CHARACTERIZATION OF A CROSSLINK BETWEEN XYLOGLUCAN AND RHAMNOGALACTURONAN FROM COTTON CELL WALLS

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LIST OF ABBREVIATIONS

ANTS	8-Aminonaphthalene-1,3,6-trisulphonate
APTS	9-Aminopyrene-1,3,6-trisulphonate
AGP	arabinogalactan protein
ara	arabinose
CZE	capillary zone electrophoresis
DEAE	diethylaminoethyl
EG	endoglucanase
EPG	endopolygalacturonase
fuc	fucose
gal	galactose
galA	galacturonic acid
glc	glucose
GRP	glycine-rich protein
HG	homogalacturonan
HyPro	hydroxyproline
kD	kilodalton
MALDI-TOF	matrix assisted laser desorption/ionization time of flight
MS	mass spectrometry
man	mannose
NMR	nuclear magnetic resonance

PRP	proline-rich protein
rha	rhamnose
RG	rhamnogalacturonan
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
XET	xyloglucan endotransglycosylase
XG	xyloglucan
XGA	xylogalacturonan
xyl	xylose
µl	micro liter
µg	micro grams
mg	milli grams
min	minutes
mL	milli liter
mM	milli molar

CHAPTER I

INTRODUCTION

The dynamic plant cell wall serves as an extracellular matrix which determines the shape and size of the cell by maintaining the rigidity of the cell (1). It is one of the most vital plant components with varied physical and chemical properties. It plays various roles ranging from i) providing the support and mechanical strength to the cell, ii) acting as a barrier against microorganism invasion thus avoiding injury and infection of the cell (1), iii) maintaining the differentiation of the cell and tissue during cell growth, iv) metabolic role such as transportation, secretion and cell signaling (1, 2) and v) producing defensive chemicals against infection caused by bacteria or fungi, hence displaying the plant disease resistance mechanism (3). All these unique properties make cell wall a crucial subject to be explored in detail at the structural, functional and molecular level.

Plant cell walls are distinguished as primary cell wall and secondary cell wall which differ in their composition and functions. Primary cell wall is thin, semi-permeable and is formed during the early stages of the cell growth. It is mechanically stable, flexible to accommodate the enlargement and elongation of the cell and easily extensible in a hypotonic medium avoiding the rupture of cell membrane. Secondary cell wall is laid down next to the primary cell wall after the cell growth is completely ceased. Primary

cell wall is mainly composed of polysaccharides in large amounts and proteins in very small amounts. The polysaccharides are distinguished into cellulose, hemicelluloses, and pectin present in varying amounts. Primary cell walls have less cellulose and higher pectin, whereas, it is vice versa in secondary cell walls. The secondary cell wall along with polysaccharides contains lignin, which provides more strength and mechanical support to the cell.

Cell wall polysaccharide components

Cellulose is the most common carbohydrate present in the plant cell wall. It is found in secondary walls accounting for nearly 40- 45% of total dry weight of cell wall whereas it is 20- 30 % of total dry weight in primary cell walls (4). It is an unbranched polymer having β-1-4-D glucan residues which aggregates into microfibrillar structures ranging from 5µm – 7µm in length and 30nm in width. These microfibrils are fomed by the hydrogen bonding between the hydroxyl group of the glucose residue and oxygen of the same or neighboring chain. It is considered that the cellulose synthesis requires β-1-4-D glucan synthases or cellulose synthase complex (CesA) forming a rosette like structure having 6 subunits arranged hexagonally giving rise to nearly 36 parallel cellulosic polysaccharide chains. These chains aggregate by forming hydrogen bonds between intra and inter cellulosic chains generating the single cellulose microfibril (5, 6, and 7). Such a cellulosic microfibril acts as a skeletal frame work for the cell, giving it shape and strength (4). The cellulosic chains of primary cell walls are high in molecular weight, highly crystalline and oriented parallel to other cellulosic micro fibrils. The cellulosic chains of the secondary cell walls are also high in molecular weight, but they consists two different forms of cellulosic chains, one showing parallel orientation whereas, the other

one is rare form which shows the antiparallel parallel orientation to other cellulosic chains. (8, 9, and 10).

Hemicelluloses are heteropolymeric polysaccharide present in most of the plant cell walls. The major types of hemicelluloses includes xyloglucan (XG) contributing around 20% of the total dry weight of primary cell wall and glucuronarabinoxylans contributing around 20% - 35% of the total dry weight of secondary cell wall (4). Minor types of hemicelluloses includes mannans (glucomannan, galactoglucomannan), xylans (arabinoxylan, glucuronoxylan), and arabinogalactan. Usually it is seen that hemicelluloses are most abundant in secondary cell walls of both monocot and dicot plants as compared to primary cell walls. Hemicelluloses have β-1-4-D glucan residues or β-1-4-D xylan as a cellulosic-like backbone branched with different sugars such as xylose, galactose, mannose, rhamnose, and arabinose. Hemicellulosic polymers are easily solubilized by alkaline solvents. They do not aggregate forming microfibrils but the backbone can interact with cellulose and hence it is also called as ‘cross-linking glycan’.

Xyloglucan (XG) is one of the most abundant hemicellulosic polysaccharides accounting for nearly 20% of total dry weight of dicot cell walls (11, 12) and 2% - 5 % of total dry weight of monocot cell walls (13, 14, 15). XG has a β-1-4-D glucan residue backbone with α-1-6-D xylose side chains attached to the 0-6 position of the glucose residues (11). Some of these xylose side chains are sometime altered at O-2 position with galactosyl or fucosyl-galactosyl residues. Glucose residues of β-1-4-D glucans are O-acetylated sometimes. A nomenclature of XG denoting the distribution of side chains was proposed by Fry et al (16). Typically three consecutive glucose residue C-6 positions are substituted by xylose moieties forming the ‘XXXG’ structure, where X represents a

xylose branch and G represents an unbranched glucose residue. The second and third xylose residues of 'XXXG' may get substituted at C-2 position by a terminal β-1-2-D galactosyl residue represented by L or β-1-2-D galactosyl linked with α-1-2-L fucosyl residue represented by F, forming different XG subunits such as XXLG, XXFG, and XLFG. Schematic structures of XG subunits are shown in Figure 1a. Regardless of the structural variability seen in various species, it is considered that the role of XG in plant growth and development is maintained throughout all the species. It functions as a structural backbone in the cellulose-XG network, an energy supply for plant seeds (17, 18), and a fucosylated xyloglucan acts as a signaling molecule during the expansion and growth of cell walls initiated by auxin (19, 20, and 21). But recently while studying *Arabidopsis* mutants, results showed that the xyloglucan with galactose side chains along with rhamnogalacturonan II (RG II) plays a major role in wall strengthening as compared to the fucosylated xyloglucan (22).

Pectin is highly complex in structure as well as function. It is a major component of the primary cell walls accounting for nearly 30 - 35% of total dry weight. Pectin plays an important role in expansion, elongation and shape of the cell, growth and development of the entire plant, avoiding the cell wall rupture under turgor pressure and maintaining the wall porosity for cell-cell adhesion, signaling with the help of enzymes present in the cell wall, e.t.c. Pectins are characterized by the presence of higher amounts of galacturonic acid along with a few other sugars like arabinose, rhamnose, galactose and xylose in lower concentrations. Pectic polysaccharides are classified into five different structural classes including homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II), xylogalacturonan (XGA), and apiogalacturonan (AP).

These structures are grouped into 2 different groups called smooth regions (HG), and hairy region (branched with side chains RG I, RG II, XGA) (23).

Homogalacturonan (HG) is one of the simplest pectic regions which accounts for nearly 60% of pectic regions in the plant cell wall. It is a polymer composed of α-1-4-D galactosyluronic acid residues (24). Usually most of the carboxyl groups of the galA residues have undergone methylesterification (25) whereas the hydroxyl groups can undergo acetylation at O-2 and O-3 position (26, 27). The unmethylated HG blocks (nearly more than 10 continuous residues) form rigid, stable and insoluble calcium-pectate gels in the presence of calcium ions accounting for nearly 70% of the pectic gel present in the plant cell wall (28, 29). Figure 1.2 shows the schematic structure of HG.

Rhamnogalacturonan I (RGI) accounts for nearly 20-35% of pectic polysaccharide. It has a backbone made up of nearly 100 repeating disaccharide units (1-2)-α-L-rhamnosyl-(1-4)-α-D-galacturonic acid (1,30,31). Usually the galA residues of the RG I backbone undergo acetylation at O-2 and O-3 position (32), whereas rha residues of RG I backbone undergoes substitution at C-4 with different neutral sugars such as ara and gal (33). These side chains mostly include β-1-4-D-galactan, α-1-5-L-arabinan with many α-1-3-arabinose or arabinan branches, arabinogalactan I and arabinogalactan II (17, 34). Arabinogalactan I sidechain typically includes β-1-4-galactan along with α-1-3-arabinose branches, whereas arabinogalactan II sidechain includes β-1-3-galactan along with β-1-6-galactose or α-1-3-arabinose branches (35). RG I has different side chains like arabinan and galactan according to the differential stage of cell growth. Experimental results in transgenic plants expressing rhamnogalacturonan lyase cleaving the RG backbone shows a significant influence on plant growth and development (36). RG I is proposed to act as

a bridging molecule between different pectins such as RG II, HG, in form of sidechains. But, the final structure of all these connections is still a question. Figure 1.2 shows a schematic structure of RG I.

Rhamnogalacturonan II (RG II) is the most complex pectin as compared to all other pectic polysaccharides. It accounts for nearly 5% of the pectic polysaccharide. Though RG II is structurally most complex, still its structure is highly conserved in all the plant species suggesting its importance in the wall function (37). RG II and RG I seem to be of similar type, but structurally and functionally they are different from each other. RG II comprises of an HG backbone of around 9 α-1-4-D galactosyluronic acid residues with four complex branches denoted from A-D having 12 different sugars arranged in 20 different ways at O-2 or O-3 position of galA residues. Unusual sugars linked to RG II include 2-O-methyl L-fucose, 2-O-methyl D-xylose, D-apiose (38), 3-C-carboxy-5-deoxy-L-xylose (L-aceric acid) (39), 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) (19), 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha) (40). Sidechain A and B are very complicated oligosaccharides and are attached to the backbone via a D-apiose residue, whereas the Kdo and Dha containing disaccharide are less complicated, forming sidechain C and D, respectively. RG II forms dimers by self association via boron-diester bond formation between the apiosyl residues of side chain of two RG II molecules. And this dimerization of RG II via boron is significant for various roles such as strengthening of the cell wall, plant growth and development (37, 41, and 42).Figure 1.2 shows the schematic structure of RG II.

Xylogalacturonan is generally ignored as a minor type of pectic polysaccharide. XGA has a backbone substituted at O-3 position of galA residue with a non-reducing

terminal xylose moiety and sometimes this xylose residue is linked with one more xylose at O-4 position (43, 44). The amount of xylosylation of the HG in XGA varies from species to species. Figure 1.2 shows schematic structure of XGA.

Apiogalacturonan (AP) is present in aquatic plant cell walls (reported first in duckweed) (45). It has an HG backbone with β-apiose sometimes on both the 2 and/ or 3 position of α-1-4-D galactosyluronic acid.

Cell Wall proteins Along with different types of polysaccharides, cell walls also contain structural proteins and enzymes playing a crucial role in different functionalities of the cell wall such as, growth, development, extensibility, signaling, protecting against pathogens, and environmental abnormalities like stress, drought, and many more. Five different groups of cell wall proteins include: extensins, glycine-rich protein (GRP), proline-rich proteins (PRP), arabinogalactan proteins (AGP), and solanaceous lectins. All the above proteins except GRP belong to hydroxyproline-rich glycoprotein (HGRP) family. Extensins are the most abundant and widely studied proteins comprised of the characteristic repeating sequence of Ser $(HyPro)_4$. They are involved in the extension growth of the cell walls and hence named as extensins (46, 47). Higher accumulation of extensin is seen in the cell walls in response to mechanical stress, attack by pests, oligosaccharide elicitors or ethylene (48). GRPs are generally divided into 2 classes, one localized in vascular tissue of the plant and other localized in the cytoplasm. They are characterized by the presence of repetitive structure containing around 70% of glycine in form of Gly-X where X can be glycine and sometimes alanine or serine (49). GRPs present in vascular tissue are thought to have easy passage from xylem to protoxylem and are proposed to take part in the repair system during the elongation of protoxylem (50).

GRPs present in the cytoplasm deal with wound healing and drought tolerance. PRPs are subdivided into 2 classes and are characterized by the Pro-Pro repeats along with a few other amino acid repeats. One subclass of PRPs is localized in the extracellular matrix of the plant cell and plays an active role in strengthening and development of cell wall (17, 51). The other subclass of PRPs is the plant nodulins, which are produced in response to the infection when nodules are produced by nitrogen fixing bacteria (52). AGPs are localized in the extra cellular matrix of the cell. Mostly they play a structural role by forming a cushion between the cell wall and plasma membrane, and sometimes they are also involved in cell-cell recognition (48). Along with these proteins there are numerous enzymes including polysaccharide degrading enzymes such as endoglucanase, pectinase, and cellulase, enzymes involved in cell wall modification such as esterases, xyloglucan endotransglycolyases (XETs), and expansins. Expansins are involved in loosening of the cell wall mediated by the acidic condition, forming the space between the XG and cellulosic microfibrils of the cell and allowing water uptake due to wall stress relaxation causing the cell expansion.

Cross-linkages between the cell wall polymers

All the polymers of the cell walls explained above can exhibit some kind of cross-linkages between each other as was reviewed by Fry et al. (53). The linkages present between the polymers might include, a) Hydrogen bonds between cellulosic and hemicellulosic polymers, b) ionic bonds between pectins and proteins, c) formation of calcium bridge between pectic polysaccharides especially HG polymers, d) apiosyl borate ester bridges between the two RG II, e) glycosidic linkages between pectin and hemicellulosic (XG) residues, between pectins e.g. RG I and HGRPs (e.g. Extensin), f)

ester linkages between the COOH group of galA acid and the OH group of other polymers, g) phenolic couplings such as formation of intramolecular isodityrosine bridge in HGRPs (e.g. Extensin), and formation of diferulate bridges between feruloylated sugars and h) formation of amide linkages between pectins and proteins as postulated recently by Fry et al. All these linkages have been studied extensively but few are yet to be supported by the definite experimental results.

Brief overview of Cell wall models

The first model was proposed by Keegstra *et al.*, in the early 1970's, while studying the connection between the different polysaccharide components of sycamore cell walls. They postulated that xyloglucan was covalently linked to pectic components via arabinan or arabinogalactan and adsorbed non-covalently on the cellulosic surface forming a monolayer. However, in recent models the possibility of XG being linked to pectic components is disregarded due to lack of experimental evidence (54, 17). The models use a two or three network cell wall model of the growing plant cell wall, in which the cellulose-xyloglucan load bearing network is embedded in a pectin matrix (17). Pectin regions are either shown as unlinked (54), or in a secondary network interacting via calcium bonds as shown in the egg-box model (55), or just present between the less esterified HG regions (17). The third network they considered was between pectins and cell wall proteins. But in last few years some evidences has shown pectins being covalently crosslinked mostly to hemicelluloses (e.g. XG or xylans).

Cellulose-xyloglucan network

Cellulose and xyloglucan, the important load bearing structural components of the plant cell, form an extensive hydrogen bonded network called cellulose-xyloglucan

network. There have been different views on this network. Earlier it was hypothesized that the crystalline cellulosic microfibrils are encapsulated by almost 65% of water (56), along with matrix formed by the combination of hemicelluloses, pectins and glycoproteins (12) together forming a network in the cell, 2) the current model postulates the presence of the hemicellulosic (XG or arabinoxylan) interconnections between the cellulosic framework forming the cellulose-hemicellulose network (17). Cellulosic microfibrils are usually 20-40 nm apart from each other and XG are usually 700 nm long. There are chances of one single XG to span and interlink several adjacent microfibrils, which was experimentally supported and was captured using electron microscopy (57, 58). Such cross-links may restrict the free movement of cellulosic microfibrils giving structural rigidity and strength to the cell wall. Hence all these findings lead to the recent view on this network which states that the surface of cellulosic microfibrils is coated with xyloglucan, along with additional cross-links of XG forming a lattice like structure. The binding of XG to the cellulosic microfibrils takes place by 3 distinct domains of XG. The first domain which is supposed to be strongly bound to cellulosic microfibrils is soluble and extractable only by strong alkaline solutions. The second domain which is supposed to be loosely bound to cellulosic microfibrils forming the cross-linking tethers is susceptible to the enzymatic treatment e.g. endoglucanase. The third domain which is supposed to be entrapped between the cellulosic microfibrils is neither solubilized by strong alkali nor by enzyme treatment (59). The side chains of XG are thought to be moderating its binding to the cellulosic microfibrils. With the use of conformational dynamics simulation of *in vitro* binding assays of pea cell walls, it was considered that the fucosylation of the side chains of XG makes XG backbone straight in conformation,

thus increasing its accessibility to microfibrils (60). Hence these differential linkages of XG to cellulosic microfibrils make this network very unique in maintaining the structural integrity and regulating the mechanical properties of wall.

