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Structural Characterization of Arabinan
from *Pinus radiata* Cambium Tissue

Zhenning Du

2000

Errata

Page Position

- 3 Line 1 *Rosa glauca* should read *Rosa glauca*
- 4 Line 15 Pectin and Hemicellulose should read pectin and hemicellulose
- 12 Line 9 *Rosa* should read *Rosa*
- 13 Line 27 *Rosa glauca* should read *Rosa glauca*
- 23 Line 4 (1998) should be placed after Youl *et al*
- 34 Fig. 2.2 The X axis should read galacturonic acid
- 42 Fig. 2.4 Acetaes should read Acetates
- 44 Line 13 Fractins should read Fractions
- 98 Line 14 Albersheim, P. *et al* should read Albersheim, P., An, J., Freshour, G., Fuller, M.S., Guillen, R., Ham, K.S., Hahn, M.G., Huang, J., O'Neill, M., Whitcombe, A. and Williams, M.V.
- Line 22 rhamnogalacteronan should read rhamnogalacturonan
- Line 25 *Multiflorum* should read *multiflorum*
- Line 31 The issue number (No.2) should be deleted
- 99 Line 3 pp.po-2 should read Abstracts (Posters), p. 2.
- 100 Line 3 *Maximum* should read *maximum*
- Line 8 *Althaea officinalis* should read *Althaea officinalis*
- Line 14 *lupinus albus* should read *Lupinus albus*
- 101 Line 13 The issue number (No.10) should be deleted
- 103 Line 6 *Sylvestris* should read *sylvestris*
- Line 20 The title of this paper should read: "An improved procedure for methylation analysis of oligosaccharides and polysaccharides."
- 104 Line 8 The issue number (No.19) should be deleted
- Line 12 *Phytophthora* should read *Phytophthora*
- Line 18 *larix* should read *Larix*
- 105 Line 32 *Napus* should read *napus*
- 106 Line 16 Universidy should read University
- Line 27 monosaccharide should read nonasaccharide
- 108 Line 5 protopectin should read protopectin
- Line 10 *Actinia deliciosa* should read *Actinidia deliciosa*
- Line 17 *Abies balsamea* should read *Abies balsamea*
- Line 21 *Abies balsamea* should read *Abies balsamea*
- Line 31 *Solanum tuberosum* should read *Solanum tuberosum*
- 109 Line 17 *Brassica campestris* should read *Brassica campestris*
- Line 26 This reference is to Fry SC and not Stephen CF
- Line 29 *Brassica olearacea* var *capitata* should read *Brassica oleracea* var *capitata*
- Line 32 *Brassica oleracea* should read *Brassica oleracea*
- 110 Line 2 *Vinca rosea* should read *Vinca rosea*
- Line 25 No. should be deleted
- Line 36 *Aspergillus niger* should read *Aspergillus niger*
-

M. E. Brock

Structural Characterization of Arabinan
from *Pinus radiata* Cambium Tissue

A thesis presented in partial fulfilment of the
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ABSTRACT

Cambium tissue isolated from *Pinus radiata* was frozen, thawed, washed with cold phosphate buffer, then extracted with phosphate buffer by passage through a French pressure cell. The washings and French press extract were dialyzed.

The pectic components partially purified by chromatography on DEAE-cellulose. These polymers bound weakly to DEAE-cellulose at pH 8.0. In both extracts, an early acidic peak of high arabinose and galactose content was separated from a late acidic peak with mostly uronic acid. Within the early acidic peak, successive fractions showed an increase in uronic acid and rhamnose content.

Treatment of the extracts with boiling water caused a decrease in binding of the arabinose-rich polymer to the DEAE cellulose reflected by earlier elution, and a minor decrease in average molecular size, consistent with β -elimination of the pectic backbone.

The acidic fractions were then further fractionated by gel filtration on Superose and Superdex FPLC columns. The earliest of these acidic fractions had ratios of Ara:Gal:Rha = 30:16:1, this suggesting relatively long side chains of about 46 residues, and were mostly excluded by the Superdex-peptide column.

The native molecular mass of the arabinose-rich polymers was determined by size exclusion chromatography and molecular masses of extract A and B were observed to be 40-100 kDa and up to >500 kDa, respectively by Superose and Superdex FPLC columns.

Other characterization studies on the arabinan involved quantitative analysis of the monosaccharides derived by sulfuric acid hydrolysis and quantitation of the sugar linkages using methylation analysis. Methylation analysis suggested a highly branched arabinan structure and the presence of terminal galactosyl residues on a branched arabinan core.

From the results, a tentative structure has been suggested for the arabinose rich polymers. Arabinose was mainly present as 3-, 5-, 3,5-, and terminal linked residues. Galactose was mainly present as terminal residues.

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LIST OF ABBREVIATIONS

$A_{400/450\text{nm}}$	Absorbances at 400 and 450nm
AG	Arabinogalactan
AGPs	Arabinogalactan proteins
Ara	Arabinose
°C	Degrees celsius
ca.	Approximately
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
ETOH	Ethanol
FID	Flame-ionization detector
FPLC	Fast protein liquid chromatography
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography
Glc	Glucose
HCl	Hydrochloric acid
HOAc	Acetic acid
HRGPs	Hydroxyproline-rich glycoproteins
kDa	Kilodaltons
Man	Mannose
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
pH	$-\text{Log}[\text{H}^+]$
RG	Rhamnogalacturonan

Rha	Rhamnose
TFA	Trifluoroacetic acid
TMSI	Trimethylsilyl-imidazole
UV	Ultra violet light

SI units (System International) are not included in the list above.

1. Chapter One: Introduction

1.1. Arabinans in *Pinus radiata* Cambium Tissue, Merit for Study

It is hard to say for certain just how or when *P. radiata* was first introduced into New Zealand. Weston (1957) speculated that miners who traveled the world from one gold rush to another in the early years of the 19th century might have introduced the seed from California, USA, to Australia, and thence to New Zealand. Kininmonth and Whitehouse (1991) suggested that some wealthy settler who wished to create park-like surroundings in his new environment shipped out the first seedlings from England (where radiata pine had been grown since the early 1830s). Whatever the case, a number of importations of both seedlings and seed, mainly from Australia, had ensured that *P. radiata* was well established in New Zealand since 1865 (Weston, 1957).

The Royal Commission on Forestry of 1913 recommended *P. radiata* as the most suitable of the introduced tree species for extensive planting in New Zealand because of its ease of propagation, rapid height growth, high volume production, adaptability to a wide range of sites, and unique wood quality. Since then, there have been two major periods of intensive plantation development in New Zealand. The first time was from the 1920s to early 1930s. The second time was from the early 1960s until the present day. Today it is the most important plantation forestry species in New Zealand and makes a significant contribution to the New Zealand economy. Although it occupies only 4.5% of the land area of New Zealand, it accounted for most of the domestic demand for forest products and provided 13.5% of New Zealand's total export earnings (NZ\$ 2.6 billion) in the year to March 1994 (Walter and Smith, 1995). This figure is predicted to increase to perhaps 30% by the year 2010 (Ministry of Forestry, 1990).

The successful plantation of *P. radiata* in New Zealand is not only a result of a climate which benefits tree growth, but also a result of innovative management, tree improvement practices and a lot of research work including plant biochemistry research. For example, Linder and Rook (1984) state that there are basically two ways of increasing yield from trees:

- Increase the net uptake of carbon dioxide, thus increasing total carbohydrate production; and

- Channel more of these resources into stem growth, either by silviculture or by genetic selection.

In all plants that contain specialized tissues, growth and formation of new cells are localized in regions of cell division known as meristems. Meristems that build up the fundamental structure of a plant are called primary meristems. The growing points of a tree, which gives rise to stem, branches and roots, contain apical meristems, and the vascular cambium and phellogen, which produce radial growth, are lateral meristems (Kininmonth and Whitehouse, 1991).

Iqbal (1995) considered the concept of the "cambium" in the following: "While every vascular plant requires at least a shoot meristem, the cambium (a lateral meristem) is not indispensable. The cambium develops from provascular elements between phloem and xylem of the primary vascular system, forms a continuous sheath around the xylem core of stems and roots, and extends in the form of strips into leaves if they have secondary growth." The cambium acts as a meristematic template for the secondary vascular tissues (xylem and phloem) and by doing so bridges the gap between these tissues. Therefore, the cambium is supposed to play an active physiological role even when in the dormant stage (Riding and Little, 1984, 1986).

The *P. radiata* cambium wall differs from previously studied primary walls in its higher lignin content and in the rather tight binding of much of the pectic material within the wall (Andrew and McKee, unpublished). Tracheid length and wood quality is dependent on the cambium. Therefore the primary wall structure, the polymers, and polymer cross-linking, which may affect growth and elongation, are important in providing a basis for improving wood quality through environmental and hormone regulation. This cannot be fully understood until the polysaccharides of the primary wall and their structures have also been characterized.

A notable feature of the *P. radiata* cambium tissue is its abundant water-soluble arabinan (Andrew and McKee, 1997). Arabinan occurs in various plant materials, notably in seeds, fruits, and roots. Isolation and investigation of arabinan structures from cotyledons of red gram (Swamy and Salimath, 1991), roots of marsh mallow (Capek *et al.*, 1983), roots of horsebean (Joseleau *et al.*, 1983), cabbage (Stevens and Selvendran, 1980), grape juice (Villetaz *et al.*, 1981), apple juice (Churms *et al.*, 1983),

and inner bark of *Rosa glauca* stems (Joseleau *et al.*, 1977) have been described. Arabinans are generally associated with pectins.

In *P. radiata*, wood quality and the resistance of leaves and wood to infection by pathogens are determined by a number of factors, important among which is the nature of the constituent polysaccharides. These polysaccharides include the pectic polysaccharides, which are major constituents of the cell walls of many higher plants and play an important role in both the elongation of plant cells and defense against invading fungal pathogens.

Pectin is believed to control the porosity of walls. It allows smaller molecules to enter the wall and possibly the cell. Disruption of the pectin alters the porosity (Andrew, *pers. comm.*). Pectin is important as a first line of defense against pathogens (fungi, bacteria). Fungi and bacteria have pectin enzymes and can degrade pectin to oligogalacturonides. These oligogalacturonides are "elicitors." They elicit a defense response, which prevents the further invasion of the pathogen (Davis *et al.*, 1986a; 1986b; Davis and Hahlbrock, 1987).

Although we have some understanding of the function of pectin, the role of arabinans in seeds and plant cell walls is unknown. To date, arabinans have not received much attention although they have some interesting physicochemical properties (Cros *et al.*, 1994).

Some preliminary work done in our laboratory suggests that *P. radiata* arabinan differs markedly from most other arabinans studied in that, as well as 1→5-linked residues, it has high levels of 1→3-linked and 1→3,5-branched residues (Little *et al.*, 1980; Fenemor, 1984; Andrew and McKee, 1997; Andrew and Little, 1997). The arabinan is present not only in cell walls, but also as soluble polysaccharides in the cells.

1.2. The Constituents of Cell Walls

The plant cell is surrounded by a cell wall, which serves as protection against injury such as desiccation and osmotic shock (Duffus and Duffus, 1984). There are two general types of plant cell walls: primary and secondary. Primary cell walls are first laid down by young, undifferentiated cells that are still growing. The primary cell walls provide the first barrier to pests; they physically control the rate of cell growth and

form the basic structural backbone of growing plant cells and tissues. These walls can best be described as reinforced multi-component gels (Bacic *et al*, 1988). Secondary cell walls, as distinct from the primary cell walls, are formed by cells that have stopped or are stopping growth and that are differentiating into cells with specialized functions (Darvill *et al*, 1980).

Primary cell walls are composed of cellulose fibrils embedded in relatively large amounts of an amorphous mixture of polysaccharides and glycoproteins. Cellulose is relatively more abundant in secondary cell walls, and these walls often contain significant amounts of lignin. Cells no longer grow once lignin is added to their walls (Albersheim *et al*, 1996). Generally, the polymers of primary cell walls are easier to isolate than those of secondary cell walls (York *et al*, 1985).

The major classical cell wall fractions have been described as follows (Preston, 1974):

- Pectic substances, which are extracted by boiling water and hot ammonium oxalate.
- Hemicelluloses, which are extracted usually by 4N KOH at room temperature.
- Cellulose, the residue after Pectin and Hemicellulose extraction and often extracted with 72% sulfuric acid.
- Protein which is often removed with polysaccharide fractions.
- Lignin which is removed by acid-chlorite treatment.

Cellulose, hemicellulose, pectic polysaccharide, structural protein, and lignin have been identified as the major components of the plant cell wall. Secondary cell walls have greatly increased levels of cellulose together with hemicellulose and lignin.

Albersheim *et al* (1994) reported that primary cell walls are composed of about 20% cellulose microfibrils, 70-80% non-cellulosic polysaccharides, and up to 10% structural glycoproteins. Structural elucidation of primary cell wall polymers is essential to dissecting and understanding their roles in wall structure and other physiological functions.

Because the study of arabinans is related to the pectic polysaccharide and protein, they will be specifically discussed in the following sections. Cellulose and hemicellulose

will be described only briefly, while lignin is a characteristic component of secondarily thickened walls and is therefore not further discussed.

1.2.1. Cellulose

Cellulose is the fibrillar component and forms the basic structure of cell walls in all higher land plants. It usually increases from about 20-30% of the dry weight of the primary cell wall to 40-90% of the secondary cell wall (Stephen, 1988). All primary cell walls depend on cellulose microfibrils for tensile strength. In order for primary cell walls to grow, the cellulose microfibrils must move relative to one another and the microfibrils must be kept from combining to form large, intractable aggregates of the type present in secondary cell walls (Albersheim *et al.*, 1996).

Chemically, cellulose is a simple linear polymer of β -(1 \rightarrow 4)-linked glucopyranosyl residues. Each glucose residue is rotated at 180° relative to its nearest neighbor giving a repeat unit structure of cellobiose, and glucan chains exist as extended ribbons with a 2-fold screw axis stabilized by intramolecular hydrogen bonding. (Bacic *et al.*, 1988). The width, degree of polymerization, and crystallinity of the cellulose microfibrils are highly variable and depend on the source and age of the tissue. The microfibrils are highly crystalline and make up an important part of the framework of the cell walls of all higher plants (Talmadge *et al.*, 1973). The degree of polymerization of the primary wall cellulose is relatively low and heterogeneous (2,000-6,000) however that of the secondary wall cellulose is higher and more homogeneous, about 13,000-16,000 (Cote, 1977).

1.2.2. Hemicelluloses

Hemicelluloses are defined as alkali-soluble cell wall polysaccharides, so named because of their close association with cellulose and because they were once thought to be cellulose precursors. Hemicelluloses bind tightly, via multiple hydrogen bonds, to the surface of cellulose microfibrils. It is believed that the hydrogen bonding to the cellulose microfibrils by the cellulose-like backbone of a hemicellulose chain is eventually sterically interrupted by carbohydrate side chains of the hemicellulose (Levy *et al.*, 1991). For the most part, hemicelluloses are composed of D-glucose, D-mannose, D-xylose, L-arabinose residues joined together in different combinations and in various

glycosidic linkages to give the hemicellulose polysaccharides (Ericson and Elbein, 1980). The nature of the hemicelluloses isolated from different tissues varies considerably. Hemicelluloses of the secondary cell walls have been divided into two broad groups: the glucomannans and xylans, whereas the xyloglucan, arabinoxylan and mixed linked glucans are components of all primary cell walls, although the relative amounts of these hemicelluloses varies from plant to plant (Albersheim *et al.*, 1996, Fenemor, 1982).

Variations on basic structure render it valuable to discuss particular hemicellulose types individually.

a. Glucomannans and Galactoglucomannans. These polysaccharides occur in significantly larger proportions in the lignified secondary cell walls of coniferous gymnosperms (12-15%) than angiosperms (3-5%) (Bacic *et al.*, 1988). They consist of chains of randomly arranged D-glucose and D-mannose residues linked β -(1 \rightarrow 4) in the main chain.

The glucomannans are the main non-cellulosic polysaccharides present in cell wall of mature softwood and they tend to be insoluble in water and if resistant to weak alkaline extraction they can be extracted with alkali-borate solution (Northcote, 1972), and subsequently fractionated with Ba(OH)₂. These polysaccharides are structurally similar to cellulose and seem to be closely associated with the cellulose molecules in the cell wall. For gymnosperms, the glucose to mannose ratio is approximately 1:3, whereas in angiosperms the ratio is approximately 1:2 (Bacic, *et al.*, 1988).

The water-soluble galactoglucomannans are isolated in low yields from softwoods. The galactoglucomannans are a homogeneous polymer and usually have a galactose: glucose: mannose ratio of 1:1:3 (Timell, 1965). The structure is the same as the alkali-soluble glucomannans, the main difference being the increase in terminal side chain galactose residues, which may account for the water-soluble properties of the polymer.

b. Xylans and Arabinoxylans. The xylans are abundant noncellulosic polysaccharides in the majority of angiosperms where they account for 20-30% of the dry weight of woody tissues (Aspinall, 1980). They are mainly secondary wall

components but they are also found in the primary walls of some plants. In gymnosperms, where galactoglucomannans and glucomannans form the major hemicelluloses, xylans are less abundant (8%) and more difficult to isolate in pure form (Timell, 1965). The general structure of xylans is that of an essentially linear main chain of D-xylopyranose residues joined by β -(1 \rightarrow 4) links. The xylans have a degree of polymerization of 150-200. Attached to these chains are L-arabinofuranose, D-glucuronic acid, or its 4-methylether and D-galactose. L-Arabinosyl units are generally furanosyl, (1 \rightarrow 3)-linked to the xylan, while uronic acid residues may be linked through (1 \rightarrow 2) or (1 \rightarrow 3) links to the main chain. The (1 \rightarrow 2) linkage is the usual form (Timell, 1965). Xylans in hardwoods generally lack arabinose, but instead commonly bear acetyl groups at C-3 of the xylose residues. The modes of attachment of single unit (4-O-methyl-) D-glucuronic acid and L-arabinofuranose residues are well established for many xylans, but the lengths and exact structures of the more extended sidechains remain to be established with precision (Timell, 1964; 1965).

An acidic arabinoxylan is a major hemicellulose present in monocot seedling primary cell walls (Darvill *et al.*, 1978; Buchala, 1974; Wada and Ray, 1978). These arabinoxylans always contain a linear (1 \rightarrow 4)-linked xylan backbone. Similar arabinose-rich arabinoxylans have been isolated from barley aleurone cell walls (McNeil *et al.*, 1975) and found in several species of cultured monocot cells (Burke *et al.*, 1974). Arabinoxylans have been detected in the extracellular polysaccharides of sycamore, sugar cane, wheat, tobacco and periwinkle (Keegstra *et al.*, 1973; Burke *et al.*, 1974; Akiyama and Kato, 1982; Takeuchi and Komamine, 1980b), and an arabinoxylan has been isolated in a highly pure form from tobacco extracellular polysaccharides (Akiyama *et al.*, 1984). This xylan (4% arabinosyl and 89.5% xylosyl residues) was purified first by anion exchange and then by cellulose chromatography.

- c. **Mixed-Link Glucan.** Glucans consisting of a mixture of β -(1 \rightarrow 3)-linked and β -(1 \rightarrow 4)-linked glucosyl residues have been observed in primary cell walls of several plants. These mixed-link β -glucans have been found in nearly all of the cell wall preparations obtained from monocots, and are thought to be important structural matrix components (Darvill *et al.*, 1980; Fenemor, 1982). The mixed-link

glucans comprise a family of polymers, which are heterogeneous with respect to molecular size and fine structure, varying with tissue, age, and source. They generally contain approximately 30% (1→3)- and 70% (1→4)-β-D-glucosidic linkages with more than 90% of the molecule consisting of (1→3)-β-D-linked celotriosyl and cellotetraosyl residues (Woodward *et al.*, 1983a,b; Kato and Nevins, 1984a,b). Individual (1→3)- and (1→4)-linked residues, blocks of more than three contiguous (1→4)-linked residues, and regions of contiguous (1→3)-linked residues may also be present.

Woodward *et al.* (1983a) reported that the 40°C water-soluble (1→3, 1→4)-β-D-glucan from barley endosperm walls contains runs of between 4 and 14 contiguous (1→4)-linked but no contiguous (1→3)-residues. In contrast, water-soluble and water-insoluble (1→3, 1→4)-β-D-glucans of *Zea mays* shoot walls contain regions of two, three, or four contiguous (1→3)-linked residues as well as blocks of more than three contiguous (1→4)-linked residues (Kato and Nevins, 1984a,b).

Fincher and Stone (1986) showed that the molecular weight estimates for (1→3, 1→4)-β-D-glucans range from 20,000-1,000,000. In previous reports (Igarashi and Sakurai, 1965; Woodward *et al.*, 1983b), however, where determined by sedimentation equilibrium ultracentrifugation, they are in the range of 200,000-300,000 corresponding to the degree of polymerization of 1200-1850. The (1→3, 1→4)-β-D-glucans are asymmetrical molecules assuming an extended conformation in aqueous solution.

- d. Xyloglucans.** Xyloglucans are major hemicelluloses of dicot primary cell walls and perhaps the most thoroughly understood of the noncellulosic polysaccharides (Bauer *et al.*, 1973). Many of these polysaccharides have been detected by the formation of a characteristic blue stain on reaction with iodine. The resulting use of the term amyloid is misleading in implying a nonexistent structural relationship to amylose (Aspinall, 1980).

Xyloglucan is the predominant hemicellulose in the primary wall of *P. radiata* as well as of dicots. It is believed to cross-link cellulose microfibrils and to establish a strong 3-dimensional network (Fry, 1989). The actual evidence for this model is currently being hotly debated (Melton *et al.*, 1997). Xyloglucan is envisaged as the

load-bearing component of the wall, and extension growth involves wall loosening which has been suggested to be due to a turnover of xyloglucan. Xyloglucan turnover is one of the early events associated with auxin-stimulated growth.

Xyloglucan resembles cellulose but the chains are much shorter and they are substituted. Turnover is catalyzed by several enzymes and involves changes in molecular weight. One of the breakdown products of xyloglucan is a nonamer called XXFG, a potent oligosaccharin, or bioactive oligosaccharide. It is an auxin antagonist at nanomolar concentrations, and may have a regulatory role during growth. (McDougall and Fry, 1991).

The structure of the xyloglucans has been determined by a combination of methylation analysis and chromatographic separation of the oligosaccharides produced by partial enzymatic digests (Darvill *et al.*, 1980). Members of the xyloglucan family have a linear extended backbone of (1→4)-linked β -D-glucopyranosyl residues substituted at C(O) 6 with α -D-xylopyranosyl residues. However, some heterogeneity in their fine structure exists. The heterogeneity resides in the substituents on the xylopyranosyl residues and includes β -D-galactopyranosyl-(1→2)-, α -L-arabinofuranosyl-(1→2)- and α -L-fucopyranosyl-(1→2)- β -D-galactopyranosyl-(1→2)-side chains (Darvill *et al.*, 1980; McNeil *et al.*, 1984).

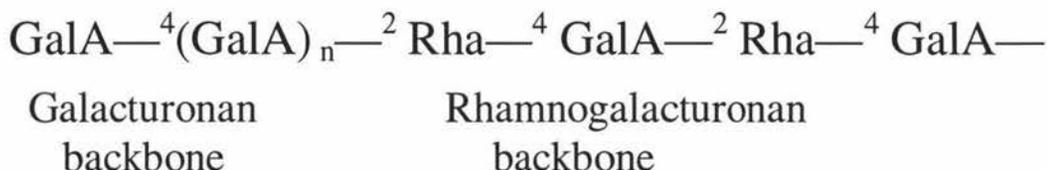
The molecular weight of xyloglucans from different plants and even within the same plant varies widely from 7,600 for the heteroglucan from sycamore suspension culture medium to 180,000 for the heteroglucan from walls of suspension cultured soybean cells (Bauer *et al.*, 1973; Hayashi *et al.*, 1981). Xyloglucans may interact with other wall components either by noncovalent (hydrogen bonding to cellulose) or possibly covalent interactions (with pectic polysaccharides). In previous reports, it was demonstrated that xyloglucan was also the major hemicellulose of the *P. radiata* hypocotyl cell walls. From the results, a tentative structure has been suggested for the xyloglucan, which showed that this component was a fucogalactoxyloglucan of similar structure to those reported from dicots (Andrew and Little, 1997).

1.3. Pectic Substances

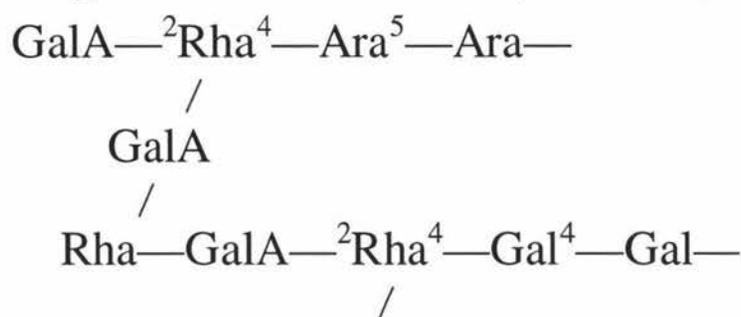
Pectic substances are a group of closely associated polysaccharides from the primary cell walls and intercellular regions of higher plant (Voragen *et al.*, 1995). They are deposited mainly in the early stages of growth when the area of the wall is increasing. The functions of pectin are diverse and include ill-defined roles in cell expansion, cell adhesion, cell wall porosity, and plant development and defense (Carpita and Gibeaut, 1993; Jarvis, 1984). The pectic polysaccharides are probably the most complex class of wall polysaccharides (Bacic, 1988) and comprise a family of acidic polymers like homogalacturonans, rhamnogalacturonans with several neutral polymers like arabinans, galactans and arabinogalactans attached to it. They are usually extracted from walls with water or aqueous solutions of chelating agents such as ethylenediaminetetraacetic acid (EDTA) or ammonium oxalate, and purified preparations of polysaccharide hydrolases have also been used for extraction (Bacic, 1988; Voragen *et al.*, 1995; O'Neill *et al.*, 1990).

