BIOSYNTHESIS AND ASSEMBLY OF PECTIN AND GLUCURONOARABINOXYLAN IN PLANTS

by

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DECLARATION

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

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Signature

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Date

LIST OF ABBREVIATIONS

%	percent
AG	arabinogalactan
AGP	arabinogalactan protein
APS	ammonium persulphate
Ara	arabinose
BD	blue dextran
Bq	Bequerel
BSA	bovine serum albumin
°C	degrees centigrade
cm	centimeter
CMC	Cyclohexyl-3-(2-mopholinoethyl)carbodiimide metho-p-toluensulfonate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
Egase	endo-glucanase
EXT	endoxyloglucan transferase
Fuc	fucose
g	grams
g	gravity
Gal	galactose
GalA	galacturonic acid
GAX	glucuronarabinoxylan
GDP-	guanidine diphospho-
Glc	glucose
GlcA	glucuronic acid
GT	glucuronyltransferase
GTC	guanidinium thiocyanate

HCl	hydrochloric acid
HGA	homogalacturonan
hr	hour
HRGP	hydroxyproline-rich glycoproteins
kDa	kilodalton
mA	milliampere
Man	mannose
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MPa	megapascal
MT	methyltransferase
NaOH	sodium hydroxide
nm	nanometer
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PC	paper chromatography
PGA	homogalacturonan
Prep	preparation
RG	rhamnogalacturonan
Rha	rhamnose
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
TEMED	N,N,N′,N′-tetramethylethylenediamine
TGN	*trans* Glogi network
TLC	thin layer chromatography
UDP-	uridinie diphospho-
v/v	volume for volume
w/v	weight for volume

XET	xyloglucan endotransglycosylase
XG	xyloglucan
XGT	xyloglucan glucosyl transferase
XRP	xyloglucan-related proteins
XT	xylosyltransferase
XTR	XET-related
Xyl	xylose

ABSTRACT

Nascent pectin and glucuronarabinoxylan (GAX), synthesized *in vitro* by membrane-bound enzymes from etiolated pea (*Pisum sativum* L.) epicotyls, were found to bind to pea xyloglucan in a pH-dependent manner. The binding was maximum at low pH (3-4), and decreased to almost zero at pH 6. The binding seemed to occur instantaneously, to be non-covalent, and to require both terminal fucose residues of xyloglucan, in addition to the non-reduced acid residues of GAX and pectin. Removal by protease of the proteins attached to nascent pectin and GAX, greatly reduced the maximum binding and abolished the pH-dependence. The proteins involved seem to have approximate molecular weights of 14 and 94 kDa.

The pH-dependent binding of nascent pectin and GAX is not completely specific to xyloglucan, since some binding occurred to a range of other matrix polysaccharides, though at a lower level than to pea xyloglucan.

Newly-deposited pectin was extracted from peas that were incubated with radioactively labelled sucrose. It was shown to behave in a similar manner as nascent pectin, exhibiting the same pH-dependent binding pattern to xyloglucan. Protease treatment of pectin decreased the binding, indicating the possible presence of proteins attached to pectin in the cell wall, and revealing the role of those proteins in the interaction of pectin with other matrix polysaccharides, particularly xyloglucan.

The significance of the binding of nascent and newly-deposited plant cell wall polysaccharides to xyloglucan is not very clear. The pH-dependence of the binding suggests a functional interaction with the mechanisms that control growth, since the wall pH decreases when elongation growth is initiated. The proteins involved would play a significant role in cell-wall assembly and cell-wall elongation.

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CHAPTER 1

INTRODUCTION

I. THE PLANT CELL WALL AND ITS ROLES

The plant cell wall is a multilayered network of complex polysaccharides and glycoproteins linked to each other and to the plasma membrane (Roberts, 1989). Cell wall composition and therefore construction varies between species and between tissues. Even within a single wall, there exist zones, or domains of different architecture: the middle lamella, plasmodesmata, channels, pit-fields and cell corners (Roberts, 1990). Temporal and spatial changes occur as elongation and differentiation take place. The earliest formed layer is the middle lamella, derived from the cell plate which is laid down at cell division. The middle lamella is extremely thin and thickens at the cell corners. The next layer to be formed is the primary cell wall that continues to be deposited as long as the cell is growing in surface area; however, the thickness of this layer is maintained at approximately 0.1-1.0 μm (Brett and Waldron, 1996). Many cells limit themselves to these two layers, whereas other specialized cells further deposit a third layer, namely the secondary wall, at the onset of differentiation. This layer differs morphologically and chemically from the primary wall, and it can be deposited with varying thickness at different regions of the cell wall.

The primary and secondary walls consist of two major phases: a microfibrillar phase made of cellulose microfibrils embedded in a non-crystalline phase called the matrix phase, or simply the wall matrix. This latter phase consists of a wide variety of polysaccharides, some of which can be extracted using a chelating agent or hot dilute acid, namely pectins. The remaining are referred to as

hemicelluloses. All the wall components interact to form a living, dynamic structure that acts and reacts in response to cellular commands to grow or differentiate. Therefore, the wall is not viewed as an inert excrescence, but more as an extracellular, pleiomorphic organelle of unique structure and enzymatic complement (Lamport, 1965); it performs a great diversity of functions in the life of the plant.

The major role that was first described is its structural role; the wall provides the strength and the shape of the cell due to various polysaccharides in its structure. Xyloglucans, the major hemicelluloses present in the primary walls of dicotyledons (McNeil *et al.*, 1984), are long enough to produce cross-links between cellulose microfibrils and thereby contribute to the overall strength of the wall (Hayashi, 1989). Pectins are also believed to be required for cell wall strength (McCann *et al.*, 1992; Shedletzky *et al.*, 1992; Iiyama *et al.*, 1994), especially in their unesterified Ca^{++}- cross bridged form (Jarvis, 1984). In addition to its structural role, the plant cell wall has many other functional properties, largely dictated by its polysaccharide and protein composition (Swords and Staehelin, 1993). The wall largely contributes to the control of cell growth, due to the presence of xyloglucans that hydrogen-bond to the surface of cellulose microfibrils, holding adjacent microfibrils together, thereby limiting cell expansion (Fry, 1988). As elongation takes place several modifications of xyloglucans are observed such as an increase in turnover (Labavitch and Ray, 1974; Lozovaya *et al.*, 1996), and a decrease in their average size (Talbott and Ray, 1992b; Talbott and Pickard, 1994). Moreover, xyloglucans exhibit 'catalytic' control of growth (Fry, 1989), because some of the by-products of

their degradation include specific oligosaccharides that inhibit cell expansion (York *et al.*, 1984; Fry, 1986b), and auxin-stimulated growth (McDougall and Fry, 1988). These oligosaccharides, also called oligosaccharins, can serve as hormone-like regulatory molecules as well (Ryan and Farmer, 1991). When applied back to living plants, they exhibit several potent biological activities such as evoking defense responses (Nothnagel *et al.*, 1983; Walker-Simmons and Ryan, 1986), influencing protein synthesis (Yamazaki *et al.*, 1983), amino-acid uptake (Fry, 1989) and morphogenesis (Tran Thanh Van *et al.*, 1985). In some cases, xyloglucan oligosaccharides were observed to act as signaling molecules promoting growth of wheat coleoptiles (Vargas-Rechia *et al.*, 1998). Xyloglucan could also act as a food reserve in seeds of different dicotyledon plant genera such as *Impatiens* and *Annona* (Reid, 1985).

Another group of matrix polysaccharides that contribute to different cell wall functions are the pectic polysaccharides, some of which are the primary determinants of pore size, ion exchange (Doong *et al.*, 1995) and sieving properties of the cell wall (Baron-Epel *et al.*, 1988). Other pectic polysaccharides, such as the unesterified polygalacturonate fragments, are known to induce synthesis of antimicrobial molecules as a plant defense against pathogens (Ryan and Farmer, 1991). They also regulate tissue morphogenesis in thin cell layer explants (Tranh Thahn Van *et al.*, 1985; Eberhard *et al.*, 1989). In addition to these diverse contributions, pectins are believed to be required for cell-cell adhesion (Stephenson and Hawes, 1994) and cell-cell communication (Darvill *et al.*, 1992; Mohnen and Hahn, 1993; Cote and Hahn, 1994).

The proteins of the plant cell wall also contribute to some of its functions. For instance, hydroxyproline-rich glycoproteins (HRGP), commonly referred to as extensins, are known to play a primarily structural role (Swords and Staehelin, 1993). Furthermore, the presence of free HRGP coating the surface of airspaces could provide a passive agglutination defense mechanism against pathogenic bacteria (Sequeira *et al.*, 1977; Leach *et al.*, 1982; Mellon and Helgeson, 1982).

The last function of the plant cell wall to be mentioned is its role in decreasing herbivory; deposition of lignin and silica in the wall renders plant tissue unpalatable to many predators (Gali-Muhtasib *et al.*, 1992).

To sum up, the cell wall plays important roles in plants including provision of strength and shape to the cell, and rigidity to the whole plant, control of cell growth, protection against pathogens and herbivores, participation in cell-cell adhesion, cell-cell communication and food storage in some seeds.

II. MAJOR COMPONENTS OF THE PLANT CELL WALL

As mentioned earlier, the plant cell wall is made of two major phases, the microfibrillar phase composed mainly of cellulose, and the matrix phase made of hemicelluloses (including xyloglucans, xylans, glucomannans, galactomannans, and callose), pectic polysaccharides, proteins, lignins and phenolic compounds (Brett and Waldron, 1996). Out of all these polymers, xyloglucans, pectins and xylans are of major interest in our present investigation, and will be discussed in the following sections.

A. Xyloglucans

Extensive studies have been carried out on xyloglucans (XG) because of their essential role in cell wall elongation due to their strong association to cellulose (Fry, 1989), the role of XG oligosaccharide fragments in growth (York *et al.*, 1984), and the use of XG as a viscosity-increasing reagent in food stuffs due to their gel form (Yamagaki *et al.*, 1998). Gel formation and self-association seem to depend on the degree of galactose substitution (Shirakawa *et al.*, 1998). Xyloglucans are the principal hemicellulose of the primary wall of dicotyledonous plants, forming 20-25% of its dry weight (McNeil *et al.*, 1984); in grasses, they make up about 2-5% (Kato and Matsuda, 1985). Xyloglucans were shown to occur uniformly across the thickness of the wall of a whole cell (Hayashi and Maclachlan, 1984), in addition to their presence in the middle lamella (Moore *et al.*, 1986).

The backbone of xyloglucans is a straight chain polymer of β(1-4)-linked D-glucopyranose residues, about 0.15 to 1.5μm long (Fry, 1989). In dicots, 60-75% of the glucose residues have an α-D-xylopyranose residue attached to carbon-6 (Bauer *et al.*, 1973; Joseleau and Chambat, 1984) and showing some regularity, whereby every three xylosylated glucose residues are followed by one unsubstituted glucose (Hayashi and Maclachlan, 1984). About 30-50% of the xylose residues have, attached to their position 2, a β-D-galactopyranose residue or, more rarely, an L-arabinofuranose residue (Fry, 1989). In onions (Redgwell and Selvendran, 1986) and fir (Fry, 1989), many of the galactose residues were found to bear at their position 2 an α-L-fucopyranose residue. The fucose residue was suggested to be an essential residue in XG for the interaction of XG with cellulose microfibrils (Levy *et al.*, 1991). *Arabidopsis* mutants were identified that contain less than 2% of the normal amounts of fucose in their primary walls (Zablackis *et al.*, 1996), or completely deficient in fucose residues (Reiter *et al.*, 1993). In both cases, the plants were able to survive, however they were characterized by smaller stature, and fragility of their stems.

Xyloglucan of grasses is similar to that of dicotyledons, except that the former contains fewer xylose side chains (36-38% of the glucose residues are xylosylated), far fewer galactosyl residues are present, and almost no terminal fucose (Kato and Matsuda, 1985). Xyloglucan produced by suspension-cultured cells contains O-acetyl residues at position 6 of galactosyl residues (Maruyama *et al.*, 1996; Sims *et al.*, 1996), or at position 6 of glucose residues (Sims *et al.*, 1996). It is postulated that these O-acetyl residues have a role in cell elongation (York *et al.*, 1988; Maruyama *et al.*, 1996) and that they are not usually detected

in molecules isolated from cell walls because of their lability in the strong alkaline conditions used for solubilisation of xyloglucans (Renard *et al.*, 1995). A few recent studies have been performed on XG extracted from gymnosperms, and revealed close similarities with XG from dicots with the exception of additional mannose residues (Andrew and Little, 1997; Kakegawa *et al.*, 1998). Xyloglucan is a major storage cell wall polysaccharide in many dicotyledonous seeds (Hayashi, 1989). It is mobilized after germination due to the action of several enzymes (Edwards *et al.*, 1985; Crombie *et al.*, 1998). Seed xyloglucans have important commercial uses: tamarind xyloglucan is used as a viscosifier and stabilizer (Reid and Edwards, 1995). Detarium xyloglucans have pharmaceutical applications in controlling drug release, modifying texture, in addition to their nutritional and therapeutic benefits, notably in the treatment of metabolic disorders such as diabetes mellitus and hyperlipidaemia (Wang *et al.*, 1997). The backbone of seed XGs consists like all XG of β-linked glucose residues to which single unit xyloses are attached. Some xylose residues are further substituted at O-2 by galactose residues (Buckeridge *et al.*, 1997), and the presence of arabinose-containing side-chains appears likely (Niemann *et al.*, 1997). No fucose is present in any of the seed XG studied to date.

Heterogeneity of xyloglucans is widely seen in FAB mass spectroscopy and NMR studies, and more recently, matrix-assisted laser desorption/ionization time-of flight mass spectrometry post-source decay (MALDI-TOFMS PSD) is being used in fine structural analysis of xyloglucans (Yamagaki *et al.*, 1997). This heterogeneity results from differences in molecular mass, distribution of additional branching side chains, or levels of substituted xylosyl residues (Hayashi, 1989). For example, generally, almost 75% of the backbone is

branched, with the exception of only 30% observed in rice seedlings, 40% in solanaceous plants, and 65% in apple pommace (Spronk *et al.*, 1998). In xyloglucan extracted from apple pommace, it is suggested that every fourth glucose residue in the backbone remains unbranched (Spronk *et al.*, 1998). Xyloglucan from Persimmon fruit cell walls showed a molar ratio of Glc:Xyl:Gal:Fuc of 10.0:6.0:3.4:1.4, with a low degree of polymerization of side-chains (Cutillas-Iturralde *et al.*, 1998). Xyloglucan extracted from *Phaseolus aureus* hypocotyls contained Glc:Xyl:Gal:Fuc in a molar ratio of 10:7:2.5:1 (Kato and Matsuda, 1976). An acidic xyloglucan was isolated from grape skins of weight-average M_r of 35 kDa; it was shown to be a linear chain of xylopyranosyl and glucopyranosyl residues linked by β(1-4) glycosidic bonds. Side-chains contained 4-O-methylglucuronopyranosyl acid, L-arabinofuranosyl and xylopyranosyl residues attached at position 2, in a ratio of one residue for every 10 units of xylose in the main chain, and L-arabinofuranosyl and glucopyranosyl residues attached at position 6 of the units of glucose, in a ratio of one residue for every four glucoses in the main chain (Igartuburu *et al.*, 1997). Xyloglucan extracted from potato is characterized by the presence of two adjacent unbranched glucosyl residues, in comparison to three in barley and rice (Vincken *et al.*, 1996a).

The biosynthesis of xyloglucan occurs in the Golgi apparatus (Moore and Staehelin, 1988), through the contribution of several enzymes. Xyloglucan glucosyl transferase (XGT), adds glucose residues from the precursor UDP-glucose (Brummell *et al.*, 1990; White *et al.*, 1993a) to position 4 of the terminal residue of the glycosyl chain at the non-reducing end (Hayashi, 1989). A protein primer might be involved (Campbell *et al.*, 1988). Xylose side chains are added

by the enzyme xylosyltransferase (XT), that uses UDP-xylose as a precursor (White *et al.*, 1993b), and possibly requires a protein primer (Campbell *et al.*, 1988). Up till 1994, not much was known about the enzymes that add the galactose and arabinose residues, though UDP-galactose and UDP-arabinose were presumed to be the substrates used, based on the abundance of both nucleotides during cell wall formation (Gibeaut and Carpita, 1994). Recently, the galactosyl transferase enzyme was solubilized from pea microsomal membranes: the preferred galactose acceptor locus was the first xylosyl glucose from the reducing end of the subunits (Faik *et al.*, 1997). The fucosyltransferase enzyme then transfers fucose residues from GDP-fucose to the formed chain (Brummel *et al.*, 1990; Baydoun *et al.*, 2000); a 60 kDa protein that exhibits this activity was purified from pea epicotyls (Perrin *et al.*, 1999).

The location and action of these enzymes within the Golgi is still under debate. Some investigators show that the assembly of the glucan backbone occurs exclusively in the *trans* cisternae (Moore *et al.*, 1991), whereas the terminal fucosyl residues on the trisaccharide chains of XG are partly added in the *trans* cisternae and partly in the *trans* Golgi network (TGN) (Zhang and Staehelin, 1992). Another group of investigators claim that the glucose-xylose backbone is initiated in lighter dictyosomal membranes or the *cis* cisternae, continues through the *medial* cisternae, whereas fucosylation is initiated in the *trans* cisternae and is completed in the Golgi secretory vesicles (Brummel *et al.*, 1990). A third group discovered that even fucosylation occurs in lighter dictyosomal membranes which probably correspond to the *cis* cisternae of the Golgi Apparatus (Baydoun *et al.*, 2000).

One issue that is also controversial in xyloglucan biosynthesis is the coordination between the different enzymes involved, specially XGT and XT since the galactosyl and fucosyl transferases may act independently (Driouich *et al.*, 1993). Two models of assembly were proposed (Campbell *et al.*, 1988): the first is the 'imprecise' synthesis, whereby the backbone is synthesized independently of the xylose side-chain, and the xylosyl units are then added in amounts proportional to the availability of UDP-xylose. The second model accounts for 'precise' synthesis, whereby XGT and XT work in tandem, sequentially adding glucose and xylose residues. XG synthesis in *Phaseolus vulgaris* cells (Campbell *et al.*, 1988) and in pea cells (Gordon and Maclachlan, 1989) follows the first model, whereas studies on soybean cells support the 'precise' biosynthetic model (Hayashi, 1989).

B. Xylans

Xylans are heterogeneous polymers of the secondary walls of dicotyledonous plants, and are the main component of hemicelluloses deposited during differentiation of the xylem (Northcote 1972; Bolwell 1993). Glucuronoxylans, the most abundant xylans in dicots, have a backbone of β(1-4)-linked xylose residues; 4-O-methyl glucuronic acid residues are attached to 10% of carbon-2 of the xylose residues through an α(1-2)-linkage (Timmel, 1964), with some regularity in the pattern of glucuronidation (Nishitani and Nevins, 1991). Xyloses are acetylated at carbon-2 or carbon-3, but the acetyl content is quite variable (Gregory *et al.*, 1998). In addition, some arabinose side-chains might be added on carbon-3 (Baydoun *et al.*, 1989b). In the primary walls of dicotyledonous

plants, small amounts of glucuronoarabinoxylans (GAX) are present which are similar in structure to glucuronoxylans, but with more arabinose side chains (Darvill *et al.*, 1980).

In grasses, xylans are the major hemicellulose of primary walls (McNeil *et al.*, 1984); these have additional O-acetylated and O-feruloylated oligosaccharides as side-chains (Wende and Fry, 1997a). It was shown that complex feruloylated side-chains of arabinoxylans are universal in the Gramineae (Wende and Fry, 1997b).

Gymnosperm xylans contain fewer arabinosyl units and are not acetylated (Gregory *et al.*, 1998).

The synthesis of glucuronoxylans has been extensively studied. Different enzymes are involved in this process, namely XT, glucuronyltransferase (GT), and a methyltransferase (MT). XT activity was determined in immature corn cob (Bailey and Hassid, 1966), in bean (*Phaseolus vulgaris*) (Bolwell and Northcote, 1981), in pea (*Pisum sativum*) (Baydoun *et al.*, 1989b), in differentiated xylem cells of sycamore trees (Dalessandro and Northcote, 1981), flax and horse-chestnut (Gregory *et al.*, 1998); in all these studies the substrate used is UDP-xylose. The enzyme is Golgi localized (Waldron and Brett, 1987; Gibeaut and Carpita, 1990), chiefly in the regions of low and medium density, which probably correspond to the cis and medial cisternae (Baydoun and Brett, 1997). A xylosyltransferase of 38 kDa was partially purified from French bean (*Phaseolus vulgaris*), and appears to be an early induced form of xylan synthase (Rodgers and Bolwell, 1992); no lipid or proteinaceous intermediate were found (Gregory *et al.*, 1998). A glucuronyltransferase that was first identified in corn cobs (Kauss, 1967), is also involved in glucuronoxylan synthesis in peas

(Waldron and Brett, 1983). This enzyme is localized in the Golgi apparatus (Delarge *et al.*, 1991), mainly in the *cis* cisternae (Hobbs *et al.*, 1991b), with minor activity in the endoplasmic reticulum, where glucuronoxylan synthesis requires a 20 kDa protein primer (Crosthwaite *et al.*, 1994). The glucuronic acid residues are added to every sixth xylosyl unit of the backbone (Carpita and Gibeaut, 1993). They are transferred from UDP-glucuronic acid (Waldron and Brett, 1983) to a nascent xylan chain, and not to a preformed one (Baydoun *et al.*, 1989b, Hobbs *et al.*, 1991a). A primer is involved in this process; in the Golgi, it consists of a 36-45 kDa protein (Waldron *et al.*, 1989) that seems to be covalently bound to the nascent polysaccharide (Crosthwaite *et al.*, 1994), and that may have a role in transporting the polysaccharide through the endomembrane system (Baydoun *et al.*, 1991). In an attempt to purify GT, the enzyme was solubilized in Triton X-100; however, the product of the solubilized enzyme was slightly altered (Waldron *et al.*, 1989).

The enzyme that methylates the oxygen on C-4 of the glucuronic acid units was first identified in immature corn cobs (Kauss and Hassid, 1967), and in mung beans (Kauss, 1969). The activity of the enzyme was detected in the endomembrane system of the cell, using S-adenosyl-methionine (SAM) as the donor of the methyl group (Baydoun *et al.*, 1989a). Its activity seems to be highest in the light and medium density cisternae of the Golgi apparatus (Baydoun *et al.*, 1999).

C. Pectin

Pectins are the most abundant plant polysaccharides after cellulose (Hoagland, 1996). They are important polysaccharides with applications in foods,

pharmaceuticals, and a number of other industries (Thakur *et al.*, 1997). Their importance in the food sector lies in the ability of pectins to form gels in the presence of calcium ions (Morris *et al*, 1982) or a solute (usually a sugar) at low pH (Walkinshaw and Arnott, 1981). Also, considerable evidence suggests that dietary supplementation with pectins may reduce levels of serum total cholesterol, and low density lipoprotein cholesterol, in addition to moderation of the glucose response (Baker, 1994); however, pectins are bulky, often difficult to consume, and otherwise non-nutritious (Baker, 1997).

Pectins are produced during the initial stages of primary cell wall deposition and make up about one third of the dry substances of the primary wall of dicots and some dicotyledonous plants (Northcote, 1972; Jarvis *et al.*, 1988). The highest concentrations of pectins occur in the middle lamella, with a gradual decrease from the primary cell wall toward the plasma membrane (Thakur *et al.*, 1997); however those present in the primary wall contain more oligosaccharide side-chains that are much longer than the ones observed in the pectin of the middle lamella (Sakai *et al.*, 1993). Pectins are present in much lower amounts in the Graminae family (Wada and Ray, 1978). Until 1996, there were three recognized kinds of pectins, namely homogalacturonan, rhamnogalacturonan I (RG I), and RG II. A new class of pectins was then discovered called xylogalacturonan (Carpita *et al.*, 1996).

Homogalacturonans (usually abbreviated as PGA) are made up of α(1-4)-linked galacturonic acid (GalA) residues that are partly methylesterified. RG I is composed of a backbone of α(1-4)-linked galacturonic acid and α(1-2)-linked rhamnose. Many of the galacturonic acid units are methylesterified, in addition to some (1-5)-linked, and (1-4)-linked galactose side chains that may attach to the

rhamnose residues. RG II is a minor component of primary walls of dicots; it is a complex structure, composed of galacturonic acid, rhamnose, arabinose and galactose residues in a ratio of 10:7:5:5 (Brett and Waldron, 1996). Xylogalacturonans have a homogalacturonan backbone with subtending groups of nonreducing terminal xylose units attached to the O-3 position of about half of the GalA units (Carpita *et al.*, 1996). The most abundant pectin is PGA/RG I, which is composed of covalently linked blocks of PGA and RG I (Zhang and Staehelin, 1992).

Even though the enzymes involved in pectin synthesis are not well characterized, immunolabelling studies using different epitopes aided in localizing pectin synthesis subcellularly in the Golgi apparatus (Moore and Staehelin, 1988). The PGA/RG I backbone is assembled in the cis and medial cisternae of cortical cells (Zhang and Staehelin, 1992), and in the trans cisternae and the TGN of epidermal cells and peripheral root cap cells (Lynch and Staehelin, 1992). A polygalacturonate 4-α-galacturonosyltransferase activity has been identified in membranes of tobacco cell-suspension cultures, transferring galacturonic acid residues from UDP-GalA to a homogalacturonan chain (Doong *et al.*, 1995). Methylesterification of the carboxyl groups of the galacturonic acid residues occurs in the Golgi apparatus (Vannier *et al.*, 1992), and more precisely in the cis and medial cisternae (Baydoun *et al.*, 1999), using S-adenosyl-methionine (SAM) as a donor for the methyl group (Kauss, 1969). However, it is not clear whether one enzyme both synthesizes and methylates PGA or whether the GalA-transferase and the methyltransferase exist as separate enzymes (Goubet *et al.*, 1998). Arabinose-containing side chains of polygalacturonic acid domains are added to the backbone in the trans cisternae (Zhang and Staehelin, 1992;

Driouich *et al*, 1993). The synthesis seems to occur in units of 30 nm that are assembled at a later stage, *in muro*, into longer polymers (McCann *et al.*, 1992). An interesting feature that was observed during pectin synthesis is that some RG I type polysaccharides synthesized in the cis and medial subcompartments, may have the potential to leave from the medial cisternae to be packaged into secretory vesicles (Moore *et al.*, 1991).

III. ASSEMBLY OF CELL WALL COMPONENTS

As mentioned earlier, the plant cell wall is composed of a wide variety of components that interact to form a dynamic structure. However, determining the means of such interaction has been a challenging issue (Brett and Waldron, 1996).

Cellulose chains are synthesized through the action of the enzyme cellulose synthase, whose terminal complexes form rosettes in the plasma membrane (Brown *et al.*, 1994), and which uses UDP-glucose as a precursor (Delmer, 1983; Inouhe *et al.*, 1986). In tobacco membranes, a 4-linked primer glucan was reported to be attached to the newly-synthesized glucan, though no evidence was given for a direct chemical linkage between the glucan and the primer (Blaschek *et al.*, 1983). Newly-formed glucan chains are thought to pass through a transitional liquid crystalline state, whereby glucuronoxylan molecules that possess surface charges and flexible side-chains, favor the formation of a helicoidal mode of assembly (Reis *et al.*, 1991); hence glucuronoxylans are also called twisting agents, or helper molecules (Vian *et al.*, 1986; Roland *et al.*, 1989; Abeysekera and Willison, 1990), in addition to their antifloc role (Vian *et al.*, 1994). Intermolecular hydrogen-bonds occur between several adjacent cellulose chains, causing them to adhere strongly to one another in overlapping parallel arrays, forming bundles of 60 –70 cellulose molecules; these organized bundles are called cellulose microfibrils, and are arranged in parallel arrays, 20 to 40 nm apart (Alberts *et al.*, 1989). However the orientation of cellulose chains with respect to each other has been a controversial issue: several investigators

support parallel alignment of chains (Hieta *et al.*, 1984), while others suggest an anti-parallel alignment (Chanzy and Henrissat, 1985). One observation suggests that within an individual microfibril, all chains are parallel, but adjacent microfibrils may be anti-parallel (Delmer, 1987). Several studies support this model (Brown and Montezinos, 1976; Revol and Goring, 1983).

Hemicelluloses are synthesized in the Golgi apparatus and are carried to the plasma membrane through secretory vesicles that bud off from the TGN (Moore *et al*, 1991; Driouich *et al.*, 1993). The mechanism of packaging in the Golgi has never been studied in detail (Gibeaut and Carpita, 1994). Surprisingly, 70 % of the polysaccharide content of the vesicles consists of a water-soluble, type II arabinogalactan protein (AGP) (Gibeaut and Carpita, 1991) which is a hydroxyproline-rich glycoprotein. AGPs had no well-characterized function in plants (Fincher *et al.*, 1983), though some studies had suggested that one function may be to serve as chaperons of hemicelluloses during the process of secretion (Gibeaut and Carpita, 1991; Carpita *et al.*, 1996). Recent studies indicate that AGPs influence development, cell proliferation and expansion (Casero *et al.*, 1998). Also, some AGPs were shown to be modified by the addition of a glycosylphosphatidylinositol anchor, and seem to be involved in signal transduction pathways (Oxley and Bacic, 1999).

Another controversial issue in cell wall synthesis is whether polymerization of polysaccharides is completed in the Golgi: some polymers such as XG are up to 700 nm long (McCann *et al.*, 1992), and the diameter of secretory vesicles does not exceed 100 nm. The polysaccharides are either intricately packaged, or small polymers would be linked together at the cell surface or *in muro* due to the action of enzymes such as XETs (discussed later). How polymer-laden vesicles migrate

to the cell wall of the plant is not known, but some studies present the wall as a virtual continuum of the cytoskeleton (Wyatt and Carpita, 1993) and it was observed that at sites of active wall deposition, vesicles are directed to the plasma membrane by microtubules. Annexins, a group of proteins discovered in several plants, have also been shown to play a role in exocytosis (Carroll *et al.*, 1998). However, other signals and receptors at the membrane surface may be involved in recognition of the sites for incorporation. Part of the control for vesicle fusion is mediated by the ionic atmosphere at the membrane, and calcium ions are necessary for the fusion to occur (Northcote, 1989). Fusion of the vesicles with the membrane necked pores up to about 60 nm in diameter. During discharge, each vesicle was observed to from a flat disc-shaped structure perpendicular to the plane of the membrane (Staehelin, 1987).

Studies on the deposition of helicoidal walls introduced a new concept of how cellulose microfibril orientation may be controlled: walls are assembled and oriented by a spontaneous self-assembly process that is characteristic of cholesteric liquid crystals (Delmer, 1987). Such self-assembly would however require rigid, long molecules that possess short and flexible side-chains. Since cellulose does not fulfil these criteria, other matrix polysaccharides would have to interact with the microfibrils. It was suggested that these cross-links are xyloglucan molecules (McCann *et al.*, 1990). In woody tissue, it was observed that xyloglucan molecules maintain a flat ribbon-like structure in solution that allows them to associate strongly with the regular array found in the cellulose surface (Houtman and Atalla, 1996). It was also observed that the backbone glucan of both tamarind and pea xyloglucan takes the extended two-fold helical conformation similar to that of cellulose (Ogawa *et al.*, 1990); it was suggested

that the XG glucan backbone aligns in parallel with cellulose at the microfibril surface, and that they are combined with each other by hydrogen bonds. To further clarify this model of interaction, xyloglucan molecules extracted from onion cell walls were found to be up to 400 nm in length, long enough to hydrogen-bond to more than two microfibrils (McCann *et al.*, 1992). Direct microscopic evidence was later provided for the generation of cross-bridges between cellulose ribbons produced in the presence of xyloglucan (Whitney *et al.*, 1995). It seems that more than one XG molecule may contribute to a single cross-link between adjacent microfibrils (McCann *et al.*, 1992). Binding studies suggest that xyloglucan may also be entrapped within the cellulose microfibril during its formation and can only be released if swelling of the microfibril happens (Hayashi, 1989). Therefore, two types of interactions between XG and cellulose exist: a relatively weak interaction formed at the surface of the microfibril, and a stronger interaction formed by the xyloglucan that is entrapped within the crystalline core of the cellulose (Edelmann and Fry, 1992). It is hypothesized that the strongly adhered xyloglucans were carried in secretory vesicles that fused with the plasma membrane at the vicinity of the terminal complex; the molecules were directly drawn into the bundle of crystallizing cellulose microfibrils. The other more loosely attached xyloglucan molecules were released from the vesicles into the free cell wall space, away from the terminal complexes (Nishitani, 1998). Recent studies imply that the XG/cellulose network provides a balance of extensibility and strength that is not achievable with cellulose alone (Whitney *et al.*, 1999).

Some studies showed that the binding site of XG to cellulose is initiated by the fucosylated trisaccharide side-chain th

at flattens out an adjacent region of the XG backbone; upon contact with the cellulose microfibril, this region spreads by step-wise flattening of successive segments of the backbone (Levy *et al.*, 1991). Other studies were performed on fucogalactoxyloglucan from apple pommace and revealed that the fucosyl residue is close in space to the glucan backbone, and that this is consistent with the xyloglucan molecule adopting a 'twisted' conformation (Watt *et al.*, 1999). However, when non-fucosylated XG is used, cross-bridges are still formed indicating that fucose residues are not essential for network formation (Whitney *et al.*, 1995). Other findings indicate that xyloglucan binds to cellulose as a mono-layer and that fucosyl residues only contribute to the increase in adsorption affinity (Hayashi *et al.*, 1994). These studies also indicate that side-chains of the XG molecules may interfere with the binding to cellulose, therefore, parts of chain region containing these residues would be disconnected around cellulose and would be located in the cross-linking regions. Recent findings suggest that the quality and quantity of potential cellulose-binding sites on XG are elevated due to the presence of trisaccharide side-chains versus mono- or disaccharide side-chains (Levy *et al.*, 1997).

Scientists now have a more or less clear idea of the cellulose-XG network, but it is quite challenging to determine how this network will interact with the rest of the wall components. Xyloglucan is expected to interact with the rest of the matrix polysaccharides in the wall. It was earlier suggested that it is covalently attached to other polysaccharides such as glucuronoxylans or pectin (Bauer *et al.*, 1973); however later work (Monro *et al.*, 1976; Darvill *et al.*, 1980) refuted the idea of XG-glucuronoxylan covalent bonding, and the evidence for XG-pectin bonding was based on the effects of bond-splitting agents whose specificity is not

well established (Chambat *et al.*, 1984). Evidence was presented for the presence of xyloglucan-arabinoxylan complexes in cell walls of rice endosperms (Shibuya and Misaki, 1978), though the evidence does not prove the existence of covalent bonding between the polymers (Fry, 1989). By 1992, the concept of a "covalently cross-linked meshwork of matrix molecules" was replaced by a new model of primary cell wall structure presented by Talbott and Ray (Talbott and Ray, 1992a); the model suggests that xyloglucan is not linked glycosidically either to xylans or to pectic polysaccharides. Xyloglucan as mentioned in other models, binds strongly to cellulose microfibrils and leaves unbound loops and ends of the XG molecule projecting into the matrix. Arabinogalactan (AG) constitutes a second matrix layer around the microfibrils, outside the xyloglucan coat. The outermost layer (AG) would interact with the pectins, which are visualized to form a gel that occupies the spaces between the coated cellulose microfibrils. This model postulates non-covalent association between matrix polymers. To further complicate things, very recent studies have reported the occurrence of a range of closely associated acidic xylans, xyloglucans and pectic polysaccharides, possibly in covalent association, extracted from cauliflower cell walls (Femenia *et al.*, 1999). In an earlier investigation, it was shown that xylan-xyloglucan 'complexes' of apparent molecular weights of 2000 and 100 kDa are present in extracts prepared from olive-pulp cell wall material (Coimbra *et al.*, 1995); the investigators suggest that the polysaccharides are in covalent association.

All recent models of wall architecture have suggested that the pectin network is independent from the cellulose-xyloglucan network (McCann *et al.*, 1994), and that a mechanical role is unlikely in the presence of a normal cellulose-

xyloglucan network (Virk and Cleland, 1990). However, a recent investigation shows that there is a net orientation of both cellulose and pectin in the walls of onion epidermal cells, indicating that pectin may, in fact, contribute to the mechanical and structural properties of the cell network (Chen *et al.*, 1997). 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 a hydrophilic filler that prevents the aggregation and collapse of the cellulose network (Jarvis, 1992). Modifications in pectin molecules could influence the fixed-charge density and/or porosity of the pectin gel (Carpita and Gibeaut, 1993), hence affecting the porosity of the cell wall to macromolecules (Baron-Epel *et al.*, 1988). Calcium is readily bound to the free carboxyl groups of pectin that are produced by the action of pectin esterases in the wall (Fry, 1986a), and would hence link pectin chains together. In addition to calcium bridging, pectins may be linked to each other by various covalent bonds (Cosgrove, 1997) that include ester-linkages (Brown and Fry, 1993) through phenolic dimers such as diferulic acid (Wallace and Fry, 1994; Wende and Fry, 1997): much of the wall's ferulic acid is linked to hydroxy groups of specific sugars in specific polysaccharides (Fry and Miller, 1989). Other types of linkages include borate diesters that cross-link two RG II molecules (Carpita *et al.*, 1996; Kobayashi *et al.*, 1996). It was also suggested that a putative role for AGPs, also called extensins (Kieliszewski and Lamport, 1994), would be as a pectin-binding protein (Baldwin *et al.*, 1993). However, our knowledge on AGP functions is still not very clear.

As a conclusion, it is still premature to describe a precise model of the primary plant cell wall. Not enough proof exists on the type and abundance of bonds that

link hemicelluloses to each other and to the pectic polysaccharides. Nevertheless, it seems likely that H-bonds, Ca-bridges, other ionic bonds, coupled phenols, and ester bonds all play a role in building the wall (Fry, 1986a), in addition to other cross-links that may also be involved.

IV. PLANT CELL WALL GROWTH

The placement, magnitude and orientation of wall extensibility in addition to the structural characteristics of the plant cell wall are critical in determining plant form, size and its mechanical properties (Xu *et al.*, 1995). The cell wall undergoes dramatic changes in architecture during elongation and differentiation, as well as more subtle changes from moment to moment during cell-wall turnover (McCann *et al.*, 1992). The wall is strong and stable enough to withstand physical stresses generated by cell turgor pressure that are in the order of 0.3 to 1 MPa (Cosgrove, 1997). Hence, wall stress relaxation is a requirement for cell enlargement (Cosgrove, 1993a). During stress relaxation, cells loosen their walls to allow water uptake; however it was observed that water transport is too rapid to be a major growth limitation (Cosgrove, 1993b). Hence, along with stress relaxation, polymer creep takes place in cell walls of growing cells (discussed later). Creep refers to a time-dependent irreversible extension that happens due to slippage of polysaccharides relative to each other in the cell wall (Cosgrove, 1997). Since the cellulose-xyloglucan network is a major determinant of the mechanical properties of the cell wall (Whitney *et al.*, 1999), a crucial role was conferred on xyloglucan in the wall-loosening process that initiates cell expansion during plant growth or tissue softening during fruit ripening (Cutillas-Iturralde *et al.*, 1998). Support for this idea comes from different lines of evidence:

a- changes in size distribution of xyloglucan in gravitropically responding epicotyls (Talbott and Ray, 1992b)

b- increase in turnover of xyloglucans in auxin-stimulated cells (Labavitch and Ray, 1974)

c- decrease in the average size of xyloglucans in auxin-stimulated cells (Nishitani and Matsuda, 1981; Lorences and Zarra, 1987)

d- increase in the average size of xyloglucans in cases where a decrease in elongation is expected (Talbott and Ray, 1992b)

e- antibodies (Hoson *et al.*, 1991) and lectins (Hoson and Matsuda, 1987) that bind to small xyloglucan fragments inhibit auxin-induced elongation

f- degradation of xyloglucan during ripening of different fruits (Cutillas-Iturralde *et al.*, 1998)

The expanding wall undergoes a decrease in pH (Brummel and Maclachlan, 1989) along with wall loosening, though it maintains its thickness (Passioura, 1994). Three protein families were hypothesized to act on xyloglucan to promote this acid-related growth mechanism, namely endo-glucanases (EGases), xyloglucan endotransglycosylases (XETs) and expansins.

A. Endoglucanases

Endoglucanases are a family of enzymes known to break the β(1-4)-glucan backbone of xyloglucan (Watt *et al.*, 1999). All plant EGases characterized to date are encoded by a large single multi-gene family (Nishitani, 1998). Seven genes coding for members of this family have been cloned from tomato. They show tissue-specific expression: Cel1 is associated with abscission, Cel2 is expressed in ripening fruit, Cel4 is highly expressed in pistil tissue undergoing cell expansion (Brummel *et al*, 1997a), whereas Cel7 is up-regulated by auxin

(Catala *et al.*, 1997) and shown to be active during fruit expansion but not ripening. Most of the EGases possess signal sequences that destine them to the endoplasmic reticulum and then to the apoplast (Nishitani, 1998). Three EGases were found to contain hydrophobic sequences typical of integral membrane proteins (Brummel *et al.*, 1997b); one of these, Cel3, is localized in the Golgi and plasma membranes (Brummel *et al.*, 1997b).

It was observed that endoglucanases differ in their mode of action towards xyloglucans (Vincken *et al.*, 1994). Their activity was blocked when there was an additional substitution of the glucan backbone with arabinose residues (Kiefer et al., 1990). Some types of endoglucanases are influenced by the presence of galactosyl (Vincken *et al.*, 1996a) or fucosyl side-chains (York *et al.*, 1995; Vincken *et al.*, 1996b).

