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The Characterisation of Nascent Pectin Complexes in Pea Plants

By

Hind Dunya Rizkallah

A dissertation presented to the University of Glasgow for the degree of
Doctor of Philosophy.

March, 2005

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DECLARATION

The work reported in this thesis is my own and is original except where specific reference is made.

Hind Dunya Rizkallah,

23 May 2005

ABSTRACT

The structure and properties of a nascent pectin-xyloglucan complex in etiolated pea epicotyls were investigated. Membrane pellets were prepared and incubated with UDP- $[^{14}\text{C}]$ galactose, and were extracted with six different reagents: Tris-HCl buffer (pH 7.4); 50mM EDTA/50mM PO_4 (pH6.8) at 100°C; 50mM EDTA/50mM PO_4 (pH6.8) at 25° C; phospholipase C with 100 μl of 0.1M Tris-HCl pH 7.4 at 25° C; trypsin at 25° C and 0.1% Triton X-100 at 25° C. The best extractant used to solubilize the pectin-complex from the pellets was 50mM EDTA/50mM PO_4 pH 6.8 at 100°C.

Aqueous solutions of pectic polyuronides tend to associate covalently into multichain aggregates. Because of the tendency of pectins to aggregate in solution, the effect of a number of eluents on the behaviour of the complex on gel filtration was studied. 10mM EDTA/10mM PO_4 /1M NaCl (pH 6.8) was chosen as the best eluent to minimise this aggregate formation. On gel filtration using Sepharose CL-2B with this eluent, the complex eluted with a K_{av} of around 0.8, corresponding to a molecular size approximately 200 kDa, as judged by dextran standards. In other solvents tested, aggregation appeared to occur. On Sepharose CL-4B and CL-6B columns using the same EDTA/Pi/NaCl solvent, the apparent molecular size was significantly reduced by a xyloglucan-specific endoglucanase, confirming the presence of a xyloglucan rather than a glucan in the complex. Polygalacturonase caused a greater decrease in apparent molecular size, to 10-20 kDa, while endo-1, 4- β -galactanase converted the radioactivity to $[^{14}\text{C}]$ galactobiose and $[^{14}\text{C}]$ galactose.

When the [^{14}C] galactose-labelled complex was incubated in solution with 3MM paper, the radioactivity was almost completely absorbed onto the paper over a period of about 6 hours. This confirmed the presence of xyloglucan attached to pectin.

There is a possibility of the presence of a mannan in the complex, which is indicated by the decrease in the molecular weight through treatment with mannanase. However, this is not conclusive since mannanase could be contaminated with other polysaccharidase enzymes.

The results of experiments involving treatment of the complex with trypsin, as well as attempts to attach the complex to a protein specific gel (Affi-Gel 15), showed that there is no definite evidence of any protein present in the nascent [^{14}C] pectin complex when labelled in the galactan side-chain.

Evidence was also obtained that the [^{14}C] galactan is bound to the complex through arabinan; this is indicated by the acid-labile nature of the linkage to the rest of the pectin complex.

The results of the research are discussed in relation to other relevant research. A new model for the structure of the nascent pectin-xyloglucan complex is proposed.

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LIST OF ABBREVIATIONS

Ara	arabinose
AGP	arabinogalactan protein
BD	blue dextran
Bq	Bequerel
BSA	bovine serum albumin
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EPN	10mM EDTA/PO ₄ /1M NaCl
Fuc	fucose
gm	grams
g	gravity
Gal	galactose
GalA	galacturonic acid
Glc	glucose
GF	gel filtration
HGA	homogalacturonan
H.Mwt	high molecular weight
hr	hour
HRGP	hydroxyl-rich-glycoprotein
kDa	kilodalton
Man	mannose

μm	micrometer
μl	microliter
mM	millimolar
Mwt	molecular weight
PC	paper chromatography
PG	polygalacturonase
PGA	polygalacturonan
PME	pectin methyl esterase
Prep	preparation
RG I	rhamnogalcturonan I
RG II	rhamnogalcturonan II
Rha	rhamnose
rpm	revolutions per minute
TFA	Trifluoroacetic acid
UDP-	uridine diphospho-
v/v	volume for volume
w/w	weight for weight
XET	xyloglucan endotransglycosylase
XG	xyloglucan
Xyl	xylose

Chapter 1

Introduction

1.1. The Plant Cell Wall

All higher plant cells are encased in a cell wall, which defines the cell's shape and contributes to the structural integrity of the entire plant. The primary cell wall, i.e. the wall surrounding expanding cells, consists of cellulose microfibrils embedded in a hydrated amorphous matrix of hemicelluloses, pectins, and glycoproteins (Darvill *et al.*, 1980).

However now, the plant's primary cell wall is considered as a metabolically dynamic network of cellulose and cross-linking glycans embedded in a gel matrix of pectic substances and reinforced with structural proteins and aromatic substances (McCann & Roberts, 1991; Carpita & Gibeaut, 1993). While the plasma membrane will synthesize cellulose, the noncellulosic polysaccharides, which are integrated with the cellulose microfibrils, are synthesized in the Golgi apparatus, packaged in secretory vesicles, and exported to the surface. Primary cell walls control the shape and size of plant cells. Knowledge of the architecture and assembly of such cell walls is of considerable interest in connection with attempts to understand and manipulate plant growth and morphogenesis (Pauly *et al.*, 2001).

The thickness of primary cell wall is approximately constant at 0.1-1.0 μm . All the wall layers consist of two phases, a microfibrillar phase and a matrix phase with immense possibilities for covalent and noncovalent modifications (Fry, 1986). The microfibrillar phase is composed of extremely long, thin structures called microfibrils. The microfibrils are made up of cellulose molecules, which are unbranched β 1, 4-glucans. The number of sugar residues in a cellulose molecule is up to at least 15,000; the chain length of such a large, insoluble molecule is difficult to measure due to possible enzymic and mechanical degradation during analysis.

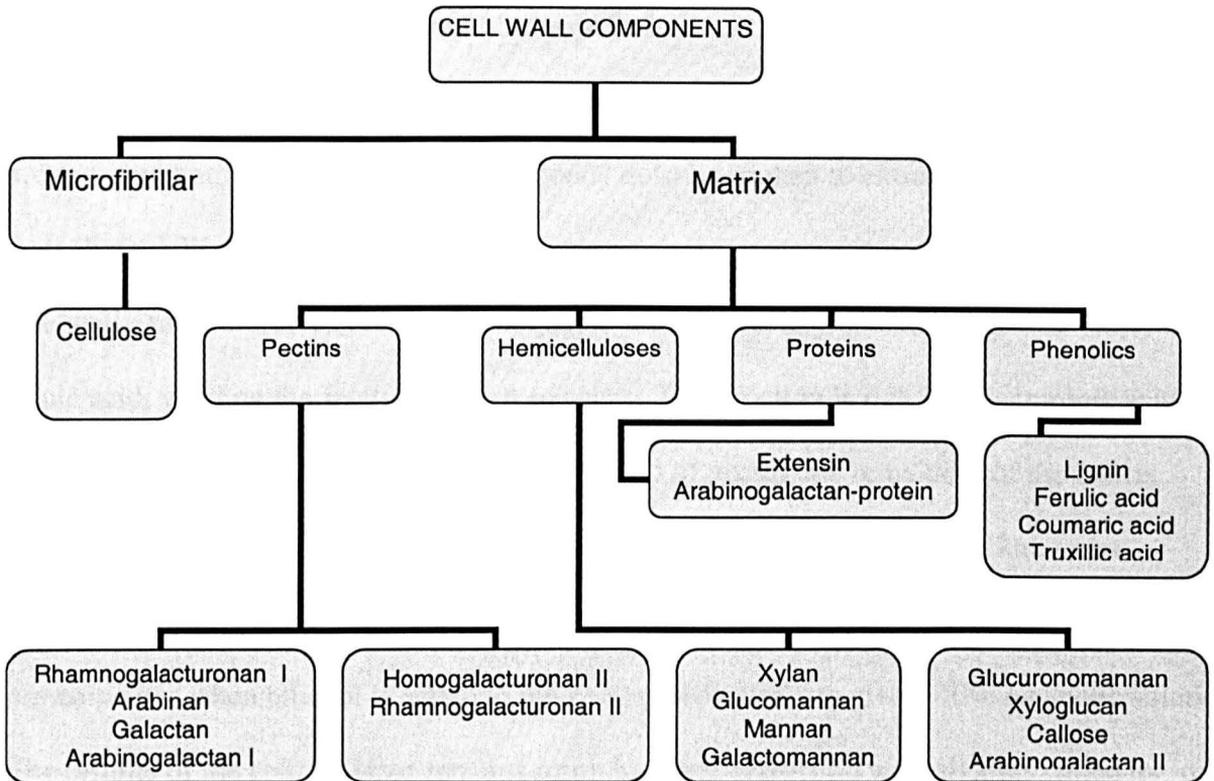


Fig.1.1. Wall components (Brett & Waldron, 1996).

The Matrix Phase:

The non-crystalline phase of the cell wall is called the wall matrix. It consists of a variety of polysaccharides, proteins and phenolic compounds. Their composition varies in different parts of the wall, in different types of cell and in different species. While purifying the matrix there are few constraints, as certain number of bonds must be broken to extract components from the wall (Brett & Waldron, 1996).

The wall can be extracted either with hot, aqueous solution of a chelating agent or with hot, dilute acid, yielding the fraction known as pectin. This fraction is rich in galacturonic acid, rhamnose, arabinose and galactose. After removal of pectin, the remainder of the matrix polysaccharides can be extracted using alkaline solution, yielding a fraction known as hemicellulose 'A' which precipitates when the alkaline extract is neutralized and 'B' which reprecipitates when ethanol is added to the neutralized extract to give a 70% ethanolic solution. The residue of the cell wall that remains insoluble after extraction with alkali is called α -cellulose, and contains the cellulose microfibrils (Brett, 2000). However, some polysaccharides are not clearly partitioned into one class. That is why some workers prefer not to divide them into pectin and hemicellulose. The major role of polysaccharides is to give the cell its shape and structure.

Proteins and Glycoproteins:

Cell walls contain mostly glycosylated proteins, with hydroxyproline as an abundant amino acid. The most extensively studied cell wall protein is known as extensin, which is hydroxyproline-rich glycoprotein (HRGP). The presence of free HRGP coating the surface of airspaces could provide a passive agglutination defense mechanism against pathogenic bacteria (Leach *et al.*, 1982).

The enzymes located in the cell wall include peroxidase, invertase, cellulase, acid phosphatase, pectinase, pectin methylesterase and malate dehydrogenase. Some exoglycosidases and some endoglycanases are present. It has been suggested that proteins may act as primers for polysaccharides and for cell wall polysaccharides in particular (Campbell *et al.*, 1988). Protein primers are thought to be involved in the synthesis of glycogen (Whelan, 1976) and starch (Crosthwaite *et al.*, 1974).

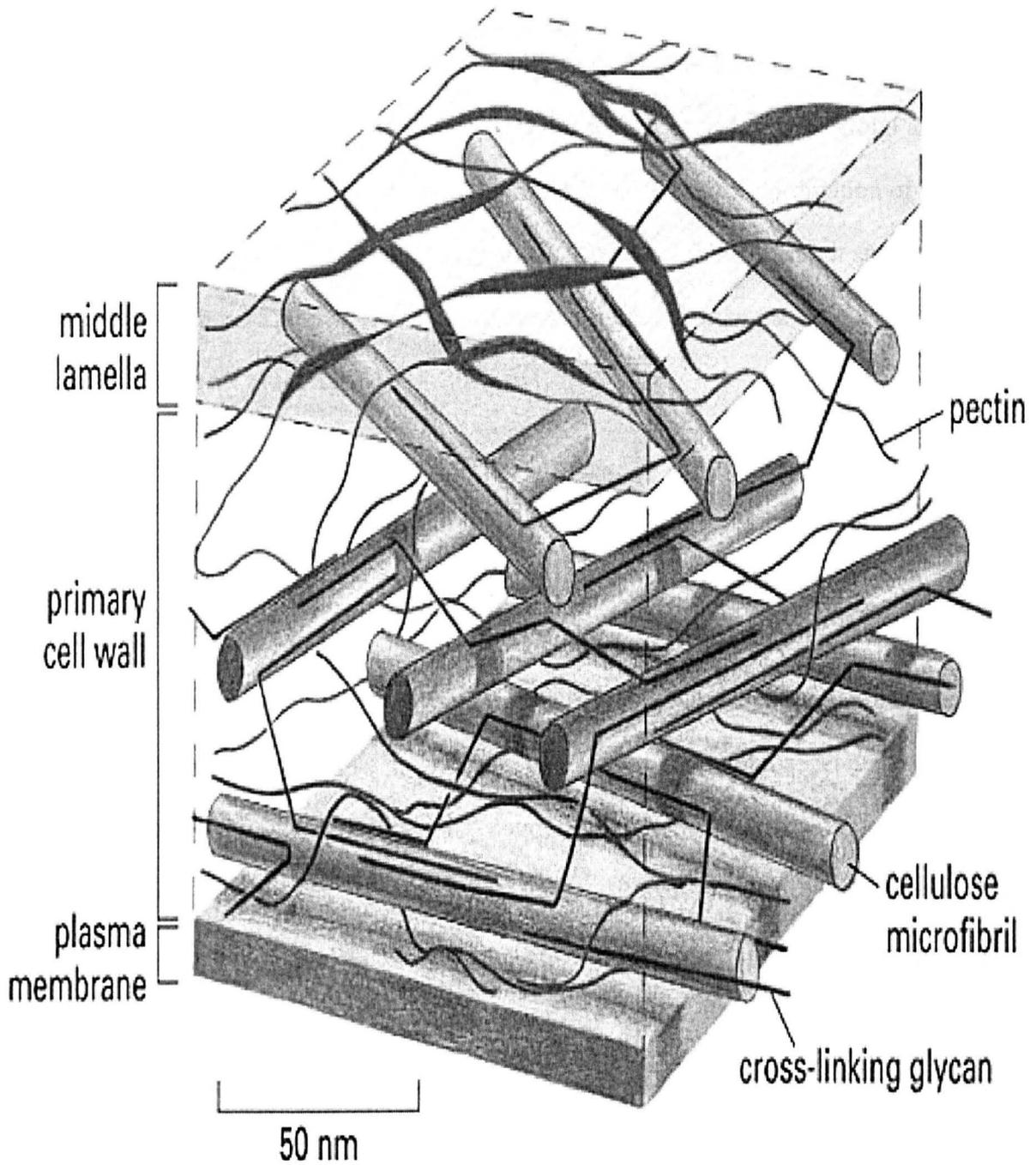


Fig.1.2. Model of the primary cell wall showing the two major polysaccharide networks.
(Alberts *et al.*, 2002)

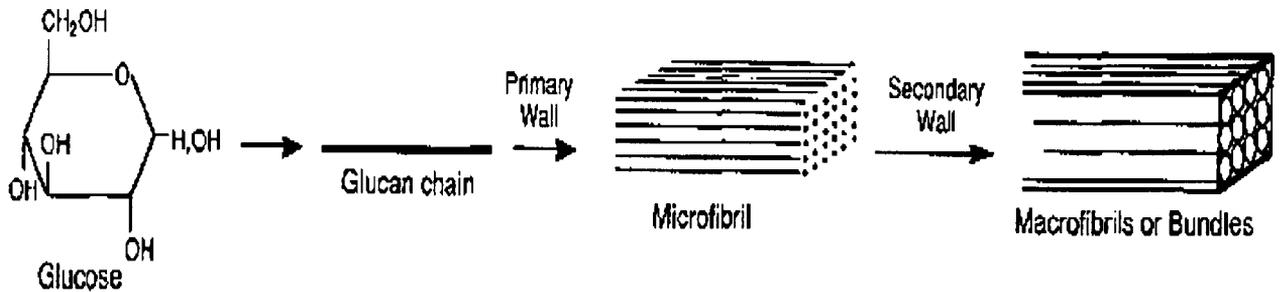
1.2. Cell Wall Polysaccharides.

Nucleotide-sugars are the substrates for the synthesis of the vast majority, if not all, of the cell wall polysaccharides. Synthesis of the noncellulosic polysaccharides at the Golgi apparatus, especially those containing numerous kinds of sugars, requires the coordination of synthase activity with the uptake of nucleotide sugars from the cytosol. This is why it seems nearly impossible to synthesize polysaccharides *in vitro* that correspond to the fine structure of those made *in vivo* (Delmer *et al.*, 1995).

As mentioned earlier, the wall layer consists of two phases, a microfibrillar phase and a matrix phase. The microfibrillar phase is a result of the polymerization of glucose residues from a substrate such as UDP-glucose to form the homopolymer β -1, 4-D-glucan. The β -1, 4-glycosidic linkage creates a linear extended glucan chain in which every other glucose residue is rotated $\sim 180^\circ$ with respect to its neighbour. This means that cellobiose, and not glucose, is the basic repeating unit of the molecule and contrasts with other glucan polymers such as starch (α -1, 4-glucan) or callose (β -1, 3-glucan) in which the disaccharide is not the repeating unit and the chains are not perfectly extended but assume less ordered, helical configurations.

The β -1, 4-glucan chains of cellulose interact with each other to exist as a composite of many chains called a microfibril. Chain length can vary among organisms, ranging from a low of ~ 2000 up to $\sim 20,000$ glucose residues. Microfibril width can also vary among organisms, in general ranging from ~ 36 chains to the very large fibrils of cellulosic algae, which can contain more than 200 chains and are so highly organized that they can diffract as a single pure crystal.

As plant cells mature and deposit a thick secondary wall, microfibrils are found associated into macrofibrils or bundles (Delmer *et al.*, 1995)



Steps in the Assembly of Native Cellulose.

Fig.1.3.(Delmer *et al.*, 1995).

Cellulose in most primary walls exists as elementary fibril that forms a complex with xyloglucan. This network contributes ~70% of the total strength to normal primary walls (Shedletzky *et al.*, 1992). The cellulose chains are held in a crystalline lattice within the microfibril, giving rise to a considerable tensile strength. Cellulose is insoluble, chemically stable, and relatively immune to enzymatic attack (Cosgrove, 1997b).

As suggested by Alberts *et al.*, 2002, the plant's primary cell wall is depicted as a network of cellulose and cross-linking glycans embedded in a gel matrix of pectic substances and reinforced with structural proteins and aromatic structures.

Pectin is the most abundant class of macromolecule within the matrix of type 1 primary cell walls. Pectin is greatly reduced or absent in non-extendable secondary cell walls and is the only

major class of plant polysaccharide to be largely restricted to primary cell walls (Willats *et al.*, 2001).

Pectins are a family of complex polysaccharides that contain 1, 4-linked α -D- galactosyluronic acid (Galp A) residues (Ridley *et al.*, 2001). Gal A occurs in two major structural features that form the backbone of three polysaccharide domains that are thought to be found in all pectin species: homogalacturonan (HGA), rhamnogalacturonan-I (RGI), and rhamnogalacturonan-II (RGII). It is thought that these three polysaccharide domains are covalently linked to form a pectic network throughout the primary cell wall matrix and middle lamellae (Mohnen, 1999).

HGA is a linear homopolymer of (1 \rightarrow 4)- α -linked-D-galacturonic acid and is thought to contain some 100-200 GalA residues. HGA is an abundant and widespread domain of pectin and appears to be synthesized in the Golgi apparatus and deposited in the cell wall in a form that has 70-80% of GalA residues methyl esterified at the C-6 carboxyl (Mohnen, 1999).

In addition to HGA, an acidic pectin domain consisting of as many as 100 repeats of the disaccharide (1 \rightarrow 2)- α -L-rhamnose- (1 \rightarrow 4)- α -D-galacturonic acid has been isolated from a wide range of plants and is known as RG-I. The highly branched nature of RG-I has led to it being known as the hairy region of pectin, in contrast to HGA domains that are known as the smooth region (Mohnen, 1999).

The capacity of HGA to participate in gel formation and to contribute to cell wall stiffening is regulated by the action of pectin methyl esterases (PMEs). PMEs remove methyl-ester groups from HGA resulting in stretches of acidic residues that can associate with other HGA chains by calcium cross-links. The variation of methyl esterification of HGA within cell walls was investigated using the first generation of anti-pectin monoclonal antibodies. The anti-HGA

probe 2F4 recognizes a calcium-cross-linked dimer of HGA, while JIM5 and JIM7 bind to a range of methyl-esterification states of HGA (Mohnen, 1999).

Rhamnogalacturonan-II (RGII) has a short HGA backbone with complex side chains. RGII occurs widely in all primary cell walls. RGII is attached to HGA as it is released by PG action. Rhamnogalacturonan-I (RGI) is not structurally related to RG-II (Ridley *et al.*, 2001). RGI is highly variable both in its fine structure and in its occurrence within cell walls. The two antibodies, LM5 and LM6 recognize, respectively, 1→4-linked β -D-galactan (Jones *et al.*, 1997) and 1→5-linked α -L-arabinan (Willats *et al.*, 1998) in the side chains of RG-I. Firstly, galactan and arabinan epitopes can occur in different regions of organs indicating that there is no single RG I structure that occurs throughout the cell walls of an organ or plant (Willats *et al.*, 1998). Secondly, when galactan and arabinan do occur in the same cell, they can have distinct, spatially restricted locations within the cell wall (Jones *et al.*, 1997). RG-I and RG-II are most probably covalently attached to HGA, and possibly each other, by glycosidic linkages but relative positions are unknown. The calcium cross-linking of HGA domains and borate ester dimerization of RG-II may contribute to the integrity of the pectic network surrounding cellulose microfibrils. Observations made with antibodies indicate that the proportions of HGA, RG-II and RG-I vary within a particular region of the pectic network (Willats *et al.*, 2001). Pectic polysaccharides have considerable potential for interaction with ions and low-molecular-weight compounds. At least 46 glycosyltransferases are required for the synthesis of the pectic polysaccharide structures that can be found in HGA, RG-I and RG-II, based on the assumption of one linkage for one enzyme (Perrin *et al.*, 2001).

Hemicelluloses are a heterogeneous group of noncrystalline glycans that are tightly bound in the wall. The most abundant hemicellulose in primary walls of dicots is xyloglucan, a polymer consisting of a backbone of 1 → 4 linked β-D-glucopyranose residues with short chains containing xylose, galactose and often a terminal fucose (Fry, 1989).

XG in pea primary wall is considered to bind to the surface of the cellulose microfibrils and to intercalate to some extent between cellulose chains in non-crystalline portions of the microfibrils, leaving unbound loops and ends of the XG chains projecting into the matrix.

XG is a polymer consisting of a backbone of 1 → 4 linked β-D-glucopyranose residues with short chains containing xylose, galactose and often a terminal fucose (Fry, 1989).

1.3. Cell Wall Models.

Early models of the primary cell wall postulated that xyloglucans and pectic polysaccharides, as well as cell-wall protein, were covalently linked together to form a continuous macromolecular network (Keegstra *et al.*, 1973). This model is now generally regarded as out-of-date, and some models emphasize the predominance of non-covalent linkages between polymers (Carpita and Gibeaut, 1993).

One popular model of dicot primary cell walls proposes two entangled but distinct polymer networks: a cellulose-xyloglucan network held together by hydrogen-bonds, and a network of pectic polysaccharides held together partly by Ca^{++} bridges (McCann and Roberts, 1991; Rizk *et al.*, 2000). In this model there is no significant interaction between these two networks by hydrogen bonds, ionic interactions or covalent bonds. In an earlier model, Albersheim had proposed a structure for the primary cell walls of dicots in which the xyloglucans and pectic polysaccharides, as well as the structural glycoproteins are linked to each other covalently to form a single tree-like macromolecule containing all the polymers of the wall matrix (Albersheim, 1976).

Since 1973, however, it has become widely recognized that some hemicelluloses (glucuronoarabinoxylans) in dicot primary cell walls contain uronic acid residues and that some pectins contain xylose and 2-o-methylxylose residues (Prade *et al.*, 1999). These discoveries undermined the original evidence for Albersheim's cell-wall model.

Further evidence for hemicellulose-pectin conjugates, has been provided from studies in which these 2 classes of polysaccharide were coextracted from the wall and found to be inseparable

from each other, by ethanol -precipitation, gel-permeation chromatography or ion-exchange chromatography (Chambat *et al.*, 1984).

In recent studies 'pectic-xylan-xyloglucan' complexes were reported in lower stems of cauliflower (Femenia *et al.*, 1999). They also reported a complex of xylan-xyloglucan complexes in olive fruit pulp (Coimbra *et al.*, 1995) and xylan-pectin complexes in both asparagus stem and olive seed hull; in each case phenolic material may have contributed to the cross-linking between the different polysaccharides present in the complex. Evidence of the interpolymeric linkage showed that treatment with endo-xylanase, which degraded the xylan moiety, simultaneously caused a decrease in the apparent Mwt of some of the pectic and xyloglucan components, indicating that they have been linked to the xylan. Treatment of the complex with endo-polygalacturonase, which degraded the pectic moiety, caused a concomitant decrease in the apparent Mwt of some of the xylan and xyloglucan components, again consistent with a hemicellulose-pectin association. There is evidence that up to 12% of the xyloglucan in the walls of suspension cultured rose cells is attached covalently (probably via an arabinan/galactan domain) to homogalacturonan. About 30 % of the xyloglucan in these walls is linked to acidic polymers (Thompson & Fry, 2000).

In cauliflower stems, the use of *endo*- xylanase and *endo*-polygalacturonase resulted in the degradation of the xylan and pectic polysaccharides moieties, and caused a decrease in the molecular weight of the xyloglucan moieties, thus demonstrating the strong, probably covalent interactions between the different polymer species (Femenia *et al.*, 1999).

The galactan-RGI-PGA complex in sycamore suspension-cultured cells may be linked to xyloglucan, a neutral hemicellulose. (Keegstra *et al.*, 1973).

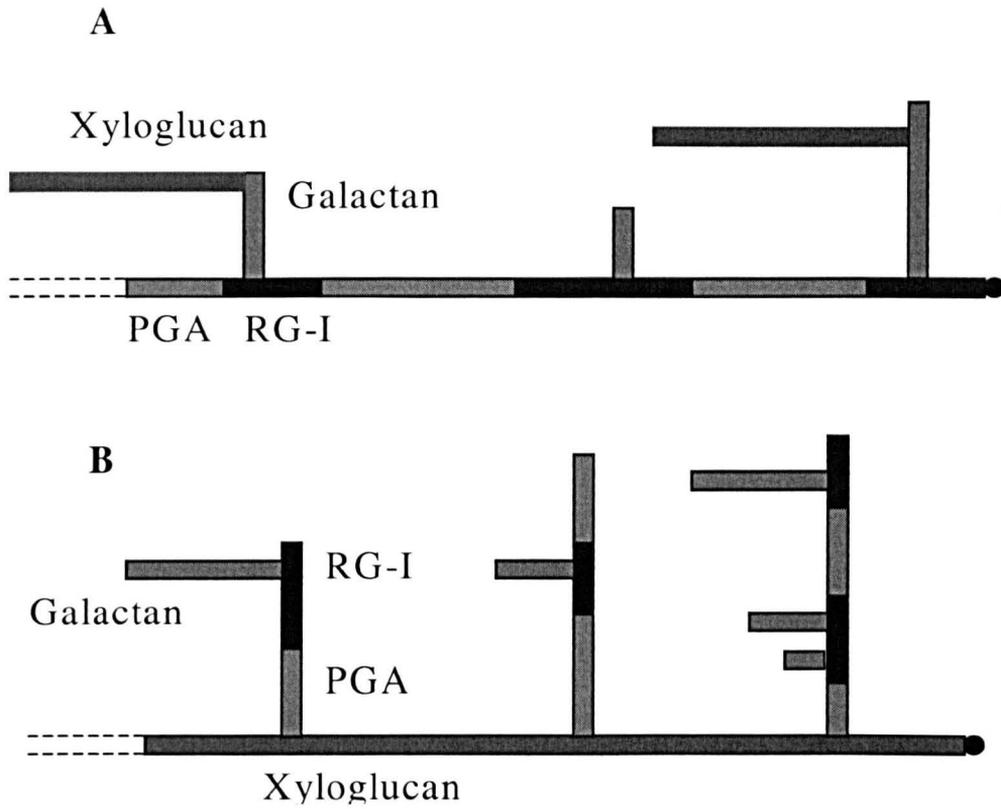


Fig.1.5 A, B. A. Arrangement based on the model of Keegstra *et al.* (1973)

B. Arrangement of galactan, RG-I and xyloglucan biosynthetic complex as suggested by Abdel-Massih *et al.* (2003).

The early model of sycamore cell walls proposed a linkage between the reducing end of xyloglucan and the arabinogalactan side-chains of pectin (Fig 1.5A; Keegstra *et al.*, 1973).

The alternative possibility presented by Abdel-Massih *et al.* (2003) suggested that xyloglucan forms the backbone of the molecule (Fig 1.5B).

1.4. The Golgi Complex.

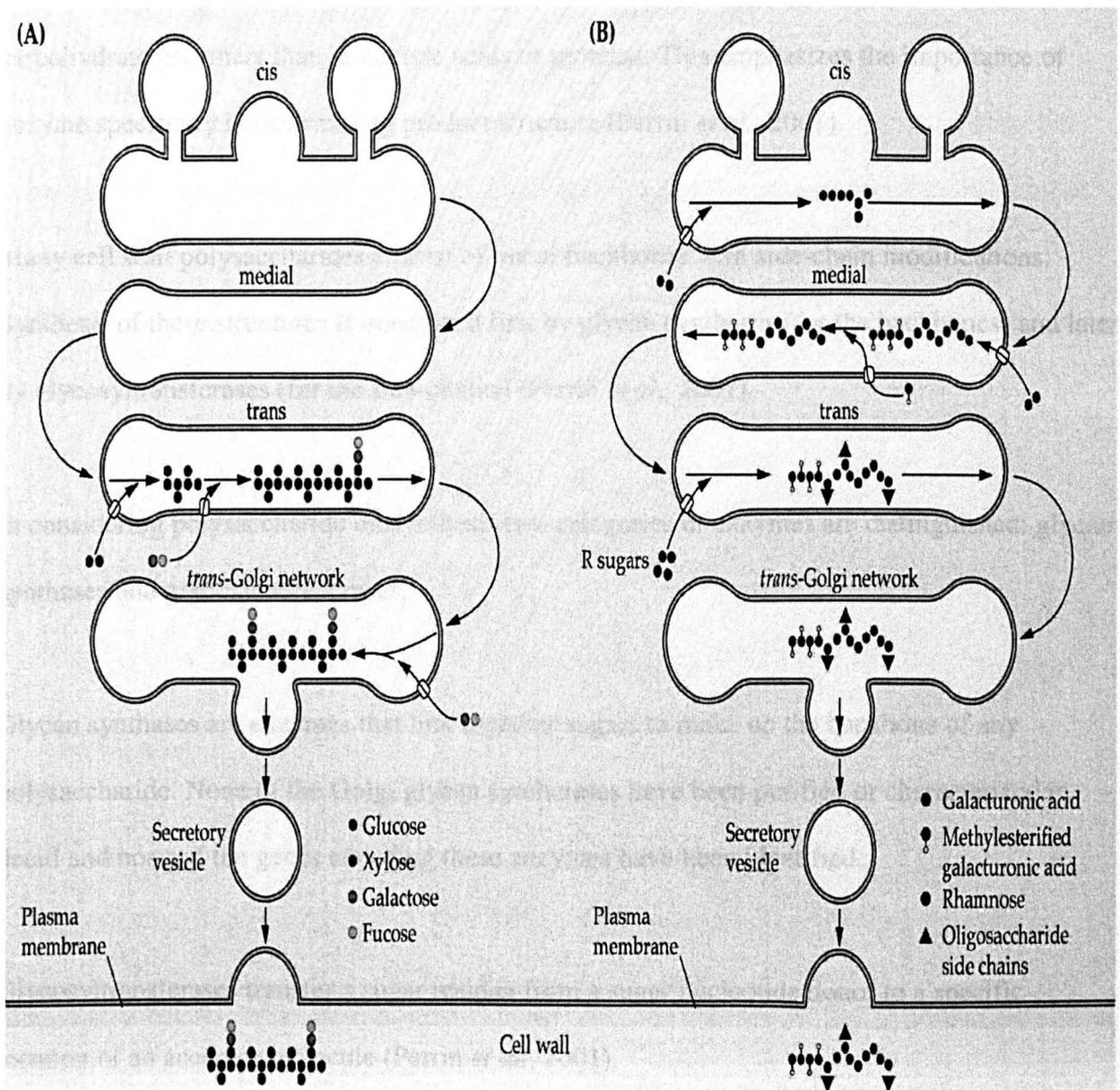
The plant Golgi apparatus is composed of many small stacks of cisternae. The stacks are dispersed throughout the plant cell cytoplasm singly or in small groups. This distribution can be illustrated by immunofluorescence labeling with the monoclonal antibody JIM84. This monoclonal antibody (JIM84) recognizes a glycoprotein epitope present in the Golgi and also in the plasma membrane of some species (Dupree and Sherrier, 1998). The size of individual plant Golgi stacks varies between different cell types and species; they are usually composed of three to eight cisternae. Within a single cell the cisternal number is uniform.

The plant Golgi apparatus synthesizes a wide range of wall polysaccharides and proteoglycans (Dupree and Sherrier, 1998). They also carry out O-linked glycosylation of hydroxyproline rich glycoproteins (HRGPs) and arabinogalactan proteins (AGPs) and modification of N-linked glycans on glycoproteins synthesized in the endoplasmic reticulum (ER) (Nebenführer and Staehelin, 2001). Subfractionation of the Golgi apparatus indicated that the majority of the galactan galactosyltransferase (galactan synthase) was found in the low-density membranes, while the glycoprotein galactosyltransferase was present in equal amounts in all three subfractions (Baydoun *et al.*, 2001). The two abundant classes of Golgi-synthesized polysaccharides, the pectins and hemicelluloses, can constitute between 50 and 80% of the dry weight of the cell wall (Brett and Waldron, 1996).

There is strong evidence that pectin biosynthetic enzymes are present in the Golgi and pectin biosynthesis occurs in the Golgi endomembrane system (Sterling *et al.*, 2001).

A single Golgi stack can synthesize both pectins and hemicelluloses. It therefore contains a wide range of enzymes. Even within a single cell, several different polysaccharides are synthesized at one time. The Golgi is involved in returning escaped proteins back to the ER, sorting of proteins and polysaccharides to the cell wall or vacuoles and in organizing the compartmentation of its own enzymes by retention or retrieval mechanisms (Dupree and Sherrier, 1998).

Fig.1.6. Golgi apparatus enzymes' location. Doblin *et al* 2002.



1.5. Biosynthesis of cell-wall polysaccharides especially pectin and galactans.

The biosynthesis of polysaccharides is different from the synthesis of other biopolymers.

During protein and nucleic acid biosynthesis, a nucleic acid template determines the sequence of monomers within the polymer. As for the sequence of sugars within a polysaccharide, it is determined by the specificity of enzymes that form the bonds between monosaccharides. Each sugar monomer has several hydroxyl residues that may serve as the attachment site for the next sugar in the chain; consequently, a much greater diversity in structure is possible in carbohydrate polymers than in nucleic acids or proteins. This emphasizes the importance of enzyme specificity in determining product structure (Perrin *et al.*, 2001).

Many cell wall polysaccharides consist of linear backbones with side-chain modifications.

Synthesis of these structures is conducted first by glycan synthases (for the backbones) and later by glycosyltransferases (for the side-chains) (Perrin *et al.*, 2001).

In considering polysaccharide biosynthesis two categories of enzymes are distinguished: glycan synthases and glycosyltransferases.

Glycan synthases are enzymes that link together sugars to make up the backbone of any polysaccharide. None of the Golgi glycan synthetases have been purified or characterized in detail and none of the genes encoding these enzymes have been identified.

Glycosyltransferases transfer a sugar residue from a sugar nucleotide donor to a specific location of an acceptor molecule (Perrin *et al.*, 2001).

There may be at least nine different galactosyltransferases required to synthesize different linkages in pectin (Mohnen, 1999). Using mung bean membranes Panayotatos and VILLEMEZ (1973) demonstrated the incorporation of radioactive galactose from UDP- [¹⁴ C] galactose to water-soluble and alkali-soluble polysaccharides. Goubet and Morvan (1993, 1994) using flax membranes reported incorporation of UDP- [¹⁴ C] galactose into a 70%-ethanol-insoluble product, which was partially soluble in water and partially in alkali. The solubility properties of the products suggest that [¹⁴ C] Gal was incorporated into both pectin and non-pectic polysaccharides (Geshi *et al.*, 2000).

The nucleotide sugars required for pectin biosynthesis are believed to be synthesized on the cytosolic side of the Golgi and transported into the Golgi lumen by specific nucleotide-sugar: nucleoside monophosphate antiporters. The nucleotide-sugar is used as a substrate by the glycosyl- transferase and the glycosyl residue is transferred onto a growing polysaccharide chain (Ridley *et al.*, 2001).

1.6. Cell Wall Assembly.

The wall polymers, after being secreted, need to be arranged and bonded; the processes that they follow is either self-assembly or enzyme-mediated assembly. The wall of enlarging plant cells is composed of approximately 30% cellulose, 30% hemicellulose, and 35% pectin, with perhaps 1-5% structural protein, on a dry weight basis. New cell walls originate from the cell plate formed by the phragmoplast during cytokinesis of plant cells. The process where wall polymers are added to and then integrated into existing wall is: synthesis → secretion → integration and assembly → stress relaxation. To this a fifth possible stage may be added where wall polymers may become cross-linked leading to loss of extensibility (Cosgrove, 1997a). The formation of cellulose involves the synthesis of the glucan and the crystallization of multiple glucans into a microfibril (Cosgrove, 1997b). Intermolecular hydrogen-bonds occur between several adjacent cellulose chains, forming 60-70 cellulose molecules, arranged in parallel arrays, 20 to 40 nm apart (Alberts *et al.*, 1989).

