PARTIAL STRUCTURAL CHARACTERIZATION OF A PECTIN EXTRACTED FROM WATERMELON CELL WALLS BY MILD ALKALI

By

LIYING YU

Bachelor of Science

Hangzhou University

Hangzhou, The P. R. of China

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Thesis Approved:

Andrew Mort
Thesis Advisor

Margaret L. Pierce

H. Olin Spivey

Thomas C. Collins
Dean of the Graduate College

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LIST OF ABBREVIATIONS

2-AP	2-aminopyridine
ANTS	8-aminonaphthalene-1,3,6-trisulphonate
Ara	arabinose
CZE	high performance capillary zone electrophoresis
DM	degree of methylesterification
EPG	endopolygalacturonase
Fuc	fucose
Gal	galactose
GalA	galacturonic acid
GC	gas chromatography
Glc	glucose
HG	homogalacturonan
HF	hydrogen fluoride
HPLC	high performance liquid chromatography
LSIMS	liquid secondary ion mass spectroscopy
Man	mannose
MHR	modified hairy region
MS	mass spectrometry
M.W.	molecular weight
Rha	rhamnose
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
SCB	sodium cyanoborohydride
Xyl	xylose
Vi	included volume
Xyl	xylose

CHAPTER I

INTRODUCTION

Pectins are probably the most complex polysaccharides known, and they are present in virtually all higher plants, and certain fresh water algae (Anderson & King, 1961). The structures of pectins have been the subject of many investigations for many years (O'Neill et al., 1990), because pectin is important as (1) a structural and functional component in the cell walls of higher plants (Carpita et al., 1993); (2) a soluble food fiber with significant nutritional benefits, and a substantial source of texture in fresh and processed fruits and vegetables (Fincher et al., 1981).

Basically, pectins comprise a family of acidic polymers including homogalacturonans and rhamnogalacturonans. Neutral sugar side chains are also present (Stoddart, 1984). O'Neill et al. (1990) stated that three pectic polysaccharide regions, homogalacturonan (HG), rhamnogalacturonan I (RG-I), and a substituted galacturonan referred to as rhamnogalacturonan II (RG-II), have been isolated from the primary cell walls of plants. The HG region is partially methyl-esterified, and it perhaps contains some rhamnose (Rha) and a low proportion of other neutral sugars (Darvill, et al., 1980). RG-I is pictured as a long repeating sequence of alternating L-rhamnose and D-galacturonic acid residues with a variety of L-arabinosyl-, D-galactosyl-, and L-fucosyl-containing side chains (O'Neill et al., 1990). RG-II is a small region which has a complex glycosyl-residue and glycosyl-linkage composition, including some unusual sugars (O'Neill et al., 1990). Based mainly on enzymic degradation, De Vries et al. (1982) proposed that apple pectins consist of highly carboxyl-methylated linear HG regions (smooth regions) and

highly branched rhamnogalacturonan regions ("hairy" regions) which contained most of the neutral sugars. Hairy regions can be isolated from cell walls of a variety of fruit and vegetable tissues by the action of a crude mixture of pectolytic and cellulytic enzymes (Schols et al., 1994).

Research on cell walls in Dr. A. Mort's laboratory deals mainly with the study of cotton. Several polysaccharides of cotton cell walls have been isolated and partially characterized (Komalavilas and Mort, 1989; EL Rassi et al., 1991; Mort et al., 1993). In the studies of pectins from cotton suspension culture cell walls, Dr. A. Mort's laboratory found that a 50% methyl-esterified HG segment, resistant to endo-polygalacturonase (EPG) digestion, associating with the Rha-rich pectic region (RG I), contained about one xylose (Xyl) residue per three galacturonic acid (GalA) residues (An, 1992). During analysis of the modified hairy region of apple pectin, which was released from the apple fruit by the combined action of pectinase and cellulases, the research group of A. Voragen in Holland found this EPG-resistant pectic fraction having a ratio of GalA : Rha much higher than that (molar ratio of GalA : Rha is 1) expected for an isolated RG I. Even after de-esterification with alkali, the HG sections of the complex were still resistant to EPG digestion, and this pectic fraction of apple pectin also contains some Xyl (Schols et al., 1990). Based on these results, we proposed that this pectin fraction is likely a complex of RG I and HG segments in which some neutral sugars (Xyl?) are directly covalently linked to GalA and protect the HG segments from digestion by enzymes.

Since only small quantities of the complex of RG I and HG segments could be obtained from the cotton cell walls, we investigated other resources from which we might be able to obtain large enough quantities of samples. Watermelon fruit produces a large amount of a relatively uniform cell type in the mesocarp. We were fortunate that one of our collaborators (Dr. Niels O. Maness of Department of Horticulture and Landscape Architecture of OSU) had prepared a large amount of cell walls from ripe watermelon fruit, and that these cell walls contained abundant pectic polysaccharides (about 62 mole% of the

sugar is GalA). Since we only wanted the oligomers, our initial approach was to treat the intact watermelon cell walls directly in HF and then to purify out the desired oligomers. To make the oligomers suitable for high resolution anion-exchange chromatography, we used mild alkali to saponify them to remove the methyl ester groups from the HG segments. However, we had great difficulty in resolubilizing the oligomer mixture after saponification, neutralization and drying. To circumvent these steps, we decided to saponify the intact watermelon cell walls before HF treatment. The saponification treatment alone solubilized a considerable portion of the pectin, and yielded, in about 15% by the weight of the treated watermelon cell walls, a pectic portion with a high degree of substitution with xylose (the molar ratio of GalA and Xyl is 7 : 1).

In this research, we isolated a pectin portion which is rich in Xyl from cell walls of ripe watermelon fruit by mild alkali and partially characterized its structure using chemical and biochemical methods. We also used it to characterize the effects of xylosylation on the activity of EPG and to produce fragments of the xylogalacturonan for further characterization.

CHAPTER II

LITERATURE REVIEW

Pectin Occurrence and Function

Pectic substances are carbohydrate macromolecules naturally existing in all higher plants. They are prominent structural constituents of primary cell walls and of the middle lamella in non-woody tissues, but are absent from the secondary walls of more mature tissues (Brillouet, 1987). Their synthesis beginning from UDP-D-GalA and taking place in the Golgi system (Karr, 1976; Mohnen, 1995) is performed mainly during the early stages of growth, in young enlarging cell. Compared with the young, actively growing tissues, lignified tissues are low in their content of pectic substances.

Pectic polysaccharides are quite easy to extract from plant materials. They occur mainly as water-insoluble pectin, but some occur as water-soluble polysaccharides. Water-soluble pectic polysaccharides are usually extracted from cell walls by water and aqueous buffer (Bacic et al., 1988), and the majority of water-insoluble pectins can be extracted using more powerful extractants, such as aqueous calcium-chelating agents, dilute acid, dilute alkali and pectic enzymes (Stoddart, 1984). In general, when more powerful extractants are used, the pectins extracted contained more neutral sugars (Schols, 1995).

Pectins make up to about 30% of the polysaccharides in the primary cell walls of most higher plants (Fry, 1988). The precise functions of pectins in plants are still open to discussion. The amount and the nature of pectic substances play an important role in

regulating the firmness of fruits and vegetables during growth, ripening, storage and processing (De Vries et al., 1984). One of the most characteristic changes during the ripening of fleshly fruit is softening, and this change is attributed to enzymatic degradation and solubilization of the pectic polysaccharides (Sakai et al., 1993). During the cooking process of green beans, the major cleavage reaction leading to vegetable softening is probably β-eliminative breakdown of highly methylated HG regions of the pectin (Stallesmits, 1995). Pectic substances also act as a structural barrier to pathogens, and pest infections can induce the enzymic de-esterification of the pectin and the formation of insoluble calcium-pectate (Weintraub et al., 1961). In addition to that, pectic polysaccharides are the major polysaccharides in the intercellular layer (middle lamella) responsible for cell cohesion (Pilnik, 1981). The ion exchange capacity of pectic substance can be used by the roots of plants to absorb calcium ions from the soil (Oades, 1978). It was also discovered that oligogalacturonides of specific size can play an important role in plant development and can regulate plant morphogenesis (Albersheim, 1989); cell-wall pectic fragments from suspension-cultured sycamore cells can inhibit the formation or alter the position of roots, cause marked tissue enlargement or induce flower formation on tobacco thin-layer explants (Eberhard et al., 1989).

Uses of Commercial Extracted Pectins

Pectins are mainly used in the food industry for their strong gelling power. More than 50% of the world's pectin production is used in making jams, jellies, marmalades and confectionery as a gelling agent (Ikkala, 1985). Low esterified pectin gives gelation in the presence of Ca^{2+}-ions, and high esterified pectin gives gelation in the presence of sugar and acid. In addition, pectin is also used as stabilizer, thickener, and fat replacer in dairy products, fruit drinks, fruit and tomato pastes. Moreover, pectic substances have a nutritional function as natural food fiber. Pectins can lower the cholesterol level of serum

and/or liver (Jenkins et al,. 1979) and decreases the amount of glucose in the serum of diabetic or obese subjects (Williams et al., 1980).

Pectins can also be used in medical preparations and possesses many pharmacological activities. Pectin has a haemostatic and anti-fibrinoloytic effect, and it has been used as blood plasma extender and for the treatment of gastric diseases (Neukom, 1967). Pectin use as a prophylactic in poisoning with heavy metals and radioactive elements has been reported (Waldron-Edward et al., 1965).

Structure of Pectin

The chemical structure of pectic substances has been intensively studied for more than sixty years because of their great importance in many fields. The structures of pectic substances extracted in mild conditions from various plants and fruits have been particularly well investigated (Mankarios et al., 1980; DeVries, 1982; Ishii, 1982; Bouau et al, 1984; Konno et al., 1986; Redgwell et al., 1986; Saulnier et al., 1987; Komalavilas and Mort, 1989; O'Neill et al, 1990; Schols et al., 1993).

In general, pectic substances are branched heteropolysaccharides in which the backbone contains L-rhamnosyl residues and α–(1→4)–linked D-galacturonic acid residues. Neutral sugars, mainly arabinose (Ara) and galactose (Gal), are found as side chains of varying length (Aspinall, 1980), and rhamnosyl residues are the main branching points (Albersheim, 1978). Until now, three different kinds of pectin fragments are isolated from the cell walls: Homogalacturonan (HG), Rhamnogalacturonan I (RG I), and Rhamnogalacturonan II (RG II).

Homogalacturonan (HG) (Smooth) Regions

The homogalacturonan (smooth) regions consist of predominantly α–(1→4)–linked D-galacturonic acid residues. The pyranose rings of D-galacturonic acid probably

occur mainly in the chair form $^4C_1$, corresponding to the most stable conformation of D-Gal (Morris et al., 1975).

The HG region, which is partially methyl-esterified, contains some Rha and a low proportion of other neutral sugars. It was proposed that at least some Xyl residues appear to be linked directly to the GalA residues of smooth regions (Thibault et al., 1993). The occurrence of xylogalacturonan in soluble pectins has been previously observed (Bouveng, 1965, Massiot et al., 1988). It was reported in literature that Xyl residues might be linked directly to GalA residues at C-2 or C-3 position (Bouveng, 1965).

The HG region of pectin can be extracted from plant cell walls by acid hydrolysis, mild alkali treatment and pectic enzyme digestion. However, depending on the extraction method used, some modification of the polymer may occur. -23 ^{0}C HF solvolysis can also be used to isolate a HG region from cell walls. At that condition, galacturonosyl linkages is completely stable to solvolysis in HF (Mort et al., 1990). By making use of their differential solubility in water vs. 80 % ethanol after HF solvolysis of the cell walls at -23 ^{0}C, a homogalacturonan with 50 % esterification was obtained from cotton suspension culture cell walls (Mort et al., 1990).

The degree of polymerization of HG is not exactly known. The data of Chambat et al. (1980) demonstrated that a HG with a degree of polymerization greater than 100 could be obtained from the cell walls of Rosa cell cultures with base treatment. From the results of acid (0.5 N HCl, 3 h, 100 ^{0}C) hydrolysis of citrus, apple, and sunflower pectins, Powell et al. (1982) suggested that the polygalacturonic backbone was formed of repetitive uronate sequences of constant length (~ 25 residues) between single Rha residues. Thibault et al. (1993) found the lengths of the HG segments from apple, beet, and citrus a minimum of 72-100 units using acid (0.1 M HCl, 72 h, 80 ^{0}C) hydrolysis. Carpita et al (1993) even stated that HG region might contain up to about 200 GalA units and be 100 nm long.

The distribution and degree of methyl esterification (DM) in HG regions varies in different plant cell walls. The DM is defined as the number of moles of esterified carboxyl groups per 100 moles of GalA. Normally, pectins are called high methoxyl pectins when the value for DM is above 50. High DM was found in citrus HG (Devries et al., 1986), whereas a much lower degree (DM is about 15) was found in cotton suspension culture cell walls (Maness and Mort, 1989). It was reported that an average degree of esterification of apple and orange pectin is about 70% (Speirs, 1979). Voragen et al. (1986) reported more detailed data about the degree of esterification as follows: apple (71%), mango (68%), citrus fruits (64%), sugar beet (55%), potato (31%), sunflower (17%), and pear (13%). Kravtchenko (1992) analyzed the mono-, di-, and tri-galacturonic acid produced by the action of EPG on commercially extracted apple pectins. He found the proportion of mono-, di-, and tri-galacturonic acid produced is much higher than that expected from a random distribution of methy esters, and suggested that unesterified galacturonan residues are arranged as small blocks (non-random distribution) over the HG region. For native apple high DM pectins, using purified pectin lyase extensively digestion, a non-random distribution of the methyl ester groups was also found by comparing the chromatograms of gel filtration and ion-exchange chromatography to those of pectins in which a random distribution of the methyl esters is assumed (De Vries et al. 1986). Using chemical methods to produce oligosaccharides containing the contiguous non-esterified GalA residues, Mort et al. (1993) found that the distribution of non-esterified GalA in a pectin fraction with 50% esterification obtained from cotton suspension culture cell walls by -23 ^{0}C HF treatment is far from random.

Rhamnogalacturonan I (RG I) regions

RG I is pectic polysaccharide that is released from the cell walls by the action of highly purified EPG (English et al., 1972). It is composed of D-galacturonosyl, L-rhamnosyl, L-arabinosyl, D-galactosyl, and small amounts of L-fucosyl residues (McNeil

et al., 1980). The backbone of RG I is composed of the diglycosyl repeating unit (→4-α-) D-Gal*p*A-(1→2)-α-L-Rha*p*. The length of this alternating structure is unknown (McNeil et al., 1984).

Acetyl groups have been reported to be present in the RG I type of polymer. Komalavilas and Mort (1989) reported the isolation of GalA-Rha dimers from cotton suspension culture cell walls using HF hydrolysis of which about 30% of the galacturonsyl residues were acetylated at C-3, but Lerouge et al. (1993) found enough acetates that every GalA in RG I from the walls of suspension-cultured sycamore cells could be acetylated at C-2 or C-3.

In suspension-cultured sycamore cell walls, approximately 50% of the 2-linked rhamnosyl residues are substituted at C-4 with oligoglycosyl side chains composed primarily of arabinosyl, galactosyl or a small amount of fucosyl residues (McNeil et al., 1985). These side chains appear to be irregular, ranging from 1 to 15 residues in length, and at least 30 different ones have been identified by GC-MS analysis (Lau et al., 1987).

Branched arabinans are known to be directly linked to the rhamnosyl residues of the RG I backbone. These arabinans are mostly 5-linked arabinofuranosyl units forming short chains (DP 2-20), but arabinosyl units of the arabinans can be connected to each other at virtually every position, the C-2, C-3, and the C-5, forming a diverse group of branched arabinans (Lerouge et al., 1993).

Two types of arabinogalactans are also isolated from plants. Arabinogalactan I is composed of β-(1→4)-linked Gal, and Arabinogalactan II constitute a broad group of (1→3), (1→6) linked Gal chains connected to each other. It is commonly accepted that both types are substituted by terminal and (1→3)-linked Ara side chains, and that they occur as side chains on RG I. However, it is not clear if they are directly linked to GalA residues or to Rha of the RG I (Ralet et al., 1994).

Rhamnogalacturonan II (RG II) regions

RG II is structurally very different from RG I, and it is completely solubilized from suspension cultured sycamore cell walls upon digestion with EPG (Darvill et al., 1978). It appears that RG II is covalently linked in the primary cell walls through a series of 4-linked GalA residues (McNeil et al., 1984). Less than 4% of the cell walls of dicots and less than 1% of the cell walls of monocots consists of RG II (O'Neill et al., 1990). RG II has a complex glycosyl-residue and glycosyl-linkage composition. There are at least 20 differently-linked glycosyl residues in this polysaccharide that has ~30 glycosyl residues in total, which include such unusual residues as aceric acid, 2-keto-3-deoxyoctulosonic acid, D-apiose, 2-O-methyl-L-fucose, 2-O-methyl-D-xylose, and 3-deoxy-D-lyxo-2-heptulosaric (Darvill et al., 1985; Stevenson et al., 1988). In contrast to RG I, in which the rhamnosyl residues of its backbone are frequently branched though C-4, RG II has a backbone of an α-(1$\rightarrow$4)-linked octa- or nonagalacturonic acid with side chains attached to position 2 and/or position 3 of most of residues (Stevenson et al., 1988). RG II has been identified in cell walls isolated from peas, pinto beans and tomato seedings based on the unusual glycosyl residues found (Darvill et al., 1985).

Hairy (MHR) Regions

Another model for pectic molecules was proposed by the group of Pilnik , in which the pectins were considered to contain HG regions (smooth region) comprising 90% of the GalA residues and 'hairy' regions which contained most of the neutral sugars (De Vries et al., 1982). Hairy region of pectin was first recognized from apple fruit tissues by Barrett and Northcote in 1965. Hairy regions of pectins were obtained from pectin fractions using complete digestion with pectate lyases and pectin lyases. They have been found in pectins isolated from apple (De Vries et al., 1982), onion (Ishii, 1982), and grape berries (Saulnier et al., 1987). Modified hairy regions (MHR) are obtained by the liquefaction process, in

which the juice is released from the apple pulp by the combined action of pectolytic and cellulolytic enzymes, followed by ultrafiltration, dialysis, and lyophilization (Schols et al. 1990b). Schols et al. (1994) isolated MHR from various plant cell walls materials and established that their structures were similar to the hairy regions described by De Vries et al. (1982).