The pectic polysaccharides are built on a pectin backbone, i.e. a polymer of galacturonic acid, which may be methylesterified or bound to calcium. Rhamnose residues occur in the chain, usually in blocks, so we get galacturonan blocks and rhamnogalacturonan blocks. Pectic polysaccharides may form a 3-D network interwoven with the cellulose-xyloglucan network. The structural pectic polysaccharides are summarized in the Figure 1.1.

From studies on pectins from many sources, it has become clear that pectin is not a homopolysaccharide (Voragen *et al.*, 1995). Pectins are esterified galacturonans or more commonly rhamnogalacturonans in which the α -(1 \rightarrow 4)-linked D-galacturonan chains are interrupted at intervals by the insertion of α -L-rhamnopyranose residues that carry arabinan or galactan side chains. Other constituent sugars are attached in sidechains and include D-galactose, D-xylose, and, less frequently, L-fucose, D-glucuronic acid, the rather rare sugars, 2-O-methyl-D-xylose, 2-O-methyl-L-fucose, and the branched chain sugar D-apiose. Most of these sugars occur in short sidechains, but the more common neutral constituents, D-galactose and L-arabinose, are also found in multiple units. At the same time, account must be taken of the fact that arabinans, and galactans or arabinogalactans may be isolated from pectin-rich sources (Aspinall, 1980).

**Galacturonan backbone:**

Homogalacturonan	modified by: Acetyl substituents Methyl-esterification
Xylogalacturonan	(Xyl on 3-)
Apiogalacturonan	(Api on 2-)
Rhamnogalacturonan II	(complex)

Rhamnogalacturonan backbone (GalA-Rha)_n:**Rhamnogalacturonan I (on Rha 3-):**

Arabinan	(mostly 5-linked)
Galactan	(mostly 4-linked)
Arabinogalactan	(Type I: Gal 4-linked) (Type II: Gal 3,6-linked)

Figure 1.1 Pectic Polysaccharides

The arabinans, whether occurring as side chains to pectins or as polymers associated with pectin, mostly consist of (1→5)-linked α -L-arabinofuranosyl residues.

Individual classes of the pectic substances will be discussed below.

1.3.1. Homogalacturonans

Homogalacturonans are defined as polymers consisting predominantly of α -(1→4)-linked galacturonosyl residues (McNeil *et al.*, 1984). They have been isolated from various plant tissues such as sunflower heads and seeds (Zitko and Bishop, 1965; 1966), sisal (Aspinall and Rodrigues, 1958), rice endosperm cell walls (Shibuya and Nakane, 1984), suspension cultured primary cell walls from *Rosa* (Chambat and Joseleau, 1980) and sycamore (McNeil *et al.*, 1984), and from apple pectin (Barrett and Northcote, 1965).

Homogalacturonans are usually extracted from plant material by mild acid treatment (Zitko and Bishop, 1966; Thibault *et al.*, 1993). Depending on the extraction method used, some modification of the polymer may occur. Uninterrupted homogalacturonan regions with a degree of polymerisation of approximately 70-100 have been isolated from various plant tissues like carrot (Konno, 1986), apple, beet and citrus (Zitko and Bishop, 1966).

An important feature of galacturonans is the esterification of the galacturonic acid residues with methanol and /or acetic acid (Schols and Voragen, 1996). Many (~70%) of the carboxyl groups of the galactosyluronic acid residues of primary cell wall homogalacturonan are methyl-esterified. The degree of methyl-esterification is variable (Voragen *et al.*, 1995; Morris, 1986; Williamson *et al.*, 1990). It is possible that the degree of esterification of homogalacturonans decreases in proportion to the amount of time the homogalacturonan is resident in the wall. The variously esterified forms of homogalacturonan appear to be concentrated in specific regions of primary cell walls (McCann *et al.*, 1994). For example, the middle lamella appears to contain sparsely methyl-esterified homogalacturonan (Moore *et al.*, 1986; Geitmann *et al.*, 1995). Homogalacturonans that are less than ~50 % methyl-esterified readily form gels, especially in the presence of calcium, which is present in primary cell walls. The fewer the methyl ester groups and the more the distribution of methyl esters is in blocks, the

greater the propensity of the homogalacturonan chains to form gels (Morris, 1986; Williamson *et al.*, 1990). Homogalacturonans are also modified by acetyl substituents. Acetyl groups are usually only present in low amounts in pectins from e.g. apple and citrus, but are present in much higher amounts in pectins from sugar beet (Rombouts and Thibault, 1986) and potato (Voragen *et al.*, 1986a).

1.3.2. Rhamnogalacturonan

a. Rhamnogalacturonan I. Rhamnogalacturonan I (RG-I) has a backbone composed of as many as 100 repeats of the disaccharide [\rightarrow 2)- α -L-rhamnosyl- (1 \rightarrow 4)- α -D-galactosyluronic acid-(1 \rightarrow] (McNeil *et al.*, 1980; Lau *et al.*, 1985). Arabinosyl- and galactosyl-rich side chains are attached to O-4 of the rhamnosyl residues, although the proportion of rhamnosyl residues with attached side chains varies from ~20% to ~80% depending on the source of the polysaccharide. By specific degradation of the uronic acids with a lithium treatment (Mort and Bauer, 1982; Lau *et al.*, 1988a), the presence of at least 30 different side chains has been indicated. Some side chains consisted of at least 15 glycosyl residues (McNeil *et al.*, 1980; 1982; Lau *et al.*, 1988b). Approximately one half of the (1 \rightarrow 2)-linked-L-rhamnosyl residues are branched at O-4 with side chains averaging about seven glycosyl residues in length composed of D-galactosyl and L-arabinosyl units. Small amounts of L-fucosyl groups have been found linked to L-rhamnosyl units (McNeil *et al.*, 1980). Occasionally, the side chains are terminated by glucuronosyl, or 4-O-methyl glucuronosyl residues and xylosyl residues always account for about 1% of RG-I, but their locations in the molecule have not been ascertained (An *et al.*, 1994). The xylosyl residues may be attached to the galactosyluronic acid residues of the backbone. On the basis of their sugar and glycosyl residues, it appears that pectin fragments similar to RG-I are present in the cell walls of many higher plants. For example, suspension-cultured rice and corn (Thomas *et al.*, 1989), Douglas fir (Thomas *et al.*, 1987), *Rosa glauca* (Chambat *et al.*, 1984), carrots (Konno *et al.*, 1986), kiwi fruit (Redgwell *et al.*, 1988), tomato tissue (Pressey and Himmelsback, 1984), rice endosperm (Shibuya and Nakane, 1984), apple tissue and pectins (Voragen *et al.*, 1986b; Renard, *et al.*, 1991; De Vries *et al.*, 1982), and sugar beet pulp and pectins (Raynal *et al.*, 1991; De Vries *et al.*, 1982), although the nature and quantity of the side chains vary. Much remains to be learned about the

structures and distribution of the side chains of this large family of polysaccharides (Albersheim *et al.*, 1996).

- b. Rhamnogalacturonan II.** Rhamnogalacturonan II (RG-II) is a quantitatively minor component of plant cell walls and has an extremely complex structure (Voragen, 1995). RG-II has a backbone of galacturonic acid, with side chains containing rhamnose and a number of other sugars.

RG-II is a low molecular weight (~4.8 kDa) complex polysaccharide, which has 11 different sugars in more than 20 different linkages (O'Neill *et al.*, 1990). The structure of RG-II is highly conserved, as apparently identical RG-II molecules have been obtained following *endopolygalacturonase* treatment of the primary cell walls of rice, onion, Douglas fir, sycamore, grape, and apple, and strong evidence of the presence of this polysaccharide has been obtained in other cell walls (Albersheim *et al.*, 1996).

RG-II is separated from the other pectic polysaccharides solubilized by the *endopolygalacturonase* by anion exchange and gel permeation chromatography. RG-II has a 'homogalacturonan' backbone composed of about nine (1→4) linked α -D-galactosyluronic acid residues (Whitcombe *et al.*, 1995). The number of residues in the backbone may depend on the particular glycosidic bond of homogalacturonan that the *endopolygalacturonase* cleaves when solubilizing RG-II. It is believed that the homogalacturonan is covalently linked to the O-1 and /or O-4 of the terminal reducing and non-reducing galactosyluronic acid residues of the galacturonan backbone of RG-II.

The elucidation of the primary structures of RG-I and RG-II has been summarized by O'Neill *et al.* (1990). RG-II was found to consist of approximately 30 glycosyl residues, among them the rare sugars mentioned earlier. It contains a high proportion of rhamnosyl residues, which occur (1→3) and (1→2,3,4) linked and as terminal units, in contrast to rhamnogalacturonan I where they are (1→2) and (1→2,4) linked (Voragen, 1995).

There are four different complex side chains, which are attached to O-2 or O-3 of four of the backbone residues and two of these chains start with apiose. These side chains sterically prevent *endopolygalacturonase* from cleaving the backbone, which

explains why intact RG-II is released from cell walls by *endopolygalacturonase*. Indeed, RG-II is highly resistant to glycanase digestion (Darvill *et al.*, 1980). Ishii *et al.* (1999) reported that RG-II forms a borate ester crosslinked dimer, which involves apiose of each monomer. RG-II binds heavy metals, which can then increase rate of dimer formation with borate. In the cell wall, RG-II is bound to homogalacturonan, and probably constrained to favor dimer formation. In RG-II, boron links two RG-II molecules via a borate diester bond. Dimeric RG-II is stable at low pH. The widespread occurrence and conserved structure of RG-II have led to the suggestion that borate ester cross-linked RG-II is required for the development of a normal cell wall. These cells have a high specific-growth rate, no significant lag phase, and reproducible changes in their wall pore size during the transition from the growth phase to the stationary phase (Fleischer, *et al.*, 1998). RG-I and RG-II both contain rhamnosyl and galactosyluronic acid residues, but these polysaccharides are not structurally related.

1.3.3. Galactan

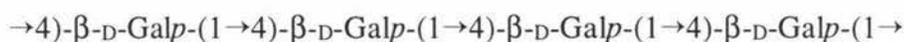
Galactans have been isolated from citrus pectin (Labavitch *et al.*, 1976), white willow (Toman *et al.*, 1972), and beech (Meier, 1962). The pectic galactans are primarily β -(1 \rightarrow 4)-linked polymers. The 4-linkage has been established by methylation analysis. The galactosidic linkages were shown to be in the β -anomeric configuration by the fact that these linkages are susceptible to hydrolysis by an endo-1-4- β -galactanase and by their low positive optical rotation (Labavitch *et al.*, 1976). Furthermore, oligosaccharides produced from the intact galactan by partial acid hydrolysis (Toman *et al.*, 1972) are susceptible to further hydrolysis by a β -galactosidase. Finally, the β -configuration of some of the galactosidic linkages of oligosaccharides derived from a galactan by partial acid hydrolysis (Meier, 1962) has been established by chromatographic comparison to known standards.

No homogalactan has been isolated directly from a primary cell wall, although analysis of the primary walls of suspension-cultured sycamore cells (Talmadge *et al.*, 1973) has indicated the probable presence of β -(1 \rightarrow 4)-linked D-galactan chains in neutral pectic side chains. Those galactans that have been studied have degrees of polymerization ranging from 33 (Toman *et al.*, 1972) to 50 (McNeil and Albersheim, 1975). These

values were obtained by vapor pressure osmosis and by comparing the ratio of terminal to internal sugars as obtained by methylation analysis.

β -(1 \rightarrow 4)-linked D-galactans, devoid of arabinose residues but containing 5-10% of uronic acid (glucuronic and /or galacturonic acid) residues, are found in compression wood of gymnosperms, for example, red spruce (Schreuder *et al.*, 1966) and tamarack (Jiang and Timell, 1972). In these polysaccharides, it is likely that the uronic acid residues are end groups.

The β -(1 \rightarrow 4)-linked D-galactan from citrus pectin is a commercial product (Evans and Linker, 1973), and similarly constituted polysaccharides have been extracted from seeds, leaves, bark, tubers and, in crude form, from delignified compression wood. The structure is shown in the Figure 1.2.



**Figure 1.2 Structure of Galactan. Galp, Galactopyranose
(From Stephen, 1983)**

Darvill *et al.* (1980) showed that galactans have been obtained which contain 6-linked galactosyl residues in addition to 4-linked residues. In two of the cases studied, the 6-linked residues accounted for approximately 4% of the polymer and are, therefore, quantitatively minor components of the polysaccharides. On the other hand, beech galactan is a polysaccharide with a major content of 6-linked galactosyl residues although the amount of the polysaccharide accounted for by the 6-linked residues had not been known well. The fact that 6-linked and 4-linked galactosyl residues are present in a single polymer has been established by the isolation of the trisaccharide: Gal (β 1 \rightarrow 6)-Gal (β 1 \rightarrow 4)-Gal.

Type II arabinogalactan is a highly branched polysaccharide with ramified chains of β -D-galactopyranose residues joined by (1 \rightarrow 3) and (1 \rightarrow 6) linkages. The (1 \rightarrow 3) linkages predominate in the interior chains, and the (1 \rightarrow 6) linkages occur mainly in the exterior chains, which are generally terminated by L-arabinofuranosyl and to some extent by L-arabinopyranosyl residues (Aspinall, 1980; McNeil *et al.*, 1984; Clarke *et al.*, 1979). Pectins with type II arabinogalactans covalently attached have been found, for example, in apple (Aspinall and Fanous, 1984; De Vries *et al.*, 1983; Selvendran, 1985), rapeseed (Siddiqui and Wood, 1977; Larm *et al.*, 1976), lemon (Aspinall and Cottrell, 1970), beet (Guillon and Thibault, 1989), and grape (Saulnier *et al.*, 1988). The type II arabinogalactans and arabinogalactan-proteins are water-soluble and are therefore likely to be extracted together with the pectic type I arabinogalactans. Type II arabinogalactans and arabinogalactan-proteins are thought not to be wall components but constituents of the extracellular space and possibly associated with the plasma membrane (Fincher *et al.*, 1983).

1.3.5. Arabinan

Arabinan polysaccharides have been isolated from the cell walls of many plant materials (Eriksson *et al.*, 1996), such as apples (Voragen *et al.*, 1987; Schols. *et al.*, 1990), sugar beet (Guillon and Thibault, 1989; Guillon *et al.*, 1989), suspension-cultured sycamore cell walls (Talmadge *et al.*, 1973), rapeseed hulls (Siddiqui and Wood, 1977), apricots (Siliha, 1985), carrots (Massiot *et al.*, 1988), cabbage (Stevens and Selvendran, 1984), onions (Ishii, 1982; Redgwell and Selvendran, 1986), pears (Bobsky and Schobinger, 1986), and pea hulls (Ralet *et al.*, 1993). However, most of these arabinans have contained small proportions of other glycosyl residues of uncertain significance and pure arabinans have been obtained only from mustard seeds (Rees and Richardson, 1966), the inner bark of *Rosa glauca* (Joseleau *et al.*, 1977), and a methylated primary cell wall polysaccharide fraction of suspension-cultured sycamore cells (Darvill *et al.*, 1980). The degree of polymerization of two arabinans from *Rosa Glauca* is 34 and 100 (Joseleau *et al.*, 1977). Methylation analysis of the primary walls of suspension-cultured pea cells suggested the presence of a similar polymer.

Arabinans are branched polysaccharides with a backbone of α -(1 \rightarrow 5)-linked L-arabinofuranosyl residues, other α -L-arabinofuranosyl units being attached to varying numbers of residues at the O-2 and /or O-3 position (Voragen *et al.*, 1995). These may be generally represented by the partial structure shown in Figure 1.4. Several arabinans contain linkages additional to those shown in Figure 1.4, notably sidechains attached by α -(1 \rightarrow 2)- L-arabinofuranosyl linkages to branch points in the backbone, which may or may not also, carry (1 \rightarrow 3)-linked units. An alternative mode of attachment has been proposed by Stevens and Selvendran (1984), for the neutral arabinan from cabbage (*Brassica oleracea*) pectic polysaccharide fractions. From methylation studies, they postulate that the arabinan is attached to the acidic rhamnogalacturonan through the C (O) 4 of the (1 \rightarrow 2)-linked rhamnopyranosyl residues via one to three (1 \rightarrow 4)-linked β -D-galactopyranosyl residues. The structural investigation of these isolates has mainly involved methylation analysis and ^{13}C NMR spectroscopy.

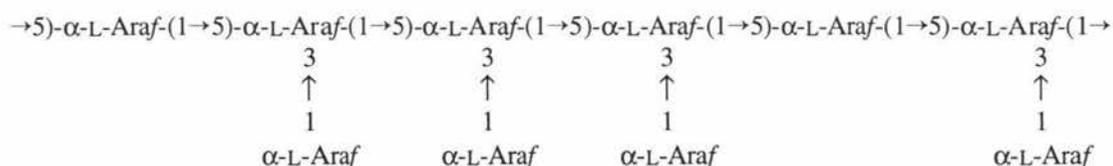


Figure 1.4 General Structure of Higher Plant Arabinans.

Araf, Arabinofuranose.

(From Brinson and Dey, 1985)

Arabinans are regarded as forming part of the neutral pectic fraction of the cell wall, and they have been isolated under strongly basic conditions, e.g., extraction with hot limewater. The so-called sugar beet arabinan is such a polysaccharide preparation (Hough and Powell, 1960; Hullar, 1965), but it is probably a degradation product in which other constituent sugars including galacturonic acid originate from the residual stubs of the rhamnogalacturonan chains of a pectin. Further evidence for the heteropolysaccharide nature of the sugar beet preparation was obtained by the isolation

of *O*- α -L-arabinofuranosyl-(1 \rightarrow 3)-*O*- β -D-galactopyranosyl-(1 \rightarrow 1)-glycerol from a Smith periodate degradation (Hullar, 1965).

The role of arabinans in seeds and plant cell walls is not known, but may be related to cohesion and bonding (Aspinall, 1973; Albersheim *et al.*, 1973; Kikuchi *et al.*, 1996). The linear arabinans can produce a spreadable gel that is similar to gelling maltodextrins (Kasapis *et al.*, 1992; Cros *et al.* 1994). Their rheological properties and resistance to human digestive enzymes suggest the possible use of α -(1 \rightarrow 5)-arabinans as a fat substitute and they have been employed in ice cream and in chilled or frozen desserts (Cooper *et al.*, 1992). These polymers have also been used in the pharmaceutical and cosmetics industries (Cros *et al.*, 1994).

1.4. Protein

Proteins are quantitatively important components of the cell wall matrix. Primary walls may contain up to 10% protein (Bacic *et al.*, 1988). Some of them are only loosely bound and may be located in the periplasmic space (inner wall surface), whereas others are more tightly bound but can be eluted by agents such as 1 M NaCl, 3 M LiCl or 0.5 M CaCl₂ (Bacic, *et al.*, 1988). This suggests that they may be held in the wall by ionic forces. Others are not removed by these treatments and are often referred to as covalently bound, although nonextractability is not proof of covalent linkage. It is sometimes difficult to decide whether protein in wall preparations is true wall protein or adventitious cytoplasmic protein deposited during wall isolation (Harris, 1983).

There are several types of structural proteins in walls, but among these the family of hydroxyproline-rich glycoproteins (HRGPs, extensins) are the best-characterized class. Arabinogalactan-proteins (AGPs) are found in most higher plants and in many of their secretions (Fincher and Stone, 1983). A possible structure of a cell wall glycoprotein segment is given in Figure 1.5, taken from Clarke *et al.* (1979).

1.4.1. Arabinogalactan-protein

Arabinogalactan-proteins are a group of macromolecules characterized by a high proportion of carbohydrate in which galactose and arabinose are the predominant monosaccharides; there is also a low proportion of protein, typically containing high

levels of hydroxyproline (Fincher and Stone, 1983). In some situations, they are isolated as polysaccharides free from associated protein; in other situations, they occur in covalent association with protein, either refers to this group as proteoglycans, in which the protein component carries polysaccharide substituents (Gottschalk, 1972; Reid and Clamp, 1978) or as glycoproteins, in which the protein component is substituted by one or more oligosaccharide residues (Marshall, 1972).

Arabinogalactan-proteins containing arabinose and galactose have been isolated from aqueous extracts of plant material by the classical methods of salt and solvent precipitation, and by ion exchange chromatography (Churms, 1970) and affinity chromatography (Andrew and Stone, 1983), and precipitation by complexing with the β -glucosyl (Yariv) antigen, a specific (and diagnostic) method of recovering arabinogalactan-proteins from tissue extracts (Clarke *et al.*, 1979). Jermyn and Yeow (1975) isolated high MW polymers containing carbohydrate and protein, in the ratio approximately 8:1. They have been isolated from seeds representing all taxonomic groups of higher plants (Jermyn and Yeow, 1975), as well as from other plant tissues (Clarke *et al.*, 1978) and filtrates of cultured callus cells (Anderson *et al.*, 1977). In each case, the protein was remarkable for its high hydroxyproline content and the major monosaccharides were arabinose and galactose.

Various means are available for obtaining the protein and carbohydrate components of the proteoglycans free from each other. However, each of these procedures has limitations as concurrent reactions leading to alteration or elimination of the other component may occur. The protein portion may be removed enzymically, by alkaline degradation or by hydrazinolysis (Clarke *et al.*, 1979). Peptide bonds are cleaved during hydrazinolysis while the carbohydrate chains are resistant to such treatment. However, glycosyl-serine linkages are also vulnerable and serine is lost through β -elimination.

Arabinogalactan proteins found in plant tissues are typified by the ryegrass (*Lolium multiflorum*) arabinogalactan-protein, which is found both in the cells and the media of suspension cultured *Lolium* endosperm (Anderson *et al.*, 1977). The *Lolium multiflorum* arabinogalactan-protein has a MW of 200,000-300,000 and contains approximately 95% carbohydrate associated with 5% protein. The carbohydrate component of the glycoprotein is an arabinogalactan. The structure of the

the plasma membrane, they may be receptors of external signals such as the low molecular weight β -glucan elicitors of the phytoalexin response (Albersheim and Valent, 1978). Recently, attention has been focused on their structure and their potential role in growth and development of plants. Youl *et al* have presented evidence that two members of a major class of arabinogalactan-proteins, from styles of *Nicotiana glauca* and cell suspension cultures of *Pyrus communis* respectively, undergo C-terminal processing involving glycosylphosphatidylinositol membrane anchors. An examination of the deduced amino acid sequences of other classical arabinogalactan-protein backbones shows that glycosylphosphatidylinositol-anchors may be a common feature of this class of arabinogalactan-proteins (Youl *et al*, 1998).

1.4.2. Hydroxyproline-rich Glycoprotein

Hydroxyproline-rich glycoproteins are important structural proteins in cell walls. They are characteristically found in dicotyledon primary walls (Roberts *et al.*, 1985; Wilson and Fry, 1986). Extensin levels increase in response to physiological stress and attack by pathogens. The name 'extensin' was adopted to convey a suggested involvement of the glycoprotein in the control of elongation growth. In these polymers, arabinose oligosaccharides are glycosidically linked to hydroxyproline and the galactose residues to serine as shown in Figure.1.6. The primary cell walls of dicots contain between 5 and 10% protein (Lamport, 1970; Preston, 1964; Talmadge *et al.*, 1973). The cell wall protein is exceptionally rich in hydroxyproline (20%). The wall protein also has a relatively high content of alanine, serine, and threonine. Fragments of the hydroxyproline-rich glycoprotein obtained from the primary cell walls of dicots invariably contain arabinosyl and galactosyl residues.