Even though EGases exhibit hydrolytic activity towards β(1-4)-linked glucan chains, they do not always act efficiently on xyloglucan (Hayashi and Ohsumi, 1994). Hence the real roles of EGases in xyloglucan breakdown during cell wall elongation are still not very well established (Nishitani, 1998). A possible role would be to generate low molecular weight xyloglucan acceptors needed for XET activity (discussed in the following section) (Cutillas-Iturralde and Lorences, 1997; Faik *et al.*, 1998).

B. Xyloglucan endotransglycosylases

Xyloglucan endotransglycosylases (XET), also known as endoxyloglucan transferases (EXT) (Xu *et al.*, 1995), are members of a large family of proteins known as xyloglucan-related proteins (XRP) (Nishitani, 1997). Based on

phylogenetic trees produced from their amino acid sequence, they can be classified into three subfamilies, whereby each family includes two or more protein members for a single plant species (Nishitani, 1998). Different members have shown to exhibit different enzymatic actions towards xyloglucans (Rose *et al.*, 1997). Some are hydrolases that cut a XG molecule and transfer the split end to a water molecule (de Silva *et al.*, 1993), others exhibit a transferase activity to transfer it to the hydroxyl group of the non-reducing terminus of another xyloglucan molecule (Zurek and Clouse, 1994; Xu *et al.*, 1995; de Silva *et al.*, 1994), whereas some enzymes exhibit both activities (Schroder *et al.*, 1998). Also, different XETs from the same plant species can require different XG substrates, for example, in seeds of nasturtium, *XET1* acts on fucosylated xyloglucan and may play a role in cell wall elongation, whereas *NXG1* acts on nonfucosylated xyloglucan and would function in mobilization of the seed stored XGs (Rose *et al.*, 1996).

The first XET activity was detected in the extracts of the growing portions of dicotyledons, monocotyledons and bryophytes (Fry *et al.*, 1992; Fry, 1995). The enzyme was able to transfer a high molecular weight portion of a xyloglucan molecule to a xyloglucan-derived nonasaccharide. The first XET enzyme was then purified from a bean plant (Nishitani and Tominaga, 1992): a 33 kDa glycoprotein that can catalyze the endo-type hydrolysis of xyloglucan and the linking of the newly generated reducing end to the non-reducing end of another xyloglucan molecule. The enzyme required the glucosyl and xylosyl main XG backbone structure of the donor and acceptor, but galactosyl and fucosyl chains were not required for the acceptor activity. XET activity was also detected in suspension culture of carrot cells undergoing cell elongation, in comparison to

much less activity detected in nonexpanding cells (Hetherington and Fry, 1993). When comparing cDNAs of XETs from five different plant species, it was observed that 71-90 % of the amino sequence of the mature proteins is conserved. Also the consensus sequence for N-linked glycosylation and four cysteine residues are conserved (Okazawa *et al.*, 1993). TCH4, an XET isolated from *Arabidopsis*, also shares these conserved features (Xu *et al.*, 1995): the expression of the enzyme was restricted to expanding tissue and organs undergoing cell wall modification. More XET enzymes were later discovered in *Arabidopsis* to form an XET-related (XTR) gene family whose cDNAs share between 46-79% sequence identity. The predicted XTR proteins share 37-84% identity (Xu *et al.*, 1996). Six cDNA clones identified from kiwi fruit also share 93-99% nucleotide identity and seem to belong to a family of closely related genes (Schroder *et al.*, 1998).

Even though XETs are thought to be ubiquitous among higher plants, they exist in different forms (Fry, 1995): in kiwi fruit (Redgwell and Fry, 1993) and spinach (Potter and Fry, 1994) they seem to be ionically bonded to the wall. In carrot (Hetherington and Fry, 1993) and *V.angularis* cells (Nishitani and Tominaga, 1992) they are soluble in the apoplast. In pea stems, they are neither ionically bonded nor trapped in the apoplast (Fry *et al.*, 1992).

XET could catalyze two enzymatic processes:

a- Molecular grafting (Nishitani, 1998): annealing of newly secreted XG molecules to existing chains during cell wall deposition and assembly. A free xyloglucan molecule is cut, generating a reducing end that can be connected to the non-reducing end of an anchored XG molecule, causing chain elongation (Thompson *et al.*, 1997). Also, The anchored XG can be split

generating a reducing free terminal that will be joined to the free XG molecule. Hence, XG chains anchored on the cellulose microfibrils can be extended in both directions by the action of XETs.

b- Polymer creep (Nishitani, 1998): cutting and reforming xyloglucan tethers to facilitate wall loosening and expansion. Cleavage of XG cross-links by XETs with hydrolase activity could cause increased mobility of the cellulose-XG network. However, it was suggested that XET could generate a free end in the cross-linking XG and connect it to the free end of an anchored xyloglucan, causing an interchange in the cross-links. This process can happen repeatedly at a high rate, rendering the cellulose-xyloglucan framework sensitive to any mechanical stress applied, or to turgor pressure that can easily cause wall extension if applied.

Due to these two activities of XETs, they have been suggested to be involved in several essential physiological processes (Fry, 1997) namely cell expansion (Fry *et al.*, 1992; Xu *et al.*, 1995), mobilization of storage XG in seeds after germination (Fanutti *et al.*, 1993; Farkas *et al.*, 1992), and in fruit softening during ripening of persimmon (Cutillas-Iturralde *et al.*, 1994), tomato (Maclachlan and Brady, 1994) and kiwi (Redgwell and Fry, 1993; Schroder *et al.*, 1998). This is in addition to their role in deposition of new xyloglucan molecules in the wall i.e. in cell wall assembly.

However, some studies showed that XETs from cucumbers are neither sufficient nor necessary for wall extension. Other proteins, with no XET activity were capable of highly inducing cell wall extension (McQueen-Mason *et al.*, 1993). This is when expansins came into the picture of wall extensibility.

C. Expansins

Expansins were first discovered when two proteins from cell walls of growing cucumber seedlings were shown to possess the ability to induce cell elongation without hydrolytic breakdown of the wall (McQueen-Mason *et al.*, 1992). They were designated Ex29 and Ex30 according to their molecular masses (McQueen-Mason and Cosgrove, 1994). A 27 kDa expansin was then characterized from tomato leaves that was able to cross-react with the antibody raised against cucumber expansin. The protein was shown to be responsible for acid-growth (Keller and Cosgrove, 1995). Two 25 kDa expansins (Cho and Kende, 1997a) occur in the cell walls of rice internodes and are involved in mediating rapid internodal elongation (Cho and Kende, 1997b). Similar results had been obtained in the coleoptile of oat seedlings (Cosgrove and Li, 1993). It was observed that the pH optimum of expansin activity is between 3.5 and 4, and that the amounts of expansins needed for reconstitution of acid-induced growth is similar the amount of expansin normally found in the walls of growing tissue (McQueen-Mason, 1995). Both observations support the evidence that expansins are responsible for the acid-induced extension of plant cell walls. Expansins also seem to function in cell wall disassembly because of the high abundance of expansin mRNA in ripening fruits (Rose *et al.*, 1997).

Expansins turned out to be unique in their sequence identity, their mechanism of action and their physical effects on the wall:

Expansin cDNAs have been cloned from cucumber, rice, Arabidopsis, peas, tomato and pine (Cosgrove, 1997). They seem to exhibit tissue-specific expression. However, all the predicted proteins are highly conserved, with up to

70-90% similarity in their sequences. A region of the proteins near the amino terminus contains eight conserved cysteines, and the carboxy terminus bears a series of conserved tryptophan, arginine and glycine residues. Also, a cluster of lysine and arginine in the middle of the proteins, in addition to evenly spaced aspartic acids along the backbone, are conserved (Cosgrove, 1996). Studies on expansins show that they account for the pH-sensitive components of wall stress relaxation that is essential for cell elongation, though expansins do not have any hydrolytic activity i.e. they do not hydrolyze the major pectin or hemicelluloses of the wall (McQueen-Mason and Cosgrove, 1995). Expansins were able to break the non-covalent adhesion between cellulose microfibrils in paper (McQueen-Mason and Cosgrove, 1994), so it was suggested that expansins bind at the interface between cellulose microfibrils and matrix polysaccharides to induce extension by reversibly disrupting non-covalent bonds and hence causing polymer creep (discussed in the previous section) (Cosgrove and Durachko, 1994). However, neither pectins nor xyloglucan appear to be the site of expansin binding (McQueen-Mason and Cosgrove, 1995). A possible mechanism of action of expansins would be the following (Cosgrove, 1997): the plant cell wall contains 'hot spots' where expansins can act to weaken the adhesion between the microfibrils and matrix polysaccharides. Expansin activity, which is modulated by secretion of the enzymes and by redox potential and pH changes in the wall, will induce stress relaxation and polymer creep needed for water uptake and cell enlargement.

A controversial issue was whether or not to consider expansins as enzymes. It was first suggested that they are not enzymes since they neither make nor break covalent bonds. However, by definition, enzymes are "biological catalysts that

speed up the rate of a reaction by lowering its activation energy". Expansins operate in catalytic rather than stoichiometric quantities, and they speed up wall extension in the absence of other factors such as heat, so they must be lowering the activation energy of the process. Hence, expansins can be considered as enzymes (McQueen-Mason, 1995).

However, it was observed that not all walls are susceptible to the activity of expansins. It seems that, as cells mature, their walls lose susceptibility to expansin action, probably due to new cross-links in the wall such as phenolic cross-bridges. These might modify the action sites or "hot spots" of expansins or make them inaccessible to the enzymes (Cosgrove, 1996).

Even though most scientists have considered proteins as the main means of loosening the plant cell wall, some recent studies have revealed the role of OH radical in promoting non-enzymic scission of plant cell wall polysaccharides, and in particular, xyloglucan (Fry, 1998). The OH radical is produced due to the presence of O_2, Cu^{2+} and the electron donor ascorbate that is present in the apoplast of plant cells. Even though OH radical is known to be detrimental to life, however it is very short-lived, and is considered to be a site-specific oxidant to promote cell wall loosening during germination, growth and fruit ripening (Fry, 1998).

V. INTRODUCTION TO EXPERIMENTAL WORK

The cell wall undergoes dramatic changes in architecture during elongation and differentiation (McCann *et al.*, 1992), in addition to the frequent changes that occur as newly-synthesized matrix polymers are incorporated into the wall. Even though the mechanisms of these changes are still unclear, a crucial role has been conferred on xyloglucan in all of the theories that explain the changes occurring in plant cell walls. When the pH of the wall drops below 4, which is characteristic of a growing wall, nascent glucuronoxylan was shown to bind maximally to cell wall preparations and to hemicelluloses extracted from cell walls of pea seedlings (Brett *et al.*, 1997). Xyloglucan is a possible candidate of the plant cell wall polysaccharides to interact with the nascent glucuronoxylan. The present study addresses this issue by first characterizing nascent EDTA-soluble polysaccharides prepared from pea epicotyls. The binding of these polysaccharides to xyloglucan extracted form the third internodes of pea stems is then studied. Characteristics, requirements and specificity of the binding are also investigated. In the last section, the interaction of xyloglucan with EDTA-soluble polysaccharides from pea cell walls is reported.

CHAPTER 2

MATERIALS AND METHODS

Plant material

Peas (*Pisum sativum*, variety Meteor) were obtained from Sharpes International, Sleaford, Lincs, NG34 7HA, UK. They were soaked overnight at room temperature and grown on damp vermiculite at 25°C in continuous darkness. For particulate enzyme preparation, the peas were grown for 6 days. For xyloglucan extraction, the growth period was 8-9 days.

Particulate enzyme preparation

The procedure was similar to that of Waldron and Brett (1987). Epicotyls (6-9 cm long) were cut off, the hooks discarded and the remainder of the tissue cooled on ice. All subsequent operations were carried out at 0-4°C. Epicotyls (30g) were homogenized using a pestle and mortar in Tris-Mes buffer (10mM. pH 6.0, 30 ml). The homogenate was strained through 2 layers of muslin, and the filtrate was centrifuged in a DuPont Ultracentrifuge at 100,000 *g* for 30 minutes. The supernatant was discarded, and the pellet was homogenized in cold homogenization buffer (1.2 ml) using a glass teflon tissue homogenizer, to obtain the particulate enzyme preparation.

Preparation of the nascent [^{14}C]-polysaccharides

Particulate enzyme preparation (0.191 ml) was incubated with UDP-[U-^{14}C]-GlcA (3.53 kBq, 1.7 μM), UDP-Xyl (1mM) and $MnCl_2$ (10 mM) in a total volume of 200μl at 25°C, for 4 hours unless stated otherwise. The reaction was terminated by

the addition of 96% (v/v) ethanol (1 ml) and centrifugation in an MSE microcentaur microfuge at 10,000*g* for 5 minutes. The particulate material was washed three times with 70% (v/v) ethanol (1 ml), and then extracted twice with 50 mM EDTA/50mM sodium phosphate buffer (0.5 ml, pH 6.8) for 5 minutes at 100°C. The EDTA/phosphate extracts were combined, passed through a column of Sephadex G-100 or G-50 (10 x 140 mm), and eluted with water. Material eluted at the void volume, referred to as the EDTA-soluble [^{14}C]-polysaccharides, was collected for analysis and binding experiments. The particulate material remaining after the EDTA-extraction was further treated with 4% KOH/0.1% $NaBH_4$ (0.5 ml) for 2 hours at 25°C. The alkali-soluble fraction was neutralized with acetic acid, then passed through the same column, eluting with water. Material eluting at the void volume, referred to as alkali-soluble [^{14}C]-polysaccharides, was also used for analysis and binding assays.

Preparation of nascent [^{14}C-Xyl]-polysaccharide

The procedure used to prepare [^{14}C]-polysaccharide was followed, except that the incubation medium contained UDP-[U-^{14}C]-Xyl (8.3 kBq; made up to 0.1 mM by addition of non-radioactive UDP-Xyl), $MnCl_2$ (10 mM) and particulate enzyme preparation (50 µl), in a total volume of 100 µl (Baydoun and Brett, 1997).

Preparation of nascent [^{14}C]-polysaccharides under mild conditions

Particulate enzyme preparation (191 µl) was incubated with UDP-[^{14}C]-GlcA (3.53 kBq, 1.7 µM), UDP-Xyl (1mM) and $MnCl_2$ (10 mM) in a total volume of 200 µl at

25°C, for 4 hours. To avoid the use of ethanol, the reaction was terminated by centrifugation at 10,000*g* for 10 minutes. The particulate material was extracted twice with 50 mM EDTA/50 mM sodium phosphate buffer (0.5 ml, pH 6.8) for 30 minutes, at 25°C. The EDTA-extracts were combined and passed through the column previously used. The material eluting at the void volume was used for certain binding assays.

Preparation of cell walls from peas

Cell walls were prepared as described by Brett *et al* (1997). 6-7 days old peas were harvested, the seedlings and hooks excised. All remaining steps were carried out at 4°C. The epicotyls were chopped using a razor blade, homogenized in an equal weight of 10 mM oxalic acid/10 mM sodium phosphate buffer (pH 5) using a pestle and mortar, and then strained through 2 layers of muslin. The filtrate was divided into 1.5ml fractions and centrifuged at 3000*g* for 5 minutes. Cell wall pellets were washed once with 0.5ml buffer and used for binding assays.

Preparation of [^{14}C]-polymers from cell walls

6-days old pea stems were excised and each 10 were placed in a petri dish, arranged in a radial arrangement so that their bases meet. At the point of intersection 20 µl of [^{14}C]-sucrose (0.148 MBq) that were diluted with 80 µl of water was added, the dishes covered and kept at 25°C. In case of dryness, 100 µl of water was added. After 24 hours, the hooks were removed, and cell wall preparations were obtained according to the method of Brett *et al* (1997). Cell wall pellets were washed once

with 0.5 ml of buffer, and then extracted twice with 50 mM EDTA/50mM sodium phosphate buffer (0.5 ml, pH 6.8) for 5 minutes at 100°C. The EDTA/phosphate extracts were combined, passed through a column of Sephadex G-100 (5 x 140 mm), and eluted with water. Material eluted at the void volume, referred to as the EDTA-soluble [^{14}C]-polysaccharides, was collected for analysis and binding experiments.

Extraction of xyloglucan

Xyloglucan extraction was based on modifications of the methods of Hayashi and Maclachlan (1984) and that of Ogawa *et al.* (1990). Third internodes (50g) were harvested from 8-9 days old peas and extracted three times with 70% (v/v) ethanol (150 ml) for 30 minutes at 70°C. The tissue was then chopped with a razor blade, homogenized using a pestle and mortar in 75 ml of Tris-HCl buffer (0.1 M, pH 7.0), and centrifuged at 8000*g* for 10 minutes. The pellets were extracted three times with EDTA buffer (0.1 M, pH 7.0, 75 ml), for 30 minutes at 85°C, and three times with 4% KOH/0.1% $NaBH_4$ (75ml) for 1 hour at 25°C in a shaking incubator at 100 rpm. The insoluble material ("cell wall ghosts") was obtained by centrifugation at 8000*g* for 10 minutes, and extracted twice with 24% KOH/0.1% $NaBH_4$ (30 ml) for 4 hours at 25°C in the incubator. The 24% KOH-soluble fractions were combined and neutralized with acetic acid. Ethanol was added to a final concentration of 70% (v/v), and the xyloglucan was allowed to precipitate overnight. The precipitated material was suspended in 10 mM phosphate buffer (5 ml), treated with freshly prepared amylase (2 ml) for 24 hours at 40°C, followed by protease (Type XIV,

Sigma) for another 24 hours at 37°C. In later experiments, the pH was adjusted to 4.5 and the xyloglucan was further treated with xylanase (100 units,Type I, Megazyme) for 16 hours at 40°C. When performing enzyme treatments, the samples were covered with a thin layer of toluene to prevent bacterial contamination.

Xyloglucan content was determined by the iodine-sodium sulphate method (Kooiman, 1960; Hayashi *et al.*, 1980): 1 ml of xyloglucan material was added to 0.5 ml of 0.5% iodine solution (in 1% aqueous KI) and 5 ml of sodium sulphate solution (20%). The reaction was left for 1 hour in the dark at 25°C, and the absorbance was read at 640 nm.

Xyloglucan preparations were suspended in 70% (v/v) ethanol and stored in the deep-freeze.

Assay of binding of [^{14}C]-polysaccharide to xyloglucan

Solid xyloglucan (about 1mg/incubation) was resuspended in 0.5 ml incubation buffer (10 mM oxalate/phosphate, adjusted to the appropriate pH with HCl or NaOH), mixed with 0.5 ml [^{14}C]-polysaccharides (generally 15-20 Bq, unless stated otherwise), and incubated for 5 minutes at 25°C. The incubation was terminated by centrifugation for 5 minutes at 10000*g*. The pellets were washed once with buffer of appropriate pH (0.5 ml), centrifuged for 5 minutes, resuspended in 0.4 ml water and their radioactivity content determined by liquid scintillation counting. The assays of binding of [^{14}C]-polysaccharides to cell wall ghosts and to other polysaccharides were performed similarly, using 1 mg of cell wall ghosts or polysaccharides per incubation. All binding experiments were carried out in duplicate. Results are

expressed as the mean +/- the difference between the experimental values and the means.

Reduction of uronic acid groups of [^{14}C]-polysaccharide

The method of Kim and Carpita (1992) was followed with some modifications. EDTA-soluble [^{14}C]-polysaccharides (3 ml) were diluted to a final volume of 10 ml and the pH was adjusted to 4.75 with dilute HCl. Cyclohexyl-3-(2-mopholinoethyl)carbodiimide metho-p-toluensulfonate (CMC) powder (250 mg) was added, keeping the pH constant by a dropwise addition of dilute HCl. After 2 hours, the solution was chilled to ice temperature. Imidazole (HCl) (2.5 ml, 4M, pH 7.0) was added with rapid stirring followed by two batches of 200 mg $NaBH_4$ each. After 1 hour, the excess $NaBH_4$ was removed by a dropwise addition of glacial acetic acid. The solution was then dialyzed against distilled water. After 40 hours the solution was rotoevaporated to dryness, and then dissolved in water to a final volume of 3 ml to be used for binding assays.

Complete acid hydrolysis

[^{14}C]-polysaccharides and xyloglucan samples were acid hydrolyzed using trifluoroacetic acid (2M, 120°C, 1 hour). The hydrolysate was then centrifuged for 10 minutes at 10000*g*. The supernatant was rotoevaporated and analyzed by paper chromatography or thin layer chromatography.

Paper chromatography

System A: For the separation of uronic acids, the acid hydrolysate was rotoevaporated several times from water and then treated with NaOH (0.1 M, 10 µl) for 1 hour to hydrolyse lactones produced (Waldron, 1984). The sample was then neutralized using dilute acetic acid and directly applied on Whatman paper no.3. The solvent used consisted of ethyl acetate: pyridine: acetic acid: water (5:5:1:3), and the tank was equilibrated in a solution of ethyl acetate: pyridine: water (40:11:6). Paper chromatography was carried out for 30 hours. Radioactive samples were cut in 1 cm strips to which 1 ml of scintillation fluid was added and the radioactivity content determined using scintillation counting. Marker sugars that were applied parallel to the samples were detected using silver staining (Fry, 1988): paper strips were dipped in solution A, dried for 3-5 minutes and then dipped in solution B.

Solution A: 0.2 g of silver nitrate was dissolved in 0.4 ml of water and added to 26 ml of acetone with rapid stirring.

Solution B: 1.5 ml of NaOH (10 M) was added to 100 ml of absolute ethanol with rapid stirring.

System B: For the separation of xylose from fucose, the acid hydrolysate was applied on Whatman no.1, run in a solvent of phenol:water (100:39 (w/v)) for 18 hours (Bowles and Northcote, 1972). Monosaccharides and marker sugars were detected using silver staining.

System C: For the separation of the rest of monosaccharides, the samples were applied on Whatman no.3 paper, run in a solvent of ethyl acetate:pyridine:water

(8:2:1) for 24 hours (Fry, 1988). Monosaccharides obtained and external marker sugars were detected using silver staining.

Thin layer chromatography (TLC)

For a good separation of uronic acids, the radioactive hydrolyzed material was subjected to the NaOH treatment described above. The samples were then applied on a silica gel coated TLC plate purchased from Sigma, run in a solvent of butan-1-ol:ethyl acetate:water (7:1:2) for 18-22 hours (Fry, personal communication). Lanes containing the radioactive samples were cut in 0.5 cm strips and their radioactivity content was determined by liquid scintillation counting. External marker sugars that were run in parallel lanes on the same plate were detected using silver staining.

Enzyme treatments

Endo-1,4β-glucanase from Trichoderma longibrachiatum (Megazyme): Cellulase treatment (5 units/incubation) was carried out in sodium acetate (10mM, pH 5) at 50°C for 16 hours.

Pectin lyase from Aspergillus japonicus (Sigma): Pectin lyase treatment (13 units/incubation) was carried out in sodium acetate (10mM, pH 4) at 40°C for 16 hours.

Pectinase from Aspergillus niger (Sigma): Pectinase treatment (10 units/incubation) was carried out in sodium acetate (10mM, pH 4.5) at 25°C for 16 hours.

Xylanase from *Trichoderma viride* (Megazyme): Xylanase treatment (40 units/incubation) was carried out in sodium acetate (10mM, pH 4.5) at 40°C for 16 hours.

Proteinase K from Tritirachium album (Sigma): Protease treatment (5 units/incubation) was carried out in Mes (10mM, pH 7) at 37°C for 16 hours.

Galactanase from Aspergillus niger (Megazyme): Galactanase treatment (15 units/incubation) was carried out in Na-acetate (10mM, pH 4.5) at 40°C for 24 hours.

Amylase from Porcine Pancreas (Sigma): Amylase treatment (15 units/incubation) was carried out in Mes (10mM, pH 7) at 20°C for 18 hours.

All enzyme treatments were terminated by boiling for 10 minutes.

Alkali treatment

To determine the identity of radioactive polymers used, the high molecular weight nascent polysaccharides were treated with 1M NaOH/ 1M $NaBH_4$ at 25°C or 100°C for 10 hours. The reaction was terminated by a dropwise addition of acetic acid to reach neutralization. Products were analyzed by column chromatography.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Material binding to xyloglucan at pH 4 was detached from xyloglucan by incubation with oxalic acid/phosphate buffer (10 mM, pH 6). The unbound material was treated with pectin lyase, dialyzed against water for 24 hours, and then rotoevaporated to

reduce its volume. It was then analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The sample was mixed with (1:1) with sample buffer that consisted of Na_2CO_3 (0.1mM), DTT (0.1mM), and 0.2 ml of a mixture containing 5% (w/v) SDS, 30% (w/v) sucrose and 0.1% (w/v) bromophenol blue. The sample was boiled at 100°C for 5 minutes and then applied to a gel prepared as follows (Hames and Rickwood, 1981):

A 12.5 % resolving gel was prepared using 12.5 ml of 30% acrylamide-0.8% bisacrylamide, 3.75 ml of resolving gel buffer, 0.3 ml of 10% (w/v) SDS, 11.95 ml of water, 15 µl of N,N,N′, N′-tetramethylethylenediamine (TEMED) and 1.5 ml of 1.5% (w/v) ammonium persulphate (APS).

The stacking gel was prepared by mixing 2.5 ml of 30% acrylamide-0.8% bisacrylamide, 5 ml of stacking gel buffer, 0.2 ml of 10% (w/v) SDS, 11.3 ml of water, 15 µl of TEMED and 1.0 ml of 1.5% (w/v) APS.

The resolving gel buffer consisted of 36.3 g of Tris and 48ml of HCl (1M), brought to a final volume of 100ml with water and the pH adjusted to 8.8.

The stacking gel buffer consisted of 6.0 g of Tris dissolved in 40 ml of water, titrated to pH 6.8 with HCl (1M), and brought to a final volume of 100ml with water.

The reservoir buffer contained 0.025 M Tris, 0.192 glycine and 0.1% SDS, adjusted to pH 8.3.

The gel was run at a constant current of 65 mA until the bromophenol blue reached the end of the gel. Pectin lyase enzyme (same as the one used for the sample) and a mixture of molecular weight markers (Sigma) were treated in an identical manner to the sample and run parallel with it.

Silver staining of SDS-polyacrylamide gel

Since Coomasie staining was not sensitive enough to detect any bands, silver staining was used. The gel was immersed in fixation solution containing 100 ml ethanol, 25 ml acetic acid and 125 ml of water for 30 minutes. It was then placed for 40 minutes in an incubation solution that was prepared as follows: 17 g of sodium acetate and 0.5 g of sodium thiosulphate were added to 75 ml of ethanol, 1.3 ml of glutaraldehyde, and the volume made up to 250 ml with water. The gel was washed three times with water and left overnight soaking in water. It was then transferred to a staining solution containing 0.25 g silver nitrate, 50 µl formaldehyde dissolved in 250 ml of water. After 40 minutes, the gel was placed in developing solution consisting of 6.25 g sodium carbonate and 25µl formaldehyde dissolved in 250 ml of water. When the protein bands appeared clearly, the reaction was stopped by immersing the gel in a 1.45% (w/v)EDTA (disodium) solution for 5-10 minutes.

Radioactivity determination

Pellets suspended in water, PC strips and TLC strips were mixed with Ultima-Flo AF (Packard Instrument Company, Meriden, CT 06450), a biodegradable scintillation fluid. Radioactivity content was determined using an LKB 1217 liquid scintillation counter.

An entry of '10'on scales that are labelled 'Bqx10"indicate an actual reading of '1'.

CHAPTER 3

Characterization of the nascent [^{14}C]-polysaccharides and of xyloglucan

Introduction

The present investigation aims at studying the interaction between nascent EDTA-soluble polysaccharides and xyloglucan extracted from peas. This chapter deals with the identification of the polymers that constitute the nascent EDTA-soluble uronate-[^{14}C]-polysaccharides, and the xyloglucan extracted according to the method of Hayashi and Maclachlan (1984).

A. Characterization of the radioactively labelled residues by PC and TLC

Complete acid hydrolysis followed by paper chromatography of the nascent EDTA-soluble and KOH-soluble polysaccharides using system A (see Materials and Methods) revealed the presence of GlcA and GalA labelled residues in both products (Fig.1 and 2). The peak that appears 17-20 cm from the origin in figure 2 is probably due to some disaccharides that were not were not completely broken down into monosaccharides. Since uronic acid marker sugars ran quite close together in the system used, the acid hydrolysate was also analyzed by TLC, where better separation was observed. EDTA-soluble products contain labelled GalA and GlcA residues in a ratio of almost 2:1, indicating the presence of more pectins than xylans in the EDTA-soluble fractions (Fig.3). KOH-soluble products contain more GlcA residues (~60%) (including the peak between18-21, that are probably due to the presence of lactone) (Waldron, 1984).

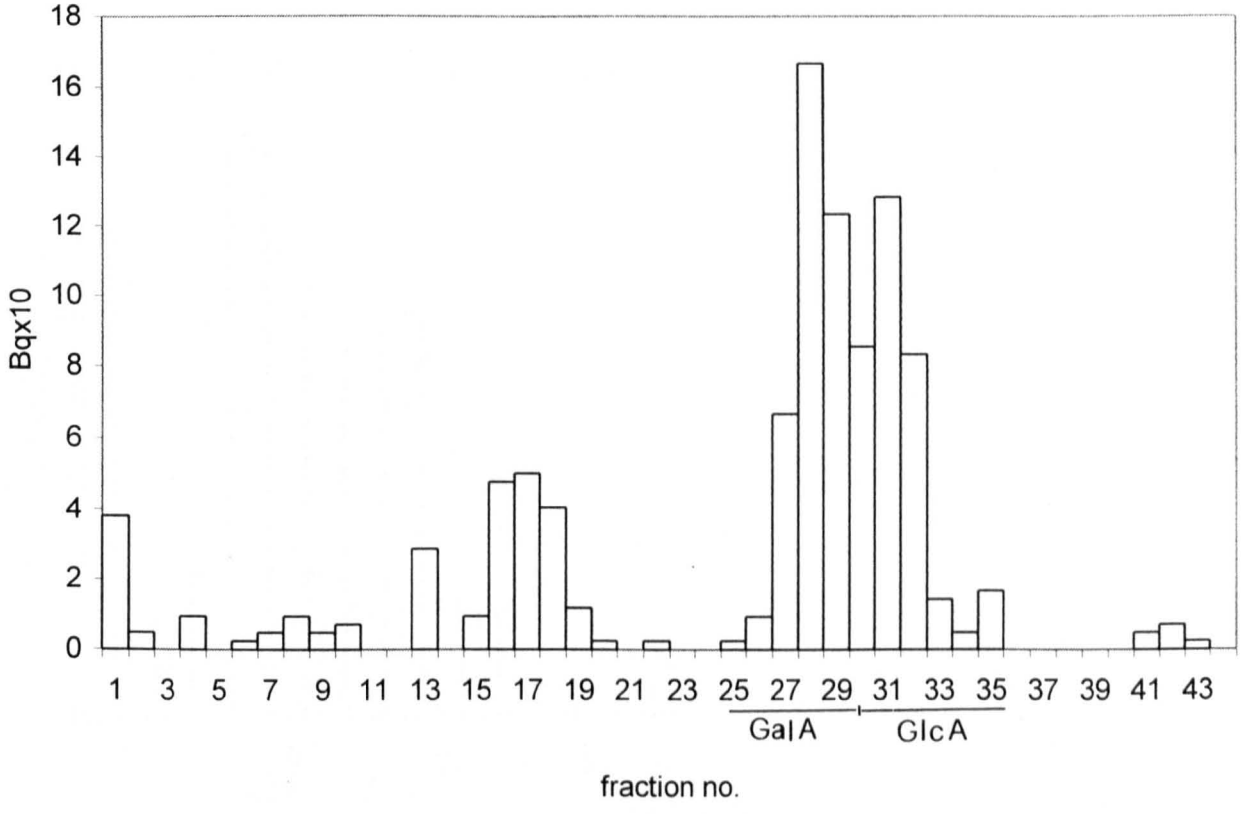

Figure 1. PC (system A) of total acid hydrolysate of nascent EDTA-soluble ^{14}C-polysaccharides

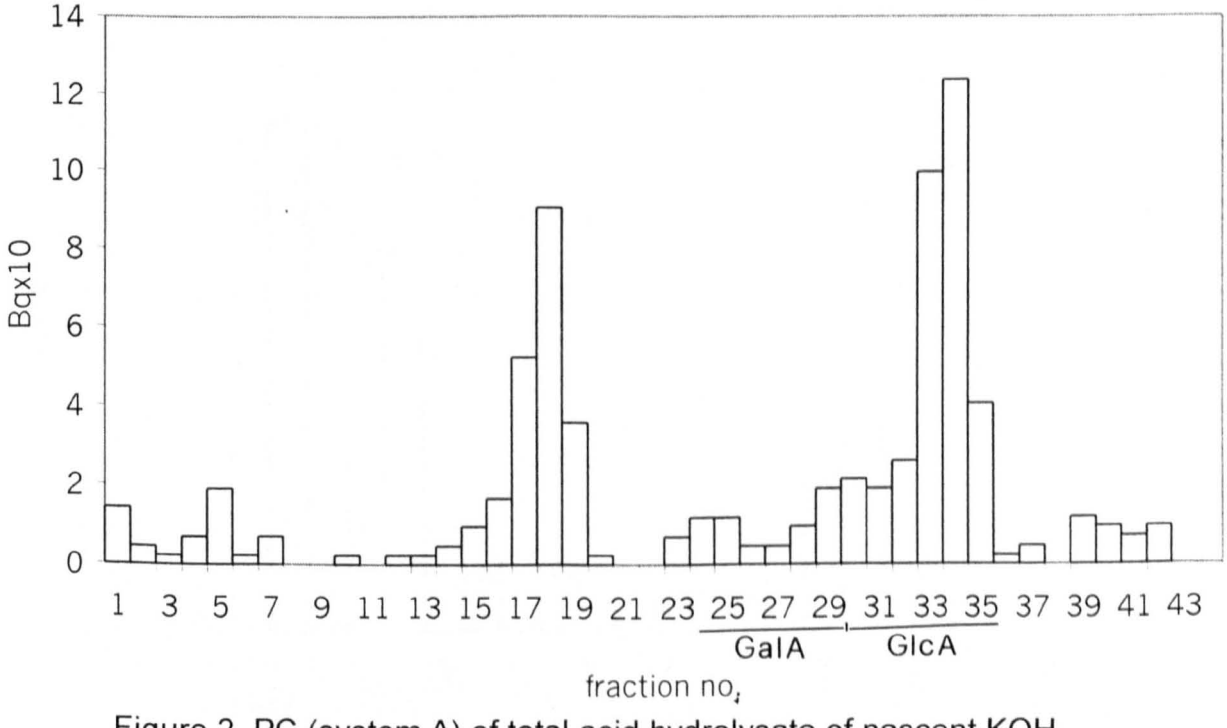

Figure 2. PC (system A) of total acid hydrolysate of nascent KOH-soluble ^{14}C-polysaccharides

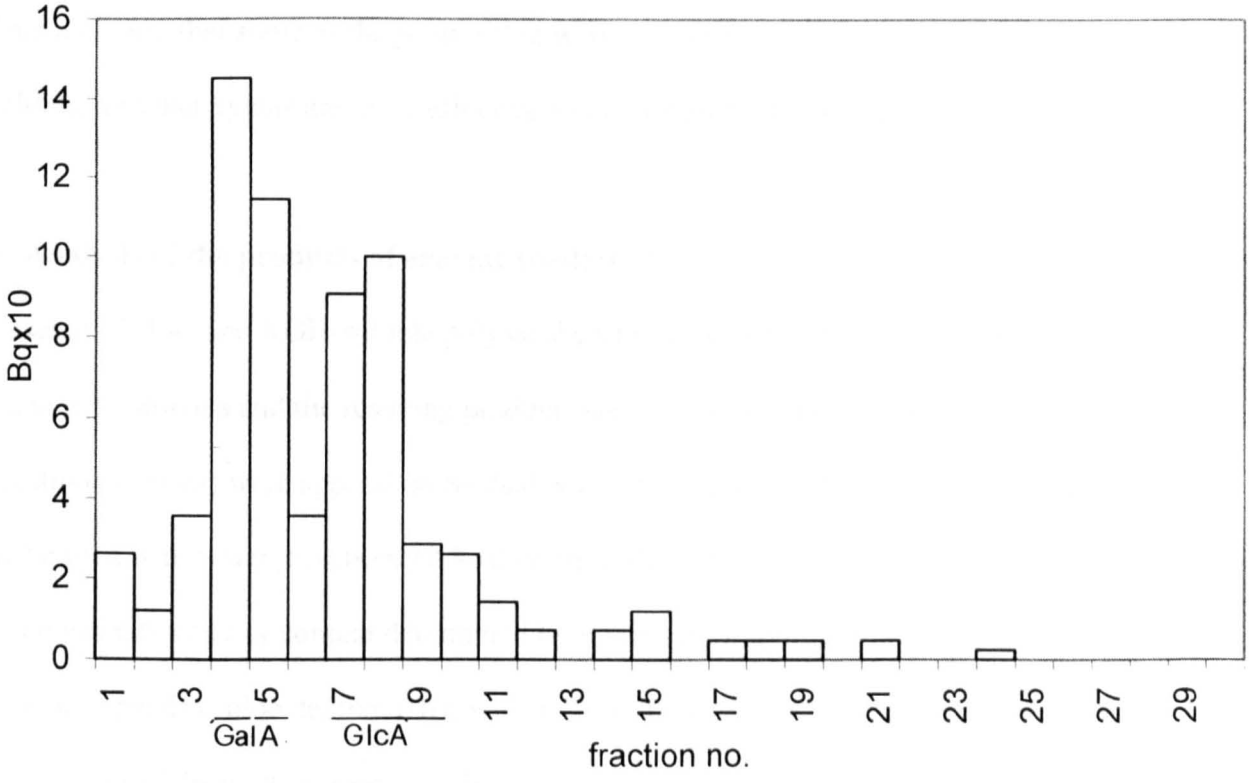

Figure 3. TLC of total acid hydrolysate of nascent EDTA-soluble ^{14}C-polysaccharides

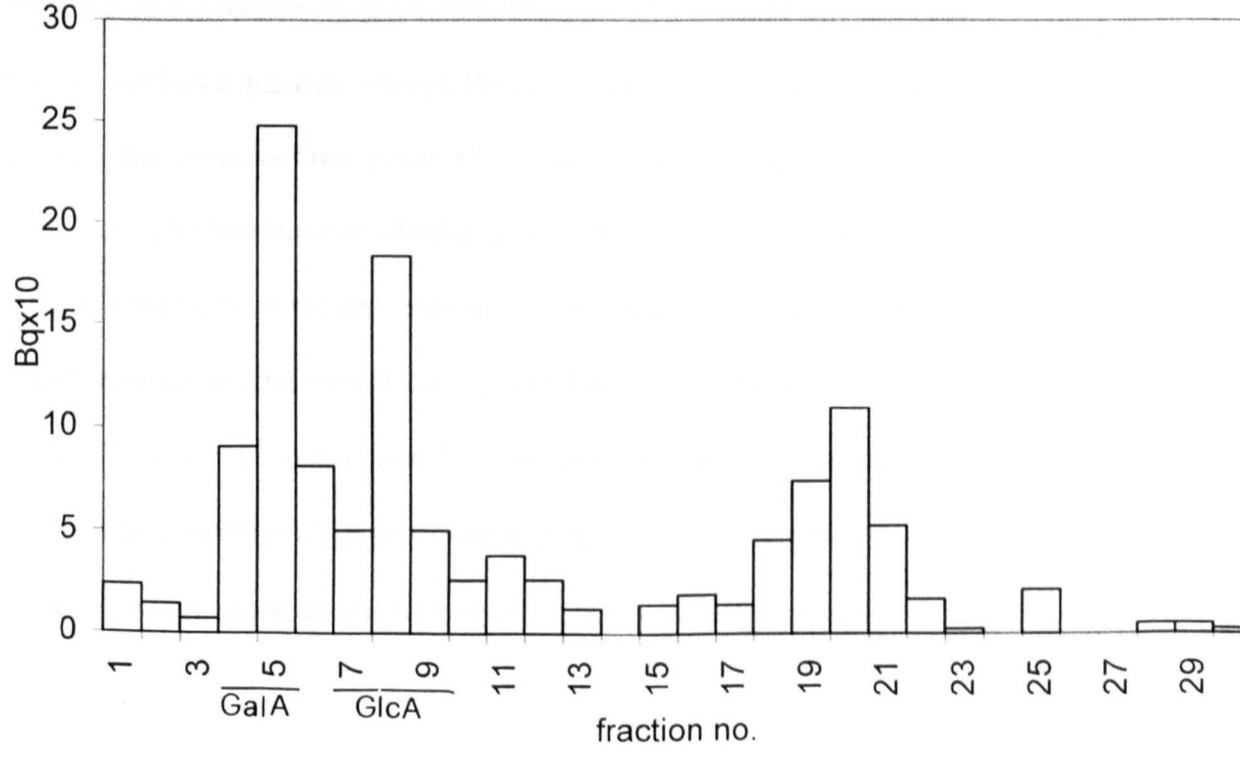

Figure 4. TLC of total acid hydrolysate of nascent KOH-soluble ^{14}C-polysaccharides

This indicates that some of the pectins that were not extracted with EDTA are alkali-soluble, and that xylans are more effectively solubilized by 4% KOH (w/v) (Fig.4).

B. Analysis of the products of enzyme treatments

Nascent EDTA- and KOH-soluble polysaccharides were subjected to different enzyme treatments and the resulting products analyzed by column chromatography. Fractions (0.6 ml) were applied on Sephadex G-100 and Biogel P-2 columns (15x1.5 cm) eluted with water. Fractions (0.5 ml) were collected using a fraction collector and their radioactivity content determined by liquid scintillation counting. In the case of Sephadex, blue dextran (BD) and DNP-lysine were used as high and low molecular weight markers respectively. When using Biogel P-2, $CoCl_2$ was used instead of DNP-lysine since DNP-lysine sticks to Biogel.