Whereas RG I is considered to consist always of alternating Rha and GalA (the ratio GalA : Rha is 1), the hairy region of pectin isolated from apple has a ratio of GalA : Rha of 4.8 (De Vries et al., 1982). It is clear that these pectic fragments, obtained after complete degradation of pectins by pure pectic enzymes, do not contain just the "homogeneous" backbone of Rha and GalA residues as suggested by O'Neill et al. (1990) for RG I (the ratio GalA : Rha is 1). Now some researchers believe, a more complex distribution of short rhamnogalacturonan units over the whole hairy regions of the molecule, contribute to this breakdown pattern. Dr. A. Mort suggested that MHR (or Hairy region) is likely a complex of RG I and HG segments in which some neutral sugars (Xyl?) are covalently linked to GalA and protect HG segments in MHR from digestion by EPG. Voragan et al. (1993) proposed an adapted model of the Modified Hairy Regions in which MHR consists of three subunits: a xylogalacturonan subunit (Xyl directly linked to GalA in HG segment) interrupted by a subunit consisting of an arabinan-rich segment of the rhamnogalacturonan backbone (typical RG I with long arabinan side chains) and a subunit in which typical rhamnogalacturonan oligomers are dominantly present.

Hydrogen Fluoride in Pectin Structure Analysis

Hydrogen fluoride (HF) can be used for selective cleavage of glycosidic bonds at subzero temperature. Cleavage of each glycosyl linkage in a polysaccharide is highly dependent on the nature of the sugar involved, the sugar's anomeric configuration, the neighboring residues, the temperature of the reaction, and the time of the reaction (Mort et al., 1989a). Specific fragments of polysaccharides can be obtained by using HF at specific

reaction temperature. At -23^{0}C, uronic acid linkages, β-glucosidic linkages, methyl and acetyl esters are stable, but all the other glycosidic linkages in cell walls are unstable in HF treatment (Mort et al., 1991). If the reaction temperature is reduced, fewer glycosidic linkages are cleaved and larger fragments of the polysaccharides can be obtained. Mort et al. (1989b) reviewed the lability of various glycosidic linkages at different temperatures. Recently, more details of highly selective cleavage of furanosyl linkages in HF were reported (Qi et al., 1993).

Endo-Polygalacturonase (EPG)

This enzyme is produced by numerous fungi and bacteria, by a few yeasts (Ravelomanana et al., 1986), by higher plants, and some plant-parasitic nematodes (Riedel and Mai, 1971). Generally, EPGs are optimally active at acidic pH (4.0-6.0), and at a temperature of 30-40 ^{0}C.

The activity of this enzyme is to catalyze the hydrolysis of interior α–(1→4)–linked D-galacturonic acid residues. Substitutions of methoxyl groups on the pectic acid molecule will influence the activity of EPG (Jansen and McDonnell,1945). If the DM increases, the rate and extent of hydrolysis decreases. The best substrate for the action of EPG is low esterified or de-esterified polygalacturonic acid. Free carboxyl groups seem to be necessary for catalytic activity (Koller and Neukom, 1969). The EPG we used requires four contiguous non-methylesterified galacturonate residues to be able to act (Chen & Mort, 1995) and is hindered or blocked by rhamnosyl residues (Rouau, 1984; Saulnier, et al., 1987) or acetyl esters at C-2 and/or C-3 of the galacturonate residues (Rexova-Benkova et al., 1976). Figure 1 shows the scheme of a partially methylesterified HG region digestion by EPG based on results obtained in Dr. A. Mort's laboratory.

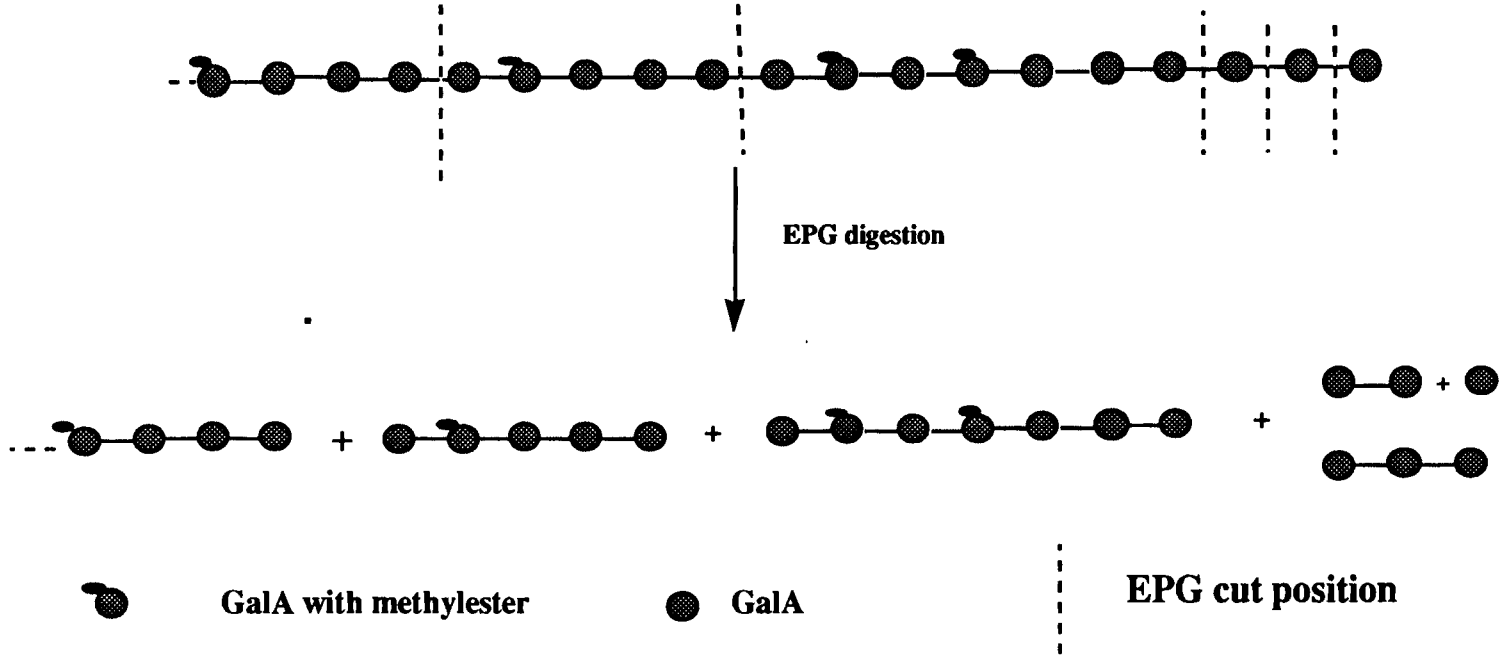

Figure 1: A scheme of a partially methylesterified HG region digested by EPG.

CHAPTER III

MATERIALS AND METHODS

Extraction of Mild-Alkali-Extract From Watermelon Cell Walls

Watermelon cell walls were kindly provided by Dr. Niels O. Maness' laboratory, which prepared them from ripe watermelon fruit. Watermelon mesocarp tissues were placed on ice and diced into small pieces, and then homogenized on ice in Tris saturated phenol to give enzymically inactive watermelon cell walls (Hegde, 1995). The solids were collected on two layers of mira cloth and washed with water until the phenol smell was gone. These crude cell walls were further washed with chloroform : methanol (1:1, V/V) and acetone until a fluffy consistency was obtained. The acetone washed cell walls residue was dried in an oven at 60 ^{0}C and stored in a brown colored bottle.

To isolate a mild-alkali-extract, dry watermelon cell walls were suspended in a solution of 0.1 N NaOH, and allowed to react with stirring at room temperature for 15 minutes. The pH, as indicated by pH paper, was kept constant by addition of 0.1 N NaOH. To ensure complete reaction, the treatment was continued overnight at 4 ^{0}C in the cold room. The soluble portion was separated by centrifugation at 10,000 RPM for 20 minutes in a GS-A rotor, and the supernatant solution was collected. The precipitate was further washed by resuspending in water, and recentrifuged two times under the same condition. All the supernatant solution was collected, and combined with the previous supernatant solution, followed by neutralization to pH 7.0. This solution was dialyzed

against distilled water one day (at least three times water changed) in a 10,000-12,000 daltons cut off membrane, and then dried by lyophilization.

Endopolygalacturonase Treatment of Mild-alkali-extract

Endopolygalacturonase (EPG) was bought from the Megazyme Company. Dry mild-alkali-extract was digested with excess EPG (approximately 0.05 units/mg sample) by suspending in 50 mM, pH 5.2 ammonium acetate buffer, and incubated at room temperature with very gentle stirring at least two days. A few drops of toluene were added to inhibit bacterial growth.

Isolation of EPG-digestion-resistant Fragments

After the mild-alkali-extract was completely degraded by EPG, the final solution was fractionated by a Dionex Bio-LC system with a continuous permanganate postcolumn detector (Thomas and Mort, 1994) and a semi-preparative Carbopac PA-1 HPLC anion-exchange column (9 × 250 mm). Gradient elution of different fragments was accomplished with 1 M NH_4Ac, pH 5.2 buffer mobile phase. Samples were injected into the system equilibrated at 30 mM NH_4Ac (97% water, 3% 1 M NH_4Ac), and the sample components were eluted after a 2 minute lag period using a gradient of NH_4Ac from 30 mM to 400 mM over 13 minutes, then to 650 mM over 30 minutes, and to 1000 mM within 15 minutes, with a final 5 minutes hold at 1000 mM to wash column. The initial gradient conditions were then maintained until a stable baseline was obtained prior to injection of subsequent samples. Three major peaks were collected as FracA, FracB, and FracC. The fractions eluted later than FracC were collected, and designated EPG-digestion-resistant fragments. After drying by lyophilization, they were retreated with the EPG, and then chromatographed by the semi-preparative Carbopac PA-1 HPLC anion-exchange column (9 × 250 mm) again.

HPLC Gel Filtration Chromatography

EPG-digested-mild-alkali-extract from watermelon cells wall was separated on two Fracto-Gel gel filtration columns of HW55(s) and HW40(s) (Supelco Inc., Bellefonte, PA) using a Dionex reagent pump system for elution. A solution of EPG degradation products was first chromatographed on a 10×500 mm HW55(s) gel filtration column (fractionation range for dextrans: 1000-200,000 daltons) at the flow rate of 1 ml/min in 50 mM ammonium acetate, pH 5.2 buffer, and the material eluted in the included volume of HW55(s) was further chromatographed on a 22×500 mm HW40(s) gel filtration column (fractionation range for dextrans: 100-7000 daltons) at the flow rate of 2 ml/min in the same buffer solution. The eluate was monitored by a Shodex R1-71 refractive index detector, and fractions were collected each minute on a Gilson Fraction Collector. Pooled fractions were lyophilized.

EPG-digestion-resistant fragments was separated on Fracto-Gel gel filtration columns of HW50(s) (fractionation range for dextrans: 500-20,000 daltons, Supelco Inc., Bellefonte, PA) using a Dionex Reagent pump system for elution at the flow rate of 1 ml/min in 50 mM ammonium acetate, pH 5.2 buffer. The eluate was monitored by a Shodex R1-71 refractive index detector, and fractions were collected each minute on a Gilson Fraction Collector. Pooled fractions were lyophilized.

Glycosyl-Composition Analysis

The monosaccharide composition of all the samples was determined by GC analysis of the trimethylsilyl methyl glycoside derivatives. Methanolysis and derivatization were performed by a modification of the method of Chaplin (1982). About 50 to 100 μg of completely dry sugars were weighed on a Cahn 29 electrobalance and placed in screw-cap glass vials with Teflon®-lined lids along with 100 nmoles of myo-inositol as an internal standard. Two hundred μl of 1.5 M methanolic HCl and 50 μl of methyl acetate were

added to the samples, then the vials were tightly sealed and heated at 80 ^{0}C in a heating block overnight. After removing the samples from the heating block and cooling them to room temperature, a few drops of t-butanol were added to each vial to aid in the removal of the residual methanolic HCl. The samples were then evaporated to dryness under a stream of nitrogen gas. Trimethylsilylating reagent was prepared fresh in an exhaust hood by mixing 1 part of Tri-Sil Concentrate (Pierce Chemical Company, Rockford, IL) with 3 parts of anhydrous pyridine. Fifty µl of this reagent was added to the dried samples and allowed to react for a minimum of 15 minutes to effect derivatization at 30-35 ^{0}C in a heating block. The derivatized samples were then evaporated under a gentle stream of nitrogen gas just to dryness, and then redissolved in 50 µl of isooctane. A 1 µl aliquot of this solution plus 1 µl isooctane were injected into a fused silica capillary column (DB-1, J & W Scientific, CA) installed in a Varian 3300 GC.

Determination of the Degree of Methyl Esterification (DM) of Pectin by Reduction

Determination of the degree of methylesterification of pectin was performed by the method of Mannes et al. (1990). First, the methylesterified GalA residues were reduced to Gal, then the sample sugar composition was analyzed by GC. DM was determined as the ratio of Gal produced by reduction divided by the total GalA in the original sample. The procedure was as follows:

Reduction of Methyl Esterified GalA Residues

One hundred µg of watermelon cell walls were suspended in 20 µl of 1 M imidazole-HCl buffer, pH 7.0, and cooled on ice. Four mg of sodium borohydride was then added and the samples were incubated for 1 hour on ice. Glacial acetic acid (10 µl/ 4 mg borohydride) was added to the cooled samples to decompose the excess borohydride. Thirty µl of redistilled water was then added, the reduced pectin was precipitated by adding 3-4 vol of 95% ethanol (200 µl). The samples were further desalted by resuspending in

water and reprecipitated two times with 95% ethanol, and then they were lyophilized for glycosyl composition analysis by GC.

Determination of DM

$$DM=\frac{[Gal(R)-Gal(N)]}{[Gal(R)-Gal(N)]+GalA(R)}*100,$$

where N is native sample and R is reduced sample, and [Gal(R)-Gal(N)] represents methyl-esterified GalA in the native sample. Gal and GalA values are expressed as residues per residue of Rha present in samples.

Hydrogen Fluoride (HF) Treatment of Different Samples at -12^{0}C

Mild-alkali-extract and EPG-digestion-resistant fragments were treated with anhydrous HF at the selected temperature (-12 ^{0}C to -15 ^{0}C). The detailed description of the procedure of selective HF solvolysis apparatus is described by Mort (Mort et al., 1989b, Qi et al., 1993). The desired amount of sample was put in a Teflon reaction vessel with a stirring bar. The whole apparatus was evacuated and leaks in any part of the system were checked for and sealed. The reaction vessel was immersed in a cooling bath of 95% ethanol. Temperature was maintained by an immersion cooler (CC-100, NESLAB Instruments, Inc.) and an offsetting regulated immersion heater (FTS system, Inc., NY). The reaction was started by forcing the desired volume of liquid HF into the reaction vessel through Teflon tubing connecting the vessels. The HF was distilled into an equilibration vessel, and cooled with a liquid N_2-acetone bath prior to its use in the react vessel. The reaction was allowed to continue for 30 minutes at the desired temperature, and then stopped by immersion in liquid N_2 until the HF solidified. Ether, cooled with liquid N_2, was introduced into the reaction vessel, and HF was allowed to thaw slowly. The vessel was frequently swirled to carry away the heat generated by the exothermic formation of the HF-ether complex. After reaching close to room temperature the HF-ether mixture was

evaporated under vacuum into a liquid N_2-cooled vessel on the HF apparatus. The remaining material was dissolved into 100 mM CH_3CO_2H (1 ml/10mg samples), and incubated at 80 ^{0}C overnight to hydrolyze the fluoro groups from the reducing ends of the oligosaccharides. Samples then were dissolved in water and freeze dried.

Labeling of the Reducing End of Oligosaccharides with 2-Aminopyridine

The samples were labeled with 2-aminopyridine (2-AP) at their reducing end by the condensation reaction described by Maness et al. (1991). Samples (10 µg to 1 mg) were weighed on a Cahn 29 electrobalance, placed in screw-cap glass vials, then aqueous 2-AP labeling reagent was added (a minimum of 50 µl per mg sugar). The reagent was prepared by dissolving 1 g of 2-AP in ca. 1 ml of water and adjusting the pH to 7.0 with glacial acetic acid, final volume 2.4 ml. The vials were sealed securely with Teflon®-lined caps, and then incubated at 70 ^{0}C in a heating block overnight. After removing samples from the heating block and cooling them to room temperature, excess 2-AP was removed by solid phase extraction. Aliquots (100 µl) of the 2-AP reaction mixture were diluted 20-fold with 0.44 M CH_3CO_2H (total volume 2.0 ml), and applied to the preconditioned solid phase extraction column [A 500 mg cation Extract-Clean column (Alltech, Deerfield, IL) rinsing with 20 ml concentrated ammonia solution , and then washing with water until the pH fell to 5-6 before the samples were applied]. The eluate was collected within 2 ml total volume, and used directly for HPLC analysis.

Labeling of Oligosaccharides for Capillary Zone Electrophoresis (CZE) Analysis

The sugars were labeled with 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) and sodium cyanoborohydride (SCB) at their reducing end as described by Mort and Chen (1995). Half to one mg of samples were weighed on a Cahn 29 electrobalance, placed in screw-cap glass vials, then 225 µl of ANTS (23 mM in 3 % w/w CH_3CO_2H) and 25 µl of SCB (1 M) were added to the reaction vials. The vials were sealed securely with Teflon®-

lined caps, and incubated at 90 ^{0}C in a heating block for ca. 1 hour. After cooling to room temperature, they were ready for CZE analysis.

Glycosyl-Linkage Analysis

The glycosyl-linkage composition of mild-alkali-extracts was determined by GC-MS of the partially methylated alditol acetates performed mainly as described by An (1991).

Methylation

Completely dried samples (about 500 μg) were flushed with nitrogen gas for 2 minutes in screw-cap glass vials. One hundred μl of trimethyl phosphate (Aldrich Chemical Co., Milwaukee, WI) was added using a dry glass syringe while continuing with nitrogen flushing. The vials were then sonicated until the samples dissolved. After cooling them to room temperature, fifteen μl of 2,6-di-(tert-butyl)-pyridine (Aldrich Chemical Co.) and 10 μl of methyl trifluoromethanesulfonate (Aldrich Chemical Co.) were added with nitrogen flushing, and were allowed to react at 50 ^{0}C in a heating block for 2 hours (Kuo et al., 1986). The methylated oligosaccharide was purified on a Sep-Pak C_{18} cartridge as described below.

Extraction of Methylated Sugars

Sep-Pak C_{18} Cartridges (Waters Associates Inc., Milford, MA) were used for the extraction of methylated sugars (Mort et al., 1983). The Sep-Pak cartridges were preconditioned with (1) 5 ml of ethyl acetate, (2) 5 ml of methanol, and (3) 15 ml of water before the samples were applied. The samples were diluted with 5 ml of water to ensure a high enough solvent polarity for the sugars to be adsorbed on the cartridge, and then this solution was applied slowly to the cartridge with a 10 ml glass syringe. The cartridge was next washed with a total of 15 ml of water, and the methylated sugars were eluted with 3

ml of methanol-chloroform (1:1, v/v). The eluted sugars were dried by evaporating the solvent in a SpeedVac concentrator.

