AN INVESTIGATION OF GALACTURONYLTRANSFERASE ACTIVITY INVOLVED IN PECTIN BIOSYNTHESIS.

by

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Abbreviations.

Ace A	Aceric acid
Api	Apiose
Ara	Arabinose
B.D.	Blue dextran
Bq	Bequerel
BSA	Bovine serum albumin
CETAB	Cetyltrimethylammonium bromide
CDP	Cytosine disphosphate
^{o}C	Degrees centigrade
DNP-lysine /DNP-L	2,4-Dinitrophenyl-L-lysine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
Fuc	Fucose
g	x gravity
Gal	Galactose
Gal A	Galacturonic acid
GDP	Guanosine diphosphate
Glc	Glucose
Glc A	Glucuronic acid
hr	hour
LDAO	Lauryldimethylamine-N-oxide
Mes	2(N-Morpholino)ethanesulphonic acid
min	minute
M.W.	Molecular weight
PMSF	Phenylmethyl sulphonyl fluoride
PVP	Polyvinylpyrrolidone
Rha	Rhamnose

Abbreviations continued/....

S.D.	Standard deviation
S.E.	Standard error
SDS	Sodium dodecylsulphate
TDP	Thymidine diphosphate
TFA	Trifluoroacetic acid
T.L.E.	Thin layer electrophoresis
Tris	Tris (hydroxymethyl) aminoethane
UDP	Uridine diphosphate
v/v	Volume for volume
w/v	Weight for volume
%	Percent

Abstract.

A particulate enzyme preparation, obtained from epicotyls of etiolated pea (Pisum sativum) seedlings, exhibited galacturonyltransferase activity. The enzyme preparation had the ability to incorporate galacturonic acid from UDP-galacturonic acid into pectin. The product is thought to be either homogalacturonan or rhamnogalacturonan I or both. The optimum conditions required by the particulate enzyme preparation were investigated. Galacturonyltransferase activity was shown to be higher in the region of elongation of the epicotyl but the whole epicotyl did contain galacturonyltransferase activity. Solubilisation of galacturonyltransferase activity was attempted. Partial solubilisation was achieved using the detergent LDAO but further work is required to confirm this. The effect of the presence of UDP-rhamnose and GDP-rhamnose on galacturonyltransferase activity was also investigated but no evidence was found to indicate co-operation between galacturonyl-transferase and rhamnosyltransferase.

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Chapter 1

INTRODUCTION

The majority of plant cells, in contrast to animal cells, are surrounded by a cell wall. The cell wall is an organelle which encloses the protoplast. Only a few plant cells do not possess a cell wall and these include the motile spores in algae and fungi, and the sexual cells of both lower and higher plants. The cell wall acts as the major factor in determining the plant cell's shape. It also provides the cell with mechanical strength and therefore gives support to the tissue. Cell walls are not static structures. As the cell elongates, the wall must expand and new components must be incorporated into the existing wall structure. Therefore, the cell wall is involved with the growth and development of plant cells. The cell wall also functions as a protective barrier to pathogens. However, some pathogens attack the cell wall by secreting enzymes which degrade the components of the wall (Bateman and Millar, 1966). Cell wall polysaccharides can also function as storage material to the cell (Meier and Reid, 1982). The cell wall is therefore of great importance and much work has been completed on the structure of the wall.

The cell wall is generally thought to be composed of microfibrils which are embedded in a matrix of polysaccharides, proteins and glycoproteins. Lignin and water can also be present in the cell wall. Polysaccharides account for around 60 to 95% of the cell wall. The polysaccharide composition of the cell wall varies according to the plant species and also the cell type.

The polysaccharides present in the cell wall can be separated according to their solubility properties and three main classes have become evident:-

1. Pectic substances:- the polysaccharide component of the cell wall which is soluble in water, chelating agents or dilute acid.

2. Hemicellulose:- the polysaccharides which are soluble in dilute alkali.

3. Cellulose:- the insoluble residue that remains following the pectin and hemicellulose extractions.

An alternative classification employed is the division of cell wall polysaccharides into two classes:-

1. Cellulose.

2. Non-cellulosic polysaccharides.

This classification is sometimes employed because the techniques used to extract the pectic and hemicellulosic polysaccharides do not always result in distinct classes.

Jensen (1960) and Jensen and Ashton (1960) working on onion root tip cells have demonstrated that, at a very early stage of differentiation, differences exist in the chemical composition of the cell walls between cells of the protoderm,the cortex and the provascular tissue. All the cell walls contain cellulose, hemicellulose and pectic polysaccharides but they are present in different ratios.

During the growth and development of the plant cell, the polysaccharides incorporated into the wall vary, and this alters the properties of the wall (Thornber and Northcote, 1961 a, b). The change in polysaccharide composition can be correlated with a variation in the function of the wall.

The cell wall can be divided into three main regions - the middle lamella, the primary wall and the secondary wall. The primary walls of adjacent cells are united by a common layer which is known as the

middle lamella. This is a thin layer which functions as an intercellular matrix holding groups of cells together. Although the predominant constituent of the middle lamella is pectin; hemicellulose and cellulose are also present. The cellulose microfibrils are thought to be distributed in a random orientation.

The primary wall is the first proper wall to be formed and, depending on the function of the cell, may be the only wall present. Primary walls are formed by undifferentiated cells that are still growing. As elongation occurs, the primary wall must retain its strength, therefore more polysaccharide material must be incorporated in order to compensate for the increase in cell wall area. The primary cell wall is composed of pectic substances, hemicellulose, and cellulose microfibrils. Originally the cellulose microfibrils were thought to be present in a random orientation, but recent work has indicated that the microfibrils may be arranged in a more ordered manner (Neville and Levy, 1985).

The secondary wall is often thicker and more rigid than the primary wall, resulting in greater protection and support to the cell. Deposition of the secondary wall is initiated when elongation ceases. Cells which form secondary walls are generally incapable of elongation, and are usually differentiating into cells with specialised functions. The secondary wall is composed mainly of hemicellulose and cellulose microfibrils which are present in a parallel arrangement. If pectic substances are present in the secondary wall, it is only as a very minor component. Electron microscopy has revealed distinct layers within the secondary walls of cells and, although the microfibrils have a tendency towards a parallel orientation within any one layer, this orientation varies between adjacent layers (Albersheim, 1965).

The primary cell walls of a variety of higher plants appear to have many features in common, but this is not true of secondary walls which can vary greatly in composition from one cell type to another (Albersheim, 1976).

Cellulose.

Cellulose is a ubiquitous component, forming the framework of all higher plant cell walls. It is composed of D-glucopyranosyl residues linked in the $\beta(1\text{-}4)$ configuration. Due to the linkage, the chain forms a two-fold screw axis. Glucan chains form paracrystalline structures known as microfibrils. Microfibrils are the only microscopically discrete components of the wall (Albersheim, 1965). Hydrogen bonding occurs both between and within chains in the microfibril and this contributes to its insolubility (Gardner and Blackwell, 1974 a, b). Within the microfibril, crystalline regions occur which indicates the uniformity of the glucan chains. X-ray diffraction studies by Gardner and Blackwell (1974 a, b), using the alga Valonia ventricosa, indicate that the glucan chains within the cellulose fibres are arranged in a parallel manner. It is likely that the glucan chains within microfibrils of higher plants are also arranged this way.

However, within the microfibril, amorphous regions also occur which are disorganised to varying degrees. Monosaccharides other than glucose are invariably found associated with the cellulose fraction and this may be the cause of these amorphous regions (Northcote, 1958). These non-glucan monosaccharides may be covalently attached to the glucan polymer, or, alternatively, their presence could be due to either the occurrence of separate non-glucan chains within the microfibrillar structure or the physical absorption of hemicellulosic polysaccharides onto the surface of the microfibril (Northcote, 1958). The latter

suggestion may be correct, as it has been demonstrated by Bauer et al. (1973) that hemicellulosic polysaccharides are associated with cellulose microfibrils.

Cellulose microfibrils are present in both primary and secondary walls. There are many reports giving varying degrees of polymerisation, however cellulose is generally reported to have a degree of polymerisation of approximately 10,000 (Northcote, 1972).

Hemicellulose.

The nature of the hemicellulose present in the cell wall varies according to the species and also the type of cell from which it was isolated. Commonly occurring hemicelluloses include the xylans, xyloglucan, gluco and galactogluco-mannans and the non-cellulosic β-D-glucans.

Xylans:-

Xylans form a major component of the hemicellulosic polysaccharides of angiosperms. D-Xylopyranose residues linked by $\beta(1\text{-}4)$ bonds form the backbone of this molecule (Aspinall, 1959). This polymer has a degree of polymerisation between 150 and 200. Terminal 4-O-methyl-O-glucuronic acid residues are present as side chains linked by $\alpha(1\text{-}2)$ bonds. There is some dispute over the distribution of the side-chains (Waldron and Brett, 1985). It is not clear whether they are randomly arranged or whether they are distributed in an ordered sequence. Side-chains occur in the ratio of 1 glucouronic acid unit to 10 xylose units. In situ, approximately 50% of the xylose residues are acetylated at either C-2 or C-3 (Aspinall, 1980).

Xylans also occur in large amounts in the cell walls of monocotyledonous plants (Aspinall, 1959). However, these polysaccharides contain side-chains

composed of single L-arabinofuranosyl residues linked by α (1-3) bonds as well as α (1-2) 4-O-methylglucuronic acid residues. Arabino (4-O-methylglucurono-) xylans also occur in small amounts in the hemicellulosic component of the gymnosperms (Timell, 1965).

Xyloglucans:-

Polysaccharides composed of glucose and xylose units - the xyloglucans - were first found to occur in seeds (Kooiman, 1961). It has also been demonstrated that xyloglucans form a major component of the cell walls of suspension-cultured dicotyledonous plants (Aspinall et al., 1969). Kooiman (1961), working on tamarind seeds, studied the structure of xyloglucan and, more recently, Bauer et al. (1973) examined the xyloglucans present in sycamore suspension-cultured cell walls. Xyloglucans are composed of a β(1-4) glucopyranose backbone with xylopyranose side-chains linked into approximately 50% of the glucose residues. The xylose residues are linked via α(1-6) bonds. The xylose side-chains can be extended by the addition of β(1-2) galactopyranose units. Terminal fucopyranosyl units can also be present linked to the galactose units by α(1-2) bonds.

Mannans:-

Glucomannans and galactoglucomannans are the major hemicellulosic component of the gymnosperms (Timell, 1965). These polymers are composed of a chain of randomly arranged D-mannopyranose and D-glucopyranose residues which are linked by β(1-4) bonds.

In galactoglucomannans, galactopyranose units are present as single unit side-chains attached onto the main chain by α(1-6) linkages. The ratio of galactose: glucose: mannose is approximately 1:1:3. In vivo, some of the mannose residues are acetylated. The polysaccharide is composed of approximately 100 glycosyl residues.

Glucomannans, isolated from gymnosperms, are also composed of $\beta(1-4)$ linked mannose and glucose units in the ratio 3 : 1. Galactose residues are always a very minor component of this polymer. The degree of polymerisation is approximately 200 and the mannose residues may be acetylated.

In angiosperms, glucomannans occur as a minor component of the hemicellulosic polysaccharides (Timmell, 1964). Glucose and mannose are present in the ratio 1 : 2. No galactose residues are linked to this polymer. Glucomannans isolated from angiosperms contain approximately 100 glycosyl residues.

Non-cellulosic Glucans:-

In this fraction, two main components occur - mixed β-glucans and callose. Mixed glucans are found in the cell walls of monocotyledonous plants (Darvill et al., 1980). Mixed glucans are composed of linear chains of D-glucopyranose units which are joined by both β (1-3) and β (1-4) linkages (Buchala and Wilkie, 1970). The proportion of $\beta(1-3)$ to $\beta(1-4)$ linkages is approximately 1 : 2 but this ratio is directly related to the age of the tissue from which the polymer was isolated - the older the tissue, the greater the occurrence of 4-linked residues (Buchala and Wilkie, 1971).

Callose is a linear polysaccharide composed of $\beta(1-3)$ linked glucopyranose units (Northcote, 1969). This polymer is not present in all tissues, but is a common constituent of phloem and wound tissue.

Pectic Substances.

The pectic substances are composed of a complex mixture of both acidic and neutral polysaccharides and they constitute approximately 35% of the primary cell walls of dicotyledonous plants (Darvill et al., 1980).

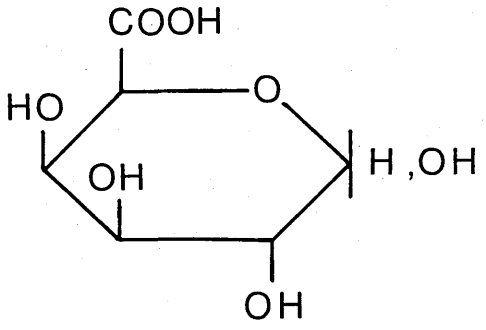
Although much of the more recent work concerning the structure of the pectic polysaccharides has been performed using cell wall preparations from suspension cultures, the pectic polysaccharides present in the cell walls of a variety of higher plants have previously been studied. Information on the structure of the pectic substances has been obtained by analysing the pectic polysaccharides extracted from cell wall material. The use of the enzyme, endopolygalacturonase, is becoming increasingly popular for extracting pectic polysaccharides (English et al., 1972).

The acidic component of the pectic substances is characterised by the presence of both galacturonic acid and rhamnose units. The structure of these sugars is illustrated in figure 1.1. Other glycosyl residues are also present and these include, in particular, arabinose and galactose units. Table 1.1. shows the glycosyl composition of pectic substances isolated from a variety of higher plants.

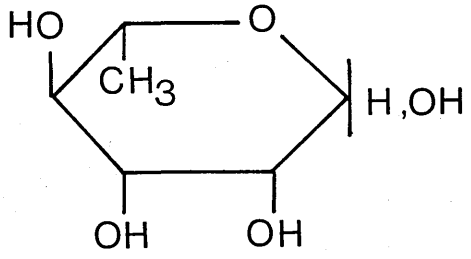
The basic structure of galacturonorhamnan consists of a backbone composed of α(1-4) linked D-galacturonopyranosyl units and (1-2) linked L-rhamnopyranosyl units. Experiments by Talmadge et al. (1973) indicated that the rhamnosyl residues are present in the β-configuration. The presence of 2-linked rhamnosyl residues causes a kink in the linear galacturonan chain which results in rhamnogalacturonan having a zig-zagged shape (Rees and Wight, 1971). A variable proportion of the carboxyl groups of the galacturonic acid units are esterified to form methyl esters.

It can be seen from table 1.1 that glycosyl residues other than galacturonic acid and rhamnose occur in pectic polysaccharides, and these are present as side-chains. Arabinose and galactose units tend to predominate. Experiments involving methylation analysis by

Figure 1.1
Structures of galacturonic acid and rhamnose



D-Galacturonic acid



L-Rhamnose

TABLE 1.1.

Glycosyl composition of pectic substances isolated from a variety of higher plants.

Reference	Plant Source	Glycosyl residues
Aspinall et al.(1967)	Soybean	Galacturonic acid, rhamnose, galactose, xylose, fucose, glucuronic acid
Aspinall et al.(1968a)	Lemon	Galacturonic acid, rhamnose, arabinose, galactose, glucuronic acid, xylose, fucose
Aspinall et al.(1968b)	Lucerne	Galacturonic acid, rhamnose, galactose, arabinose, glucose, glucuronic acid, fucose, xylose, 2-O-methylxylose, 2-O-methylfucose
Aspinall and Jiang (1974)	Rapeseed	Galacturonic acid, rhamnose, galactose, arabinose, xylose, fucose, glucuronic acid
Barrett and Northcote (1965)	Apple	Galacturonic acid, rhamnose, arabinose, galactose, xylose, fucose, 2-O-methylxylose, 2-O-methylfucose
Eda et al.(1983)	Tobacco	Galacturonic acid, rhamnose, arabinose, galactose
Ishii (1982)	Onion	Galacturonic acid, rhamnose, arabinose, galactose
Stevens and Selvendran (1984)	Cabbage	Galacturonic acid, rhamnose, arabinose, galactose
Wada and Ray (1978)	Oat	Galacturonic acid, rhamnose

Talmadge et al. (1973) using cell walls from sycamore suspension cultures have demonstrated that approximately 50% of the rhamnose units are branched, having substituents attached at C-4 in addition to C-2. This indicates that this is one site of attachment of side-chains. Galacturonic acid residues have been isolated which are 3,4-linked, indicating that side-chains are also linked via galacturonic acid (Aspinall et al., 1967; Rees and Wight, 1969).

It has been concluded from recent work using suspension-cultured cell walls that treatment with endopolygalacturonase solubilises three distinct acidic polysaccharides, all containing galacturonic acid. These three polysaccharides are known as rhamnogalacturonan I, rhamnogalacturonan II and homogalacturonan. The three polysaccharides can be separated on the basis of charge using ion-exchange chromatography and by size using gel filtration chromatography. Subsequent experiments have yielded much information on the structure of these polysaccharides.

Rhamnogalacturonan I:-

McNeil et al. (1980) have shown that rhamnogalacturonan I accounts for 7% of suspension-cultured sycamore cell walls. The backbone of this polymer consists of 4-linked galacturonic acid units and 2-linked rhamnose units in the ratio 2 : 1. These residues appear to occur in an ordered sequence (Lau et al., 1983). Experiments have indicated that this polymer does not contain any long continuous regions of 4-linked galacturonic acid units (McNeil et al., 1980). Studies using gel filtration chromatography have demonstrated that the polysaccharide has a degree of polymerisation of approximately 2000 (McNeil et al., 1980).

Methylation analysis (McNeil et al., 1980; Talmadge et al., 1973)

has demonstrated that approximately 50% of the rhamnose residues are linked at position 2 and position 4. Therefore, C-4 of the rhamnosyl residues is a branching point onto which side-chains are linked. Side-chains are always present and are mainly composed of arabinosyl and galactosyl residues. The side-chains appear to be relatively short in length averaging six sugar units, however, the actual length can vary from one residue to over twelve sugar units (Lau et al., 1983). McNeil et al. (1982) confirmed the complexity of these polysaccharides when they demonstrated that at least seven different side-chains can be linked to the rhamnose units. Only the first sugar residue was identified so the number of different side-chains occurring could be much greater. It has also been shown that at least 14 differently linked L-arabinosyl and D-galactosyl residues are present in rhamnogalacturonan I (McNeil et al., 1980). Albersheim has proposed that rhamnogalacturonan I is a family of polysaccharides - each with the same backbone but with differing side-chains (McNeil et al., 1982).

Rhamnogalacturonan II:-

Rhamnogalacturonan II is structurally a very complex polysaccharide and constitutes 3 to 4% of suspension-cultured sycamore cell walls (Darvill et al., 1978). Rhamnogalacturonan II has been isolated from seven different cell wall preparations of sycamore suspension-cultured cells, and similar amounts of the characteristic sugar residues present in rhamnogalacturonan II have been found in cell walls of pea, pinto-bean and tomato (Darvill et al., 1978). Although galacturonic acid and rhamnose are the main constituents of this polymer, table 1.2.reports the variety of glycosyl residues and their linkages found in rhamnogalacturonan II. In contrast to rhamnogalacturonan I, terminal-linked rhamnose and 3-linked

TABLE 1.2. (from Darvill et al, 1978)

Glycosyl composition and linkage of rhamnogalacturonan II.

Sugar residue	Glycosidic linkage
Galacturonic acid	terminal
	4
	3,4
Rhamnose	terminal
	3
	2,4
	3,4
	2,3,4
Arabinose	terminal
	2
Galactose	terminal
	3
	2,4
Apiose	3
2-O-methylfucose	terminal
Fucose	3
	3,4
2-O-methylxylose	terminal
Glucuronic acid	2
Glucose	4

rhamnose are the most common rhamnosyl residues found in rhamnogalacturonan II. Rare sugars such as apiose, 2-O-methyl fucose and 2-O-methyl xylose are present in the polysaccharide (Darvill et al.,1978). A previously unidentified glycosyl residue present in rhamnogalacturonan II has been characterised by Spellman et al.(1983) and has been shown to be 3-C-carboxyl-5 deoxy-L-xylose. It has been proposed that this monosaccharide be named aceric acid.

Gel filtration analysis has indicated that there are approximately 50 sugar residues in the polysaccharide (Darvill et al., 1978). The structure of the molecule is not known, however, Darvill et al. (1978) demonstrated, using methylation analysis, that many of the residues are terminal. This indicates that it is a highly branched polymer. In order to elucidate the structure of rhamnogalacturonan II, it has been subjected to selective acid hydrolysis of the glycosidic linkages of the apiosyl residues (Spellman et al., 1983 b) This resulted in the isolation of a heptasaccharide with the following structure:-

α-L-Rha(1-2)α-L-Ara(1-4)α-D-Gal(1-2)β-L-AceA(1-3)β-L-Rha(1-3)Api
2

α-L-Fuc

However, no galacturonic acid residues are present in the oligosaccharide and it is possible that the heptasaccharide unit is present as a side-chain onto a 4-linked galacturonan backbone.

Homogalacturonan:-

Homogalacturonan regions can also occur in suspension - cultured sycamore cell walls. McNeil et al. (1980) demonstrated that endopolygalacturonase releases polysaccharide material composed almost entirely of galacturonic acid residues. Studies on suspension-cultured cell walls of *Rosa glauca* have also demonstrated the isolation of a homogalacturonan (Chambat and Joseleau, 1980).

Methylation analysis experiments indicated that the galacturonyl residues are linked by $\alpha(1\text{-}4)$ bonds. The polymer is thought to have a degree of polymerisation greater than 100.

The degree of polymerisation of all three polysaccharides has been estimated using gel filtration chromatography. However, the values given are only approximate. The three polysaccharides were obtained following treatment with endopolygalacturonase and extraction with this enzyme may cause partial degradation of the polysaccharides.

Neutral Fraction.

Neutral polysaccharides containing L-arabinose and D-galactose units are always present in the pectic fraction (Barrett and Northcote, 1965). Pectic arabinogalactans are generally thought to be composed of a chain of $\beta(1\text{-}4)$ galactopyranose residues onto which side-chains composed of $\alpha(1\text{-}3)$ and $\alpha(1\text{-}5)$ arabinofuranose units are linked. The ratio of galactose to arabinose residues varies according to the species from which the pectic arabinogalactan was obtained (Darvill et al., 1980). Although pectic arabinogalactans have been isolated from a variety of tissues, it has not yet been established whether separate arabinans and galactans occur in cell walls.

Stoddart et al.(1967), working on sycamore tissue, demonstrated that in actively dividing cells, the pectic fraction is strongly acidic and only traces of neutral glycosyl residues are present. However, in more mature cell walls, side-chains composed of arabinose and galactose units are linked to the rhamnogalacturonan chain (Barrett and Northcote, 1965). Stoddart et al. (1967) concluded that the composition of pectic substances varies according to the stage of development of the cell wall and this may be directly related to the function of pectin in the cell wall.

Stoddart and Northcote (1967) have investigated the metabolic interactions of the various components of the pectic substances using sycamore tissue. Pulse-chase experiments using radioactive arabinose demonstrated that initially the neutral arabinogalactan polymer was the only component to become labelled. However, if the pectic substances were analysed following a longer time period, a weakly acidic fraction was labelled with radioactivity. This weakly acidic fraction is thought to be analogous to the rhamnogalacturonan chain with side-chains of galactose and arabinose covalently linked. These results suggest that the neutral constituent of pectin, the arabinogalactan, is related to the side-chains of galactosyl and arabinosyl units which are covalently linked to the rhamnogalacturonan chain in the more mature cell walls (Stoddart and Northcote, 1967).

The way in which polysaccharides are linked in the cell wall has also been investigated. Work on the pectic substances has revealed that these polysaccharides are linked via a variety of different types of bonds, including glycosidic bonds, H-bonds, calcium bridges and phenolic bridges (Fry, 1986). Knee (1978) demonstrated that some pectin can be extracted from the cell wall using cold water, therefore, these polysaccharides can not be covalently bound.

The addition of chelating agents is commonly employed to extract the pectic polysaccharides. It is thought that the polysaccharides are extracted by these compounds due to the presence of ionic bonds between the carboxyl groups of the galacturonic acid residues and calcium ions. Due to their divalent nature, calcium ions will bind to two carboxyl groups resulting in calcium bridges cross-linking galacturonan chains. Jarvis (1982) demonstrated that a substantial proportion of the pectic substances was extracted by cyclohexanediamine tetraacetic acid (CDTA)

at room temperature. This indicates that calcium bridges do occur *in vivo*. It has also been demonstrated by Thom *et al.* (1982) that cross-linking of pectic polysaccharides occurs *in vitro* in the presence of calcium ions. However, highly methylesterified galacturonans cannot form these linkages due to the methyl group on carbon - 6.

Cross-linking of pectic polysaccharides may also occur by oxidative coupling of their phenolic substituents (Fry, 1986). Cell walls isolated from suspension cultures of spinach contain ferulic acid (Fry, 1982). Experiments have indicated that at least 60% of the ferulate present in the cell walls is linked to either galactose or arabinose (Fry, 1982). Two feruloyl disaccharides of the following structures were isolated:-

3-O (3-O-feruloyl-α-L-arabinopyranosyl)-L-arabinose

4-O (6-O-feruloyl-β-D-galactopyranosyl)-D-galactose

It was concluded from further work by Fry (1983) that the arabinose and galactose units linked to the feruloyl residues belong to the pectic substances. It is possible that peroxidases present in the wall catalyse oxidative coupling resulting in cross-linking of pectic polysaccharides via diferulate bridges (Fry, 1986).

The pectic substances have been the subject of much research in recent years. Two main areas of research are in progress at the moment. The first is concerned with the role of the pectic polysaccharides in host-pathogen interactions. When a plant is subjected to attack by micro-organisms, phytoalexins are produced. It is thought that compounds known as elicitors are responsible for phytoalexin production.

Albersheim and his co-workers have studied the structure of the pectic polysaccharides. The complex structure of the pectic polysaccharides led Albersheim to investigate the possibility of other functions for these polysaccharides apart from a structural role. PIIF (Proteinase Inhibitor Inducing Factor) is an elicitor which regulates the synthesis and accumulation of two proteinase inhibitors. Ryan et al. (1981) have demonstrated that fragments of endopolygalacturonase - treated cell walls and PIIF are similar in structure. Rhamnogalacturonan I has been shown to contain PIIF activity.

It has been demonstrated from studies using soybean hypocotyls (Hahn et al., 1981) that a fragment of a pectic polysaccharide is an elicitor for phytoalexin accumulation. Experiments by Nothnagel et al. (1983) have indicated that the elicitor is composed of $\alpha(1\text{-}4)$ linked galacturonic acid and is 12 sugar residues in length. However, it may be that in vivo the elicitor is a slightly modified oligogalacturonide.

Studies by West and his co-workers have demonstrated that polygalacturonase activity elicits casbene synthetase activity in castor bean seedlings. Casbene is thought to function as a phytoalexin in castor bean (Bruce and West, 1982; West et al., 1982). It appears that pectic cell wall fragments released by the action of endopolygalacturonase function as elicitors for casbene synthesis (Bruce and West, 1982). Jin and West (1983) demonstrated that, for elicitor activity, fragments of poly-galacturonic acid at least 10 or 11 units in length are required. Therefore, it is possible that the pectic polysaccharides have a defensive role against invasion by micro-organisms.

The second main area of research is concerned with the role of cell wall degrading enzymes during the ripening of fruit. Much of the research has concentrated on tomato, and experiments have demonstrated that during ripening there is an increase in soluble polyuronide

(Gross and Wallner, 1979). This is thought to be due to the action of polygalacturonase enzymes which increase in activity during ripening (Poovaiah and Nukaya, 1979). Further investigations using different fruits have revealed that other cell wall degrading enzymes such as cellulase may also be involved in the fruit ripening process (Ben-Arie et al., 1979; Awad and Young, 1979).

Biosynthesis of Cell Wall Polysaccharides.

Less work has been completed concerning the biosynthesis of cell wall polysaccharides compared to that concerned with the structure of polysaccharides. Nucleoside diphosphate sugars are known to be intimately involved in the biosynthesis of cell wall polysaccharides. Sugar nucleotides have a wide distribution in nature (Hassid et al., 1959).

Investigations studying the biosynthesis of non-cellulosic wall polysaccharides have demonstrated that uridine-diphosphate sugars are the most common glycosyl donors (table 1.3.). However, other sugar nucleotides also play a role in polysaccharide biosynthesis. Work on the biosynthesis of the mannans has revealed GDP-mannose and GDP-glucose as the substrates (Villemez, 1971).

Sugar nucleotides can be synthesised from the monosaccharide, ATP and the corresponding nucleoside triphosphate. Glucose-1-phosphate and sucrose can also act as precursors to all five glucose nucleotides.

Enzymic interconversion of the uridine-diphosphate sugars can occur via the pathway shown in figure 1.2. The enzymes which catalyse these reactions are widely distributed in plants.

Myo-inositol may also act as a precursor to some of the sugar nucleotides (Loewus et al., 1962; Roberts and Loewus, 1966). Enzymic cleavage of the myo-inositol ring results in the formation of the acidic and pentose-

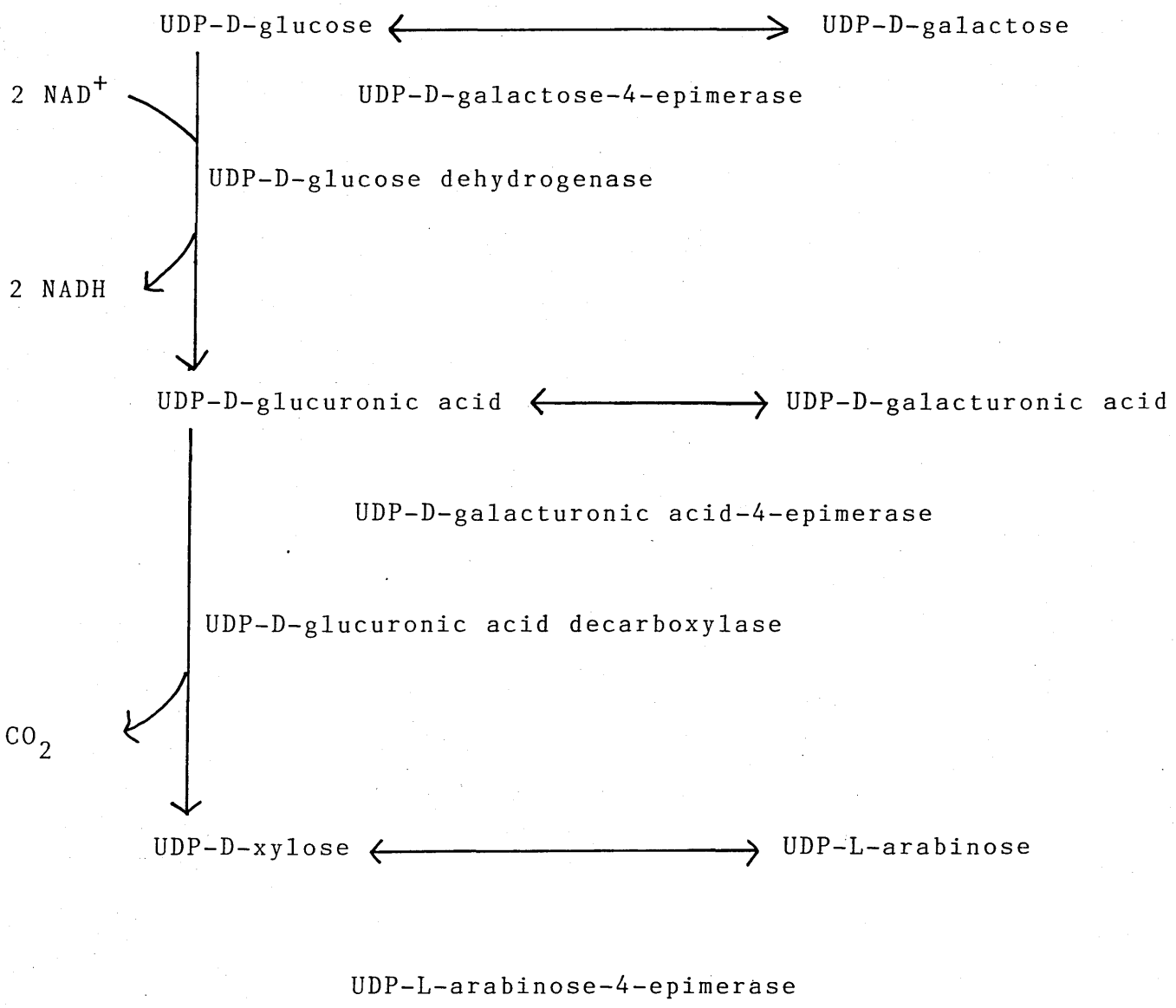
TABLE 1.3.

Nucleotide sugars used as glycosyl donors for the synthesis of the non-cellulosic cell wall polysaccharides.

Reference	Substrate	Product
Panayotatos and Villemez (1973)	UDP - galactose	Galactan
Villemez *et al.* (1965)	UDP-galacturonic acid	Galacturonan
Odzuck and Kauss (1972)	UDP-arabinose	Arabinan
Waldron and Brett (1983)	UDP-glucuronic acid	Glucuronoxylan
Ray (1980)	UDP-xylose	Xyloglucan
Villemez (1971)	GDP-mannose	Glucomannan
Villemez (1971)	GDP-glucose	Glucomannan
Ben-Arie *et al.* (1973)	UDP-xylose	Glucuronoxylan
Feingold *et al.* (1958)	UDP-glucose	β(1-3) Glucan

Figure 1.2

Enzymic interconversion of the UDP-sugars



containing sugar nucleotides.

The enzymes involved in the synthesis of cell wall polysaccharides are known as glycosyltransferases. Glycosyltransferases catalyse the transfer of the glycosyl unit from the sugar nucleotide to an acceptor molecule, and this leads to the formation of a polysaccharide (Hassid, 1967). Glycosyltransferases are generally recovered in the particulate fraction of the cell indicating that they occur in association with membranes. The enzymes are either present in membrane-bound organelles or are actually bound to membranes.

The involvement of lipid intermediates in the biosynthesis of cell wall polysaccharides is still uncertain. Anderson et al.(1965) have demonstrated the presence of a glycolipid intermediate in the biosynthesis of bacterial cell wall polysaccharides. In this system, the individual sugars are transferred from the sugar nucleotides to the lipid, therefore the polymer is assembled on a lipid carrier. Kauss (1969) has isolated a particulate enzyme from Phaseolus aureus which catalyses the formation of a mannosyl lipid from GDP-mannose. The mannosyl lipid is easily hydrolysed, indicating that it has a similar group potential to GDP-mannose. However, this is not conclusive evidence that there is the involvement of a lipid intermediate in polysaccharide biosynthesis as this compound may be involved in glycoprotein synthesis. The involvement of lipid intermediates in glycoprotein biosynthesis is well established and it has been shown that oligosaccharides linked to a lipid moiety can act as substrates in the biosynthesis of glycoproteins (Parodi and Leloir, 1979). Therefore, there is evidence that plant membranes have the ability to transfer the glycosyl unit from the corresponding sugar nucleotide to lipid-linked oligosaccharides. There is, however, no evidence to suggest that these lipid-linked oligosaccharides function in the biosynthesis of cell wall

polysaccharides (Maclachlan,1985).

Little attention has been given to the possibility of the involvement of a proteinaceous intermediate in the biosynthesis of the non-cellulosic cell wall polysaccharides. The outer root cap cells of maize secrete a mucus and much work has been completed using this system. The mucus consists of polysaccharides and analysis of the polysaccharides has revealed that they are similar in composition to the pectic polysaccharides (Wright and Northcote, 1974). Further work by Green and Northcote (1978) has indicated that these polysaccharides are synthesised attached to proteins. An investigation into the synthesis of starch by Tandecarz et al. (1975) indicated that a glycoprotein may be involved in this process also. Therefore, it is possible that glycoproteins may be involved as intermediary compounds in the biosynthesis of cell wall polysaccharides.

Glucuronoxylan biosynthesis has been studied in detail by Waldron and Brett (1985). There is evidence that a water-soluble product (thought to be glucuronoxylan) is attached to protein. Therefore, a protein may function as a primer in cell wall biosynthesis.

The matrix polysaccharides of the cell wall are synthesised within the cell, therefore secretion of these molecules to the wall must occur (Northcote, 1985). Different techniques have been employed to determine the site of synthesis of these polysaccharides. Auto-radiography experiments on root tips of wheat using radioactively-labelled glucose indicated that the golgi apparatus functions in the biosynthesis of polysaccharides. Pulse-chase experiments demonstrated that the radioactively-labelled material was transferred to the wall via golgi-derived vesicles (Northcote and Pickett-Heaps, 1966). Bowles and Northcote (1972) investigated the route of ^{14}C-glucose in maize root cap cells and, from their experiments, they also concluded

that the golgi apparatus functions in the biosynthesis of the non-cellulosic cell wall polysaccharides.

Ray has investigated the location of glycosyltransferases. A dictyosome fraction isolated by Eisinger and Ray (1972) demonstrated glycosyltransferase activity. The polysaccharides formed by this fraction were reported to be similar in composition to the pectic substances and the hemicelluloses. The two enzyme systems, xylosyltransferase and glucosyltransferase, involved in xyloglucan biosynthesis, have both been found associated with the golgi dictyosomes (Ray, 1980). Waldron and Brett (1987), investigating the biosynthesis of glucuronoxylan, demonstrated that glucuronyltransferase activity is also associated with the golgi dictyosome fraction. Bolwell and Northcote (1983) have demonstrated that xylosyltransferase activity involved in xylan synthesis and arabinosyltransferase activity involved in arabinan synthesis are associated with the endoplasmic reticulum as well as the golgi apparatus. However, experiments in vivo using bean suspension cultures have indicated that the synthesis of these polymers is confined mainly to the golgi apparatus (Bolwell and Northcote, 1983). Therefore, it can be concluded that the golgi apparatus is involved in the biosynthesis of the matrix polysaccharides, however, the involvement of the endoplasmic reticulum in this process is uncertain at the moment. It may be that the endoplasmic reticulum is important in the initiation of polysaccharide biosynthesis (Bolwell and Northcote, 1983).

The incorporation of pectin, hemicellulose and cellulose into the cell wall varies according to the stage of development of the cell. During the formation of the cell wall, pectic substances are in demand. The primary wall requires the incorporation of pectin, hemicellulose and cellulose microfibrils, whereas secondary wall formation requires the incorporation of hemicellulose and cellulose. Therefore, the cell

wall polysaccharides incorporated into the wall vary according to the stage of development of the cell.

Dalessandro and Northcote (1977 a, b) investigated the enzymes involved in the interconversion of sugar nucleotides to determine whether they reflected the changes seen in the synthesis of the cell wall polysaccharides during development. The results demonstrated that the enzymic activities only vary slightly during differentiation, therefore the activities of these enzymes are not responsible for the immediate regulation of polysaccharide biosynthesis. Throughout growth, the cell retains its ability for the interconversion of of UDP-sugars (Dalessandro and Northcote, 1977 a).

Recent studies have concentrated on the activity of the glycosyltransferase enzymes for regulation of pectin and hemicellulose biosynthesis. Bolwell and Northcote (1981), working on bean hypocotyls and bean callus tissue, demonstrated that xylosyltransferase activity increased at the onset of differentiation, whereas arabinosyltransferase activity increased during cell division and cell growth. Galacturonyltransferase activity has also been determined in cambial cells, differentiating xylem cells and differentiated xylem cells isolated from sycamore trees (Bolwell et al., 1985). The results indicated that there is a significant decrease in galacturonyltransferase activity which can be correlated with the cessation of the incorporation of the pectic substances. Therefore, it appears likely that the biosynthesis of the non-cellulosic cell wall polysaccharides is regulated at the glycosyltransferase stage.

Biosynthesis of Galacturonan.

Villemez et al. (1965) reported that a particulate enzyme preparation obtained from Phaseolus aureus seedlings catalyses the incorporation of galacturonic acid from UDP-galacturonic acid into polygalacturonic

acid. It was further demonstrated by Villemez et al. (1966) that the enzyme system from Phaseoulus aureus is specific for UDP-galacturonic acid. It was reported that there was no incorporation of D-galacturonic acid residues from the other ribonucleoside diphosphate D-galacturonic acid derivatives. However, investigations by Lin et al. (1966) reported that a particulate enzyme preparation from tomatoes had the ability to utilise both UDP-galacturonic acid and TDP-galacturonic acid as donors of galacturonic acid for polygalacturonic acid biosynthesis, although UDP-galacturonic acid is the more efficient donor.

The possibility of UDP-methyl-D-galacturonic acid acting as a precursor of methyl polygalacturonate has also been investiaged by Villemez et al. (1966). However, there was no significant incorporation of methyl-D-galacturonic acid. It is evident, therefore, that methyl esterification of polygalacturonic acid occurs after formation of the chain.

Experiments by Sato et al. (1958) using radish plants demonstrated that the methyl ester groups on the polygalacturonic acid chain can be formed by a transmethylation reaction using methionine as the methyl donor. Wu and Byerrum (1958) continued these investigations and reported that formate, formaldehyde,glycine, and, to a lesser extent, serine could all act as precursors for methyl groups to polygalacturonic acid. A study using parsley leaves (Roberts et al., 1967) indicated that methanol could also be utilised. It was concluded that these compounds feed into a common C-1 pool and that S-adenosine-L-methionine is the immediate donor of methyl groups (Kauss, 1974). A particulate enzyme preparation, very similar to the preparation used for the synthesis of polygalacturonic acid, contains an enzyme capable of catalysing the transfer of the methyl group of S-adenosine-L-methionine to polygalacturonic

acid (Kauss and Hassid, 1967).

Therefore, these results demonstrate that the biosynthesis of methylpolygalacturonic acid involves the action of two enzymes - a galacturonyltransferase which is involved in the formation of the polygalacturonic acid chain and the subsequent action of a methyltransferase which transfers methyl groups from S-adenosine-L-methionine to the polygalacturonic acid chain.

Further investigations by Kauss et al. (1969) revealed that the addition of pectin methylesterase to the particulate enzyme preparation containing methylpolygalacturonan did not result in the liberation of methyl groups. However, addition of detergent, which causes disruption of the integrity of membranes, results in the liberation of methyl ester groups. This occurs even if no methylesterase is added, indicating that there is endogenous pectin methylesterase. These results show that the enzymes necessary for the formation of methylpolygalacturonan and the product itself are enclosed in a lipid complex.

No work has been published regarding the possible role of rhamnose nucleotides in the biosynthesis of rhamnogalacturonan.

Solubilisation of Membrane Proteins.

The enzymes involved in the biosynthesis of the pectic substances and the hemicellulosic polysaccharides are isolated in the particulate fraction, and therefore occur in association with membranes. Membranes consist mainly of lipids and proteins, however the proportion of protein to lipid varies depending on the metabolic activity of the membrane. The current model for the structure of membranes is the fluid mosaic model. In this model, the phospholipids form a bilayer with the polar groups facing outwards and the hydrophobic chains facing towards the centre. Two main categories of membrane proteins exist - these are

extrinsic and intrinsic proteins. Extrinsic proteins are bound by ionic interactions and can be removed from the membrane either by chelating agents or by altering the ionic strength or the pH. However, intrinsic proteins interact with the hydrocarbon chains of the membrane lipids and can only be solubilised by disruption of the lipid bilayer.

