PARTIAL CHARACTERIZATION OF PEA ROOT MUCILAGE

By

ANGELA RENEE JONES

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Thesis Approved

Andrew Mont
Thesis Advisor

Margaret Pierce

James D Oerkby

Wayne B Powell
Dean of the Graduate College

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LIST OF ABBREVIATIONS

ANTS	8-Aminoaphthalene-1,3,6-trisulphonate
AQC	6-Aminoquinolyl-N-hydroxysuccinimidyl
Ara	Arabinose
Araf	Arabinofuranosyl
AGP	Arabinogalactan/protein
Arap	Arabinopyranosyl
C^0	Celius
Cm	Centimeter
EDA	Ethylenediamine
FITC	Fluorescein Isothiocynate
Fucp	Fucopyranosyl
Fuc	Fucose
Galp	Galactopyranosyl
Gal	Galactose
GalA	Galuronic Acid
Glc	Glucose
Glcp	Glucopyranosyl
GlcA	Glucronic Acid
kV	Kilovolts
µg	Microgram
µL	Microliter
µm	Micrometer
M	Moles/Liter
MALDI/MS	Matrix Assited Laser Desorption/Ionization Mass Spectrometry
Manp	Mannopyranosyl
Man	Mannose
mg	Milligrams
mL	Millimeters
mM	Millimolar
min	Minutes
nm	Nanometers
nmoles	Nanomoles
ppm	Parts per Million
PFG	Pulse Field Gradient
Rha	Rhanmose
SCB	Sodium Cyanoborohydride

tAraf	Terminal Arabinofuranosyl
Xyl	Xylose

CHAPTER I

INTRODUCTION

Mucilage is predominately high molecular weight polysaccharide. It is secreted primarily from the root cap and epidermal cells that coat the root surfaces. These cells may extend various distances from the root tip to the rhizosphere forming a primary site for colonization by microbes. Mucilage is thought to form a lubricant protecting the root cap from abrasion and aiding the passage of the root tip as it forces its way into the soil.

Mucilage is a slimly layer that is on the root surfaces of all higher plants. A variety of terminologies have been used for this layer such as, "mucigel, gelatinous material, mucilage, and a gel-like sac material". The different terminology goes with not knowing/understanding what mucilage is. In 1976, Foster showed that the outer layer of mucilage comes from the root cap and the inner layer is exuded from epidermal cells (Foster, 1976). There is evidence that both outer root cap cells and epidermal cells of the root actively produce and secrete mucilage. Mucilage is found primarily on the root tip and has been seen on root hairs (Oades,1978). Greaves and Darbyshire (Greaves, et al, 1972) showed this in 1972, by using the absorption of cationic dyes such as ruthenium red and methylene blue to determine the location of mucilage.

When observing mucilage on a microscope, the thickness of mucilage varies with plant species, environmental conditions, and how it is prepared. Mucilage is known to shrink due to various preparation techniques. Mucilage can range anywhere from 1 to 10 μm in thickness (Oades, 1978). O'Brien (1972) showed that mucilage layers on maize, oats, wheat, peas, and willow were about 10 μm thick.

Geaves and Darbyshire (1972) reported that the thickness of mucilage on a range of plants was influenced by presence of bacteria. They showed that in the presence of microorganisms, mucilage ranged from 0.5 to 8 μm thick. The presence of microorganisms also enhances the appearance of the boundary of the mucilage. Mucilages on roots of grasses from irrigated pastures were 20-50 μm thick (Foster, 1976). Roots of aquatic plants that have been observed were shown to have mucilages that 1 mm thick.

Mucilage may have some important roles attributed to both physical and chemical properties of soil. Jenny and Grossenbacher (1963) showed that mucilage was closely associated with the surface of the clay particles and suggested that this contact facilitates exchange of ions between clay particles and plant root. An example of an ion associated with mucilage is zinc. Mucilage provided the continuity between plant root and "ped" surfaces necessary to account for the uptake of zinc ions in a dry soil (Oades, 1978).

Mucilage can exert selectivity on the uptake ions by plants. It is believed that mucilage may act as the first stage in the selective absorption of potassium ions over sodium ions by plants. The rate at which ions move through a hydrophilic gel such as mucilage is affected by the degree of the hydration of the ions. The more hydrated an ion, the more will its movement to the epidermal cell wall be retarded. Such ions as sodium will be more retarded than potassium ions (Oades, 1978).