Pectin network

Literature has repeatedly shown that when the cell walls are treated with endopolygalacturonase (EPG) it degrades the non-methylated HG, and yields a mixture of RG I, RG II. Requirement of such a treatment to isolate the pectic components from each other, as well as from the walls, indicates the presence of covalent linkage between the pectic polysaccharides. There is no definite answer as to how these pectic components are exactly linked to each other, but available data helps us in creating a tentative model which suggests HG and RG II are linked to each other via their backbones, and are side chains on RG I extending out perpendicularly (61, 62). Glycosidic linkage between these pectic components connecting them through the backbone is just one possibility. Other linkages include calcium cross-links between HG, boron ester cross-linking between RG II forming dimers, and covalent cross-linkages with phenolic and other compounds. HG has a self associating property depending on the degree of methylesterification. Methylesterification can be controlled by the enzyme pectin methylesterase (PME), which influences the formation of pectic gel. Pectic gels are formed due to the formation of the calcium bridges between the de-esterified HGs generating the expanded junction zone which prevents the free passage of wall proteins (28, 29). RG I backbone has rhamnose which is sometimes branched by arabinan, galactan or arabinogalactan which may cross-link with other wall polymers like XG, xylans, lignin and proteins. RG II molecules dimerises by the formation of borate diester bonds linking the apiosyl moiety

present in sidechain and thus forming a structure which strengthens the cell wall (37, 41, and 42).

Pectins do crosslink with hemicellulose, wall proteins and phenolics adding more structural and functional complications to the cell wall. A linkage between XG and pectin was hypothesized in the first cell wall model given by Keegstra *et al*. Sometimes it is seen that sequential extraction of suspension cell walls by chelating agents and strong alkali solubilizes only a low amount of pectin and XG, but if an EPG pre-digestion is followed by strong alkali extraction then there is a possibility of getting a good amount of pectin-XG complex solubilized. This pectin-XG complex can be co-eluted by an anion exchange column, and on treatment with endoglucanase enzyme it digests most of XG but not RG (63, 11). All this evidence points to the possibility of covalent cross-linkage existing between pectin and XG. To investigate the linkages between these regions is a big challenge due to its complex structural heterogeneity and presence of various domains. Use of cloned enzymes with high specificity may help in resolving the mystery of the connecting element and thus giving a better understanding of the cross-linking between the pectic regions with hemicelluloses and its architectural framework in the cell wall.

AIM AND OUTLINE OF THESIS

The aim of this project was to determine and characterize the crosslink present between XG-RG. The presence of cross-links connecting different wall polymers, such as cellulose/xyloglucan network and pectic network may have significant effect on physical properties of cell wall which directly or indirectly regulates the cell growth. Gaining knowledge and insight about these cross-links would be beneficial to understand

architecture and functionality of the whole cell wall. Preliminarily results during isolation of XG-RG complex from intact cell wall illustrated that strong alkali extraction (24% KOH and 0.1% $NaBH_4$) of EPG pretreated cell walls solubilizes around 80-90% of the RG and XG present in the cell wall (64). Evidences shows the presence of two types of XG, unlinked XG and XG covalently crosslinked to RG I (chapter 2). Various chromatographic techniques were used for isolation XG-RG complex. Chapter 3 illustrates the use of cloned enzymes with specifically acts on XG-RG complex to determine the crosslink. Enzymes used for degradation were arabinosidase, arabinanase, endoglucanase. Fragments produced after the enzymatic degradations were analyzed using mass spectrometry, and NMR spectroscopy. Arabinosidase treatment does remove the arabinan side branches. Along with free arabinose, unlinked XG also elutes out as a non adsorbed fraction when applied on PA1 column. The removal of unlinked XG is not a result of arabinosidase degradation, hence showing the probable chances of XG being non-covalently associated to pectic regions. Arabinanase treatment does cleave the α-1-5 linked arabinofuranosidic bonds present in polymers containing arabinan. This enzymatic degradation does dissociate a significant quantity of XG from RG illustrating arabinan as a major crosslink between XG and RG.

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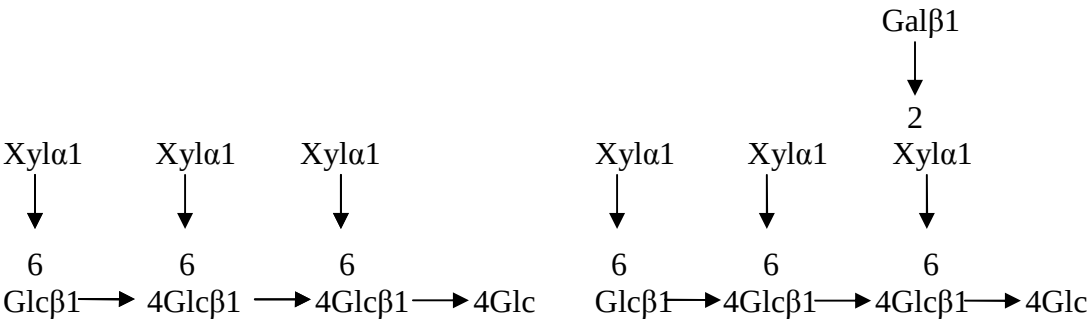
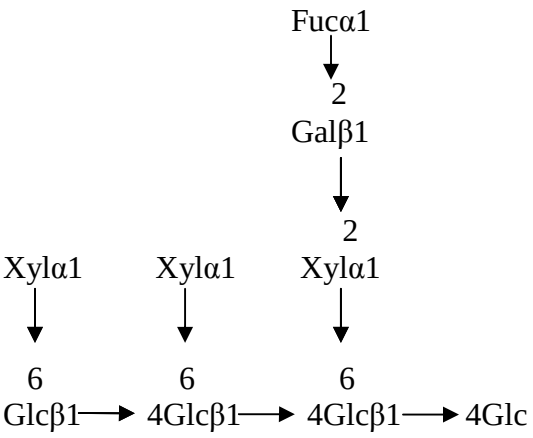
Figure 1.1 Schematic structures of xyloglucan (XG) subunits. (Subunits are designed based on nomenclature proposed by Fry et. al. In the figure G represents unbranched glucose, X represents xyl, L represents gal-xyl and F represents fuc-gal-xyl.

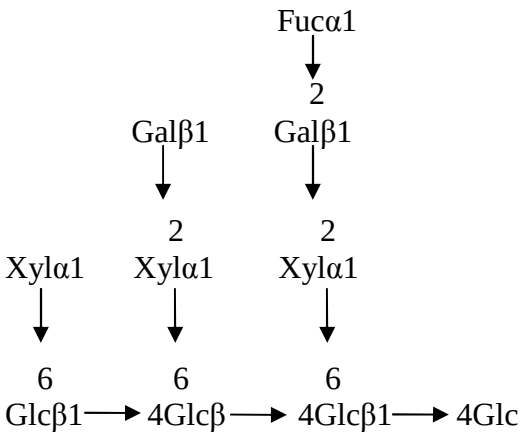


XXXG

XXLG

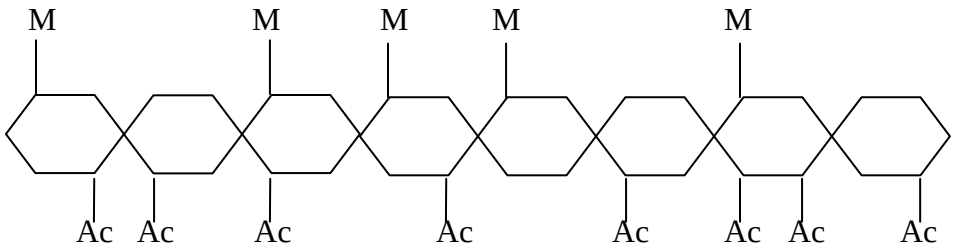


XXFG

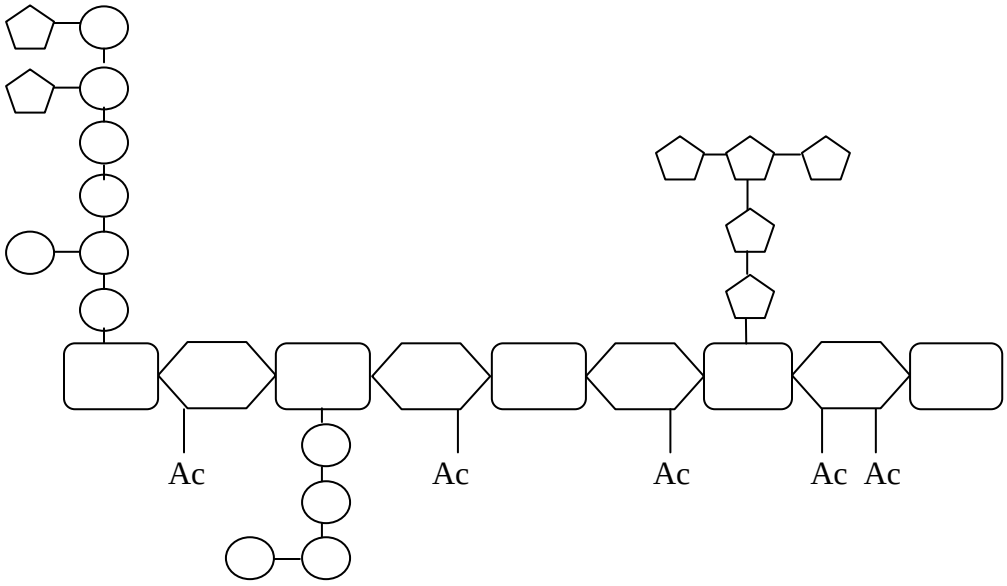


XLFG

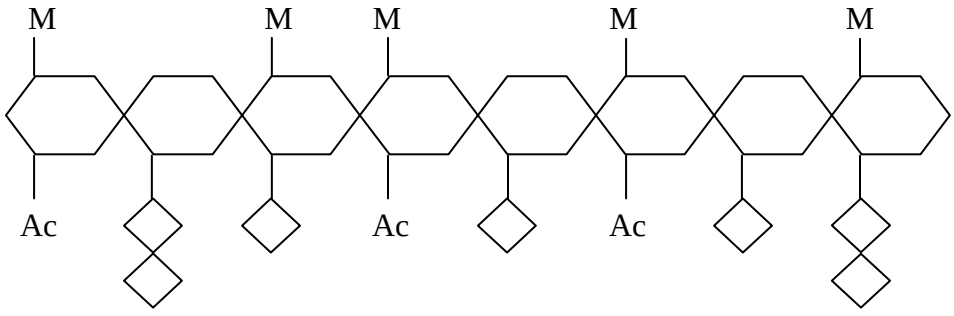
Figure1.2 Schematic structures of HG, RG I, XGA.



Homogalacturonan

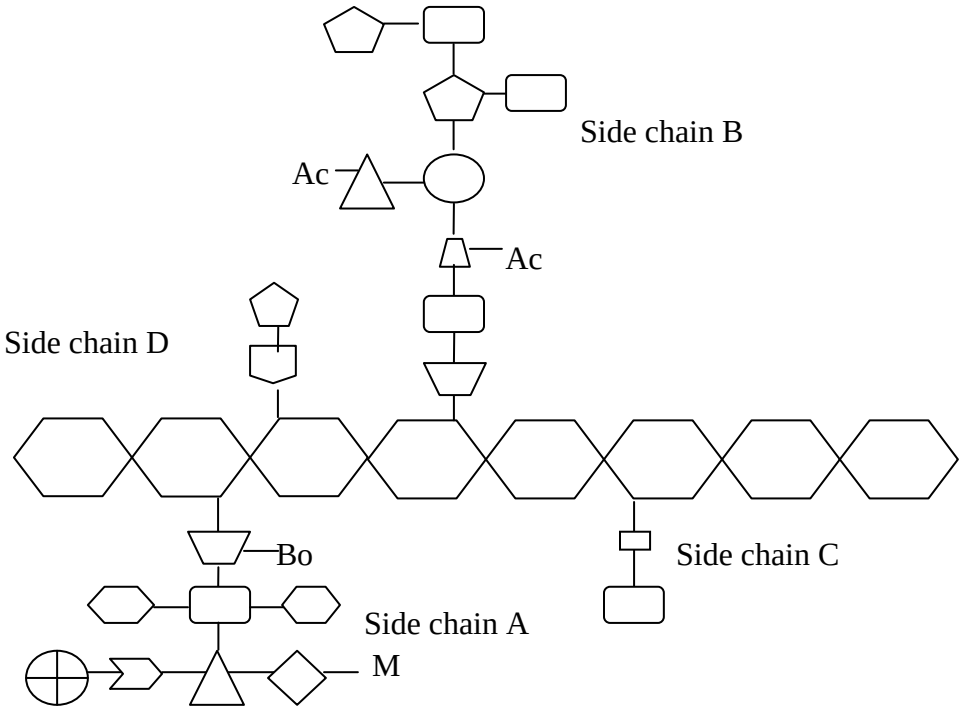


Rhamnogalacturonan I



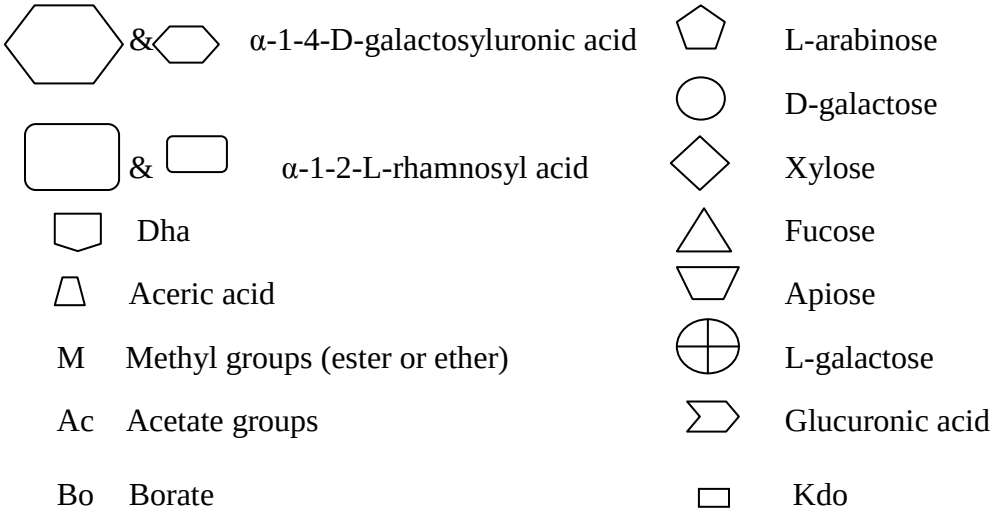
Xylogalacturonan

Figure1.2 Schematic structures of RG II



Rhamnogalaturonan II

Key to structures



CHAPTER 2

EXTRACTION AND ISOLATION OF XG-RG COMPLEX FROM COTTON CELL WALLS

INTRODUCTION

In an early model of the primary cell wall proposed by Keegstra and co-workers in early 70's, it was postulated that xyloglucan was covalently linked to pectic components via arabinan or arabinogalactan and adsorbed non-covalently on the cellulosic surface forming a monolayer (1). This model was proposed based on the evidence found while studying the connection between the different polysaccharide components of sycamore cell walls (1). A new model was proposed in the early 90's by Carpita and Gibeaut while studying cell wall architecture of different flowering plants. They stated that cellulose-xyloglucan network is embedded in a pectic matrix, where as pectin which has four distinguished regions such as HG, RG I, RG II and XGA forms an independent network (2). However, when we were trying to solubilize rhamnogalacturonan from cell walls of cotton suspension cultures by a sequential digestion with endopolygalacturonase (EPG) followed by the extraction with strong alkali, we found convincing evidence showing nearly half of the xyloglucan is covalently crosslinked to rhamnogalacturonan (3). A similar kind of covalent cross linkage was found between extensin (cell wall protein rich in hydroxyproline) and rhamnogalacturonan in cell walls of cotton suspension cultures (4).

MATERIALS AND METHODS

Initiation of Cotton suspension culture

Cotton suspension cultures were initiated using the method explained by Ruyack et al (5). Leaves and stems from 30 to 90 days old cotton plants (*Gossypium hirsutum L.* Acala 44 & OK 1.2) were used as explants. Explants were surface sterilized for about 2-5 minutes by washing the stem and leaves with 100 ml of 70% ethanol along with 0.1% Tween-20. Ethanol was then removed and explants were washed for about 10 minutes with 100 ml of 2.15% aqueous sodium hypochlorite with 0.1% Tween-20 followed by several washing with autoclaved distilled water under sterile conditions. Leaves and stems were cut into small rectangular and longitudinal pieces, respectively, using sterile surgical blades. These explants were then transferred on to Schenk and Hildebrandt (SH) media slants containing ten times more concentrated pyridoxine. Sterilization of explants and transferring of explants to media was done in the laminar flow hood. These media slants were maintained under continuous light at 30 ^{O}C. After 4 weeks undifferentiated cell growth was seen on explants and some were excised and sub-cultured to new media. Suspension cultures were developed by transferring 3-4 g of callus tissue from agar medium to 50 ml of Schenk and Hildebrandt (SH) liquid medium in 125 ml culture flasks. These flasks were kept on a shaker at 130 rpm under continuous light at 30 ^{O}C.