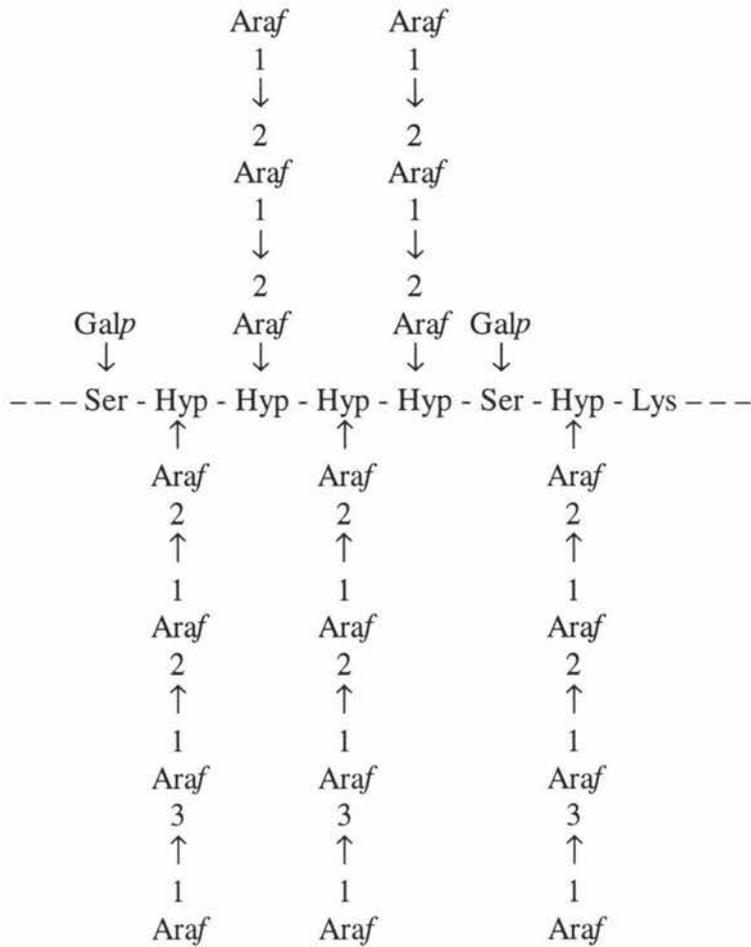


Figure 1.6 Possible Structure of Cell Wall Glycoprotein Segment

(From Clarke *et al.*, 1979)

In the glycoproteins of higher plant cell walls, carbohydrate is linked to protein through Ara-*O*-Hyp (Lamport, 1977) and Gal-*O*-Ser (Cho and Chrispeels, 1976; Lamport *et al.*, 1973). The carbohydrate consists of oligomers of three or four arabinofuranosyl residues. Hydroxyproline is usually glycosylated, although unsubstituted hydroxyproline residues occur in cell wall glycoproteins from some monocots (Lamport and Miller, 1971). The degree of glycosylation changes during development (Klis and Eeltink, 1979).

The hydroxyproline-rich glycoprotein fragments used for these studies have generally been isolated from the walls of suspension-cultured sycamore (*Acer pseudoplatanus*) and tomato (*Lycopersicon esculentum*) cells. A series of hydroxyproline-arabinosides have been isolated from such wall preparations (Darvill *et al.*, 1980). Extensins occur as extended rodlike molecules coated on the surface with covalently attached sugar residues. Chen and Varner (1985a, b) obtained cDNA and genomic clones for a wound-induced extensin from carrot and thus the full sequence of the 306-amino acid protein (molecular weight 34,000). There are no Gln, Asn, Asp, Trp, or Cys residues in the protein but it contains numerous repeat sequences, the most abundant of which is the pentapeptide (Ser-Hyp-Hyp-Hyp-Hyp).

The covalent attachment of arabinose and galactose to the hydroxyproline-rich glycoprotein of primary cell walls is a generally accepted fact (Lampport, 1970; Lampport *et al.*, 1973). However, the available evidence suggests that the hydroxyproline-rich glycoprotein is not covalently attached to any of the other cell wall polymers. The evidence does not rule out the possible existence of strong, noncovalent bonding between the hydroxyproline-rich glycoprotein and the other wall polymers. The hydroxyproline-rich glycoprotein is secreted by suspension-cultured sycamore cells into their culture medium (Keegstra *et al.*, 1973).

A composite model of a portion of the hydroxyproline-rich structural glycoprotein of dicot primary cell walls is depicted in Figure 1.6.

Hydroxyproline-rich glycoprotein has also been isolated from suspension-cultured monocot cells. The total amount of protein in the walls of suspension-cultured monocots and in the walls of maize coleoptiles is equal to or larger than the total amount of protein in the walls of suspension-cultured dicots (Burke *et al.*, 1974).

Several of the hydroxyproline-rich glycoproteins extracted from plant tissues have carbohydrate-binding activity; these glycoproteins have the characteristic of lectins. These lectins or lectin-like glycoproteins have components, which are similar to those of the cell wall hydroxyproline-rich glycoproteins (Darvill *et al.*, 1980).

1.5. Aims of the Thesis

Arabinans in plants are usually associated with pectins, as side chains on rhamnose residues, but their function is unknown. The arabinan is present not only in cell walls, but also as a soluble polysaccharide in the cells. Its abundance in the early stages of cell wall development is somewhat an enigma. Our laboratory has studied this area for a number of years. As a result of early studies, some preliminary structural information on the arabinans of *P. radiata* has been obtained. A highly branched arabinan has been found as part of the pectic fraction from *P. radiata* callus tissue (Andrew *et al.*, 1999), and preliminary work by Andrew and McKee (1997) has suggested the same type of compound to be present in vascular cambium of the same species.

The aim of this project is to isolate and purify an arabinan or arabinose-rich fraction from the cambium of *P. radiata* and to do some detailed analysis of its structure. This area would greatly facilitate understanding of the function of arabinans in plant cells and cell walls.

The arabinan from *P. radiata* cambium tissues was studied for the following reasons:

- a. *P. radiata* is economically important to New Zealand both as a building material and to the pulp and paper industry and make a significant contribution to the export earnings. Any further knowledge gained on the polysaccharide composition of tissues in pine is advantageous because these polysaccharides relate to the growth and development of plants.
- b. The arabinan content of *P. radiata* is relatively high and the presence of 3-linked and 3,5-branch residues is distinctive, but almost nothing is known of its function in any plants.
- c. There is a lot of soluble pectin in cambium tissue and it is easy to isolate. Several neutral and acidic fractions have been identified in preliminary work. The neutral fractions are relatively enriched in arabinan. The acidic fractions are more typical of rhamnogalacturonan with arabinogalactan side-chains.
- d. It should enable a comparison with results on other types of *P. radiata* tissues such as callus tissue.

- e. The knowledge from this project will be useful to the further understanding of function of arabinans in plant cells and cell walls.

2. Chapter Two: Materials and Methods

2.1. Isolation of Arabinan from *P. radiata* Cambium Tissue

2.1.1. Chemicals

Where possible, analytical grade chemicals and reagents supplied by either BDH or Sigma were used in this part of the study. Water used was purified by ion exchange (de-ionized water). All solutions were stored at 4°C or room temperature unless otherwise stated. The pH was measured using a digital pH meter (Model 3032 pH Meter).

2.1.2. Extraction

Materials

- Cambium tissue, scraped from under the bark of a mature tree of *P. radiata* and supplied by the Forest Research Institute, was stored frozen until required, with occasional thawing and re-freezing for other purposes.
- Potassium phosphate buffer. A: 0.2 M solution of monobasic potassium phosphate (27.22 g. of KH_2PO_4 in 1000 ml.). B: 0.2 M Solution of dibasic potassium phosphate (34.84 g. of K_2HPO_4 in 1000 ml.). This B phosphate solution was used to adjust 0.2 M KH_2PO_4 to pH 7.0.
- Sodium azide. (NaN_3)

Details of Procedures

Cambium tissue (about 25-50 g.) was thawed in 0.1 M potassium phosphate buffer, pH 7.0, temperature 0-4°C, then was washed with about 5 vol. (v/w, i.e. 125 ml per 25 g tissue) of cold phosphate buffer, pH 7.0; this was decanted or centrifuged to remove washings and stored as (A); this was repeated with a further 5 vol. of phosphate buffer; and then 1 vol. of buffer, and both the washings were combined with the initial batch (A).

The washed tissue was passed through a French pressure cell at 2000-3000 psi to shear the cells; passing the cell homogenate directly into 4 vol. (v/w) of phosphate buffer.

The homogenate was examined under a microscope to ensure that cell disruption was complete and re-run through the French pressure cell if necessary.

The homogenate was centrifuged at 2000 g for 10 min, in a Sorvall refrigerated centrifuge (3.2 K in RTH 750 bucket rotor); the supernatant was stored as (B). The pellet was washed with 5 vol. (v/w) phosphate buffer and centrifuged at 1000 g. for 5 min. The supernatant was added to the initial batch B.

About 10 ml of each of washing (A) and extract (B) was set aside for assays, as below.

The remainder of the samples A and B were dialyzed separately against 20 vol. cold distilled water, overnight at 4°C. About 100 ml was used in the dialysis bag, and then the water was about 2 litre.

An aliquot of the dialysate (10 ml) was stored for subsequent assays.

The dialysis was repeated against a further 20 vol. cold distilled water. This dialysis should give a 400x dilution of any monosaccharides present, as these passed out of the bag into the wash solution (they were not retained in the bag with the polysaccharides).

2.2. Estimation of Main Components

2.2.1. Determination of Total Carbohydrate

Materials

- Aqueous phenol (5% solution).
- Rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and galacturonic acid standards.
- Concentrated sulfuric acid.

Principle and Procedure

The amount of total carbohydrate was determined by the phenol-sulfuric method (Dubois *et al.*, 1956) modified by Takeuchi and Komamine (1980a) with glucose as standard.

Duplicate 0.4 ml samples were used, containing up to 50 μg of carbohydrate, in wide (19 mm) glass tubes, along with blank (0.4 ml water) and standards (glucose, to 50 μg in 0.4 ml). To each tube, 0.4 ml 5% aqueous phenol (Analytical grade) was added and 2.4 ml concentrated sulfuric acid (Analytical grade) was added rapidly using a Zipette dispenser, then the tubes were spun on a vortex mixer and allowed to cool for 10 minutes to room temperature. Standards contained between 10 and 50 μg of glucose from stock solutions, which could be stored frozen over a period of several months. The absorbance was read at 490 nm in a Hitachi U-1100 spectrophotometer. All solutions including blanks were read against distilled water and blank values subtracted from sample readings.

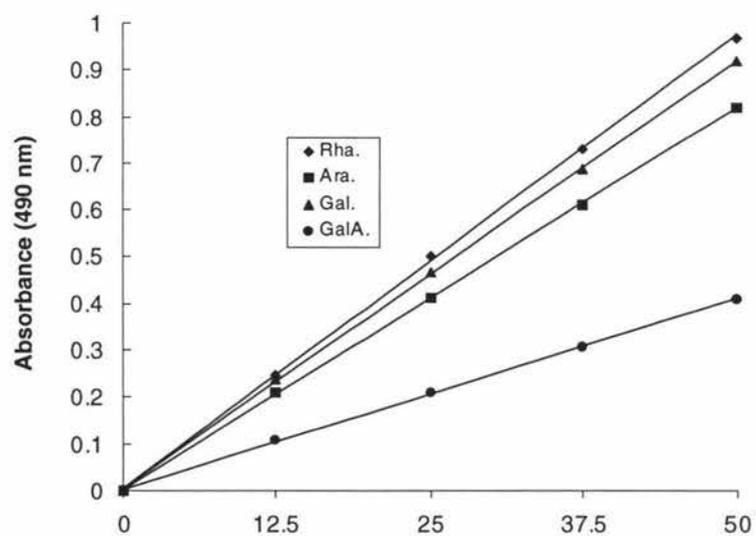
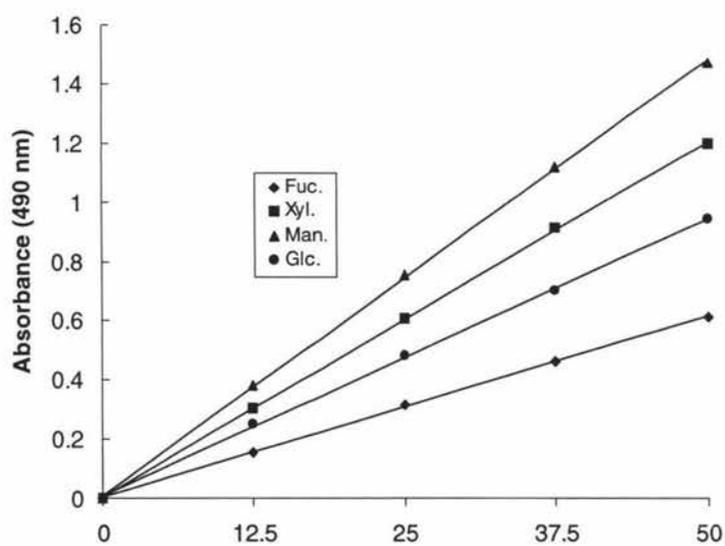
Rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, and galacturonic acid standards (10-100 μg) all gave linear standard curves (Figure 2.1a,b). The absorbances found for 50 μg of each of these standard sugars are shown in Table 2.1.

Table 2.1 Values of Standard Monosaccharides

Standard 50 μg	Rha.	Fuc.	Ara.	Xyl.	Man.	Gal.	Glc.	Gal A*
Absorbance (490 nm)	0.97	0.61	0.82	1.20	1.47	0.92	0.94	0.41

* All uronic acid found by spectrophotometric analysis was assumed to be galacturonic acid (see text).

Figure 2.1 Phenol-Sulfuric Colorimetric Standard Curves

Sugars (µg)
(A)Sugars (µg)
(B)

Correction was applied to spectrophotometric assay for total sugar. In the spectrophotometric assay, each sugar has a different absorbance at the wavelength read. The standard curves obtained for sugars are shown in Figure 2.1. The Anthrone assay (Hodge and Hofreiter, 1962) was also reported to give different absorbances for different sugars at a particular wavelength. This limits the usefulness of these assays in determining accurately the amount of total sugar present in a hydrolysate or fraction. It was therefore necessary to determine a "theoretical standard absorbance" dependent on the composition of the sample. The sugar composition was based on the neutral sugar analysis by gas chromatography, summed with total uronic acid (Section 2.2.2) and each sugar was expressed as a percentage of the total thus determined. The "theoretical absorbance" of e.g. 50 μg polysaccharide was then determined by multiplying the absorbance of 50 μg of each pure sugar by its percentage composition value. The sum of absorbances for each sugar present gave the expected "theoretical absorbance" for 50 μg of sample carbohydrate. By using this value for a standard absorbance, with the actual absorbances obtained in the assay for aliquots of fractions, a closer agreement was obtained between colourimetry and results for the sugar content of a fraction based on combined gas chromatography and uronic acid results, than if glucose only was used as the standard in the spectrophotometric assay.

2.2.2. Determination of Uronic Acid

Materials

- Sodium chloride-boric acid solution. Dissolve 2 g of NaCl and 3 g of boric acid (H_3BO_3) in 100 ml of water.
- Dimethylphenol solution. Dissolve 0.1g of 3,5-dimethylphenol [$(\text{CH}_3)_2\text{C}_6\text{H}_3\text{OH}$] in 100 ml of glacial acetic acid as the colorimetric reagent.
- Galacturonic acid.
- Concentrated sulfuric acid.

Apparatus

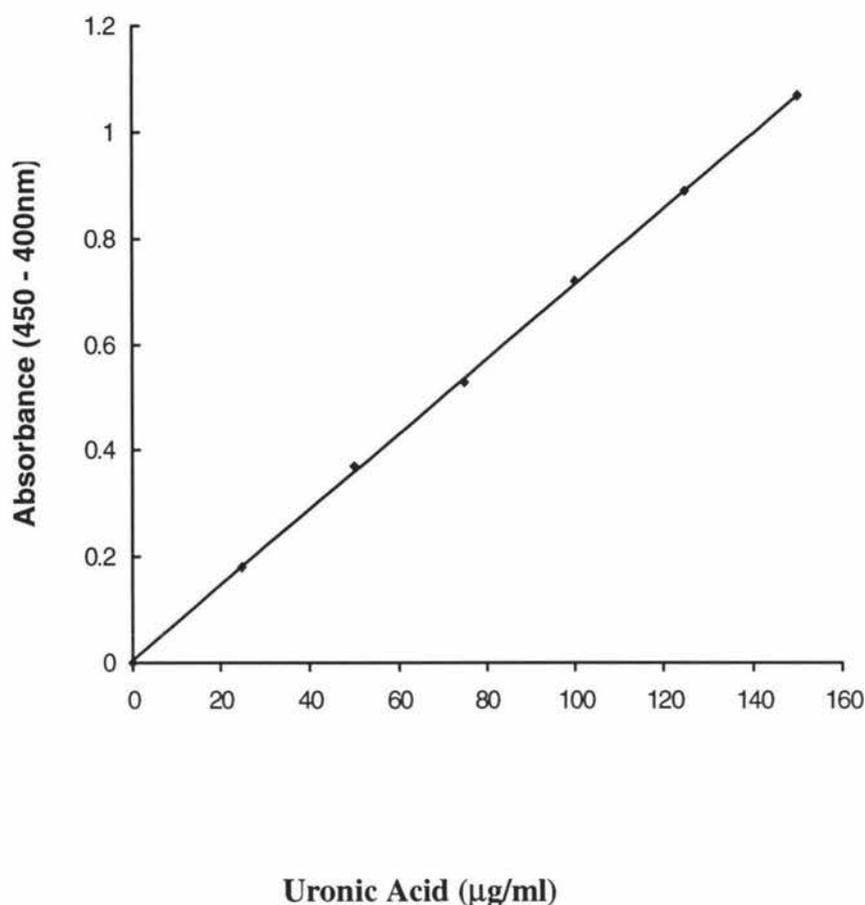
Samples were reacted with H₂SO₄ in 15x150 mm glass tubes held in a water bath. All reagents were dispensed to stock bottles. Absorbances were measured with a double-beam spectrophotometer (Hitachi U-1100) using 3-cm cells.

Procedure

Uronic acid was estimated spectrophotometrically using galacturonic acid as standard. The method used was a modification of the method of Scott (1979) by Englyst *et al.* (1984, 1994).

The standard solutions were made as follows. 50 mg of galacturonic acid (dried to constant mass under reduced pressure) was weighed and placed in a 100 ml calibrated flask. This was diluted to volume with 1 M sulfuric acid to give a 500 µg/ml galacturonic acid solution. To prepare the uronic acid standard solution, the above sugar solution was placed in separate tubes and diluted to 10 ml with 1 M sulfuric acid to give standard of 25, 50, 100 and 150 µg/ml of galacturonic acid. Only the 100 µg/ml standard was required for routine analysis, and it could be kept at 5°C for several weeks. Results were duplicated and standard curves are shown in Figure 2.2.

0.15 ml of blank solution (1M sulfuric acid), 0.15 ml of each of the standard solutions, and 0.15 ml of the samples were placed into separate tubes (20-30 ml capacity). The samples were diluted as necessary to contain no more than 150 µg/ml of uronic acids. To each tube, 0.15 ml of sodium chloride solution-boric acid solution was added and the solution mixed. 2.4 ml concentrated sulfuric acid (Analytical grade) was then added rapidly using a Zipette dispenser and the tube vortex mixed immediately. The tubes were placed in a water bath at 70°C and left for 40 min. Then the tubes were removed and cooled to room temperature in water. To each tube, 0.1 ml of dimethylphenol solution was added and the tube vortex mixed immediately. After exactly 15 min, the absorbance at 400 and 450 nm were measured in the spectrophotometer against the blank solution. The timing for measurement of the absorbance of standards and samples should be identical. In practice, this was achieved by adding the chromogenic reagent at 1 min intervals. The readings at 400 nm and at 450 nm were subtracted to correct for interference from hexoses.

Figure 2.2 Standard Curve for Uronic Acid**Calculation of Uronic Acid**

The amount of uronic acids (expressed as grams of polysaccharide per 100 g of sample) was calculated as:

$$\text{Amount} = (AtVtDC \times 100 \times 0.91) / AsMt$$

Where At is the difference in absorbance of the sample solution, Vt is the total volume of sample solution (in ml), D is the dilution of the sample solution, C is the concentration of the standard (here 100 µg/ml), As is the difference in absorbance of the 100 µg/ml standard, Mt is the mass (in mg) of the sample and 0.91 is the factor for converting experimentally determined values for monosaccharides to polysaccharides.

2.2.3. Determination of Protein

Principle

Most proteins exhibit a distinct ultraviolet light absorption maximum at 280 nm, due primarily to the presence of tyrosine and tryptophan (Layne, 1957). Since the tyrosine and tryptophan content of various enzymes varies only within reasonably narrow limits, the absorption peak at 280 nm can be used as a rapid and a fairly sensitive measure of protein concentration. Unfortunately, nucleic acid, which is apt to be present in enzyme preparations, has a strong ultraviolet absorption band at 280 nm. Nucleic acid, however, absorbs much more strongly at 260 nm than at 280 nm, whereas with protein the reverse is true. Layne has taken advantage of this fact to eliminate, by calculation, the interference of nucleic acid in the estimation of protein.

Procedure

The absorbance of an appropriately diluted protein solution is obtained at both 280 nm and 260 nm.

Calculation

According to this method, the following equation was used (Layne, 1957):

$$\text{Protein concentration (mg/ml)} = 1.55 D_{280} - 0.76 D_{260}$$

Where D_{280} and D_{260} are absorbance at 280 and 260 nm, respectively.

2.2.4. Determination of Pentoses

Materials

- Cysteine hydrochloride (the cysteine hydrochloride is freshly prepared as a 3% aqueous solution). Dissolve 30 mg of Cysteine in 1 ml of water.
- Arabinose (100 $\mu\text{g/ml}$).

- Concentrated sulfuric acid (analytical grade). It has been found that unknown impurities in the concentrated H₂SO₄ increase the breakdown of sugars, especially hexoses. It is therefore advisable to use a very pure acid, which is low in nitrogen and arsenic.

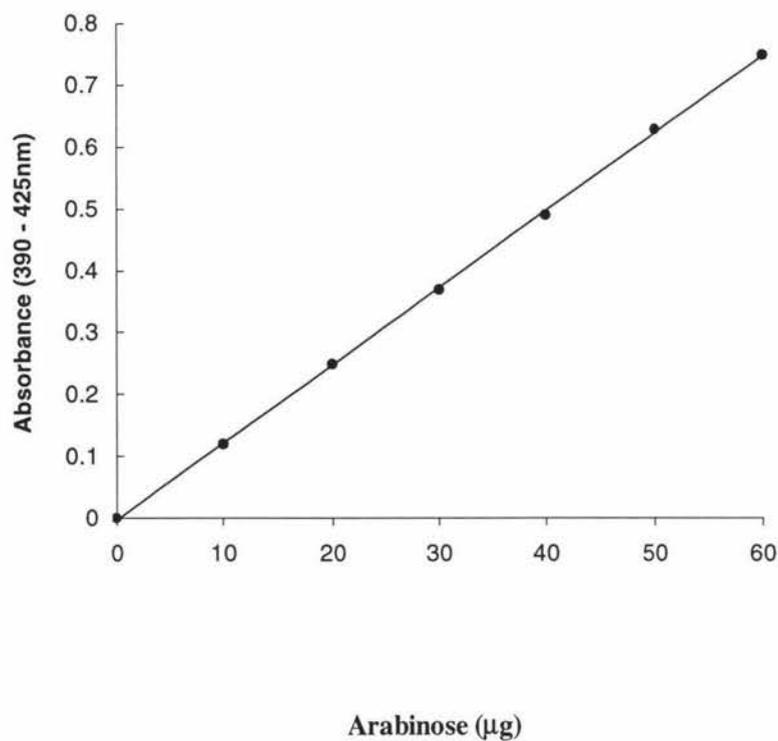
Principle

There are several alternate procedures, which may be of value under special circumstances for the determination of pentoses. The cysteine-H₂SO₄ reaction has been used for determination of pentoses. Cysteine reacts with various classes of sugars to form reaction products of characteristic absorption and stability. The so-called basic cysteine reaction described here represents the application of this principle to the quantitative determination of small amounts of pentose in the presence of other classes of sugars (Ashwell, 1957).

Procedure

To 0.6 ml of a solution containing 10-60 µg of pentose (arabinose) was added, with cooling in tap water, 2.4 ml of concentrated sulfuric acid using a Zipette dispenser (2x1.2 ml). The mixture was shaken vigorously and replaced in a cold water bath. Care was taken to treat all samples in an identical manner. When the reaction mixture was cooled to room temperature, the decomposition of the sugar ceased, and the reaction product remained unchanged for hours. In order to avoid excessive formation of air bubbles, it was desirable to allow the sample to sit at room temperature for 2 hours with frequent shaking before adding 0.06 ml of the cysteine solution. The samples were read exactly 15 minutes after addition of the cysteine. Maximum absorption was at 390nm. In the presence of hexose, pentoses were determined quantitatively by $\Delta D_{390-425}$.

$\Delta D_{390-425}$ is a quantitative measure of pentoses in the solution and is proportional in the range of 10-100 µg. Since various pentoses have different values of $\Delta D_{390-425}$, it is not possible by this reaction alone to determine their total amount in solutions containing more than one of these sugars. However, it could be used to advantage for screening of fractions prior to gas chromatography analysis.

Figure 2.3 Standard Curve for Pentoses**Table 2.2 Absorbance of Reaction of Cysteine-sulfuric Acid with Various Sugars**

Sugar	(µg)	15 min		30 min		60 min	
		390nm	425nm	390nm	425nm	390nm	425nm
Ara	60	0.744	0.027	0.892	0.030	0.979	0.036
GalA	10	0.248	0.054	0.275	0.052	0.286	0.057
GalA	60	0.987	0.237	1.074	0.246	1.116	0.264
Gal	10	0.102	0.106	0.106	0.111	0.110	0.121
Gal	60	0.771	0.781	0.809	0.831	0.857	0.900
Glc	10	0.130	0.139	0.138	0.147	0.148	0.159
Glc	60	0.757	0.787	0.795	0.840	0.842	0.939

2.3. Monosaccharide Analysis of Carbohydrate Fractions

The most convenient way of analyzing the polysaccharide composition of fractions from the plant cell wall is by hydrolysis of the glycosidic linkages to produce the component monosaccharides, which can then be analyzed by gas chromatographic techniques (Albersheim, *et al.*, 1967; Anderson and Stone, 1978; Englyst, *et al.*, 1992,1994). Hydrolysis has been used to study the sugars present in the native polysaccharide of fractions, and to cleave methylated polysaccharides to analyze the linkage of components. Partial acid hydrolysis of polysaccharides to monosaccharides and oligosaccharides often affords further information on linkages existing in the polysaccharides. However, in this thesis, the primary interest is the complete hydrolysis to monosaccharides of a fraction, and their derivatization and analysis.