Mucilage is believed to be important reserve of various nutrients. This may be due to mass flow of ions towards the root resulting in a concentration in the rhizosphere, i.e. "the association with mucilage may be coincidental" (O'Brien, 1972).

The mucilaginous material is an important source of protons at the root surface as

it is probably exuded in the acid form. It has been suggested that the vesicles that carry mucilage to the plasmalemma prevent it from reacting with cytoplasmic components. However, mucilage is not only seen as a proton source but as a good cation exchange because it contains carboxyl groups in a polyuronide (Paull et al., 1975). In a growing root tip, that is continuously moving through soil pores, and is distributing mucilage over surface in the soil can result in ion exchange between carboxyl groups and soil minerals.

Mucilage has been indicated to lubricate and protect the roots. As a lubricating process, the continuous exudation of mucilage material by the root cap cells may result in reduced friction between the growing root tip and the soil. As a protecting layer, mucilage protects the plant against infection, desiccation or mechanical damage. Roots may be protected from the attack of pathogens by the mucilage layer as a result of active competition against a slow growing pathogen by saprophytic species which rapidly colonized mucilage (Oades, 1978). By mucilage being a highly hydrated material it protects the root tip from desiccation.

Mucilage is thought to be an essential polysaccharide to the root. It functions to help in the defense mechanism against pathogens and help protect the root tip in penetration through the soil. It is very important that we have an understanding of its chemical structure, because of the functions that mucilage is believed to have. With an understanding of mucilage chemical structure, we will be able to understand more of the symbiotic relationships between plant and microbes. It can help to us understand the texture of the soil and the rhizosphere. For progression in the agricultural field, this project could be a helpful tool in the future.

CHAPTER II

LITERATURE REVIEW

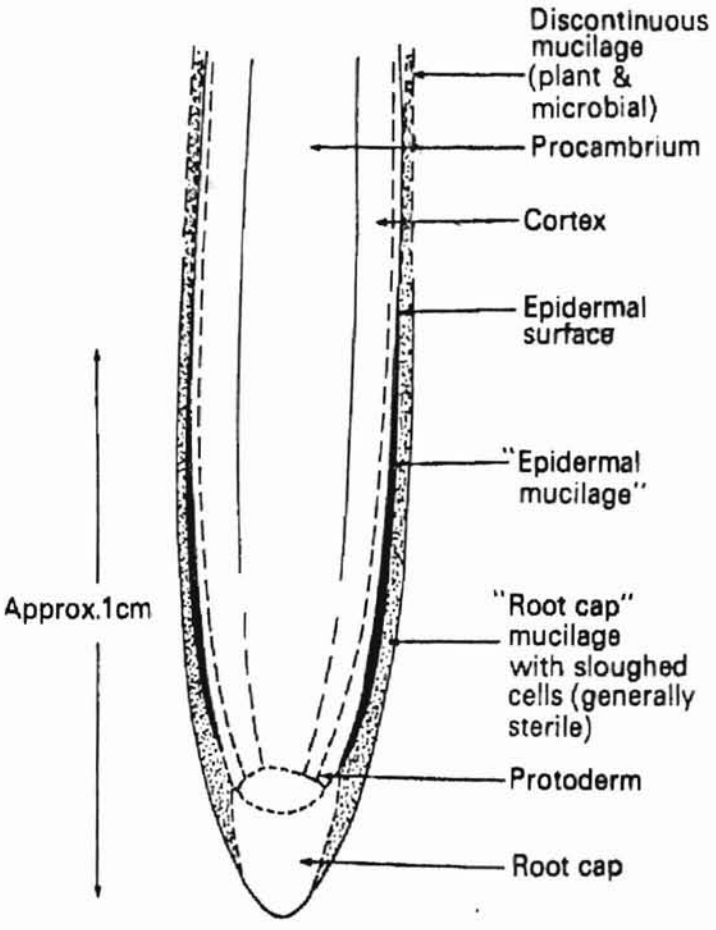
Mucilage is predominantly a water-soluble polysaccharide that is found in most roots of higher plants. Little work has been done on the chemical structure and function of mucilage. The primary role of mucilage is thought to be protection at the root tip and zone of elongation from abrasion during root penetration through the soil.

Mucilage is thought to be similar in structure arabinogalactan proteins. In studies done on mucilage, it was found that its sugar content was predominately arabinose and galactose. Linkages show as Type II arabinogalactan characteristics. Through other evidence, it is believed that mucilage is derived from arabinogalactan proteins.