Preparation of cell walls from suspension culture

Cotton suspension culture was used to prepare cotton suspension cell walls. Suspensions cultures were grown for about 2 weeks and cells were then harvested from it by filtration. The cell wall preparation method was described by Komalavilas and Mort (6) where cells were collected on fine nylon cloth (35 µm pore size , Small Parts Inc.,

Miami Lakes, FL, USA), and washed sequentially with 10 vol of 100 mM and 6 vol of 500 mM potassium phosphate buffer (pH 7.0). Cells were then suspended in 1vol of 500 mM potassium phosphate buffer (pH 7.0) and crushed using a Polytron homogenizer (Brinkmann Instruments, NY) on ice for about 12 mins at full speed with stopping after every 3 minutes for cooling. The cells were then washed in sequential pattern with 5 vol of 500 mM potassium phosphate buffer (pH 7.0), followed by 10 vol of distilled water, 5 vol of 1:1 chloroform-methanol, 10 vol of acetone. Cell walls obtained after rinsing with acetone were air dried and then further dried under vacuum at room temperature.

Endopolygalacturonase digestion of cell walls

Dried Cell walls were suspended in 50 mM ammonium acetate buffer (pH 5.2) making the final concentration approximately to 10 mg/ml. Fifteen Units of EPG enzyme/ gm of cell wall digesting HG, was added to the mix. The EPG was cloned from *Aspergillus nidulans* open reading frame AN8327.2 and expressed in *Pichia pastoris* (7).Mixture was kept at 37 ^{O}C for 24 hours with gentle stirring. A few drops of toluene were added to cell wall mixture to avoid fungal growth. After 24 hours the cell wall and enzyme mixture was centrifuged at 13,200 g for 15 min. Pellets were washed twice with water and were centrifuged at 13,200 g for 15 mins. Supernatants and pellets were pooled separately and were freeze dried 3 to 4 times to remove salts.

Strong Alkali Extraction

KOH (24%) along with $NaBH_4$ (0.1%) was applied to EPG digested cell walls making the final concentration of walls approximately 10mg/ml. This mixture was kept overnight at room temperature with gentle stirring. The mixture was then centrifuged at 13,200 g for 15 mins and pellet was washed with water twice and centrifuged again at

13,200 g for 15 mins. The supernatants were mixed together and neutralized with concentrated glacial acetic acid to pH 5.5. After neutralization, the mixture was desalted by dialyzing the mixture overnight against water using membrane of molecular weight cutoff of 12,000 to 14,000 (Spectrum Medical industries, Inc., Los Angeles, CA, USA). The supernatants were then ultra-filtered using 10 kD cutoff membrane and were then freeze dried.

Endoglucanase digestion of EPG digested cell walls

EPG digested cell walls (300 mg) were suspended in 50 mM ammonium acetate buffer (pH 5.2) bringing the final concentration to 10 mg/ml. XG specific endoglucanase enzyme (0.5 U) cloned from *Aspergillus nidulans* from open reading frame AN 0452.2 expressed in *Pichia pastoris* was added to the mixture. Mixture was kept at 37 ^{O}C for 24 hours with gentle stirring. After 24 hours the mixture was centrifuged at 13,200 g for 15 mins. Pellets were washed twice with water and were centrifuged at 13,200 g for 15 mins. Supernatants and pellets were pooled separately and were freeze dried 2 to 3 times to remove salts.

Formation of xyloglucan subunits using endoglucanase

Tamarind xyloglucan (127 mg) was suspended in 10 ml of 50 mM ammonium acetate buffer pH 4.5. XG specific endoglucanase enzyme (1 U) cloned from *Aspergillus nidulans* from open reading frame AN 0452.2 expressed in *Pichia pastoris* was applied to mixture. The mixture was incubated at 37 ^{O}C for an hour and was incubated for 3 min in boiling water to kill the enzyme. Mixture was then freeze dried and resuspended in 2 ml of ammonium acetate buffer (pH 4.5). The resuspended mixture was fractionated using gel filtration chromatography on Toyopearl HW-50S (Supelco Inc., Bellefonte, PA,

USA) packed in a 500 x 22.5 mm stainless steel column (Alltech associates Inc) using 50 mM ammonium acetate buffer pH(5.2) with flow rate of 2 ml/min (8). Sample eluted by buffer was analyzed using a Shodex Rl-71 refractive index. Fractions were collected every minute using a Gilson Fraction collector and they were freeze dried 2 to 3 times to remove salts. Peaks were analyzed by capillary zone electrophoresis (CZE). Then 1 mg of dimers of repeat units of tamarind was labeled with 5 ul of 8-aminopyrene-1,3,6-trisulfonic acid (APTS 100 mM in 15% acetic acid) along with 20 ul of 1 M $NaBH_3CN$ (1 M in Me_2SO) and heating the reaction mixture at 80 ^{O}C for 1 hour. After heating around 200 ul of water was added the mixture was fractionated using Toyopearl HW-40S (Supelco Inc., Bellefonte, PA, USA) packed in 150 x 10 mm stainless steel column (Alltech associates Inc) using a combination of 75% of 50 mM ammonium acetate buffer (pH 5.2) and 25% of acetonitrile with flow rate of 1 ml/min. The sample eluted by buffer was monitored using a Shimadzu R-535 fluorescence HPLC detector. Fractions were collected every minute. Peaks were collected and analyzed on CZE. The single and double repeating units of oligosaccharides were collected and freeze dried (8).

Anion exchange chromatography

This technique was applied to separate neutral polysaccharides from acidic polysaccharides. Anion exchange columns such as PA1 (22 x 250 mm & 9 x 250 mm Carbo Pac, Dionex), DEAE (22.5 x 250 mm Poros 50 DEAE, Perspective Biosystems) were used. The column was eluted with an increasing ammonium acetate gradient (3 min hold at 30 mM, increased from 30 mM to 1 M over the next 37 min, and final increment from 1 M to 2 M over the next 20 min) (pH 5.2) with flow rate of 5 min/ml or 2 ml/min for the 9 mm column. A Sedex 55 evaporative light-scattering detector (S.E.D.E.R.E.

France) was used to analyze 1/25th of the eluant, split by an analytical adjustable flow splitter of Analytical Scientific Instruments.

Sugar composition analysis using Gas Liquid Chromatography (GLC)

Sugar compositions were examined using gas liquid chromatography analysis of trimethylsily methyl glycosides. Samples were methanolyzed and derivatized using the method developed by Chaplin (1982) and modified by Komalavilas and Mort (6).

Approximately 100 µg of sample was weighed using a Cahn 29 electrobalance (Instrument Group Walnut Creek division, Walnut Creek, CA, USA) and was collected in 4 ml glass vial having Teflon lined screw caps. Inositol (100 nmol) was added to sample as an internal standard and the mixture was dried in speed vacuum. Methanolic HCl (200 ul of 1.5 N) and methyl acetate (100 ul) were added to the vial. The vial was properly screw capped and kept at 80 ^{O}C for at least 3 hours or overnight. After heating, the vials were cooled to room temperature and 4 to 6 drops of *t*-butanol were added to each vial. Samples were then completely dried under nitrogen gas and 50 µl of freshly prepared trimethylsilylating reagent was added to each vial. Trimethylsilylating reagent is 5:1:1 of anhydrous pyridine (Sigma-Aldrich Inc, USA): hexamethyldisilazane (Sigma-Aldrich Inc, USA): chlorotrimethylsilane (Sigma-Aldrich Inc, USA). The sample was derivatized with trimethylsilylating reagent at room temperature for 15 min, dried under nitrogen gas and then resuspended in 50-100 µl of isooctane. Derivatized sample (1 µl) was separated on Durabond-1 liquid phase fused silica capillary column (J & W Scientific, Inc., Rancho Cordova, CA, USA) installed in a Varian 3300 & 3350 (Sunnyvale, CA, USA). Sample was injected at 105 ^{O}C and oven temperature was held at 105 ^{O}C for next 1 min, followed by increase to 160 ^{O}C at a rate of 10 ^{O}C/min and held to 160 ^{O}C for next 4 min, then

raised to 220 ^{O}C at a rate of 2 ^{O}C/min, and final temperature to 240 ^{O}C at a rate of 10 ^{O}C/min. column was held to 240 ^{O}C for 10 min before returning to starting conditions. Peaks obtained by the separation of the trimethylsilyl sugar samples were recorded and integrated on an in house data acquisition system and custom integration program (both constructed by Dr. Jerry Merz).

Capillary Zone Electrophoresis (CZE)

Sample (25 µg) was labeled with 10 µl of 23 mM ANTS (3:17 v/v acetic acid: water) in the presence of 1 µl of 1 M $NaBH_3CN$ (1 M in Me_2SO) by heating at 80 ^{O}C for 1 hour (9). A custom built instrument having a laser-induced fluorescence detector was used to examine the samples separated at 18 kV. The sample was excitated using a helium-cadmium laser and detection was done using an intensified charge-coupled device (ICCD) camera (10). The column used for separation of oligosaccharide was a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). NaH_2PO_4 buffer (pH 2.5, 0.1 M) was used as a running buffer, and it was also used to rinse the capillary between runs. Buffer and sample was injected into the capillary by gravity driven flow for 10-12 seconds. 18 kV was applied across the electrophoretic system where the cathode (negative electrode) was placed on the injection side.

Samples were also derivatized using 8-aminopyrene-1,3,6-trisulfonic acid (APTS 100 mM in 15% acetic acid) along with 1 M $NaBH_3CN$ (1 M in Me_2SO) and heating the reaction mixture at 80 ^{o}C for 1 hour. These samples were analyzed using a BioFocus 2000 (Bio-Rad Laboratories) capillary electrophoresis system with enhanced laser-induced fluorescence detector. Column used for oligosaccharide separation was similar to the one used in custom built instrument (a fused-silica capillary with 50 µm internal

diameter and 31 cm in length). Helium pressure (4.5 psi) was applied to inject the sample at the cathode end (negative electrode). A voltage of 15 kV/70-100 µA was applied across the electrophoretic system and system was maintained at 20 ^{o}C. NaH_2PO_4 buffer (pH2.5, 0.1 M) was used as a running buffer and 1 M NaOH was used for rinsing the capillary after each injection to prevent the carryover of sample. Labeled oligomeric samples were excited at 488 nm and emission spectra were collected using a 520 nm band pass filter (11).

MALDI-TOF MS

Samples were dissolved in water making the final concentration approximately 10 µg/µl. 2,5-dihydroxybenzoic acid dissolved in MeOH/water (65% + 35% v/v) bringing the final concentration 10 mg/ml was used as a matrix. Matrix (0.25 µl) and samples (0.25 µl) were spotted on the 96 well plate used for MALDITOF-MS. Maltodextrin (10 mg/ml) was used as a standard for the calibration. Spectra were obtained in the positive ion mode using a Voyager DE-PRO (Applied Biosystems) MALDI-TOF MS (8).

NMR spectroscopy

Sample digested with different enzymes were freeze dried and dissolved in D_2O. 1H NMR spectra of these samples was recorded using either a Varian Unity Inova 400 or 600 NMR spectrometer.

RESULTS

Isolation of XG-RG complex by EPG digestion and KOH extraction

Figure 2.1 illustrates the scheme used for the sequential extraction and isolation of the XG-RG complex from intact cell walls by EPG digestion and KOH extraction. One gram of cell walls (OK1.2 denoted as green cells and Acala 44 denoted as white cells)

was digested by 15 units of EPG enzyme (cloned). Extensive digestion by EPG results in solubilization of around 30% by weight of green cells and 15% by weight of white cells in water. When the sugar composition of EPG soluble fraction was analyzed results showed the presence of galA probably in the form of monomer, dimer and trimer in higher amounts along with complex pectic component RG II and rhamnose rich pectic component RG I in a very small amount. The soluble fraction had a very low amount of xylose, but it does show the presence of fucose, galactose and glucose hinting xyloglucan being linked to acidic sugars (Table 2.1). Treatment with strong alkali (24% KOH) along with 0.1% $NaBH_4$ the residual part of EPG digests solubilizes around 30-35% by weight of white and green cell walls, which is actually around 25% of the starting cell wall material. The solubilized fractions consists of unlinked XG, XG linked with RG I, XGA, xylan, arabinose, and starch which precipitates when the alkali extracted complex is dissolved in water before injecting on the anion exchange column (Table2.1). The presence of starch was detected using ^1H NMR spectra (Inova 400 NMR spectrometer) and was indicated by a high amount of glucose joined by α-1-4 linkages. The unsolubilized fraction of EPG digest and strong alkali extract was predominantly cellulose.

We examined the hypothesis proposed by Keegstra et al. that there is cross-linkage between xyloglucan and pectins in the sycamore cell suspension cultures (1). Cotton suspension cell cultures were used for this study and the experimental data showed cross-linkage between xyloglucan and pectins is probable. The strong alkali extract was chromatographed on 2 different anion exchange columns for isolation of the XG-RG complex. First it was chromatographed on a DEAE porous HQ anion exchange

column using an ammonium acetate gradient and evaporative light scattering detector. Figure 2.2 shows the chromatogram obtained by the fractionation of EPG digested and KOH extracted cell wall on DEAE column. The sugar composition of 3 peaks (A-1, A-2, A-3) obtained by fractionating the strong alkali extract of cell wall on DEAE column were analyze and given in Table 2.2. A-1, the unadsorbed fraction predominantly contains neutral sugars eluted with 0.03 M NH_4Ac buffer (pH 5.2) showed the presence of glucose in higher percentage along with xyloglucan. A-2 fraction weak acidic sugars eluted with 0.5 M NH_4Ac buffer (pH 5.2) was composed of mostly xylans. This fraction was low in quantity and accounted for 2-5% of the total extract. A-3, acidic fraction, eluted with 1 M NH_4Ac buffer (pH 5.2) and showed the combination of xyloglucan, RG I and XGA. The presence of xylose and glucose along with galactose and fucose in lesser amount illustrated the presence of xyloglucan. Presence of rhamnose and galacturonic acid illustrated the presence of RG I. XGA was seen closely associated with RG I (XGA is a modified HG and EPG resistant pectic component) (12).

The adsorbed peak A-3 from the DEAE column was again fractionated using a PA1 anion exchange column. The use of two different anion exchange column helped in getting rid of unlinked xyloglucan and other neutral sugars from the XG-RG complex. The basic difference between these two columns is that DEAE column is made up of porous material and large amount of samples can be applied on it, whereas PA1 column is made up of non porous beads and has higher resolution as compared to DEAE column. Hence refractionation of A-3 peak would give us well isolated XG-RG complex along with XGA. Figure 2.3 shows the chromatogram obtained by refractionating the A-3 peak on the PA1 column. The unadsorbed peak B-1 eluted with 0.03 M NH_4Ac buffer (pH 5.2)

shows the presence of xyloglucan analyzed by sugar analysis. This peak accounts for only a little material as compared to the adsorbed peak, B-4. Peaks B-2 and B-3 eluted at around 0.25-0.30 M NH_4Ac buffer (pH 5.2) showed very little amount of sugars (like arabinoxylan and arabinose containing polymers (e.g. extensin) and were probably mostly salt. The sugar composition indicated mostly arabinoxylan and arabinose. The peak eluted with 1-1.5 MNH_4Ac buffer (pH 5.2) gave a crooked shape peak but the sugar composition indicated the presence of XG, RG I and XGA. Figure 2.3 shows a combination of 3 peaks (B-4, B-5, and B-6), because the sugar composition of all the peaks eluted adjacent to each other were similar expect the later peaks had a higher galA content as they were more acidic in nature and the later material eluted out with higher molarity of buffer. (Refer Table 2.3 for detailed sugar composition of peaks obtained on PA1 column). If we take into account the recovery of glucose or fucose present in the adsorbed peak as compared to strong alkali extract, we found almost half of it is recovered, which gives us the clear indication of xyloglucan being present in adsorbed peak. We considered only the recovery of glucose or fucose because xylose and galactose are present in other polymers also. The chromatographic pattern suggests xyloglucan being associated with an acidic polymer such as RG I, as it binds to the anion exchange column and elutes out later with higher molar concentration of buffer.

Detection of xyloglucan by endoglucanase assay

The presence of xyloglucan in the adsorbed and unadsorbed fraction of DEAE and PA 1 anion exchange column was illustrated by endoglucanase assay. It has been shown that endoglucanase can digest cotton xyloglucan (13). Each peak fraction (A-1, A-3, B-1, B-4) was digested by endoglucanase enzyme and the products formed by

digestion were derivatized with ANTS and analyzed by capillary electrophoresis using the custom built instrument. Single and double repeating units of oligosaccharide formed by digesting tamarind xyloglucan by endoglucanase were used as standards. Figure 2.4 shows the comparative study between the electropherograms obtained by these adsorbed and unadsorbed peaks compared to the tamarind xyloglucan subunits.

EPG-Endoglucanase digestion

Previously it has reported in some cases that the XG and RG are together in EPG predigested cell walls. To illustrate this we tried endoglucanase treatment on EPG predigested cell walls (scheme explained in figure 2.5). From 300 mg of EPG predigested cell walls around 50% was solubilized and fractionated on PA1 anion exchange column. Fractionation gave 5 peaks (C-1, C-2, C-3, C-4 and C-5) which were analyzed by ANTS labeling and CZE and GC analysis of their sugar composition. Table 2.4 gives the detailed sugar composition of each peak and figure 2.6 shows the electropherograms obtained by each peak of EPG-EG digest separated on PA1 column. The unadsorbed peak C-1 eluted with 0.03M NH_4Ac buffer (pH 5.2) mainly consisted of XG fragments which was shown by the electropherograms obtained by capillary electrophoresis and also by sugar composition. The XG subunits formed in peak C1 were also analyzed using MALDI-TOF MS. It showed similar peaks to tamarind XG such as XXXG, XXLG, XLXG, and XLLG along with few more like XXFG and XLFG. Peaks obtained in MS spectra (figure 2.7) were analyzed and assigned the subunit depending on the mass of the peak as follows: peak with m/z 1085 XXXG, peak with m/z 1247 has 2 possibilities XXLG and XLXG, peak with m/z 1393 XXFG, peak with m/z 1409 XXLG and peak with m/z 1555 XLFG (6). Peak C-2 and C-3 eluted with 0.7 M and 0.75 M NH_4Ac buffer

(pH 5.2) mainly consists of galA dimer and trimer respectively. Peak C-4 and C-5 eluted with 1 M and 1.4 M NH_4Ac buffer (pH 5.2) mainly consists of RG along with traces of XG indicated by the presence of small amounts of fucose. The sugar composition of peak C-4 and C-5 showed RG being released by endoglucanase digestion and hence illustrates the possibility that part of RG is interconnected to the XG. Refer figure 2.5 for scheme of EPG-EG extraction, figure 2.6 for electropherograms obtained by each peak labeled with ANTS and Table 2.4 for the detailed sugar composition of each peak.