The solubilisation of membrane proteins can be achieved using detergents. Detergents are compounds which contain both hydrophobic and hydrophilic regions. Due to their amphiphilic nature, in aqueous medium, detergent molecules form micelles when the concentration of detergent is greater than the critical micelle concentration and the temperature is above the critical micelle temperature. Below these values, the detergent molecules exist as monomers. Micelles are colloidal aggregates in which the non-polar regions of the molecules are sequestered into the centre and the polar groups face outwards. The size of the micelle varies according to the detergent. Detergents are able to solubilise proteins because their ability to form micelles enables them to simulate the native environment of the protein. However, the ability of proteins to retain their biological activity on the addition of detergents is variable. Although some proteins only require the interface between aqueous medium and a region of hydrocarbon, it appears that to retain the biological activity of other proteins, specific head groups or specific alkyl chains are needed (Tanford and Reynolds, 1976).

The structures of the detergents used in this project are shown in figure 1.3. The detergents used can be divided into three main categories:-

1. Alkyl ionic detergents
2. Non-ionic detergents
3. Steroidal detergents

Ionic detergents:- Ionic detergents possess charged groups as well as a hydrophobic region. Ionic detergents tend to denature proteins as they can bind onto proteins resulting in conformational changes and this often leads to the loss of biological activity. Common ionic detergents include sodium dodecylsulphate (SDS), cetyltrimethylammonium bromide (CETAB) and lauryldimethylamine-N-oxide (LDAO).

Non-ionic detergents:- Although the detergents in this group do not contain any charged groups, both polar and non-polar regions occur in the detergent molecules. Non-ionic detergents have been used successfully to solubilise some membrane proteins. Detergents belonging to the Triton series and the Brij series are examples of non-ionic detergents.

Steroidal detergents:- Steroidal detergents are considerably different structurally from the other types of detergents. They have a steroid ring structure and do not contain clearly separated polar and non-polar regions. The bile salts and digitonin are included in this group of detergents.

To date, two enzyme systems involved in cell wall biosynthesis have been solubilised using the detergent Triton X-100. Heller and Villemez (1972) reported the solubilisation of a mannosyltransferase which catalyses the incorporation of mannose from GDP-mannose into a mannan. The solubilised enzyme preparation was obtained by extracting the particulate enzyme preparation with Triton X-100. A glucuronyltransferase enzyme involved in glucuronoxylan biosynthesis has been solubilised by Waldron (1984) using 1% Triton X-100.

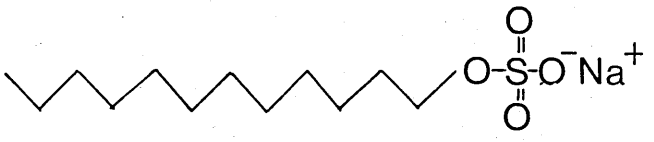
Solubilisation is the first main step in purifying particulate enzyme preparations. Once the enzyme system is solubilised, it can be purified further using techniques such as gel filtration chromatography, ion-exchange chromatography and gel electrophoresis.

Figure 1.3

Structures of detergents

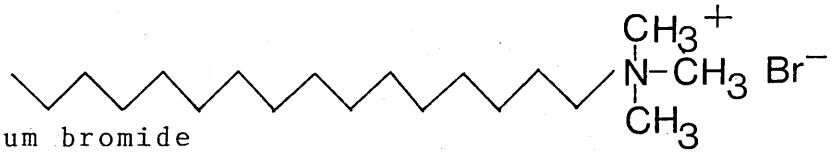
SDS

Sodium dodecylsulphate



CETAB

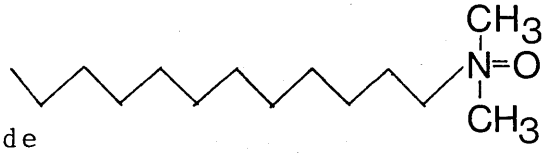
Cetyltrimethylammonium bromide



LDAO

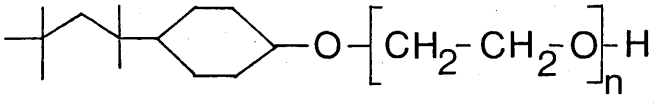
N-N-Dimethyldodecylamine oxide

(Lauryldimethylamine-N-oxide)



Triton X Series

Polyoxyethylene p-t-octyl phenol



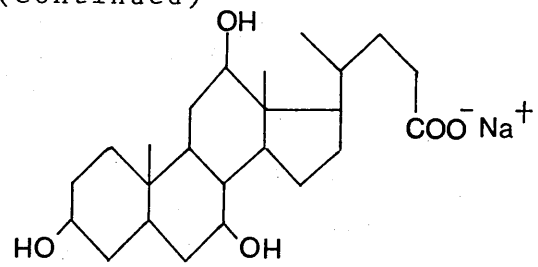
Brij Series

Polyoxyethylene alcohol

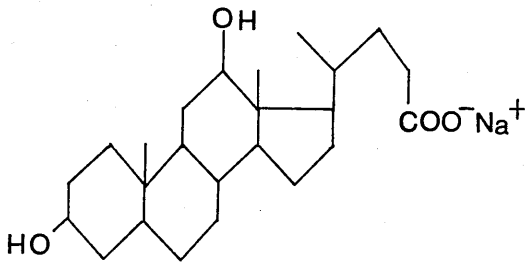
Figure 1.3

Structures of detergents (continued)

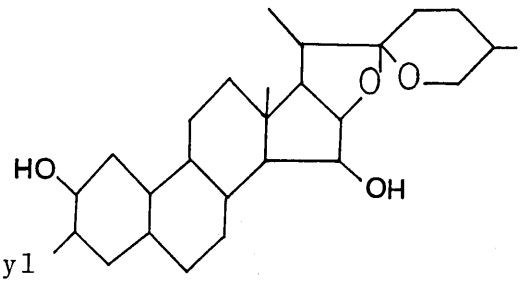
Cholate



Deoxycholate



Digitonin



Glu-Glu-Gal-Gal-Xyl

Introduction to experimental work

The aim of this project was to investigate the biosynthesis of the galacturonan polysaccharides. It was hoped to investigate many aspects of the enzyme galacturonyltransferase including the distribution of galacturonyltransferase activity in the epicotyl, the solubilisation of the enzyme and, also, the possibility of co-operation occurring between galacturonyltransferase and rhamnosyltransferase.

Previous work on *Phaseolus aureus* has indicated the presence of a galacturonyltransferase enzyme which is capable of transferring the galacturonic acid unit from UDP-galacturonic acid into polygalacturonic acid (Villemez *et al.*, 1965). Little work has been completed on this enzyme system and this may be due to UDP-^{14}C- galacturonic acid not being available from a commercial source. Therefore, initially it was necessary to prepare UDP-^{14}C-galacturonic acid using UDP-^{14}C-glucuronic acid as the substrate. The preparation of ^{14}C-labelled UDP-galacturonic acid made it possible to study the biosynthesis of the galacturonan polysaccharides.

Chapter 2

MATERIALS AND METHODS

Chemicals and column materials.

Radioactive - labelled compounds, UDP-[U-^{14}C]- glucuronic acid (10.58 GBq mmol $^{-1}$) and ^{14}C-sucrose (20.72 GBq mmol $^{-1}$), were purchased from Amersham International, England. Sugars, sugar nucleotides and nucleoside - phosphomorpholidates were obtained from Sigma Chemical Company, U.S.A. All the Sephadex and Sepharose products were obtained from Pharmacia Fine Chemicals, Sweden. The ion-exchange resins used (Amberlite resin IRA-400, Amberlite resin IR 120 and Dowex 50 W - X 8) were all purchased from BDH Chemicals, England. Cellulose powder was purchased from Macherey-Nagel, Germany. Molecular sieve 4A was supplied by BDH Chemicals, England. Celite was purchased from Sigma Chemical Company, U.S.A. Triton X-100 and digitonin were both purchased from Koch Light Limited, England. SDS, CETAB and Brij - 35 were obtained from BDH Chemicals, England. Deoxycholic acid was supplied by Sigma Chemical Company, U.S.A., and LDAO was purchased from Fluka A G, Switzerland. Pectinase, proteinase K and protease were all supplied by Sigma Chemical Company, U.S.A.

Germination of peas.

Seeds of Pisum sativum, variety Alaska, obtained from Sinclair McGill (Ayr, Scotland) were soaked overnight in tap water, planted in trays containing vermiculite and grown in darkness for 6-7 days at 22 ± 1 °C.

Preparation of UDP-[U-^{14}-C]- galacturonic acid.

The procedure used to convert UDP-glucuronic acid to UDP-galacturonic acid is described by Feingold et al.(1960). All operations were carried out at 0-4°C. The epicotyls of the etiolated seedlings were harvested and cut into 2 cm sections. The tissue (25g) was homogenised with 100 ml 10mM sodium-potassium phosphate buffer, pH 7.0, using a Polytron homogeniser (PCU2, Kinematica). The material was homogenised twice at speed 3 for 10 seconds. The homogenate was filtered through 4

layers of muslin, and then centrifuged at 3000g for 5 minutes in a MSE 18 High Speed Centrifuge. The supernatant was then centrifuged at 18000g, and the resulting pellets were resuspended in 0.3 ml Tris (hydroxymethyl)-aminoethane (Tris) buffer (100 mM) adjusted to pH 7.5 with HCl.

The incubation mixture consisted of the particulate enzyme preparation (15 µl) and UDP-[U-^{14}-C]- glucuronic acid (0.185 MBq) resulting in a total volume of 30 µl . The mixture was incubated at 37°C for 20 minutes. The reaction was terminated by the addition of 70% (v/v) ethanol (150 µl) and then centrifuged in an Eppendorf Microfuge at 10000 g for 3 minutes. UDP-^{14}C-galacturonic acid was separated from UDP- ^{14}C-glucuronic acid and other products of the reaction by thin layer electrophoresis (T.L.E.) in pyridine : acetic acid : H_2O (0.1 : 1 : 9 v/v) at 300 volts for 4 hours. The T.L.E. plate was cut into 50 x 5 mm strips and the strips were then analysed for radioactivity. The peak of radioactivity co-incident with UDP-galacturonic acid was recovered.

Enzyme preparations.

1. Particulate enzyme preparation:- All operations were conducted at 0-4°C. Epicotyls of etiolated pea seedlings were cut into sections approximately 2 cm in length. The tissue (25g) was homogenised in 100 mls 2(N-morpholino)-ethanesulphonic acid (Mes) buffer (25mM), adjusted to pH 6.0 using NaOH, and containing 1% (w/v) bovine serum albumin (BSA) plus 5mM dithiothreitol (DTT), using a Polytron homogeniser - two bursts at Speed 3 for 10 seconds. The homogenate was filtered through 4 layers of crossed muslin. The filtrate was centrifuged at 97000 g for 30 minutes in a Sorvall Ultracentrifuge OTD-65B using an AH 627 swing-out rotor. The pellets were resuspended in the homogenisation buffer using a glass homogeniser and the

suspension was centrifuged again at 97 000 g for 30 minutes using the Sorvall Ultracentrifuge with the AH-627 swing-out rotor. The two pellets were resuspended in a total volume of 1 ml homogenisation buffer.

2. Solubilised enzyme preparation:- The procedure for the isolation of the particulate enzyme preparation was followed using 50g epicotyl tissue and 200 ml 25 mM Mes-Na buffer, pH 6.0, containing 1% (w/v) BSA and 5mM DTT. After the second centrifugation step, the resulting pellets were resuspended in homogenisation buffer containing detergent (10 ml) using a glass homogeniser. The suspension was centrifuged in the Sorvall Ultracentrifuge OTD-65B using a T865.1 fixed angle rotor at 285 000 g for 2 hours. The particulate material was resuspended in homogenisation buffer containing detergent (2 ml).

Incubations

1. Particulate enzyme preparation:- The standard incubation mixture consisted of 50 µl particulate enzyme preparation, UDP-^{14}C galacturonic acid (90 Bq) and 10 mM $MnCl_2$ resulting in a total volume of 100 µl. Incubations were carried out at 25°C for 10 minutes. Any variation of the standard incubation mixture is described in the text. The reactions were terminated by the addition of 70% (v/v) ethanol (1 ml) and then centrifuged in the Eppendorf Microfuge at 10 000 g for 3 minutes.

2. Solubilised enzyme preparation:- To test for enzymic activity in the supernatant, incubations consisted of 80 µl solubilised enzyme preparation, UDP-^{14}C-galacturonic acid (280 Bq) and 10 mM $MnCl_2$ in a total volume of 100 µl. The pellet was also tested for galacturonyltransferase activity, and the incubations consisted of 50 µl particulate enzyme preparation, UDP-^{14}C-galacturonic acid (90 Bq) and 10 mM $MnCL_2$ in a total volume of 100 µl.

The incubations were carried out at 25°C for 30 minutes. The reactions were terminated by the addition of 70% (v/v) ethanol (1 ml), and then either cellulose powder or boiled membrane preparation was added as a carrier. The incubations were left overnight, and then centrifuged at 10 000 g for 3 minutes in the Eppendorf Microfuge.

Isolation of general polysaccharide fraction

The pellets were washed three times in 70% (v/v) ethanol (1 ml). The ethanol-insoluble material was extracted twice with H_2O (0.5 ml) at 100°C for 15 minutes. The residue was taken as the general polysaccharide fraction.

Pectin extraction

Pectin was extracted following the procedure of Stoddart et al. (1967). The general polysaccharide material was extracted using buffer containing 50 mM ethylenediaminetetra-acetic acid (EDTA) and 50 mM NaH_2PO_4 at pH 6.8. Extractions were carried out twice using 0.5 ml 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, at 100°C for 15 minutes, and the corresponding supernatants were combined.

Cadoxen extraction

The procedure used to prepare Cadoxen is based on the method of Wood and McCrae (1978). Cadoxen was prepared by stirring ethylene-diamine (28g), cadmium oxide (10g) and H_2O (72 ml) for 3 hours at room temperature, and then at 4°C for 18 hours. The supernatant was decanted and this solution is Cadoxen.

The material remaining following the pectin extraction was extracted with Cadoxen (0.5 ml) at 25°C for 30 minutes. This procedure was repeated and the corresponding supernatants were combined.

Isolation of polyprenylphosphate-sugars

To investigate the possibility of lipid intermediates in the biosynthesis of galacturonan, polyprenylphosphate-sugars were extracted as described by Brett (1981). Incubations containing the particulate enzyme preparation, UDP-^{14}C-galacturonic acid (180 Bq) and 10 mM $MnCl_2$ were carried out for 10 minutes. The reactions were terminated by the addition of 2 ml $CHCl_3$: CH_3OH (3 : 2 v/v), resulting in a mixture containing $CHCl_3$: CH_3OH : H_2O in the ratio of 12 : 8 : 1 (v/v), and then centrifuged at 10 000 g for 3 minutes in the Eppendorf Microfuge.

Initially, the supernatant was taken and 4 mM $MgCl_2$ (400 µl) was added to give a solution with a composition of $CHCl_3$: CH_3OH : H_2O of 12 : 8 : 5 (v/v). The solution separated into two phases at this stage - sugars and nucleoside diphosphate sugars were present in the upper phase whereas polyprenylphosphate-sugars would be present in the lower phase. The lower phase was recovered and, to remove any H_2O-soluble compounds, it was washed three times with Folch theoretical upper phase solution ($CHCl_3$: CH_3OH : H_2O : $MgCl_2$ (1 M) = 18 : 294 : 282 : 1 v/v) (Brett, 1981). As the washed lower phase would contain any polyprenylphosphate-sugars, the solution was dried under nitrogen and then analysed for radioactivity.

The precipitate was also taken as any polyprenylpyrophosphate-oligosaccharides would be present in this fraction. The precipitate was washed twice with $CHCl_3$: CH_3OH (2 : 1 v/v) and then once with CH_3OH. The material was then washed five times with H_2O to remove any H_2O-soluble material. The polyprenylpyrophosphate-oligosaccharides were extracted with two washes of $CHCl_3$: CH_3OH : H_2O (10:10:3 v/v). The extracted material was dried under nitrogen, and then analysed for radioactivity. The pellet remaining after the second extraction was also analysed for the presence of radioactive material.

Total acid hydrolysis

The material to be hydrolysed was placed in a sealed Reacti-vial (Pierce, Pierce Chemical Company, USA) and trifluoroacetic acid (TFA) was added to give a concentration of 2M TFA. Hydrolysis was conducted in an autoclave at 120°C for 1 hour. The hydrolysate was dried under vacuum overnight in the presence of NaOH pellets and then redissolved in the required volume of H_2O.

Polygalacturonase treatment

Pectinase (Sigma Chemical Company, USA) was dissolved in 100 mM sodium acetate buffer, pH 4.0, to give a solution of 1 $mg.ml^{-1}$ concentration. The material to be treated was incubated with the pectinase preparation (0.5 ml) for 21 hours at 25°C. As a control, 100 mM sodium acetate buffer, pH 4.0, (0.5 ml) was added to identical material. To terminate the reactions, the samples were incubated at 100°C for 10 minutes.

Purification of the pectinase preparation

In order to purify the pectinase preparation, a solution of 12 $mg.ml^{-1}$ concentration was run through a column of Sepharose 6B-CL (270 mm x 10 mm) which was equilibrated with 100 mM sodium acetate buffer, pH 4.0, and 25 fractions of 1 ml volume were collected. To test for polygalacturonase activity, 0.5 ml of each fraction was added to 1% (w/v) polygalacturonic acid dissolved in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, (1.5 ml). The viscosity of the solution was measured using a Volac semi-microviscometer after 0 and 10 minutes. Enzymic activity was measured by a reduction in the viscosity of the solution. The fractions containing polygalacturonase activity were combined to give the purified pectinase preparation.

Thin layer electrophoresis

Thin layer electrophoresis was employed to separate uronic acids and also sugar nucleotides. Marker sugars (10 µl, 10 $mg.ml^{-1}$) were also spotted onto the silica-gel coated plates (Polygram, Macherey-Nagel, Germany). Electrophoresis was conducted at 300 volts for 4 hours in pyridine : acetic acid : H_2O (0.1 : 1 : 9 v/v), pH 3.5. Location of sugars:- sugars and sugar nucleotides were located by spraying the plate with 0.5% (w/v) α-naphthol in methanol. The plate was dried at 70°C, then resprayed with 10% (v/v) H_2SO_4 in methanol, then dried again at 70°C. Sugars and sugar nucleotides were detected as blue/purple spots.

Location of radioactive samples : To analyse the plates for regions of radioactivity, the plate was divided into 5 mm x 50 mm strips. The strips were added to vials containing toluene scintillant (Harris and Northcote, 1970) and counted for radioactivity.

To recover radioactive samples, the strips were washed free of toluene scintillant by washing five times in toluene. The remaining toluene was removed by evaporation. The silica-gel was transferred into vials and washed in H_2O three times. The H_2O washings were combined and dried under nitrogen to give the radioactive material.

Paper chromatography

Separation of sugar nucleotides from sugar phosphates was achieved using descending paper chromatography on Whatman No. 1 paper. The solvent system (I) was composed of ethanol : ammonium acetate (1M) (7 : 3 v/v) and the chromatogram was run for 60 hours.

For the separation of glycerol, erythritol and galactose, solvent system (II) comprising of ethyl acetate : pyridine : H_2O (8:2:1 v/v) was used. This was performed by descending paper chromatography on

Whatman No.1 paper for 10-12 hours.

Identification of compounds :- To detect sugar nucleotides, the chromatogram was placed under an ultra-violet light (wavelength = 254 nm). Sugar nucleotides were visible as dark spots on a light background.

To detect sugar phosphates, the method of Hanes and Isherwood (1949) was used. The chromatogram was sprayed with a solution containing 60% (w/w) perchloric acid (5 ml), 1M HCl (10 ml), 4% (w/v) ammonium molybdate (25 ml) and H_2O (60 ml). The chromatogram was dried, and then exposed to an ultra-violet lamp (wavelength = 254 nm) for 15 seconds. Sugar phosphates were seen as yellow spots.

Sugars were detected using the method of Trevalyan et al.(1950). The paper chromatogram was initially run through a bath containing acetone saturated with silver nitrate. The paper was dried, then run through a bath containing 0.5% (w/v) NaOH in 96% (v/v) ethanol. Sugars were detected as brown spots.

Ion-exchange chromatography

Ion-exchange chromatography was conducted using a column (10 mm x 80 mm) of Amberlite resin IRA-400 in the acetate form. The extract was loaded onto the column and 2 column volumes of H_2O were run through to elute the neutral material and the cations. The anionic material was eluted with 2 column volumes of 0.1 M HCl. The neutral and cationic material was dried under reduced pressure, then re-dissolved in H_2O (1 ml). The anionic material was also dried under reduced pressure, and then left overnight in a dessicator with NaOH pellets to remove all traces of HCl. The material was redissolved in H_2O. To separate the cationic material from neutral material, a column (10 mm x 80 mm) of Amberlite resin IR 120 in the H^+ form was used. The material was loaded onto the column and the neutral

material was eluted with 2 column volumes of H_2O. To elute the cationic material, 2 column volumes of 0.1 M HCl were run through the column. The neutral material was dried, then redissolved in H_2O (1 ml). The cationic material was dried under reduced pressure, then left overnight in a dessicator to remove any HCl present. The material was then redissolved in H_2O (1 ml).

Gel filtration chromatography

Gel filtration chromatography was carried out at room temperature on a column composed of Sephadex G-100 (180 mm x 7.5 mm) which was equilibrated in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8. Twenty fractions of 0.5 ml volume were collected. To determine where molecules of different molecular weights were eluted, markers were run through the column. Blue dextran (M.W. = 2 000 000) was eluted between fractions 6 and 9 whereas 2,4 - dinitrophenyl - L - lysine (DNP-lysine) which has a molecular weight of 367, was eluted in fractions 13 - 20.

Gel filtration was also carried out in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, on a column of Sepharose 6B-CL (450 mm x 10 mm) at room temperature. Eighty fractions of 0.5 ml were collected. Markers were run through the column and blue dextran (M.W.= 2 000 000) was eluted in fractions 25 - 28 and DNP-lysine (M.W. = 367) in fractions 69-80.

A second column composed of Sepharose 6B-CL (270 mm x 10 mm) equilibrated with 100 mM sodium acetate buffer, pH4.0, was also used. Twenty-five fractions of 1 ml volume were collected. The markers, blue dextran and DNP-lysine, were eluted in fractions 8-10 and 20-24 respectively.

A column of Sepharose 2B-CL (450 mm x 10 mm) was also used for gel filtration chromatography. The column was equilibrated with 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, and was run at room temperature.

Fifty fractions of 2ml were collected. Blue dextran was eluted in fractions 14-34 and DNP-lysine in fractions 38-46.

Preparation of pectin

Etiolated pea epicotyls (50g) were boiled in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, (50 ml) for 10 minutes. The material was then homogenised using a mortar and pestle. The homogenate was filtered through 4 layers of muslin followed by filtration through Whatman No.1 filter paper. The supernatant was dialysed against H_2O for 5 days before use.

The pectin prepared from the epicotyls was subjected to acid hydrolysis. Four aliquots (212 µl) of pectin were hydrolysed with 2M TFA at 100°C for varying periods of time - 0, 1, 10 and 30 minutes. Following hydrolysis, the material was dried under vacuum, then redissolved in H_2O (100 µl).

The pectin obtained from the epicotyls was also freeze-dried using an Edwards freeze-dryer. The powder was redissolved in H_2O (1 ml).

Enzymic hydrolysis of polygalacturonic acid

Polygalacturonic acid (10 mg), supplied by Sigma Chemical Company, USA, was incubated with the pectinase preparation (1 ml) for 50 minutes at 25°C. The reaction was terminated by incubating at 100°C for 10 minutes. The supernatant was dried under vacuum, then redissolved in H_2O (1 ml).

Proteinase digest

Proteinase K and protease (both obtained from Sigma Chemical Company USA) were dissolved in 10 mM Tris-HCl buffer, pH 7.5, to give

solutions of 5 $mg.ml^{-1}$ concentration. The material to undergo proteinase digestion was resuspended in H_2O (0.5 ml) and incubated with proteinase K preparation (5 µl) or protease preparation (5 µl) for 3 hours at 25°C. The samples were incubated at 100°C for 10 minutes to terminate the reactions.

Extraction of the particulate enzyme preparation using 10% (w/v) Triton X-100

The procedure used to obtain the particulate enzyme preparation was followed using 75g epicotyls. Following the second centrifugation step, the pellets were resuspended in 25 mM Mes buffer containing 1% (w/v) BSA, 5 mM DTT and 10% (w/v) Triton X-100, pH 6.0 (14 ml). The suspension was centrifuged for 30 minutes at 97 000 g in the Sorvall Ultracentrifuge OTD-65B using the TST 41.14 swing-out rotor. The supernatant was recovered and frozen at -18°C until use.

Extraction of homogenate using buffer containing 1M NaCl

Pea epicotyls (40 g) were homogenised in 25 mM Mes buffer containing 1% (w/v) BSA, 5 mM DTT and 1M NaCl, pH 6.0, using a mortar and pestle. The homogenate was filtered through 4 layers of muslin and the filtrate was centrifuged at 97 000 g for 30 minutes in the Sorvall Ultracentrifuge OTD-65B using the TST 41-14 swing-out rotor. The supernatant was divided into two aliquots. One aliquot was frozen until use. The second aliquot was dialysed overnight against 25 mM Mes buffer containing 1% (w/v) BSA, 5 mM DTT, pH 6.0, and then frozen at -18°C.

^{14}C - Sucrose feeding experiment

Pea epicotyls were harvested and the sections 6 of the epicotyls were isolated (figure 5.1.). Forty sections were incubated with 92.5 K Bq sucrose (10 mM, 20 ml) in the dark at 25°C. Following

an incubation period of 2 hours, the sections were washed using unlabelled sucrose. The sections were then incubated in unlabelled sucrose (100 mM, 20 ml) for 0, 2 and 4 hours in darkness at 25°C. Following the chase incubation periods, the sections were homogenised in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, (1 ml) using a mortar and pestle. The pectic polysaccharides were then extracted by incubating the homogenised material at 100°C for 15 minutes. The material was then filtered through Whatman No.1 filter paper. The filtrate was dried by rotary evaporation and then analysed for incorporation of radioactivity.

In order to analyse the material into which ^{14}C was incorporated, the experiment was repeated. However, in this experiment, the sections were incubated in 3.7 MBq sucrose (10 mM, 20 ml) for 8 hours. The procedure used to extract the pectic polysaccharides was then followed and the resulting filtrate was dialysed against H_2O for 4 days. The material was rotary evaporated to dryness, and then hydrolysed using 2M TFA. Analysis of the products was carried out by thin layer electrophoresis.

Preparation of rhamnose nucleotides

The synthesis of rhamnose nucleotides was performed using an adaptation of the phosphomorpholidate procedure (Moffat, 1965). The method involves reacting rhamnose-phosphate with the appropriate nucleoside-5-phosphomorpholidate. The reaction results in the formation of a pyrophosphate bond, thereby forming the corresponding rhamnose nucleotide.

Initially, it was necessary to synthesise rhamnose-phosphate using rhamnose as the substrate. The first stage involved acetylating rhamnose using pyridine as a catalyst. (Wolfrom and Thompson,1963). Rhamnose (5 g) was added to anhydrous pyridine (30 ml) and the

suspension was cooled to 0°C. Acetic anhydride (26 ml) was then added dropwise. The suspension was stirred at 0°C for 2 hours and was then allowed to stand overnight at room temperature. The solution was added to a mixture of ice and H_2O (200 ml) and a slurry formed. The H_2O was removed by rotary evaporation leaving rhamnose tetra-acetate.

The procedure described by MacDonald (1972) was then followed to form rhamnose-phosphate. Phosphoric acid (15 g), dried over anhydrous magnesium percholate in a dessicator, was added to the rhamnose tetra-acetate and the material was rotated under diminished pressure for 2 hours at 50°C. The material, which was of a syrup consistency, was cooled and 2M lithium hydroxide (200 ml) at 0°C was added. The flask was shaken to disperse the syrup, then left overnight at 25°C. The precipitated lithium phosphate was removed by filtration through Celite. To remove lithium ions, the solution was run through a column (15 mm x 300 mm) of Dowex 50 W-X8 (H^+). Two column volumes of H_2O were passed through the column. The eluate was run into a stirred solution of 3.8 M KOH (50 ml). The pH of the resulting solution was adjusted to 9.5 using Dowex 50W-X8(H^+) resin. The resin was removed by filtration and the solution was concentrated by rotary evaporation. Absolute ethanol was added, and then removed by rotary evaporation six times. The residue was collected by centrifugation at 4000 g for 5 minutes using a MSE bench-top centrifuge. The rhamnose-phosphate was then dried in a dessicator for 2 days.

The phosphomorpholidate procedure was then followed. Rhamnose-phosphate (50 mg) and the appropriate nucleoside-5 - phosphomorpholidate (50 mg) were dried separately in a dessicator. Dimethylsulphoxide (2 ml), which had been dried using molecular sieve 4A, was added to each flask. The two suspensions were mixed

together, and anhydrous pyridine (1 ml) and trioctylamine (1 ml) were added to increase the solubility of the compounds. The suspension was left stirring at 60°C for 18 hours. The solvents were then evaporated off under nitrogen. The solid material was extracted with diethyl ether, and then dried in the dessicator to give the corresponding rhamnose nucleoside.

Purification of UDP-rhamnose

In order to remove any uridine-phosphomorpholidate present in the UDP-rhamnose preparation, the material was subjected to thin layer electrophoresis. This technique separates UDP-rhamnose from uridine-phosphomorpholidate. UDP-rhamnose (2 mg) was run on the thin layer electrophoresis system and the material which ran co-incident with UDP-rhamnose was eluted. The concentration of the eluted UDP-rhamnose was estimated by comparison of absorbance measurements with UDP-glucose. The absorption maxima of UDP-glucose is 262 nm, therefore the absorbance of a known concentration of UDP-glucose was measured at 262 nm in a Pye Unicam SP-8-500 uv/vis Spectrophotometer. The absorbance of UDP-rhamnose at 262 nm was measured and compared to that of UDP-glucose.

Dialysis

Dialysis tubing, supplied by Medicell International Limited, London, was used to dialyse solutions. Solutions were dialysed against H_2O at 4°C for 4-5 days. The H_2O was changed twice daily.

Estimation of radioactive material.

Radioactivity, in the form of ^{14}Carbon, was counted in a Packard Scintillation Counter (Model 3380). The material to be counted was either suspended in H_2O (0.5 ml) or, if in solution, aliquots up to a volume of 1 ml were taken and 5 ml xylene scintillant

(Liquid scintillant Unisolve 1, Koch Light Limited, England) was added.

Toluene scintillant (Harris and Northcote, 1970) was used to count strips of the silica-gel plates as it was then possible to recover the radioactive material. Toluene scintillant was prepared by adding 2,5-diphenyloxazole (10g) to toluene (2.5 L).

Presentation of results

In this project, all measurements were carried out in duplicate. The results are expressed in terms of the mean plus or minus the standard error of the mean (SE). The formula for the standard error is as follows:-

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

where S.D. = standard deviation

n = number of measurements

The standard deviation is calculated using the formula:-

$$S.D. = \sqrt{\frac{\sum(x-\bar{x})^2}{n}}$$

where x = each measurement

$\bar{x}$ = mean

n = number of measurements

Chapter 3

INVESTIGATION OF GALACTURONYLTRANSFERASE ACTIVITY IN PEA EPICOTYLS

I. Preparation of UDP - [U - ^{14}C] galacturonic acid.

UDP - [U - ^{14}C] - galacturonic acid is not available commercially, therefore it was necessary to prepare it using UDP - [U-^{14}C] - glucuronic acid as the substrate. The method used for the preparation of UDP - galacturonic acid from UDP - glucuronic acid is based on the method of Feingold et al. (1960). The particulate enzyme preparation has the ability to convert UDP - glucuronic acid to UDP - galacturonic acid, UDP - xylose and UDP - arabinose (Feingold et al., 1960).

In a typical preparation, UDP - glucuronic acid (0.185 MBq) was incubated with the enzyme preparation in a total volume of 30 µl. After termination of the reaction, UDP - galacturonic acid was separated from the other sugar nucleotides using thin layer electrophoresis. Figure 3.1. shows a typical profile obtained following thin layer electrophoresis of the product. The radioactive material which ran co-incident with UDP - galacturonic acid was eluted. Conversion of UDP - glucuronic acid to UDP - galacturonic acid occurred with an efficiency of 15 - 20%.

In order to confirm that UDP - galacturonic acid was being synthesised, the product isolated as UDP - galacturonic acid was hydrolysed with 2 M TFA and then analysed using thin layer electrophoresis. Electrophoresis of the hydrolysate resulted in a radioactive peak co-incident with the mobility of galacturonic acid (figure 3.2.). This is the expected product following hydrolysis of UDP - galacturonic acid. There also appears to be some material which has a mobility corresponding to that of glucuronic acid. However, in the experiments concerning the incorporation of galacturonic acid from UDP - galacturonic acid into polysaccharide material, ^{14}C - glucuronic acid was never found to be present in the polysaccharide material.

Figure 3.1.

Typical profile obtained following thin layer electrophoresis of the product formed following the incubation of UDP-^{14}C- glucuronic acid with the enzyme preparation used to convert UDP-glucuronic acid to UDP-galacturonic acid. The incubation was terminated by the addition of ethanol and the material was analysed directly.

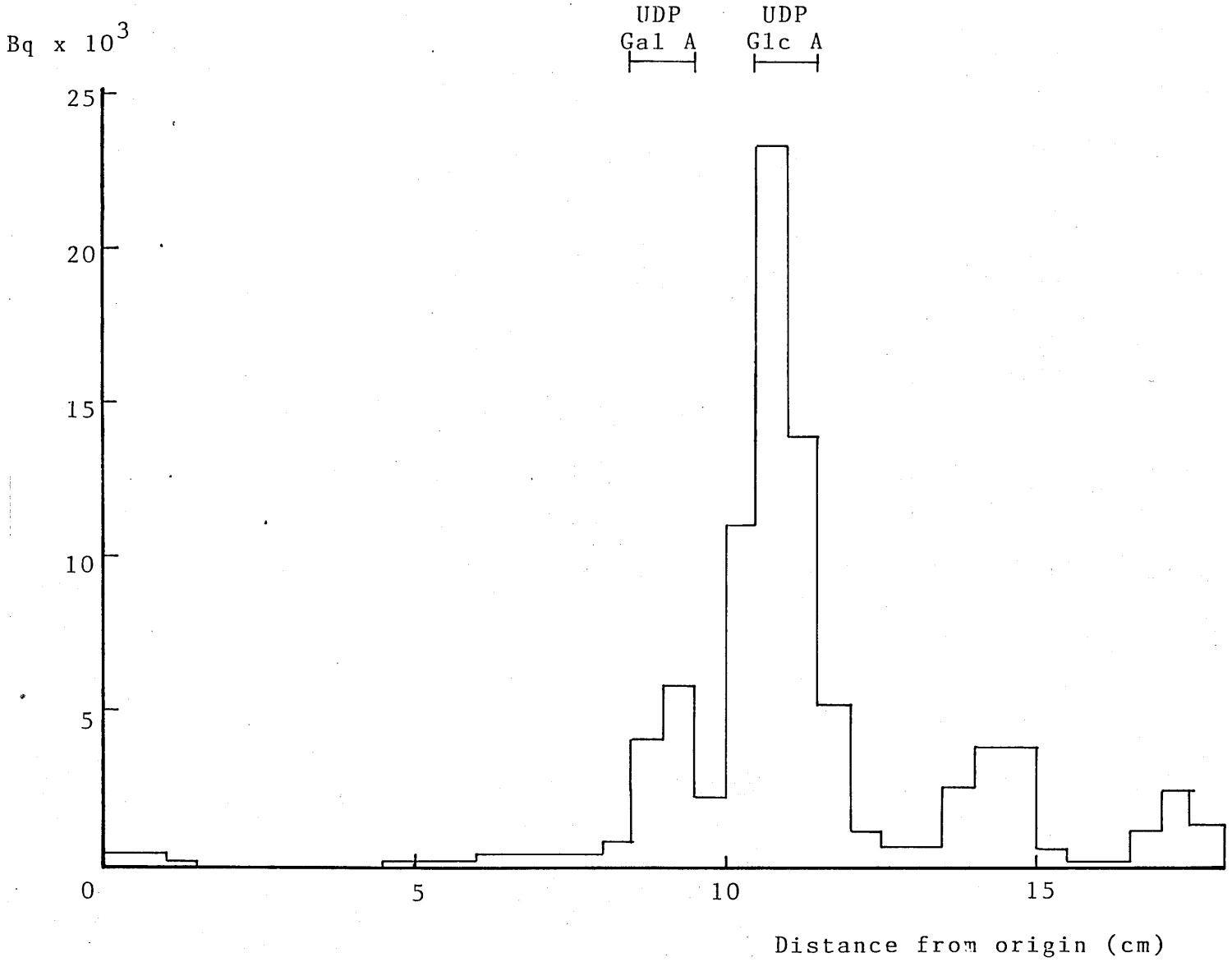
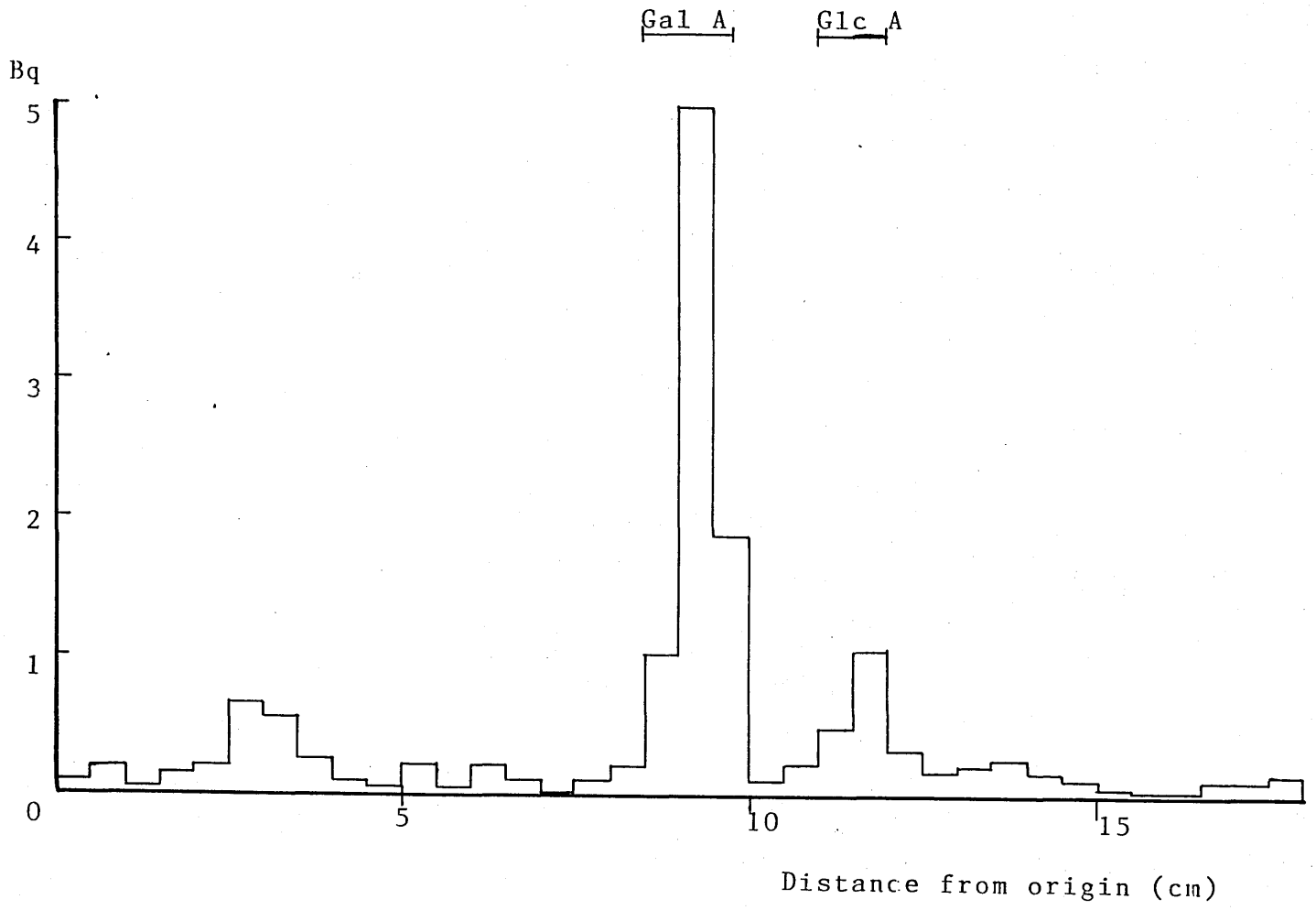

UDP
Gal A
UDP
Glc A
Bq x 10^3
25
20
15
10
5
0
5
10
15
Distance from origin (cm)

Figure 3.2.

Thin layer electrophoretic analysis following total acid hydrolysis of the UDP-^{14}C- galacturonic acid prepared as described in the text using UDP-^{14}C- glucuronic acid as the substrate.


Gal A
Glc A
Bq
5
4
3
2
1
0
5
10
15
Distance from origin (cm)

The time-course of the reaction was investigated to determine the optimum period of incubation for UDP - galacturonic acid synthesis. The results, shown in figure 3.3., indicate that the reaction reaches an equilibrium after an incubation period of 10 minutes.

II Incorporation of Radioactivity from UDP - [U - ^{14}C] - galacturonic acid into Polysaccharide Material.

In order to obtain information on the synthesis of the pectic substances, UDP-galacturonic acid, labelled with ^{14}C in the sugar moiety of the compound, was incubated with the particulate enzyme preparation which was obtained as described in the materials and methods chapter. The reaction was terminated, and the incorporation of radioactivity into the general polysaccharide fraction was then analysed. Initially, the optimum conditions required by the enzyme preparation were investigated in order to achieve maximum incorporation of galacturonic acid into polysaccharide material.

Time-course of incorporation of galacturonic acid into polysaccharide material

The time-course of incorporation of ^{14}C-galacturonic acid into the general polysaccharide fraction was investigated first. A typical time-course of the reaction is shown in figure 3.4. The results indicate that the particulate enzyme preparation obtained from Pisum sativum has the ability to catalyse the incorporation of galacturonic acid from UDP-galacturonic acid into polysaccharide material. However, it can be seen from figure 3.4. that the reaction terminates after approximately 30 minutes. To determine the reasons for the termination of the reaction, the time-course was repeated. After an incubation period of 30 minutes, more UDP-galacturonic acid

Figure 3.3.

Time-course of the % conversion of UDP- glucuronic acid to UDP-galacturonic acid. UDP-galacturonic acid was prepared by incubating UDP-glucuronic acid with the enzyme preparation obtained as described in Chapter 2.