Where mucilage is found

By looking at Figure 1, we can see a simplified picture of the anatomy of a growing root tip. The picture indicates that mucilage production is located all along the epidermal and root cap. It appears that the root cap is the major site of exudation (Paull et al., 1975). However, the continuous mucilage layers over the epidermal surface of the root suggest that the epidermal cells of the root excrete some mucilage as well. There is evidence that both the outer root cap cells and epidermal cells of the growing root actively produce and secrete mucilage (Oades, 1978). During root growth, these cells are "physically released from the cap". Once they are physically released they still maintain a fairly good metabolic rate and continue to secrete mucilage. These cells were renamed by Hawes (1990) as "border cells". She did not agree that the released cells should be

Figure 1: Anatomy of Growing Root Tip



*Reproduced with permission from Oades, 1978.

called root cap cells since, these cells had a pattern of gene of gene expression that is very distinct from other plant tissue (Hawes, 1994). She named them "border cells", because that was appropriate to both their location, forming a sheath over a portion of the root surface, and to their inferred function, which is to create a buffer or interface between the root and the adjacent soil (Hawes, 1990).

Figure 2a (Foster et al., 1983) shows young root cap cells (border cells) that have been physically released from the cap. The cells are surrounded by mucilage and are presumably starting to secrete it from all sides. Figure 2b shows an older root cap cell. When the old cells start to die they lose turgor, autolyse, collapse, and bacteria start colonizing the mucilage.

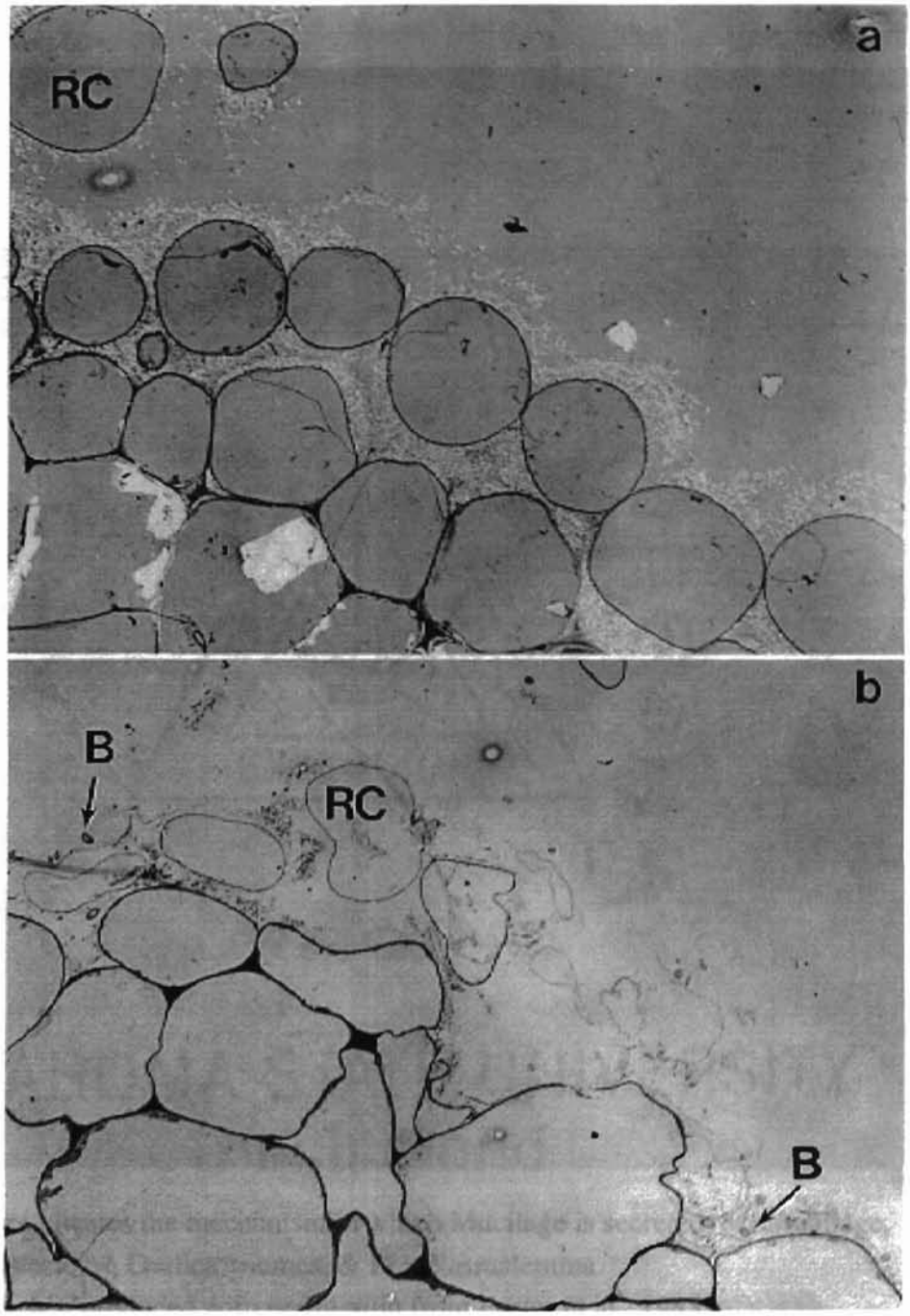
Figure 3 (Foster et al., 1983) shows a detailed picture of a border cell from pine. Here we see the mechanism by which mucilage is secreted. The cytoplasm of the cell is filled with dictyosomes that secrete the mucilage into small membrane-bound vesicles. These vesicles fuse with the plasmalemma. The membrane will break down and the mucilage is deposited into the free space of the cell wall (Foster et al., 1983).