Hemicelluloses are not organized into crystalline arrays like cellulose, yet they appear through spectroscopy studies to lie in an orientation parallel to the cellulose microfibril (Séné *et al.*, 1994). This is consistent with the conclusion that xyloglucan can bind tightly to the surface of the cellulose microfibril (Hayashi, 1989).

Both cellulose and hemicellulose fractions become spontaneously aggregated into an ordered network (Roland *et al.*, 1977). This is shown when cellulose is regenerated in vitro; it spontaneously forms fibers, known as rayon. Likewise, when the hemicellulose fraction of the

wall was dissolved and subsequently precipitated, it spontaneously aggregated into ordered network that resembles the native wall (Roland *et al.*, 1977).

Pectins form dispersed network. Pectins are subject to a number of modifications that alter their conformation and linkage in the wall. Many of the acidic residues are esterified during biosynthesis in the Golgi, and the methyl ester groups may be removed by esterases in the wall (McCann *et al.*, 1994). De-esterification causes pectin chains to assemble into expanded highly hydrated gel networks linked by Ca^{++} ions (Lapasin and Priel, 1995). In addition to calcium bridging, pectins may be linked to each other by various covalent bonds (Wallace and Fry, 1994).

Numerous enzymes are associated with cell walls (Fry, 1995). Some can modify the major polysaccharides of the plant wall, e.g. endoglucanase, xylosidases, pectinases, pectin methyl esterases, and xyloglucan endotransglycosylases(XET). Others can act to modify substrates in the wall, e.g. invertase, peroxidases, phosphatases, and various dehydrogenases.

The enzymes involved in glycoprotein and cell-wall-polysaccharide biosynthesis are located within the secretory pathway. Many of these enzymes are type II membrane proteins possessing a single hydrophobic segment that spans the membrane and functions as a signal-anchor sequence (Keegstra and Raikhel, 2001).

XET has the ability to cut the backbone of xyloglucans and to join the newly formed reducing end to the nonreducing end of an acceptor xyloglucan. XET also has the ability to integrate newly synthesized xyloglucan into the wall (Okazawa *et al.*, 1993).

Both xylan and xyloglucan, hydrogen bond to cellulose, suggesting that non-covalent bonding of newly-synthesized matrix polymers with nascent cellulose is likely. In the case of xyloglucan there is evidence that transglycosylation occurs, linking newly formed xyloglucan to existing wall xyloglucan by covalent bonds (Brett *et al.*, 1997).

Recent models suggest that the pectin network is independent from the cellulose-xyloglucan network (McCann *et al.*, 1994). The presence and physical state of pectin at the time when cellulose microfibrils are deposited into the wall may affect extensibility of the wall (Chanliaud and Gidley, 1999). Pectins may also act as hydrophilic filler that prevents the aggregation and collapse of the cellulose network (Jarvis, 1992).

Thompson and Fry. (2000) have presented strong evidence that xyloglucan does not form close non-covalent associations with pectin and that the pectin-xyloglucan complexes found in rose cell walls were covalently linked. Rizk *et al.* (2000) have shown that nascent pectin binds non-covalently to xyloglucan by a mechanism that depends on the presence of an “assemblin” protein.

1.7. The use of antibodies specific to pectin.

Monoclonal antibodies to epitopes occurring in homogalacturonan and side chains of rhamnogalacturonan I have been used in an immunolocalization study of cell wall architecture of developing pea cotyledons. Homogalacturonan (HGA) is a polymer of 1→4- α -linked galacturonic acid. Rhamnogalacturonan I (RGI) has a backbone of galacturonic acid alternating with rhamnose residues. Antibodies have proven useful for the localization of HGA and other pectic epitopes in relation to cell wall architecture (Willats and Knox, 1999). Monoclonal antibodies recognizing epitopes in pectin with low (JIM5) and high (JIM7) degrees of esterification have been used to locate these epitopes by indirect immunofluorescence and immunogold electron microscopy. The low-ester epitope was located to the inner surface of the primary cell walls adjacent to the plasma membrane, in the middle lamella and to the outer surface at intercellular spaces. The epitope containing more highly methyl-esterified pectin was located evenly throughout the cell wall (Knox *et al.*, 1990).

It is noted that monoclonal antibody JIM5 recognizes a low-ester epitope of HGA (VandenBosch *et al.*, 1989) that may occur in both free HGA and HGA attached to branched components. JIM5 is generated subsequent to immunization with carrot protoplasts (Knox *et al.*, 1990). 2F4 is a monoclonal antibody generated subsequent to immunization with a conjugate of HG coupled to methylated bovine serum albumin (BSA). It recognizes a calcium-dependent conformation of HG (Liners *et al.*, 1992). The major observation made with these three antibodies has been the heterogeneous distribution of pectin epitopes. The use of these antibodies indicated that low-ester pectin (indicated by JIM5 and 2F4 epitopes) is located in

regions of the middle lamella and the linings of intercellular spaces. However it was indicated that highly-esterified pectin was observed throughout primary cell walls (Knox, 1997).

A monoclonal antibody LM5 to an epitope of (1 →4)-β-galactan recognizes the side chains in the branched regions of pectic polysaccharides. Immuno-dot-assay showed that LM5 binds to a galactan-rich pectin from lupin (Jones *et al.*, 1997) and LM6 binds to arabinan-rich pectins from sugar beet (Willats *et al.*, 1998) and that both antibodies bind to citrus pectic polysaccharides. However, LM5 and LM6 do not recognize a sample of HGA that is recognized by the anti-pectic monoclonal antibody JIM5 (Knox *et al.*, 1990).

1.8. Aims of the research.

Previous work had established that the 1, 4- β -galactan that forms part of pectin in etiolated pea epicotyls is synthesized by a UDPGal: 1,4- β -galactan galactosyltransferase that is localized in the Golgi apparatus (Baydoun *et al.*, 2001). Also, that this 1, 4- β -galactan is synthesized as part of a nascent pectin complex that contains homogalacturonan, RGI and either xyloglucan or glucan (Abdel-Massih *et al.*, 2002). Similar nascent pectin complexes also contain a protein termed assemblin (Rizk *et al.*, 2000).

Thus the objectives of the study were:

- 1- To determine the optimum procedures for the extraction of the nascent pectin-XG complex formed in pea stems.
- 2- To optimize methods for the determination of the molecular weight of the nascent pectin-xyloglucan complex, and to avoid formation of pectin aggregates.
- 3- To analyze the composition of the nascent pectin-xyloglucan complex using specific polysaccharidases.
- 4- To determine whether nascent pectin-XG complex binds to anti-pectin antibodies. If so, then to clarify the overall structure of the complex.
- 5- To determine whether nascent pectin-XG complex contains protein, using specific coupling reagents to bind the complex through its protein component to a solid support. If successful, then to treat the bound complex with specific hydrolases to clarify the overall structure of the complex.
- 6- To characterize the binding of nascent pectin-XG complex to cellulose paper; to use the binding of the complex to cellulose to clarify the overall structure of the complex using

specific hydrolases .

Chapter 2

Materials and Methods

2.1. Plant material

Peas (*Pisum sativum*, variety *Meteor*) were obtained from Thomas Dagg & Sons, 16 Bath St, Glasgow G2, UK . They were soaked overnight in water at room temperature and grown on compost at 25°C in continuous darkness for 6 days (for particulate membrane preparation).

2.2. Particulate enzyme preparation

Membranes were prepared and fractionated as described previously (Hobbs *et al.*, 1991; Baydoun *et al.*, 2001; Abdel-Massih *et al.*, 2003) with minor modifications. Epicotyls (100gms) were harvested before the second internode had begun to elongate i.e. 6-9 cm long .The hooks were discarded and the remainder of the tissue cooled on ice. The epicotyls (100gms) were chopped and homogenized using a pestle and mortar in 35 ml of homogenization buffer that contained 10mmol.l⁻¹ Tris-HCl (pH 7.4), 10mmol.l⁻¹ KCl, 1.5 mmol.l⁻¹ MgCl₂, and 10mmol.l⁻¹ dithiothreitol. The homogenate was strained through four crossed layers of muslin. The residue was then rehomogenised in 50 ml of homogenization buffer and strained again. The two filtrates were combined and centrifuged at 27,000g (15,000 rpm) for 10 min in a Sorvall RC-5B centrifuge. The supernatant was recentrifuged at 100,000g (27,000 rpm) for 1 hour in a Sorvall OTD-65B ultracentrifuge using an AH629 swing-out rotor. The resulting pellets were then resuspended in 500μl of resuspension buffer/pellet (50mmol.l⁻¹ Mes, pH5.5) and subjected to ten strokes in a glass Teflon tissue homogeniser, to obtain the particulate enzyme preparation. This was kept on ice to be used within 20 minutes to ensure that the temperature is kept at 4°C throughout the whole preparation.

2.3. Preparation of radioactive [¹⁴C-Gal]-polysaccharide for analysis

Standard incubations for galactan synthesis contained UDP- [U-¹⁴C] galactose (1.4 KBq, 1.4 μmol.l⁻¹), MnCl₂ (10 mmol.l⁻¹), uridine-diphospho-galactose (UDP-galactose 2 mmol.l⁻¹) and the particulate membrane preparation (80 μl) in a total volume of 200 μl. The incubations were carried out at 25°C for 1 hour, and terminated by the addition of 90% (v/v) ethanol (1 ml). The pellet was then washed three times with 70% ethanol (1 ml) and once with water (1 ml). Pectins were then extracted by boiling for 10 minutes with 50 mmol.l⁻¹ EDTA/PO₄ buffer (pH 6.8).

2.4. Enzyme Treatments

10 units of each enzyme were used, at the pH and temperature specified by the Sigma or Megazyme catalogue. For each enzyme, one unit of activity was the amount of enzyme required to produce one micromole of product per minute under the specified assay conditions. Reactions were stopped by boiling for 15 minutes at 100°C. Control incubations were processed as above with the appropriate buffer in the absence of the enzyme.

1. Endo-1,4-β-D-galactanase: from *Aspergillus niger* (Megazyme), 10 units in 50 mmol.l⁻¹ sodium acetate buffer, pH 4.5 at 40°C for 2 hours.
2. Endo-1,4-β-D-glucanase or cellulase: from *Trichoderma longibrachiatum* (Megazyme), 10 units in 50 mmol.l⁻¹ sodium acetate buffer, pH 4.5 at 40°C for 2 hours.
3. Endo-1,4-polygalacturonase: from Megazyme, 10 units in 50 mmol.l⁻¹ sodium acetate buffer, pH 4.0 at 40°C for 2 hours.

4. Endo-1,4- β -D-xylanase: from *Trichoderma viride* (Megazyme), 10 units in 50mmol.l⁻¹ sodium acetate buffer, pH 4.5 at 40°C for 2 hours.
5. Endo- β -Mannanase: from *Bacillus* (Megazyme), 10 units, pH 8.8 at 40°C for 2 hours.
6. Xyloglucan-specific endo-1, 4- β -D-glucanase: from *Aspergillus aculeatus*, a kind gift from Dr Kirk Schnorr, Novozymes A/S, Kroghoejvej 36, DK-2880 Bagsvaerd, Denmark. Experimentally tested for specificity by Pauly et al. (1999b). 10 μ of 10mg/ml of enzyme in 50mmol.l⁻¹ sodium acetate buffer, pH 5.5 at 40°C for 2 hours.
7. Protease from *Aspergillus saito* (Sigma P2143), or protease from *Streptomyces griseus* (Sigma P5147): 1.5ml protease prepared by adding 10mg of the protease in 0.2M glycine-HCl pH 2.5 incubated for 2 hours at 37° C.

2.5. Gel Permeation Chromatography (gel filtration)

Supernatants obtained from EDTA extractions or from enzyme treatments were applied to columns (Bio-rad 1.4x42 cm) of Sepharose CL2B or CL6B or columns (1.4x26cm) of Sepharose CL4B eluted with water or 10mM EDTA/PO₄/1M NaCl (pH 6.8) (EPN). Samples were collected using a fraction collector and assayed for their radioactivity. Blue dextran (average molecular weight 2,000,000) and CoCl₂ (formula weight 238) were used as high-and low-molecular-weight markers, respectively. Dextran standards were detected using the phenol-sulphuric acid method (Dubois *et al.*, 1956), where 0.4 ml of carbohydrate was added to 0.4ml of 5% phenol and mixed thoroughly in a wide glass tube. Then 2 ml of concentrated H₂SO₄ was added directly

onto the surface of the sample. The tube was left to cool and the absorbance was read at 490nm.

2.6. Preparation of Microsomal Pectin

Microsomes were prepared according to the method of Hobbs *et al.*, (1991).

Supernatant was obtained as described in “Particulate Membrane Preparation” section until the centrifugation at 27,000g for 10minutes. The supernatant was centrifuged onto a cushion of 40% (w/w) sucrose solution (made up in 10mmol.l⁻¹ Tris-HCl, pH7.4, containing 0.1 mmol.l⁻¹ MgCl₂, 1mmol.l⁻¹ EDTA and 10mmol.l⁻¹ dithiothreitol) at 100,000g for 1 hour in a Sorvall OTD-65B ultracentrifuge using an AH629 swing-out rotor. The membranes at the interface were collected and adjusted with a sucrose solution (10mmol.l⁻¹ Tris-HCl, pH7.4, containing 5 mmol.l⁻¹ MgCl₂, 100mmol.l⁻¹ KCl and 10mmol.l⁻¹ dithiothreitol and 2.26mmol.l⁻¹ sucrose) to a final sucrose concentration of 40% (w/w).

To obtain the microsomal pectin, NaCl buffer (1mmol.l⁻¹ Mes/NaOH, pH6/1 mmol.l⁻¹ NaCl) was added to the preparation, and it was centrifuged for one hour at 100,00g. The resulting pellet was resuspended twice in 50mmol.l⁻¹ EDTA-phosphate buffer (pH6.8) at 70°C for 30minutes. The extracts were then collected after centrifugation at 4000g for 10minutes.

2.7. Total Acid Hydrolysis

Galactosylated products were hydrolyzed with 2mol.l⁻¹ trifluoroacetic acid (TFA) at 120°C for 1 hour. Samples were rotoevaporated to dryness to evaporate off all TFA, dissolved in 100µl water and applied to Whatman No3 paper chromatogram.

Descending paper chromatography was carried out for 24-30 hours in ethyl acetate/pyridine/water 8:2:1 (Baydoun *et al.*, 1989). For detection of marker sugars, chromatograms were then sprayed with aniline hydrogen phthalate (Fry, 1988), dried first in a fume hood for 5 minutes, then in an oven at 105°C for another 5 minutes. Marker sugars (glucose, galactose, arabinose or others) were run parallel to the hydrolyzed material. Monosaccharides obtained and external marker sugars also were detected using silver staining. Radioactive products were detected by cutting the chromatogram into 1 cm strips for estimation of radioactivity by scintillation counting.

2.8. Partial acid hydrolysis.

Galactosylated products were hydrolyzed with 0.1 mol.l⁻¹ trifluoroacetic acid (TFA) at 100°C for 1 hour. Samples were rotoevaporated to dryness to evaporate off all TFA, dissolved in 100µl water and applied to Whatman No3 paper chromatogram.

Descending paper chromatography was carried for 24-30 hours in ethyl acetate/pyridine/water 10:4:3 (Panayotatos and Villemez, 1973). To detect non-radioactive sugars, chromatograms were then sprayed with aniline hydrogen phthalate (Fry, 1988), dried first in a fume hood for 5 minutes, then in an oven at 105°C for another 5 minutes. Galactobiose and galacto-oligosaccharide standards were generated by partial acid hydrolysis of 1, 4-β-galactan (Megazyme). Radioactive products were detected as described in section 2.7.

2.9. Pectin Extraction.

Whole etiolated stems were chosen for they formed no leaves and had lots of dividing cells. 200gms of the above mentioned stems were harvested and extracted three times with 70 % (v/v) ethanol (600ml) for 30 minutes at 70°C. The tissue was then chopped with a razor blade and washed with water, and centrifuged at 8000g for 10 minutes. The pellets were extracted twice with 0.1mmol.l⁻¹ Na-EDTA buffer (pH 7.0) at 85°C for 30minutes. The two extracts were combined and dialyzed against three changes of distilled water (1hour, overnight, 1hour) in a cold room with constant stirring.

2.10. Immuno-dot assay: this was as explained in section 4.2.2.

2.11. Use of Ligands to Immobilize Proteins: this was as described in section 5.2.

2.12. Paper binding methods I and II: these were as described in section 6.3.

Chapter 3

Determination of the molecular weight of the nascent pectin complex.

3.1 Introduction

The experiments described in this chapter aimed at determining the molecular weight of the nascent pectin complex through the use of Sepharose gel exclusion chromatography and different eluents. In order to achieve this, it was first necessary to find a suitable extracting medium to solubilize the radioactive polysaccharides from the particulate membrane preparation.

3.1.1 Previous work.

It was reported that incubation of Golgi membranes from etiolated pea stems with UDP-U- $[^{14}\text{C}]$ galactose resulted in the incorporation of $[U-^{14}\text{C}]$ galactose into the 1,4- β -galactan sidechains of a pectin-xyloglucan complex. The molecular weight of the complex was concluded to be at least 10^4 kDa, as it was excluded from the gel on passing through a CL-2B column eluted with water (Abdel-Massih *et al.*, 2003).

Talbott & Ray (1992) reported that pectic polyuronides extracted from the primary walls of third internodes of etiolated peas have a peak molecular mass of about 1100 kDa, without appreciable material below 500kDa, relative to dextran standards. Xyloglucan, the principal hemicellulosic polymer, has a molecular mass of about 30 kDa with a secondary peak of approximately 300 kDa. Aqueous solutions of pectic polyuronides tend to associate covalently into multichain aggregates (Goldberg *et al.*, 1989). According to Talbott & Ray (1992), their results showed that when pea pectin solutions are concentrated by evaporation, some aggregation occurs.

Because of possible aggregation before the initial GF (gel filtration), the molecular weight values obtained for pectic polymers may overestimate the size of their primary structures.

3.1.2 Background to methods of pectin extraction.

The wall can be extracted either with hot solution of a chelating agent or with dilute acid, yielding pectin (Brett & Waldron, 1996). Three major pectic polysaccharides have been characterized and are thought to occur in all primary plant cell walls. These are homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Albersheim *et al.*, 1996; O'Neill *et al.*, 1990; Mohnen, 1999). HG is a polymer consisting of (1-4)- α -linked D-galactosyluronic acid residues and these can be partially methyl-esterified and/or acetylated. The degree of methyl-esterification is highly variable in relation to cells, tissues and within individual cell walls and can greatly influence the pectic network and cell wall properties (Willats *et al.*, 2001). HG is thought to be synthesized and deposited in the cell wall in a highly methyl-esterified form (Mohnen, 1999). *In muro* de-esterification by pectin methyl esterases results in de-esterified HG which is implicated in cell-to-cell adhesion and other aspects of cell development (Willats *et al.*, 2001).

3.1.2.1. EDTA

EDTA a chelating agent that extracts polymers held only by Ca^{++} bridges. It extracts much of the pectin from the walls (Fry, 1988). Extraction of dicot cell wall preparations with calcium-chelating agents brings some pectin into solution, while only limited cell separation occurs under these conditions (McCartney & Knox, 2002). The presence of further, presumably covalent, intermolecular linkages must be proposed (Jarvis *et al.*, 2003). Heating methyl-esterified pectins in EDTA at pH 6 to pH 8 may cause backbone breakage by β -elimination (Fry, 1988). This can be reduced by prior deesterification with cold alkali.

3.1.2.2. NaOH

Mild alkaline extraction, under conditions suitable for cleaving ester linkages, solubilizes a substantial pectic fraction (Goldberg *et al.*, 1996) and also separates some of the cells that are not separated by chelators (Jarvis *et al.*, 2003).

NaOH containing NaBH₄ is used to extract high proportion of total hemicelluloses with de-acylation and some pectins with de-esterification. Low temperature NaOH (0°C) deesterifies galacturonic acid. Higher temperature NaOH breaks the polygalacturonic backbone by β-elimination if methyl ester groups are present (Fry, 1988).

3.1.2.3. Use of enzymes

Enzymic cleavage of pectic galacturonan chains is an efficient method of pectin solubilization (Keegestra *et al.*, 1973).

3.1.3 Use of Gel Filtration (GF) or Size Exclusion Chromatography (SEC)

The term gel filtration (GF) refers to the ability of a porous gel to "filter" or separate a mixture of macromolecules according to their individual hydrated sizes. The hydrated size of a molecule is typically a function of its molecular weight, branching, the ambient pH, ionic strength, and some other factors (ambient detergents, urea or equivalents, chelators, or chaotropic agents).

The size of the gel pores is a continuous function of the agarose concentration.

The Gel Exclusion Limit: Regardless of the agarose concentration chosen, there is always a molecular size for proteins, polysaccharides and DNA, which will not enter the pores of the gel. This is the smallest molecule, which is excluded from the gel and is therefore called the exclusion limit. All molecules larger than the exclusion limit, expressed in Daltons (for proteins

and polysaccharides), will also be excluded from the gel and will pass through the column in the "void volume". Sepharose CL-2B has an agarose concentration of 2 %, CL-4B of 4% and CL-6B of 6 %. (Agarose beads technologies).

CL-2B Sepharose has an exclusion limit of 40,000 kDa (Lévesque, 2001). This means that anything larger than this will come out of the column in the void volume (this includes membranes and anything that is integrated/secreted in them). Smaller molecules will be retained in the gel beads and move out of the column more slowly, thus will appear in the later fractions. Therefore, for sepharose CL-2B,

A) Excluded fractions contain those molecules which are too large to go through the pores and move between and around the beads (> 40,000 kDa) (including membranes + associated molecules). This will be the void volume.

B) Retained fractions contain those molecules which are small enough to pass through the gel pores (< 40,000 kDa) (Andrew, 2002).

3.2 Materials and methods.

GFC was performed with a Sepharose CL-2B column (Bio-rad 1.4 x42cm), or CL-4B, or in later chapters, with CL-6B columns. Supernatants obtained from various extractions were loaded onto the column with a Pasteur pipette and were eluted with different eluents at room temperature (approximately 22° C). The column was equilibrated with an equal volume of eluent before applying the sample. Blue dextran (average molecular weight 2000 kDa) and CoCl₂ (formula weight 238 Da) were used as high and low molecular weight markers respectively. Dextran standards were detected using the phenol-sulphuric acid method (Dubois *et al.*, 1956).

3.3 Rationale behind these experiments.

The best reagent to be used for the extraction of the pectin-complex from the membrane preparation was to be determined. Because of the known tendency of pectins to aggregate in solution, the effect of a number of eluents on the behaviour of the complex on gel filtration chromatography was studied as well.

3.4 Experiments to determine the best extracting reagent.

Membrane pellets were prepared and incubated with UDP- [¹⁴C] galactose according to the procedure described by Abdel-Massih et al. (2003) and were extracted with different reagents. Five different extractants were used.

3.4.a. 0.5 mls of 0.1M HCl was added to a pellet and was incubated for 2 hours at 25

3.4.b. 0.5ml of 6M NaOH containing 1% NaBH₄ was added, and incubated at 0° C for 1 hr, followed by 37° C for 1 hr. At the end of the incubation, 0.25 ml of glacial acetic acid was added to neutralize the supernatant. Similar treatment but with longer incubation time led to the extraction of all the xyloglucan (Fry, 1988).

3.4.c. 1mg of phospholipase C with 100 μl of 0.1M Tris-HCl pH 7.4 was added and was incubated at 37° C for 2 hours. Phospholipase C may be used to release phosphatidylinositol (PI)-linked proteins from membranes.

3.4.d. 0.5 mls of 0.1 M Tris-HCl buffer (pH 7.4) as control.

3.4.e. 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 100° C.

After centrifugation, the supernatants were then each passed through a CL-4B column eluted with 10mM EDTA/10mM PO₄/1M NaCl. Fig.3.1 shows that the highest incorporation of radioactivity into the supernatant containing the pectin complex was with the EDTA extraction, where 62 % of the radioactivity was found as high molecular weight material relative to the

total counts. EDTA also extracted high molecular weight radioactive material that was not extracted by NaOH (section 3.5.6).

Other extraction experiment including:

3.4.a' 0.5 mls of 0.1 M Tris-HCl buffer (pH 7.4) as control .

3.4.b' 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 25°C.

3.4.c' 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 100° C,

3.4.d' 10 units of bovine trypsin in HCl pH 7.4.

3.4.e' 1mg of phospholipase C with 100 µl of 0.1M Tris-HCl pH 7.4 was added and was incubated at 37° C for 2 hours.

3.4.f' 10 units of triton X-100 were used and the highest incorporation of radioactivity into the supernatant containing the pectin complex was with the trypsin and with EDTA extraction at 100° C, where around 50 % of the radioactivity was found as high molecular weight material relative to the total counts (Fig 3.2).

3.4.1 Experiments after extraction with EDTA

All the subsequent experiments used the EDTA extracted material, as described in the above section.

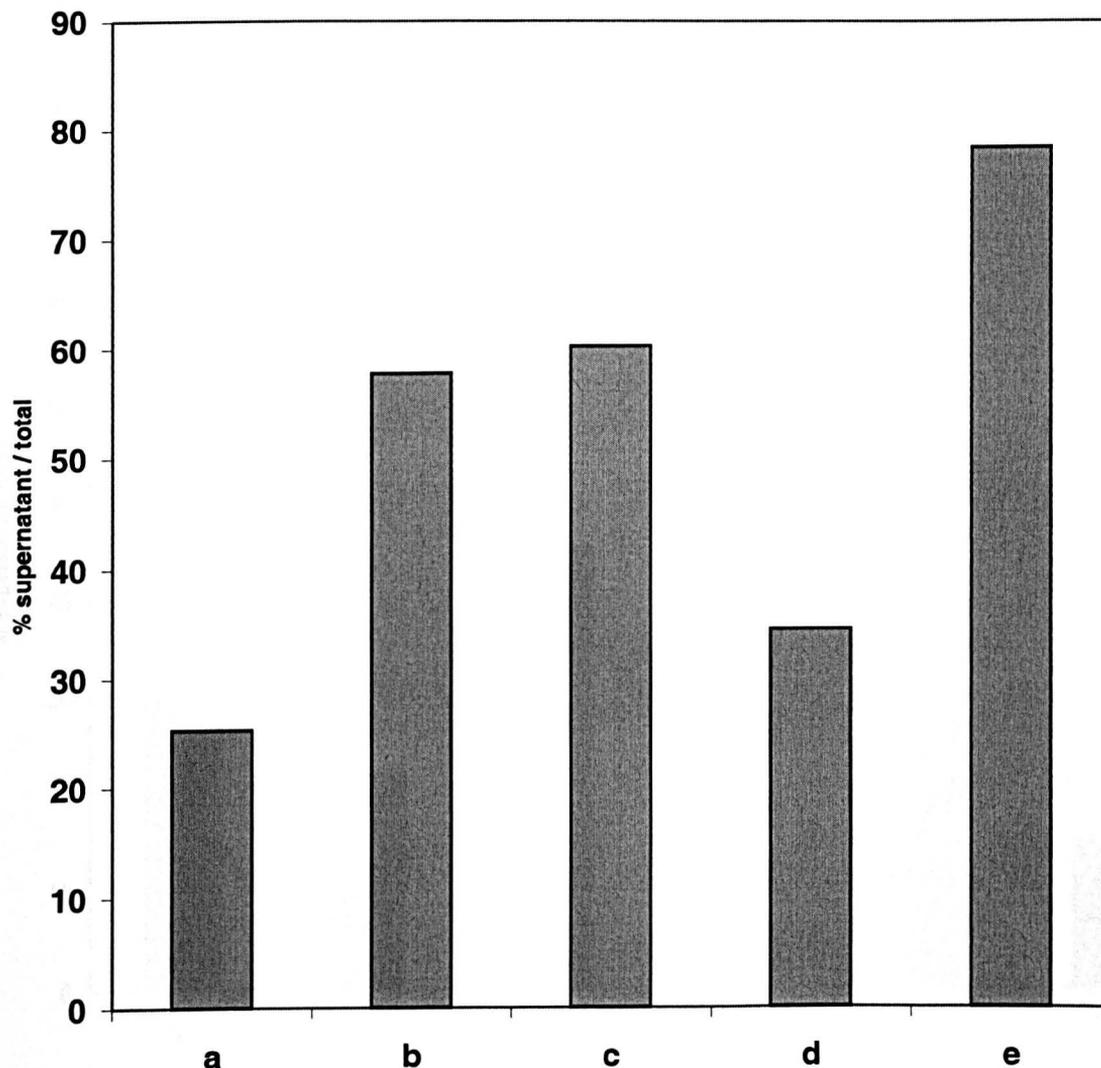


Fig 3.1 Experiments to determine the best extractant (Section 3.4). The supernatant was then passed through CL-4B and eluted with EPN.

a. 0.5 mls of 0.1M HCl was added to a pellet and was incubated for 2 hours at room temperature.

b. 0.5ml of 6M NaOH containing 1% NaBH₄ was added, and incubated at 0° C for 1 hr, followed by 37° C for 1 hr.

c. 1mg of phospholipase C with 100 μl of 0.1M Tris-HCl pH 7.4 was added and was incubated at 37° C for 2 hours.

d. 0.5 mls of 0.1 M Tris-HCl buffer (pH 7.4) as control.

e. 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 100° C.

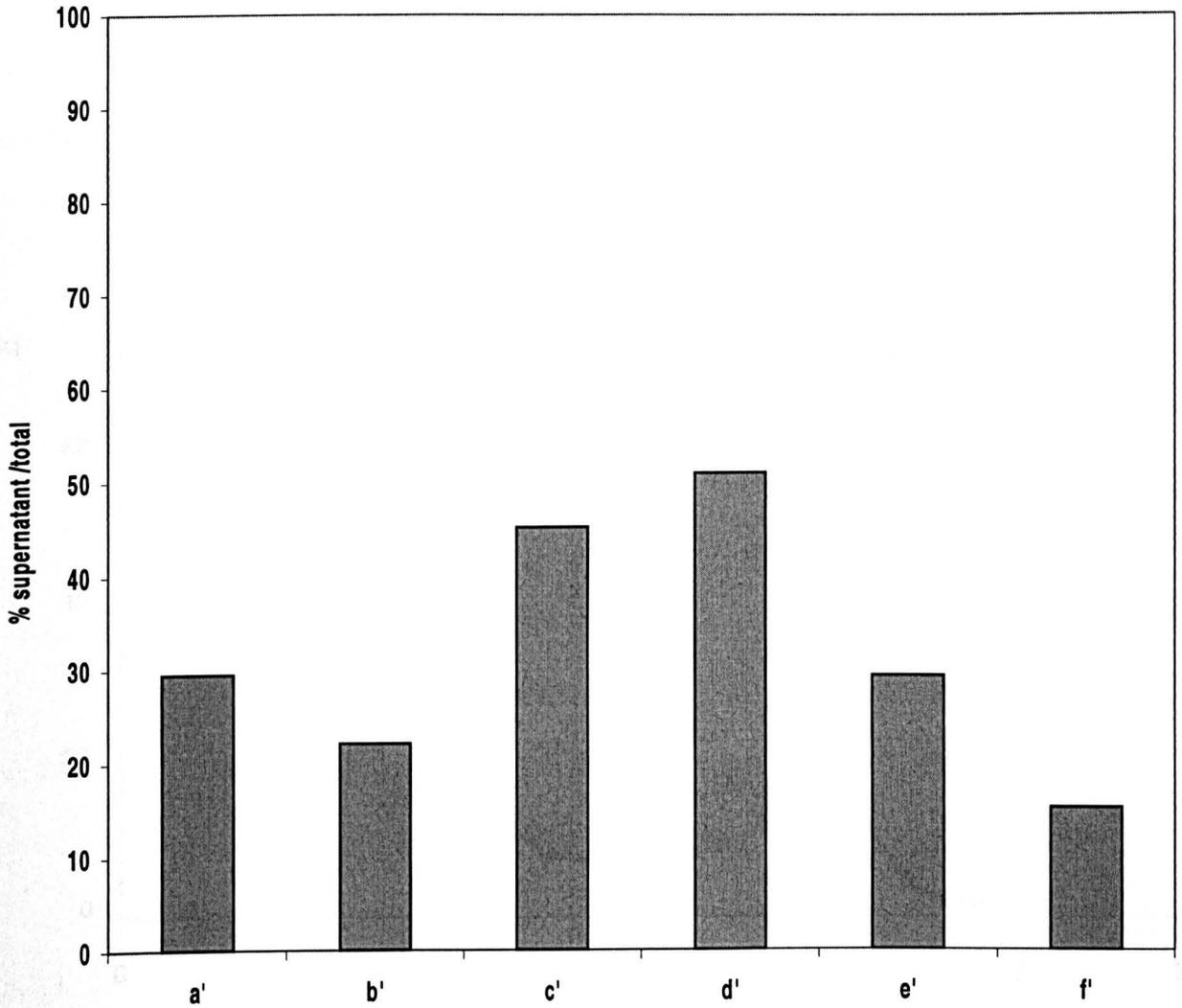


Fig 3.2 Experiments to further determine the best extractant (Section 3.4). The supernatant was then passed through CL-4B and eluted with EPN.

3.4.a' buffer alone.

3.4.b' 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 25°C.

3.4.c' 0.5 mls of 50mM EDTA/50mM PO₄ (pH6.8) for 10 minutes at 100° C.

3.4.d' 10 units of bovine trypsin in HCl pH 7.4.

3.4.e' 1mg of phospholipase C with 100 μl of 0.1M Tris-HCl pH 7.4 was added and was incubated at 37° C for 2 hours.

3.4.f' 10 units of triton X-100.

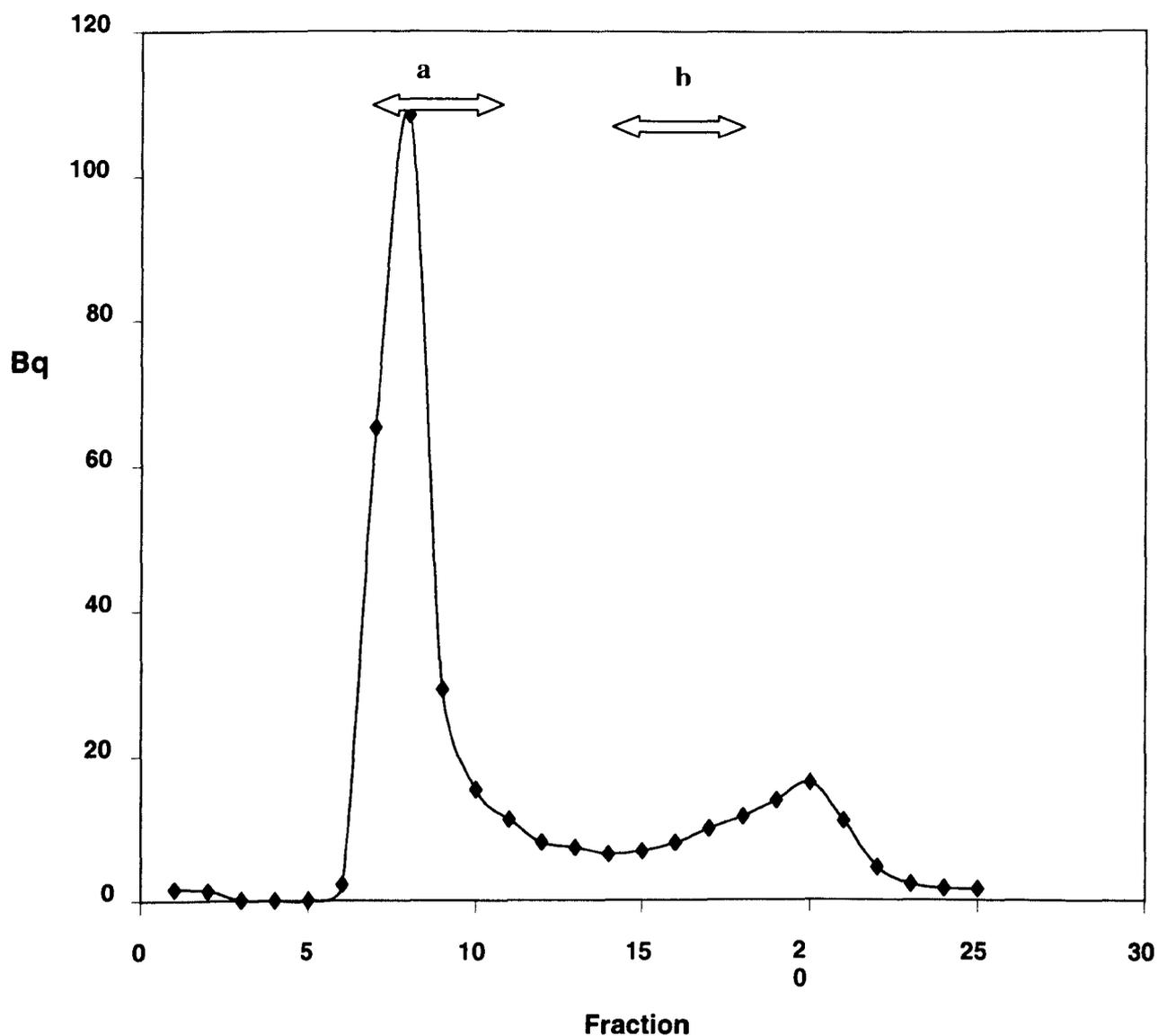


Fig. 3.3 Gel filtration on Sepharose CL-2B column of radioactive material obtained by EDTA/phosphate extraction of the particulate enzyme material (section 3.5). The sample was eluted with water.

a. Blue Dextran high molecular weight marker.

b. Cobalt chloride low molecular weight marker.