The 50% unsolubilized part was further extracted with strong alkali (24% KOH along with 0.1 $NaBH_4$) and fractionated on DEAE column giving XG in unadsorbed peak and RG along with traces of XG in adsorbed peak. The presence of XG in unadsorbed and adsorbed fraction hinted at the possibility of XG remaining tightly associated with cellulose microfibrils and being inaccessible to the enzymatic degradation. The presence of RG shows the chances of it being linked to XG associated with cellulose, or may be to some other insoluble material such as extensin.

DISCUSSION

It is well established that 24% KOH extraction is one of the conventional methods used to break the strong hydrogen bond present within XG and cellulose by swelling the cellulose microfibrils and resulting in the extraction of XG from the cell walls (14). Lower concentration of KOH like 4% and 10% were tried and tested for XG extraction from Pea suspension cell wall, but it was found to be inefficient for XG extraction from the pea cell walls (15). Chelating agents such as CDTA, EDTA have been used to extract HGs bounded by calcium. But all these methods failed to extract the XG present in cell wall when they were used alone or even in combination. It was seen in rose suspension

cell walls, that when chelating agents were used in combination with strong alkali extraction nearly half of the XG from the original wall and one fourth pectic acids were extracted (16).

In the case of cotton cell walls we have seen that EPG digestion followed by 24 % KOH extraction solubilizes almost 90% of total XG and pectin components. One possibility for the success of this scheme is that EPG digests the HG pectic component and allows the extraction of XG linked RG by strong alkali. Removal of HG pectic component reduces the chances of any kind of bond formation like calcium bridges creating the physical entanglement or HG connecting RG I causing the hindrance in solubilization of other wall polymers and hence making the whole complex insoluble by alkali solution. We have seen two types of XG in cotton cell wall, unlinked XG and RG linked XG. Nearly half of XG is unlinked and can be removed in the unadsorbed peak during fractionation on DEAE and PA1 anion exchange columns (A-1 and B-1). Sugar composition of both the peaks shows the presence of xylose, glucose, fucose and galactose- the characteristic sugar combination of XG. These peaks along with the linked XG (adsorbed peaks having XG) when analyzed on CZE after endoglucanase digestion and compared with tamarind xyloglucan subunits show the presence of the same subunits. One gm of intact cell wall yields around 60 mg of the XG-RG complex. Although strong alkali extraction is a very harsh treatment, the XG-RG complex was intact when extracted. Hence it shows that the XG-RG complex must have a very strong bond within them such as glycosidic bonds. Endoglucanase digestion of EPG predigested cell walls released around two thirds of the total RG and XG oligomers, where as remaining one third of RG and some of XG was extracted using strong alkali. If RG was

not linked to XG but was just stuck to XG physically, endoglucanase treatment would not have liberated RG fragment by cleaving the XG fragment. These results illustrate the possibility of covalent linkage between XG and RG.

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Figure 2.1 Flow chart of scheme used for sequential extraction of XG-RG complex from intact cell walls.

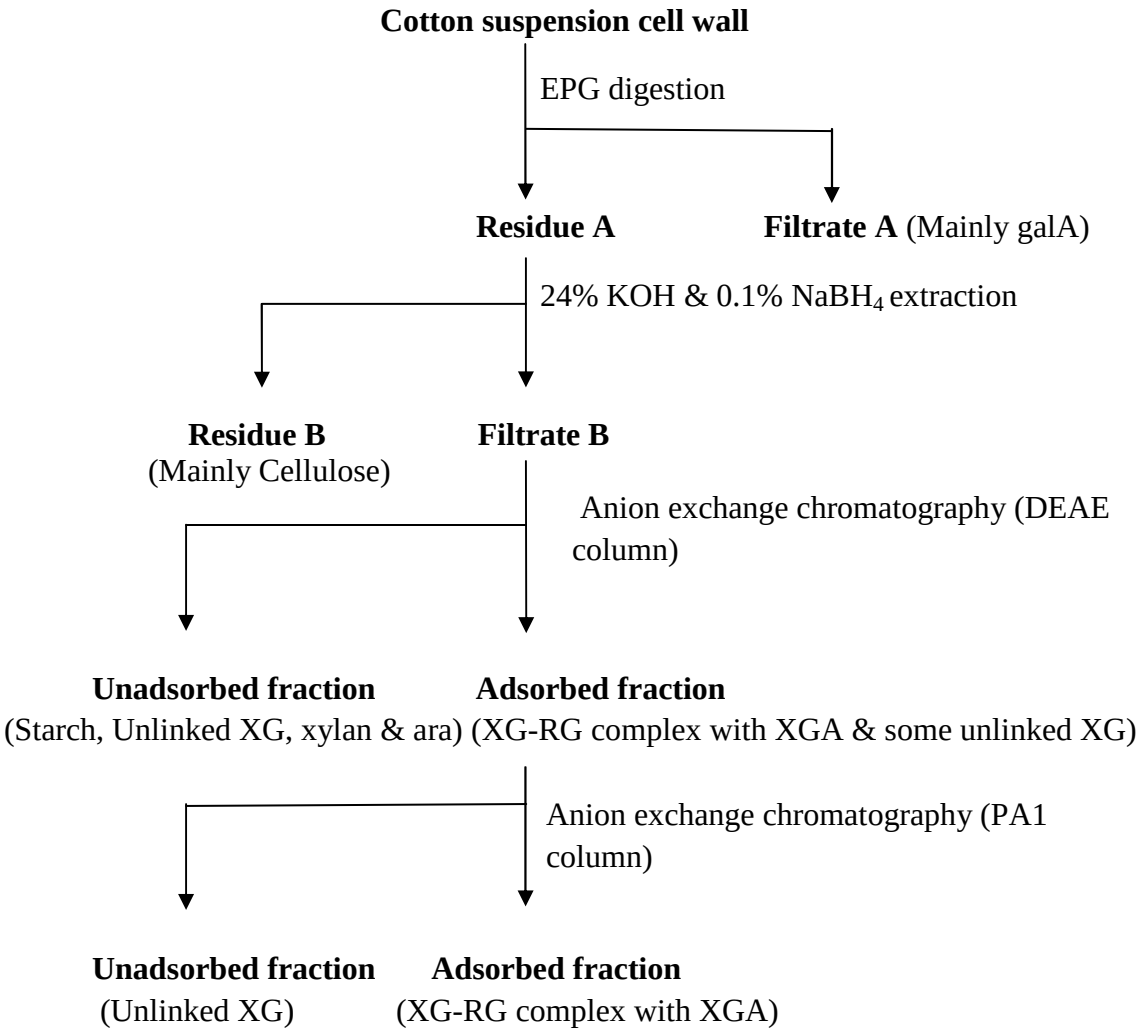


Figure 2.2 EPG digested and KOH extracted cell wall fractionated on DEAE anion exchange column.

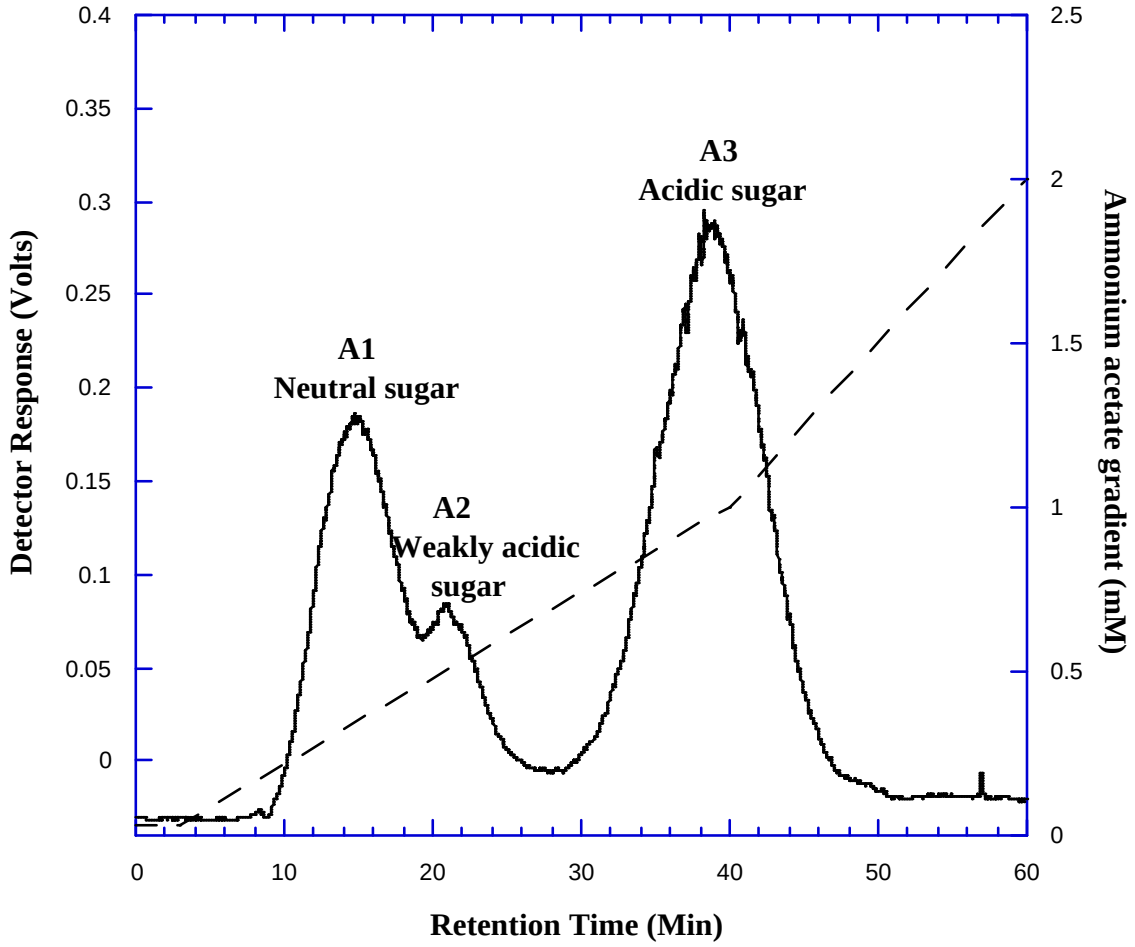


Table 2.1 Sugar compositions of fractions obtained during EPG and KOH extraction.

Name of the sugar	Intact cell wall(GCS) Mole%	EPG(S) (GCS) Mole%	EPG (R) (GCG) Mole%	KOH (S) (GCS) Mole%	KOH (GCS) Mole%
Ara	12.6	8.7	12.7	11.5	16.5
Rha	5.4	4.4	5.2	5.5	9.1
Fuc	1.8	0.7	2.1	2.2	3.2
Xyl	10.6	3.8	12.0	12.7	20.0
GalA	20.1	60.4	16.6	14.8	13.3
Man	0.5	1.5	0.6	0.2	0.2
Gal	9.0	13.4	8.4	9.4	14.4
Glc	40.0	7.1	42.2	43.8	23.5

Name of the sugar	Intact cell wall(WCS) Mole%	EPG(S) (WCS) Mole%	EPG (R) (WCS) Mole%	KOH (S) (WCS) Mole%	KOH (WCS) Mole%
Ara	16.0	10.0	13.4	13.6	17.4
Rha	6.3	5.2	4.9	6.2	10.0
Fuc	1.3	0.7	0.9	1.3	1.8
Xyl	10.1	4.5	10.1	11.4	16.2
GalA	18.7	56.2	13.7	17.8	21.0
Man	0.4	1.4	0.8	0.2	0.3
Gal	7.9	13.9	7.5	8.4	14.0
Glc	39.4	8.3	48.8	41.1	18.8

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green cell walls (OK1.2) WCS: White cell walls (Acala 44)
S: soluble fraction R: residual fraction

Table 2.2 Sugar compositions of EPG digested and KOH extracted cell wall on DEAE column

Name of the sugar	A-1 GCS Mole%	A-2 GCS Mole%	A-3 GCS Mole%	A-1 WCS Mole%	A-2 WCS Mole%	A-3 WCS Mole%
Ara	13.7	15.0	14.9	15.5	16.8	15.7
Rha	n.d	2.7	12.3	n.d	2.9	12.6
Fuc	4.5	0.8	2.7	4.1	1.3	2.8
Xyl	22.6	38.6	12.0	21.5	35.8	11.7
GalA	n.d	12.4	34.4	n.d	12.0	35.2
Gal	13.8	12.3	15.1	11.6	13.8	14.2
Glc	44.8	18.2	8.6	47.1	17.4	7.6

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green Cell walls (OK1.2)
WCS: White Cell walls (Acala 44)
n.d: not detected

Figure 2.3 Refraction of the DEAE adsorbed peak EPG digested and KOH extracted on PA1 anion exchange column.

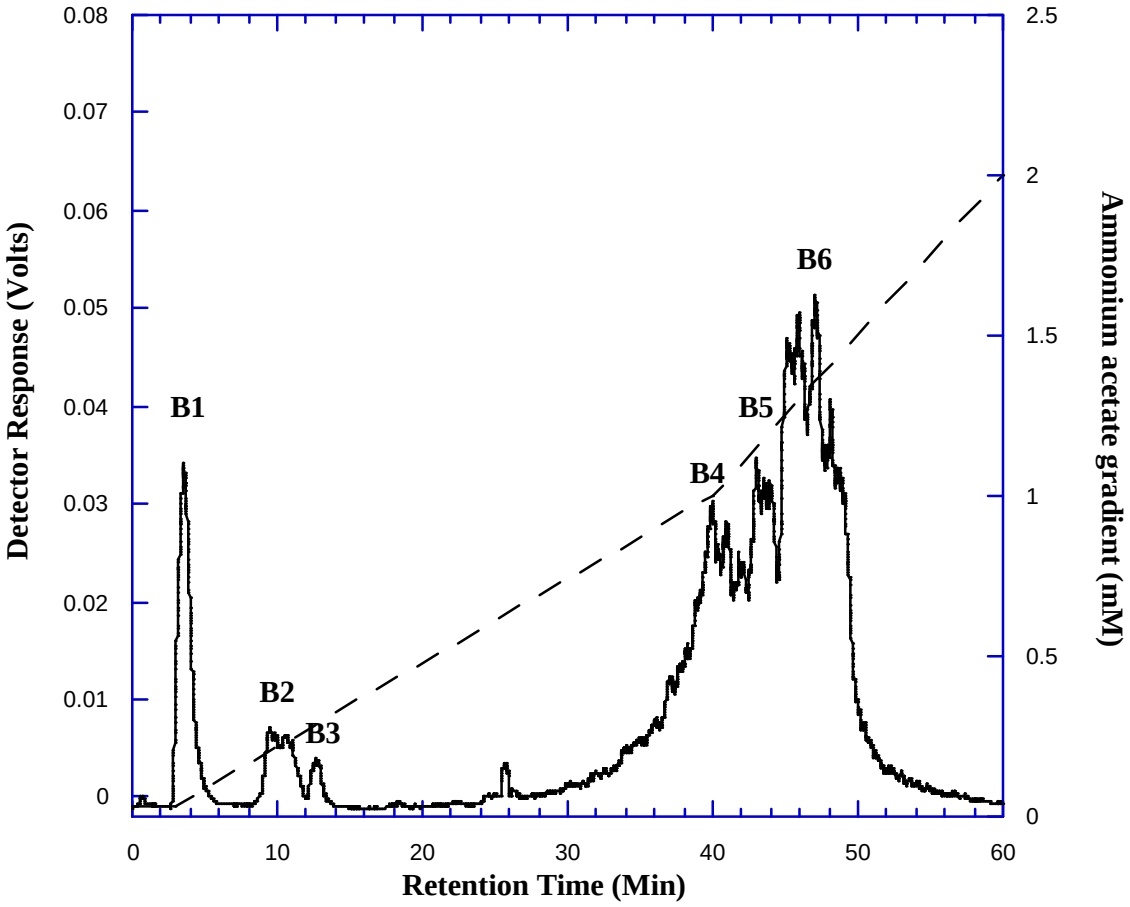


Table 2.3 Sugar compositions of refractionation of adsorbed DEAE peak on PA1 anion exchange column.