2.3.1. Reagents and Apparatus

High-purity reagents and distilled, de-ionized water were used throughout the method.

- Gas chromatography was performed with both GC-8A Shimadzu (column A: SP2340) and Hewlett Packard 5890 series II (column B: Econowax; column C: BPX70), fitted with a flame-ionization detector (FID).
- Internal standard solution. 2 mg/ml. Weigh 50 mg of allose (dried to constant mass under reduced pressure using vacuum oven) to the nearest 0.1 mg. Make up to 100 ml with 50% saturated benzoic acid to give a 2 mg/ml solution. The mixture is stable at 4°C for several months.
- Stock sugar mixture. Accurately weigh, to the nearest 0.01 mg, 10 mg of rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose and galacturonic acid respectively into a calibrated flask and make to the mark with 50% saturated benzoic acid.
- Ammonia solution, 12 M. This reagent must be kept in a well-stoppered bottle and should be replaced when more than 1.2 ml is required to neutralize 3 ml of hydrolysate.

- Ammonia solution-sodium borohydride solution. Accurately weigh 0.5 mg of sodium borohydride (NaBH₄) into a 5 ml calibrated flask, add 1.25 ml 12 M ammonia solution, make to the mark, and mix thoroughly. Prepare immediately before use.
- Bromophenol blue solution, 0.4 g/L (BDH).
- Potassium hydroxide solution, 7.5 M. Dissolve 42 g of potassium hydroxide in 100 ml of water.

2.3.2. Acid Hydrolysis of Polysaccharides

The bulk of the hydrolyses in this thesis have been performed with 2 M H₂SO₄ at 100°C for 2 hours. Take 0.5 ml sample into capped tubes, and then add 0.5 ml 2 M sulfuric acid and mix. Place the tubes in a boiling water-bath and leave for 2 hours, timed from when boiling recommences. Remove the tubes and cool them to room temperature by placing the tubes in tap water. The hydrolysates are neutralized with 0.2 ml 12 M ammonia solution and the pH checked using pH paper (pH>10).

2.3.3. Preparation of Alditol Acetates

The monosaccharides released by acid hydrolysis of the polysaccharide were analyzed by gas chromatography as their volatile alditol acetate derivatives. The method used was based on that of Albersheim *et al.* (1967) and modified by Englyst *et al.* (1992, 1994).

The alditol acetate derivatives were prepared for chromatography by following two methods. Method one: Add 0.1 ml of internal standard (2 mg/ml allose) to 0.2 ml of the cooled hydrolysates in the 125 mm test tube; vortex mix. Add 0.1 ml of the ammonia solution-sodium borohydride solution and vortex mix. Leave the tubes in a water-bath at 40°C for 40 min, then remove, add 0.1 ml of glacial acetic acid, and mix again. After 3 min, add 0.1 ml of 1-methylimidazole to each. Add 3 ml of acetic anhydride and vortex mix immediately. Leave the tubes for 10 min for the reaction to proceed (the reaction is exothermic and the tubes will become hot). Add 1 ml of absolute ethanol, Vortex mix and leave for 5 min. Add 2 ml of water, vortex mix and leave for 5 min. Add 0.25 ml of Bromophenol Blue solution. Place the tubes in ice-water and add 3 ml

of 7.5 M potassium hydroxide solution; a few minutes later add a further 3 ml of 7.5 M potassium hydroxide solution, cap the tubes and mix by inversion (must be well stoppered: glad-wrap or screw cap). Leave until the separation into two phases is complete (10-15 min) or centrifuge for a few minutes. If top layer is inadequate, add extra ethyl acetate (0.5 ml) and mix again. Transfer the top (colorless) layer cleanly to an Eppendorf tube (no water should be transferred).

Method two: To 0.2 ml of hydrolysate add 0.1 ml of internal standard (2 mg/ml allose) and vortex mix. Add 0.1 ml of the ammonia solution-sodium borohydride solution and vortex mix again. Leave the tubes in a water-bath at 40°C for 40 min, then remove, add 0.1 ml of glacial acetic acid, and mix again. After 3 min, add 0.1 ml of 1-methylimidazole to each. Add 3 ml of acetic anhydride and vortex mix immediately. Leave the tubes for 10 min at 20°C (room temperature). Add 2 ml of water, vortex mix, and when cooled add 1 ml of dichloromethane, agitate the contents vigorously on a vortex mixer, and centrifuge for a few minutes to separate the mixture into two phases. Remove the bulk of the upper phase by aspiration and discard it, then transfer the lower phase to an Eppendorf tube, seal and store it at -20°C.

2.3.4. Gas Chromatography of Alditol Acetates

Gas chromatography was routinely performed using column A (SP2340). The following conditions are used: Injector temperature, 240°C; column temperature, 190°C; detector temperature, 240°C; carrier gas, nitrogen 750KPa; hydrogen 50KPa; air 200KPa. A good separation was achieved, under these conditions, of all the monosaccharides encountered except rhamnose and fucose.

Column A (SP2340); 3% SP2340 on 100/120 Supelcoport, ID: 3 mm.

Column B (Econowax); Phase: EC-WAX, Length: 30 meters, ID: 0.32 mm, Film Thickness: 0.25 µm.

Column C (BPX70); 70% Cyanopropyl (equiv.) Polysilphenylene-siloxane, Length: 30 meters, ID: 0.25 mm, Film Thickness: 0.25 µm.

Authentic alditol acetates of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose were prepared and used to identify the peaks of the alditol acetates of the

carbohydrate samples in the gas chromatography. The method used was based on a comparison of the retention time of the unknown component with that obtained from a known compound analyzed under identical conditions (Table 2.3, Figure 2.4).

Table 2.3 Relative Retention Times of Alditol Acetates

Sugar	Relative Retention Time		
	SP2340	Econowax	BPX70
Rhamnose	0.54	0.52	0.41
Fucose	0.56		0.43
Arabinose	0.73	0.64	0.60
Xylose	0.88	0.69	0.79
Allose	1.00	1.00	1.00
Mannose	1.06	1.18	1.07
Galactose	1.13	1.30	1.16
Glucose	1.20	1.26	1.29
	*190-240°C	*150-210°C	*150-210°C

* Column operating temperatures

2.3.5. TMS sugars

This method was designed to assist in the routine determination of sugars in the extracts. It was modified after Brittain and Schewe(1971).

Reagents and Apparatus

- Gas chromatography was performed with GC-8A Shimadzu, fitted with a flame-ionization detector (FID).
- OV17 column: 2% OV17 on Gas Chrom Q 80/100 Mesh.
- Tri-Sil[®]Z Reagent [a mixture of trimethylsilyl-imidazole (TMSI) in dry pyridine].

Procedure

0.5 ml sugar solution was evaporated to a glassy-syrup in an open screw-cap vial. 0.1ml Tri-Sil[®]Z Reagent (TMS-Imidazole in pyridine) was added then heated at 60°C until the sugar was dissolved completely. 1 µl sample was injected into the OV17 column. GC was performed with a temperature program: 170-290°C.

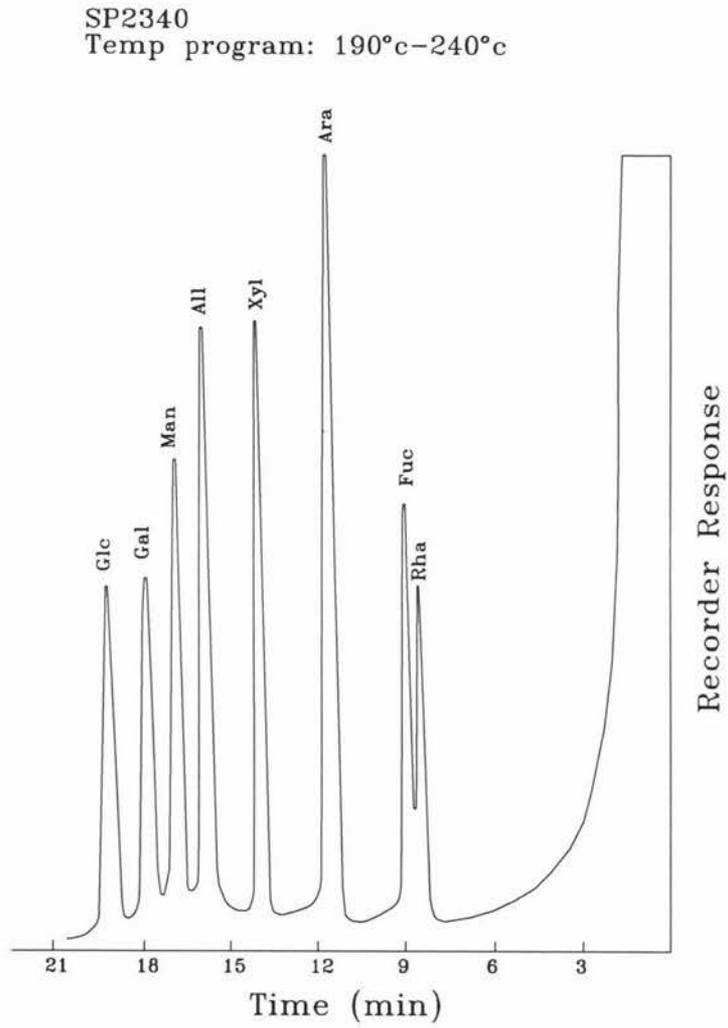


Figure 2.4 The Separation by Gas Chromatography of Monosaccharides as Alditol Acetaes

2.4. Purification of Arabinan

2.4.1. DEAE-Cellulose Chromatography

The separation in ion exchange chromatography is obtained by reversible adsorption. Most ion exchange experiments are performed in two main stages. The first stage is sample application and adsorption. Unbound substances can be washed out from the exchanger bed using a column volume of starting buffer. In the second stage, substances are eluted from the column, separated from each other. DEAE-cellulose is ideal for exchange of polysaccharides.

Materials and Apparatus

- DEAE-Cellulose, (Diethylaminoethyl cellulose). DE-32. Whatman.
- The Automated Econo Chromatograph System. BIO-RAD.

Procedure

The column was pre-equilibrated with 0.01 M ammonium bicarbonate and then eluted with the same solution, followed by gradient elution with ammonium bicarbonate from 0.01 to 0.5 M, then from 0.5 to 2.0 M. Fractions were collected by an automatic fraction collector. The protein was monitored by measuring the absorbance at 280 nm using the Econo UV Monitor.

2.4.2. Fast Protein Liquid Chromatography (FPLC)

Gel filtration or gel permeation or size exclusion chromatography separates different biomolecules based on their stoke radius, which depends on the native mass, and overall shape. The larger molecules are excluded from the pores and are eluted first from the column; the medium molecules have access to a portion of the pores and move down the column at a rate depending on their size. The smallest molecules are able to penetrate the pores and therefore are the last to be eluted from the column.

Materials and Apparatus

- Advanced Protein Purification System (FPLC), Waters 650 E. MILLIPORE.

- Superose 6 HR 10/30 column. Pharmacia.
- Buffer composition: 50 mM NaOAc, 10 mM NaCl, pH 5.0 (FPLC grade).
- Superdex-peptide column. Pharmacia.
- Eluent composition: Pyridine: Acetic Acid: H₂O = 1:1:98 (FPLC grade).
- Microtitre plate (Nunc, A/S, Roskilde, Denmark).

Procedure

Both the above gel filtration columns were used for operation with the fast protein liquid chromatography (FPLC) system. Samples from the DEAE-cellulose column were freeze-dried to remove NH₄HCO₃ and redissolved in about 1.0 ml water. Aliquots of 0.5 ml were applied to the FPLC columns. The column flow rate was 0.5 ml/min. Fractions (0.5 ml) were automatically collected in tubes, and samples (50 µl) taken from the eluted fractions were assayed for total carbohydrate (section 2.2.1).

2.5. Methylation Analysis of Polysaccharide Fractins

Methylation analysis is one of the most important methods for determining the type of glycosidic linkages in polysaccharides. Samples of an appropriate fraction were methylated using a method modified from that described by Harris *et al.*, (1984) and Ciucanu and Kerek (1984).

Materials and Apparatus

- Gas chromatography was performed with both GC-8A Shimadzu and Hewlett Packard 5890 series II, fitted with a flame-ionization detector (FID).
 - (SP2340); 3% SP2340 on 100/120 Supelcoport, ID: 3 mm.
 - (OV225); 3% OV225 on 100/120 Gas Chrome Q, ID: 3 mm.
- (BPX70); 70% Cyanopropyl (equiv.) Polysilphenylene-siloxane, Length: 30 meters, ID: 0.25 mm, Film Thickness: 0.25 µm.

Procedure

A solution of the sample (about 1 mg) was dried down in a Teflon-lined screw capped glass tube. Three pellets of sodium hydroxide were placed in a dry mortar and 1.8 ml DMSO was added in an atmosphere of nitrogen. The pestle was used to grind the sodium hydroxide until a slurry was formed with the DMSO. 0.25 ml of the DMSO/NaOH slurry was transferred to each vial with a Pasteur pipette. The solution was kept at room temperature in a nitrogen atmosphere and stirred for 10 minutes. Methyl iodide (0.2 ml) was added dropwise with cooling. The reaction mixture was mixed vigorously. The reaction was quenched by careful, dropwise addition of about 1 ml of water. 2 ml chloroform was added, mixed thoroughly, and the mixture allowed settling into two layers. The upper aqueous layer was removed and discarded. The lower chloroform layer was washed several times with water until the water being removed was completely clear, then the chloroform layer was dried down under a stream of nitrogen.

2 ml trifluoroacetic acid (0.5 ml) was added to the methylated sample and it was then autoclaved for 1 hour at 121°C. After cooling, the sample tube was placed in a water bath (40°C), and evaporated to dryness with a stream of nitrogen.

0.1 ml freshly prepared solution of 10% sodium borohydride in 2 M ammonia was added to the dry hydrolysate. It was then reduced in a water bath for 60 minutes at 40°C. The residue was dissolved in 0.1 ml acetic acid and left 3 minutes, then 0.1 ml methylimidazole was added and the resulting mixture was acetylated with 1 ml acetic anhydride for 10 minutes. Water was added to the reaction mixture which, after removal of solvent, was taken up in 1 ml chloroform for analysis by gas chromatography.

Gas chromatography was performed on columns. Identifications were made by comparison with standards (Figure 2.5-2.7).

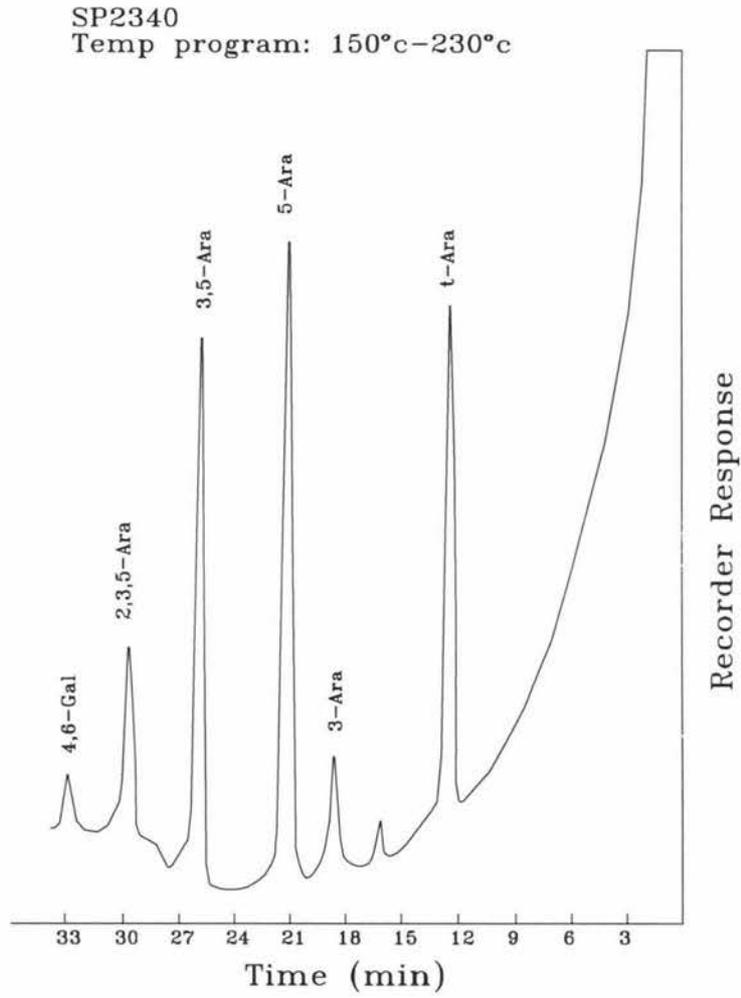


Figure 2.5 The Separation by Gas Chromatography of Methylated Sugar Derivatives (SP 2340)

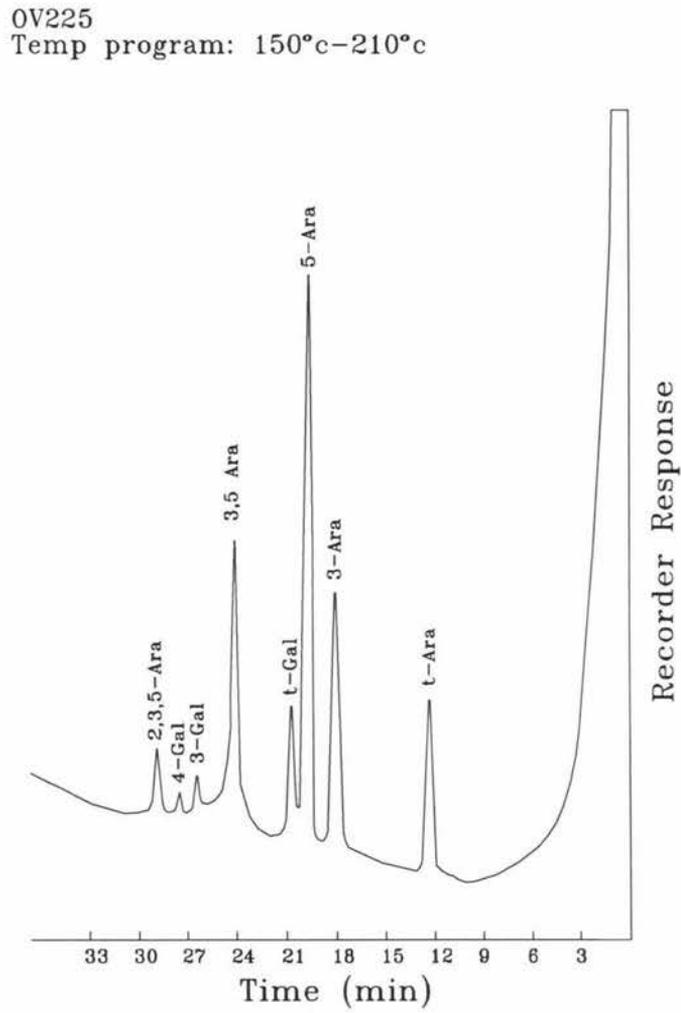


Figure 2.6 The Separation by Gas Chromatography of Methylated Sugar Derivatives (OV 225)

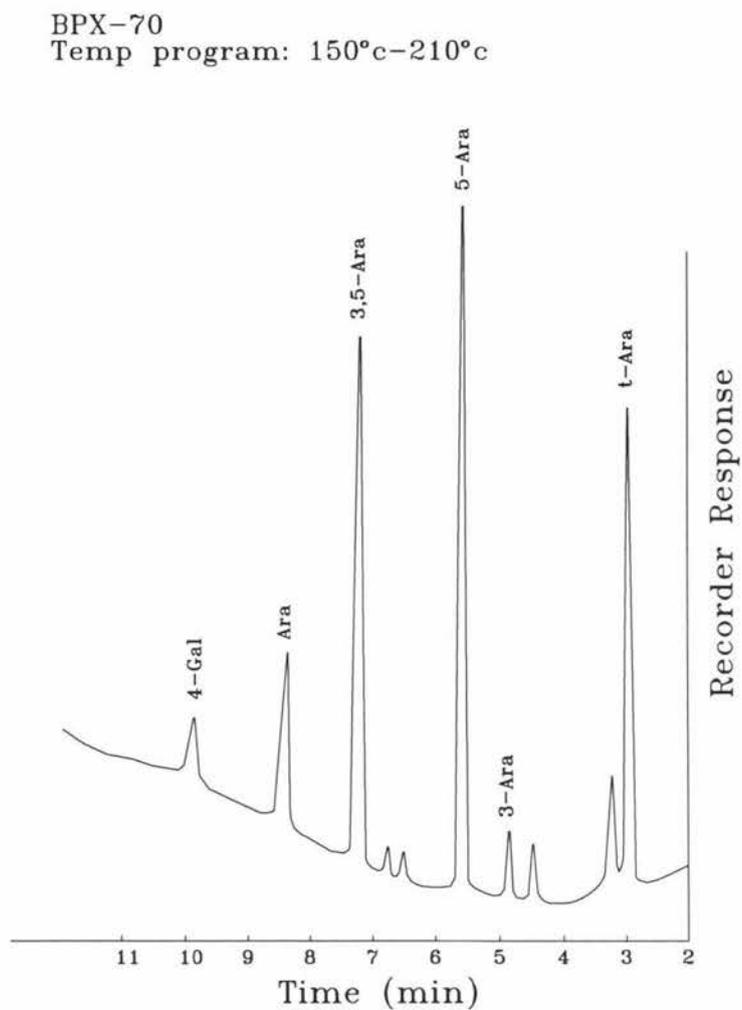


Figure 2.7 The Separation by Gas Chromatography of Methylated Sugar Derivatives (BPX-70)

3. Chapter Three: Results

3.1. Introduction

Traditionally, extraction procedures for pectic substances involve hot water, EDTA, oxalate or chlorite/acetic acid (Oosterveld, et. al., 1996). These extraction procedures often consist of several steps; each of which has a low yield, but an additional large amount can be extracted from cells by cold buffer. In *P. radiata* cambium, this can amount to about 40% of the total (Andrew and McKee, unpublished). Because hot water is known to cause degradation of pectin by β -elimination with minimal degradation of the arabinan side chains, this is a potential method for improving the yield of arabinan.

As a first step in this study of arabinan in *P. radiata* cambium tissue, it was important to establish optimum conditions for fractionation. Ion-exchange chromatography is particularly valuable in separating the acidic polysaccharides from the neutral polysaccharides and is also useful in separating those polysaccharides that contain differing amounts of acidic residues.

It is important to be able to determine which chromatography column fractions contain polysaccharides and, specifically, which fractions are enriched in arabinan. It is also important to detect the presence of protein in the fractions. In this work, the pectic components that were extracted using cold buffer were partially purified by chromatography on DEAE cellulose. The molecular size of the pectic polysaccharides was estimated by using gel filtration chromatography, on both Superose and Superdex FPLC columns.

The determination of total carbohydrate, uronic acid, and pentose were carried out by facile and sensitive colorimetric procedures as detailed in chapter two.

Monosaccharide composition of fractions was determined by gas chromatography as the corresponding alditol acetate derivatives with allose as internal standard. The glycosyl-linkage composition was determined by methylation analysis. The detailed procedure has been described in Section 2.5.

3.2. Extraction and Separation of Arabinan from *P. radiata* Cambium Tissue

3.2.1. Extraction Sequences and Preliminary Fractionations

The extraction procedure used for the *P. radiata* cambium tissue was based on that of Andrew and McKee (1997 and unpublished).

