IMPROVEMENT OF METHODS FOR PECTIN CHARACTERIZATION AND STRUCTURAL CHARACTERIZATION OF A METHYL ESTERIFIED TETRAMER OF GALACTURONIC ACID FROM PECTIN

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NOMENCLATURE

2AP	2-aminopyridine
ANTS	8-aminonaphtalene-1,3,6-trisulfonate
APTS	9-aminopyrene-1,4,6-trisulfonate
DHB	2,5-dihydroxybenzoic acid
MSA	5-methoxysalicylic acid
SCB	sodium cyanoborohydride
TFA	tri-fluoroacetic acid
THAP	2,4,6-trihydroxyacetophenone monohydrate
THF	tetrahydrofuran
Nafion	perfluorinated ion-exchange resin
Ara	Arabinose
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic Acid
Glc	Glucose
Man	Mannose
Rha	Rhamnose
Xyl	Xylose
CZE	capillary zone electrophoresis
GLC	gas liquid chromatography
HPLC	high performance liquid chromatography
LIF	laser induced fluorescence
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
NMR	nuclear magnetic resonance
PSD	post source decay
TOF	time of flight
Arabinanase	endo-arabinanase
Ara-sidase	α-L-arabinofuranosidase
EPG	endo-polygalacturonase
Galactanase	endo-1,4-β-galactanase
galactosidase	β-galactosidase
RGase	rhamnogalacturonase (correctly termed an RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase)
HG	homogalacturonan
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
RG	rhamnogalacturonan
XGA	xylogalacturonan

CHAPTER 1

1.1 GENERAL INTRODUCTION

Plant cell walls are very complicated structures that play very important roles, although not many of them are understood in the life of a plant. This important dynamic extra-cellular matrix, composed of complex polysaccharides, structural proteins and aromatic substances, is said to maintain the rigidity of the cell, therefore, providing it with its stable shape by the means of its mechanical strength (Pérez et al., 2000). The functions of the wall are also biological. Other major roles attributed to plant cell walls are: limiting the cell expansion, porosity control, barrier to digestion, and cell adhesion, which makes it an important component in plant morphogenesis (Fry, 1989). Moreover, it is believed that plant cell walls also participate in cell-to-cell signaling by the release of signal molecules named oligosaccharins or oligosaccharides (Pérez et al., 2000; McCann et al. 2001).

Plant cell walls are divided into primary and secondary subtypes. Primary cell walls are synthesized during the early stage of differentiation, when the young cells still retain the capacity to divide and elongate. Secondary cell walls are laid down on the inner surface of the primary walls after growth stops, and they are characterized by their enrichment in cellulose micro-fibrils and lignification (Stumpf, 1980).

Plant cell wall carbohydrates form the most abundant natural compounds on earth, which implies that they are our most important renewable natural resource. Their omnipresence in agricultural products gives the plant cell wall carbohydrates a major role in nutrition. The plant cell wall polymers, which vary amongst the source and other

conditions, are arranged in various carbohydrates structures that include cellulose, hemicellulose (xyloglucan and arabinan) and pectin (Pérez et al., 2000).

Cellulose is an unbranched polymer of glucose residues joined by β-1,4-linkages. The β configuration allows cellulose to form very long straight chains, which is optimal for the construction of fibers with a high tensile strength. These characteristics give cellulose an important structural role in plants, making it responsible for the ability of plants to withstand mechanical stress and osmotic pressure (Stryer, 1995).

Hemicellulose is comprised of those polysaccharides non-covalently associated with cellulose, such as xyloglucan, various xylans, heteroxylans and arabinan (Stumpf, 1980). Xyloglucans, which are present in the cell walls of virtually all higher plants, are structurally related to cellulose, but the presence of side chains at the O-6 of their (1→4)-linked β-D-Glc p residues makes them very different from cellulose in terms of their physical properties. Approximately 75% of the residues in the backbone bear a glycosyl side chain at O-6, which invariably is α-D-Xyl p. In addition, 50% of the times, this side chain contains moieties at its O-2, such as β-D-Gal p or α-L-Fuc p- (1→2)-β-D-Gal p (York et al., 1996). Arabinans are mainly composed of α-L-arabinofuranosyl residues, generally arranged in (1→5) linked chains, with varying numbers of residues substituted with other α-L-arabinofuranosides at the O-2 and/or O-3 position (Beldman et al. 1997).

Pectins are the most abundant complexes in the primary walls, yet we still do not have a clear understanding of their functions. They are loosely defined as "polymers rich in galacturonic acid and their associated side-chains" (McCann et al., 2001), which comprise 20-50 % of the wall (McCann et al., 2001). Pectins comprise a family of acidic polymers, like homogalacturonans and rhamnogalacturonans, with several neutral

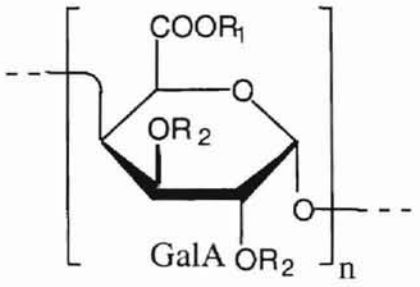
polymers such as arabinans, galactans and arabinogalactans attached to them (Strasser et al. 2001). So far, 3 pectic polysaccharides complexes have been isolated and structurally characterized from primary cell walls: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) (Ridley et al., 2001). In addition, a fourth pectic polysaccharide, xylogalacturonan (XGA), found near RGI, has emerged in the last decade as a minor type (Schols et al., 1995). The 3 main polysaccharides (HG, RGI and RGII) are solubilized by endo-polygalacturonase (EPG) treatment of primary cell walls, which has led to the belief that they are all covalently linked together (Ishii et al., 2001).

HG is a homopolymer of (1→4) α-D-GalA with a varying degree of carboxyl groups methyl-esterified (Pérez et al., 2000, Daas et al., 1998). The GalA residues may also be acetyl-esterified at C2 and C3 (Ridley et al., 2001). RGI is composed of alternating L-rha and D-GalA residues, forming a repeating (1→2)-α-L-rhamnosyl-(1→4)-α-D-GalA unit. Some of the GalA units, as many as one third, are acetylated at the O-3 position (Komalavilas et al., 1989). L-arabinosyl and D-galactosyl rich side chains are attached to approximately 2/3 of the rhamnose residues of this backbone, which are responsible for terminating or limiting inter-chain association (Pérez et al., 2000). Occasionally, the side chains are terminated by L-fucosyl, D-glucuronosyl or 4-O-methyl-D-glucuronosyl residues (Strasser et al., 2001). The most abundant arabinans appear to be homopolysaccharides of mainly (1→5)-linked-α-L-arabinofuranosyl units. They form helical chains with further branching at the 2- and 3- positions. There are 2 types of arabinogalactans: type I, the most abundant and composed of (1→4)-linked β-D-galactan side chains with α-L-arabinofuranosyl units attached to the O-3 of the galactosyl units; and type II, the most complex and composed of (1→3)- linked β-D-galactan chain

substituted with short (1→6)- linked β-D-galactan chains which in turn carry additional branches of (1→3)- and/or (1→5)- linked α-L-arabinofuranosyl residues (Pérez et al., 2000). RGII is a complex low molecular weight polysaccharide. It exists primarily as a dimer (dRG-II-B) that is covalently linked by a 1:2 borate diol ester. This cross-linking forms a matrix that may be involved in regulating the pore size and some of the physical properties of the primary walls. The backbone of RGII contains at least 7 (1→4)-linked α-D-galactopyranosyluronic acid residues, with 4 structurally distinct oligoglycosyl side chains (Vidal et al., 2000). According to Whitcombe et al. (1995), RGII contains 11 different glycosyl residues including unusual monosaccharides, seldom observed methyl-etherified sugars and unusual glycosidic linkages. XGA contains a homogalacturonan backbone with a non-reducing terminal Xyl attached at the O-3 position of many of the GalA residues. The structures of the 4 pectic regions are illustrated in Fig. 1-1 and Fig. 1-2.

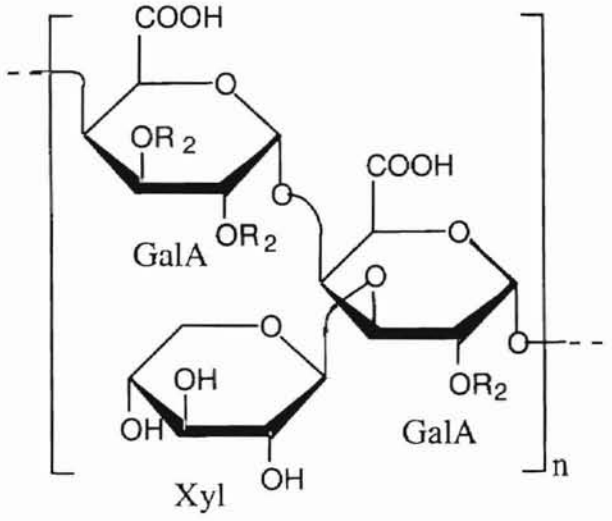
The distribution of the mentioned pectic regions is varied and variable, from species to species and at different developmental stages and conditions. As reviewed by Ridley et al., unesterified HG is localized to the middle lamella, to cell corners, and around air spaces whereas esterified HG is typically present throughout the wall. In addition, Ridley et al. infer that the presence and location of the arabinan and galactan side chains of RGI are often correlated with stages of cell and/or tissue development.

The absence of pectic polysaccharides in secondary walls has led to the belief that pectin synthesis has to be regulated in a temporal, spatial, and developmental specific manner. It is likely that both the synthesis of HG and RGI begin in *cis* Golgi and continues into the medial Golgi, while the esterification of HG appears to occur in the



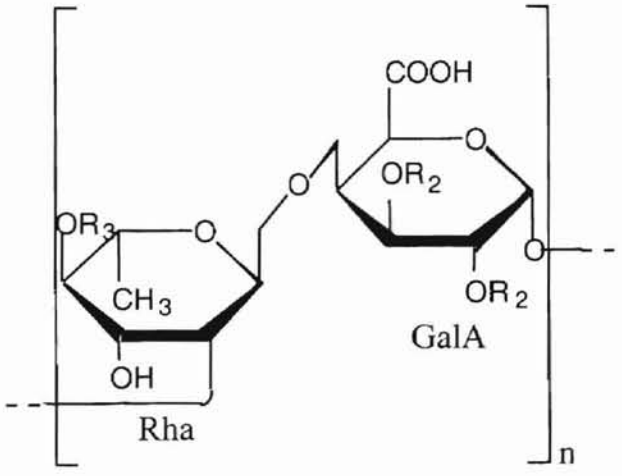
Homogalacturonan

R_1=H or methyl esters
R_2=H or acetyl



Xylogalacturonan

R_1=H or methyl esters
R_2=H or acetyl



Rhamnogalacturonan I

R_2=H or acetyl
R_3=H, gal, galactan,arabinan or arabinogalactan

Fig 1-1 Structure representation of HG, XGA and RGI

Fig 1-2 Structure representation of RGII (from Ridley et al. 2001)

medial and *trans* Golgi. In addition, more extensive branching of pectins appears to occur in the *trans* Golgi cisternae. The final pectin assembly might occur as the Golgi vesicles are transported to the plasma membrane and the pectin is inserted into the wall (Ridley et al., 2001).

All cell wall polymers are assembled together through many covalent (glycosidic, ester, etc) and non-covalent (hydrogen and ionic) cross-links to form a strong dynamic wall. Many models have been proposed to explain how the cross links of these polymers make up the plant cell wall. Peter Albersheim's group was the first one, in 1973, to propose a cell wall model. Albersheim's model describes the whole cell wall as a macromolecule, in which all non-cellulosic polymers are covalently linked except for the hydrogen bonding of xyloglucans to the cellulose micro fibrils. This model also suggests covalent cross-linking between pectin to wall proteins (Keegstra et al., 1973). In 1993, Carpita and Gibeaut proposed a new cell wall model for primary walls of most flowering plants. This new model, which accounts for details such as the directions that the micro fibrils move in relation to each other during growth, is comprised of 3 structurally independent but interacting domains. These are a cellulose-xyloglucan network, which interacts with and within the pectin network, and a third and more independent network of structural proteins. The components of these networks can change independently depending on developmental state or in response to special kinds of stress. The pectic polysaccharides are involved in diverse structures. The helical chains of the homogalacturonan pectic region can condense with Ca^{2+} to form what is known as 'eggbox' junction zones by linking 2 anti-parallel chains. This new model takes into account both cell division and cell expansion by emphasizing that the cell shape is

established by the orientation of synthesis of the cellulose micro fibrils, while the rate of cell expansion and the extent that the micro-fibrils are pulled apart in the longitudinal axis is dictated by the dynamic interaction between the cellulose and the non-cellulosic polysaccharides networks (Carpita et al., 1993).

Determining the structures of the complex pectic polysaccharides is very important for further understanding of the pectin function and its biosynthesis. This very challenging task, blurred by under-developed applicable methods, needs degradation methods, either chemical or biochemical, to break the polysaccharides into manageable pieces. Enzymatic digestion seems to be the answer to the quest for a less harsh biochemical degradation method. Due to the heterogeneity in the composition and structure of pectin, a wide range of enzymes is required for the biodegradation of these polysaccharides. Unfortunately, there are few purified enzymes commercially available, and even these retain extraneous activities to a varying extent. Commercially available enzymes include both endohydrolases, which attack polysaccharides in mid-chain and yield oligosaccharide products, and exohydrolases, which attack from the non-reducing end to give monosaccharides (Fry, 1989). Commercially available enzymes can be used when their side activities are not expected to compromise the results, since their major activity is known. For example, endo-(1→5)-α-L-arabinanases [(1→5)-α-L-arabinan (1→5)-α-L-arabinohydrolase] cleaves the (1→5)-α-L-arabinofuranosyl backbone of arabinans in a random manner releasing arabino-oligosaccharides. The extent and rate of branched arabinan hydrolysis is affected by the degree of substitution on the arabinan backbone by (1→3) or (1→2)-α-L-arabinofuranosyl residues. Pitson et al. suggested that this enzyme needs at least one unsubstituted α-L-arabinofuranosyl residue on each side

of a substituted residue for hydrolysis to occur (Pitson et al., 1997). When extraneous activities are expected to compromise the results, then purer cloned enzymes can be used. For example, Fu et al. reported a recombinant RGase of *B. fuckeliana* that cleaves at the glycosidic bond between GalA and Rha, the backbone of the RGI region of pectin. This recombinant RGase preferentially cleaves 4 to 6 residues from the reducing end of the $(GR)_{n>5}$ substrate, but a multiple attacks mechanism is absent. This cloned enzyme is devoid of extraneous activities. In addition, Mutter et al. reported another RGase (correctly termed an RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase) from *A. aculeatus* , which is able to cleave oligomers of 5 Rha-GalA units or more, and it cleaves at 4 or 6 residues from the non-reducing end Rha. In addition, this RGase shows a multiple attack mechanism of action.