Xylanase treatment of the EDTA-soluble polysaccharides caused the breakdown of ~20 % of high molecular weight material as shown by passage through Sephadex G-100 and ~20% after passage through Biogel P-2 (Fig.5 and 6). Pectin lyase treatment caused the breakdown of more than 55% in both columns (Fig.5 and 7). This further reveals the high abundance of labelled pectins in the EDTA-soluble fractions. To verify the specificity of the enzymes on the substrates, the high molecular weight fractions remaining after xylanase and pectin lyase treatments (after passage through Biogel P-2) were collected separately. They were then subjected to complete acid hydrolysis followed by thin layer chromatography for the analysis of labelled residues. After xylanase treatment, the only labelled residue detected in the high

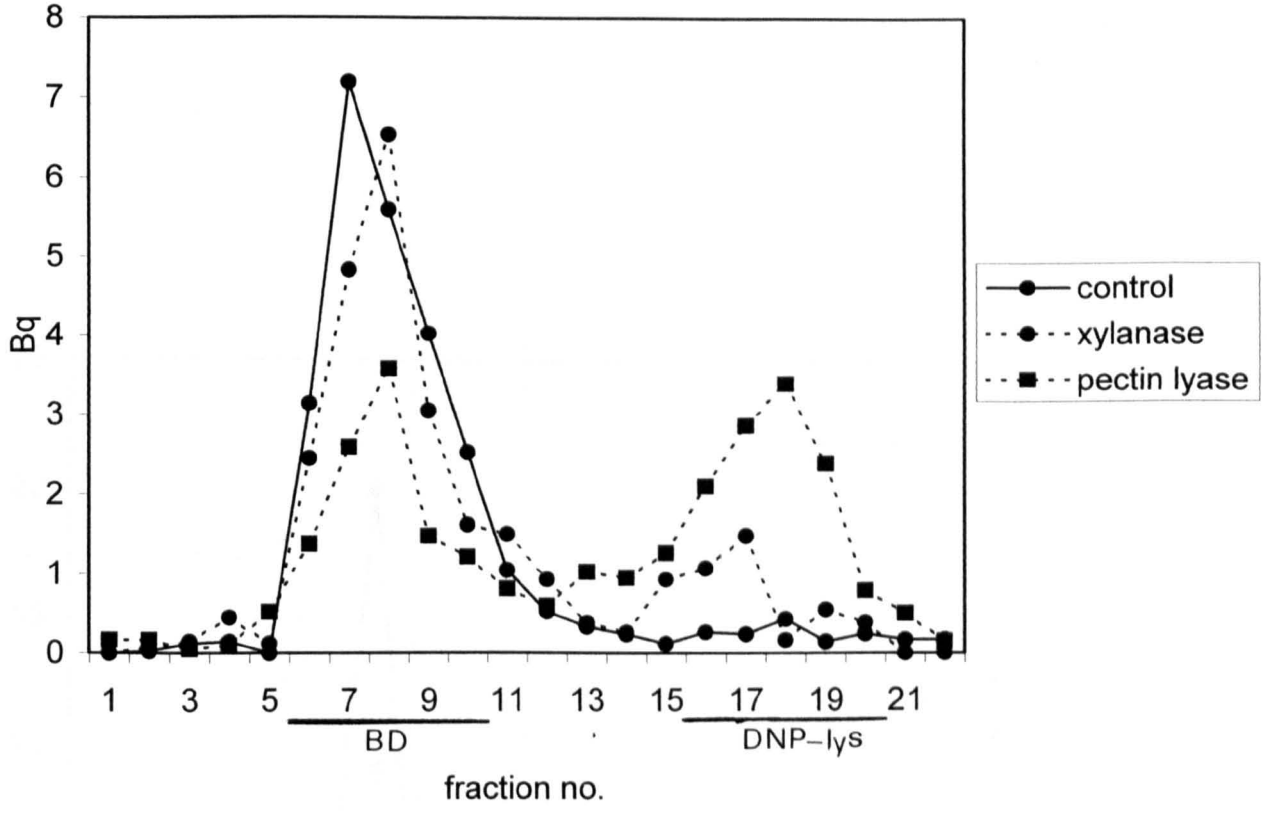

Figure 5. Gel filtration on Sephadex G-100 of xylanase and pectin lyase products of nascent EDTA-soluble ^{14}C-polysaccharides

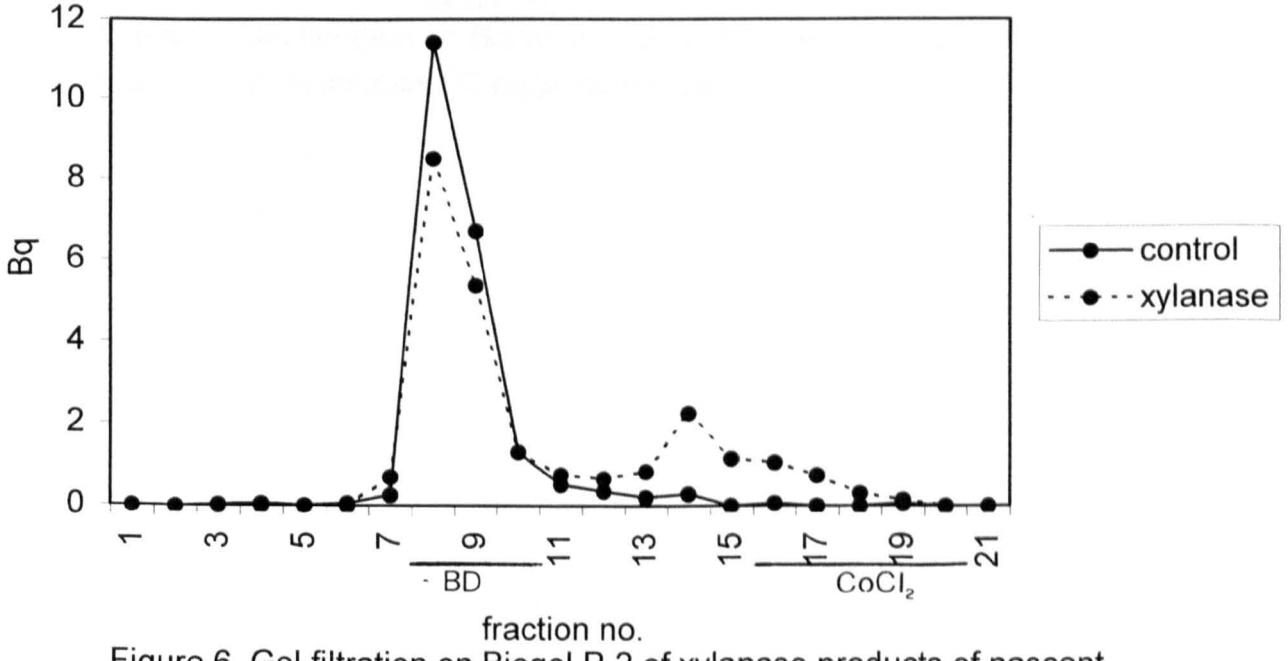

Figure 6. Gel filtration on Biogel P-2 of xylanase products of nascent EDTA-soluble ^{14}C-polysaccharides

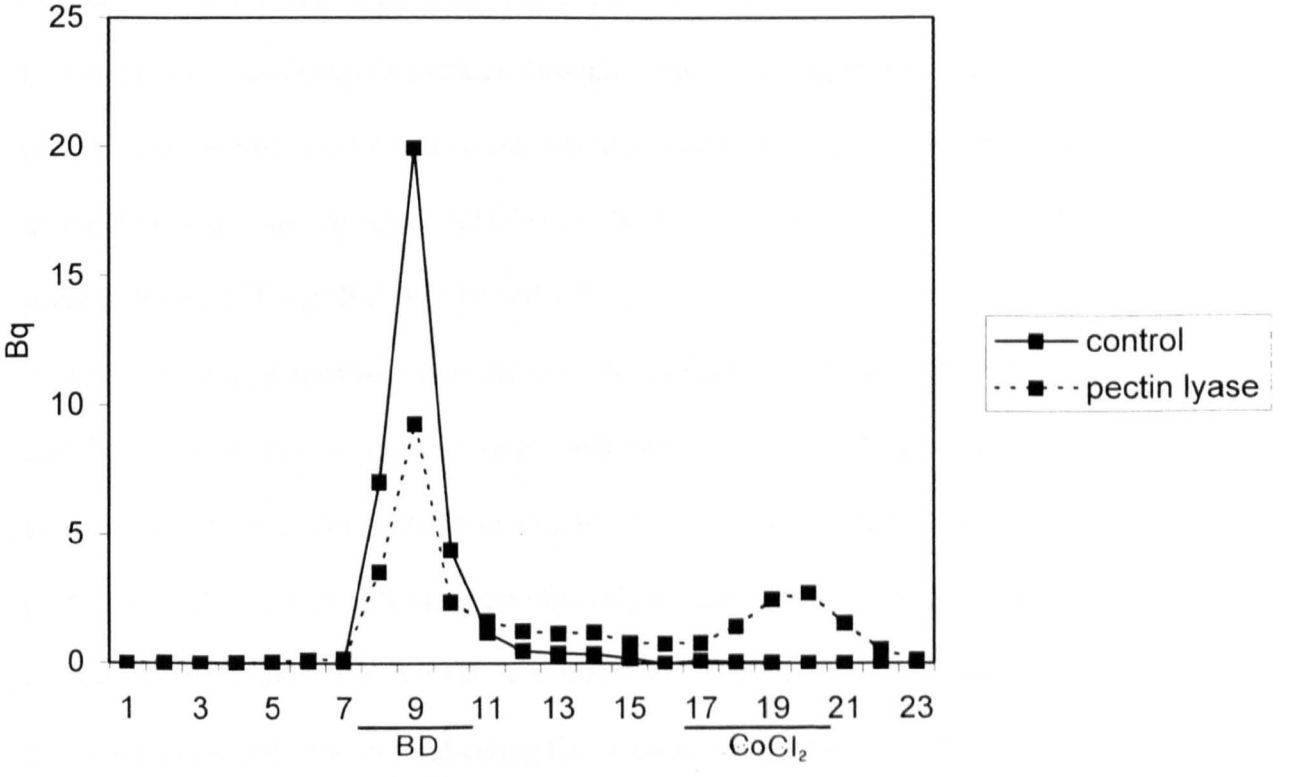

Figure 7. Gel filtration on Biogel P-2 of pectin lyase products of nascent EDTA-soluble ^{14}C-polysaccharides

molecular weight material was GalA residues (Fig. 8), whereas after pectin lyase treatment, labelled GlcA residues were the major residues detected (Fig. 9).

In the case of the KOH-soluble polysaccharides, only 16% were broken down due to xylanase treatment when passed through Sephadex G-100 although 57 % seemed to be broken down as shown by passage through Biogel P-2 (Fig.10 and 11). Due to pectin lyase treatment, a 52% decrease was observed in the high molecular weight material after passage through Sephadex G-100 compared to a 40% decrease after passage through Biogel P-2 (Fig.10 and 12).

Pectinase treatment surprisingly resulted in the complete degradation of both EDTA and KOH-soluble high molecular weight material (Fig.13 and 14). In an attempt to explain this unexpected result, the low molecular weight material resulting from pectinase treatment was collected, acid hydrolyzed and analyzed by PC using system A (see Materials and Methods). GlcA as well GalA residues seem to be released by pectinase treatment (Fig.15), indicating the contamination of the enzyme with hemicellulases that are probably breaking xylans along with pectins. This would explain the complete breakdown of all high molecular weight material.

C. Analysis of the products of alkali treatment

Nascent EDTA- and KOH-soluble polysaccharides were subjected to strong alkali treatment at 25°C and 100°C, and the products were passed through a Sephadex G-100 column as described above. At room temperature, 29 % of EDTA-soluble polysaccharides and 25% of KOH-soluble polysaccharides were broken down (Fig.16). At 100°C, 80% of EDTA-soluble polysaccharides and 58% of KOH-

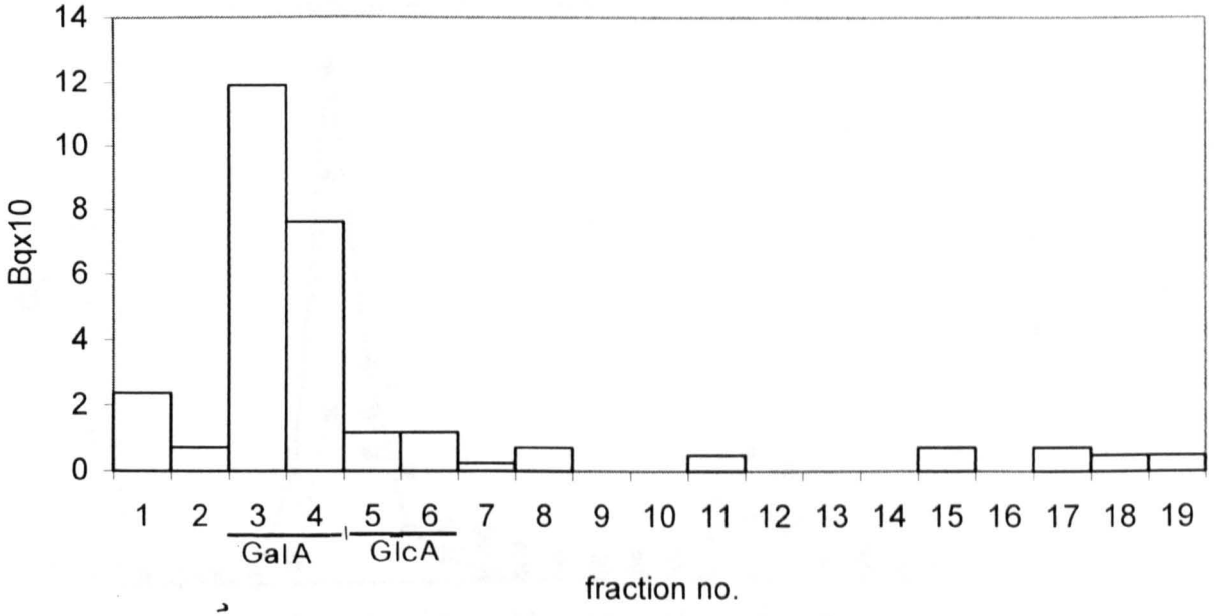

Figure 8. TLC of total acid hydrolysate of high molecular weight material remaining after xylanase treatment of nascent EDTA-soluble polysaccharides

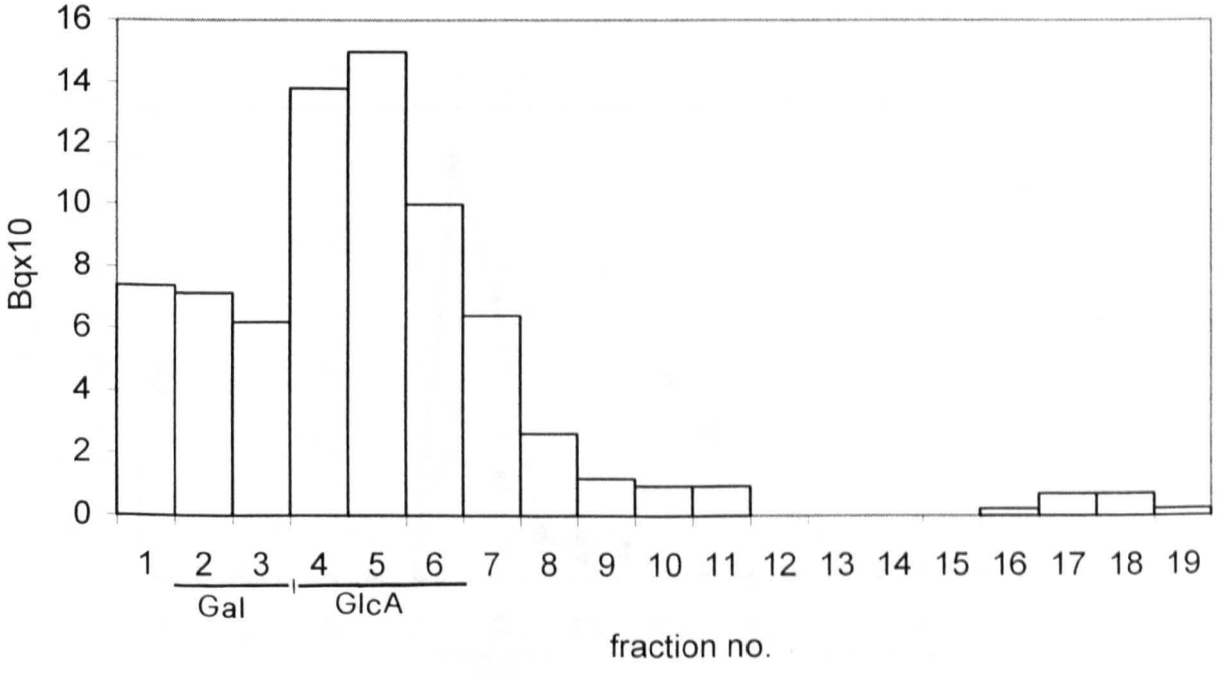

Figure 9. TLC of total acid hydrolysate of high molecular weight material remaining after pectin lyase treatment of nascent EDTA-soluble polysaccharides

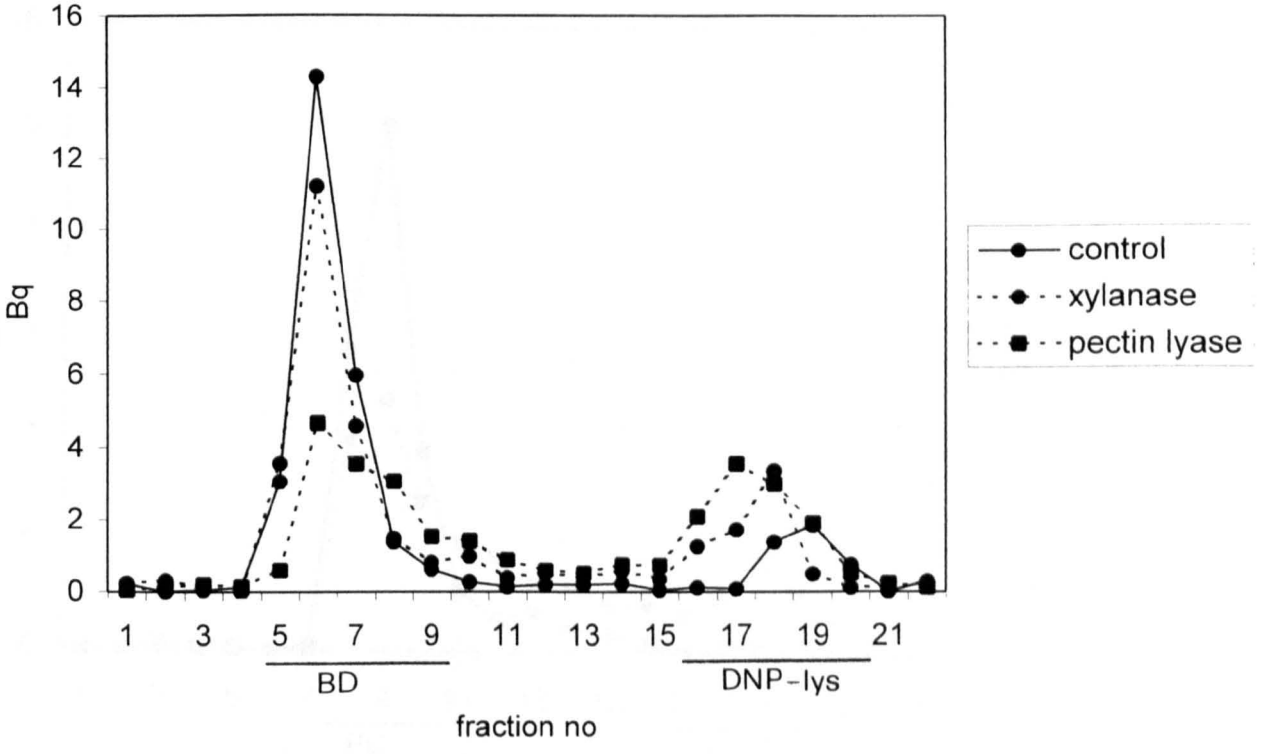

Figure 10. Gel filtration on Sephadex G-a100 of xylanase and pectin lyase products of nascent KOH-soluble ^{14}C-polysaccharides

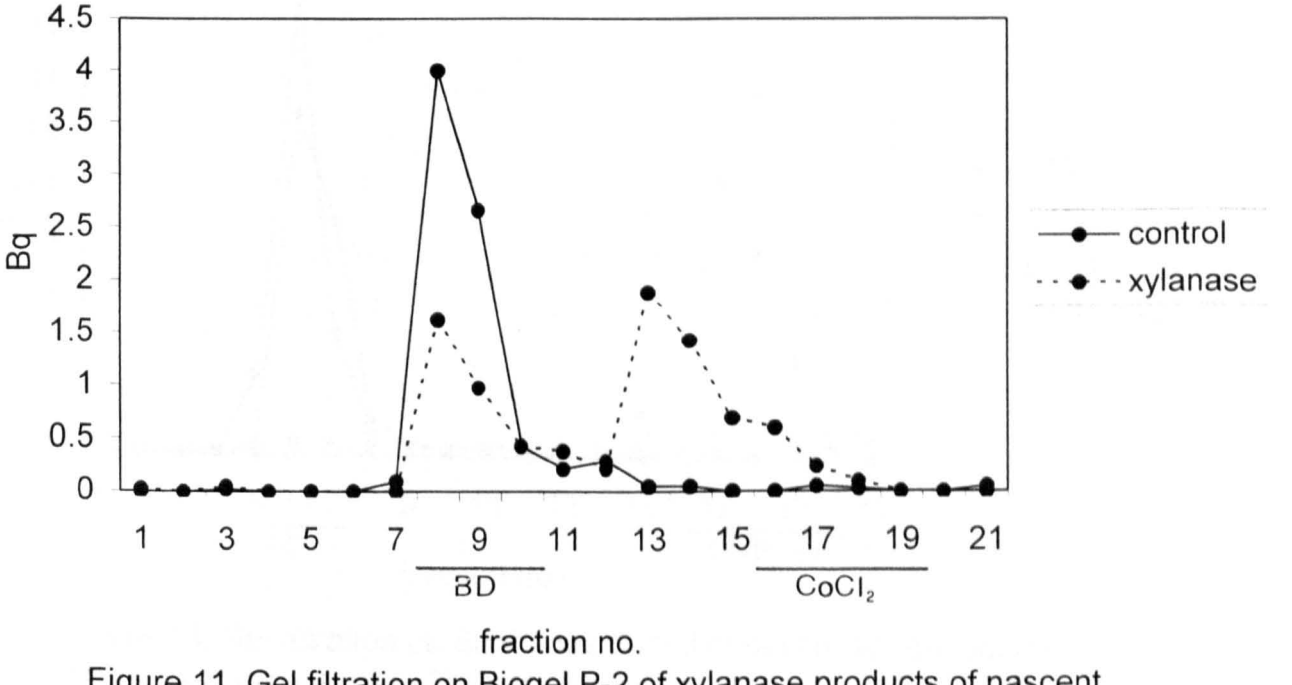

Figure 11. Gel filtration on Biogel P-2 of xylanase products of nascent KOH-soluble ^{14}C-polysaccharides

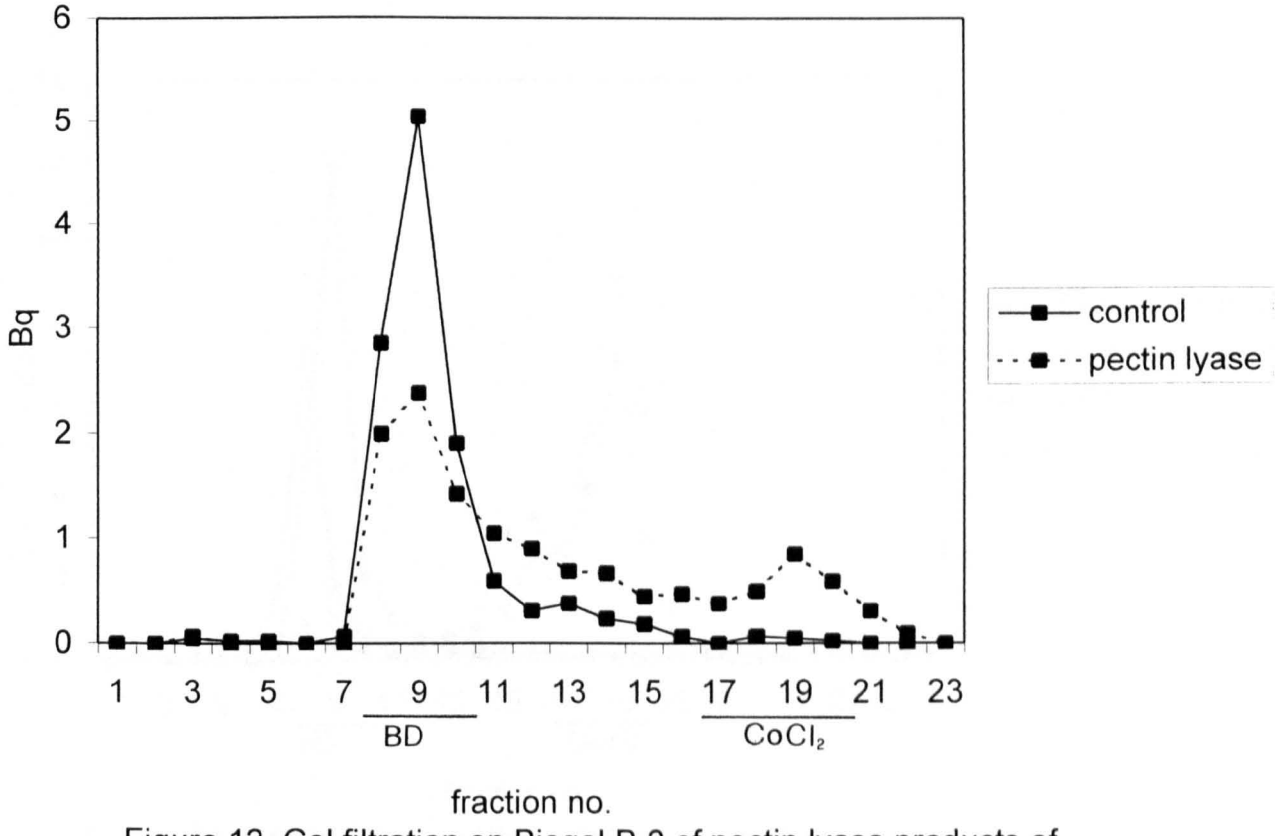

Figure 12. Gel filtration on Biogel P-2 of pectin lyase products of nascent KOH-soluble ^{14}C-polysaccharides

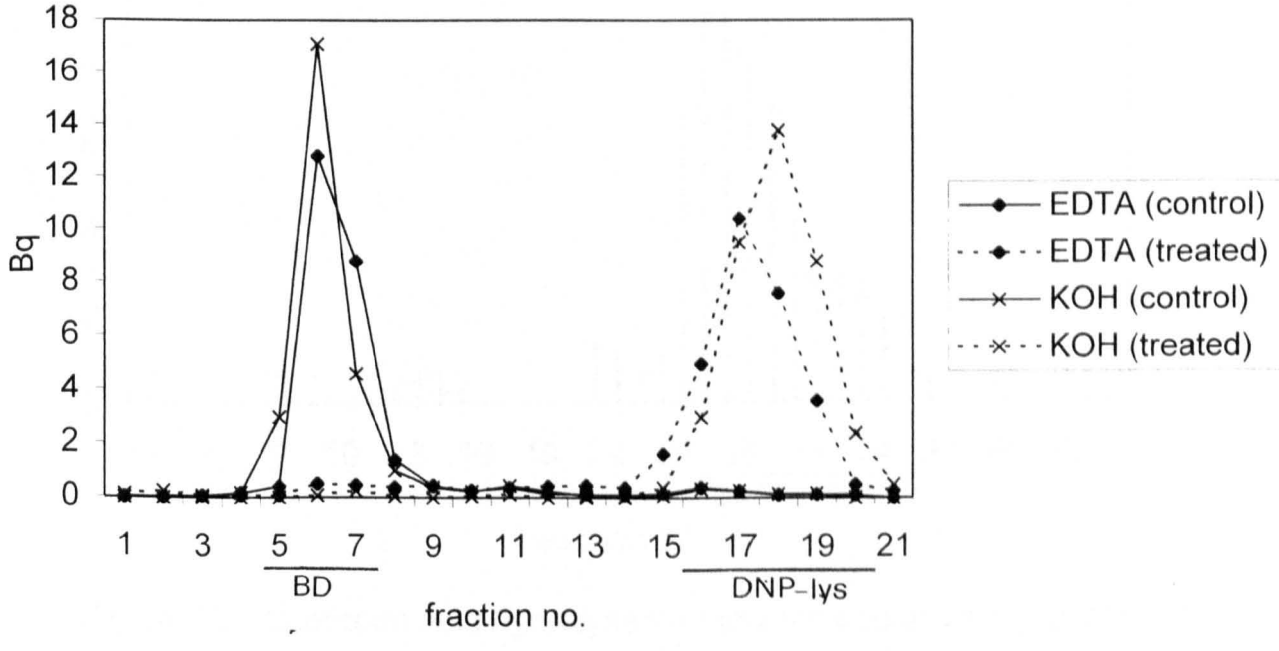

Figure 13. Gel filtration on Sephadex G-100 of pectinase products of EDTA- and KOH-soluble ^{14}C-polysaccharides

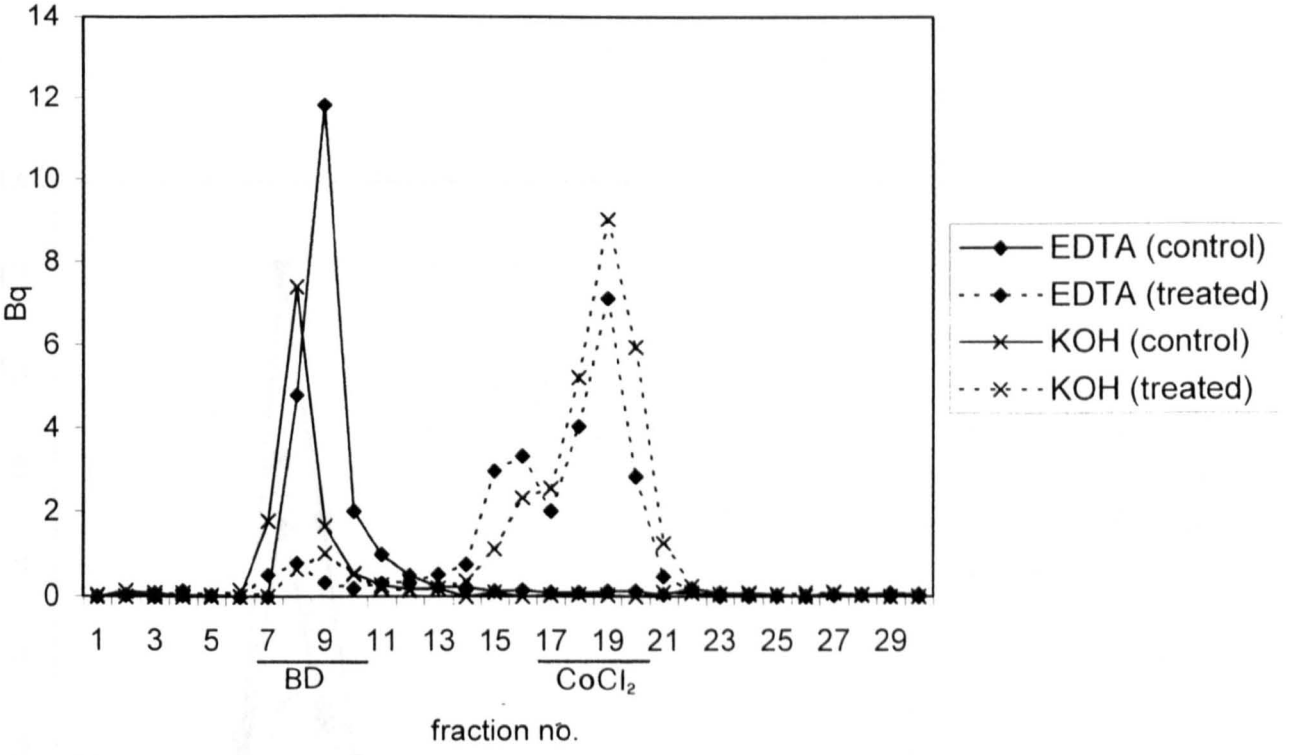

Figure 14. Gel filtration on Biogel P-2 of pectinase products of nascent EDTA- and KOH-soluble ^{14}C-polysaccharides

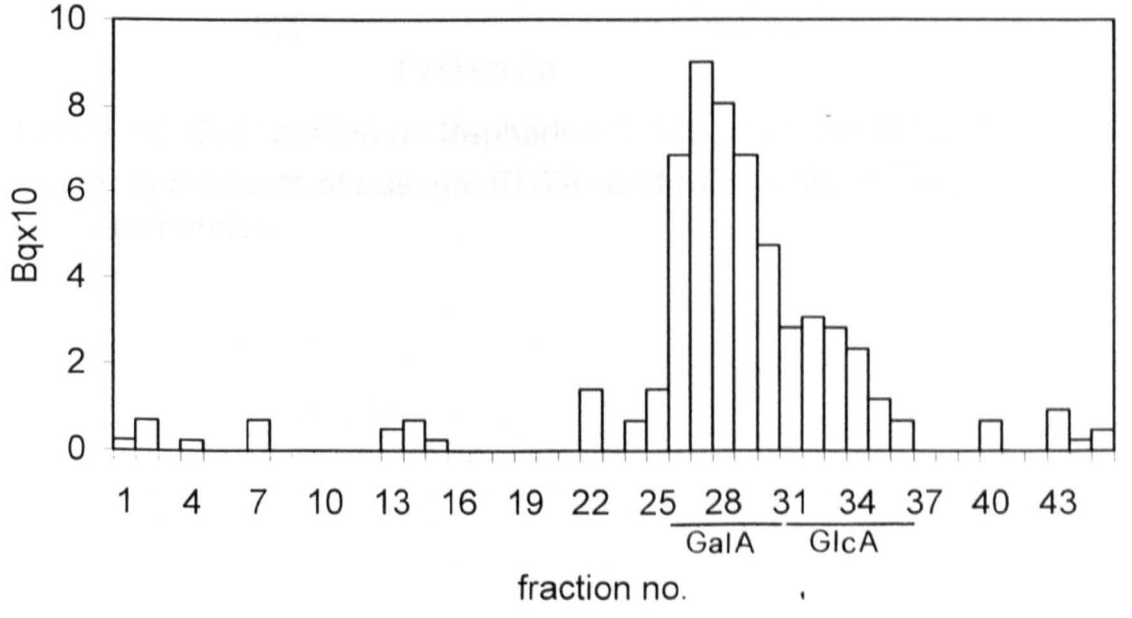

Figure 15. PC of total acid hydrolysate of low molecular weight product material after pectinase treatment of EDTA-soluble ^{14}C-polysaccharides

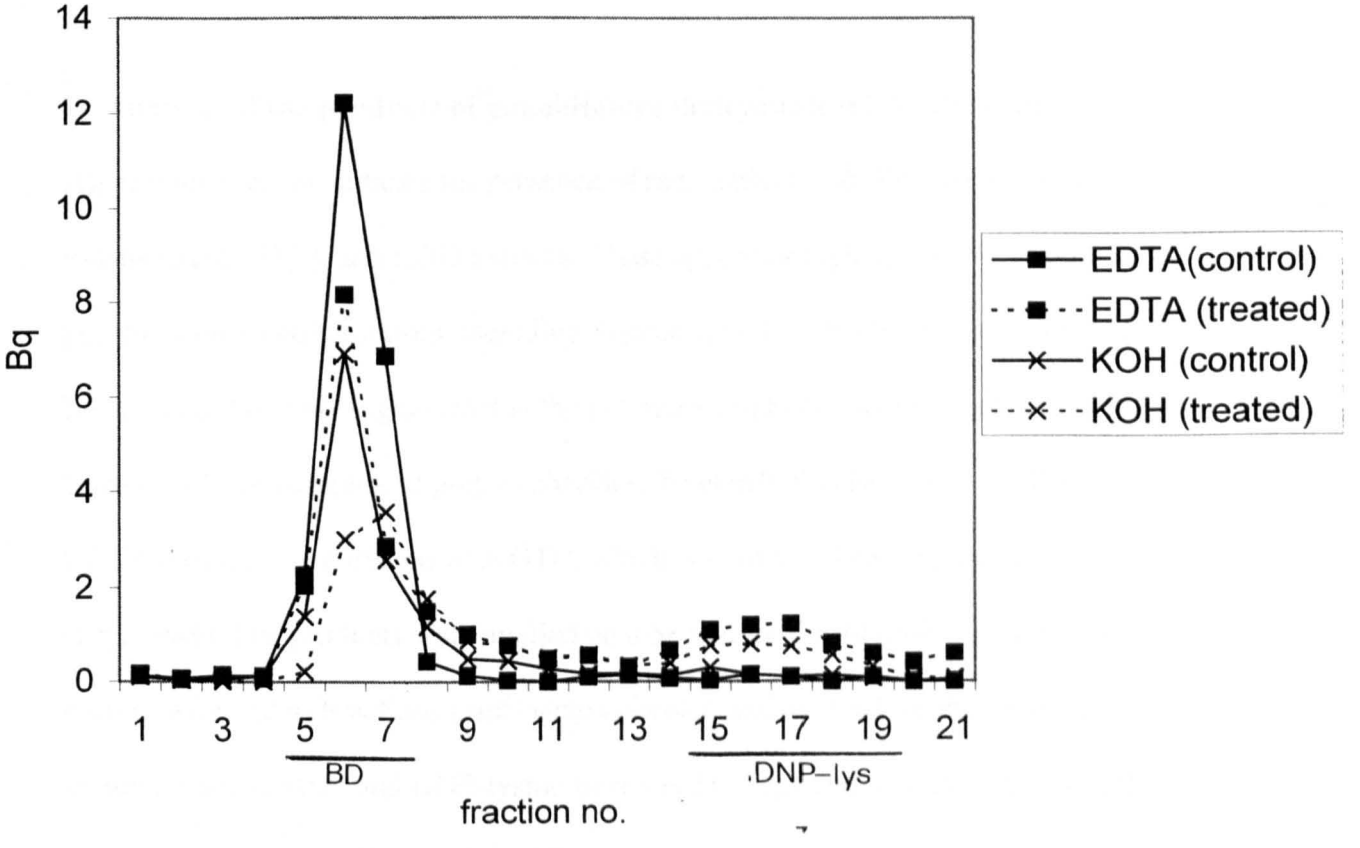

Figure 16. Gel filtration on Sephadex G-100 of strong alklai (25 degrees) products of nascent EDTA- and KOH-soluble ^{14}C-polysaccharides

soluble polysaccharides were broken down probably by β-elimination (Fig.17). This indicates the presence of pectins in both EDTA- and KOH-soluble fractions, though in higher amounts in the EDTA-extracts.

D. Analysis of the products of guanidinium thiocyanate (GTC) treatment

All previous results indicate the presence of radioactively labelled pectins and xylans in the EDTA and KOH extracts. These appear as high molecular weight polymers on all column used, including Sephadex G-100 that has a high exclusion limit (100,000). This suggested that the polymers might be associating together forming a large complex of polysaccharides. To clarify this issue, the labelled EDTA-extracts were treated with GTC, which is known to break non-covalent bonds (Fry, 1988). The products were applied on a Sepharose CL-6B column (40x2 cm), eluting with water. Fractions (1ml) were collected and assayed for their radioactivity content. Blue dextran and DNP-lysine were used as high and low molecular weight markers respectively. Most of the radioactivity was recovered in the intermediate molecular weight region, indicating the probable non-covalent association between pectins and xylans (Fig.18).