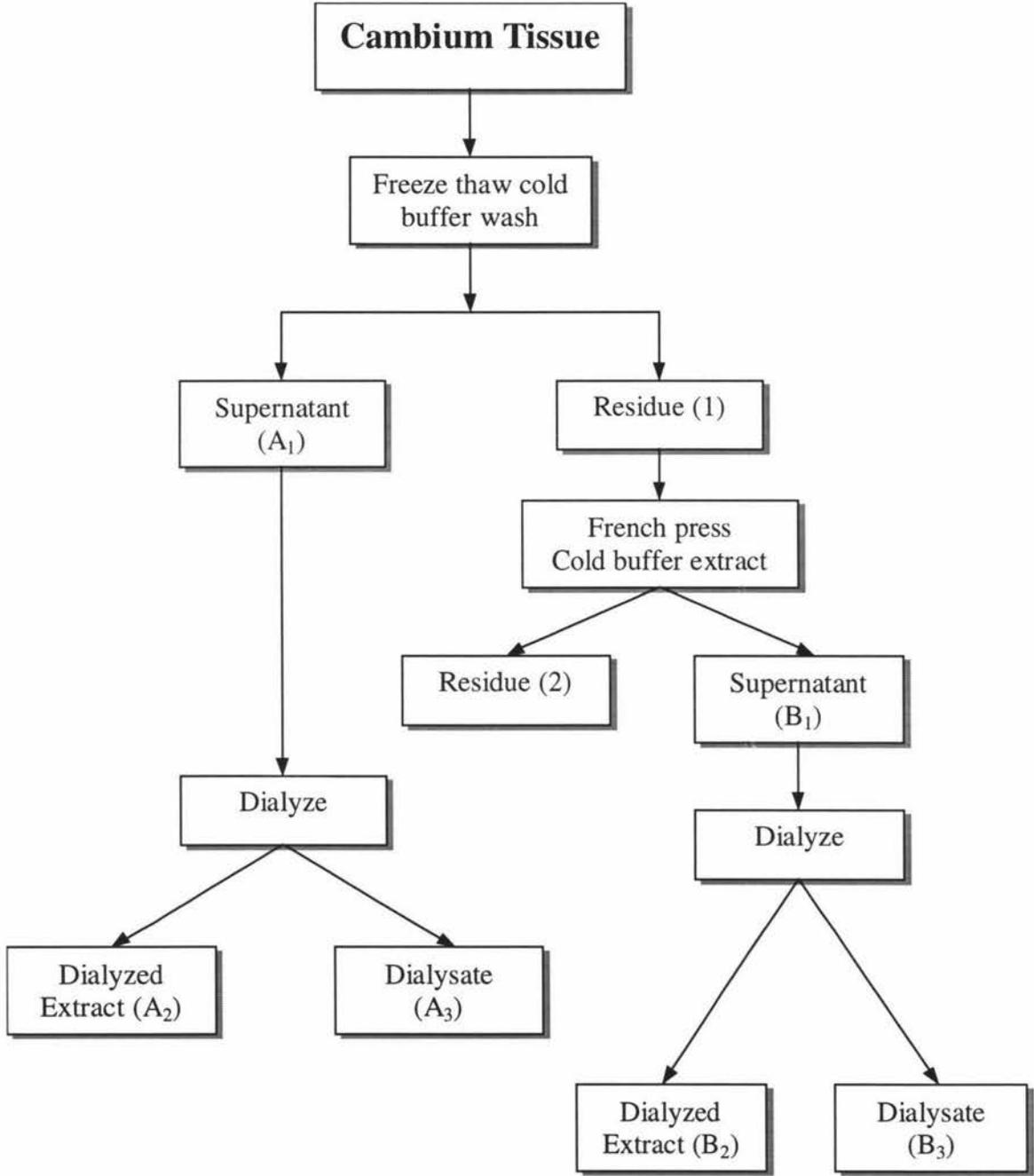


Figure 3.1 Cambium Tissue Extraction Scheme

The extraction scheme of *P. radiata* cambium tissue is shown in Figure 3.1. Mild extraction conditions were employed to isolate the water-soluble pectic polysaccharides with the minimum of modification or degradation. Two batches of cambium tissue were extracted. Practical details concerning the extraction sequences used for the first preparation (Batch 1), performed on a small amount of *P. radiata* cambium tissue, are as follows:

Cambium tissue (25 g) isolated from *P. radiata* was frozen, thawed in 25 ml 0.1 M potassium phosphate buffer, pH 7.0, then washed twice with 125 ml of phosphate buffer at 4°C. Because the polysaccharides are most stable at or near pH 7.0 (Whistler and Sannella, 1965), the cold buffer was adjusted to pH 7.0 before use. The washings were pooled as extract A₁ (265 ml), then the extract A₁ was dialyzed two times, first against about 4.7 L cold distilled water, then against 1.0 L cold distilled water, to yield dialyzed extract A₂ and dialysate, extract A₃.

The residue, suspended in 25 ml phosphate buffer, pH 7.0, was passed through a French Press at 2000 psi to rupture the cells, and the cell homogenate collected directly in 100ml of phosphate buffer, and then centrifuged at 2000 g for 10 minutes. The supernatant was stored and the pellet was resuspended in 80ml phosphate buffer and recentrifuged in order to extract completely. The combined supernatants were collected as extract B₁ (185 ml), which was dialyzed two times against about 2.4 L cold distilled water, then against 1.0 L cold distilled water to give dialyzed extract B₂ and dialysate B₃.

Before dialysis, about 10ml portions of extracts A₁ and B₁ were set aside at 4°C for analysis. After dialysis, extracts A₂ and B₂ were stored at 4°C with sodium azide (1mg/10ml) and portions of extracts A₃ (200 ml) and B₃ (160 ml) were concentrated by freeze drying for further purification and analysis.

The pectic polysaccharide components were then analyzed by polysaccharide fractionation and quantitative analysis of the monosaccharides comprising each of the extracts taken.

The second preparation (Batch 2) was a scale up of the previous batch, using 50 g cambium tissue. The ensuing procedure was the same as for Batch 1.

3.2.2. Analytical Data

In order to find an arabinan-rich fraction, the sugar contents and monosaccharide composition from extracts were analyzed. Batch 1 was used to find the best protocol for scaling up. Most subsequent work was performed using the extracts from Batch 2.

Table 3.1 summarizes the gross analytical features of Batch 1 obtained from analysis for total carbohydrate, uronic acid, and pentose.

**Table 3.1 Gross Analysis of Sugar Contents in Extracted Fractions:
Batch 1 (mg/25g 4°C Frozen Cambium)**

Fraction*	Component (mg/25g)			
	Carbohydrate ¹	Carbohydrate ²	Uronic Acid ³	Pentose ⁴
A ₁	1780	1474	40	188
A ₂	149	313	16	92
A ₃	1296	851	3	..**
B ₁	176	241	44	66
B ₂	88	151	27	60
B ₃	67	34	1	2

* The fraction numbers are the same as in the extraction scheme (Fig. 3.1).

** Not detected.

¹ Using glucose as standard (corrected as described in section 2.2.1). ² GC data plus uronic acid. ³ Using galacturonic acid as standard. ⁴ Using arabinose as standard.

The data in Table 3.1 show that extracts A₂ and B₂ had relatively higher proportions of pentose and uronic acid than the other extracts.

Carbohydrate was determined by two methods: the phenol-sulphuric method (section 2.2) and gas chromatography (section 2.3). The former, using glucose as standard, gives low values for pectin and arabinose-rich fractions (extracts A₂, B₁, and B₂); The latter using allose as standard, shows the data may be slightly low as they were not corrected for possible losses on hydrolysis.

Table 3.2 Monosaccharide % of Total Carbohydrate in *P. radiata* Cambium Tissue Extracts (Batch 1)

Fraction	Component (Wt %)*					
	Ara	Gal	Xyl	Glc	Man	Uronic Acid
A ₁	9.7	6.6	1.8	70.0	9.2	2.7
A ₂	38.7	22.7	-**	20.1	13.4	5.1
A ₃	3.8	3.6	1.2	81.7	9.3	0.4
B ₁	37.8	17.0	-	27.0	-	18.2
B ₂	59.6	22.5	-	-	-	17.9
B ₃	5.9	-	-	91.2	-	2.9

* % of total carbohydrate

**Not detected

The results in Table 3.2 show that the water-soluble polysaccharides in extracts A₂ and B₂ (Batch 1) contained significant amounts of arabinose. The polysaccharides in extract B₂ consisted mainly of arabinose (59.6%), galactose, and galacturonic acid, while the polysaccharides in extract A₂ contained relatively less arabinose (38.7%) and galacturonic acid. The high proportion of glucose in extracts A₃ and B₃ suggest that these extracts contain small molecular size glucan, oligosaccharides, and/or glucose monomer. The high proportion of glucose in extract A₂ can be partly accounted for by incomplete dialysis. In the two dialysis steps, a maximum of 98% of low molecular weight material was removed, most of it glucose (see extract A₃ in Table 3.2). The remaining undialysed glucose would amount to about 20 mg/ 25 g (calculated from data in Table 3.1) which was about 10% of the total extract A₂. The apparent presence of mannose in Extracts A₂ and A₃ may indicate the presence of fructose because reduction of fructose in the analytical procedure gives rise to equal amounts of mannitol and glucitol. See also section 3.2.3.

**Table 3.3 Gross Analysis of Sugar Contents in Extracted Fractions:
Batch 2 (mg/50g 4°C Frozen Cambium)**

Fraction*	Component (mg/50g)			
	Carbohydrate ¹	Carbohydrate ²	Uronic Acid ³	Pentose ⁴
A ₁	2279	1587	58	400
A ₂	327	364	27	150
A ₃	1469	818	4	250
B ₁	350	374	85	172
B ₂	224	232	49	132
B ₃	108	92	2	25

* The fraction numbers are the same as in the extraction scheme (Fig. 3.1).

¹. Using glucose as standard (corrected as described in section 2.2.1) ². GC data plus uronic acid. ³. Using galacturonic acid as standard. ⁴. Using arabinose as standard.

A summary of the gross analytical features of the Batch 2 obtained from analysis for total carbohydrate, uronic acid, and pentose is presented in Table 3.3. The sugar components of the each extract were very similar to Batch 1.

From the Tables 3.1 and 3.3, it appears that some losses occurred on dialysis. For example, the total carbohydrate recovered in extracts A₂ and A₃ was less than that in the original extract A₁. These losses were probably due to the following: (1) some loss of material may have occurred during transfer; (2) some samples were too dilute for accurate analysis; (3) the second dialysates were not included in the assays.

Table 3.4 Monosaccharide % of Total Carbohydrate in *P. radiata* Cambium Tissue Extracts (Batch 2)

Fraction	Component (Wt %)*					
	Ara	Gal	Xyl	Glc	Man	Uronic Acid
A ₁	15.1	8.3	2.6	59.0	11.3	3.7
A ₂	40.1	15.9	-**	20.9	15.7	7.4
A ₃	11.5	-	5.0	69.6	13.4	0.5
B ₁	35.3	16.6	-	25.4	-	22.7
B ₂	53.5	25.4	-	-	-	21.1
B ₃	4.3	-	-	93.5	-	2.2

* % of total carbohydrate

**Not detected

The monosaccharide compositions of Batch 2 are shown in Table 3.4. The sugar analysis here on the extract B₂ demonstrated the presence of arabinose (53.5%), galactose (25.4%), and galacturonic acid. The sugar components in extract A₂ were mainly arabinose (40.1%), glucose (20.9%), galactose(15.9%) and mannose with a smaller amount of galacturonic acid. The high proportion of glucose present in extract A₂ probably reflects incomplete dialysis as for batch 1 (Table 3.2).

Table 3.5 Total Recovery of Sugars in Extracts A₁ and B₁ from Cambium Tissue Using Cold Buffer (wet weight)

Sample	Monosaccharide composition of crude extracts (mg/g cambium) (%Recovery in parenthesis)*					
	Ara	Gal	Xyl	Glc	Man	Uronic Acid
Cambium	11.5	8.4	1.6	80.0	10.1	9.6
Batch 1	9.4(81.7)	5.6(66.7)	1.0(62.5)	43.8(54.8)	5.4(53.5)	3.4(35.4)
Batch 2	7.5(65.2)	3.9(46.4)	0.8(50.0)	20.8(26.0)	3.6(35.6)	2.9(30.2)

*Expressed as percent of total in cambium.

In order to determine the efficiency of the isolation procedure for arabinan, the yields of carbohydrate constituents in batches 1 and 2 were compared with the total yield of sugar components from unextracted cambium. For this analysis, 100 mg cambium was subjected directly to the monosaccharide analysis procedure as described for the extracts.

Table 3.5 shows the sugar recovery rate of monosaccharide units in extracts A₁ and B₁ of Batch 1 and Batch 2 from *P. radiata* cambium tissues using cold buffer. The arabinose had a high recovery in both Batch 1 and Batch 2. This shows that arabinose was mostly present in the cold buffer soluble fraction, whereas other sugars tended to remain in the residual unextracted cell wall fractions. Arabinan may be partly soluble in cytoplasm and not cell wall-bound.

The results from Table 3.2, Table 3.4, and Table 3.5 indicate that the *P. radiata* cambium tissues were rich in pectic type polymers and the extracts A₂ and B₂ were arabinan-rich fractions.

Further investigation for this project was carried out to purify the arabinan, and analyze its structure in line with the aims of the thesis. Most subsequent work was performed using the extracts A₂ and B₂ from Batch 2.

3.2.3. Comparison between Extract A and Extract B

In the work reported here, the fractions extracted from *P. radiata* cambium tissues (Batch 2 samples) were divided into two groups, referred to as extract A (A₁, A₂, and A₃) and extract B (B₁, B₂, and B₃) (see Figure 3.1). The following points of comparison between extract A and extract B emerge from the results presented in this chapter.

Before dialysis, in the monosaccharide compositions of extract A₁, the results from Table 3.2 and Table 3.4 showed that glucose was the most abundant monosaccharide, arabinose was the second most abundant monosaccharide, next to the "mannose" and galactose with smaller amounts of uronic acid and xylose. In contrast, in the extract B₁, arabinose was the most abundant monosaccharide whereas glucose was the second most abundant monosaccharide, followed by uronic acid and galactose. The "mannose" peak on the gas chromatogram probably in part was derived from fructose. A sample of extract A₂ was analyzed for soluble sugar by gas chromatography of the TMS derivatives (as described in chapter two). The major peak coincided on an OV17 gas chromatography column with the major peak from fructose, but further tests would be needed to confirm identity.

A possible alternative source of the mannose peak in extract A₁ is water-soluble galactoglucomannans. Mannose is usually present only in secondary cell walls. It does not normally occur in pectins and, in this work, it was removed on further fractionation (section 3.3). Its origin was therefore not further pursued.

After dialysis, the cold buffer extracts A₁ and B₁ were each divided into two fractions: extracts A₂ and A₃, and extracts B₂ and B₃. The high proportion of uronic acid in tables

3.2 and 3.4 indicates that extracts A₂ and B₂ contained polysaccharides enriched in the pectin.

3.3. Purification of the Arabinan by Ion-exchange Chromatography

In the previous section of this chapter, extracts A₂ and B₂ extracted from *P. radiata* cambium tissues were found very high in arabinose. In order to characterize these arabinose-rich polymers, it was necessary to purify them as much as possible.

This section reports the results of efforts to separate some of these components by ion-exchange chromatography on a DEAE-cellulose column and different elution programs and treatment methods for these samples were tried in this work.

3.3.1. Ion Exchange Chromatography on DEAE-Cellulose Column

Ion exchange chromatography on DEAE-cellulose was used to further purify the extracts A₂ and B₂ using gradient elution with ammonium bicarbonate, pH 8.0.

In order to determine suitable chromatographic conditions, a small size DEAE-cellulose column was used for preliminary experiments.

The dialyzed extracts A₂ and B₂ were turbid and in order to clarify the solution, they were heated at 100°C for 15 minutes. It was also expected that the potential recovery from the column would be increased because any insoluble matter might lead to reduced recovery from the column and could impede the flow.

The heated dialyzed sample was applied to a column (Whatman DE-32, 10 x 100mm, 7.9 ml bed volume) of DEAE-cellulose as described in section 2.4.1. The total carbohydrate and uronic acid content were analyzed as described in section 2.2. The results are plotted as elution profiles in the following figures.

3.3.2. DEAE-Cellulose Chromatography Using Different Elution Programs

In order to find the optimum column elution program, two different elution gradients were used. Figure 3.2 and Figure 3.3 show the results of two elution programs, program 1, and program 2 respectively.

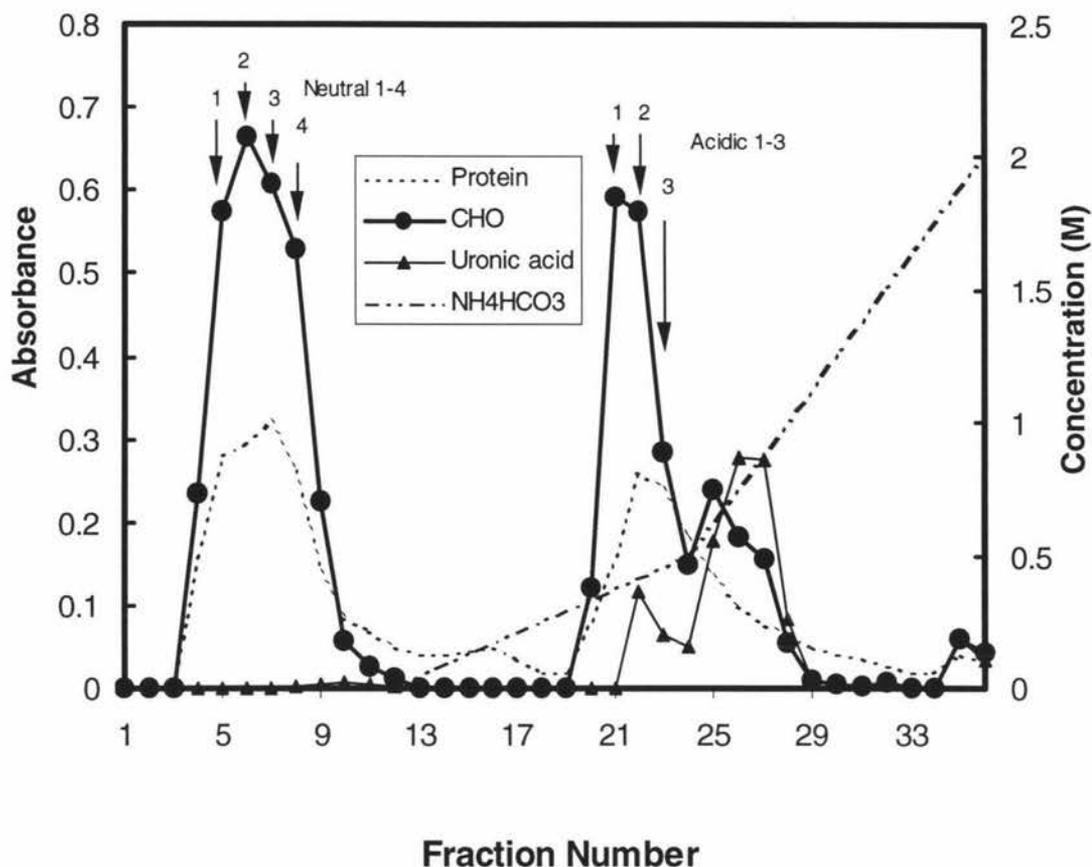


Figure 3.2 DEAE Cellulose Chromatography of Boiled Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (A₂₋₁)

Extract A₂ (20 ml) was heated at 100°C, 15 minutes, then applied to a column of DEAE-cellulose (10 x 100 mm), and the column eluted at a flow rate of 1 ml/min; 5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program 1 was: 0.01 M NH₄HCO₃ (40 ml); 0.01-0.5 M NH₄HCO₃ (60 ml); 0.5-2.0 M NH₄HCO₃ (60ml). Total carbohydrate (CHO), uronic acid, and protein were shown as absorbance, whereas NH₄HCO₃ was shown as concentration. The arrows indicate the fractions that were analyzed for monosaccharide composition.

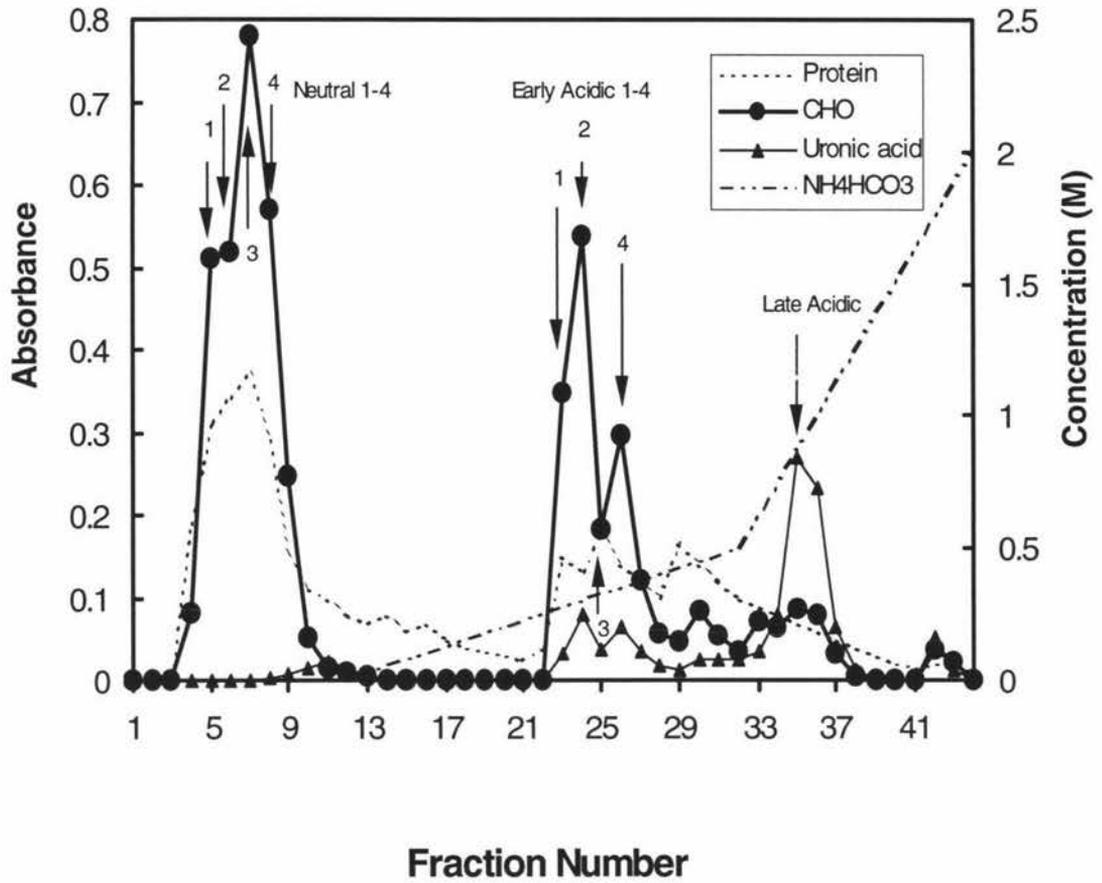


Figure 3.3 DEAE Cellulose Chromatography of Boiled Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient ($A_{2,2}$)

Extract A_2 (20 ml) was heated at 100°C, 15 minutes, then applied to a column of DEAE-cellulose (10x100 mm) and the column eluted at a flow rate of 1 ml/min; 5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program 2 was 0.01 M NH_4HCO_3 (40 ml); 0.01-0.5 M NH_4HCO_3 (100 ml); 0.5-2.0 M NH_4HCO_3 (60 ml). Other details are as in Fig.3.2.

Figure 3.2 shows a typical elution profile for extract A₂ on DEAE-cellulose. In this case, three major fractions were separated: an unbound neutral fraction, an early acidic fraction, and a late acidic fraction.

Figure 3.3 shows the elution profile for extract A₂ on DEAE-cellulose with the elution program 2. In this elution program, four major fractions were separated: an unbound neutral fraction, two early acidic fractions, and a late acidic fraction.

Table 3.6 Monosaccharide Composition of Fractions of Extract A₂ Separated on DEAE-Cellulose Run no. A₂₋₁ as in Fig. 3.2

Fraction	Yield (mg) ^a	Component (Wt %)*						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glu	Man
Neutral 1 (fr.5)	1.57	-**	9.3	12.5	-	3.1	49.9	25.2
Neutral 2 (fr.6)	1.88	-	10.3	12.6	-	9.8	44.8	22.5
Neutral 3 (fr.7)	1.78	-	10.6	12.9	tr***	3.6	48.6	24.3
Neutral 4 (fr.8)	1.61	-	9.4	10.7	tr	4.3	53.1	22.5
Acidic 1 (fr.21)	2.57	3.2	55.0	31.5	tr	8.0	2.3	-
Acidic 2 (fr.22)	2.37	4.4	44.3	33.1	11.9	4.6	1.7	-
Acidic 3 (fr.23)	1.14	3.8	42.6	36.0	14.1	-	3.4	-

^a In 5ml fraction

* % of total carbohydrate

** Not detected

*** Trace (less than 0.5%)

Table 3.7 Monosaccharide Composition of Fractions of Extract A₂ Separated on DEAE-Cellulose Run no. A₂₋₂ as in Fig. 3.3

Fraction	Yield (mg) ^a	Component (Wt %)*						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glc	Man
Neutral 1(fr.5)	1.74	-**	12.1	15.3	-	4.8	40.2	27.7
Neutral 2(fr.6)	1.50	-	10.5	13.4	-	8.8	42.5	24.8
Neutral 3(fr.7)	1.72	-	10.3	13.5	-	4.9	45.1	26.2
Neutral 4(fr.8)	1.34	-	12.6	11.8	tr***	2.7	47.5	25.4
Early Acidic 1(fr.23)	1.33	1.9	55.2	31.3	6.0	2.6	3.0	-
Early Acidic 2(fr.24)	1.78	2.7	47.9	30.8	10.9	5.7	2.0	-
Early Acidic 3(fr.25)	0.92	3.5	46.4	33.8	9.3	4.4	2.6	-
Early Acidic 4(fr.26)	1.23	4.2	43.0	34.5	12.7	2.4	3.2	-
Late Acidic (fr.35)	1.00	-	6.4	2.5	86.8	1.2	3.1	-

^a In 5ml fraction

* % of total carbohydrate

** Not detected

*** Less than 0.5%

Table 3.6 shows the results of the selected fractions from Fig.3.2 using the elution program 1. Glucose and mannose are dominant in the neutral fractions, next to the galactose and arabinose as well as a smaller amount of xylose. Within the acidic fractions (from acidic fraction 1 to 3), successive fractions show the sugar compositions with decreasing ara/gal ratio and increasing uronic acid content.