The un-masking of pectin structure is an unquestionable need, and the future only holds progress in isolating and characterizing pectic polysaccharides. The achievement of this open-ended task will require the combined use of carbohydrate chemistry, biochemistry, molecular biology and genetics.

1.2 INTRODUCTION TO CARBOHYDRATE METHODS

Enzymes have specificity that allows them to be useful tools to degrade polymers. The specific activity of each enzyme is measured in units, where one unit of enzyme activity is defined as the amount of enzyme required to release one micromole of the reducing monosaccharide equivalent per minute under the manufacturer's defined assay conditions. Despite its usefulness, most commercial enzymes have extraneous activities that need to be taken into account if they might interfere with the expected results. EPG

(endo-polygalacturonase) catalyzes the hydrolysis of interior α-(1→4)-linked D-galacturonic acid residues. The EPG used (from *A. niger*, Lot 00901, specific activity 890 U/mg protein) requires 4 contiguous non-methylesterifed galacturonate residues upon which to act. The best substrate for the action of EPG is low esterified or de-esterified polygalacturonic acid, which makes it a great choice of enzyme to degrade the saponified HG region of pectin. The cloned RGase (rhamnogalacturonase, correctly termed an RG α-D-galactopyranosyluronide-(1,2)-α-L-rhamnopyranosyl hydrolase) used preferentially cleaves 2 or 3 repeating units of RG (4 or 6 residues) from the reducing end of the $(GR)_{n>5}$ substrate. RGase is of very useful in degrading the RGI backbone. The following enzymes are also very useful in degrading the side chains found in the RGI region of pectin. Ara-sidase (α-L-arabinofuranosidase) (from *A. niger*, Batch MAF80601, specific activity 40.3 U/mg protein) catalyzes the hydrolysis of the α-L-arabinofuranosyl residues from the arabinan side chains of both types I and II arabinogalactans. Arabinanase (endo-(1→5)-α-L-arabinanase) (from *A. niger*, Batch MAR00301, specific activity 8.0 U/mg protein) cleaves the (1→5)-α-L-arabinofuranosyl backbone of debranched arabinans in a random manner releasing arabino-oligosaccharides. Galactanase (endo-1,4-β-galactanase) (from *A. niger*, Batch MGA00901, specific activity 408 U/mg protein) catalyzes the hydrolysis of the de-branched galactan side chains . Galactosidase (β-galactosidase) (from *A. niger*, Lot 60102, specific activity 112.3 U/mg protein) catalyzed the hydrolysis of the galactan side-chains.

Carbohydrate labeling is very useful in their sensitive detection. A number of fluorescent tags have been reported, which contain aromatic ring systems and share some

common features. Anumula (2000) suggests that the fluorescent tag's aromatic ring system must be substituted at the *ortho* position to an amine or nitrogen heterocycle to be fluorescent and useful. The fluorescent labeling of both monosaccharides and oligosaccharides is generally carried out by reductive amination at the sugar's reducing end (Anumula, 2000). Figure 1-3 shows a representative reductive amination in a labeling reaction using ANTS. Labeled oligosaccharides coupled with other analytical techniques such as CE and HPLC form invaluable tools for the carbohydrate researcher.

CE (capillary electrophoresis) is a micro-column (fused-silica columns) separation technique very suitable for the detection of analytes in very small volumes. The commercial availability of its automated system adds to its research suitability. The separation is based in the charge/mass ratio, which is very helpful in the characterization or elucidation of complex samples. Different experimental conditions, i.e. pH of the running buffer, influence the migration behavior of the analytes, therefore should be optimized and maintained constant. Figure 1-4 shows a schematic representation of the principles of capillary electrophoresis.

MALDI (matrix-assisted laser desorption/ionization) is a laser-based soft ionization method that has proven to be one of the most successful ionization methods for mass spectrometric analysis and investigation of large molecules. Its distinguishing feature is that the sample is embedded in a chemical matrix that greatly facilitates the production of intact gas-phase ions from large, nonvolatile, and thermally labile compounds such as peptides and proteins, synthetic polymers, oligonucleotides, oligosaccharides, lipids, and large inorganic compounds. A laser beam (generally from a

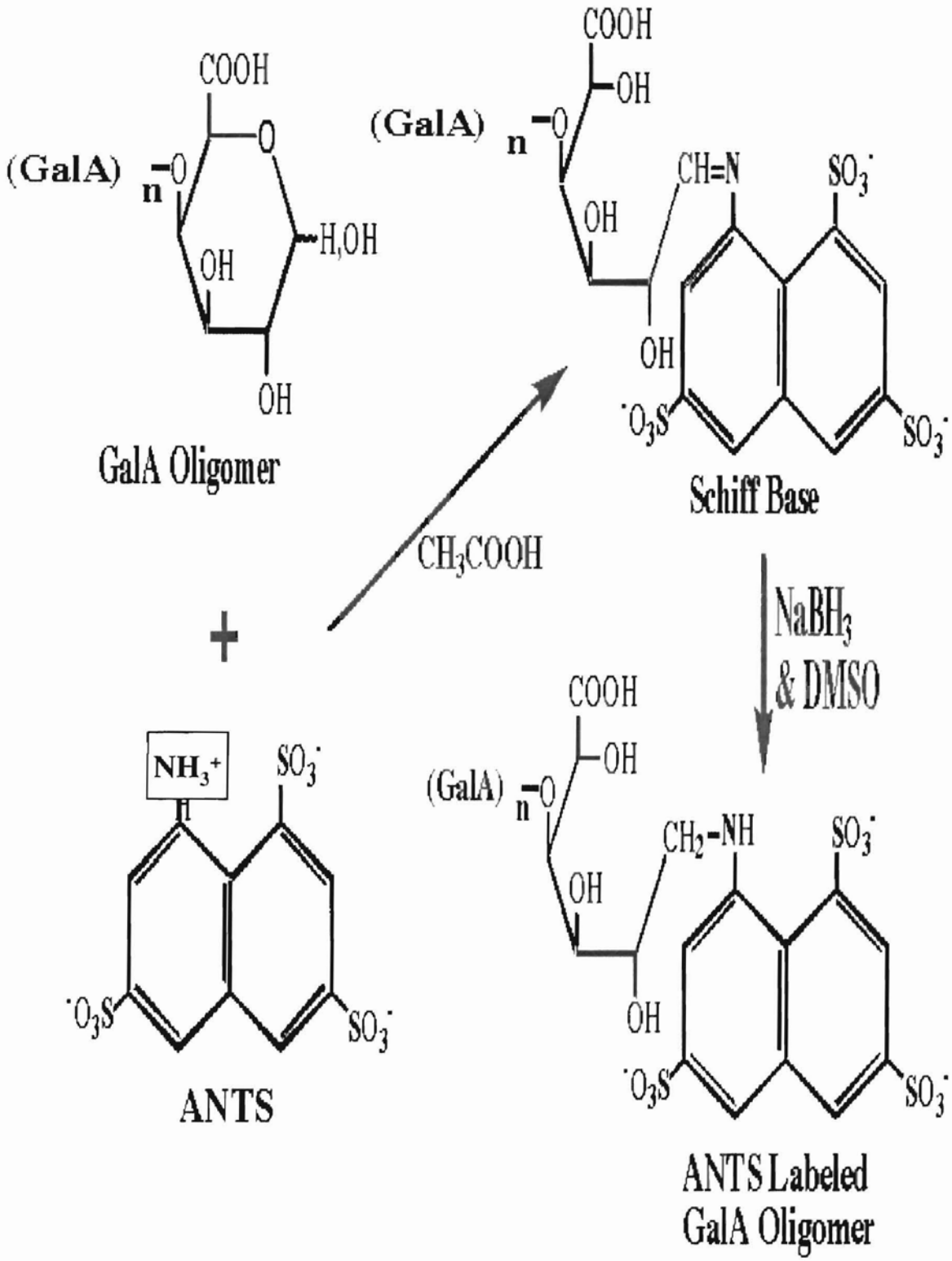


Fig. 1-3 Representative reductive amination in a labeling reaction using ANTS

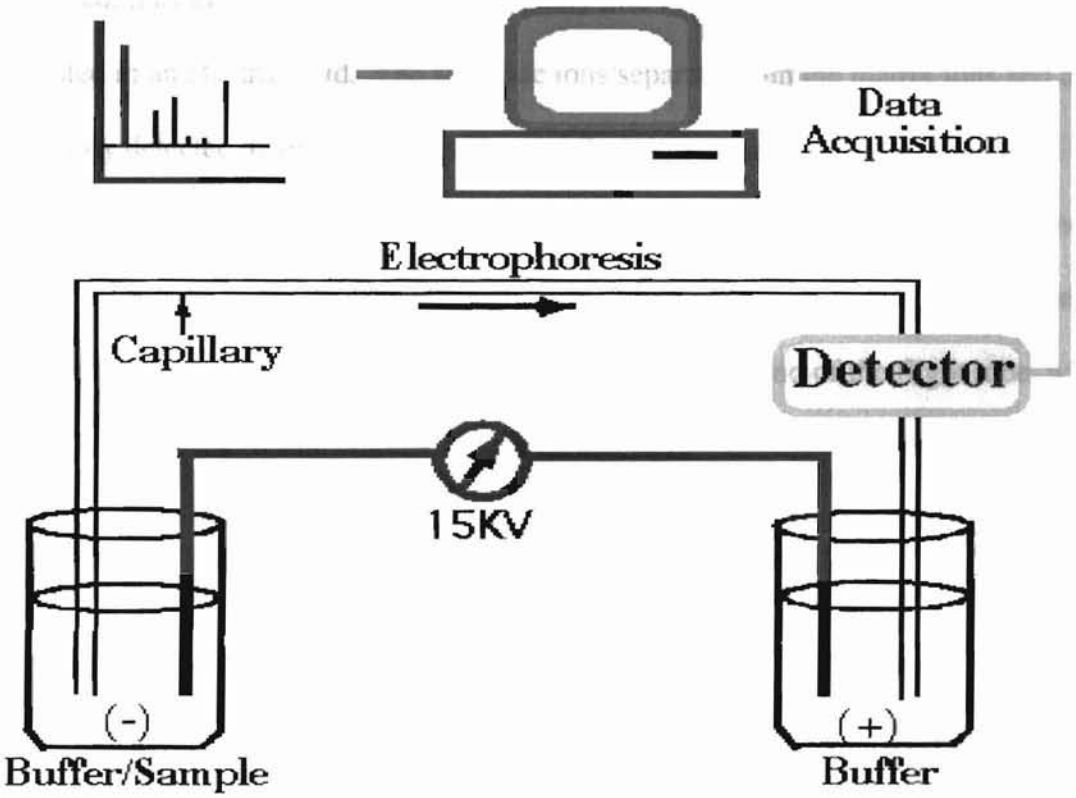


Fig 1-4. General Representation of capillary electrophoresis

nitrogen UV laser, 337 nm) serves as a desorption and ionization source. The matrix plays a key role in this technique, and although the mechanism remains uncertain, it is thought to absorb the laser light energy and transfer it to the substrate, which may cause a small part of the target substrate to vaporize. Once the sample molecules are vaporized and ionized, they are transferred electrostatically into a mass spectrometer where they are accelerated in an electric field. The substrate ions separate from the matrix ions and are individually detected, usually by TOF (time-of-flight) mass spectrometry. In linear mode, the ions travel down a linear flight path and their mass/charge ratio is determined by the times it takes for them to reach the detector. In a reflectron mode, the instrument has an ion mirror, which is essentially a potential field, at the end of the flight tube, which reflects the ions back to a detector. Those ions that have more kinetic energy penetrate farther into the field and hence are slowed down relative to fragment ions that do no penetrate as far into the mirror. Hence all the ions are separated. The reflectron mode permits higher mass accuracy and resolution, since it compensates for similarly charged ions having slightly different overall energies. Analysis by MALDI mass spectrometry may be divided into two steps. The first step involves preparing a sample by mixing the analyte with a molar excess of matrix. The typical matrix for use with UV lasers is an aromatic acid with a chromophore that strongly absorbs the laser wavelength. The second step of the MALDI process involves desorption of bulk portions of the solid sample by a short pulse of laser light.

The MALDI matrix must meet a number of requirements simultaneously:

a) be able to embed and isolate analytes (e.g., by co-crystallization)

b) be soluble in solvents compatible with analyte

c) be vacuum stable

d) absorb the laser wavelength

e) cause co-desorption of the analyte upon laser irradiation

f) promote analyte ionization

The MALDI method has been developed empirically and despite its widespread use, the factors that determine success or failure of MALDI experiments are not yet fully understood (information compiled from websites from Sigma-Aldrich/Fluka, Analytix and Kratos companies).

NMR (nuclear magnetic resonance) is a very useful technique to determine chemical structures, including the composition and sequence of units in polysaccharides. In a simplified way we can explain this technique by saying that a sample in a magnetic field can absorb electromagnetic radiation in the radio frequency (rf) region at frequencies that depend in the characteristics of the sample. A plot of the frequencies of the absorption peaks versus peak intensities constitutes a NMR spectrum (Silverstein et al., 1998). More in depth, we can say that the basis of this behavior relies on the resonance of the spins of subatomic particles. Atomic nuclei are composed of nucleons, i.e., protons and neutrons. Each of these particles shows a property named "spin" (behaving like an angular momentum) that adds up to the total spin of the nucleus (which might be zero, due to pair-wise cancellation). When in an NMR magnet, this spin interacts with an external magnetic field, comparable to a compass-needle in the Earth's magnetic field (for spin-1/2 nuclei). The most important nuclei in organic chemistry are the spin-1/2 isotopes ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P (with different isotopic abundance). As spin-1/2 nuclei they can assume two states in a magnetic field, α ($m_I = -1/2$) and β ($m_I = +1$

/2). How much energy can be absorbed by a large ensemble of spins (like our NMR sample) depends on the population difference between the α and β state. Furthermore, resonance frequencies of the same isotopes in different molecular surroundings differ by several ppm (parts per million). Two-dimensional (2D) NMR experiments have greatly increased the usefulness of NMR for structural identifications. Developed homonuclear and heteronuclear correlations, in both long and short ranges, comprise what is called 2D NMR. This introduction will focus only on the 2D NMR used in this project. COSY, COrrelation SpectroscopY correlates directly coupled ^{1}H to ^{1}H, while TOCSY (TOtally Correlated SpectroscopY) correlates relayed coherence transfer from ^{1}H to ^{1}H. HMQC (Heteronuclear Multiple Quantum Coherence) correlates directly attached ^{1}H-^{13}C, while HMBC (Heteronuclear Multiple Bond Coherence) correlates long range (2 and 3 bond) ^{1}H-^{13}C couplings, which allows us to correlate quaternary carbons with nearby protons (Silverstein et al., 1998; Sanders et al., 1997).