3.5 Experiments to determine the best eluent to determine the molecular weight of the complex.

Fig.3.3 shows the profile of a sample of the EDTA/Phosphate extracted material (800 microliters) passed through CL-2B and eluted with water. The high molecular weight material was collected from fractions 5-10 together with or just before the elution position of the blue dextran marker. This material will be referred to as [^{14}C] Gal-HMwt-complex.

3.5.1 Effect of eluting the [^{14}C] Gal-HMwt-complex with water.

[^{14}C] Gal-HMwt-complex was passed through the same column of CL-2B and eluted again with water. The profile (fig.3.4) shows that the radioactive material was not running consistently on CL-2B, since 2 peaks instead of one were observed, probably due to a non-covalent association breaking down. There is a possibility that the initial EDTA extraction was not enough to break the Ca^{++} crossbridges and other noncovalent bonds between pectin molecules.

3.5.2 Effect of eluting the [^{14}C] Gal-HMwt-complex with 8 M urea.

The effect of adding an equal volume of double strength 8 M Urea to [^{14}C] Gal-HMwt-complex before applying to the CL-2B column and eluting with 8 M urea was studied. Urea (8M), a strong chaotropic agent, would be expected to weaken interpolymeric hydrogen bonds between xyloglucan and either an acidic wall polysaccharide or the agarose of the CL-2B column (Thompson & Fry, 2000). Fig 3.5 shows 2 peaks with urea, suggesting that urea was causing the dissociation of pectin aggregates.

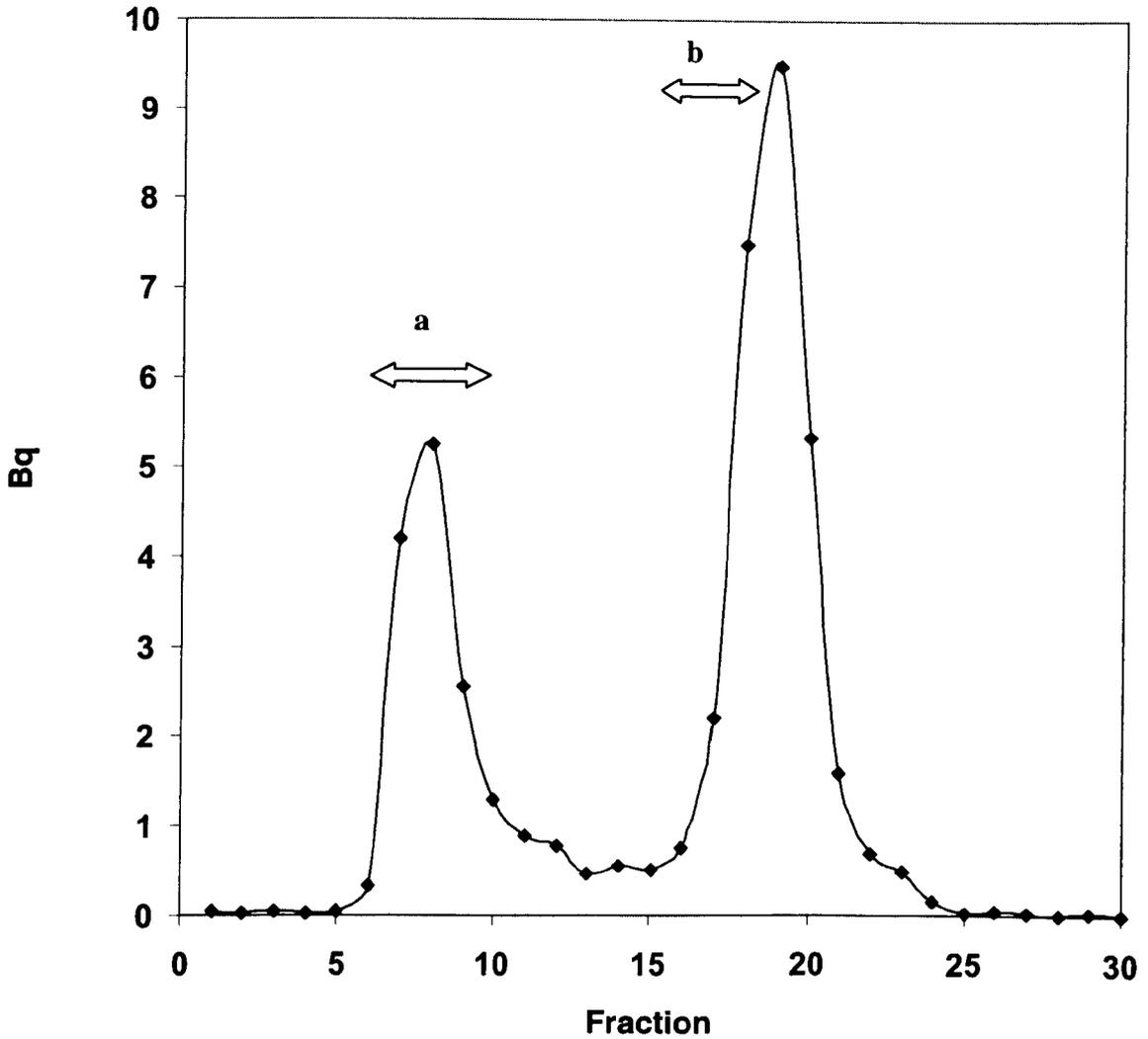


Fig.3.4 Second gel filtration on Sepharose CL-2B column of [^{14}C] Gal-HMwt-complex (section 3.5). Both columns were eluted with water.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.

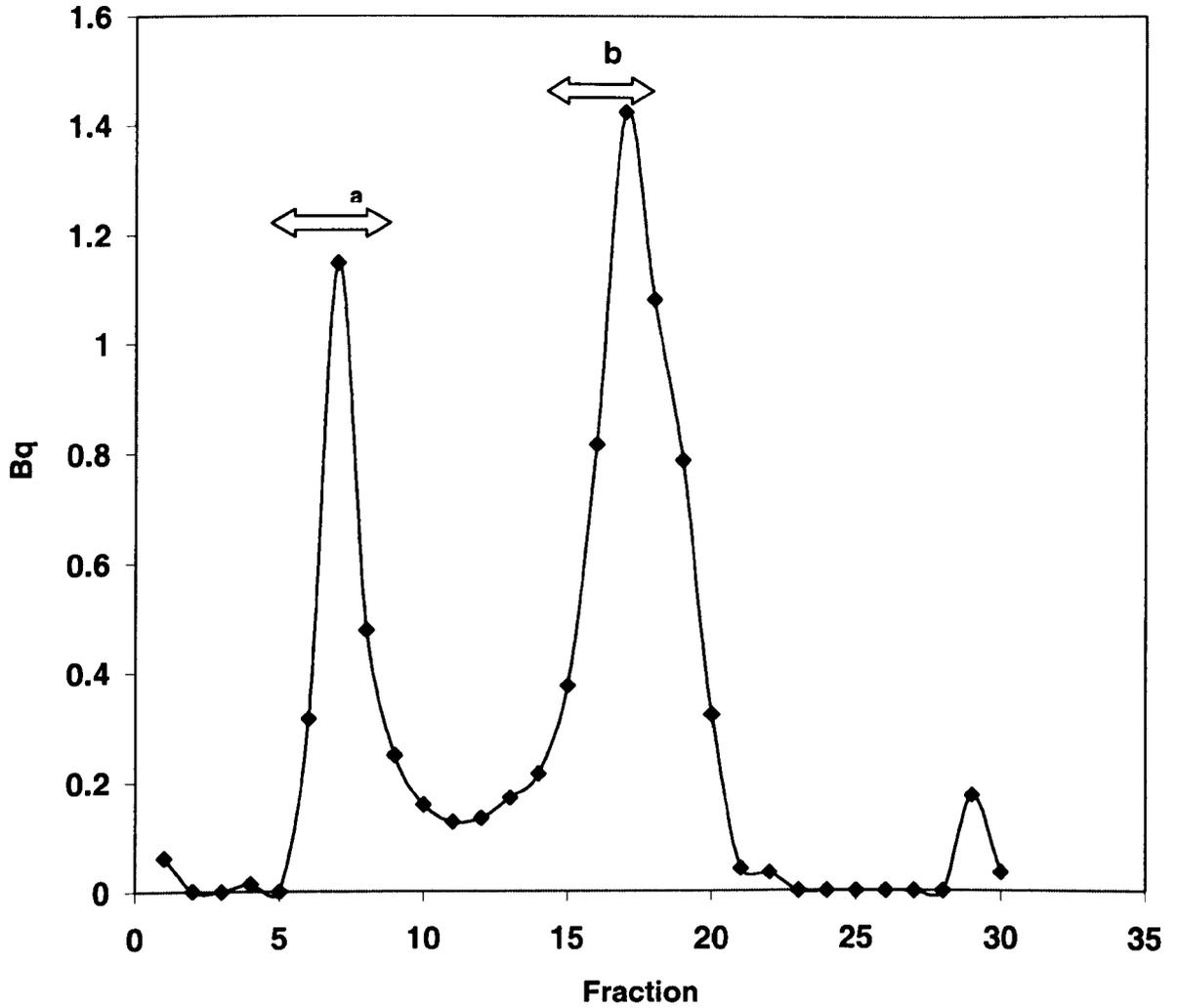


Fig.3.5 Effect of 8M urea on gel filtration on Sepharose CL-2B column of [^{14}C] Gal-HMwt-complex (section 3.5).

To the [^{14}C] Gal-HMwt-complex, an equal volume of 16 M urea was added. The treated sample was eluted with 8 M urea.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.

3.5.3 Effect of using glycine (pH2.5) and glycine (pH9.5) to elute the [¹⁴C] Gal-HMwt-complex.

Glycine is the simplest amino acid, with a hydrogen atom as a side chain. Using glycine with pH 2.5 will lead to changing of the charge in the complex, therefore any interaction due to charge will change. The pKa of pectin is reported to be around 3.5, meaning that with an eluent of pH 2.5 the pectin will be protonated and change its nature. Using glycine pH (9.5) may abolish any residual acidic charges and the pectin will be fully deprotonated. Fig 3.6 shows that recovery is very low at pH 2.5. Radioactivity was probably adsorbed onto the column, since subsequent washing of the column with EPN led to elution of the remaining radioactivity (Fig 3.6). When eluted with glycine (pH 9.5) the profile is comparable to that with water, where the complex was found to elute with a high apparent molecular weight.

To investigate further if the effect of adding glycine (pH 2.5) was due to the pH of the glycine, the [¹⁴C] Gal-HMwt-complex was mixed with an equal volume of 0.2M HCl to give a final concentration of 0.1M HCl (pH 1), incubated for 30 min or 2 hours at 22 °C, then passed through a CL-4B column eluted with 10mM EDTA/10mM PO₄/1M NaCl (Fig 3.7). Untreated [¹⁴C] Gal-HMwt-complex eluted at the void volume of CL-4B under these conditions (see section 3.5.6). Exp 3.8 shows that there were only 26 % of the complex eluted as high molecular weight material and the total amount of radioactivity was observed to decrease. When the HCl- treated [¹⁴C] Gal-HMwt-complex was neutralized with NaOH after the two hours incubation, the profile was similar to that of untreated material and of material treated only with acetate buffer (pH 7.4) (Fig. 3.8). It can be seen that 84% of the material was eluted as high molecular weight material, similar to that observed with no HCl treatment.

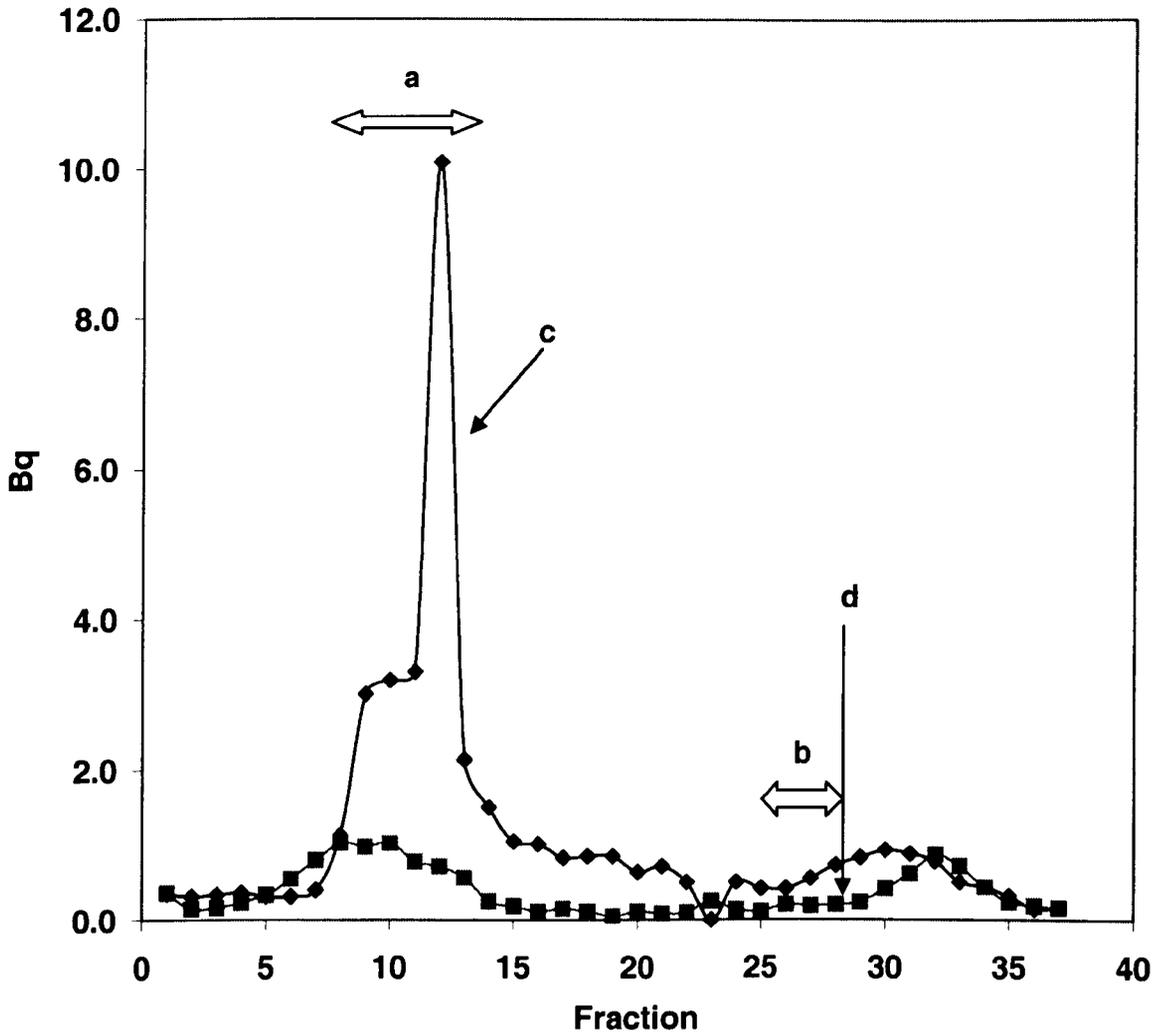


Fig.3.6 Effect of glycine buffers at pH 9.5 and pH 2.5 on gel filtration on Sepharose CL-4B column of [^{14}C] Gal-HMwt-complex (section 3.5).

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** To the [^{14}C] Gal-HMwt-complex, an equal volume of 10mM glycine buffer of the pH 9.5 was added, and it was applied to the column and then eluted with 10mM glycine buffer at pH 9.5.
- d.** To the [^{14}C] Gal-HMwt-complex, an equal volume of 10mM glycine buffer of the pH 2.5 was added, and it was applied to the column and then eluted with 10mM glycine buffer at pH 2.5.

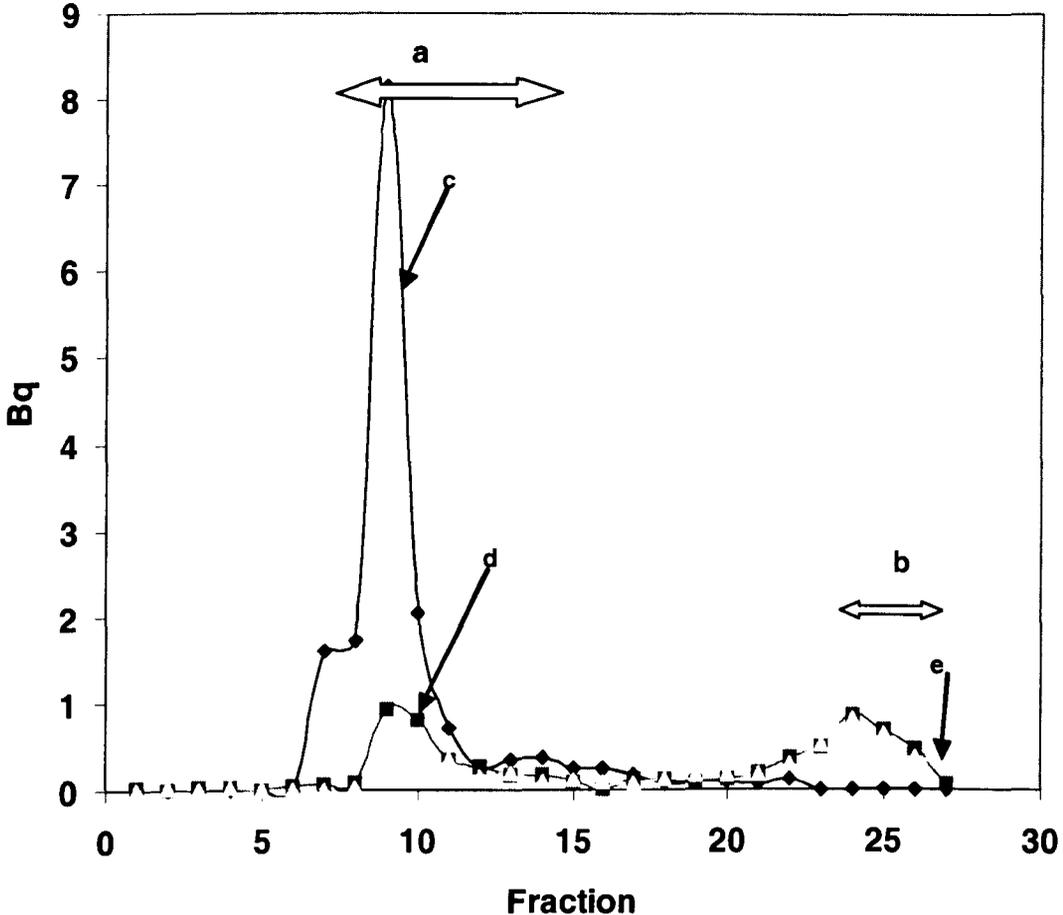


Fig.3.7 Effect of acid on gel filtration on Sepharose CL-4B column of of [^{14}C] Gal-HMwt-complex (section 3.5).

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** A sample of [^{14}C] Gal-HMwt-complex (section 3.5) was passed through CL-4B using EPN as eluent.
- d.** An equal volume of double strength (0.2 M) HCl was added to [^{14}C] Gal-HMwt-complex (section 3.5).It was then incubated for 30 minutes at room temperature, and then passed through CL-4B using EPN as eluent.
- e.** An equal volume of double strength (0.2 M) HCl was added to [^{14}C] Gal-HMwt-complex (section 3.5).It was then incubated for 2 hours at room temperature, and then passed through CL-4B using EPN as eluent.

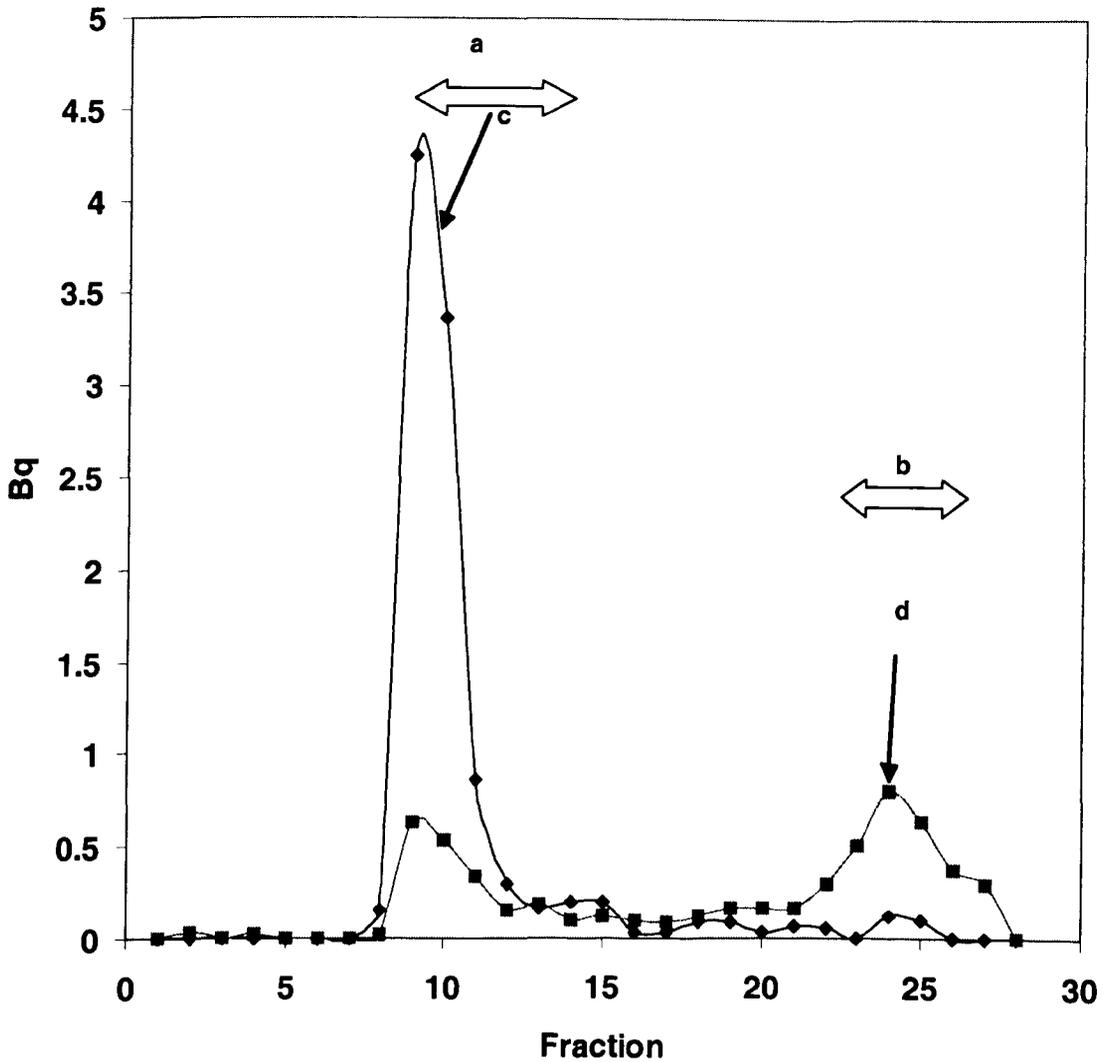


Fig.3.8 Effect of acid on gel filtration on Sepharose CL-2B column of [^{14}C] Gal-HMwt-complex (section 3.5).

a. Blue Dextran high molecular weight marker.

b. Cobalt chloride low molecular weight marker.

c. An equal volume of double strength (0.2 M) HCl was added to [^{14}C] Gal-HMwt-complex. It was then incubated for 2 hours at room temperature, and then neutralized with 4 N NaOH before being passed through CL-4B using EPN as eluent.

d. An equal volume of double strength (0.2 M) HCl was added to [^{14}C] Gal-HMwt-complex. It was then incubated for 2 hours at room temperature before being passed through CL-4B using EPN as eluent.

3.5.4 Effect of using Pyridine/acetic acid/water (1:1:23) to elute the [¹⁴C] Gal-HMwt-complex.

Thompson & Fry (1997) found that this eluent disrupted complexes of xyloglucan, which would otherwise elute with an erroneously high apparent Mwt. The [¹⁴C] Gal-HMwt-complex was mixed with an equal volume of double strength eluent then passed through CL-2B using this eluent. (Fig 3.10) shows that 71% of the radioactivity was in the high molecular weight material (fractions 11-17).

3.5.5 Effect of using 10mM EDTA/10mM PO₄/1M NaCl to elute the [¹⁴C] Gal-HMwt-complex.

EDTA /PO₄ was selected because EDTA is a chelating agent that may chelate any calcium in the complex and was also used to extract the complex. To this preparation of EDTA /PO₄, 1 M NaCl was added to further decrease the strength of non-covalent interactions. Fig 3.9 shows that the majority (66%) of the complex was found to be partially included in the CL-2B gel when eluted with 10mM EDTA/10mM phosphate/1M NaCl. In this eluent, the mean K_{av} was about 0.8, corresponding to a mean molecular size of 200 kDa, as compared to the behaviour of dextran markers (Fig 3.11). Variations may occur between different batches and preparations, which was taken into account by running markers for every column. 10mM EDTA/phosphate/1M NaCl (EPN) was therefore selected for subsequent experiments since it gave consistently lower molecular weights than the other eluents tested.

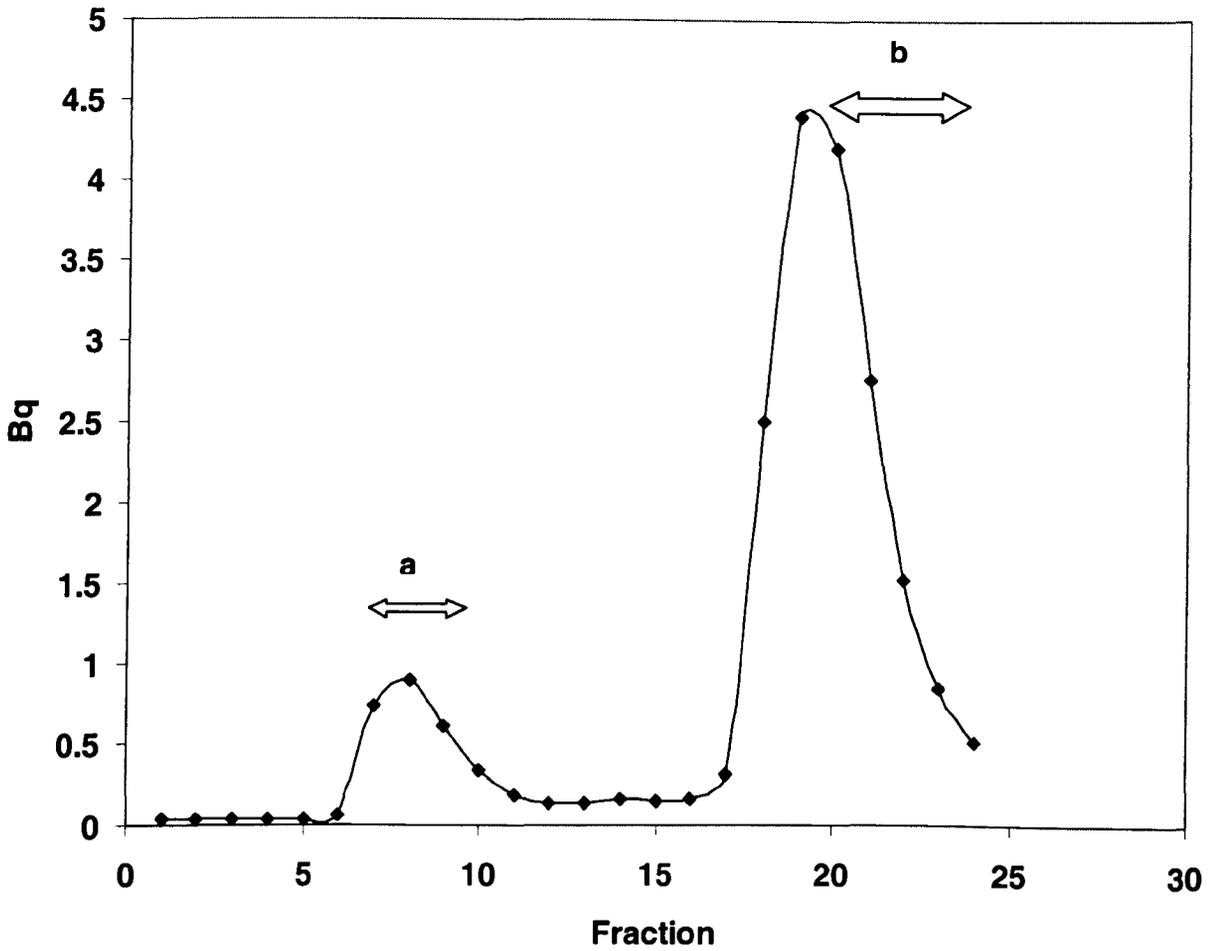


Fig.3.9 Gel filtration on Sepharose CL-2B column, using 10mM EDTA/10mM PO₄/1M NaCl (EPN) as eluent, of [¹⁴C] Gal-HMwt-complex (section 3.5).

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.

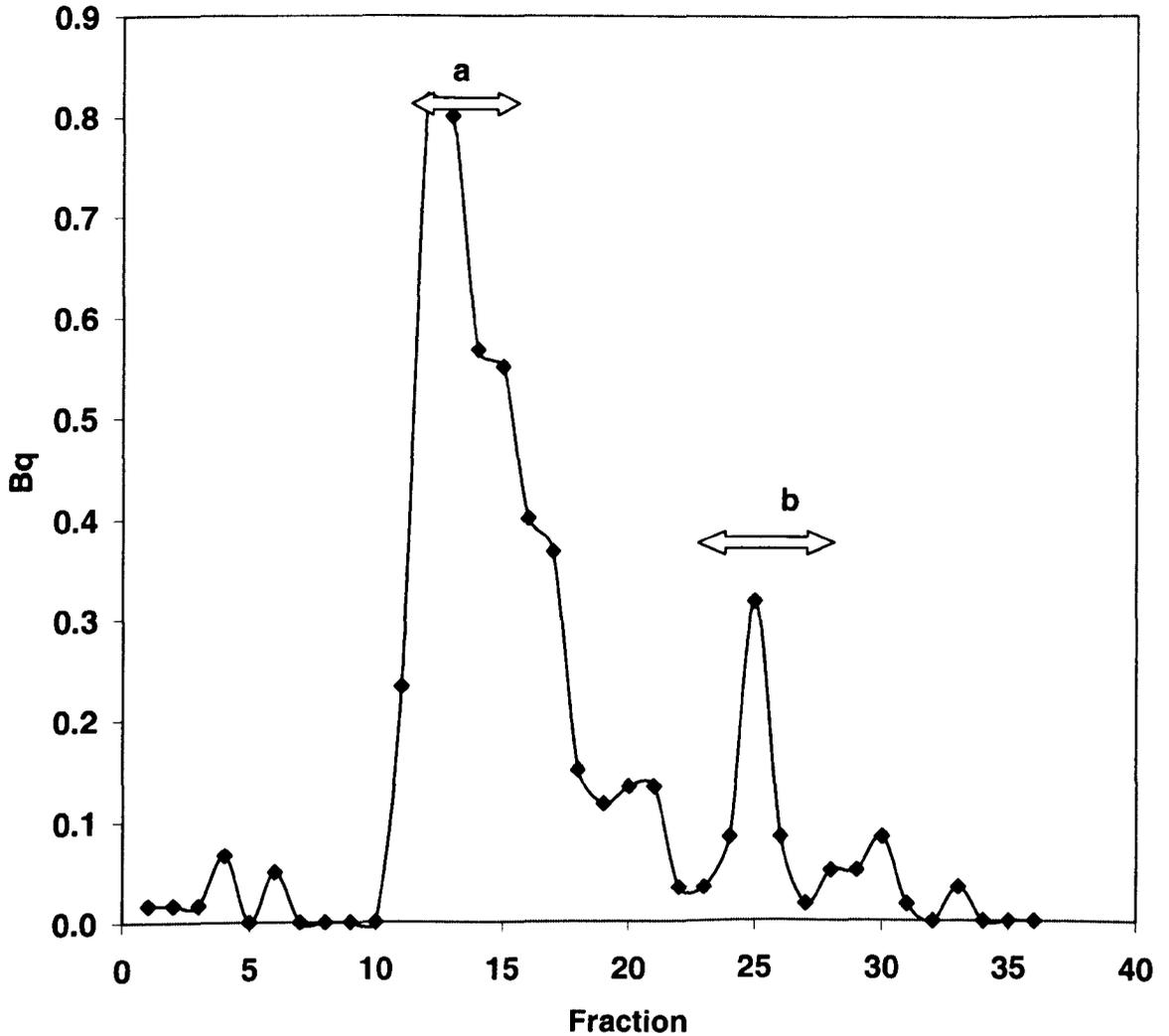


Fig.3.10 Gel filtration on Sepharose CL-2B column, using pyridine/acetic acid/water (1:1:23) as eluent, of ^{14}C Gal-HMwt-complex (section 3.5).

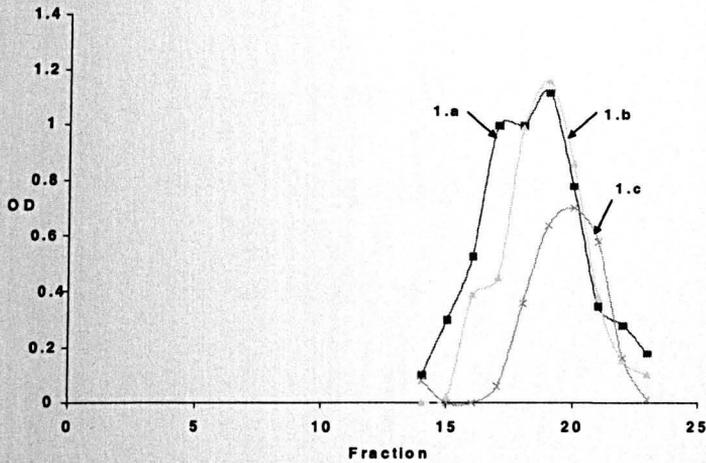
^{14}C Gal-HMwt-complex was mixed with equal volume of pyridine: acetic acid: water (2:2:23). They were then passed through a Sepharose CL-2B column, eluted with pyridine : acetic acid : water in a ratio of 1:1:23.

a. Blue Dextran high molecular weight marker.

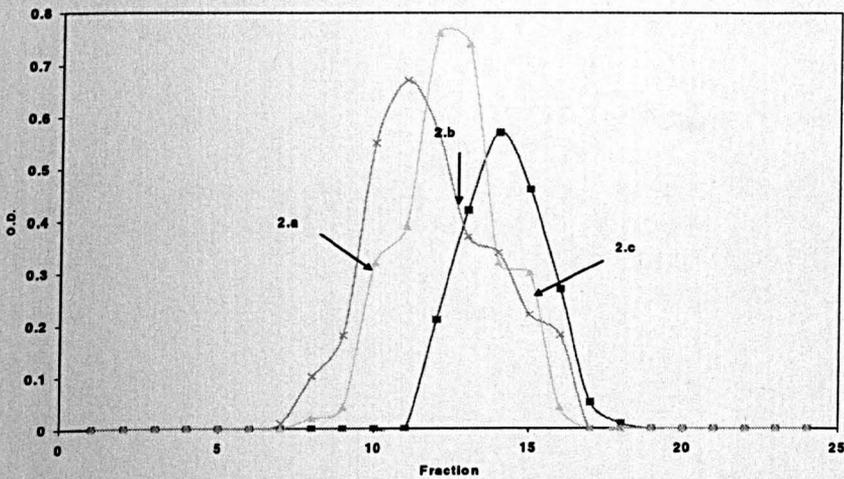
b. Cobalt chloride low molecular weight marker.

3.5.6 Effect of extracting with NaOH followed by further extraction with EDTA on the [¹⁴C]Gal-HMwt-complex.

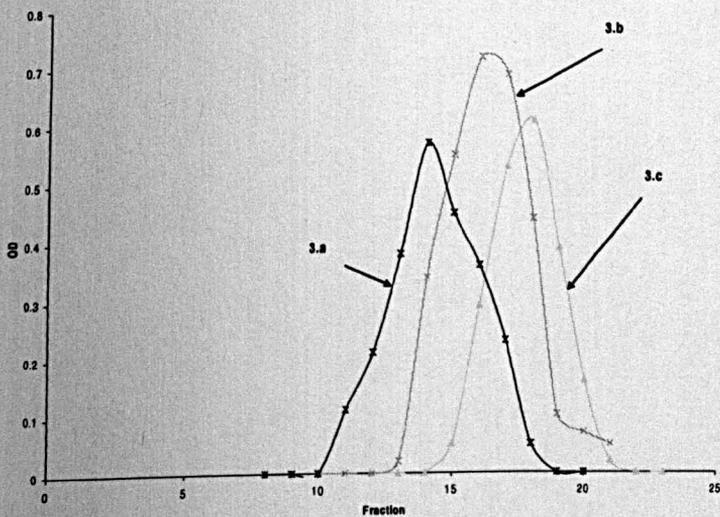
Fig 3.1 shows that after extraction with NaOH and gel filtration on Sepharose CL-2B, only 14% of the radioactivity was in the high molecular weight material relative to the total. The pellet from the NaOH extraction was then extracted with EDTA/phosphate, and the high molecular weight peak from this EDTA extraction was taken and 1ml was re-run down Sepharose CL-2B in 10mM EDTA/10mM PO₄/1M NaCl as eluent. This treatment could have resulted in an increase in the molecular weight compared to direct extraction with EDTA, if direct extraction with EDTA/phosphate had resulted in some breakage of the pectin backbone by beta-elimination. However, the profile in Fig 3.12 shows that there was little, if any, increase in molecular weight compared to direct extraction with EDTA/phosphate.