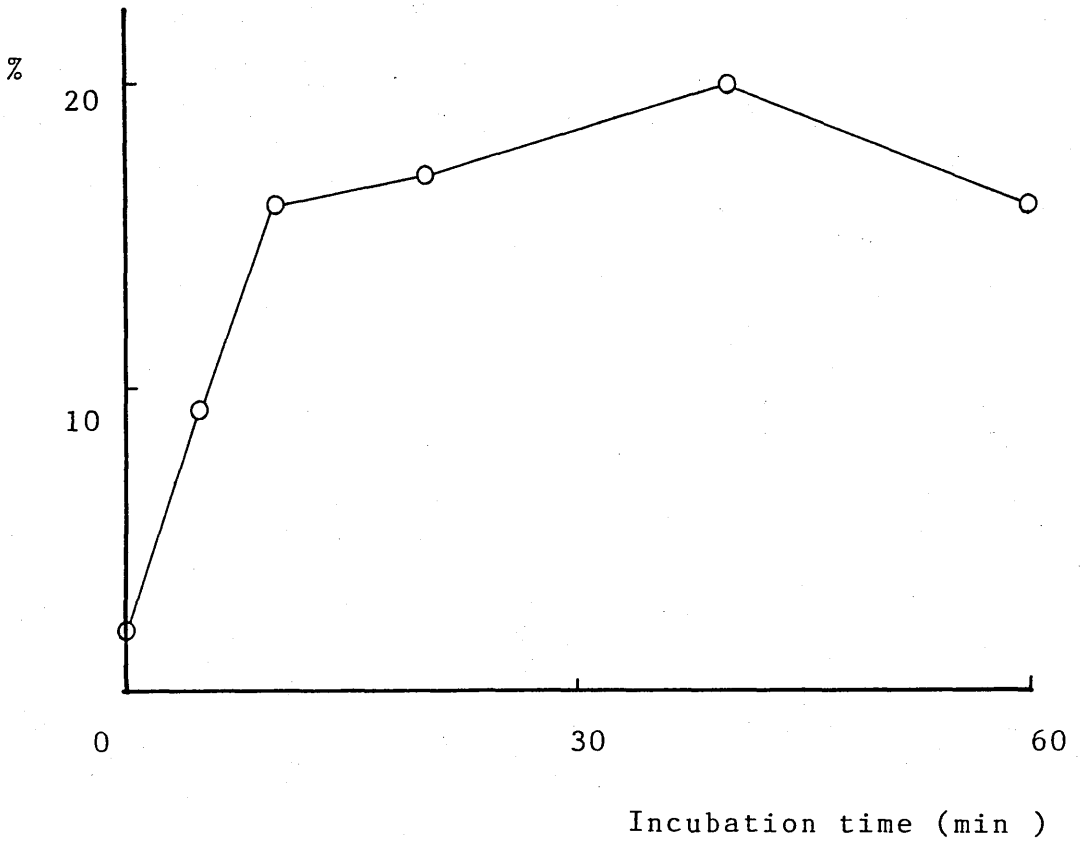
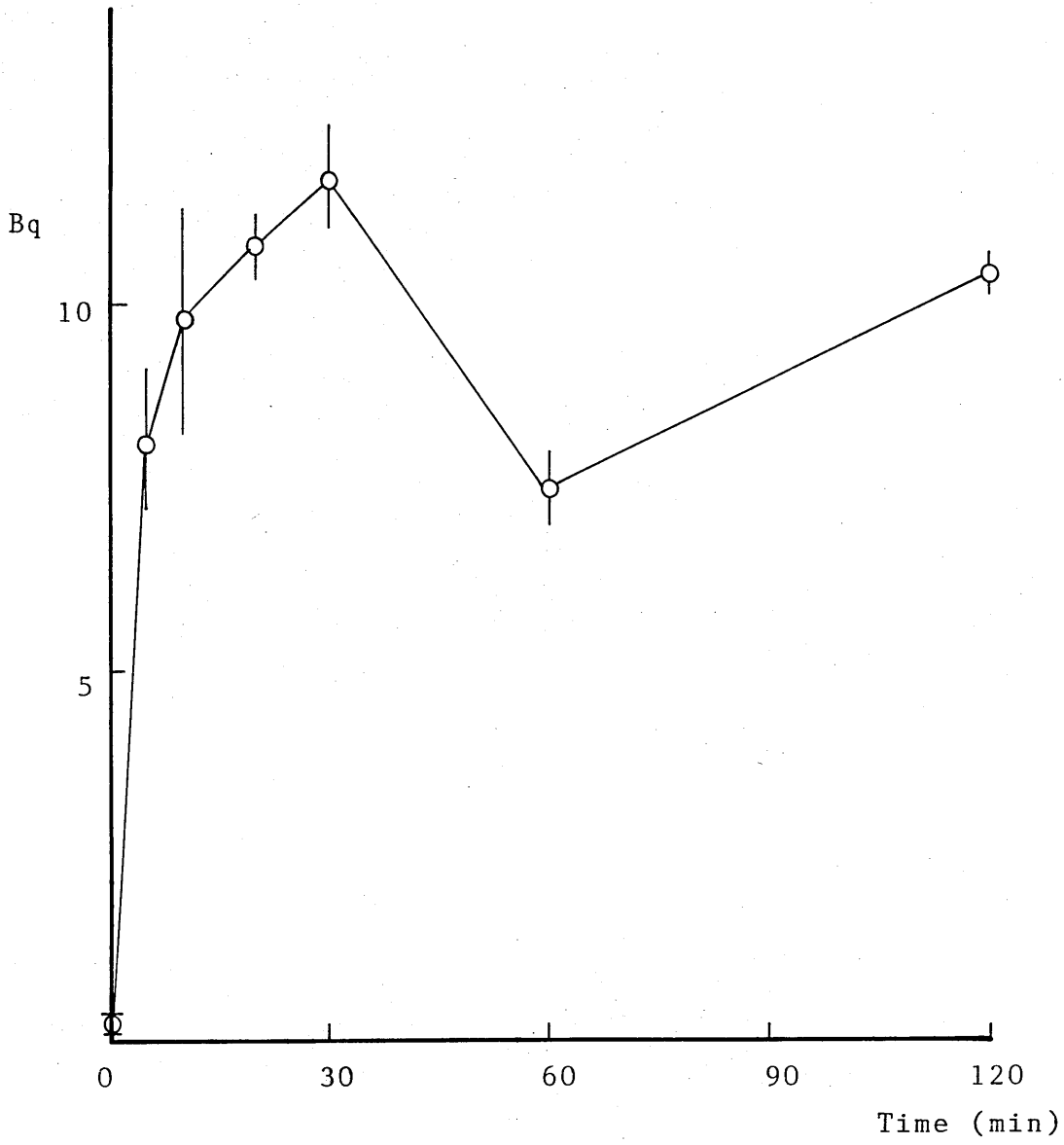

%
20
10
0
30
60
Incubation time (min)

Figure 3.4.

Time-course of incorporation of radioactivity from UDP-^{14}C - galacturonic acid into the general polysaccharide fraction.


Bq
10
5
0
30
60
90
120
Time (min)

(90 Bq) was added to the incubation mixture. A time-course was also conducted following the pre-incubation of the enzyme preparation for 30 minutes at 25°C. The results (figure 3.5.) demonstrate that, on addition of UDP-galacturonic acid after 30 minutes, incorporation increases immediately. This suggests that lack of an acceptor, onto which the galacturonic acid units are binding, is not a limiting factor in the reaction. The results also demonstrate that the particulate enzyme preparation has retained some of its enzymic activity. Following pre-incubation of the enzyme preparation for 30 minutes, galacturonyltransferase activity was retained although it was reduced to approximately 40% of its normal level. Denaturation of the particulate enzyme preparation may be accelerated in the absence of any substrate. Therefore, it is likely that the amount of UDP-galacturonic acid is the limiting factor. It may be that UDP-galacturonic acid is rapidly degraded. In an attempt to prolong the lifetime of the UDP-galacturonic acid in the incubation mixture, a time-course was conducted in which non-radioactive-labelled UDP-galacturonic acid was present in the incubation mixture, in addition to the ^{14}C-labelled UDP-galacturonic acid (145 Bq), to give a total concentration of 1.5 μM . The results (figure 3.6.) indicate that termination of the reaction occurs after 10 minutes. It is possible that the enzymes responsible for the degradation of UDP-galacturonic acid have a high Km value, therefore increasing the concentration of UDP-galacturonic acid ten-fold does not result in a reduction of the amount of UDP-^{14}C-galacturonic acid degraded. It is also of interest to note that, following an incubation period of 120 minutes, the amount of product has decreased. The degradation of the product appears to be more pronounced when the concentration of UDP-galacturonic acid is increased. It is possible that the increased amount of polysaccharide material available results in increased activity of enzymes catalysing the degradation of the

Figure 3.5.

Time-course of incorporation of radioactivity from UDP-^{14}C - galacturonic acid into general polysaccharide material demonstrating the effect of the addition of more UDP-^{14} C - galacturonic acid (90 Bq) to the incubation mixture after an incubation period of 30 minutes. The graph also illustrates the effect of pre-incubation of the particulate enzyme preparation at 25^{o}C, prior to use, on the incorporation of radioactivity from UDP-^{14}C- galacturonic acid into the general polysaccharide fraction.

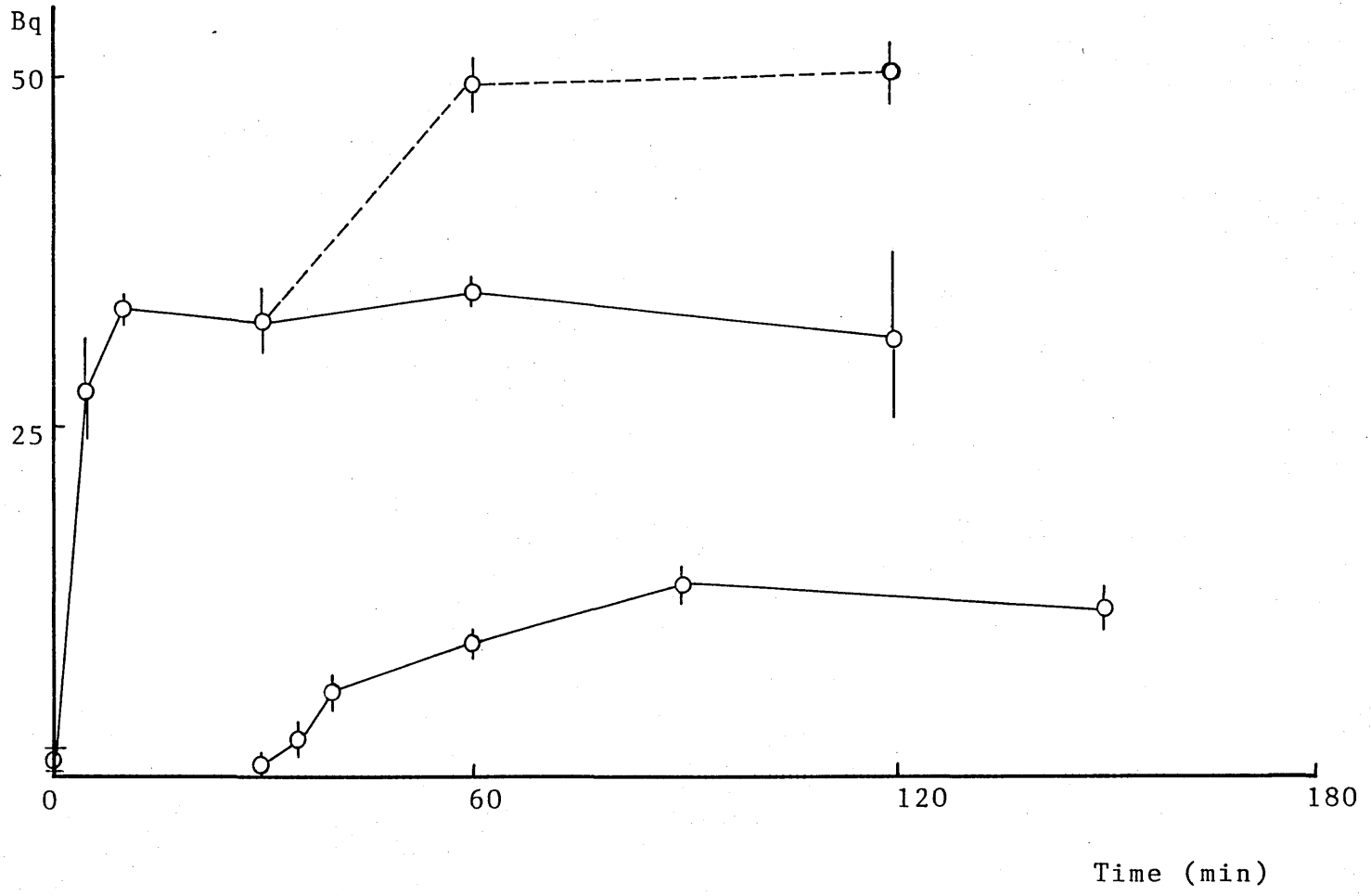
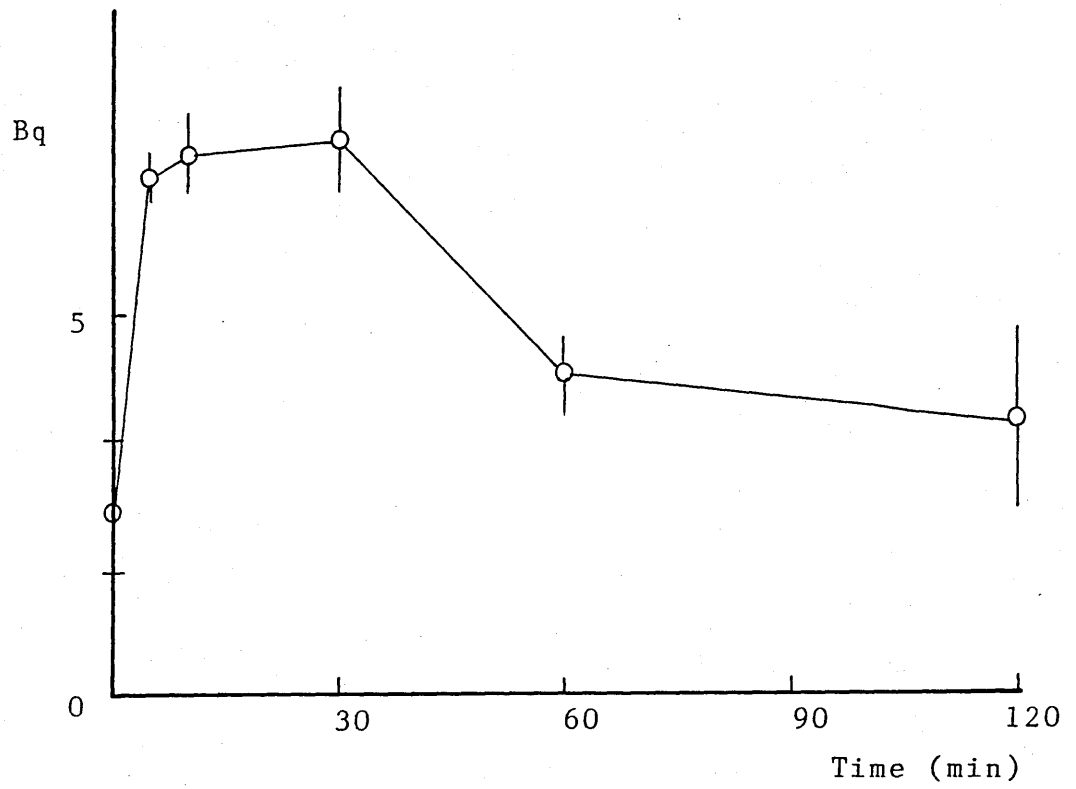

Bq
50
25
0
0
60
120
180
Time (min)

Figure 3.6.

Effect of increasing the concentration of UDP-galacturonic acid on the time-course of incorporation of galacturonic acid from UDP-galacturonic acid into the general polysaccharide fraction. Incubations contained UDP-galacturonic acid (145 Bq) and unlabelled UDP-galacturonic acid was added to give a concentration of 1.5 μM.


Bq
5
0
30
60
90
120
Time (min)

polysaccharide material.

The effect of divalent cations on the incorporation of ^{14}C-galacturonic acid.

The effect of the presence of co-factors in the incubation mixture has been examined. Many enzymes involved in the biosynthesis of the non-cellulosic polysaccharides are stimulated by the presence of divalent cations (Waldron and Brett, 1983). Firstly, the effect of different concentrations of manganese ions was investigated. The concentration of manganese ions was varied from a control containing no manganese ions to 100 mM. From the results, shown in figure 3.7., it was concluded that the presence of manganese ions increases enzymic activity and that the optimum concentration of manganese ions, required by the enzyme, is approximately 10 mM. In view of the stimulatory effect of manganese ions, different cations were examined to determine their effect on the enzyme system. All the cations examined were salts of chloride and their effect was investigated when present at a concentration of 10 mM. The results (figure 3.8.) demonstrate that, although manganese ions resulted in the greatest promotory effect, the presence of magnesium, cobalt and nickel ions also stimulated incorporation. However, the presence of calcium ions appears to have no significant effect on incorporation of galacturonic acid into polysaccharide material.

The effect of the concentration of UDP-galacturonic acid on the incorporation of radioactivity into polysaccharide material

The influence of the substrate concentration on the reaction has been investigated. This involved the addition of non-radioactive UDP-galacturonic acid to the standard incubation mixture. The results,

Figure 3.7.

Effect of the concentration of manganese ions present in the incubation mixture on the incorporation of radioactivity from UDP-^{14}C - galacturonic acid into general polysaccharide material following an incubation period of 10 minutes.

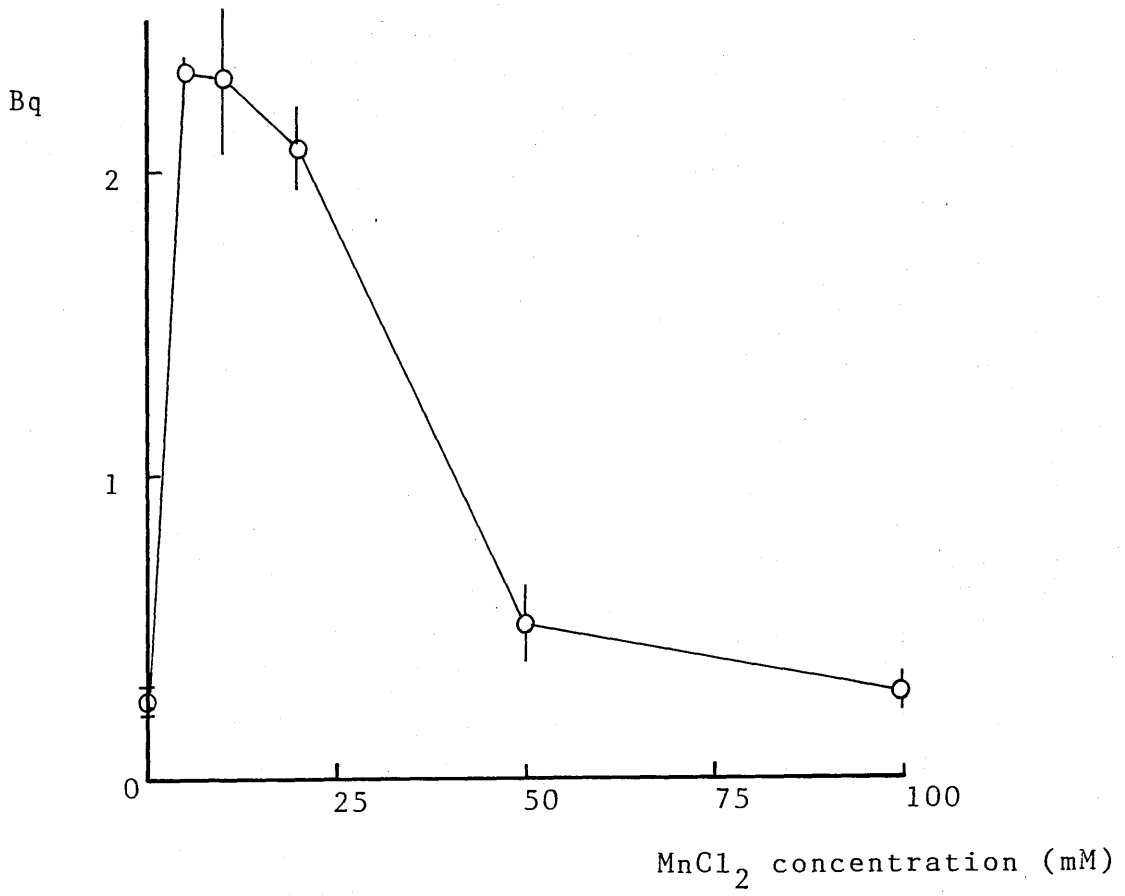
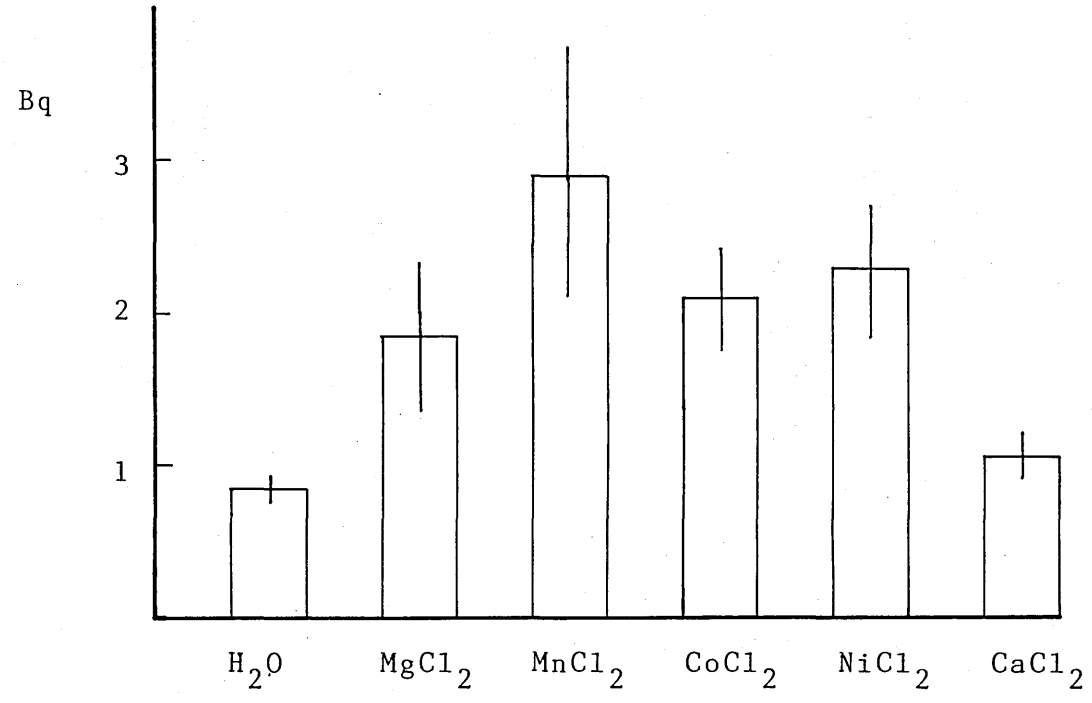

Bq
2
1
0
25
50
75
100
MnCl2 concentration (mM)

Figure 3.8.

Effect of the presence of divalent cations in the incubation mixture on the incorporation of radioactivity from UDP - ^{14}C - galacturonic acid into the general polysaccharide fraction following an incubation period of 10 minutes.


Bq
3
2
1
H_2O
$MgCl_2$
$MnCl_2$
$CoCl_2$
$NiCl_2$
$CaCl_2$

shown in figure 3.9 and table 3.1., demonstrate that increasing the substrate concentration results in increased incorporation of galacturonic acid into polysaccharide material. However, due to the dilution of the radioactively-labelled UDP-galacturonic acid, the increase in incorporation is not reflected in the measurement of radioactivity.

To examine the kinetic properties of the enzyme, an Eadie-Hofstee plot was constructed (figure 3.10.). However, the results do not give a linear plot, but a biphasic curve. The biphasic nature of the plot may indicate the involvement of two enzymes. If this is so, then the two enzymes have very different kinetic properties. Another possibility is that the enzyme does not follow Michaelis-Menten kinetics. This is not uncommon for membrane-bound enzymes. Although it has been attempted to investigate the kinetic properties of the enzyme galacturonyltransferase using the Michaelis-Menten equation, even if a linear plot had been constructed, this would not necessarily have given true values. The reaction that the enzyme is catalysing is a two-substrate reaction. In order to obtain a true Km value for one substrate, the second substrate must be present at a saturating concentration. However, there is no information regarding the second substrate, the acceptor molecule, in the reaction.

The effect of pH on the incorporation of radioactivity into the polysaccharide fraction

The effect that pH has on incorporation of galacturonic acid into polysaccharide material has also been examined. The buffer used was 100 mM Tris-Mes-Glycine buffer, and this was added to the incubation mixture at pH values ranging from 2 to 10. The results (figure 3.11.) indicate that the incorporation of galacturonic acid peaks at approximately pH 6.0. However, from pH 8.0 to pH 10.0, there is a

Figure 3.9.

Effect of UDP-galacturonic acid concentration on the incorporation of radioactivity from UDP-^{14}C - galacturonic acid into the general polysaccharide fraction following an incubation period of 10 minutes.

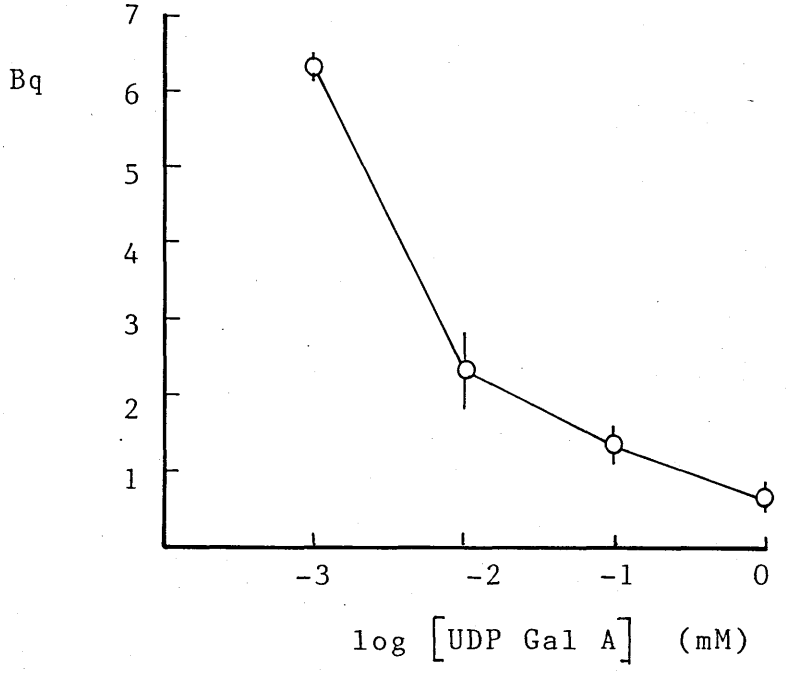

Bq
7
6
5
4
3
2
1
-3
-2
-1
0
log [UDP Gal A] (mM)

TABLE 3.1.

Effect of UDP-galacturonic acid concentration on the incorporation of galacturonic acid from UDP - ^{14}C - galacturonic acid into general polysaccharide material following an incubation period of 10 minutes.

UDP-galacturonic acid concentration (mM)	Incorporation (pmol)
1.5×10^{-4}	0.9 ± 0.15
1×10^{-3}	4.5 ± 0.05
1×10^{-2}	16.5 ± 3.8
0.1	91.0 ± 3.0
1	407.5 ± 37.5

Figure 3.10.

Construction of an Eadie-Hofstee plot, drawn in order to investigate the kinetic properties of the enzyme system.

In the Eadie-Hofstee plot, the slope of the line gives -Km and the point where the line meets the y-axis gives Vmax.

The velocity of the reaction (v) was measured by the pmoles of galacturonic acid incorporated into the general polysaccharide fraction following an incubation period of 10 minutes and $[S]$ is the concentration of UDP-galacturonic acid present in the incubations. (mM)

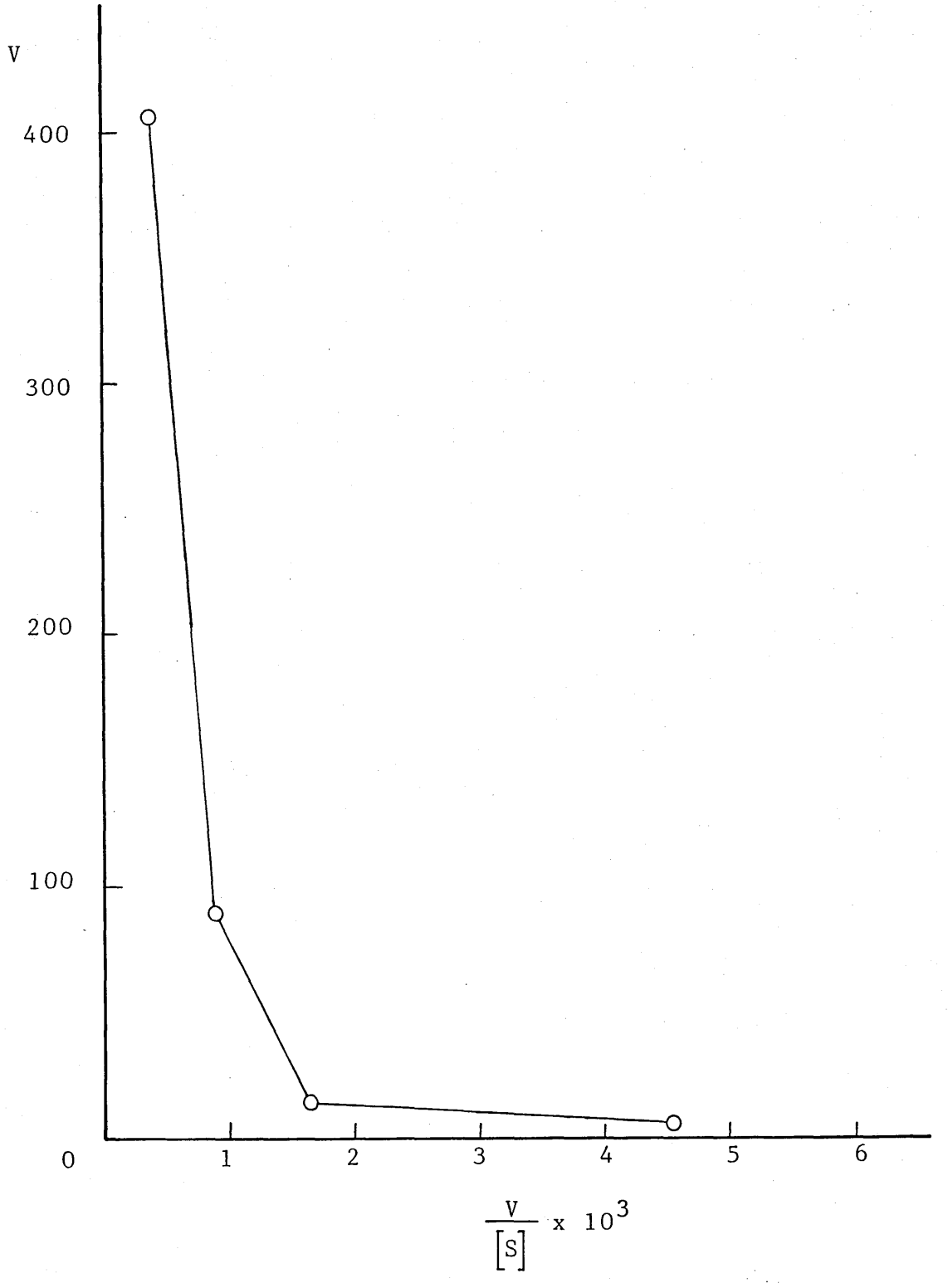
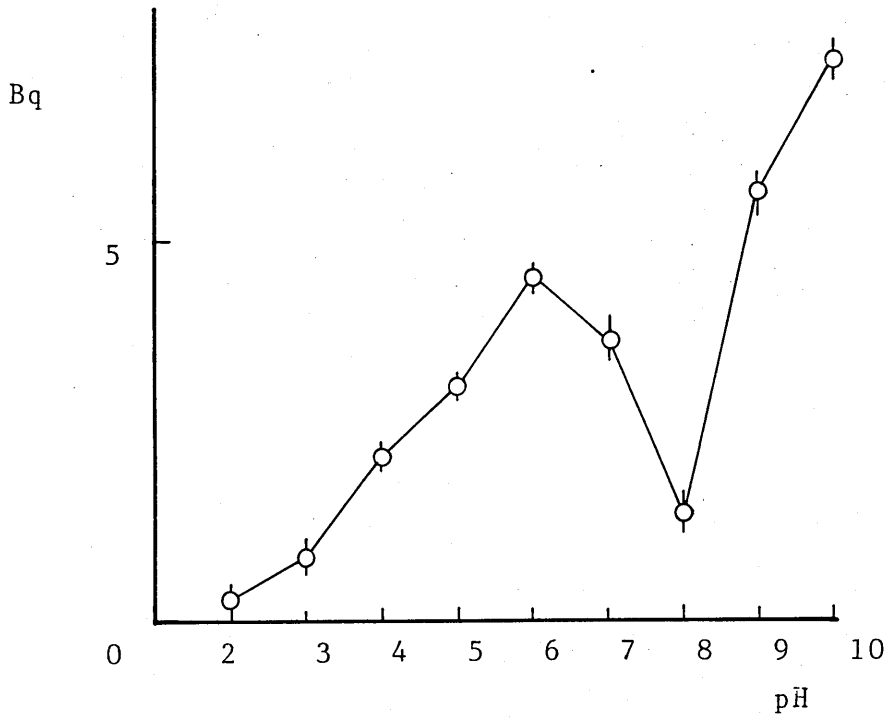

V
400
300
200
100
0
1
2
3
4
5
6
V / [S] x 10^3

Figure 3.11.

The effect of pH on the incorporation of galacturonic acid into general polysaccharide material. Incubations were carried out for 10 minutes.


Bq
5
0
2
3
4
5
6
7
8
9
10
pH

substantial increase of radioactivity incorporated into the polysaccharide fraction. This apparent increase in the incorporation of galacturonic acid at the high pH values was investigated further. Initially, an experiment was conducted to determine into which polysaccharide fraction the galacturonic acid units are incorporated. Incubations were carried out at both pH 6.0 and pH 10.0. Firstly, pectin was extracted from the general polysaccharide material using 50mM EDTA/NaH_2PO_4 buffer, pH 6.8. Then, a cadoxen extraction was performed which is reported to extract all polysaccharides, including cellulose (Wood and MacRae, 1978). A small pellet remained following these extractions. The different fractions were then analysed for radioactivity. The results, in table 3.2., show that following incubations at both pH values of 6.0 and 10.0, the galacturonic acid units are incorporated into material extracted by 50mM EDTA/ NaH_2PO_4 buffer, pH 6.8.

To obtain an approximate value for the molecular weight of the material into which the galacturonic acid units are incorporated, the experiment was repeated. However, 230 Bq UDP-^{14}C-galacturonic acid was added to the incubation mixtures. The material extracted by 50mM EDTA/NaH_2PO_4 buffer, pH 6.8, was run through a column of Sephadex G-100 (7.5mm x 180mm). It can be concluded from the results (figure 3.12.) that incubating UDP-galacturonic acid with the enzyme preparation at pH 6.0 results in the incorporation of galacturonic acid into high-molecular-weight material, whereas incubating UDP-galacturonic acid with the particulate enzyme preparation at pH 10.0 does not result in the incorporation of galacturonic acid into high-molecular-weight material. Therefore, pH 6.0 is the optimum pH value required by the enzyme involved in polysaccharide synthesis.

TABLE 3.2.

Amount of radioactivity present in the different fractions after extracting the general polysaccharide fraction with EDTA/NaH_2PO_4 buffer and Cadoxen following incubations at pH6.0 and pH 10.0 for 10 minutes.

Fraction	Radioactivity (Bq)	
	pH 6.0	pH 10.0
EDTA/NaH_2PO_4 extract	2.42 ± 0.43	16.4 ± 1.5
Cadoxen extract	0.03 ± 0.02	0.03 ± 0.03
Pellet	0.12 ± 0.05	0.26 ± 0.02

TABLE 3.3.

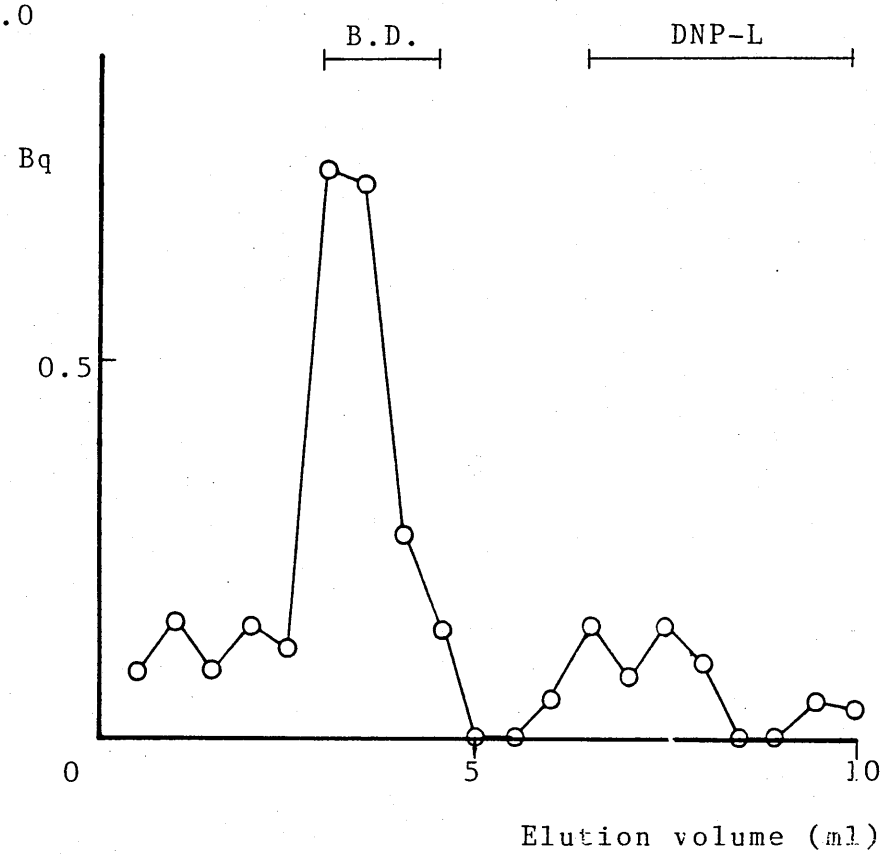
Effect of freezing the particulate enzyme preparation at -20°C on galacturonyltransferase activity. Incubations were carried out for 10 minutes and the general polysaccharide fraction was analysed for incorporation of radioactivity.

Time frozen (days)	Incorporation (Bq)
0	15.11
1	14.20
7	7.0

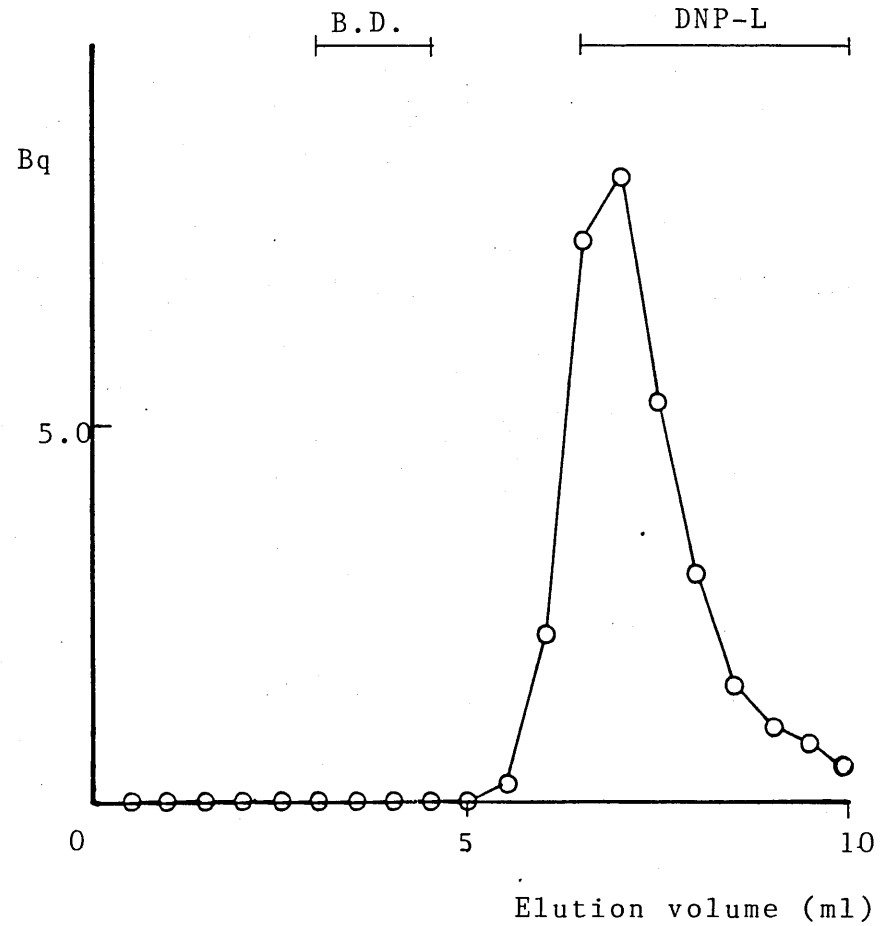
Figure 3.12.

Sephadex G-100 profiles of the products formed following the incubation of UDP-^{14}C - galacturonic acid with the particulate enzyme preparation at a) pH 6.0 and b) pH 10.0. The incubations were carried out for 10 minutes and the EDTA-soluble fractions were analysed.

a)pH 6.0



b)pH 10.0



The effect of freezing the particulate enzyme preparation on the enzymic activity.

The effect of freezing the particulate enzyme preparation at -18°C before use was also investigated. The results, in table 3.3., demonstrate that freezing the preparation for 7 days results in a 50% decrease in enzymic activity. This indicates that the enzyme preparation is not very stable to freezing.

The effect of different homogenisation and resuspension buffers used to obtain the particulate enzyme preparation on galacturonyltransferase activity

The homogenisation and resuspension buffers employed in the enzyme preparation procedure have been investigated in order to obtain buffers which resulted in maximum enzymic activity. Different buffers, including Mes, Tris-Mes and Mops, all at pH 6.0, have been used in the preparation of the enzyme. The buffers were used at a concentration of 10mM and 1% BSA was present in each buffer. It can be seen from the results, in table 3.4., that Mes and Tris-Mes are equally effective and it was decided to continue the experiments using Mes buffer. Enzyme preparations have been obtained using Mes buffer at different concentrations varying from 1mM to 100mM. The results (figure 3.13.) indicate that 25mM Mes is a suitable concentration for the preparation of the enzyme.

The importance of the presence of BSA in the homogenisation and resuspension buffer was examined. Particulate enzyme preparations were obtained in the presence and absence of 1% BSA in both the homogenisation and resuspension buffers. The results (figure 3.14.) indicate that the particulate enzyme preparation, obtained when BSA is present in both buffers, exhibited the highest enzymic activity.

Figure 3.13.

Effect of the concentration of Mes buffer used in the preparation of the enzyme on the incorporation of galacturonic acid into the general polysaccharide fraction. BSA (1% (w/v)) was present in the buffers.