1.3 AIM AND OUTLINE OF THESIS PROJECT

Pectin is without a doubt very important for humankind for its function in the plant cell wall and its uses, mainly in the food industry. Despite its importance, its structure has not yet been totally deciphered. For that reason, one of the many aims of Dr. Mort's lab has been to attempt to clarify the chemical nature of pectin linkages and the sequence of the interconnections among its pieces. It has been postulated that pectin is a network of interconnected regions: HG, RG I, RG II and XGA, where RGI constitutes a large percentage of the total pectin. RGI has different side chains of unspecified functions, but it is believed to play an important role in cell wall. In addition,

pectin fragments from the HG region have been shown to regulate a number of responses in plants (Daas et al, 1998). Although the importance of these HG fragments is indubitable, their chemical structure still is unknown and needs to be further pursued. Past researchers from Dr. Mort's lab have encountered some arabinose rich RGI fractions, whose actual existence and function was not determined. The functions and interrogatives left open from previous studies, have determined the aims of this thesis project. The main aim of this thesis study was to attempt to isolate and characterize the chemical nature of an arabinose rich side chain linkage to the main chain of RGI. Another aim of this thesis study comprised the improvement of techniques used. It did not take long to get involved in the characterization of a methylated tetramer of galacturonic acid, which quickly comprised another aim in this project. The isolation attempts of the arabinose rich RGI fragment involved many sequential enzymatic digestions and chemical separations using various chromatographic methods. In addition, anion-exchange HPLC was used to isolate the methylated tetramer of GalA. Characterizations made great use of a variety of techniques such as CZE, HPLC, GLC, NMR and MALDI-TOF MS.

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CHAPTER 2

MATERIALS AND METHODS

2.1 BREAKDOWN OF POLYSACCHARIDES

2.1.1. ENZYMATIC DIGESTION

Enzymes used :

endo-polygalacturonase (EPG) (Megazyme, Ireland)

endo-1,4-β-D-galactanase (Megazyme, Ireland)

β-galactosidase (Megazyme, Ireland)

endo-arabinanase (Megazyme, Ireland)

α-L-arabinofuranosidase (Megazyme, Ireland)

Cloned RGase (Fu et all, 2001)

2.1.2. Obtaining HMW and LMW from commercial citrus pectin

Commercial citrus pectin (10 g), from Sigma Co., St. Louis, MO, was dissolved in 15 ml of methanol (or ethanol) and 300 ml of NP H_2O. The dissolved pectin was saponified by adjusting its pH to 11, with NaOH at room temperature, and then to pH 4 with glacial acetic acid. The saponified pectin was then EPG digested as per Zhan et all, 1998.
In order to fractionate the supernatant according to digestion size, the combined supernatants were ultra-filtered using a YM10 or YM30 (10 and 30 k MW cutoff respectively) membranes (Amicon, Inc., Beverly, MA) into two major fractions, a low molecular weight fraction (LMW) and a high molecular weight fraction (HMW). In order to rid the HMW of the dimer and trimer of GalA, approximately 0.5 g of dry HMW

was treated with 20 ml of 0.5 M imidazole solution (pH 7), stirred for 2 hrs at room temperature and ultra-filtered again through the YM30. The imidazole treated HMW was washed at least 5 times with NP H_2O. The imidazole-LMW was discarded. The usable HMW (imidazole treated) and LMW (previous to imidazole treatment) were lyophilized and saved for further use. From 10 g of initial dry pectin, approximately 30% remained insoluble (EPG residue), 50% went to the LMW and 13 % went to the HMW.

2.1.3. RGase digestion

Approximately 10 mg of the obtained HMW was incubated with the purified RGase in 50 mM sodium acetate pH 4.0 buffer, stirring, at 37 ^{0}C for 24 hrs. The amount of RGase added was adequate to digest 6 μmol of linear $(RG)_8$ in 1 hr. One hundred of the complex contains the equivalent of 12 μmol of $(RG)_8$. The digestion mixture was heated at 90 ^{0}C for 15 min to inactivate the enzyme before chromatographic separation.

2.1.4 Digestion by other various enzymes – general conditions used.

A known amount of substrate, weighed and placed in a 1.5 ml microfuge tube, was dissolved in a minimum amount, 0.5 ml, of 50 mM ammonium acetate, pH 4, buffer. The amount of enzyme used was roughly calculated based on the sugar composition of the fraction and the specific activity of each enzyme. For example, for the use of endogalactanase the calculation was based on the amount of gal in the sample (HMW on this case). With the μmole of gal in the sample and the specific activity of this enzyme, which is provided by the manufacturer, the amount of enzyme needed was calculated. The mixture was incubated in a water bath at 40 °C for 24 hr. The enzymes were

inactivated by placing them in an 80 °C water bath for 30 min. The digest was centrifuged at 10,000 rpm for 15 min to separate the residue. The supernatant was separated through a cutoff membrane of a specified size (see figure 3-4 for details).

2.2 FRACTIONATION OF DIGESTED POLYSACCHARIDES

High Performance Liquid Chromatography

Neutral and acidic polysaccharides were separated on anion exchange columns: PA1 (Carbo Pac) or DEAE (Poros 50 DEAE, Perseptive Biosystems). The column was eluted with an ammonium acetate gradient, which differed for each separation, with a flow rate of 2 ml/min using a Dionex Bio-LC carbohydrate system (Dionex Corporation, Sunnyvale, CA). A continuous post-column detection of underivatized polysaccharides by reaction with permanganate was used, as devised by Thomas and Mort, 1994. The obtained fractions were collected using a fraction collector. Chromatograms were recorded on a chart recorder and also in a Macintosh microcomputer via a Data Logger connected to the detector (Merz and Mort, 1992). Collected fractions were frozen and lyophilized a minimum of two times to rid them of the salt in the elution buffer.

2.3 DERIVATIZATION

2.3.1. ANTS Derivatization

Approximately 100 µg of substrate, weighted on a Cahn 29 electrobalance and placed in a 500 µl Eppendorf tube, was heated, in a heating block, at 90 ^{0}C for at least 1 hr in a mixture of 20 µl of 23 mM ANTS (Molecular Probes, Oregon, USA) (in 3:17 v/v

of acetic acid:water) and 2 µl of 1 M SCB in THF (method adapted from Evangelista et al., 1995).

2.3.2. APTS Derivatization

Approximately 100 nmol of substrate, weighted on a Cahn 29 electrobalance and placed in a 500 µl Eppendorf tube, was heated, in a heating block, at 80 ^{0}C for at least 1 hr in a mixture of 2 µl of 0.1 M APTS (Molecular Probes, Oregon, USA) (in 25% acetic acid) and 10 µl of 1 M SCB in THF (method adapted from Evangelista et al., 1995).

2.3.3. 2AP Reversible Derivatization

Oligosaccharides were derivatized by condensation reaction with 2-aminopyridine by dissolving approximately 1 mg of the sample, weighted on a Cahn 29 electrobalance and placed in a 1.5 ml Eppendorf tube, in a minimum of 50 µl of labeling reagent and incubating it at 70 ^{o}C for a minimum of 3 hours. The labeling reagent was prepared by dissolving 1g of 2-aminopyridine in 1 ml of NP H_2O and adjusting the pH to approximately 7 with 1 ml of glacial acetic acid. After removing samples from the heating block and cooling them to room temperature, excess labeling reagent was removed by solid phase extraction. The labeled sample was diluted 20 fold with 0.44 M acetic acid (final pH 4-5) and then applied to a pre-conditioned solid phase extraction column of 500 mg cation-exchange Extract-Clean column from Alltech (Deerfield, IL, USA). The sample was eluted with H_2O. The columns were pre-conditioned by converting them to the ammonium form using approximately 5 ml of concentrated ammonia solution and rinsed with H_2O to a pH 5-6 before sample application. After

usage, the columns were converted back to the ammonium form for later re-use (Maness et al., 1991).

2.4 SUGAR COMPOSITION ANALYSES

2.4.1. Gas Liquid Chromatography

Sugar compositions were determined by GLC analysis of the trimethylsilyl methyl glycosides. Methanolysis and derivatization were performed using the protocol of Chaplin (1982) as modified by Komalavilas and Mort (1989). About 100 μg of sample was weighed on a Cahn 29 electro balance and the exact amount was recorded. Sample was placed in a 4 ml glass vial with a Teflon-lined screw lid. One hundred nmoles of inositol were added as an internal standard and dried in a speed-vacuum centrifuge. Two hundred μl of 1.5 M methanolic HCl and 50 μl of methyl acetate were added to each vial. The vial was sealed tightly and placed in a heating block at 80 °C for at least 3 hours. After cooling to room temperature, a few drops of t-butanol were added to each vial and the sample was dried under a stream of N_2. Fifty μl of a 3:1 Trimethylsily: Pyridine mixture were added to the sample and allowed to react for 15 minutes at room temperature. The derivatized sample was then evaporated gently under a stream of N_2 and re-dissolved in 100 μl of isooctane. The trimethylsilyl sugar derivatives were separated on a DB-225 fused silica capillary column (30 m x 0.25 mm i.d., Durabond-1 liquid phase; J & W Scientific Inc., Rancho Cordova, CA) installed in a Varian (Sunnyvale, CA) 3300 gas chromatography equipped with an on column injector and FID detector.

One µl of isooctane and 1 µl of sample was injected at 105 °C. The program was held at 105 °C for 1 minute. Then the temperature was raised to 160 °C at a rate of 10 °C/min and held for 4 min, then raised to 220 °C at a rate of 2 °C/min, finally raised to 240 °C at a rate of 10 °C/min and held for 10 min. Peaks were integrated on a Varian 4290 integrator. Individual monosaccharides calculation was achieved by using a 'in-house' program based on the areas obtained relative to the internal standard area.

2.4.2. Monosaccharide composition analysis for small amounts of sample

Ten to 100 ng of polysaccharide sample was injected into one of the 5 wells of the specially designed vial, along with 1 nmol of 3-O-methyl glucose as an internal standard. This step was done for each sample. The sample and the internal standard were evaporated away in a speed vacuum centrifuge in about 2 min. TFA was used to hydrolize the polysaccharides to monosaccharides, so 1.5 µl of 2 M TFA was added to each well. The vial was then heated at 121 °C for 1 hr by placing it in a specially designed aluminum holder. This holder had a Teflon lined cap, which was held to the base by 6 screws. After cooling, the acid was evaporated, in about 5 min, in a speed vacuum centrifuge. The monosaccharides were then derivatized with a fluorescent label to be later separated by CZE and quantitated. One µl of the derivatization agent, 3.0 mg/ml anthranilic acid in 4% sodium acetate 2% borate solution in methanol, was then added to each well and the vial was heated at 80 °C for 2hr by placing it into the holder as previously described. After cooling, the methanol was evaporated, and the samples, taken up in 20 µl of NP H_2O, were transferred into a microfuge tube. The analytical separation was done by using a BioRad Biofocus 2000 CZE instrument. The detection

was achieved by LIF. The instrument used permitted the subsequent quantitation of the individual monosaccharides by using an 'in-house' program based on the areas obtained relative to the internal standard area. Between each sample run, there was a 0.1 M NaOH wash followed by a H_2O wash. A 385 nm cutoff filter was used to block scattered UV laser light. Each run lasted 30 min at 15 kV, with a constant 21 °C temperature. The samples were run using a pH 7.0 200 mM borate and 50 mM phosphate buffer, which complexes the monosaccharides at any 2 vicinal OH at a time, adding possible negative charges, and therefore, helping with the separation by allowing the drifting time to be based mainly on the type of the sugar molecule. In addition, the borate buffer used helped the electrosmotic flow of the capillary. Some important steps were taken into account for a successful analysis of the samples, which were:

1) The flat head syringe used was rinsed many times before and in between samples to prevent cross-contamination.
2) Each little well was rinsed first with 0.1 M NaOH, then H_2O and later with distilled H_2O using a flat head syringe. The H_2O rinsing was repeated at least 7 times to completely rid the well of any NaOH.
3) A tight seal of all the sample wells was assured by replacing the liner of the cap holder with a new Teflon liner every time the holder was used. The new liner created a tight seal of all the sample wells when screwed onto the base.

This method kept the volume low, by adding the minimum amount necessary to the samples and by using a volatile acid for the hydrolysis. In addition, the result was maximized by the use of LIF detection, which is known for its high sensitivity, and CZE, which allowed very small samples to be injected. (Yuan and Mort, unpublished)

2.5 DETERMINATION OF SIZE

2.5.1. Initial estimation by Capillary Zone Electrophoresis

Samples were run on a custom-built capillary electrophoresis instrument with laser-induced fluorescence detector, which used a helium-cadmium laser for excitation and an intensified charge-coupled device camera for detection (Merz and Mort, 1998). A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (355 μm OD) was used as the separation column for oligosaccharides. The capillary was 50 cm in length, with 26 cm to the detection window. 0.1 M NaH_2PO_4, pH 2.5, was used as a running buffer. The capillary was rinsed with running buffer after each run and samples were introduced by gravity-driven flow for several seconds. Electrophoresis was conducted at 18 kV with the negative electrode on the injection side.

2.5.2. Final determination of mass by MALDI-TOF MS

The matrices used originated from different sources. The MSA (CAS 2612-02-4), THAP (CAS 480-66-0) and Nafion (CAS 31175-20-9) used were purchased from Aldrich Chem. Co., WI. DHB (CAS 303-38-8) was purchased from Acros Organics, NJ. The ammonium citrate (CAS 3012-65-5) used was from Fisher, NJ. MALDI-TOF MS instrument configurations have been focused on the study of proteins, which made its use on polysaccharides a learning journey of trial and error. To circumvent the lack of detailed configurations for polysaccharides, best instrument configurations (BICs) were developed based on scarce published and unpublished details, and were saved in the OSU's instrument database for further usage. In addition, many matrices and co-matrices, in various combinations, were tried for the different types of polysaccharides

(acidic, neutral and labeled) used in this research project. Comparative examples of a THAP and nafion/THAP used for the analysis of acidic polysaccharide $(GR)_4$ are shown in figure 2-1. THAP, when used with nafion showed an improved signal/noise ratio and limited salt adducts. Furthermore, THAP also improved the signal/noise ratio for neutral oligosaccharides, as compared to DHB, which is shown in figure 2-2.

The practical use of the MALDI-TOF VOYAGER DE-PRO WITH DELAYED EXTRACTION TECHNOLOGY carried many considerations. Some very important ones are:

1) The instrument's sensitivity to contaminants, including plasticizers from the plastic-ware used, was very high. Undesired signals from plasticizers were avoided by the use of methanol/ethanol washed, and autoclaved, plastic-ware used during sample preparation. In addition, taking into account the instrument's sensitivity, extreme care was taken while spotting the plate to avoid extraneous contamination.