E. Characterization of xyloglucan

The identity of extracted xyloglucan was studied using the iodine-sulphate method (Kooiman, 1960), before pelleting with ethanol. Xyloglucan was also cellulase (endo-1,4β-glucanase) treated and the products passed through the 40 cm Sepharose CL-6B column previously mentioned. Fractions (1.5 ml) were collected and their

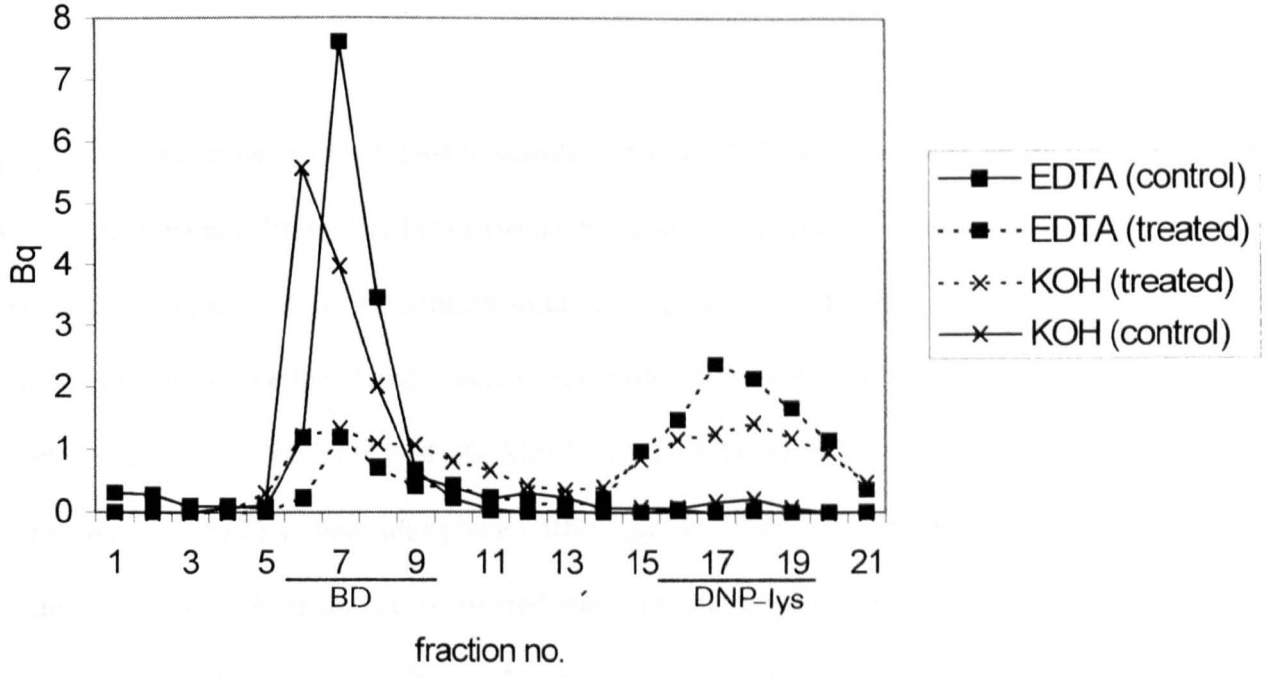

Figure 17. Gel filtration on Sephadex G-100 of strong alkali products (100 degrees) of nascent EDTA- and KOH-soluble ^{14}C-polysaccharides

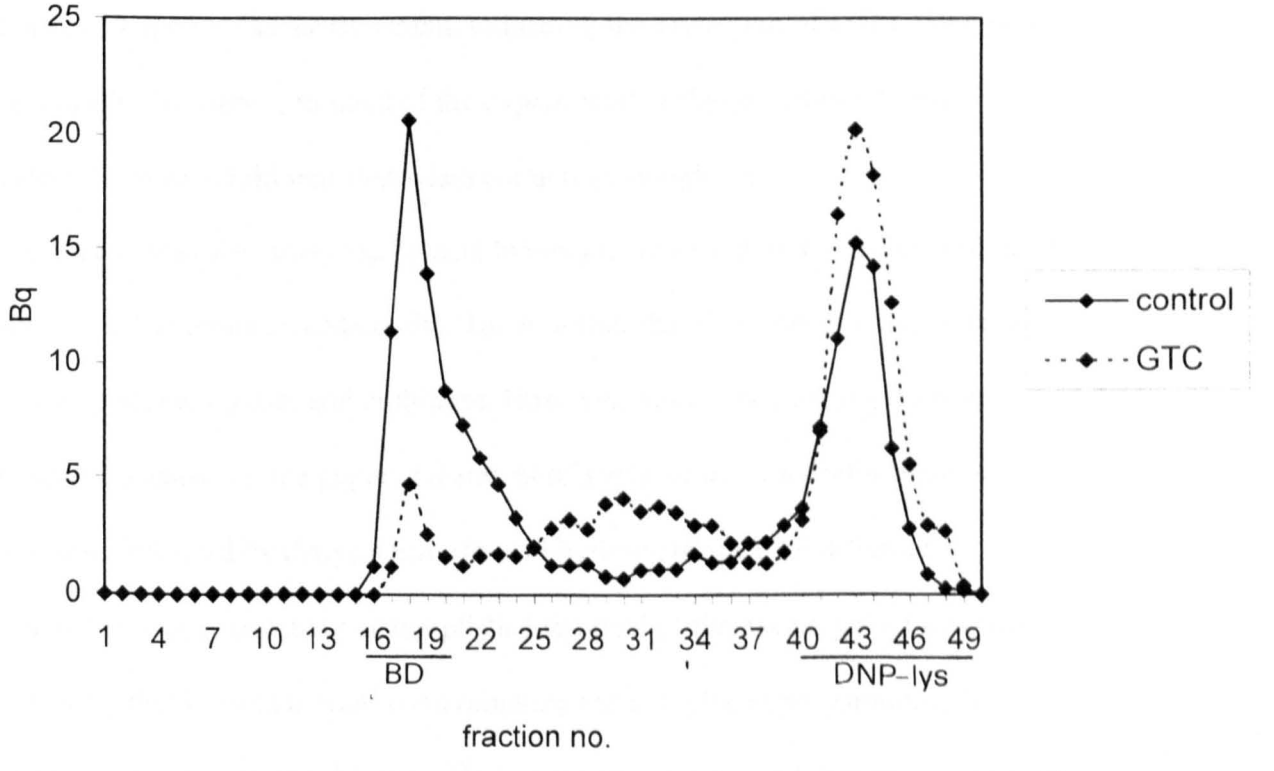

Figure 18. Gel filtration on Sepharose CL-6B of GTC products of ^{14}C-ETDA-extract

xyloglucan content determined. Due to cellulase treatment, the peak accounting for xyloglucan presence disappeared (Fig.19) confirming the presence of xyloglucan.

The effect of pH and salt on xyloglucan solubility was also investigated. Xyloglucan pellets (pelleted from ethanol and washed once with water) were washed twice (~1 minute) with 1 ml of NaCl (1M, pH 4), $MgCl_2$ (1M, pH 4), $AlCl_3$ (1M, pH 4) and 10mM oxalate/10mM sodium phosphate buffers (pH 4,5 and 6). Control pellets were washed with water. Supernatants recovered after centrifugation at 10000*g* were assayed for xyloglucan content. It was shown that neither pH nor high salt concentration affect xyloglucan solubility (Table 1).

It was suggested that the method being used to extract xyloglucan is resulting in some contamination of xylan. Xyloglucan was hence xylanase treated and the effect on the weight obtained was studied. The amount of xyloglucan obtained decreased about 50% upon xylanase treatment, indicating the extraction of xylan along with the xyloglucan. Hence, in most of the experiments, xylanase treatment was performed as an additional step when extracting xyloglucan.

Xyloglucan was also analyzed by acid hydrolysis followed by PC using systems A and C (see Materials and Methods). The monosaccharides present were as expected: xylose, glucose, fucose, and arabinose. However, large amounts of galactose residues appeared on the paper. Treatment of xyloglucan with pectin lyase or pectinase followed by dialysis prior to acid hydrolysis made no difference. Treatment with galactanase accomplished removal of the excess galactose residues indicating that galactans were contaminating the xyloglucan preparations. An attempt to remove all residues by cellulase treatment was tried, however it was

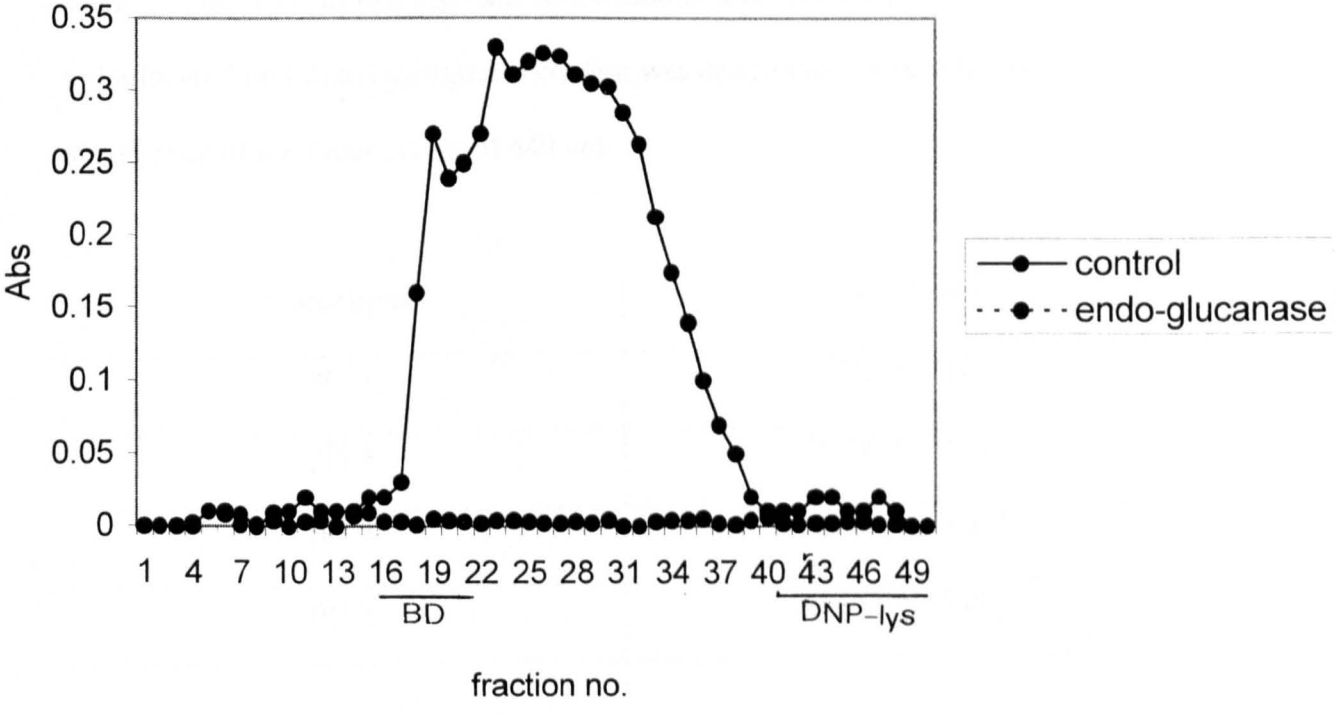

Figure 19. Gel filtration on Sepharose CL-6B of endo-glucanase products of xyloglucan

Table 1. The effect of pH, high salt concentration and GTC on the solubility of xyloglucan. Solubilized xyloglucan content was determined by reading the absorbance of the supernatants at 640 nm.

Conditions	Absorbance
pH 3	0.255 +/- 0.005
pH 4	0.260 +/- 0.010
pH 5	0.265 +/- 0.005
pH 6	0.255 +/- 0.005
Control	0.241 +/- 0.008
NaCl (1M)	0.240 +/- 0.001
$MgCl_2$ (1M)	0.242 +/- 0.002
$AlCl_3$ (1M)	0.240 +/- 0.002
GTC (5.5 M)	(5)

unsuccessful because the enzyme itself is contaminated with monosaccharide residues.

Conclusion

Nascent EDTA-soluble [uronate-^{14}C]-polysaccharides consist of radioactively labelled pectins and xylans, whereas pectins occur in higher amounts. KOH-soluble polysaccharides have the same composition, though xylans are present in slightly higher amounts.

The method used to prepare xyloglucan resulted in the extraction of xylans and galactans along with xyloglucan. However, the contaminants could be easily removed by xylanase and galactanase treatments.

CHAPTER 4

Characteristics and requirements of the binding of nascent [^{14}C]-polysaccharides to xyloglucan

Introduction

Nascent EDTA-soluble polysaccharides were shown to consist of pectin and xylan. This section describes the binding of these polysaccharides to xyloglucan. The requirements and characteristics of the binding are also investigated.

A. Effect of pH on binding

Binding of nascent EDTA-soluble [uronate-^{14}C]-polysaccharides to pea xyloglucan was pH-dependent, showing highest binding (~25%) at pH 3-4 (Fig.20). Binding decreased sharply above pH 4, reaching negligible values at pH 6. A similar pattern was observed upon binding of EDTA-soluble [Xyl-^{14}C]-polysaccharides to xyloglucan (Fig.21). The effect of different pH's on xyloglucan solubility was studied: no effect was observed on xyloglucan solubility due to a change in pH (Table 1). KOH-soluble [^{14}C]-polysaccharides also showed the same pH-dependent binding to pea xyloglucan (Fig 22). Since no major difference was observed between EDTA- and KOH-soluble polysaccharides, all remaining experiments were performed using EDTA-soluble polysaccharides (except where specified).

B. Time course of binding

Standard binding assays were performed at pH 4, 25°C for various times up to 15 minutes. Centrifugation was carried out for only one minute to stop the reaction. The

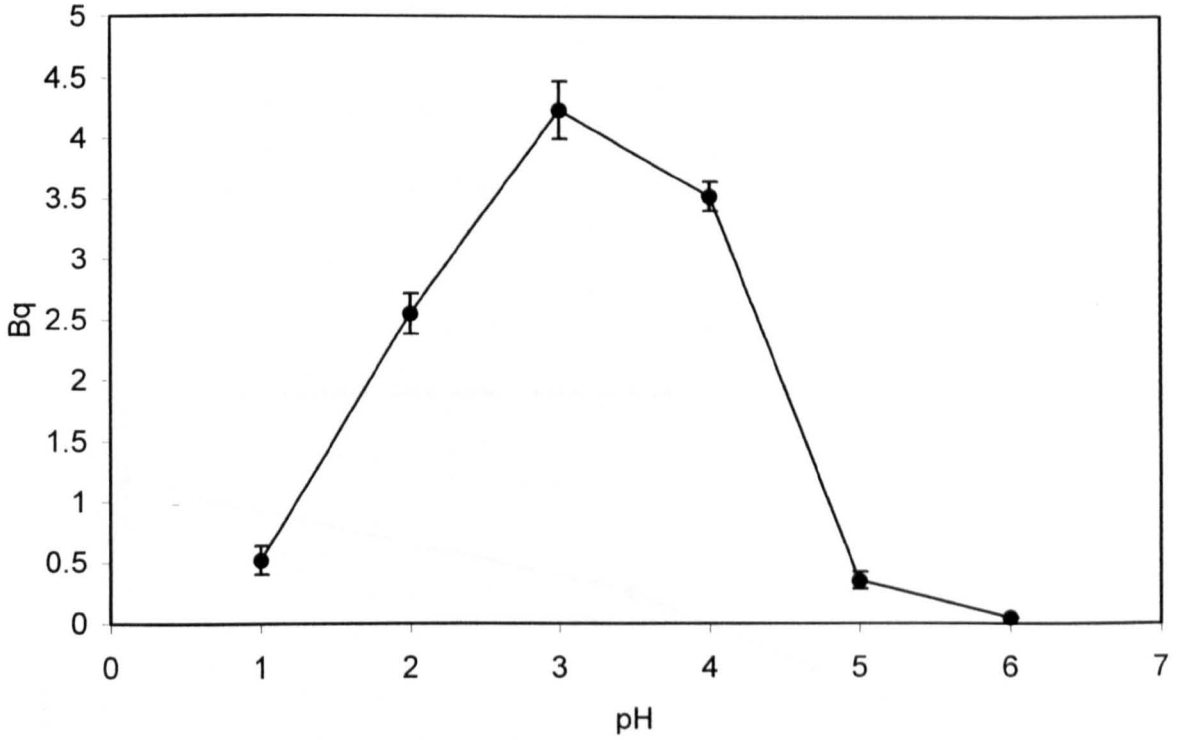

Figure 20. Effect of pH on binding of nascent EDTA-soluble ^{14}C-polysaccharides to xyloglucan

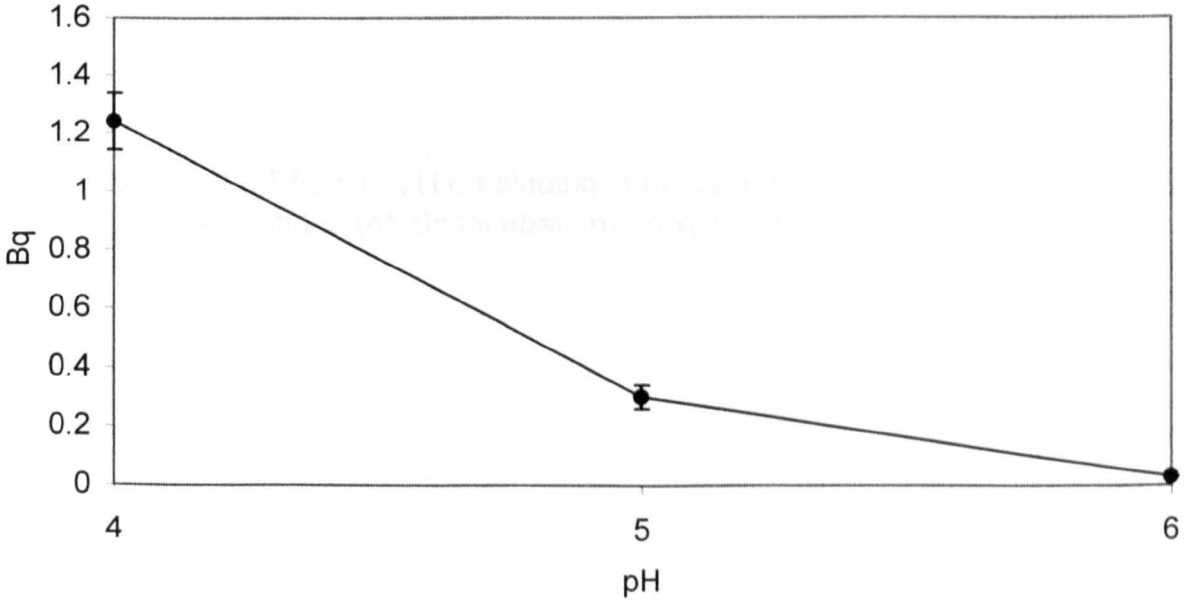

Figure 21. Effect of pH on binding of nascent EDTA-soluble [Xyl-^{14}C]-xylans (9.5 Bq/incubation) to xyloglucan

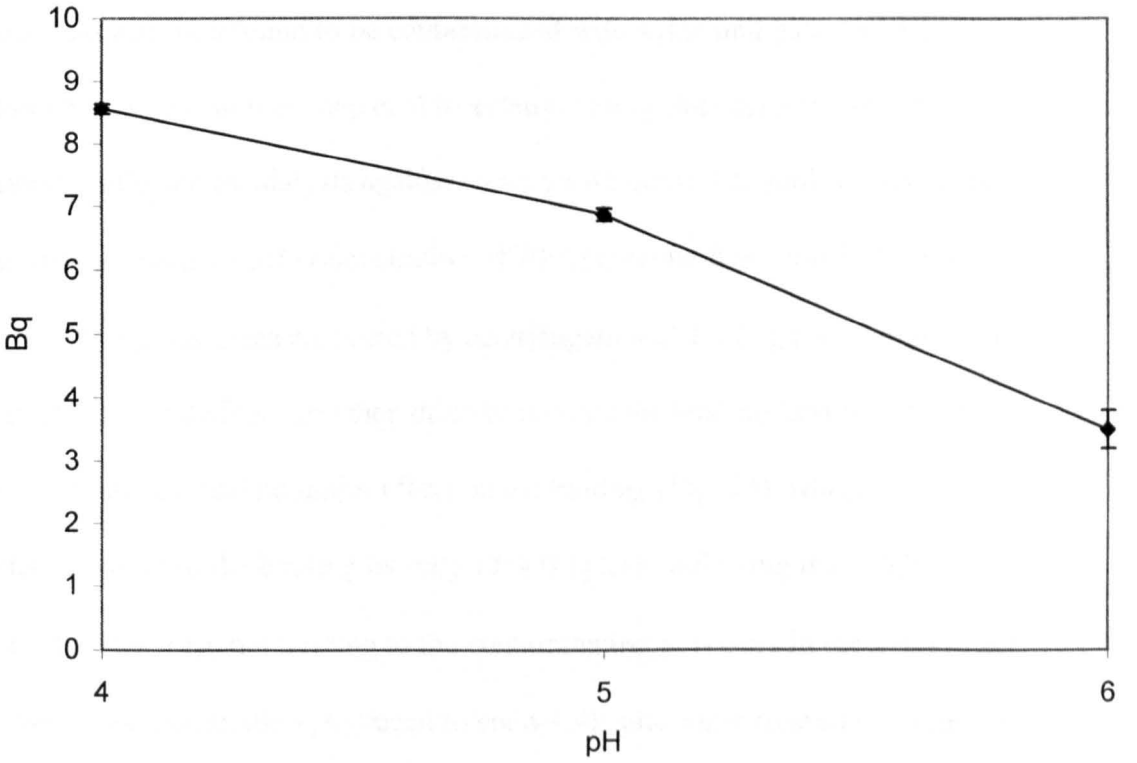

Figure 22. Effect of pH on binding of nascent KOH-soluble uronate-^{14}C-polysaccharides (45 Bq/incubation) to xyloglucan

binding seems to be instantaneous (Fig. 23), indicating that the association between xyloglucan and the EDTA-soluble polysaccharides is probably non-covalent. In all other experiments, standard binding assays were carried out for 5 minutes.

C. Effect of xylanase and galactanase treatments of xyloglucan on binding

Since xyloglucan was found to be contaminated with xylan and galactan (Chapter 3, Section E), xyloglucan was subjected to xylanase and galactanase treatments separately, followed by dialysis against water for 48 hours. Ethanol was added to the xyloglucan to reach a final concentration of 70% (v/v) and then stored at 4°C during 48 hours. Xyloglucan was recovered by centrifugation at 13000g for 10 minutes in a Sorvall RC-5B centrifuge, and then dried to be used for binding assays at pH 3. Xylanase treatment had no major effect on the binding (Fig. 24), whereas galactanase reduced the binding by only 11% (Fig.25), indicating that [^{14}C]-polysaccharides were not binding to the contaminating polymers in the xyloglucan prep. When subjecting the xyloglucan to endo-1,4β-glucanase treatment, more than 70% of the binding was abolished, whereas when subjected to both galactanase and endo-1,4β-glucanase treatment (xylanase treatment was already included in the method of xyloglucan extraction),very low levels of binding were detected (Fig. 25).

D. Effect of varying the amounts of xyloglucan and [^{14}C]-polysaccharides on binding

To study the relationship between the amount of pea xyloglucan present and the amount of binding to nascent polysaccharides, binding assays were performed at

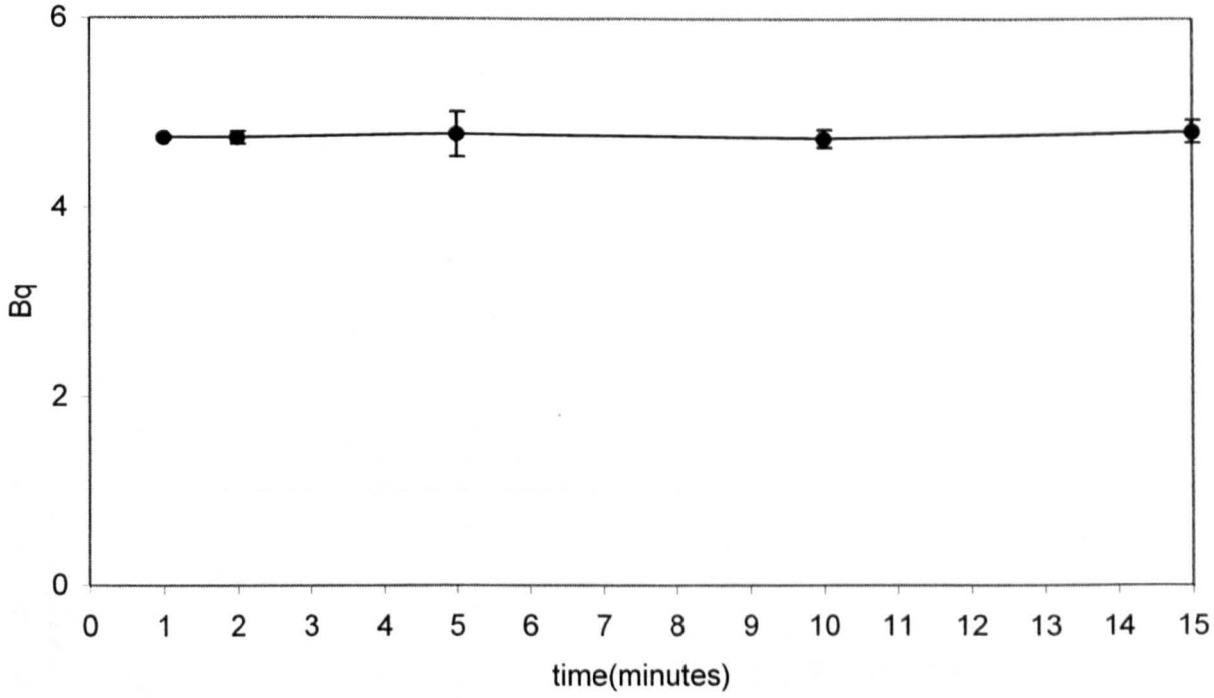

Figure 23. Time course of binding of nascent EDTA-soluble ^{14}C-polysaccharides to xyloglucan

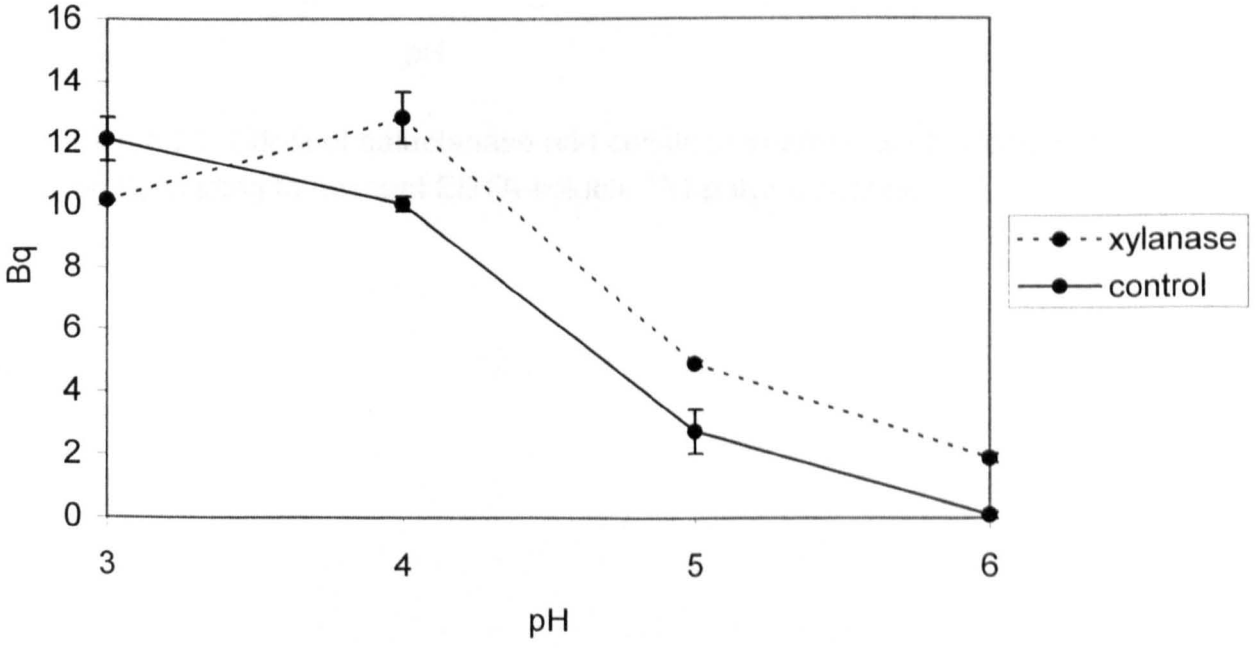

Figure 24. Effect of xylanase treatment of xyloglucan on its binding to nascent EDTA-soluble ^{14}C-polysaccharides

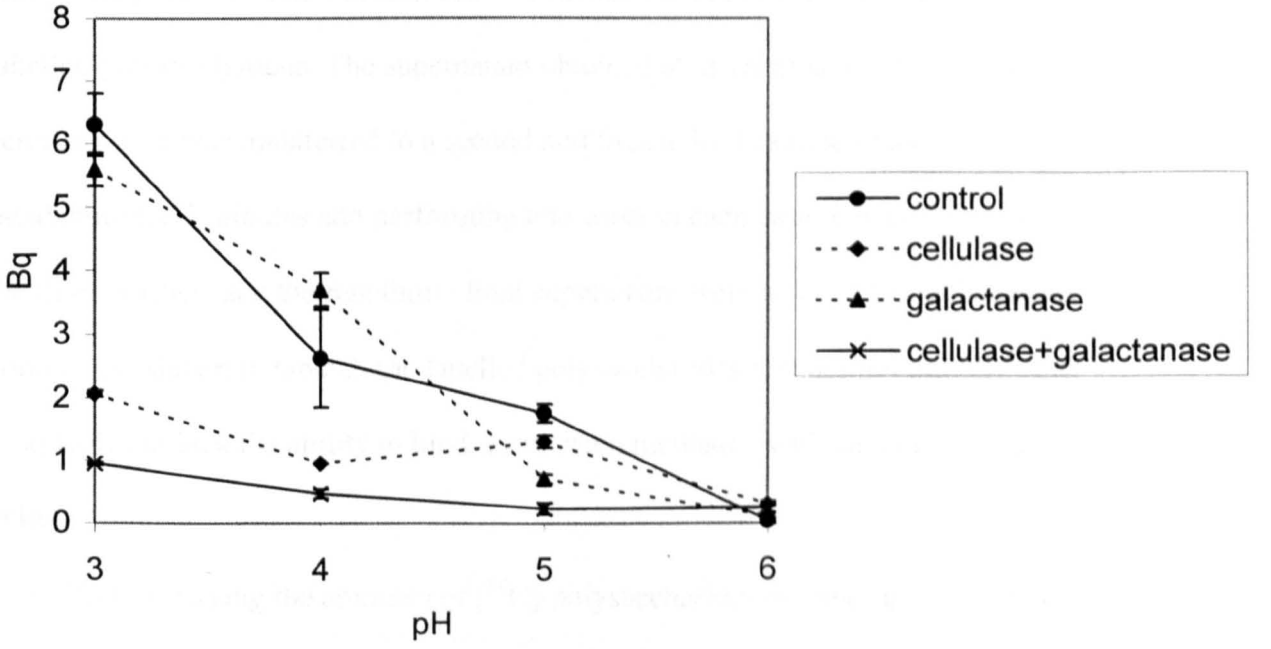

Figure 25. Effect of galactanase and cellulase treatments of xyloglucan on its binding to nascent EDTA-soluble ^{14}C-polysaccharides

pH 4, using a constant amount of [^{14}C]-polysaccharides with varying amounts of xyloglucan. Binding increased with increasing xyloglucan, with saturation at high xyloglucan levels (Fig.26). Maximum binding was about 30% of the [^{14}C]-polysaccharides in the incubation. To confirm that the unbound labelled polysaccharides lacked the ability to bind to xyloglucan the following experiment was performed: a standard incubation was carried out at pH 4 using 45 Bq of labelled polysaccharides. The supernatant obtained after terminating the reaction by centrifugation was transferred to a second and then a third sample of xyloglucan, incubating for 5 minutes and performing one wash in each case. Each of the pellets, the three washes, and the remaining final supernatant were assayed for radioactivity content. As shown in table 2, the labelled polysaccharides that did not initially bind to xyloglucan lack the ability to bind, even when incubated with an intact xyloglucan pellet.

The effect of varying the amounts of [^{14}C]-polysaccharides on binding was tested at pH 4, using a constant amount of xyloglucan with increasing amounts of [^{14}C]-polysaccharides. Binding was proportional to the amount of labelled polysaccharides present (Fig. 27).

E. Effect of NaCl, $MgCl_2$, $AlCl_3$ and guanidinium thiocyanate

Standard binding assays were performed at pH 4, with one wash with pH 4 buffer. To study the nature of the binding and test its lability to high salt, the pellet was further washed twice with NaCl, $MgCl_2$ or $AlCl_3$ (pH 4, 1ml). Control pellets were washed twice with oxalate-phosphate buffer (pH 4). Radioactivity in the pellets and

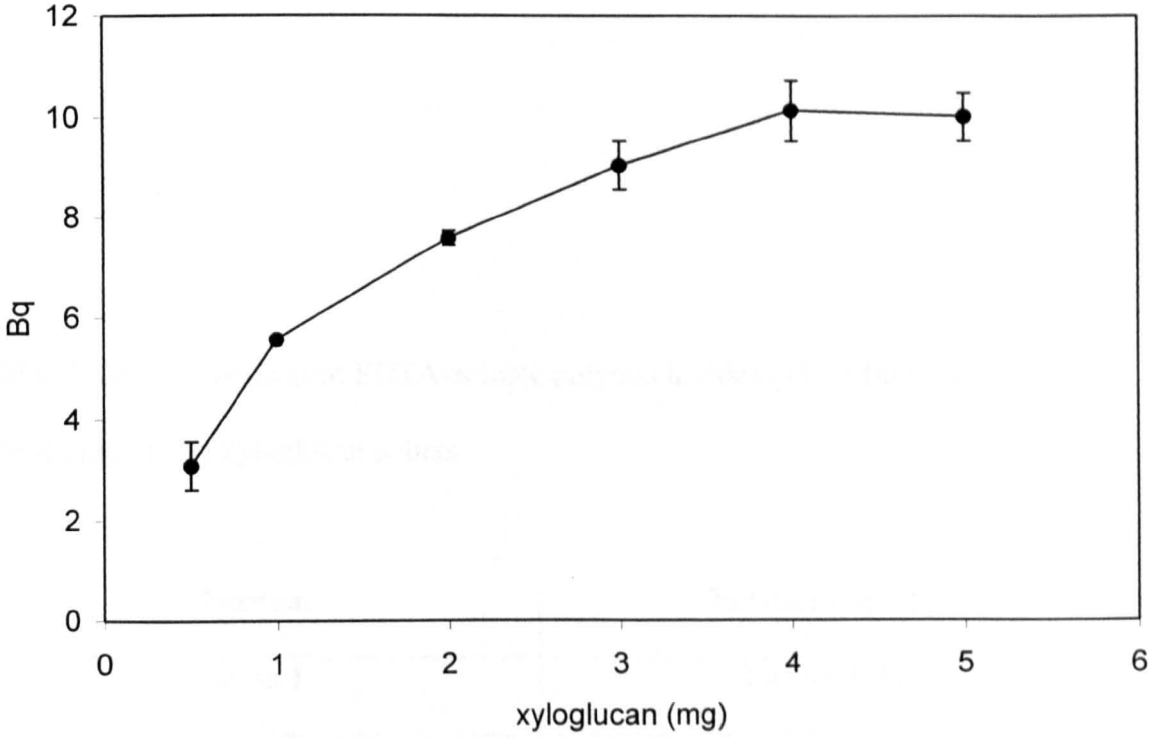

Figure 26. Effect of varying amounts of xyloglucan on binding to nascent ^{14}C-polysaccharides

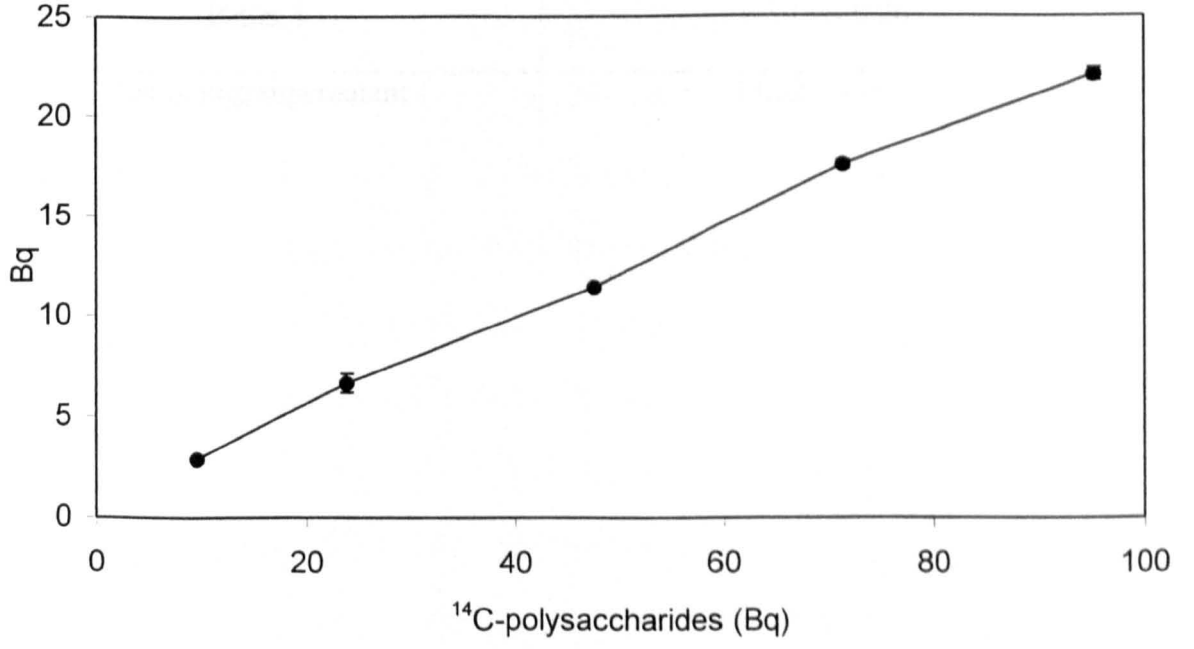

Figure 27. Effect of varying amounts of nascent ^{14}C-polysaccharides on binding to xyloglucan

Table 2. Binding of nascent EDTA-soluble polysaccharides (45.22 Bq) to three consecutive fresh xyloglucan pellets

Fraction	Radioactivity (Bq)
Wash 1	1.45 +/- 0.12
Pellet 1	21.18 +/- 0.36
Wash 2	0.83 +/- 0.05
Pellet 2	1.78 +/- 0.12
Wash 3	0.74 +/- 0.24
Pellet 3	0.59 +/- 0.26
Remaining supernatant	14.52 +/- 0.33

the combined washes was determined. The lability of the bond to guanidinium thiocyanate (GTC) was also tested by incubating the washed pellets with GTC (5.5M, pH 4, 1 ml) for 18 hours at 25°C. Controls were incubated with oxalate-phosphate buffer (pH 4), under the same conditions. Pellets were recovered by centrifugation (10000g, 5 minutes). Radioactivity in the supernatant and the pellet was determined.

NaCl, $MgCl_2$ and $AlCl_3$ released 46%, 53% and 70% of the radioactivity, respectively, into the supernatant (Table 3), suggesting that the binding of [^{14}C]-polysaccharides to xyloglucan was partly due to ionic bonds. The effect of salt treatments on xyloglucan solubility was studied using the iodine-sulphate method (Kooiman, 1960), and it was observed that xyloglucan solubility is not affected by high salt concentrations (Table 1).

H-bonding appears to also have an essential role in the binding of [^{14}C]-polysaccharides to xyloglucan, since almost all the radioactivity (86%) was released by GTC treatment (Table 3). However, one cannot rule out the possibility that the release was due to solubilization of xyloglucan by GTC, since the effect of GTC on xyloglucan solubility could not be investigated. This was basically because the absorbance of the supernatant after the treatment could not be determined due to the presence of the high concentration of GTC (Table 1).

F. Effect of mild acid treatment of xyloglucan on binding

Fig.28 shows that tamarind xyloglucan (Megazyme), which lacks fucose, bound [^{14}C]-polysaccharides much less effectively than pea xyloglucan. To test the effect

Table 3. The effect of NaCl, $MgCl_2$, $AlCl_3$ and GTC on binding of EDTA-soluble [^{14}C]-polysaccharides to xyloglucan

Treatment	Radioactivity bound (Bq)	%Decrease
Control	1.75 +/- 0.20	-
NaCl (1M)	0.95+/- 0.01	46
$MgCl_2$ (1M)	0.82 +/- 0.01	53
$AlCl_3$ (1M)	0.53 +/- 0.02	70
Control	1.81 +/- 0.19	-
GTC (5.5M)	0.25 +/- 0.06	86

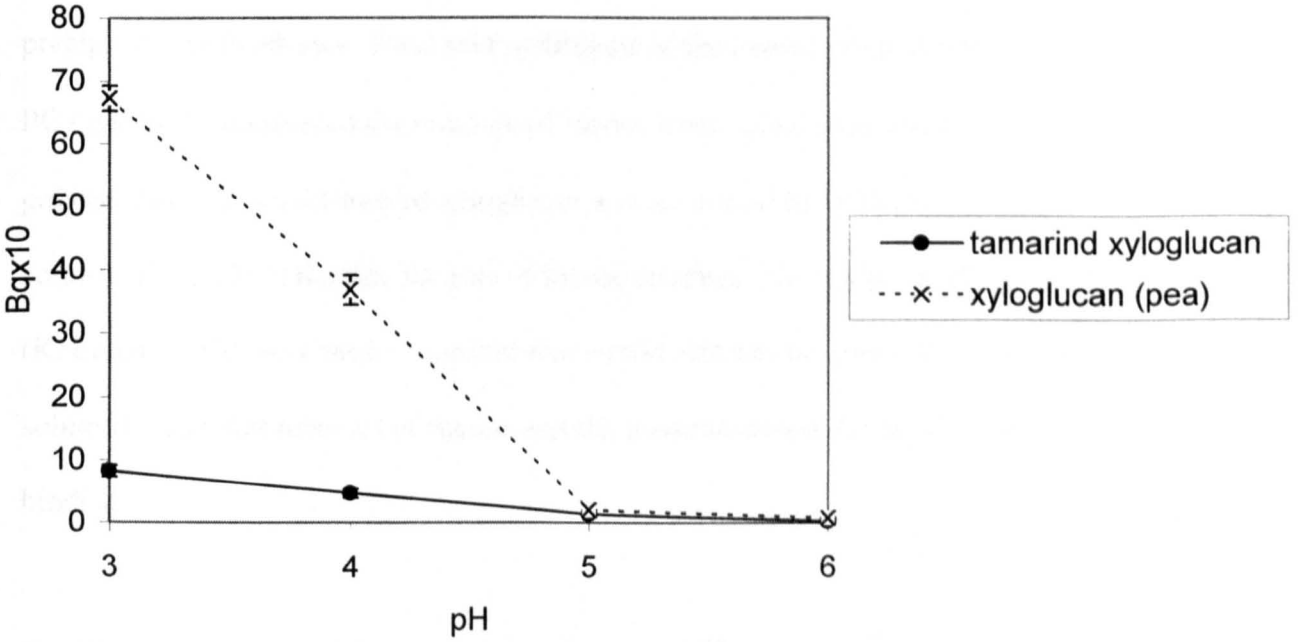

Figure 28. Effect of pH on binding of nascent EDTA-soluble ^{14}C-polysaccharides to tamarind xyloglucan

of removing fucose from pea xyloglucan, the process of xyloglucan extraction was modified by keeping the extracted xyloglucan samples in solution (avoiding precipitation with ethanol). After neutralization of the 24% KOH extract with acetic acid, it was treated with mild acid (10mM oxalic acid, 100°C, 2 hours) to remove fucose (Hayashi and Maclachlan, 1984; Maruyama *et al.*, 1996), and then precipitated with ethanol. Total acid hydrolysis of the treated product followed by PC (system B) confirmed the removal of fucose from xyloglucan. Binding of [^{14}C]-polysaccharides to acid-treated xyloglucan was decreased by 60% compared to controls (Fig. 29), reflecting the role of fucose residues. The iodine-sulphate method (Kooiman, 1960) was used to confirm that oxalic acid has no effect on xyloglucan solubility, and that removal of fucose was the possible reason for the decrease in binding.