Reduction of Uronic Esters to Alcohols

After the methylated sugars were dried, 50 µl of 2 M lithium borohydride in tertrahydrofuran was added, and the mixture was allowed to react for 2 hours at 50 ^{0}C in a heating block. When the reaction was over, the excess of lithium borohydride was decomposed by addition of glacial acetic acid (10 µl). The mixture was diluted with 5 ml of water, and purified by passage through a preconditioned Sep-Pak C_{18} cartridge as described above. The sugars were eluted with 3 ml of methanol-chloroform (1:1, v/v), and dried by evaporating the solvent in a SpeedVac Concentrator.

Hydrolysis

The above samples were hydrolyzed by adding 100 µl of 88% formic acid and reacting for 1 hour at 100 ^{0}C in a heating block. After evaporating the acid with a nitrogen stream, 100 µl of 2 N trifluoroacetic acid was added, and hydrolysis was continued for 1.5 hour at 121 ^{0}C. After cooling to room temperature, 250 nmoles inositol was added, and the mixture was dried in a SpeedVac Concentrator.

Reduction

Ten µl of 1 M ammonium hydroxide and 100 µl of 0.3 M potassium borohydride in DMSO (freshly prepared) were added to the above samples, and allowed to react for 1.5 hour at 40 ^{0}C. After reaction, excess potassium borohydride was decomposed by adding 10 µl of glacial acetic acid (Harris et al., 1984).

Acetylation

Twenty µl of methylimidazole and 200 µl of acetic anhydride were added to the reduced samples. The reaction was allowed to proceed for 10 minutes at room

temperature. Excess acetic anhydride was decomposed by adding 5 ml of distilled water. The alditol acetates were purified using preconditioned Sep-Pak C_{18} cartridges as described above. The alditol acetates were eluted in 2-3 ml of methylene chloride. The water on top of the methylene chloride was sucked out with a pasteur pipet, and the remaining water was absorbed by adding anhydrous sodium sulfate. After evaporation of methylene chloride with a nitrogen stream, the samples were dissolved in ethyl acetate for GC-MS analysis.

Analytical Methods

Gas Liquid Chromatography

The trimethylsilyl derivatives of sugars were separated on a fused silica capillary column (30 m, 0.25 mm i.d, Durabond-1 liquid phase, J&W scientific, Inc., Rancho Cordova, CA) installed in a Varian 3300 gas-chromatography equipped with an on-column injector and FID detector. Two µl aliquots of the samples were injected at 105 ^{0}C. After 1 minute the temperature was raised at the rate of 10 ^{0}C/min to 160 ^{0}C. After a hold of 4 minutes, the temperature was raised at a rate of 2 ^{0}C/min until 220 ^{0}C was reached, whereupon the temperature was raised to 240 ^{0}C at a rate of 10 ^{0}C/min, and held there for 10 minutes to clean the column. Peaks were integrated using a Varian 4290 integrator.

Dionex CarboPac PA-100 Anion-Exchange Chromatography

The 2-AP reducing end-labeled oligosaccharides were chromatographed on a CarboPac PA-100 HPLC anion-exchange column (4 × 250 mm) using a Dionex Bio-LC Carbohydrate System (Dionex Corporation, Sunneyvale, CA). The system consisted of a gradient pump module, an eluent degassing module, and a RF-535 variable excitation and emission wavelength fluorescence detector (Shimadzu, Kyoto, Japan). Labeled oligosaccharides were detected by fluorescence with an excitation wavelength of 290 nm, and an emission wavelength of 350 nm. The elution buffer consisted of solvent A (water), and solvent B (Phosphate buffer, 1 M, pH 7.0). A flow rate of 1 ml/min was used.

Samples were injected into the system equilibrated with 50 mM phosphate buffer (95 % water, 5 % solvent B), and sample components were eluted after a 3 minutes lag period using a gradient of phosphate buffer from 50 mM to 270 mM over 47 minutes, to 350 mM over 40 minutes, to 430 mM over another 65 minutes, then to 500 mM within 5 minutes, with a final 5 minutes hold at 500 mM to wash the column. The initial conditions were then maintained until a stable baseline was obtained prior to injection of subsequent samples.

Dionex CarboPac PA-1 Anion-Exchange Chromatography with a Continuous Permanganate Postcolumn Detector

The mild-alkali-extracts and EPG-digestion-resistant fragments treated by -12 ^{0}C to -14 ^{0}C HF were chromatographed on CarboPac PA-1 anion-exchange column (4 × 250 mm) using a Dionex Bio-LC carbohydrate system (Dionex Corporation, Sunneyvale, CA) with a continuous permanganate postcolumn detector. Detailed description of the detection system is given by Thomas and Mort (1994). A Dionex variable wavelength detector was used to monitor the chromatography at 525 nm by taking advantage of the bleaching of the permanganate which is proportional to the sugar concentration. The flow rate was 1 ml/min. The elution buffer consisted of solvent A (water), and solvent B (ammonium acetate buffer, 1 M, pH 5.2). Samples were injected into the system equilibrated with 30 mM NH_4Ac (97 % water, 3% solvent B), and the sample components were eluted after a 2 minutes lag period with a 90 minutes linear gradient of NH_4Ac from 30 mM to 1000 mM with a final 5 minutes hold at 1000 mM to wash the column. The initial conditions were then maintained until a stable baseline was obtained prior to injection of subsequent samples.

High Performance Capillary Zone Electrophoresis (CZE)

The capillary zone electrophoresis system used in this research was a custom-built

instrument which included a Spellman Model CZE 1000 R high voltage power supply (Plainview, NY, USA) with positive and negative polarity and a model "FL-750 HPLC Plus" spectrofluorescence detector (McPherson Instrument, Acton, MA, USA) equipped with a cell for on-column capillary detection. A detailed description of the instrument is given by Mort and Chen (1995). Samples derivatized with ANTS were applied directly to uncoated fused silica open tubular capillary by hydrodynamic injection without removal of excess reagent. The conditions of separation were as follows: 100 mM sodium phosphate buffer at pH 2.5, current 60 μA, voltage 17 KV. Labeled oligosaccharides were detected by fluorescence with excitation wavelength of 364 nm. A cut-off filter permitted detection of emission beyond 440 nm (Mort and Chen, 1995). Chromatographic data were collected using custom-built data loggers provided by Dr. Merz and Dr. Mort (1992).

Gas Chromatography and Mass Spectrometry (GC-MS)

The partially methylated alditol acetate derivatives of sugars were subjected to GC-MS (HP 5989B Engine) to identify their glycosyl-linkage compositions. One to three μl of sample dissolved in ethyl acetate was separated on a DB-225, fused-silica, capillary column with a capillary injection-system used for on-column injection and analyzed by GC-MS using EI modes. The GC oven temperature program was as follows: Injection at 80 ^{0}C, hold for 4 minutes at 160 ^{0}C, and then raise the temperature at 2 ^{0}C/min to 220, and hold there for 10 minutes to clean the column. The linkages of the different sugar residues were identified based on their mass fragmentation patterns.

^{1}H-NMR Spectroscopy

^{1}H spectra of samples were recorded on an Varian Unity Plus 600 MHz n.m.r. spectrometer by Dr. Feng Qiu (Department of Chemistry, CUNY College of Staten Island). Samples were lyophilized twice from D_2O to eliminate exchangeable protons. Chemical shifts are reported in ppm and were measured by reference to water (4.800 ppm).

CHAPTER IV

RESULTS AND DISCUSSION

Extraction of Mild-alkali-extracts from Watermelon Cell Walls

Watermelon cell walls, which were prepared from the ripe watermelon fruit were kindly provided by Dr. Niels O. Maness' laboratory. The sugar composition of the watermelon cell walls is shown in Table 1. Galacturonic acid (GalA), xylose (Xyl), arabinose (Ara), galactose (Gal), glucose (Glu), and rhamnose (Rha) are the main sugar residues, and mannose (Man) is the minor sugar residue. The degree of methylation (DM) was about 45 %. It is clear these watermelon cell walls contained abundant pectic polysaccharides and that some of galacturonosyl residues carry a methyl group. Due to the large amount water washing during the cell walls preparation, no water soluble pectins were expected to remain in the intact watermelon cell walls.

In order to isolate a pectin without seriously changing its structure, mild alkali (0.1 N NaOH) and low temperature, which are normally used to saponify the methyl esters in the pectin, was chosen for extraction. The watermelon cell walls were treated with 0.1 N NaOH, followed by centrifugation, neutralization, dialysis and lyophilization. As outlined in Figure 2, 200 mg of watermelon cell walls was stirred with about 20 ml of 0.1 N NaOH at room temperature for 15 minutes, then incubated in a 4 ^{0}C cold room overnight with stirring. The soluble portion was separated by centrifugation. The supernatant containing the mild-alkali-extract was collected, neutralized, dialyzed against water to remove salts, and then freeze-dried. Under this rather mild extraction condition and low

TABLE 1

MOLE PERCENT OF SUGARS IN WATERMELON CELL WALLS AND
IN THE MILD-ALKALI-EXTRACT OF THE WATERMELON CELL WALLS

Material	Ara	Rha	Xyl	GalA	Man	Gal	Glc	*wt%	wt(mg)
Intact Walls	10.1	4.2	15.8	62.4	0.8	6.7	8.0	33.8	200
Alkali Extract before dialysis (100 mM NaOH)	7.6	3.3	10.5	72.3	0.4	7.7	1.4	32.9	60
After Dialysis (12,000 cut off membrane)	7.1	3.2	11.0	76.1	0.3	7.2	1.4	51.4	30
Residue	12.9	3.9	19.6	46.9	0.8	9.1	5.2	32.3	145

* wt% (weight percent sugar) was calculated from the weight of sugar detected by GC for a known weight of sample

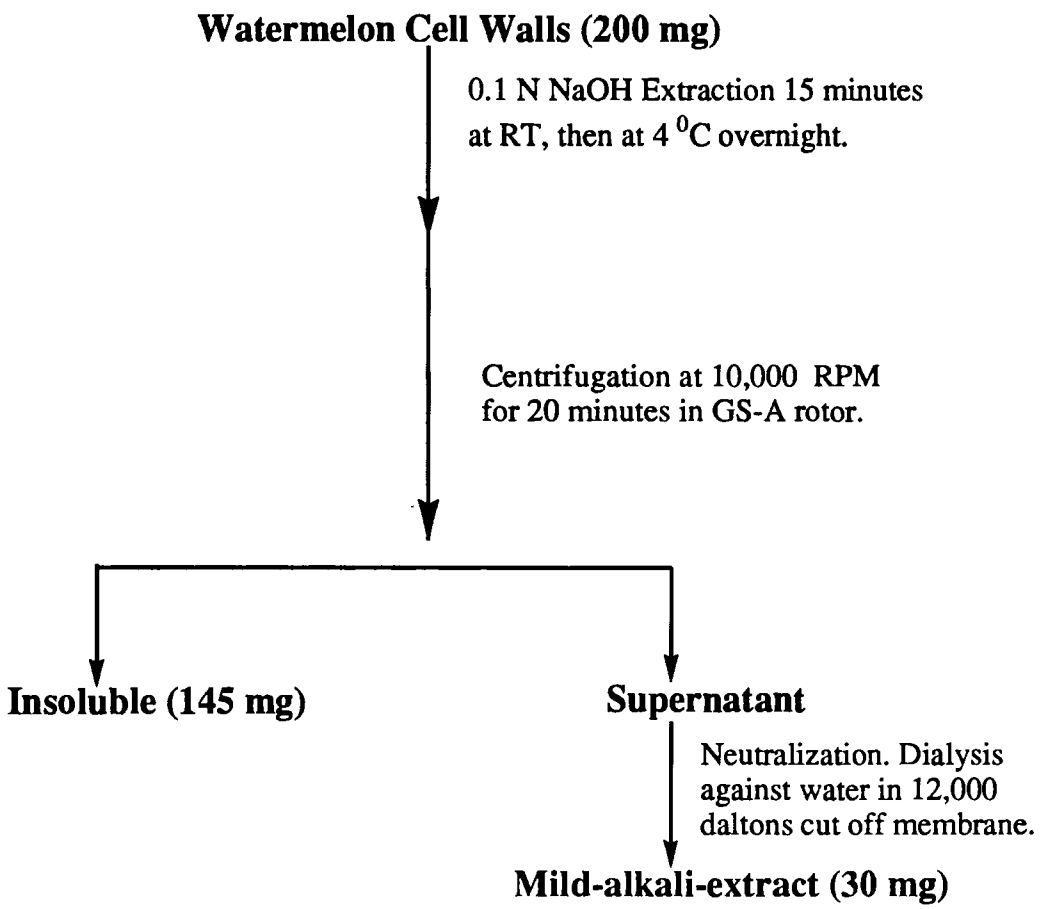

Figure 2. Flow chart of isolation of mild-alkali-extract from watermelon cell walls

temperature that was used, it can be expected that only ester linkages are cleaved and some hydrogen bonds disrupted without changing the polysaccharide structures (Rombouts et al., 1986; Rouau et al., 1984).

The mole percentage and the weight percentage of the sugars in the extract are shown in Table 1. The extract, accounting for about 15 % by weight of the treated cell walls, comprised about 28 % of the GalA present in the watermelon cell walls. It contains predominantly GalA (about 76 mole% of the sugar), but is also rich in Xyl, Ara, Gal, and Rha. It is obviously a pectin-like polysaccharide preparation, and the mild-alkali-extract was isolated from water-insoluble pectin. This extracted pectin portion has a higher Xyl content (11 mole% of the sugar) than pectins previously described (Schols, 1995; Mankarios et al., 1980; DeVries, 1982; Ishii, 1982; Konno et al., 1986; Redgwell et al., 1986; Saulnier et al., 1987). It contains about one Xyl for every seven GalA.

Before and after dialysis against water in a 10,000-12,000 daltons cut off membrane, the sugar composition was almost the same although some of the sample was lost during dialysis. Closely checking the weight percentage sugar increasing after dialysis, we can conclude that the majority of the weight lost was salt. This result indicates that the mild-alkali-extract is high molecular weight with a predominant non-methylesterified GalA content. It also has a significant content of neutral sugars with equal amounts of Ara and Gal, and a high Xyl content. It is clear that this mild-alkali-extract contains a large proportion of HG segments plus some RG I. This mild-alkali-extract has limited solubility due to the large amount of GalA (presumably HG regions). Therefore a reliable molecular weight distribution from HPLC gel filtration chromatography could not be obtained.

EPG Degradation of Mild-alkali-extract

EPG hydrolysis was performed on the mild-alkali-extract from watermelon cell walls in ammonium acetate buffer (50 mM, pH 5.2) with excess enzyme at room temperature for at least 48 hours. EPG we used requires four adjacent non-methylesterified

galacturonate residues for activity (Chen & Mort, 1995) and is hindered or blocked by rhamnosyl residues (Rouau, 1984; Saulnier, et al., 1987) or acetyl ester groups at C-2 and/or C-3 of the GalA units (Rexova-Benkova et al., 1976). Pectin molecules are made of a combination of branched (Rha-rich pectic region [RG I]) and unbranched galacturonan region (HG region). EPG only can degrade HG regions that have more than four adjacent non-methylesterified GalA residues. After complete EPG digestion of the mild-alkali-extract, all of the HG segments that were previously de-methylesterified, were expected to be hydrolyzed to mono-, di-, and tri-galacturonic acids. The polymeric materials remaining (EPG-digestion-resistant fragments) were expected to be the Rha-rich pectic region (RG I-like polysaccharides).

Characterization of EPG Degradation Products

EPG Degradation Products on Anion-exchange Chromatography

After the mild-alkali-extract was completely digested by excess EPG, The mixture was fractionated using a semi-preparative Carbopac PA-1 HPLC anion-exchange column on a Dionex Bio-LC system with a continuous permanganate postcolumn detection system. Figure 3 shows the elution pattern of the EPG digest of the mild-alkali-extract from watermelon cell walls on the semi-preparative Carbopac PA-1 (9×250 mm) HPLC anion-exchange column. Three major peaks were collected as Frac A, Frac B, and Frac C. The fractions eluting later than Frac C were collected and designated as EPG-digestion-resistant fragments. The fractions were pooled as indicated in Figure 2 and analyzed for their sugar compositions (Table 2).

From the elution pattern from the PA-1 column, it can be concluded that after exhaustive digestion by excess EPG, oligomers are produced in a range of sizes, and these EPG-digestion-resistant fragments are not homogeneous according to charge. The separation is not improved by changes in elution condition. One possible reason is the great similarity in structure of the various oligomers. Another reason is that EPG

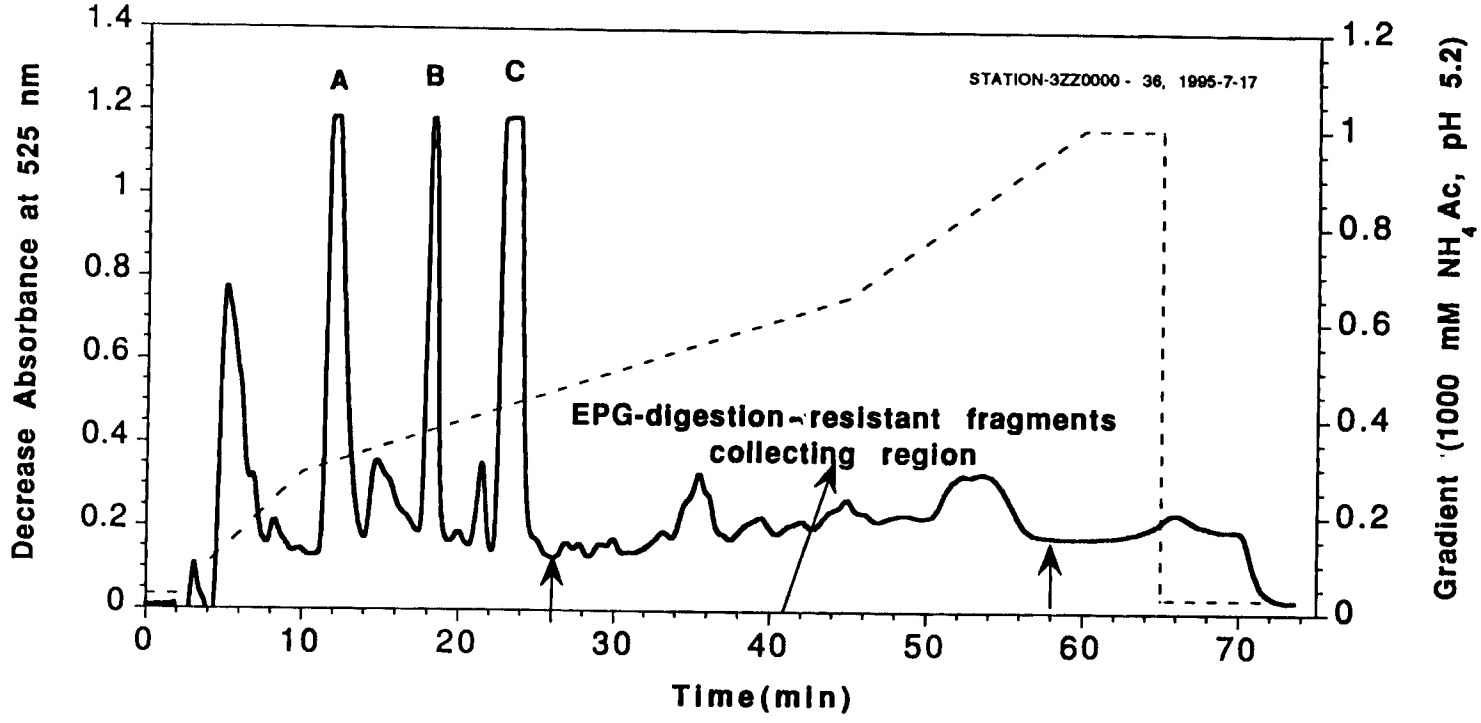

Figure 3. The HPLC chromatogram of the mild-alkali-extract digested by EPG using a Carbopac PA-1 HPLC anion-exchange column (9 × 250 mm) with a continuous permanganate postcolumn detector.