2) Salt adduct formation happened readily. Salt adduct formation was decreased by desalting samples and matrices as well. Desalting was achieved by adding 10 µl of Dowex -50W 50X8-200 beads in the ammonium form to the samples and matrices solutions used. The mixture of beads and solution (sample or matrix) was allowed to react for at least 15 min at room temperature. It was found that matrices that were allowed to sit with the beads for more than 12 hr showed less salt adducts. The beads were stored at 4 °C in H_2O, so care was taken to only add beads from the bottom of the flask to prevent further dilution of the sample (by just adding the supernatant H_2O). Some matrices found to work best with polysaccharides, and their compositions, are presented in the table 2-1. Other co-matrices used and their compositions follow:

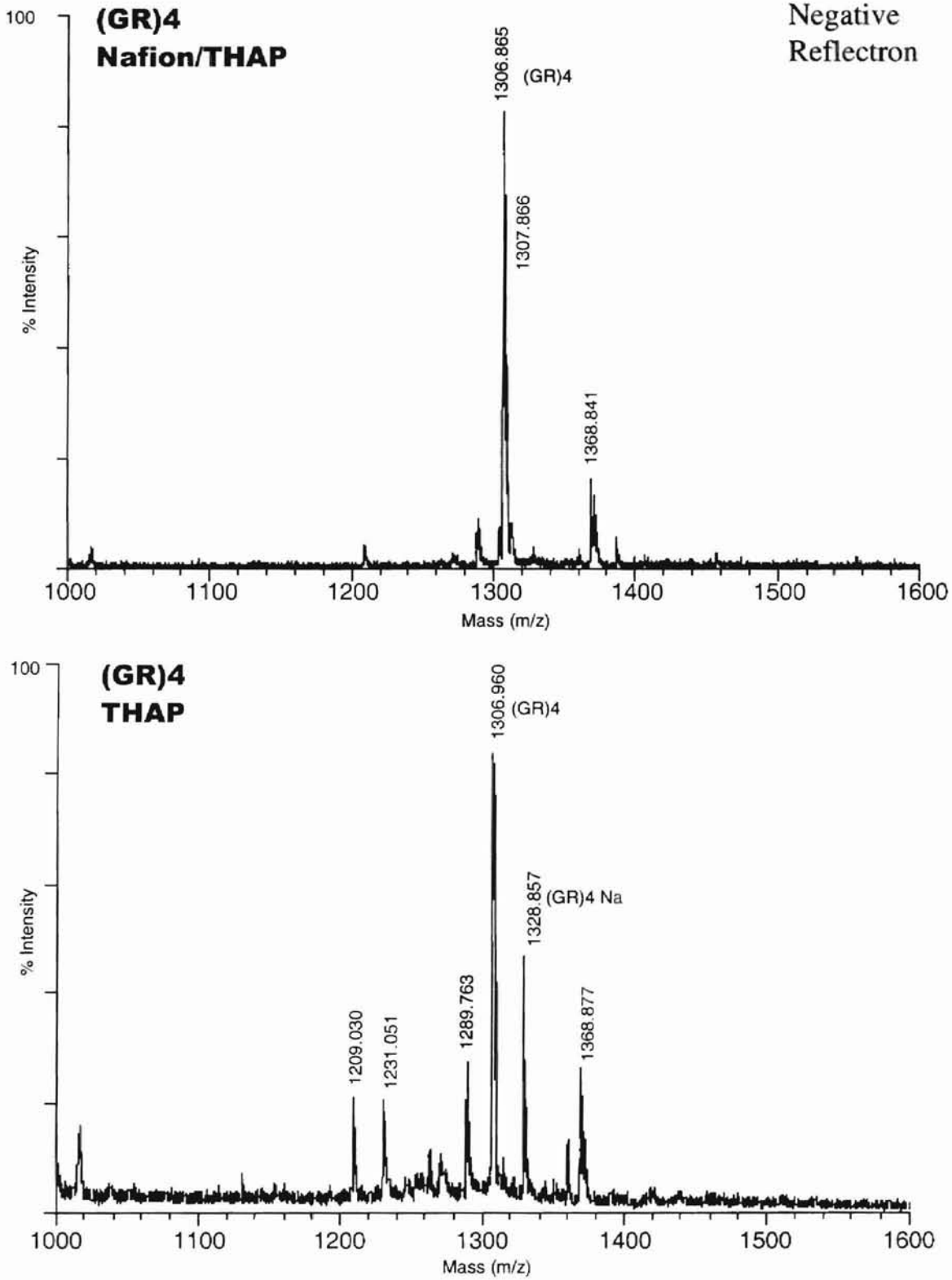

Fig 2-1. Comparison of different matrices used in MALDI-TOF MS for (GR)4

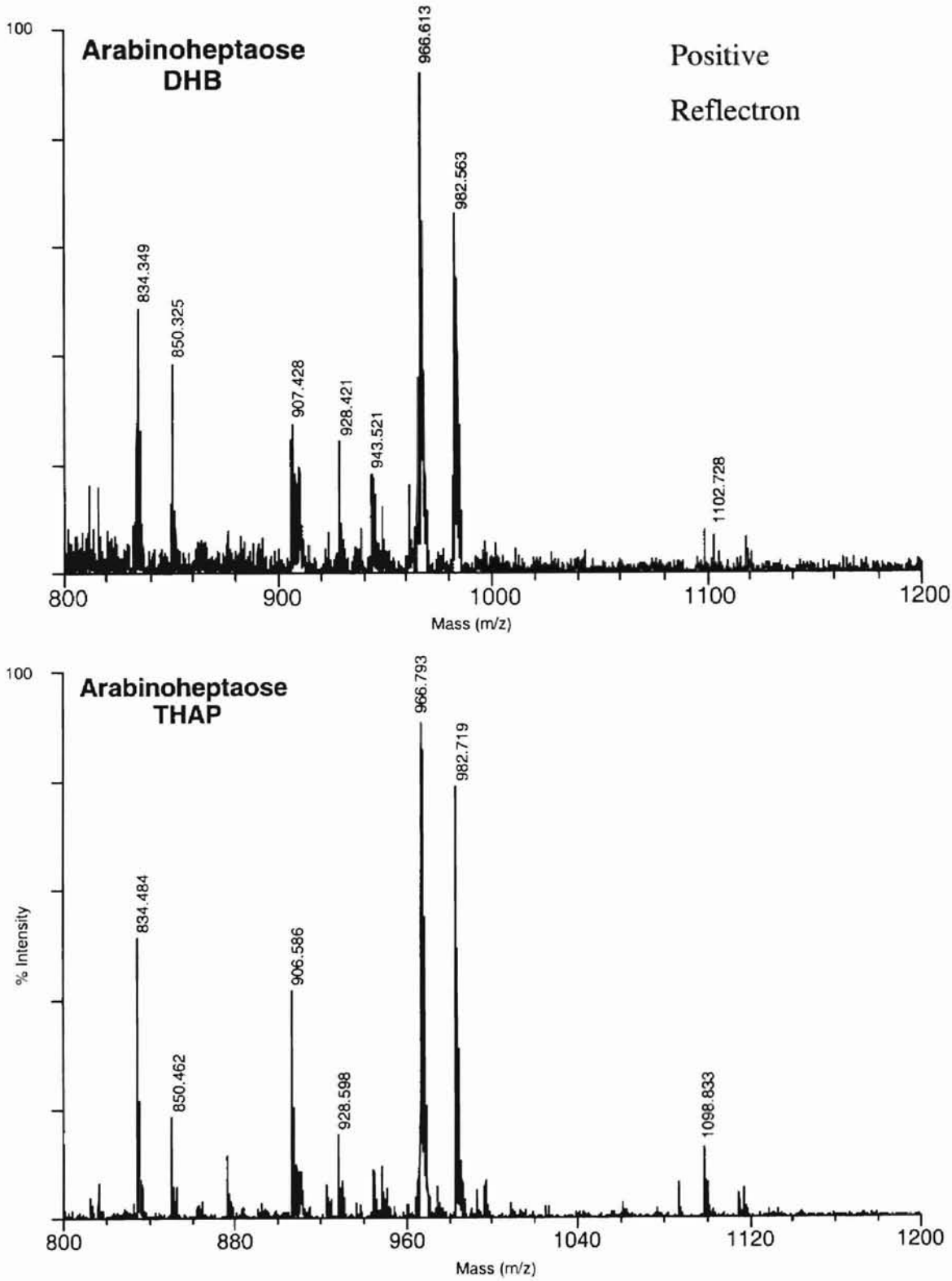

Fig 2-2. Comparison of different matrices used in MALDI-TOF MS for Arabinoheptaose

Matrix	abbreviation used	composition
DHB	D1	10 mg/ml in H2O
	D2	10 mg/ml in 0.1% TFA/acetonitrile (70:30)
	D3	20 mg/ml in ethanol/H2O (50:50)
THAP	T0	100 mg/ml in methanol
	T1	20 mg/ml in methanol
	T2	10 mg/ml in H2O/acetonitrile (50:50)
	T3	10 mg/ml in methanol/H2O (50:50)
	T0 Nc (pre-mixed)	4:1
MSA	M1	1 mg/ml in ethanol/H2O (50:50)
	M2	20 mM in ethanol/H2O (50:50)
	D M (pre-mixed)	9:1

Table 2-1 Matrices used and their composition.

Polysaccharide type	Matrix	Mode
Acidic (i.e. GR oligomers)	ToNcAc	reflectron - negative
Neutral (galactans, arabinans, etc)	D2	reflectron - positive
APTS and ANTS labeled oligosaccharides	NTo	reflectron - negative
2AP labeled oligosaccharides	ToNcAc	reflectron - positive

Table 2-2 Matrices and modes for different polysaccharides types.

- Ammonium Citrate [$(NH_4)_2 HC_6H_5O_7$]

Ac = 20mM sol in H_2O

- Nitrocellulose (30 mg/ml in acetone)

Nc = 15 mg/ml in acetone/2 propanol (50:50)

- Nafion perfluorinated ion exchange resin, 5 wt.% solution in mixture of lower aliphatic alcohols and water.

N = nafion diluted in ethanol (50:50)

3) Different matrices and modes were used with different carbohydrate types. The best matrices and modes found are presented in the table 2-2.

4) Various sample-spotting techniques were tried, and it was found that one of the simplest ones gave the best and most consistent results. This simple technique consisted in spotting 0.5 μl of matrix first and secondly 0.5 μl of the sample. Mixing was on the plate with the pipette tip. When spotting various layers, the same technique was applied. For example 0.5 µl of co-matrix was applied and let dry, secondly 0.5 µl of the matrix was applied and mixed on plate with 0.5 µl of the sample as previously described.

5) Dryness of the sample was very important, and two methods were tried, air and vacuum drying. Air-drying worked for almost all matrices used. An exception was the D/M mixes, which required vacuum drying both to speed the drying and to form better crystals.

7) The cleanliness of the plate proved to be very important, so care was applied when washing the plate. The plate was washed with distilled water, acetone, and ethanol as many times as necessary for the complete removal of any residue left on it. To avoid scratching the plate when scrubbing was needed, a chemwipe was used very lightly.

Detailed instructions, prepared by this author, on how to use the MALDI-TOF VOYAGER DE-PRO WITH DELAYED EXTRACTION TECHNOLOGY (OSU, Department of Biochemistry and Molecular Biology, 3rd floor) for carbohydrate analysis are given in the Appendix A.

(Korner et al., 1998; Jacobs et al., 2001; Talbo et al., 1996; Harvey 1993; Harvey 1999; Gusev et al., 1995; Papac et al., 1996)

2.6 STRUCTURE DETERMINATION

^{1}H, ^{13}C, both 1D and 2D (COSY, TOCSY, HMQC, HMBC) NMR spectra of the samples were recorded on a Varian Unity Plus 600 MHz NMR spectrometer by Dr. Feng Qiu at the OSU's NMR shared facility using the standard pulse sequence with water pre-saturation. Samples were dissolved in D_2O. The pH of the solution was 6. The spectra were acquired at 25°C, except for the TOCSY spectrum, which was acquired at 12°C.

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CHAPTER 3

PARTIAL ISOLATION AND CHARACTERIZATION ATTEMPTS OF AN ARABINOSE RICH FRAGMENT FROM COMMERCIAL CITRUS PECTIN

3.1 DIGESTIONS

From the commercial citrus pectin, HMW and LMW fractions were obtained. In the beginning of this study, the HMW and LMW fractions were obtained as per Zhan et al. 1998, using an YM10 cutoff membrane and without an imidazole treatment. The HMW fraction was the one used for further analysis, but its high content of GalA dimer and trimer masked the expected results during the separations stage. In order to circumvent that problem, and based upon previous experiences described in Mort et al. 1991, the use of imidazole was decided. The usage of the imidazole treatment, whose mode of action is not well understood, helped the dimer and trimer of GalA to ultrafiltrate to the LMW fraction and rid the HMW analysis of their interference. Since the desired fraction was theorized to be bigger than 10k MW, and since the interference of the lower oligomers of GalA was solved, the use of a bigger cutoff membrane was decided upon, and a 30k MW cutoff membrane was then used (YM30). The obtained LMW was not used and therefore discarded. The HMW fraction obtained was lyophilized prior to further characterization. A schematic representation of a generalized digestion scheme is given in figure 3-1. The different HMW fractions obtained by the different schemes described differed greatly in their GalA content, in their dimer and trimer forms, which was proven by the lack of interference in their later chromatographic separations, which is represented in table 3-1. The obtained results show a marked improvement in ridding

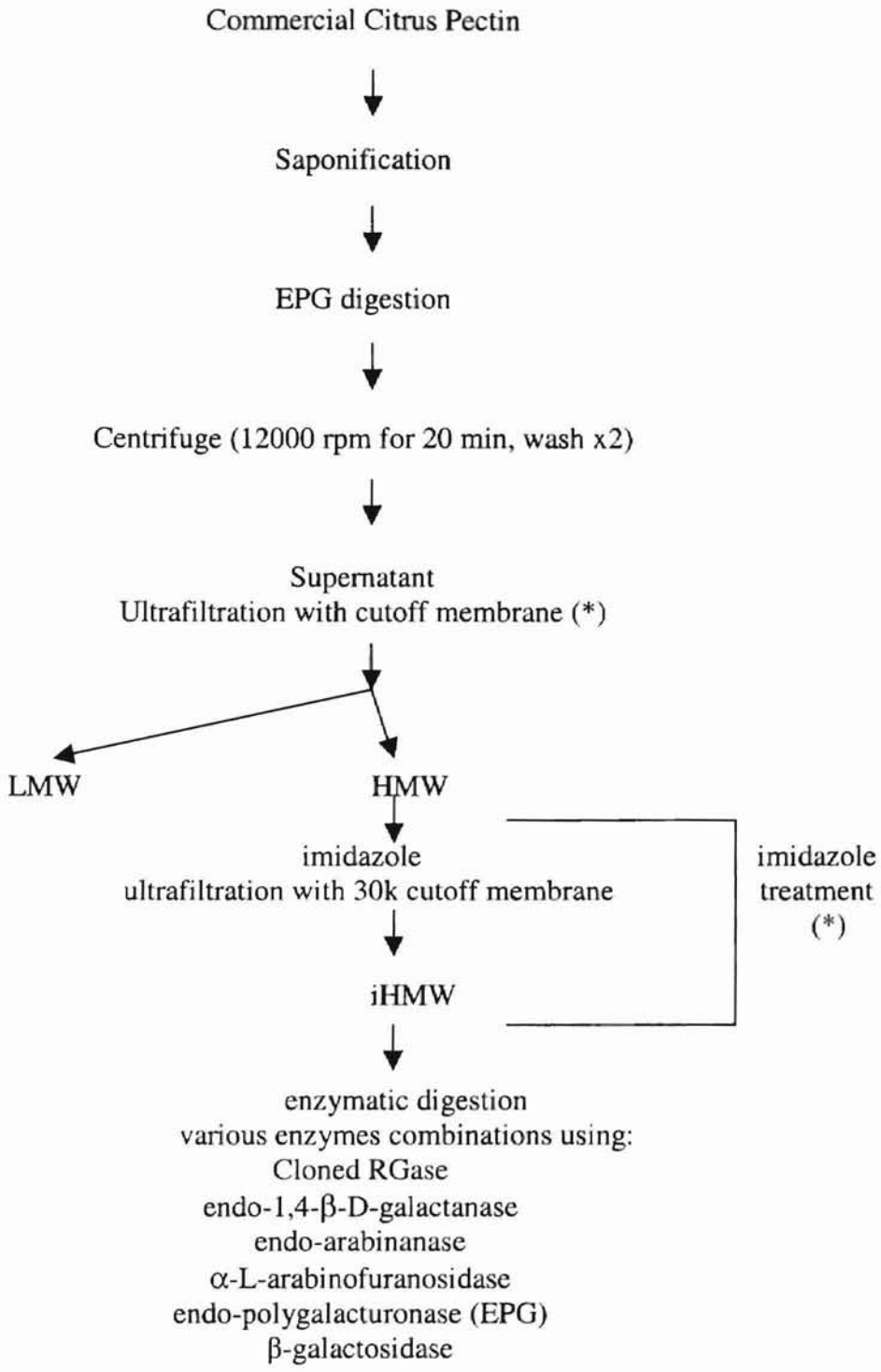

Fig 3-1 Schematic representation of a generalized digestion scheme
Note: (*) represents optional treatment and the changes that it incurs in cutoff membrane selection.