Name of the sugar	B-1GCS Mole%	B-2GCS Mole%	B-3 GCS Mole%	B-4 GCS Mole%	B-5 GCS Mole%	B-6 GCS Mole%	XG-RG Complex (B4, B5, B6)
Ara	9.6	19.1	18.3	17.3	14.5	7.1	14.6
Rha	0.5	2.9	1.8	14.5	14.2	7.3	12.8
Fuc	5.1	1.9	1.3	3.1	2.1	0.8	2.5
Xyl	29.1	36.3	31.9	17.4	15.0	6.8	12.8
GalA	n.d	7.3	10.9	20.3	32.2	63.3	35.9
Gal	12.3	17.4	22.2	18.7	14.2	11.6	13.8
Glc	43.5	15.1	13.6	8.7	7.8	3.5	7.4

Name of the sugar	B1WCS Mole%	B2WCS Mole%	B3WCS Mole%	B4 WCS Mole%	B5 WCS Mole%	B6 WCS Mole%	XG-RG Complex (B4, B5, B6)
Ara	10.4	28.1	27.4	21.4	17.4	16	16.8
Rha	0.2	1.4	4.2	12.8	15.8	12.3	14.3
Fuc	4.7	1.3	1.6	3.3	2.1	1.3	2.0
Xyl	30.3	35.6	32.5	12.4	13.5	9.8	12.7
GalA	n.d	7.1	12.0	19.8	29.5	40.6	33.5
Gal	9.5	15.8	13.8	22.5	16.9	15.8	14.3
Glc	44.9	10.7	8.5	7.8	5.4	4.2	6.4

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green cell walls (OK1.2) WCS: White cell walls (Acala 44)
n.d: not detected

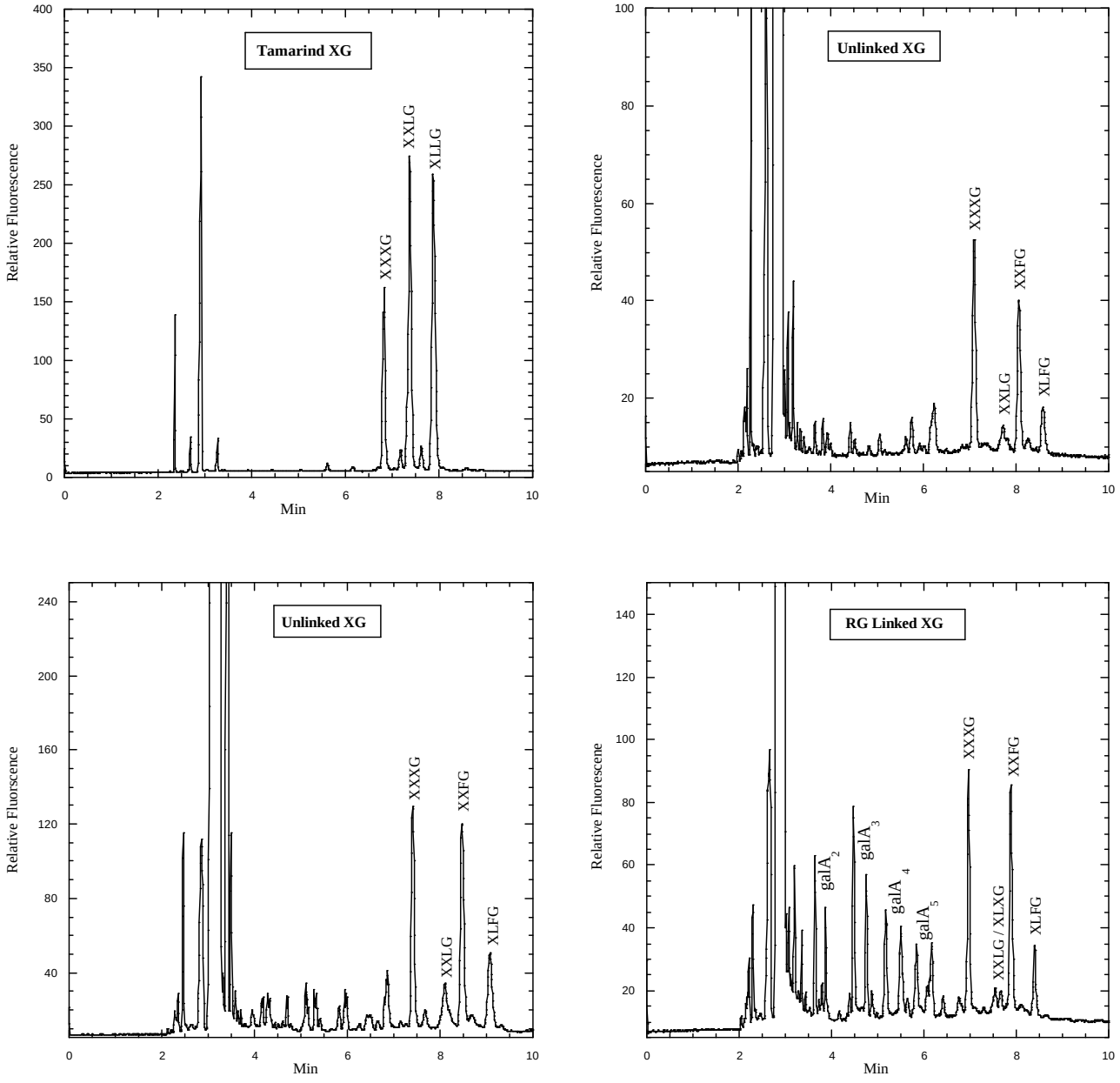
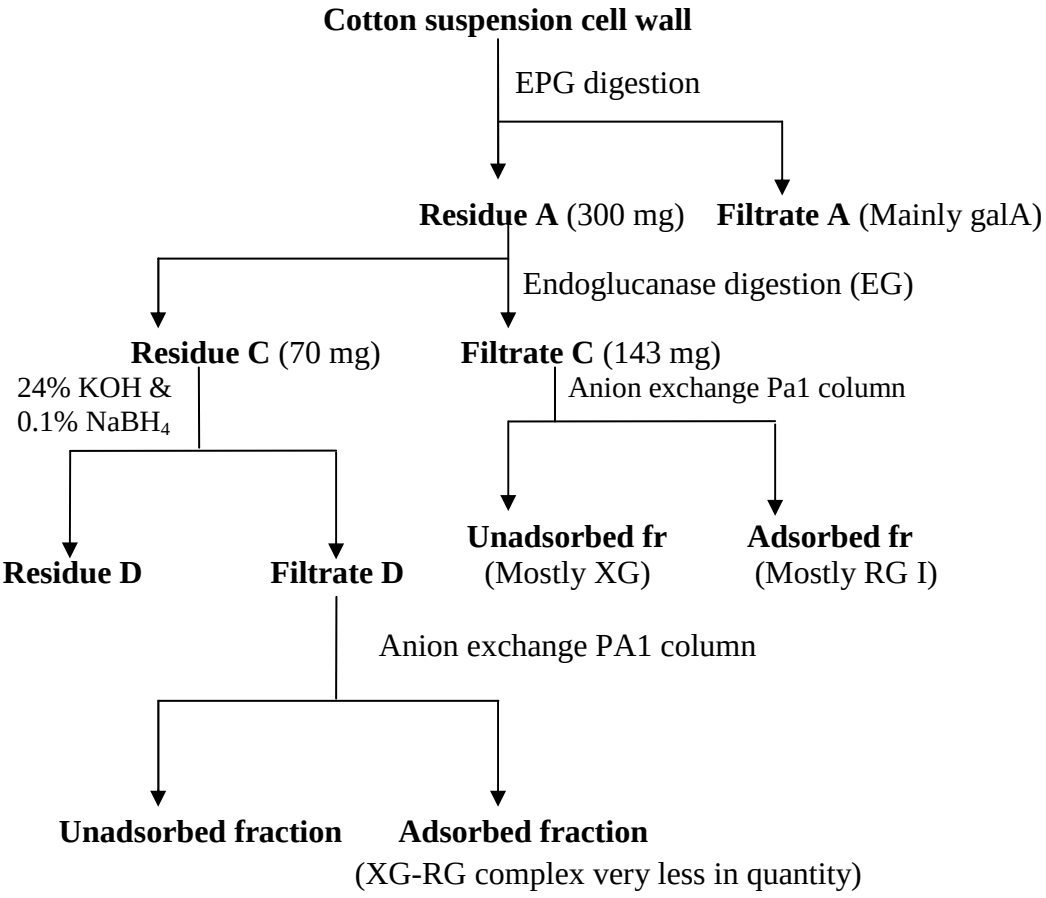


Figure 2.4 Electropherograms of ANTS labeled XG subunits obtained by the endoglucanase digestion of Tamarind XG, Unlinked XG and RG linked XG obtained by DEAE and PA1 anion exchange column.

Figure 2.5 Flow chart of scheme used for EPG-EG extraction



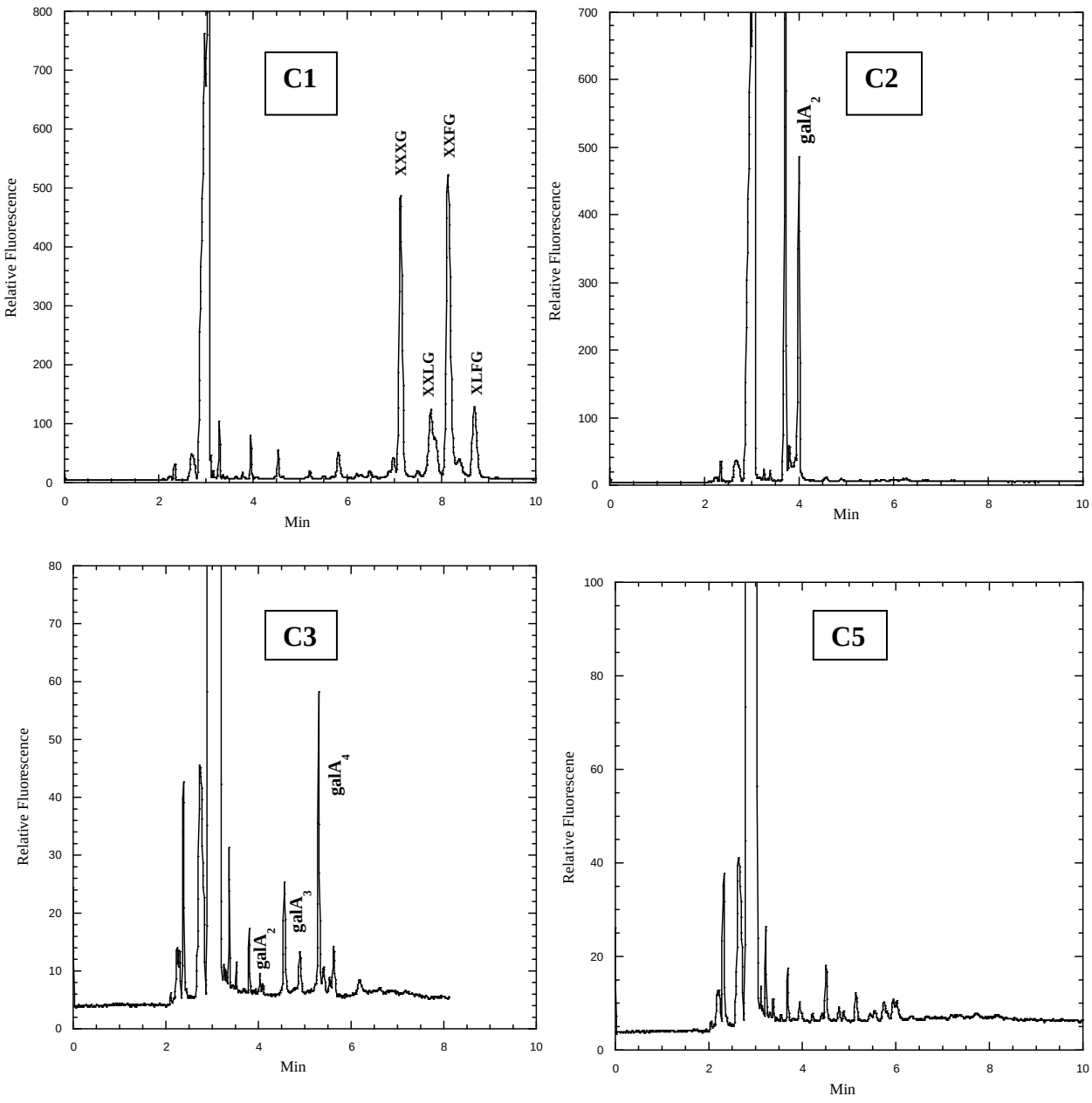


Figure 2.6 Electropherograms of peaks obtained by endoglucanase digestion of EPG pretreated cell walls. C-1 shows XG subunits, C-2 shows dimers of galA, C-3 shows trimers of galA and C- 5 shows no XG subunits.

Table 2.4 Sugar composition of EPG pretreated cell walls solubilized by endoglucanase and fractionated on PA1 column.

Name of the sugar	C-1 Mole%	C-2 Mole%	C-3 Mole%	C-4 Mole%	C-5 Mole%
Ara	1.4	2.1	5.9	20.1	18.8
Rha	n.d	n.d	n.d	9.4	17.7
Fuc	6	0.5	1.3	0.6	0.9
Xyl	37.3	3.3	8.1	4.0	8.8
GalA	1.8	83.7	65.6	47.2	35.3
Gal	9.9	6.8	12.4	14	16.1
Glc	43.6	3.6	6.7	4.7	2.4

Mole%: molar percentage of sugars accounted for in the GLC analysis.
n.d: not detected

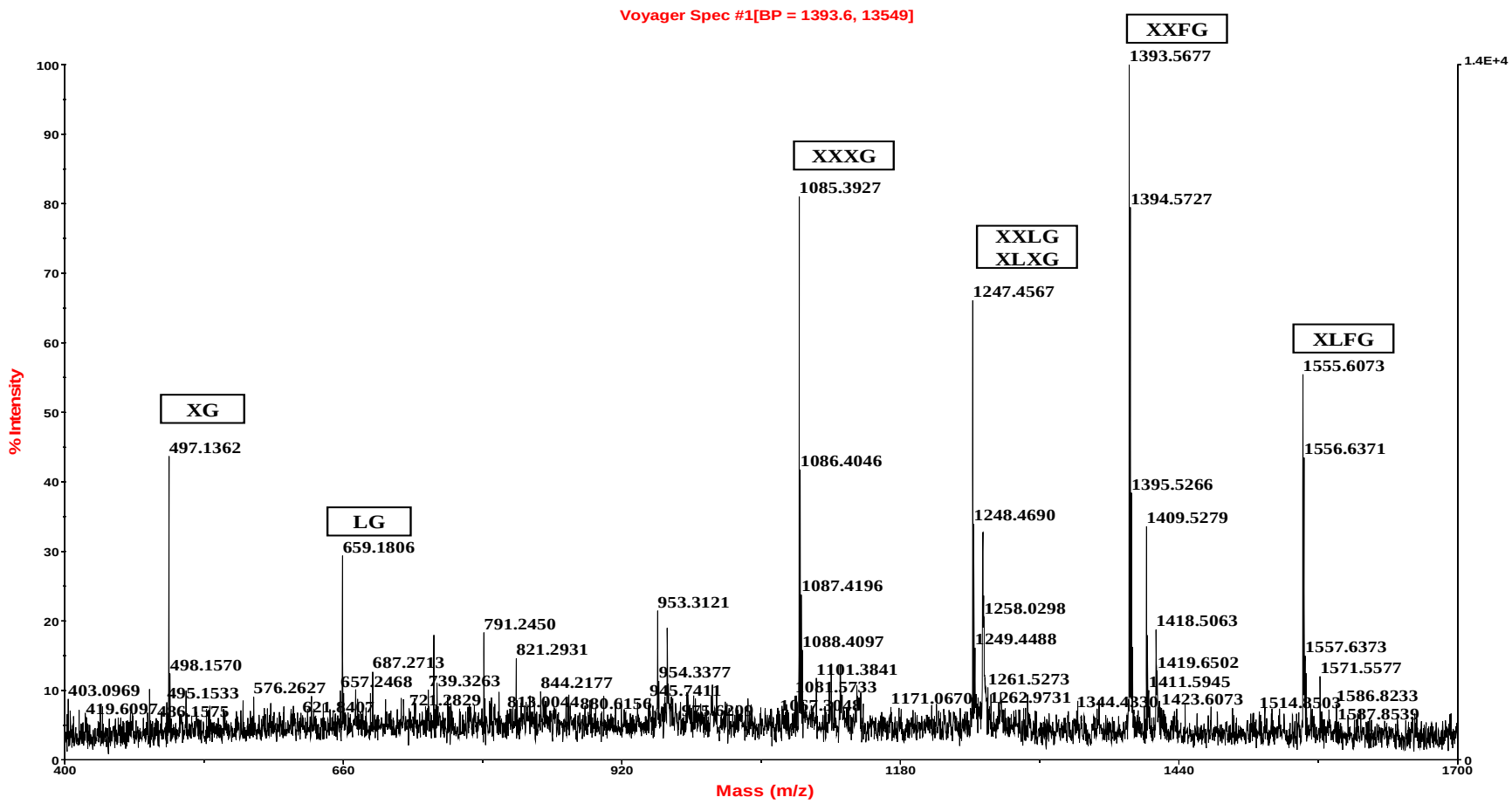


Figure 2.7 MALDI-TOF MS spectra of peak C1 formed by EPG-EG digest and separated on PA1 column

CHAPTER 3

ENZYMATIC DEGRADATION OF THE XG-RG COMPLEX

INTRODUCTION

To have a better understanding about the structural complexity and heterogeneity of pectic polysaccharide, characterization of the linkages between the pectic regions as well as to other polysaccharides present in primary cell walls is very crucial. To characterize the crosslink one needs to first isolate and extract the XG-RG complex from the intact cell walls without destroying the crosslink present within the complex. After isolation, application of cloned enzymes having high specificity would degrade the crosslink of the complex releasing the smaller pectic fragments for further characterization using mass spectrometry and NMR. Chemical and enzymatic degradation are extensively used in investigating the cell wall linkages and structures. A chemical method such as partial acid hydrolysis in combination with HPLC releases smaller oligomers for further characterization by NMR and Mass spectrometry. But this method is less preferred over enzymatic degradation because it lacks specificity towards one type of glycosidic linkages releasing complex products which are difficult for further characterization. Cloned enzymes are pure and have a unique activity on a particular substrate. Commercial enzymes are, at best of questionable purity. One also needs to remember that to isolate and characterize the desired crosslink present within the complex an adequate amount of starting material is required. To investigate the crosslink

between the XG-RG complex, a series of enzymes including arabinosidase, arabinanase, endoglucanase were used. Arabinosidase removes the arabinosyl side branches, whereas, arabinanase degrades linear regions of α-1-5 linked arabinan. Both these enzymes play key role in identifying the proposed arabinan or arabinogalactan linkage between the XG-RG complex (1). Endoglucanase degrades the xyloglucan polymer into subunits such as XXXG, XXLG, XXFG, and XLFG (2). It has been proven in previous chapter that endoglucanase digestion cleaves the XG releasing RG indicating the possibility of covalent linkage between the XG-RG complex. Figure 3.1 shows the scheme of the sequential use of each enzyme for investigating the crosslink between XG-RG complex.