The flow rate of elution buffer was 2 ml/min. Solvent A was water, and solvent B was 1M ammonium acetate (1 M, pH 5.2). Samples were injected into the system equilibrated at 30 mM NH_4Ac (97 % water, 3% solvent B), and sample components were eluted after a 2 min lag period using a gradient of NH_4Ac from 30 to 280 mM over 8 min, then to 650 mM over 35 min, and to 1000 mM over 15 min, with a final 5 min hold at 1000 mM to wash the column. Fractions 26-58 min were collected as EPG-digestion-resistant Fragments. Fractions 11-13 min as Frac A, Fractions 18-19 min as Frac B, and Fractions 23-25 min as Frac C.

TABLE 2

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT FRACTIONS FROM PA-1 ANION EXCHANGE CHROMATOGRAPHY OF THE MILD-ALKALI-EXTRACT TREATED BY EPG

Residue	Unbound Sugars	Frac A	Frac B	Frac C	EPG-digestion-resistant Fragments
Ara	24.8	4.8	3.0	1.2	7.6
Rha	11.2	0.2	0.8	0.9	4.8
Xyl	27.0	3.4	1.2	1.1	16.6
GalA	7.9	84.5	92.1	94.7	68.0
Man	1.3	0.7	0.3	0.4	/
Gal	22.0	6.0	2.6	1.1	5.0
Glc	5.7	0.3	/	0.7	/

degradation products contained RG I like polysaccharides. These polymeric materials can't be separated on PA-1 anion-exchange column, and they elute as a broad peak.

As shown in Table 2, the EPG-digestion-resistant fragments contained GalA (~68 mole%), Xyl (~16.6 mole%), Ara (~7.6 mole%), Rha (~4.8 mole%), and Gal (~5.0 mole%). The molar ratio of GalA and Xyl is about 4:1, and the ratio of GalA and Rha is about 14:1. This sugar composition revealed that EPG-digestion-resistant fragments don't resemble only the RG I-like polysaccharide, but are a mixture of HG fragments with RG I-like polysaccharides with high content of Xyl. Further fractionation of the EPG-digestion-resistant fragments on an HPLC HW50(s) gel filtration chromatography (Figure 4) shows a peak excluded from the gel filtration column containing most of the Rha, Ara, and Gal residues whereas peaks eluting in the fractionation range of the column are mainly GalA and Xyl residues (Table 3). This result indicates that EPG-digestion-resistant fragments contains some high molecular weight RG I like polysaccharides (GalA : Rha is 3 : 1), and some low molecular weight HG segments with considerable Xyl residues (xylogalacturonan?) that originated from the HG regions.

To check that the EPG digestion was complete, the EPG-digestion-resistant fragments collected from the PA-1 column were digested with EPG for another 48 hours, and then separated on PA-1 anion-exchange chromatography again. The chromatogram (figure not shown) showed that no more mono-, di-, or tri-galacturonic acid were obtained and that the first EPG digestion was complete.

Identification of Major Oligosaccharies by High Performance CZE.

Based on the sugar composition analysis, Frac A, Frac B and Frac C contain predominantly GalA (Table 2), and from previous studies (Thibault, 1983, Mort and Chen, 1995), we also know that the end products of complete digestion of homogalacturonic acid by EPG are mono-, di- and tri-galacturonic acid. The identification of Frac A, Frac B, Frac C as mono-, di-, and tri-galacturonic acid, respectively, were confirmed by HPLC and high

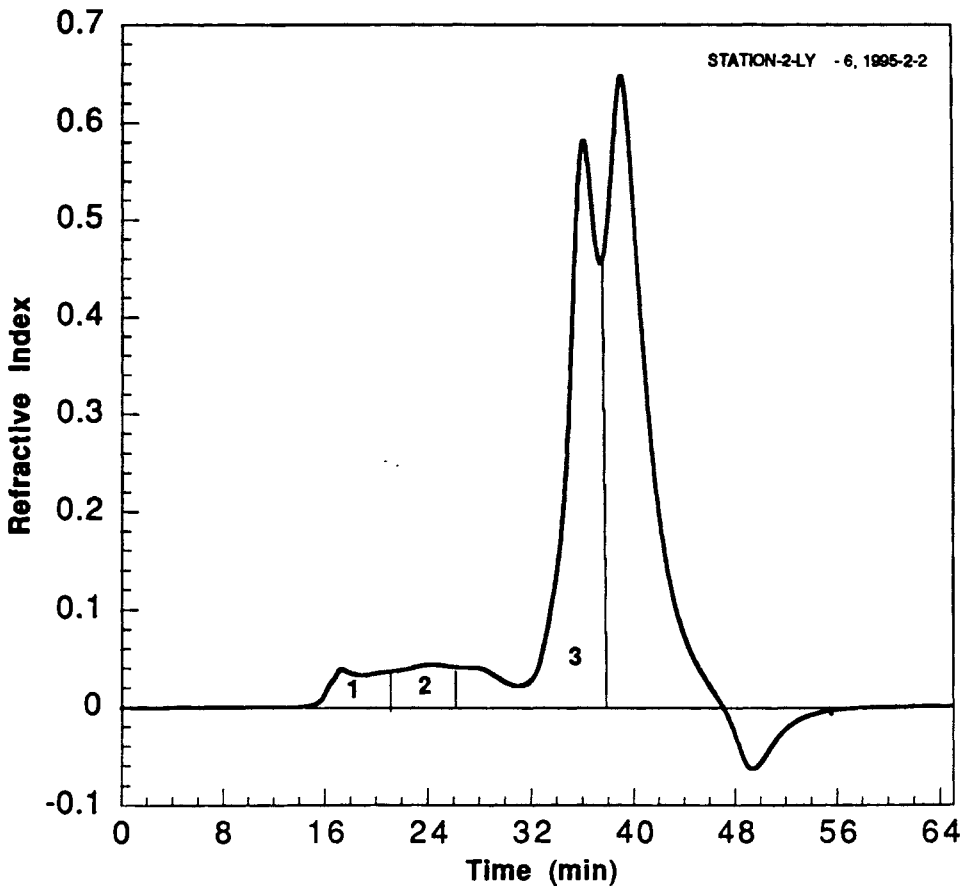

Figure 4: Chromatography on an HW50(s) HPLC gel filtration column of the EPG-digestion-resistant fragments. Tubes 13-20 were pooled into Fraction 1, tubes 21-27 were pooled into Fraction 2, tubes 28-38 into Fraction 3, and tubes 39-50 were assumed to be salt.

TABLE 3

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT FRACTIONS FROM HW50(s) GEL FILTRATION CHROMATOGRAPHY OF EPG-DIGESTION-RESISTANT FRAGMENTS

Residue	EPG-Digestion Resistant Fragments	Frac 1	Frac 2	Frac 3
Ara	7.6	13.5	6.6	3.8
Rha	4.8	12.8	5.8	2.5
Xyl	16.6	18.7	23.2	17.3
GalA	68.0	37.9	59.8	75.0
Gal	5.0	12.3	4.5	1.3
Glc	/	/	0.2	1.1

performance capillary zone electrophoresis. In the HPLC PA-1 anion-exchange chromatogram (Figure 5), Frac A, Frac B, and Frac C have the same retention times as mono-, di-, and tri-galacturonic acid, respectively. Figure 6, Figure 7, and Figure 8 are electropherograms of Frac A, Frac B, and Frac C, respectively. Comparing to electropherograms of known GalA oligomers derived from non-methylesterified pectic acid, they also have the same relative retention time as mono-, di-, and tri-galacturonic acid (Figure 9). That means that Frac A, Frac B, and Frac C were predominantly mono-, di-, and tri-galacturonic acid, respectively. That fracC, tri-galacturonic acid, is found to be the most abundant final product (Figure 3) and no tetra-galacturonic acid is found, also indicate that the EPG action on the mild-alkali-extract was complete (Thibault, 1983).

Determination of the Glycosyl-Linkage Composition of Xyl and GalA in the EPG-digestion-resistant Fragments by GC-MS

The glycosyl-linkage compositions of the Fraction 2 from HW50(s) gel filtration chromatography of EPG-digestion-resistant fragments (Figure 4) were determined by GC-MS of the partially methylated alditol acetate derivatives after reduction of the GalA residues. The total ion current chromatogram of the sugar derivatives is shown in Figure 10. Three major peaks were found besides the internal standard Inositol. Peaks with retention time of 9.78 minutes, 21.24 minutes, and 23.90 minutes were identified as 2,3,4-tri-O-methyl-xylose, 2,6-di-O-methyl-galactose and 2,3,6-tri-O-methyl-galactose, respectively. These results show that almost all the Xyl residues are terminal. The galacturonosyl residues, which were reduced to Gal, were (1→4)-linked or (1→3,4)-linked. Since no other major sugars were found in this mixture (Fraction 2 contains 23.2 mole% Xyl and 59.8 mole% GalA) and GalA is known to be in a (1→4)-linkage in the HG region, the terminal Xyl residues are most likely attached to C-3 of the (1→4)-linked GalA backbone as a single residue sidechain.

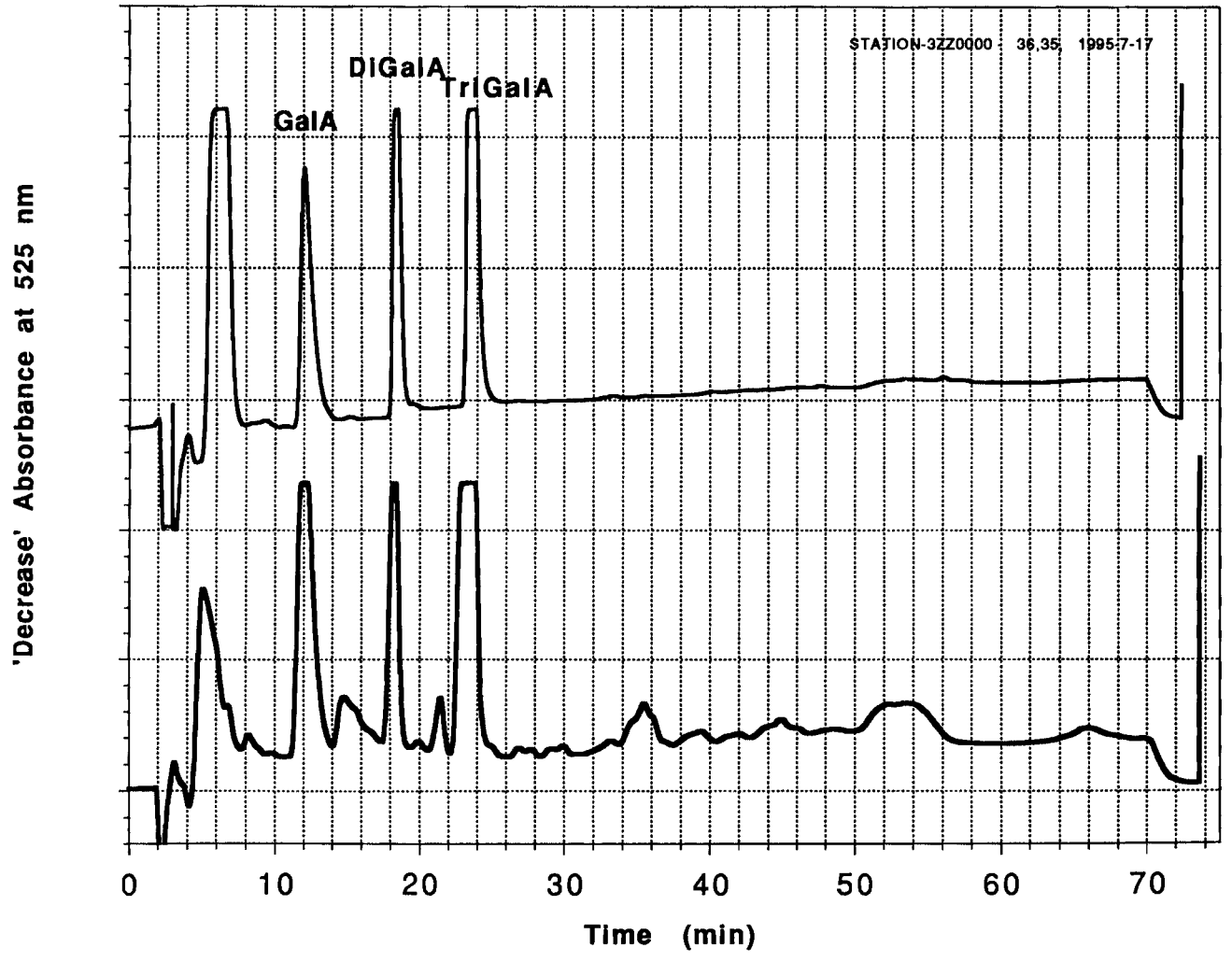

Figure 5. The comparison of HPLC chromatograms of GalA oligomers and the EPG-digested mild-alkali-extract.

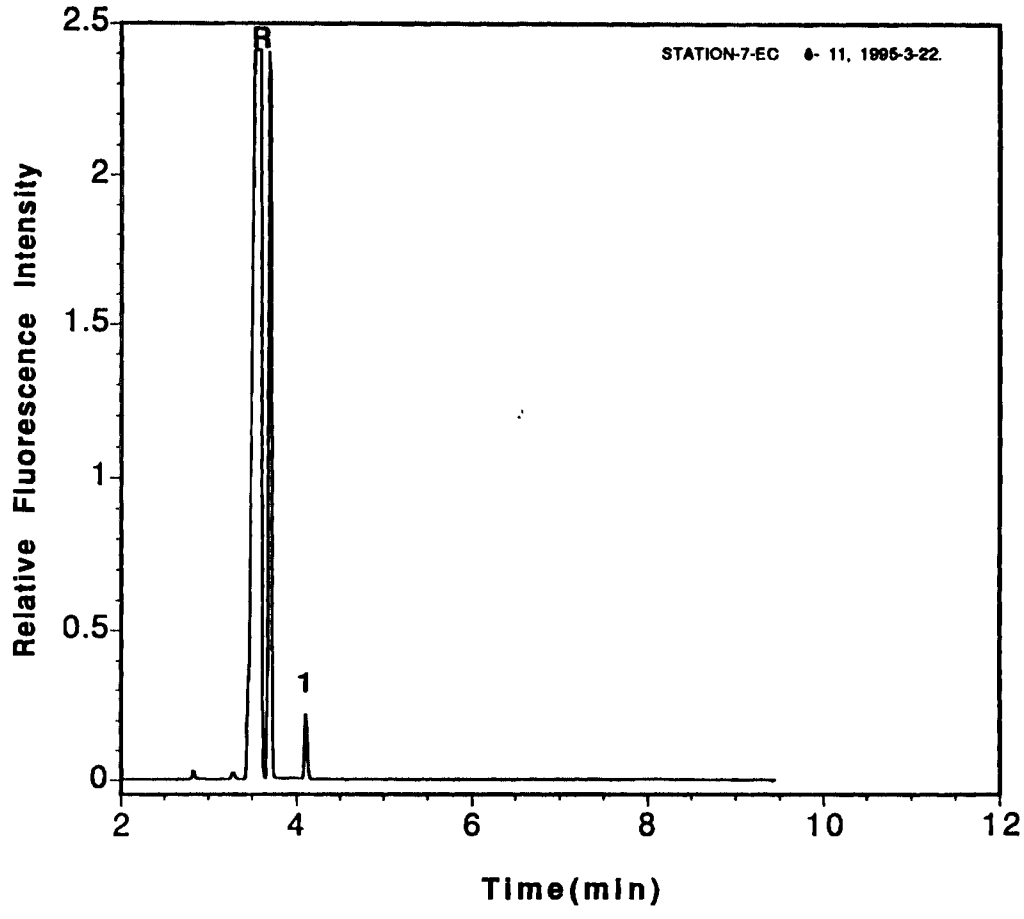

Figure 6. Electropherogram of ANTS derivative of Frac A of Figure 3 separated on an uncoated fused silica open tubular capillary. Running buffer: 0.01 M phosphate, and pH 2.5; Hydrodynamic injection; Applied voltage: 17KV; Detection: Fluorescence Detector with an excitation wavelength of 364 nm, and a cut-off filter permitted detection of emission beyond 440nm. R=reagent, and the relative retention time of 1 = 1.15.