	HMW - original	HMW - 10-I-30	HMW - 30-I-30
Relative mol of			
GalA	3.31	2.45	1.33
Ara	0.28	0.73	0.54
Gal	1.74	1.64	1.39
Glc	0.16	0.12	0.05
Xyl	0.20	0.10	Tr.

Table 3-1 Comparison of the monosaccharide composition of the different HMW fractions.

Notes: The moles are relative the rha mole content in the sample.
Tr.-denotes trace amount.

	scheme 1	scheme 2	scheme3
saponification	methanol	methanol	ethanol
cutoff membrane	YM10	YM30	YM30
imidazole treatment	N	Y	Y
enzyme(s) 1	RGase	galactanase arabinanase ara-sidase	galactanase ara-sidase
enzyme(s) 2	galactanase arabinanase ara-sidase	RGase	RGase
enzyme(s) 3	-	-	arabinanase
separation	PA1	PA1	PA1

Table 3-2 Details of digestion schemes used

the HMW fraction from the dimer and trimer of GalA. A representation of the HMW fraction obtained is shown in figure 3-2. Three different digestion schemes were approached in trying to obtain the desired RGI arabinose-rich fraction. The digestion schemes details are shown in table 3-2. The described digestions schemes varied in enzyme types and combinations. A representation of an expected result from HMW digestion with RGase is shown in figure 3-3. Figure 3-4 shows the scheme followed to obtain HA, HB, HC and HD and their respective LA, LB, LC and LD. Figure 3-5 depicts the comparative sugar composition of HA, HB, HC and HD fractions.

3.2 SEPARATIONS

After each digestion, the digest was separated into fractions according to their mass/charge ratio by HPLC through a PA1 anion-exchange column. Many separated fractions were obtained, but we focused on some with specific retention times, based on the theorized size of the expected arabinose-rich fraction, taking into account the decrease in its charge due to the neutral side chains. Furthermore, we assumed that the existence of a minimal number of neutral side chains on the expected fraction would decrease the charge/mass ratio, therefore the interesting fraction would elute earlier than its debranched $(RG)_n$ counterpart. Each obtained fraction was labeled with ANTS as described in chapter 5 and subjected to CZE. The obtained electropherograms were compared with standards' electropherograms and tentative size determinations and identifications were deduced.

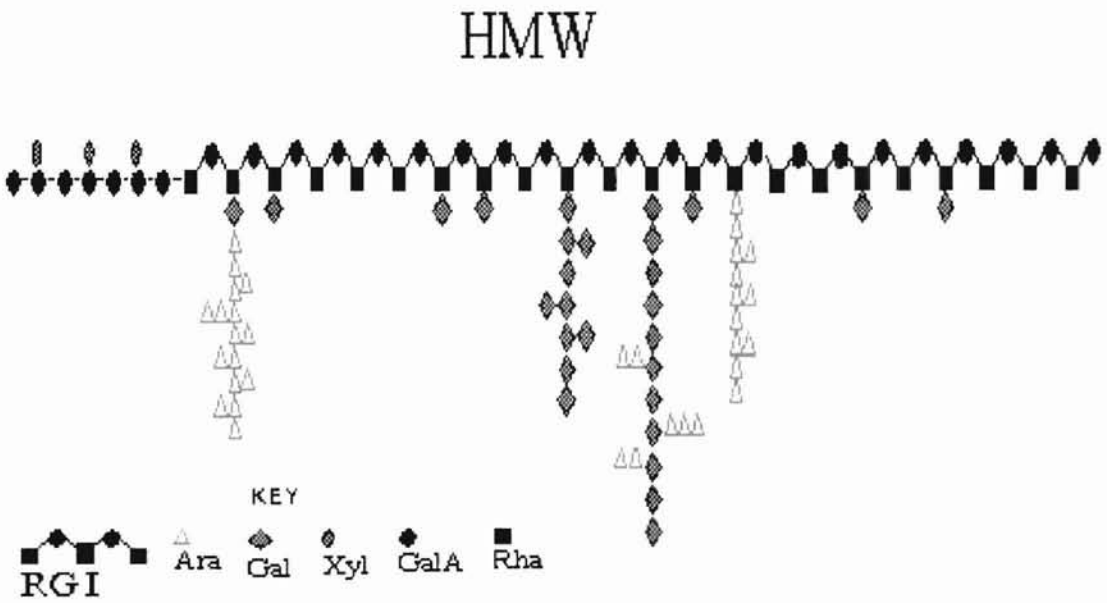

Fig 3-2 Representation of the HMW fraction obtained

Fig 3-3 Expected HMW fragments after RGase digestion

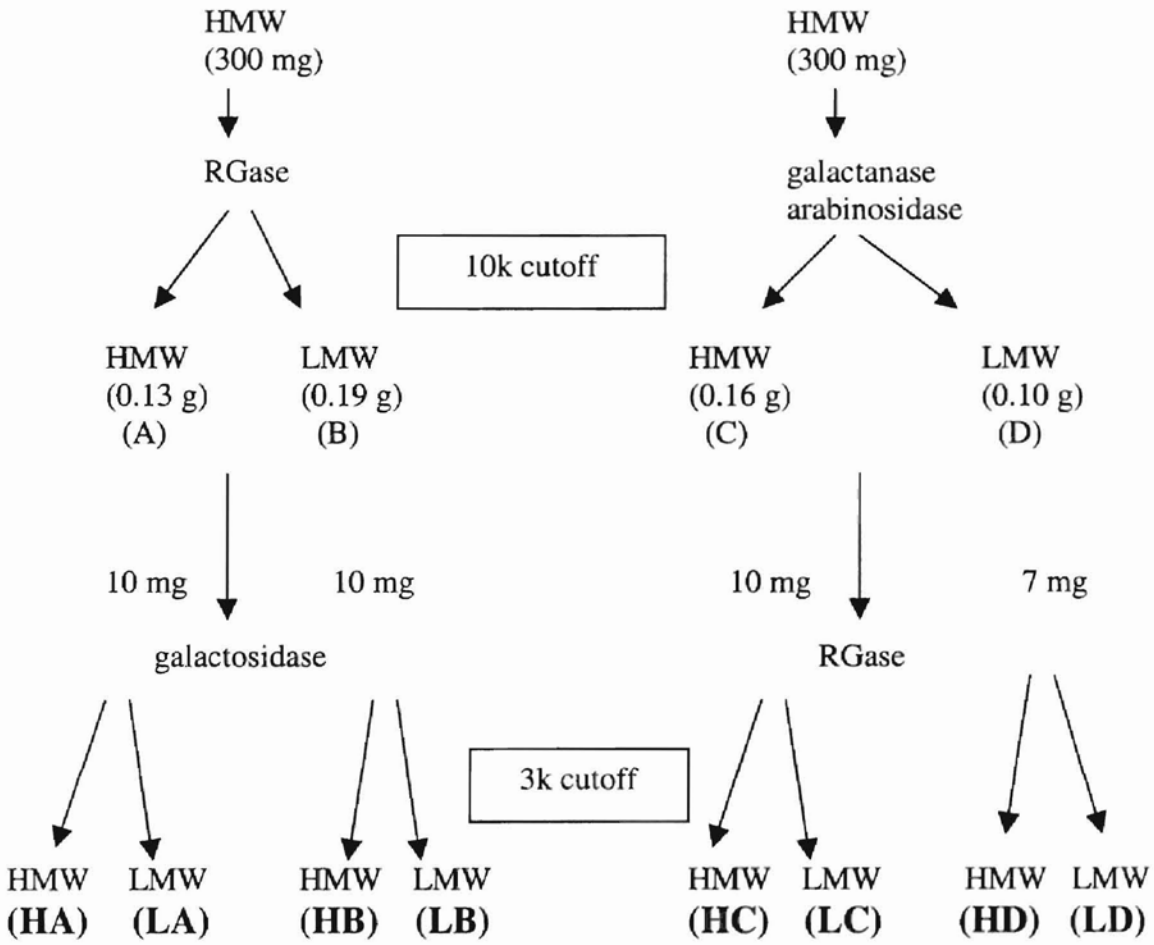

Fig. 3-4. Schemes to obtain HA, HB, HC and HD

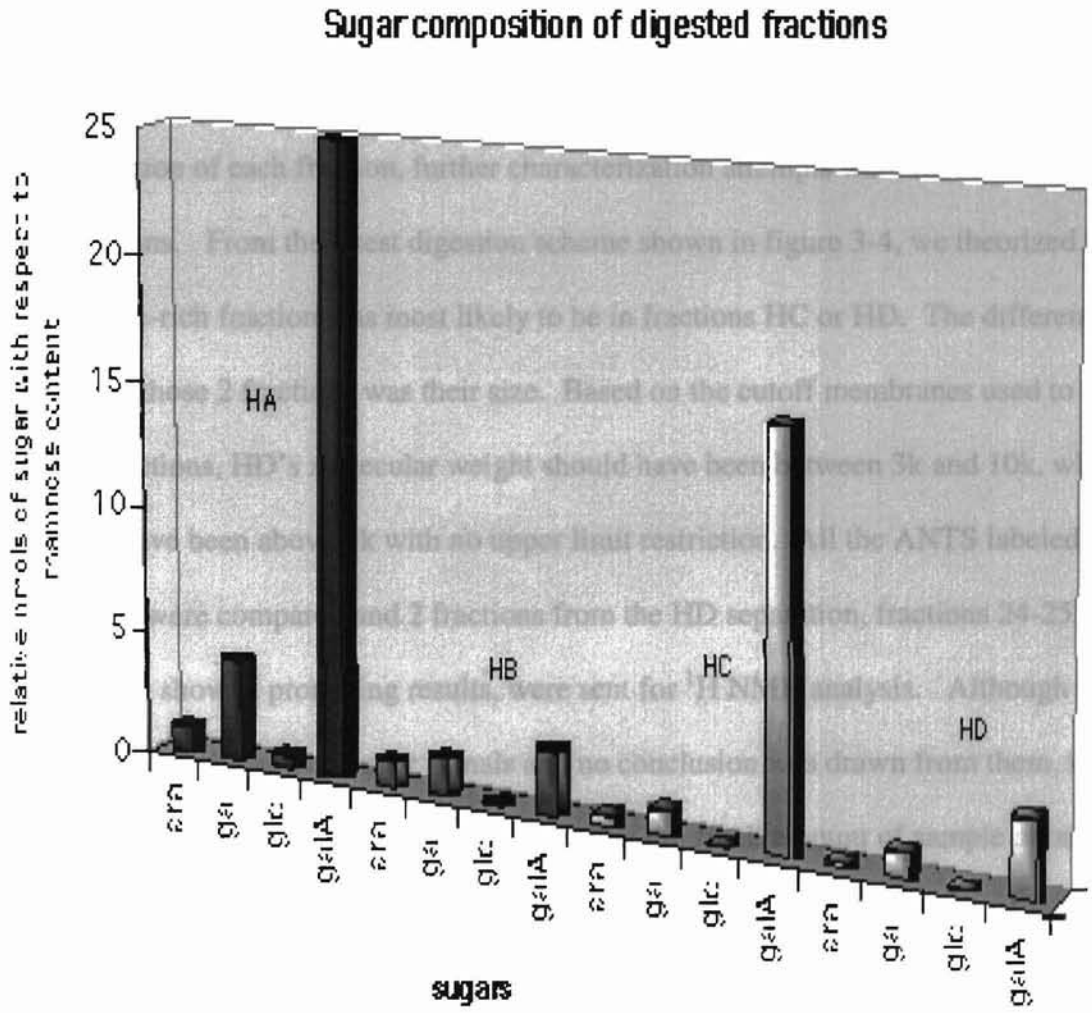

Fig 3-5. Sugar composition of fractions HA, HB, HC and HD.

3.3 TENTATIVE CHARACTERIZATIONS

All the fractions obtained were labeled with ANTS and electrophoresed. In addition, the monosaccharide composition of each fraction was determined by either GC, if there was enough sample, or the method for small amounts, if the amount of sample was not enough to spare between 50-100 μg, as described in chapter 2. Based on the comparison of the electropherograms with standards, and the monosaccharide composition of each fraction, further characterization attempts were focused on a handful of fractions. From the latest digestion scheme shown in figure 3-4, we theorized that the arabinose-rich fraction was most likely to be in fractions HC or HD. The difference between those 2 fractions was their size. Based on the cutoff membranes used to obtain those fractions, HD's molecular weight should have been between 3k and 10k, while HC should have been above 3k with no upper limit restriction. All the ANTS labeled PA1 fractions were compared and 2 fractions from the HD separation, fractions 24-25 and 29-31, which showed promising results, were sent for ^{1}H NMR analysis. Although the NMR results showed no sugar signals and no conclusion was drawn from them, we are confident that the lack of sugar signals was due the small amount of sample obtained after the HPLC separation. Due to the high amount of buffer needed to elute the sugars held in the PA1 column, the many lyophilizations to rid them of the salt, and reasonable loss, sometimes the small amounts of sample become just too small for further characterizations. The separations that led to the promising fractions should be repeated with the aim of obtaining more sample. With more sample in hand, we are confident that future characterizations of those fractions will shed more information on the linkage between the arabinose rich side chains to the rhamnogalacturonan backbone of RGI.

