins. Standard curves were prepared using galacturonic acid. Estimates for each sample were routinely made from 3 to 5 assays.

3. Hydrolysis

One ml samples containing 0.1 mg carbohydrate dissolved in 2M TFA were heated at 100 degree C for 6 hrs. in sealed screw-cap bottles. Acid was removed by drying under vacuum over potassium hydroxide, and the samples were redissolved in 0.1 ml water. The monosaccharide composition

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of these was determined by TLC analysis on silica-gel plates by the method of Menzies et al. (298). Triplicate hydrolysis was carried out on each sample. The monosaccharide composition of these was carried out in duplicate as described below.

4. TLC

Four test samples and 4 standard samples (each containing 0.4 mg/ml ribose) were applied as bands 1.5 cm long and 0.5 cm apart along a line of origin 1.0 cm above the lower edge of the plate. For most samples 5 μ l were applied evenly along the band using a Hamilton syringe. In some cases where low concentrations of sugars were present, more samples were applied by superimposing further aliquots after drying. Plates were developed twice in a solvent mixture containing ethyl acetate, pyridine, acetic acid and water in the ratio 60:30:10:10. Colour development was carried out by dipping in a 4-aminobenzoic acid (PABA) solution in methanol (14g/litre containing orthophosphoric acid 15ml/litre) and heating in an oven at 120 degrees C for 5 minutes. Chromatograms were then cut into strips and scanned along the axis of chromatographic development by reflectance densitometry. Peak heights were measured from scan profiles and corrected by applying an internal marker (ribose) factor which compensated for variations in samples. Standard graphs relating corrected peak heights to concentration were constructed for each chromatogram and test sample sugar concentrations were read from these using the corresponding corrected test peak heights.

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Samples which contained excessive amounts of polysaccharides and/or protein were treated with 3 times their volumes of ethanol in microcentrifuge tubes. After centrifugation, supernatants were concentrated by blowing with nitrogen prior to TLC analysis.

D. Soluble Polysaccharide Analysis

1. Preparation of Soluble Fraction

All operations were carried out at 4 degrees C. Equal amounts of tissue (20g) were taken from the pulp of 5 mangoes and homogenized in 50 mM sodium acetate buffer, pH 5.0 (1g/ml). This was centrifuged at 10,000 g for 1 hr. after which total carbohydrate content of the supernatant was determined by the phenol/sulphuric acid method (296).

2. Bio-Gel P-150 Gel-Filtration

One ml of sample was applied to a Bio-Gel P-150 column (76 x 0.8cm) and eluted with 50 mM acetate buffer pH 5.0 at a rate of 0.1ml/min. The column was water jacketed and run at room temperature with tap water flowing through the jacket. Fractions of 1 ml were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296) and/or uronic acid by the method of Blumenkrantz ^{and Asboe Hansen} (295). Fractions of peaks containing carbohydrate were pooled and freeze-dried prior to further analysis. The column was

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calibrated using the following as standards: Dextran T10 (MW 10,000), Dextran T40 (MW 40,000), Dextran T70 (MW 70,000), glucose, and blue dextran.

3. Bio-Gel P-2 Gel-Filtration

One ml of sample was applied to a Bio-Gel P-2 column (74 x 1.4 cm) equilibrated in 50 mM sodium acetate buffer, pH 5.0 and eluted at a rate of 0.1 ml/min with the same buffer. Fractions of 2.0 ml were collected and assayed for carbohydrate by the phenol/sulphuric acid method (296).

4. DEAE Sephadex

DEAE Sephadex A 25 was allowed to swell in distilled water and repeatedly washed with 0.5 M sodium hydroxide on a Buchner funnel until free of chloride ions (tested using silver nitrate). Excess alkali was washed off with water after which the gel was treated with excess acetic acid (0.5 M). Equilibration with 50 mM sodium acetate buffer pH 5.0 was finally carried out after acid was washed off with distilled water.

Samples (4ml) were applied to a column (11 x 2.5 cm) of the equilibrated gel which was then washed through with buffer (200 mls). Gradient elution of bound material was carried out using 200 mls equilibrating buffer and 200 mls

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0.8 M sodium chloride in buffer. Four ml fractions were collected and assayed for carbohydrate. Fraction of peaks containing carbohydrate were pooled and freeze-dried after removal of salt by dialysis against distilled water.