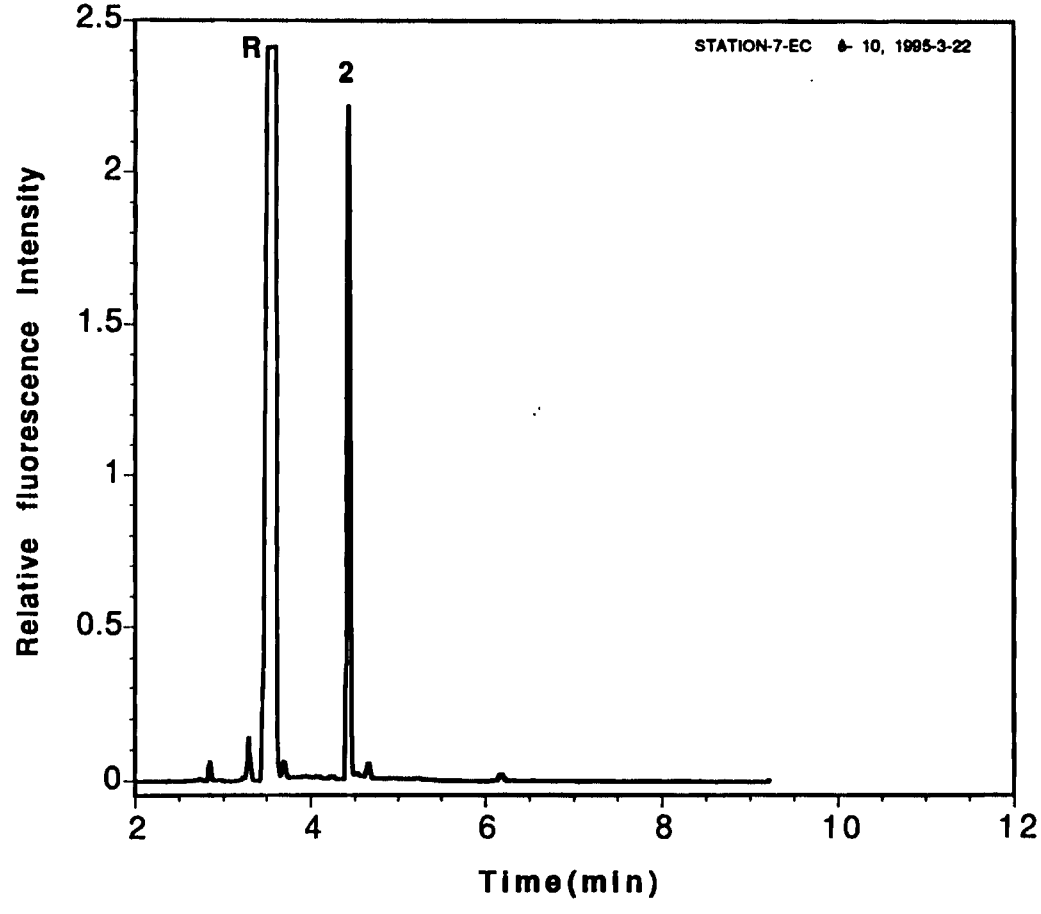

Figure 7. Electropherogram of ANTS derivative of Frac B of Figure 3 separated on an uncoated fused silica open tubular capillary at the same operation condition as in Figure 6. R = reagent, and the relative retention time of 2 = 1.23.

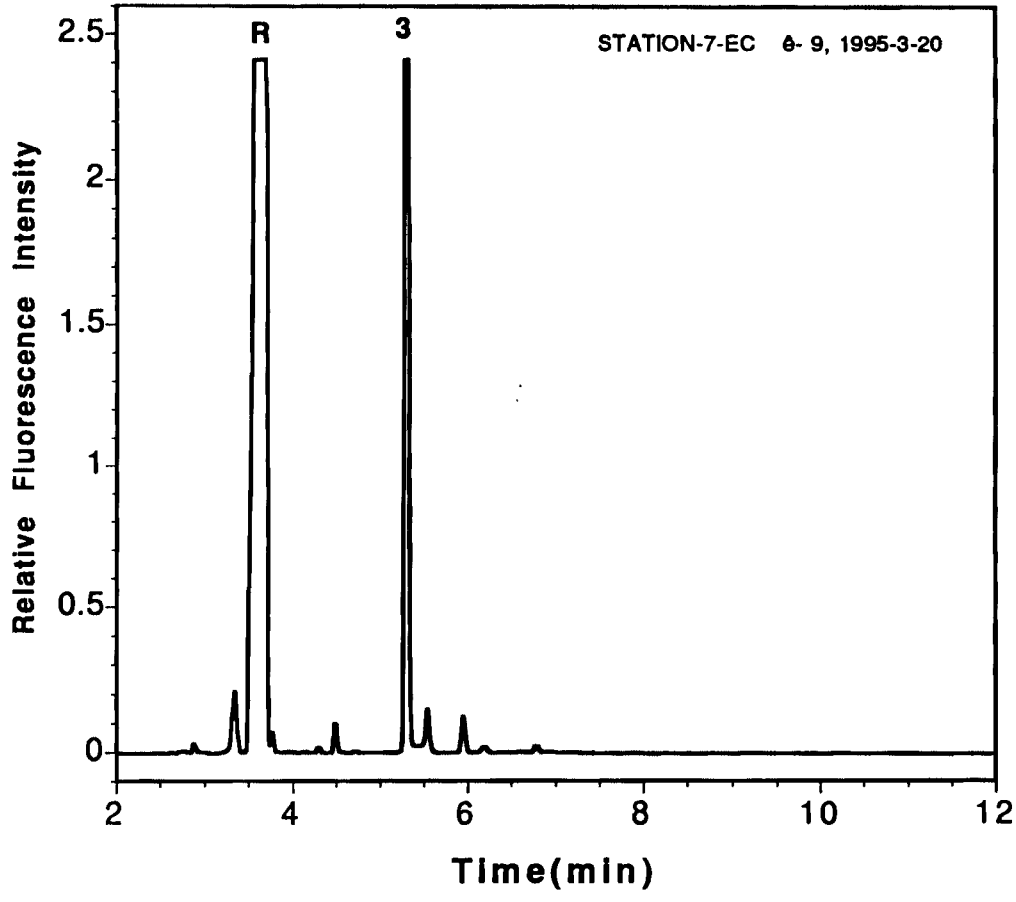

Figure 8. Electropherogram of ANTS derivative of Frac C of Figure 3 separated on an uncoated fused silica open tubular capillary at the same operation condition as in Figure 6. R= reagent, and the relative retention time of 3 = 1.5.

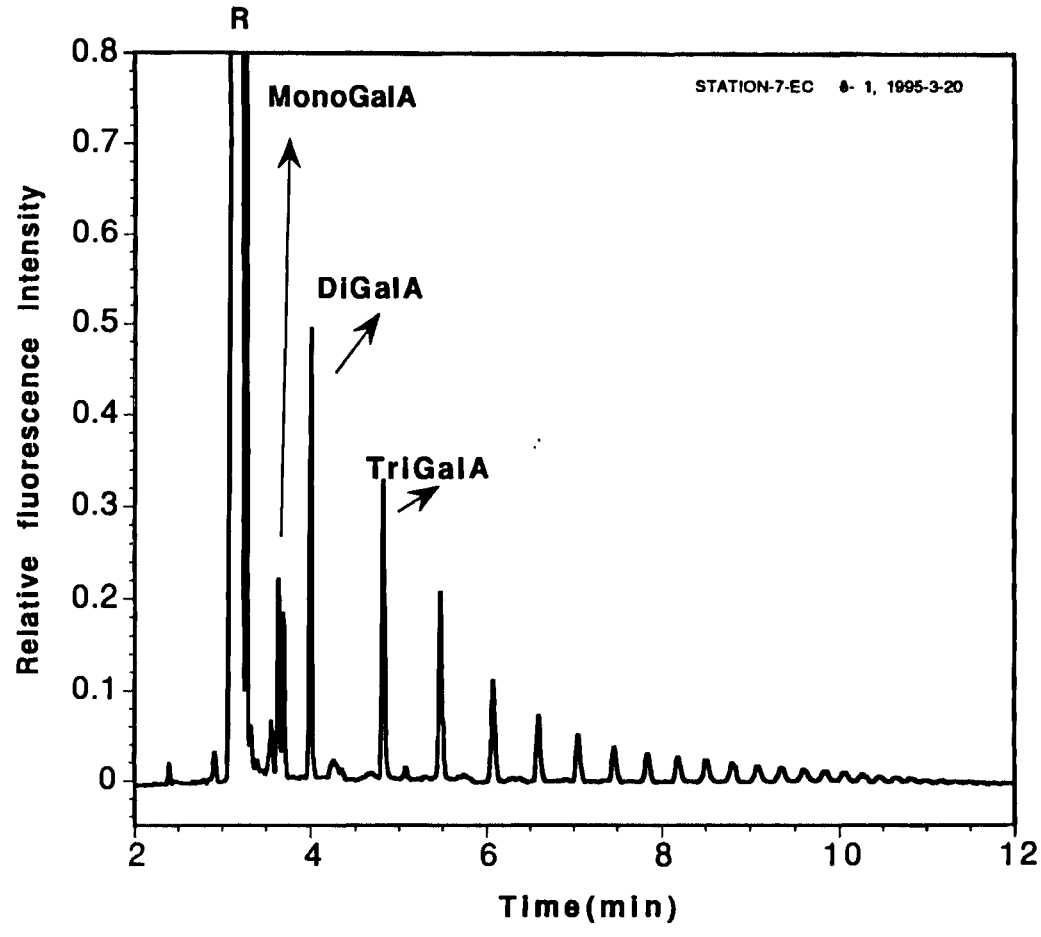

Figure 9. Electropherogram of ANTS derivatives of known GalA oligomers from non-methylesterified pectic acid separated on an uncoated fused silica open tubular capillary at the same operation condition as Figure 6. R= reagent, and the relative retention time of MonoGalA = 1.15, of DiGalA = 1.23, and of TriGalA = 1.51.

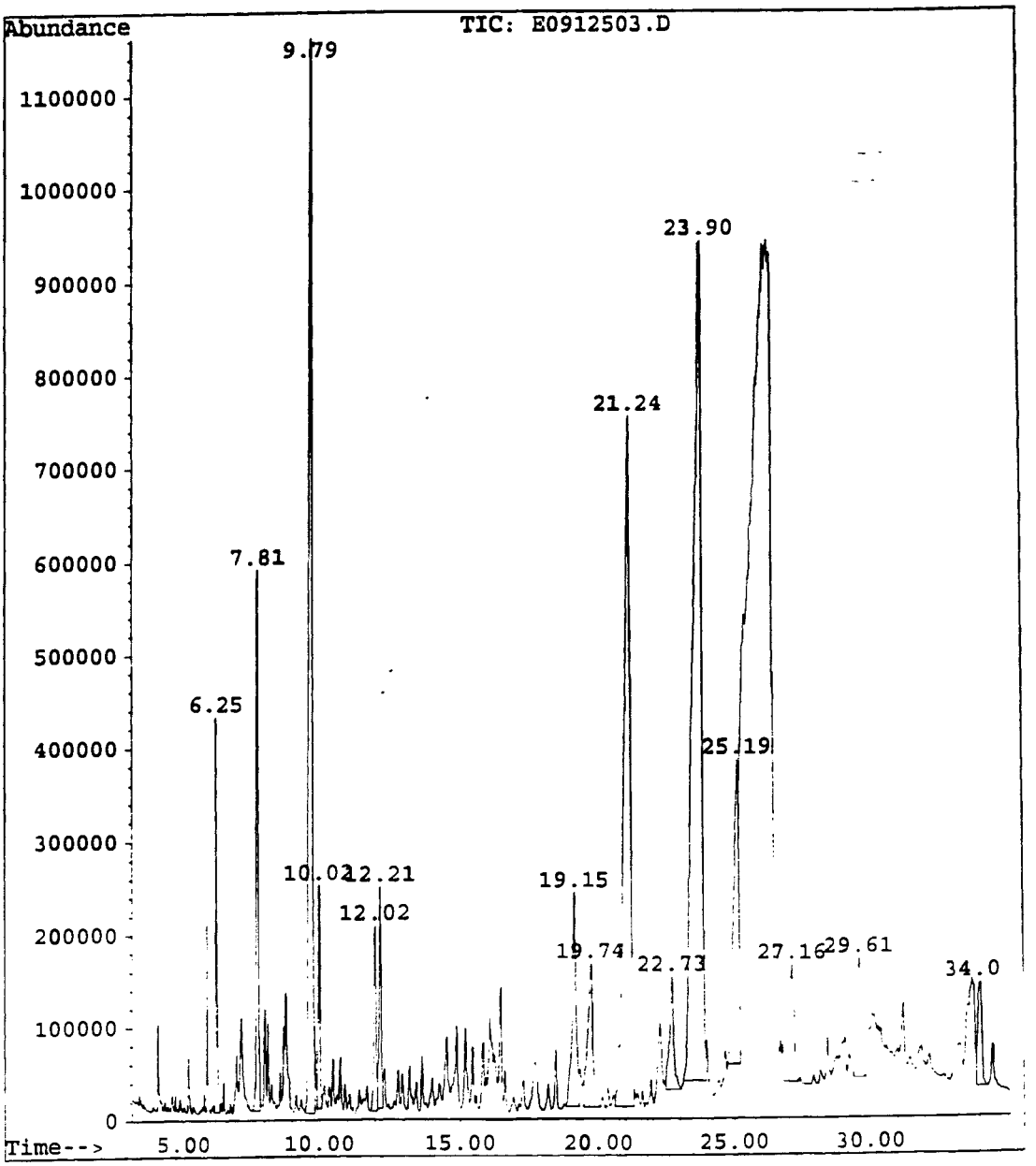

Figure 10. Total ion current of the partially methylated alditol acetate derivatives of EPG-digestion-resistant fragments isolation by anion-exchange chromatography from EPG treated mild-alkali-extract from watermelon cell walls. Peaks were identified from the mass spectra of the these signals: 9.79 min: Terminal Xylose; 21.24 min: 1,4-GalA; 23.90 min: 1,3,4-GalA; 25.19 min: inositol.

Determination of the Length of HG Backbone in Mild-alkali-extract and in EPG-digestion-resistant Fragments

Current work in Dr. A. Mort's laboratory shows that -12 ^{0}C to -15 ^{0}C anhydrous HF treatment selectively cleaves the glycosidic linkages present in pectic polysaccharide samples except for those of GalA. Under this condition, there is only a small amount of cleavage of GalA linkage (Mort et al., unpublished). All GalA residues in the mild-alkali-extract are unesterified because of the conditions used for extraction. To determine the sizes of contiguous stretches of (unesterified) GalA in the mild-alkali-extract before and after an exhaustive EPG digestion, the mild-alkali-extract and EPG-digestion-resistant fragments were treated with anhydrous hydrogen fluoride at -12 ^{0}C to -15 ^{0}C for 30 minutes. This treatment was designed to remove neutral sugar side chains of the HG segments and cleave the backbone at the locations of Rha residues. As expected, these samples clearly showed a series of peaks corresponding to those expected for HG fragments on an HPLC PA-1 anion-exchange column (4 × 250 mm) with a permanganate postcolumn detection system (Figure 11, Figure 12). The number above the peaks corresponds to the degree of the polymerization (dp) of the oligosaccharide in that peak. It can be concluded that -12 ^{0}C HF treatment removed all neutral sugar side chains of the HG segments and destroyed the RG I like polysaccharide backbone, leaving the homogalacturonic backbone relatively intact. From the elution pattern, it is seen that the intact mild-alkali-extract has much longer HG fragments than those of the EPG-digestion-resistant fragments. However, due to the poor resolution of long GalA fragments, the length of the longest GalA fragments are not revealed by this chromatography.

A second type of chromatography was employed to determine the lengths of the homogalacturonic acid backbone of these two samples. Dr. A. Mort's laboratory found that after a –12 ^{0}C treatment with HF, the reducing ends of GalA can be labeled with 2-aminopyridine, and this treatment results in a homologous series in which the length of each oligomer in the series can be deduced. These two samples were derivatized with 2-

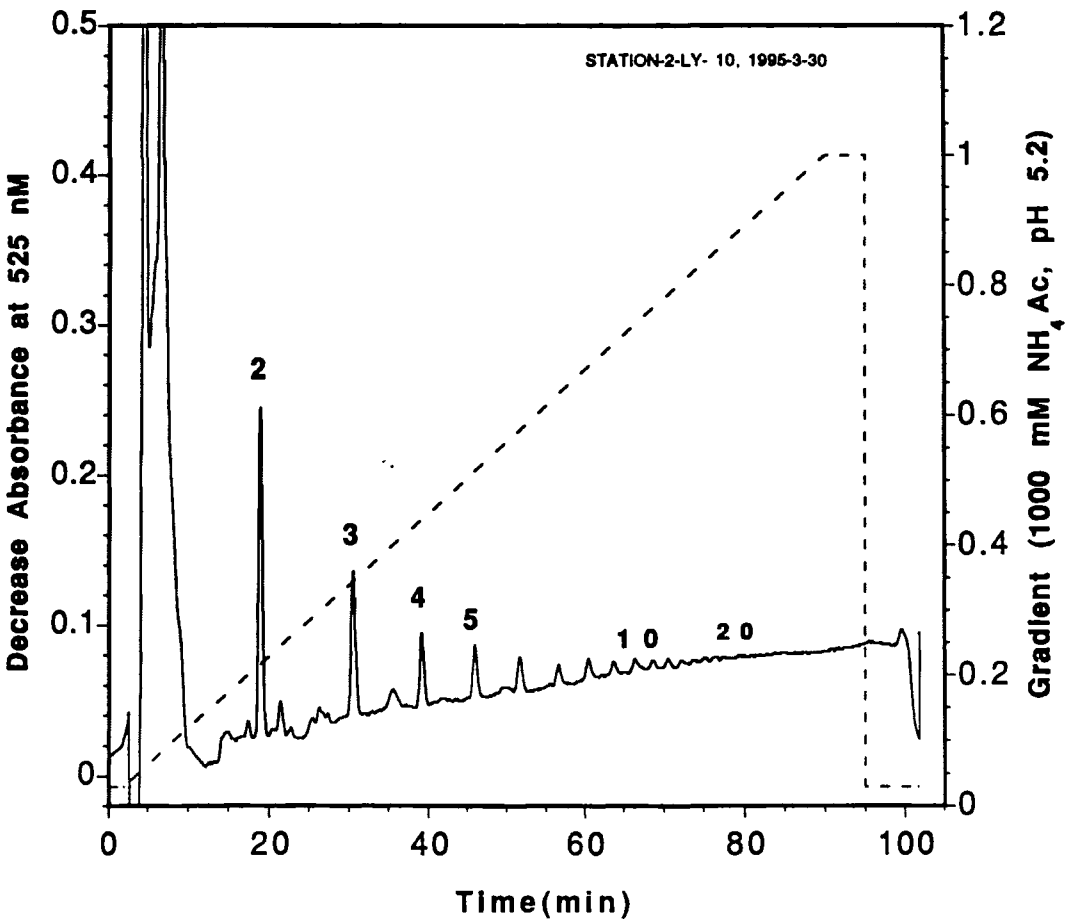

Figure 11. The HPLC anion-exchange chromatogram of EPG-digestion-resistant fragments treated by -12 ^{0}C HF. The numbers above the peaks correspond to the dp of the oligosaccharide in that peak.

Sample was applied on (4 × 250 mm) Carbopac PA-1 anion-exchange column using a 90 min linear gradient consisting of 30 mM to 1 M ammonium acetate at a flow rate of 1 ml/min.