The epidermal cells secrete and produce mucilage as well. As indicated earlier, the majority of mucilage is on the root tip. Epidermal cells are surrounded by mucilage too. Figure 4 (Foster et al., 1983) shows a root cap cell and epidermal cell from wheat. The root cap cell is covered with mucilage and the epidermal cells have remnants of mucilage from the cap-epidermis interface or boundary.

Synthesis of Mucilage

Mucilage is synthesized the same as are all other cell wall polysccharides. The

Figure 2: chickpea Root Cap Cells

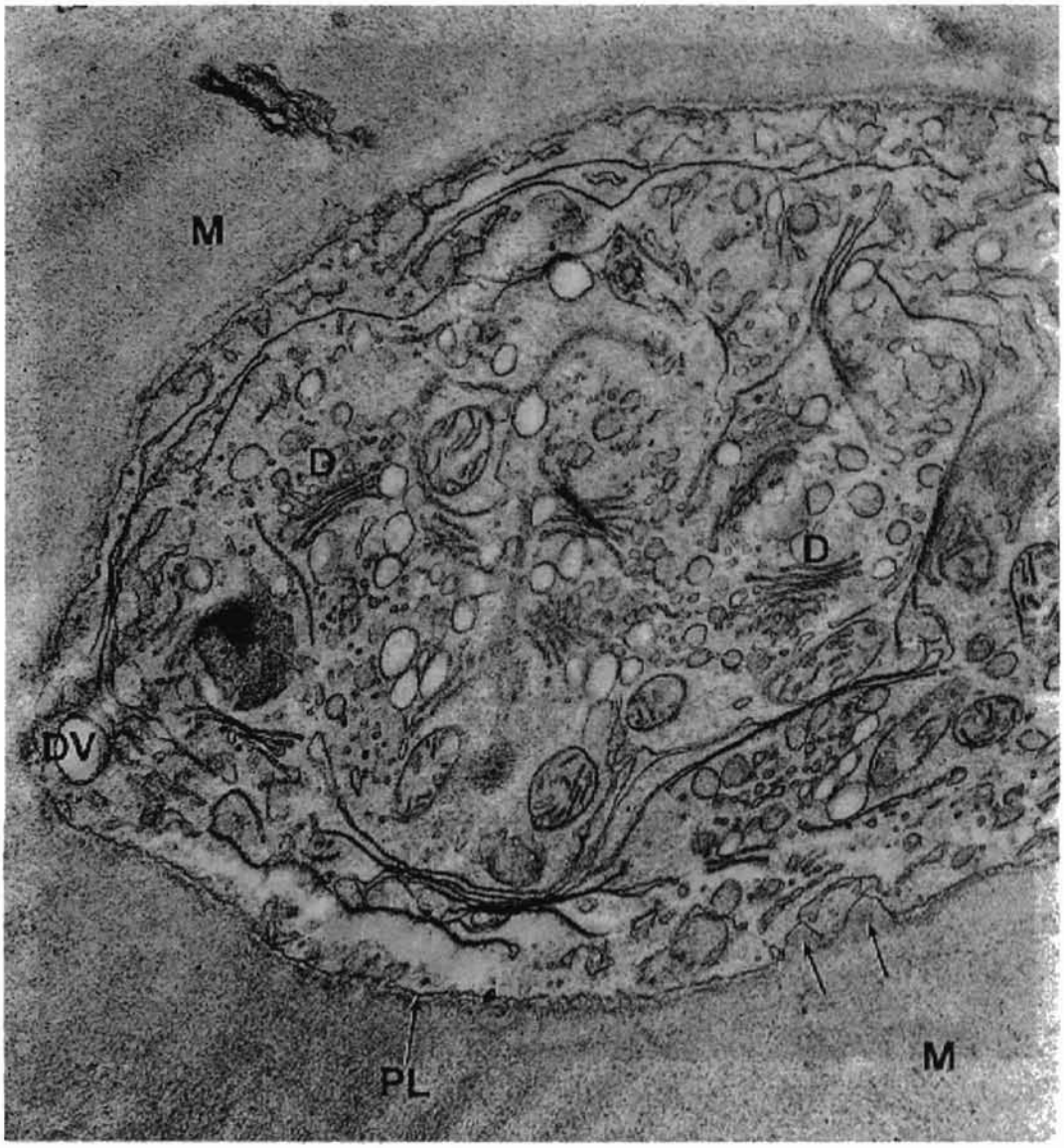


A. Young Cap cells secreting mucilage on all sides. RC=Root Cap

B. Old Root Cap cells that are losing turgor, and beginning to auto lyse and collaspe. Bacteria are beginning to colonize the mucilage. B=Bacteria