E. 3 M LiCl Enzyme Preparation

1. Preparation

All manipulations were carried out at 4 degrees C. Mango pulp was homogenized in 50 mM sodium acetate buffer, pH 5.0 (1g/ml) with a Silverson blender and centrifuged at 23,000g for 30 minutes. The pellet was resuspended in water (0.25g/ml), stirred continuously for 10 minutes and the resulting suspension was again centrifuged at 23,000g for 30 mins. The supernatant was discarded and the process was repeated once more. The pellet was next incubated for 6 hrs. with continuous stirring in 50 mM acetate buffer pH 5.0 (1.25g/ml) containing 3 M LiCl. This was centrifuged (10,000g) for 1 hr. and the pellet was again incubated in high salt solution (1.25 g/ml, for 18 hrs.). The 2 supernatants were pooled and dialysed for 24 hours against 5 litres of 50 mM sodium acetate buffer pH 5.0, during which period the buffer was changed 3 times. Samples were next concentrated (approximately 10-fold) by dialysis against a saturated solution of polyethylene glycol, after which they were dialysed against 3 litres 50 mM sodium acetate buffer, pH 5.0 for 2 hours.

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2. Activity Against Cell-Walls

Unripe mango cell-walls (5 mg) were incubated at 30 degrees C with 0.6 ml of 3 M LiCl enzyme preparation plus 0.8 ml 50 mM sodium acetate buffer, pH 5.0 in microcentrifuge tubes. For incubations at pH 6.0 and 7.0, 50 mM potassium phosphate buffer was used. A small crystal of thymol was added to prevent bacterial growth. After appropriate periods of incubations, 30 μ l samples were removed and assayed for carbohydrate. Controls were done using enzyme heated at 100 degrees C for 8 minutes.

3. Protein Estimation

This was carried out by the method of Bradford (300). Samples of 0.1 ml were added to 0.5 ml reagent and absorbance values at 575 nm were determined after 5 mins. Calibration curves were prepared using BSA standard. Where necessary, samples were concentrated by freeze-drying prior to analysis. Protein content of samples were determined routinely using 3 to 5 assays.

4. β -Galactosidase

Aliquots of 50 μ l substrate (PNPG or ONPG 10 mg/ml in water) were added to enzyme and buffer (pre-incubated at 30 deg. C for 3 mins.) to give final volumes of 430 μ l. Incubation times were usually of 20 mins. duration, after which reactions were terminated by the addition of 1 ml 0.3 M sodium

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carbonate. Absorption values at 405 nm were determined. Activity values were routinely determined in triplicate.

5. Viscometric Assay

Samples of enzyme preparations (0.6ml) were added to 9.4 ml of substrate solutions (1% in 50 mM sodium acetate buffer, pH 5.0) preincubated at 30 degrees C in an Ubbelohde viscometer. Viscosity measurements were made immediately and after incubation at various time intervals to a maximum of 24 hrs.

F. β -Galactosidase

1. Preparation of Crude Fractions

Preparations were obtained from mango cell-walls as for the preparation of the 3 M LiCl enzyme preparation. However, after supernatants were combined dialysis was carried out against 50 mM sodium acetate buffer pH 4.4. Samples were not concentrated with polyethylene glycol. Soluble fractions prepared as for the preparation of soluble polysaccharides.

2. DEAE Sephadex Ion-Exchange Chromatography.

Samples were dialysed against appropriate buffers and then applied to a DEAE Sephadex A 25 column (2.1 x 4.7cm) equilibrated with the same buffer as previously described. Sodium acetate (50 mM) was used to prepare buffer of pH values

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lower than 6.0. For higher values, 50 mM potassium phosphate (pH 6.0) and 50 mM tris/HCl (pH 9.0) were used. Fractions of 3 mls were collected and assayed for β -galactosidase activity. Activity values were routinely determined in triplicate.