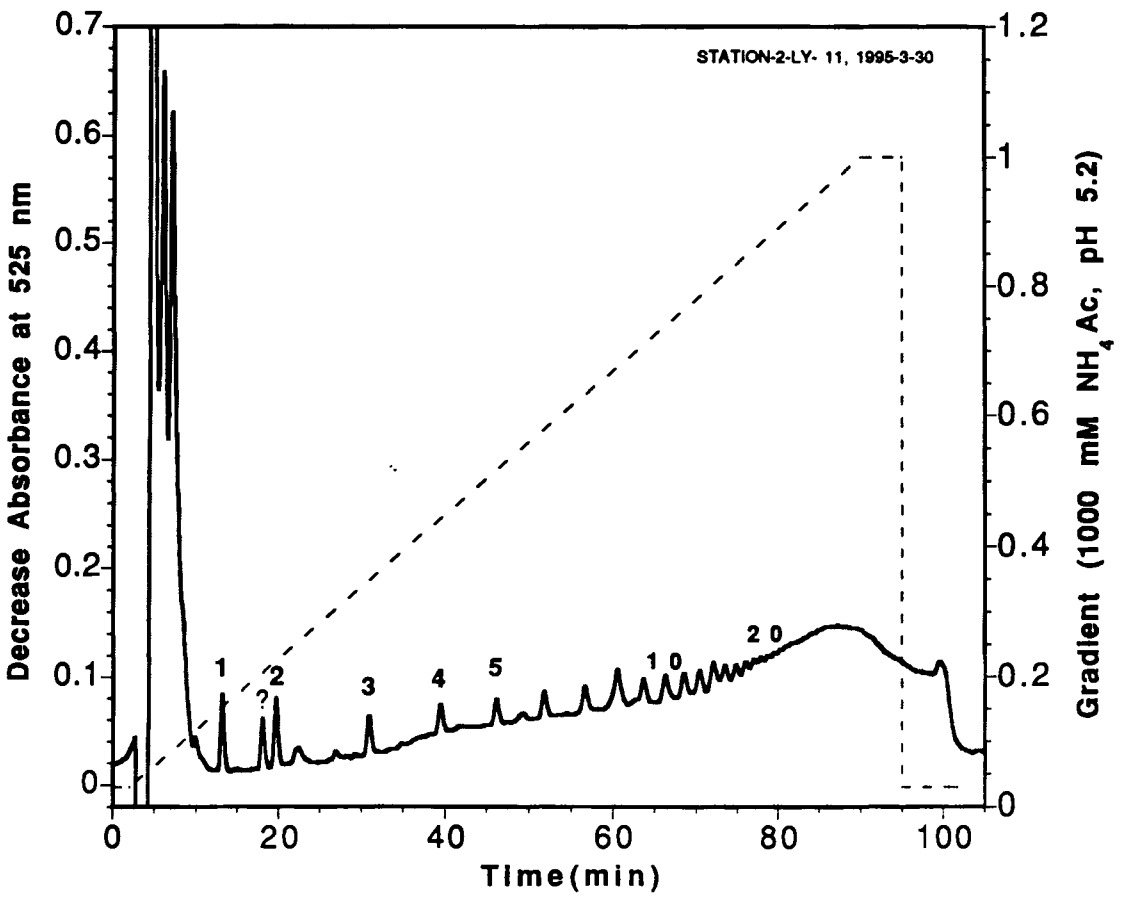

Figure 12. The HPLC chromatogram of mild-alkali-extract from watermelon cell walls treated by -12 ^{0}C HF. HPLC parameters are the same as in Figure 11.

aminopyridine and separated using an HPLC PA-100 anion-exchange Column (4 × 250 mm) with a fluorescence detector. Figure 13 and Figure 14 show the results. By comparing to a chromatogram of a pectic acid which contains a wide range of GalA oligomers (Fig. 15), we estimate the length of homogalacturonic of backbone of mild-alkali-extract to be up to 65-70 GalA residues. The longest length of GalA EPG-digestion-resistant fragments range up to 25-30 residues.

Since the EPG we used is not able to hydrolyze tri-galacturonic acid (Thibault, 1983, Mort and Chen, 1995), and requires four contiguous non-methylesterified GalA residues as a substrate in partially esterfied pectins (Chen and Mort, 1995), it is likely that four adjacent GalA residues without Xyl substitution in HG segments are needed by the enzyme for activity. We hypothesized that Xyl substitution of HG segments was responsible for EPG resistance and that there are no more than three contiguous GalA residues between two Xyl side chains after complete digestion with EPG. The 4 : 1 molar ratio of GalA and Xyl in EPG-digestion-resistant fragments is in good agreement with this assumption.

Isolation and NMR Analysis of Xylogalacturonan Fragments

Fractionation of the EPG Degradation Products on HPLC HW55(s) and HW40(s) Filtration Chromatography

From the above results, we can conclude that the mild-alkali-extract contains a combination of branched Rha-rich pectic regions and HG segments substituted with some single terminal Xyl residues. Figure 16 shows the representative structure of a mild-alkali-soluble pectin in watermelon cell walls. The methyl ester groups in HG region are expected to be remove by the mild alkali when this pectin fraction is extracted from the watermelon cell walls. There is a range of sizes of xylogalacturonan fragments formed from the mild-alkali-extract by an exhaustive EPG digestion. A small fragment was isolated to get more structural details of the xylogalacturonan.

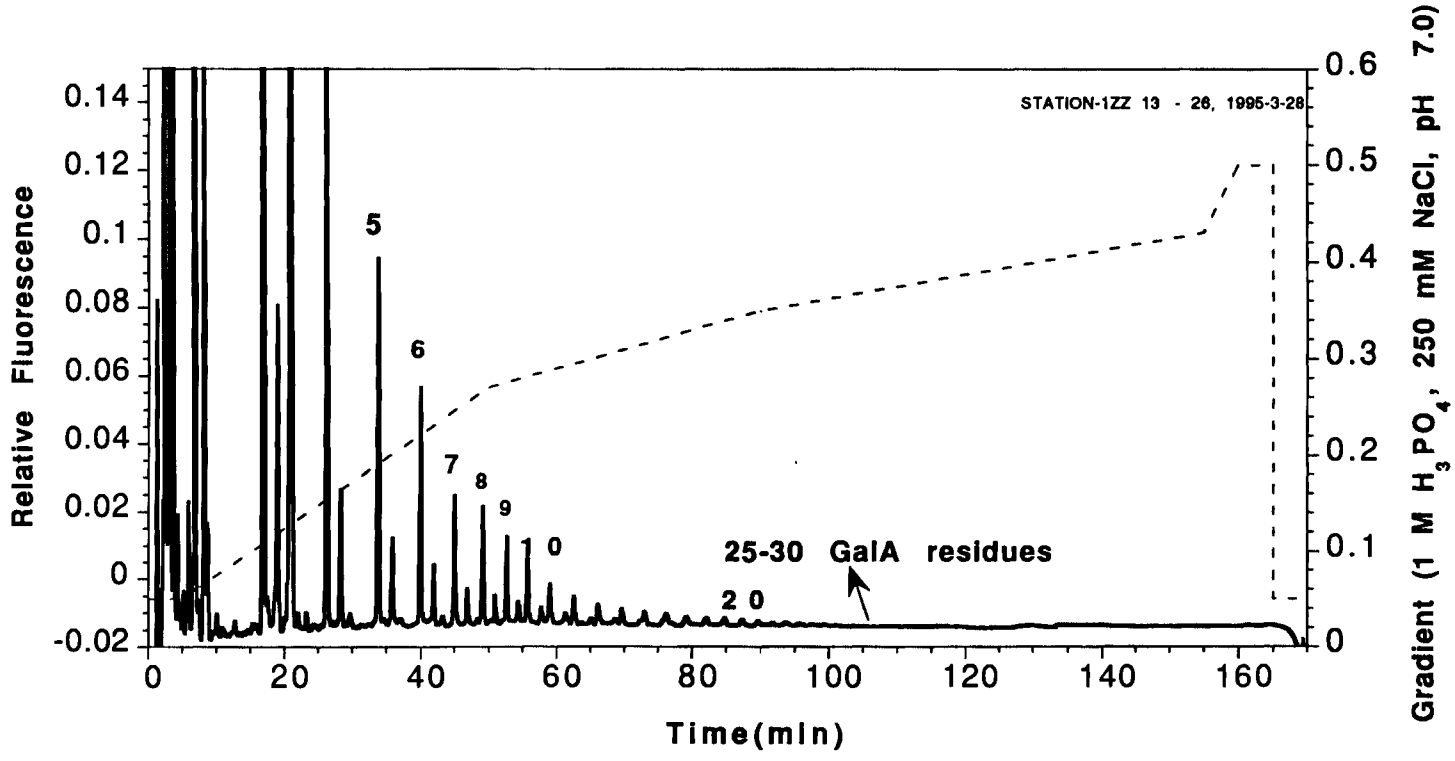

Figure 13. The HPLC chromatogram of EPG-digestion-resistant fragments, treated by -12 ^{0}C HF and derivatized with 2-AP, on a Carbopac PA-100 anion-exchange column (4 × 250 mm).

The flow rate of elution buffer was 1 mL/min. Solvent A was water, and solvent B was potassium phosphate (1 M, pH 7.0). Samples were injected into the system equilibrated at 50 mM potassium phosphate (95% water, 5% solvent B), and sample components were eluted after a 3 min lag period using a gradient of potassium phosphate from 50 mM to 270 mM over 47 min, to 350 mM over 40 min, to 430 mM over another 65 min, then to 500 mM over 5 min, with a final 5 min hold at 500 mM to wash the column.

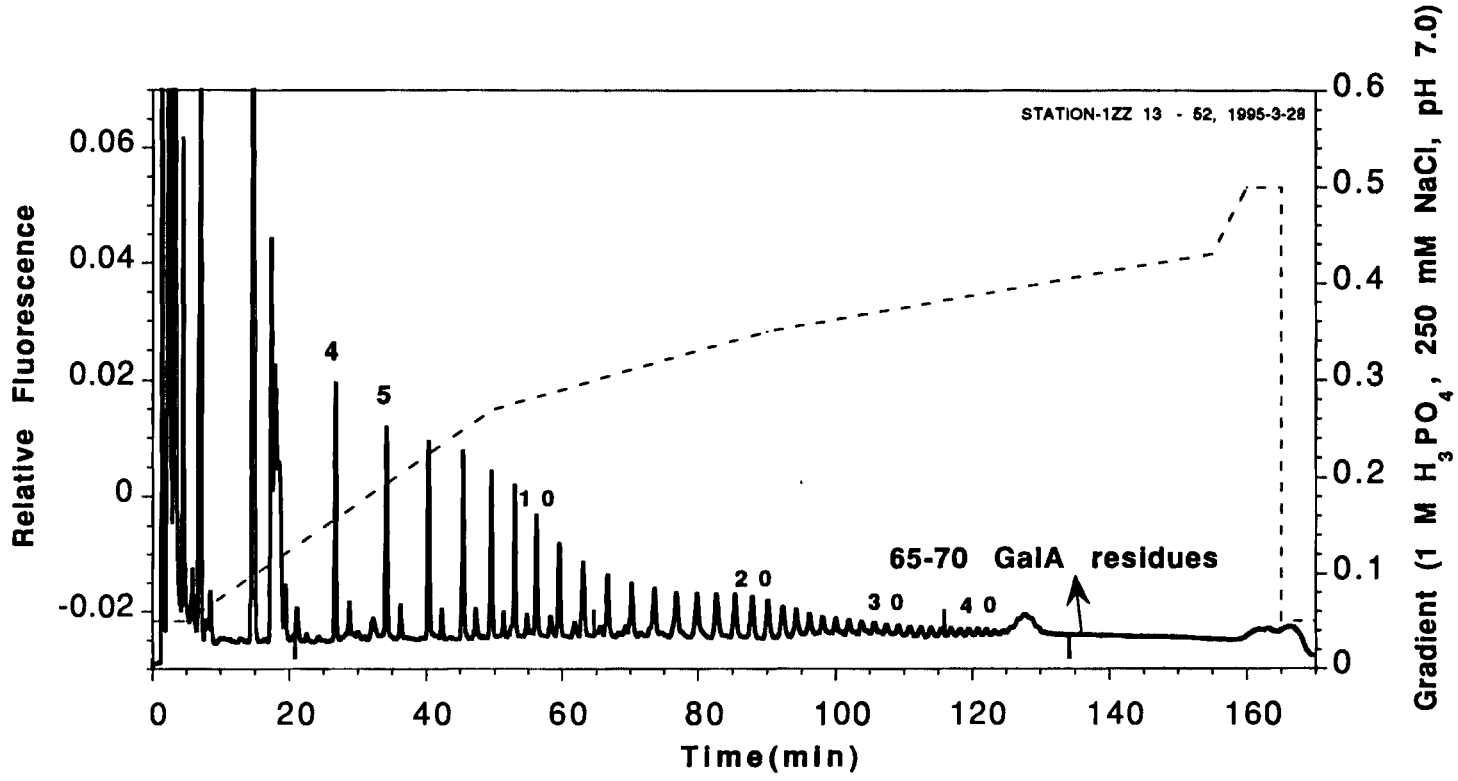

Figure 14. The HPLC chromatogram of mild-alkali-extract, treated by -12 ^{0}C HF and derivatized with 2-AP, on a Carbopac PA-100 anion-exchange column (4 × 250 mm). The HPLC parameters are the same as in Figure 13.

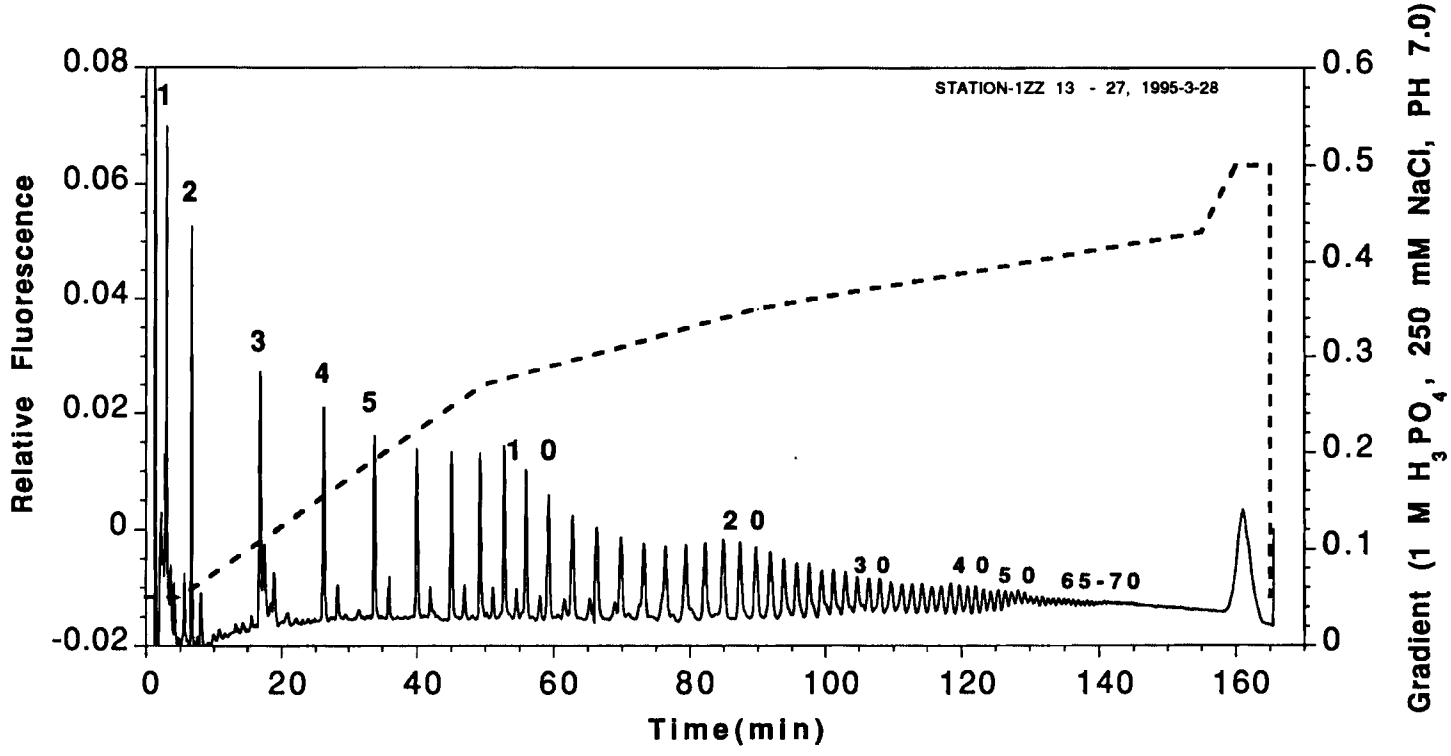

Figure 15. The HPLC chromatogram of pectic acid derivatized with 2-AP, which contains a wide range of GalA oligomers, on a Carbopac PA-100 anion-exchange column (4 × 250 mm). The HPLC parameters are the same as in Figure 13.

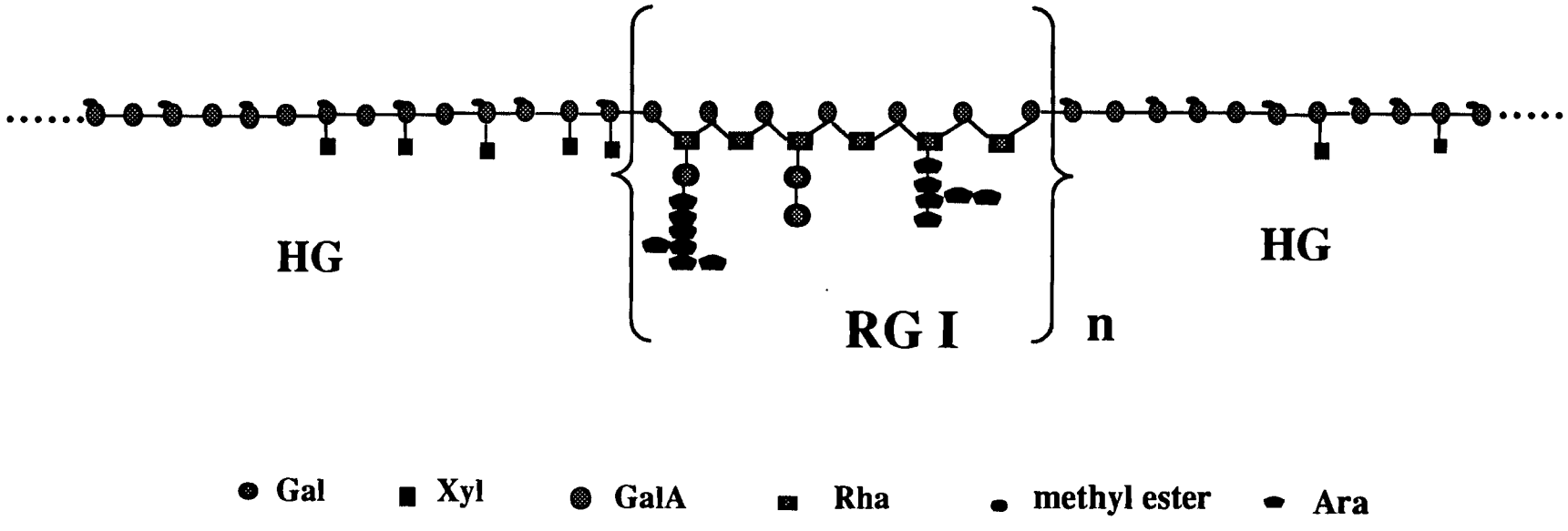

Figure 16: A representative structure of a mild-alkali-soluble pectin in watermelon cell walls. The HG region, which is partially methyl-esterified (DM = 45%), substituted with some single terminal Xyl residues. The length of the diglycosyl alternating structure (n) is unknown. [This representative pectin structure was modified from a structure in Henk Schols (1995)].