* Figure 2 was reproduced with permission from Foster et al., 1983

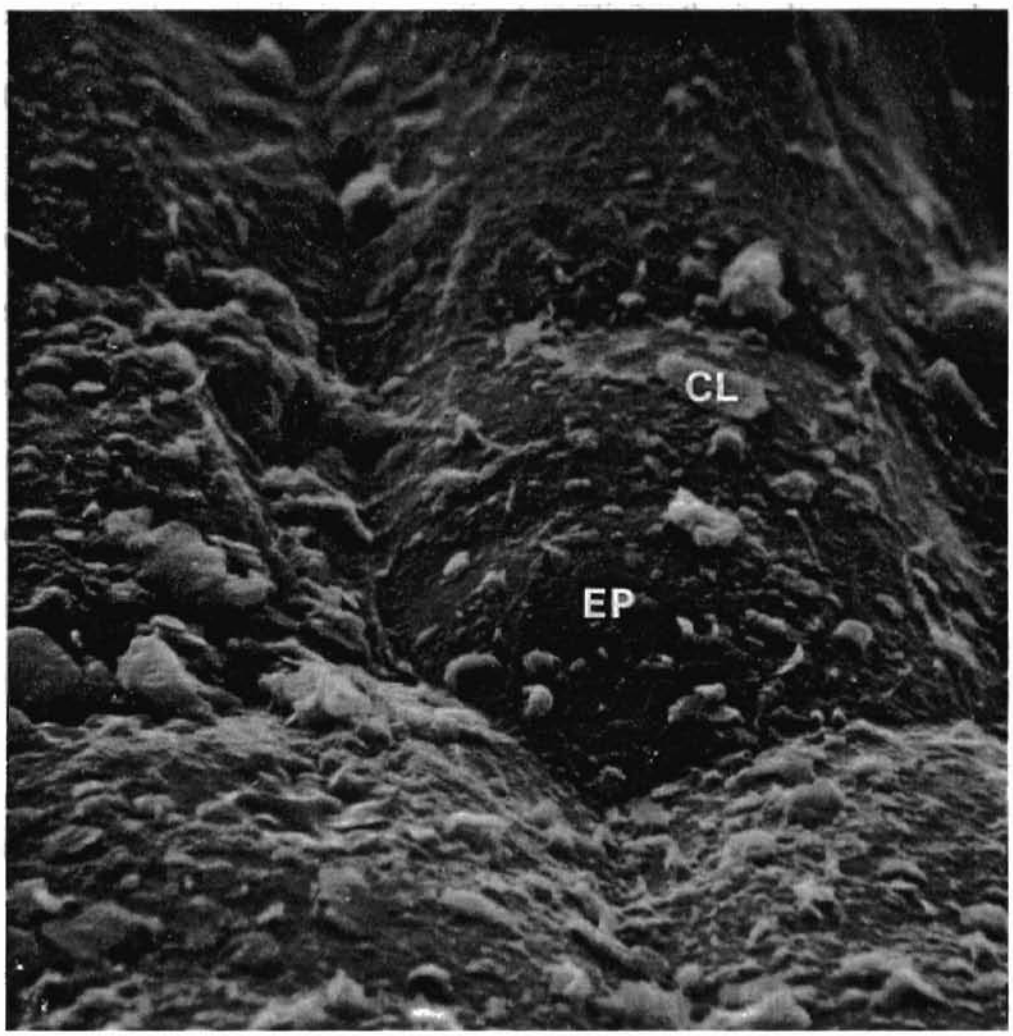
Figure 3: Pine Root Cap Cell



This picture indicates the mechanism in which Mucilage is secreted. M=Mucilage, DV=bound vesicles, D=dictoysomes, & PL=Plasmalemma.

* Figure 3 was reproduced with permission from Foster et al., 1983

Figure 4: Boundary between Root Cap Cell and Epidermal Cells of a Wheat Root



The picture show the root cap cells are covered with mucilage and the epidermal cells have remnants of mucilage from the cap-epidermis interface. EP=Epidermal cells and CL=Clay particles.

* Figure 4 was reproduced with permission from Foster et al., 1983

membrane system consists of the endoplasmic reticulum, Golgi bodies, vesicles, and the plasmalemma, and serves both for the synthesis and transport of polysaccharide. Synthesis of mucilage, similar in composition to pectin synthesis, also appears to be due to Golgi apparatus and endoplasmic reticulum of the root cap cells.

The Golgi apparatus consists of dictyosomes. Dictyosomes are composed of tublar cisterna. When mucilage destined for excretion is accumulated, secretretory vesicles develop and the vesicles separate and become free in the cytoplasm. Once in the cytoplasm the vesicle will move toward the cell membrane and fuse with it. The contents are then discharged as droplets from the protoplast at the root tip (Oades, 1978). This vesicle prevents the mucilage from reacting with the cytoplasm.

Sugar composition

There has been little work on characterizing mucilage. Over the years only four studies plus my study have been done. Bacic, Moody, and Clarke (1986) found that maize mucilage is 94% carbohydrate and 5% protein. The sugar compositions of monosaccharide contain 6 neutral sugars. Galactose and fucose, the two abundant sugars are followed by glucose, xylose, and