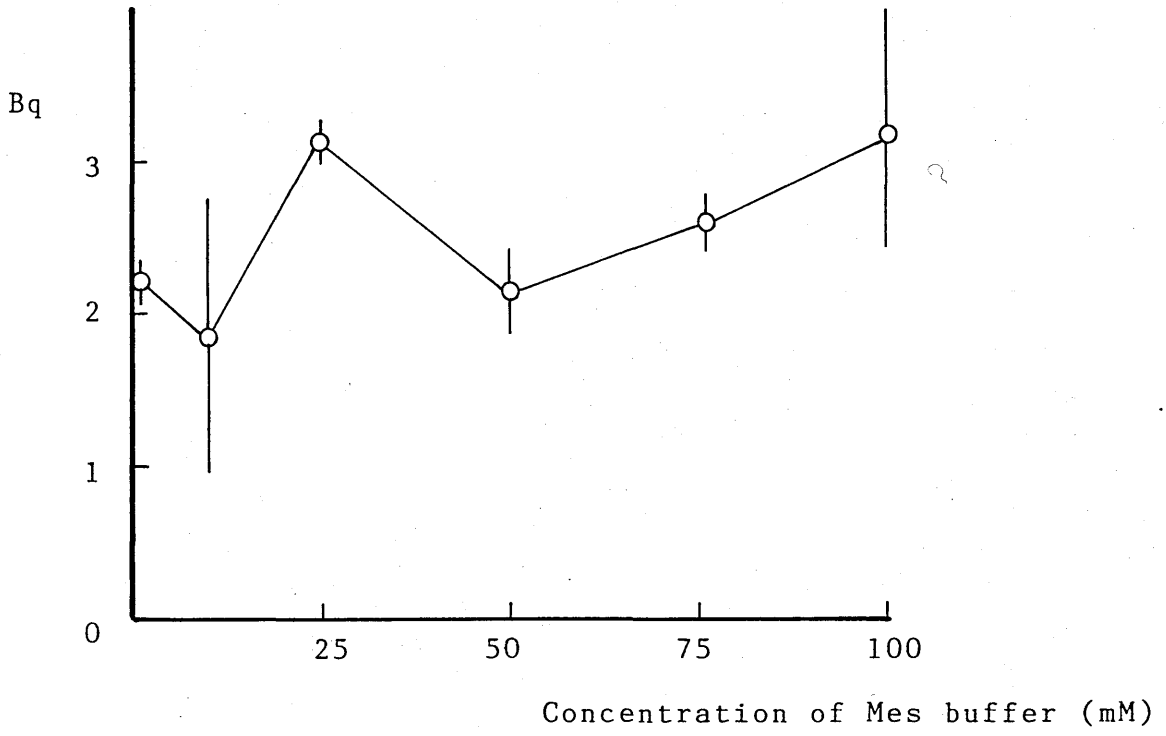

Bq
3
2
1
0
25
50
75
100
Concentration of Mes buffer (mM)

Figure 3.14.

Effect of the presence of 1% (w/v) BSA in the homogenisation and/or resuspension buffer (25 mM Mes buffer, pH 6.0) used in the preparation of the enzyme on the incorporation of galacturonic acid from UDP-^{14}C- galacturonic acid into general polysaccharide material. The different permutations tried were:-

a = BSA present in the homogenisation and the resuspension buffers.

b = BSA absent from both the homogenisation and resuspension buffers.

c = BSA present in the homogenisation buffer but not present in the resuspension buffer.

d = BSA present in the resuspension buffer but not the homogenisation buffer.

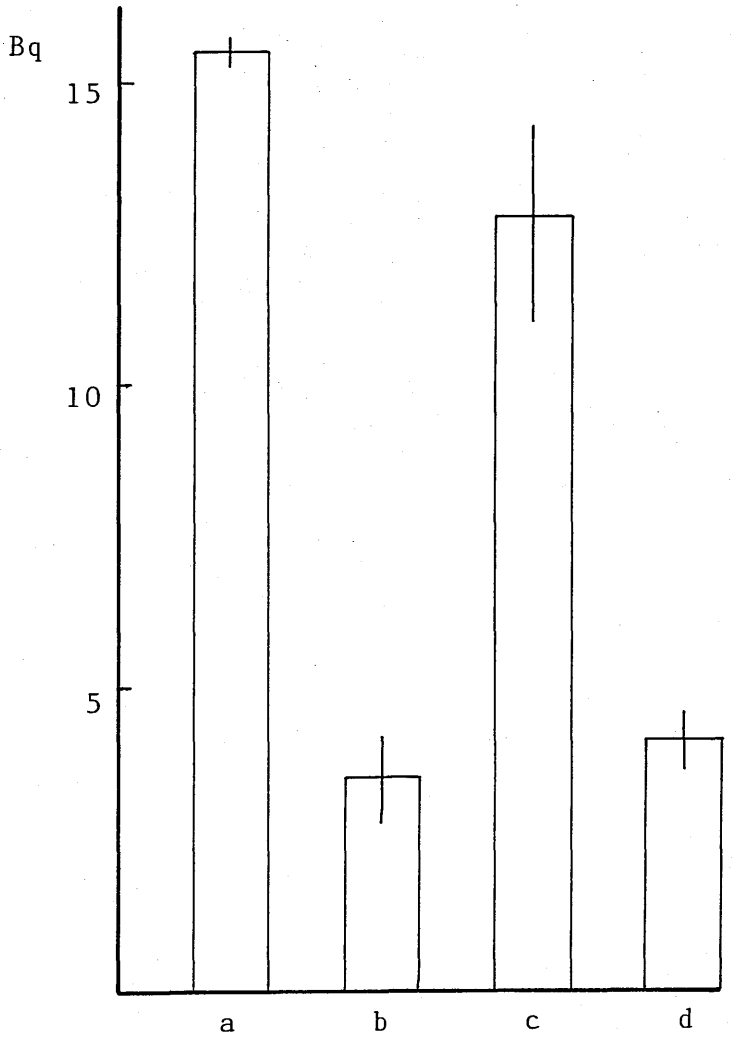

Bq
15
10
5
a
b
c
d

TABLE 3.4.

Effect of different buffers (all at pH 6.0 and a concentration of 10mM) employed in the preparation of the particulate enzyme on the incorporation of ^{14}C- galacturonic acid into the general polysaccharide fraction. BSA (1% w/v) was present in the buffers.

Buffer	Incorporation (Bq)
Tris - Mes	3.32 ± 0.12
Mops	1.75 ± 0.77
Mes	3.06 ± 0.27

TABLE 3.5.

Effect of the concentration of BSA added to the homogenisation and resuspension buffer (25 mM Mes buffer, pH 6.0) on the incorporation of radioactivity into the general polysaccharide fraction

BSA concentration (w/v)	Incorporation (Bq)
0.1	3.62 ± 0.19
1	15.09 ± 0.49

The absence of BSA from both the homogenisation and resuspension buffers reduced enzymic activity to approximately 25% of its normal level. It can also be concluded, from the results, that the presence of BSA in the homogenisation buffer is particularly important for enzymic activity to be retained.

The presence of BSA in the homogenisation and resuspension buffer has been tested at concentrations of 0.1% and 1%. The results (table 3.5.) demonstrate clearly that the presence of 1% BSA in the buffer results in greater enzymic activity.

It has previously been reported (Villemez et al., 1966) that the presence of sucrose in the buffers, used in the preparation of a galacturonyltransferase enzyme, obtained from Phaseolus aureus, resulted in greater enzymic activity. However, on investigation, the presence of 0.4M sucrose was not shown to enhance the activity of the pea epicotyl preparation, but in fact appeared to cause slight inhibition (table 3.6.).

In addition to the presence of BSA in the homogenisation and resuspension buffer, the presence of other protective agents was investigated. Polyvinylpyrrolidone (PVP), dithiothreitol (DTT) and phenylmethyl sulphonyl fluoride (PMSF) were all tested to determine if they improved the activity of the enzyme preparation. PVP is thought to improve the stability of enzymes by removing phenolic compounds which can denature enzymes. DTT is a reducing agent and will help to maintain -SH groups in a reduced state, whereas PMSF is a protease inhibitor. From the results (figure 3.15.) it can be concluded that the presence of DTT greatly enhances the activity of the enzyme preparation.

The presence of DTT in the buffer was tested at final concentrations of 0.5, 5 and 50mM. The results (table 3.7.) demonstrate that 5mM DTT results in the highest enzymic activity.

Figure 3.15.

Effect of the addition of protective agents to the homogenisation and resuspension buffer used in the preparation of the particulate enzyme on the incorporation of galacturonic acid into the general polysaccharide fraction. The protective agents employed are listed below:-

a = Control

b = 1% (w/v) PVP

c = 5mM DTT

d = 0.1 mM PMSF

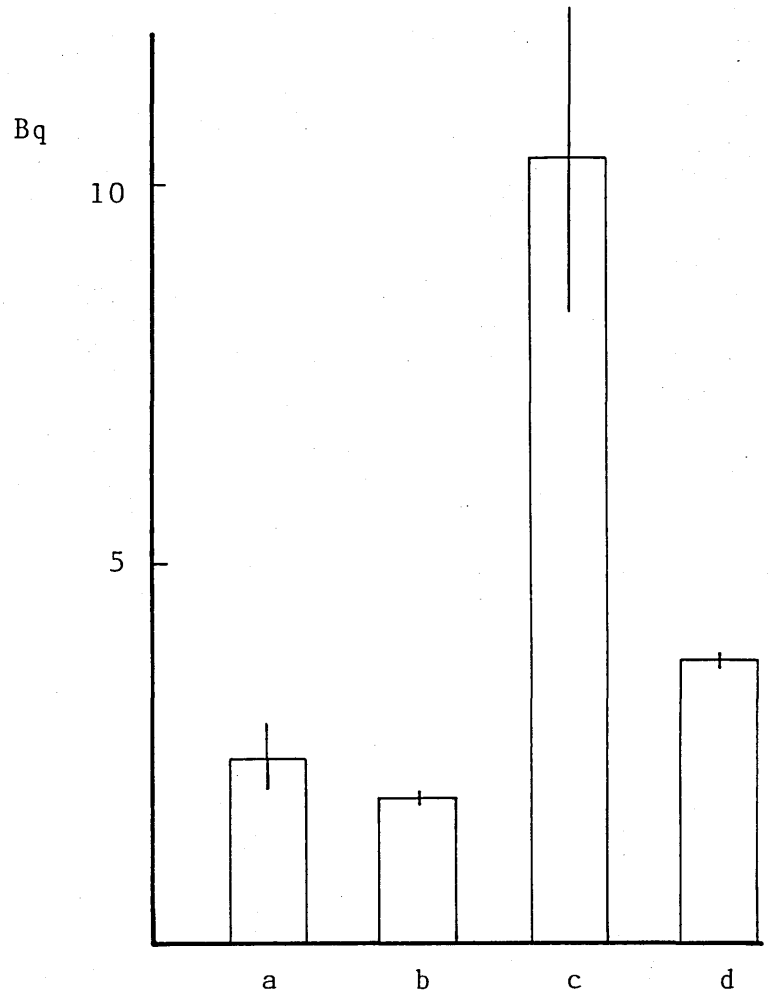

Bq
10
5
a
b
c
d

TABLE 3.6.

Effect of the presence of 0.4 M sucrose in the homogenisation and/or resuspension buffer on the incorporation of radioactivity from UDP-^{14}C - galacturonic acid into general polysaccharide material following an incubation period of 10 minutes.

Buffer	Incorporation (Bq)
Sucrose absent	6.56 ± 0.34
Sucrose present in resuspension buffer	5.44 ± 0.24
Sucrose present in homogenisation and resuspension buffers	4.20 ± 0.36

TABLE 3.7.

Effect of the concentration of DTT in the homogenisation and resuspension buffer (25 mM Mes buffer plus 1% (w/v) BSA at pH 6.0) used in the preparation of the enzyme on the enzyme activity.

DTT concentration (mM)	Incorporation (Bq)
0.5	8.29 ± 0.28
5	10.82 ± 0.06
50	7.63 ± 0.41

Therefore, from this set of experiments, it was concluded that a homogenisation and resuspension buffer comprising of 25mM Mes buffer at pH 6.0 containing 1% BSA and 5mM DTT results in optimal enzymic activity.

III Identification of the Polysaccharide Material

Cell wall polysaccharides can be separated according to their solubility properties, and the pectic substances are characteristically extracted by hot H_2O and chelating agents such as EDTA. It has previously been demonstrated that the polysaccharides into which the galacturonic acid units are incorporated, following the incubation of UDP-galacturonic acid with the particulate enzyme preparation, are extracted by EDTA, indicating that they are pectic polysaccharides. The following experiments were conducted in order to identify the polysaccharides into which the galacturonic acid units are incorporated.

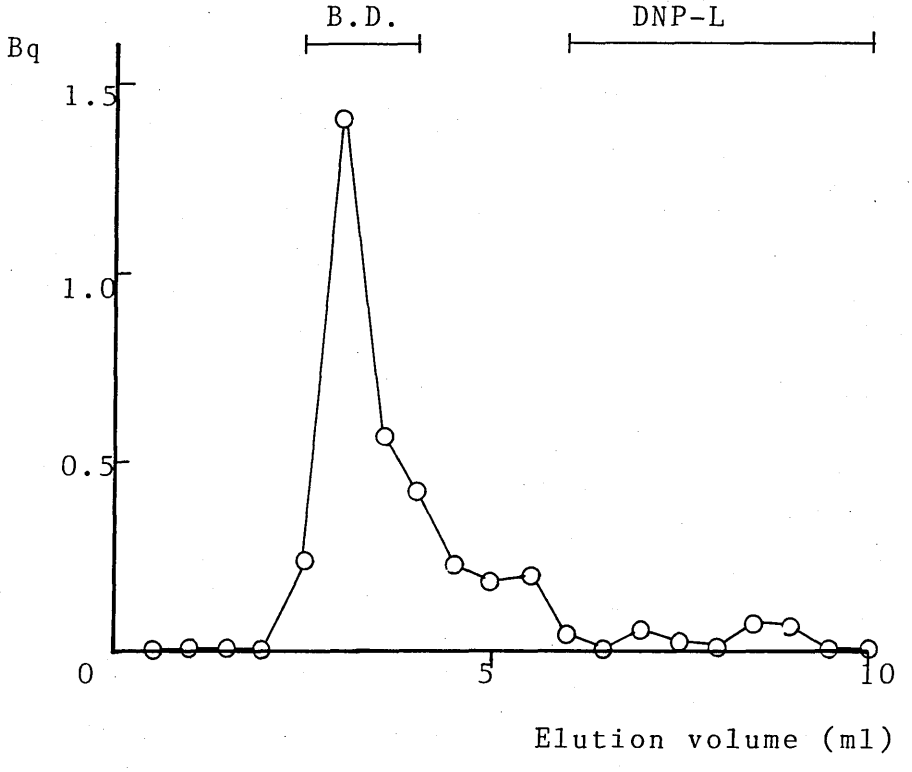
To analyse the polysaccharides, the particulate enzyme preparation was incubated with UDP-[U-^{14}C]-galacturonic acid. The reaction was then terminated and the general polysaccharide fraction was recovered. The material was either analysed directly or extracted with 50mM EDTA/NaH_2PO_4 buffer, pH 6.8, and the extract analysed.

Determination of the molecular weight of the product

Initially, the molecular weight of the product was investigated using gel filtration chromatography. UDP-[U-^{14}C]-galacturonic acid (230 Bq) was incubated with the particulate enzyme preparation and the resulting EDTA extract was run through a column of Sephadex G-100 (180mm x 7.5mm). The results, shown in figure 3.16., demonstrate that the radioactive material is excluded from the column. The exclusion limit of polysaccharides for Sephadex G-100 is approximately 10^5 daltons for

Figure 3.16.

Gel filtration using Sephadex G-100 of the polysaccharide material extracted by EDTA/NaH_2PO_4 buffer, obtained following the incubation of UDP-^{14}C- galacturonic acid (230 Bq) with the particulate enzyme preparation.


B.D.
DNP-L
Bq
1.5
1.0
0.5
0
5
10
Elution volume (ml)

dextrans (Pharmacia Handbook), therefore it can be concluded that the product is of high molecular weight.

In order to obtain further information on the molecular weight of the product, the experiment was repeated and the EDTA extract was analysed using a column of Sepharose 6B-CL (450mm x 10mm). In this experiment, 370 Bq UDP-[U-^{14}C]-galacturonic acid was present in the incubations. The exclusion limit for Sepharose 6B-CL is approximately 10^6 daltons for dextrans (Pharmacia Handbook). Some of the polysaccharide product is excluded from this column (figure 3.17.) but there is also some material of intermediate molecular weight.

Identification of the constituent radioactive monosaccharides of the product using total acid hydrolysis

Identification of the constituent monosaccharides of the polymer was attempted using acid hydrolysis. UDP-galacturonic acid (185 Bq) was incubated with the particulate enzyme preparation and the resulting general polysaccharide fraction was hydrolysed using 2M TFA. To identify the radioactive monosaccharides, thin layer electrophoresis was employed. The results, shown in figure 3.18., demonstrate that the main radioactive zone migrated at the same rate as galacturonic acid. However, a smaller peak of radioactivity just past the origin, was also evident. The possibility of galacturonic acid forming a lactone was excluded by eluting the radioactive material, and then incubating it with 2M NaOH for 1 hour. The material was analysed again using thin layer electrophoresis. There was no change in the mobility of the compound, indicating that it was not a lactone of galacturonic acid.

Figure 3.17.

Sepharose 6B-CL profile of the polysaccharide material extracted by 50mM EDTA/NaH_2PO_4 buffer, pH 6.8, obtained following the incubation of UDP-^{14}C- galacturonic acid (370 Bq) with the particulate enzyme preparation.

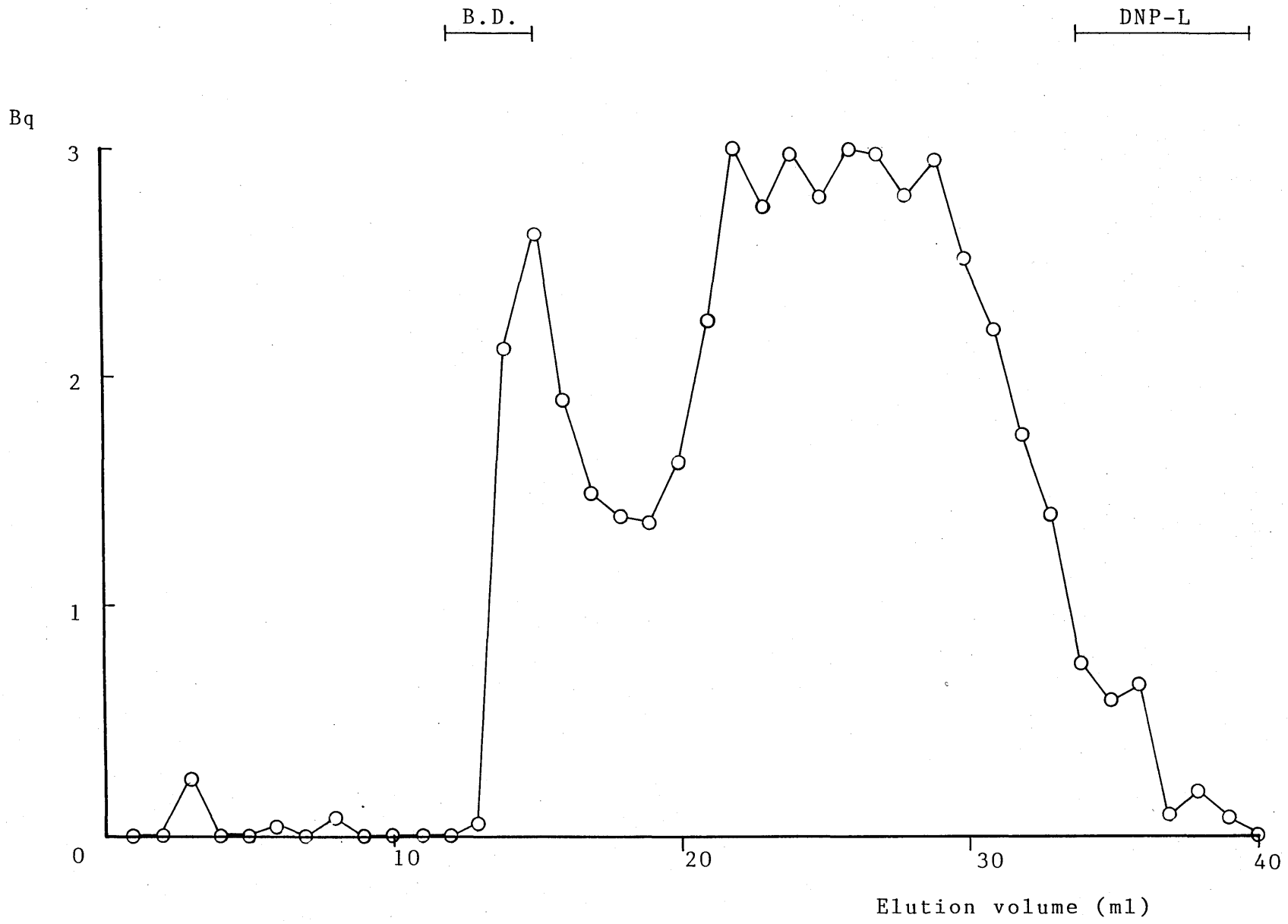
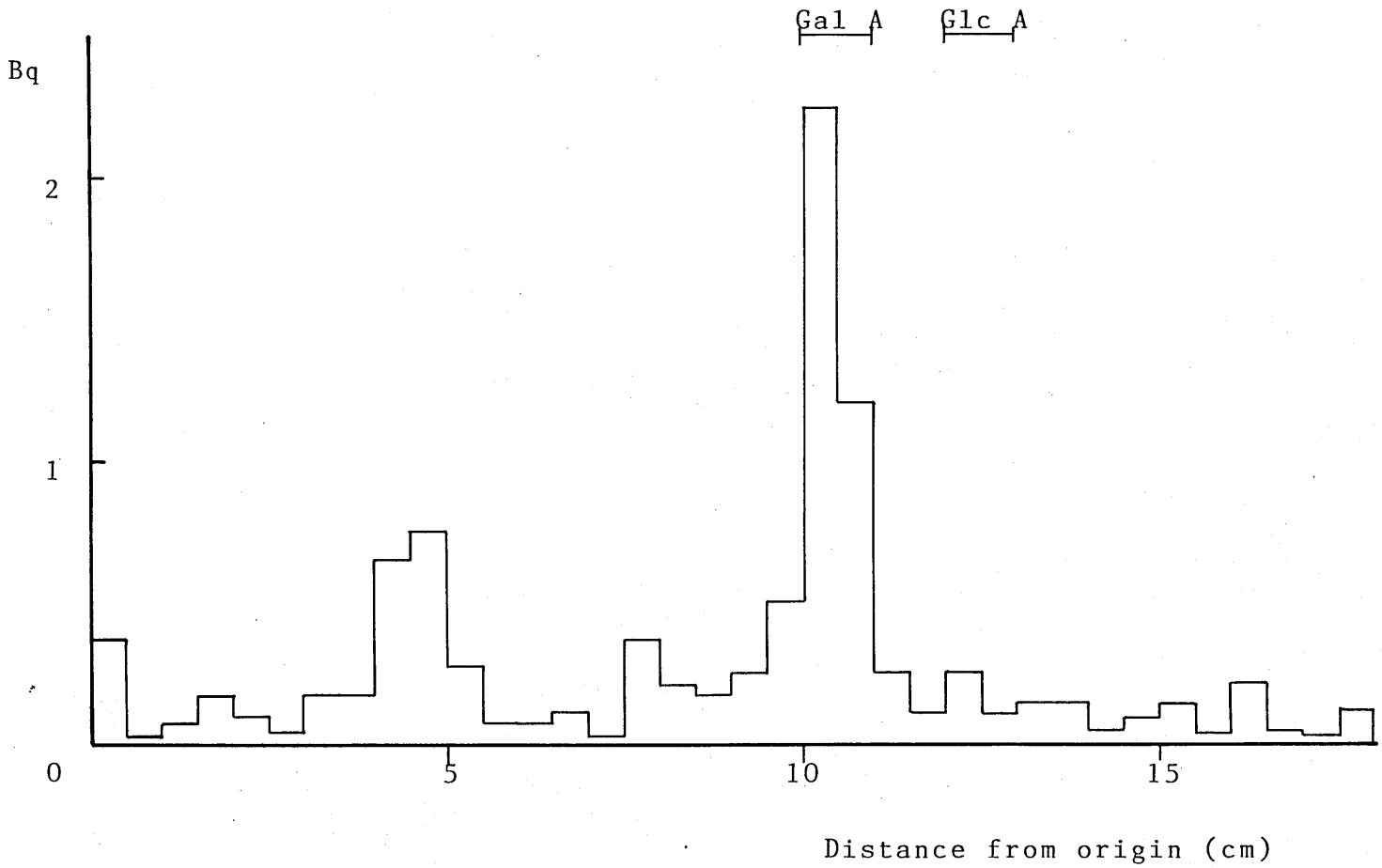

B.D.
DNP-L
Bq
3
2
1
0
10
20
30
40
Elution volume (ml)

Figure 3.18.

Identification using thin layer electrophoresis of the radioactive monosaccharides present following total acid hydrolysis of the general polysaccharide fraction, obtained after incubating UDP-galacturonic acid (185 Bq) with the particulate enzyme preparation.


Gal A
Glc A
Bq
2
1
0
5
10
15
Distance from origin (cm)

The results of this experiment indicate that galacturonic acid units are incorporated into polysaccharide material, however the experiment does not provide any information on any non-radioactive monosaccharides which may be present in the polysaccharide material.

Degradation of the product using a commercial polygalacturonase preparation.

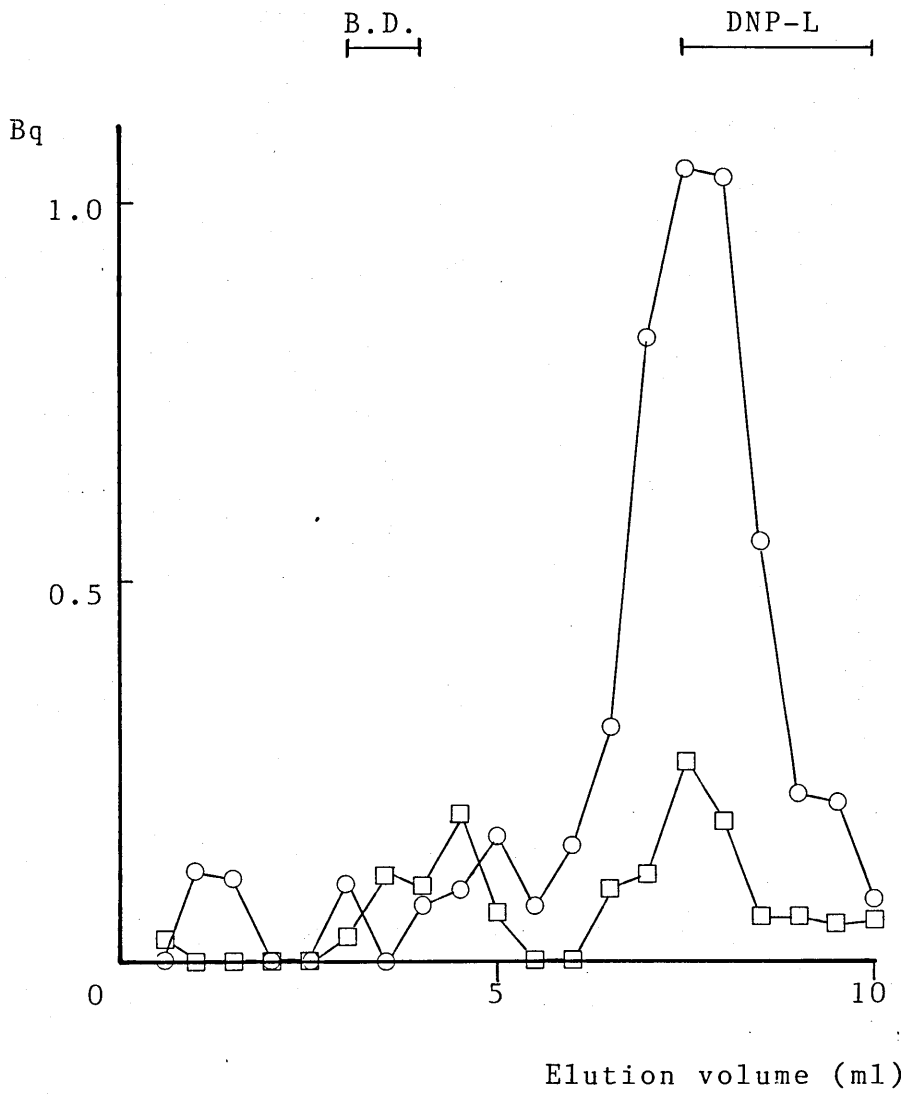
To confirm that the polysaccharide material is polygalacturonan, the effect of treating the material with a commercial polygalacturonase was investigated. UDP-galacturonic acid (370 Bq) was incubated with the particulate enzyme preparation and the resulting general polysaccharide fraction was incubated with a commercial endo-polygalacturonase, isolated from a Rhizopus species.

Polygalacturonases have the ability to hydrolyse the $\alpha(1\text{-}4)$ glycosidic bonds of polygalacturonic acid chains and the preferentially attack galacturonan chains with a low degree of methyl esterification (Doesburg, 1973). The molecular weight of the resulting material was analysed using a column of Sephadex G-100 (180mm x 7.5mm) and the results (figure 3.19.) indicate that the previously high-molecular-weight polysaccharide has been degraded into low-molecular-weight material.

As the polygalacturonase is obtained commercially, the purity of the

Figure 3.19.

Gel filtration of the general polysaccharide material (obtained following the incubation of UDP-^{14}C-galacturonic acid (370 Bq) with the particulate enzyme preparation) which had been treated with either polygalacturonase for 21 hours at 25°C (○——○) or, buffer for 21 hours at 25°C (□——□). Following treatment, the solubilised material was analysed using a column of Sephadex G-100. Polygalacturonase was dissolved in 100 mM sodium acetate buffer, pH 4.0, to give a concentration of 1 mgml^{-1}


B.D.
DNP-L
Bq
1.0
0.5
0
5
10
Elution volume (ml)

preparation is not known. Therefore, to remove any contaminants of different molecular weights, the polygalacturonase preparation was run through a column of Sepharose 6B-CL (270mm x 10mm). The fractions containing polygalacturonase activity were combined and used to treat the general polysaccharide fraction, obtained following the incubation of UDP-galacturonic acid (460 Bq) and the particulate enzyme preparation. The molecular weight of the resulting material was again analysed using gel filtration chromatography. Analysis was completed using a Sephadex G-100 column (180mm x 7.5mm) and the results (figure 3.20.) demonstrate that the polysaccharide was broken down into low-molecular-weight material by the action of polygalacturonase.

The end-products produced following treatment with polygalacturonase are generally monomer, dimer or trimer units or mixtures of these (Doesburg, 1973). In order to identify the components into which the polysaccharide had been degraded, the low-molecular-weight material was subjected to thin layer electrophoresis. However, the results obtained were not conclusive (figure 3.21.). The radioactive zone may correspond to that of a disaccharide or a trisaccharide. Whatever the precise nature of the enzyme-treated product, the degradation of the polysaccharide by the pectinase indicates that it is a pectin.

Further analysis of the product using ion-exchange chromatography

To confirm further that the polysaccharide is polygalacturonan, the charge present on the polysaccharide was determined using ion-exchange chromatography. Polygalacturonan is known to be a negatively-charged compound due to the carboxyl group on carbon 6. In the cell wall, methyl groups can be ester-linked to the carboxyl group on carbon 6

Figure 3.20.

Gel filtration of the general polysaccharide material (obtained following the incubation of UDP-^{14}C- galacturonic acid (460 Bq) with the particulate enzyme preparation) which has been treated with either purified polygalacturonase for 21 hours at 25°C (○——○) or, buffer for 21 hours at 25°C (□——□). Following treatment, the solubilised material was analysed using Sephadex G-100. The purified polygalacturonase was prepared as described in Chapter 2.

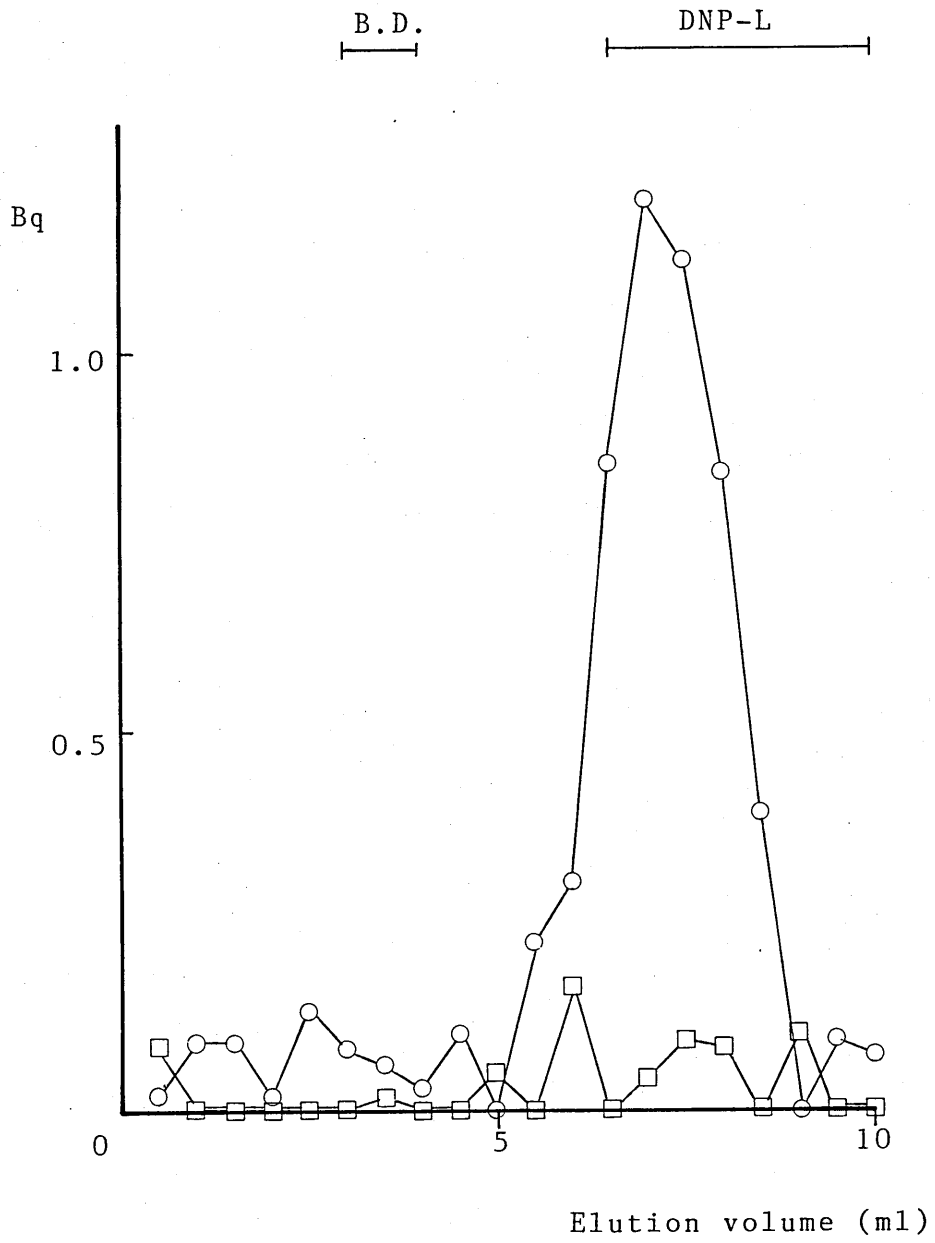
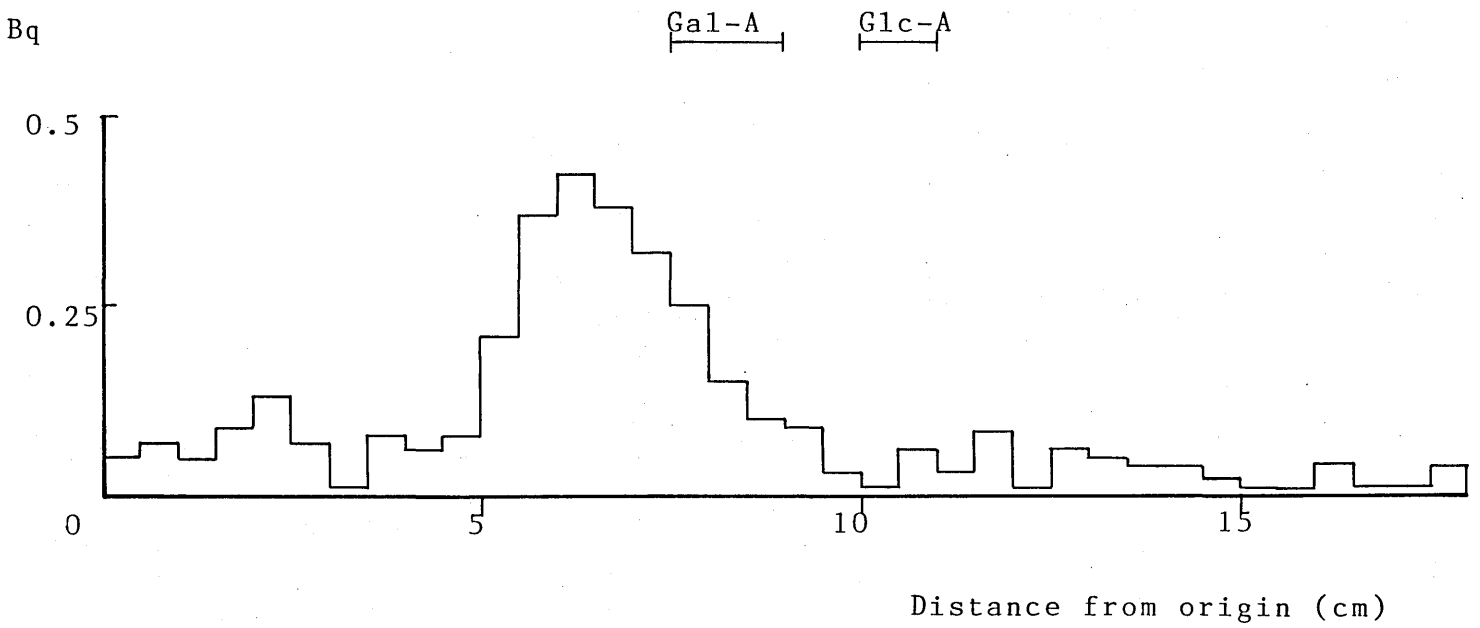

B.D.
DNP-L
Bq
1.0
0.5
0
5
10
Elution volume (ml)

Figure 3.21.

Thin layer electrophoretic analysis of the low-molecular-weight material obtained following treatment of the general polysaccharide fraction with the purified polygalacturonase preparation. The general polysaccharide material, obtained after incubating UDP-^{14}C-galacturonic acid (460 Bq) with the particulate enzyme preparation, was treated with the purified polygalacturonase preparation for 21 hours at 25°C. The solubilised material was analysed using Sephadex G-100 and the low-molecular-weight material was subjected to thin layer electrophoresis. The polygalacturonase preparation was purified, as described in Chapter 2.


Bq
Gal-A
Glc-A
0.5
0.25
0
5
10
15
Distance from origin (cm)

of the galacturonic acid units, therefore in vivo the polysaccharide may not be negatively charged. However, in this system, it is likely that at least the newly-formed, radioactive polysaccharide material is not methylated and therefore it would be expected to have a negative charge. UDP-galacturonic acid (460 Bq) was incubated with the enzyme preparation and the resulting EDTA extract was dialysed, before being analysed on a column of Amberlite IRA-400 resin. The results, table 3.8., indicate that the polysaccharide material is negatively-charged.

Analysis of the radioactive material present in the H_2O extractions

The H_2O extractions have also been investigated to determine whether any high-molecular-weight material is extracted by the H_2O. Knee (1978) demonstrated that pectic substances can be extracted by H_2O, therefore it was of interest to examine the H_2O extract for high-molecular-weight material. The H_2O extractions were included in the standard extraction procedure in order to remove radioactive UDP-galacturonic acid which had not been completely removed in the ethanol washes.

Determination of the molecular weight of the radioactive material present in the H_2O extractions

The particulate enzyme preparation was incubated with UDP-galacturonic acid (230 Bq) and the H_2O extracts were analysed on a column of Sephadex G-100 (180mm x 7.5mm). The results (figure 3.22.) demonstrate that there is both low-and high-molecular-weight material present in the H_2O extract.

TABLE 3.8.

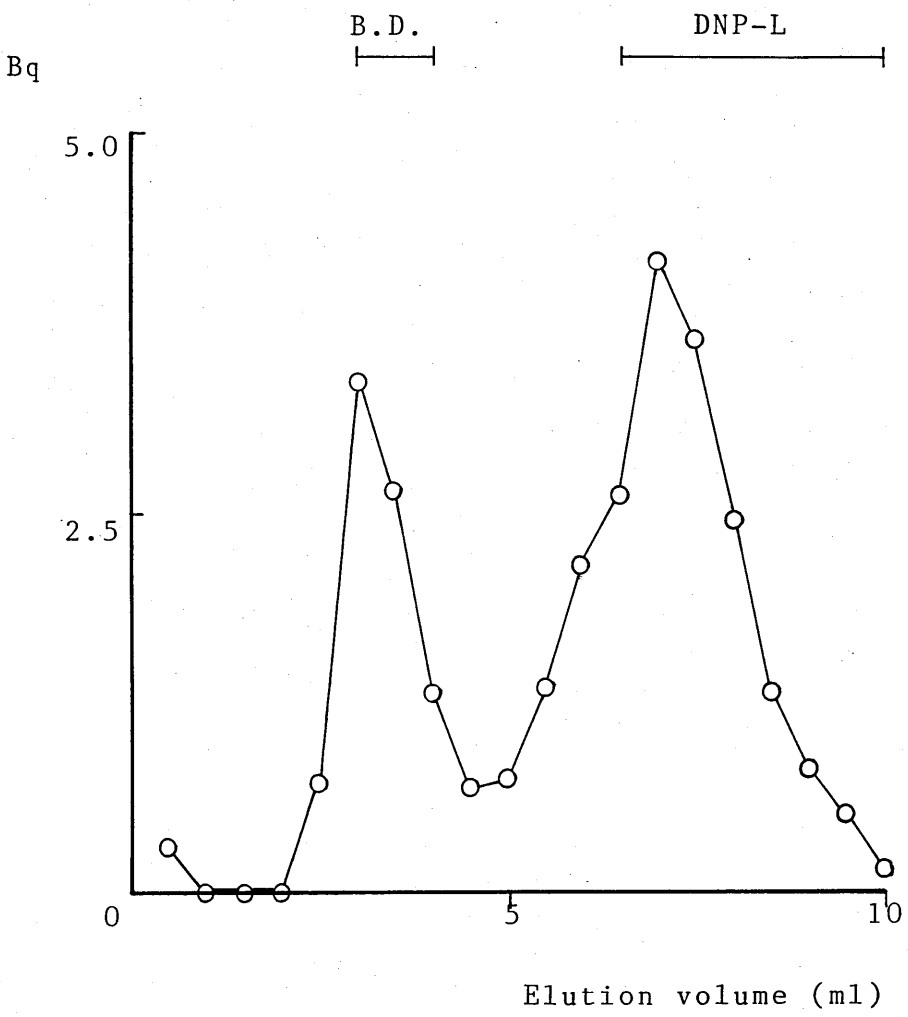
Analysis of EDTA - soluble material using a column of ion-exchange resin Amberlite IRA - 400.

The EDTA-soluble material was extracted from the general polysaccharide material obtained following the incubation of UDP-^{14}C - galacturonic acid (460 Bq) with the particulate enzyme preparation for 10 minutes.

Fraction	Radioactivity (Bq)
Anionic material	4.06
Cationic and neutral material	0.70

Figure 3.22.