MATERIALS AND METHODS

Arabinosidase

Arabinosidase or α-L-arabinofuranosidase belongs to glycoside hydrolase family GH62 which characteristically hydrolyses the glycosidic bonds of α-L-arabinofuranosides (Figure 3.2a). This enzyme was cloned from *Aspergillus nidulans* open reading frame AN1571.2 and expressed in *Pichia pastoris* (3).

Enzymatic Digestion of XG-RG complex with arabinosidase

Approximately 100 mg of XG-RG complex was suspended in 50 mM NH_4Ac buffer (pH 5.2) making the final concentration to 10 mg/ml and was incubated with 0.1 U of purified arabinosidase at $37^{O}C$ for 24 hours. One hundred milligram of the complex contains around 106 µmole of arabinose and 0.3 U of arabinosidase is sufficient to digest 106 µmole in 6 hours. After 24 hours the reaction mixture was heated at 90 ^{O}C for 15 min to inactivate the enzyme and sample was freeze dried. During the 24 hour of digestion, aliquots of 5 µl were collected at every 2 hour and 2 µl was derivatized with ANTS to determine the progress and completion of the reaction by monitoring the liberation of free

arabinose compared with cellobiose as internal standard using capillary electrophoresis (results not shown).

Three hundred microgram of the APTS labeled double repeat units of oligosaccharide obtained from tamarind xyloglucan were incubated with 0.01 U of arabinosidase at 37^{O}C. Aliquots were collected after 15 mins, 1 hour, 2 hour, 4 hour and enzyme was inactivated by heating at 90^{O}C for 10 mins. Samples were then analyzed using the automated CZE.

Arabinanase

Endo-1,5-α-L-arabinanase belongs to glycoside hydrolase family GH43 which characteristically hydrolyses the α-1-5 linked arabinofuranosidic bonds present in pectic arabinans (Figure 3.2b) (4). This enzyme was cloned from the hyperthermophilic bacterium *Thermotoga petrophila* open reading frame Tpet0637.1 and expressed in *Escherichia coli.* This protein was insoluble when *E. coli* grew at 37^{O}C and hence purification was difficult. Literature has many such examples of insoluble proteins which can be recovered as a soluble form by slowing down the expression so that the proteins get sufficient time to fold correctly. This can be done by inducing the expression at comparatively low temperature for a longer period of time. Usually protein was induced at 37^{O}C and was grown for around 4 hours. We tried inducing the expression at 30^{O}C for 8 hours. Cells were lysed and analyzed on SDS-PAGE, which illustrated protein was soluble.

Purification of arabinanase enzyme

Cells were harvested by centrifuging the 150 ml culture at 10,000 g for 30 mins and were washed thoroughly and further purified using Qiagen protocol modified by

Roberts Matts (5). Harvested cells were resuspended in a primary lysis buffer (0.40 ml 1M Tris pH8, 1.20 ml 5 M NaCl, 0.160 ml PMSF, 0.40 ml PIC, 17.8 ml deionized water) and 26 mg lysozyme was added to the cells. Bottle containing the resuspended cells was kept stirring in cold room for an hour. Secondary lysis buffer (1.28 ml 1M Tris pH 8, 0.128 ml 1 M $MgCl_{2,}$ 32 mg DNAase, 0.51 ml PMSF, 0.065 ml PIC, 59.5 ml deionized water) was added to the bottle and was kept stirring for 30 mins more. Solution was then sonicated for 4 to 6 mins with regular interval of 30 sec and 2.15 ml of 5 M NaCl/ 26 ml final volume and 1.45 ml imidazole elution buffer/ 26 ml final volume were added and centrifuged at 10,000 g for 30 mins. Supernatant was applied on Ni^{2+}- chelating affinity media packed in a 100 mm x 9 mm stainless steel column (Ni sepharose 6 fast flow resin from GE Healthcare, Piscataway, NJ, USA). Column was packed and washed with imidazole wash buffer (50 mM Tris (pH 8), 300 mM NaCl, 15 mM imidazole, (pH 8). The solution was pumped and was bound to the column and the flow through solution was collected. After binding the column was washed with 25 ml of imidazole wash buffer followed by 20 ml of wash buffer without imidazole, and later with elution buffer at a flow rate of 1 ml per min. (50 mM Tris (pH 8), 300 mM NaCl, 250 mM imidazole, (pH 8). Fractions were collected at every 0.5 ml and were monitored by a UV detector. The purified protein sample was concentrated to 20 mg/ml using centriprep filters (Millipore). The purified protein was further analyzed on 10% SDS-PAGE gel and stained with Commassie blue.

Enzyme activity assay

DNS (dinitrosalicyclic acid reagent) method which determines the amount of reducing sugar formed from various polysaccharide substrates was used to determine the

activity of arabinanase (6). Twenty microlitre of substrate (1% debranched arabinan in buffer) and 20 µl of 50 mM NH_4Ac (pH 5.2) was incubated with 10 µl of arabinanase at 80^OC for 30 min. DNS reagent (40 µl) was added and then boiled at 100^OC for 5 min to stop the reaction. Color change produced by the reaction was measured at 550 nm using a micro plate reader (InfiniteM200, TECAN, Switzerland) (7). A standard curve was prepared using Arabinose. Enzyme activity was defined in terms of Units/ml which was calculated depending on the amount of enzyme required to liberate reducing sugar at rate of 1 µmol/min. Activity of enzyme was also monitored by CZE. One hundred micrograms of debranched arabinan was incubated for different time intervals, 15 mins, 1 hour, 2 hour and 4 hour with 5 µl of arabinanase and then aliquots were labeled with APTS.

Enzymatic Digestion of XG-RG complex with arabinanase

Approximately 100 mg of XG-RG complex treated with arabinosidase and fractionated on PA1 column, was suspended in 50 mM NH_4Ac buffer (pH 5.2) bringing the final concentration to 10 mg/ml and was incubated with 0.2 U of purified arabinanase at 80^OC for 24 hours. One hundred milligrams of the complex contains around 94 µmole of arabinose and 0.2 U of arabinanase is sufficient to digest 94 µmole in 8 hours. After 24 hours, the reaction mixture was incubated at -20^OC to inactivate the enzyme and the sample was freeze dried. To test the ability of the enzyme to degrade XG, three hundred microgram of the APTS labeled double repeat units of oligosaccharide obtained from tamarind xyloglucan was incubated with 0.05 U arabinanase at 80^OC. Aliquots were collected after 15 mins, 1 hour, 2 hour and 4 hour respectively and enzyme was

inactivated by incubating samples at -20^{O}C for 20 min. Samples were then analyzed using the automated CZE. (Figure 3.2 shows the electropherograms obtained).

MALDI-TOF MS

Samples were dissolved in water brining the final concentration to approximately 10 µg/µl. 2,5-dihydroxybenzoic acid dissolved in MeOH/water (65% + 35% v/v) bringing the final concentration to 10 mg/ml was used as a matrix. The matrix (0.25 µl) and samples (0.25 µl) were spotted on the 96 wall plate used for MALDI-TOF MS. Maltodextrin (10 mg/ml) was used as a standard for making the calibration while analyzing the sample. Spectra were obtained in the positive ion mode using a Voyager matrix-assisted laser desorption time of flight mass spectrometer.

NMR spectroscopy

Samples digested with different enzymes were freeze dried and dissolved in D_2O. 1H NMR spectra of these samples were recorded using either a Varian Unity Inova 400 or 600 NMR spectrometers.

For Anion exchange chromatography, capillary electrophoresis, gas chromatography for sugar composition, ANTS and APTS derivatization refer chapter 2.

RESULTS

Digestion of XG-RG complex with Arabinosidase

Arabinosidase treatment on the APTS labeled double repeats of oligosaccharide obtained from tamarind XG was done to examine the specificity of the enzyme. The electropherograms obtained from reaction mixture aliquots of different time points did not show any significant change in the structure of double repeats (Figure 3.2 shows the comparative results of Tamarind XG treated with various enzymes). The quantity of

enzyme applied on the substrate was sufficient to show the enzymatic activity within 2 hours. The experimental evidence proved the inactivity of enzyme on XG and assured that it does not digest XG present in the complex even after a prolonged incubation time. Action of arabinosidase on the complex was closely monitored by collecting aliquots from the reaction mixture at every 2 hours and examining the reaction progress by the liberation of free arabinose formed by the enzyme on CZE. The NMR spectra of intact XG-RG complex, when compared with the one treated with enzyme, showed the significant reduction in arabinosyl side chains. The loss of peaks, at 5.16 ppm and 5.12 ppm corresponding to t-arabinofuranose and 3, 5-linked arabinofuranose illustrated the efficiency of the enzyme towards the degradation of arabinosyl side chains present in the complex. XG-RG complex treated with enzyme was heated at 80^{O}C for about 15-20 mins to inactivate the enzyme and was later freeze dried and rechromatographed on a PA1 column generating 6 peaks (D-1 to D-6) (refer figure 3.3). Unadsorbed Peak D-1 eluted with 0.03 M NH_4Ac buffer (pH 5.2) mainly consisted arabinose (approx. 40% of the total fraction weight) along with XG in small quantity. The peak accounts for about 10-12% of the total weight of the sample. Peak D-2 eluted with 0.25 M NH_4Ac buffer (pH 5.2) was mostly salt with very low quantity of sugars in it. Peak D-3,D-4, D-5, D-6 eluted from 0.65 M to 1.3 M NH_4Ac buffer (pH 5.2) consisted of RG I along with XG and XGA. Sugar composition of these peaks is similar but it differs in galA percentage. These peaks show the presence of XG illustrated by the presence of fucose and galactose, and RG I illustrated by the presence of rhamnose and galacturonic acid. For our convenience we mixed all of them and labeled those as XG-RG complex after the arabinosidase treatment (refer table 3.1 for detailed sugar composition of each peak obtained on PA1

column). The unadsorbed and adsorbed peaks (D-1, D-3, D-4, D-5, and D-6) were treated with endoglucanase and were analyzed on CZE after ANTS labeling. Electropherograms obtained for these samples were compared with those of the subunits of tamarind XG, and results showed the presence of similar XG subunits. The adsorbed peak was also analyzed using MALDI-TOF MS. It showed the similar peaks to tamarind XG such as XXXG, XXLG, XLXG, and XLLG along with XXFG and XLFG. Peak obtained in MS spectra (figure 3.4) were analyzed and assigned to a subunit type depending on the mass of the peak as follows: peak with m/z 1085- XXXG, peak with m/z 1247 has 2 possibilities XXLG and XLXG, peak with m/z 1393- XXFG, peak with m/z 1409-XXLG and peak with m/z 1555-XLFG (8). These results were similar to those obtained for peak C1 obtained by EPG-EG digestion (figure 2.7). The spectra also showed many other peaks with different masses which were hard to assign.

Digestion of arabinosidase treated XG-RG complex with arabinanase

Arabinanase treatment on the APTS labeled double repeat oligosaccharide obtained from tamarind XG was done to examine the specificity of the arabinanase. The electropherograms obtained from the reaction mixture aliquots of different time points did not show any significant change in the structure of the double repeats (Figure 3.2). The quantity of enzyme applied on the substrate was sufficient to show the enzymatic activity within 2 hours. The experimental evidence proved that it does not digest the XG present in the complex even after prolonged incubation time. Commercial debranched arabinan, on treatment with arabinanase, formed dimer, trimer and tetramer units showing the efficiency of arabinanase to cleave the α-1-5 linked arabinan releasing smaller units. After the arabinanase treatment, the XG-RG complex sample was incubated at -20^{O}C for

30 mins to inactivate the enzyme, was freeze dried, and rechromatographed on PA1 column giving 5 different peaks (E-1 to E-5)(figure3.5). The unadsorbed peak E-1 eluted with 0.03 M NH_4Ac buffer (pH 5.2) is mostly XG based on sugar composition, endoglucanase assay and manual CZE. Presence of XG in the unadsorbed peak in high percentage indicates arabinan does link XG and RG I. Peak E1 when analyzed using MALDI-TOF MS after endoglucanase digestion, the spectra obtained did show the presence of cotton XG subunits. Peaks obtained in MS spectra (figure 3.6) were analyzed and assigned to a subunit type depending on the mass of the peak as follows: peak with m/z 1085-XXXG, peak with m/z 1247 has 2 possibilities XXLG and XLXG, peak with m/z 1393-XXFG, peak with m/z 1409-XXLG and peak with m/z 1555 XLFG (8). Peak E-2 eluted at 0.25 M was mostly salts and had a very low amount of sugar in it. Peaks E-3 to E-5 eluted with 0.6 M to 1.3 M NH_4Ac buffer (pH 5.2) mainly consisted of RG in it with very low amounts of XG. All these 3 peaks showed similar sugars except varying galA content. Adsorbed peaks (i.e. E3 to E5) had minor amounts of XG, when they were treated with endoglucanase and analyzed on CZE. But it was not detected by MALDI-TOF MS.

DISCUSSION

Sequential use of cloned enzymes with high specificity on the XG-RG complex did show results which point to the crosslink between the XG and RG being through a branched α, 1-5 linked arabinan. Treatment of the XG-RG with arabinosidase shows liberation of free arabinose as one would expect for this enzyme, cleaving the terminal arabinose braches from branched arabinan. The specificity of enzyme was cross examined by applying it on APTS labeled double repeats of tamarind XG. Even after a prolonged incubation period,

the enzyme did not show any significant effect on the tamarind XG double repeats. The release of a little XG found in the unadsorbed peak D-1 on treatment with arabinosidase is not the outcome of the enzymatic degradation, but the XG indicates that some XG has a non covalent cross-linkage to the pectic region. MALDI-TOF MS and CZE shows the presence XG subunits in the XG-RG complex treated with arabinosidase. Further treatment of this pretreated XG-RG complex with arabinanase showed the dissociation of much of the XG from RG. The arabinanase enzyme cleaves the internal arabinan linkages, present between the polymers. The results obtained by arabinanase treatment did show the dissociation of XG from RG and generation of free arabinose and arabinobiose. The experimental evidence illustrates the removal of significant amount of XG from RG, justifying our hypothesis of arabinan being a major cross-linkage between the XG and RG I. Use of some more cloned enzymes such as galactanase, xylogalacturonase, and rhamnogalacturonase will give us more details on the linkage structures. By using galactanase enzyme we could check the possibility of an arabinogalactan also being a cross-link as proposed in the Keegstra *et. al.* plant cell wall model.

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Figure 3.1 Flowchart illustrating the sequential degradation of XG-RG complex with enzymes like arabinosidase and arabinanase.