Table 3.7 shows the monosaccharide compositions of the selected fractions from Fig.3.3. The early acidic fractions, which have high arabinose and galactose content were separated from a late acidic fraction with mostly uronic acid. Within the early acidic fractions (from early acidic fraction 1 to 4), successive fractions show a general increase in uronic acid and rhamnose content and a decrease in arabinose as in Table 3.6. This suggests that a higher uronic acid content lead to the stronger binding to the column and later elution by buffer. The fraction Early Acidic 1 (shown in Figure 3.3) contained the highest proportion of arabinose followed by galactose and smaller amounts of uronic acid. The ratios of Ara:Gal:Rha for this fraction were 29:16:1.

The results from Tables 3.6 and 3.7 are almost the same for the early acidic fractions (with the notable exception of a lower uronic acid content in acidic fraction 1 of Table 3.6). The difference between the two elution programs is principally the much better separation of early from late acidic fraction by program 2. Also, program 2 shows two peaks for the early acidic fractions, but the significance of the double peak is unclear.

The significant amount of arabinose present in the early acidic fractions suggested that the early acidic fraction could be a good resource for further study.

To further confirm the preceding results, another two larger samples of extract A₂ were fractionated on a DEAE-cellulose column. The elution program used was scaled up from program 2.

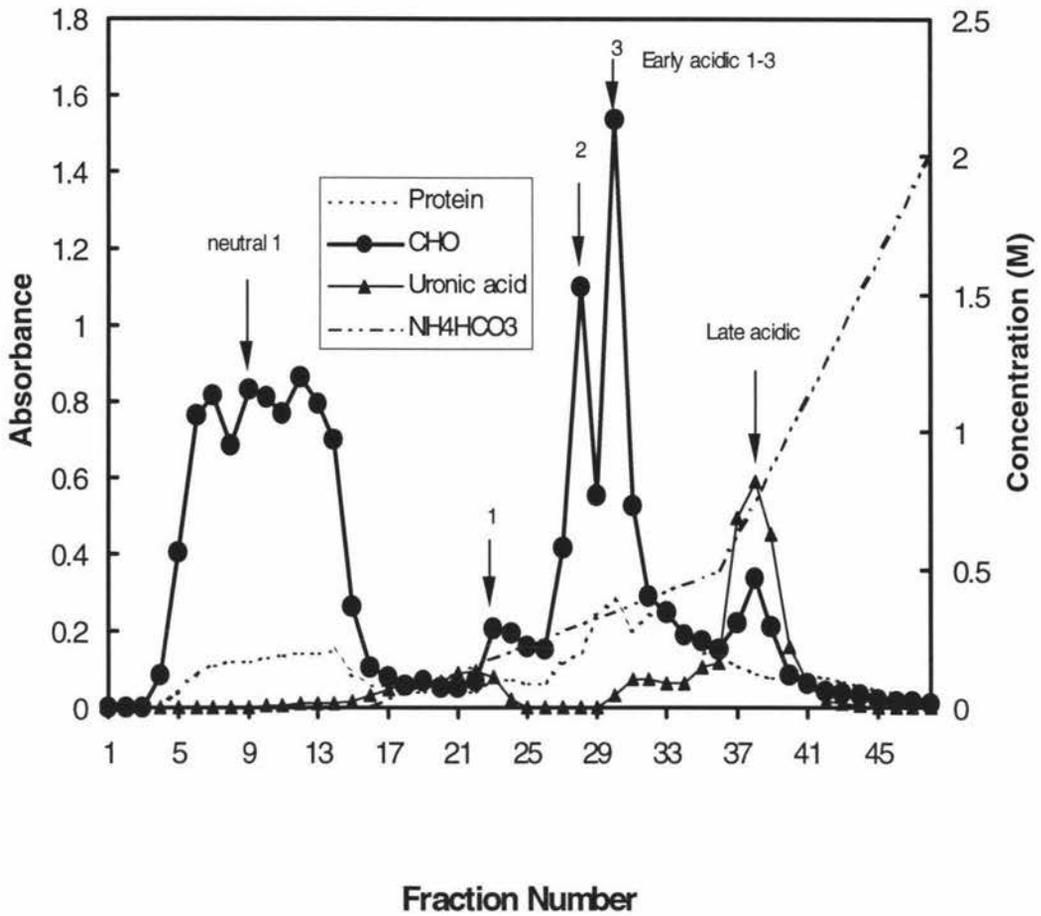


Figure 3.4 DEAE Cellulose Chromatography of Boiled Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (A_{2-3A})

Extract A₂ (75 ml) was heated at 100°C, 15 minutes, then applied to a column of DEAE-cellulose (15 x 130 mm), and the column eluted at a flow rate of 1.5 ml/min; 7.5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program was 0.01 M NH₄HCO₃ (45 ml); 0.01-0.5 M NH₄HCO₃ (150 ml); 0.5-2.0 M NH₄HCO₃ (90 ml). Other details as in Fig. 3.2.

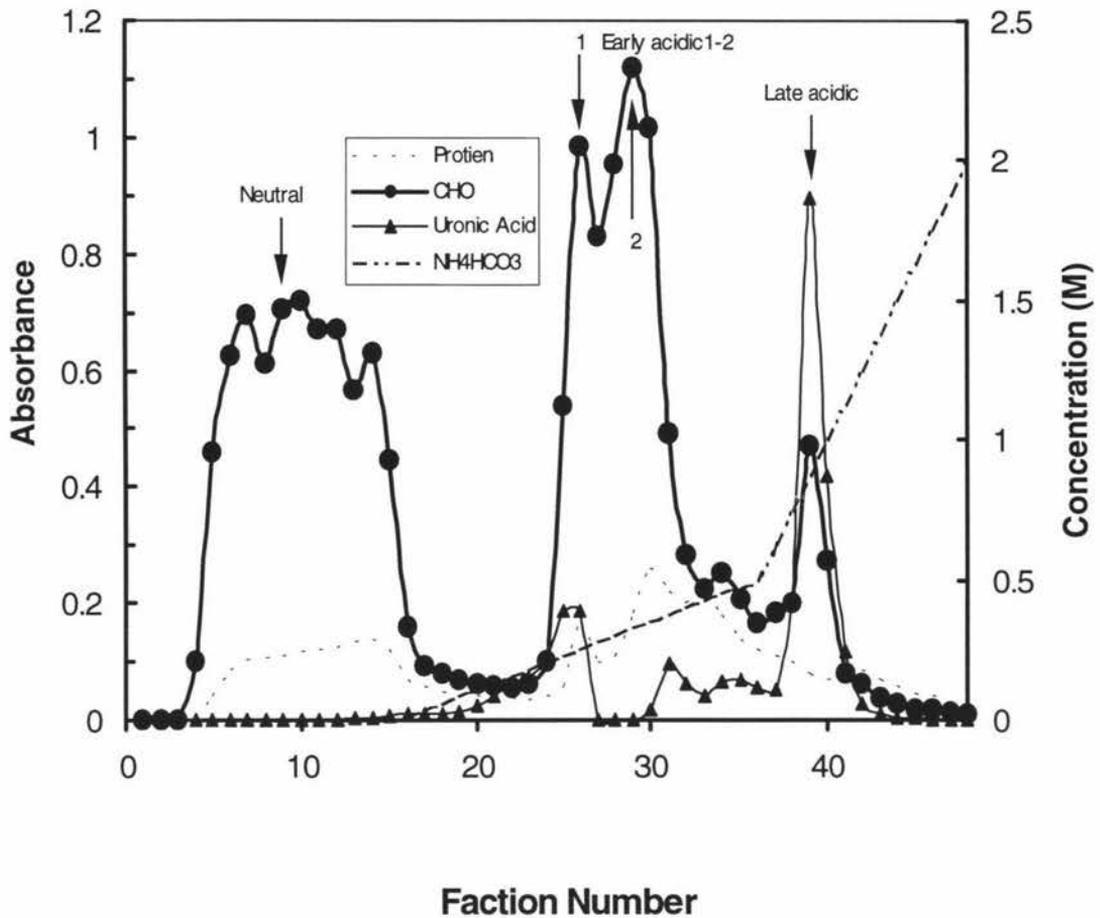


Figure 3.5 DEAE Cellulose Chromatography of Boiled Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (A_{2-3B})

Extract A₂ (75 ml) was heated at 100°C, 15 minutes, then applied to a column of DEAE-cellulose (15 x 130 mm), and the column eluted at a flow rate of 1.5 ml/min; 7.5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program was 0.01 M NH₄HCO₃ (45 ml); 0.01-0.5 M NH₄HCO₃ (150 ml); 0.5-2.0M NH₄HCO₃ (90 ml). Other details as in Fig.3.2.

Figures 3.4 and 3.5 show the elution profiles obtained from extract A₂. Both of them are heated at 100°C for 15 minutes. It can be seen in these figures that they are similar to A₂₋₂. However, the positions of the peaks are slightly different between the two runs. This may be due to the use of different batches of DEAE-cellulose. The former was run with fresh DEAE-cellulose, whereas the latter used the regenerated of DEAE-cellulose.

Table 3.8 Monosaccharide Composition of Fractions of Extract A₂ Separated on DEAE-Cellulose Run no. A_{2-3A} as in Fig. 3.4

Fraction	Yield (mg) ^a	Component (Wt %)*						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glu	Man
Neutral (fr.9)	2.65	-**	22.6	16.4	-	6.1	32.5	22.2
Early acidic 1(fr.23)	1.68	-	44.8	34.3	14.9	-	6.0	-
Early acidic 2(fr.28)	5.94	2.0	64.5	27.3	tr	6.2	-	-
Early acidic 3(fr.30)	7.06	3.0	54.4	35.4	1.4	5.8	-	-
Late acidic (fr.38)	3.11	-	6.3	2.5	86.8	1.2	3.2	-

^a In 7.5ml fraction

* % of total carbohydrate

** Not detected

Tables 3.8 and 3.9 show the monosaccharide analytical results. Both show similar trends to those in Table 3.7 in that the early acidic fractions contained high proportions of arabinose and the other fractions have similar relative proportions of all sugars. Early acidic 1 (fr.23) in A_{2-3A} is anomalous in that it has a low ara/gal ratio. This may be due to the fraction being an early early acidic fraction. Contrary to the trend observed with A₂₋₁ and A₂₋₂, early acidic 2 in both columns has lower uronic acid content than the preceding fractions. This low uronic acid content is discussed in section 3.5.1.

Table 3.9 Monosaccharide Composition of Fractions of Extract A₂ Separated on DEAE-Cellulose Run no. A_{2-3B} as in Fig. 3.5

Fraction	Yield (mg) ^a	Component (Wt %)*						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glu	Man
Neutral (fr.9)	1.88	-**	16.4	16.5	-	4.9	34.1	28.1
Early acidic 1(fr.26)	5.35	1.2	61.7	15.6	12.2	6.7	2.6	-
Early acidic 2(fr.29)	4.72	1.5	57.0	35.6	tr	3.7	2.3	-
Late acidic (fr.39)	3.20	-	6.2	-	93.8	-	-	-

^a In 7.5ml fraction

* % of total carbohydrate

** Not detected

3.3.3. DEAE-Cellulose Chromatography of Unheated Extracts-Comparison with Heated Extracts

In the preceding runs, the samples applied (extracts A₂) were heated at 100°C for 15 minutes to aid solubility. It is possible that this treatment may degrade the pectin backbone and facilitate enrichment of the arabinan (Fry, 1988). To test this, further chromatograms were run with unheated samples and the fractions from the heated and unheated samples were compared by gel filtration (see section 3.5.2)

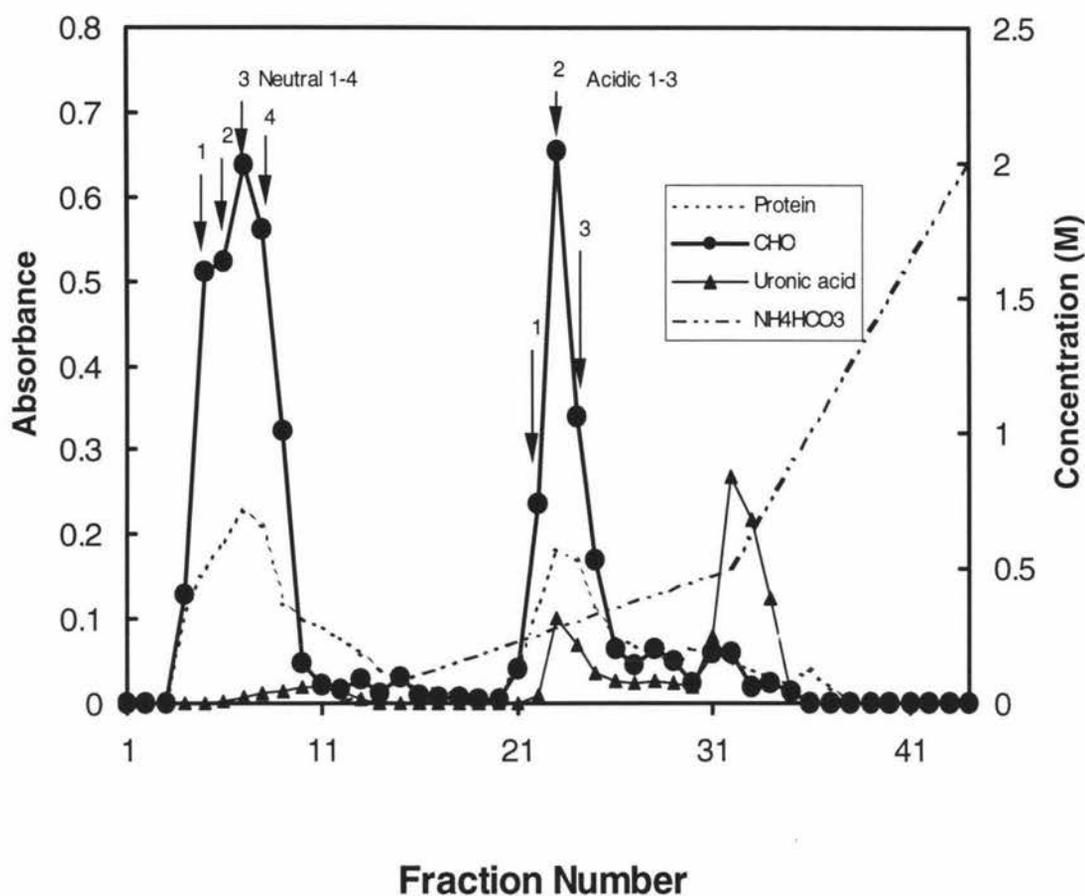


Figure 3.6 DEAE Cellulose Chromatography of Cold Buffer Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (A_{2.4})

20 ml of unheated extract A₂ was applied to a column of DEAE cellulose (10x100mm), and the column eluted at a flow rate of 1 ml/min; 5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program 2 was: 0.01 M NH₄HCO₃ (40 ml); 0.01-0.5 M NH₄HCO₃ (100 ml); 0.5-2.0 M NH₄HCO₃ (60 ml). Other details as in Fig.3.2.

Table 3.10 Monosaccharide Composition of Fractions of Extract A₂ Separated on DEAE-Cellulose Run no. A₂₋₄ as in Fig. 3.6

Fraction	Yield (mg) ^a	Component (Wt %)*						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glc	Man
Neutral 1 (fr.5)	1.06	-**	12.2	19.3	-	-	68.5	-
Neutral 2 (fr.6)	1.52	-	12.1	13.3	tr***	-	46.5	28.1
Neutral 3 (fr.7)	1.70	-	13.5	18.9	tr	1.9	42.0	23.7
Neutral 4 (fr.8)	1.62	-	9.2	14.7	tr	2.7	47.3	26.1
Acidic 1 (fr.22)	1.60	1.6	57.8	39.1	1.5	-	-	-
Acidic 2 (fr.23)	2.47	2.0	46.9	37.7	10.3	3.0	-	-
Acidic 3 (fr.24)	1.63	2.5	42.4	44.1	10.8	-	-	-

^a In 5 ml fraction

* % of total carbohydrate

** Not detected

*** Less than 0.5%

Figures 3.3 and 3.6 show the elution profiles obtained from extract A₂ using program 2. The former shows the effect of a heat treatment at 100°C for 15 minutes, the latter shows the result of separation using unheated extract A₂. It appears from the figures that the heat treatment caused a splitting of the early acidic peak and a slight retardation of the late acidic peak.

The monosaccharide analytical data are shown in Tables 3.7 and 3.10. Both of them indicate that the early acidic fractions contained high proportions of arabinose and the other sugars also have similar relative amounts.

3.3.4. Elution Profile for Extract B₂

Previous work just used extract A₂, but the extract B₂ is also important, and it can be compared with extract A₂ using the same elution program.

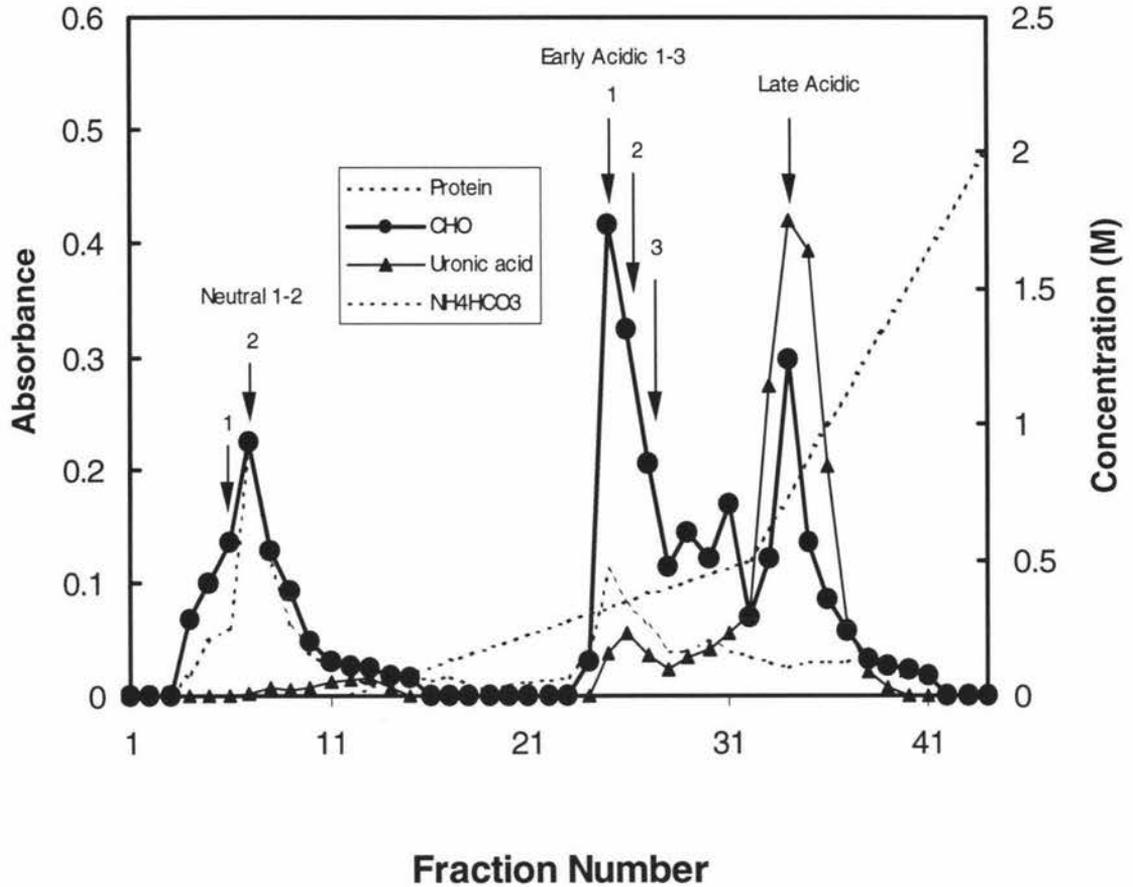


Figure 3.7 DEAE Cellulose Chromatography of Boiled Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (B₂₋₁)

Extract B₂ (20 ml) was heated at 100°C, 15 minutes, then applied to a column of DEAE-cellulose (10 x 100 mm), and the column eluted at a flow rate of 1 ml/min; 5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program was: 0.01 M NH₄HCO₃ (40 ml); 0.01-0.5 M NH₄HCO₃ (100 ml); 0.5-2.0 M NH₄HCO₃ (60 ml). Other details as in Fig.3.2.

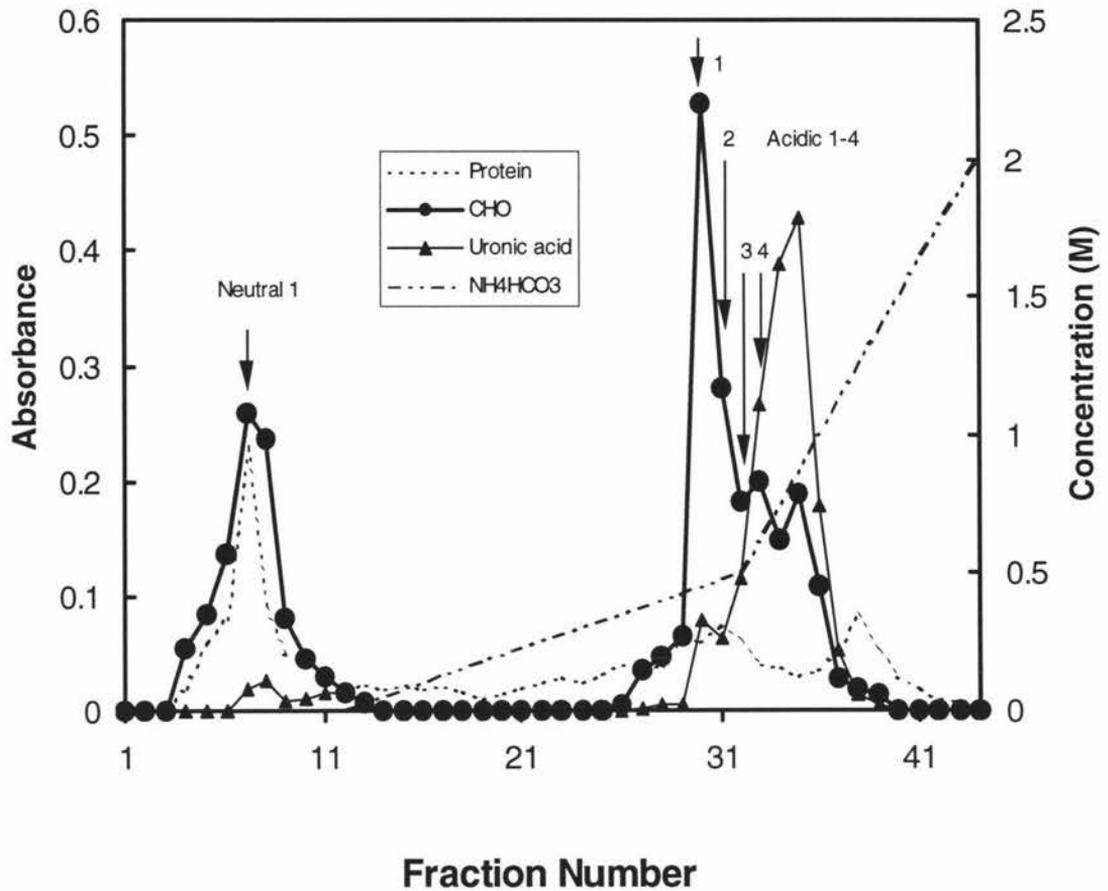


Figure 3.8 DEAE Cellulose Chromatography of Cold Buffer Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (B_{2.2})

20 ml of unheated extract B₂ was applied to a column of DEAE-cellulose (10x100mm), and the column eluted at a flow rate of 1 ml/min; 5 ml fractions were collected. Column fractions were assayed as described in the text. The elution program 2 was: 0.01 M NH₄HCO₃ (40 ml); 0.01-0.5 M NH₄HCO₃ (100 ml); 0.5-2.0 M NH₄HCO₃ (60 ml). Other details as in Fig.3.2.

Table 3.11 Monosaccharide Composition of Fractions of Extract B₂ Separated on DEAE-Cellulose Run no. B₂₋₁ as in Fig. 3.7

Fraction	Yield (mg) ^a	Component (Wt %)*		
		Ara	Gal	Uronic Acid
Neutral 1 (fr.6)	0.21	55.8	44.2	-
Neutral 2 (fr.7)	0.16	53.5	46.5	-
Early Acidic 1 (fr.25)	1.34	64.3	28.6	7.1
Early Acidic 2 (fr.26)	1.30	55.4	33.6	11.0
Early Acidic 3 (fr.27)	0.87	53.5	35.0	11.5
Late Acidic (fr.34)	1.41	6.9	18.8	74.3

^a In 5 ml fraction

* % of total carbohydrate

** Not detected

Figures 3.7 and 3.8 show the elution profiles obtained with extract B₂. The former shows the profile for a sample treated at 100°C for 15 minutes, the latter shows the result of separation using unheated extract B₂. It is clear that the heat treatment caused the early acidic peak to move to an earlier position on the elution profile. This is consistent with the hypothesis that heat treatment has caused degradation of the pectin backbone, causing a lowering of the uronic acid content and therefore weaker binding and earlier elution of the arabinan-rich fraction. The effect with extract A₂ (section 3.3.3) was less marked.

Data from monosaccharide analysis of fractions from extract B₂ with and without heat treatment are given in Table 3.11 and Table 3.12 respectively. It can be seen in these tables that both of the early acidic fractions contained highest arabinose content and that, within the acidic fractions (from acidic fraction 1 to 4), successive fractions showed an increase in uronic acid and decrease in arabinose.