1.a. 181K M.Wt Blue Dextran passed through CL-2B with EPN.
1.b. 76K M.Wt Blue Dextran passed through CL-2B with EPN.
1.c. 40K M.Wt Blue Dextran passed through CL-2B with EPN.



2.a. 181K M.Wt Blue Dextran passed through CL-4B with EPN.
2.b. 76K M.Wt Blue Dextran passed through CL-4B with EPN.
2.c. 40K M.Wt Blue Dextran passed through CL-4B with EPN.



3.a. 181K M.Wt Blue Dextran passed through CL-6B with EPN.
3.b. 76K M.Wt Blue Dextran passed through CL-6B with EPN.
3.c. 40K M.Wt Blue Dextran passed through CL-6B with EPN.

Fig.3.11 Gel filtration on different Sepharose columns.

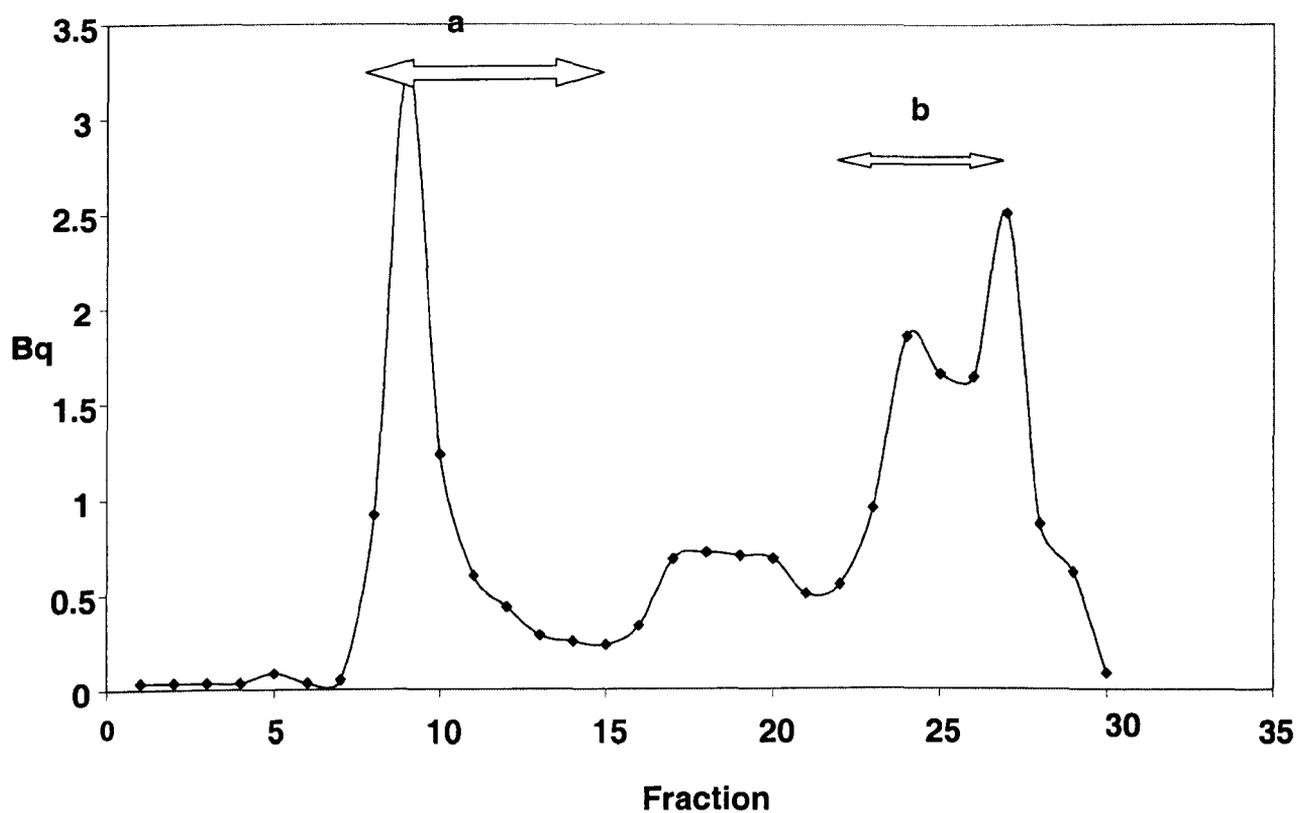


Fig.3.12 Gel filtration on Sepharose CL-2B, eluting with EPN, of [^{14}C] Gal-HMwt-complex (section 3.5) of material extracted by EDTA/phosphate from the pellet remaining after NaOH extraction.

a. Blue Dextran high molecular weight marker.

b. Cobalt chloride low molecular weight marker.

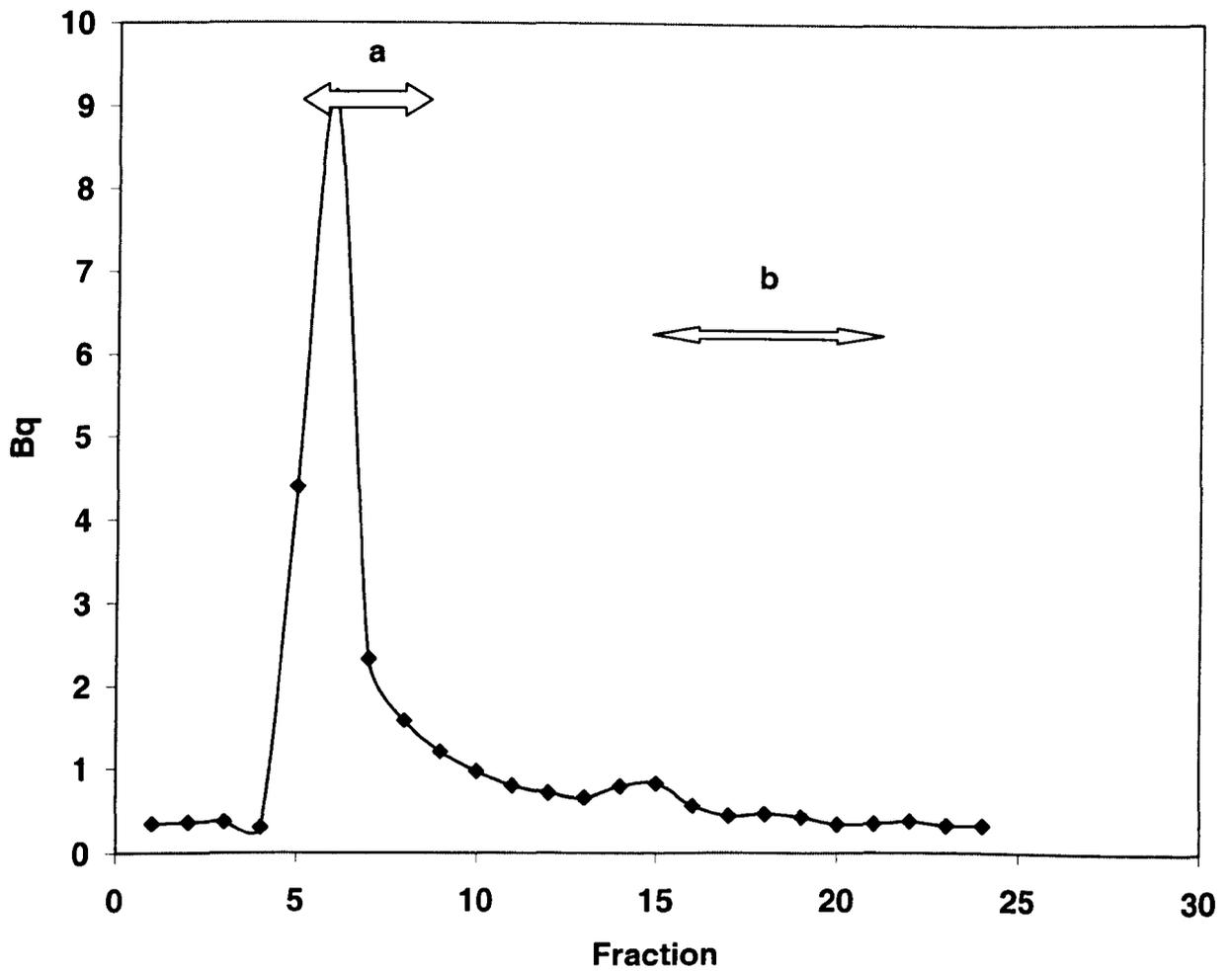


Fig.3.13 [^{14}C] Gal-HMwt-complex (section 3.5), passed through CL-4B using EPN as eluent.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.

3.5.7 Effect of using Sepharose CL-4B to determine the molecular weight of the complex using 10mM EDTA/10mM PO₄/1M NaCl as eluent.

[¹⁴C] Gal-HMwt-complex was eluted through Sepharose CL-4B using EPN (Fig 3.13). The majority of radioactivity eluted at the void volume (fractions 6-7) i.e. fractions where the blue dextran elutes, suggesting a molecular weight close to 10³ kDa, which is the exclusion limit of polysaccharides on SepharoseCL-4B.

3.6. Conclusion

The best extractant used to solubilize the pectin-complex from the pellets was 50mM EDTA/50mM PO₄ pH 6.8. This confirms the data reported by Abdel-Massih *et al.*, (2003). It can be concluded from these experiments that the best eluent used to elute the [¹⁴C]-Gal pectin-complex was EPN. It was also concluded that the average molecular weight of this complex was in the range of 200-1000 kDa as judged by GF performed using Sepharose CL-2B as well as CL-4B.

It can also be concluded that elution in acid conditions led to the binding of the radioactivity to the gel (Fig.3.8).

Chapter 4

Composition of the high molecular weight pectin complex.

4.1 Introduction

The research described in this chapter aimed at studying the composition of the high molecular weight pectin complex through several enzymatic treatments, followed by GF to determine whether enzyme action changed the molecular weight of the complex. The composition of the non-radioactive microsomal pectin was also studied, using monoclonal antibodies specific to pectin epitopes.

4.1.1 Previous work

Abdel-Massih *et al.* (2003) reported that the molecular weight of the complex was decreased when treated with endo- β -1, 4-glucanase, polygalacturonase, RGase A and endo- β -1, 4-galactanase and then analyzed by GF eluting with water. Abdel-Massih *et al.* (2003) concluded that the 1,4- β -galactan formed in their *in vitro* system was attached to a complex containing homogalacturonan, RGI and xyloglucan. However, their experiments left open the possibility that the nascent pectin was attached to a 1,4- β -glucan rather than xyloglucan. Treatment with a xyloglucan-specific endoglucanase that breaks the glucan backbone only of xyloglucan would be able to distinguish between xyloglucan and 1,4- β -glucan (Pauly *et al.*, 1999).

Pectin consists of three structurally well-characterized polysaccharides: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (O'Neill *et al.*, 1990). These polysaccharides are believed to be covalently linked to one another because they are all solubilized by endo-polygalacturonase treatment of primary cell wall (Ishii and Matsunaga, 2001).

Xyloglucan consists of cellulose-like β -1, 4-linked glucose backbone which is frequently substituted with various side chains. A xyloglucanase cleaves the beta-1, 4-glycosidic linkages

in the backbone of xyloglucan. Endo- β (1-4) glucanases also break the xyloglucan backbone. Handford *et al.* (2003) reported that mannans are of particular significance in both lignified and non-lignified thickened cell walls.

JIM5 is an antibody that binds to a range of methyl-esterification of homogalacturonan (Knox *et al.*, 1990; Willats *et al.*, 2000) Another monoclonal antibody, LM7, binds specifically to HGA with a non-block-wise distribution of methyl esters (Willats *et al.*, 2001).

4.2 Methods

4.2.1 Enzyme treatment.

The [^{14}C] Gal-HMwt-complex, described in section 3.5, was treated with different enzymes and then passed through different sized columns and eluted with EPN. 10 units of each enzyme were used, at the pH and temperature specified by the different suppliers, as described in section 2.4. For each enzyme, one unit of activity was the amount of enzyme required to produce one micromole of product per minute under the specified assay conditions. Enzyme treated samples were then applied to columns (1.4 x 42 cm) of Sepharose CL-2B or CL-6B or columns (1.4 x 26cm) of Sepharose CL-4B, and eluted with EPN buffer (see section 3.5.5). 2 ml fractions were collected using a fraction collector, ecoscint (bio-degradable scintillant) was then added to the samples, and radioactivity was measured using a scintillation counter.

4.2.2. Immuno-dot assay.

Various concentrations of microsomal preparation were tested as well as different standards (see section 2.6). Pectins with different degrees of esterification (34%, 56% and 89%) as well as different concentrations of each of the above were dissolved in water to a concentration of 1

mg/ml and applied as 1- μ l to nitrocellulose membrane. Nitrocellulose membranes were air-dried at room temperature for at least 30 minutes. After blocking for 1 hour in phosphate-buffered saline (PBS) containing 5% fat-free milk (5%M/PBS), membranes were incubated for 90 mins in primary antibodies diluted in 5%M/PBS. JIM5 and LM7 were used as 1/10 dilution of hybridoma supernatants. After washing, membranes were incubated for 1.5 hour in secondary antibody anti-rat horseradish peroxidase conjugate (Sigma, D-4293) diluted 1/1000 in 5%M/PBS. Finally, membranes were washed in water for 1 hour where all unbound anti-rat IGG- horseradish peroxidase was washed off (Willats *et al.*, 2001). The membrane was then incubated with DAB and urea hydrogen peroxide (supplied in tablet form as D-4293). This allowed the peroxidase to bring about the reaction between DAB and hydrogen peroxide, which produces the colour.

4.3 Rationale

After determining the apparent molecular weight of the pectin complex (see chapter 3), different enzymes were used to determine the composition of this complex. If a decrease in the apparent molecular weight of the complex occurred, this indicated that the complex contained bonds susceptible to breakage by the enzyme used. Monoclonal antibodies were used to clarify the composition of nascent pectin (non-radioactive) extracted from pea microsomes.

4.4 Effect of treatment with Polysaccharidases

4.4.1 Effect of adding β -1,4-Endoglucanases to the [14 C]Gal-HMwt-complex.

β -1, 4-Endoglucanases degrade polysaccharides possessing β -1, 4-glucan backbones such as cellulose and xyloglucan. [14 C] Gal-HMwt-complex was treated for 2 hours with 10 units of β -

1, 4-Endoglucanases. Then treated samples were passed through different Sepharose columns. Fig 4.1 using the Sepharose CL-2B column shows that most of the cellulase product is at the low molecular weight region. Fig 4.2 using the Sepharose CL-4B column shows that the product is partially included in the gel, with an apparent molecular weight of about 20 kDa.

4.4.2 Effect of treating the [¹⁴C] Gal-HMwt-complex with endo-polygalacturonase.

Endo-polygalacturonase breaks the covalent bond within homogalacturonan (HG), at non-methylated galacturonic acid residues. [¹⁴C] Gal-HMwt-complex was treated for 2 hours with 10 units of endo-polygalacturonase. The sample was then passed through different columns. Fig 4.1 The Sepharose CL-2B column showed that the product of the enzyme was fully included in the gel. Sepharose CL-4B exclusion column showed that the products were partially included in the gel (Fig 4.2). Fig 4.3 the enzyme treated complex was passed through Sepharose CL-6B, and 82% of the product was partially included in the gel, with an apparent molecular weight of about 10 kDa.

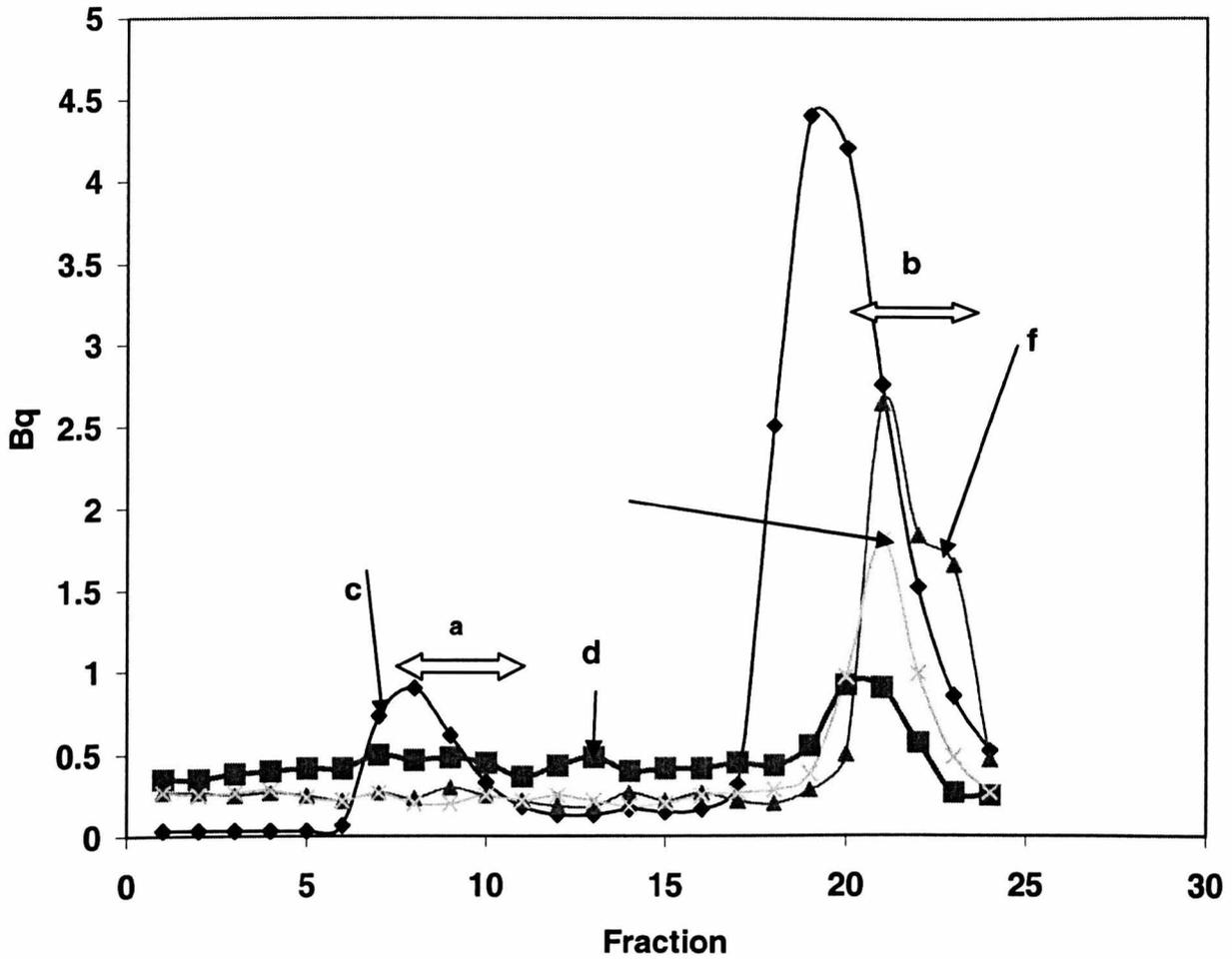


Fig 4.1 Gel filtration of $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) on Sepharose CL-2B column, using EPN as eluent, treated with different polysaccharidases.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** Control, treated with buffer only.
- d.** Treated with cellulase.
- e.** Treated with Polygalacturonase.
- f.** Treated with galactanase.

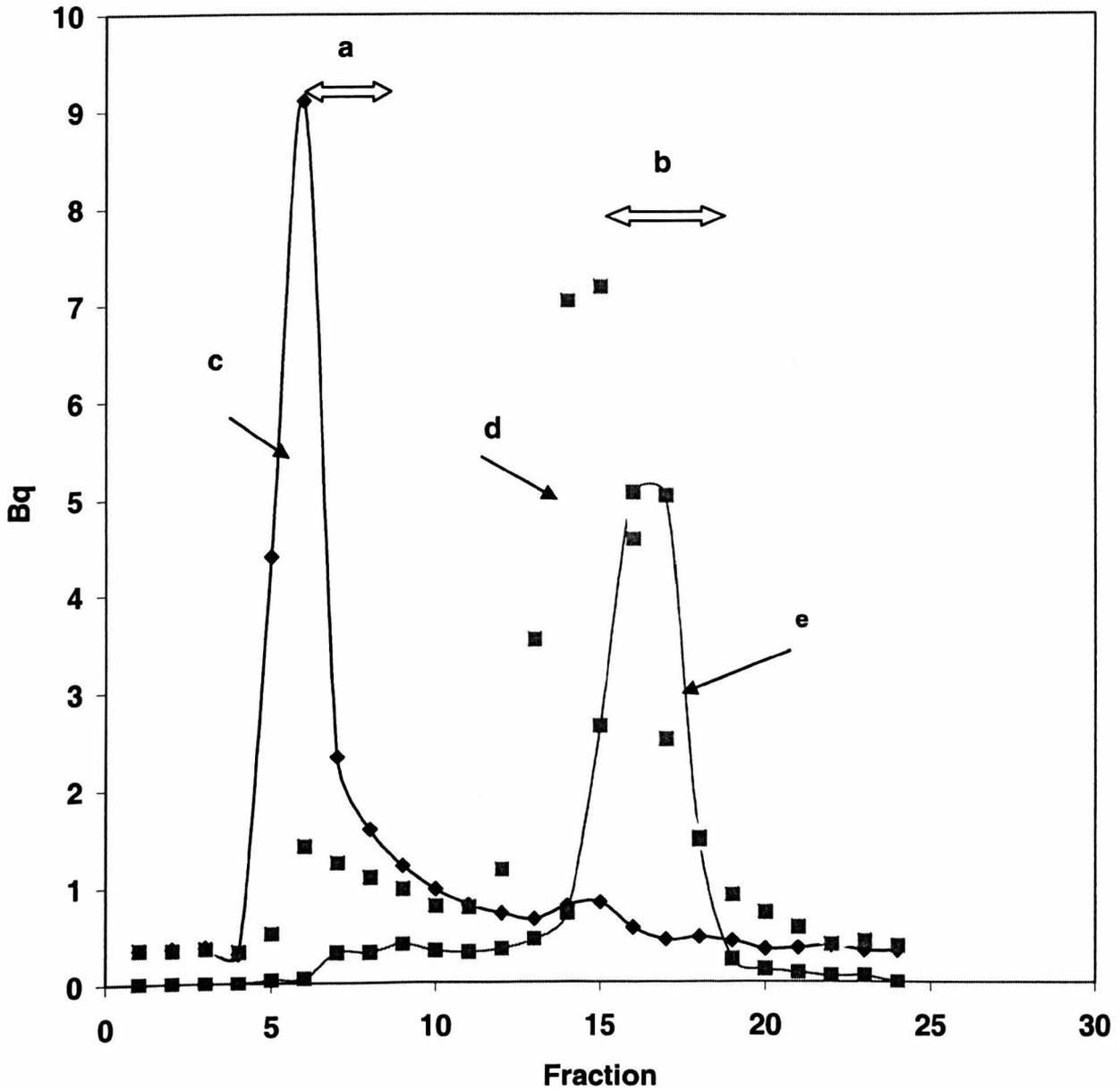


Fig 4.2. Gel filtration on Sepharose CL-4B column, using (EPN) as eluent, of [^{14}C] Gal-HMwt-complex (section 3.5), treated with different polysaccharidases.

- a. Blue Dextran high molecular weight marker.
- b. Cobalt chloride low molecular weight marker.
- c. Control, treated with buffer only.
- d. Treated with cellulase.
- e. Treated with Polygalacturonase.

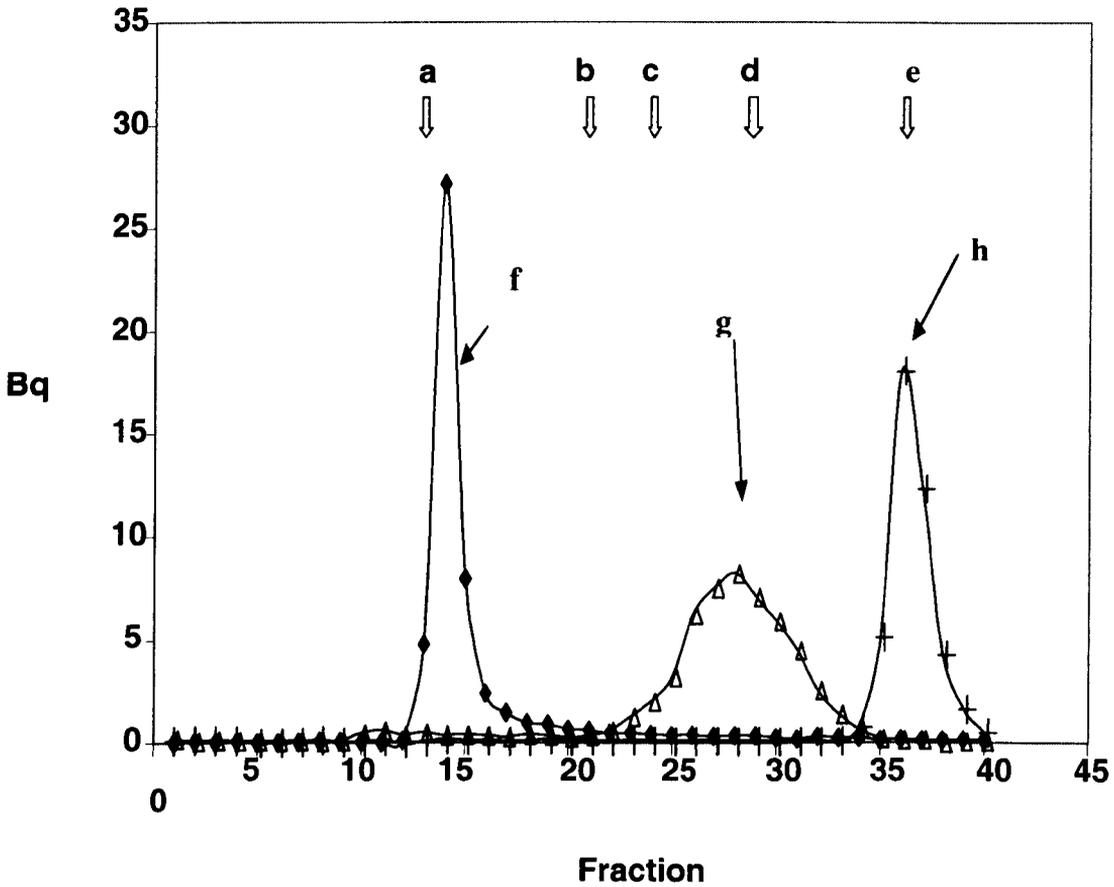


Fig 4.3 Gel filtration on Sepharose CL-6B column, using (EPN) as eluent, of [^{14}C] Gal-HMwt-complex (section 3.5), treated with different polysaccharidases.

- a.** Blue Dextran high molecular weight marker.
- b.** 76K dextran.
- c.** 40K dextran.
- d.** 10K dextran.
- e.** Cobalt chloride low molecular weight marker.
- f.** Control, treated with buffer only.
- g.** Treated with Polygalacturonase.
- h.** Treated with galactanase.

4.4.3 Effect of treating the [¹⁴C] Gal-HMwt-complex with galactanase.

A sample of [¹⁴C] Gal-HMwt-complex was treated with 10 units of galactanase for 2 hours.

The treated sample was then passed through different bead sized columns. On a Sepharose CL-2B column the radioactivity was totally included as low molecular weight products (Fig 4.1).

Fig 4.3 shows that 86 % of the radioactivity was totally included as low molecular weight product on GF in Sepharose CL-6B.

Paper chromatography was performed on galactanase-treated [¹⁴C] Gal-HMwt complex, and

Fig 4.4 shows that the products contained major amounts of radioactivity co-chromatographing with galactotriose, some with galactobiose and traces with galactose.

4.4.4 Effect of treating the [¹⁴C] Gal-HMwt-complex with mannanase.

The [¹⁴C] Gal-HMwt complex was treated with mannanase for 2 hours. Fig 4.5 shows that the product was totally included in Sepharose CL-4B. Fig 4.6 shows that the product was partially included in the Biogel P10, with an apparent molecular weight of about 1000 Da.

Because of the very low apparent molecular weight of the products of mannanase digestion, it was possible that β-1, 4-galactanase contaminated the mannanase. Paper chromatography showed that the products were not as small as galactobiose (Fig 4.4). However, the presence of a very small amount of galactanase cannot be excluded.

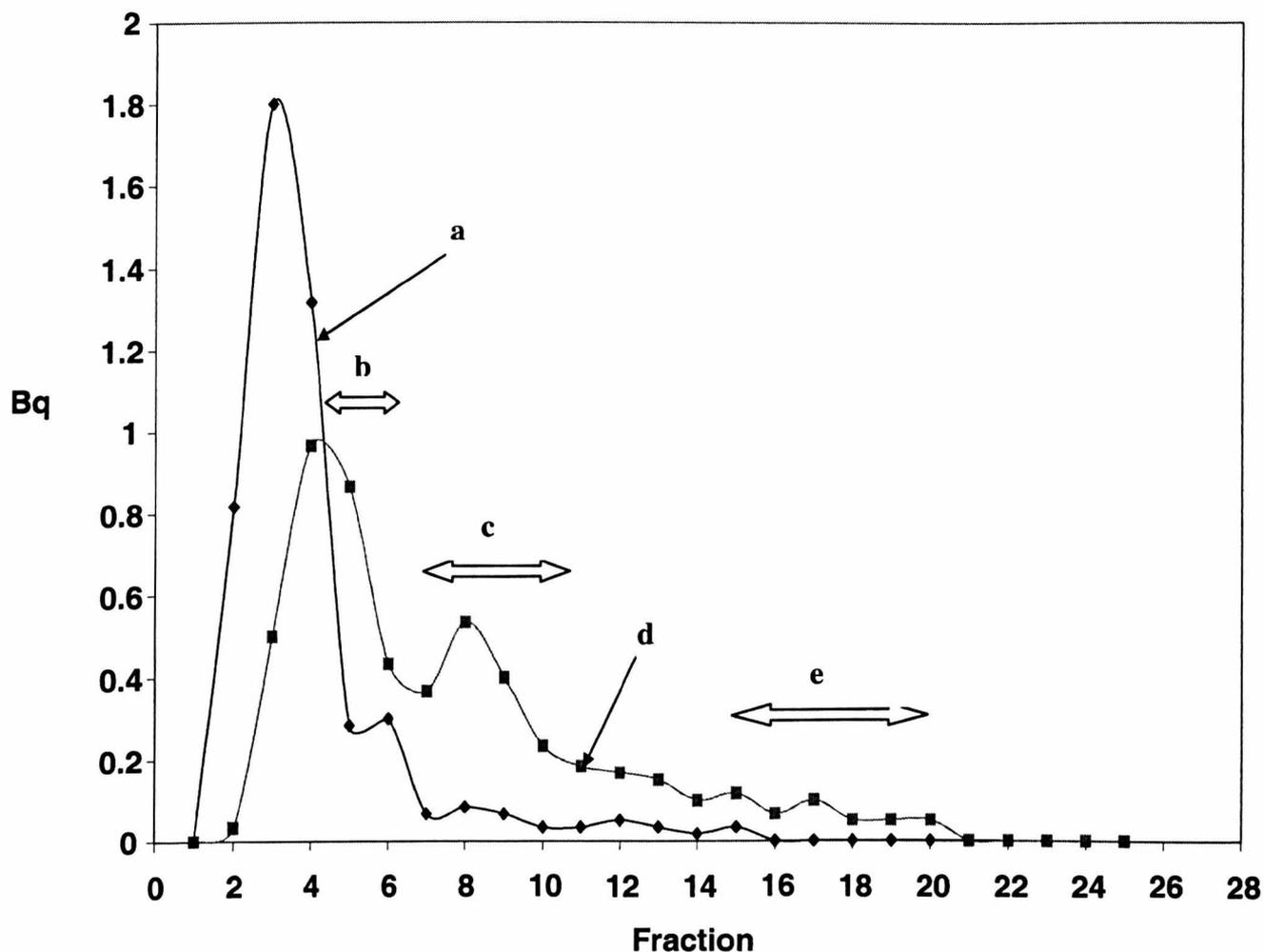


Fig 4.4 Paper chromatography of the products of galactanase and mannanase treatment of [^{14}C] Gal-HMwt-complex (section 3.5). Mannanase as well as galactanase treated samples were reduced to a volume of 200 microliters each. 100 microliters of each were spotted on 3 MM paper and run in pyridine/ ethylacetate /water 10:4:3 for 18 hours.

- a. Mannanase treatment.**
- b. Galactotriose product.**
- c. Galactobiose product.**
- d. Galactanase treatment.**
- e. Galactose product.**

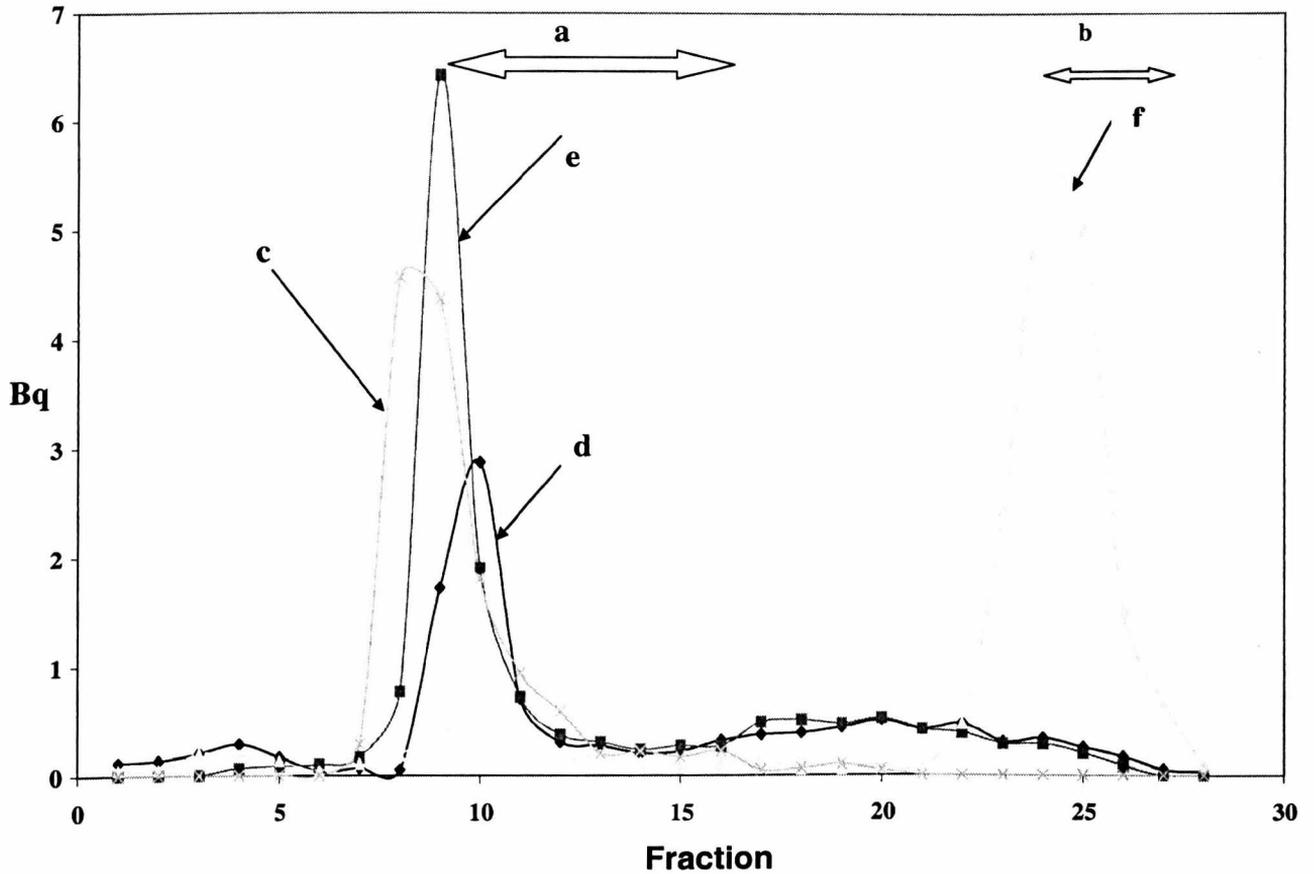


Fig 4.5 Gel filtration on Sepharose CL-4B column, of [^{14}C] Gal-HMwt-complex (section 3.5) using EPN as eluent, treated with mannanase or xylanase.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** Control, pH 8.8 treated with buffer only.
- d.** Control, pH 8.8 treated with buffer only.
- e.** Xylanase treatment.
- f.** Mannanase treatment.