G. Effect of reduction of uronic acid residues of [^{14}C]-polysaccharides on binding

The effect of treatment designed to reduce the uronic acid residues of [^{14}C]-polysaccharides prior on subsequent binding to pea xyloglucan was investigated. Treatment resulted in a considerable decrease in the binding at pH 4, and the abolition of the pH-dependence of binding (Fig. 30).

H. Effect of protease treatment of [^{14}C]-polysaccharides on binding

Nascent GAX is synthesized attached to a protein of approximately 40 kDa (Crosthwaite *et al.*, 1994). The role of proteins attached to pea [^{14}C]-polysaccharides

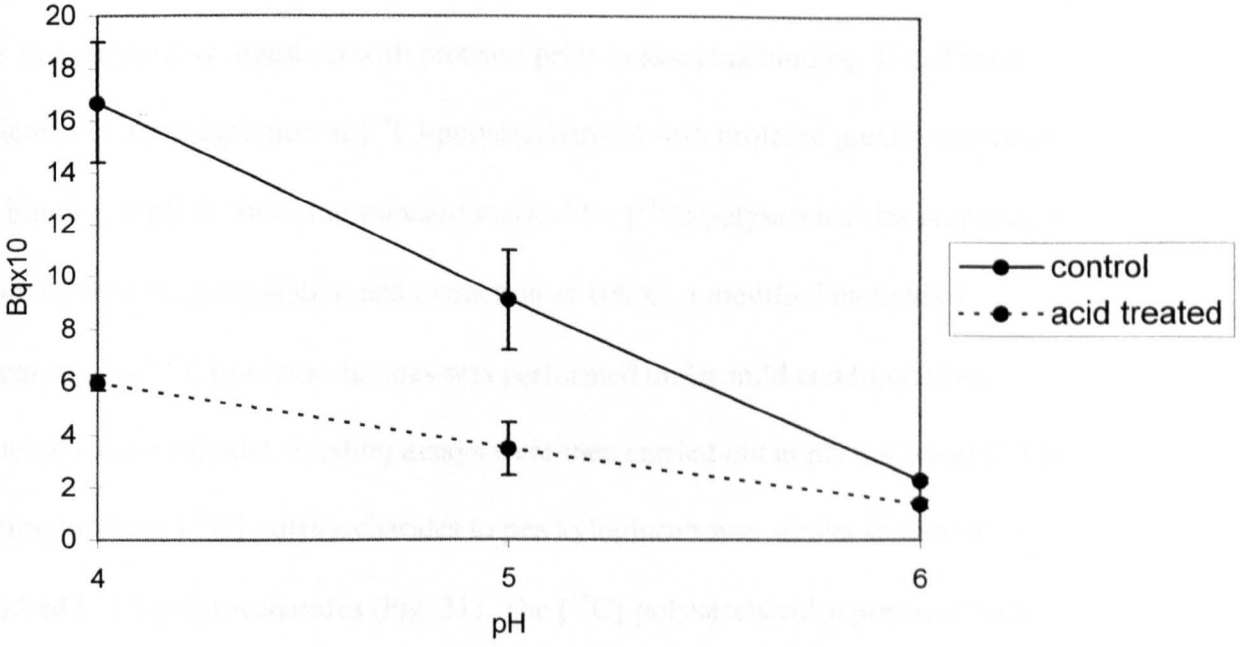

Figure 29. Effect of mild acid treatment of xyloglucan on the pH-dependent binding to nascent EDTA-soluble ^{14}C-polysaccharides

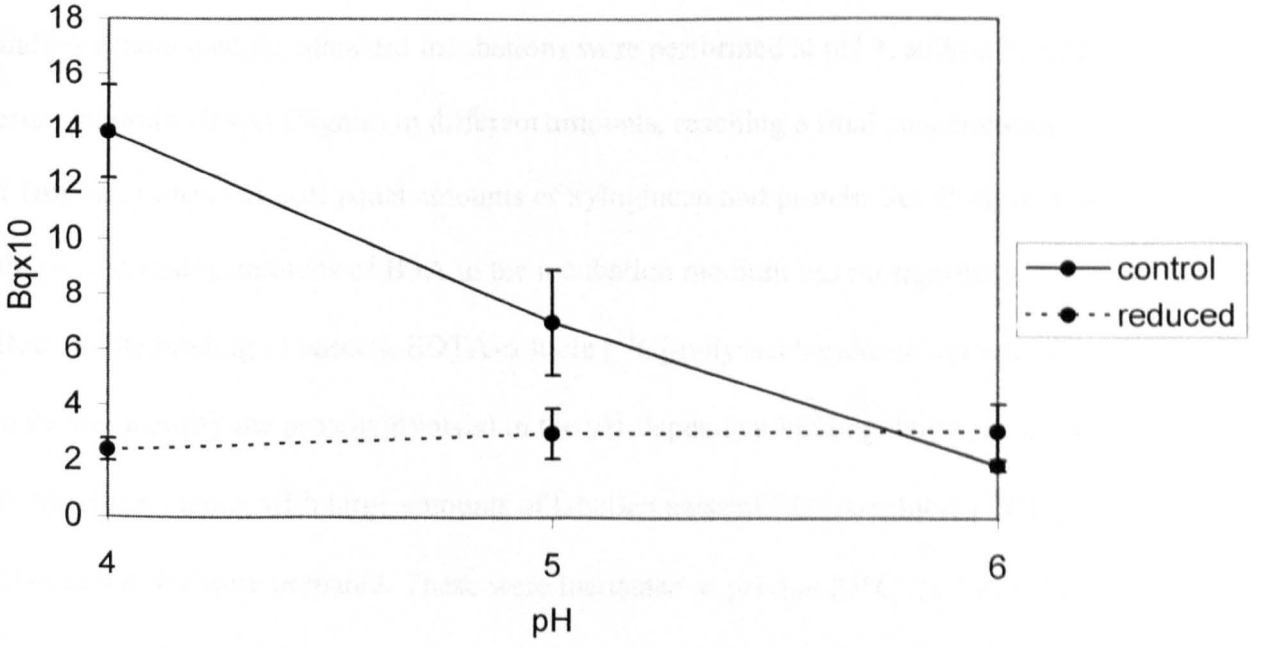

Figure 30. Effect of reduction of uronic acid of nascent EDTA-soluble ^{14}C-polysaccharides on their binding to xyloglucan

was investigated by digestion with protease prior to assaying binding. Initial tests indicated that pre-digestion of [^{14}C]-polysaccharides with protease greatly decreases the binding at pH 4. Since the standard method for [^{14}C]-polysaccharides preparation involved ethanol precipitation and extraction at 100°C, a modified method of preparation of [^{14}C]-polysaccharides was performed under mild conditions (see Materials and Methods). Binding assays were then carried out at pH 3,4,5 and 6. The binding of these [^{14}C]-polysaccharides to pea xyloglucan was similar to that of standard [^{14}C]-polysaccharides (Fig. 31). The [^{14}C]-polysaccharides prepared under non-denaturing conditions were treated with proteinase, and binding assays were then performed at pH 4,5 and 6. The binding to pea xyloglucan at pH 4 was decreased by 68% by proteinase, and the pH dependence of binding was abolished (Fig. 32), indicating the essential role of protein(s) in the pH-dependent binding of [^{14}C]-polysaccharides to xyloglucan. To test whether the effect of the protein on the binding is non-specific, standard incubations were performed at pH 4, adding bovine serum albumin (BSA) (Sigma) in different amounts, reaching a final concentration of 1mg/ml, to have almost equal amounts of xyloglucan and protein. As illustrated in table 4, increasing amounts of BSA in the incubation medium has no significant effect on the binding of nascent EDTA-soluble [^{14}C]-polysaccharides to xyloglucan. To further identify the protein involved in the pH-dependent binding, large amounts of xyloglucan, along with large amounts of labelled nascent EDTA-soluble [^{14}C]-polysaccharides were prepared. These were incubated at pH 4 at 25°C for 5 minutes and the xyloglucan pellets were then washed once with pH 4 buffer. Material bound to xyloglucan was detached from xyloglucan by incubating twice with pH 6 buffer

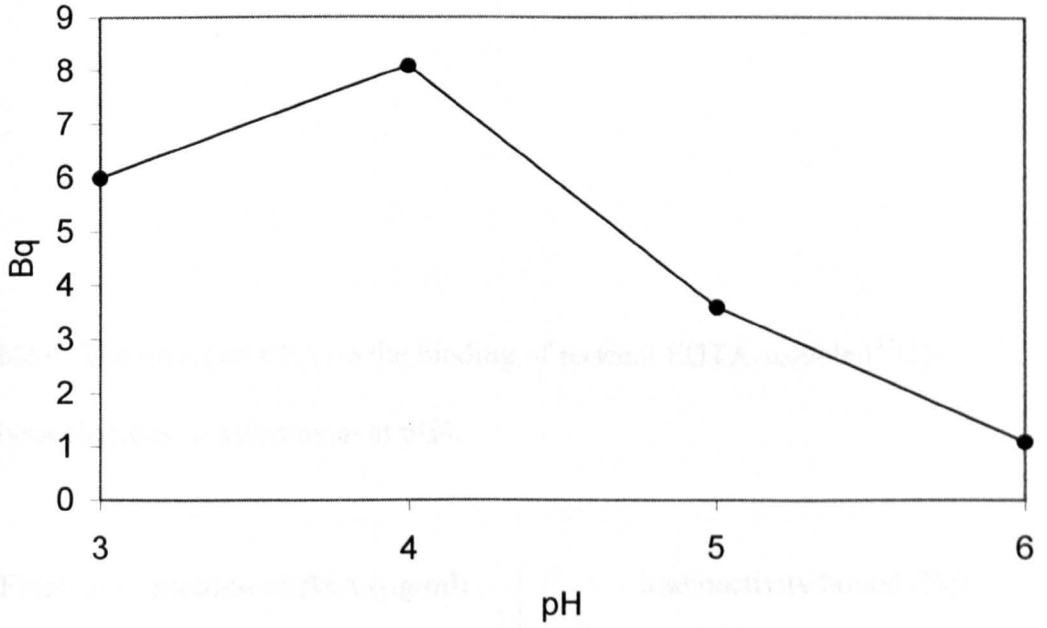

Figure 31. Effect of pH on binding of nascent EDTA-soluble polysaccharides prepared under non-denaturing conditions to XG

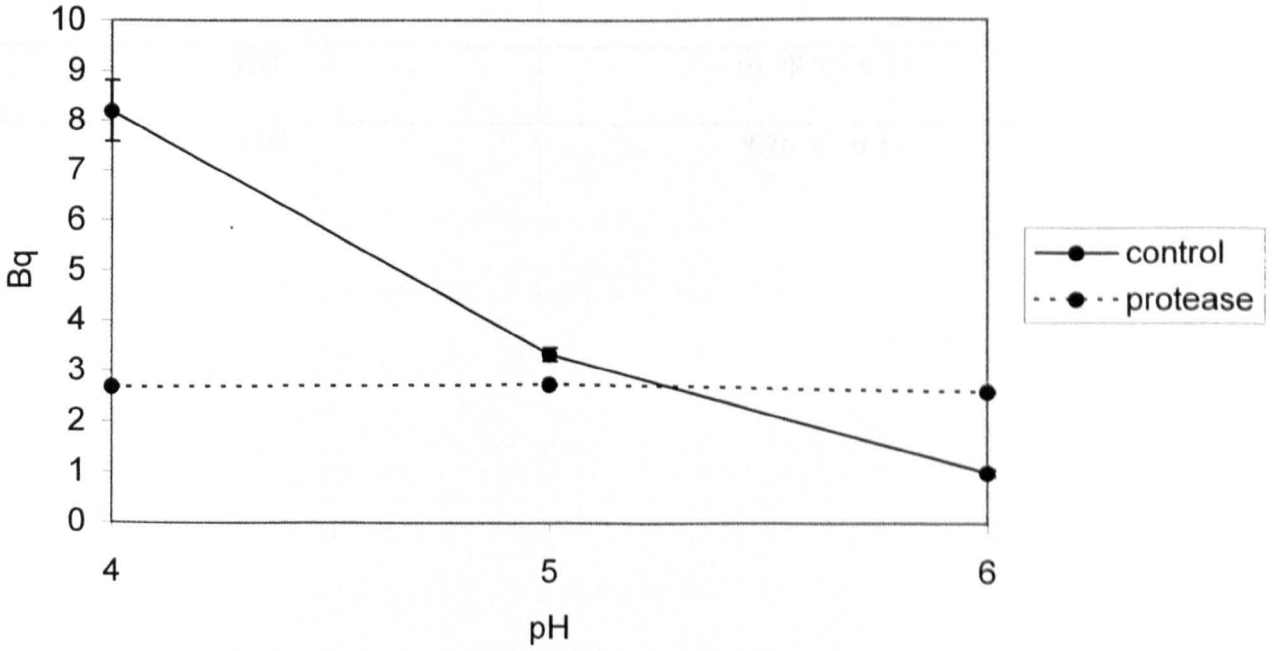

Figure 32. Effect of protease treatment of nascent EDTA-soluble ^{14}C-polysaccharides on their binding to xyloglucan

Table 4. The effect of BSA on the binding of nascent EDTA-soluble [^{14}C]-polysaccharides to xyloglucan at pH4.

Final concentration of BSA (μg/ml)	Radioactivity bound (Bq)
0	10.40 +/- 0.33
10	10.16 +/- 0.31
30	10.61 +/- 0.21
100	9.76 +/- 0.36
300	10.98 +/- 0.19
1000	9.76 +/- 0.37

for 5 minutes. pH 6 washes were combined and subjected to pectin lyase treatment since pectin lyase degrades most of the high molecular weight polysaccharides (Fig. 5 and 7). The remaining material was run on a 12% SDS-polyacrylamide gel, in parallel with the pectin lyase enzyme that was still present in the sample being analyzed. Two enzymes were used, both purchased from Sigma: when using the first enzyme the bands that appear in the middle account for the pectin lyase enzyme (lane A), whereas two other proteins in the sample (lane B) seem to be involved in the pH-dependent binding, one that has a molecular weight of about 94 kDa. The other appears as a faint band of very low molecular weight (Fig. 33). When using the other pectin lyase enzyme, the enzyme does not appear on the gel (lane B), the high molecular weight band is barely visible in the sample (lane A), whereas the low molecular weight band appears clearly and seems to be about 14 kDa (Fig. 34).

I. Effect of xylanase, pectin lyase and pectinase treatments of the [^{14}C]-polysaccharides on their binding to xyloglucan

Nascent EDTA-soluble [^{14}C]-polysaccharides were shown to consist of labelled pectins as well as xylans (Chapter 3). To determine which of the two polysaccharides was responsible for the pH-dependent binding to xyloglucan, the EDTA-soluble polysaccharides were subjected to xylanase and pectin lyase treatments separately, prior to performing the binding assays to xyloglucan. For both treatments, the effect of protein removal by protease was investigated to determine which of the two polymers is associated with the protein shown to be responsible for the pH-dependent binding. As revealed in figures 35 and 36, xylans and pectins

Figures 33 and 34. SDS PAGE of protein released from EDTA-soluble material bound to xyloglucan.

EDTA-solubilized material was prepared as described in Materials and Methods (page 38) and bound to xyloglucan (page 39) at pH 4.0. The bound material was pelleted in a microfuge (13,000*g*) and resuspended in buffer at pH 6 to release the pectin/xylan fraction. The supernatant was treated with pectin lyase (page 43), boiled with SDS-sample buffer (page 44). It was then loaded onto a linear 20x20cmx2mm polyacrylamide gel and electrophorized at 65 mA for approximately 3 hours.
Figure 33: lane A (20 µl of pectin lyase enzyme) and lane B (~20µg sample).
Figure 34: lane A (~20µg sample) and lane B (20µl of pectin lyase enzyme).
Outer lanes show molecular weight markers . Protein sizes are expressed in kDa at the side of the figures.

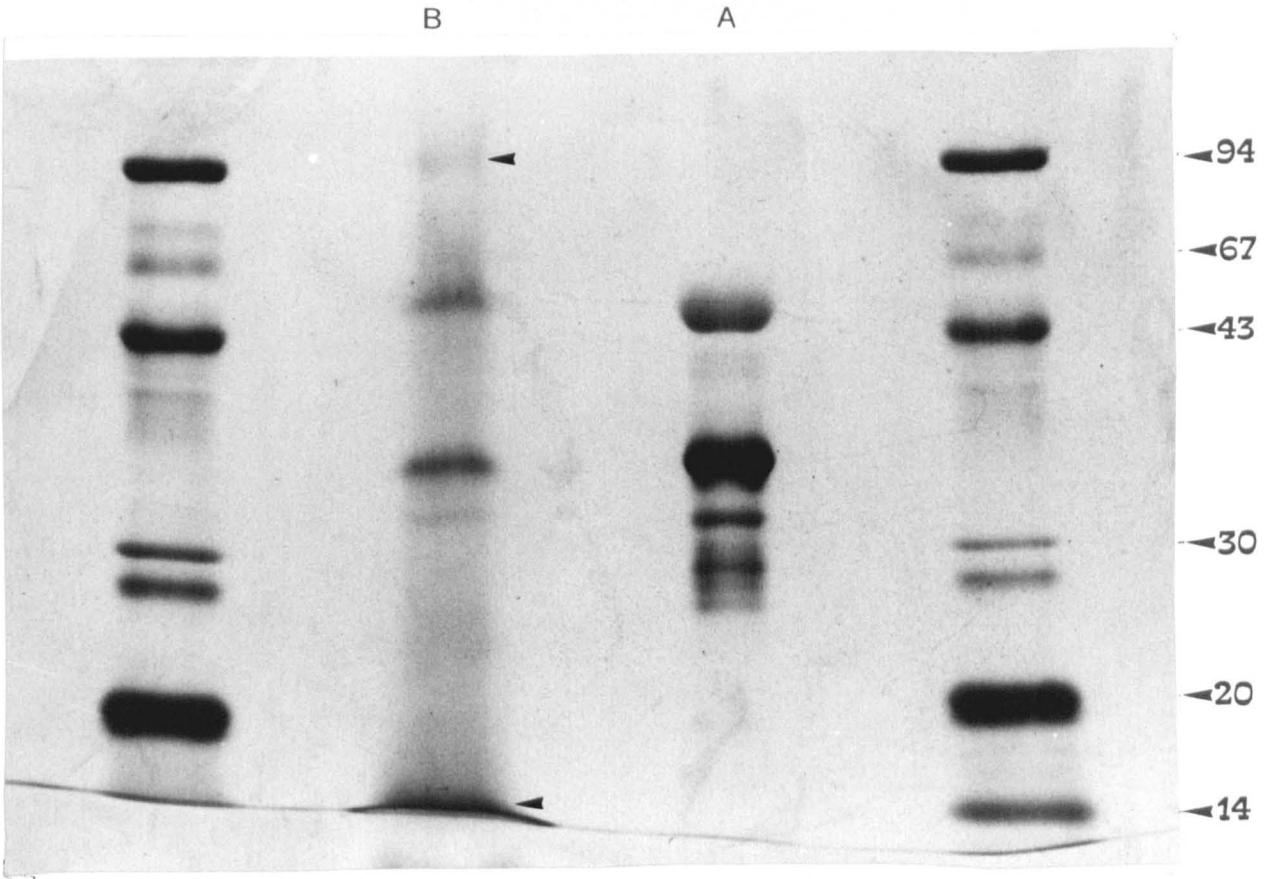

Figure 33. SDS-PAGE of material unbound from xyloglucan at pH 6

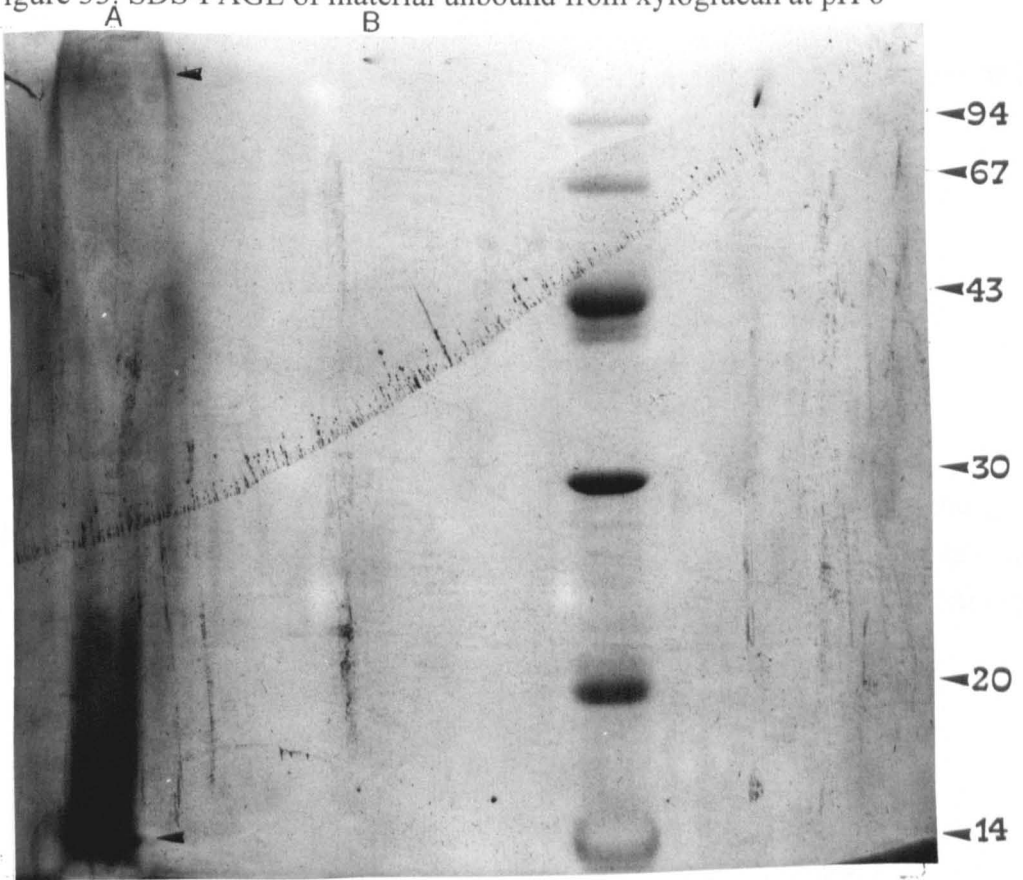

Figure 34. SDS-PAGE of material unbound from xyloglucan at pH 6

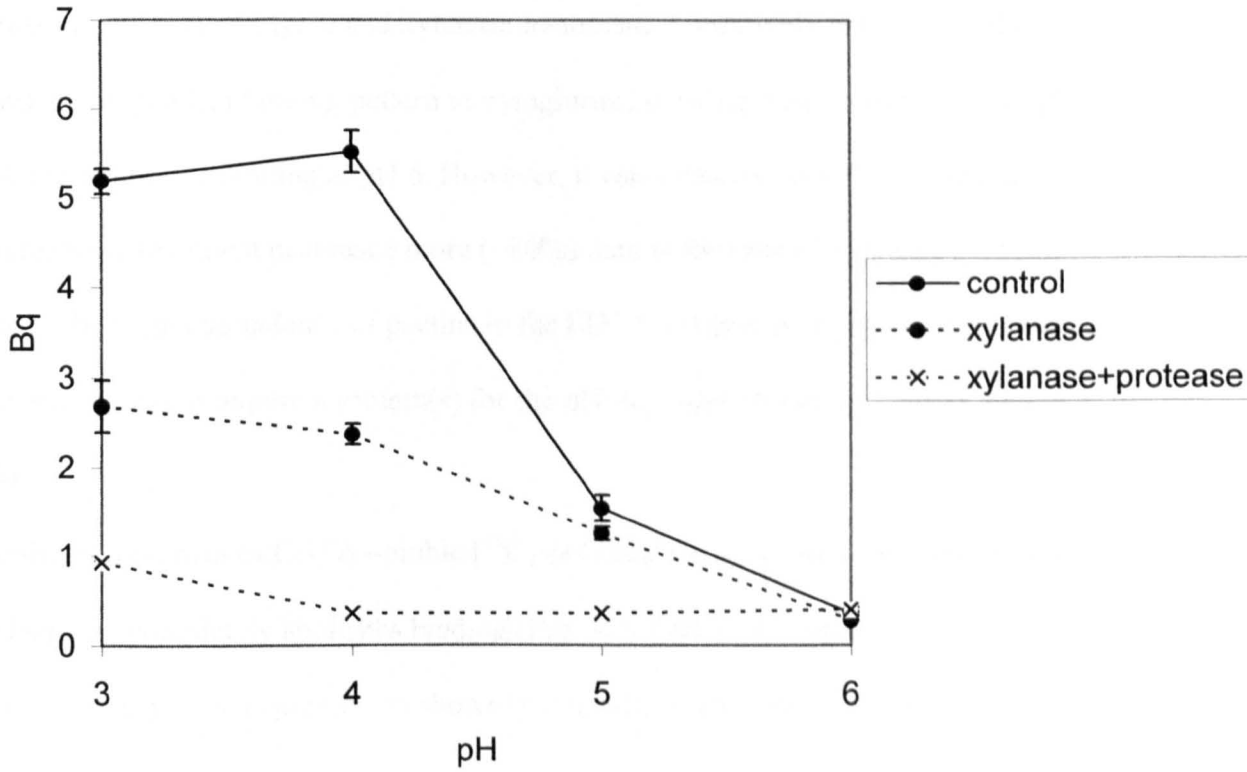

Figure 35. Effect of xylanase and protease treatment of nascent EDTA-soluble ^{14}C-polysaccharides on their binding to xyloglucan

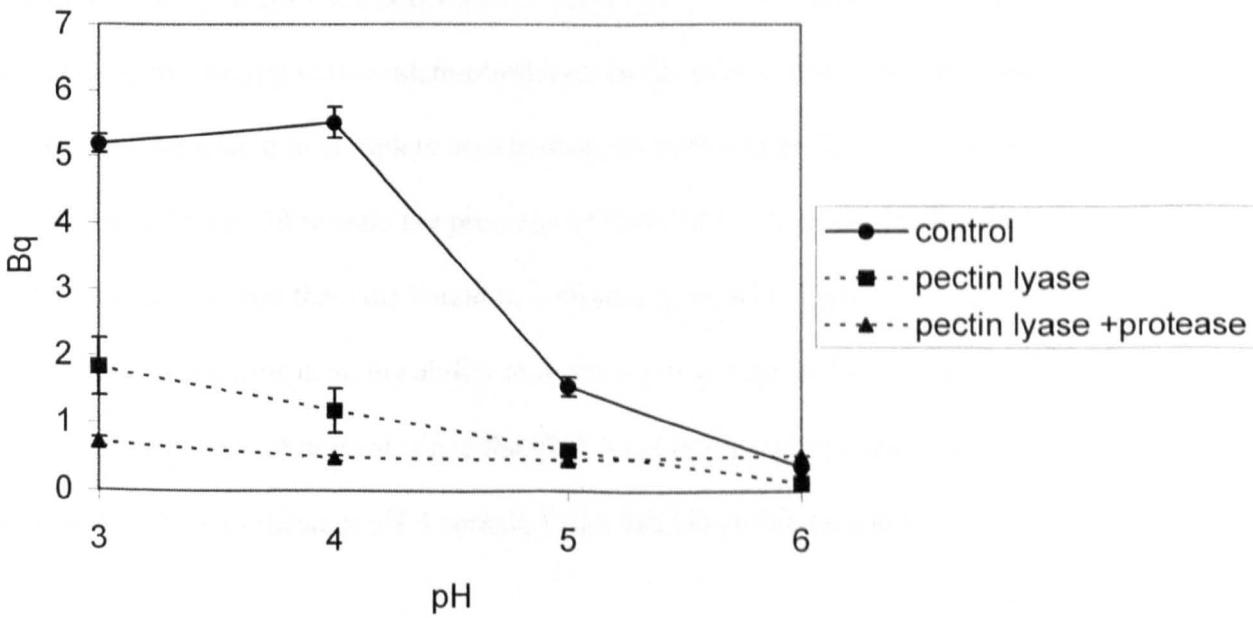

Figure 36. Effect of pectin lyase and protease treatment of nascent EDTA-soluble ^{14}C-polysaccharides on their binding to xyloglucan

remaining after pectin lyase and xylanase treatments respectively both follow the same pH-dependent binding pattern to xyloglucan, showing maximum binding at pH 3-4 and negligible binding at pH 6. However, it was observed that the binding after pectin lyase treatment decreased more (~80%) than in the case of xylanase (~55%), due to the higher abundance of pectins in the EDTA-extracts (Chapter 3). Also, both polymers seem to require a protein(s) for the pH-dependent binding (Figures 35 and 36).

Pectinase treatment of EDTA-soluble [^{14}C]-polysaccharides prior to incubating with xyloglucan completely abolishes binding (Fig. 37). This is not surprising since pectinase enzyme was previously shown to degrade xylans along with pectins (Fig. 15).

J. TFA hydrolysis and TLC of [^{14}C]-polysaccharides binding to xyloglucan

Standard binding assays were performed at pH 4. Material bound to xyloglucan was extracted by incubating with oxalate-phosphate buffer (pH 6, 1ml). The unbound material was subjected to complete acid hydrolysis followed by TLC on silica gel coated plates. Figure 38 reveals the presence of labelled GlcA as well as GalA residues in the material that was bound to xyloglucan, confirming the finding that both xylans and pectins have the ability to bind to xyloglucan at pH 4. As expected, more GalA residues are present, since the EDTA-soluble polysaccharides that were incubated with xyloglucan at pH 4 contain GalA and GlcA residues in a 2:1 ratio (Fig. 3).

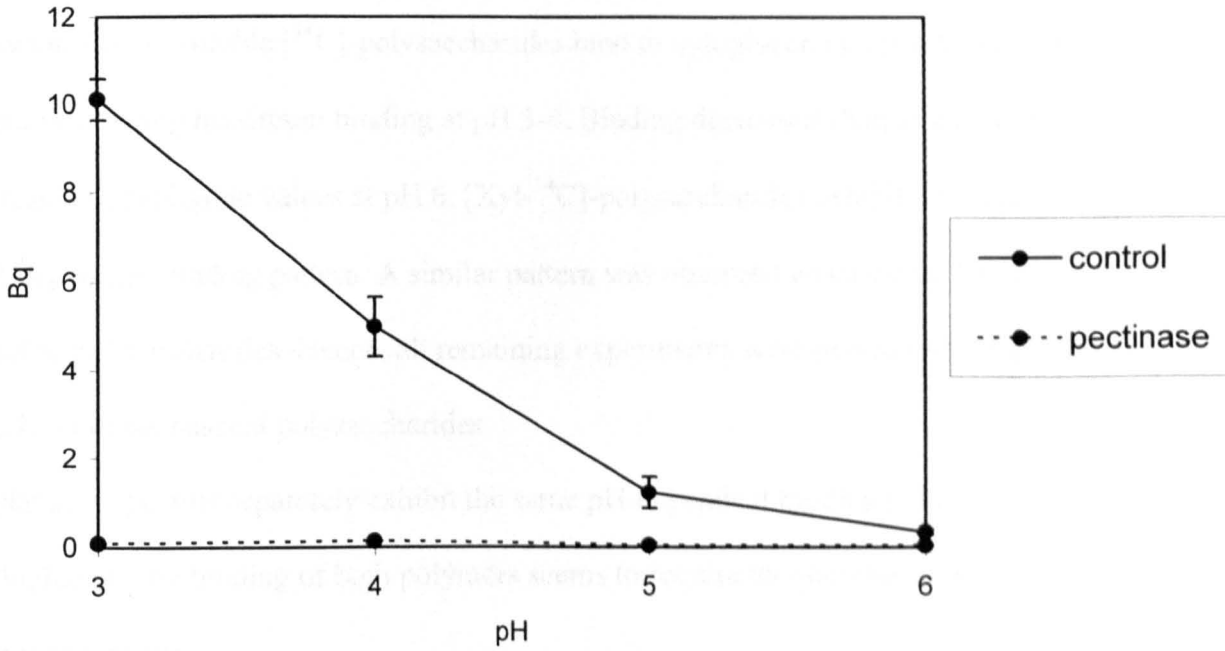

Figure 37. Effect of pectinase on binding of nascent EDTA-soluble ^{14}C-polysaccharides to xyloglucan

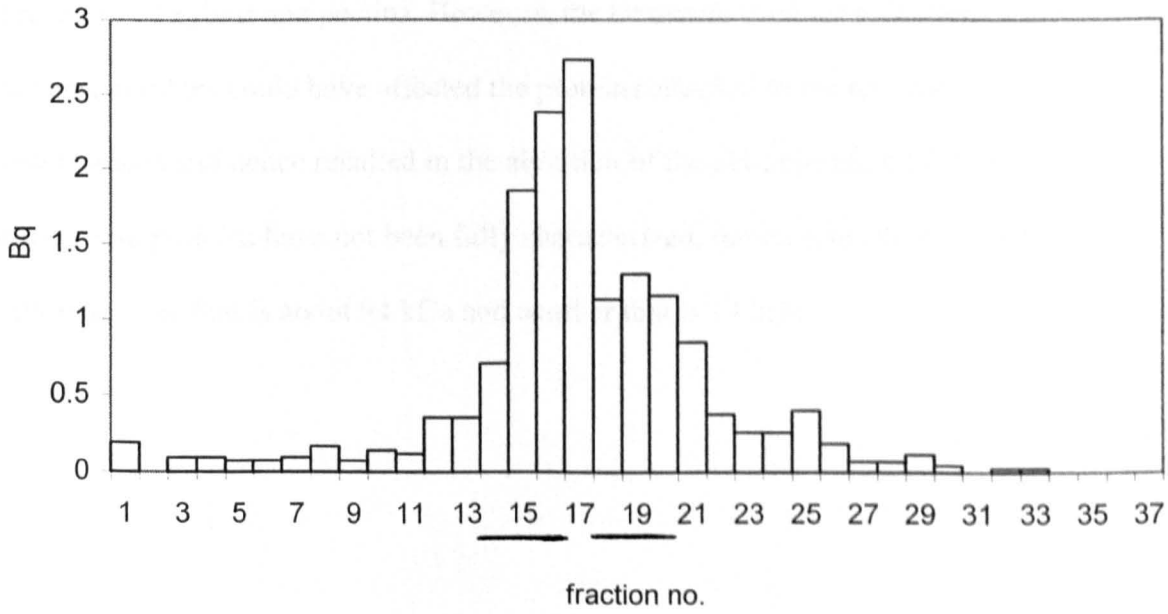

Figure 38. TLC of total acid hydrolysate of nascent EDTA-soluble ^{14}C-polysaccharides unbound from xyloglucan

Conclusion

Nascent EDTA-soluble [^{14}C]-polysaccharides bind to xyloglucan in a pH-dependent manner showing maximum binding at pH 3-4. Binding decreased sharply above pH 4, reaching negligible values at pH 6. [Xyl-^{14}C]-polysaccharides exhibit the same pH-dependent binding pattern. A similar pattern was observed when using, KOH-soluble polysaccharides. Hence, all remaining experiments were performed using the EDTA-soluble nascent polysaccharides.

Xylans and pectins separately exhibit the same pH-dependent binding pattern to xyloglucan. The binding of both polymers seems to require the proteins to which they are attached.

The binding of nascent EDTA-soluble [^{14}C]-polysaccharides to xyloglucan seems to be instantaneous and mostly non-covalent. It seems to require fucose residues of xyloglucan, the proteins attached to the nascent polymers and the unreduced uronic acid residues of xylans and pectins. However, the treatment used for reduction of the uronic acid residues could have affected the proteins attached to the nascent polysaccharides and hence resulted in the abolition of the pH-dependent binding pattern. These proteins have not been fully characterized, but it seems they consist of two proteins, one that is about 94 kDa and another that is 14 kDa.

CHAPTER 5

Binding of nascent EDTA-soluble polysaccharides to polysaccharides other than xyloglucan

Introduction

Nascent EDTA-soluble polysaccharides exhibit pH-dependent binding to xyloglucan extracted from pea epicotyls, with highest binding at pH 3-4, and almost zero binding at pH 6. It is not clear whether this binding pattern is unique to xyloglucan. In this chapter, the binding of the nascent polysaccharides to "cell wall ghosts", cell walls, hemicellulose preparations and commercial polysaccharides is described.

A. Binding to "cell wall ghosts"

When preparing xyloglucan from pea stems, the pellets remaining after extraction with 4% KOH/0.1% $NaBH_4$ are referred to as "cell wall ghosts" and consist of cellulose microfibrils to which xyloglucan is attached. Binding assays were performed using these cell wall ghosts (1mg/incubation) and nascent EDTA-soluble polysaccharides. The binding pattern obtained is very similar to the one observed when binding the polysaccharides to xyloglucan, with high levels of binding at pH 3-4, and negligible binding at pH 6 (Fig. 39).

B. Binding to pea cell walls

Cell wall pellets were freshly prepared as described by Brett *et al.* (1997). They were incubated with nascent EDTA-soluble polysaccharides that were xylanase and pectin lyase treated (about 60 Bq/incubation before enzyme treatment). As expected, both

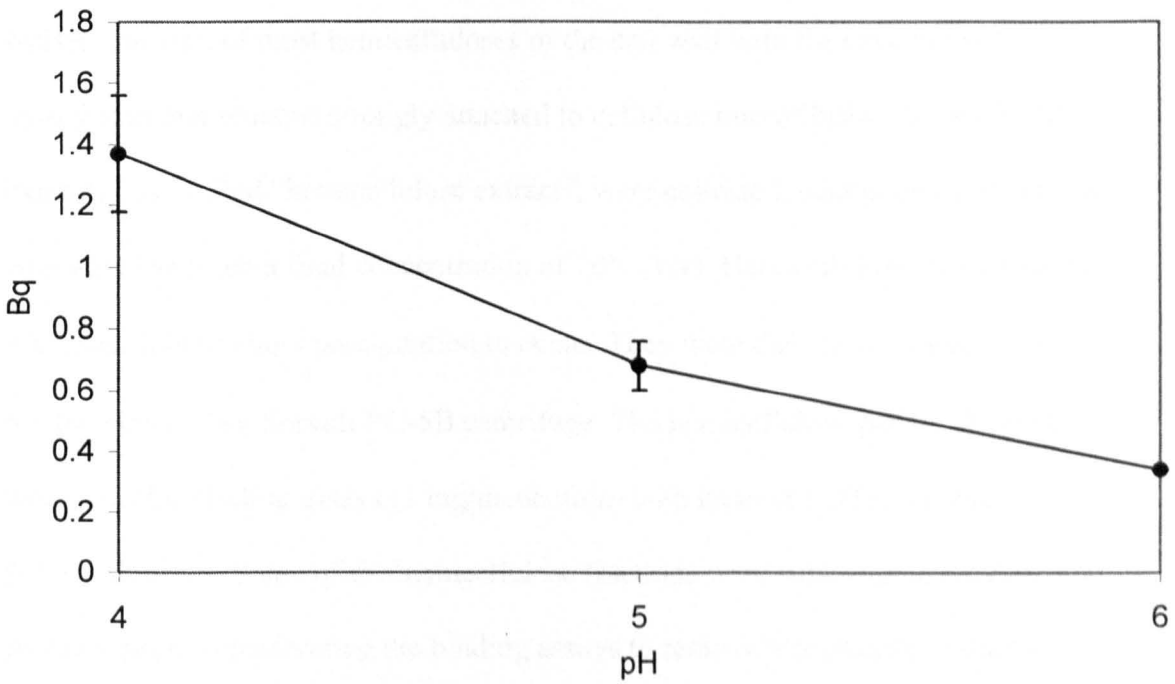

Figure 39. Effect of pH on binding of nascent ETDA-soluble ^{14}C-polysaccharides to cell wall ghosts

xylans and pectins bind to cell walls in a pH-dependent manner as when binding to xyloglucan (Fig. 40).

C. Binding to hemicelluloses

When preparing xyloglucan from pea stems, the material extracted by 4%KOH/0.1% $NaBH_4$ consists of most hemicelluloses of the cell wall with the exception of xyloglucan that remains strongly attached to cellulose microfibrils. The 4% KOH extracts, also called "hemicellulose extract", were collected, neutralized and ethanol was added to reach a final concentration of 70% (v/v). Hemicelluloses were kept at 4°C overnight to allow precipitation to occur. They were then centrifuged at 13000*g* for 10 minutes in a Sorvall RC-5B centrifuge. The hemicellulose pellets obtained were used for binding assays (1 mg/incubation) with nascent EDTA-soluble polysaccharides. Some of the hemicellulose fractions were subjected to amylase and protease prior to performing the binding assays to remove any protein or starch contamination.