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CHAPTER 4

CHARACTERIZATION OF METHYL ESTERIFIED TETRAGALACTURONIDE FROM COMMERCIAL VP PECTIN

INTRODUCTION

VP is an old nomenclature kept here when referring to the commercial apple pectin classic AY 802 (Lot 201137, 38-40 % degree of methylesterification). This particular apple pectin used was a gift from Dr. Benjamin Jones of Campbell Soup Company (Camden, NJ), who purchased it from Atomergic Chemicals Corporation, Plainview, New York.

3.1 SEPARATION

Commercial VP pectin was dissolved in NP H_2O, and its pH adjusted to 4 with glacial acetic acid. EPG digestion was done overnight at room temperature, and afterwards the digest was centrifuged to remove the insoluble material. The supernatant of the EPG digested VP pectin was lyophilized before further use. The EPG digested VP pectin, 40 mg in 2 ml of NP H_2O, was then separated on a HPLC using a PA1 anion-exchange column. The ammonium acetate elution gradient used was mostly linear up to the 2 M wash. It started at 0.3% 1 M at 5 min and it was linearly increased to 1% 1 M at 80 min. The column was washed with 2 M ammonium acetate for 10 min and the run ended by returning the column to the original conditions. The fractions were collected and desalted by repetitive lyophilization for further characterization. The obtained HPLC chromatogram is shown in figure 4-1. The separation was based on mass/charge ratio. The retention time of the peak of interest, between the dimer and trimer of GalA,

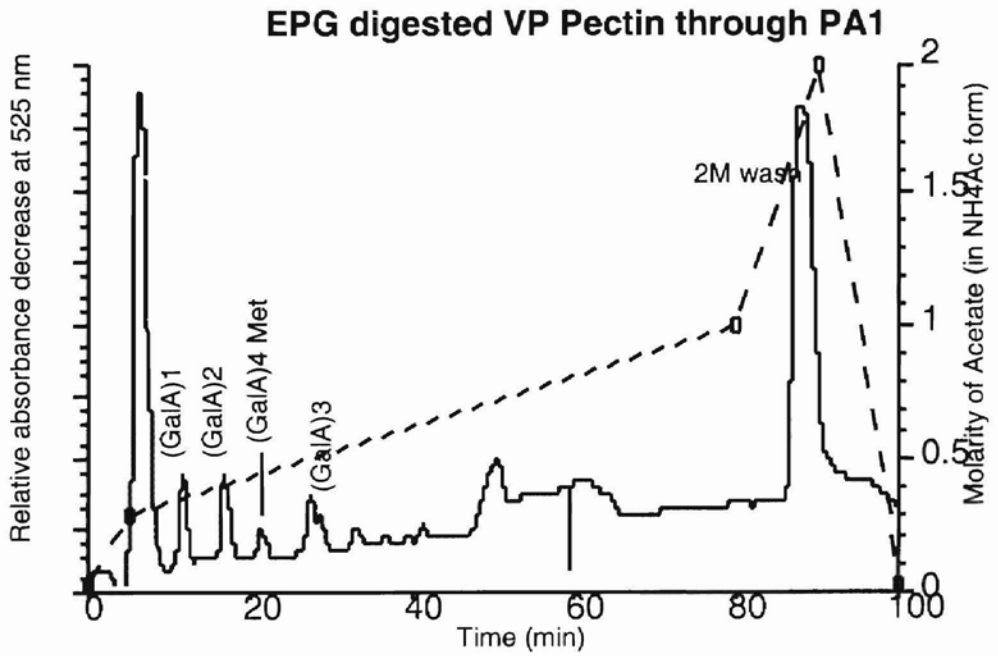

Fig 4-1 HPLC chromatogram of EPG digested VP pectin using a PA1 anion-exchange column

indicated the possibility of it being partially methyl-esterified, since we can assume that it was separated on the basis of its overall charge in respect to its mass. According to Daas et al (1998), the dimer of GalA has an overall charge of –2, while both the trimer and the methylated tetramer have an overall charge of –3. The difference between the later two lies on their mass, and thus the difference in their observed retention times.

3.2 CHARACTERIZATION

The first few desalted and dried fractions were labeled with ANTS and electrophoresed, while the rest, after being desalted and dried, were reserved for possible future work. The obtained electropherograms were compared to standards' electropherograms. The first peak was determined to be composed of neutral sugars and presented no further interest. The second, third and fifth peaks were determined, by comparison, to be the monomer, dimer and trimer of GalA respectively, and thus labeled $(GalA)_1$, $(GalA)_2$, and $(GalA)_3$ on figure 4-1. The fourth peak had an interesting retention time between the dimer and trimer of GalA, indicating that its mass/charge ratio was comparable to both of the above but it was none of them. In addition, in agreement with the previous statement, when subjected to CZE analysis, P4 eluted after $(GalA)_3$. This interesting peak, assumed to be a methylated counterpart of either the trimer or tetramer of GalA, was referred to as P4. The mass of the sodiated adduct of P4 was determined by MALDI-TOF MS to be 759.075, as shown in figure 4-2. This mass (minus the sodium adduct) fits the mass of a methylated GalA tetramer, which is in agreement with the above HPLC elution pattern, the CZE data and the data from literature reviewed (see

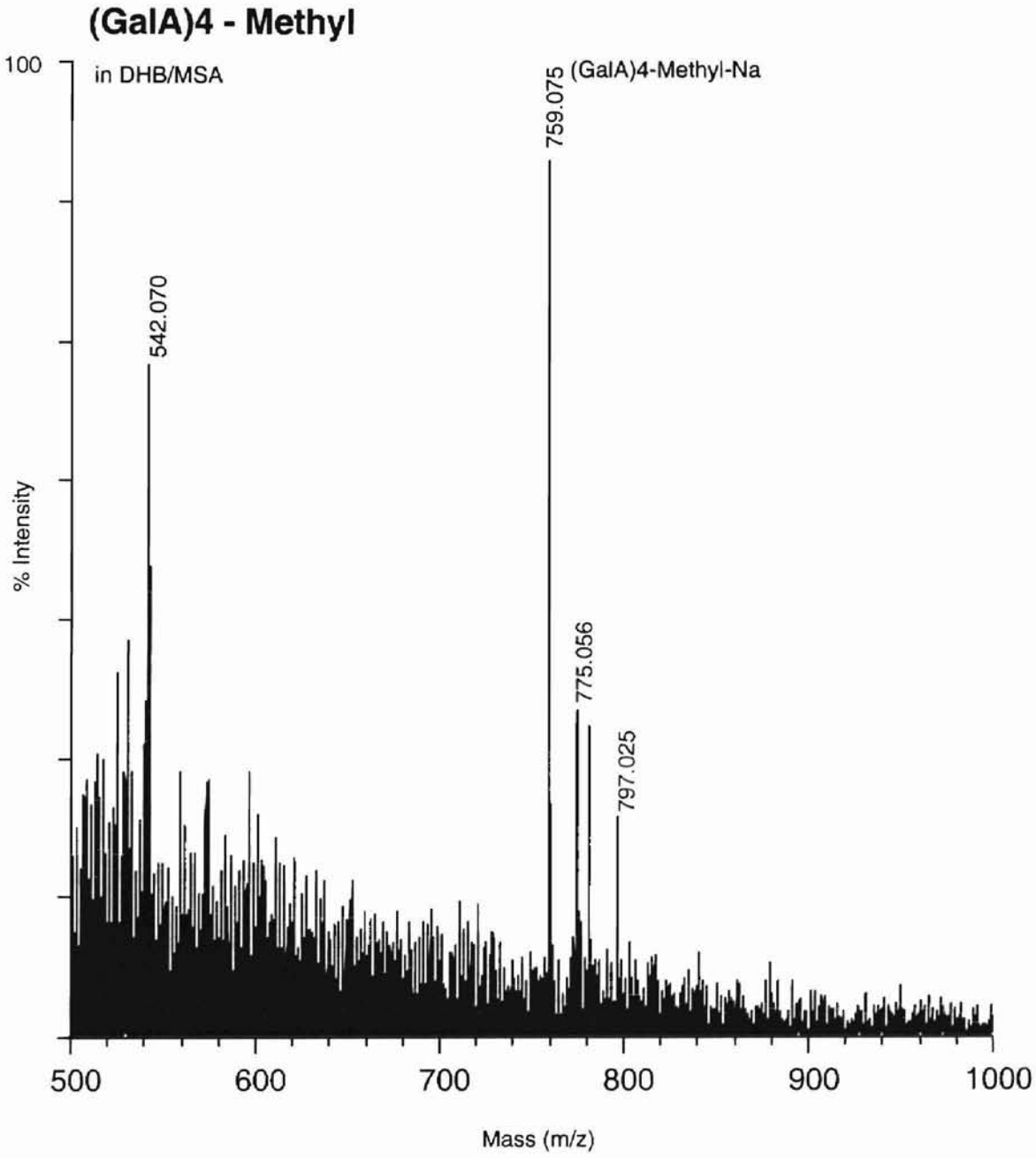

Fig 4-2. MALDI-TOF MS of fraction P4

Daas et al., 1998). Although this assignment was very tangible, more proof was needed. Data to demonstrate first, that P4 was, in fact, a methylated GalA tetramer, and second, to show on which of the GalA residues was the methyl on, was acquired by NMR. The 1D ^{1}H NMR spectrum of P4, was very complex with many overlapping signals, and it only showed with certainty that the oligomer was methyl esterified by the singlet of the methyl signal at approximately 3.83 ppm. An arbitrary nomenclature of the GalAs of the tetramer was defined to ease the chemical shift assignments. The nomenclature was as follows: GalA I was the non-reducing end (NR), GalA II was the one next to it, GalA III the following and GalA IV represented the reducing end (R). Figure 4-3 aids in the visualization of this nomenclature. Further elucidation of the spectrum was accomplished with the help of various 2D spectra, namely, COSY, TOCSY, HMQC and HMBC. The 2D data obtained were analyzed with the help of NMR-View (Bruce A. Johnson, Merck and Co. Whitehouse Station, NJ, USA), a computer program of great value for 2D NMR signals correlation. In addition, data obtained was compared to published data from Lo et al. (1994), Mort et al. (1993) and Korner et al.(1999). Published data showed that the chemical shifts of the ends of a GalA oligomer are clearly different, from each other and from the internal residues, and somewhat easy to identify, unless some other chemical structure present distorts them. This analysis gave us the ability to assign most of the signals obtained, shown in table 4-1, and to conclude that the GalA were α linked ((1→4) α-D-GalA) and that the methyl was esterified on the carboxyl group of GalA III, as represented in figure 4-3. We started with the TOCSY spectrum, taking into account that it correlates both long and short-range spin-spin proton interactions. The H2 of GalA IV β was identified at 3.49 ppm, and following its horizontal correlation we were able to

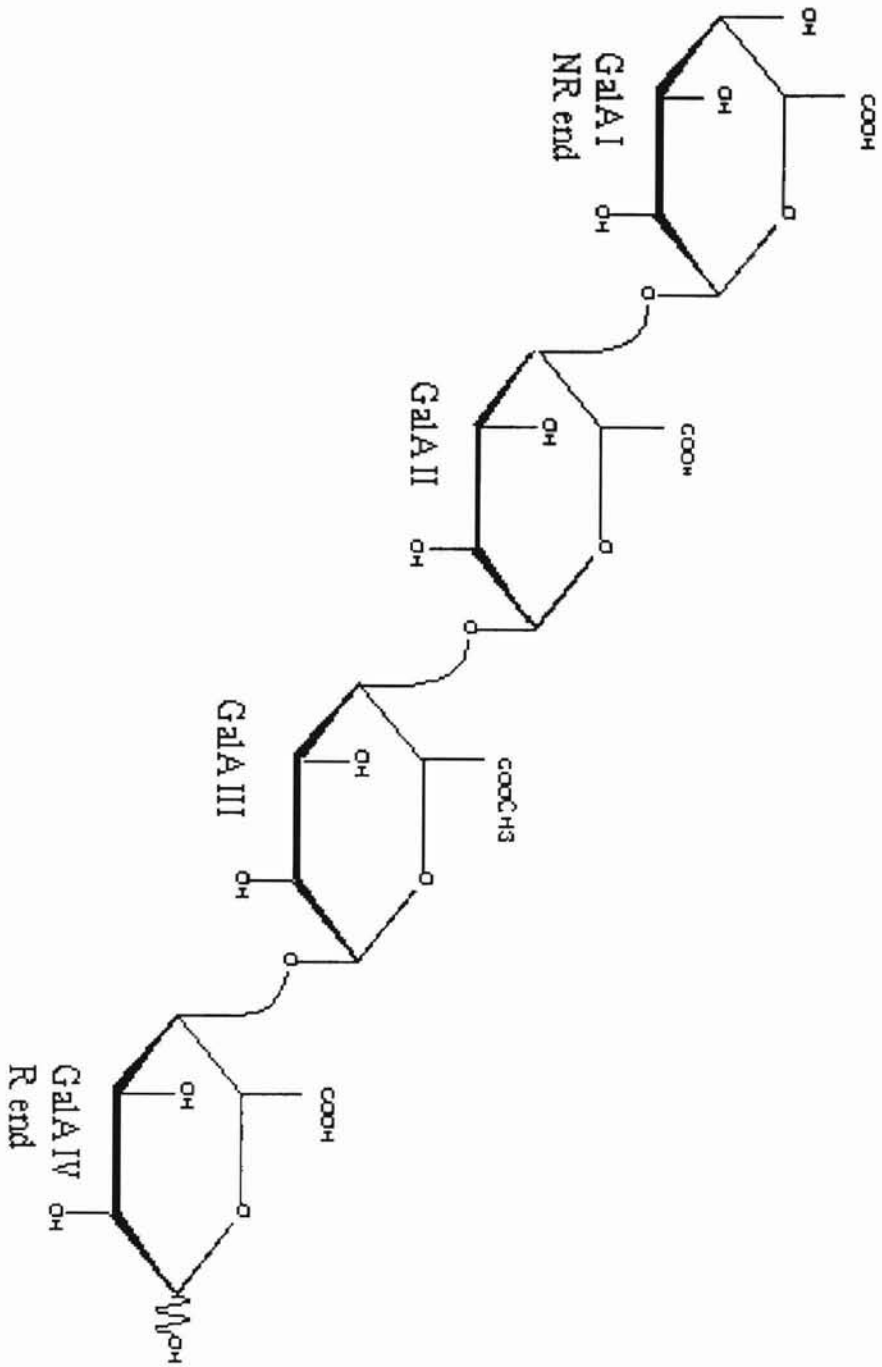

Fig 4-3 Representation of methylated tetramer of GalA

	H1	H2	H3	H4	H5		C1	C2	C3	C4	C5	C6
GalA I	5.06	3.72	3.92	4.26	4.76		99.41	69.85	69.89	71.20	72.59	176.39
GalA II	4.91	3.77	4.05	4.41	4.74		100.27	68.40	68.36	79.57	71.75	176.15
GalA III	5.15	3.74	4.41	4.55	5.19		99.93	68.45	69.00	78.68	70.75	171.33
GalA IV α	5.32	3.83	4.01	4.42	4.43		92.29	68.27	68.95	78.45	70.71	175.31
GalA IV β	4.61	3.49	3.78	4.34	4.07		96.41	71.45	72.45	78.43	74.53	174.35