Sephadex G-100 profile of the H_2O extractions included in the standard extraction procedure for the isolation of the general polysaccharide material. Incubations contained 230 Bq UDP-^{14}C-galacturonic acid.


B.D.
DNP-L
Bq
5.0
2.5
0
5
10
Elution volume (ml)

Analysis of the high-molecular-weight, H_2O-soluble material using total acid hydrolysis

The high-molecular-weight material present in the H_2O extract has been analysed. UDP-galacturonic acid (230 Bq) was incubated with the enzyme preparation and the resulting H_2O extract was dialysed to remove all low-molecular-weight material. The high-molecular-weight material was then subjected to total acid hydrolysis. Thin layer electrophoresis was employed to identify the radioactive monosaccharides present in the hydrolysate. The results (figure 3.23.) indicate that galacturonic acid units are present in the polysaccharide material. The peak of radioactive material which migrated just past the origin was not identified. The results of this experiment indicate that the enzyme preparation catalyses the incorporation of galacturonic acid into polysaccharide material which is soluble in hot H_2O.

Treatment of the H_2O-soluble polysaccharide material with a commercial polygalacturonase preparation

The polysaccharide material present in the H_2O extracts has been treated with the commercial polygalacturonase preparation. The H_2O extract, obtained following the incubation of UDP-galacturonic acid (230 Bq) with the enzyme preparation, was initially run through a column of Sephadex G-100 (180mm x 7.5mm). The high-molecular-weight material was combined and dried, before treatment with the commercial endo-polygalacturonase preparation. The molecular weight of the resulting material was analysed using a column of Sephadex G-100 (180mm x 7.5mm) and the results (figure 3.24.) demonstrate that the polysaccharide material was partially degraded by the action of polygalacturonase.

Figure 3.23.

Thin layer electrophoretic analysis of the radioactive monosaccharides resulting from total acid hydrolysis of the high-molecular-weight material present in the H_2O extracts. The H_2O extract was obtained following the incubation of UDP-^{14}C-galacturonic acid with the particulate enzyme preparation and the low-molecular-weight material present was removed by dialysis.

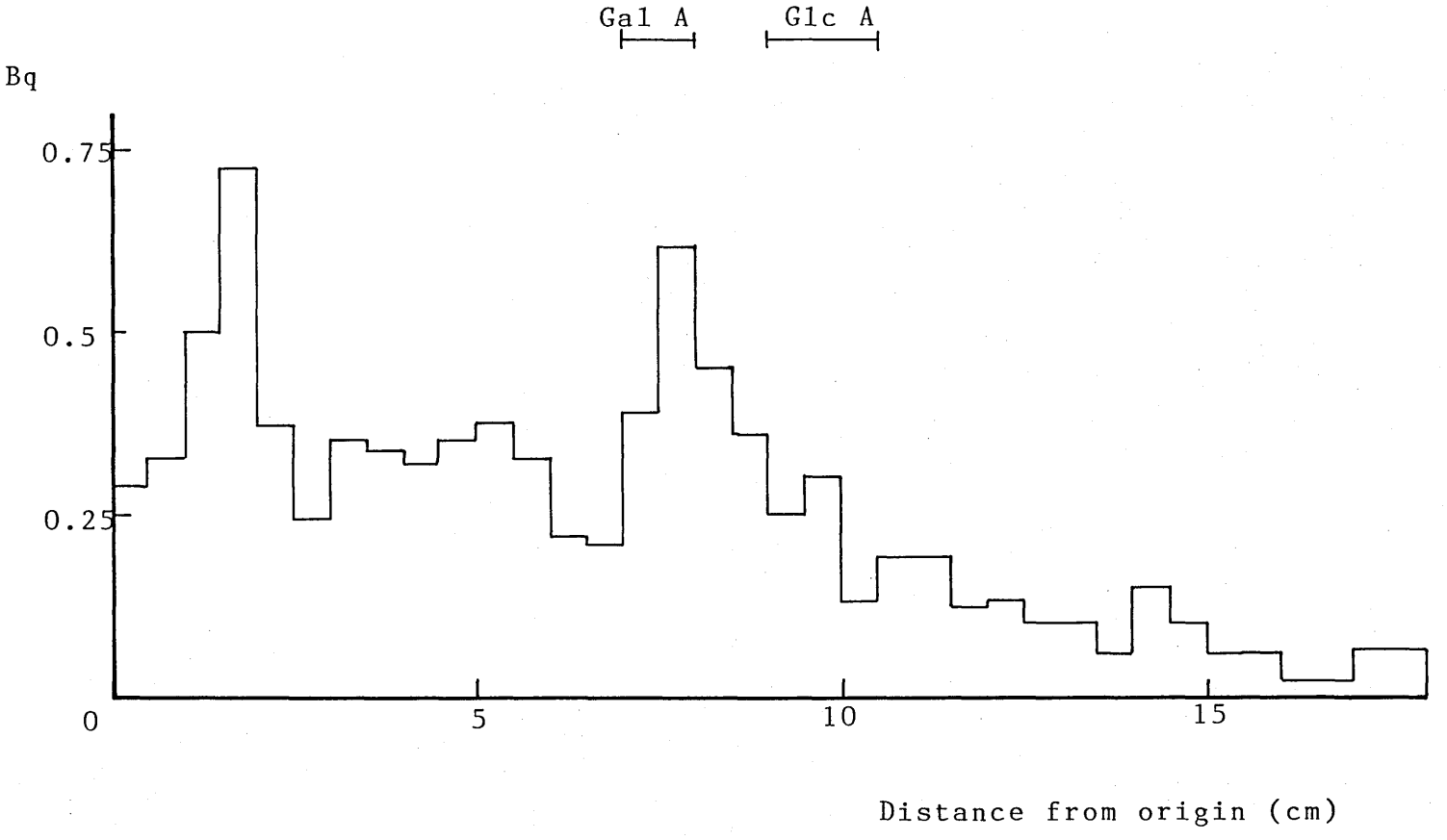
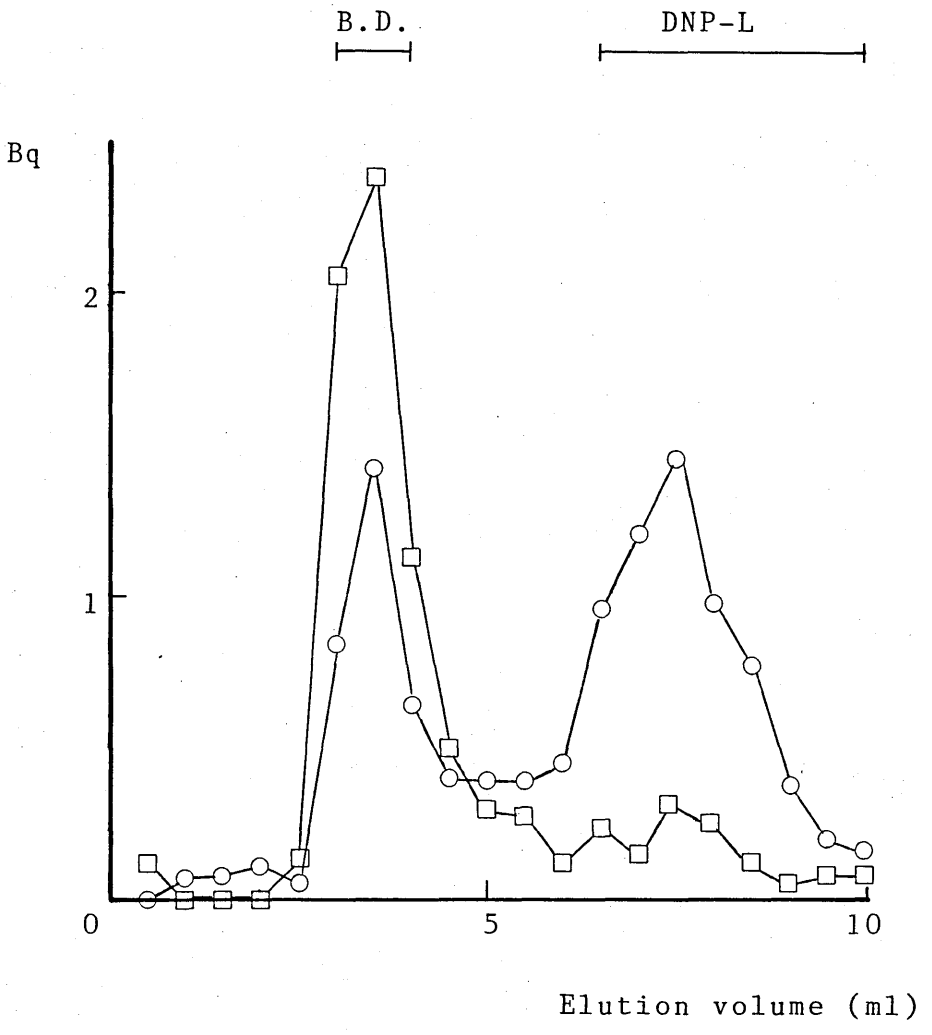

Gal A
Glc A
Bq
0.75
0.5
0.25
0
5
10
15
Distance from origin (cm)

Figure 3.24.

Gel filtration of the high-molecular-weight, H_2O-soluble material following treatment with polygalacturonase. The H_2O extract, obtained after incubating UDP-galacturonic acid (230 Bq) with the particulate enzyme preparation, was analysed on a column of Sephadex G-100. The high-molecular-weight material was either treated with polygalacturonase for 21 hours at 25°C (○—○) or with buffer (□—□). The material was then analysed using a column of Sephadex G-100.


B.D.
DNP-L
Bq
2
1
0
5
10
Elution volume (ml)

Discussion.

From the results, reported in this chapter, it can be concluded that a galacturonyltransferase enzyme is present in pea epicotyls. The enzyme, which is membrane-bound, is capable of catalysing the incorporation of galacturonic acid from UDP-galacturonic acid into polysaccharide material. Some degradation of the polysaccharide product can occur. The optimum conditions required by the enzyme have been determined, and the product, into which the galacturonic acid units are incorporated, was characterised using a variety of techniques.

At this stage, it is not clear into which pectic polysaccharide the galacturonic acid units are being incorporated. Results from gel filtration chromatography have indicated that all the polysaccharide material is of high molecular weight. It is probably not rhamnogalacturonan II, since this polysaccharide is complex, composed of nine monosaccharides linked by a variety of different bonds, and therefore the synthesis of this polymer would involve the action of many enzymes and the presence of many substrates would be required.

The product may be homogalacturonan. However, there is no information regarding the possibility of other monosaccharides being present in the product. Therefore, the possibility remains that galacturonic acid units may have been incorporated into rhamnogalacturonan I.

Alternatively, galacturonic acid may be incorporated into more than one polysaccharide. The fact that the polysaccharide material can be separated into H_2O - soluble and EDTA - soluble material may support the latter suggestion. However, this apparent distinction of the polysaccharide material may not be real but result from overlapping

of the extraction procedures.

As the precise structure of the product is not known, the terms galacturonan and polygalacturonan will be used in this thesis as a general term for the galacturonic acid-containing polysaccharides.

Chapter 4

INVESTIGATION OF POSSIBLE ACCEPTORS AND INTERMEDIATES INVOLVED IN THE BIOSYNTHESIS OF GALACTURONAN

Introduction

UDP-$[U-^{14}C]$- galacturonic acid, $MnCl_2$ and the particulate enzyme preparation are the only components present in the standard incubation mixture. No acceptor molecules are added to the incubation mixture; therefore, if the enzyme system is not synthesising galacturonan de novo then, in order for the reaction to proceed, acceptor molecules must be present in the membrane preparation. In an attempt to obtain further information regarding the reactions involved in the biosynthesis of polygalacturonan, different molecules were added to the standard incubation mixture to determine if they had the ability to act as acceptor molecules for galacturonic acid units.

The molecules investigated for their ability to act as exogenous acceptors were added to the standard incubation mixture and, following incubation with the particulate enzyme preparation, the general polysaccharide fraction was analysed for any variation in the incorporation of galacturonic acid. The incorporation of galacturonic acid into the polysaccharide material extracted by the hot H_2O was also determined. The H_2O extracts were analysed using a Sephadex G-100 column (180 mm x 7.5 mm) and the incorporation of galacturonic acid into high-molecular-weight material was determined.

The effect of the addition of different forms of polygalacturonic acid on the enzyme system

Initially, commercial polygalacturonic acid was added to the incubation mixture in a variety of different forms which are listed below:-

1. 1% polygalacturonic acid which had been dissolved in 50 mM EDTA/ NaH_2PO_4, pH 6.8 (30 µl).
2. 1% polygalacturonic acid which had been dissolved in 50 mM EDTA/ NaH_2PO_4, pH 6.8, and then dialysed to remove the EDTA solution (30 µl).

3. Polygalacturonic acid (10 mg) following treatment with 1 mg polygalacturonase (30 µl , see Materials and Methods Chapter).

4. Solid polygalacturonic acid (1 mg)

5. Solid polygalacturonic acid (1 mg) plus 10% Triton X-100 (10 µl)

6. 1% polygalacturonic acid which had been dissolved in 50 mM EDTA/ NaH_2PO_4, pH 6.8, and then dialysed (20 µl) plus 10% Triton-X-100 (10 µl).

Two control incubations were also conducted. In the first, only H_2O was added, and in the second, 10% Triton X-100 (10 µl) was added. Triton X-100 was added to some incubations in an attempt to increase contact between the polygalacturonic acid and the enzyme system by its action as a detergent.

The results, in figure 4.1., demonstrate that most of the different forms of polygalacturonic acid added to the incubation mixture resulted in a decrease in the incorporation of galacturonic acid.

The addition of 1% polygalacturonic acid, dissolved in 50mM EDTA/ NaH_2PO_4 buffer, pH 6.8, and then dialysed, did not appear to inhibit the enzyme system, although no promotory effect was detected either. Different concentrations (0.25, 0.5, and 1%) of polygalacturonic acid, prepared in this way, were added to the standard incubation mixture (30µl). Following incubation with the particulate enzyme preparation, the polysaccharide fractions were analysed. From the results, illustrated in figure 4.2., it appears that the greater the concentration of polygalacturonic acid present in the incubation mixture, the greater the inhibition on the enzyme system.

<u>The effect of the addition of pectin on the enzyme system</u>

Pectin (30 µl), prepared from pea epicotyls, has also been added to the incubation mixture to determine if it could function as an

Figure 4.1.

Effect of the addition of polygalacturonic acid, in a variety of different forms, on the incorporation of galacturonic acid into both the general polysaccharide fraction (clear bars on chart) and the high-molecular-weight, H_2O-soluble fraction (crossed bars on chart) following a 10 minute incubation period.

The numbers on the bar chart correspond to the addition of the following compounds to the standard incubation mixture:-

1 = H_2O

2 = 1% polygalacturonic acid which had been dissolved in EDTA buffer, and then dialysed to remove the EDTA buffer.

3 = 1% polygalacturonic acid which had been dissolved in EDTA buffer.

4 = Solid polygalacturonic acid

5 = Polygalacturonic acid following treatment with polygalacturonase.

6 = 10% Triton X-100

7 = Solid polygalacturonic acid plus 10% Triton X-100

8 = 1% polygalacturonic acid, which had been dissolved in EDTA buffer and then dialysed, plus 10% Triton X-100.

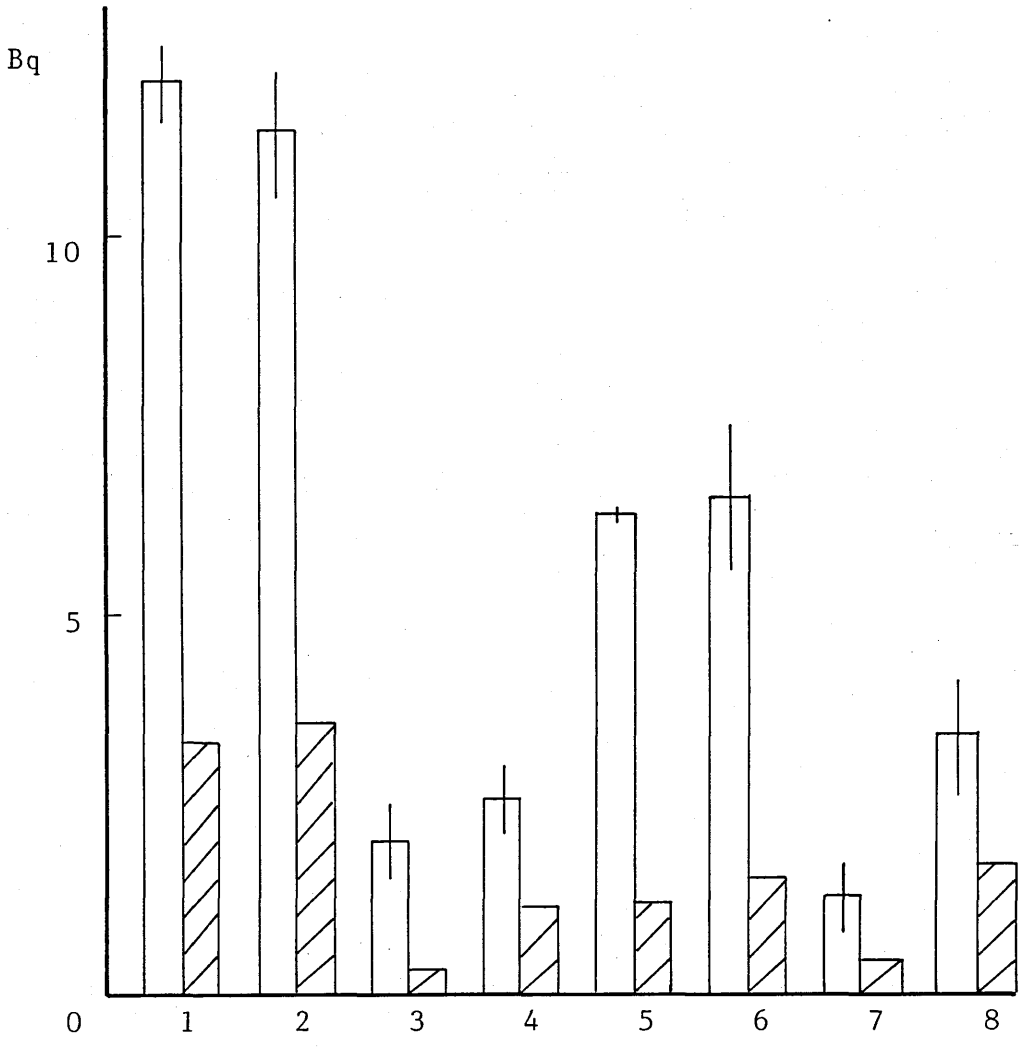
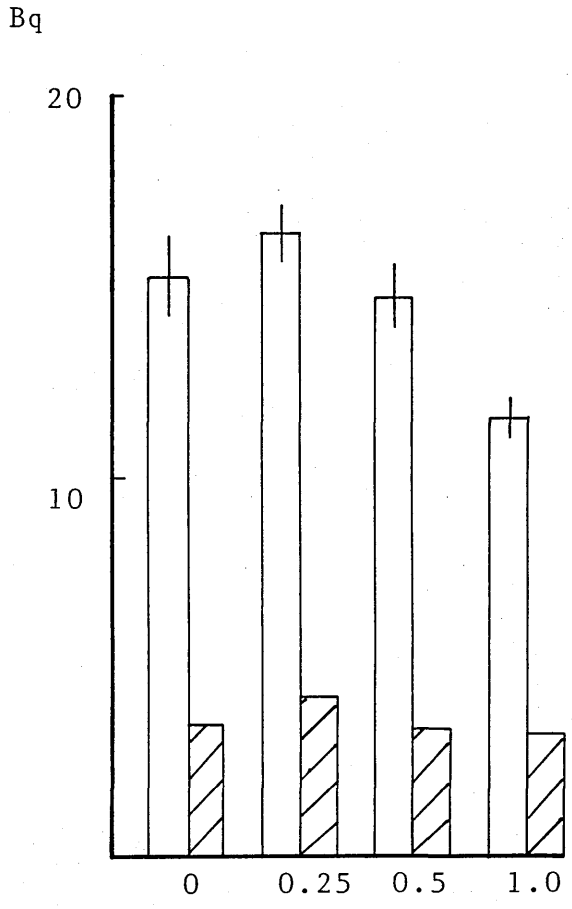

Bq
10
5
0
1
2
3
4
5
6
7
8

Figure 4.2.

Effect of the addition of different concentrations of poly-galacturonic acid (which had been dissolved in 50 mM EDTA/NaH_2PO_4 buffer, pH 6.8, and then dialysed against H_2O) to the incubation mixture on the incorporation of radioactivity from UDP-^{14}C - galacturonic acid into the general polysaccharide material (clear bars on chart) and the high-molecular-weight, H_2O- soluble fraction (crossed bars on chart) following an incubation period of 10 minutes.


Bq
20
10
0
0.25
0.5
1.0
Concentration of polygalacturonic acid (%)

acceptor. Analysis of the polysaccharide fractions (table 4.1.) demonstrated that the addition of pectin had no significant effect on incorporation of galacturonic acid.

In order to concentrate the pectin preparation, it was freeze-dried, and then redissolved in H_2O (1 ml). This increased the concentration of pectin by a factor of twenty. Addition of the freeze-dried pectin preparation (30 µl) to the incubation mixture caused a significant decrease in the incorporation of galacturonic acid units (table 4.2.). Therefore, addition of the more concentrated pectic material resulted in the inhibition of the incorporation of galacturonic acid into galacturonan.

More pectic material was prepared from pea epicotyls and then subjected to acid hydrolysis. The pectin was hydrolysed with 2 M TFA at 100°C for varying periods of time - 0, 1, 10, and 30 minutes. The hydrolysed pectin preparations were then added to the incubation mixture (30 µl) and, following incubation with the enzyme preparations, analysis of the resulting polysaccharide fractions (figure 4.3.) revealed that the longer the pectic material had been hydrolysed, the greater its inhibitory effect on the enzyme system. Therefore, it appears that increasing the number of pectic oligosaccharide fragments results in greater inhibition of galacturonyltransferase activity.

<u>The effect of the addition of boiled membrane preparation on the enzyme system.</u>

Boiled membrane preparation has also been examined to determine whether it contains any acceptor molecules. However, the results, shown in table 4.3., demonstrate that the addition of boiled membrane preparation (30 µl) to the standard incubation mixture has no significant effect on the incorporation of galacturonic acid. One

TABLE 4.1.

Effect of the presence of pectin (prepared from pea epicotyls) in the incubation mixture on the incorporation of ^{14}C-galacturonic acid into both the general polysaccharide fraction and the high-molecular-weight, H_2O-soluble material following an incubation period of 10 minutes.

Fraction	Incorporation (Bq)	
	No pectin added	Pectin added
General polysaccharide fraction	24.14 ± 3.13	27.54 ± 0.03
High M.W., H_2O - soluble fraction	3.96 ± 0.25	4.08 ± 0.41

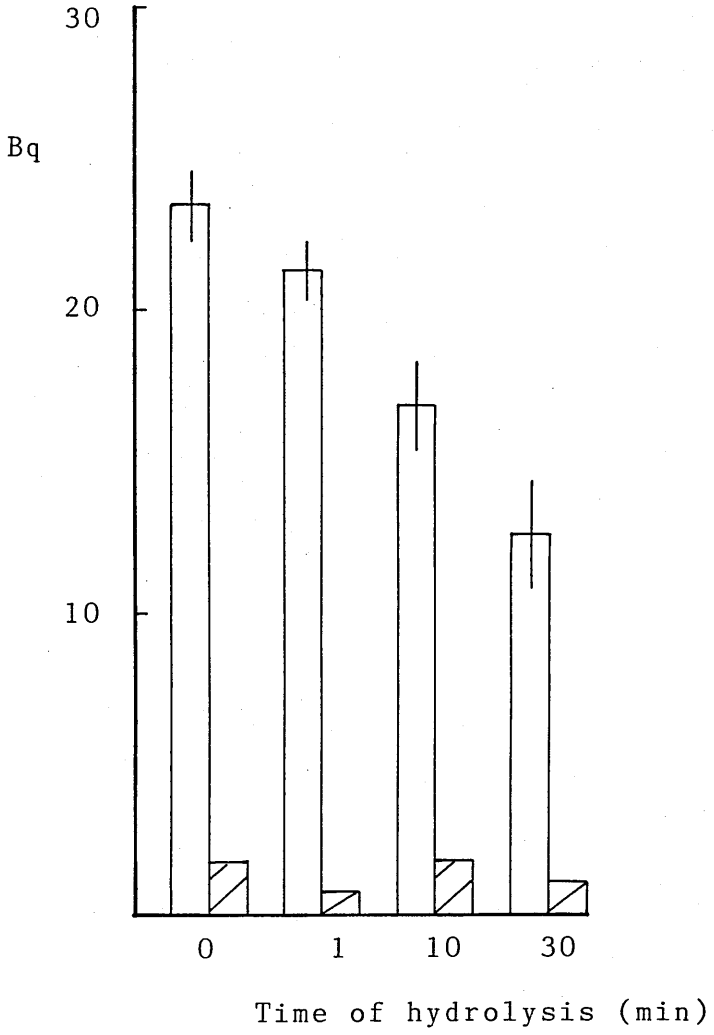
TABLE 4.2.

Effect of the addition of freeze-dried pectin to the incubation mixture on the incorporation of ^{14}C-galacturonic acid into both the general polysaccharide fraction and the high-molecular-weight, H_2O-soluble fraction following an incubation period of 10 minutes.

Fraction	Incorporation (Bq)	
	No freeze-dried pectin added	Freeze-dried pectin added
General polysaccharide	19.81 ± 0.23	1.85 ± 0.49
High M.W., H_2O-soluble fraction	1.83 ± 0.23	0.30 ± 0.01

Figure 4.3.

Effect of the addition of hydrolysed pectic material to the incubation mixture on the incorporation of galacturonic acid from UDP-galacturonic acid into both the general polysaccharide fraction (clear bars on chart) and the high-molecular-weight, H_2O-soluble material (crossed bars on chart) following an incubation period of 10 minutes. Pectic material was prepared from pea epicotyls and then subjected to hydrolysis using TFA at 100°C for varying periods of time.


30
Bq
20
10
0
1
10
30
Time of hydrolysis (min)

possible explanation for the apparent absence of acceptor molecules in this preparation may be that the boiling procedure has altered the structure of the acceptor molecule.

An investigation into the possibility of lipid intermediates.

The possibility of the involvement of lipid intermediates in the biosynthesis of cell wall polysaccharides has been suggested (Maclachlan, 1985). Therefore, it was of interest to investigate whether lipid intermediates play a role in the biosynthesis of polygalacturonan. The particulate enzyme preparation was incubated with UDP-galacturonic acid (185 B_q) for 10 minutes. The incubations were then extracted as described in Chapter 2.

The results, shown in table 4.4., demonstrate that there was no significant incorporation of radioactive material in the extract containing polyprenylphosphate sugars or in the extract containing polyprenylphosphate oligosaccharides (Brett, 1981). The radioactive material remained in the pellet. These results suggest that lipid compounds are not intermediates in the biosynthesis of galacturonan.

An investigation into the possibility of the involvement of a protein intermediate.

The possibility of the involvement of proteinaceous intermediates in the biosynthesis of galacturonan has also been investigated. Initially, the product was examined for any evidence indicating the attachment of protein onto the polysaccharide material. The polysaccharide material, obtained following the incubation of UDP-galacturonic acid with the particulate enzyme preparation, was treated with either protease, proteinase K or buffer. The molecular weight of the material was then estimated using gel filtration chromatography. A reduction in the molecular weight of the material following protease or proteinase K treatment would indicate the

TABLE 4.3.

Effect of the addition of boiled membrane preparation to the incubation mixture on the incorporation of ^{14}C-galacturonic acid from UDP-galacturonic acid into both the general polysaccharide fraction and the high-molecular-weight, H_2O-soluble fraction following an incubation period of 30 minutes.

Fraction	Incorporation (Bq)	
	No boiled membrane preparation added	Boiled membrane preparation added
General polysaccharide fraction	23.66 ± 1.77	24.99 ± 2.61
High M.W., H_2O-soluble fraction	1.12	0.95

TABLE 4.4.

Result of an investigation into the possibility of the involvement of lipid intermediates during the biosynthesis of galacturonan. Polyprenylphosphate-sugars and polyprenylphosphate - oligosaccharides were extracted as described by Brett (1981) following a 10 minute incubation period of UDP-galacturonic acid with the particulate enzyme preparation

Extract	Radioactivity incorporated (Bq)
Polyprenylphosphate-sugar extract	1.07 ± 0.22
Polyprenylphosphate-oligosaccharide extract	0.19 ± 0.07
Pellet remaining	21.30 ± 4.56

attachment of a protein onto the polysaccharide.

UDP-galacturonic acid (500 B_q) was incubated with the enzyme preparation for 10 minutes. The resulting EDTA extracts were combined and dialysed against H_2O. The material was dried under vacuum and then treated with either proteinase K, protease or buffer. The molecular weight of the resulting material was analysed on a column of Sepharose 6B-CL (450mm x 10 mm). The results, illustrated in figures 4.4.,4.5.,and4.6., demonstrate that, following all three treatments, the polysaccharide material is excluded from the column. Therefore, the results are inconclusive as any reduction in molecular weight may not be detected due to the exclusion of the material from the column.

The experiment was repeated using 750 Bq UDP-galacturonic acid, and analysis of the molecular weight of the treated material was completed using a column of Sepharose 2B-CL (450 mm x 10 mm). This column separates polysaccharides with a molecular weight in the range of 10^5 to 20 x 10^6 (Pharmacia Handbook). From the results (figures 4.7., 4.8. and 4.9.) it would appear that there is no difference in the molecular weight of the proteinase K-treated, protease-treated and the buffer-treated material. However, the radioactive material was excluded from the column over a relatively large volume indicating a range of molecular weights. To ensure that there was no difference in the molecular weight of the treated material, the experiment was repeated. Incubations containing 1000 B_q UDP-galacturonic acid were conducted for 10 minutes. In this experiment, all the EDTA extracts were combined and dried under vacuum. The extract was run on a column of Sepharose 2B-CL (450 mm x 10 mm) and 25 fractions of 4 ml volume were collected. The material was divided into two parts. Fraction A comprised of the material excluded in fractions 9 to 15

Figure 4.4.

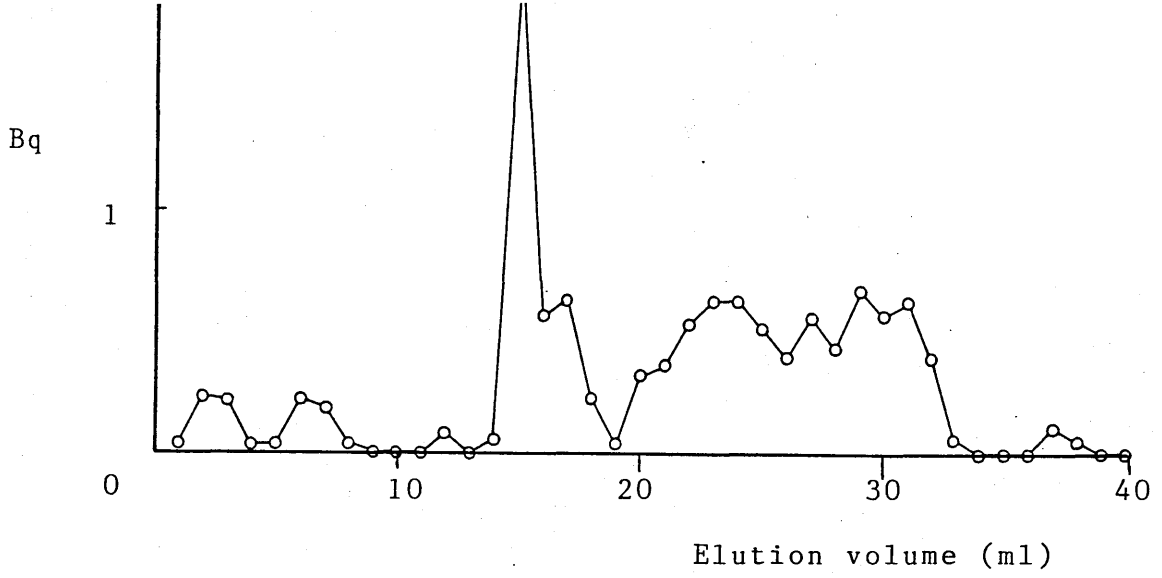
Sepharose 6B-Cl profile of polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following treatment of the extract with buffer (10 mM Tris-HCl buffer, pH 7.5). The EDTA-soluble material was obtained following the incubation of UDP-^{14}C-galacturonic acid (500 Bq) with the particulate enzyme preparation. The EDTA-extracted material was dialysed against H_2O and then treated with buffer for 3 hours at 25°C.

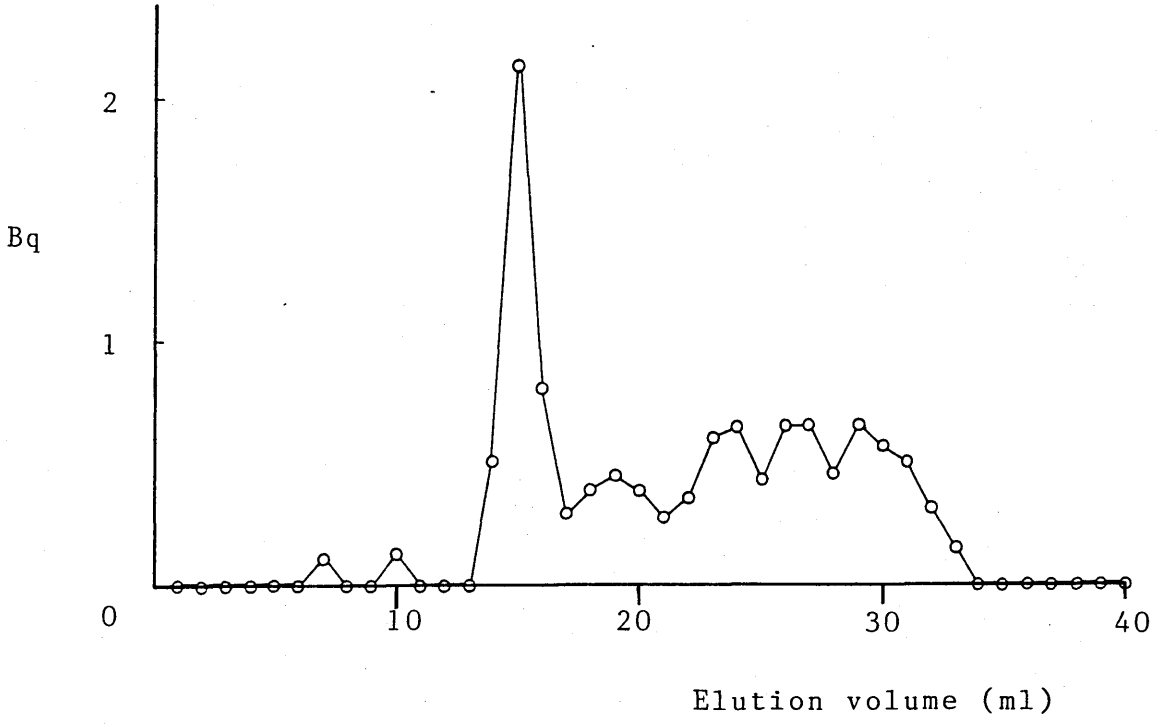
Figure 4.5.

Sepharose 6B-Cl profile of polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following treatment with proteinase K. The EDTA-soluble material was obtained after incubating UDP-^{14}C-galacturonic acid (500 Bq) with the particulate enzyme preparation. The EDTA extract was dialysed and then treated with proteinase K, which was dissolved in 10 mM Tris-HCl buffer, pH 7.5, (5 $mg.ml^{-1}$ concentration) for 3 hours at 25°C.

Figure 4.6.

Sepharose 6B-Cl profile of polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following treatment of the material with protease. The EDTA-soluble material was obtained following the incubation of UDP-^{14}C-galacturonic acid (500 Bq) with the particulate enzyme preparation. The EDTA extract was dialysed before treatment with protease which was dissolved in 10 mM Tris-HCl buffer, pH 7.5, (5 $mg.ml^{-1}$ concentration) for 3 hours at 25°C.


Bq
1
0
10
20
30
40
Elution volume (ml)


Bq
2
1
0
10
20
30
40
Elution volume (ml)

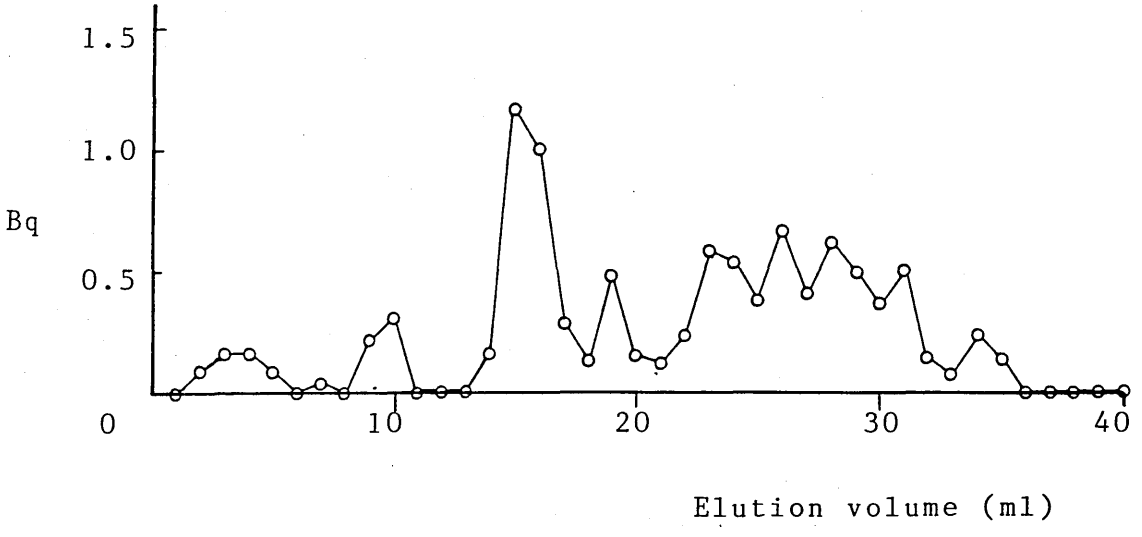

Bq
1.5
1.0
0.5
0
10
20
30
40
Elution volume (ml)

Figure 4.7.

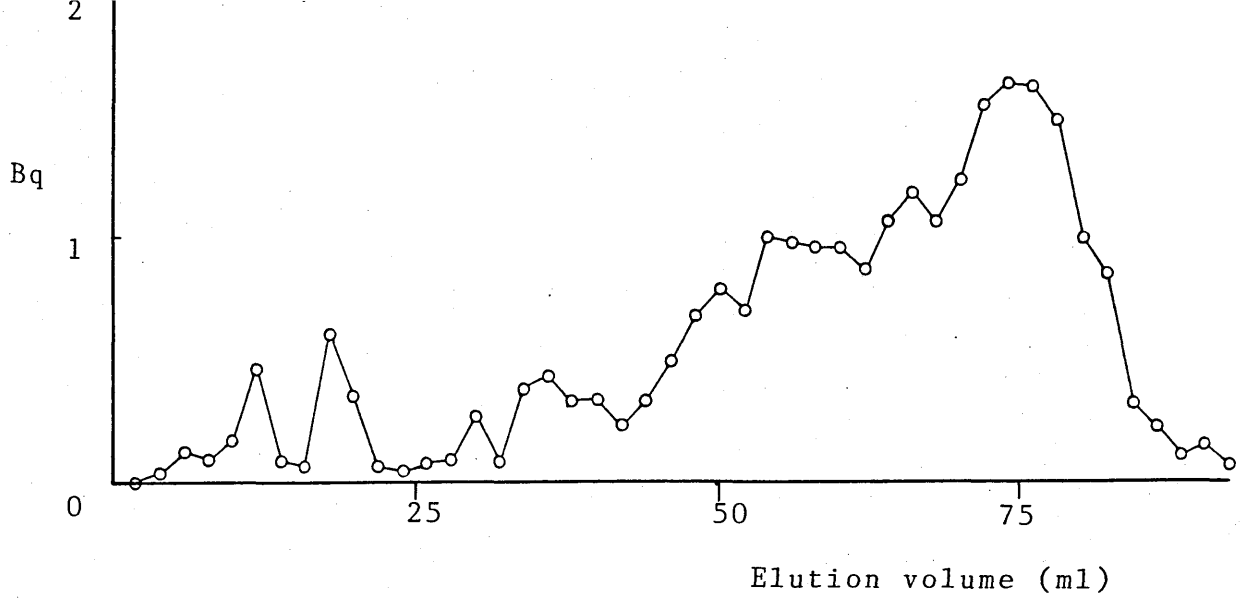
Gel filtration analysis using Sepharose 2B-CL of the polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following treatment with buffer (10mM Tris-HCL, pH 7.5) for 3 hours at 25°C. The EDTA extract was obtained following the incubation of UDP-^{14}C-galacturonic acid (750 Bq) with the particulate enzyme preparation and was dialysed before use.

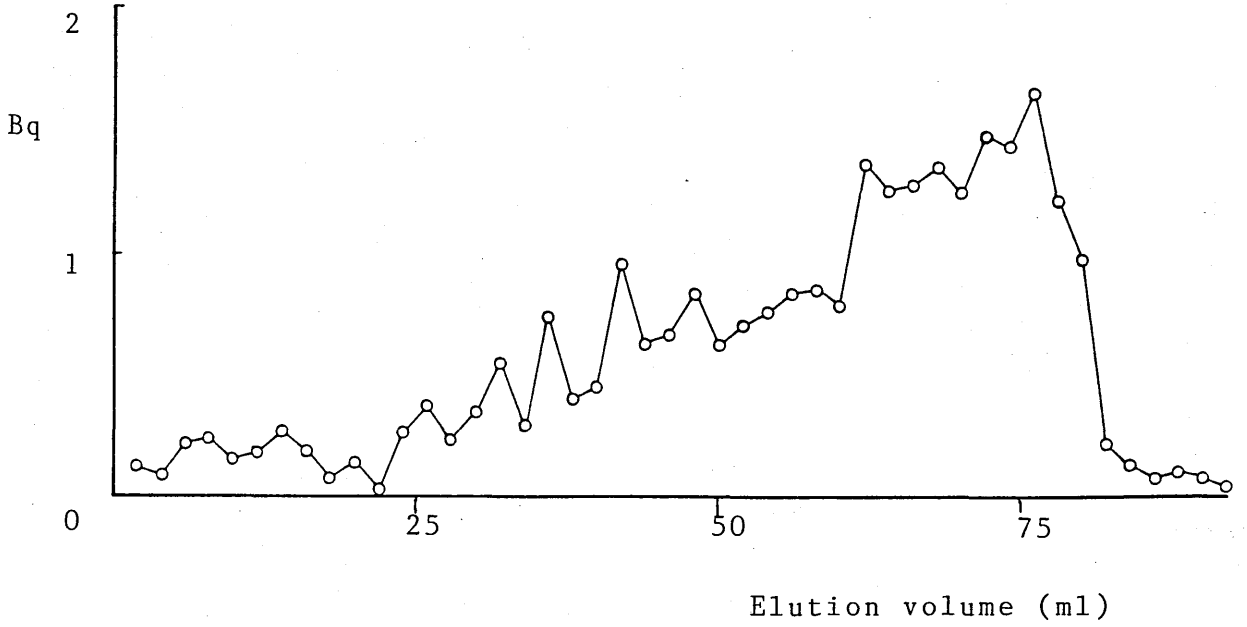
Figure 4.8.