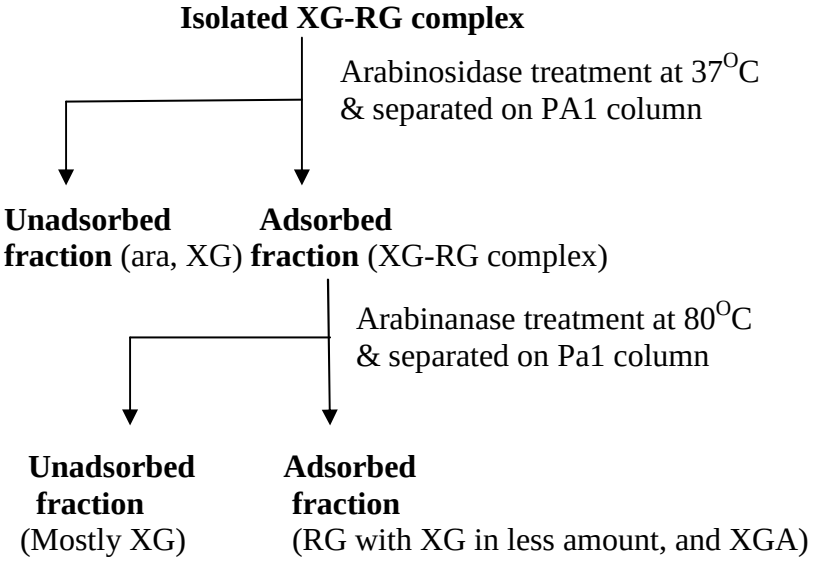
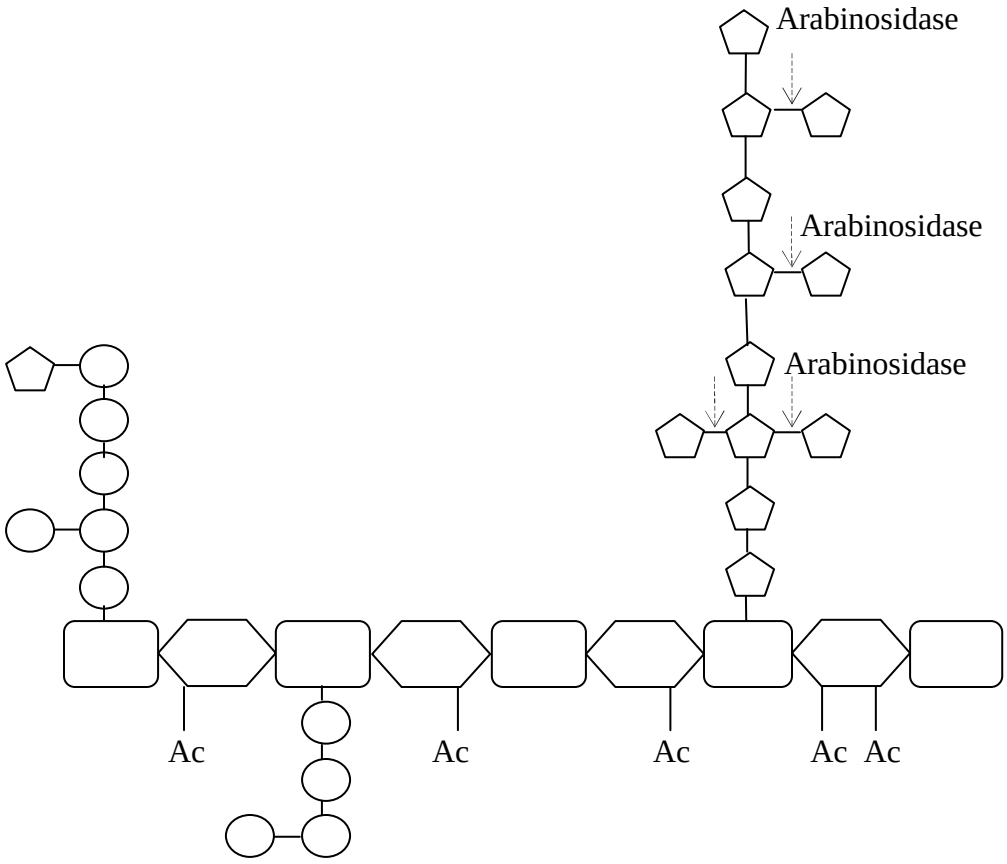
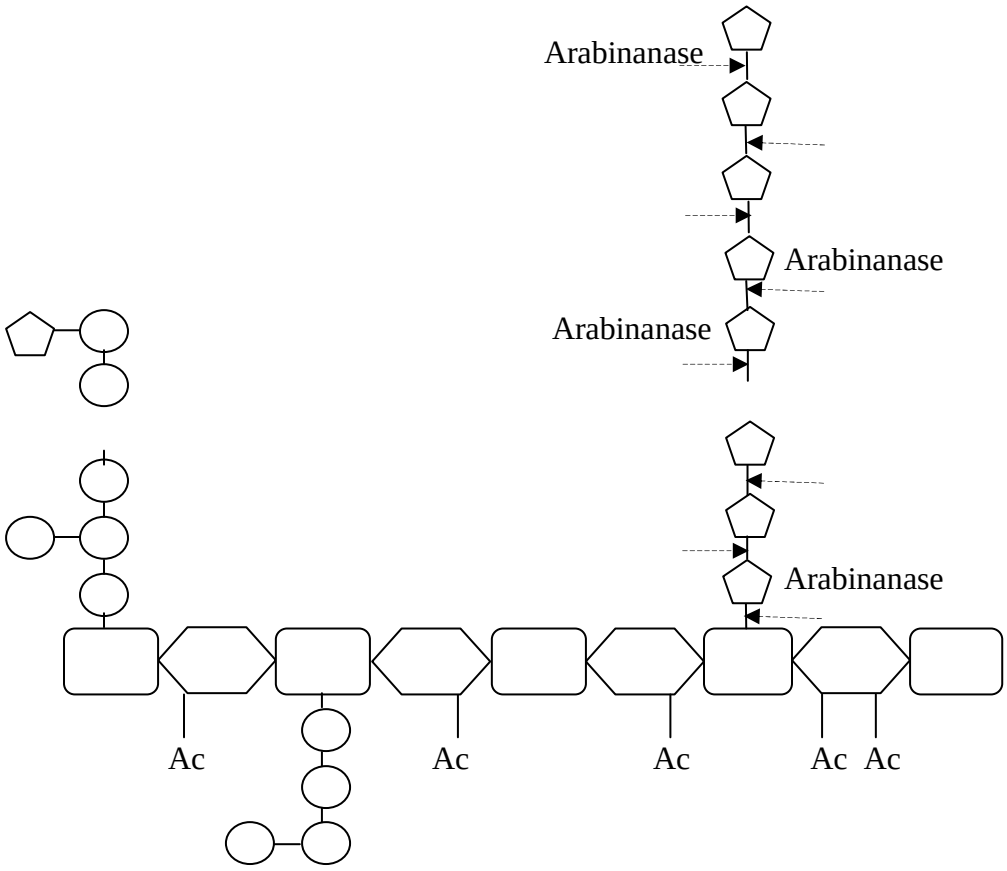


Figure 3.2a Schematic representation of arabinosidase activity on RG I.



Arabinosidase activity on Rhamnogalacturonan I

Figure 3.2b Schematic representation of arabinanase activity on RG I.



Arabinanase activity on Rhamnogalacturonan I

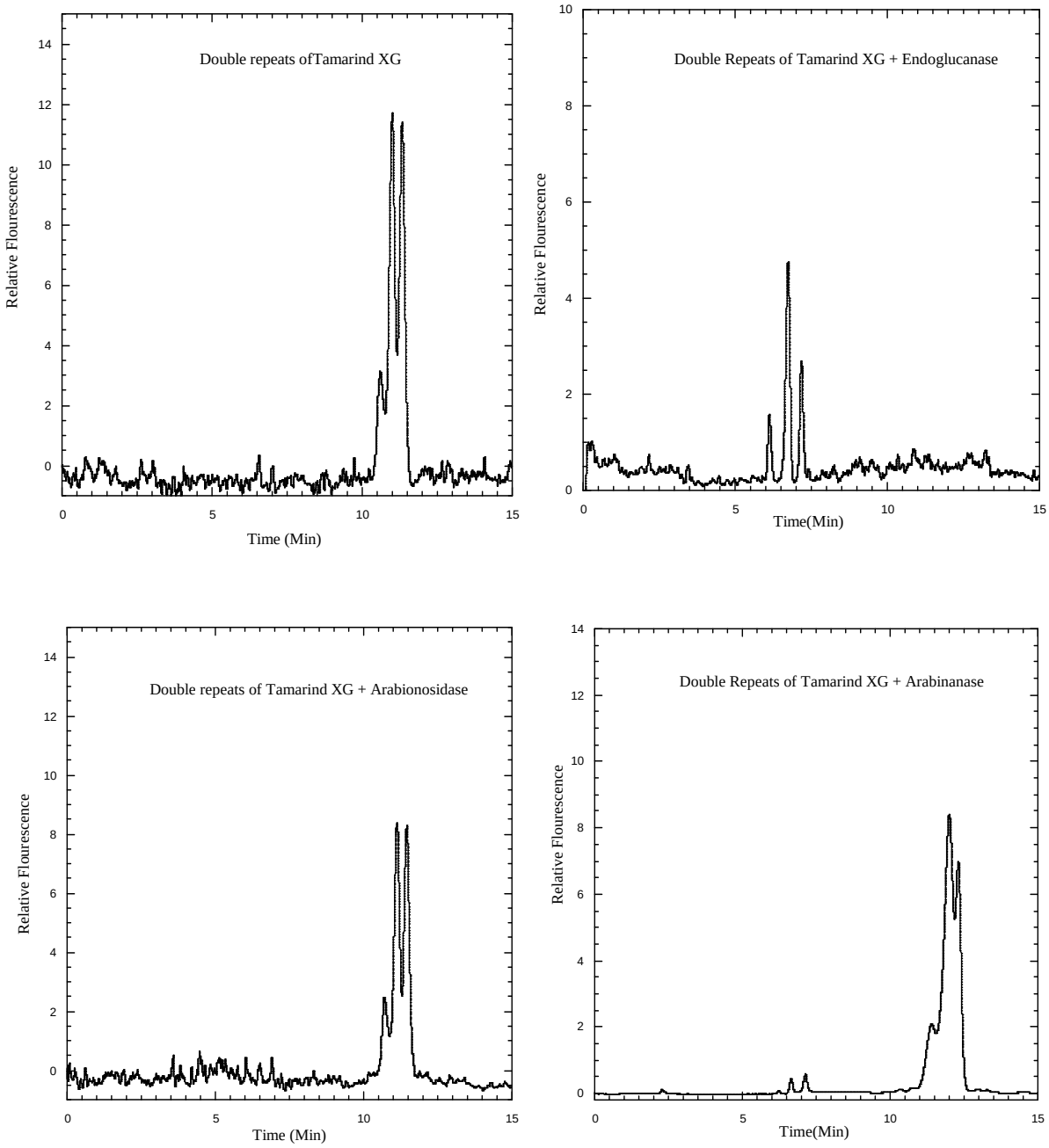


Figure 3.3 Electropherograms obtained by APTS labeled fractions of Tamarind XG double repeats treated with Endoglucanase, Arabinosidase and Arabinanase.

Figure: 3.4 Separation of the XG-RG complex digested with Arabinosidase on a PA1 column.

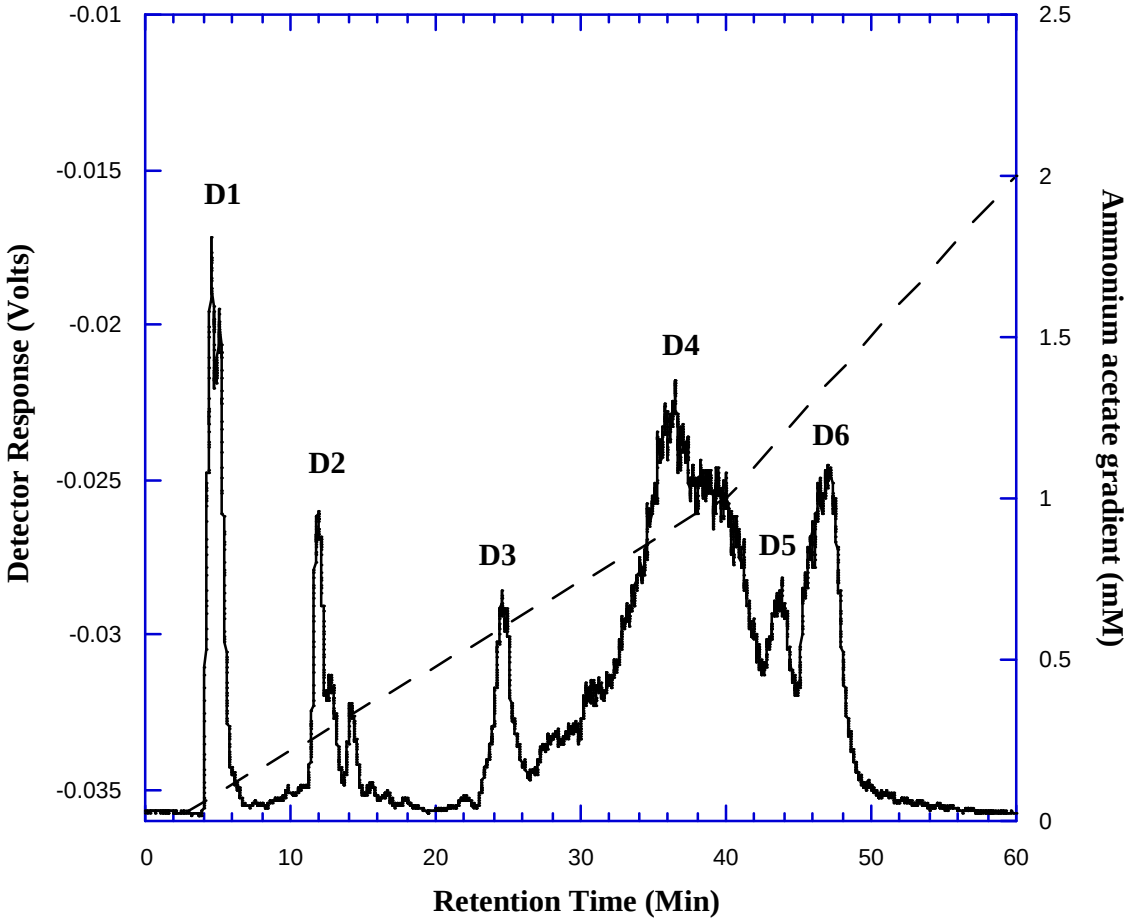


Table 3.1 Sugar compositions of peaks obtained by digestion of XG-RG complex with arabinosidase

Name of the sugar	D-1 GCS Mole%	D-2 GCS Mole%	D-3 GCS Mole%	D-4 GCS Mole%	D-5 GCS Mole%	D-6 GCS Mole%	XG-RG Complex (D4, D5, D6, D7)
Ara	45.2	7.8	9.9	12.3	8.5	5.9	9.3
Rha	n.d	4.1	10.5	17.7	19.1	11.1	14.3
Fuc	3.5	1.5	1.3	0.5	0.7	0.8	1.4
Xyl	18.3	10.6	6.3	7.5	8.7	7.9	9.2
GalA	n.d	14.3	36.1	38.2	43.0	57.7	45.3
Man	1.7	22.5	0.8	0.4	0.2	0.1	0.2
Gal	11.3	20.8	13.6	19.6	16.4	8.6	15.5
Glc	20	18.6	21.5	3.8	4.1	8	4.8

Name of the sugar	D-1 WCS Mole%	D-2 WCS Mole%	D-3 WCS Mole%	D-4 WCS Mole%	D-5 WCS Mole%	D-6 WCS Mole%	XG-RG Complex (D4, D5, D6)
Ara	39.1	10.1	14.9	15.7	11.2	8.6	11.4
Rha	n.d	4.8	11.9	19.5	14.7	10.8	15.9
Fuc	4.9	2.1	1.3	0.5	0.7	0.8	1.3
Xyl	19.8	15.2	5.6	9.5	12.1	8.6	10.4
GalA	n.d	13	31.5	32.3	42.7	51.4	40.7
Man	1.5	18.3	0.3	0.5	0.3	0.1	0.2
Gal	9.6	17.7	16.6	18.5	13.8	11.2	15.8
Glc	25.3	18.8	17.9	3.5	4.5	8.5	4.3

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green cell walls (OK1.2) WCS: White cell walls (Acala 44)
n.d: not detected

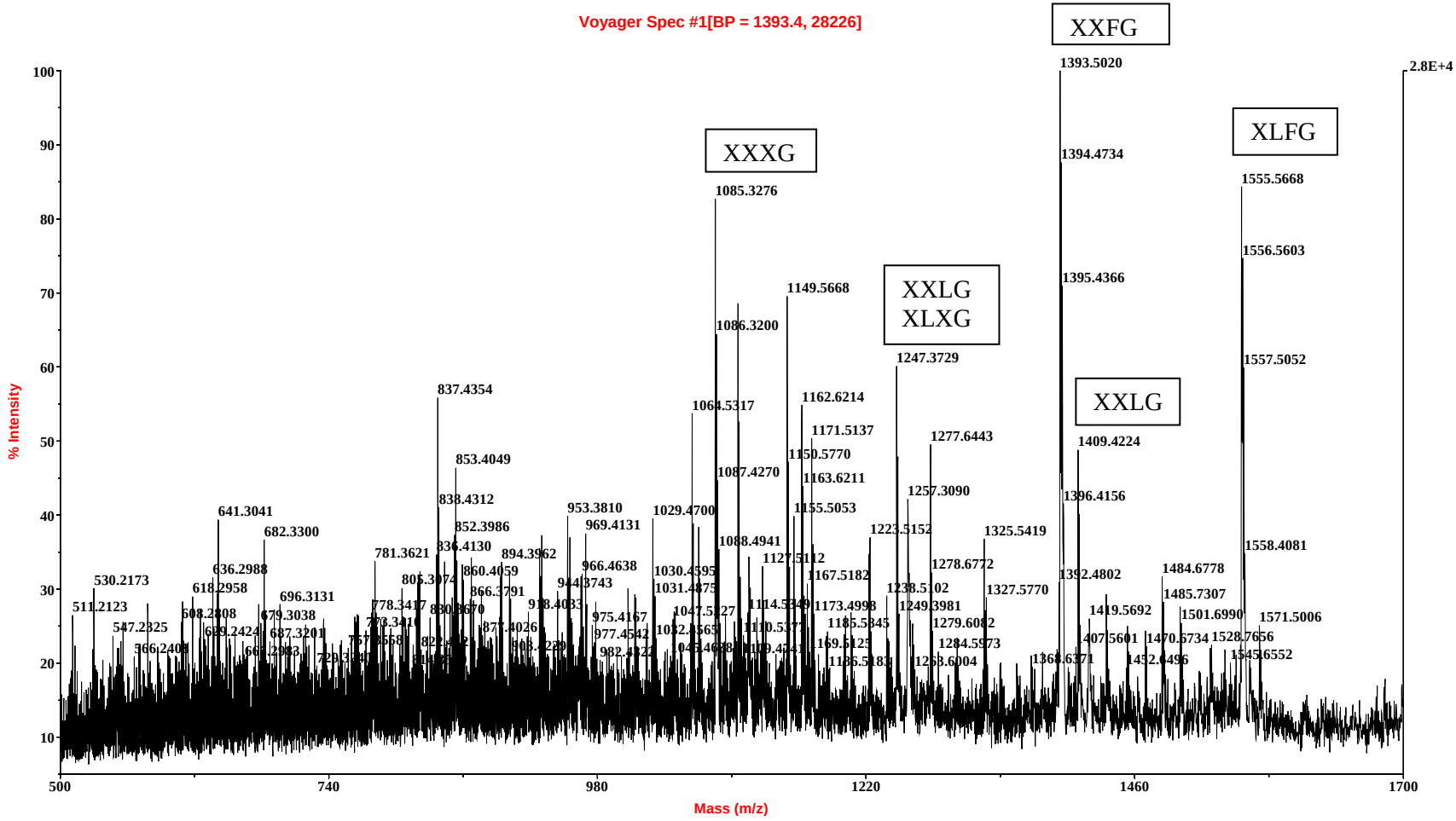


Figure 3.5 MALDI-TOF MS spectra of adsorbed fraction obtained by arabinosidase treatment separated on PA1 column and digested with endoglucanase

Figure 3.6 Separation of the predigested XG-RG complex with arabinanase on a intermediate size PA1 column with flow rate of 2ml/min.