The binding pattern obtained was slightly different from the one obtained when binding to xyloglucan pellets; however, much higher binding was still observed at pH 3 than at pH 6 (Fig. 41). Removal of proteins and starch decreased the binding, however, the pattern observed was still pH-dependent. One explanation for this pH-dependence in binding could be due to the contamination of the hemicellulose extract with some xyloglucan that is easily extracted by 4% KOH. To check this possibility, hemicellulose fractions were subjected to endo-1,4β-glucanase treatment after protease and amylase treatments, prior to binding to the nascent EDTA-soluble

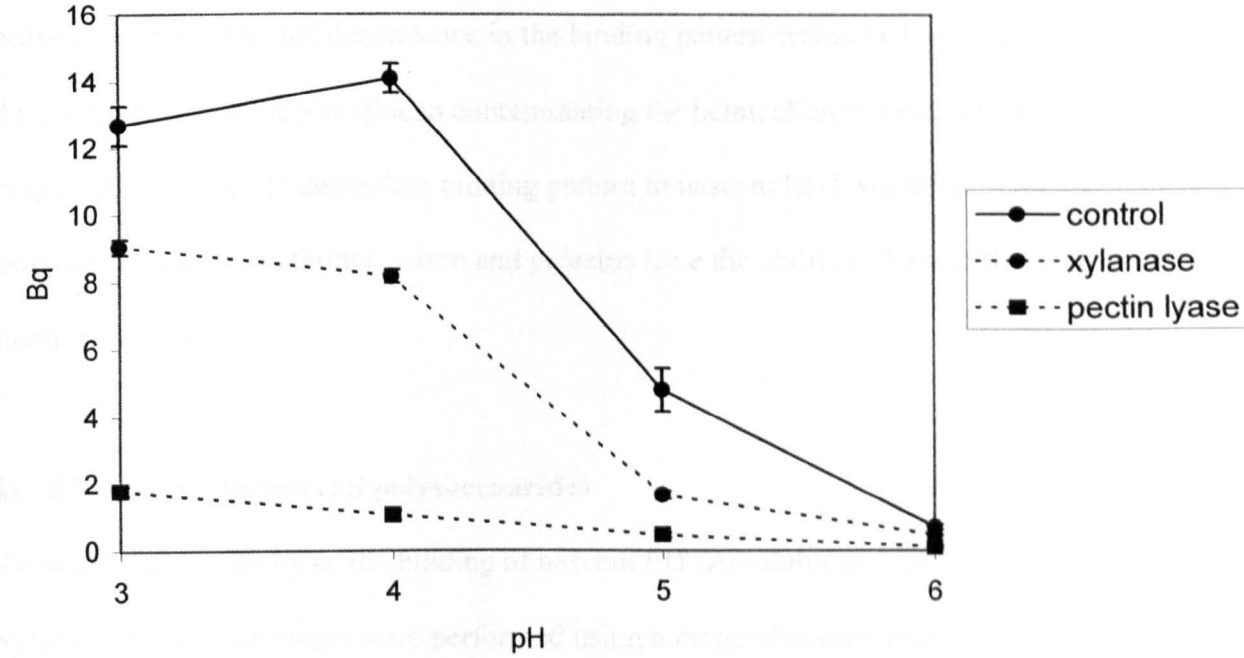

Figure 40. Effect of xylanase and pectin lyase treatment of nascent EDTA-soluble ^{14}C-polysaccharides on binding to cell walls

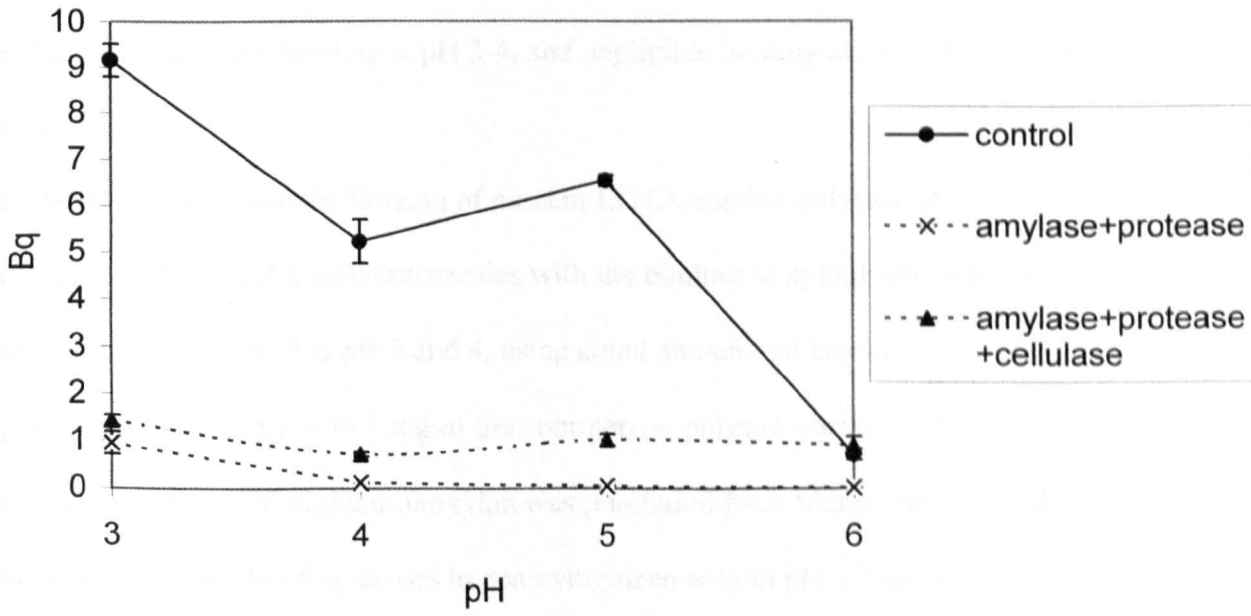

Figure 41. Binding of nascent EDTA-soluble ^{14}C-polysaccharides to 4% KOH-extractable hemicelluloses from peas

polysaccharides. The pH dependence in the binding pattern seems to disappear (Fig. 41), indicating that the xyloglucan contaminating the hemicellulose extract was responsible for the pH-dependent binding pattern to nascent EDTA-soluble polysaccharides, even though starch and proteins have the ability to bind to the hemicelluloses.

D. Binding to commercial polysaccharides

To study the specificity of the binding of nascent EDTA-soluble polysaccharides to xyloglucan, binding assays were performed using a range of commercially-available polysaccharides, namely pectic galactan from potato (Megazyme), xylan from birchwood (Sigma), pectin from citrus fruits (Sigma) and cellulose (Sigma, Type 101). All of these polymers were incubated with labelled polysaccharides (about 25 Bq/incubation) as usually performed when using xyloglucan.

With the exception of pectin (Fig. 42), the binding seems to be pH-dependent, exhibiting maximum binding at pH 3-4, and negligible binding at pH 6 (Fig. 43,44 and 45).

To be able to compare the binding of nascent EDTA-soluble polysaccharides to commercially available polysaccharides with the binding to xyloglucan, binding assays were performed at pH 3 and 4, using equal amounts of labelled polysaccharides along with 1 mg of the commercial polysaccharides and 1 mg of pea xyloglucan. The methylglucuronoxylan was purchased from Sigma. As revealed in table 5, maximum binding occurs to pea xyloglucan at both pH's 3 and 4.

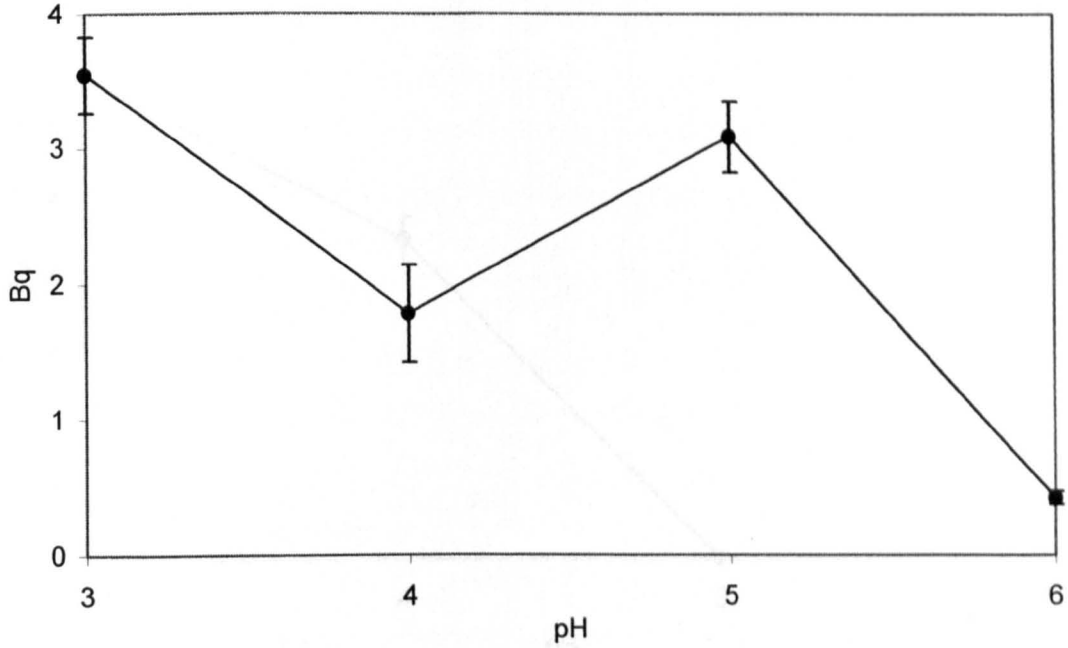

Figure 42. Binding of nascent EDTA-soluble ^{14}C-polysaccharides to pectin

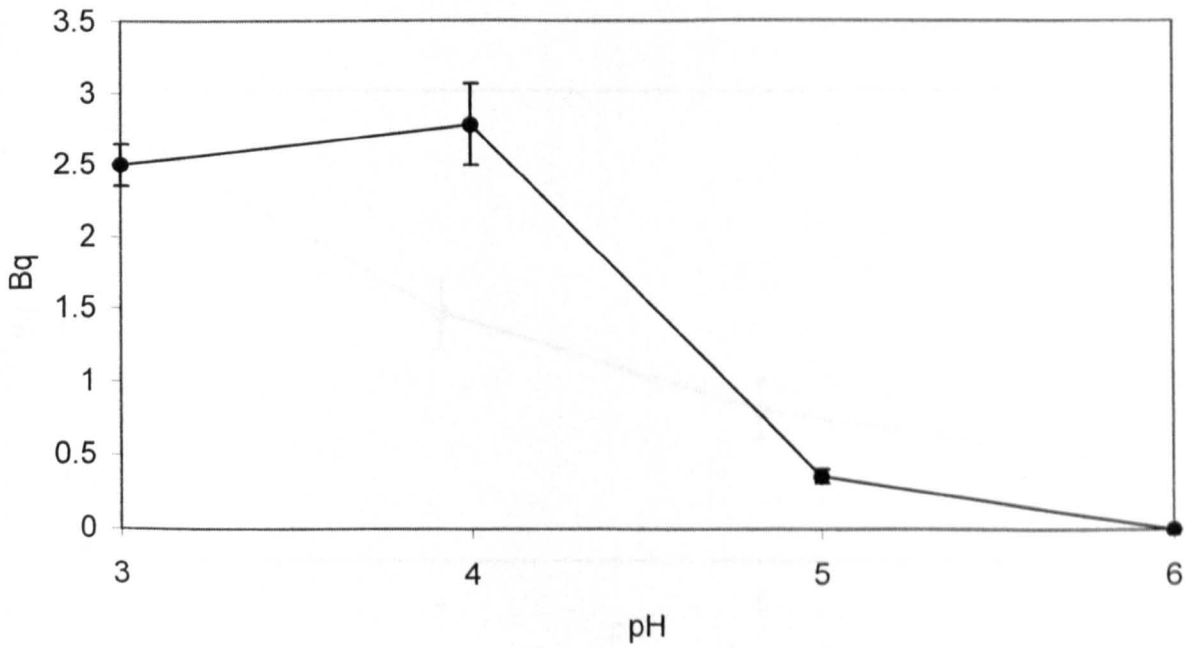

Figure 43. Binding of nascent EDTA-soluble ^{14}C-polysaccharides to pectic galactan

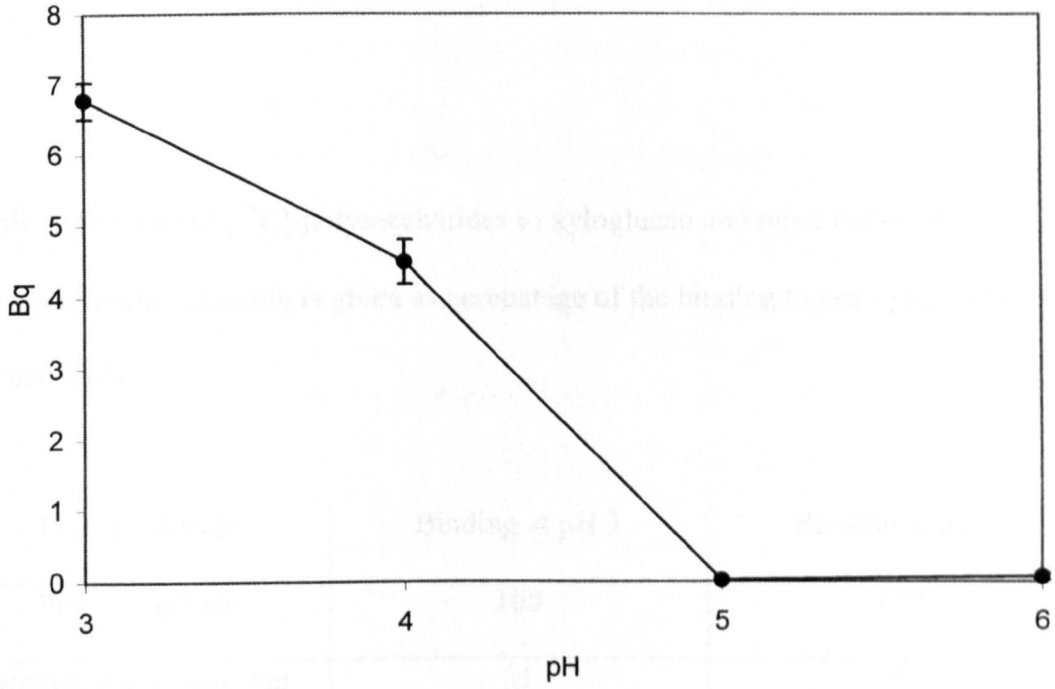

Figure 44. Binding of nascent EDTA-soluble ^{14}C-polysaccharides to xylan

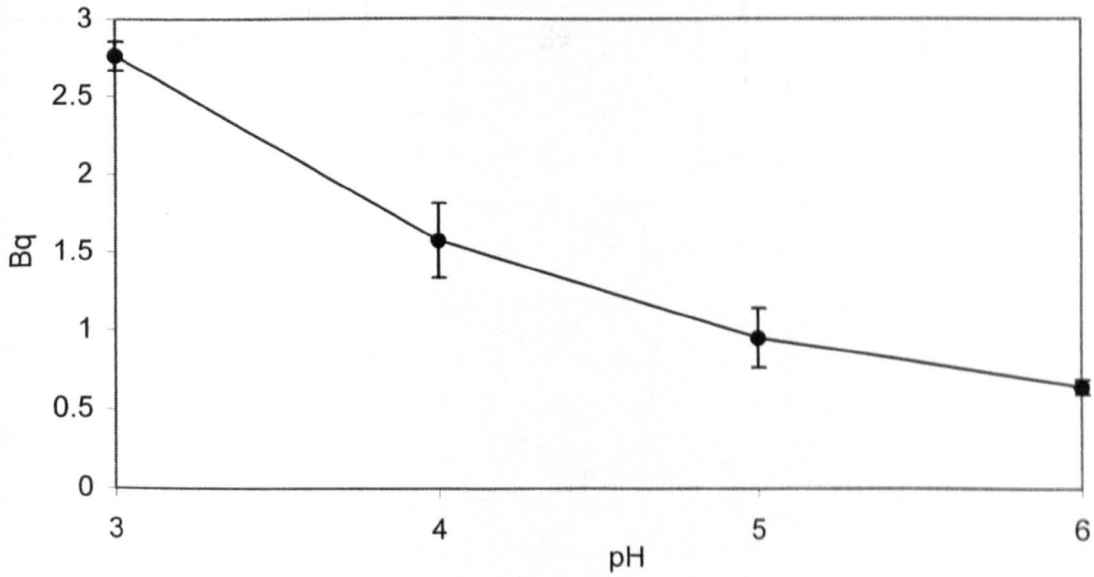

Figure 45. Binding of nascent EDTA-soluble ^{14}C-polysaccharides to cellulose

Table 5. Binding of [^{14}C]-polysaccharides to xyloglucan and other cell-wall polysaccharides. Binding is given as percentage of the binding to pea xyloglucan at the same pH.

Polysaccharide	Binding at pH 3	Binding at pH 4
Pea xyloglucan	100	100
Methylglucuronoxylan	31	50
Xylan	67	43
Pectin	44	14
Cellulose	47	29
Tamarind xyloglucan	12	13
Galactan	29	27

Conclusion

The binding of nascent EDTA-soluble [^{14}C]-polysaccharides to 4% KOH-soluble hemicelluloses prepared from pea cell walls does not follow the same pattern as the one observed when binding to xyloglucan. The same pH-dependent binding pattern was observed when binding to cell walls, cell wall ghosts, cellulose, and a range of commercially available polysaccharides. However, the binding of the nascent polymers occurs in highest levels to pea xyloglucan.

CHAPTER 6

Preparation of radioactively labelled xylans and studying their binding to xyloglucan

Introduction

Nascent EDTA-soluble polysaccharides were shown to consist of pectin and xylan (Chapter 3). The conditions used to prepare these polysaccharides were altered in an attempt to prepare nascent [^{14}C]-xylans, with no contamination of labelled pectin. This chapter identifies the labelled products obtained under different conditions to establish a method for the production of labelled xylan, and then describes the binding pattern and requirements of xylan to xyloglucan.

A. Extraction of EDTA-soluble polysaccharides under different conditions

EDTA-soluble polysaccharides were extracted using EDTA/phosphate buffer (pH 6.8) at 100°C for 5 minutes twice. To check that this is the method that extracts the highest amount of polysaccharides possible, different extraction modes were tried: water and EDTA at 25°C, 40°C and 100°C. The extracts were passed through the Sephadex G-100 column previously used, eluting with water. Fractions (0.5ml) were collected and assayed for radioactivity content. The high molecular weight marker (blue dextran) eluted in fractions 6-10. As revealed in figure 46, the highest amount of high molecular weight material was extracted when using EDTA buffer at 100°C. Hence, this method of extraction was adopted throughout all the work, except when preparing polysaccharides under mild conditions.

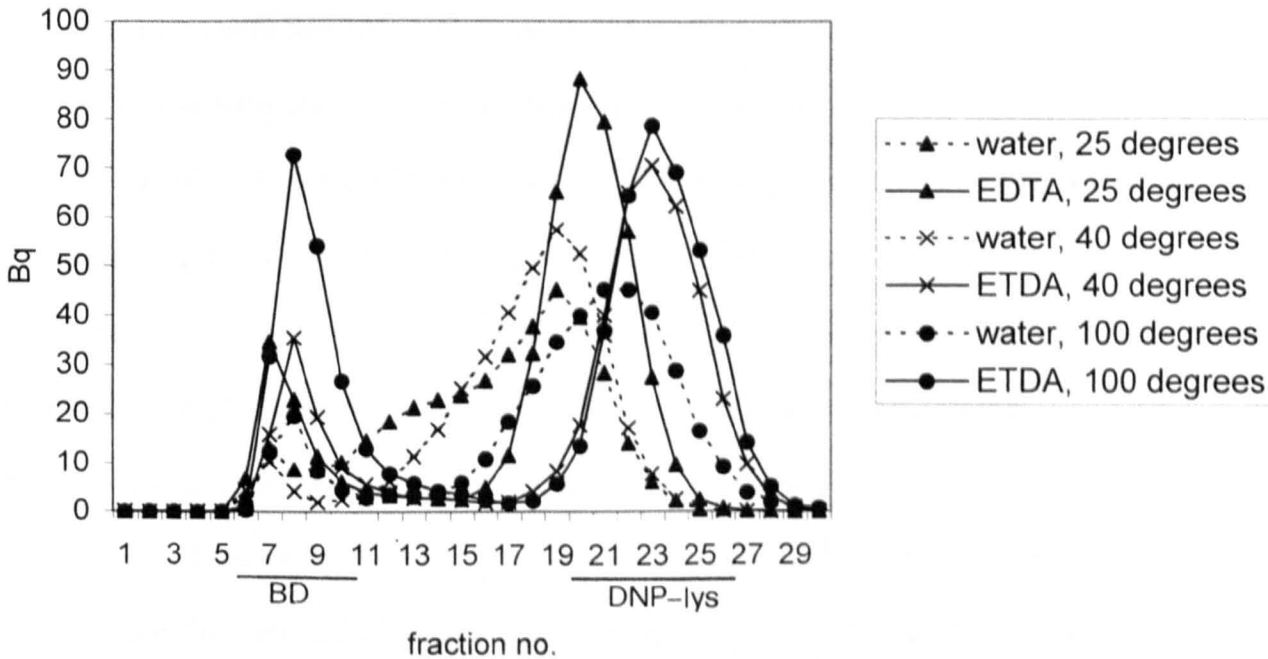

Figure 46. Gel filtration on Sephadex G-100 of EDTA and water extracts of membrane preparations

B. Preparing EDTA-soluble polysaccharides under different conditions

The EDTA-soluble polysaccharides prepared under the conditions stated in Materials and Methods consisted of radioactively labelled pectins and xylans (Chapter 3). Incubation conditions were adjusted to obtain only labelled xylans i.e. polysaccharides with labelled GlcA residues only. Incubation of the membrane preparations with the radioactive donor was carried out at pH 6 and 7, for 1, 2 and 4 hours, with and without the addition of non-radioactive UDP-GalA (1mM) to reduce the effect of epimerases on the radioactive donor UDP-GlcA. Reactions were terminated as usual and extracted with EDTA buffer (pH 6.8). EDTA extracts were passed through a Sephadex G-100 column, the high molecular weight material collected and assayed for radioactivity content. Incorporation of radioactivity was greatly reduced due to the addition of non-radioactive UDP-GalA residues (Table 6). To determine the nature of the labelled polysaccharides in the high molecular weight fractions prepared under different conditions, they were subjected to complete acid hydrolysis followed by thin layer chromatography. As revealed in figures 47-50, labelled GalA as well as GlcA residues are present in all products. Only in the case of incubation with non-radioactive UDP-GalA residues for 4 hours at pH 6, GalA residues are not detected (Fig. 51); the peak appearing in fractions 21-24 would probably be due to the presence of lactones. Reducing the time of incubation to only 1 hour was also tried. In this case also, only GlcA residues labelled residues were

Table 6. Amounts of radioactively labelled polysaccharides prepared under different incubation conditions

Incubation conditions	Radioactivity (Bq)
pH 6, 1 hr	37.13
pH 6, 2 hr	38.20
pH 6, 4 hr	37.13
pH 6, 4 hr, +UDP-GalA (1mM)	5.09
pH 7, 1 hr	34.27
pH 7, 2 hr	34.39
pH 7, 4 hr	33.56
pH 7, 4 hr, +UDP-GalA (1mM)	4.95

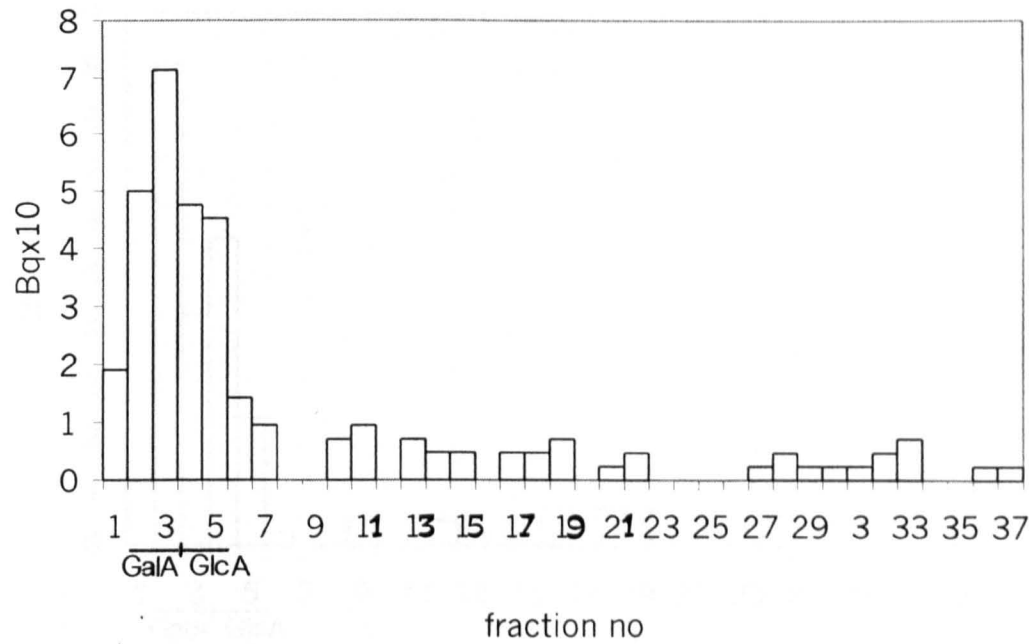

Figure 47. TLC of total acid hydrolysate of EDTA-soluble products prepared at pH 6 for 1 hour

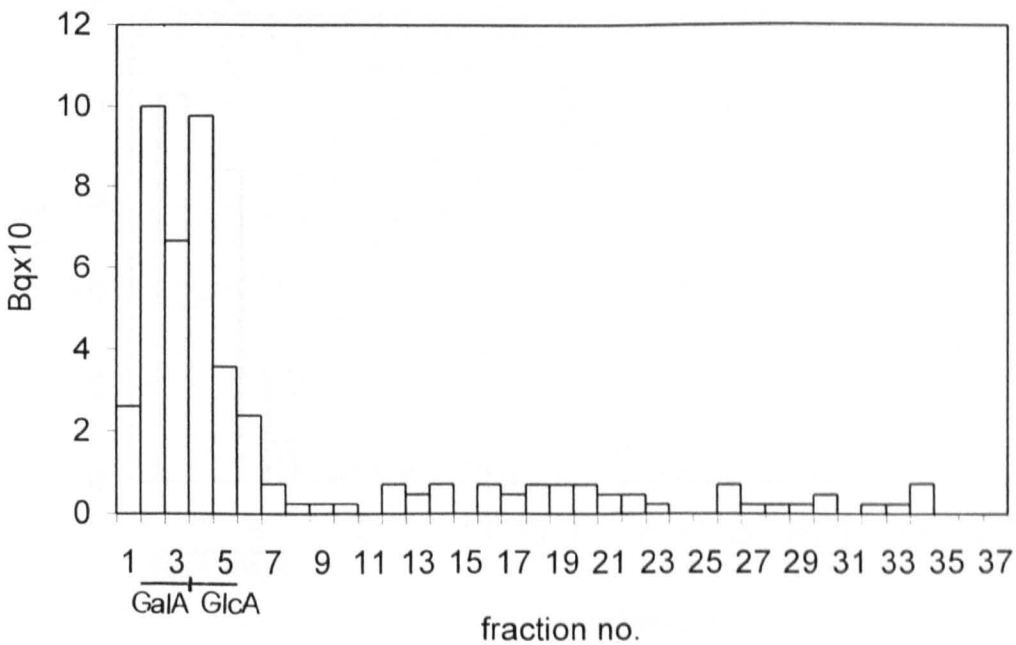

Figure 48. TLC of total acid hydrolysate of EDTA-soluble products prepared at pH7 for 1 hour

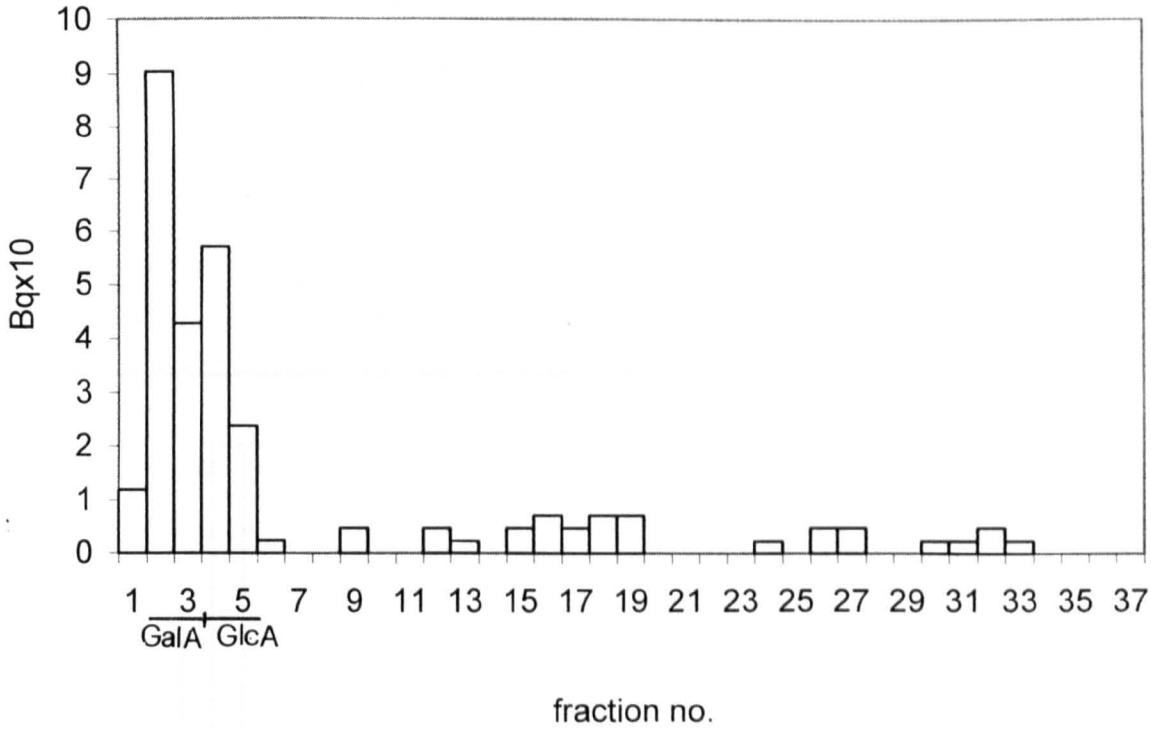

Figure 49. TLC of total acid hydrolysate of EDTA-soluble products prepared at pH7 for 2 hours

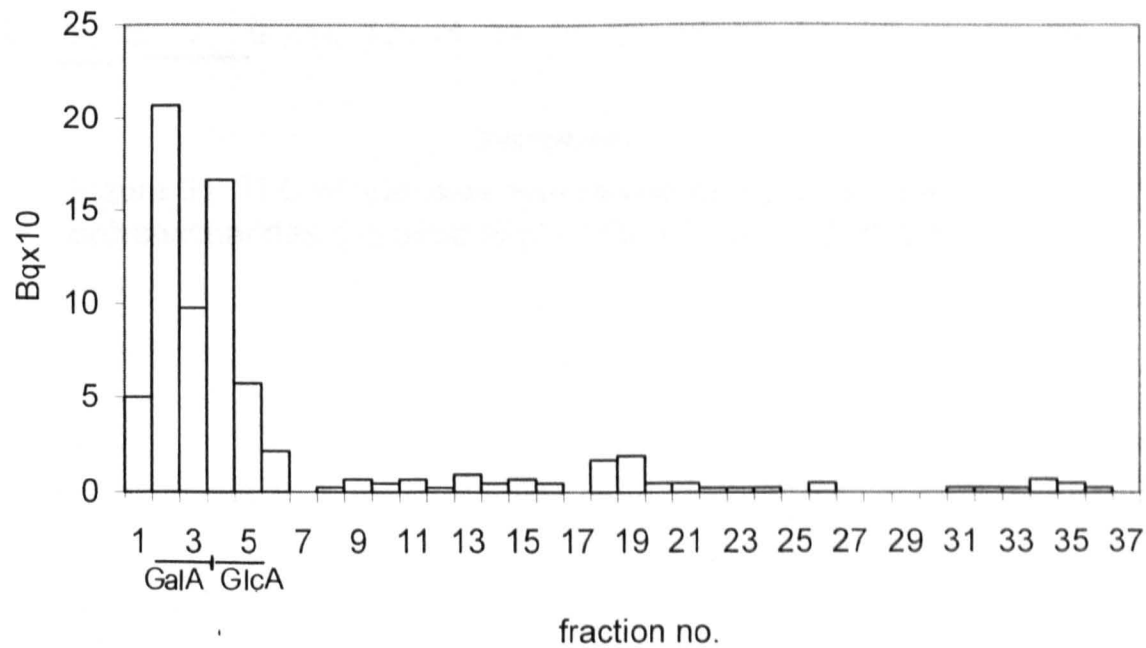

Figure 50. TLC of total acid hydrolysate of EDTA-soluble products prepared at pH 6 for 4 hours

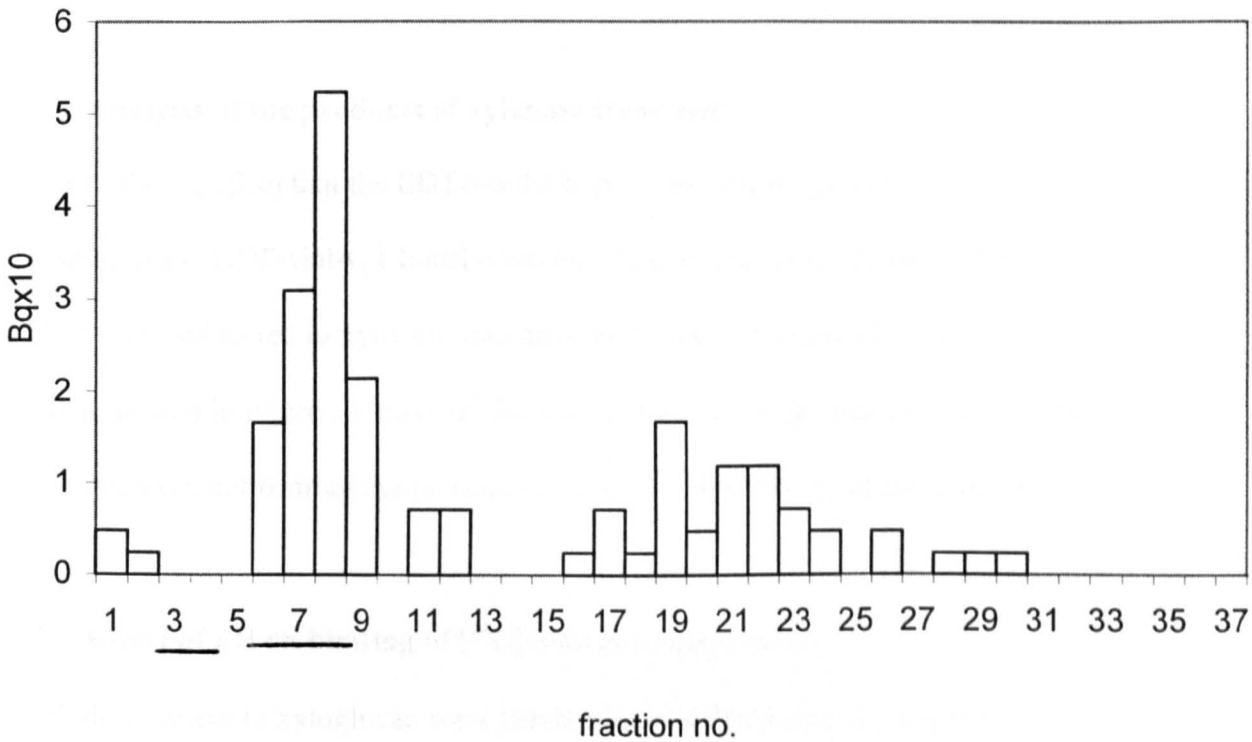

Figure 51. TLC of total acid hydrolysate of EDTA-soluble polysaccharides prepared at pH 6 for 4 hours (+UDP-GalA)

detected, with even more incorporation of GlcA residues in the high molecular weight material, indicating the presence of only labelled xylans (Fig. 52). Hence, the following incubation conditions were used to prepare labelled xylans: pH 6, UDP-GalA (1mM), for 1 hour.

C. Analysis of the products of xylanase treatment

To further confirm that the EDTA-soluble polysaccharides prepared under specific conditions (+UDP-GalA, 1 hour) consist of mainly radioactively labelled xylans, they were subjected to xylanase treatment and passed through a Biogel-P2 column. As indicated in figure 53, most of the high molecular weight material was degraded by xylanase, confirming the presence of mainly radioactively labelled xylans.

D. Effect of pH on binding of [^{14}C]-xylans to xyloglucan

Binding assays to xyloglucan were performed at different pH's, using the [^{14}C]-polysaccharides where the radioactivity is present as only[^{14}C]-GlcA. The binding pattern obtained is very similar to the one observed when using EDTA-soluble polysaccharides, confirming that xylans, on their own, bind to xyloglucan in a pH-dependent manner with highest binding at pH 3 and negligible binding at pH 6 (Fig. 54).