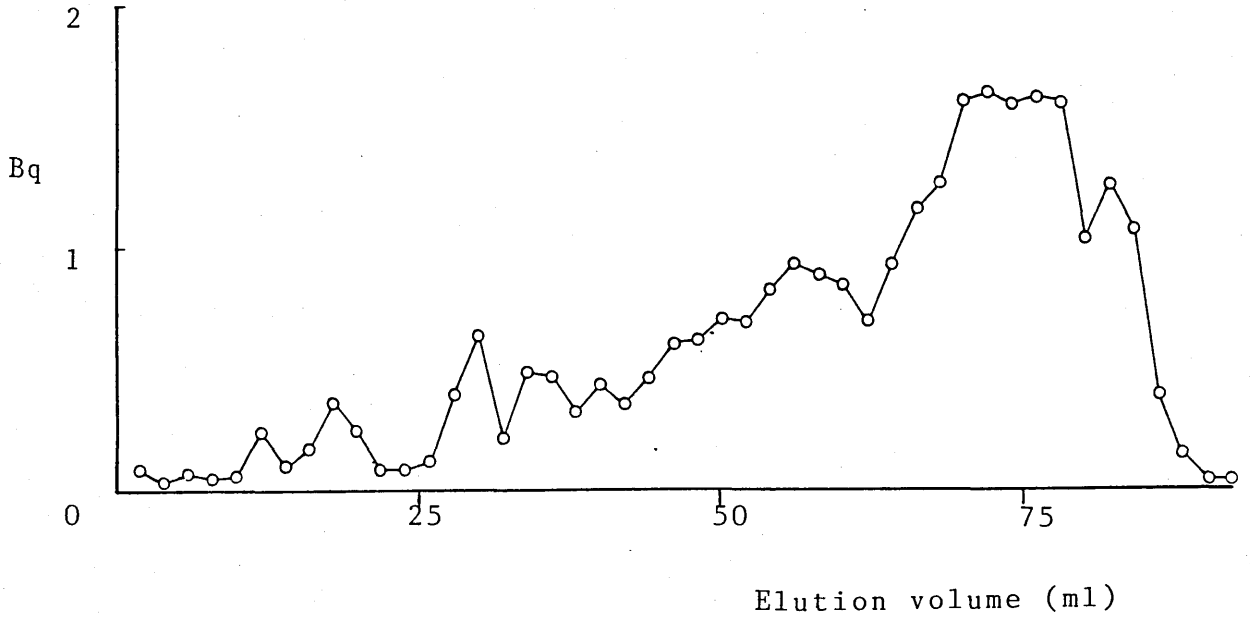
Gel filtration analysis using Sepharose 2B-CL of the polysaccharide fraction extracted by EDTA/NaH_2PO_4 buffer following treatment with proteinase K. The EDTA extract was obtained following the incubation of UDP-^{14}C-galacturonic acid (750 Bq) with the particulate enzyme preparation. The EDTA extract was dialysed then treated with proteinase K (which was dissolved in 10mM Tris-HCl buffer, pH 7.5, to give a concentration of 5mg.ml^{-1}) for 3 hours at 25°C.

Figure 4.9.

Gel filtration analysis using Sepharose 2B-CL of the polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following treatment with protease. The EDTA extract was obtained following the incubation of UDP-^{14}C-galacturonic acid (750 Bq) with the particulate enzyme preparation. The EDTA extract was dialysed before treatment with protease (which was dissolved in 10mM Tris-HCl, pH 7.5, to give a concentration of 5mg.ml^{-1})for 3 hours at 25°C.


2
Bq
1
0
25
50
75
Elution volume (ml)


2
Bq
1
0
25
50
75
Elution volume (ml)


2
Bq
1
0
25
50
75
Elution volume (ml)

and fraction B comprised of the material in the fractions 16 to 18 (figure 4.10.) The two fractions were dialysed against H_2O and then treated with either proteinase K or buffer. The molecular weight of the resulting material was estimated using the Sepharose 2B-CL column. The results (figures 4.11., 4.12, 4.13.and 4.14.) indicate that there is no difference in the molecular weight of the proteinase K-treated material and the buffer-treated material.

One further experiment was carried out to determine whether protein is bound to the polysaccharide material. UDP-galacturonic acid (185 B_q) was incubated with the particulate enzyme preparation for 10 minutes. The general polysaccharade fraction obtained was treated with the proteinase K preparation (50 μl). As a control, buffer was added to identical material. Following an incubation period of 3 hours, the pellet and supernatant were analysed for radioactive material. The results (table 4.5.)demonstrate clearly that treatment with proteinase K does not result in the liberation of more radioactive material than treatment with buffer.

Although these results do not provide any evidence to indicate that protein is bound to the polysaccharide, this does not rule out the possibility of the involvement of protein intermediates. It may be that the protein becomes dissociated from the polysaccharide. Therefore, in order to further investigate the possibility of a protein intermediate, the particulate enzyme preparation was extracted with buffer containing 10% Triton X-100. Detergents can solubilise some membrane-bound proteins and it was thought that the presence of the detergent Triton X-100 may cause the release of a membrane-bound protein which functions as an acceptor molecule. The Triton X-100-soluble material (30 μl) was added to the standard incubation mixture and, following incubation with the particulate enzyme preparation, analysis of the resulting polysaccharide fractions indicated that

Figure 4.10.

Sepharose 2B-CL profile of the polysaccharide material extracted by EDTA/NaH_2PO_4 buffer. The EDTA-soluble material was obtained by combining the EDTA extractions from ten incubations all containing 1000 Bq UDP-^{14}C-galacturonic acid.

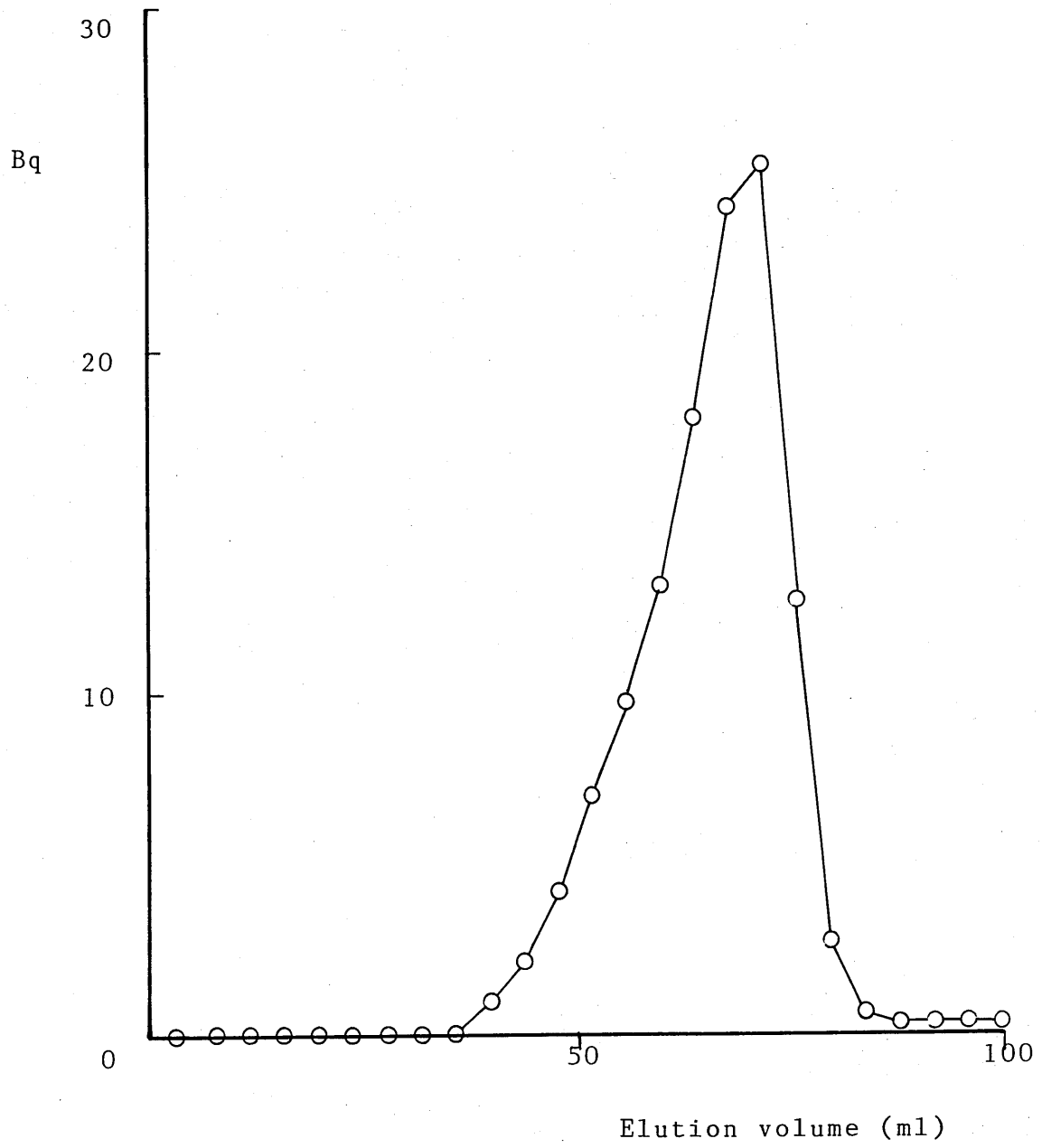
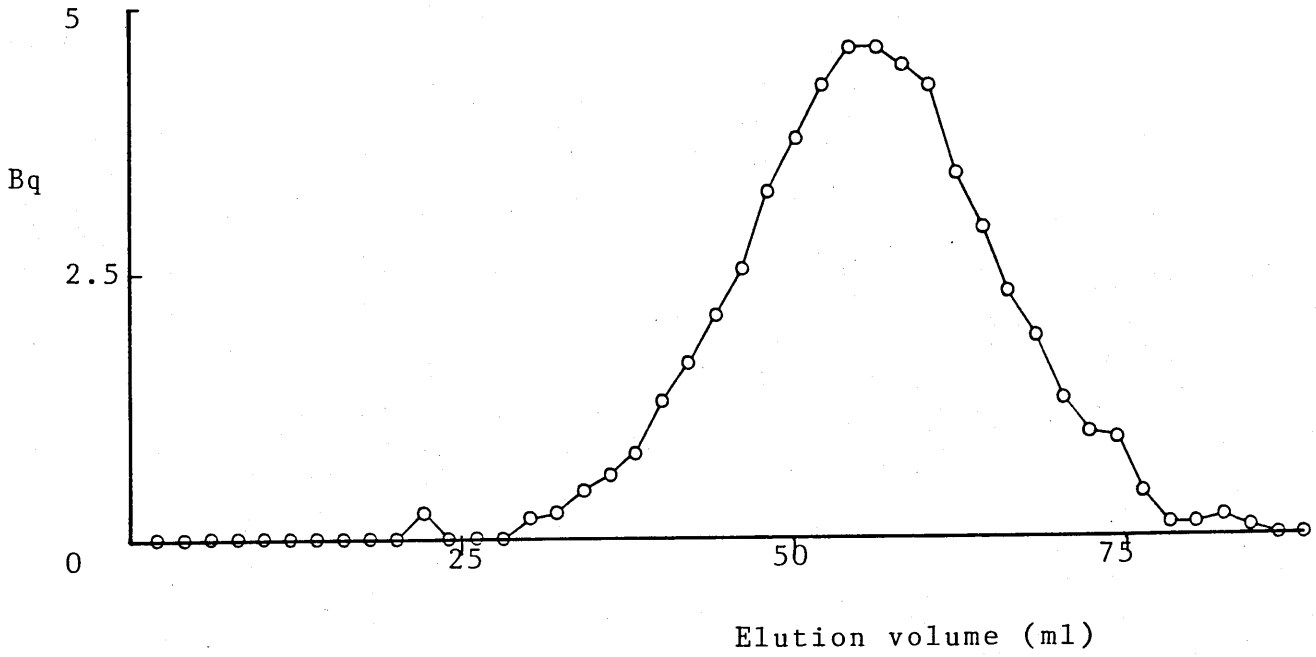

30
Bq
20
10
0
50
100
Elution volume (ml)

Figure 4.11.

Gel filtration analysis using Sepharose 2B-CL of fraction A after treatment with 10mM Tris-HCl buffer, pH 7.5, for 3 hours at 25°C. Fraction A was the material eluted in fractions 9-15 following analysis of EDTA-soluble material using Sepharose 2B-CL (figure 4.10.).

Figure 4.12.

Gel filtration analysis using Sepharose 2B-CL of fraction A following treatment with proteinase K for 3 hours at 25°C. The proteinase K was dissolved in 10mM Tris-HCL buffer, pH 7.5, to give a concentration of $5 mg.ml^{-1}$. Fraction A was the material eluted in fractions 9-15 following analysis of the EDTA-soluble material using Sepharose 2B-CL (figure 4.10.).


5
Bq
2.5
0
25
50
75
Elution volume (ml)

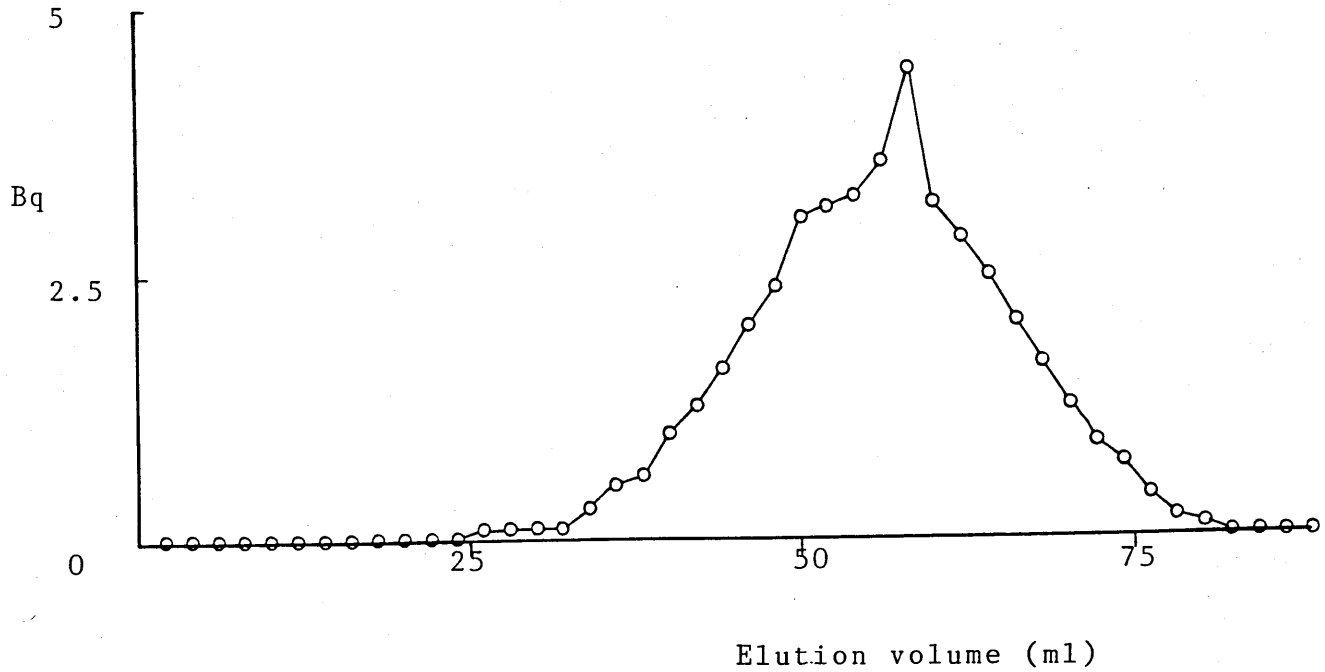
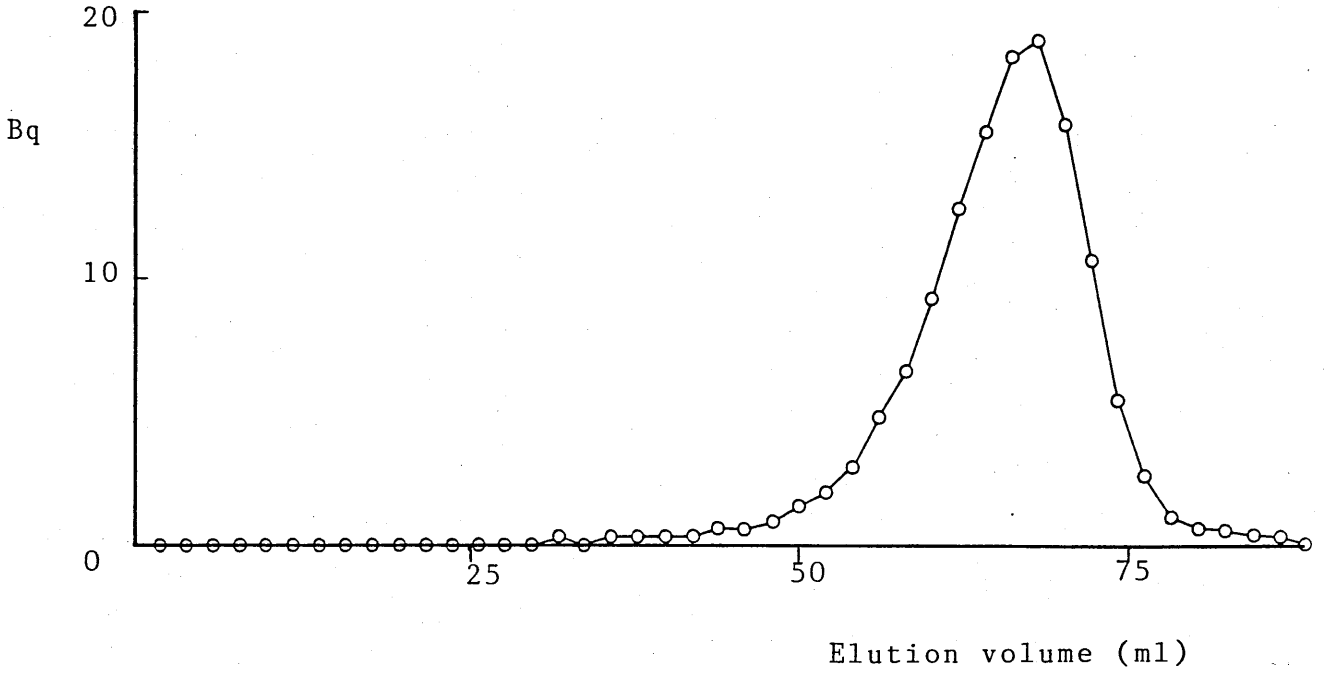

5
Bq
2.5
0
25
50
75
Elution volume (ml)

Figure 4.13.

Gel filtration analysis using Sepharose 2B-CL of fraction B following treatment with 10mM Tris-HCl buffer, pH 7.5, for 3 hours at 25°C. Fraction B was the polysaccharide material eluted in fractions 16-18 following analysis of the EDTA-soluble material using Sepharose 2B-CL (figure 4.10.).

Figure 4.14.

Gel filtration analysis using Sepharose 2B-CL of fraction B following treatment with proteinase K (which was dissolved in 10mM Tris-HCl buffer, pH 7.5, to give a concentration of 5 $mg.ml^{-1}$) for 3 hours at 25°C. Fraction B was the material eluted in fractions 16-18 following analysis of the EDTA-soluble material using Sepharose 2B-CL (figure 4.10.).


20
Bq
10
0
25
50
75
Elution volume (ml)

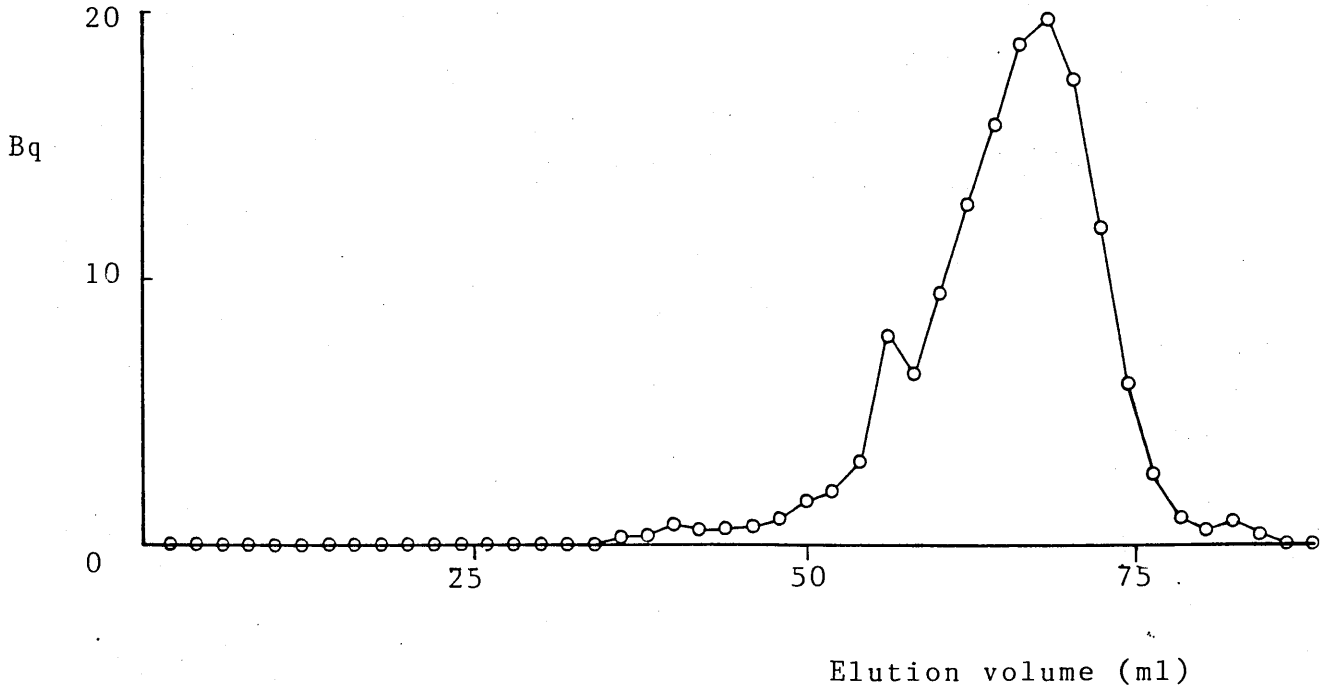

20
Bq
10
0
25
50
75
Elution volume (ml)

TABLE 4.5.

Distribution of radioactivity between the supernatant and the pellet following the incubation of the general polysaccharide fraction with proteinase K preparation. The general polysaccharide fraction was obtained following a 10 minute incubation of UDP-galacturonic acid with the particulate enzyme preparation.

Treatment	Distribution of radioactivity (%)	
	Supernatant	Pellet
Proteinase-treated	17.5	82.5
Buffer-treated	26.5	73.5

incorporation of galacturonic acid was greatly reduced in the presence of the Triton X-100-extracted material (table 4.6.). The presence of the detergent Triton X-100 does reduce enzymic activity by approximately 50% (figure 4.1.). However, the inhibitory effect exhibited by the Triton X-100-soluble material was greater than the loss of enzymic activity expected in the presence of Triton X-100.

Extrinsically-bound proteins can be extracted in the presence of high concentrations of salt solutions. Therefore, in order to determine whether the acceptor molecule is an extrinsic membrane protein, epicotyls were homogenised in buffer containing 1 M NaCl. The soluble and particulate fractions were separated by centrifugation as described in the Materials and Methods Chapter. An aliquot of the NaCl-solubilised material was dialysed to remove the NaCl solution.

The dialysed and non-dialysed extracts (30 µl) were added to the standard incubation mixture to examine whether the addition of either material increased incorporation of galacturonic acid into galacturonan. Following analysis of the polysaccharide fractions, the results (table 4.7.) indicate that the presence of the dialysed extract inhibited incorporation of galacturonic acid, whereas no difference in galacturonic acid incorporation was noted in the presence of the non-dialysed material. The NaCl-soluble material was dialysed to remove NaCl as it was thought that the presence of NaCl may interfere with galacturonyltransferase activity. However, from the results, it would appear that dialysis has removed a low molecular weight component, the presence of which inhibits the action of some high molecular weight compound. This high molecular weight compound inhibits the incorporation of galacturonic acid into galacturonan.

TABLE 4.6.

Effect of the addition of Triton X-100-solubilised material to the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide fraction and the high-molecular-weight, H_2O-soluble fraction following an incubation period of 30 minutes. The Triton X-100-solubilised material was obtained by extracting the particulate preparation with buffer containing 10% Triton X-100.

Fraction	Incorporation (Bq)	
	No Triton X-100-soluble material added	Triton X-100-soluble material added
General polysaccharide fraction	26.12 ± 2.17	2.83 ± 0.08
High M.W., H_2O-soluble fraction	2.71	0.90

TABLE 4.7.

Effect of the addition of NaCl-solubilised material and NaCl-solubilised material, which had been dialysed, to the incubation mixture on the incorporation of ^{14}C-galacturonic acid into both the general polysaccharide fraction and the high-molecular-weight, H_2O-soluble fraction following an incubation period of 30 minutes. The NaCl-solubilised material was obtained by extracting homogenised pea epicotyls with buffer containing 1M NaCl. The solubilised material was used directly, or dialysed against buffer (25mM Mes buffer, pH 6·0, with 1% BSA and 5mM DTT) and then used.

Fraction	Incorporation (Bq)		
	No NaCl-soluble material added	NaCl-soluble material added	NaCl-soluble material which had been dialysed
General polysaccharide fraction	26.12 ± 2.17	25.33 ± 0.57	6.31 ± 1.05
High M.W., H_2O-soluble fraction	2.71	1.75	0.39

Discussion

The experiments in this chapter were carried out in order to obtain information on the nature of the acceptor molecule into which the galacturonic acid units are incorporated. From the results, it appeared that the presence of both commercial polygalacturonic acid and pectin could result in the inhibition of galacturonyl-transferase activity. The reason for this inhibitory effect may be due to the pectic compounds binding onto the enzyme molecule and thereby inhibiting its action. Another possible explanation is that the presence of the pectic compounds may be resulting in feedback inhibition of the enzyme galacturonyl-transferase.

No molecules, thought to resemble the final product, appeared to function as acceptor molecules. One reason for this may be that a specific compound is required to function as an acceptor molecule. For example, there may be a requirement for a certain degree of methyl esterification for the compound to be able to act as an acceptor molecule.

Alternatively, the polysaccharide into which the galacturonic acids are incorporated, may be bound to the enzyme molecule. Therefore, enzymic activity would not increase in the presence of more acceptor molecules.

The possibility of the occurrence of lipid or protein intermediates was also examined. However, no evidence was found to indicate the involvement of either molecule in the biosynthesis of galacturonan.

Chapter 5

DISTRIBUTION OF GALACTURONYLTRANSFERASE ACTIVITY IN THE PEA EPICOTYL

Introduction

This chapter is concerned with the distribution of the enzyme system, galacturonyltransferase, in the pea epicotyl. Pectic polysaccharides are major constituents of both the middle lamella and the primary cell wall but not the secondary cell wall. Therefore, net synthesis of pectic polysaccharides would be expected to occur in the region of elongation, however net synthesis of polygalacturonan is unlikely elsewhere in the epicotyl. Although net synthesis of polygalacturonan is unlikely, some polygalacturonan synthesis may occur, involved in turnover of polysaccharides.

Results

Initially, experiments were completed to determine where elongation occurs in the epicotyl. Epicotyls of 6-8 cm in length were divided into 6 sections of 1 cm length as illustrated in figure 5.1. The sections were incubated on H_2O-saturated filter paper under dark conditions at 22°C for 24 hours. After this period, the length of the sections was measured. The results (table 5.1.) indicate that elongation is confined to the top 2 cm of the epicotyl, with most growth occurring in the top 1 cm.

To confirm these results, intact epicotyls of the same stage were marked at 1 cm intervals down the epicotyl. The seedlings were left to grow under dark conditions at 22°C. After 24 hours, the epicotyls were examined to determine in which region growth had occurred. The results, in table 5.2. , confirm that most elongation occurs in the top 1 cm of the epicotyl with some growth occurring in section 2.

These experiments established that elongation of the epicotyl is confined to the top 2 cm of the epicotyl. Therefore, it was of interest to examine the different sections of the epicotyl to

Figure 5.1.

Diagram of the pea epicotyl divided into 6 sections all 1cm in length.

Section number

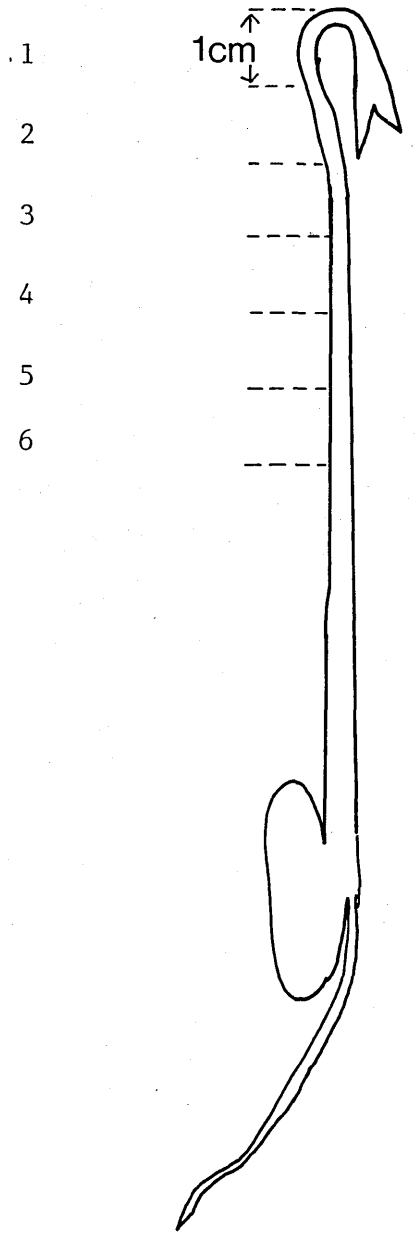


TABLE 5.1.

Elongation of epicotyl sections, isolated from different regions of the epicotyl, following the incubation of the sections in H_2O for 24 hours in darkness.

Epicotyl section.	% Elongation
1	19.5 ± 2
2	11.5 ± 1
3	3 ± 1
4	3 ± 1
5	0
6	0

TABLE 5.2.

Elongation pattern of the different regions of 7-day old, intact epicotyls over a period of 24 hours.

Epicotyl section.	% Elongation
1	122 ± 11
2	8 ± 4
3	0
4	0
5	0
6	0

determine the distribution of the enzyme system, galacturonyl-transferase.

Epicotyls (6-8 cm) were harvested and divided into sections of 1 cm length down the epicotyl. Particulate enzyme preparations were isolated from each section. The procedure used for the isolation of the particulate enzyme preparation was followed with the exception that the material was homogenised using a mortar and pestle instead of the Polytron homogeniser. This method of homogenisation was used because of the smaller volume of buffer (approximately 40 ml) used to obtain the enzyme preparations. The six enzyme preparations were then analysed for galacturonyltransferase activity. Incubations contained 90 Bq UDP-galacturonic acid, and the general polysaccharide fraction was analysed for incorporation of galacturonic acid following incubation periods of 0, 5, 10 and 30 minutes.

The results (figure 5.2.) indicate that the top 2 sections of the epicotyl incorporate more galacturonic acid units into polysaccharide material than the other sections. However, all the enzyme preparations have the ability to incorporate galacturonic acid into polysaccharide material. The results are also illustrated as enzymic activity per g fresh weight of tissue (figure 5.3.) and enzymic activity per g dry weight of tissue (figure 5.4.). These results demonstrate more accurately that galacturonyltransferase activity is higher in the top 2 sections of the epicotyl compared to the lower sections.

To ensure that the enzyme preparations are catalysing the incorporation of galacturonic acid into galacturonan, partial analysis of the product was completed. Particulate enzyme preparations, isolated from sections 1 and 6, were incubated with UDP-^{14}C - galacturonic acid and the resulting polysaccharide material was subjected to gel filtration chromatography and total acid hydrolysis.

Figure 5.2.

Enzymic activity present in the particulate enzyme preparations obtained from the different regions of the epicotyl. Enzymic activity was analysed as the incorporation of radioactivity from UDP-^{14}C-galacturonic acid into the general polysaccharide fraction following incubation periods of 0, 5, 10 and 30 minutes.

Symbol	Incubation time (minutes)
○	0
●	5
■	10
□	30

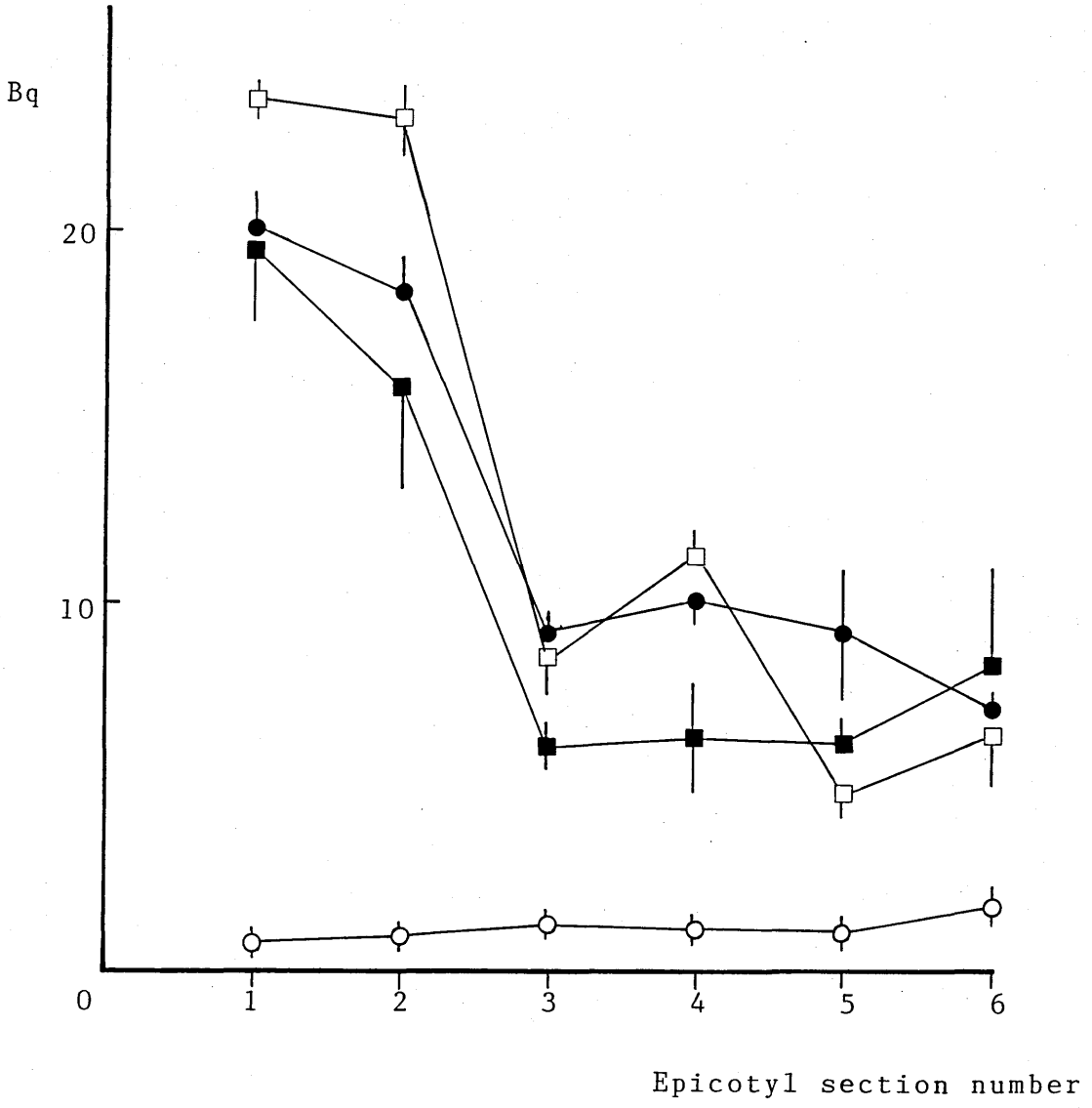

Bq
20
10
0
1
2
3
4
5
6
Epicotyl section number

Figure 5.3.

Enzymic activity, expressed as activity per g fresh weight of tissue, present in the particulate enzyme preparations isolated from the different regions of the epicotyl. Enzymic activity was analysed as incorporation of galacturonic acid into the general polysaccharide fraction following an incubation period of 5 minutes.

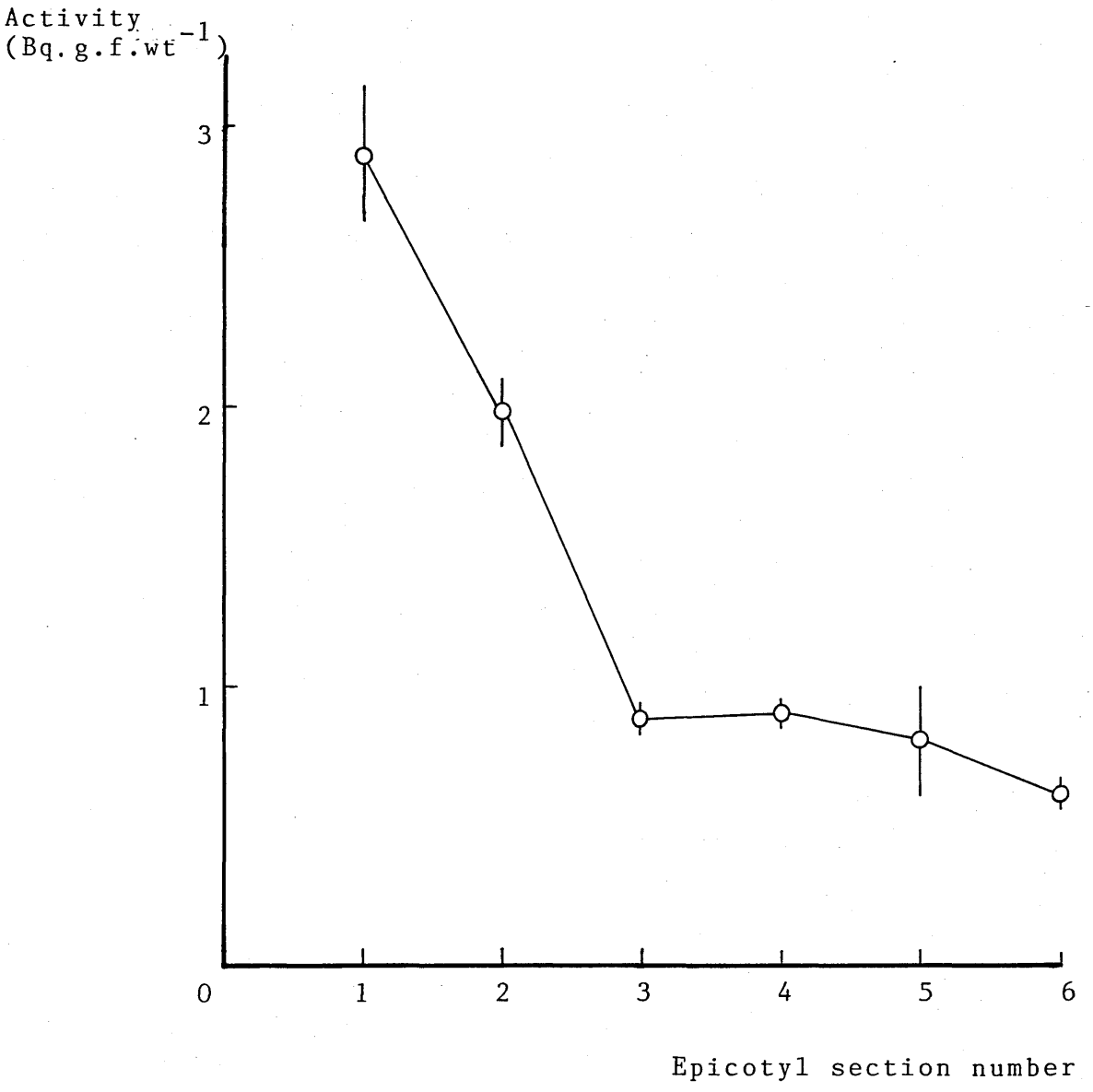
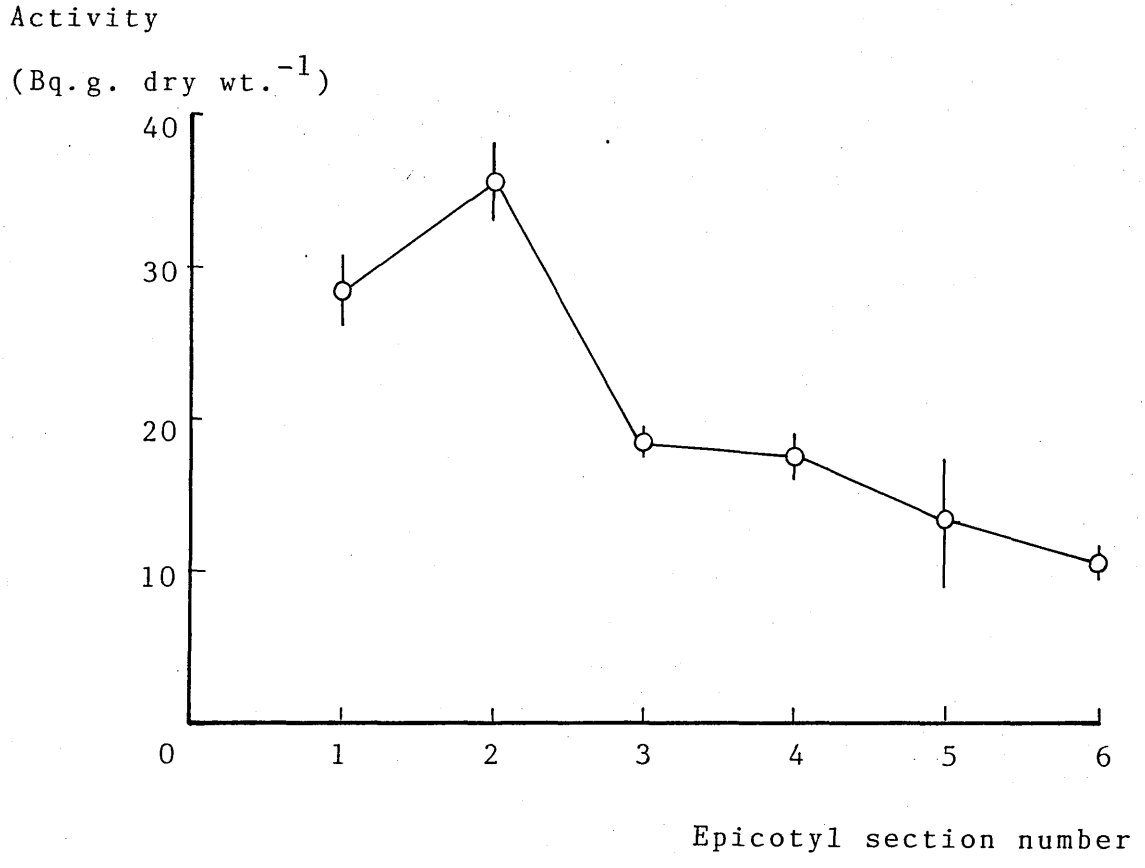

Activity
(Bq.g.f.wt^{-1})
3
2
1
0
1
2
3
4
5
6
Epicotyl section number

Figure 5.4.