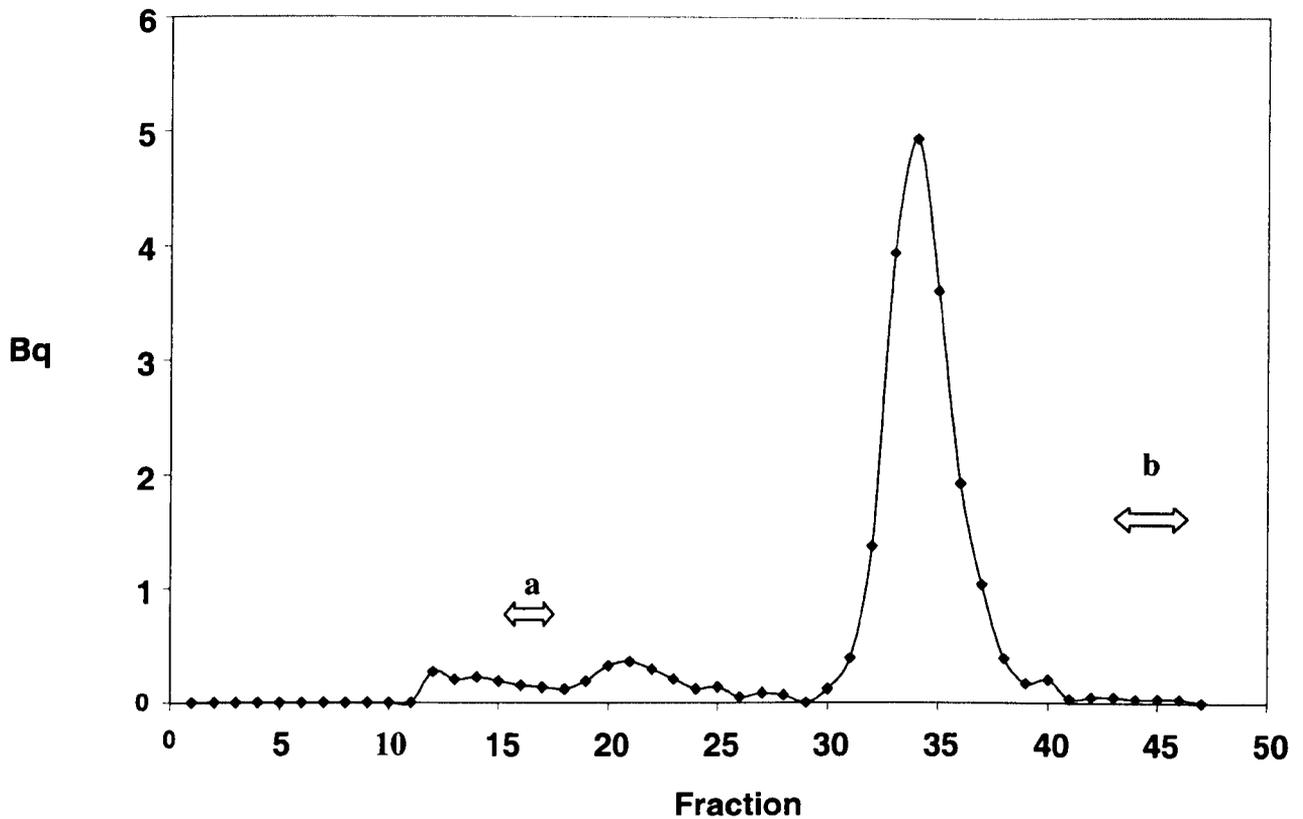


Fig 4.6 Gel filtration on Biogel P10 of products of mannanase treatment.
 The experiment shown in Fig 4.5 was repeated, except that Biogel P-10 was used in place of Sepharose CL4B. On the basis of K_{av} values derived from Fig 6 of Abdel-Massih *et al.* (2003), a 4kD Dextran would elute in fraction 25 on this column, while xyloglucan oligosaccharides (average M.Wt. approximately 1200) would elute in fraction 39.

- a. Blue Dextran high molecular weight marker.**
- b. Cobalt chloride low molecular weight marker.**

4.4.5 Effect of treating the [¹⁴C] Gal-HMwt-complex with xylanase.

β -1, 4-xylanase is an enzyme that is used to break down β -1, 4- linked xylans (including arabinoxylan, glucouronxylan and GAX). Pectin-xylan-xyloglucan complexes have been reported in the cell walls Femenia *et al.* (1999). Fig 4.5 showed that treatment with xylanase led to the product being totally excluded from the gel on GF in Sepharose CL-4B. Thus xylanase had no apparent effect on the molecular weight of the complex. Hence, xylans are probably not present in the complex. For further accuracy, xylanase activity ought to be checked by incubation with xylan and the products monitored.

4.4.6 Effect of treating the [¹⁴C] Gal-HMwt-complex with xyloglucanase.

Xyloglucanase cleaves xyloglucan to xyloglucan subunit oligosaccharides (Pauly *et al.*, 1999). Treating the [¹⁴C] Gal-HMwt-complex with xyloglucanase led to partial inclusion of the products in the gel when passed through the Sepharose CL-4B column (Fig 4.7). The apparent molecular weight was about 20 kDa, similar to that obtained after endo-1, 4- β -glucanase treatment. To give more details about the component, the peak from xyloglucanase treatment should be further treated with galactanase.

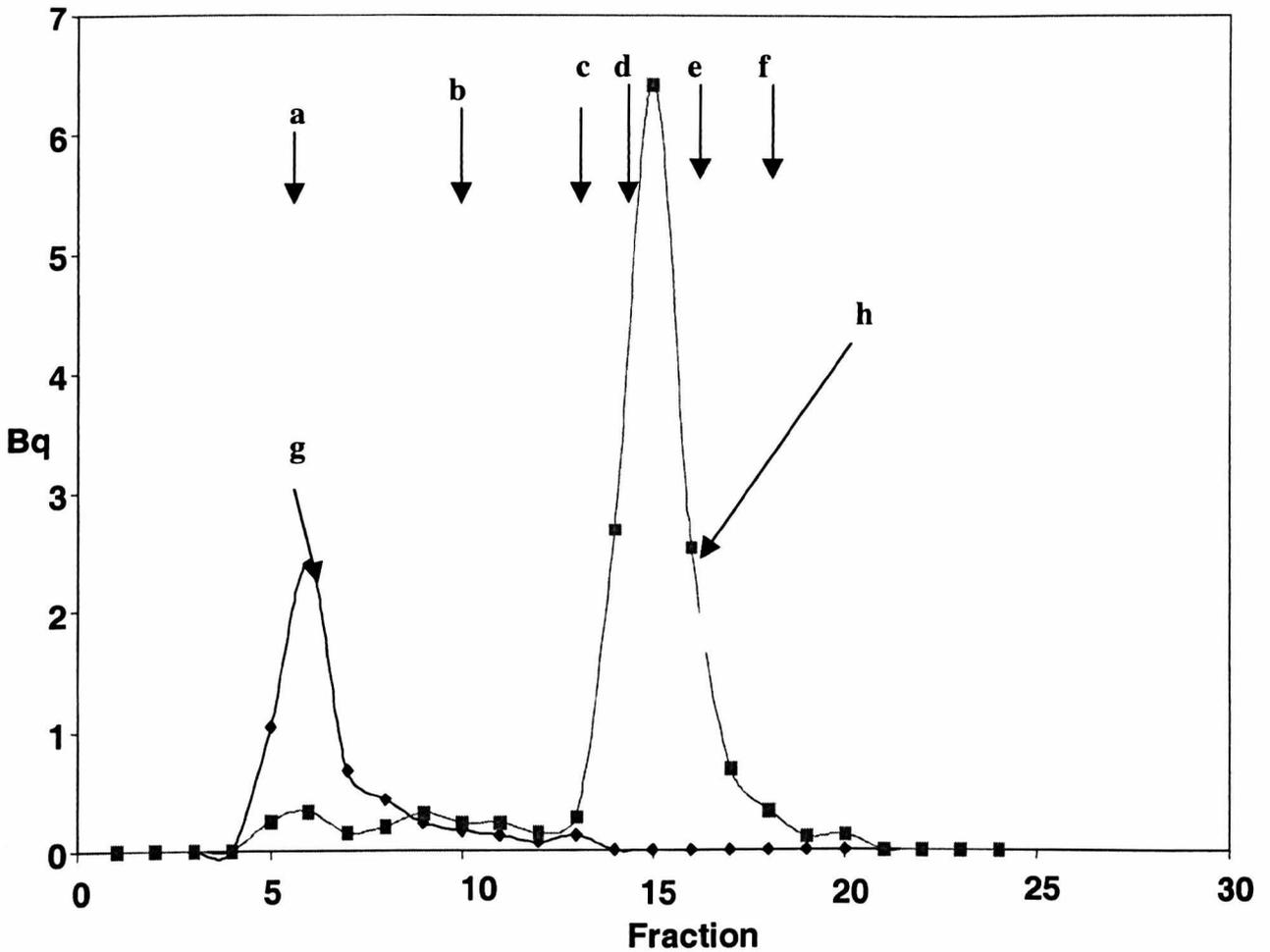
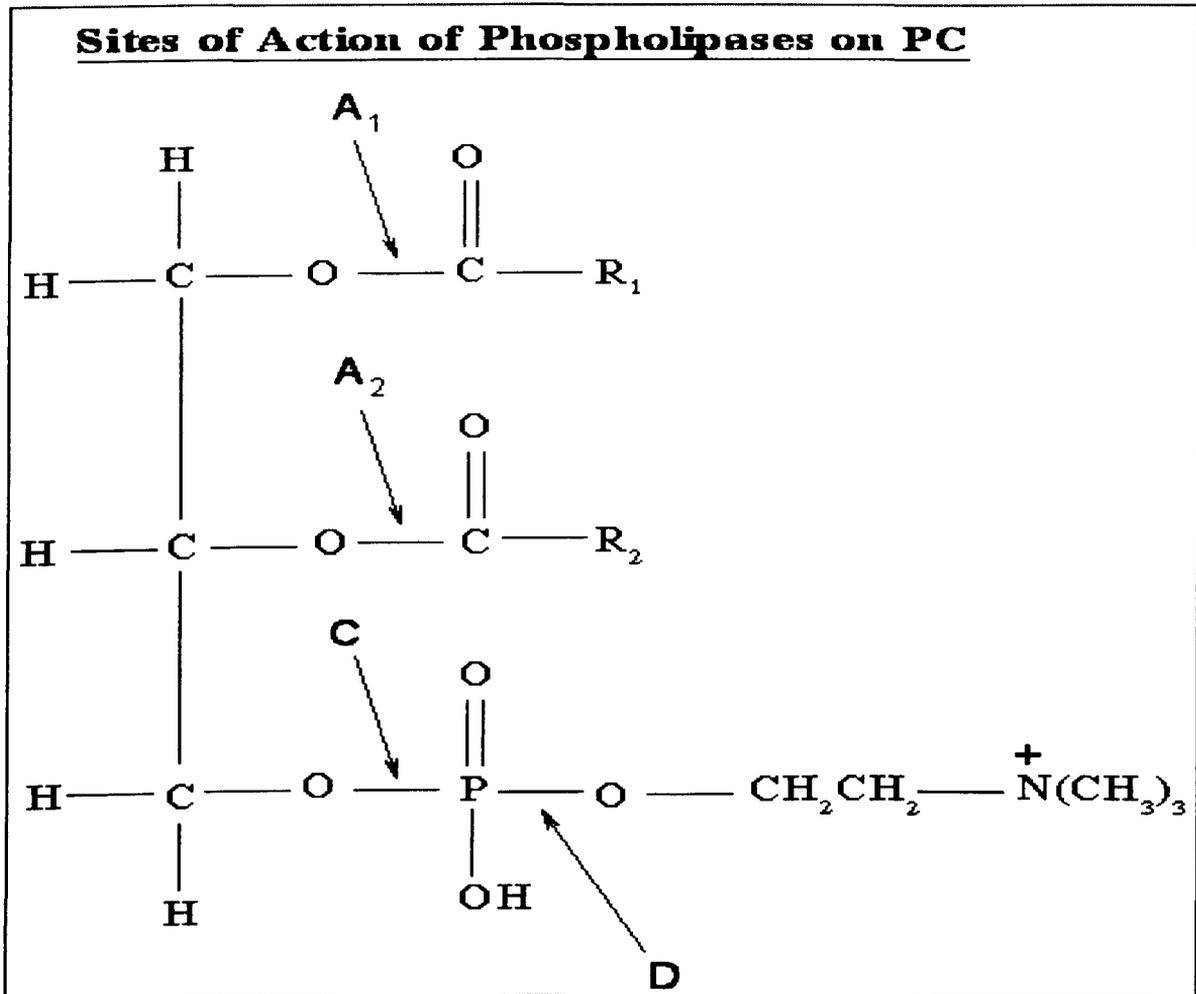


Fig 4.7 Gel filtration on Sepharose CL-4B column, of [^{14}C] Gal-HMwt-complex (section 3.5) using EPN as eluent, treated with xyloglucanase or buffer.

- a.** Blue Dextran high molecular weight marker.
- b.** 181K Dextran.
- c.** 76K Dextran.
- d.** 40K Dextran.
- e.** 10K Dextran.
- f.** Cobalt chloride low molecular weight marker.
- f.** Control, treated with buffer only.
- g.** Treated with xyloglucanase.

4.5 Effect of treatment with phospholipase C

There are 4 positional lipases: phospholipase A₁, A₂, C and D (Fig 4.8).



<http://www.uoguelph.ca/~hlee/418chap7.htm>.

In this chapter, phospholipase C (Sigma P-7633) was used in order to determine whether it caused a decrease in the molecular weight of the complex due to the removal of a possible lipid attached in this nascent pectin complex. While there are no previous reports of cell-wall polysaccharides being attached to lipid, such an attachment might occur during biosynthesis and/or transport in the endomembrane system. This could be either a direct attachment or via

protein (Eisenhaber *et al.*, 2003). Phospholipase C's activity should be checked by treating phospholipids and observing the products.

5 units of phospholipase C were dissolved in 100 μ l of 0.1 M Tris-HCl pH 7.3. They were then added to the [14 C] Gal-HMwt-complex and incubated for 2 hours at 37°C. The enzyme-treated samples as well as buffer-treated sample were then passed through a Sepharose CL-4B column eluted with EPN. Fig 4.9 shows the profile of the enzyme treatment as well as the control with the corresponding buffer alone. Both treatments had the same effect, indicating that phospholipase C had little effect on breaking down the complex into smaller subunits. Most of the radioactivity is at the high molecular weight position. Hence, there is no evidence for attachment of the complex to a lipid. It is possible that such an attachment could not be detected by this method, because of the relatively small size of the lipid.

4.6 Effect of treatment with trypsin

Trypsin is a protease that exhibits specificity towards basic amino acids (eg. Lys, Arg) on the carboxyl side of the susceptible bond. It is most active at around pH 8.0. Trypsin (10 units) from bovine pancreas (Sigma T-4665) dissolved in 100 μ l of 0.1M Tris-HCl pH 7.3 were added to the [14 C] Gal-H.Mwt-complex and incubated for 2 hours at 37°C. Trypsin activity should be checked by treating a protein with trypsin and checking the products.

The enzyme-treated sample, as well as the buffer treated sample, was then passed through a Sepharose CL-4B column eluted with EPN. Fig 4.10 shows the profile of the enzyme treatment as well as the control with the corresponding buffer alone. Both treatments had the same effect, indicating that trypsin had little effect on breaking down the complex into further subunits. Most

of the radioactivity is at the high molecular weight position. Hence, there was no evidence for the attachment to protein.

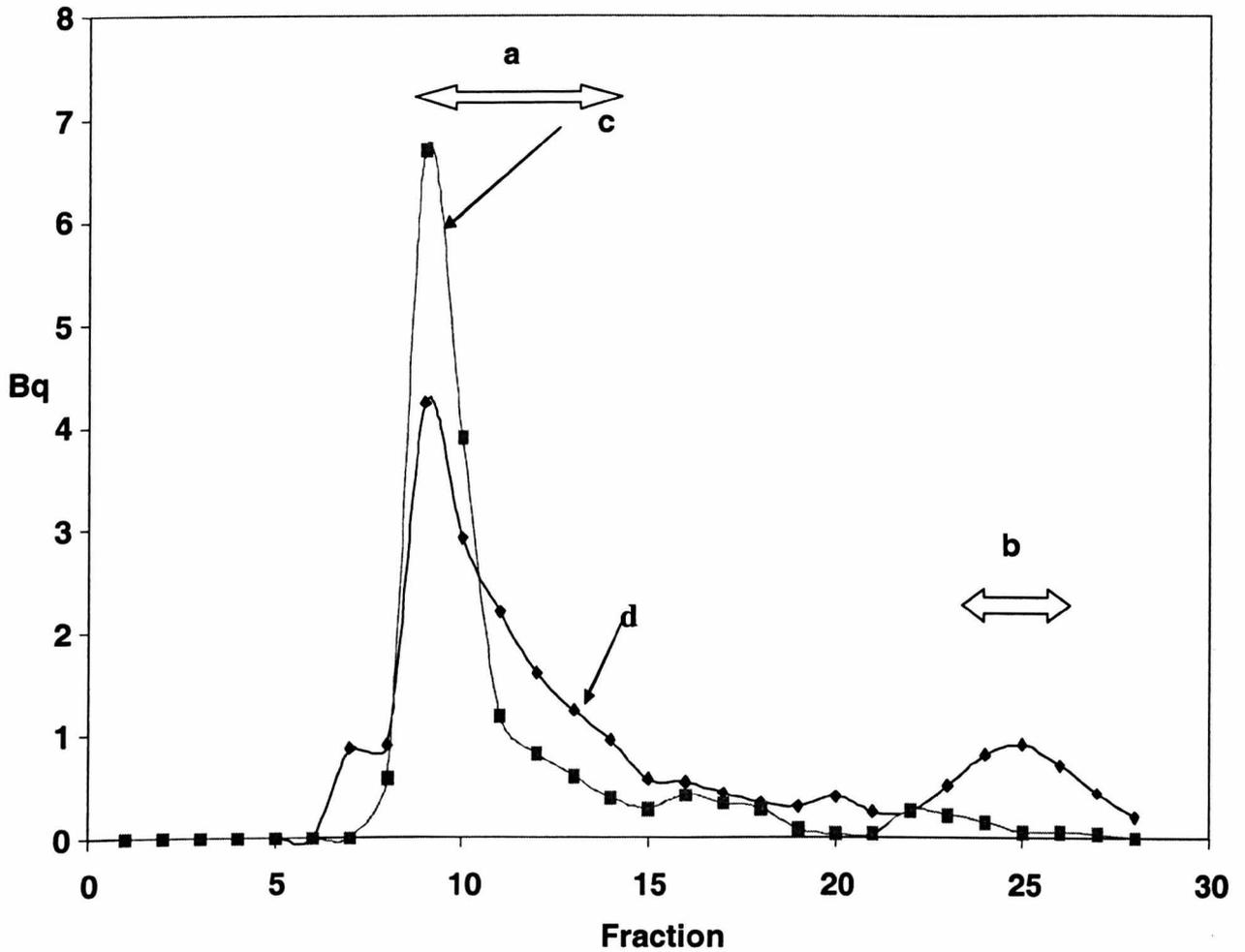


Fig 4.9 Gel filtration on Sepharose CL-4B column, of [^{14}C] Gal-HMwt-complex (section 3.5) using EPN as eluent, treated with phospholipase C or buffer.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** Phospholipase C treatment.
- d.** Control, treated with buffer.

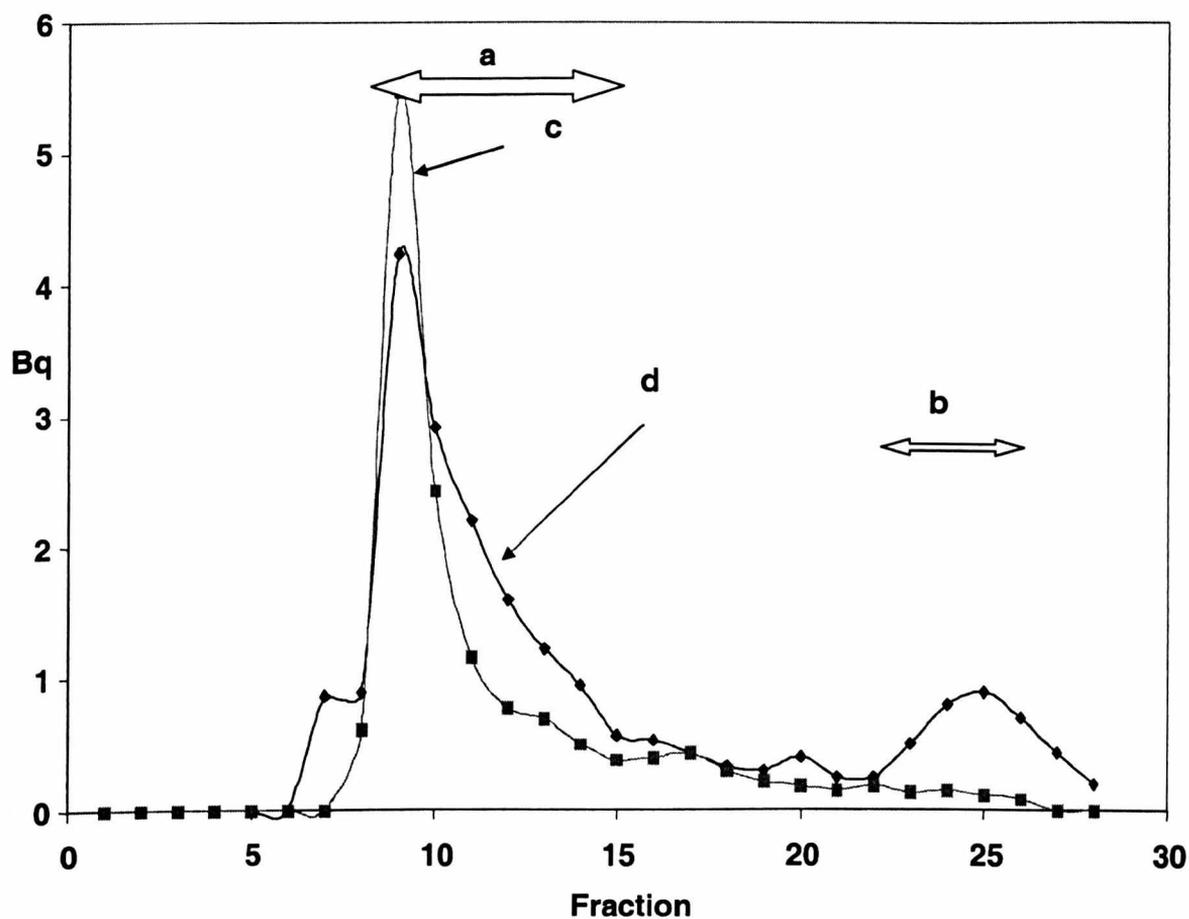


Fig 4.10 Gel filtration on Sepharose CL-4B column, of $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) using EPN as eluent, treated with trypsin or buffer.

- a.** Blue Dextran high molecular weight marker.
- b.** Cobalt chloride low molecular weight marker.
- c.** Trypsin treatment.
- d.** Control, treated with buffer.

4.7. Experiments using monoclonal antibodies.

4.7.1. Three pectins with different degree of methylation were studied with immunoblotting, using antibody JIM5.

Immunoblotting with JIM5 showed that JIM5 epitope was present in all 3 samples, and most abundant in the 34% methylated pectin (Fig 4.11). As expected, intensity of staining was greatest at the highest pectin concentrations.

4.7.2. Three pectins with different degree of methylation as well as pea microsomal pectin were studied, using antibody JIM5.

When the experiment described in the previous section was repeated, with the addition of pea microsomal pectin, the pea microsomal pectin also showed some staining, indicating that the JIM 5 epitope was present. The intensity of staining was less than any of the three commercial partially esterified pectins. However, when the pectin concentrations were determined using Dubois carbohydrate assay, it was found to be $250.86\mu\text{g/ml}$.

Hence, the JIM5 epitope was present in significant amounts in the pea microsomal pectin.

4.7.3. Three pectins with different degree of methylation as well as pea microsomal pectin were studied, using antibodies JIM5 and LM7.

The experiment described above was repeated again, but this time both JIM5 and LM7 were used as probes separately. Only the highest concentration of pea microsomal pectin was

included, and three concentrations of cell wall pectin from Arabidopsis cell suspension were also analysed. This Arabidopsis pectin was prepared by Lolita Quota, another Ph.D. student in our laboratory. It was found that both antibodies bound to the pea microsomal pectin. JIM 5 also bound to the Arabidopsis pectin, but LM7 showed no visible binding either to the commercial pectins or the Arabidopsis pectin.

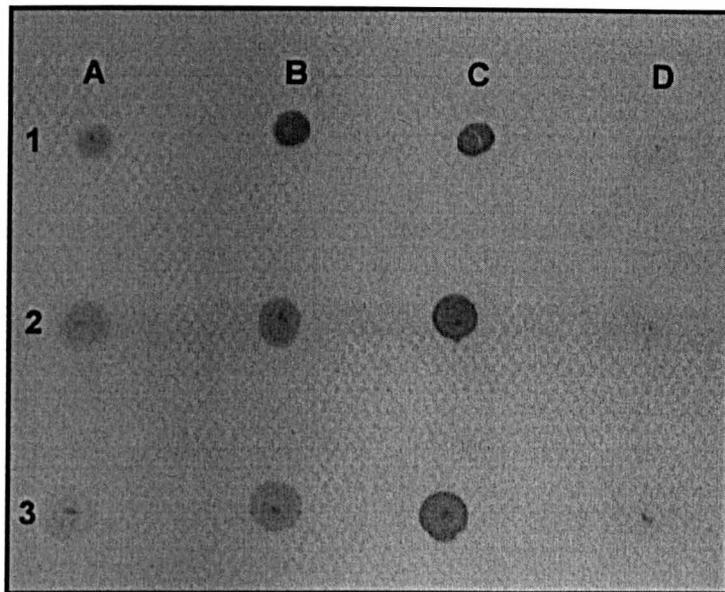
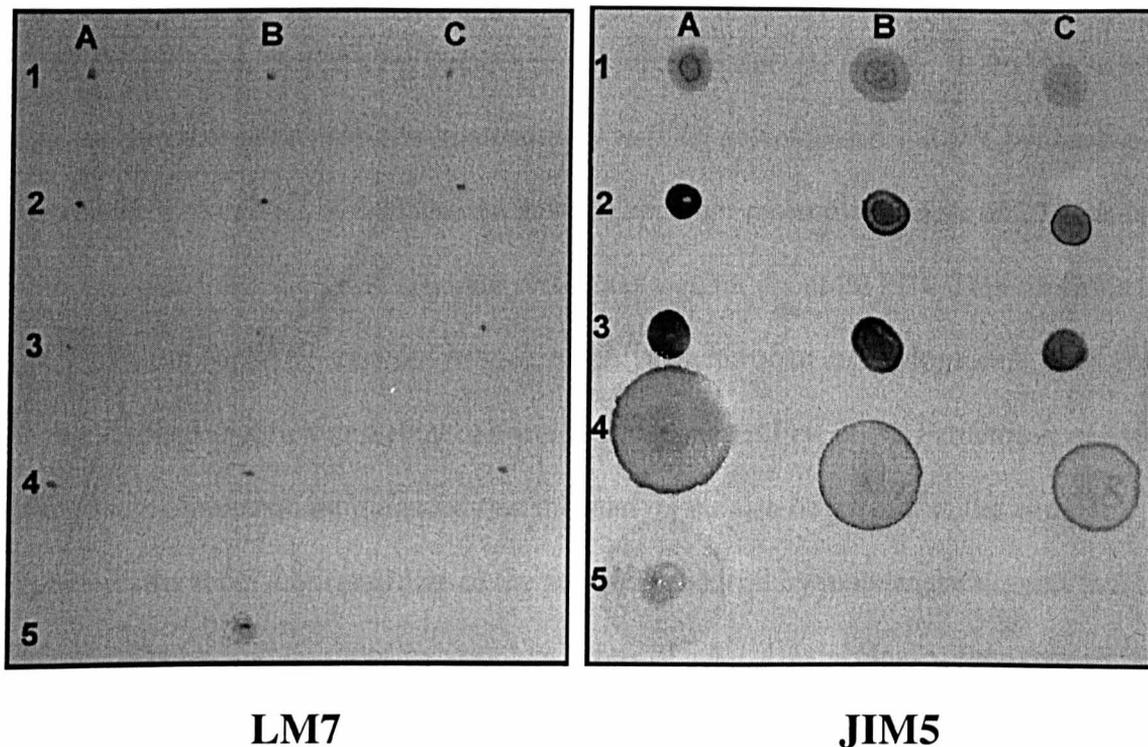


Fig 4.11 Three pectins with different degrees of methylation, as well as pea microsomal pectin were studied with respect to the degree of binding to JIM5.

- 1A** corresponds to 10 μ l of 0.6 mg/ml 89 % esterified pectin.
- 1B** corresponds to 10 μ l of 0.6 mg/ml 65 % esterified pectin.
- 1C** corresponds to 10 μ l of 0.6 mg/ml 34 % esterified pectin.
- 1D** corresponds to 10 μ l of 0.6 mg/ml pea microsomal pectin.
- 2A** corresponds to 10 μ l of 0.4 mg/ml 89 % esterified pectin.
- 2B** corresponds to 10 μ l of 0.4 mg/ml 65 % esterified pectin.
- 2C** corresponds to 10 μ l of 0.4 mg/ml 34 % esterified pectin.
- 2D** corresponds to 10 μ l of 0.4 mg/ml of pea microsomal pectin.
- 3A** corresponds to 10 μ l of 0.2 mg/ml 89 % esterified pectin.
- 3B** corresponds to 10 μ l of 0.2 mg/ml 65 % esterified pectin.
- 3C** corresponds to 10 μ l of 0.2mg/ml 34 % esterified pectin.
- 3D** corresponds to 10 μ l of 0.2mg/ml of pea microsomal pectin.



LM7

JIM5

Fig 4.12 Three pectins with different degrees of methylation, as well as pea microsomal pectin were studied with respect to the degree of binding to LM7 as well as JIM5.

- 1A corresponds to 10 μ l of 0.6 mg/ml 89 % esterified pectin.
- 1B corresponds to 10 μ l of 0.4 mg/ml 89 % esterified pectin.
- 1C corresponds to 10 μ l of 0.2 mg/ml 89 % esterified pectin.
- 2A corresponds to 10 μ l of 0.6 mg/ml 65 % esterified pectin.
- 2B corresponds to 10 μ l of 0.4 mg/ml 65 % esterified pectin.
- 2C corresponds to 10 μ l of 0.2 mg/ml 65 % esterified pectin.
- 3A corresponds to 10 μ l of 0.6 mg/ml 34 % esterified pectin.
- 3B corresponds to 10 μ l of 0.4 mg/ml 34 % esterified pectin.
- 3C corresponds to 10 μ l of 0.2mg/ml 34 % esterified pectin.
- 4A corresponds to 10 μ l of 0.6mg/ml Arabidopsis pectin.
- 4B corresponds to 10 μ l of 0.6mg/ml Arabidopsis pectin.
- 4C corresponds to 10 μ l of 0.6mg/ml Arabidopsis pectin.
- 5B corresponds to pea microsomal pectin in case of LM7.
- 5A corresponds to pea microsomal pectin in case of JIM5.

4.8. Conclusion

Polygalacturonase caused a big decrease in apparent molecular size, to 10-20 kDa, while endo-1, 4- β -galactanase converted the radioactivity to [^{14}C] galactobiose and [^{14}C] galactose.

Treatment with endo-1, 4- β -glucanase gave an apparent molecular weight of 20 kDa and that with xyloglucanase the same apparent molecular weight, about 20 kDa. This confirms that the [^{14}C] galactan is part of complex containing polygalacturonan and xyloglucan. Such complexes have also been reported in mature cell walls (Thompson & Fry, 2000; Femenia *et al.*, 1999).

The effects of mannanase indicated that mannan might also be present in the complex.

However, the relatively small size of the product produced by mannanase suggest that this enzyme may be contaminated by very low levels of a β -1,4-galactanase, even though no direct evidence for this could be obtained. Alternatively, mannan may be present in the complex. If so, it would need to be close to galactan in the complex, in order to give rise to relatively small radioactive fragments when degraded with mannanase. However, this is not conclusive since mannanase could be contaminated with other polysaccharidases enzymes.

No evidence was obtained for attachment to lipid, protein, or xylan.

Immuno blotting showed that the microsomal pectin contained epitopes binding to both JIM5 and LM7. These epitopes have been reported to contain pectin with low degree of esterification in case of JIM5, and non-blockwise esterified pectins with LM7.

Chapter 5

Experiments to determine whether the nascent complex is attached to protein.

5.1 Introduction.

The present investigation aims at determining whether the nascent pectin complex, prepared and radioactively labeled by incubation with UDP- [U-¹⁴C] galactose as described by Abdel-Massih *et al.* (2003), is covalently attached to protein.

5.1.1 Previous work.

The matrix polysaccharides of the higher-plant cell wall are synthesized in the endomembrane system, chiefly in the Golgi apparatus, and transported to the cell wall by Golgi vesicles (Brett & Waldron, 1996). It has been suggested that proteins act as primers for cell wall polysaccharides (Campbell *et al.*, 1988). Proteins may act as an anchor to the nascent matrix polysaccharides to the membrane during their passage from the Golgi to the cell wall (Crosthwaite *et al.*, 1994).

Cell walls contain a variety of different proteins, most of which are glycosylated. The most abundant ones contain hydroxyproline (Brett & Waldron, 1996). Hydroxyproline-rich glycoproteins (HRGP), commonly referred to as extensins, are known to play a primarily structural role (Swords and Staehelin, 1993). The cell wall also contains lectins (proteins that bind specifically to certain sugars without acting enzymically on them). There are a set of enzymes that are also located on the cell wall.

In pea epicotyls, nascent xylans are linked to protein (Crosthwaite *et al.*, 1994). Two different proteins may be involved; one is attached to xylan in the ER and the other is attached to xylan in the Golgi apparatus. Baydoun *et al.* (1991) reported that nascent glucuronoxylan formed by a general pea membrane fraction is linked to protein. Campbell *et al.* (1988) reported that nascent xyloglucan is attached to protein. This protein attached to xyloglucan was considerably larger than the protein attached to glucuronoxylan (Crosthwaite *et al.*, 1994). It was reported by Rizk *et al.* (2000) that

protease treatment decreased the binding of nascent pectin to xyloglucan, suggesting that pectin is synthesized attached to a protein.

5.1.2 Use of Affi-Gel as a protein-binding matrix.

In affinity chromatography, a ligand with specific affinity for the molecule to be isolated is covalently attached to a solid matrix in a chromatography column. A mixture of components is then applied to the column. The desired components (for example, proteins) will bind to the matrix-bound ligand while other components of the mixture that have no affinity for the ligand are washed through the column (Life Science Research products Bio-Rad). Affi-Gel 10 and Affi-Gel 15 affinity media provide spontaneous, rapid, and highly efficient coupling of ligands via primary amines. Both Affi-Gel 10 and 15 supports are N-hydroxysuccinimide esters of a derivatized crosslinked agarose gel bead support and both couple to ligands spontaneously in aqueous or non-aqueous solution. The Affi-Gel 10 support contains a neutral 10-atom spacer arm, whereas the Affi-Gel 15 support contains a cationic charge in its 15-atom spacer arm, which enhances the coupling efficiency of acidic proteins. The Affi-Gel 10 support is most efficient for coupling neutral or basic proteins with isoelectric points of 6.5 to 11. Affi-Gel 15 gel is recommended for coupling acidic proteins with isoelectric point below 6.5, due to the interactions between the charge on the protein and charge on the gel.

Ligands with free alkyl or aryl amino groups will couple spontaneously with Affi-Gel 10 or 15 supports in aqueous or non-aqueous solution. Upon addition of ligand, the N-hydroxysuccinimide is displaced, and a stable amide bond is formed. Both Affi-gel supports are suited for coupling low molecular weight ligands. In order to maintain pH control, minimum buffer strength of 10mM is recommended. Tris and glycine contain primary amino groups, which will couple to the gel and not

allow the protein in the complex itself to couple to the gel. Tris and glycine are therefore not recommended for use as buffers (tech notes 1085 chromatography bulletin).

The experiments reported in this chapter were designed to investigate whether proteins were present in the nascent pectin complex. If so, it is to be expected that the proteins would bind covalently to Affi-Gel via amino groups. However, since proteins and pectins readily form non-covalent bonds, it was also necessary to investigate the type of binding that occurred.

5.2 Methods.

Either Affi-Gel 10 or 15 gel slurry (3ml of each) was transferred to a 13.5ml polypropylene (falcon) centrifuge tube, and was allowed to settle. The supernatant was removed and discarded. The slurry was then washed with 3ml cold water; the gel was allowed to settle, then the supernatant discarded. Two ml 0.1M MOPS (pH 7.5), 0.1M MOPS (pH 7.5) + 80mM CaCl₂, or 0.1M MES (pH 4.8) were added along with high molecular weight [¹⁴C] pectin complex (approximately 1ml, 1,500cpm). Nascent [¹⁴C] Gal-HMwt-complex was prepared as described in section 2.3.

The samples were shaken for 4 hours at 4°C using an orbital shaker. Then they were allowed to settle and the supernatant was transferred to a scintillation vial (“supernatant 1”). Cold water (1.5ml) was added and mixed, then the supernatant was removed and counted (“supernatant 2”). To the remaining slurry, either scintillation cocktail was added and counted (“gel”), or the gel was treated with other reagents (“supernatant 3”) before washing with 1.5ml cold water (“supernatant 4”), and then adding scintillant to the gel.