Table 4-1. Identified chemical shifts (in ppm) of the methylated tetramer of GalA. NR end= GalA I, GalA II, GalA III, GalA IV =R end.

determine the chemical shifts of the H3 IVβ, H5 IVβ, H4 IVβ and H1 IVβ. In the same manner, H1 of GalA I was identified at 5.06 ppm, and from its correlations, H5 I, H4 I, H3 I and H2 I, were assigned. Many other signals were assigned following the same pattern of logic and correlation. From the TOCSY and the 1H data, it was relatively easy to identify the NR and the R ends signals, corroborating that those were not shifted from the values reported for simple GalA oligomers. We concluded that the methyl group had to be on one of the two internal GalA residues. The signal of the H1 of GalA II, at 4.91 ppm was up-field in comparison with other H1, usually found between 5.00 and 5.10 ppm (Lo et al., 1994). This shift was likely caused by other than the direct bonding proximity of the methyl group, since the methyl was determined to be on GalA III. The methyl group on GalA III is spatially close to the H1 of GalA II and so, probably affects its chemical shift through space. The TOCSY spectrum showed some key correlations for our structural determination. We observed that the signal for the H5 III, which was very close to the signal for H1 III, was slightly downfield at approximately at 5.19 ppm. In addition, we observed that all the horizontal correlations of the H5 III and the H1 III were almost parallel, which we took as an indication that they were on the same spin system. Furthermore, we observed that the H4 III and H4 II were well separated from each other at 4.55 and 4.41 ppm respectively. With the help of heteronuclear 2D NMR spectroscopy, we were able to deduce many more signals. The HMBC, which correlates long range H-C and the HMQC, which correlates coupled H-C signals, were of invaluable help. The ^{13}C signals from the C1 were clearly observed in the HMBC between 95 and 103 ppm. The signals for the C6 were found downfield between 160 and 180 ppm. The HMBC spectrum also showed some key correlations. It was observed that

the C6 III was correlated to the CH_3 of III, and also to the H5 III, showing that the methyl esterified GalA H5 was downshifted at 5.19 ppm in comparison to the expected at 4.75 ppm for a non-esterfied residue. Furthermore, on the HMBC it was observable that the C1 II correlated to the H4 III, an inter-residue correlation, which aided us in determining the order of the galacturonic acid residues. On the HMQC spectrum, the signal at 5.19 ppm correlated to a ^{13}C chemical shift of 70.75 ppm showing that it was not from a C1 but instead must be from the H5 III. It was observed that C5 III was correlated to the H5 III, discarding the possibility that this signal was from an H1. Based on the presented data and comparison with published one, we concluded that the methyl group was on the GalA III, the one next to the reducing end GalA, on its carboxyl group, forming a methyl ester. Figure 4-4 shows partial view of the TOCSY spectrum, while figure 4-5 shows partial view of the HMQC spectrum where the shifted C5 of GalA III can be observed. Figure 4-6 highlights the shifted signals of the C6's and the C1's on the HMBC spectrum.

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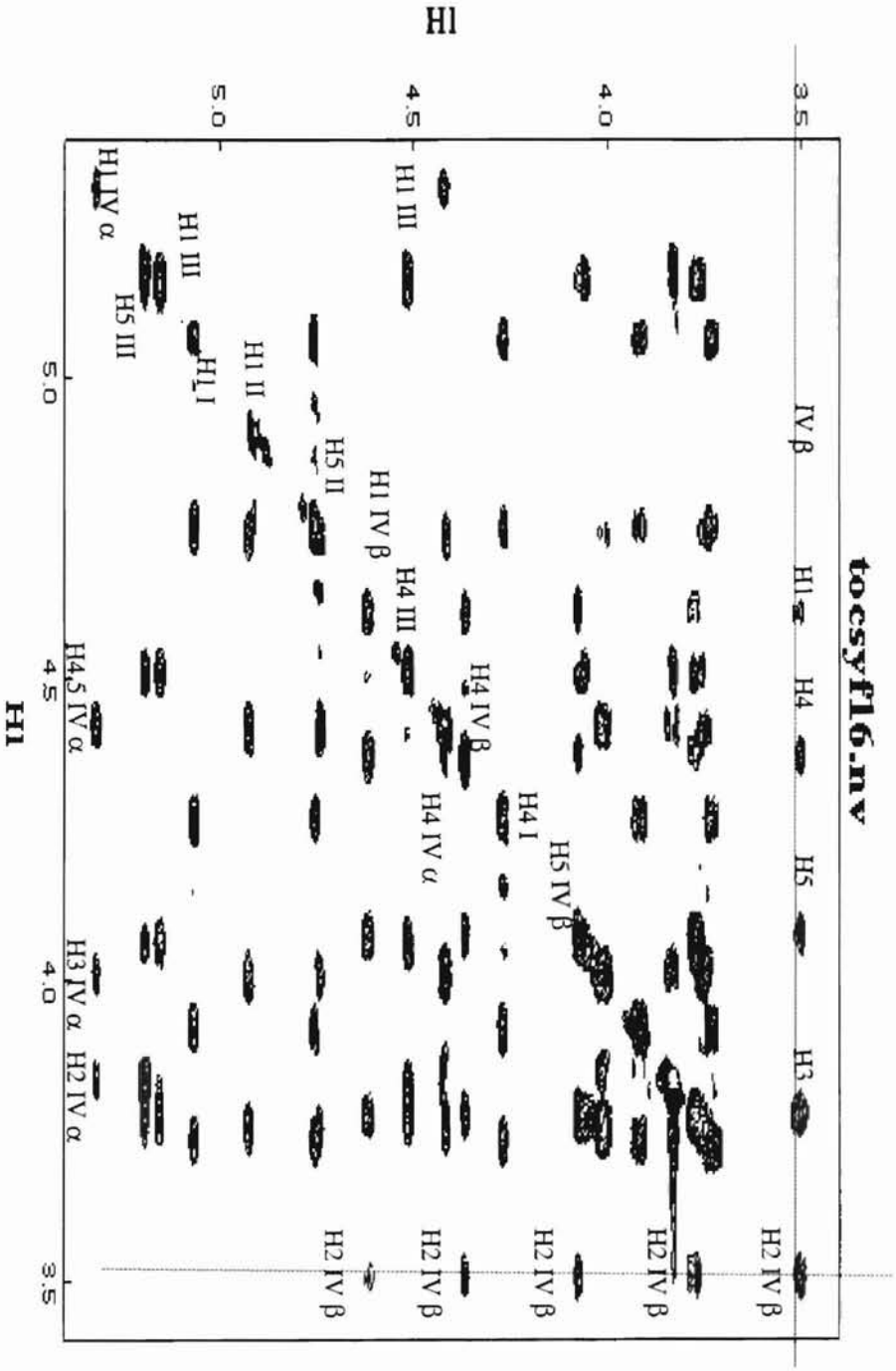

Fig 4-3. TOCSY spectrum

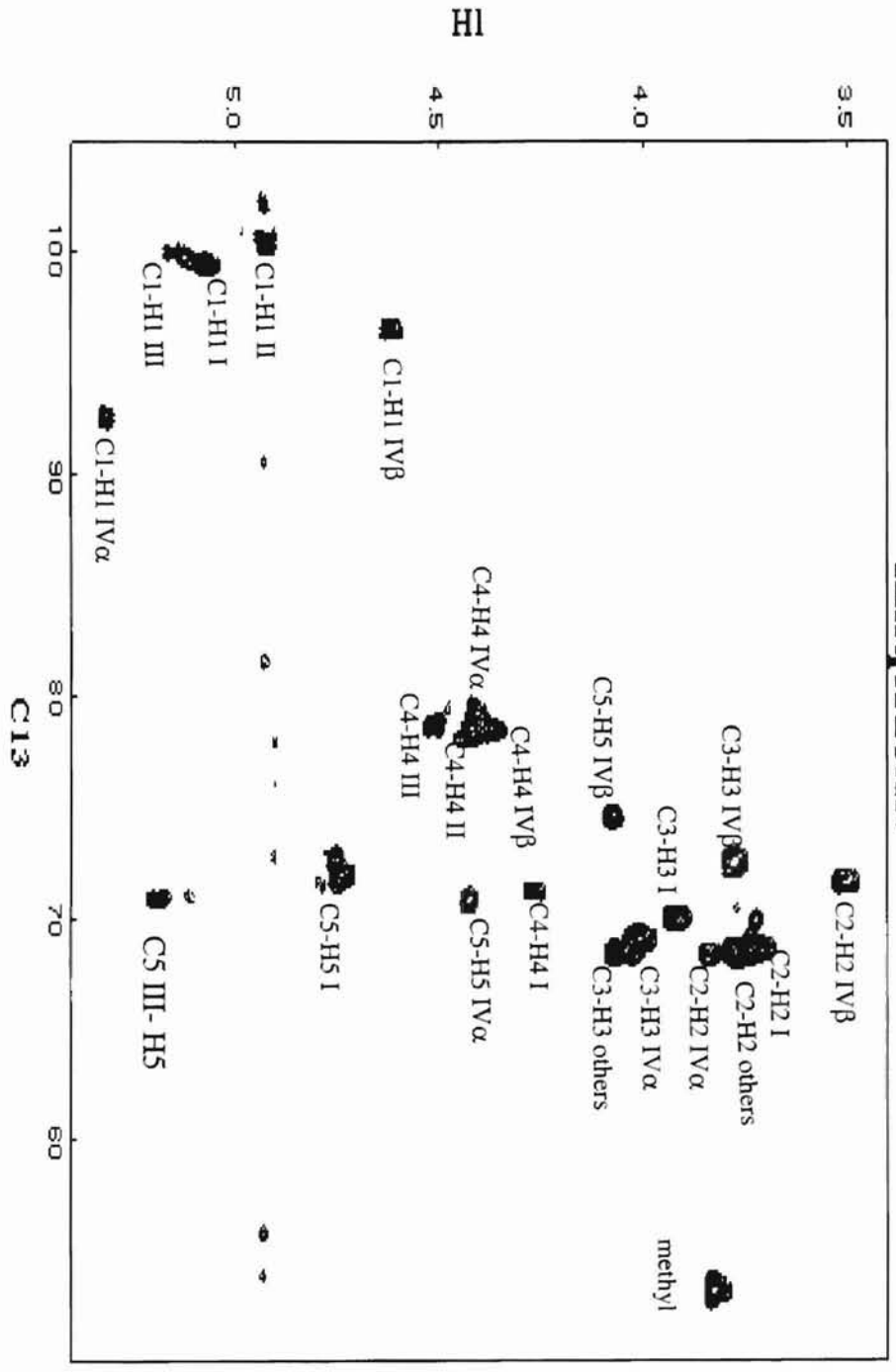

Fig 4-5. HMQC spectrum highlightening the shifted C5 of GalA III

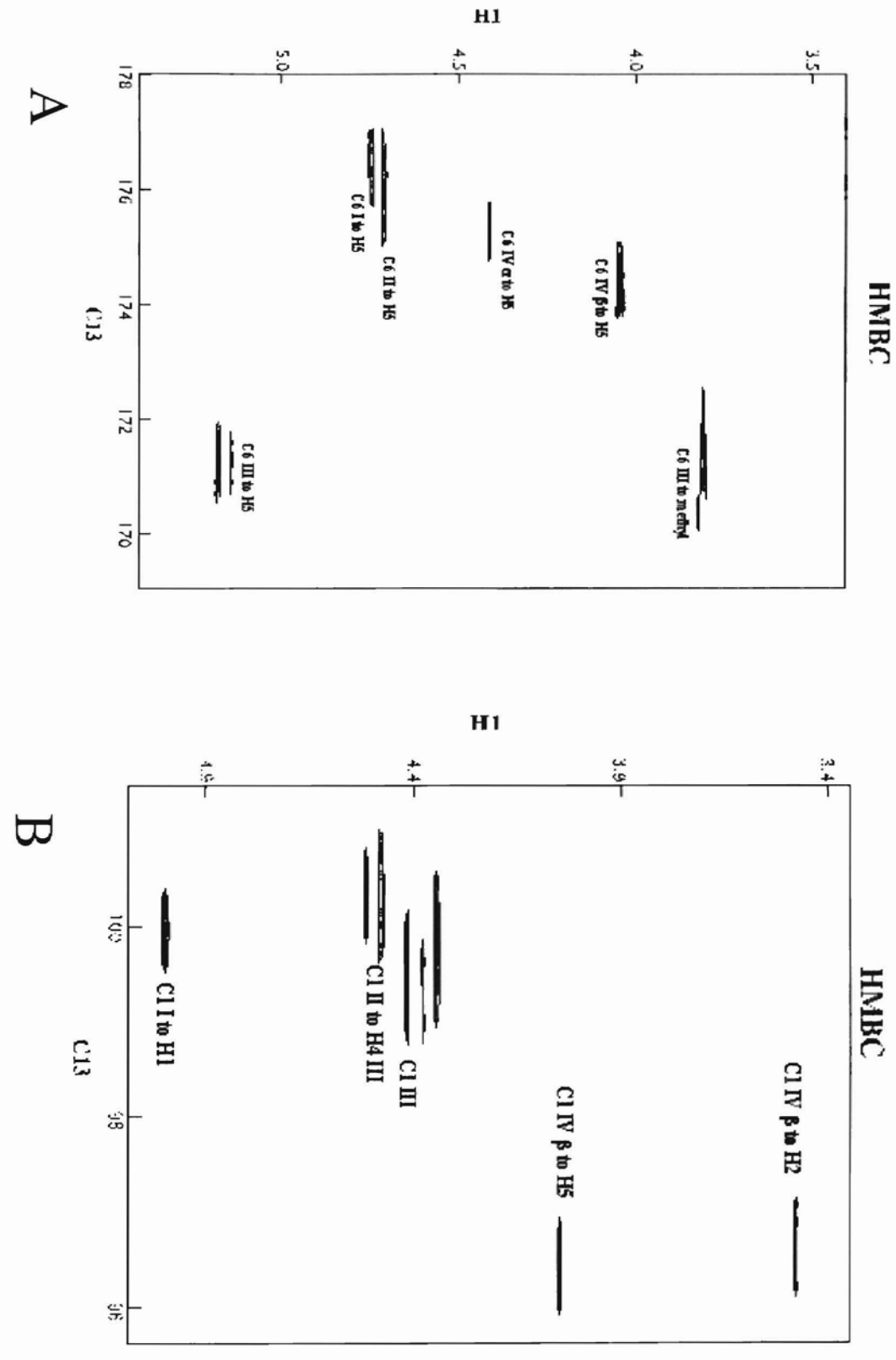

Fig 4-6 HMBC spectrum fractions showing the shifted C6 (A) and C1 (B) signals

CHAPTER 5

SUMMARY AND CONCLUSIONS

For its function in the plant cell wall and its uses, mainly in the food industry, pectin is without a doubt very important for humankind. Despite its importance, its structure remains unclear.