3. Bio-Rex 70 Ion-Exchange Chromatography

A column (2.1 x 4.7 cm) of Bio-Rex 70 was prepared after equilibration of the gel with 50 mM sodium acetate buffer pH 4.4 (according to the manufacturers instructions). Approximately 1,000 units of crude β -galactosidase preparation previously dialysed against the same buffer were applied to the column. The latter was washed with equilibrating buffer until no more carbohydrate material eluted (1.5-2 litres), and then with 50 ml 0.5 M sodium acetate buffer, pH 4.84. Gradient elution was next carried out using 75 ml 0.7 M acetate buffer pH 5.48. Finally the column was washed with 120 mls of the latter buffer followed by 50 ml of 0.5 M sodium hydroxide. A flow rate of 2.4 ml/min was employed throughout, and 2 ml fractions were collected.

4. Sephacryl S-200 Filtration

Pooled fractions of peaks containing β -galactosidase activity were concentrated by dialysis against saturated solutions of polyethylene glycol. Samples of 2 ml were applied to a Sephacryl S-200 Superfine column (2.4 x 90 cm) equilibrated with 100 mM sodium acetate buffer, pH 5.0 containing 100 mM sodium chloride and 0.02% sodium azide. Elution was carried

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out with the same buffer at a flow rate of 1.8ml/min. Fractions of 3 ml were collected and assayed for β -galactosidase activity with PNPG. The column was calibrated using grade III ovalbumin (43,000), BSA (67,000) bovine liver aldolase (158,000), bovine liver catalase (210,000) and bovine thyroglobulin (669,000).

5. Affinity Chromatography

Ten ml succinic anhydride solution (60mg/ml) were added to a 5 mg of ω -aminohexyl sepharose suspended in 10 ml water at 4 degrees C. The pH was adjusted and maintained at 6 using 10% sodium hydroxide for 4 hours. Complete reaction of amino groups was assessed by using sodium trinitrobenzene sulphate. After washing with water the gel was again suspended in 10 ml water. Ten ml of a p-aminophenyl- β -D-thiogalactopyranoside solution (3 mg/ml) were added to this and the pH of the suspension adjusted to 4.3 with 1 M hydrochloric acid. After adding 2 ml water containing 312.5 mg carbodiimide, the reaction was left to complete at room temperature for 20 hrs.. The substituted gel was washed with 5 litres of water over a period of 24 hrs. and then was equilibrated with 50 mM sodium acetate buffer pH 5.0.

Pooled fractions of peaks containing β -galactosidase activity after Bio-Rex 70 ion-exchange chromatography were applied to the column at a flow rate of 0.1ml/min. Fractions of 2 ml

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were collected and assayed for β -galactosidase activity. The experiment was repeated with enzyme preparations and column equilibrated at pH 7.0.

6. Activity Against Pectin

One ml of β -galactosidase preparations were added to 4.5 ml pectin solution in 50 mM sodium acetate buffer, pH 4.4. The latter was obtained by hot water extraction of unripe mango cell-walls as previously described. At various time intervals 1 ml samples were removed for TLC and reducing sugar analysis.

Reducing sugar analysis was carried out by the Park-Johnson ferricyanide method (306). To a 0.5 ml suitably diluted sample was added 0.5 ml of a cyanide/carbonate solution (0.65 g/litre cyanide in 50 mM sodium carbonate) followed by 0.5ml of a 0.05% potassium ferricyanide solution. This was heated in a boiling water bath for 15 mins. and then cooled in an ice-water bath. Ferric ion solution (9 mM ferric ammonium sulphate in 25 mM sulphuric acid containing 0.1% sodium monolauryl sulphate) was added to this (2.5 ml) and absorption value at 690 nm was determined after 15 mins..

A standard curve was prepared using glucose. Triplicate analyses were carried out on each sample.

1. Activity Against Cell-Walls

Unripe mango cell walls prepared as previously described

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were incubated with 0.6 ml β -galactosidase preparations and 0.7 ml 50 mM sodium acetate buffer pH, 4.4 in microcentrifuge tubes. Small crystals of thymol were added to prevent bacterial growth. Aliquots of 0.1 ml. were removed before and after incubation (following centrifugation in a microcentrifuge) at 30 degrees C for 36 hrs. . These were assayed for carbohydrate by the phenol/sulphuric acid method (296). Aliquots of 0.3 ml were also removed and assayed for monosaccharides by TLC analysis (298).

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