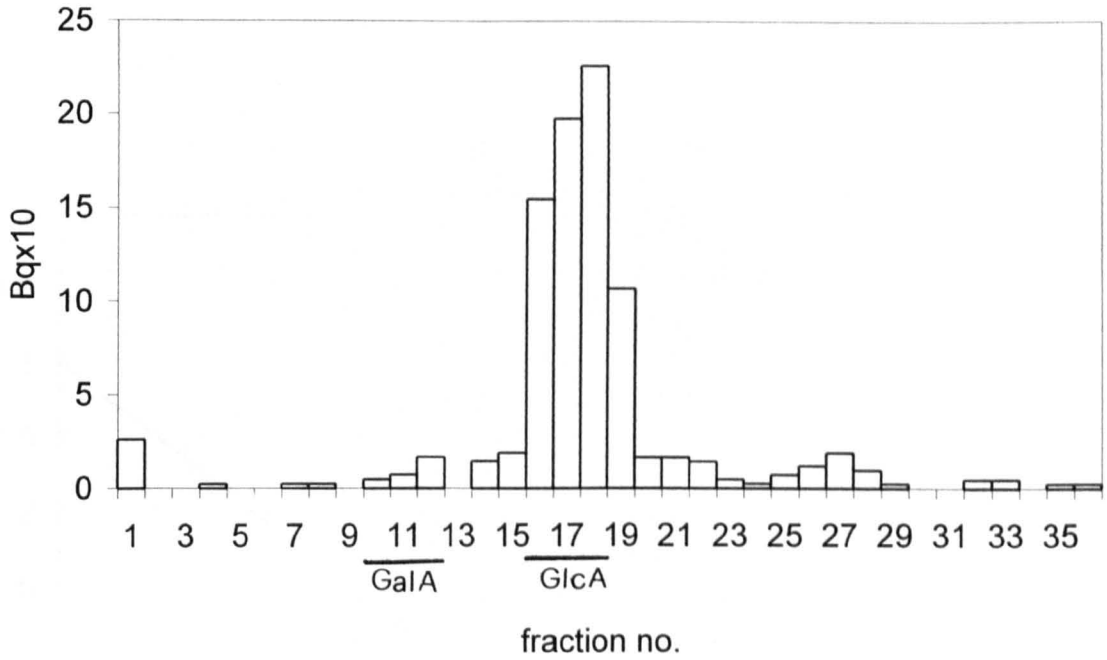

Figure 52. TLC of total acid hydrolysate of nascent EDTA-soluble ^{14}C-polysaccharides prepared at pH6 for 1 hour (+UDP-GalA)

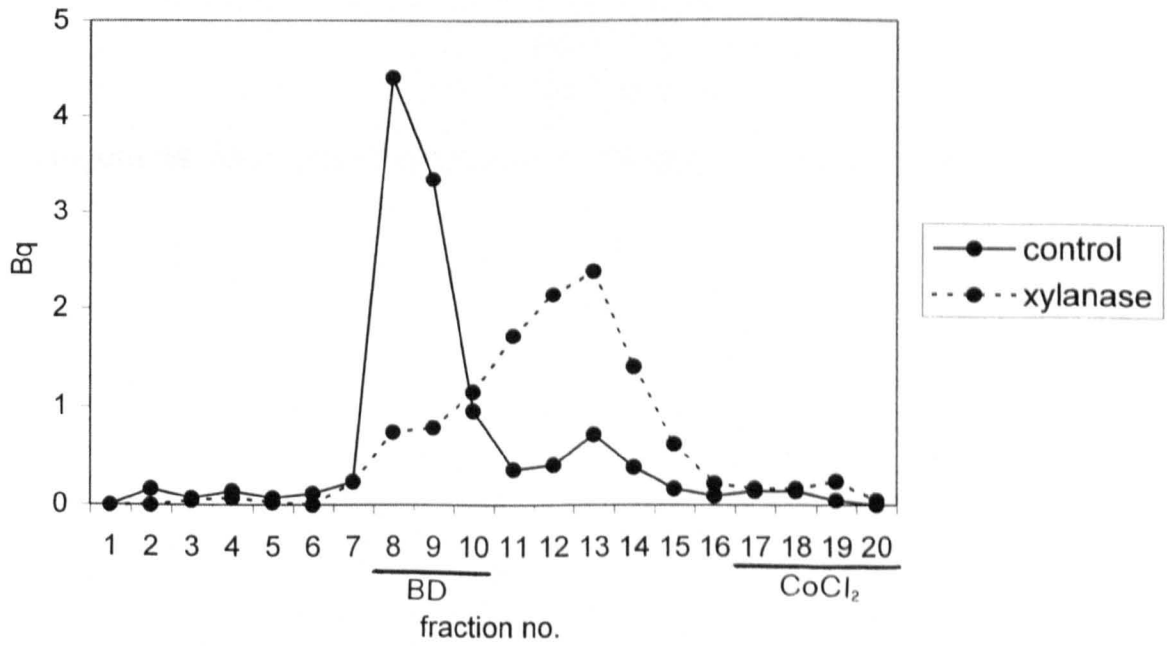

Figure 53. Gel filtration on Biogel P-2 of xylanase products of ^{14}C-GlcA-polysaccharides

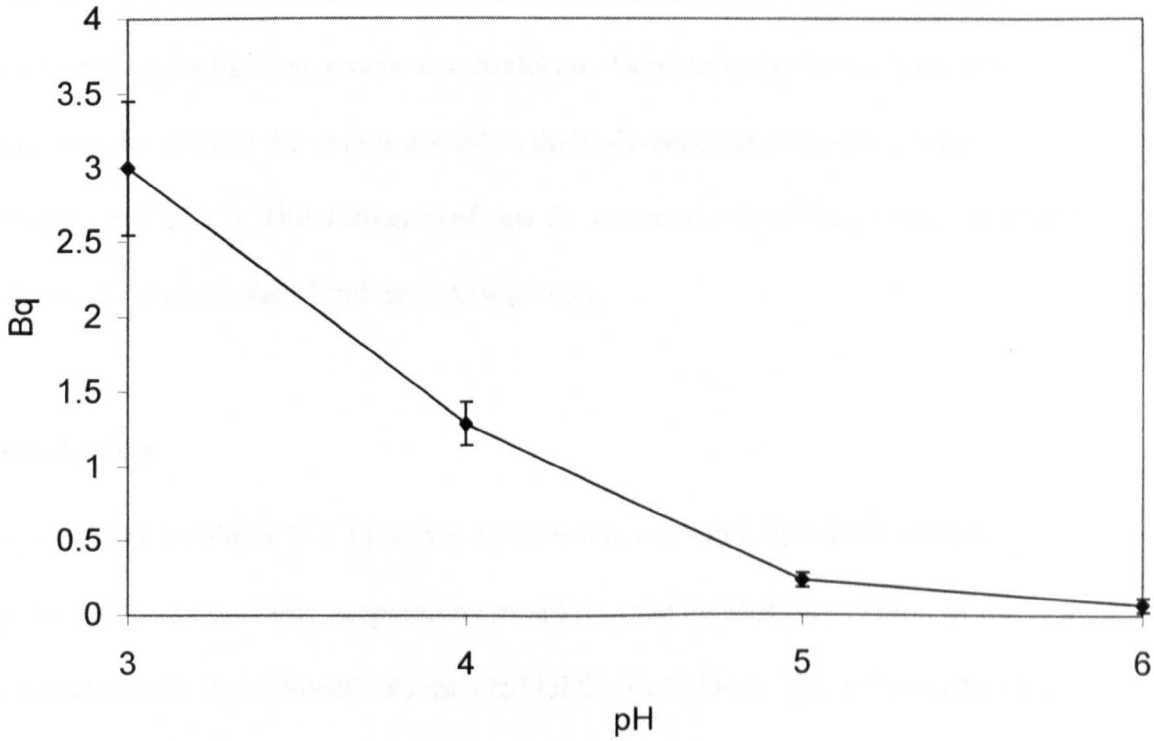

Figure 54. Effect of pH on binding of nascent ^{14}C-xylans to xyloglucan

E. Effect of protease treatment of [^{14}C]-xylans on binding to xyloglucan

To study the role of proteins, if present, in the binding of xylans to xyloglucan, [^{14}C]-xylans were prepared under mild conditions that would conserve the proteins attached to the polysaccharides. Samples were subjected to protease treatment prior to performing the binding assays to xyloglucan. As previously shown (Fig. 36), protein removal from the xylans abolishes their pH-dependent binding ability to xyloglucan (Fig. 55). This further confirms the essential role of the proteins attached to nascent xylans in their binding to xyloglucan.

Conclusion

Nascent EDTA-soluble [^{14}C]-polysaccharides that contain only labelled GlcA residues were successfully prepared by modifying the incubation medium of the pea membranes with the radioactively labelled UDP-GlcA donor. The polysaccharides obtained seem to consist of radioactive xylans without pectin contamination. These xylans bind to xyloglucan in the same pH-dependent binding pattern previously observed, and require protein(s) for the binding to occur.

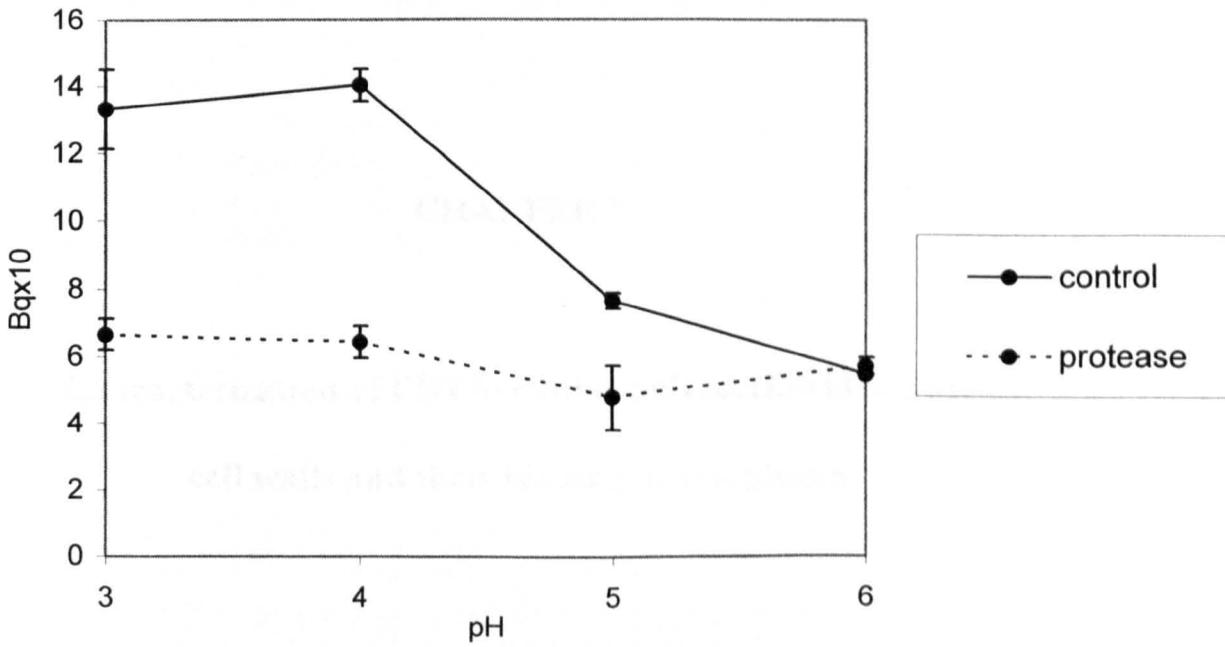

Figure 55. Effect of protease treatment of ^{14}C-xylans on their binding to xyloglucan

CHAPTER 7

Characterization of EDTA-soluble polysaccharides from cell walls and their binding to xyloglucan

Introduction

Nascent EDTA-soluble polysaccharides extracted from pea membrane preps bind to xyloglucan in a pH-dependent manner, and seem to require protein(s) for the binding to occur. However, polysaccharides might behave differently when deposited into the cell wall. To clarify this issue, EDTA-soluble [^{14}C]- polymers were extracted from pea cell walls. This chapter deals with the characterization of these polymers and their interaction with xyloglucan.

A. Characterization of EDTA-soluble polysaccharides from cell walls by PC

EDTA-extracts prepared from pea cell walls were passed through a column of Sephadex G-100, eluting with water and collecting 0.5 ml per fraction. The high molecular weight peak that runs parallel to blue dextran (fractions 5-10 in fig. 56), referred to as "[^{14}C]-polysaccharides from cell walls" was collected and used for analysis and binding assays to xyloglucan.

To determine the nature of the polysaccharides present in the EDTA-extract, the [^{14}C]-polysaccharides from cell walls were subjected to complete acid hydrolysis followed by PC using system C (Materials and Methods).The polysaccharides consist of radioactively labelled galactose, arabinose, a large amount of glucose and relatively small amounts of rhamnose and fucose residues that have the same Rf value as xylose residues (Fig.57). A significant amount of radioactivity remained at

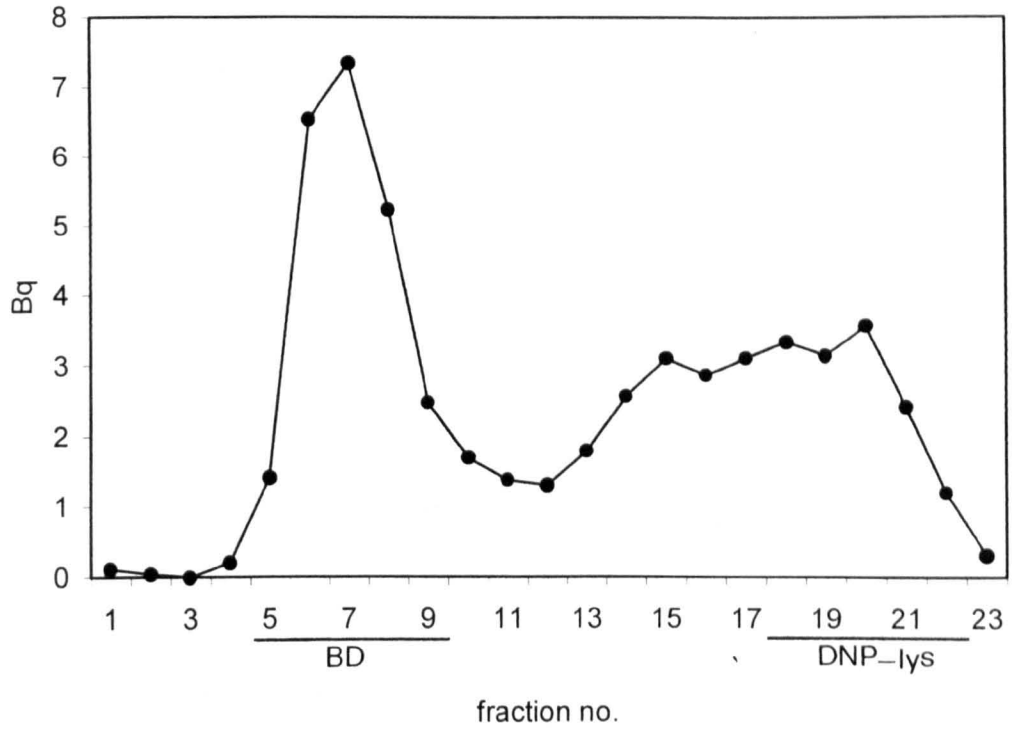

Figure 56. Gel filtration on Sephadex G-100 of EDTA-extract prepared from cell walls

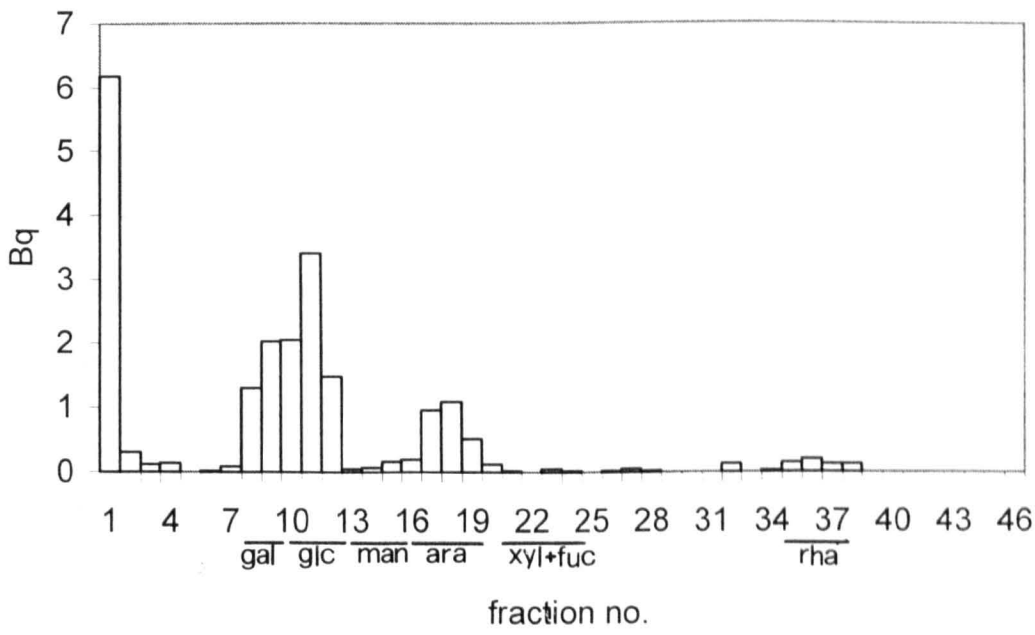

Figure 57. PC (system C) of total acid hydrolysate of EDTA-soluble ^{14}C-polysaccharides from cell walls

the origin and would probably account for uronic acids that were analyzed by using system A of PC (Materials and Methods). As revealed in figure 58, ~90% of the uronic acids are present as GalA residues, whereas only ~10% are present as GlcA residues. This reveals the high abundance of pectins in the [^{14}C]-polysaccharides from cell walls.

B. Analysis of xylanase, pectin lyase and amylase products of [^{14}C]-polysaccharides from cell walls

[^{14}C]-polysaccharides from cell walls were subjected to xylanase and pectin lyase treatments separately, and the products were analyzed by column chromatography, using the Biogel-P2 column previously used. Xylanase degrades only about 10%, whereas pectin lyase degrades about 50% of the high molecular weight material, confirming that the [^{14}C]-polysaccharides from cell walls consists of mainly pectins (Fig. 59). However, labelled glucose residues are present in relatively high amounts (Fig. 57), suggesting that starch could be extracted along with pectins. To verify this, the [^{14}C]-polysaccharides from cell walls were treated with amylase, and the products were passed through the same Biogel-P2 column. About 16% of the polysaccharides consist of starch (Fig. 60).

C. Effect of pH on binding of [^{14}C]-polysaccharides from cell walls to xyloglucan

Binding assays were performed to xyloglucan, as performed earlier, but using polysaccharides extracted from cell walls instead of nascent polysaccharides. As

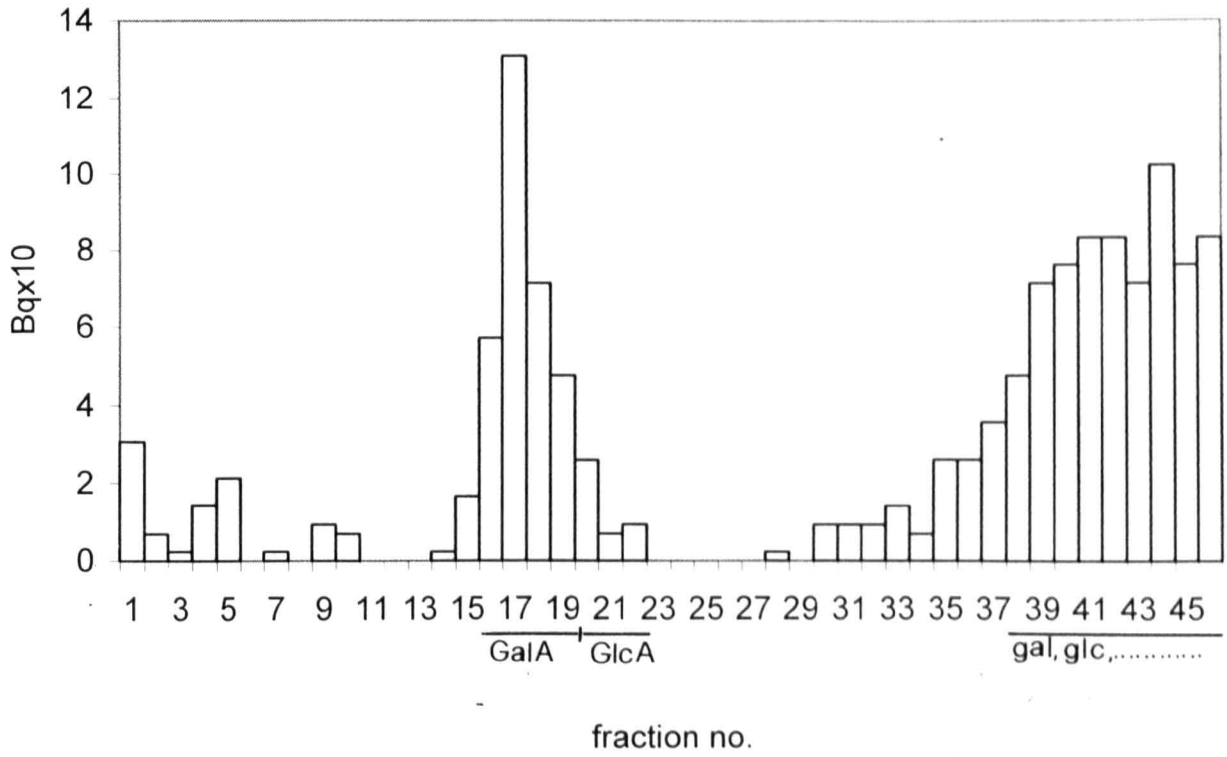

Figure 58. PC (system A) ot total acid hydrolysate of EDTA-soluble ^{14}C-polysaccharides from cell walls

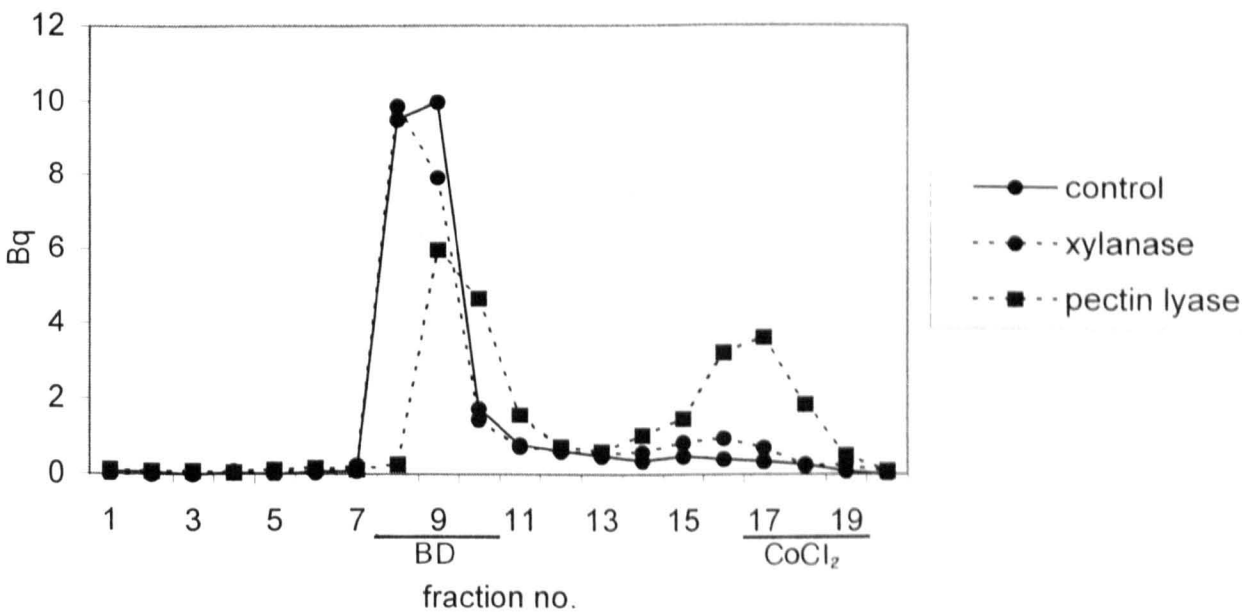

Figure 59. Gel filtration on Biogel P-2 of xylanase and pectin lyase products of EDTA-soluble ^{14}C-polysaccharides from cell walls

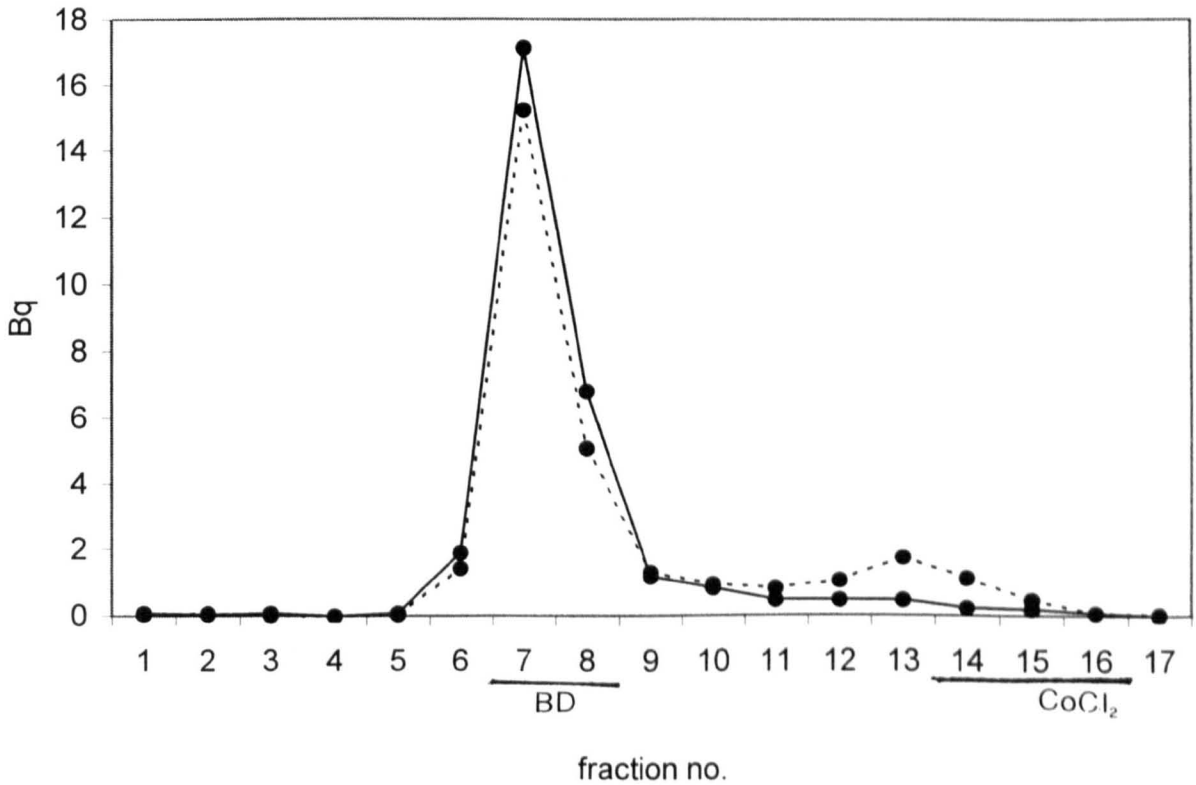

Figure 60. Gel filtration on Biogel P-2 of amylase products of ^{14}C-polysaccharides from cell walls

expected, the [^{14}C]-polysaccharides from cell walls bind to xyloglucan in a pH-dependent manner, showing maximum binding at pH 3 (17%) that gradually decreases as the pH increases (Fig. 61).

D. Effect of protease treatment of [^{14}C]-polysaccharides from cell walls on their binding to xyloglucan

To verify whether proteins are still attached to polysaccharides in cell walls and whether they play a role in the pH-dependent binding to xyloglucan, [^{14}C]-polysaccharides from cell walls were subjected to protease treatment prior to performing the binding assays to xyloglucan. As revealed in figure 62, removal of the proteins resulted in a 50% decrease in the binding, even though the binding pattern was still pH-dependent.

E. Effect of xylanase and pectin lyase treatments of [^{14}C]-polysaccharides from cell walls on their binding to xyloglucan

To determine which of the polymers in the EDTA-soluble polysaccharides extracted from cell walls have the ability to bind to xyloglucan in a pH-dependent manner, [^{14}C]-polysaccharides from cell walls were subjected to separate xylanase and pectin lyase treatments, prior to performing the binding assays to xyloglucan. A small decrease in the binding was observed upon xylanase treatment due to the minor amounts of xylans in the [^{14}C]-polysaccharides from cell walls (Sections A andB). The remaining pectins (and probably starch) still exhibit a clear pH-dependent binding pattern to xyloglucan (Fig. 63). However, a larger decrease was observed

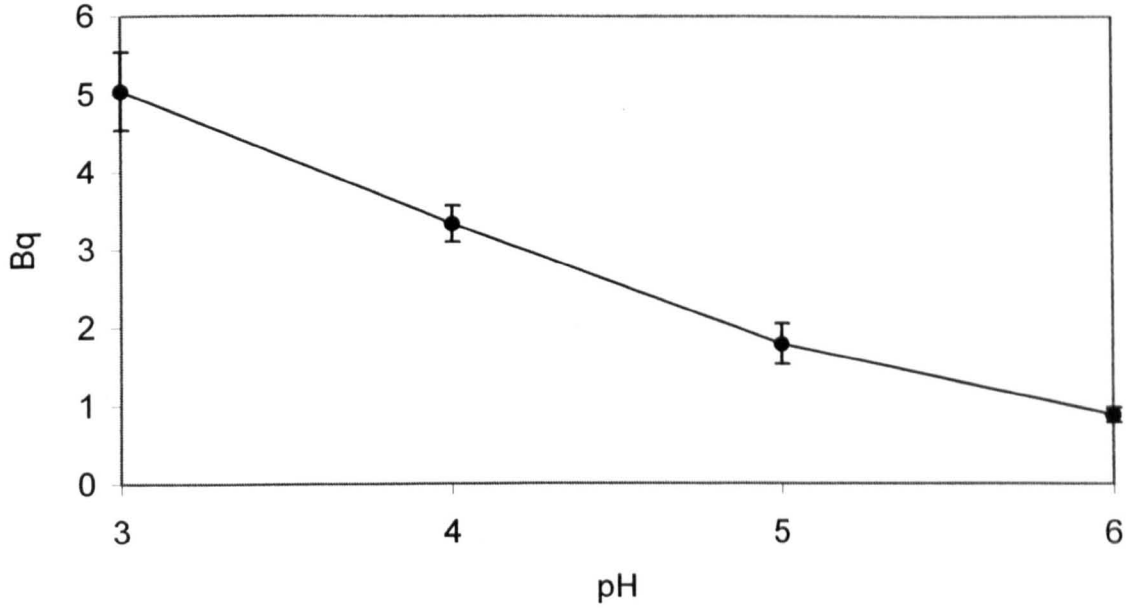

Figure 61. Effect of pH on binding of EDTA-soluble ^{14}C-polysaccharides extracted from cell walls to xyloglucan

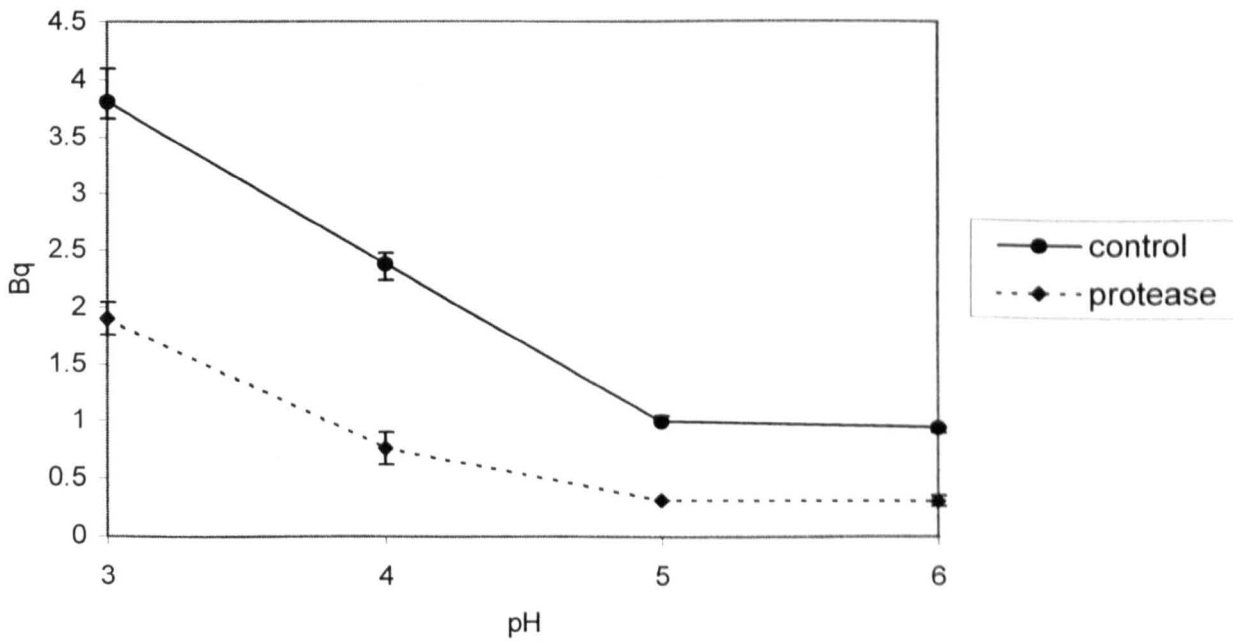

Figure 62. Effect of protease treatment of EDTA-soluble ^{14}C-polysaccharides from cell walls on their binding to xyloglucan

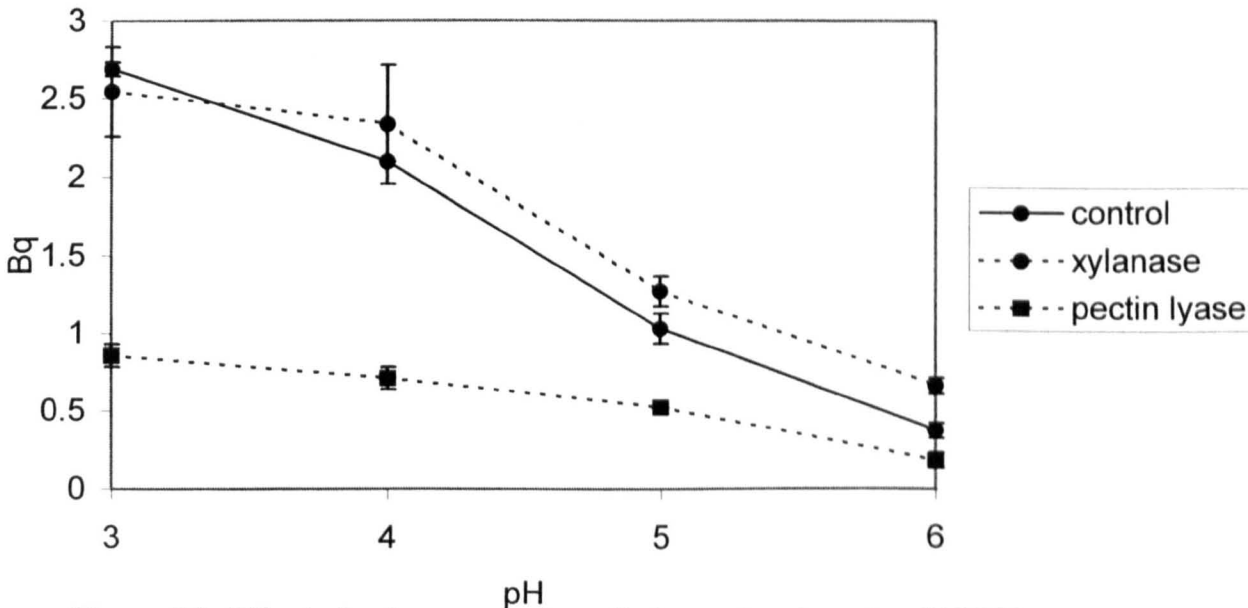

Figure 63. Effect of xylanase and pectin lyase treatments of EDTA-soluble ^{14}C-polysaccharides form cell walls on their binding to xyloglucan

upon pectin lyase treatment (Fig. 63) due to the high abundance of pectins in EDTA-extracts prepared from cell walls.

Conclusion

[^{14}C]-polysaccharides extracted from pea cell walls seem to consist of mainly pectins, with minor amounts of starch, and traces of xylans. They follow the same pH-dependent binding pattern to pea xyloglucan as the one exhibited by nascent polysaccharides. Proteins play a role in the binding, however protease treatment does not completely abolish the pH-dependent binding pattern.

CHAPTER 8

DISCUSSION

Nascent EDTA-soluble [^{14}C]-polysaccharides consist of radioactively labelled pectins and xylans, though pectins occur in higher amounts. KOH-soluble polysaccharides have a similar composition except that xylans are present in slightly higher amounts. All of the remaining experiments were performed on the EDTA-soluble fraction of polysaccharides extracted from pea membrane preparations. Both GAX and pectin bind to xyloglucan , the major hemicellulose of pea epicotyl cell walls, in a pH-dependent manner, with the highest binding at pH 3-4, which corresponds to the pH of a growing wall (McQueen-Mason, 1995). The binding decreases to almost zero at pH6. The same binding pattern had been reported to occur between nascent GAX and hemicelluloses from pea epicotyls: the binding was thought to occur via non-covalent bonds (Brett *et al.*, 1997). In the present investigation, it is revealed that even though the binding is not totally specific to xyloglucan, GAX and pectin bind maximally to xyloglucan when compared with a number of other cell wall polysaccharides. The binding seems to be instantaneous, and to depend on the amounts of XG and nascent polysaccharides present. The fucose side-chains present on xyloglucan probably play a role in the pH-dependent binding; these fucose residues have also been reported to play a role in the binding of xyloglucan to cellulose microfibrils (Levy *et al.*, 1997).

Since pre-treatment of nascent GAX with protease greatly decreases the binding at acid pH and abolishes the pH dependence of binding, both these properties must be dependent on the presence of the protein(s) attached to nascent GAX (Crosthwaite *et al.*, 1994). Protease treatment had the same effect on the binding of nascent pectin to

xyloglucan, providing indirect evidence that pectin is also synthesized attached to a protein, and that this protein has an essential role in the pH-dependent binding of nascent pectin to xyloglucan. The standard method used for preparing nascent [^{14}C]-polysaccharides involved conditions that might have denatured proteins. However, the same binding pattern and protease effect was observed when denaturing treatments were avoided, so the binding properties are likely to be due to native rather than denatured proteins. Also, the proteins seem to be specific, since no effect was observed when adding varying amounts of BSA to the incubation medium of nascent polysaccharides and xyloglucan. When investigating the molecular weight of these proteins involved, pectin was degraded in an attempt to release the protein(s) attached to it: two proteins appear on the SDS gel used, one of about 14 kDa, and the other of about 94 kDa. These proteins, could either both be attached to pectin, or one to pectin and the other to GAX, since when using different pectin lyase enzymes, the intensities of the bands observed differ, probably due to contamination of the pectin lyase with minor amounts of hemicellulases.

Reduction of the uronic acid residues of nascent polysaccharides resulted in abolition of the pH-dependent binding pattern; however, one cannot rule out the possibility that the result observed is due to alteration of the protein subjected to the harsh conditions of the treatment used (Kim and Carpita, 1992).

The reversibility of the binding observed when binding nascent GAX to cell walls indicated that the bonds involved are probably non-covalent (Brett *et al.*, 1997). The disruption of the binding by high salt concentration including guanidinium thiocyanate in this work is in accordance with this. Even though high salt

concentration does not seem to affect XG solubility, the effect observed after GTC treatment could be due to solubilisation of some XG, and not due to disrupting the bonds between xyloglucan and the nascent polysaccharides.

The significance of the binding of the nascent polysaccharides to xyloglucan remains to be established. It is likely that it plays a role in cell-wall assembly. The association between the polymers would probably not occur in the Golgi vesicles before their arrival at the plasma membrane, since the pH within the vesicles is thought to be about 6-7. The pH of the wall is more acidic, specially when undergoing growth, so the binding would only occur when the nascent polysaccharides are deposited in the wall. Recently-formed EDTA-soluble polysaccharides extracted from the cell wall seem to consist of mostly pectin, with some starch contamination. These polysaccharides follow the same pH- and protein-dependent binding pattern to xyloglucan as nascent GAX and pectin, indicating that the proteins are still present when the polysaccharides are deposited in the wall. Hence, these proteins may play a significant part in cell-wall assembly, at the point when newly-formed polysaccharides interact with each other and with newly-formed cellulose microfibrils to form the innermost wall layer. The proteins could perhaps be termed "assemblins".

The pH dependence of the binding also suggests a functional interaction with the mechanisms that control growth, since the wall pH decreases when elongation growth is initiated (McQueen Mason, 1995). This would strengthen the binding of newly-formed pectin and probably GAX to xyloglucan as the binding of xyloglucan to cellulose is gradually decreasing. Rapid growth may require strong interactions

between matrix polymers in order to maintain the cohesion of the wall. Alternatively, pectin and GAX may compete with cellulose for binding to xyloglucan: the strong binding of pectin and GAX to xyloglucan under acid conditions (pH 3-4) may interfere with xyloglucan-cellulose binding and hence help to render the cell wall more extensible, inducing cell elongation.

REFERENCES

Abeysekera, R. and Willison, M. 1990. Architecture of the fluid cellulose arrays in the epidermis of the quince seed. *Biol.Cell.* **68**: 251-257.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. 1989. Special features of plant cells. In: Molecular biology of the cell (2nd ed.). Garland Publishing Inc.New York. Pp.1137-1184.

Andrew, I.G. and Little, J.W.L. 1997. A xyloglucan in etiolated seedlings of *Pinus radiata. Phytochemistry.* **46**: 203-207.

Bailey, R.W. and Hassid, W.Z. 1966. Xylan synthesis from uridine-diphosphate-D-xylose by particulate preparations from immature corn cobs. *Proc.Natl.Acad.Sci.USA.* **56**: 1586-1593.

Baker, R.A. 1994. Potential dietary benefits of citrus pectin and fiber. *Food Technology.* **48:** 133-139.

Baker, R.A. 1997. Reassessment of some fruit and vegetable pectin levels. *J. of Food Science.* **62**: 225-229.

Baldwin, T.C., McCann, M.C. and Roberts, K. 1993. A novel hydroxyproline-deficient arabinogalactan protein secreted by suspension-cultured cells of *Daucus carota. Plant Physiol.* **103**: 115-123.

Baron-Epel, O., Gharyal, P.K. and Schindler, M. 1988. Pectins as mediators of wall porosity in soybean cells. *Planta.* **175**: 389-395.

Bauer, W.D., Talmadge, K.W., Keegstra, K. and Albersheim, P. 1973. The structure of plant cell walls. II. The hemicellulose of suspension-cultured sycamore cells. *Plant Physiol.* **51**: 174-184.

Baydoun, E.A.-H. and Brett, C.T. 1997. Distribution of xylosyltransferases within the Golgi apparatus in etiolated pea (*Pisum sativum*) epicotyls. *J.Exp.Bot.* **48**: 1209-1214.

Baydoun, E.A.-H., Usta, J.A.-R., Waldron, K.W. and Brett, C.T. 1989a. A methyltransferase involved in the biosynthesis of 4-O-methylglucuronoxylan in etiolated pea epicotyls. *J.Plant Physiol.* **135**: 81-85.

Baydoun, E.A.-H., Waldron, K.W. and Brett, C.T. 1989b. The interaction of xylosyltransferase and glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum*) epicotyls. *The Biochemical Journal.* **257**: 853-858.

Baydoun, E.A.-H., Hobbs, M.C., Delarge, M.H.P., Farmer, M.J., Waldron, K.W. and Brett, C.T. 1991. Formation of glucuronoxylan linked to protein in plant Golgi and plasma membranes. *Biochemical Society Transactions.* **19**: 250S.

Baydoun, E.A-H., Rizk., S.E. and Brett, C.T. 1999. Localisation of methyltransferases involved in glucuronoxylan and pectin methylation in the Golgi apparatus in etiolated pea epicotyls. *J.Plant Physiol.* **155**: 240-244.

Baydoun, E.A-H., Abdel-Massih, R., Dani, D. Rizk, S.E. and Brett, C.T. 2000. Galactosyl- and fucosyltransferases in etiolated pea epicotyls: product identification and sub-cellular localization. *J.Plant Physiol.* Submitted for publication.

Bolwell, G.P. 1993. Dynamic aspects of the plant extracellular matrix. *International Reviews in Cytology.* **146**: 261-234.

Bolwell, G.P. and Northcote, D.H. 1981. Control of hemicellulose and pectin synthesis during differentiation of vascular tissue in bean hypocotyl. *Planta.* **152**: 225-233.

Blaschek,W., Hass, D., Koehler, H., Semler, U. and Franz, G. 1983. Demonstration of a β-1,4-primer glucan in cellulose-like glucan synthesized *in vitro*. *Plant Physiol.* **111**: 357-364.

Brett, C.T. and Waldron, K.W. 1996. Physiology and Biochemistry of Plant Cell Walls. Chapman and Hall, 2-6 Boundary Row, London SE1 8HN, UK.

Brett, C.T., Healy, S.A., McDonald, M.S., Macgregor, C. and Baydoun, E.A.-H. 1997. Binding of nascent glucuronoxylan to the cell walls of pea seedlings. *Int.J.Biol.Macromol.* **21**: 169-173.

Brown, J.A. and Fry, S.C. 1993. Novel O-D-Galacturonoyl esters in the pectic polysaccharides of suspension-cultured plant cells. *Plant Physiol.* **103**: 993-999.