Pectin is a major complex component of primary plant cell walls with four distinguishable regions: HG, RGI, RGII, and XGA. RGI has a backbone of a repeating disaccharide (1-2)-α-L-rhamnosyl- (1-4)-α-D-galactosyluronic acid. Many of the rhamnose residues are glycosylated at the O-4 position with arabinose and/or galactose side chains. Pectin fragments from the HG region, a homopolymer of (1→4) α-D-GalA with a varying degree of carboxyl groups methyl-esterified, have been shown to regulate a number of important responses in plants. Although the importance of these HG fragments is indubitable, their chemical structure is uncharacterized and still being pursued. Commercial pectins are an abundant, cheap source of RGI and HG.

This thesis project comprised 3 main aims. The first was the attempt to isolate and characterize the chemical nature of an arabinose rich side chain linkage to the main chain. The second aim was to characterize a methylated tetramer of galacturonic acid. The last and third aim was to improve some of the techniques used to help this research project and future carbohydrate research.

RGI and HG fragments were prepared from the pectin by sequential saponification, digestion with endopolygalacturonase (EPG), and ultrafiltration through a cutoff membrane. The high molecular weight (HMW) fractions obtained in this fashion were digested by various combinations of a cloned rhamnogalacturonase (RGase) and

commercial β-galactosidase, endo-1,4-β-galactanase and α-L-arabinofuranosidase. The neutral sugars obtained by digestion were removed through an anion-exchange PA1 column. The acidic fractions separated from the digests by PA1 anion-exchange chromatography underwent subsequent analysis to determine their sugar composition by GC, and their purity by CZE. Further characterizations of interesting fractions made great use of various techniques such as CZE, HPLC, GLC, NMR and MALDI-TOF MS. Trial and error research led to some improvements in the methods used and the development of workable instrument configurations for the MALDI-TOF.

The isolation attempts of the arabinose rich RGI fragment involved many sequential enzymatic digestions and chemical separations using various chromatographic methods. Three different enzymatic schemes were tried and no conclusive results were drawn from the data obtained. Furthermore, we concluded that the enzymatic combination used was not enough to strip the RG of the side chains, since its degradation was not sufficient to decrease its molecular weight below 3000 Da in order to be found in the ultra-filtrated LMW fractions. The characterization of the methylated tetramer of GalA was based mainly on NMR spectroscopy results and from the data obtained we concluded that the methyl was on the GalA next to the reducing end (GalA III) on a methyl-ester bond on the carboxyl group. Great improvements in obtaining a more usable HMW fraction were obtained by the use of imidazole, which rid the HMW fraction of the dimer and trimer of GalA interference. MALDI-TOF MS BICs were developed for the different carbohydrate types used. In addition, matrices were found that improved the signal/noise ratio for the different carbohydrate types used.

APPENDIX A

INSTRUCTIONS FOR THE USE OF THE MALDI-TOF VOYAGER DE-PRO WITH DELAYED EXTRACTION TECHNOLOGYFOR CARBOHYDRATE RESEARCH

OSU – DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

NOTE 1 - since the instrument is very sensitive to contaminants (including plasticizers), use methanol/ethanol washed and autoclaved tips and microfuge tubes for sample preparation

NOTE 2 - since salt adducts form rather easily desalting is very important. The usage of Dowex -50W 50X8-200 beads in the ammonium form is highly recommended for desalting purposes. The beads are stored at 4°C in H_2O, so always add beads from the bottom of the flask to prevent further dilution by just adding the supernatant H_2O.

NOTE 3 - Different oligosaccharide samples require different matrices and different modes. GR oligomers - T0NcAc, reflectron, negative mode

Neutral oligomers (galactans, arabinans, etc) - D2, reflectron, positive mode

APTS and ANTS labeled oligosaccharides - NTo, reflectron, negative mode. Check table 2-2 in chapter 2.

SAMPLE PREPARATION-

Dissolve your sample in water (need above fmol conc. to be detected, pmol and above is good). For every µl of sample, add 1 µl of beads and let it sit for at least 15 min.

MATRIX PREPARATION

NOTE 4 - different matrices work better for different samples, so if you have a new sample you should try it on different matrices.

NOTE 5 - matrices seem to have salts too, so it is recommended that they will be prepared 1 day ahead of use time and stored on10 µl of beads.

DHB = 2,5-dihydroxybenzoic acid

D1= 10 mg/ml in H_2O

D2= 10 mg/ml in 0.1% TFA/acetonitrile (70:30)

THAP= 2,4,6-trihydroxy acetophenone

T0= 100 mg/ml in methanol

T1= 20 mg/ml in methanol

T2= 10 mg/ml in H_2O/acetonitrile (50:50)

T3= 10 mg/ml in methanol/H_2O (50:50)

MSA = 5-methoxysalicylic acid

M1= 1 mg/ml in ethanol/ H_2O (50:50)

M2= 20 mM in ethanol/H_2O (50:50)

D / M mixtures= 9:1

OTHER SOLUTIONS USED

Ammonium Citrate [$(NH_4)_2 HC_6H_5O_7$] (CAS 3012-65-5)

Ac = 20mM sol in H_2O

Nitrocellulose (30 mg/ml in acetone)

Nc = 15 mg/ml in acetone/2 propanol (50:50)

Nafion perfluorinated ion exchange resin (CAS 31175-20-9) 5 wt.% solution in mixture of lower aliphatic alcohols and water.

N = nafion diluted in ethanol (50:50)

T0Nc = T0/Nc (4:1)

SAMPLE SPOTTING

NOTE 6 - there are many ways to spot the samples in order to obtain better crystals, but it is always important to let the samples dry completely before using the instrument

NOTE 7 - since the matrices and samples are sitting on beads, be careful to only spot the supernatant and not the beads onto the plate. If some beads are spotted, make sure to remove them before using the plate in the instrument. Beads can be removed (not an easy task) by vacuum drying and/or blowing onto the plate (be careful of contaminant deposition onto the plate when blowing)

NOTE 8 - it is important to know which sample is where on the 100 well plate, therefore it is advisable to use a chart to record the spots, matrices used etc, as the one attached at the end of this appendix

NOTE 9 - Use the wells in the middle of the plate (in our plate be careful to avoid the scratched wells 44, 45)

SPOT A CALIBRANT EVERY TIME (the calibrant will depend on your sample size)

A NEW CALIBRATION FILE SHOULD BE CREATED EVERY TIME.

T_NcAc = 0.5 up T_Nc mix, let it dry

add 0.5 up Ac + 0.5 up sample

N-matrix = 0.5 µl dil nafion, let it dry

add 0.5 µl matrix + 0.5 µl sample

matrix-Ac = 0.5 up matrix, let it dry

add 0.5 µl Ac + 0.5 µl sample

matrix = 0.5 µl matrix + 0.5 µl sample - let them dry together

NOTE 10 - sample and matrix can be mixed on plate with the pipette tip if desired. In addition, sample can be vacuum or air-dried, which sometime it does make a difference.

RUNNING THE VOYAGER DE PRO

NOTE 11 - All our data is on the D drive in the Mort folder (D:/Mort/2001/ ...)

NOTE 12 - The joystick has 2 buttons, the one to the top (top meaning the wire side) SAVES data, the one to the lower left SHOOTS the laser.

Make sure that the computer, the computer monitor and the TV monitor are on.

1) open the VOYAGER INSTRUMENT CONTROL PANEL program (shortcut on the desktop)

2) insert spotted plate (CAREFUL WITH PIN POSITION)

2a- icon with a plate on a hand will eject plate holder (after downing the voltages and vacuum, it takes a few seconds) (or go to sample plate/eject)

2b- place plate on plate holder with care of the pin position (pin should go towards the instrument and the solid edge should face the door, in other words, the PerSeptive Biosystems symbol faces the instrument). Slide plate in until it clicks in place.

2c- click on icon again and choose 100 well plate from the drop-down plate id menu. Click on load plate (loading takes a few seconds too)

3) make sure the voltage is on (check instrument status at the bottom of the screen). If voltage is not on, turn on by using the voltage icon (looks like a lightning) (or go to instrument/turn on voltage)

4) open instrument setting (file / instrument settings - D:/Mort/2001/bics) by choosing the appropriate BIC (Best Instrument Configuration) according to the sample and matrix in

use. Note the mass range, which can be modified by just retyping the desired range (do not save this as a new bic)

5) change data storage directory to save your files in the right place. To do so click on the .. symbol, in the data storage area of the screen. Go to D:/Mort/2001/ etc and give your file to be saved a name following this format : date/matrix/sample, ex 0605T0NcAcGR4. Make sure the autosequence filename option is clicked on so you can save more than one file with the same name by creating a numerical sequence of it.

6) create an active position by entering the number of the well where that sample is spotted and pressing enter (you can also go to the number by using the drop-down menu, or by clicking in the correct well hole of the plate represented on the computer screen). Position your spot using the joystick and the drawn target on the monitor screen.

The first sample to be shot should be the calibrant, so a calibration file can be created. Since all your calibration will depend on this data, take some time to make it the best possible.

7) shoot the laser (using lower-left button on joystick) and acquire data.

Check for the intensity of the data acquired (max intensity is 6.3 x 10^4). If intensity is too low discard the data and "shop" for another "hot" spot on the sample to obtain better intensity. As a last resource up the laser power in increments of 10.

Check for peak resolution, go to tools/resolution calculator - select the peak with the mouse's right button and click ok. In the reflectron mode a resolution of >5000 should be achieved, although a resolution of >2500 is enough to create a calibration file.

8) To reduce signal/noise ratio the spectra can be accumulated by using the sum icon (looks like an epsilon), the one to the right of it (epsilon with an eraser) will clear the

accumulated spectra. Accumulate the current spectrum, shoot again, and if the new spectrum is satisfactory, accumulate it, if not, shoot again (this will erase the recently acquired spectrum). Go on until you have obtained desirable signal/noise ratio, intensity and resolution to create your calibration file.

9) save your accumulated file (and any individual file that you might need) by using the top button on the joystick.

CREATE A CALIBRATION FILE

10) open the data in DATA EXPLORER VERSION 4.0 by using the icon that has two spectra, one on top of the other (the 7th icon from the left). The use of this icon will open the last saved data file on explorer. Or you can open explorer and then manually open (file/open file/D:/Mort/2001/....) the desired file. Click on the file, click on add (add as much as 8 files at a time) and click on finish to plot the data.

11) data massaging - process/advance baseline correction- ok

process/noise-filter smooth - ok

12) create calibration file - process/mass calibration/manual calibration - choose reference file from the Mort folder (that is the only reference file that has the oligosaccharides reference in it, other reference files have only proteins). Choose a peak (make sure that you are choosing only one peak by expanding the spectrum with the mouse's left button) with the mouse's right button , and it should show you a mass in the reference file that is pretty close to your mass (make sure it corresponds to one of the oligosaccharides masses in the reference file). If you agree that the mass that you have should correspond to the mass in the reference file, press ok. Do the same for all the

peaks in your acquired spectrum to have many calibration points. When you are done doing this, click on export. This will save the file as a calibration file. Be sure that the proper name is assigned to it (date/matrix/sample) so it cannot be confused with older calibration files.

To go back to full unzoom use the double arrow icon.

BACK TO VOYAGER INSTRUMENT CONTROL PANEL

USING THE CALIBRATION FILE

13) In order for the new data to be calibrated with the calibration file just created, you need to open the file. Look at the right lower side of the screen and locate the calibration area, click on external file, and from there locate the file just created (D:/Mort/2001/.....)

BACK TO YOUR SAMPLES

14) now you are ready to shoot all your samples following steps 6,7,8. Data processing can be done through the other computer terminal to free the instrument for other users.

15) when all samples are finished eject the plate by using the hand with plate icon, press eject (this takes a few seconds).

16) after the plate is retrieved, use the same icon to LOAD NO PLATE.

17) turn the TV monitor off but leave the computer on and the program open.

DATA PROCESSING

1) Open DATA EXPLORER VERSION 4. Open the desired file(s). If more than one file was acquired for a certain sample, open them all, and decide for the best one.

2) if desired, recommended data massaging

-process/advance baseline correction- ok

-process/noise-filter smooth - ok

3) to print -file/printer setup - landscape

-file/ print/ print file with instrument settings - ok (or Ctrl + P)

pick up print outs in the 3rd floor printer.

TO PLOT DATA IN KALEIDOGRAPH

1) Open DATA EXPLORER VERSION 4. Open the desired file(s). Do all the data processing needed, including truncating the spectrum so there are less points left to transfer.
2) Go to FILE and EXPORT DATA – as ASCI (to that computer's desktop).
3) Log in to the lab computers using the NETWORK icon on the desktop, and from there choose Apple Miramar Talk (all). That will show all the computers on the network, choose the desired one to move the ASCI data to.
4) Back at your computer, drag and drop ASCI data in Kaleidograph, and graph as usual (ignore comments on too many rows).

AND FINALLY...WASHING THE PLATE

Be careful not to scratch the plate, repeat as needed.

Rinse with 1) distilled water, 2) acetone and 3) ethanol

Scrub if needed, very lightly, with a chemwipe or your gloved finger.

100 well plate chart

1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100

DATE: OPERATOR:

NOTES:

VITA

Gianna Natalia Bell-Eunice

Candidate for the Degree of

Master of Science

Thesis: IMPROVEMENT OF METHODS FOR PECTIN CHARACTERIZATION AND STRUCTURAL CHARACTERIZATION OF A METHYL ESTERIFIED TETRAMER OF GALACTURONIC ACID FROM PECTIN

Major Field: Biochemistry and Molecular Biology.

Biographical:

Personal Data: Born in Paysandú, Uruguay, on December 19, 1968. The daughter of Eduardo W. Bell Quintana and Julia B. Viola de Bell.

Education: Graduated with honors from Meade Senior High School, Ft. Meade, Maryland, in May 1986. Attended Catonsville Community College, Catonsville, Maryland, September 1993 to December 1995; intended major: Chemical Technology. Received a Bachelor of Science degree in Biochemistry and Molecular Biology and Microbiology and Molecular Genetics with a minor in Chemistry from Oklahoma State University, Stillwater, Oklahoma, in May 1999. Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in December 2001.

Experience: Employed by Oklahoma State University, Department of Biochemistry and Molecular Biology as an undergraduate and as a graduate research assistant; 1997 to present.

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