Compared with the early acidic fractions in B₂₋₁, the early acidic fractions in B₂₋₂ have higher uronic acid content, which suggest that the unheated sample may bind more strongly to the column because it is less degraded. Degradation would result in removal of some uronic acid from early acidic fractions. This in turn would lead to a greater separation of the early acidic fraction from the late acidic fraction as was observed. The neutral fraction 1 has higher uronic acid content in the unheated preparation. This may be due to error in the assay or it may represent methyl esterified (neutral) uronic acids.

Comparison of this result with that obtained with extract A₂ shows some differences, for example, the clear splitting of the early acidic peak in extract A₂ was not observed with extract B₂, but with both extracts the heating leads to better separation, which can be explained by partial degradation of the pectin backbone. This idea is further explored in section 3.5.2.

Table 3.12 Monosaccharide Composition of Fractions of Extract B₂ Separated on DEAE-Cellulose Run no. B₂₋₂ as in Fig. 3.8

Fraction	Yield (mg) ^a	Component (Wt %)*		
		Ara	Gal	Uronic Acid
Neutral 1 (fr.7)	0.55	58.3	32.6	9.1
Acidic 1 (fr.30)	2.07	63.4	26.9	9.7
Acidic 2 (fr.31)	0.92	59.1	23.5	17.4
Acidic 3 (fr.32)	0.82	37.0	27.8	35.2
Acidic 4 (fr.33)	1.08	17.6	19.6	62.8

^a In 5 ml fraction

* % of total carbohydrate

** Not detected

3.3.5. DEAE-Cellulose Chromatography of Cold Buffer Fraction (Large Scale)

For structural analysis of the high arabinose components, a scale-up fractionation on DEAE-cellulose was expected to yield better results and to provide significant samples for further work including methylation analysis.

On the basis of the results from the preceding work, extract B₂ was selected for the large scale fractionation on a DEAE-cellulose column. Compared to the preliminary work, the major differences were that the flow rate on the column was increased from 1ml/min to 1.5 ml/min and the collecting volume of fractions was changed from 5 ml to 7.5 ml.

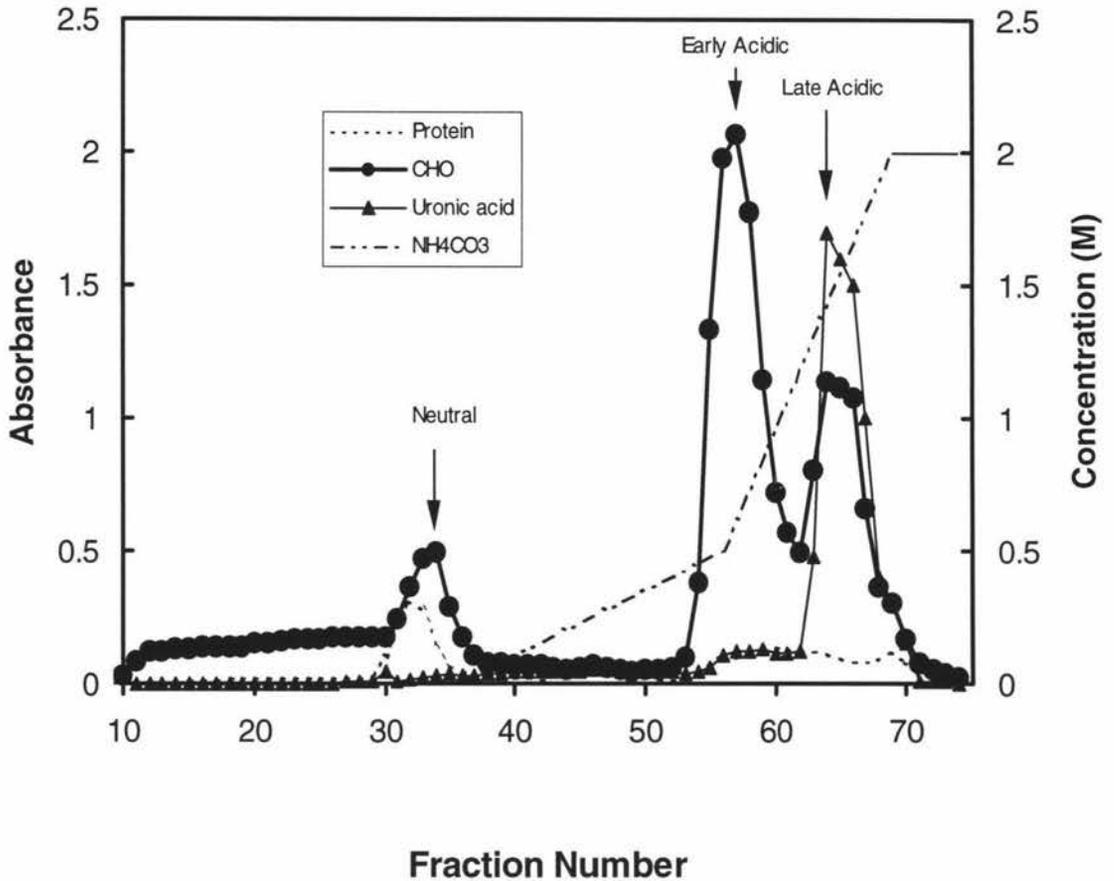


Figure 3.9 DEAE Cellulose Chromatography of Cold Buffer Extract of *P. radiata* Cambium Tissue with Ammonium Bicarbonate Gradient (B_{2.3})

Extract B₂ (195 ml) was heated at 100°C for 15 minutes, then applied to a column of DEAE-cellulose (25 x 140 mm), and the column eluted at a flow rate of 1.5 ml/min; 7.5ml fractions were collected by a timed automatic fraction collector. Column fractions were assayed as described in the text. The elution program 2 was: 0.01 M NH₄HCO₃ (75 ml); 0.01-0.5 M NH₄HCO₃ (150 ml); 0.5-2.0 M NH₄HCO₃ (90 ml); 2.0M NH₄HCO₃ (30 ml). Other details as in Fig.3.2.

Figure 3.9 shows the elution profile obtained with this scale up. As in Figure 3.7, three major fractions were separated: an unbound neutral fraction, an early acidic fraction, and a late acidic fraction. The early acidic fraction eluted as a single peak and this was clearly separated from the late acidic peak.

3.3.6. Analysis of Fractions from DEAE-Cellulose Chromatography (Scale Up)

Selected fractions from the elution profile of Figure 3.9 were analyzed for monosaccharide composition and protein concentration (see Section 2.2.3). The results are presented in Table 3.13.

Table 3.13 Monosaccharide Composition and Approximate Protein Levels of Pectic Fractions of DEAE-Cellulose run no. B_{2,3} as in Fig. 3.9

Fraction	Protein (mg/ml)	individual monosaccharides as (%) of total sugars*					
		Rha	Ara	Gal	Uronic Acid	Xyl	Glc
Neutral (fr.34)	0.39	-**	57.7	18.0	7.3	8.1	8.9
Early Acidic (fr.57)	0.27	1.1	70.2	22.3	3.6	2.8	-
Late Acidic (fr.64)	0.33	-	8.2	-	91.8	-	-

* % of total carbohydrate

** Not detected

The monosaccharide and protein analytical data for selected fractions indicated on Figure 3.9 are shown in Table 3.13. Arabinose was the most abundant monosaccharide in the early acidic fraction (70.2%). This early acidic fraction was selected from the profile (Figure 3.9) for methylation analysis. The result is shown in Section 3.5.3.

3.3.7. Recovery of carbohydrate from fractionation procedures

Data on the recovery of sugars from DEAE-cellulose chromatography are shown in Table 3.14.

Table 3.14 Recovery of Sugars by Using DEAE-Cellulose Chromatography

Sample	Heat treatment	Amount applied to column (mg)*	Amount recovered (mg)*	Recovery (%)
A ₂₋₁	+	21.1	16.8	79.6
A ₂₋₂	+	21.1	16.7	79.1
A _{2-3A}	+	79.1	66.2	83.7
A _{2-3B}	+	79.1	66.6	84.2
A ₂₋₄	-	21.1	14.8	70.0
B ₂₋₁	+	12.6	9.8	77.8
B ₂₋₂	-	12.6	7.9	62.7
B ₂₋₃	+	123.0	102.8	83.6

* Using glucose as standard.

The data in Table 3.14 show that for both extract A₂ and extract B₂, samples which had been given a 15 minutes treatment in water at 100°C, had a higher recovery rate (80%) on the DEAE-cellulose column, while samples that had not been heated, gave a lower recovery rate (60-70%).

The heat treatment was used initially to clarify the solution to ensure a better recovery of total carbohydrate. It appears that it achieved its purpose. Possibly, some insoluble material in the unheated sample was not eluted from the DEAE-cellulose column by NH₄HCO₃. This result indicates that heat treatment not only caused degradation of the pectin backbone, weaker binding and earlier elution of the arabinan-rich fraction (see section 3.3.4), but also increased the recovery from the column. This result was evident with extract B₂ (section 3.3.4), but not so much with extract A₂ (section 3.3.3).

3.4. Separation Using Ethanol Fractionation

Because the highest purity arabinan from ion-exchange chromatography was only 70% arabinose, another method was attempted for fractionation of extract B₂. This was ethanol fractionation according to the method of Fry (1988).

To 50 ml of extract B₂ was added 140 ml 96% ethanol and the final concentration of ethanol was 70%. The solution was stored at 4°C overnight. The precipitate was separated by centrifugation at 3000 g, then was dissolved in 7 ml distilled water for analysis. The supernatant was concentrated using the Rotary Evaporator (ROTAVAPOR-R, Switzerland). The residues were dissolved in 5 ml distilled water.

Table 3.15 Monosaccharide Composition of Fractions Isolated from Extract B₂ Tissue Using Ethanol Precipitation

Fraction	Carbohydrate in 50ml extract (mg)	individual monosaccharides as (%) of total sugars *						
		Rha	Ara	Gal	Uronic Acid	Xyl	Glc	Man
Precipitate	11.5	-**	43.5	18.5	34.2	-	3.8	-
Supernatant	9.0	-	42.8	13.5	20.8	8.3	6.3	8.3

* % of total carbohydrate

** Not detected

The monosaccharide analytical data are shown in Table 3.15. The major components were tentatively identified from the sugar composition in the table. The sugar compositions of the precipitate and supernatant fractions were very similar. They consisted mainly of arabinose, followed by uronic acid and galactose. Compared to the early acidic fractions from DEAE-cellulose chromatography, arabinose and galactose were present in smaller quantities, but the uronic acid amounts were much higher than with the DEAE-cellulose chromatography. Thus, the ethanol procedure was less

effective than the DEAE-cellulose chromatography at purification of the arabinan fraction.

Compared to DEAE-cellulose chromatography, the ethanol fractionation method possessed a higher recovery rate (93%), but DEAE-cellulose chromatography gave better separation.

3.5. Characterization of the Arabinan

Structural studies reported here on the arabinan from *P. radiata* cambium tissues have involved three major methods: Quantitative monosaccharide analysis, size separation of acidic fractions using FPLC and methylation analysis.

Monosaccharide analysis has involved quantitation of the neutral monosaccharides in arabinan-rich fractions. The method gives accurate quantitation combined with quick analysis of isolated fractions, and results have been reported in sections 3.2 and 3.3.

Size separation of acidic fractions using FPLC not only gives the range of the molecular weight but also can provide a basis for further purification of the arabinan-rich fractions.

Methylation analysis is probably the most important method for polysaccharide structural analysis. The process involves exhaustive methylation of polysaccharides by etherification of the hydroxyl groups. The methylated polymer is then hydrolyzed, and the methylated monomers separated, identified, and quantitatively analyzed, commonly after conversion to alditol acetates. Methylation analysis of extracted fractions provides confirmation of the presence of these polysaccharides by enabling linkages between sugar residues in the polysaccharide to be analyzed.

3.5.1. Monosaccharide Analysis

The monosaccharide compositions of selected fractions from DEAE-cellulose chromatography have been presented as wt % in section 3.3 (Tables 3.6 to 3.13). In Table 3.16, the data are shown as mole ratios.

Table 3.16 Mole Ratios of Arabinan Fractions

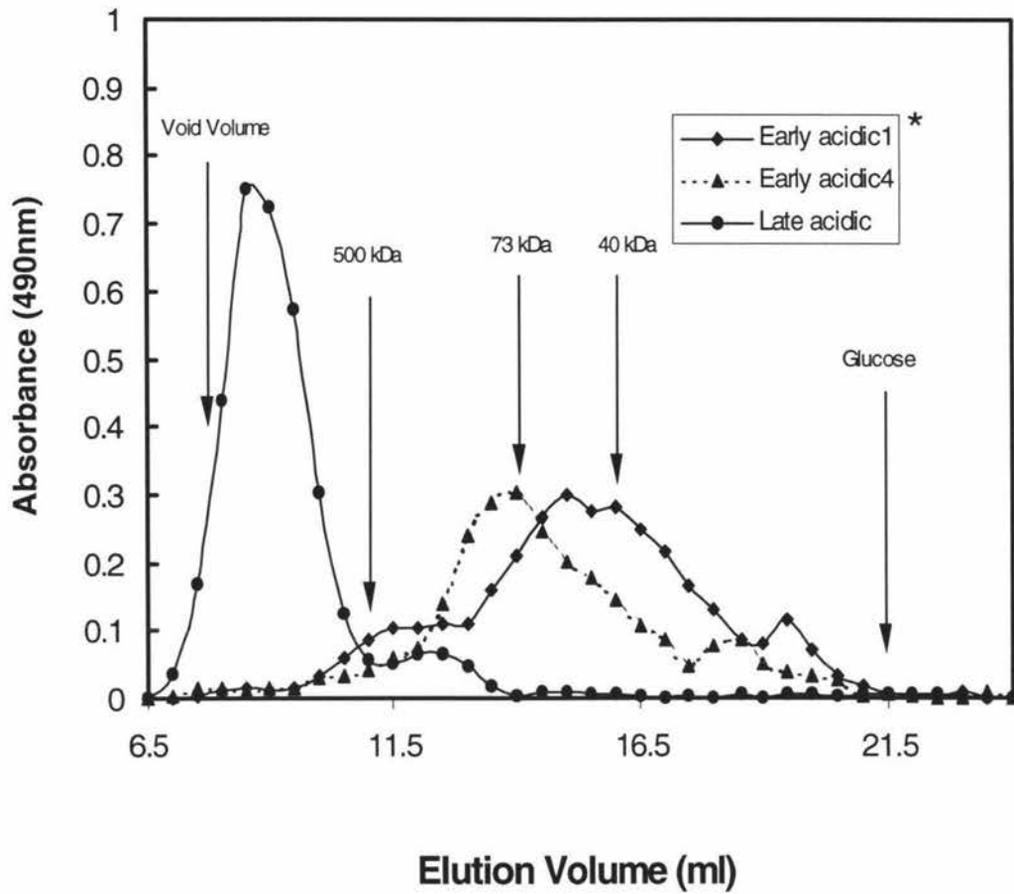
Fraction	Rha	Ara	Gal	Uronic Acid	Xyl	Glc	Man
A₂₋₁							
Acidic 1	5.3	100	47.8	tr	14.5	3.5	-
Acidic 2	9.1	100	62.3	20.8	10.4	3.2	-
Acidic 3	8.1	100	70.4	25.6	-	6.7	-
A₂₋₂							
Early acidic 1	3.1	100	47.3	8.4	4.7	4.5	-
Early acidic 2	5.1	100	53.6	17.6	11.9	3.5	-
Early acidic 3	6.9	100	60.7	15.5	9.5	4.7	-
Early acidic 4	9.0	100	66.8	22.8	5.6	6.2	-
A_{2-3A}							
Early acidic 1	-	100	63.8	25.7	-	11.2	-
Early acidic 2	2.8	100	35.3	tr	9.6	-	-
Early acidic 3	5.0	100	54.3	2.0	10.7	-	-
A_{2-3B}							
Early acidic 1	1.7	100	21.1	15.3	10.9	3.5	-
Early acidic 2	2.4	100	52.1	tr	6.5	3.3	-
A₂₋₄							
Acidic 1	2.6	100	56.3	2.0	-	-	-
Acidic 2	3.9	100	67.0	17.0	6.4	-	-
Acidic 3	5.4	100	86.7	19.7	-	-	-
B₂₋₁							
Early acidic 1	-	100	37.1	8.5	-	-	-
Early acidic 2	-	100	50.5	15.4	-	-	-
Early acidic 3	-	100	54.5	16.6	-	-	-
B₂₋₂							
Acidic 1	-	100	35.3	11.8	-	-	-
Acidic 2	-	100	33.2	22.7	-	-	-
Acidic 3	-	100	62.6	73.5	-	-	-
B₂₋₃							
Early acidic	1.5	100	26.5	3.9	4.0	-	-
Ethanol							
Precipitate	-	100	35.4	60.8	-	7.3	-
Supernatant	-	100	26.3	37.6	19.4	12.3	16.2

The data shown here are limited to those acidic fractions with greater than 30% arabinose. The ethanol fractions are also included in this table. These fractions all contained major proportions of arabinose and galactose together with minor proportions of uronic acid, and a small amount of rhamnose in some fractions. The rhamnose data were not available on some gas chromatography columns because of incomplete separation from unidentified contaminant peaks as well as poor sensitivity associated with low sample sizes.

Binding to the DEAE-cellulose column requires negative charge. In most samples, this will be the charge on uronic acid residues of the pectin backbone, but in some early acidic fractions, the uronic acid content was very low. This may indicate an error in the assay, but because it was low in a several samples it may be that the polysaccharide was covalent attached to a negatively charge protein or phenolic acids. Alternatively, the order of elution of polysaccharides may be related to the degree of methyl esterification rather than total uronic acid content.

3.5.2. Size Separation of Acidic Fractions Using FPLC

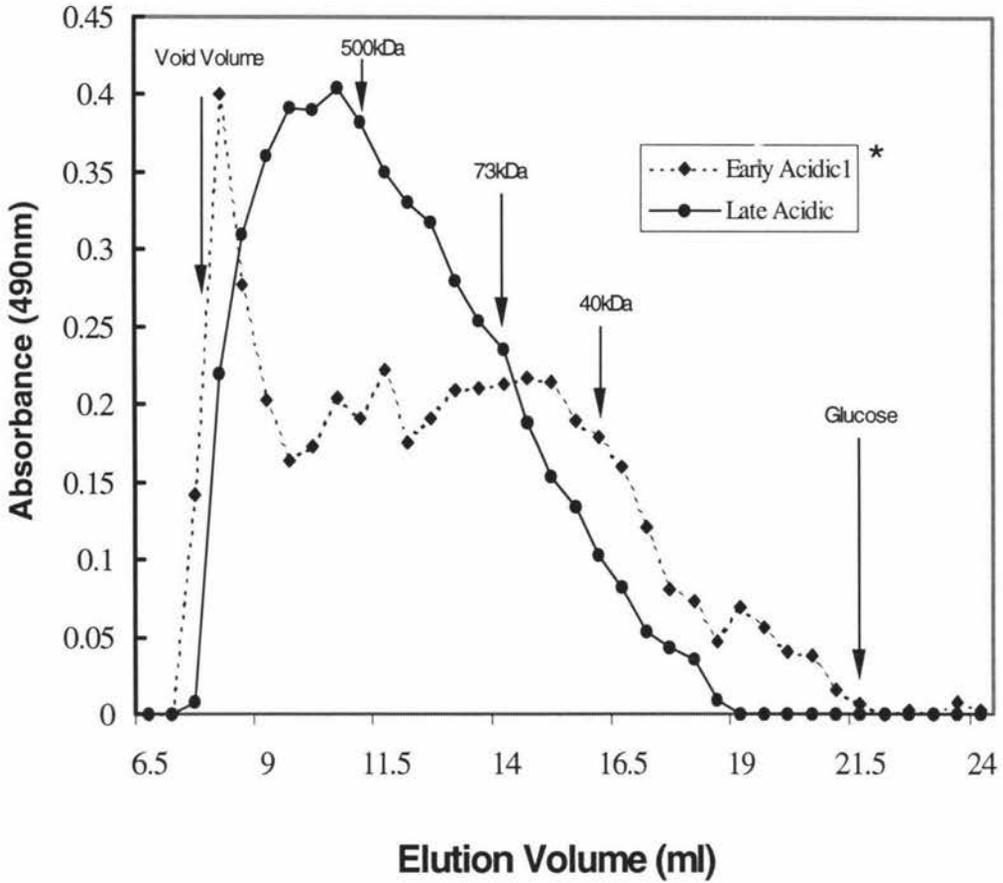
Selected fractions from DEAE-cellulose chromatography were submitted to gel filtration on a Superose 6HR 10/30 column and on a Superdex Peptide column. The fractionation procedure is described in Section 2.4.2. The results are shown in Figures 3.10 to 3.14.



*Early acidic1-Fr. 23, Early acidic4-Fr.26, Late acidic-Fr 35 as on Fig. 3.3

**Figure 3.10 Size Separation of Acidic Fractions A_{2.2}
Using FPLC Superose Column**

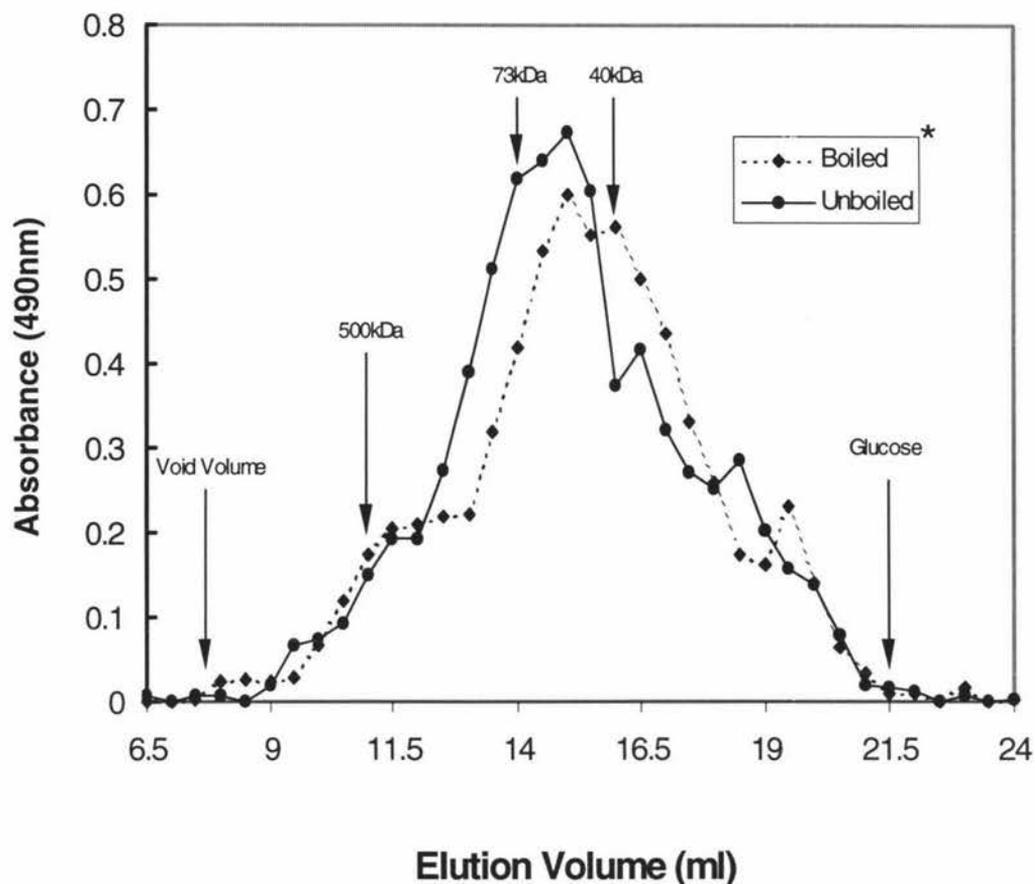
0.5 ml sample was applied to a Superose 6HR 10/30 column; buffer compositions: 50mM NaOAc, 10 mM NaCl, pH 5.0; column flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The automatic fraction collector was used and the column fractions were assayed as described in section 2.4.2.