Enzymic activity, expressed as activity per g dry weight of tissue, present in the enzyme preparations isolated from the different regions of the epicotyl. Enzymic activity was analysed as incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into the general polysaccharide fraction following an incubation period of 5 minutes.


Activity
(Bq.g. dry wt.$^{-1}$)
40
30
20
10
0
1
2
3
4
5
6
Epicotyl section number

UDP-galacturonic acid (90 Bq) was incubated with both enzyme preparations and the resulting EDTA extracts were run through a column of Sephadex G-100 (180 mm x 7.5 mm). It can be concluded from the results, in figures 5.5. and 5.6. that in both cases the material was excluded from the column indicating that it has a molecular weight greater than 10^5.

Incubations containing UDP-galacturonic acid (185 Bq) were also conducted and the general polysaccharide fractions were hydrolysed with 2M TFA. The solubilised material was dried and then identified using thin layer electrophoresis. The results (figures 5.7. and 5.8.) demonstrate that, in both cases, galacturonic acid residues were incorporated into polysaccharide material. Analysis of the thin layer electrophoresis plates also indicates that there is a peak of radioactive material which remained around the origin.
The amount of this material appears to be greater in the material synthesised by the enzyme preparation obtained from section 6 of the epicotyls. The nature of this material was not identified, however the incorporation of galacturonic acid into polysaccharide material confirms the presence of galacturonyltransferase activity in both particulate enzyme preparations.

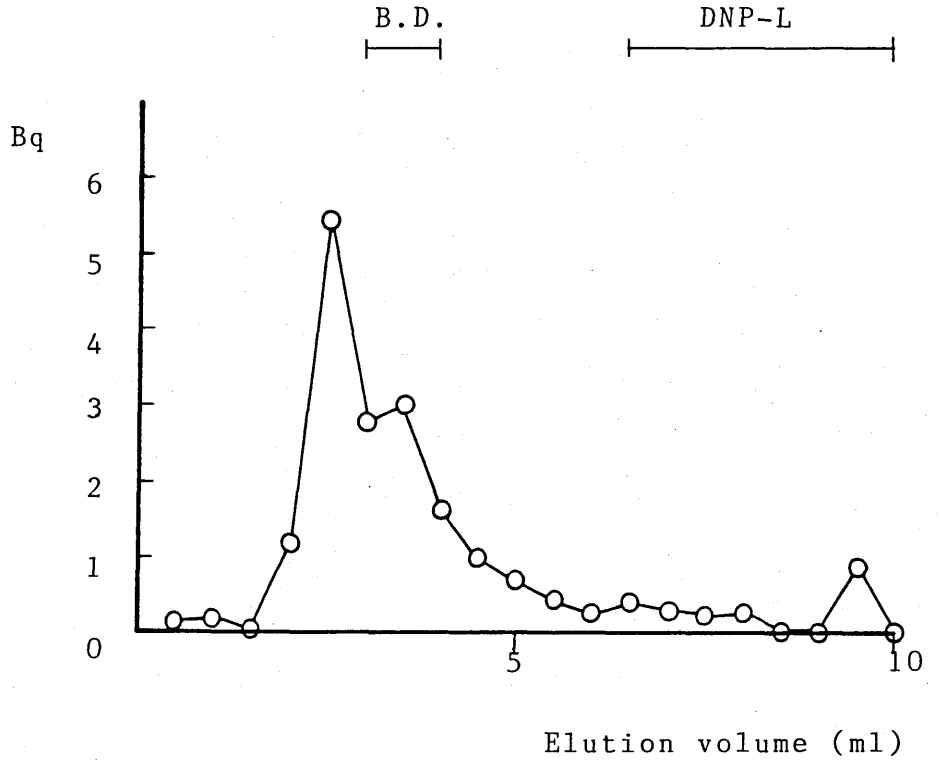
It is possible that the presence of galacturonyltransferase in the lower sections of the epicotyl is involved in turnover of galacturonan. In order to determine whether this was so, section 6 of 40 epicotyls were isolated and incubated for 2 hours with ^{14}C-sucrose. The incubations were followed by chase-periods of 0, 2 and 4 hours using unlabelled sucrose. The results, in table 5.3. , demonstrate that some radioactivity is incorporated into material extracted by EDTA/NaH_2PO_4 buffer which is indicative of pectic polysaccharides. Following chase periods with unlabelled sucrose, the amount of radioactivity present in the material extracted by EDTA/NaH_2PO_4

Figure 5.5.

Sephadex G-100 profile of polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following the incubation of UDP-^{14}C-galacturonic acid with particulate enzyme prepared from epicotyl sections 1

Figure 5.6.

Sephadex G-100 profile of polysaccharide material extracted by EDTA/NaH_2PO_4 buffer following the incubation of UDP-^{14}C-galacturonic acid with the particulate enzyme prepared from epicotyl sections 6.


B.D.
DNP-L
Bq
6
5
4
3
2
1
0
5
10
Elution volume (ml)

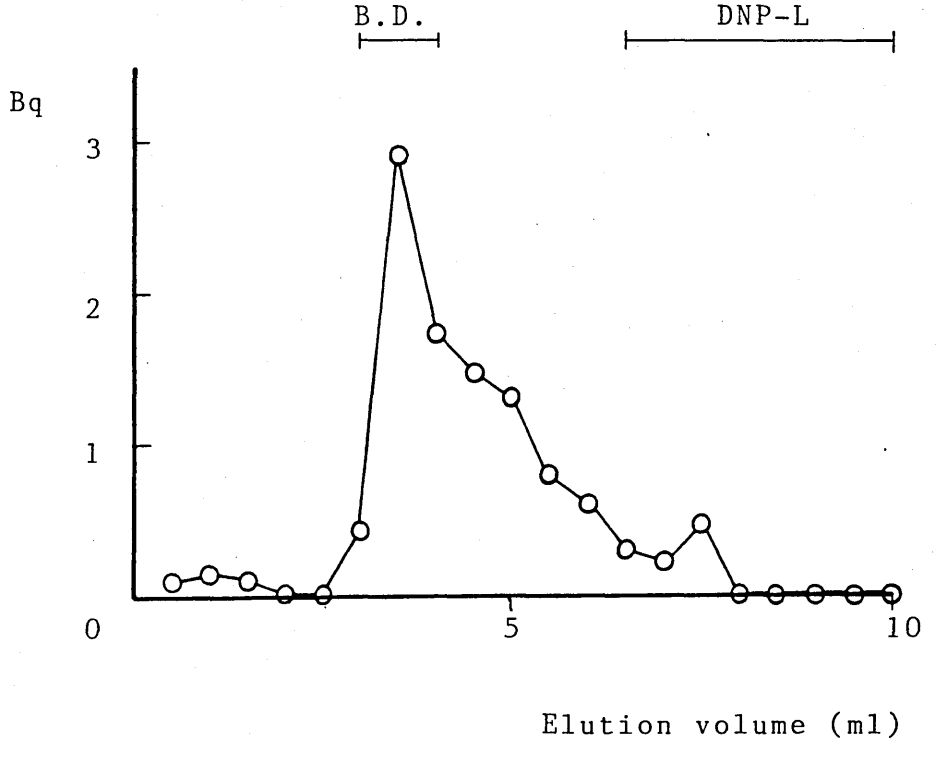

B.D.
DNP-L
Bq
3
2
1
0
5
10
Elution volume (ml)

Figure 5.7.

Identification using thin layer electrophoresis of the radioactive monosaccharides present after total acid hydrolysis of the general polysaccharide fraction obtained following incubation of UDP-^{14}C-galacturonic acid (185 Bq) with the particulate enzyme preparation isolated from epicotyl sections 1.

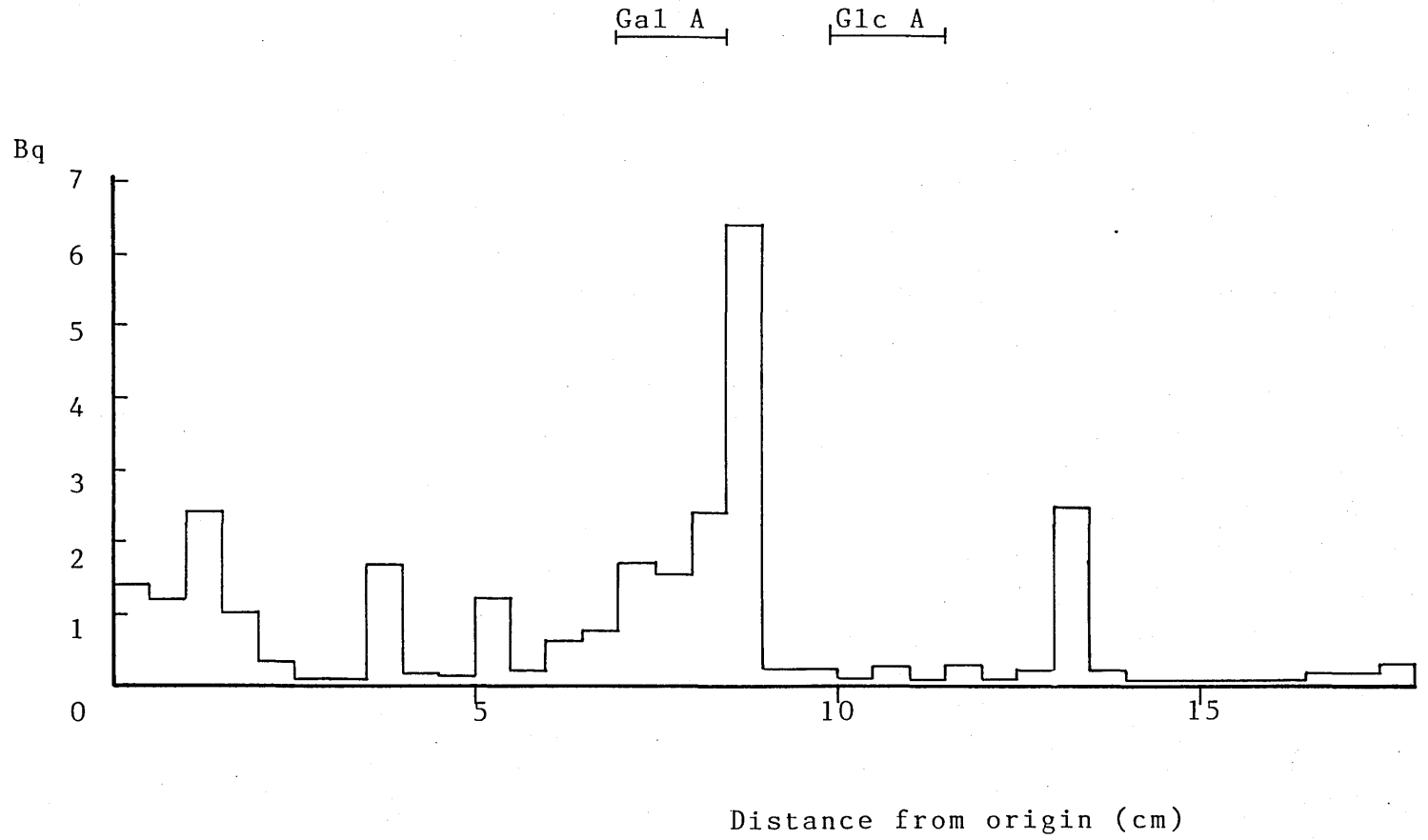

Gal A
Glc A
Bq
7
6
5
4
3
2
1
0
5
10
15
Distance from origin (cm)

Figure 5.8.

Identification using thin layer electrophoresis of the radioactive monosaccharides present after total acid hydrolysis of the general polysaccharide fraction obtained following the incubation of UDP-^{14}C-galacturonic acid (185 Bq) with the particulate enzyme prepared from epicotyl sections 6.

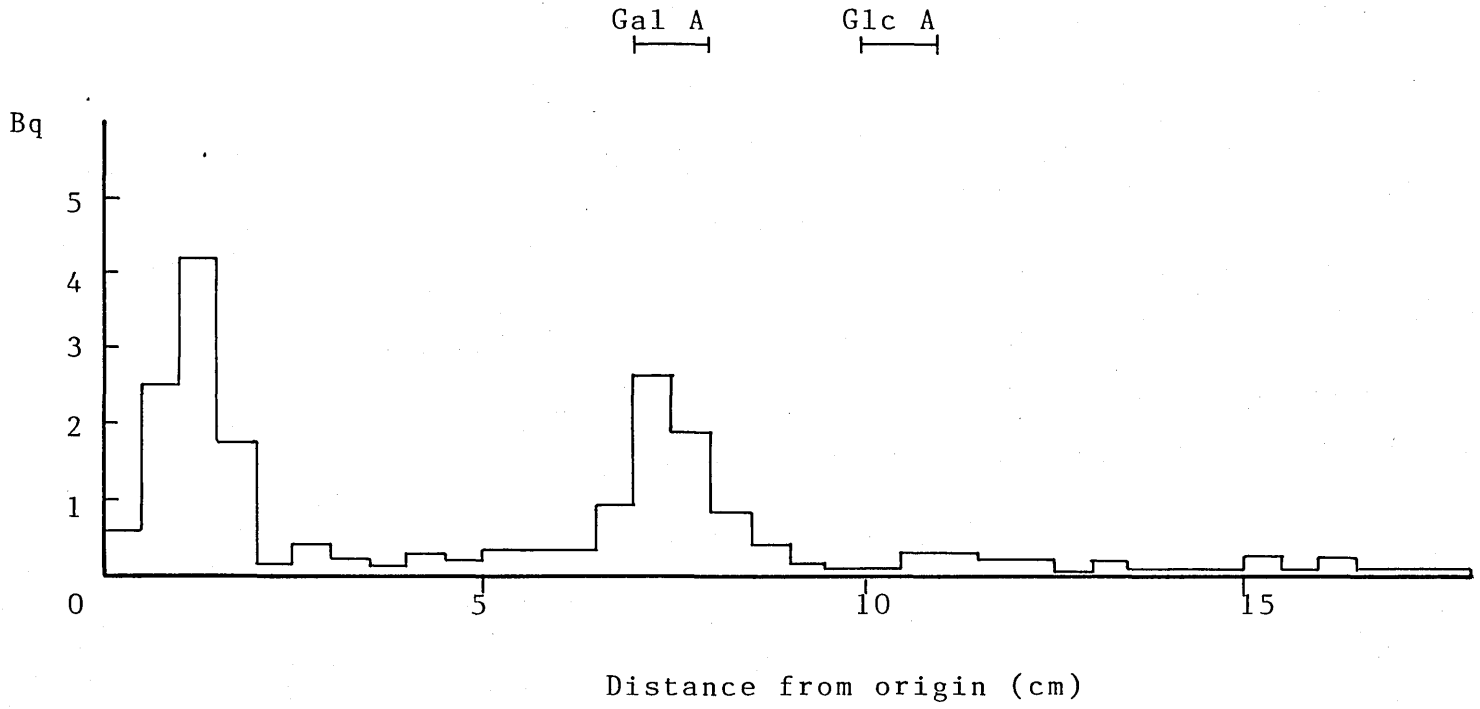

Gal A
Glc A
Bq
5
4
3
2
1
0
5
10
15
Distance from origin (cm)

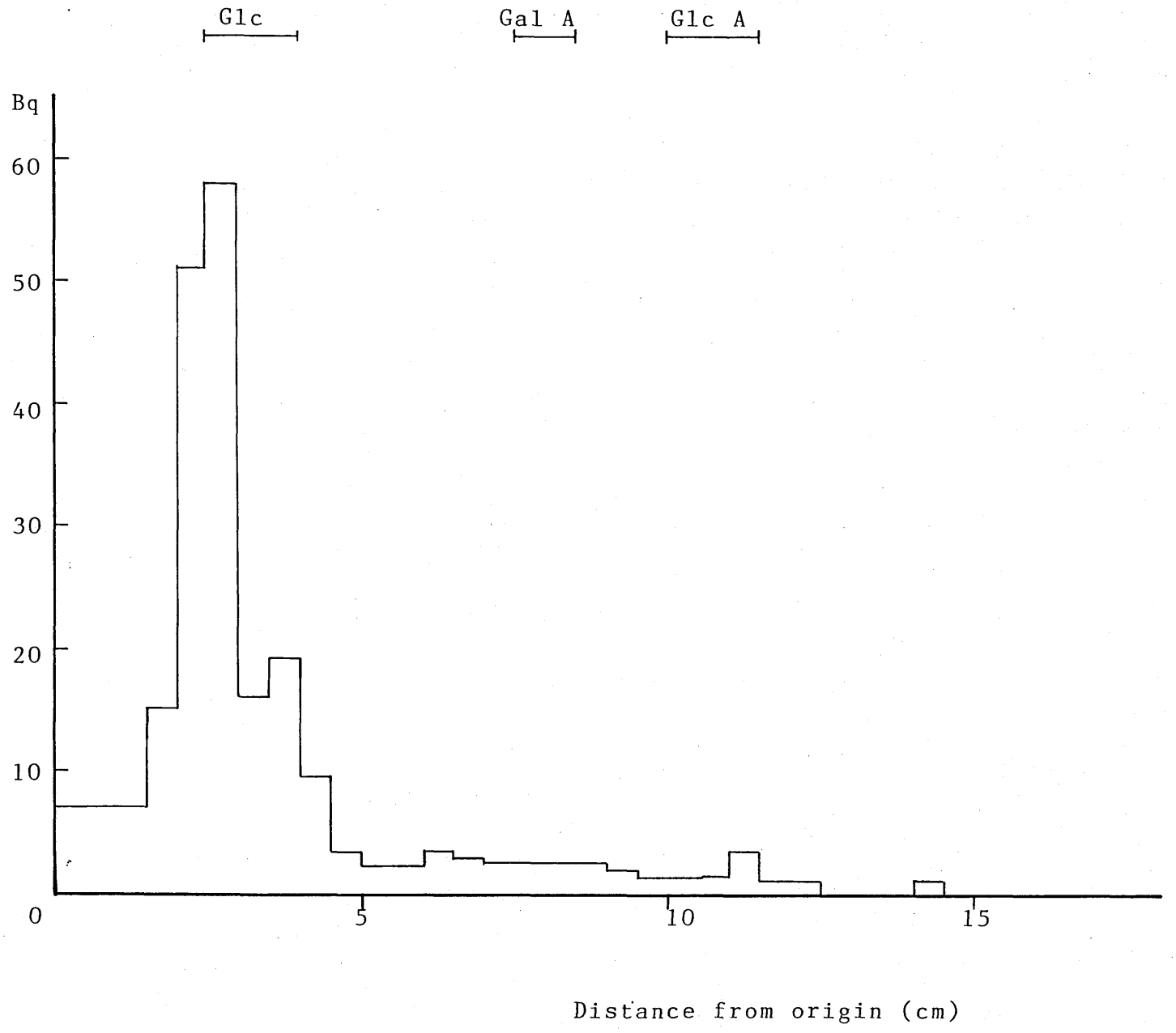
TABLE 5.3.

Incorporation of radioactivity into material extracted by EDTA/NaH_2PO_4 buffer, pH 6.8, following incubation of ^{14}C-sucrose with epicotyl segments (section 6) for 2 hours followed by chase-incubations with unlabelled sucrose for varying periods of time.

Chase-incubation period (hr)	Incorporation (Bq)
0	88.9 ± 9.1
2	12.44 ± 0.18
4	7.42 ± 1.66

Figure 5.9.

Thin layer electrophoretic analysis of the hydrolysate obtained after subjecting polysaccharide material , extracted by EDTA/NaH_2PO_4 buffer, to total acid hydrolysis. The polysaccharide material was extracted from epicotyl sections following the incubation of epicotyl sections 6 with ^{14}C-sucrose (3.7 MBq) for 8 hours.


Glc
Gal A
Glc A
Bq
60
50
40
30
20
10
0
5
10
15
Distance from origin (cm)

buffer decreased. These results suggest rapid turnover of the material.

To determine whether galacturonan is involved in this process, the radioactive-labelled product extracted by the EDTA buffer was analysed. Epicotyl sections 6 were incubated with ^{14}C-sucrose for 8 hours. The pectic polysaccharides were extracted using EDTA/NaH_2PO_4 buffer, pH 6·8, and then hydrolysed using 2 M TFA. The hydrolysed material was analysed using thin layer electrophoresis and the results (figure 5.9.) indicate the incorporation of radioactivity into the neutral sugar fraction. Therefore, these results suggest that ^{14}C is incorporated into a high - molecular - weight polymer composed of neutral sugars. There was no ^{14}C-galacturonic acid, therefore this experiment does not provide any evidence to suggest that galacturonan is involved in turnover.

Discussion

The results presented in this chapter demonstrate that galacturonyltransferase activity is higher in the upper two sections of the epicotyl compared to the lower sections. Therefore, galacturonyltransferase activity is higher in the region of elongation of the epicotyl. However, all the sections of the epicotyl examined did exhibit galacturonyltransferase activity. Therefore, the whole epicotyl has the ability to synthesise galacturonan even though elongation has ceased in the older region. It was thought that the synthesis of galacturonan in the older region of the epicotyl may be involved in turnover of cell wall polysaccharides. However no evidence was found to support this theory. Another possibility is that the enzyme may not be used *in vivo* due to lack of substrate or to the operation of some control mechanism.

Chapter 6

ATTEMPTED SOLUBILISATION OF GALACTURONYLTRANSFERASE ACTIVITY USING DETERGENTS

Introduction.

The particulate enzyme preparation used in this project is a crude membrane preparation. Solubilisation of the enzyme preparation is the first stage in its purification and this can be achieved using detergents. In order to find a suitable detergent for the solubilisation of the enzyme system, detergents were initially added to the standard incubation mixture. Following incubation with the particulate enzyme preparation, the general polysaccharide fraction was analysed to determine the effect of the addition of the detergent on galacturonyltransferase activity. If enzymic activity was retained in the presence of the detergent, then the detergent was used to try to solubilise the enzyme system.

The use of Triton X-100 in the solubilisation of the enzyme system.

The detergent Triton X-100 has proved successful in the solubilisation of two enzyme systems involved in cell wall biosynthesis (Waldron, 1984; Heller and Villemez, 1972). Therefore, different concentrations of Triton X-100 were added to the incubation mixture to investigate the effect that Triton X-100 has on the enzyme galacturonyltransferase. The results, figure 6.1., demonstrate that the addition of Triton X-100 reduces galacturonyltransferase activity by approximately 50%. An attempt was then made to solubilise the enzyme system using Triton X-100 at concentrations of 1% and 10%. It can be concluded from the results (table 6.1.) that the enzyme was not solubilised by Triton X-100 at either concentration as there was no galacturonyltransferase activity in the solubilised material.

Figure 6.1

Effect of the presence of different concentrations of Triton X-100 in the incubation mixture on galacturonyltransferase activity. Enzymic activity was analysed by the incorporation of radioactivity into the general polysaccharide fraction.

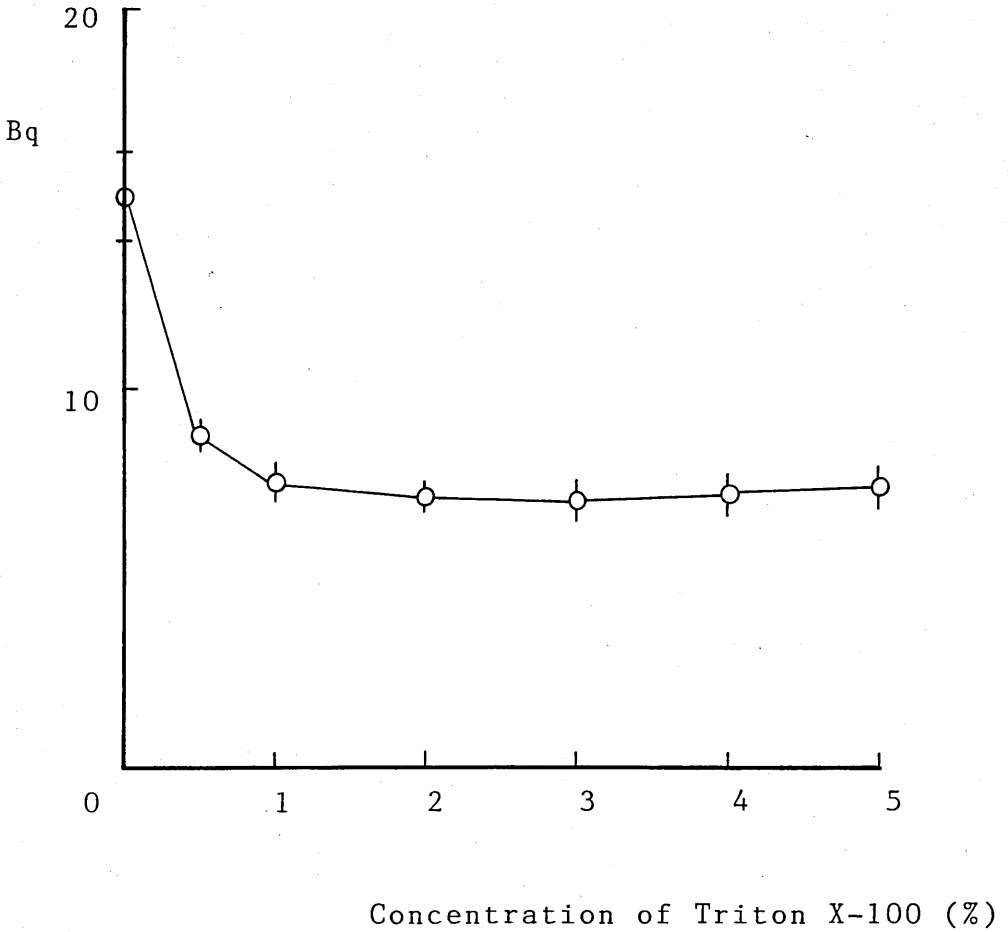

20
Bq
10
0
1
2
3
4
5
Concentration of Triton X-100 (%)

TABLE 6.1.

Distribution of enzymic activity between the supernatant and the pellet following solubilisation of the particulate enzyme with 1% and 10% Triton X - 100. Incubations were carried out for 30 minutes and the general polysaccharide fraction was analysed for radioactivity.

1% Triton X - 100.

	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	25.7	37

10% Triton X - 100.

	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	33.6	48

The effect of the addition of various detergents on the enzyme system.

Different types of detergents were then employed in order to find a detergent capable of solubilising the enzyme system. The effect that the addition of the detergents had on the particulate enzyme preparation was examined initially. The results, illustrated in figure 6.2., demonstrate the varying effects that the different detergents have on the enzyme system. The detergents SDS and cholic acid completely inhibited the activity of the enzyme. There was some enzymic activity retained in the presence of CETAB and deoxycholic acid but only when present at a concentration of 0.5%. At concentrations of 1% and 2.5%, both detergents resulted in complete inhibition of the enzyme system. However, galacturonyltransferase activity was retained in the presence of both LDAO and Brij-35, although the addition of both detergents resulted in a reduction of enzymic activity. The results also indicate that the higher the concentration of LDAO added into the incubation mixture, the greater the inhibitory effect on the enzyme system, whereas increasing the concentration of Brij-35 did not result in increased inhibition of the enzyme system.

The use of Brij-35 in the solubilisation of galacturonyltransferase activity.

In view of the above-mentioned results, Brij-35, at a concentration of 2.5%, was used to try to solubilise the enzyme system. However, the results (table 6.2.) demonstrate that galacturonyltransferase activity was retained in the pellet. Therefore, the enzyme system was not solubilised using 2.5% Brij-35.

The use of the detergent LDAO in the solubilisation of the enzyme system.

LDAO was also investigated to determine whether it resulted in the solubilisation of galacturonyltransferase activity. From the results, in table 6.3., it was concluded that, in the presence of 2.5% LDAO, the

Figure 6.2.

Effect of the addition of detergents, at different concentrations, to the incubation mixture on the incorporation of radioactivity from UDP-^{14}C-galacturonic acid into general polysaccharide material. The detergents employed are listed below:-

Symbol	Detergent
□	Brij-35
■	LDAO
●	Deoxycholic acid
△	CETAB
▲	SDS
○	Cholic acid

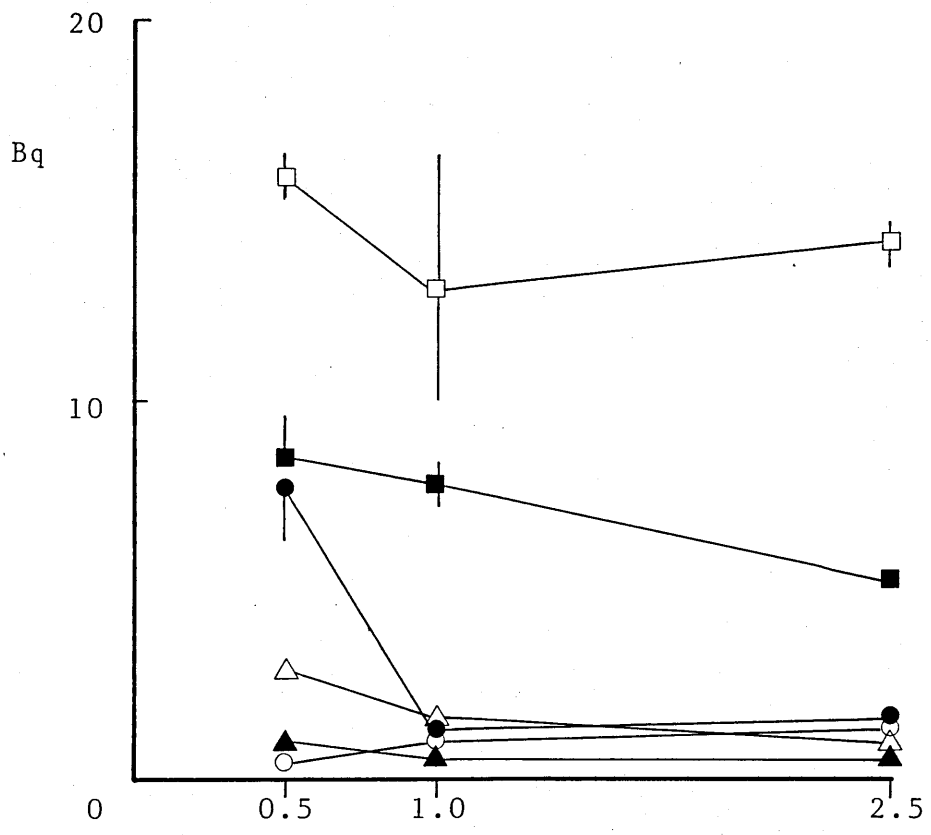

20
Bq
10
0
0.5
1.0
2.5
Concentration of detergent (%)

TABLE 6.2.

Distribution of galacturonyltransferase activity between the supernatant and the pellet following solubilisation of the particulate enzyme with 2.5% Brij-35. Incubations were carried out for 30 minutes and the general polysaccharide fraction was analysed.

	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	34.8	50

TABLE 6.3.

Distribution of galacturonyltransferase activity between the supernatant and the pellet following solubilisation of the particulate enzyme using 2.5% LDAO. Incubations were carried out for 30 minutes and the general polysaccharide fraction was analysed.

	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	12.3	18
Pellet	19.7	28

enzyme system was partially solubilised. However, when repeated, the results obtained were variable. Galacturonyltransferase activity was not consistently found associated with the solubilised material following treatment with 2.5% LDAO (table 6.4.) LDAO has also been investigated at concentrations of 1% and 5%. The results (table 6.5.) demonstrate that the use of LDAO, at these concentrations, decreased the amount of galacturonyltransferase activity present in the solubilised material.

The conflicting results obtained using the detergent LDAO may be due to the ratio of protein to detergent not being optimal. This ratio can be very important in the solubilisation of proteins. The high concentration of BSA present in the buffer may also be affecting the solubilisation process.

The attempted solubilisation of galacturonyltransferase activity using a modified enzyme preparation procedure.

Solubilisation of the enzyme system was attempted again using the detergents Triton X-100 and LDAO. However, in this set of experiments, BSA was not present in the buffer used to resuspend the particulate material following the second centrifugation step. Therefore, at the stage that the detergent is added, the concentration of BSA present in the preparation is much reduced.

Triton X-100 was used at concentrations of 1% and 10% to attempt to solubilise the enzyme system following this modified procedure. It can be seen from the results, in table 6.6., that the enzyme system was not solubilised. Galacturonyltransferase activity remained in the particulate fraction.

Solubilisation of the enzyme system was also attempted, following this modified procedure, using 2.5% LDAO. However, the results (table 6.7.) indicate that galacturonyltransferase activity was not solubilised.

TABLE 6.4.

Varying distribution values between the supernatant and the pellet obtained following solubilisation of the particulate enzyme with 2.5% LDAO. All incubations were 30 minutes and the incorporation of radioactivity into the general polysaccharide fraction was analysed.

	Relative activity			
Particulate preparation	100	100	100	100
After treatment:				
Solubilised material	18	0	0	10
Pellet	28	32	17	20

TABLE 6.5.

Distribution of galacturonyltransferase activity between the supernatant and the pellet following solubilisation of the particulate enzyme with 1% and 5% LDAO. Incubations were carried out for 30 minutes and the general polysaccharide material was analysed.

	1% LDAO	
	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	4.6	6.5
Pellet	53.8	77.0

	5% LDAO	
	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	7.0	10
Pellet	36.3	52

TABLE 6.6.

Distribution of enzymic activity between the supernatant and the pellet following solubilisation of the particulate enzyme using Triton X-100. The particulate enzyme was resuspended in resuspension buffer containing either 1% or 10% Triton X - 100 but BSA was not present. Incubations were conducted for 30 minutes and the general polysaccharide material was analysed.

	1% Triton X - 100	
	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	11.9	17

	10% Triton X - 100	
	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	16.0	23

TABLE 6.7.

Distribution of galacturonyltransferase activity between the supernatant and the pellet following solubilisation of the particulate enzyme with 2.5% LDAO. The particulate enzyme was resuspended in resuspension buffer containing LDAO but not BSA. Incubations were carried out for 30 minutes and the general polysaccharide material was analysed.

	Units of activity	Relative activity
Particulate preparation	70	100
After treatment:		
Solubilised material	2.6	4
Pellet	36.0	51

The effect of manganese ions on the solubilised enzyme preparation.

In all the previous experiments in this chapter, manganese ions have been present in the incubation mixture. To exclude the possibility of manganese ions having an inhibitory effect on the solubilised enzyme preparation, the effect of the presence of manganese ions was investigated. UDP - ^{14}C - galacturonic acid was incubated with the solubilised enzyme preparation, isolated using 2.5% LDAO, in both the presence and absence of manganese ions (10 mM). The results, in table 6.8., suggest that the presence of 10mM manganese ions has no significant effect on the activity of the enzyme preparation.

The effect of the addition of pectin on the solubilised enzyme preparation.

In order to examine whether lack of enzymic activity in the solubilised preparation was due to the absence of an acceptor molecule, pectic material was added into the incubation mixture. Pectin, prepared from pea epicotyls, was added to the incubation mixture (1 µl and 10 µl). However, the addition of pectin did not have any effect on the activity of the solubilised preparation (table 6.9.).

The use of digitonin in the solubilisation of galacturonyltransferase activity.

Digitonin has also been used in an attempt to solubilise the enzyme system. Villemez et al. (1966) reported that they had no success in solubilising galacturonyltransferase activity using digitonin. However, digitonin has been used recently in the solubilisation of glucosyltransferases (Thelen and Delmer, 1986).

Initially, the effect of the presence of digitonin in the incubation mixture on enzymic activity was determined. The results, in figure 6.3., illustrate that the presence of digitonin reduces galacturonyltransferase activity to approximately 60% of its normal level. Digitonin (2.5%) was

TABLE 6.8.

Effect of the presence of Mn^{2+} ions in the incubation mixture on the activity of the solubilised enzyme preparation obtained following the solubilisation of the particulate enzyme with 2.5% LDAO. The incubation mixture contained UDP-galacturonic acid (280 Bq) and $MnCl_2$ at a concentration of 10mM. Incubations were conducted for 30 minutes and the general polysaccharide material was analysed.

	Incorporation (Bq)
Presence of Mn^{2+}	1.71 ± 0.45
Absence of Mn^{2+}	1.92 ± 0.33

TABLE 6.9.

Effect of the addition of pectin (prepared from pea epicotyls) to the incubation mixture on the activity of the solubilised enzyme preparation obtained following solubilisation of the particulate enzyme with 2.5% LDAO. Pectin (either 1µl or 10µl) was added to the incubation mixture which contained 280 Bq UDP-galacturonic acid. Incubations were conducted for 30 minutes and the general polysaccharide material was analysed.

	Incorporation (Bq)
Pectin 1 µl	1.29 ± 1.00
Pectin 10µl	0

Figure 6.3.

Effect of different concentrations of digitonin in the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide fraction.

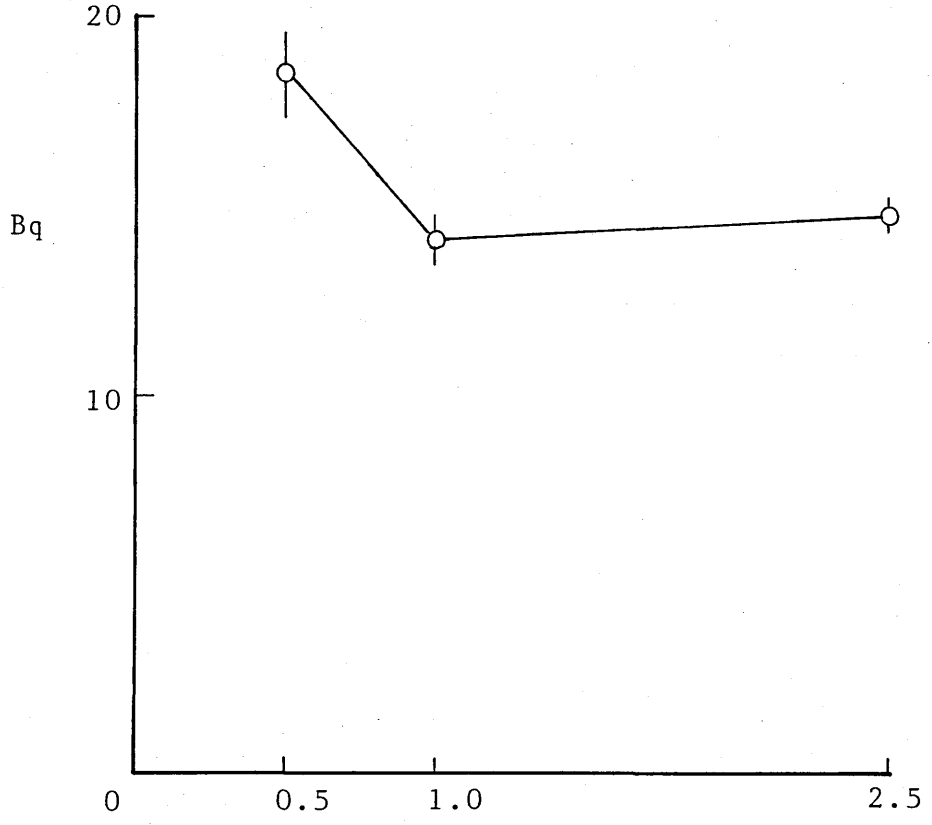

20
Bq
10
0
0.5
1.0
2.5
Concentration of digitonin (%)

TABLE 6.10.

Distribution of galacturonyltransferase activity between the supernatant and the pellet following solubilisation of the particulate enzyme using 2.5% digitonin. Incubations were carried out for 30 minutes and the general polysacchardie material was analysed.

	Units of activity	Relative activity
Particulate preparation	70.0	100
After treatment:		
Solubilised material	0	0
Pellet	3.6	5

then used in the solubilisation procedure. The results (table 6.10.) indicate that no galacturonyltransferase activity was present in the solubilised material.

Discussion.

The experiments reported in this chapter demonstrate the varying effects that the presence of detergents have on the enzyme system. Ionic detergents tend to result in the denaturation of proteins and this was shown to be true with SDS and CETAB. However, the presence of LDAO, a zwitterionic detergent, did not inactivate the enzyme preparation, although galacturonyltransferase activity was reduced to approximately 15% in the presence of 2.5% LDAO. Galacturonyltransferase activity was retained in the presence of the non-ionic detergents Triton X-100 and Brij-35, however activity was reduced to approximately 50% in the presence of Triton X-100 and to around 45% in the presence of Brij-35. The effect of the presence of some steroidal detergents was also determined. The presence of cholate completely inhibited enzymic activity whereas, although higher concentrations of deoxycholate resulted in loss of enzymic activity, some activity remained in the presence of 0.5% deoxycholate. Digitonin did not inactivate the enzyme although activity was reduced to approximately 60%. Further work was carried out using Triton X-100, Brij-35, LDAO and digitonin. Partial solubilisation of the enzyme was achieved using LDAO (2.5%). Further experimentation is necessary in order to obtain consistent results using LDAO. It may be that the ratio of protein to detergent is critical. The sensitivity of proteins to their environment is variable (Tanford and Reynolds, 1976) and it is possible that the enzyme galacturonyltransferase requires the presence of certain polar groups or alkyl chains for activity to be retained. Identification of the product formed following the incubation of UDP-galacturonic acid with the solubilised enzyme preparation would also be necessary to confirm the presence of galacturonyltransferase activity.

Chapter 7

EFFECT OF THE PRESENCE OF TWO RHAMNOSE NUCLEOTIDES ON GALACTURONYLTRANSFERASE ACTIVITY

Introduction.

One objective of this project was to investigate the role of rhamnose nucleotides in the biosynthesis of the rhamnogalacturonan polysaccharides. Rhamnose nucleotides are not available commercially, and therefore it was necessary to synthesise them. Both UDP- β -L-rhamnose and GDP- β -L-rhamnose were synthesised. Each rhamnose nucleotide was then examined to investigate whether it had the ability to function as a rhamnosyl donor. This was done by observing the effect of the addition of the rhamnose nucleotide on the galacturonyltransferase enzyme system. An increase or prolongation in galacturonyltransferase activity in the presence of either nucleotide would indicate co-operation between galacturonyltransferase and rhamnosyltransferase. Therefore, this would provide information on the rhamnosyl donor and also indicate the presence of a rhamnosyltransferase enzyme, provided that the possibility of allosteric activation of galacturonyltransferase by the rhamnose nucleotide could be eliminated.

Preparation of rhamnose nucleotides.

A common method employed for the synthesis of sugar nucleotides is the phosphomorpholidate procedure (Moffat, 1965). Barber (1962) used this method to synthesise UDP-L-rhamnose. The method involves reacting rhamnosyl-phosphate with nucleoside-5-phosphomorpholidates. The reaction results in the formation of the pyrophosphate bond thereby synthesising the corresponding rhamnose nucleotide.

The procedure used for the synthesis of rhamnose nucleotides is described in detail in the materials and methods chapter. Initially, rhamnosyl-phosphate had to be synthesised using rhamnose as the substrate.

This involved acetylating α-L-rhamnose to form α-L-rhamnose-tetra-acetate. The acetylated sugar was then phosphorylated on carbon 1. The phosphate group binds to carbon 1 preferentially as it is more reactive than the other carbons. The remaining acetyl groups were then removed resulting in L-rhamnose-phosphate. Following the phosphorylation procedure, the stable end-product is the anomer with the phosphate group on the axial position (MacDonald, 1972). Therefore, the expected product following this stage is β-L-rhamnose-phosphate.