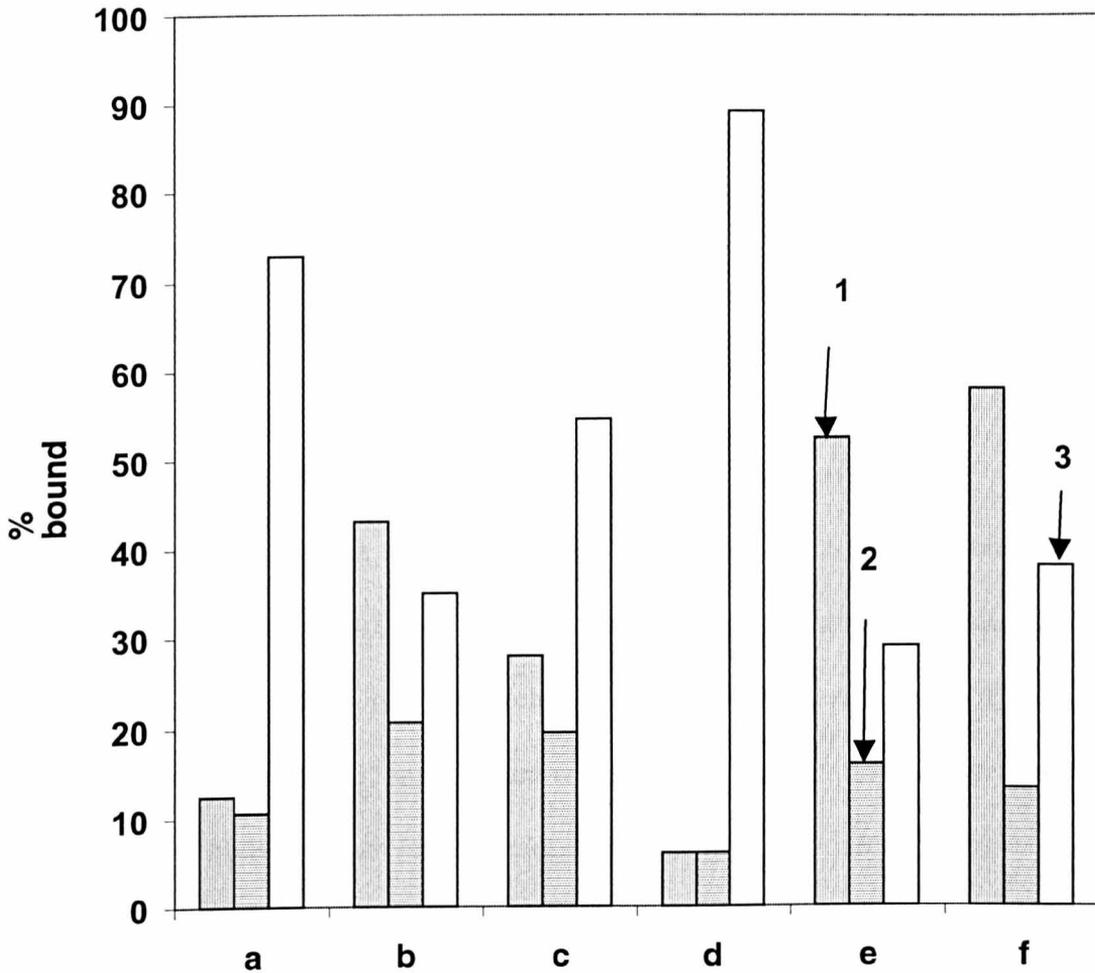


Fig 5.1 Binding of [^{14}C] Gal-HMwt-complex (section 3.5) to different types of gel and in different buffers. See section 5.2 for descriptions of different supernatants.

- a. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 10 with MOPS.
- b. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 10 with MOPS + CaCl_2 .
- c. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 10 with MES.
- d. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 15 with MOPS.
- e. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 15 with MOPS+ CaCl_2 .
- f. Binding of the [^{14}C] Gal-HMwt-complex (section 3.5) to Affi-Gel 15 with MES.

1. Supernatant 1 after first wash.
2. Supernatant 2 after second wash.
3. Gel.

5.3. Experiments

5.3.1 Experiments to determine optimum conditions for binding.

Fig 5.1 shows that a high level of binding of the [¹⁴C] pectin complex to Affi-Gel occurred. The percentage attachment of the complex to the slurry was highest (89%) with Affi-Gel15 in the presence of MOPS. The next highest was with Affi-Gel 10 with MOPS (76%). Affi-Gel 15 (with MOPS) was therefore used in all subsequent experiments.

5.3.2 Experiments to determine whether binding was non-covalent.

Each labeled pectin complex solution was incubated with MOPS and Affi-Gel 15. The gel was shaken at 4°C for 4 hours, washed, and the 1st and 2nd supernatants were removed for counting. To the slurry 1.5ml of 6M Urea; 7M Urea+1 M NaCl; 0.1 M acetic acid; or PBS+ 0.5M NaCl was added, mixed, and the 3rd supernatant was removed for counting. The gel samples were then washed with water, and both the wash (supernatant 4) and the gels were added to the scintillant and counted. All these reagents have the ability to break some non-covalent bonds. The principle is that the complex is bound first to the affigel and then treatment with these different reagents will lead to the dissociation of the complex from the Affi-Gel if it is bound by non-covalent bonds, thus liberating the complex back into solution. The results showed that there was some decrease in the binding to the gel, from 66% in the control to between 42% (with PBS+ 0.5M NaCl) and 54% (when treated with acetic acid) (Fig 5.2).

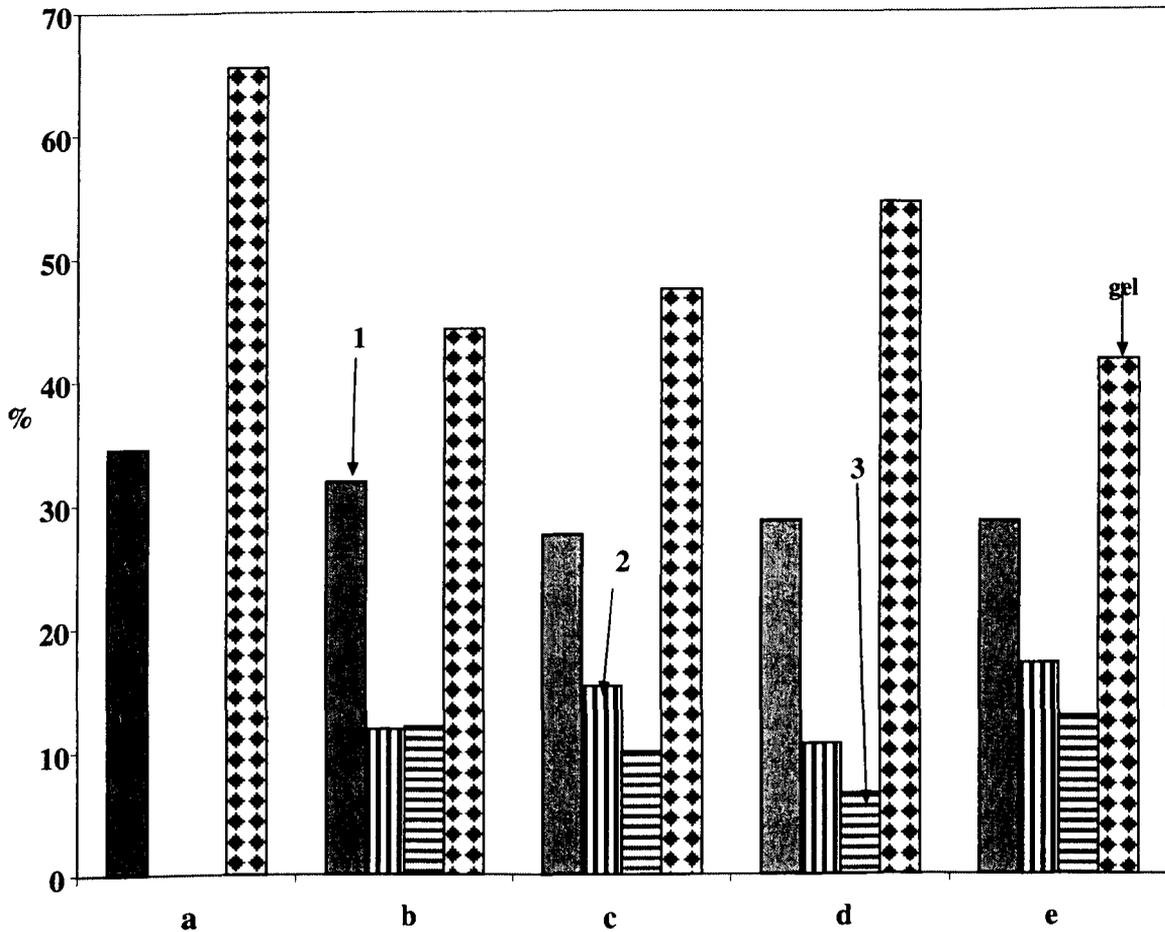


Fig 5.2 Effect of breaking non-covalent bond through the use of different reagents on $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) to Affigel 15. To 1.5 ml of Affigel 2 ml of MOPS and 0.5ml of $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) were added.

1. Supernatant 1 after 4 hrs, the radioactive solution was removed (supernatant 1).

2. Supernatant 2 after the gel was washed with water.

3. Supernatant 3 after the reagents were added.

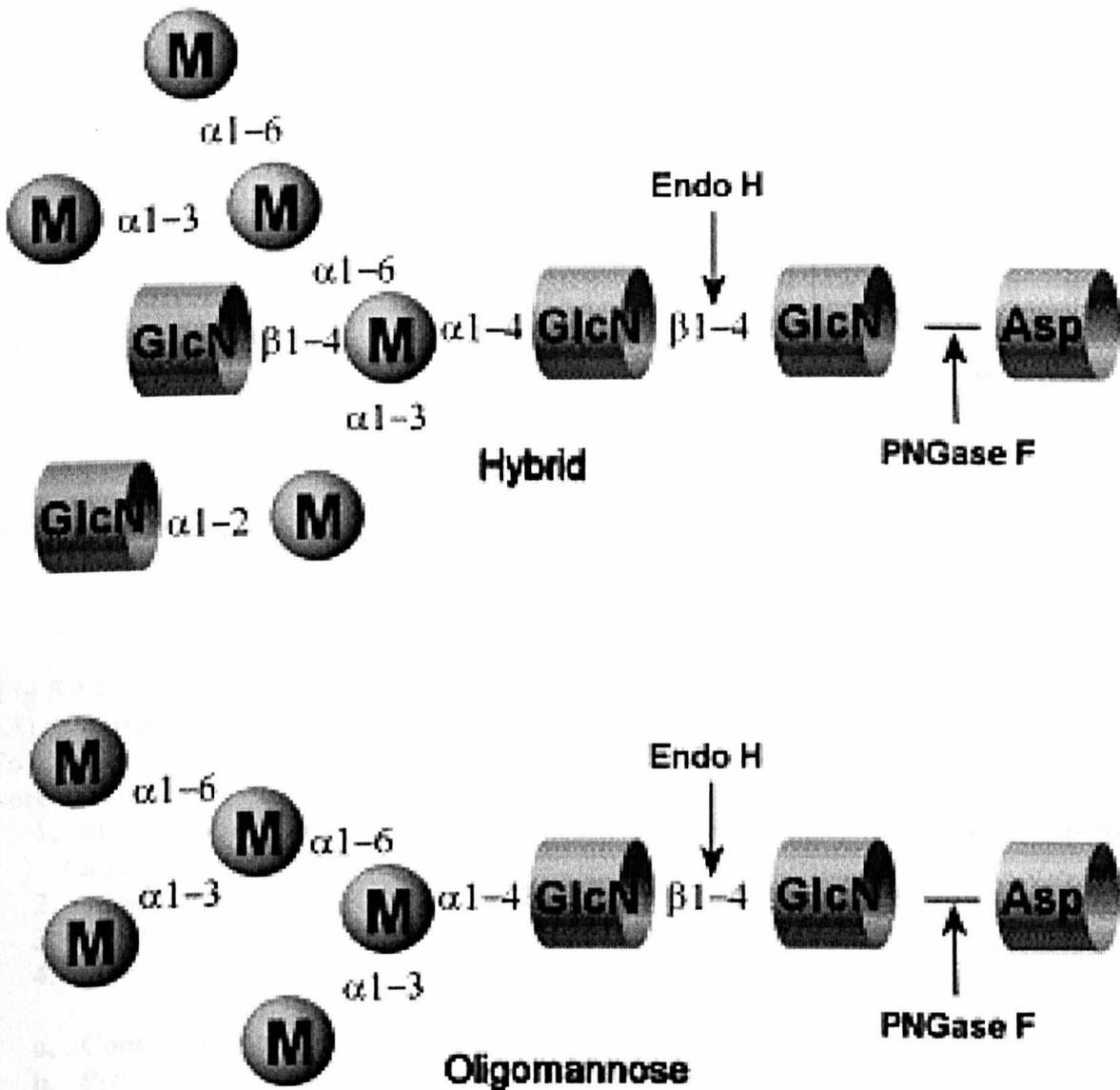
4. Gel.

- a. Control
- b. 1.5ml of 6M Urea.
- c. 7M Urea+1 M NaCl.
- d. 0.1 M acetic acid.
- e. PBS+ 0.5M NaCl.

protease is suspected to be contaminated with galactanase, which therefore might degrade the galactan (see fig 5.6 below).

Endo-H (Fig 5.3) cleaves between the N-acetylglucosamine residues of the diacetylchitobiose core of N-linked glycans. Oligomannose and most hybrid type glycans, including the core fucosylated type, are hydrolyzed by Endo-H. However complex type oligosaccharides are not.

Fig 5.3 Position of action of Endo-H.



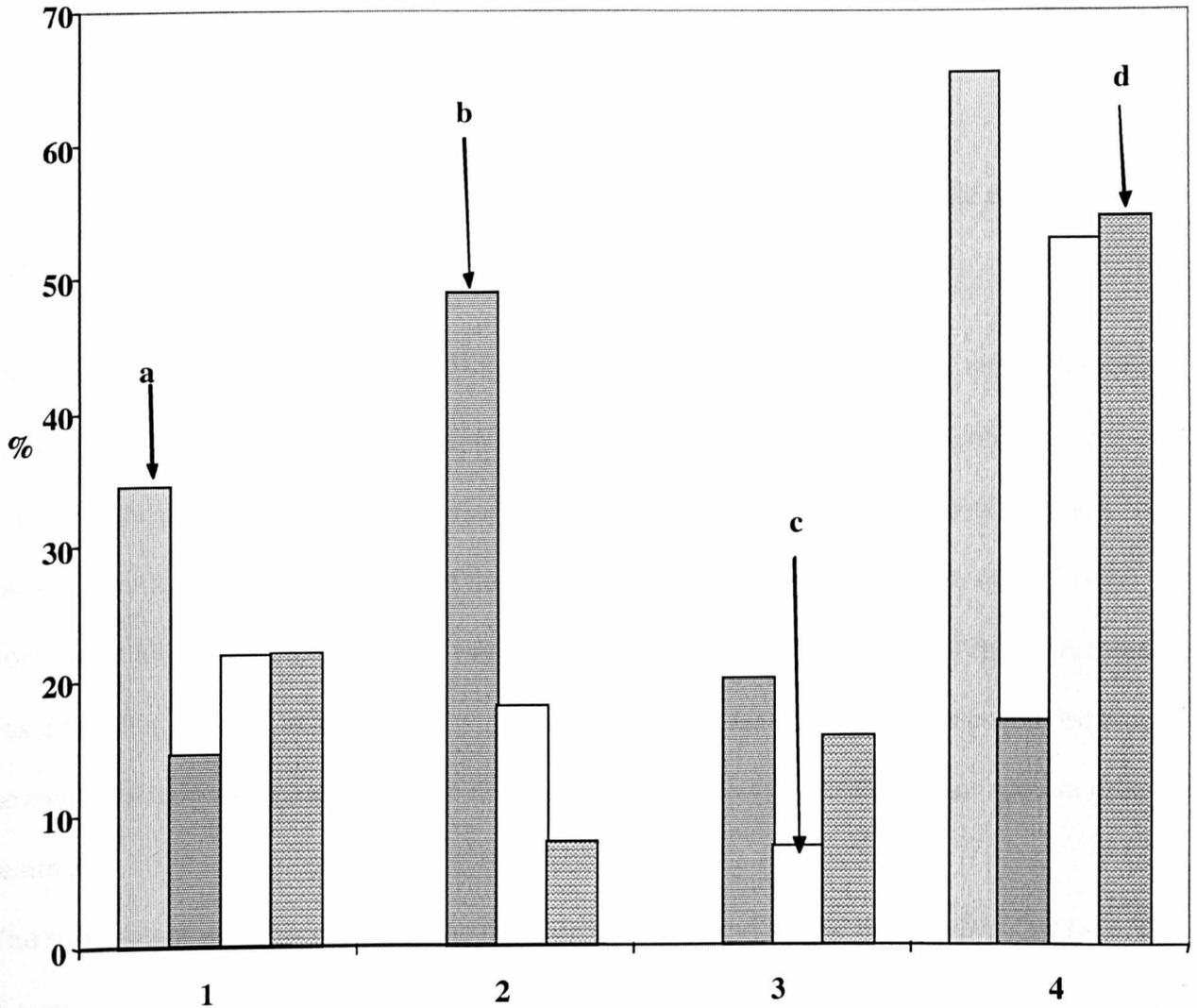


Fig 5.4 Effect of different proteases or endo-H on binding [^{14}C] Gal-HMwt-complex (section 3.5) to Affigel 15.

To 1.5 ml of Affigel 2 ml of MOPS and 0.5ml of [^{14}C] high molecular weight pectin complex were added.

1. after incubation for 4 hrs the radioactive solution was removed. The gel was washed, and supernatant 2 was discarded.
2. 1.5ml Proteases or endo-H in glycine buffer of the appropriate pH was added.
3. After washing post treatment with different reagents.
4. gel.

- a. Control group.
- b. Protease 2143 was added.
- c. Protease was added.
- d. Endonuclease-H was added.

Endo-H treatment released relatively little radioactivity, indicating that any protein involved in binding the complex to Affi-Gel did not require an endo-H- sensitive side-chain for binding.

5.3.4 Experiments to determine the effect of protease on the binding of the complex to the Affi-Gel.

Protease (P2143) was dissolved in 0.2M glycine pH (2.5). After the binding of the [¹⁴C] pectin complex to the gel, the bound complex was treated with either protease or buffer for 2 hours. The percentage binding of both the buffer-only-treated and the protease-treated gel samples showed similarities, with only 3.7% of the radioactivity bound to gel in the tube with protease alone and 2.8% binding to the control tube (Fig 5.5). The percent released after 2hrs of incubation was 29% after protease treatment compared to 25% with buffer alone. It can be concluded that the more washing took place, the more removal of the complex occurred, with or without protease treatment.

The supernatant was collected after the protease treatment and passed through Bio-Gel P-10. The elution profile showed that after protease incubation, most of the radioactivity was present as low-molecular-weight products (< 1000Da)(Fig 5.6). This suggests the formation of galactose or galactobiose as a result of treatment with enzyme. This implies that the protease was contaminated with either a β -galactosidase or an endo 1, 4- β -galactanase.

5.3.5 Effect of blocking any active ester by the use of ethanolamine.

A blocking agent for any active esters (1M ethanolamine HCl, pH8) was added one hour before incubating the Affi-Gel with the buffer and the complex. This reagent reacts with the N-ethylsuccinimide esters on Affi-Gel, preventing any subsequent coupling of protein to these groups.

The percentage of the radioactive complex bound to the gel after incubation with ethanolamine was 31%, whereas that without the addition of ethanolamine was 36% (Fig 5.7). It was concluded that ethanolamine did not abolish binding; therefore binding was not through the covalent coupling of protein in the complex to N-ethyl succinimide esters on Affi-Gel.

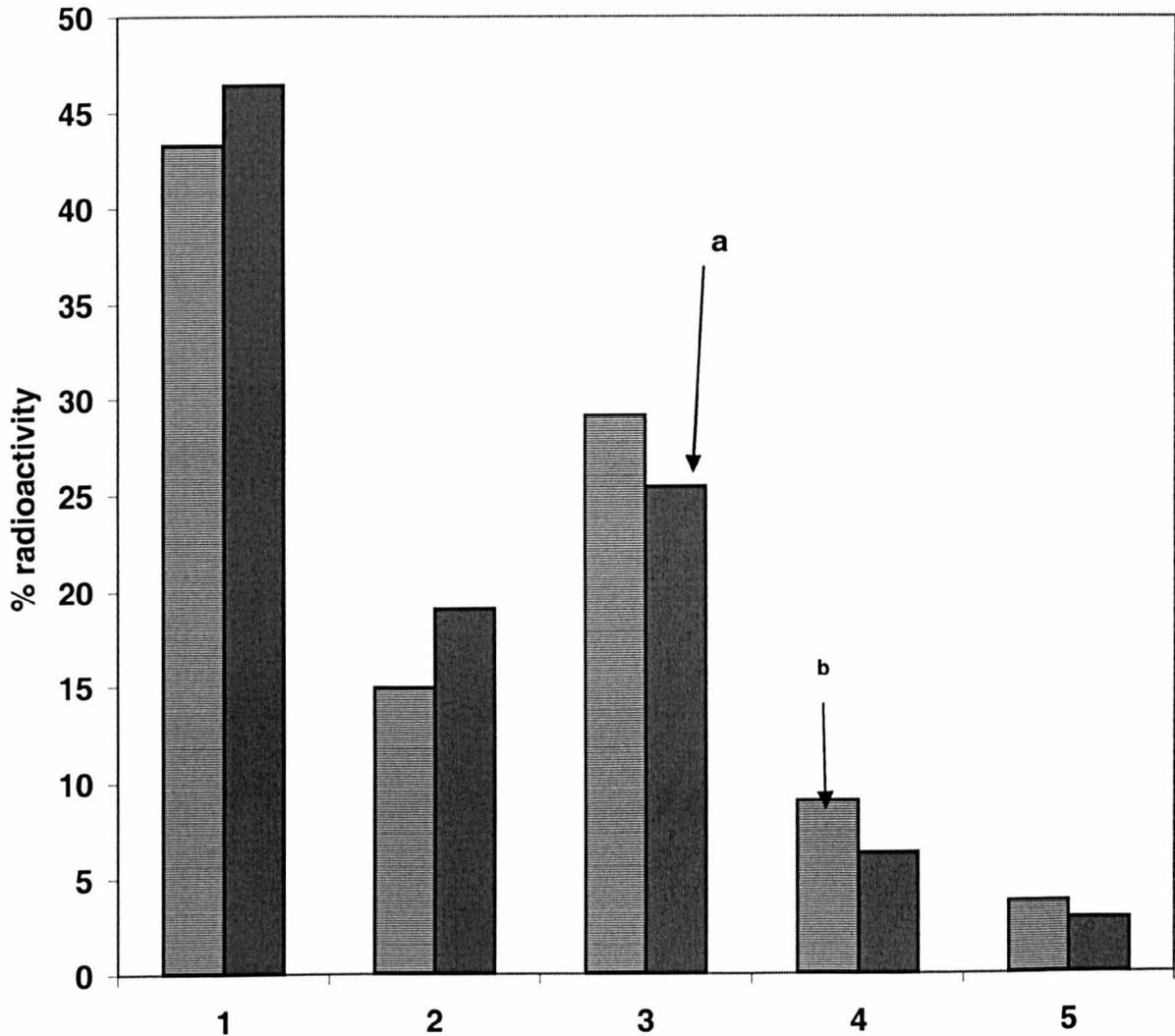


Fig 5.5 Effect of protease on binding $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) to Affigel 15 . To 1.5 ml of Affigel 2 ml of MOPS and 0.5ml of $[^{14}\text{C}]$ Gal-HMwt-complex (section 3.5) were added.

1. Supernatant after first wash.
2. After incubation for 4 hrs the radioactive solution was removed.
3. 1.5ml Proteases was added and solution removed.
4. After washing post treatment with different reagents.
5. Gel.

- a. Control group.
b. Protease

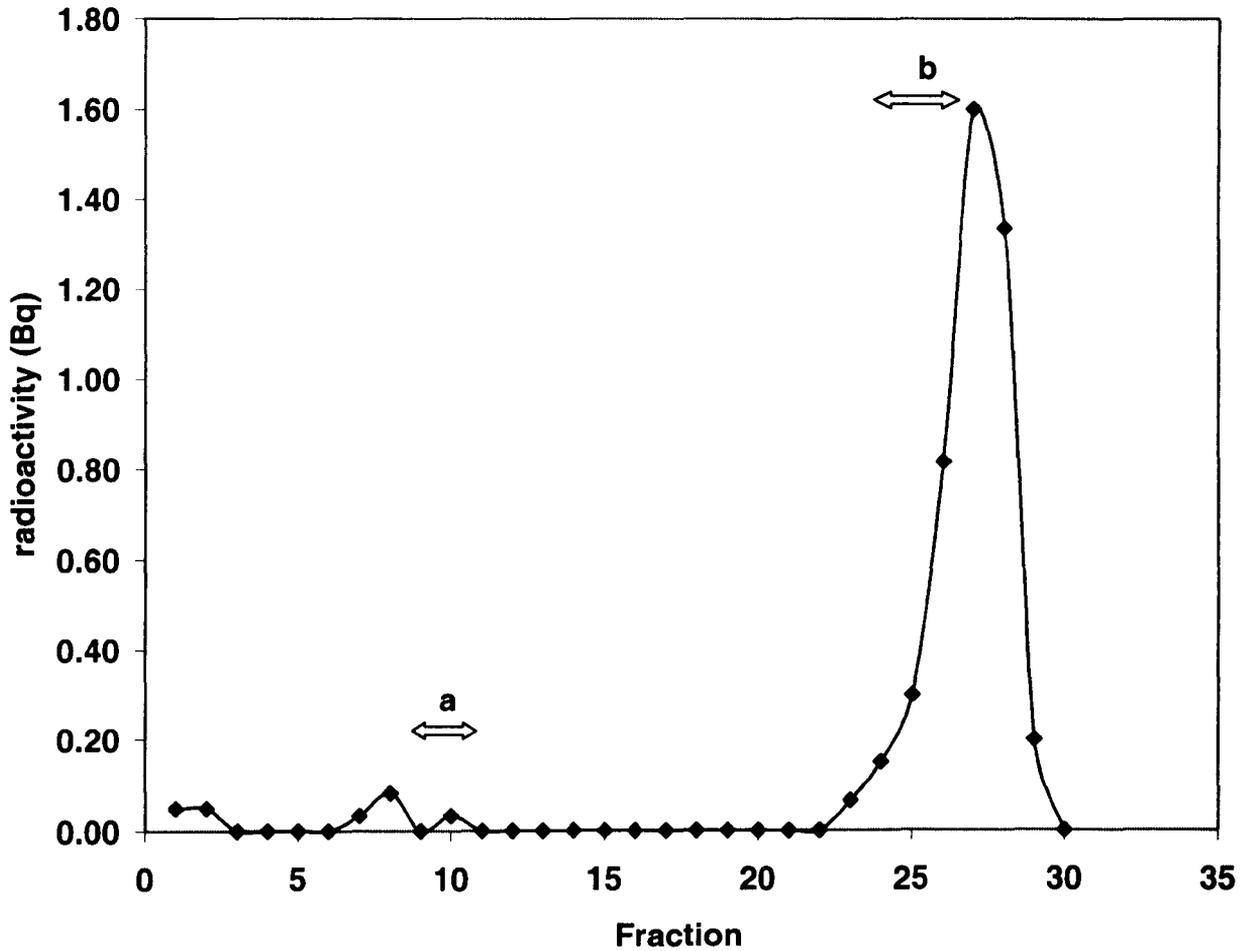


Fig 5.6 Analysis by gel filtration on Biogel P-10 of radioactivity released from Affigel by protease.

- a. Blue Dextran high molecular weight marker.**
- b. Cobalt chloride low molecular weight marker.**

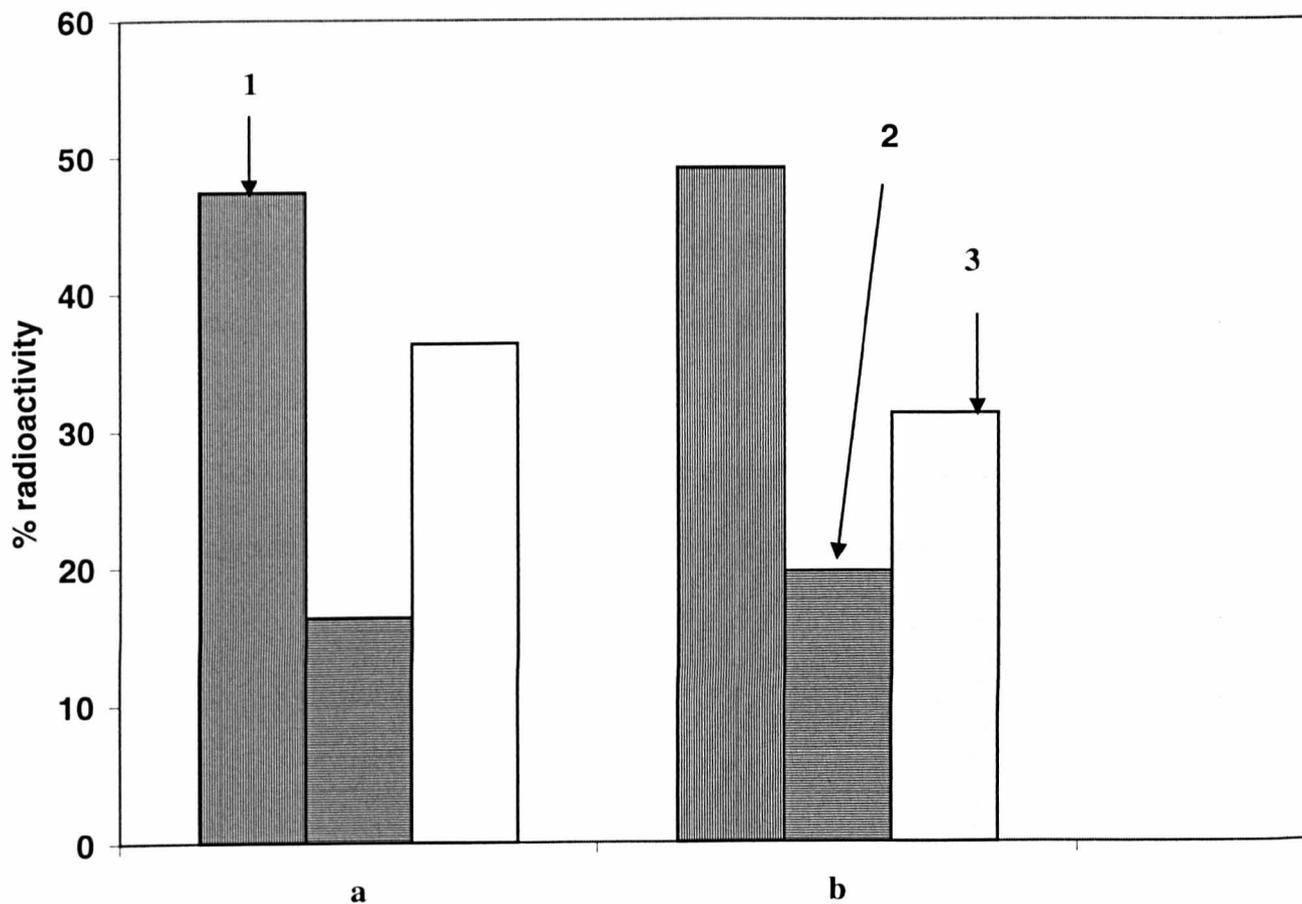


Fig 5.7 Effect of pre-treatment of Affigel with ethanolamine on subsequent binding of [^{14}C] Gal-HMwt-complex (section 3.5) to Affigel 15. Affigel 15 was incubated with 1M ethanolamine, pH 8, for 1 hr before binding to high molecular weight [^{14}C] pectin.

a. MOPS

b. MOPS+ ethanolamine.

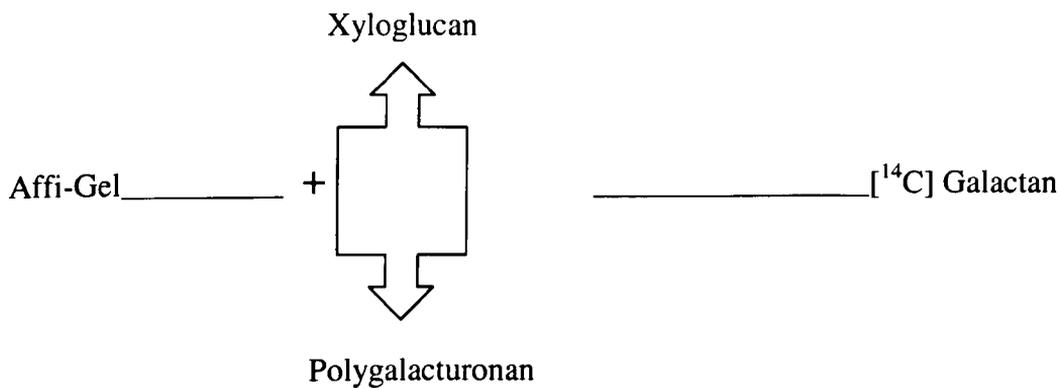
1. Supernatant 1

2. Supernatant 2

3. Gel

5.3.6 Effect of adding enzyme-treated complexes to the Affi-Gel.

This experiment was designed to investigate which part of the complex might be attached to Affi-Gel. Polysaccharidases were added to the [^{14}C] pectin complex and incubated for 2 hours at the appropriate pH. After the 2 hr incubation the enzyme treated complexes were added to different gel slurries with the proper amount of MOPS. They were shaken for 4 hours, and the radioactivity of the supernatants as well as gels was measured. Fig 5.8 showed that highest binding (25 %) was with the control where no enzyme was administered. All the enzyme-treated slurries had a decrease in the % binding to gel to 8-10%. This indicates that the part of the complex that is attached to the gel is linked to the [^{14}C]galactan through both xyloglucan and polygalacturonan.



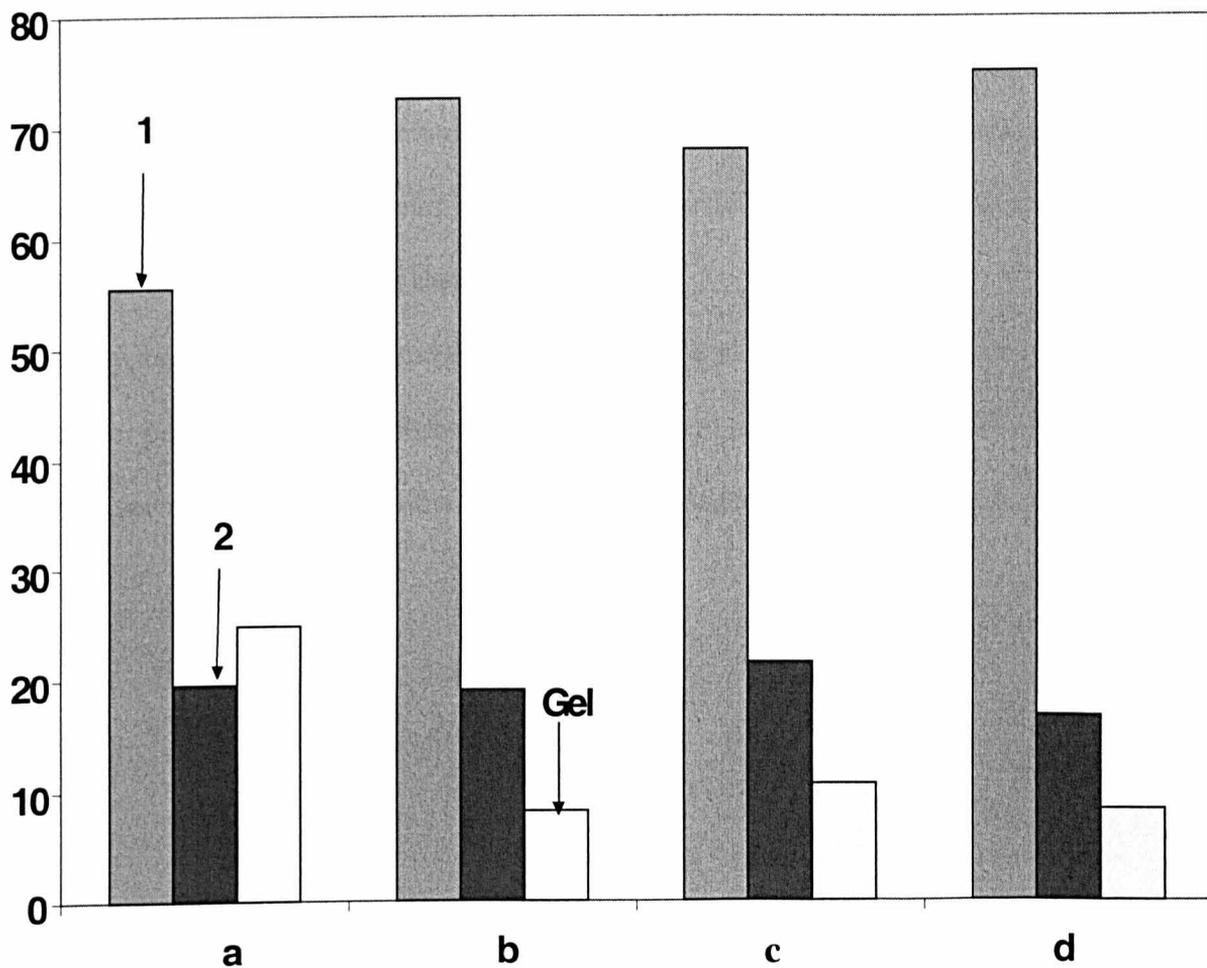


Fig 5.8 Effect of pre-treatment of [^{14}C] Gal-HMwt-complex (section 3.5) with polysaccharidases on its subsequent binding to Affigel 15.