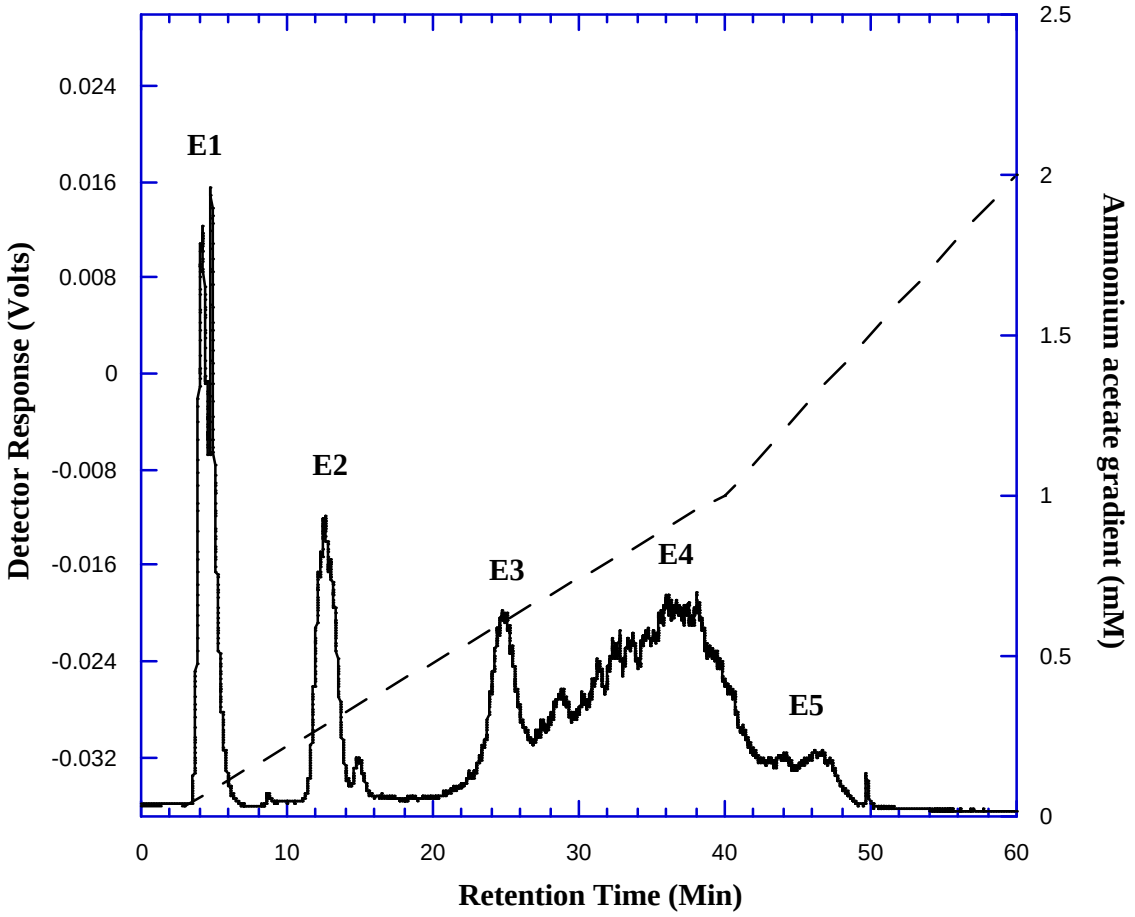


Table 3.2 Sugar compositions of peaks obtained by digestion of predigested XG-RG complex with arabinanase

Name of the sugar	E-1 GCS Mole%	E-2 GCS Mole%	E-3 GCS Mole%	E-4 GCS Mole%	E-5 GCS Mole%	XG-RG Complex (E3, E4, E5)
Ara	8.7	5.4	7.4	6.3	5.4	6.4
Rha	1.1	3.7	19.6	21.6	11.8	16.8
Fuc	7.2	0.9	1.3	0.9	0.8	0.6
Xyl	37.4	13.4	12.8	8.4	10.9	8.3
GalA	n.d	7.8	35.4	40.8	57.5	48.8
Man	1.1	46.8	0.2	n.d	0.1	0.2
Gal	14.8	9.8	18.7	16.6	10.9	16.6
Glc	29.7	11.2	4.6	5.4	2.6	2.9

Name of the sugar	E-1 WCS Mole%	E-2 WCS Mole%	E-3 WCS Mole%	E-4 WCS Mole%	E-5 WCS Mole%	XG-RG Complex (E3,E4, E6)
Ara	9.9	6.6	7.9	8.5	8.4	8.6
Rha	2.5	4.1	20.4	21.5	13.3	18.1
Fuc	6.8	0.8	1.4	0.8	0.7	0.6
Xyl	37.1	14.1	14.6	7.1	11.1	8.8
GalA	n.d	7.3	30.7	35.3	51.1	44.1
Man	1.1	43.4	n.d	0.1	0.1	0.1
Gal	17.3	11.3	20	21.8	12.4	17.3
Glc	25.3	12.4	4.3	5.0	2.9	2.4

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green cell walls (OK1.2) WCS: White cell walls (Acala 44)
n.d: not detected

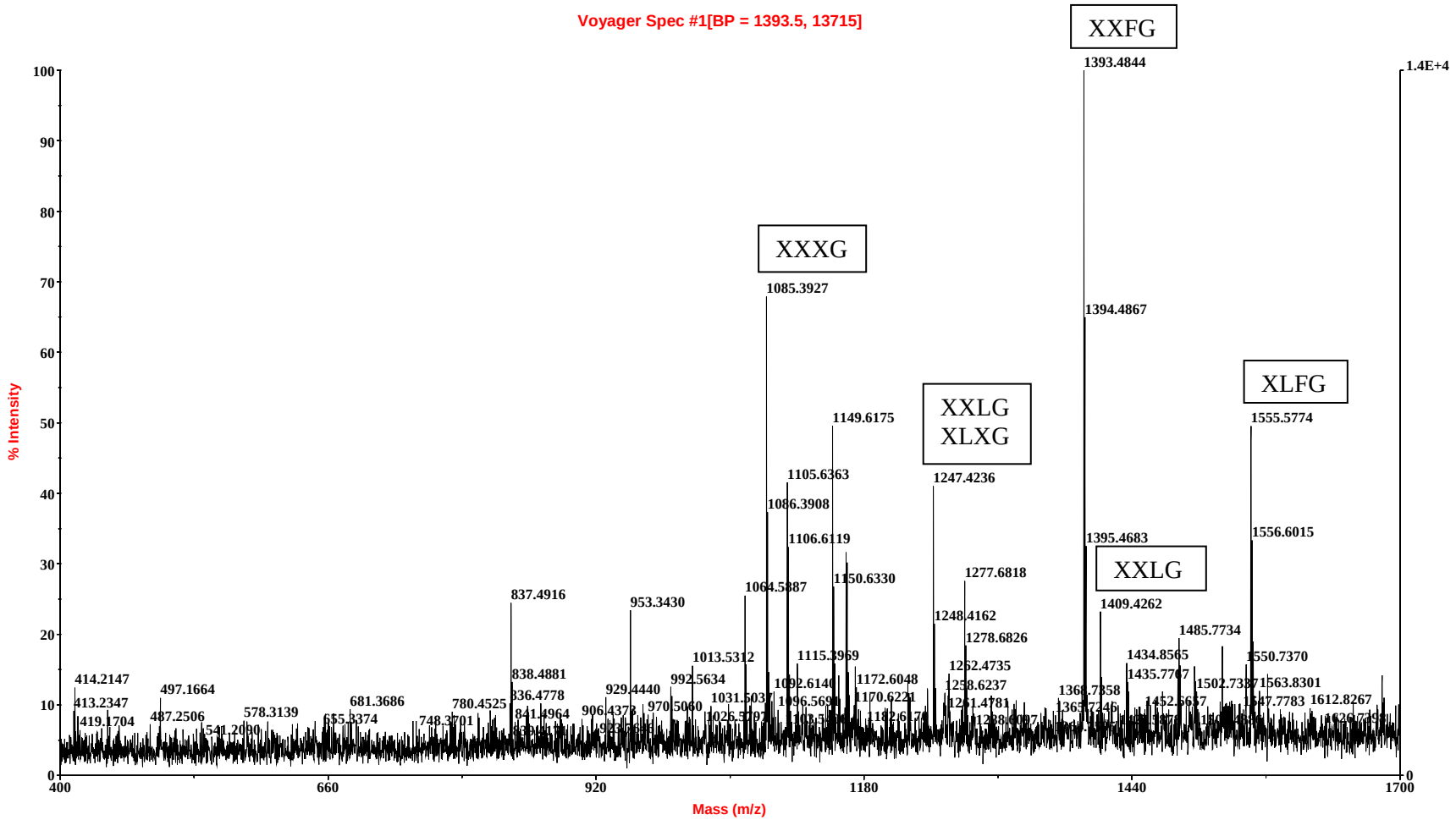


Figure 3.7 MALDITOF-MS spectra of unadsorbed peak formed by arabinanase treatment separated on PA1 column and digested with endoglucanase.

CHAPTER 4
SUMMARY AND CONCLUSION

Polysaccharide is the main ingredient in plant cell walls. Other components of plant cell walls are proteins, lignin, suberin, wax, and cutin. Six major polysaccharides present in the primary cell wall of higher plant include cellulose, hemicellulose (xyloglucan in dicots), homogalacturonan (HG), rhamnogalacturonan (RG I), rhamnogalacturonan (RG II),and xylogalacturonan (XGA). For a better understanding of the structural complexity and heterogeneity of pectic polysaccharides, it is important to study the linkages between the pectic regions as well as to other polysaccharides present in primary cell walls. The current plant cell wall model suggests a xyloglucan-cellulose network independent from the pectin network. The work carried out by Mort and group in the last few years has shown convincing results that about half of the xyloglucan is covalently linked to RG. These results are in accordance to the proposed early model of primary cell walls, where arabinan or arabinogalactan linked these two polymers. But the linkage structure between XG and RG is still unresolved. The term RG I is replaced in this thesis by RG because the sugar composition of XG-RG complex showed more galA than rha, which suggests XGA is also in the complex. The main aim of this thesis project was to determine and characterize the crosslink present between XG-RG to understand architecture and functionality of the whole cell wall. Preliminary results during isolation of XG-RG complex from intact cell wall illustrated that strong alkali extraction (24% KOH & 0.1% $NaBH_4$) of EPG pretreated cell walls solubilized around 80-90% of the RG

and XG present in the cell wall. Evidence obtained by fractionating the alkali extracts on DEAE and PA1 anion exchange column, showed the presence of two types of XG: the unlinked XG and XG crosslinked to RG. Endoglucanase digestion of EPG predigested cell walls released around two thirds of the total RG and XG oligomers, where as remaining one third of RG and some of XG was extracted using strong alkali. If RG was not linked to XG but was just stuck to XG physically, endoglucanase treatment would not have liberated RG fragment by cleaving the XG fragment. These results illustrate the possibility of covalent linkage between XG and RG. Also the Harsh treatment with strong alkali could not separate XG from RG, indicating the presence of cross-linkages between them.

Chemical and enzymatic degradation methods have been widely used in the past few years for understanding the cell wall structure. Polysaccharide degrading enzymes are of great use due to their unique feature of having high substrate specificity. Many pectin degrading enzymes are present in plants and microorganisms. These can be heterogeneously expressed in *Pichia* for ease of purification. Enzymes like endopolygalacturonase (EPG) degrade the non-esterified HG regions and make the XG-RG isolation easier. In the literature it is reported that removal of HG releases much of RG I and RG II from sycamore cell walls. But in cotton cell walls extraction with strong alkali after EPG digestion released much of RG I present in intact cell walls. This indicated the presence of some type of linkage which kept RG I retained in the cell wall after EPG digestion. The EPG solubilized part mostly consisted of galA mono, di and trimers along with RG II. Strong alkali treatment, extracted the XG-RG complex from the insoluble EPG-cell wall fraction. Nearly 90% of XG and RG present in cell wall got

extracted by strong alkali along with XGA. XG-RG complex upon treatment with arabinosidase and arabinanase enzyme showed a significant change in their structure, dissociating most of the XG from RG I. Table 4.1 shows the comparative change in sugar composition of the intact XG-RG complex, XG-RG complex treated with arabinosidase and pretreated XG-RG complex with arabinanase treatment. The trend showed by the sugar composition does fairly agree with our hypothesis of arabinan being the cross-linking factor between XG and RG. The amount of rhamnose and galactose increases indicating more and more XG getting dissociated from RG I as an outcome of enzymatic removal of arabinose side as well as internal arabinan bonds. Xylose, glucose, fucose shows decrease indicating removal of XG from the complex. Most of XG is dissociated from RG. But it appears a small amount of XG is still left with RG I indicating residual cross-linking perhaps by an arabinogalactan. Use of galactanase, XGAse and few more enzymes in the future may help us in better understanding of the crosslink. Along with the enzymatic degradation we can deduce how these cell wall polymers are connected and interact with each other. Understanding the nature of crosslinks between wall polymers will allow development of a more elaborative cell wall model showing the possible interconnections between all cell wall polymers.

Table 4.1 Comparative trend of sugar composition of XG-RG before enzymatic treatment and after enzymatic treatment.

Name of the sugar	XG-RG complex (intact) GCS Mole%	XG-RG complex with arabinosidase GCS Mole%	XG-RG complex with arabinanase GCS Mole%
Ara	14.6	9.3	6.4
Rha	12.8	14.3	16.8
Fuc	2.5	1.4	0.6
Xyl	12.8	9.2	8.3
GalA	35.9	45.3	48.8
Gal	13.8	15.5	16.6
Glc	7.4	4.8	2.9

Name of the sugar	XG-RG complex (intact) WCS Mole%	XG-RG complex with arabinosidase WCS Mole%	XG-RG complex with arabinanase WCS Mole%
Ara	16.8	11.4	8.6
Rha	14.3	15.9	18.1
Fuc	2.0	1.3	0.6
Xyl	12.7	10.4	8.8
GalA	33.5	40.7	44.1
Gal	14.3	15.8	17.3
Glc	6.4	4.3	2.4

Mole%: molar percentage of sugars accounted for in the GLC analysis.
GCS: Green cell walls (OK1.2)
WCS: White cell walls (Acala 44)

VITA

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Candidate for the Degree of

Master of Science

Thesis: CHARACTERIZATION OF A CROSSLINK BETWEEN XYLOGLUCAN AND RHAMNOGALACTURONAN FROM COTTON CELL WALLS

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Pectin is the most complex and major component of primary plant cell walls. For better understanding of its structural complexity and heterogeneity, it is crucial to know about the structure and linkages within the pectic regions as well as to other polysaccharides present in primary cell walls. Our main aim is to characterize the cross-linkage between the XG and RG.

Preliminary results show nearly half of the XG is covalently linked to RG. EPG digestion of cell walls followed by strong alkali extraction (24 % KOH & 0.1 % $NaBH_4$) solubilizes most of the XG-RG complex along with other pectic polysaccharides and unlinked hemicelluloses. The XG-RG complex can then separated from the neutral and slightly acidic sugars using ion exchange chromatography. Treatment with arabinosidase removes the arabinose from the arabinan in the XG-RG complex. Free arabinose can be separated using an ion exchange column. Further treatment of the complex with arabinanase, cleaves the arabinan linkages essentially dissociating most of the XG from RG. These finding were supported with sugar composition, CZE electropherograms, MALDI-TOF MS spectra, and NMR spectroscopy. Our experimental results significantly suggest arabinan being the crosslink present between the XG and RG complex as proposed in an earlier plant cell wall model by Keegstra *et al*.

ADVISER'S APPROVAL: Dr Andrew Mort