Brown, R.M. and Montezinos,D. 1976. Cellulose microfibrils visualization of biosynthetic and orienting complexes in association with the plasma membrane. *Proc.Natl.Acad.Sci.USA.* **73**: 143-147.

Brown, R.M., Li, L.K., Okuda, K., Kuga, S., Kudlicka, K., Drake, R., Santos, R. and Clement S. 1994. *In vitro* cellulose synthesis in plants. *Plant Physiol.* **105**: 1-2.

Brummell, D.A. and Maclachlan, G.A. 1989. Xyloglucan and derivatives. *ACS Symposium Series.* **399**: 18-32.

Brummell, D.A., Camirand, A. and Maclachlan, G.A. 1990. Differential distribution of xyloglucan glycosyl transferases in pea Golgi dictyosomes and secretory vesicles. *J.Cell Science*. **96**: 705-710.

Brummell, D.A., Bird, C.R., Schuch, W. and Bennett, A.B. 1997a. An endo-1,4-β-glucanase expressed at high levels in rapidly expanding tissues. *Plant Molecular Biology*. **33**: 87-95.

Brummell, D.A., Catala,C., Lashbrook, C.C. and Bennett, A.B. 1997b. A membrane-anchored E-type endo-1,4-β-glucanase is localized on Golgi and plasma membranes of higher plants. *Proc.Natl.Acad.Sci. USA*. **94**: 4794-4799.

Buckeridge, M.S., Crombie, H.J., Mendes, C.J.M., Reid, J.S.G., Gidley, M.J. and Vieira, C.C.J. 1997. A new family of oligosaccharides from the xyloglucan of *Hymenaea courbaril* L.(Leguminosae) cotyledons. *Carbohydrate Research*. **303**: 233-237.

Campbell, R.E., Brett, C.T and Hillman, J.R. 1988. A xylosyltransferase involved in the synthesis of a protein-associated xyloglucan in suspension-cultured dwarf-French-bean (*Phaseolus vulgaris*) cells and its interaction with a glucosyltransferase. *Biochem.J.* **253**: 795-800.

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

Carpita, N., McCann, M. and Griffing L.R. 1996. The plant extracellular matrix: News from the cell's frontier. *The Plant Cell.* Meeting report: 1451-1463.

Carroll, A.D., Moyen, C.V., VanKesteren, P., Tooke, F., Battey, N.H. and Brownlee, C. 1998. Ca^{+2}, annexins, and GTP modulate exocytosis from maize root cap protoplasts. *Plant Cell*. **10**: 1267-1276.

Casero, P.J., Casimiro, I. and Knox, J.P. 1998. Occurrence of cell surface arabinogalactan-protein and extensin epitopes in relation to pericycle and vascular tissue development in the root apex of four species. *Planta*. **204**: 252-259.

Catala, C., Rose, J.K.C. and Bennett, A.B. 1997. Auxin regulation and spatial localization of an endo-1,4-β-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. *Plant J.* **12**: 417-426.

Chambat, G., Barnoud, F. and Joseleau, J.-P. 1984. Structure of the primary cell walls of suspension-cultured *Rosa glauca* cells. I. Polysaccharides associated with cellulose. *Plant Physiol*. **74**: 687-693.

Chanliaud, E. and Gidley, M.J. 1999. In vitro synthesis and properties of pectin/*Acetobacter xylinus* cellulose composites. *Plant Journal.* **20**: 25-35.

Chanzy, H. and Henrissat, B. 1985. Unidirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action. *FEBS Letters.* **184**: 285-288.

Chen, L., Reginald, H.W. and McCann, M.C. 1997. Investigation of macromolecular orientation in dry and hydrated walls of single onion epidermal cells by FTIR microspectroscopy. *J.Molecular Structure.* **408/409**: 257-260.

Cho, H. and Kende, H. 1997a. Expansins in deepwater rice internodes. *Plant Physiol.* **113**: 1137-1143.

Cho, H. and Kende, H. 1997b. Expansins and internodal growth of deepwater rice. *Plant Physiol.* **113**: 1145-1151.

Coimbra, M.A., Rigby, N.M., Selvendran, R.R. and Waldron, K.W. 1995. Investigation of the occurrence of xylan-xyloglucan complexes in the cell walls of olive pulp (*Olea europaea*). *Carbohydrate Polymers.* **27**: 277-284.

Cosgrove, D.J. 1993a. Wall extensibility: its nature, measurement and relationship to plant cell growth. *New Phytol.* **124**: 1-23.

Cosgrove, D.J. 1993b. Water uptake by growing cells: an assessment of the controlling roles of wall relaxation, solute uptake and hydraulic conductance. *Int.J.Plant Sceince.* **154**: 10-21.

Cosgrove, D.J. 1996. Plant cell enlargement and the action of expansins. *BioEssays.* **18**: 533-540.

Cosgrove, D.J. 1997. Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. *The Plant Cell.* **9**: 1031-1041.

Cosgrove, D.J. and Durachko, D.M. 1994. Autolysis and extension of isolated walls from growing cucumber hypocotyls. *J.Exp.Botany.* **45**: 1711-1719.

Cosgrove, D.J. and Li, Z.-C. 1993. Role of expansin in developmental and light control of growth and wall extension in oat coleoptiles. *Plant Physiol.* **103**: 1321-1328.

Cote, F. and Hahn, M.G. 1994. Oligosaccharins: structures and signal transduction. *Plant Mol.Biol.* **26**: 1375-1411.

Crombie, H.J., Chengappa, S., Hellyer, A. and Reid, J.S.G. 1998. A xyloglucan oligosaccharide-active, transglycosylating β-D-glucosidase from the cotyledons of

nasturtium (*Tropaeolum majus* L) seedlings-purification, properties and characterization of cDNA clone. *The Plant Journal.* **15**: 27-38.

Crosthwaite, S.K., Macdonald, F.M., Baydoun, E.A.-H. and Brett, C.T. 1994. Properties of a protein-linked glucuronoxylan formed in the plant Golgi apparatus. *J.Exp.Botany.* **45**: 471-475.

Cutillas-Iturralde, A. and Lorences, E.P. 1997. Effect of xyloglucan oligosaccharides on growth, viscoelastic properties, and long-term extension of pea shoots. *Plant Physiol.* **113**: 103-109.

Cutillas_Iturralde, A., Zarra, I., Fry, S.C. and Lorences, P. 1994. Implication of persimmon fruit hemicellulose metabolism in the softening process. Importance of xyloglucan endotransglycosylase. *Physiol.Plant.* **91**: 169-176.

Cutillas-Iturralde, A., Pena, M., Zarra, I. and Lorences, E.P. 1998. A xyloglucan from persimmon fruit cell walls. *Phytochemistry.* **48**: 607-610.

Dalessandro, G. and Northcote, D.H. 1981. Xylan synthase activity in differentiated xylem cells of sycamore trees (*Acer pseudoplatanus*). *Planta.* **151**: 53-60.

Darvill, A.G., McNeil, M., Albersheim, P. and Delmer, D.P. 1980. The primary cell walls of flowering plants. In *Biochemistry of plants, a comprehensive treatise*,1. Ed. N. E.Tolbert, Academic Press, New York. Pp. 91-162.

Darvill, A., Augur, C., Bergmann, C. 1992. Oligosaccharins-oligosaccharides that regulate growth, development and defense responses in plants. *Glycobiology.* **2**:181-198.

de Silva, J., Jarman, C.D., Arrowsmith, D.A., Stronach, M.S., Chengappa, S., Sidebottom, C. and Reid, J.S.G. 1993. Molecular characterization of a xyloglucan-specific endo-1,4-β-glucanase (xyloglucan endo-transglycosylase) from nasturtium seeds. *Plant J.* **3**: 701-711.

de Silva, J., Arrowsmith, D., Hellyer, A., Whiteman, S. and Robinson, S. 1994. Xyloglucan endotransglycosylase and plant growth. *J.Exp.Botany.* **45**: 1693-1701.

Delarge, M.H.P., Hobbs, M.C. and Brett, C.T. 1991. A rapid method for Golgi isolation from etiolated pea epicotyls. *Biochemical Society Transactions.* **19**: 244S.

Delmer, D.P. 1983. Biosynthesis of cellulose. *Adv.Carbohydr.Chem.Bio-chem.* **41**: 105-153.

Delmer, D.P. 1987. Cellulose biosynthesis. *Ann.Rev.Plant Physiol.* **38**: 259-290.

Doong, R.L., Liljebjelke, K., Fralish, G., Kumar, A. and Mohnen, D. 1995. Cell-free synthesis of pectin. *Plant Physiol.* **109**: 141-152.

Driouich, A., Faye, L. and Staehelin, L.A. 1993. The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *TIBS.* **18**: 210-214.

Eberhard, S., Doubrava, N., Marfa, V., Mohnen, D., Southwick, A., Darvill, A. and Albersheim, P. 1989. Pectic cell wall fragments regulate tobacco thin layer explant morphogenesis. *Plant Cell.* **1**: 747-755.

Edelmann, H.G. and Fry, S.C. 1992. Effect of cellulose synthesis inhibition on growth and the integration of xyloglucan into pea internode cell walls. *Plant Physiol.* **100**: 993-997.

Edwards, M., Dea I.C.M., Bulpin, P.V. and Reid, J.S.G. 1985. Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus* L. seeds following germination. *Planta.* **163**: 133-140.

Faik, A., Chileshe, C., Sterling, J. and Maclachlan, G. 1997. Xyloglucan galactosyl- and fucosyltransferase activities from pea epicotyl microsomes. *Plant Physiol.* **114**: 245-254.

Faik, A., Desveaux, D. and Maclachlan, G. 1998. Enzymic activities responsible for xyloglucan depolymerization in extracts of developing tomato fruit. *Phytochemistry.* **49**: 365-376.

Fanutti, C., Gidley, M.J. and Reid, J.S.G. 1993. Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1→4)β-D-glucanase) from the cotyledons of germinated nasturtium seeds. *Plant J.* **3**: 691-700.

Farkas, V., Sulova, Z., Stratilova, E., Hanna, R. and Maclachlan, G. 1992. Cleavage of xyloglucan by nasturtium seed xyloglucanase and transglycosylation to xyloglucan subunit oligosaccharides. *Arch.Biochem.Biophys.* **298**: 365-370.

Femenia, A., Rigby, N.M., Selvendran, R.R. and Waldron, K.W. 1999. Investigation of the occurrence of pectin-xylan-xyloglucan complexes in the cell walls of cauliflower stem tissues. *Carbohydrate Polymers.* **39**: 151-164.

Fincher, G.B., Stone, B.A. and Clarke, A.E. 1983. Arabinogalactan-proteins: structure, biosynthesis and function. *Annu.Rev.Plant Physiol.* **34**: 47-70.

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

Fry, S.C. 1986b. In-vivo formation of xyloglucan nonasaccharide: a possible biologically active cell-wall fragment. *Planta.* **169**: 443-453.

Fry, S.C. 1988. The growing plant cell wall: chemical and metabolic analysis. John Wiley and Sons, Inc., New York.

Fry, S.C. 1989. The structure and functions of xyloglucan. *J.Exp.Botany.* **40**: 1-11.

Fry, S.C. 1995. Polysaccharide-modifying enzymes in the plant cell wall. *Annu.Rev.Plant Physiol.Plant Mol.Biol.* **46**: 497-520.

Fry, S.C. 1997. Novel 'dot-blot' assays for glycosyltransferases and glycosylhydrolases: optimization for xyloglucan endotransglycosylase (XET) activity. *The Plant Journal.* **11**: 1141-1150.

Fry, S.C. 1998. Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochem.J.* **332**: 507-515.

Fry, S.C. and Miller, J.G. 1989. Toward a working model of the growing plant cell wall. *ACS Symposium Series.* **399**: 33-46.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. and Matthews, K.J. 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity form plants. *Biochem.J.* **282**: 821-828.

Gali-Muhtasib, H.U., Smith, C.C. and Higgins, J.J. 1992. The effect of silica in grasses on the feeding behavior of the prairie vole, *Microtus ochrogaster. Ecology.* **73**: 1724-1729.

Gibeaut, D.M. and Carpita, N.C. 1990. Separation of membranes by flotation centrifugation for in vitro synthesis of plant cell wall polysaccharides. *Protoplasma.* **156**: 82-93.

Gibeaut, D.M. and Carpita, N.C. 1991. Tracing the biosynthesis of the cell wall in intact cells and plants. Selective turnover and alteration of soluble and cell wall polysaccharides in grasses. *Plant Physiol.* **97**: 551-561.

Gibeaut, D.M. and Carpita, N.C. 1994. Biosynthesis of plant cell wall polysaccharides. *FASEB J.* **8**: 904-915.

Gordon, R. and Maclachlan, G. 1989. Incorporation of UDP-[^{14}C]-glucose into xyloglucan by pea membranes. *Plant Physiol.* **91**: 373-378.

Goubet, F., Council, L.N. and Mohnen, D. 1998. Identification and partial characterization of the pectin methyltransferase "homogalacturonan-methyltransferase" from membranes of tobacco cell suspensions. *Plant Physiol.* **116**: 337-347.

Gregory, A.C.E., O'Connell, A.P. and Bolwell, G.P. 1998. Xylans. *Biotechnology and Genetic Engineering Reviews*. **15**: 439-454.

Hayashi, T. 1989. Xyloglucans in the primary cell wall. *Annu.Rev.Plant. Physiol.Plant Mol.Bio*. **40**: 139-168.

Hayashi, T. and Maclachlan, G. 1984. Pea xyloglucan and cellulose. *Plant Physiol.* **75**: 596-604.

Hayashi, T. and Ohsumi, C. 1994. Endo-1,4-β-glucanase in the cell wall of stems of auxin-treated pea seedlings. *Plant Cell Physiol.* **35**: 419-424.

Hayashi, T., Kato, Y. and Matsuda, K. 1980. Xyloglucan from suspension-cultured soybean cells. *Plant Cell Physiol.* **21**: 1405-1418.

Hayashi, T., Ogawa, K. and Mitsuishi, Y. 1994. Characterization of the adsorption of xyloglucan to cellulose. *Plant Cell Physiol.* **35**: 1199-1205.

Hetherington, P.R. and Fry, S.C. 1993. Xyloglucan endotransglycosylase activity in carrot cell suspensions during elongation and somatic embryogenesis. *Plant Physiol.* **103**: 987-992.

Hieta, K., Kuga, S. and Usuda, M. 1984. Electron staining of reducing ends evidences a parallel-chain structure in *Valonia* cellulose. *Biopolymers*. **23**: 1807-1810.

Hoagland, P.D. 1996. Films from pectin, chitosan and starch. *ACS Symposium Series*. **650**: 145-154.

Hobbs, M.C., Baydoun, E.A.-H., Delarge, M.H.P. and Brett, C.T. 1991a. Interactions between a xylosyltransferase and a glucuronyltransferase involved in glucuronoxylan synthesis in pea epicotyls. *Biochemical Society Transactions*. **19**: 245S.

Hobbs, M.C., Delarge, M.H.P., Baydoun, E.A.-H. and Brett, C.T. 1991b. Differential distribution of a glucuronyltransferase, involved in glucuronoxylan synthesis, within the Golgi apparatus of pea (*Pisum sativum* var. Alaska). *Biochem.J.* **277**: 653-658.

Hoson, T. and Matsuda, Y. 1987. Effect of lectins on auxin-induced elongation and wall loosening in oat coleoptile and azuki bean epicotyl segments. *Physiol.Plant*. **71**: 1-8.

Hoson, T., Matsuda, Y., Sone, Y. and Misaki, A. 1991. Xyloglucan antibodies inhibit auxin-induced elongation and cell wall loosening of azuki bean but not of oat coleoptile. *Plant Physiol.* **96**: 551-557.

Houtman, C.J. and Atalla, R.H. 1996. Glucomannan and xyloglucan association with model cellulose surfaces. *Abstracts Papers American Chemical Soc. Cell.* **211**: 1(124).

Igartuburu, J.M., Pando, E., Luis, F.R. and Gil-Serrano, A. 1997. An acidic xyloglucan from grape skins. *Phytochemistry.* **46**: 1307-1312.

Inouhe, M., Yamamoto, R. and Masuda, Y. 1986. Inhibition of IAA-induced cell elongation in *Avena* coleoptile segments by galactose: its effect on UDP-glucose formation. *Physiologia Plantarum.* **66**: 370-376.

Iyama, K., Lam, T.B.T. and Stone, B.A. 1994. Covalent cross-links in the cell wall. *Plant Physiol.* **104**: 315-320.

Jarvis, M.C. 1984. Structure and properties of pectin gels in plant cell walls. *Plant Cell Environment.* **7**: 153-164.

Jarvis, M.C. 1992. Control of thickness of collenchyma cell walls by pectins. *Planta.* **187**: 218-220.

Jarvis, M.C., Forsyth, W. and Duncan, H.J. 1988. A survey of the pectin content of nonlignified monocot cell walls. *Plant Physiol.* **88**: 309-314.

Joseleau, J.-P. and Chambat, G. 1984. Structure of the primary cell walls of suspension-cultured *Rosa glauca* cells. II. Multiple forms of xyloglucan. *Plant Physiol.* **74**: 694-700.

Kakegawa, K., Edashige, Y. and Ishii, T. 1998. Xyloglucan from xylem-differentiating zones of *Cryptomeria japonica. Phytochemistry.* **47**: 767-771.

Kato, Y. and Matsuda, K. 1976. Presence of xyloglucan in the cell wall of *Phaseolus aureus* hypocotyls. *Plant and Cell Physiol.* **17**: 1185-1198.

Kato, Y. and Matsuda, K. 1985. Xyloglucan in cell walls of suspension-cultured rice cells. *Plant and Cell Physiology.* **26**: 437-445.

Kauss, H. 1967. Biosynthesis of the glucuronic acid unit of hemicellulose B from UDP-glucuronic acid. *Biochim.Biophys.Acta.* **148**: 572-574.

Kauss, H. 1969. Enzymic 4-O-methylation of glucuronic acid linked to galactose in hemicellulose polysaccharides from *Phaseolus aureus. Phytochemistry.* **8**: 985-988.

Kauss, H. and Hassid, W.Z. 1967. Biosynthesis of the 4-o-methyl-D-glucuronic acid unit of hemicellulose B by transmethylation from S-adenosyl-L-methionine. *J.Biol.Chemistry.* **242**: 1680-1684.

Keller, E. and Cosgrove, J. 1995. Expansins in growing tomato leaves. *The Plant Journal.* **8**: 795-802.

Kiefer, L.L., York, W.S., Albersheim, P. and Darvill, A.G. 1990. Structural characterization of an arabinose-containing heptadecasaccharide enzymically isolated from sycamore extracellular xyloglucan. *Carbohydrate Research.* **197**: 139-158.

Kieliszewski, M.J. and Lamport, D.T.A. 1994. Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *The Plant Journal.* **5**: 157-172.

Kim, J.-B. and Carpita, N.C. 1992. Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.* **98**: 646-653.

Kobayashi, M., Matoh, T. and Azuma, J. 1996. Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol.* **110**: 1017-1020.

Kooiman, P. 1960. A method for determination of amyloid in plant seeds. *Recueil.* **79**: 675-678.

Labavitch, J.M. and Ray, P.M. 1974. Relationship between promotion of xyloglucan metabolism and induction of elongation by indoleacetic acid . *Plant Physiol.* **54**: 499-502.

Lamport, D.T.A. 1965. Cell wall proteins. *Adv.Bot.Res.* **2**: 251-258.

Leach, J.E., Cantrell, M.A. and Sequeira, L. 1982. Hydroxyproline-rich bacterial agglutinin from potato. Extraction, purification and characterization. *Plant Physiol.* **70**: 1353-1358.

Levy, S., York, W.S., Stuike-Prill, R., Meyer, B. and Staehelin, L.A.1991. Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *The Plant Journal.* **1**: 195-215.

Levy, S., Maclachlan, G. and Staehelin, L.A. 1997. Xyloglucan sidechains modulate binding to cellulose during *in vitro* binding assays as predicted by conformational dynamics simulations. *The Plant Journal.* **11**: 373-386.

Lorences, E.P. and Zarra, I. 1987. Auxin-induced growth in hypocotyl segments of *Pinus pinaster* Aiton: changes in molecular weight distribution of hemicellulosic polysaccharides. *J.Exp.Bot.* **38**: 960-967.

Lozovaya, V.V., Zabotina, O.A. and Widholm, J.M. 1996. Synthesis and turnover of cell-wall polysaccharides and starch in photosynthetic soybean suspension cultures. *Plant Physiol.* **111**: 921-929.

Lynch, M.A. and Staehelin, L.A. 1992. Domain-specific and cell type-specific localization of two types of cell wall matrix polysaccharides in the clover root tip. *J.Cell Biol.* **118**: 467-479.

Maclachlan, G. and Brady, C. 1994. Endo-1,4-β-glucanase, xyloglucanase and xyloglucan endotransglycosylase activities versus potential substrates in ripening tomatoes. *Plant Physiol.* **105**: 965-974.

Maruyama, K., Goto, C., Numata, M., Suzuki, T., Nakagawa, Y., Hoshino, T. and Uchiyama, T. 1996. O-acetylated xyloglucan in extracellular polysaccharides from cell-suspension cultures of *Mentha*. *Phytochemistry*. **41**: 1309-1314.

McCann, M.C., Wells, B. and Roberts, K. 1990. Direct visualization of cross-links in the primary plant cell wall. *J.Cell Science*. **96**: 323-334.

McCann, M.C., Wells, B. and Roberts, K. 1992. Complexity in the spatial localization and length distribution of plant cell-wall matrix polysaccharides. *J.Microscopy*. **166**: 123-136.

McCann, M.C., Shi, J., Roberts, K. and Carpita, N.C. 1994. Changes in pectin structure nad localization during the growth of unadapted and NaCl-adapted tobacco cells. *The Plant Journal*. **5**: 773-785.

McDougall, G.J. and Fry, S.C. 1988. Inhibition of auxin-stimulated growth of pea stems segments by a specific nonasaccharide of xyloglucan. *Planta*. **175**: 412-416.

McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. 1984. Structure and function of the primary cell walls of plants. *Ann.Rev.Biochem*. **53**: 625-663.

McQueen-Mason, S.J. 1995. Expansins and cell wall expansion. *J.Exp.Botany*. **46**: 1639-1650.

McQueen-Mason, S.J. and Cosgrove, D.J. 1994. Disruption of hydrogen bonding between wall polymers by proteins that induce plant wall extension. *Proc.Natl.Acad.Sci.USA*. **91**: 6574-6578.

McQueen-Mason, S.J. and Cosgrove, D.J. 1995. Expansin mode of action on cell walls. *Plant Physiol.* **107**: 87-100.

McQueen-Mason, S., Durachko, D.M. and Cosgrove, D.J. 1992. Two endogenous proteins that induce cell wall extension in plants. *The Plant Cell*. **4**: 1425-1433.

McQueen-Mason, S.J., Fry, S.C., Durachko, D.M. and Cosgrove, D.J. 1993. The relationship between xyloglucan endotransglycosylase and in-vitro cell wall extension in cucumber hypocotyls. *Planta.* **190**: 327-331.

Mellon, J.E. and Helgeson, J.P. 1982. Interaction of a hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. *Plant Physiol.* **70**: 401-405.

Mohnen, D. and Hahn, M.G. 1993. Cell wall carbohydrates as signals in plants. *Seminars in Cell Biology.* **4**: 903-102.

Monro, J.A., Penny, D. and Bailey, R.W.1976. The organization and growth of primary cell walls of lupin hypocotyl. *Phytochemistry.* **15**: 1193-1198.

Moore, P.J. and Staehelin, L.A. 1988. Immunogold localization of the cell-wall matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta.* **174**: 433-445.

Moore, P.J., Darvill, A.G., Albersheim, P. and Staehelin, A.L. 1986. Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. *Plant Physiol.* **82**: 787-794.

Moore, P.J., Swords, K.M.M., Lynch, M.A. and Staehelin, L.A. 1991. Spatial organization of the assembly pathways of glycoproteins nad complex polysaccharides in the Golgi apparatus of plants. *J.Cell Biol.* **112**: 589-602.

Morris, E.R., Powell, D.A., Gidley, M.J. and Rees, D.A. 1982. Conformations and interactions of pectins. I. Polymorphism between gel and solid states of calcium polygalacturonate. *J.Mol.Biol.* **155**: 507-516.

Niemann, C., Carpita, N.C. and Whistler, R.L. 1997. Arabinose-containing oligosaccharides from tamarind xyloglucan. *Starch Starke.* **49**: 154-159.

Nishitani, K. 1997. The role of endoxyloglucan transferase in the organization of plant cell walls. *Int.Rev.Cytol.* **173**: 157-206.

Nishitani, K. 1998. Construction and restructuring of the cellulose-xyloglucan framework in the apoplast as mediated by the xyloglucan-related protein family- A hypothetical scheme. *J.Plant Research.* **111**: 159-166.

Nishitani, K. and Matsuda, Y. 1981. Auxin-induced changes in the cell wall structure: changes in the sugar composition, intrinsic viscosity and molecular weight distribution of matrix polysaccharides of the epicotyl cell wall of *Vigna angularis.* *Physiol. Plant.* **52**: 482-494.

Nishitani, K. and Nevins, D.J. 1991. Glucuronoxylan xylanhydrolase. *J.Biol.Chemistry.* **266**: 6539-6543.

Nishitiani, K. and Tominaga, R. 1992. Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J.Biol.Chemistry.* **267**: 21058-21064.

Northcote, D.H. 1972. Chemistry of the plant cell wall. *Annu.Rev.Plant Physiol.* **23**: 113-132.

Northcote, D.H. 1989. Control of plant cell wall biogenesis. *ACS Symposium Series.* **399**: 1-15.

Nothnagel, E.A., McNeil, M., Albersheim, P. and Dell, A. 1983. Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiol.* **71**: 916-926.

Ogawa, K., Hayashi, T. and Okamura, K. 1990. Conformational analysis of xyloglucans. *Int.J.Biol.Macromol.* **12**: 218-222.

Okazawa, K., Sato, Y., Nakagawa, T., Asada, K., Kato, I., Tomita, E. and Nishitani, K. 1993. Molecular cloning and cDNA sequencing of endoxyloglucan transferase, a novel class of glycosyltransferase that mediates molecular grafting between matrix polysaccharides in plant cell walls. *J.Biol.Chemistry.* **268**: 25364-25368.

Oxley, D. and Bacic, A. 1999. Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from *Pyrus communis* suspension-cultured cells. *Proceed.Natl.Acad.Sci. USA.* **96**: 14246-14251.

Passioura, J.B. 1994. The physical chemistry of the primary cell wall: implications for the control of expansion rate. *J.Exp.Bot.* **45**: 1675-1682.

Perrin, R.M., Derocher, A.E., Barpeled, M., Zeng, W.Q., Norambuena, L., Orellana, A., Raikhel, N.V. and Keegstra, K. 1999. Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science.* **284**: 1976-1979.

Potter, I. and Fry, S.C. 1994. Changes in xyloglucan endotransglycosylase (XET) activity during hormone-induced growth in lettuce and cucumber hypocotyls and spinach cell suspension cultures. *J.Exp.Bot.* **45**: 1703-1710.

Redgwell, R.J. and Fry, S.C. 1993. Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening: implications for fruit softening. *Plant Physiol.* **103**: 1399-1406.

Redgwell, R.J. and Selvendran, R.R. 1986. Structural features of cell wall polysaccharides of onion, *Allium cepa. Carbohydrate Research.* **157**: 183-199.

Reid, J.S.G. 1985. Cell wall storage carbohydrates in seeds: biochemistry of the seed 'gums' and 'hemicelluloses'. *Advances in Botanical Research.* **11**: 125-155.

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

Reis, D., Vian, B., Chanzy, H. and Roland, J.-C. 1991. Liquid crystal-type assembly of native cellulose-glucuronoxylans extracted from plant cell wall. *Biol.Cell.* **73**: 173-178.

Reiter, W.-D., Chapple, C.C.S. and Somerville, C.R. 1993. Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis. Science.* **261**: 1032-1035.

Renard, C.M.G.C., Lemeunier, C. and Thibault, J.-F. 1995. Alkaline extraction of xyloglucan from depectinised apple pommace: optimisation and characterisation. *Carbohydrate Polymers.* **28**: 209-216.

Revol, J.-F. and Goring, D.A.I. Directionality of the fibre c-axis of cellulose crystallites in microfibrils of *Valonia ventricosa. Polymer.* **24**: 1547-1550.

Roberts, K. 1989. The plant extracellular matrix. *Curr.Opin.Cell Biol.* **1**: 1020-1027.

Roberts, K. 1990. Structures at the plant cell surface. *Curr.Opin.Cell Biol.* **2**: 920-928.

Rodgers, M. and Bolwell, G.P. 1992. Partial purification of Golgi-bound arabinosyltransferase and two isoforms of xylosyltransferase from French bean (*Phaseolus vulgaris* L.). *Biochem.J.* **288**: 817-822.

Roland, J.-C., Reis, D., Vian, B. and Roy, S. 1989. The helicoidal plant cell wall as a performing cellulose-based composite. *Biol.Cell.* **67**: 209-220.

Rose, J.K.C., Brummell, D.A. and Bennett, A.B. 1996. Two divergent xyloglucan endotransglycosylases exhibit mutually exclusive patterns of expression in Nasturtium. *Plant Physiol.* **110**: 493-499.

Rose, J.K.C., Lee, H.H. and Bennett, A.B. 1997. Expression of a divergent expansin gene is fruit-specific and ripening-regulated. *Proc.Natl.Acad.Sci.USA.* **94**: 5955-5960.

Ryan, C.A. and Farmer, E.E. 1991. Oligosaccharide signals in plants: a current assessment. *Ann.Rev Plant Physiol.Plant Mol.Biol.* **42**: 651-674.

Sakai, T., Sakamoto, T., Hallaert, J. and Vandamme, E.J. 1993. Pectin, pectinase and protopectinase: production, properties and applications. *Adv.Appl.Microbiol.* **39**:213-217.

Schroder, R., Atkinson R.G., Langenkamper, G. and Redgwell, R.J. 1998. Biochemical and molecular characterisation of xyloglucan endotransglycosylase from ripe kiwifruit. *Planta.* **204**: 242-251.

Sequeira, L., Gaard, G. and Dizoeten, G.A. 1977. Interaction of bacteria and host cell walls: its relation to the mechanism of induced resistance. *Physiology of Plant Pathology.* **10**: 43-50.

Shedletzky, E., Shumel, M., Trainin, T., Kalman, S. and Delmer, D. 1992. Cell wall structure in cells adapted to growth on the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile. *Plant Physiol.* **100**: 120-130.

Shibuya, N. and Misaki, A. 1978. Structure of hemicellulose isolated from rice endosperm cell wall: mode of linkages and sequences in xyloglucan, β-glucan and arabinoxylan. *Agricultural and Biological Chemistry.* **42**: 2267-2274.

Shirakawa, M., Yamatoya, K. and Nishinari, K. 1998. Tailoring of xyloglucan properties using an enzyme. *Food Hydrocolloids.* **12**: 25-28.

Sims, I.M., Munro, S.L.A., Currie, G., Craik, D. and Bacic, A. 1996. Structural characterisation of xyloglucan secreted by suspension-cultured cells of *Nicotiana plumbaginifolia. Carbohydrate Research.* **293**: 147-172.

Spronk, B.A., Rademaker, G.J., Haverkamp, J., Thomas-Oates, J.E., Vincken, J.-P., Voragen, A.G.J., Kamerling, J.P. and Vliegenthart, J.F.G. 1998. Dimers of a GFG hexasaccharide occur in apple fruit xyloglucan. *Carbohydrate Research.* **305**: 233-242.

Staehelin, L.A. 1987. Secretion in plant cells differs from animal cells: intermediate plasma membrane configurations visualized in ultrarapidly frozen cells. *J.Cell Bio. Suppl.* **105**: 56.

Stephenson, M.B. and Hawes, M.C. 1994. Correlation of pectin methylesterase activity in root caps of pea with root border cell separation. *Plant Physiol.* **106**: 739-745.

Swords, K.M.M. and Staehelin, L.A. 1993. Complementary immunolocalization patterns of cell wall hydroxyproline-rich glycoproteins studied with the use of antibodies directed against different carbohydrate epitopes. *Plant Physiol.* **102**: 891-901.

Talbott, L.D. and Pickard, B.G. 1994. Differential changes in size distribution of xyloglucan in the cell walls of gravitropically responding *Pisum sativum* epicotyls. *Plant Physiol.* **106**: 755-761.

Talbott, L.D. and Ray, P.M. 1992a. Molecular size and separability features of pea cell wall polysaccharides. Implications for models of primary wall structure. *Plant Physiol.* **98**: 357-368.

Talbott, L.D. and Ray, P.M. 1992b. Changes in molecular size of previously deposited and newly synthesized pea cell wall matrix polysaccharides. Effects of auxin and turgor. *Plant Physiol.* **98**: 369-379.

Thakur, B.R., Singh, R.K. and Handa, A.K. 1997. Chemistry and uses of pectin- A review. *Critical Reviews in Food Science and Nutrition.* **37**: 47-73.

Thompson, J.E., Smith, R.C. and Fry, S.C. 1997. Xyloglucan undergoes interpolymeric transglycosylation during binding to the plant cell wall *in vivo*. Evidence from $^{13}C/^{3}H$ dual labelling and isopycnic centrifugation in caesium trifluoroacetate. *Biochem.J.* **327**: 699-708.

Timmel, T.E. 1964. Wood hemicelluloses. *Advances in Carbohydrate Chemistry.* **19**: 247-302.

Tran Thanh Van, K., Toubart, P., Cousson, A., Darvill, A.G., Gollin, D.J., Chelf, P. and Albersheim, P. 1985. Manipulation of morphogenetic pathways of tobacco explants by oligosaccharins. *Nature.* **314**: 615-617.

Vannier, M.P., Thoiron, B., Morvan, C. and Demarty, M. 1992. Localization of methyltransferase activities throughout the endomembrane system of flax (*Linum usitatissimum* L.) hypocotyls. *Biochem.J.* **286**: 863-868.

Vargas-Rechia, C., Reicher, F., Sierakowski, M.R., Heyraud, A., Driguez, H. and Lineart, Y. 1998.Xyloglucan octasaccharide XXLGOL derived from seeds of *Hymenaea courbaril* acts as signaling molecule. *Plant Physiol.* **116**: 1013-1021.

Vian, B., Reis, D., Mosiniak, M. and Roland, J.-C. 1986. The glucuronoxylans and the helicoidal shift in cellulose microfibrils in linden wood: cytochemistry *in muro* and on isolated molecules. *Protoplasma.* **131**: 185-199.

Vian, B., Reis, D., Darzens, D. and Roland, J.C. 1994. Cholesteric-like crystal analogs in glucuronoxylan-rich cell wall composites: experimental approach of acellular re-assembly from native cellulosic suspension. *Protoplasma.* **180**: 70-81.

Vincken, J.-P., Beldman, G. and Voragen, A.G.J. 1994. The effect of xyloglucans on the degradation of cell-wall embedded cellulose by the combined action of

cellobiohydrolase and glucanases from *Trichoderma viride*. *Plant Physiol.* **104**: 99-107.

Vincken, J.-P., Wijsman, A.J.M., Beldman, G., Niessen, W.M.A. and Voragen, A.G.J. 1996a. Potato xyloglucan is built from XXGG-type subunits. *Carbohydrate Research.* **288**: 219-232.

Vincken, J.-P., Beldman, G., Niessen, W.M.A. and Voragen, A.G.J. 1996b. Degradation of apple fruit xyloglucan by endoglucanase. *Carbohydrate Polymers.* **29**: 75-85.

Virk, S.S. and Cleland, R.E. 1990. The role of wall calcium in the extension of cell walls of soybean hypocotyls. *Planta.* **182**: 559-564.

Wada, S. and Ray, P.M. 1978. Matrix polysaccharides of oat coleoptile cell walls. *Phytochemistry.* **17**: 923-931.

Waldron, K.W. 1984. Glucuronyltransferase activity associated with hemicellulose and pectin biosynthesis in *Pisum sativum*. Ph.D. Thesis, University of Glasgow, pp.96-97.

Waldron, K.W. and Brett, C.T. 1983. A glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum*) epicotyls. *Biocehm.J.* **213**: 115-122.

Waldron, K.W. and Brett, C.T. 1987. The subcellular localisation of a glucuronyltransferase involved in glucuronoxylan biosynthesis in pea (*Pisum sativum*) epicotyls. *Plant Science.* **49**: 1-8.

Waldron, K.W., Baydoun, E.A.-H. and Brett, C.T. 1989. The solubilization of a glucuronyltransferase involved in pea (*Pisum sativum* var. Alaska) glucuronoxylan synthesis. *Biochem.J.* **264**: 643-649.

Walker-Simmons, M. and Ryan, C.A. 1986. Proteinase inhibitor I accumulation in tomato cultures. *Plant Physiol.* **80**: 68-71.

Walkinshaw, M.D. and Arnott, S. 1981. Conformations and interactions of pectin. II. Models for junction zones in pectinic acid and calcium pectate gels. *J.Mol.Biol.* **53**: 1075-1086.

Wallace, G. and Fry, S.C. 1994. Phenolic components of the plant cell wall. *Int.Rev.Cytol.* **151**: 229-267.

Wang, Q., Ellis, P.R., Ross-Murphy, S.B. and Burchard, W. 1997. Solution characteristics of the xyloglucan extracted from *Detarium senegalense* Gmelin. *Carbohydrate Polymers.* **33**: 115-124.

Watt, D.K., Brasch, D.J., Larsen, D.S. and Melton, L.D. 1999. Isolation, characterisation, and NMR study of xyloglucan from enzymatically depectinised and non-depectinised apple pommace. *Carbohydrate Polymers*. **39**: 165-180.

Wende, G. and Fry, S.C. 1997a. O-feruloylated, O-acetylated oligosaccharides as side-chains of grass xylans. *Phytochemistry*. **44**: 1011-1018.

Wende, G. and Fry, S.C. 1997b. 2-O-β-D-xylopyranosyl-(5-O-feruloyl)-L-arabinose, a widespread component of grass cell walls. *Phytochemistry*. **44**: 1019-1030.

White, A.R., Xin, Y. and Pezeshk, V. 1993a. Separation of membranes from semiprotoplasts of suspension-cultured sycamore maple (*Acer pseudoplatanus*) cells. *Physiologia Plantarum*. **87**: 31-38.

White, A.R., Xin, Y. and Pezeshk, V. 1993b. Xyloglucan glucosyltransferase in Golgi membranes from *Pisum sativum* (pea). *Biochem.J.* **294**: 231-238.

Whitney, S.E.C., Brigham, J.E., Darke, A.H., Reid, J.S.G. and Gidley, M.J. 1995. *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *Plant J.* **8**: 491-504.

Whitney, S.E.C., Gothard, M.G.E., Mitchell, J.T. and Gidley, M.J. 1999. Roles of cellulose and xyloglucan in determining the mechanical properties of primary plant cell walls. *Plant Physiol.* **121**: 657-663.

Wyatt, S.E. and Carpita, N.C. 1993. The plant cytoskeleton-cell wall continuum. *Trends Cell Biol.* **3**: 413-417.

Xu, W., Purugganan, M.M., Polisensky, D.H., Antosiewicz, D.M., Fry, S.C. and Braam, J. 1995. Arabidopsis *TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *The Plant Cell*. **7**: 1555-1567.

Xu, W., Campbell, P., Vargheese, A. and Braam, J. 1996. The *Arabidopsis XET*-related gene family: environmental and hormonal regulation of expression. *The Plant Journal*. **9**: 879-889.

Yamagaki, T., Mitsuishi, Y. and Nakanishi, H. 1997. Structural analyses of xyloglucan heptasaccharide by the post-source decay fragment method using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Bioscience Biotechnology Biochemistry*. **61**: 1411-1414.

Yamagaki, T., Mitsuishi, Y. and Nakanishi, H. 1998. Structural analysis of xyloglucan oligosaccharides by the post-source decay fragmentation method of MALDI-TOF mass spectrometry: influence of the degree of substitution by

branched galactose, xylose, and fucose on the fragment ion intensities. *Bioscience Biotechnology Biochemistry*. **62**: 2470-2475.

Yamazaki, N., Fry, S.C., Darvill, A.G. and Albersheim, P. 1983. Host-pathogen interactions.XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [^{14}C]-leucine into proteins. *Plant Physiol.* **72**: 864-869.

York, W.S., Darvill, A.G. and Albersheim, P. 1984. Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiol.* **75**: 295-297.

York, W.S., Oates, J.E., Van Halbeek, H., Darvill, A.G. and Albersheim, P. 1988. Location of the O-acetyl substituents on a nonasaccharide repeating unit of sycamore extracellular xyloglucan. *Carbohydrate Research.* **173**: 113-132.

York, W.S., Impallomeni, G., Hisamatsu, M, Albersheim, P. and Darvill, A.G. 1995. Eleven newly characterized xyloglucan oligoglycosyl alditols: the specific effects of sidechain structure and location on ^{1}H-NMR chemical shifts. *Carbohydrate Research.* **267**: 79-104.

Zablackis, E., York, W.S., Pauly, M., Hantus, S., Reiter, W.-D., Chapple, C.C.S., Albersheim, P. and Darvill, A. 1996. Substitution of L-fucose by L-galactose in cell walls of *Arabidopsis mur 1. Science.* **272**: 1808-1810.

Zhang, G.F. and Staehelin, L.A. 1992. Functional compartmentation of the Golgi apparatus of plant cells. *Plant Physiol.* **99**: 1070-1083.

Zurek, D.M. and Clouse, S.D. 1994. Molecular cloning and characterization of brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. *Plant Physiol.* **104**: 161-170.