- a. Control.**
- b. Cellulase treatment.**
- c. Polygalacturonase treatment.**
- d. Galactanase treatment.**

- 1. Supernatant 1.**
- 2. Supernatant 2.**
- 3. Gel.**

5.3.7 Effect of treating the complex (bound to Affi-gel) with various enzymes.

The [^{14}C] pectin complexes were added to different gel slurries with the usual amount of MOPS. They were shaken for 4 hours, and then treated with different polysaccharidases. The radioactivity of the supernatants as well as the gels was measured. Fig 5.9 shows that the lowest binding (8%) to the gel was with galactanase compared to control (43%) and both cellulase and polygalacturonase (34%) treated gel. This experiment showed that relatively little reduction in binding occurred when either xyloglucan or polygalacturonan was digested. It could be that the attachment of the [^{14}C] pectin complex to the Affi-gel was through galactan.

Alternatively, once the [^{14}C] pectin complex was attached to the gel, the presence of either xyloglucan or polygalacturonan might have been sufficient to maintain the attachment. This second explanation seems more likely in view of the results described in paragraph 5.3.6.

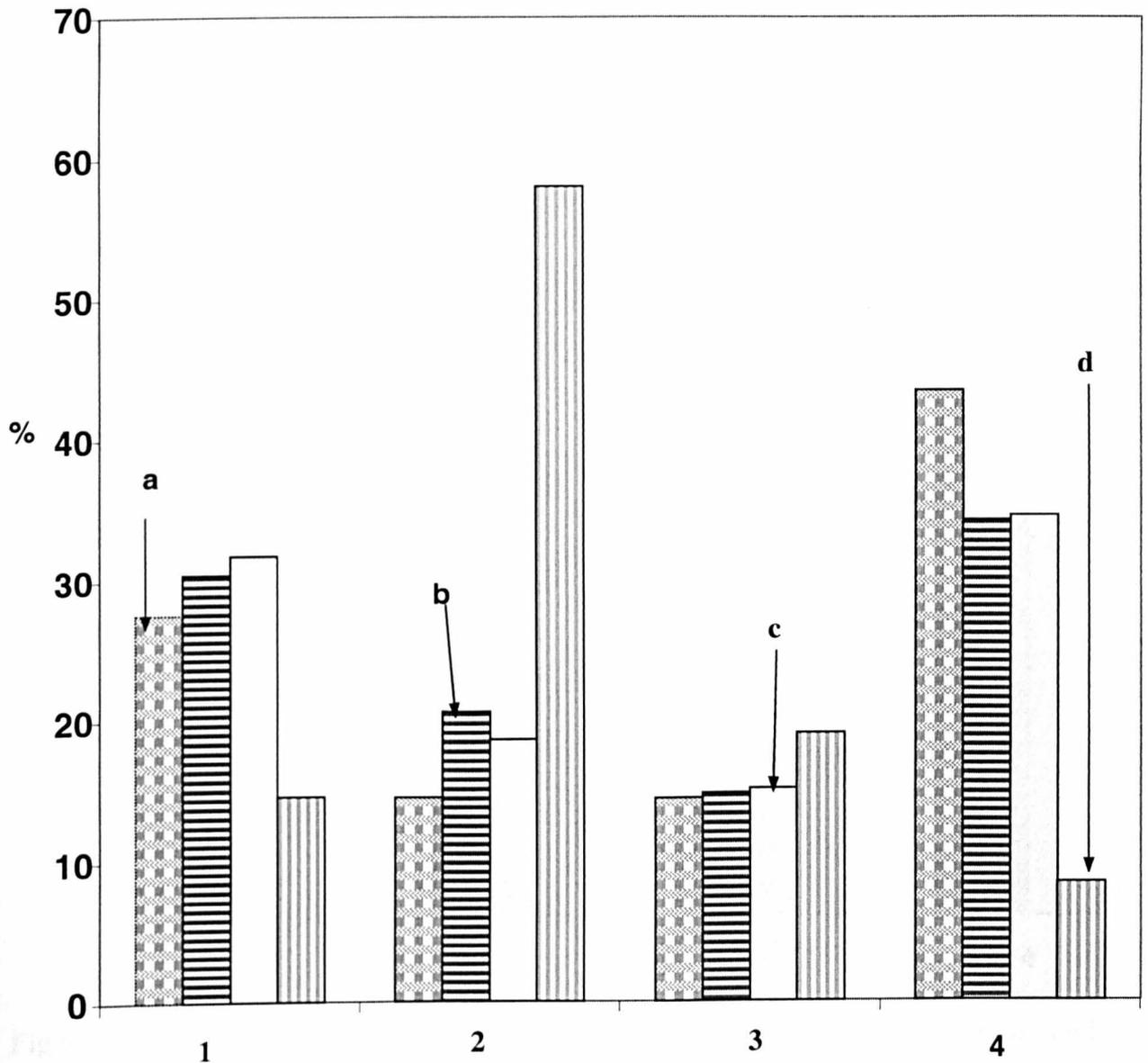


Fig 5.9 Effect of treatment of [^{14}C] Gal-HMwt-complex (section 3.5) with polysaccharidases after binding to Affigel 15.

[^{14}C] Gal-HMwt-complex (section 3.5) was linked to Affigel 15, after 4 hours it was treated with cellulase, polygalacturonase, or galactanase for a period of 2 hour.

- a. Control, buffer treated sample.
 - b. Cellulase treated sample.
 - c. Polygalacturonase treated sample.
 - d. Galactanase treated sample.
1. Supernatant 1
 2. Supernatant 3
 3. Supernatant 4
 4. Gel. No water wash was carried out between binding the complex to the gel and the enzyme treatment; hence no "supernatant 2" was obtained

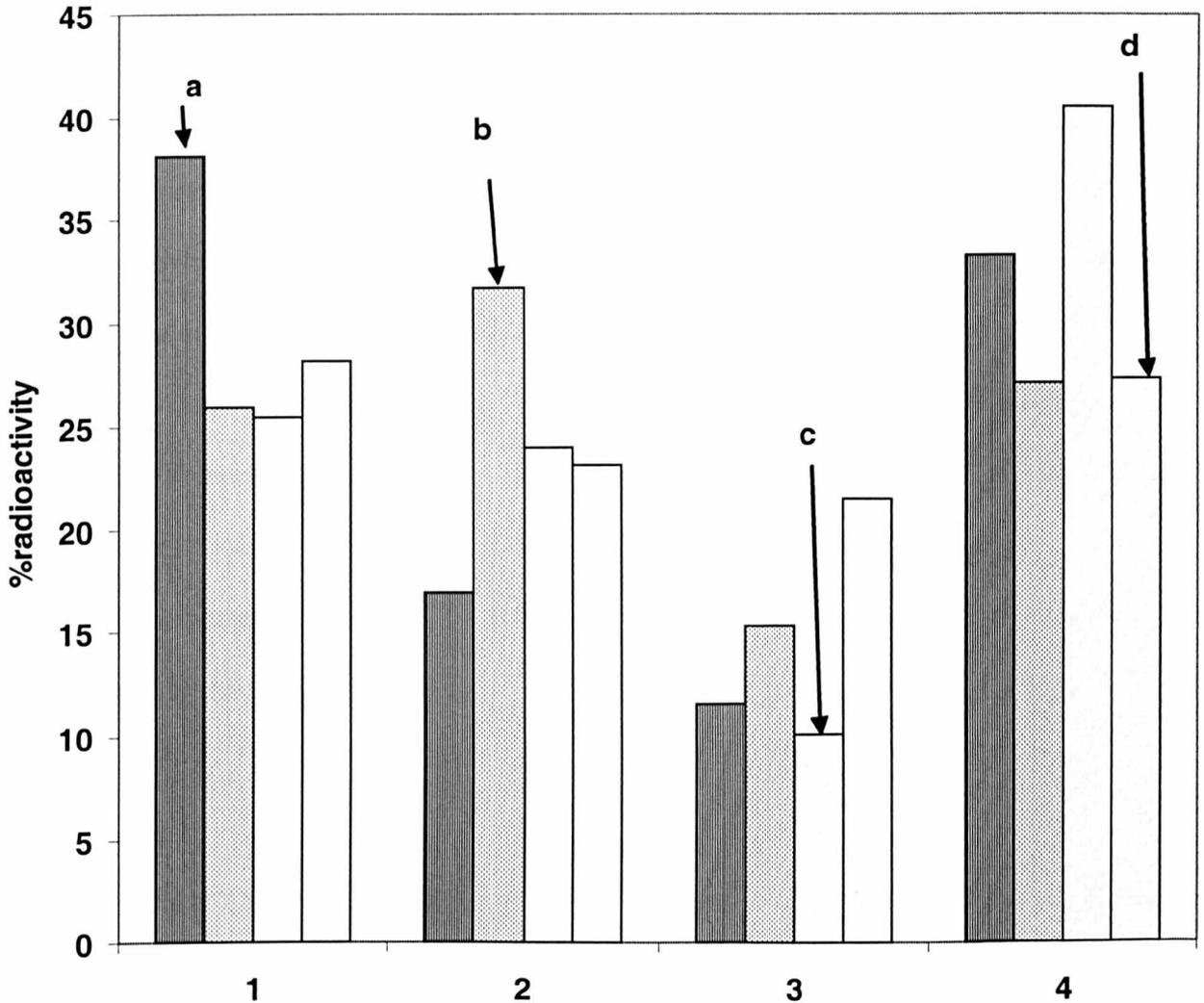


Fig 5.10 Effect of treatment of [^{14}C] Gal-HMwt-complex (section 3.5) with cellulase and polygalacturonase, separately and in combination, after binding to Affigel 15.

[^{14}C] Gal-HMwt-complex (section 3.5) was linked to Affigel 15, after 4 hours it was treated with cellulase, polygalacturonase, or a combination of cellulase and polygalacturonase for a period of 2 hour.

- e. Control, buffer treated sample.
- f. Cellulase treated sample.
- g. Polygalacturonase treated sample.
- h. A combination of cellulase and polygalacturonase.
 1. Supernatant 1
 2. Supernatant 3
 3. Supernatant 4
 4. Gel.

No water wash was carried out between binding the complex to the gel and the enzyme treatment; hence no "supernatant 2" was obtained.

5.3.8 Effect of adding endoglucanase and polygalacturonase separately or in combination to the complex bound Affi-Gel.

In order to test the hypothesis that once the [^{14}C] pectin complex was attached to the gel, the presence of either xyloglucan or polygalacturonan was sufficient to maintain the attachment, the experiment shown in Fig 5.9 was repeated, including a treatment in which the complex attached to affigel was treated with both cellulase and polygalacturonase. Fig 5.10 shows that the effect of treating the gel combined to the complex with either endoglucanase or polygalacturonase as well as their combined treatment did not greatly differ from that of the control. These results do not support the hypothesis. However, they can be explained by the possibility that xyloglucan and polygalacturonan portions of the complex might become inaccessible to added enzymes when the complex binds to the Affigel.

5.4 Conclusion

Treatment of the [^{14}C] pectin complex attached to the gel with reagents that break non-covalent bonds, led to some decrease in binding of the complex to the gel. Protease (P2143) treatment had a major effect on the binding of the complex to the gel. This suggests that protein was involved in the binding. Further investigations showed that this protease could be contaminated with enzymes that could degrade the [^{14}C] galactan. Therefore the results of protease treatment were not conclusive of protein involvement. Pre-treatment of Affi-Gel with ethanolamine showed that binding of the complex to the Affi-Gel did not require the active side-chains of the Affi-Gel. Hence there is no definite evidence for the involvement of protein in the binding process, which appears to be at least partly non-covalent. There is also no definite evidence of any protein present in the nascent [^{14}C] pectin complex.

Chapter 6

Binding of the nascent pectin complex to paper.

6.1. Introduction.

This section describes the binding of the nascent [^{14}C] Gal-HMwt-complex to cellulose paper. The requirements and conditions of the binding were also investigated.

6.2. Previous work.

It has been previously reported that xyloglucan binds to cellulose. There is a structural similarity between xyloglucan and cellulose. Since xyloglucan has a cellulose-like main chain composed of 1, 4-linked β -glucopyranosyl residues, the 1, 4- β -glucans may share a certain biosynthetic mechanism (Hayashi, 1989). XG is the major hemicellulosic component in the primary walls of dicotyledonous and non-graminaceous monocotyledonous plants (Pauly *et al.*, 1999). The most important feature of XGs is their ability to form strong, non-covalent associations with cellulose (Hayashi, 1989; Hayashi *et al.*, 1994). XGs bind to cellulose *in vitro* in a pH-independent manner (Hayashi *et al.*, 1987), suggesting that the formation of hydrogen bonds is involved in the association of these polymers (Pauly *et al.*, 1999). Features of the current cell-wall model indicate that xyloglucan binds to cellulose *in vivo* (Brett & Waldron, 1996), while pectin is not thought to do so.

Fry *et al.* (1992) showed that high molecular weight xyloglucan binds strongly to filter paper. Drying onto Whatman 3 MM paper, and then subjecting the paper to prolonged washing with water was originally introduced by Fry *et al.* (1992) as part of the assay for XET.

6.3. Materials and Methods.

Nascent [^{14}C] Gal-HMwt-complex was prepared as described in section 2.2. In the experiments shown in Figs 6.1 and 6.2, the high-molecular-weight material was obtained by passage down Biogel P10. In the experiments described in the rest of the chapter, Sepharose CL-2B was used in place of Biogel P10.

For the investigation of binding to paper, one of two basic methods was followed. The first method involved the spotting of the HMwt [^{14}C] nascent pectin complex, which had been incubated with Tris-HCl along with various enzymes, directly on a 2x2 cm piece of 3MM paper. After air-drying the samples on paper, the papers were then washed out with running tap water for a period of one hour. Then the papers were dried and added to scintillant for determining the radioactivity.

The second method, which was adapted from that of Thompson & Fry (2000), involved the addition of the HMwt [^{14}C] nascent pectin complex to Tris-HCl and water in a beaker. Then 2x2 cm pieces of the 3MM papers were soaked in water, and then placed in the above preparation, followed by shaking using an orbital shaker for different time intervals. After the specified time, the papers were removed from the corresponding beakers. The radioactivities of both the liquids and the papers were measured and reported.

6.4 Rationale.

The binding behavior of high molecular weight nascent pectin complex to 3 MM paper was characterized. The effect of unlabelled pectin, xyloglucan or different enzymes on this process was studied, to determine whether the binding was due to the presence of xyloglucan in the complex.

6.5 Effect of enzymes on the binding of nascent [¹⁴C]Gal-HMwt-complex to paper, using the first binding method.

[¹⁴C] nascent pectin complex was passed through a P-10 column and the high molecular weight material was collected. The HMwt [¹⁴C] nascent pectin complex was incubated for 2 hours with polysaccharidases as well as buffer at 37°C, and the reaction was stopped by boiling for 15 minutes. All samples were then spotted on 2x2 cm pieces of 3MM paper and left to dry. The papers were then washed out with running water for 1 hour, and left to dry. Dried papers were added to the scintillant and their radioactivity was measured. Fig 6.1 shows that about 30% of the radioactivity remained bound to the paper using this method. Treatment of the samples with galactanase led to a decrease in the radioactivity, which was expected because the radioactive label was in galactan. Treatment with endo-β 1, 4-glucanase also led to a decrease in the radioactivity bound to the paper. This suggested that the binding of the complex to the 3 MM paper was due to the presence of xyloglucan in the complex.

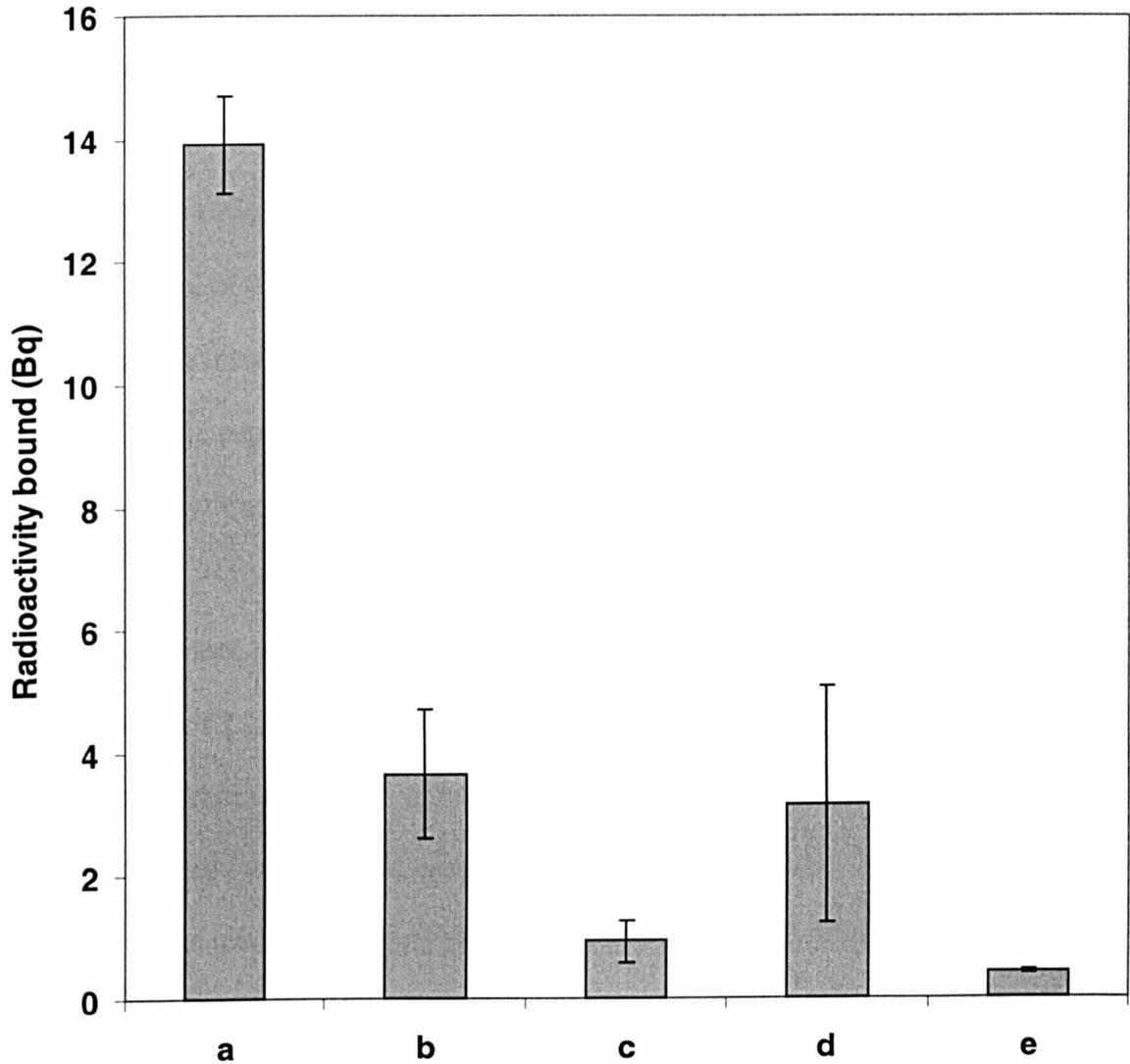


Fig 6.1 Nascent [^{14}C] Gal-HMwt-complex, collected by gel filtration on P-10 of the membrane preparation extracted with hot EDTA-phosphate (100°C), was treated with different enzymes or buffer only.

Samples were then spotted onto 3 MM paper. After drying, papers were placed under running water for 1 hour and then dried, and radioactivity bound to the paper was measured.

- a.** Unwashed control.
- b.** Washed control.
- c.** Cellulase treated sample.
- d.** Polygalacturonase treated sample.
- e.** Galactanase treated sample.

6.6. Time course of binding, using the second binding method.

All the following experiments followed the second method of adding the pectin complex to the 3 MM paper.

Nascent [^{14}C] Gal-HMwt-complex (0.8ml), prepared by passing through Biogel P-10, was added to 0.2ml of 0.1 M Tris-HCl pH 7. A circle of 3 MM paper was placed in each sample, these were incubated on an orbital shaker for different lengths of time, and the liquid as well as the paper were then tested for their radioactivity. This experiment showed that the binding of radioactivity to the paper reached a plateau at around 2-3 hour (Fig .6.2).

In another experiment, nascent [^{14}C]Gal-HMwt-complex (0.8ml), prepared by passing through Sepharose CL2B, was added to 0.2 ml 0.1M Tris-HCl pH 7 and 2.7 ml water, and this material was then divided into 4 beakers, to which 2x2 cm 3MM papers were added and incubated for 4 different periods of time. Fig 6.3 shows that the binding reached 62% of the total radioactivity after 60 minutes of incubation with the paper, and 80% after 4 hours of incubation.

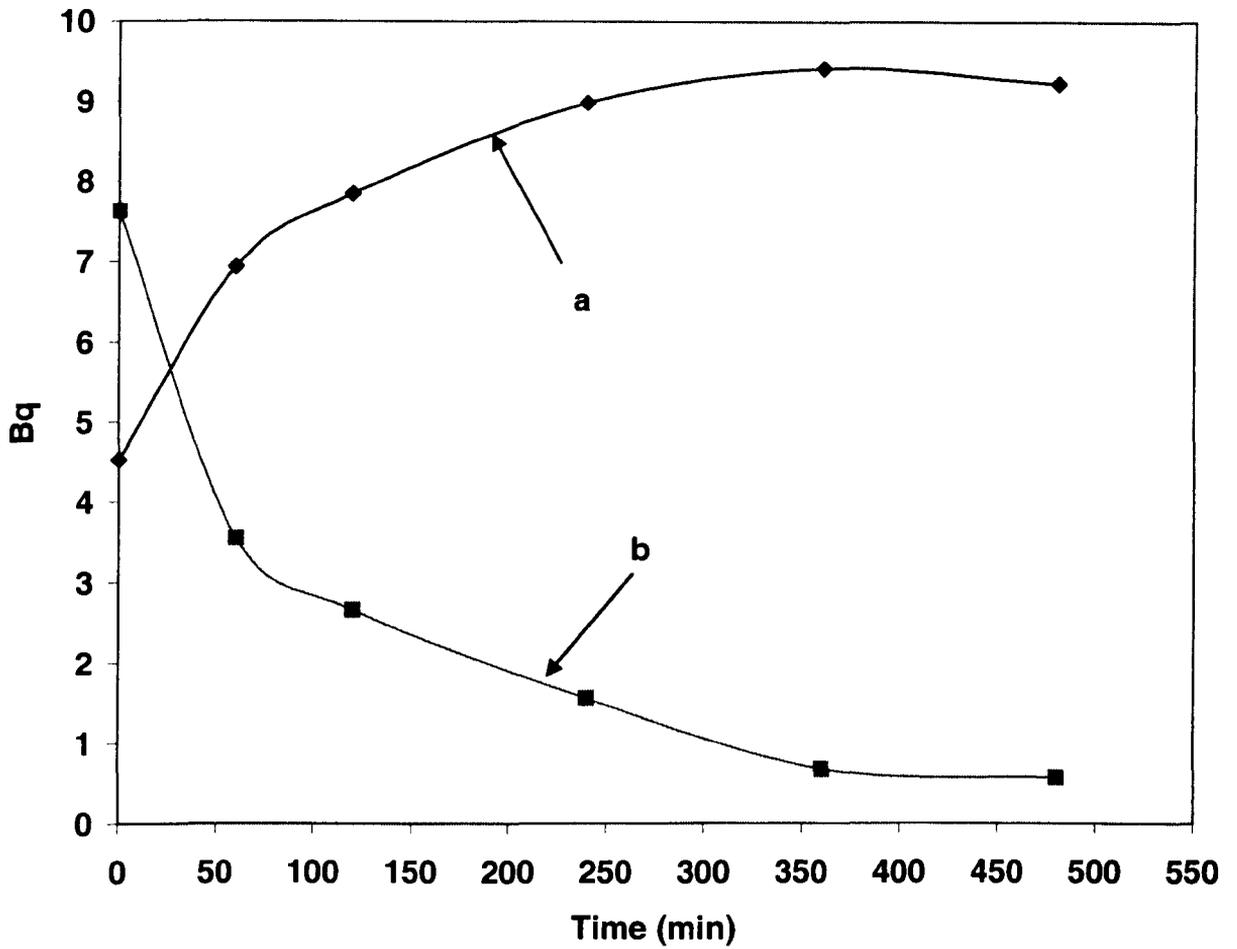


Fig 6.2 Time course of binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex collected on P-10 column.

- a. Radioactivity read on paper.
- b. Radioactivity read in the liquid.

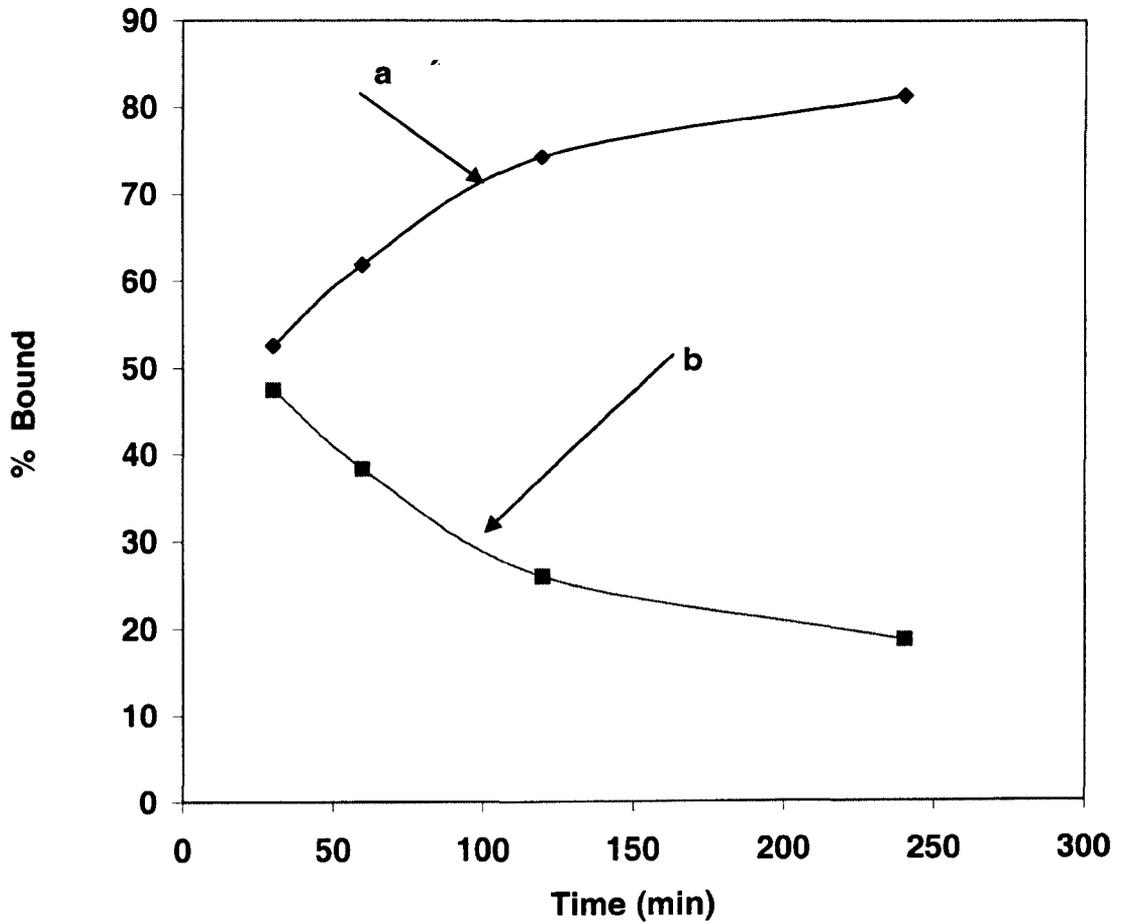


Fig 6.3 Time course of binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex collected on CI-2B.

- a.** The percentages of radioactivity bound to paper.
- b.** The percentages of radioactivity remaining in solution.

In a similar experiment carried out in duplicate, nascent [^{14}C] Gal-HMwt-complex (0.8ml), collected by passage through Sepharose CL-2B, was added to 2.4ml Tris-HCl and 21 ml water, and this material was then divided into 8 portions. 2x2 cm, 3MM papers were soaked in water, removed, and then placed in different beakers with each portion on an orbital shaker for different time intervals (Fig 6.4). This experiment shows that binding continued to increase for at least 4 hours.

6.6.1 Effect on binding of different kinds of pectin.

Though it was expected that xyloglucan was the main component responsible for binding to paper, it is possible that pectin itself may bind to paper to some extent. So the effect of unlabelled (non-radioactive) pectin on binding of the radioactive component to paper was therefore studied. The effect of adding 1% pectin from apples, citrus fruits and pectins of various degrees of esterification on binding of nascent [^{14}C] Gal-HMwt-complex to paper was studied. Fig 6.5 shows that the highest inhibition of binding of the radioactive complex to the paper was in the presence of citrus pectin.

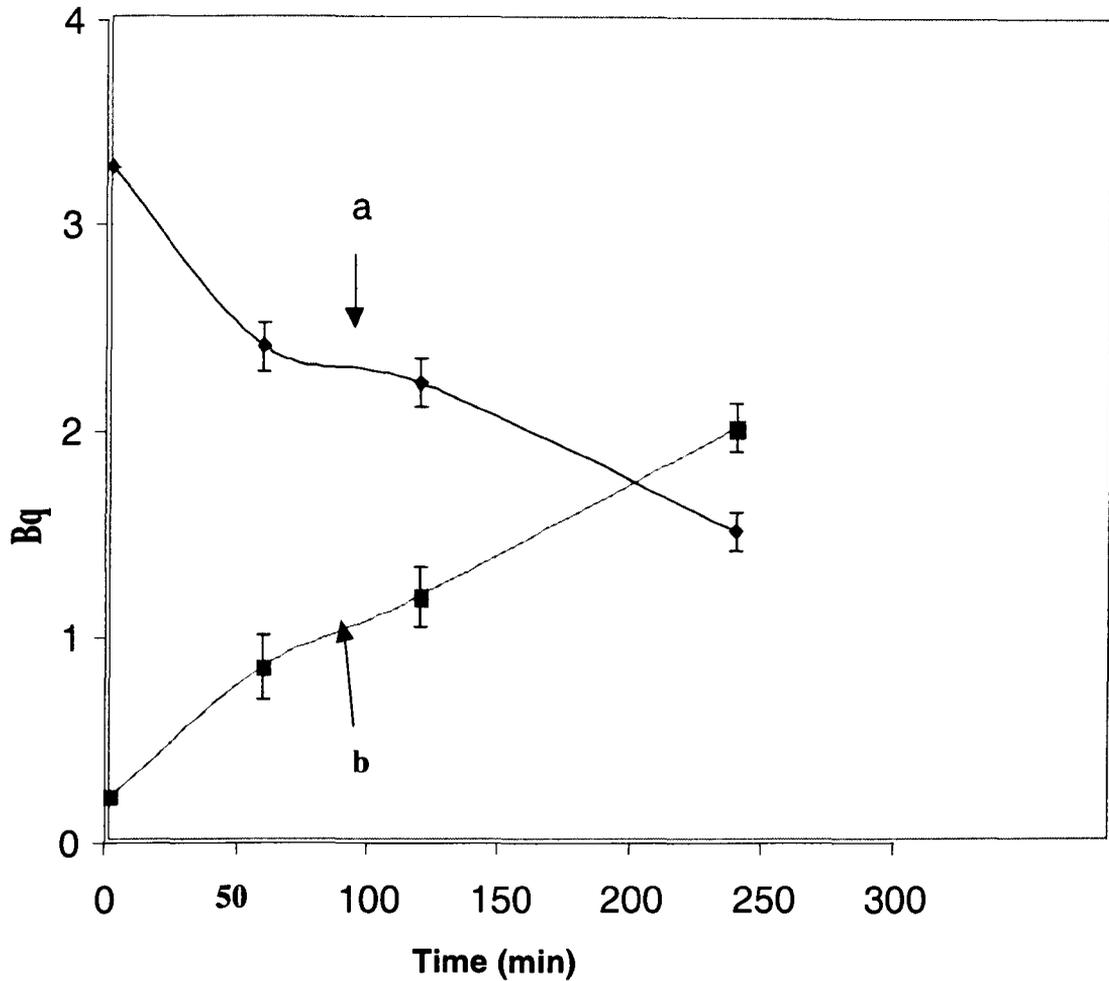


Fig 6.4. Time course of binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex collected by gel filtration on CL-2B.

Nascent [^{14}C] Gal-HMwt-complex (0.8ml) was added to 2.4ml Tris-HCl and 21 ml water, and this material was then divided into 8 portions. 3MM papers (2x2 cm) were soaked in water, removed, and then placed in different beakers with each portion on an orbital shaker for different time intervals.

- a. Radioactivity read in the liquid.
- b. Radioactivity read on paper.

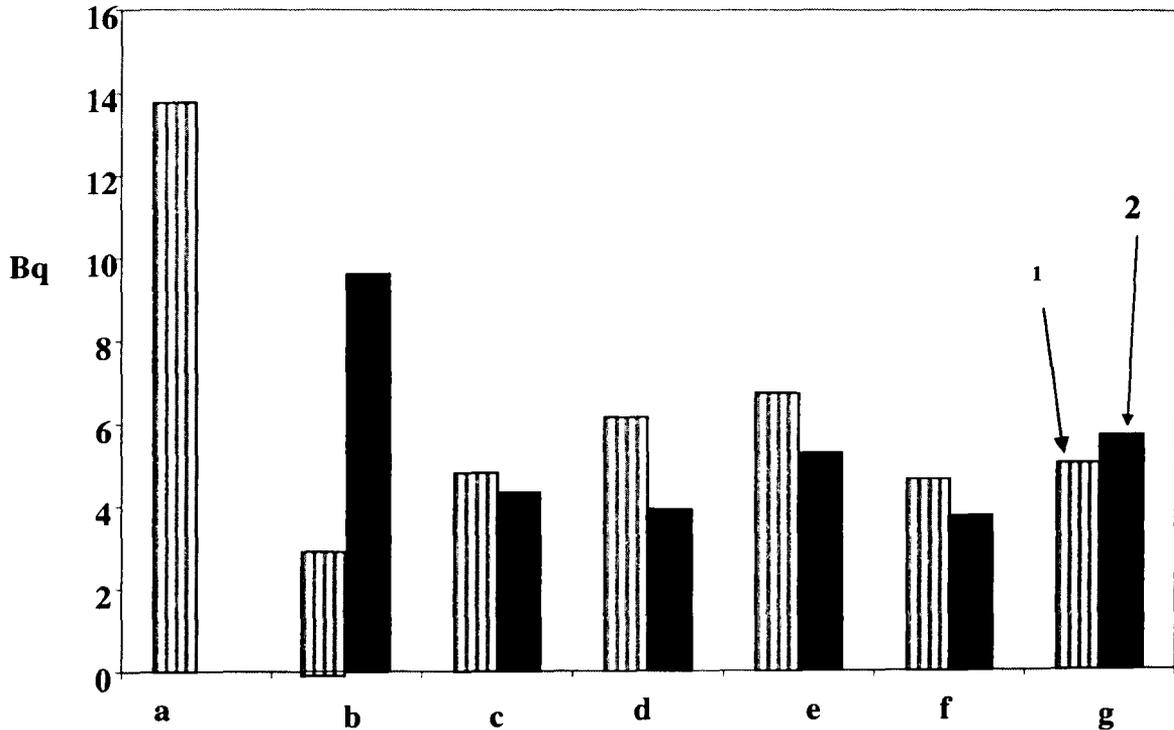


Fig.6.5 Effect of non-radioactive pectin 1% on the binding to 3 MM paper of nascent [^{14}C] Gal-H.Mwt-complex, in the presence of different types of unlabelled pectins, as well as with different levels of esterification of pectin, after 120 minutes of incubation.

1. Radioactivity read in the liquid.
 2. Radioactivity read on paper.
- a. Radioactivity of [^{14}C] Gal-H.Mwt-complex alone.
 - b. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex after 120 min of incubation.
 - c. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex + apple pectin after 120 min of incubation.
 - d. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex + citrus pectin after 120 min of incubation.
 - e. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex + 34% esterified pectin after 120 min of incubation.
 - f. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex + 68% esterified pectin after 120 min of incubation.
 - g. Radioactivity read on paper and liquid of [^{14}C] Gal-H.Mwt-complex + 98% esterified pectin after 120 min of incubation.

6.6.2 Effect of different concentrations of pectin on binding.

Citrus pectin (1.5 ml of 2%, 0.2%, 0.02%, 0.002% and 0%) was added to 0.2 ml Tris-HCl, 0.8ml radioactively labeled pectin and 0.5 ml water, covered with Nescofilm and incubated on an orbital shaker for 120 min, to make up pectin percentages of 1, 0.1, 0.01, 0.001 and 0 in the incubations.

Concentrations of 0.1% or more were needed for a significant effect to be seen (Fig 6.6). This interference could be due to competition between citrus pectin and the labeled pectin complex for binding sites on the paper. If so, then this would imply that pectin itself binds to paper to some extent. The fact that citrus pectin prevents the binding of the complex to 3MM paper is not born out in previous literature. There is a possibility that citrus pectin is impure because it might contain xyloglucan. However an alternative explanation is that citrus pectin binds directly to the labeled pectin complex in solution, thus preventing it binding to the paper. This explanation would not imply that pectin bound to paper.