To be able to get the small xylogalacturonan fragments present in the EPG degradation products, the reaction mixtures were first separated by gel filtration chromatography on HPLC HW55(s) and HW40(s) columns.

Using HW55(s) chromatography, the EPG degradation products were eluted roughly into three peaks (Figure 17). The fractions were pooled as indicated in the figure and analyzed for their sugar composition (Table 4). The sugar composition clearly shows that most of the neutral sugar units are concentrated on the large fragments that elute close to the void volume of the column (Fraction 1 and Fraction 2), and the peaks eluting in the later fractionation range of the column (Fraction 3) are composed mainly of GalA residues and Xyl residues.

Figure 18 shows the representative structure of EPG degradation products which were separated on the HW 55(s) gel filtration chromatography.

The molar ratio of GalA residues to Rha was found to be 2.0 in Fraction 1, and Xyl is also present in high concentration in this fraction (the molar ratio of GalA : Xyl is 2.3). It is concluded that this Fraction 1 represents the 'Hairy region' of pectin containing both RG I like polysaccharide and an HG with Xyl as a single unit in high density along the GalA backbone.

Fraction 2 contains less Rha, but more GalA (the molar ratio of GalA : Rha is 7). The molar ratio of GalA residues to Xyl was found to be 3.5. This fraction probably corresponds to a fraction containing less RG I like polysaccharides but associated with longer stretches of xylogalacturonans protected from the EPG by the Xyl residues.

Fraction 3 from the HW55(s) column contains mainly GalA, but also contains a relatively high concentration of Xyl. This fraction contains mono-, di-, tri-galacturonic acid, and xylogalacturonan fragments that originated from the HG segment.

Fraction 3 from the HW55(s) column was further separated by gel filtration on an HPLC HW40(s) column. Six peaks could be distinguished (Figure 19). The sugar

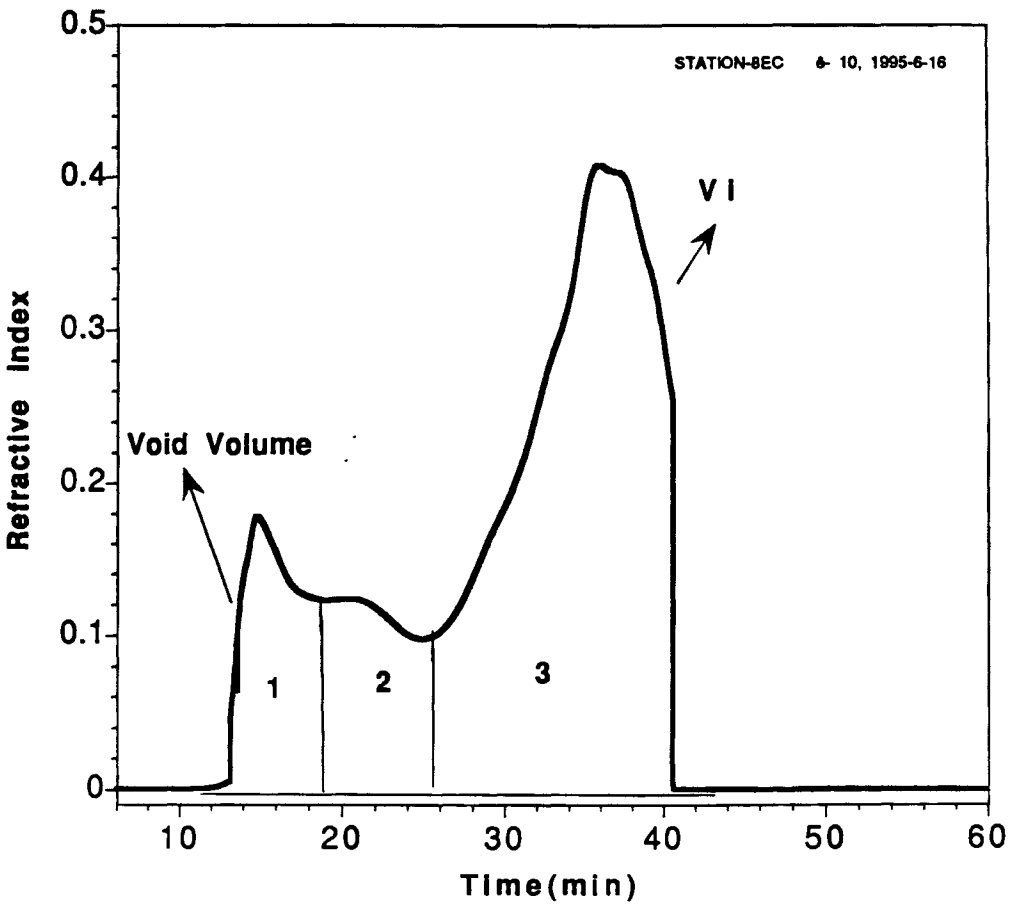

Figure 17. Chromatography on an HW55(s) HPLC gel filtration column of the mild -alkali-extract from watermelon cell walls after treatment with EPG. Tubes 9-19 were pooled into Fraction 1, tubes 20-25 were pooled into Fraction 2, and tubes 26-47 into Fraction 3.

TABLE 4

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT FRACTIONS FROM GEL FILTRATION HW-55(s) CHROMATOGRAPHY OF MILD-ALKALI-EXTRACT TREATED BY EPG

Residue	Alkali Extract	Frac 1	Frac 2	Frac 3
Ara	7.1	35.9	17.5	2.0
Rha	3.2	12.9	6.3	1.1
Xyl	11.0	10.5	12.6	12.8
GalA	76.1	24.9	45.0	81.1
Man	0.3	0.3	0.4	0.5
Gal	7.2	15.3	16.6	1.8
Glc	1.4	1.1	1.6	1.4

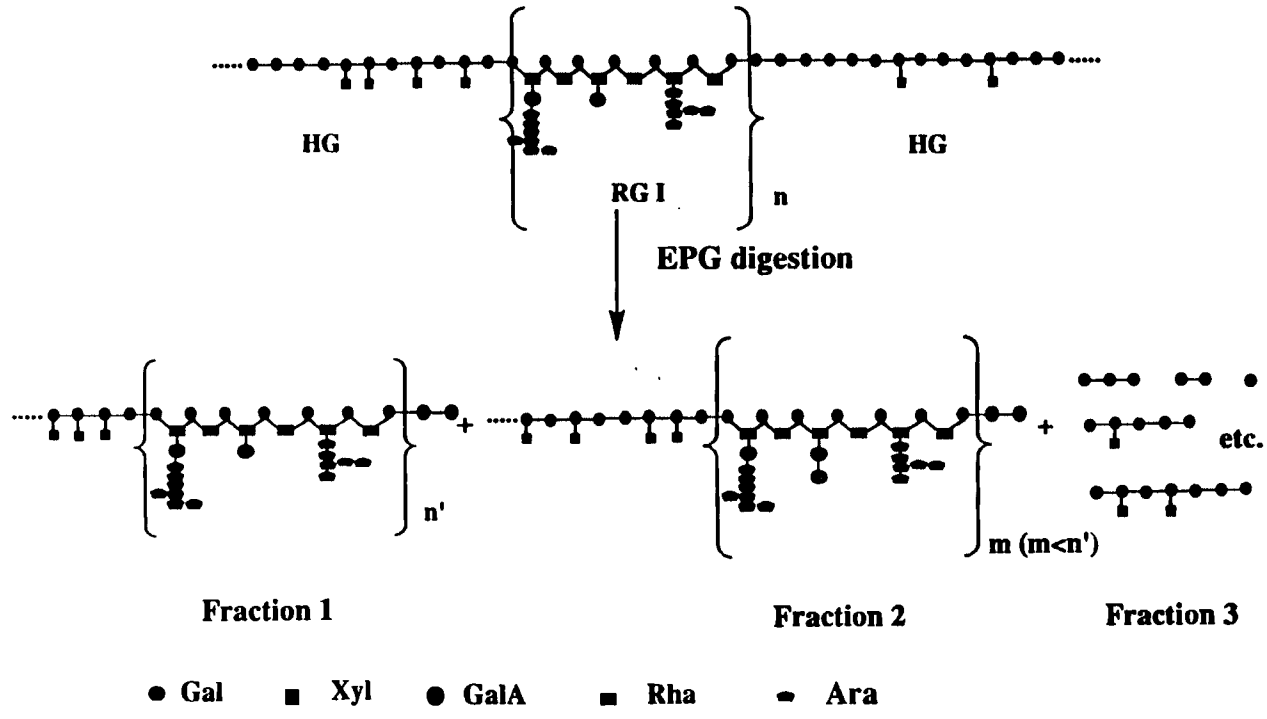

Figure 18: The representative structures of EPG degradation products separated on an HW55(s) gel filtration chromatography. Fraction 1 represents the 'Hairy region' of pectin containing both RG I like polysaccharide and an HG with Xyl as a single unit in high density along the GalA backbone. Fraction 2 corresponds to a fraction containing less RG I like polysaccharides but associated with longer stretches of xylogalacturonans protected from the EPG by the Xyl residues. Fraction 3 contains mono-, di-, tri-galacturonic acid, and xylogalacturonan fragments that originated from the HG segments.

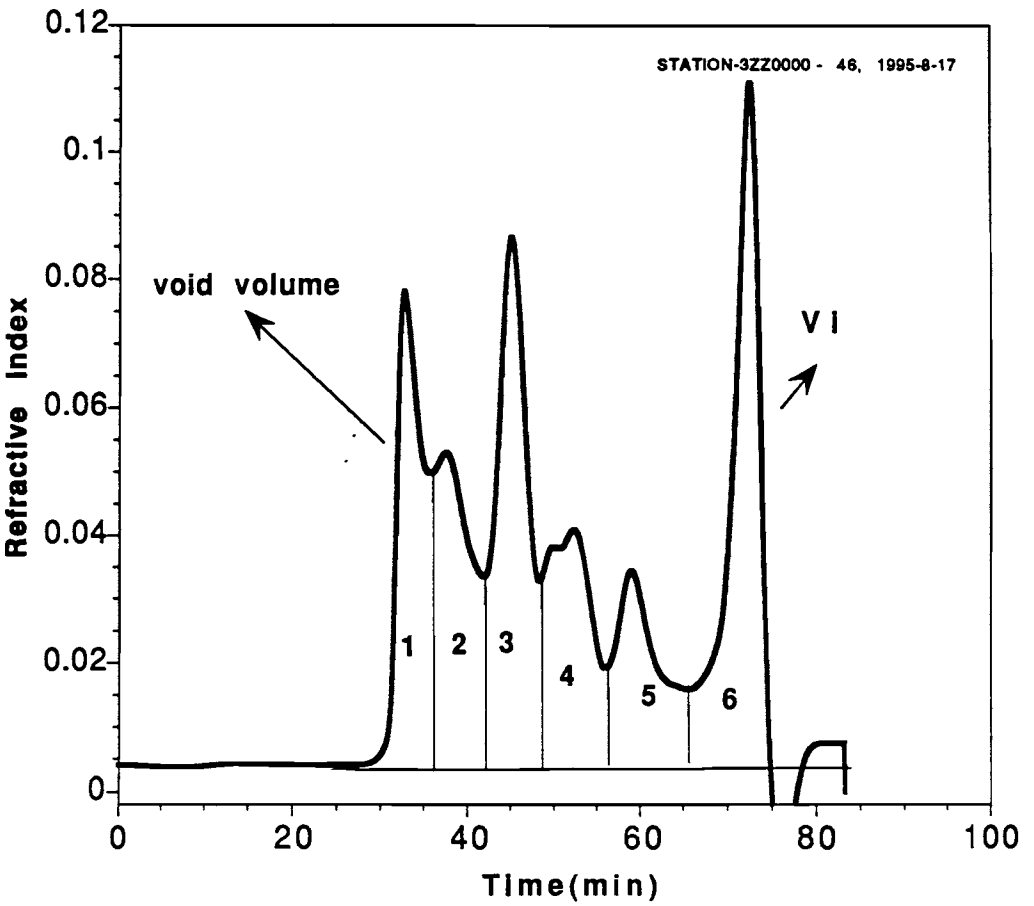

Figure 19. Chromatography on an HW40(s) HPLC gel filtration column of the Fraction 3 from the HW55(s) as indicated in Figure 17. Tubes 29-34 were pooled into Fraction 1, tubes 35-45 were pooled into Fraction 2, tubes 46-53 into Fraction 3, tubes 54-59 into Fraction 4, tubes 60-68 into Fraction 5, and tubes 69-79 into Fraction 6.

compositions of the pooled fractions are shown in Table 5. It can be seen that GalA and Xyl are the main sugars in Fractions 1 and 2, and the molar ratio of GalA residues to Xyl is between 3 and 3.5. These two fractions probably represent xylogalacturonan fragments that originated from the HG segment with single Xyl side chains. Fractions 3, 4 and 5 contain mainly GalA, and are identified as tri-, di-, and mono-galacturonic acid. Fraction 6 contained only a small amount of sugar and was assumed to be salt.

Isolation of the Xylogalacturonan Fragments by PA-1 Anion-exchange Chromatography of Fraction 2 from the HW40(s) Gel Filtration Column Chromatography

To isolate a small pure xylogalacturonan fragment, Fraction 2 from the HW40(s) gel filtration chromatography, which was thought to have a molecular size less than 10 sugars, was further separated on a Carbopac PA-1 anion-exchange column with a continuous permanganate postcolumn detector. Figure 20 shows the elution pattern of the this fraction on a semi-preparative CarboPac PA-1 anion-exchange column (9×250 mm). Due to the removal of the high molecular weight material (RG I), a better resolution between the different oligomers as compared to that in Figure 3 was obtained. Four fractions were collected and analyzed for their sugar composition (Table 6). It is shown that GalA and Xyl were the main sugars in these fractions, and their molar ratio is very similar among all four fractions.

Since Fraction B and Fraction C appeared to be large fairly homogeneous peaks, they were characterized by CZE. Figure 21 and Figure 22 show the results. They both contained one dominate peak on CZE. The earlier peak on the PA-1 column, Fraction B, eluted at the same retention time as $GalA_6$, whereas the later peak on the PA-1 column, Fraction C, eluted at the position of tri-GalA. This unexpected elution order may be a result of the substitution of Xyl on the GalA backbone.

TABLE 5

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT FRACTIONS FROM GEL FILTRATION HW40(S) OF FRAC 3 FROM HW55(S)

Residue	Frac 1	Frac 2	Frac 3	Frac 4	Frac 5
Ara	4.5	2.3	0.7	0.7	0.3
Rha	3.1	0.7	0.2	0.2	0.2
Xyl	23.8	22.2	3.9	1.6	0.8
GalA	63.8	74.1	93.8	95.6	96.1
Man	0.5	0.7	0.8	0.5	0.4
Gal	4.4	1.8	0.7	1.3	1.3
Glc	/	1.1	1.2	0.2	0.9

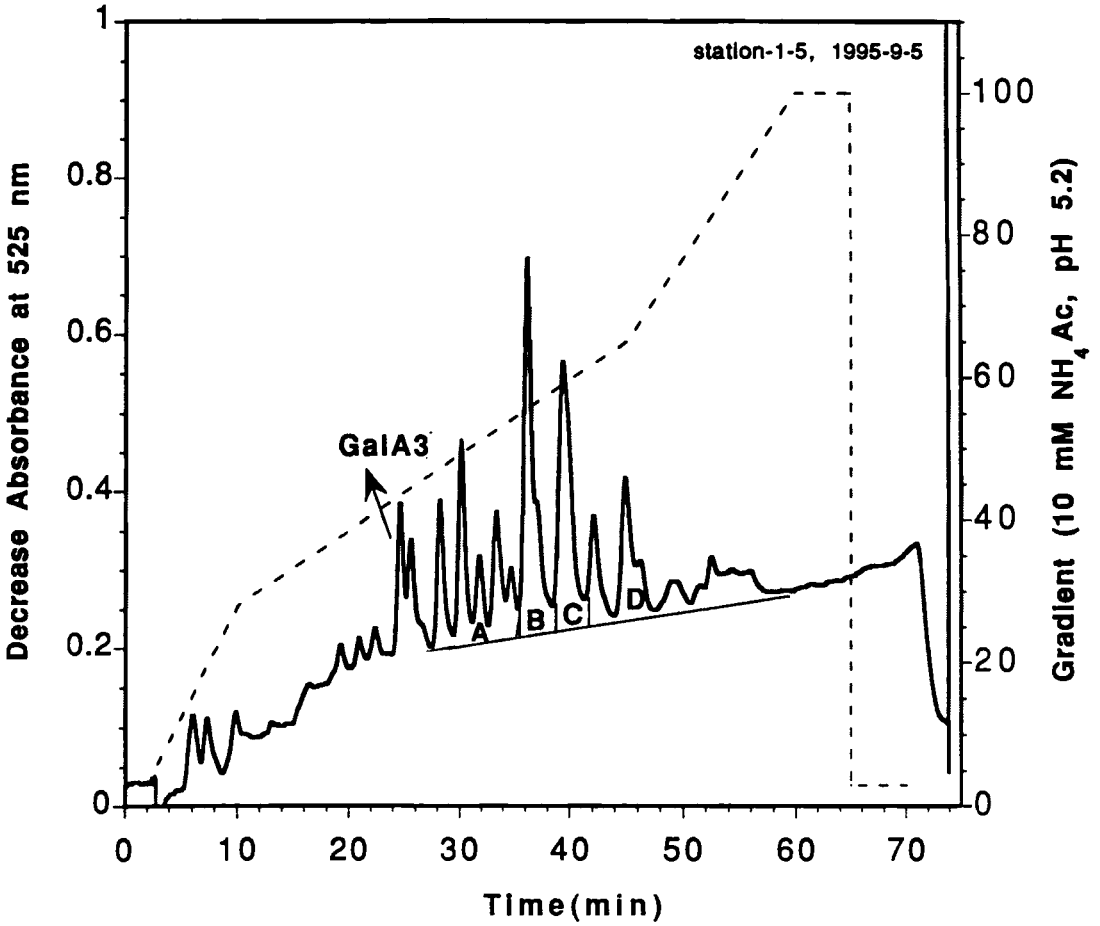

Figure 20. The HPLC PA-1 anion-exchange chromatogram of Fraction 2 from HW40(s) gel filtration column. Fractions 27-35 min were collected as Frac A, Fractions 36-38 min were collected as Frac B, Fractions 39-41 min were collected as Frac C, and Fraction 42-58 min were collected as Frac D.