*Early acidic 1-Fr.25, Late acidic-Fr.34 as on Fig. 3.7

**Figure 3.11 Size Separation of Acidic Fractions B₂₋₁
Using FPLC Superose Column**

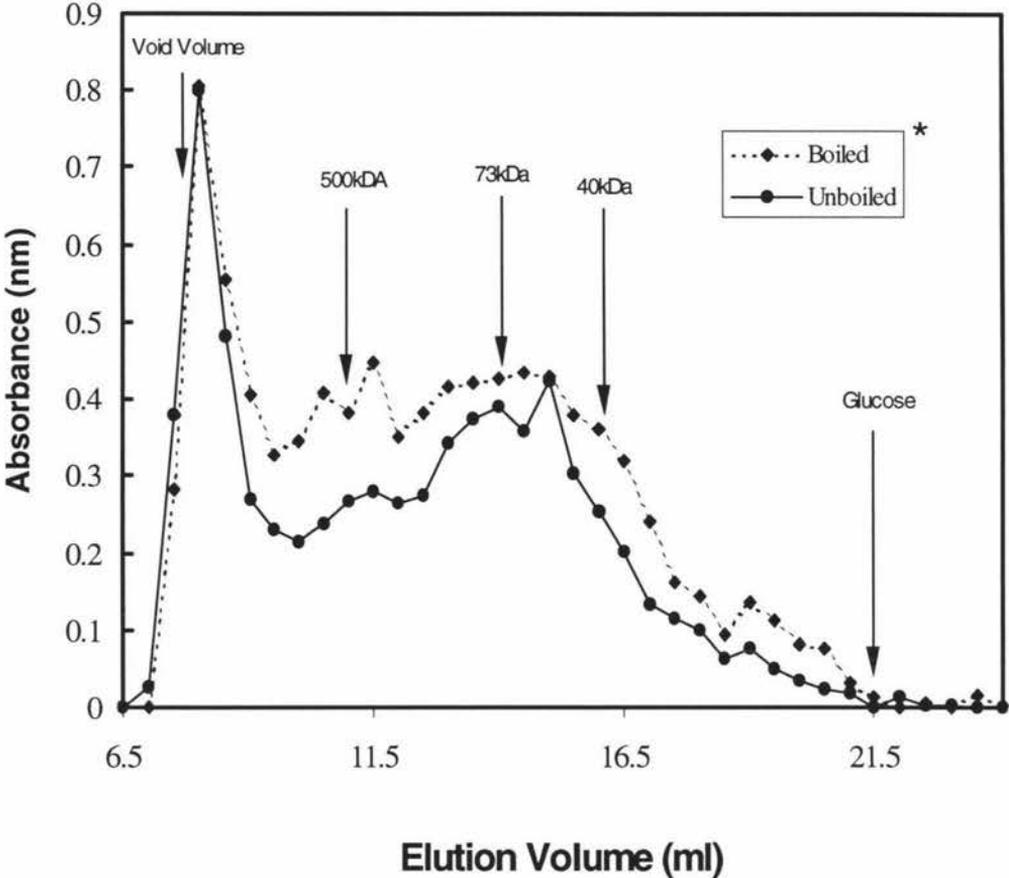
0.5 ml sample was applied to a Superose 6HR 10/30 column; buffer compositions: 50mM NaOAc, 10 mM NaCl, pH 5.0; column flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The automatic fraction collector was used and the column fractions were assayed as described in section 2.4.2.



*Boiled-Fr. 23 (Fig.3.3), Unboiled-Fr. 23 (Fig. 3.6)

Figure 3.12 Effect of Heat Treatment on Size Separation of Acidic Fractions (Fig. 3.3 & 3.6) of Extract A₂ Using FPLC Superose Column

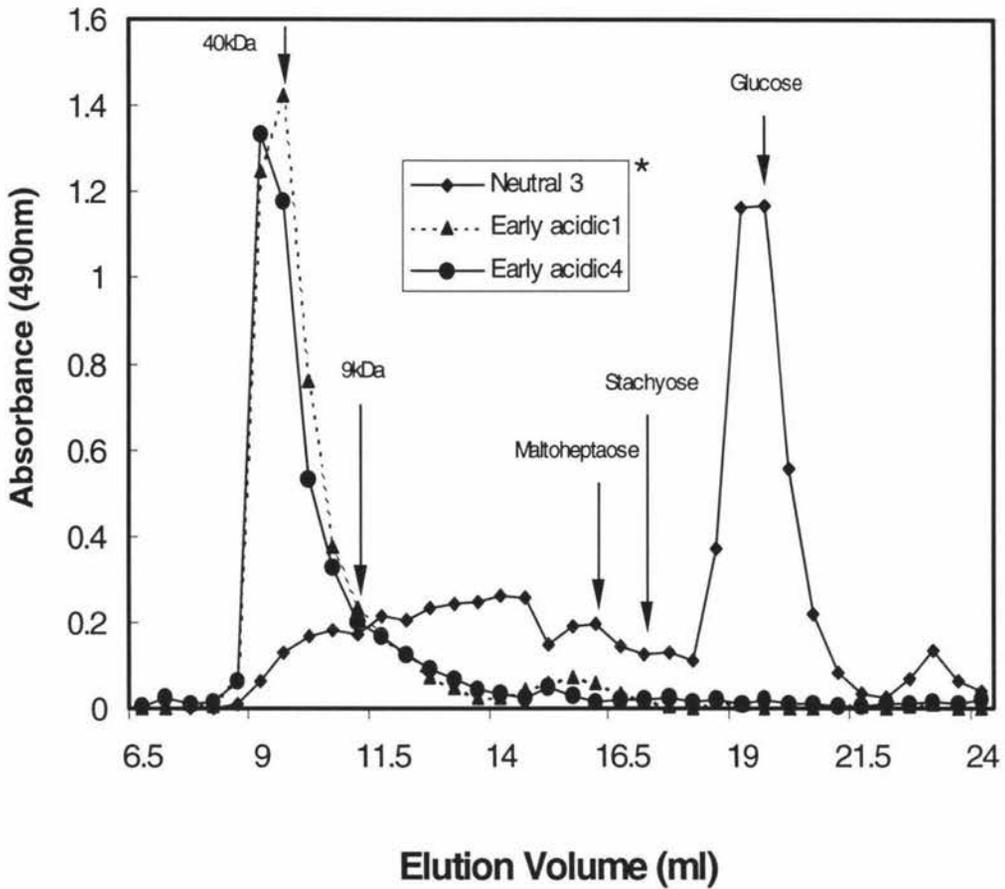
0.5 ml sample was applied to a Superose 6HR 10/30 column; buffer compositions: 50mM NaOAc, 10 mM NaCl, pH 5.0; column flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The automatic fraction collector was used and the column fractions were assayed as described in section 2.4.2.



*Boiled-Fr.25 (Fig. 3.7), Unboiled-Fr.30 (Fig. 3.8).

Figure 3.13 Effect of Heat Treatment on Size Separation of Acidic Fractions (Fig. 3.7 & 3.8) of Extract B₂ Using FPLC Superose Column

0.5 ml sample was applied to a Superose 6HR 10/30 column; buffer compositions: 50mM NaOAc, 10 mM NaCl, pH 5.0; column flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The automatic fraction collector was used and the column fractions were assayed as described in section 2.4.2.



*Neutral 3-Fr. 7, Early acidic1-Fr. 23, Early acidic 4-Fr. 26.

**Figure 3.14 Size Separation of Fractions from A_{2.2} (Fig. 3.3)
Using FPLC Superdex Column**

0.5 ml sample was applied to a Superdex-peptide column; eluent compositions: Pyridine: Acetic Acid: H₂O = 1:1:98; column flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The automatic fraction collector was used and the column fractions were assayed as described in section 2.4.2.

Figure 3.10 shows that two early acidic fractions of extract A₂ had average M_r of about 75 kDa and 50 kDa respectively. On the other hand, the late acidic fraction was of very high M_r, great than 500 kDa. In Figure 3.11, the profiles for fractions from extract B₂ are seem to be markedly different. For this extract, the early fraction (Fr.25) showed two peaks, one above 500 kDa, the other in the range from 40 kDa to 160 kDa. In contrast, the late acidic fraction was smaller than that from extract A₂.

In comparison to extract A₂, the early acidic fractions from extract B₂ had a higher M_r distribution (Figure 3.11 and Figure 3.13) This extract was prepared by rupture of the cells. The higher M_r suggests that the intracellular arabinose-rich polymers are larger than those in the washings (extract A₂) are, but late acidic fractions showed the opposite effect.

A small shift toward lower M_r was apparent on heat treatment of both extract A₂ and extract B₂ (see Figure 3.12 and Figure 3.13), these suggest that the pectin backbone was chopped to a slight extent after heat treatment, therefore, a small decrease in average M_r was seen in heat treated samples. However, the change on heating was not marked. Moreover, the samples are just single fractions, not pooled peaks.

Figure 3.14 shows the results obtained with neutral and early acidic fractions from extract A₂ using the FPLC Superdex column. The early acidic fractions were mostly excluded by the Superdex-peptide column although the M_r of the early acidic 1 fraction appeared to be smaller than that of the early acidic 4 fraction as seen clearly on the FPLC Superose column. On the other hand, the neutral fraction consisted of much smaller molecules than the early acidic fractions. Similar results for early acidic fractions were found with the FPLC Superose column (Fig. 3.10).

3.5.3. Methylation of "Arabinan-rich Fraction"

The principle and the procedure of the methylation analysis were described in section 2.5.

The fraction with the highest Ara/Gal ratio in Table 3.16 was an early acidic fraction (Fraction 57, Figure 3.9) with Ara/Gal ratio of ca.3.2:1 (Table 3.13). This fraction was selected for methylation analysis of the arabinan.

The chloroform-soluble methylated portion was hydrolyzed and derivatized and gave the chromatograms on SP2340, OV225, and BPX70 column as shown in Figure 3.15, Figure 3.16, Figure 3.17 respectively.

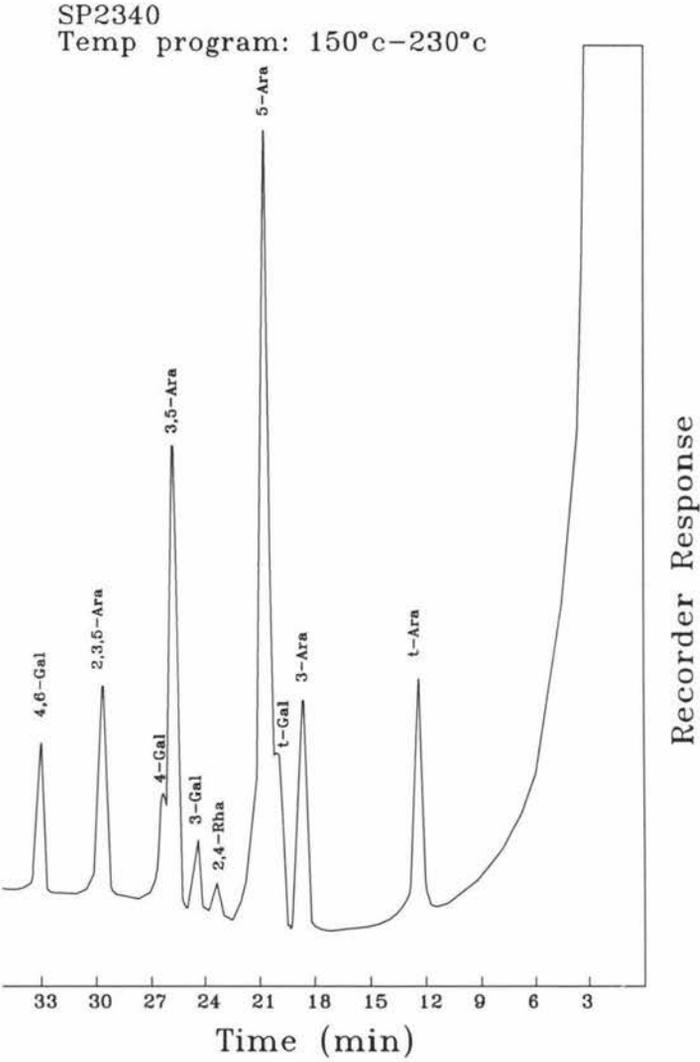
Table 3.17 Linkage Methylation Analysis of Arabinosyl Residues in Early Acidic Fraction from Extract B₂ (*P. radiata* cambium tissues)

Sugar Residues	Deduced Linkage	Composition Mole %*
Arabinose	t-	11
	3-	12
	5-	32
	3,5-	20
	2,3,5-	7
Galactose	t-	8
	3-	4
	4-	4
Rhamnose	2,4-	2

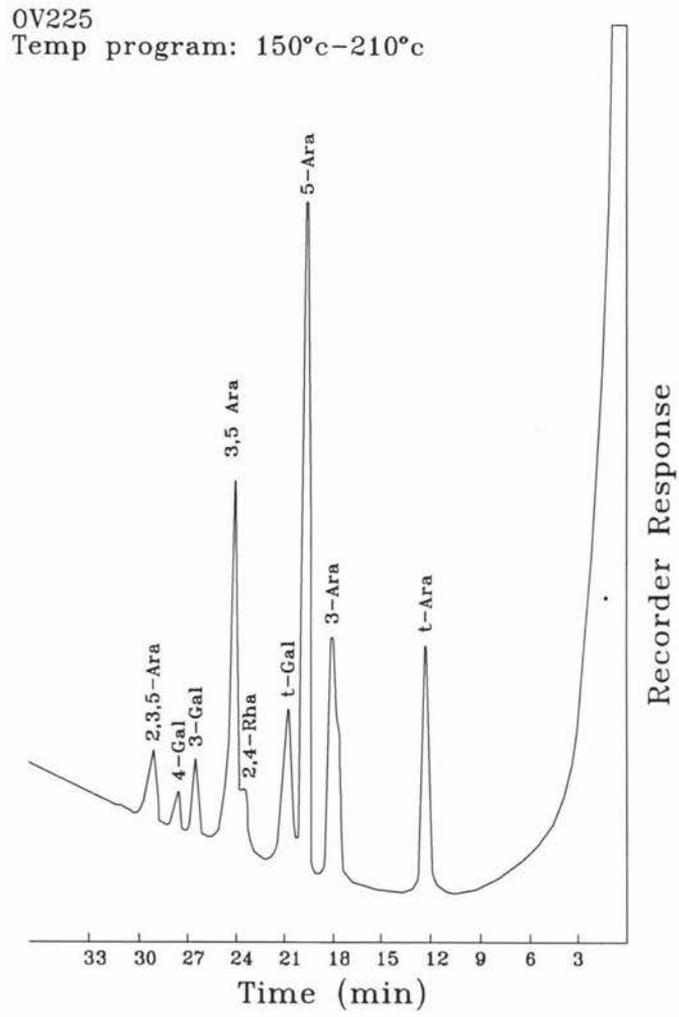
* % of total carbohydrate

The results of methylation analysis of Fraction 57 (Figure 3.9) are presented in Table 3.17. The structural analysis suggested that the arabinose was present as a branched 5-linked arabinan with high levels of 3,5-linked and terminal arabinose residues, but the level of 3-linked arabinose residues was unusual and these residues may be associated with an arabinogalactan or a rhamnogalacturonan.

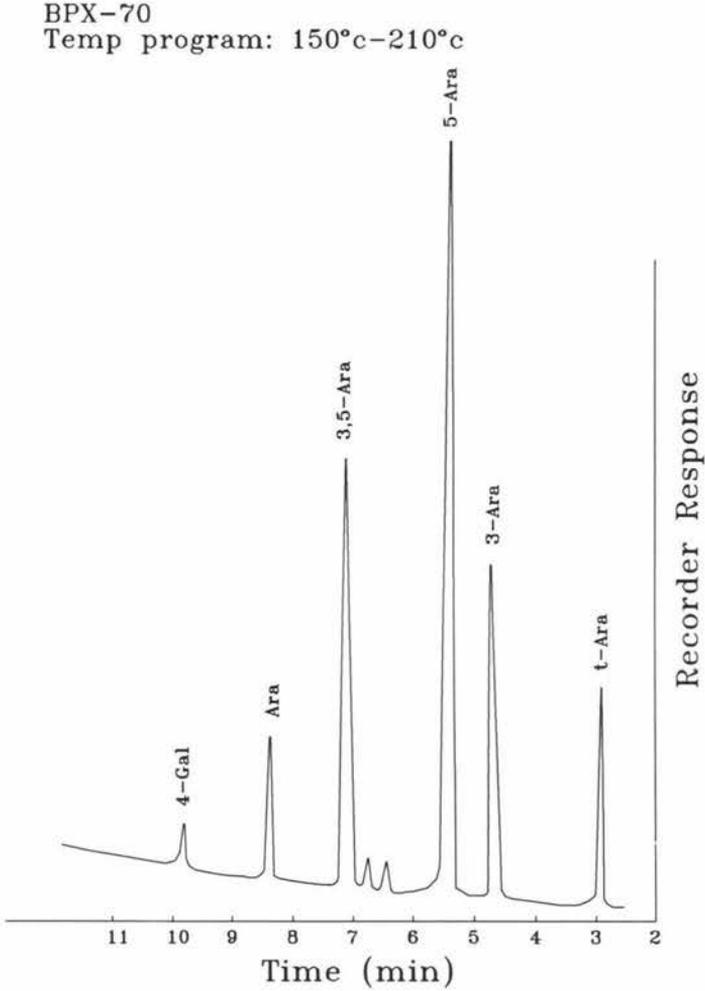
The data for galactose derivatives suggests the presence of a pectic 4-linked galactan, but also shows a high ratio of terminal galactose residues. It may be that some of the galactan residues were terminal on a branched arabinan or arabinogalactan.



**Figure 3.15 Methylation Chromatogram of Fr. 57 (B₂₋₃)
(SP2340 Column)**



**Figure 3.16 Methylation Chromatogram of Fr. 57 (B₂₋₃)
(OV225 Column)**



**Figure 3.17 Methylation Chromatogram of Fr. 57 (B_{2.3})
(BPX70 Column)**

4. Chapter Four: Discussion and Conclusion

There are three major components to this thesis. First, the water-soluble pectic polysaccharide material from *P. radiata* cambium tissue was extracted with cold phosphate buffer, and then the extracts were dialysed. Secondly, the dialysed sample was further purified using ion exchange chromatography on a DEAE-cellulose column (carbonate form), and a neutral polysaccharide and a number of acidic fractions were obtained respectively from the gradient elution with ammonium bicarbonate. Thirdly, structural studies were conducted on the arabinan using three major methods: quantitative monosaccharide analysis, size separation of acidic fractions using FPLC and methylation analysis.

4.1. General Discussion

Arabinose and galactose are major components of two types of polymer in higher plants.

The pectic polymers of all higher plants contain predominantly arabinose, galactose, and rhamnose as neutral sugar components. Pectins (rhamnogalacturonans), in primary cell walls, have a backbone of galacturonic acid and rhamnose and contain side chains of arabinose and galactose, in which the arabinan is predominantly 5-linked, while the galactan is predominantly 4-linked.

Arabinogalactans and arabinogalactan proteins are also widely distributed in higher plants (Clarke *et al.*, 1979; Fincher *et al.*, 1983). Arabinogalactan proteins are proteoglycans, whose polysaccharide side chains contain predominantly 3,6-linked galactose residues and terminal arabinose residues (Fincher *et al.*, 1983).

The arabinans in primary cell walls of some higher plants have been isolated and investigated structurally. These arabinans, whether occurring as sidechains to pectins or as polymers associated with pectin, mostly consist of (1→5)-linked α -L-arabinofuranosyl residues. No function has been assigned to arabinans, so it was of interest to study the arabinan in more detail. The arabinan found in *Pinus pinaster* by Roudier and Eberhard (1965) was predominantly (1→5)-linked with branching principally at C-3 but also at C-2. Side branches were both single residues and Ara-

(1→3)-Ara→, disaccharides. Fu and Timell (1972) observed a similar arabinan in the walls of phloem of Scots pine. Thus, these arabinans resemble the typical dicot pectic arabinans such as rapeseed arabinan (Siddiqui and Wood, 1976). A highly branched arabinan has also been found as part of the pectic fraction from *Pinus radiata* callus tissue (Andrew *et al* 1999). The linkage composition of this fraction suggests a highly branched arabinan or arabinogalactan, with relatively high content of 3-, 5- and 3,5-linked arabinose residues. The galactose residues are mainly 4-linked. The GalA/Rha ratio of the fraction was about 2.5, consistent with its identification as a rhamnogalacturonan I, with neutral side branches linked through the 4-position of rhamnose.

Preliminary work suggests that, in the arabinans from *P. radiata* cambium, unbranched 5-linked arabinan residues predominated, but these were more highly branched than the arabinan isolated from *Pinus pinaster* wood, with a much higher proportion of 3- and 3,5-linked residues (Andrew *et al.* unpublished).

Results presented in this thesis show that most of the water-soluble arabinan from *P. radiata* cambium tissue can be isolated as an early acidic fraction by DEAE-cellulose chromatography, separating from a late-eluting fraction which is probably mostly homogalacturonan. In each of the acidic fractions, arabinose and galactose are the principle neutral monosaccharides. The purified fractions have about 55 to 70% arabinose with smaller amounts of galactose. Much of the galactose may be present as terminal residues on the arabinan. Protein accounts for about 10-15%, which may be present as backbone linked to these polysaccharides. However, it remains unknown whether the protein and polysaccharide exist as independent molecules or as a single molecule.

These early acidic fractions have low uronic acid and rhamnose content, and the ratio of branched rhamnose to arabinose and galactose residues suggests an average structure of about 50 to 60 residues per rhamnose. FPLC gel filtration chromatography indicated that the polysaccharides were polydisperse with apparent molecular weights in the range from 30 to 500 kDa according to dextran standards. This could indicate a structure with about 180 to 3000 sugar residues. However, the distribution of the polymers within this range appeared to be different for each polysaccharide fraction and different treatment. These results suggest block structures with many side chains. It

is also possible that there are polymers differing in molecular size distribution, which may be in close association with various amounts of proteins.

The proportions of different linkages obtained from the methylation analysis of an early acidic fraction (Fraction 57, Fig. 3.9) were given in Table 3.17. In agreement with monosaccharide analysis, arabinose was the predominant sugar, mainly present as 3-,5-, 3,5-, and terminal linked residues. Galactose was mainly present as terminal residues. The result obtained from the methylation analysis was slightly different from that found in a previous study (Andrew and McKee, unpublished).

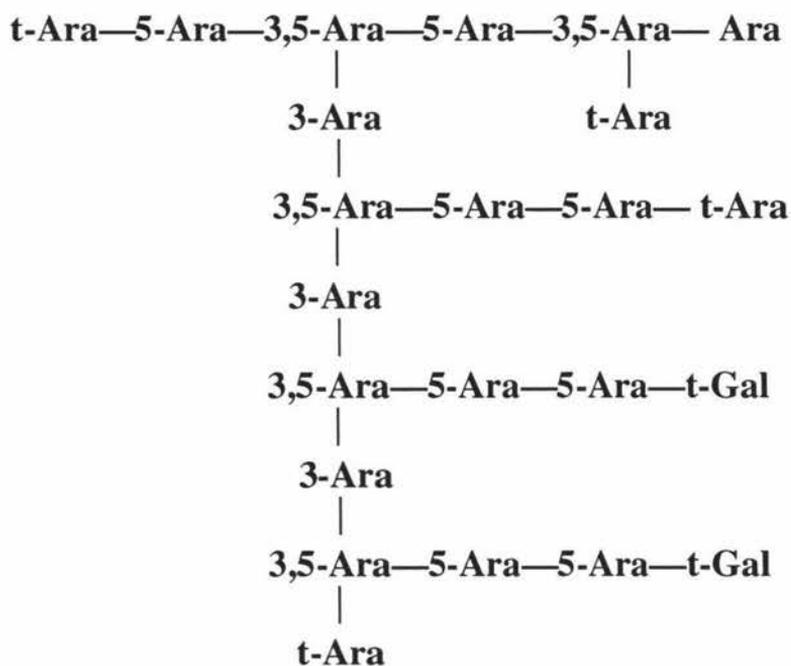


Figure 4.1 Hypothetical Structure of Arabinose-rich Molecules from *P. radiata* Cambium Tissue

On the basis of the relevant results from both the analysis of monosaccharide composition and methylation analysis, a schematic model of the neutral sugar chains was derived (Figure. 4.1). The neutral sugar chains from the acidic fraction are mainly composed of arabinan, terminal arabinose and galactose. Arabinan chains are tentatively shown to be attached directly to rhamnose residues of rhamnogalacturonan I but they may alternatively be associated with protein. 2,3,5- arabinose was disregarded in this schematic model because it may be a result of incomplete methylation.

4.2. Conclusion

To conclude, this thesis was written in an attempt to describe a highly branched water-soluble polysaccharide, of which the main structural feature is an arabinan fragment. Individual observations are summarized as following.

- Soluble arabinose-rich polysaccharide fractions were obtained (A) from washing of frozen and thawed *P. radiata* cambium tissue, and (B) from subsequent French press extraction.
- Ion-exchange chromatography of the washing and French press extract yielded an acidic arabinose-rich fraction. These polymers bound weakly to DEAE cellulose at pH 8, separating clearly from neutral and strongly acidic polymers.
- The intracellular arabinose-rich polymers obtained by French pressing (extract B, up to >500kDa) were on average much larger than those in the initial washings (extract A, mostly ca. 40-100 kDa).
- Treatment of the extracts with boiling water caused a decrease in binding of the arabinose-rich polymer to the DEAE cellulose reflected by earlier elution, and a minor decrease in average molecular size, consistent with β -elimination of the pectic backbone.
- Methylation analysis suggested a highly branched arabinan structure and the presence of terminal galactosyl residues on a branched arabinan core.

- In the arabinans from *P. radiata* cambium tissue, unbranched 5-linked residues predominated, but they were more highly branched than the arabinan isolated from *Pinus pinaster* wood, with a much higher proportion of 3- and 3,5-linked residues.

4.3. Suggestions for Future Work

The work in this thesis involved mainly the purification and structural characterization of the arabinan from *P. radiata* cambium tissue. It also provides some interesting avenues for further investigation on the arabinan structure and function in *P. radiata* cambium tissues.

An important work for future investigation is to study the distribution of galactose residues, which will be investigated by treatment with β -galactosidase. Preliminary works in our laboratory suggest that the galactose residues may be less accessible to methylation than the arabinose residues (Andrew, *pers. comm.*).

Further studies, principally involving enzymatic breakdown of the polysaccharide, change in conditions for DEAE-cellulose chromatography, NMR spectroscopy, and electrospray mass spectrometry are required to elucidate whether and how the arabinan fragment described here is linked to the galactose residues and linked to the proteins.

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