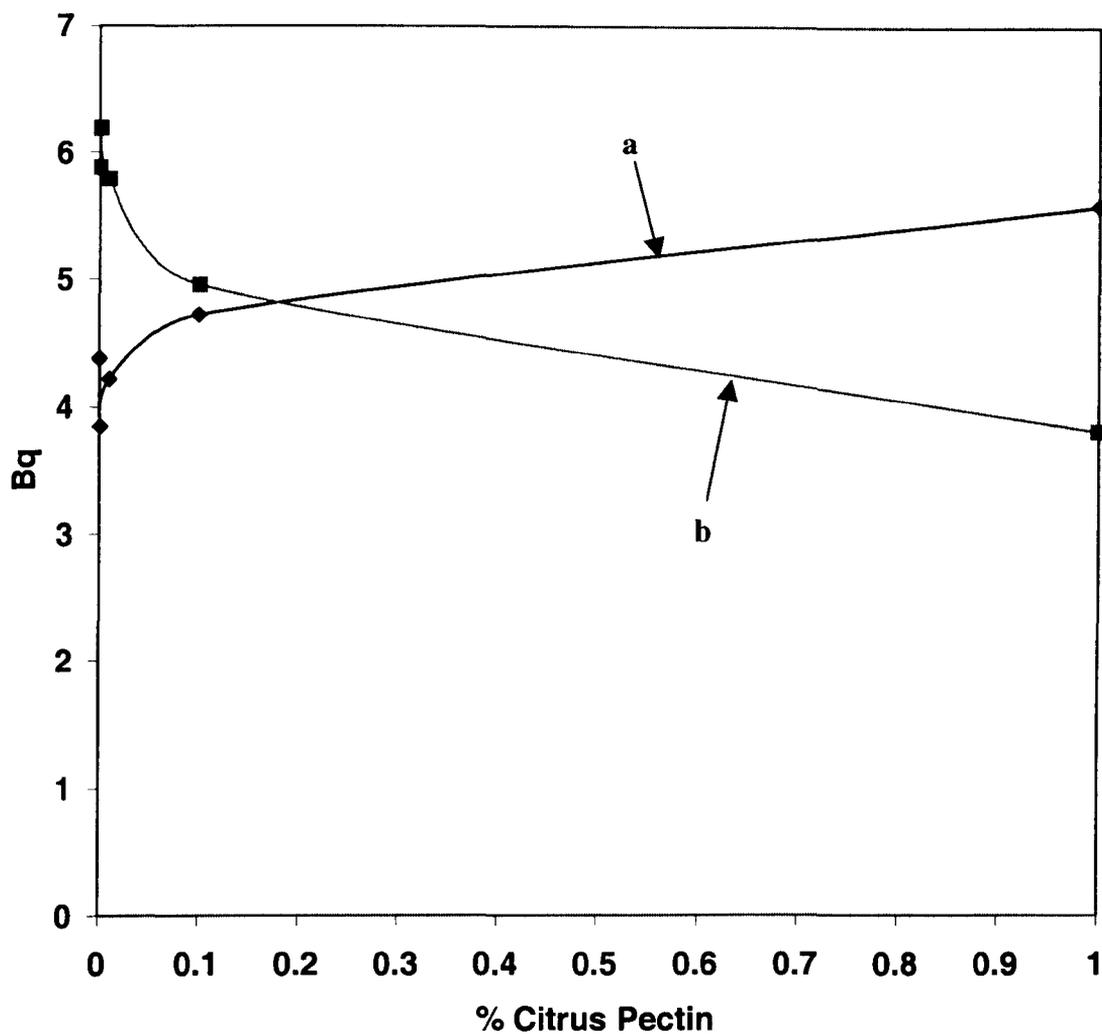


Fig.6.6 Effect of different concentrations of non-radioactive citrus pectin on the binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex. Different percentages of unlabelled citrus pectin incubated with 3 MM paper for a period of 120 minutes.

- a. Radioactivity in the paper.**
- b. Radioactivity in the liquid.**

6.6.3. Two time points of binding with prepared pea pectin.

Tris-HCl (0.1M, 1.2 ml) was added to 0.8 ml nascent [¹⁴C] Gal-HMwt-complex, collected by passage through Sepharose CL-2B, with 1.5 mls unlabeled pea pectin or water. 2x2 cm of the 3 MM papers were pre-soaked in water, added to the samples, and incubated for 2 or 240 min. Fig 6.7 shows that 57% of radioactivity was bound to the paper after 4 hours of shaking in the control. In contrast, the paper soaked in the solution containing unlabelled pectin had an attachment of 28.7% to the paper, indicating the decrease of binding in the presence of unlabelled pectin.

6.6.4. Time course of binding with xyloglucan.

To determine whether externally prepared xyloglucan addition had an effect on binding, the following experiment was performed. The radioactive complex (0.1 ml) was added to 0.5ml 2% xyloglucan, 1.5ml Tris-HCl pH 7.4 and 1ml water. Fig 6.8 shows a small increase of the absorption of the radioactivity to the paper till time 60 minutes, after which absorption leveled off. This showed that xyloglucan inhibited binding more strongly than non-radioactive pectin did.

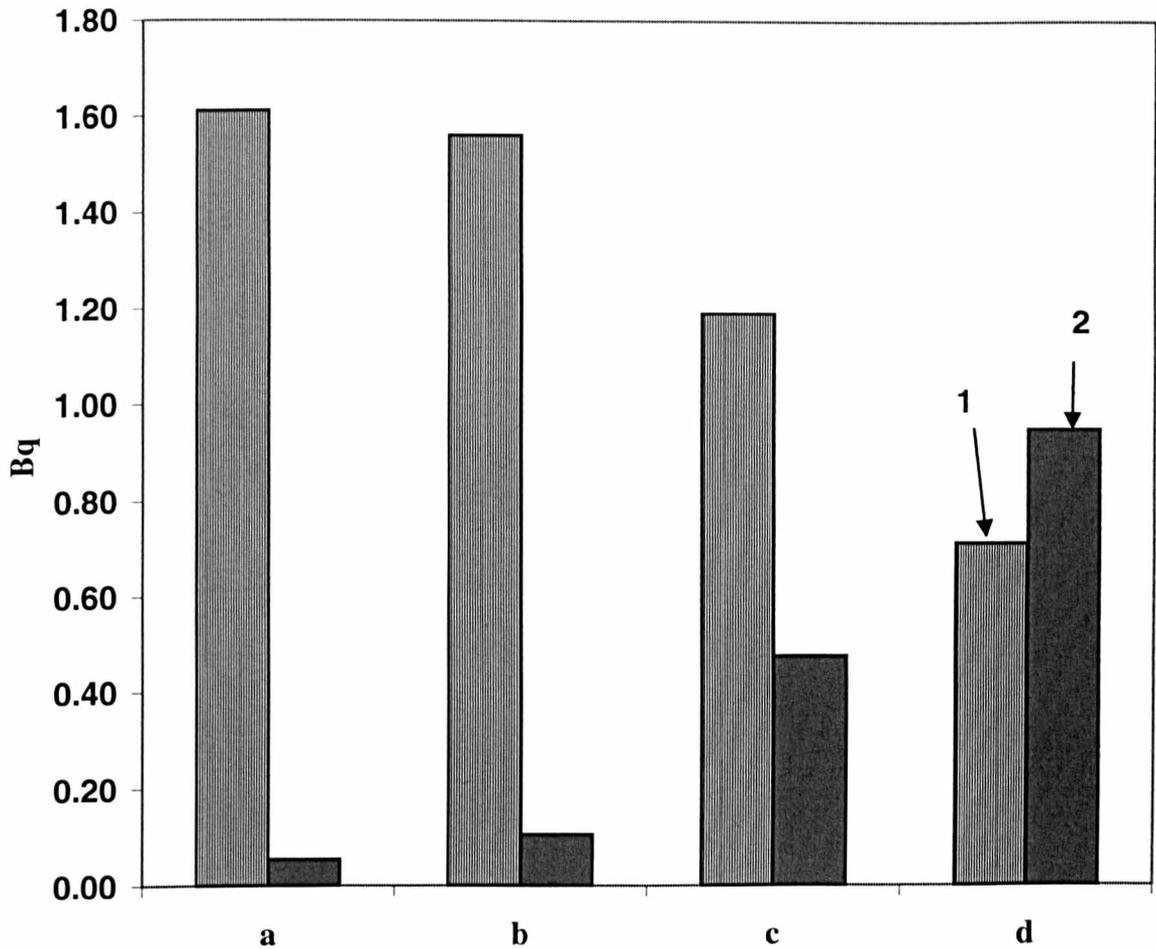


Fig.6.7 Effect of non-radioactive pea pectin on the binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex. Binding of labelled pectin in the presence of pea unlabelled pectin to 3 MM paper was measured at 2 and 240 minutes.

1. Radioactivity read in the liquid.
2. Radioactivity read on paper.

- a. Radioactivity of [^{14}C] Gal-H.Mwt-complex alone measured after 2 min.
- b. Radioactivity of [^{14}C] Gal-H.Mwt-complex with unlabelled pectin measured after 2min.
- c. Radioactivity of [^{14}C] Gal-H.Mwt-complex alone measured after 240 min.
- d. Radioactivity of [^{14}C] Gal-H.Mwt-complex with unlabelled pectin measured after 240 min.

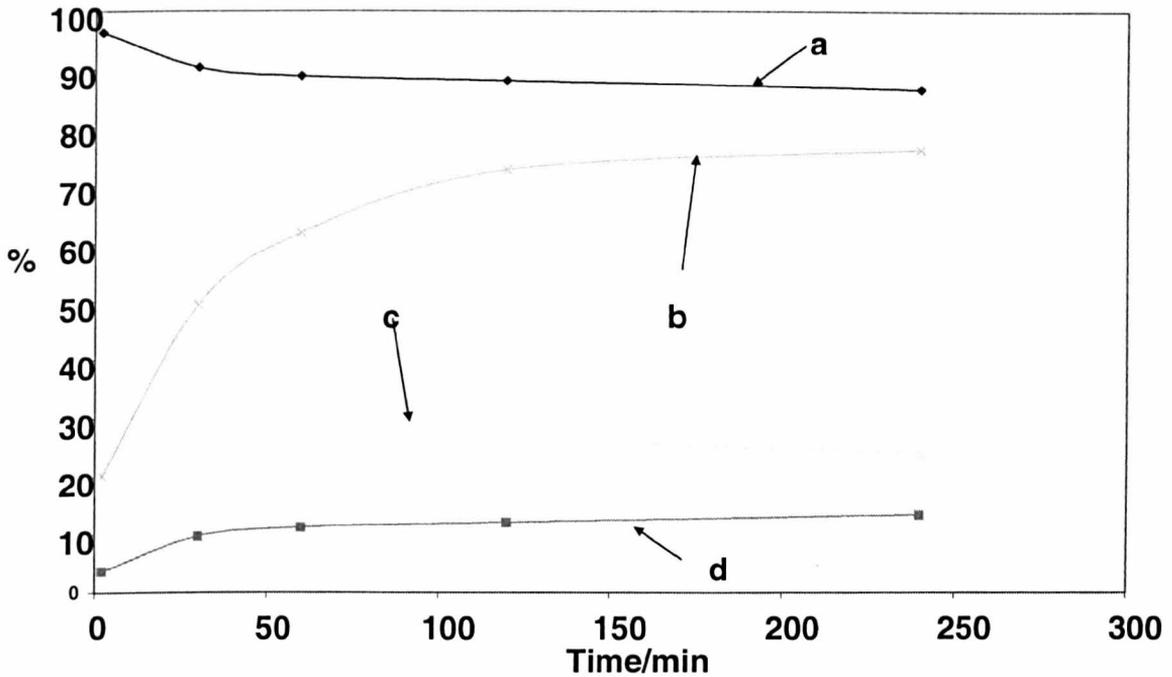


Fig.6.8 Effect of xyloglucan on the binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex.

A time course of % binding to 3MM paper in the presence of 0.2% xyloglucan was measured as well as binding in the absence of xyloglucan.

- a.** Radioactivity read in the liquid containing xyloglucan as well as the [^{14}C] Gal-HMwt-complex.
- b.** Radioactivity read in paper in the absence of xyloglucan powder.
- c.** Radioactivity read in liquid in the absence of xyloglucan powder.
- d.** Radioactivity read in the paper containing xyloglucan as well as the [^{14}C] Gal-HMwt-complex.

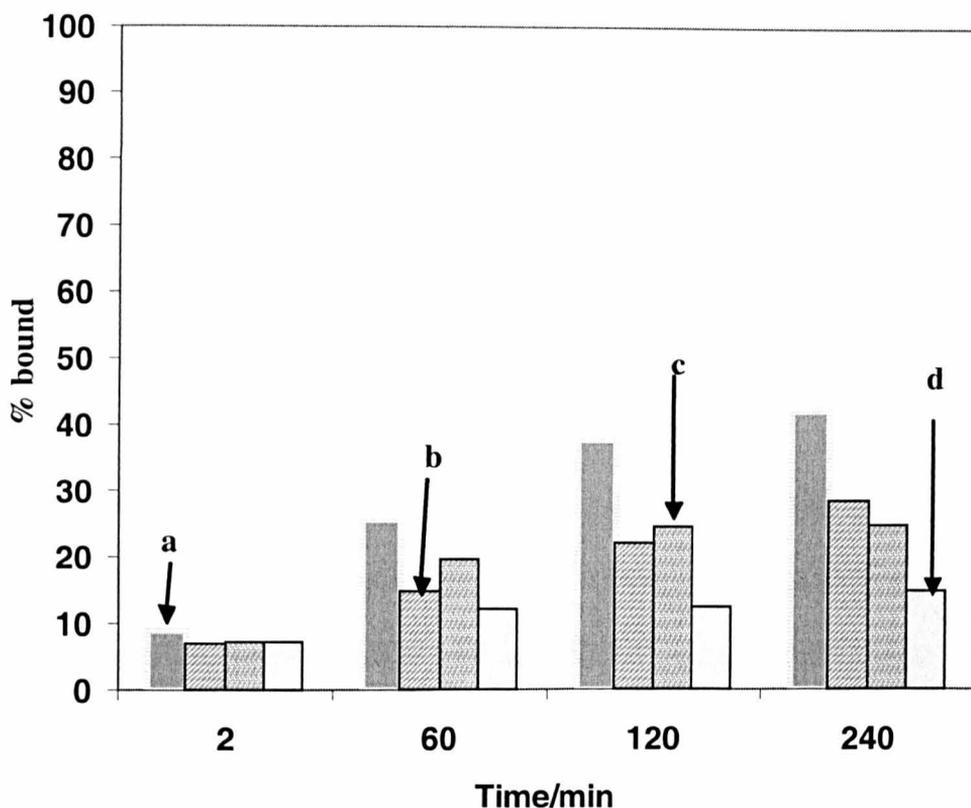


Fig 6.9 Effect of pre-treatment with enzymes on the binding to 3 MM paper of nascent [^{14}C] Gal-HMwt-complex. 0.8ml of treated sample was added to 1.6ml Tris-HCl (100mM, pH 7.4) and 10ml water. Pieces (2x2cm) of 3MM paper were soaked in water and then added to each beaker, and these were then incubated for different lengths of time.

- a. Control, treated with buffer only
- b. Cellulase treated nascent [^{14}C] Gal-HMwt-complex.
- c. Polygalacturonase treated nascent [^{14}C] Gal-HMwt-complex.
- d. Galactanase treated nascent [^{14}C] Gal-HMwt-complex.

6.7. Effect of enzymes on binding.

H.Mwt [^{14}C] nascent pectin complex was treated for 2 hours with different enzymes and then Tris-HCl was added to the various preparations, followed by 3MM paper. Fig 6.9 shows that after 60 minutes of incubation on an orbital shaker, 12 % of the galactanase-treated sample was bound to the paper, compared to 15% with endo- β 1, 4-glucanase, 20% with polygalacturonase and 26% with the control. After 240 minutes of incubation, 14% of the galactanase-treated sample was bound to the paper, compared to 28% with endo- β 1, 4-glucanase, 25% with polygalacturonase and 42% with the control.

6.8. Effect of pH on binding.

Nascent [^{14}C] Gal-HMwt-complex (0.8ml) was added to 0.2 ml 0.1 M buffer (either Tris-HCl pH 8, or acetate buffer pH's 2, 4 and 6); along with 0.5 ml water, and shaken for 60 min covered with nescofilm. Supernatants as well as papers were measured for their radioactivity. Fig 6.10 shows that paper binding was not pH dependent, because all the percentages of radioactivity binding to the paper were similar, ranging from 70-77%.

6.9. Effect of TFA treatment on binding.

Nascent [^{14}C] Gal-HMwt-complex (2ml) was added to 14.8 μl of TFA or water as control. They were left in boiling water (100°C) for 1 hour. Tubes were then dried out by blowing nitrogen gas through. Tris HCl (2 ml) was added to 18 mls of water to make up duplicate samples to study the time course of binding to 3 MM paper. Fig 6.11 shows that the binding after treatment with TFA had decreased compared to Fig 6.12 in which the complex was boiled with water as control. This indicates that mild acid treatment had an

effect on the binding behaviour. This suggests that the radioactive galactan part of the complex was linked to xyloglucan through an acid-labile linkage.

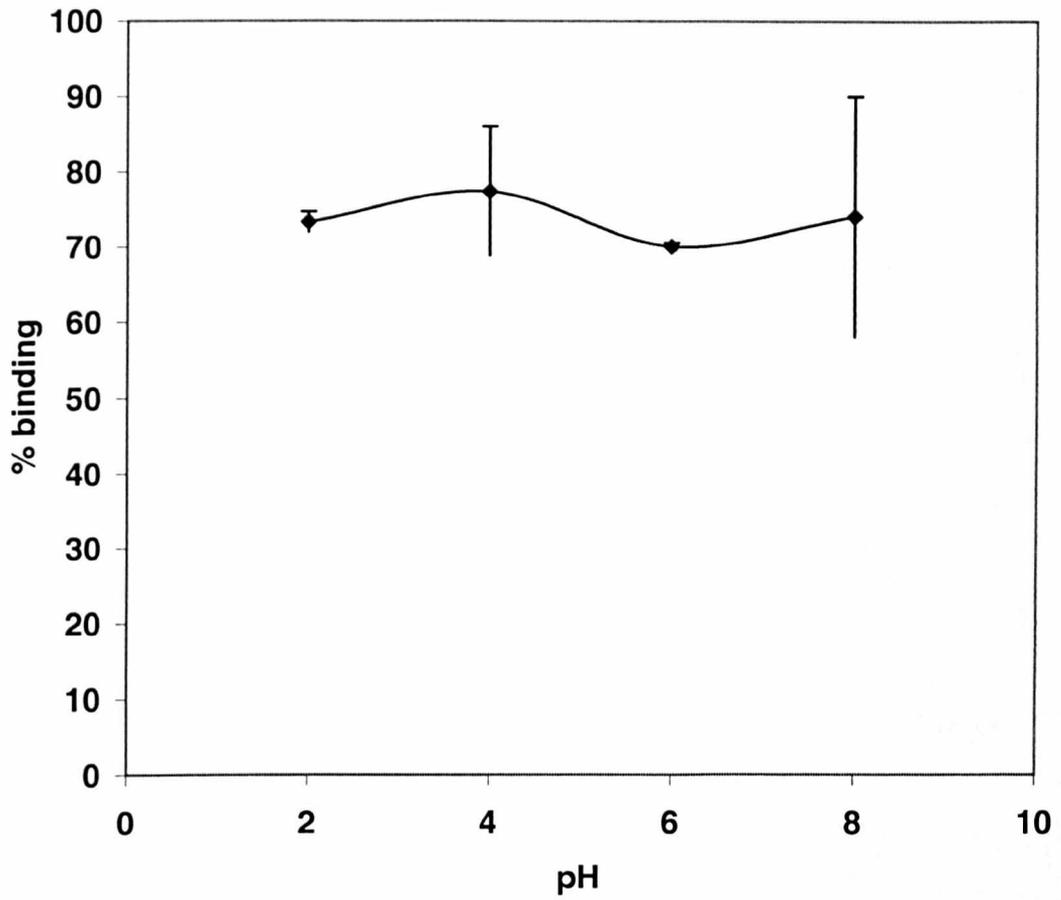


Fig.6.10 Nascent [^{14}C] Gal-HMwt-complex (0.8ml) was added to 0.2 ml 0.1 M buffer (either Tris-HCl pH 8, or acetate buffer pH's 2, 4 and 6); along with 0.5 ml water, and shaken for 60 min covered with nescofilm.

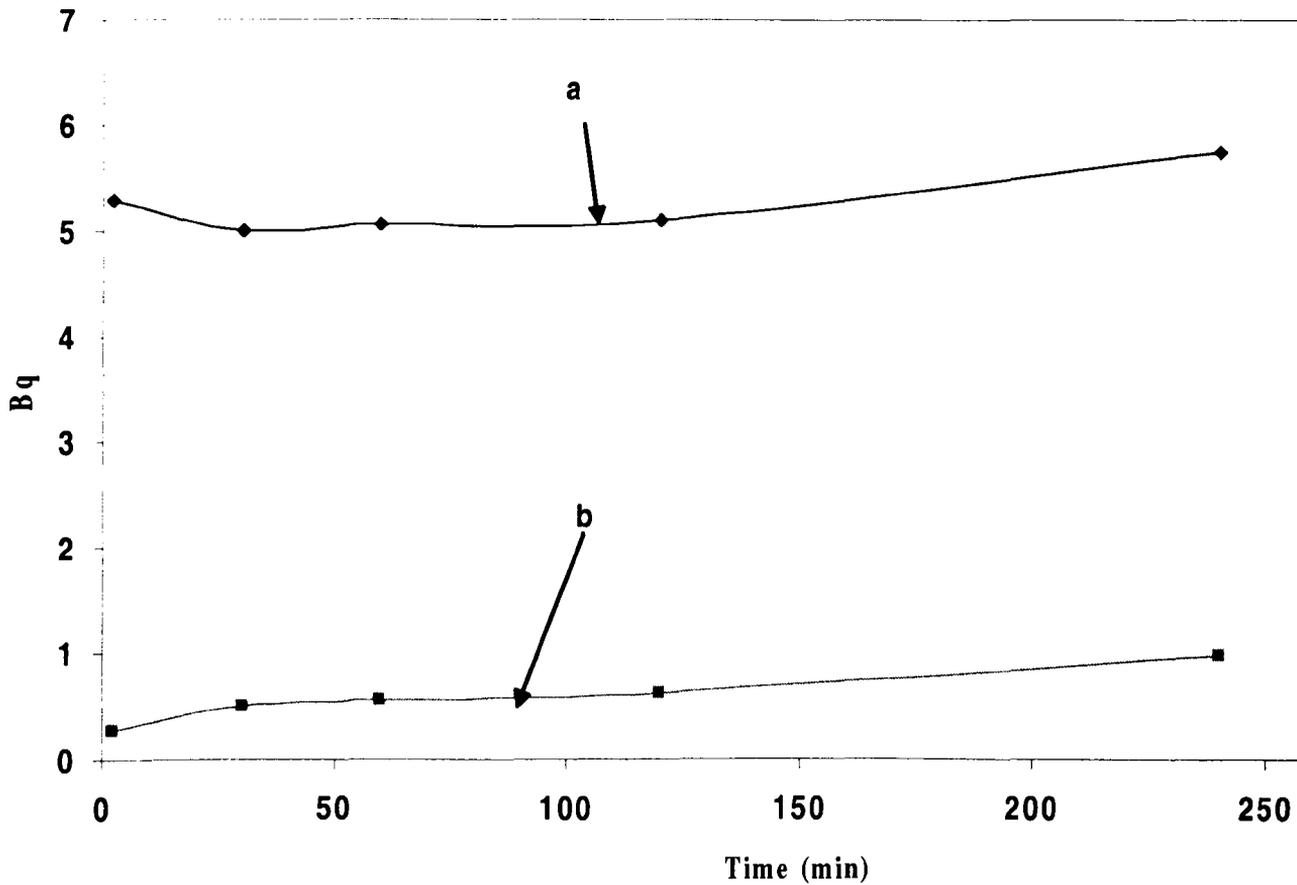


Fig 6.11 Nascent [^{14}C] Gal-HMwt-complex (2ml) was added to 14.8 μl TFA, boiled for 1 hr, and dried. The samples were then dissolved in buffer and shaken for different time intervals with paper, covered with nescofilm.

a. Radioactivity measured in the liquid.

b. Radioactivity measured in the paper.

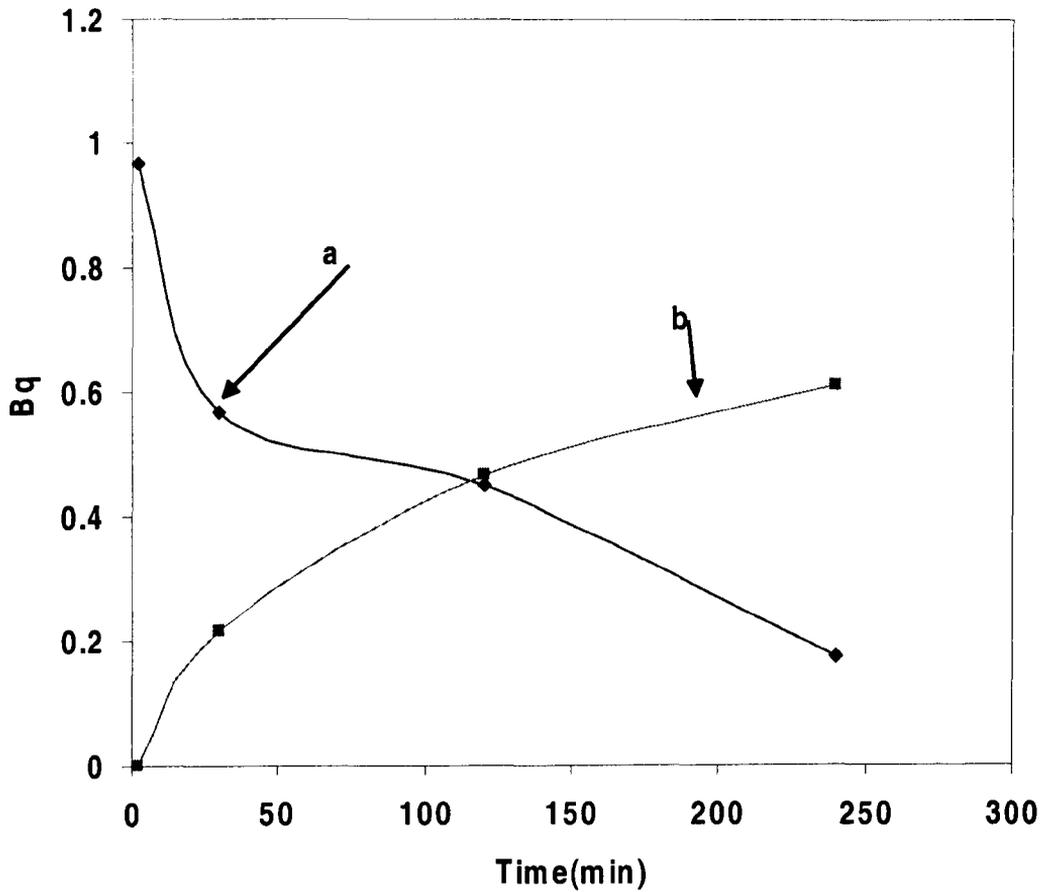


Fig 6.12 Nascent [^{14}C] Gal-HMwt-complex (2ml) was added to 14.8 μl water, boiled for 1 hr, and dried. The samples were then dissolved in buffer and shaken for different time intervals with paper, covered with nescofilm.

a. Radioactivity measured in the liquid.

b. Radioactivity measured in the paper.

6.9. Discussion and Conclusions.

1. Up to 85% of the high Mwt nascent pectin bound to paper when incubated with 3 MM paper for 4 hours or more.
2. Binding to paper was also observed when the high Mwt nascent pectin was first dried onto paper, then exposed to running tap water for 1 hour.
3. This binding provides further evidence for the presence of xyloglucan in the nascent pectin complex, since xyloglucan is known to bind to cellulose (Fry *et al.* (1992), Hayashi *et al.*, 1987).
4. Incubation of the high Mwt nascent pectin with endo- β 1, 4-glucanase before or during binding decreased the degree of binding to paper. This is in agreement with the idea that the binding is due to the presence of xyloglucan attached to nascent pectin. Incubation with polygalacturonase also decreased the degree of binding to paper, perhaps indicating that the [14 C]galactan was linked to xyloglucan via homogalacturonan.
5. The presence of non-radioactive xyloglucan in the binding assay inhibited the binding. This suggests that non-radioactive xyloglucan competes with the nascent pectin complex for binding sites on the paper.

Chapter 7

Discussion

Discussion.

A number of models for pectin structure have been proposed. Keegestra *et al.* (1973) suggested that homogalacturonan and RGI formed the backbone of the pectin complex. Another proposal was that made by Abdel-Massih *et al.* (2003) that suggested that the backbone of the complex was xyloglucan. A further structural model of pectin, where homogalacturonan as well as other sugars are present as side-chains of RGI, has also been suggested (Vinken *et al.*, 2003). In the work described in this thesis, the report of Abdel-Massih *et al.* (2003) that nascent pea pectin was attached to xyloglucan was further investigated, and further information about the structure of the pectin-xyloglucan complex was obtained.

1. To determine the optimum procedure for the extraction of the nascent pectin-XG complex formed in pea stems, membrane pellets were prepared and incubated with UDP-[¹⁴C] galactose according to the procedure described by Abdel-Massih *et al.* (2003) and were extracted with six different reagents. These were: Tris-HCl buffer (pH 7.4); 50mM EDTA/50mM PO₄ (pH6.8) at 100° C; 50mM EDTA/50mM PO₄ (pH6.8) at 25° C; phospholipase C with 100 μl of 0.1M Tris-HCl (pH 7.4) at 25° C; trypsin at 25° C and 0.1% Triton X-100 at 25° C. Results discussed in chapter 3 showed that the best extractant used to solubilize the pectin-complex from the pellets was found to be 50mM EDTA/50mM PO₄ (pH 6.8) at 100° C.

2. Aqueous solutions of pectic polyuronides tend to associate covalently into multichain aggregates (Goldberg, 1989). To optimize methods for the determination of the molecular weight of the nascent pectin-xyloglucan complex, and to avoid formation of pectin aggregates, the effect of a number of eluents on the behaviour of the complex on gel filtration was studied. Results of chapter 3 showed that 10mM EDTA/10mM PO₄/1M NaCl (pH 6.8) was the best eluent to minimise this aggregate formation.

3. To analyze the composition of the nascent pectin-xyloglucan complex using specific polysaccharidases, a number of different polysaccharide-degrading enzymes were used as described in chapter 4. On gel filtration using Sepharose CL-2B and EPN as an eluent, the complex eluted with a K_{av} of around 0.8, corresponding to a molecular size of approximately 200kDa, as judged by dextran standards. Polygalacturonase caused a big decrease in apparent molecular size, to 10-20 kDa, while endo-1,4- β -galactanase converted the radioactivity to [¹⁴C] galactobiose and [¹⁴C]galactose. The effect of endo-1,4- β -glucanase was to reduce the apparent molecular weight to 20 kDa, and that of xyloglucanase the same apparent molecular weight, about 20 kDa. This confirms that the [¹⁴C] galactan is part of complex containing polygalacturonan and xyloglucan. Such complexes have also been reported in mature cell walls (Thompson & Fry, 2000; Femenia *et al.*, 1999).

Handford *et al* (2003) reported that Golgi-rich vesicles derived from Arabidopsis callus were able to synthesize mannan polysaccharides in vitro, and that the thickened cell walls of both leaves and stems also contained abundant mannan epitopes. Use of mannanase in

our experiments showed that this enzyme also significantly decreased the apparent molecular weight of the complex, which indicated that mannan might also be present in the complex.

Some evidence was also obtained that the [^{14}C] galactan is bound to the complex through arabinan; this is indicated by the acid-labile nature of the linkage to the rest of the pectin complex. This suggests a structure for the pectin-xyloglucan complex different to all those previously proposed. A possible structure is given in Fig 7.1.

Related work carried out by other members of our research group has provided additional insights into the structure of nascent pectin as it is formed in the endomembrane system and then deposited in the cell wall. UDP-[U- ^{14}C]-galacturonic acid labelling of nascent pectin showed that the galacturonic-acid-containing backbone became labelled when the complex was of lower molecular weight than the nascent pectin studied here, in accordance with the expectation that the backbone is formed before the side-chains.

The presence of pectin attached to xyloglucan in the Golgi and during exocytosis may help to prevent either component from forming inappropriate non-covalent associations with other molecules before their deposition in the wall (Brett *et al.*, 2005). The hemicelluloses tend to associate via hydrogen bonds, which is observed through the formation of a precipitate upon neutralizing the alkaline extract from the EDTA treated cell wall. If they were to be transported alone, they would precipitate. Consequently, they are thought to be kept in solution by linkage to pectin in the complex.

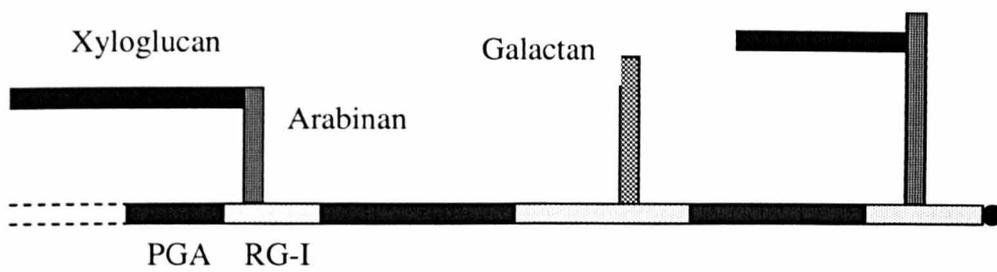


Fig 7.1. Model for possible arrangement of xyloglucan (XG), polygalacturonan (PG), rhamnogalacturonan I (RGI), galactan, and arabinan in the complex.

Other experiments on this galacturonic-acid-labelled nascent pectin indicated that xyloglucan was not attached to the backbone in this material. Hence xyloglucan may become attached to pectin at a relatively late stage of pectin biosynthesis. (Rabih Seif-el Dein; M.Sc thesis, American University of Beirut, 2004).

4. JIM5 and LM7 were used in chapter 4 in order to determine whether nascent pectin-XG complex binds to these anti-pectin antibodies.

Immuno blotting showed that the microsomal pectin contained epitopes that bind to both JIM5 and LM7. These epitopes have been reported to contain pectin with low degree of esterification in the case of JIM5, and non-blockwise esterified pectins with LM7.

However, it remains to be determined whether these epitopes are present in the pectin molecules that are linked to xyloglucan.

5. To determine whether nascent pectin-XG complex contains protein, attempts were made to attach the complex to a gel matrix, using specific coupling reagents to bind the complex through its protein component (if present) to a solid support. The solid matrix that was chosen was Affi-Gel 15. Previous work in our laboratory has indicated that galacturonic-acid-labelled nascent pectin is attached to protein (Rizk et al., 2000). More recent results using the same labelling method have confirmed this (Rabih Seif-el Dein; M.Sc thesis, American University of Beirut, 2004). However, the results of experiments reported in this thesis showed that there is no definite evidence of any protein present in the nascent [^{14}C] pectin complex when labelled in the galactan side-chain. Hence the protein may be detached from the complex before the galactan side-chains are added.

Recent work by Zoe Popper and Steve Fry used a different experimental approach and arrived at a conclusion that XG-RGI is found together as a complex. They concluded that such XGs are probably assembled *de novo* on RGI primer within the Golgi system, then secreted into the wall, where they are stable (Abstracts of the Tenth Cell Wall Meeting, Sorrento, Italy, 2004, p85). Popper and Fry used an *in vivo* experimental approach to arrive at these conclusions. To trace the metabolism of anionic xyloglucan, ^3H -arabinose was fed to Arabidopsis culture which labels xyloglucan residues. Driselase was used to obtain isoprimeverose (the characteristic digestion product of xyloglucan). Anionic acidic polysaccharides containing xyloglucan were obtained after only 8 minutes of incubation of the cells with the radioactive label. Such early formation indicates that the pectin-xyloglucan link must be formed in the Golgi, since 8 minutes is too short a time for polysaccharides to become labelled and then exported to the wall.

6. To clarify the overall structure of the complex, its binding to cellulose was studied, as described in chapter 6. Up to 85% of the high M.Wt. nascent pectin bound to paper when incubated with 3 MM paper for 4 hours or more. This binding provides further evidence for the presence of xyloglucan in the nascent pectin complex, since xyloglucan is known to bind to cellulose (Fry *et al.* 1992; Hayashi *et al.*, 1987).

Experiments carried out in our laboratory by Dr C.M. Cumming showed that [^{14}C] pectin extracted from pea cell walls with EDTA did not contain xyloglucan and did not bind to paper (Cumming CM, personal communication). This [^{14}C] pectin was prepared by incubation of intact pea stems with [^{14}C] sucrose. This showed that pectin itself does not

bind to paper. The [^{14}C] sucrose labelled pectin was also extracted with 6 M NaOH at 0°C for 6 hr followed by 37° C for 18 hours. This alkaline solution was able to extract the pectin-xyloglucan complex from the cell wall, possibly by disrupting the hydrogen bonding of the xyloglucan portion of the complex to cellulose microfibrils.

Future work

In future work, I intend to carry out the following experiments:

1. Further investigate the attachment of arabinan to the complex by treating the nascent galactose-labelled pectin-xyloglucan complex with arabinanase.
2. Collect mannanase treated high molecular weight products, and then further treat them with galactanase.
3. Bind the complex to Affi-Gel 15 and treat it with trypsin.
4. Use immunoprecipitation techniques to determine whether JIM5 and other antibodies bind to mature as well as to newly synthesized pectin, and whether the antibody-binding epitopes are present in the radioactively-labelled pectin-binding complexes.

Chapter 8

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