TABLE 6

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT FRACTIONS FROM PA1 ANION EXCHANGE COLUMN OF FRACTION 2 OF HW40(S) SEPARATION

Residue	Frac A	Frac B	Frac C	Frac D
Ara	1.8	1.2	1.7	3.2
Rha	2.7	0.6	2.1	1.9
Xyl	22.6	18.5	24.9	21.7
GalA	70.4	78.7	70.1	73.2
Gal	/	/	/	/
Glc	2.0	1.0	1.1	/

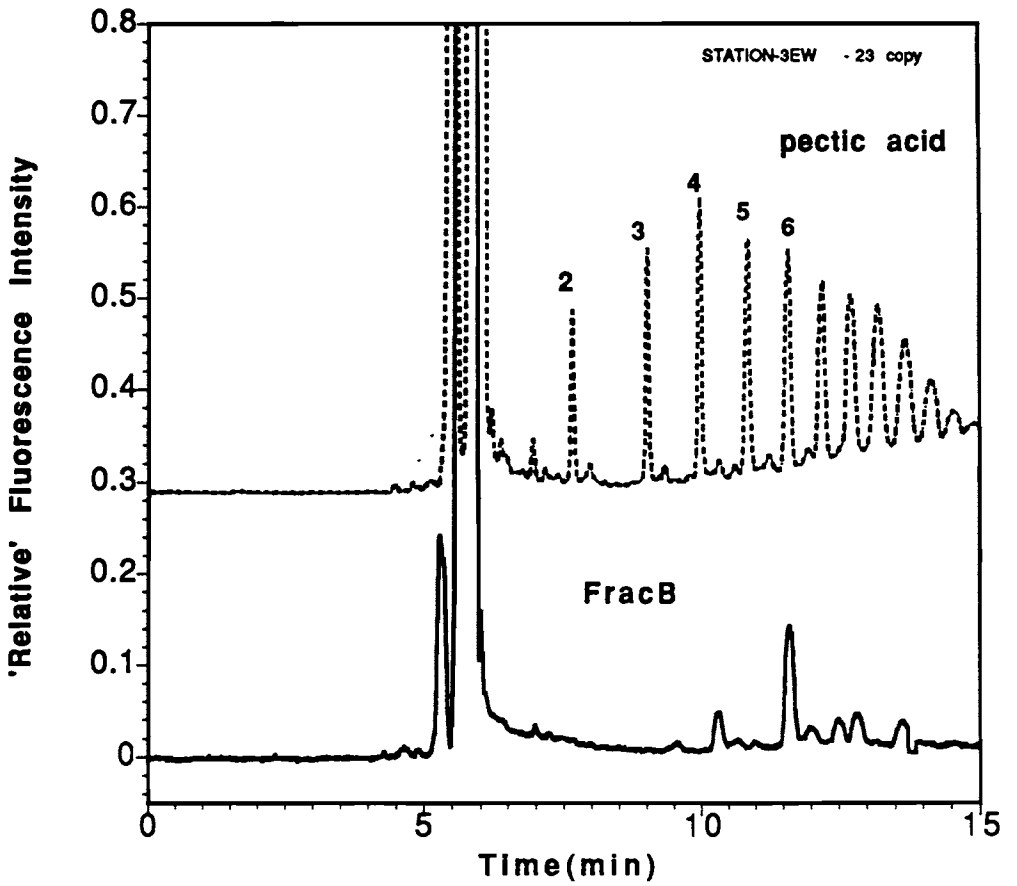

Figure 21. Electropherogram of ANTS labeled derivatives of pectic acid (upper trace) and Frac B from PA-1 anion-exchange chromatography as shown in Figure 20 (lower trace). CZE was performed on an uncoated fused silica open tubular capillary at the same operation conditions as in Figure 6.

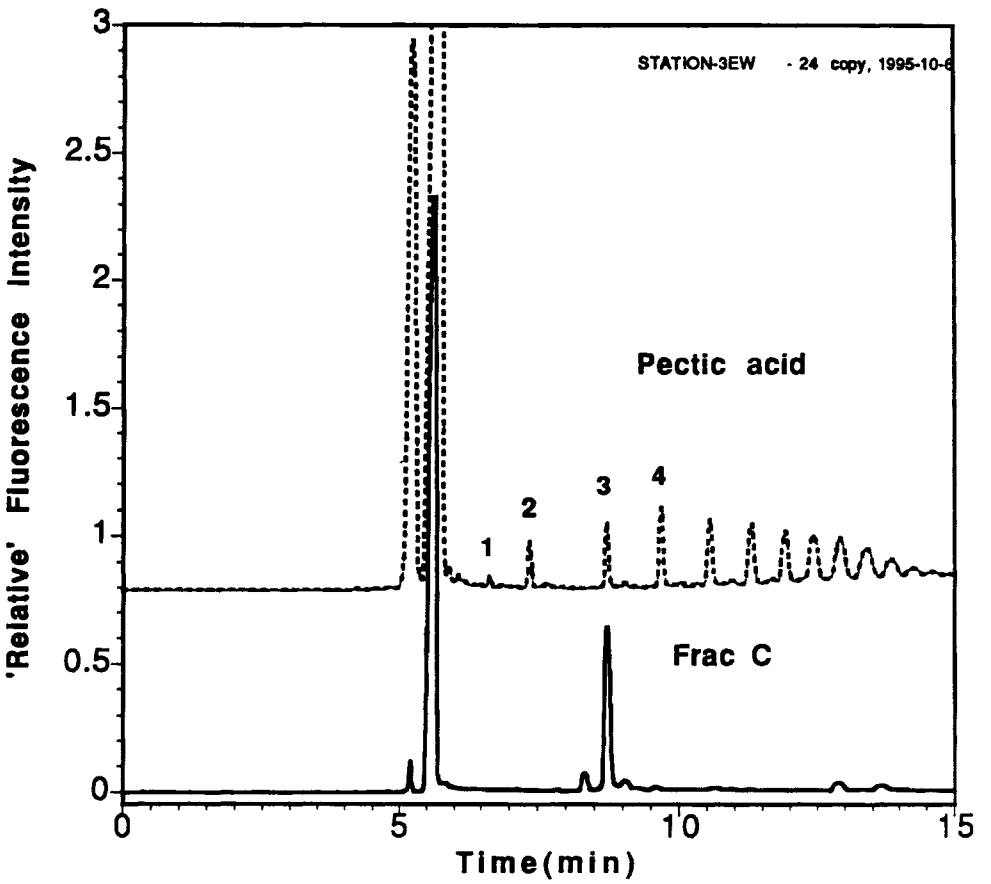

Figure 22. Electropherogram of ANTS labeled derivatives of pectic acid (upper trace) and FracC from PA-1 anion-exchange chromatography as shown in Figure 20 (lower trace). CZE was performed on an uncoated fused silica open tubular capillary at the same operation conditions as in Figure 6.

Fraction B was the closest to a relatively pure xylogalacturonan fragment, and was the most abundant of these four fractions. This fraction was chosen for further characterization by NMR spectroscopy.

Xylogalacturonan Structure from NMR Spectra

^1H spectra of this xylogalacturonan were recorded on a 600 MHz n.m.r. spectrometer. Chemical shifts were reported in ppm setting the residual HOD peak to 4.80. In Figure 23, the proton-proton correlated 2D (COSY) spectrum is shown. The good quality of the COSY spectrum allowed us to assign the ^1H chemical shifts of the main components in the xylogalacturonan fragment (Table 7).

The chemical shifts of the Xyl residues were compared to those of a known di-arabinosylxylotetraose produced from a xylan (Gruppen, et al., 1992). Two distinct sets of signals characteristic of β-Xyl were observed. These are separated into set a (i.e. Xyl^a) and set b (i.e. Xyl^b) in Table 7. It can be seen that both of the Xyl residues' ^1H chemical shifts are almost the same as those of terminal β-Xyl. Schols et al. (1995) previously reported multiple sets of β-Xyl signals in the xylogalacturonan of apple MHR. The nature of the differences between the Xyl residues is not known.

We also identified most GalA chemical shifts in the xylogalacturonan fragment. The reducing end GalA chemical shifts agree with those in tri-GalA which we obtained previously (Mort and Ryan, unpublished). By comparing the peak areas of the H-1 resonances of the reducing end GalA to the H-1 resonance for all the other GalA in this xylogalacturonan, we concluded that this xylogalacturonan is a small molecular fragment (less than seven sugars, possibly 2 Xyl plus 5 GalA).

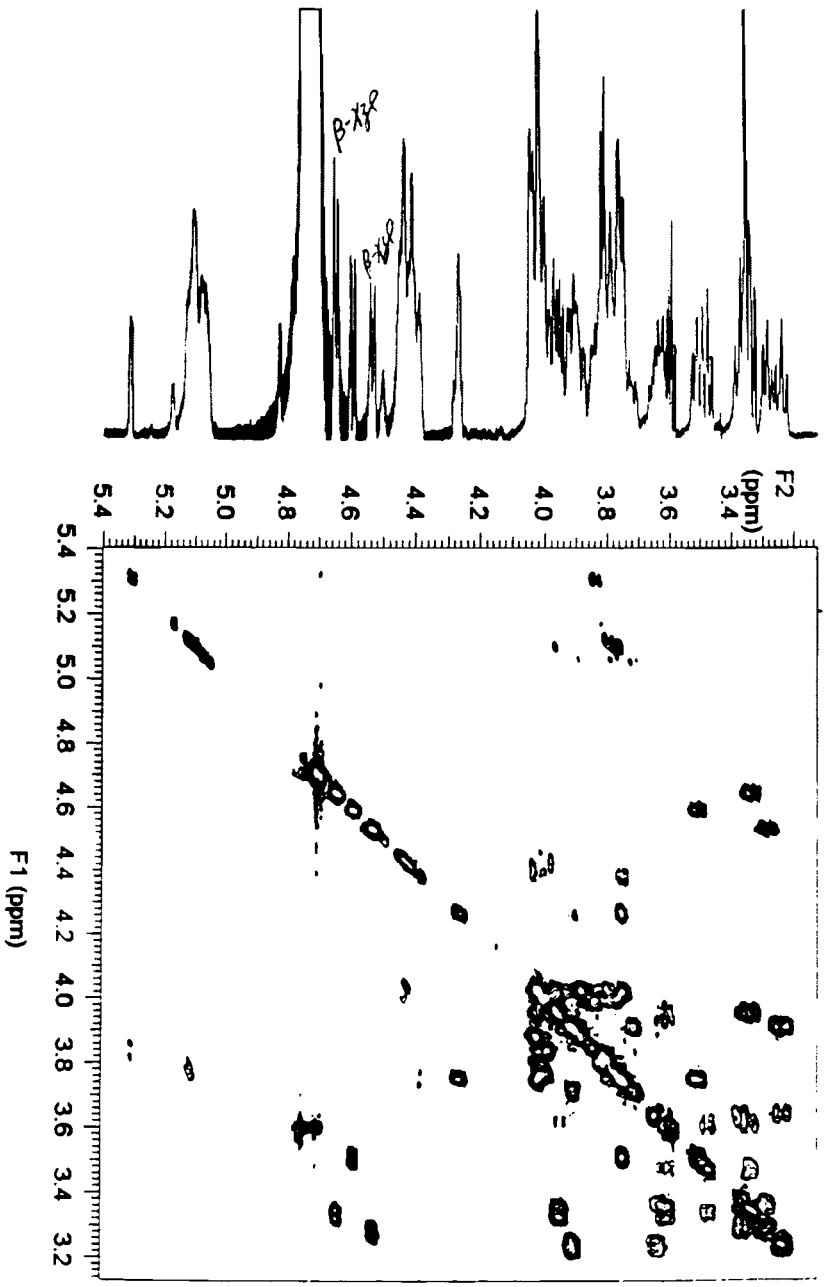

Figure 23. {^{1}H, ^{1}H} COSY spetrum of a xylogalacturonan fragment. The spectrum was recorded on a Varian Unity Plus 600 MHz n.m.r. spectrometer in D_2O. Chemical shifts are reported in ppm setting the residual HOD peak to 4.80.

TABLE 7

^{1}H CHEMICAL SHIFTS (ppm) OF XYLOSE AND GALACTURONIC ACID RESIDUES AS FOUND FOR THE XYLOGALACTURONAN FRAGMENTS

	Xyl^a	Xyl^b	*Xyl βterminal
H-1	4.64	4.52	4.43
H-2	3.33	3.27	3.24
H-3	3.48	3.38	3.41
H-4	3.64	3.60	3.59
H-5	4.00 3.30	3.92 3.24	3.91 3.27

	$GalA\beta^{(r.e)}$	$GalA\alpha^{(r.e)}$	$GalA^{(n.r)}$	$^*GalA\beta^{(r.e)}$	$^*GalA\alpha^{(r.e)}$
H-1	4.59	5.3	5.08	4.69	5.37
H-2	3.5	3.84	3.72	3.51	3.84
H-3	3.74	3.98	3.76	3.67-3.88	3.99-4.14
H-4	4.35	4.42	4.27	4.44	4.40-4.55
H-5	/	/	/	4.47	4.80

*Xyl β terminal : the chemical shifts of terminal xylose in the diarabinosylxylotetraose (Gruppen, et al. 1992).

*GalA (r.e): the chemical shifts of reducing end GalA in triGalA.

*Xyl^a or Xyl^b: two distinct sets of chemical shifts of Xyl.

CHAPTER V

SUMMARY AND CONCLUSION

Pectins are important constituents of plant cell walls, and determination of their structures is a great challenge for many researchers. The aim of this research was to partially characterize a pectin that is extracted by mild alkali from cell walls of ripe watermelon fruit, and to investigate the effects of xylosylation on the inhibition of its digestion by endoploygalacturonase.

A mild alkali with low temperature was used to isolate a pectin. Under these rather mild extraction conditions, only ester linkages in the pectin are expected to be completely cleaved. This mild alkali treatment alone solubilized a considerable portion of the pectin from watermelon cell walls, and that these gave, in about 15% yield by weight of the treated cell walls, a pectic portion comprised about 28 % of the GalA present in the watermelon cell walls. GalA was the main sugar residue in this extract, and Xyl was the second most abundant sugar residue. Based on a constant sugar composition before and after dialysis, it is mostly of high molecular weight although some of the sample was lost during dialysis. (Due to solubility problems, no reliable molecular weight data from HPLC gel filtration could be obtained).

Since this extracted pectin was characterized by a high Xyl content, and all the HG segment was previously de-esterified by the mild alkali, it was used to investigate the effects of xylosylation on the inhibition of its digestion by endoploygalacturonase. After exhaustive digestion by EPG, the mixture was separated by PA-1 HPLC anion-exchange chromatography. In addition to the expected mono-, di-, and tri-galacturonic acid that one

would obtain from the complete digestion of pectic acid, EPG-digestion-resistant fragments were produced in a range of sizes. The EPG-digestion-resistant fragments contain some high molecular weight RG I like regions with HG segments that have Xyl as side chains, and some low molecular weight xylogalacturonan fragments that originated from the HG regions. Methylation analysis revealed the presence of terminal Xyl residues, and that some of the GalA residues were linked through both the 3 and 4 positions. Since GalA is known to link in a (1 →4)-linkage in the HG region, the terminal Xyl residues are most likely to be attached to C-3 of the (1 →4)-linked GalA backbone as single residue side-chain.

Treating the entire EPG-digestion-resistant fragments with HF at -12 ^{0}C to -15 ^{0}C removes the Xyl residue side-chains and destroys the Rha-rich pectin region, leading to simple HG fragments which can be characterized by HPLC anion-exchange chromatography. We find that HG oligomers ranging to 25 or more residues in length are completely protected from EPG digestion by the xylosylation of, on average, every one in four GalA residues. Since the EPG we used is not able to hydrolyze tri-galacturonic acid (Thibault, 1983, Mort and Chen, 1995) and requires four contiguous non-methylesterified GalA residues as a substrate in partially esterified pectins (Mort and Chen, 1995), the 4 : 1 molar ratio of GalA and Xyl in EPG-digestion-resistant fragments was in good agreement with the assumption that Xyl substitution can interfer with EPG activity. Thus regions in the HG with Xyl residues closer than five GalA residues apart will be protected from EPG digestion.

A combination of gel filtration chromatography with anion-exchange chromatography was used to isolate a small xylogalacturonan fragment which only contains GalA and Xyl from the EPG degradation products. A 2D ^{1}H COSY n.m.r. spectrum showed this xylogalacturonan is a small molecular weight fragment. Two different sets of β-Xyl signals were also found from this 2D ^{1}H COSY spectrum. The nature of the differences between the two Xyl residues is not known.

Based on the above results, we conclude that the mild-alkali-extract contained a combination of branched Rha-rich pectic regions and HG regions substituted with some single terminal Xyl residues. There is a range of sizes of xylogalacturonan fragments formed from the mild-alkali-extract by an exhaustive EPG digestion. Xyl residues are directly linked β to the C-3 position of GalA residues of HG regions as single residue side chains. They are the most likely inhibitor for EPG digestion.

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VITA

Liying Yu

Candidate for the Degree of Master of Science

Thesis: PARTIAL STRUCTURAL CHARACTERIZATION OF A PECTIN EXTRACTED FROM WATERMELON CELL WALLS BY MILD ALKALI

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Hangzhou, Zhejiang, The P.R. of China, November 8th, 1964, the son of Zhirui Yu and Renying Zhang.

Education: Received Bachelor of Science Degree from Hangzhou University, Hangzhou, The P. R. of China, in July, 1986; Completed requirements for the Master of Science Degree at Oklahoma State University, U.S.A. in December, 1995.

Professional Experience: Research Assistant in Biochemistry and Molecular Biology Department, Oklahoma State University, Stillwater, Oklahoma, August 1993 to present; Research Assistant in Mass Spectrometry Facility, Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma, May 1993 to present; Teaching Assistant in Animal Science Department, Oklahoma State University, Stillwater, Oklahoma, January 1993 to May 1993; Analytical Chemist, Analysis Center of Zhejiang Food Science Institute, Hangzhou, The P.R. of China, August 1986 to July 1992.