The phosphomorpholidate procedure was then followed using uridine-phosphomorpholidate and guanosine-phosphomorpholidate to form the corresponding rhamnose nucleotides, UDP-β-L-rhamnose and GDP-β-L-rhamnose.

To ensure that UDP-rhamnose and GDP-rhamnose had been synthesised, both paper chromatography and thin layer electrophoresis were employed. Using the thin layer electrophoresis system, the rhamnose nucleotide and the corresponding nucleoside-phosphomorpholidate had different mobilities, with the rhamnose nucleotide running faster than the nucleoside-phosphomorpholidate, whereas descending paper chromatography resulted in the separation of both rhamnose nucleotides from rhamnose-phosphate.

Each rhamnose nucleotide was initially subjected to paper chromatography. This separated the rhamnose nucleotide from rhamnose-phosphate. The rhamnose nucleotide, which was identified using an ultra-violet lamp, was eluted. The eluted material was then run on the thin layer electrophoresis system. In both cases, the rhamnose nucleotide had a different mobility from its respective nucleoside-phosphomorpholidate.

The effect of the addition of UDP-β-L-rhamnose on galacturonyltransferase activity.

UDP-β-L-rhamnose was the first rhamnose nucleotide to be synthesised because UDP-sugars are the most common glycosyl donors and, also, because there appears to be an enzyme system capable of synthesising UDP-L-rhamnose. Enzyme preparations have been isolated from leaves of both red campion (Kamsteeg et al.,1978) and mung bean (Barber, 1962) which catalyse the conversion of UDP-glucose to UDP-L-rhamnose.

The effect of the addition of UDP-β-L-rhamnose on galacturonyltransferase was examined. UDP-rhamnose was added into the incubation mixture to give final concentration values of 0.01, 0.1 and 1mM. As controls, incubations were also conducted using the same concentrations of both uridine-phosphomorpholidate and rhamnose-phosphate. Incorporation of galacturonic acid into polysaccharide material was analysed following incubations of 10 and 120 minutes.

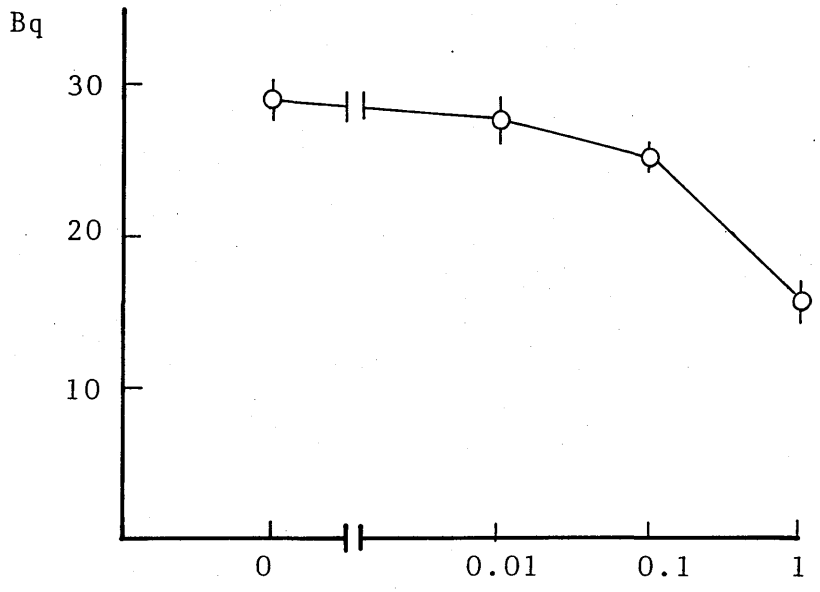
It can be seen from the results (figures 7.1., 7.2. and 7.3.) that the presence of UDP-L-rhamnose, at the concentrations investigated, does not stimulate galacturonyltransferase activity. The H_2O-soluble fractions were then investigated to examine whether the presence of UDP-L-rhamnose was stimulating the incorporation of galacturonic acid into the polysaccharide material extracted by hot H_2O. However, it was concluded from the results (table 7.1) that the presence of UDP-L-rhamnose does not increase the incorporation of galacturonic acid into the H_2O-soluble polysaccharide fraction.

From the results illustrated in figures 7.1.,7.2. and 7.3., it appears that, following an incubation period of 10 minutes, the presence of 1mM UDP-L-rhamnose inhibits galacturonyltransferase activity. A greater inhibitory effect was detected in the presence of 1mM uridine-phosphomorpholidate. It was thought that some uridine-phosphomorpholidate

Figure 7.1

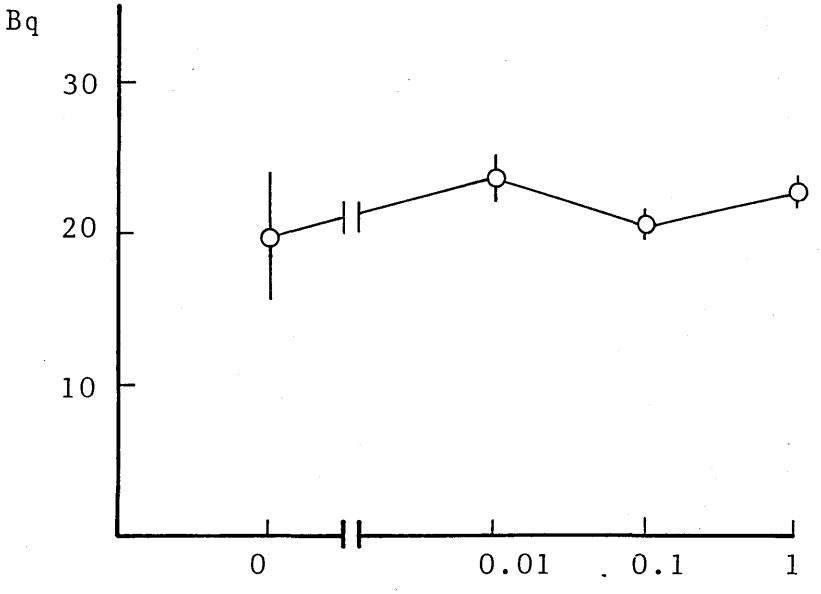
Effect of the addition of different concentrations of UDP-L-rhamnose to the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide fraction following incubation periods of 10 and 120 minutes.

10 minute incubation



Concentration of UDP-rhamnose (mM)

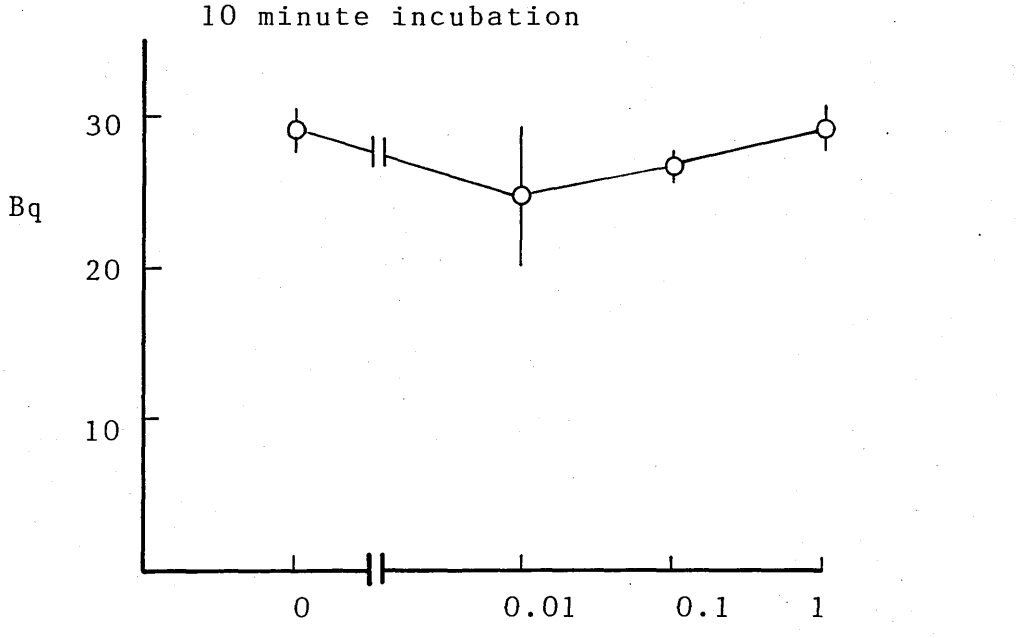
120 minute incubation



Concentration of UDP-rhamnose (mM)

Figure 7.2.

Effect of the presence of different concentrations of rhamnose-phosphate in the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide fraction following incubations of 10 and 120 minutes.


10 minute incubation
Bq
30
20
10
0
0.01
0.1
1
Concentration of rhamnose-phosphate (mM)

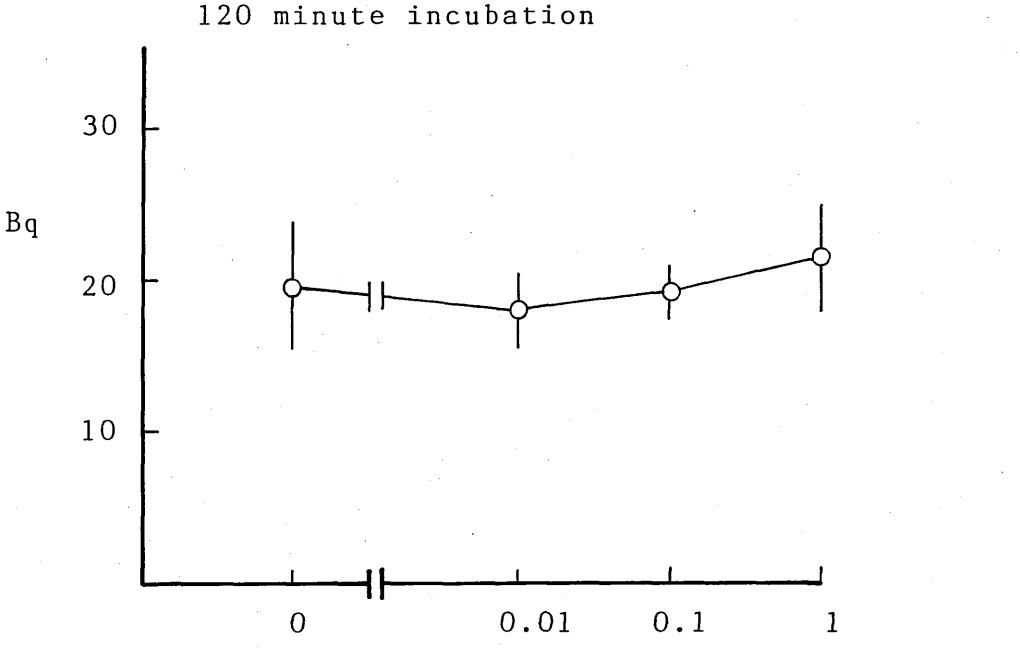
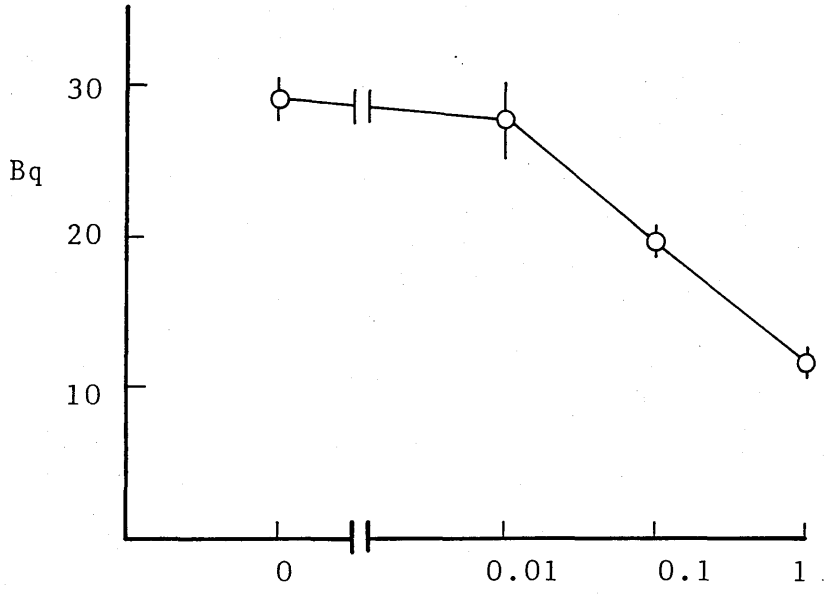

120 minute incubation
Bq
30
20
10
0
0.01
0.1
1
Concentration of rhamnose-phosphate (mM)

Figure 7.3.

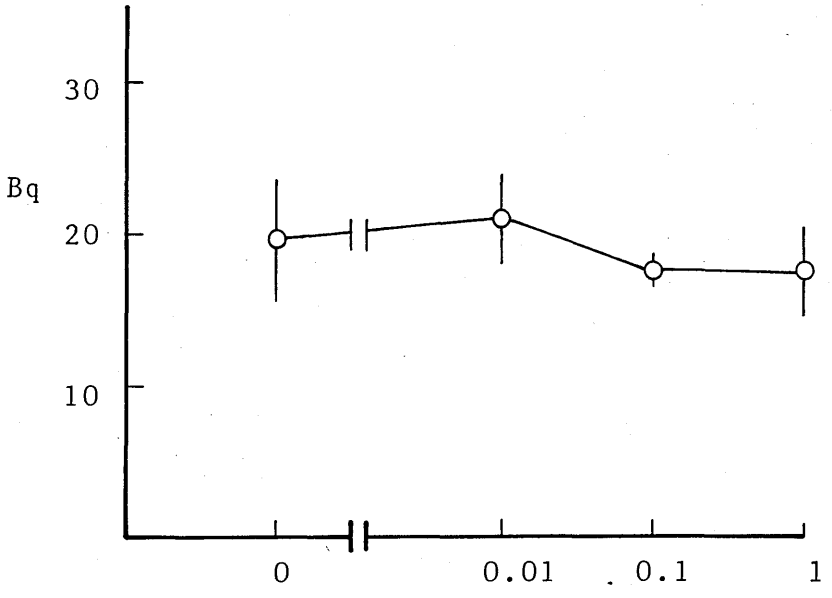
Effect of different concentrations of uridine-phosphomorpholidate in the incubation mixture on the incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into general polysaccharide material following incubations of 10 and 120 minutes.

10 minute incubation



Concentration of uridine-phosphomorpholidate (mM)

120 minute incubation



Concentration of uridine-phosphomorpholidate (mM)

TABLE 7.1.

Effect of the addition of UDP-rhamnose (at concentrations of 0.01, 0.1 and 1mM) to the incubation mixture on the incorporation of galacturonic acid into the high-molecular-weight, H_2O-soluble material following incubation periods of 10 and 120 minutes.

UDP-rhamnose concentration (mM)	Incorporation (Bq)	
	10 min incubation	120 min incubation
0	1.01	1.12
0.01	0.95	1.30
0.1	0.70	0.84
1	0.15	0.84

may be contaminating the UDP-L-rhamnose preparation. Therefore, the UDP-L-rhamnose preparation was purified as described in the materials and methods chapter. The purified UDP-L-rhamnose did not contain any uridine-phosphomorpholidate. Because of the purification process, the purified UDP-L-rhamnose could only be investigated at concentrations of 1 and 10 μM . The effect of the presence of uridine-phosphomorpholidate and rhamnose-phosphate, at these concentrations, was also investigated. The results (figures 7.4., 7.5. and 7.6.) demonstrate that the presence of purified UDP-L-rhamnose, at concentrations of 1 and 10 μM , has no effect on the incorporation of galacturonic acid. No effect on galacturonyltransferase activity was detected in the presence of either uridine-phosphomorpholidate or rhamnose-phosphate at these concentrations.

The effect of the presence of GDP- β -L-rhamnose on the incorporation of galacturonic acid.

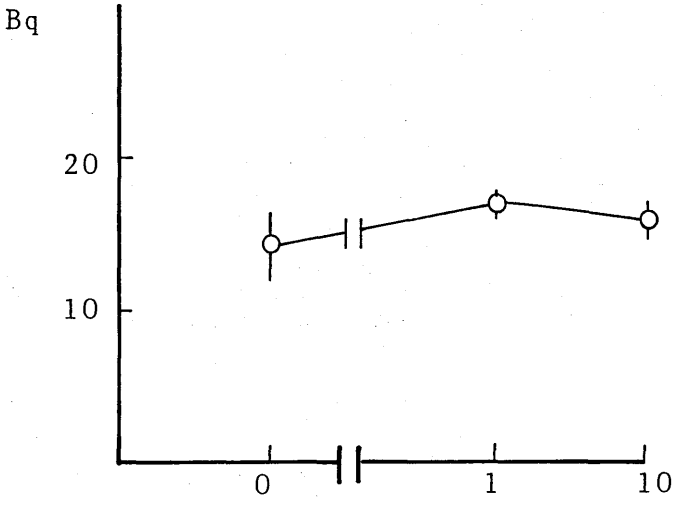
GDP-β-L-rhamnose was also investigated to determine whether it had the ability to function as a rhamnosyl donor. L-Rhamnose is equivalent to 6-deoxy-L-mannose. The conversion of GDP-mannose to GDP-D-rhamnose was shown to be catalysed by extracts obtained from the leaves of Leucaena glauca (Barber, 1968).

GDP-L-rhamnose was synthesised and then added into the standard incubation mixture at the following concentrations - 0.01, 0.1 and 1 mM. The effect of the presence of guanosine-phosphomorpholidate and rhamnose-phosphate, at the same concentrations, was also examined. As before, the incorporation of galacturonic acid into the general polysaccharide fraction was analysed following incubation periods of 10 and 120 minutes. It can be concluded from the results (figures 7.7., 7.8. and 7.9.) that the presence of GDP-L-rhamnose does not stimulate the incorporation of

Figure 7.4.

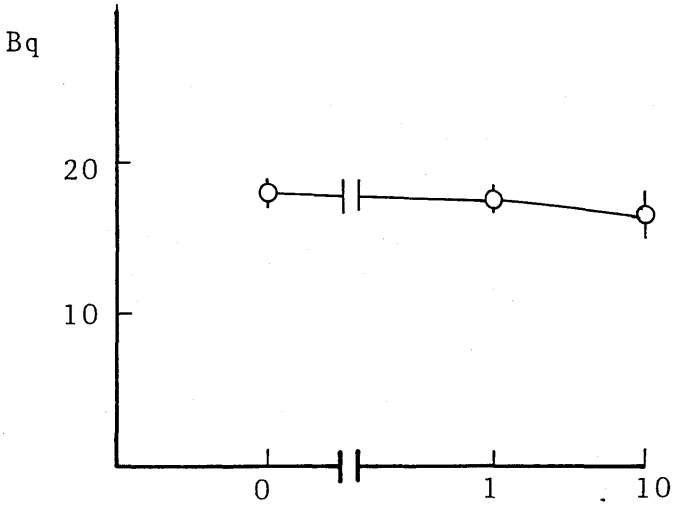
Effect of different concentrations of purified UDP-rhamnose in the incubation mixture on the incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into the general polysaccharide fraction following incubation periods of 10 and 120 minutes. The UDP-rhamnose was purified as described in Chapter 2.

10 minute incubation



Log concentration of purified UDP-rhamnose (μM)

120 minute incubation

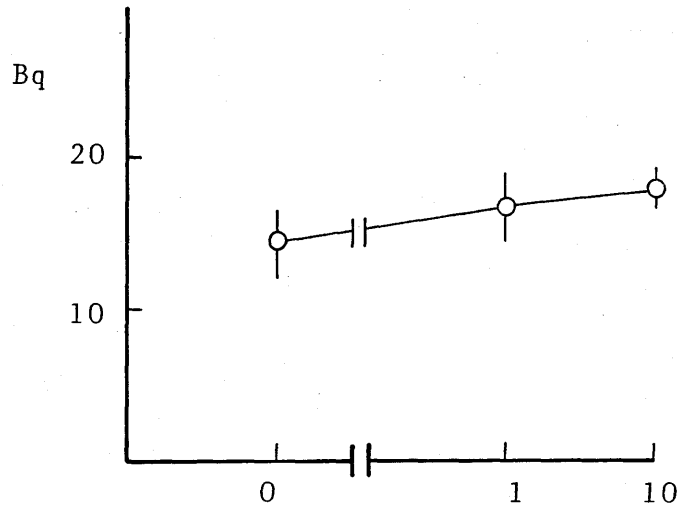


Log concentration of purified UDP-rhamnose (μM)

Figure 7.5

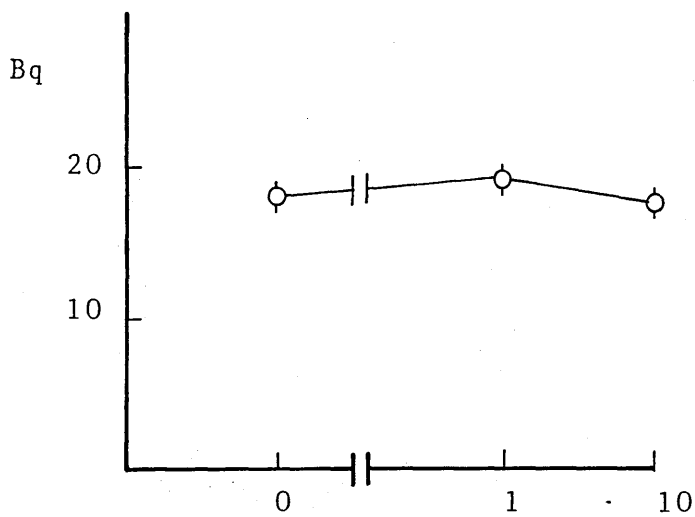
Effect of different concentrations of rhamnose-phosphate present in the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide material following incubation periods of 10 and 120 minutes.

10 minute incubation



Log concentration of rhamnose-phosphate (μM)

120 minute incubation

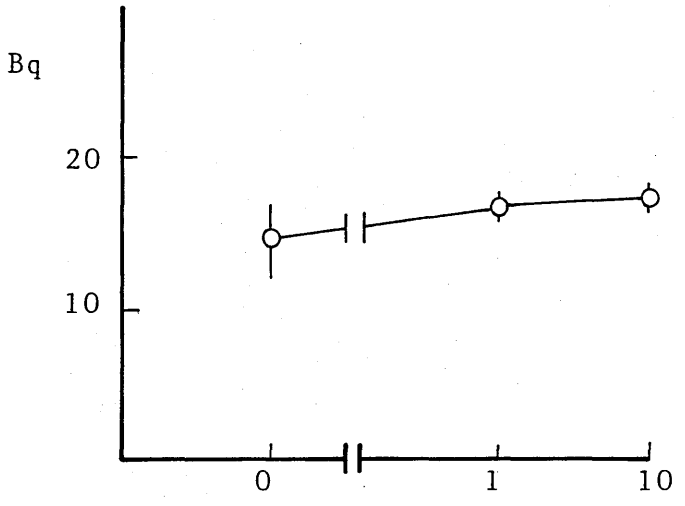


Log concentration of rhamnose-phosphate (μM)

Figure 7.6.

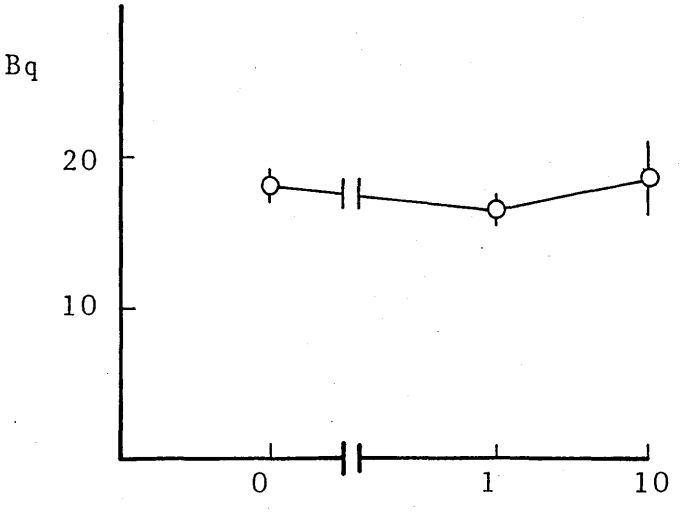
Effect of different concentrations of uridine-phosphomorpholidate present in the incubation mixture on the incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into general polysaccharide material following incubation periods of 10 and 120 minutes.

10 minute incubation



Log concentration of uridine-phosphomorpholidate (μM)

120 minute incubation

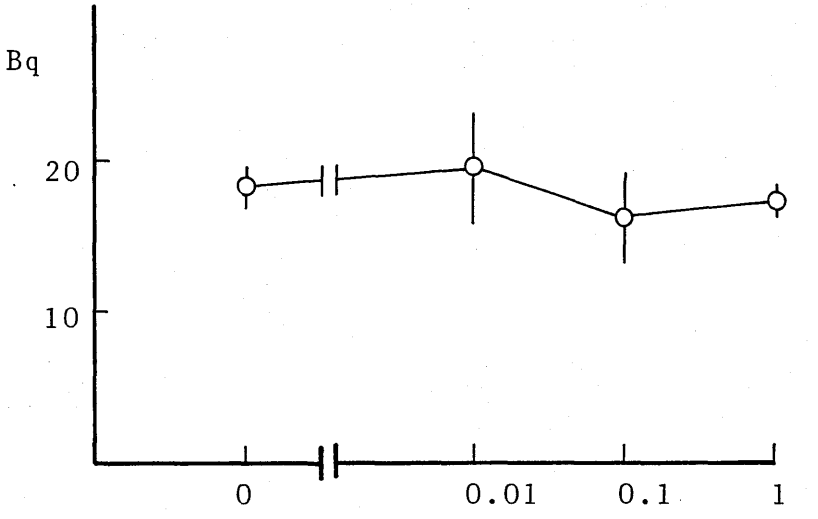


Log concentration of uridine-phosphomorpholidate (μM)

Figure 7.7

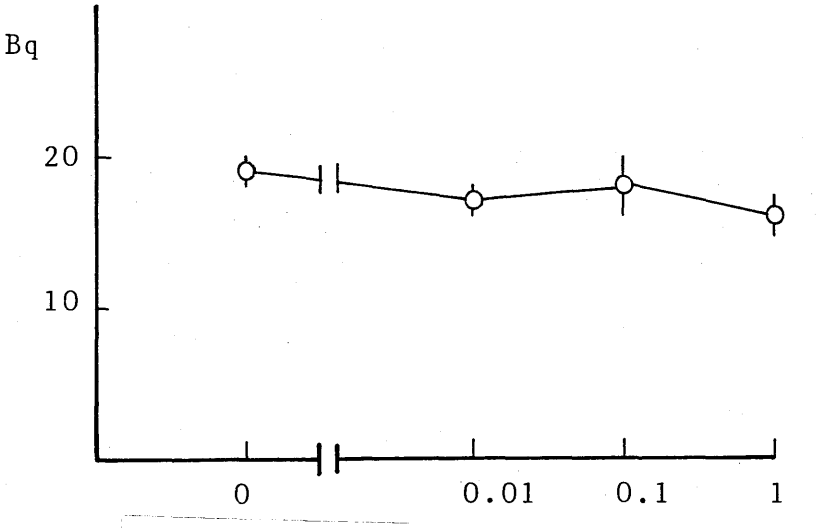
Effect of the presence of different concentrations of GDP-L-rhamnose in the incubation mixture on the incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into the general polysaccharide fraction following incubations of 10 and 120 minutes.

10 minute incubation



Log concentration of GDP-rhamnose (mM)

120 minute incubation

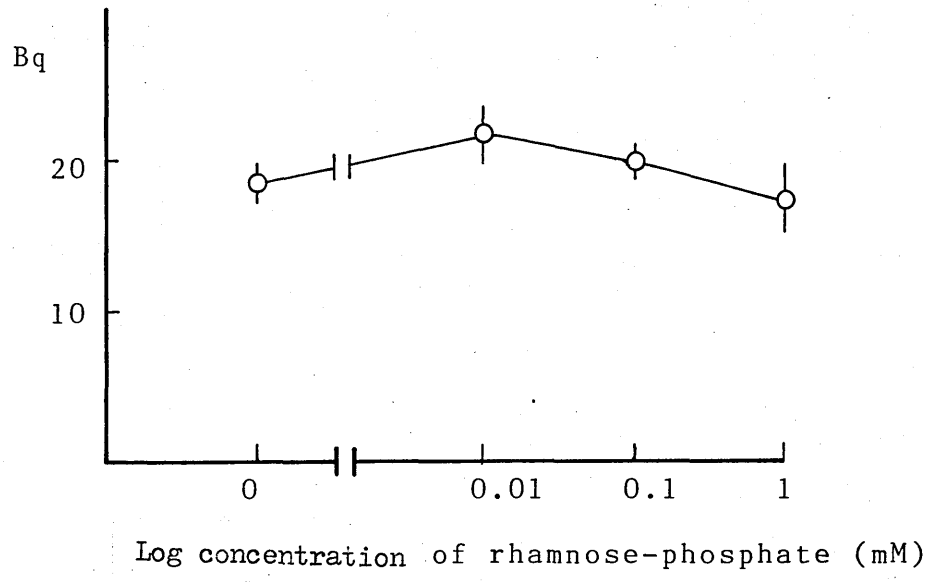


Log concentration of GDP-rhamnose (mM)

Figure 7.8.

Effect of different concentrations of rhamnose-phosphate present in the incubation mixture on the incorporation of galacturonic acid into general polysaccharide material following incubations of 10 and 120 minutes.

10 minute incubation



120 minute incubation

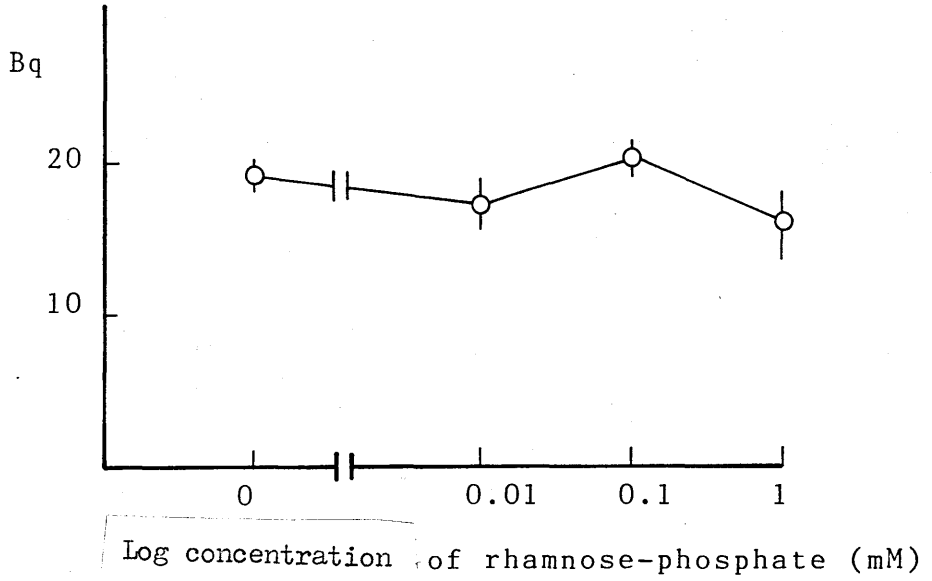
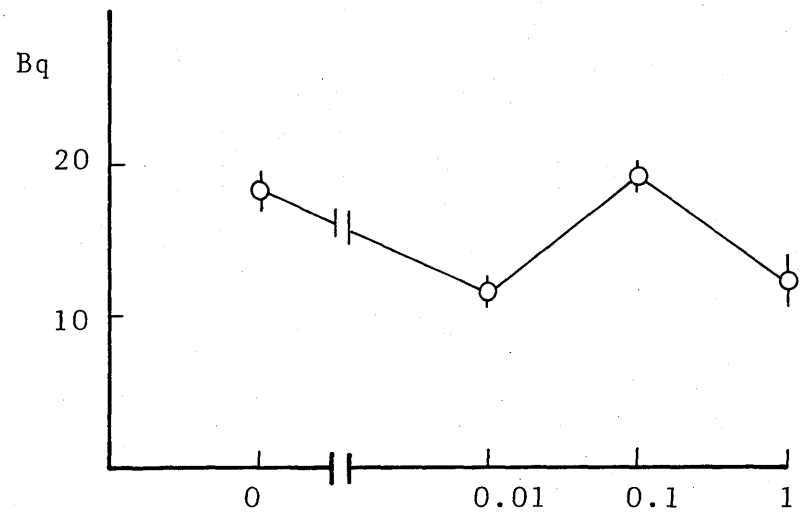


Figure 7.9.

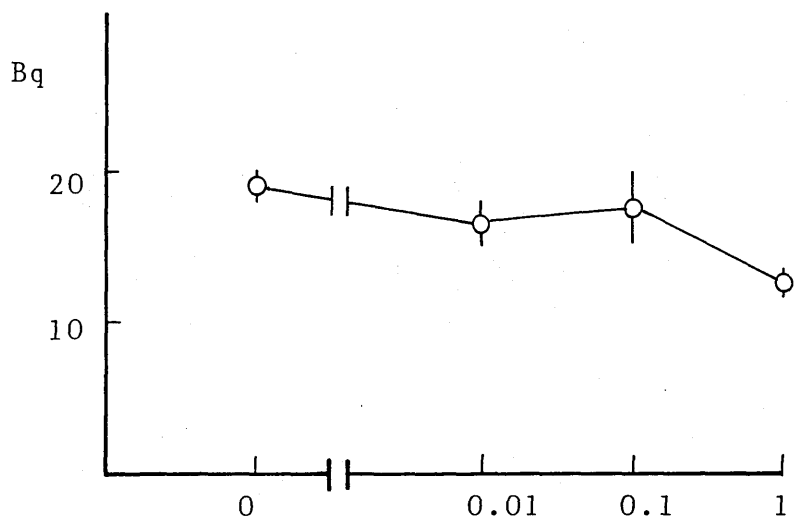
Effect of the addition of different concentrations of guanosine-phosphomorpholidate to the incubation mixture on the incorporation of galacturonic acid into the general polysaccharide fraction following incubation periods of 10 and 120 minutes.

10 minute incubation



Log concentration of guanosine-phosphomorpholidate (mM)

120 minute incubation



Log concentration of guanosine-phosphomorpholidate (mM)

galacturonic acid into the general polysaccharide fraction. The H_2O-soluble polysaccharide fraction was also analysed but the results (table 7.2.) demonstrate that incorporation of galacturonic acid was not increased in the presence of GDP-L-rhamnose.

The effect of the addition of purified UDP-β-L-rhamnose on galacturonyltransferase activity using a modified procedure to obtain the enzyme preparation.

The results from the previous experiments do not provide any evidence to indicate co-operation between galacturonyltransferase and rhamnosyltransferase. One reason for this may be that the enzyme preparation does not contain rhamnosyltransferase activity. Therefore, a modified procedure was employed to obtain the enzyme preparation. Pea epicotyls (10g) were homogenised in homogenisation buffer (5ml) and then filtered. The filtrate was used as the enzyme preparation.

Initially, an experiment was conducted to ensure that galacturonyltransferase activity could be detected in this fraction. Purified UDP-L-rhamnose was then added into the incubation mixture (1 and 10 μM). Following incubation periods of 10 and 120 minutes, the general polysaccharide fraction was analysed. The results (figure 7.10.) indicate that the presence of 1 μM UDP-β-L-rhamnose did not affect incorporation of galacturonic acid whereas the presence of 10 μM UDP-β-L-rhamnose slightly inhibited galacturonyltransferase activity.

Discussion.

The experiments reported in this chapter were carried out in order to investigate the role of rhamnose nucleotides in the biosynthesis of the rhamnogalacturonan polysaccharides. Both UDP-β-L-rhamnose and GDP-β-L-rhamnose have been examined to investigate whether either functions as the rhamnose donor. Attempts to synthesise CDP-β-L-rhamnose and TDP-β-L-rhamnose proved unsuccessful.

TABLE 7.2.

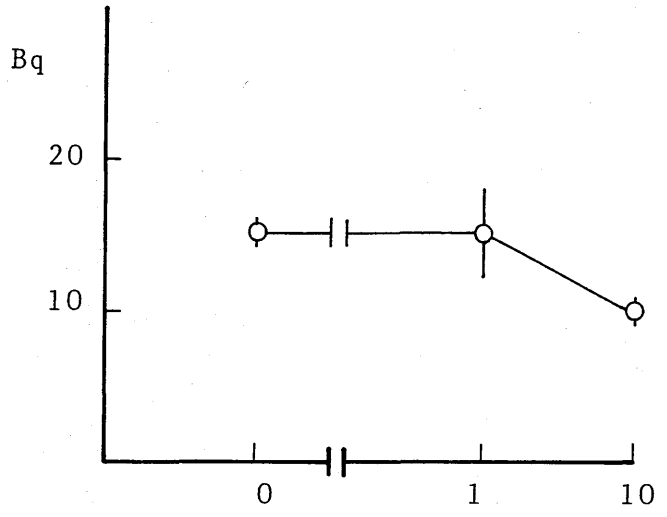
Effect of the addition of GDP-rhamnose (at concentrations of 0.01, 0.1 and 1mM) to the incubation mixture on the incorporation of galacturonic acid into the high-molecular-weight, H_2O-soluble material following incubation periods of 10 and 120 minutes.

GDP-rhamnose concentration (mM)	Incorporation (Bq)	
	10 min incubation	120 min incubation
0	0.98	1.62
0.01	0.96	1.68
0.1	0.67	1.45
1	1.26	1.90

Figure 7.10.

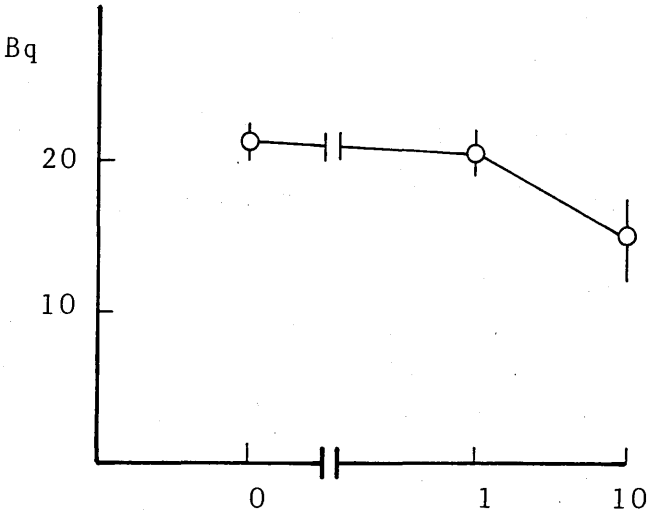
Effect of the addition of different concentrations of purified UDP-L-rhamnose to the incubation mixture on the incorporation of galacturonic acid from UDP-^{14}C-galacturonic acid into the general polysaccharide fraction following incubation periods of 10 and 120 minutes. The enzyme preparation used in this experiment was the material obtained after homogenising pea epicotyls in homogenisation buffer followed by filtration through muslin.

10 minute incubation



Log concentration of purified UDP-rhamnose (μM)

120 minute incubation



Log concentration of purified UDP-rhamnose (μM)

The addition of either UDP-β-L-rhamnose or GDP-β-L-rhamnose did not increase or prolong galacturonyltransferase activity, therefore there is no evidence to indicate co-operation occurring between galacturonyltransferase activity and rhamnosyltransferase activity.

One explanation for these results is that the enzyme preparation does not contain rhamnosyltransferase activity or, although present, it is inactive. For an enzyme to be active, specific conditions are required. It may be that the enzyme preparation is not exhibiting rhamnosyltransferase activity due to certain conditions not being met.

The second possibility is that neither UDP-β-L-rhamnose or GDP-β-L-rhamnose functions as the rhamnosyl donor. Enzymes tend to be highly specific regarding substrates and the rhamnose nucleotide, which functions as the donor, may not have been supplied. In the absence of the correct rhamnosyl donor, it is possible that galacturonyltransferase activity, which is normally involved in rhamnogalacturonan I synthesis, synthesises homogalacturonan. Due to the complex structure of rhamnogalacturonan II, it is likely that for the synthesis of this polymer, the presence of many other enzymes and substrates would be required.

Chapter 8

DISCUSSION

It can be concluded from the results presented in this thesis that a galacturonyltransferase enzyme, involved in pectin biosynthesis, is present in pea (Pisum sativum) epicotyls. The membrane-bound enzyme catalyses the incorporation of the galacturonic acid unit from UDP-galacturonic acid into galacturonan. The precise nature of the product, into which the galacturonic acid units are incorporated, is not known but it appears most likely to be either homogalacturonan or rhamnogalacturonan I or both. The nature of the acceptor molecule was also examined in an attempt to provide more information on the product of the reaction. However, no molecules were found which had the ability to act as acceptor molecules. This may be due to the requirement of a specific compound by the enzyme system or, alternatively, the polysaccharide compound may be tightly bound to the enzyme molecule therefore the addition of acceptor molecules would not result in increased enzymic activity. The possibility of the involvement of protein or lipid intermediates in the biosynthesis of galacturonan was also investigated but no evidence was found to suggest the involvement of either functioning as an intermediate.

The distribution of galacturonyltransferase activity in the epicotyl was also of interest as the pectic polysaccharides are confined to the middle lamella and the primary wall. Bolwell et al. (1985), investigating the control of cell wall polysaccharide biosynthesis using sycamore, demonstrated that galacturonyltransferase activity decreased as cells differentiated from vascular cambium to xylem. Examination of the epicotyl revealed that galacturonyltransferase activity was higher in the region of elongation of the epicotyl but all regions of the epicotyl did exhibit galacturonyltransferase activity. No evidence was found to substantiate the theory that the galacturonyltransferase activity present in the lower regions may be involved in turnover. It is possible that although

galacturonyltransferase activity is present in the lower regions of the epicotyl, it may not be used *in vivo*.

Due to the success in this laboratory of the solubilisation of glucuronyltransferase (Waldron, 1984), it was attempted to solubilise galacturonyltransferase activity. Partial solubilisation of galacturonyltransferase activity was achieved using LDAO. However, further work is necessary in order to obtain consistent results. Solubilisation of membrane-bound proteins is the first stage in purification. Once solubilised, other purification techniques can be employed. Thelen and Delmer (1986) have demonstrated that it is possible to detect enzymic activity following gel electrophoresis of digitonin-solubilised membrane preparations. Digitonin-solubilised enzyme preparations were purified using gel electrophoresis and a glucosyltransferase enzyme involved in β(1-3) glucan synthesis was then detected. This procedure allows the detection of several enzymes simultaneously and the requirements of enzyme systems can be characterised.

Another area of interest is the co-operation of glycosyltransferases in the synthesis of cell wall heteropolysaccharides (Waldron and Brett, 1985). Therefore, the possibility of co-operation occurring between galacturonyltransferase and rhamnosyltransferase was investigated. The rhamnose nucleotides had to be synthesised using rhamnose as the substrate and it was only possible to investigage UDP-β-L-rhamnose and GDP-β-L-rhamnose. However, there is no evidence to suggest that either rhamnose nucleotide stimulates galacturonyltransferase activity. This may be due to neither UDP-rhamnose nor GDP-rhamnose functioning as the rhamnosyl donor. Alternatively, the enzyme preparation employed may not contain rhamnosyltransferase activity (although a very crude preparation was used) or the enzyme may not be active due to certain requirements not being provided.

Further work in necessary in order to investigate all the rhamnose nucleotides. This may provide some evidence of co-operation between galacturonyltransferase and rhamnosyltransferase. The presence of the correct rhamnosyl donor may result in the synthesis of rhamnogalacturonan I rather than homogalacturonan.

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