arabinose, with mannose as a minor component. Maize mucilage showed small amounts of ferulic acid and related phenolics (Bacic et al.,1986). They suggested that the presence of ferulic acid in maize mucilage confirmed earlier cytochemical evidence for the presence of esterified feruloyl residues in maize mucilage (Bacic et al., 1986). Uronic acid was detected in low levels (3% mol) implying that mucilage contains a low proportion of acidic pectic polysaccharides.

Charboud (1983) analyzed the composition of maize mucilage. Bacic et al.

(1986) results agreed with Charboud. Charboud reported that mucilage contained 95 % carbohydrate and 5% protein. Its sugar composition contained the same six neutral sugars that Bacic et al. found. He reported that 40 % of the total carbohydrate present in maize root exudate was associated with mucilage.

Chaboud and Rougier (1984) did sugar composition analysis on rice mucilage. They found that rice mucilage contained the same sugars found in maize mucilage, but in different proportions. In rice mucilage they found specific lectins to bind to the mucilage on the rice roots, which was not seen in maize.

Moody et al. (1988) analyzed wheat and cowpea mucilage. They found that wheat mucilage was composed primarily of carbohydrate (95%) and a small amount of protein (5%). Cowpea contained 97.5% carbohydrate and only 3% protein. The carbohydrate of mucilage from cowpeas contained considerably higher amounts of uronic acids (11.5%), whereas maize contained only 3% and wheat contained 4%. The uronic acid was primarily galacturonic acid in cowpea mucilage, while wheat contained a mixture of both galacturonic and glucuronic acids.

Cowpea mucilage was predominantly rich in three neutral sugars: arabinose, galactose, and glucose. Fucose, xylose, mannose, and rhamnose were found in smaller amounts. Wheat mucilage predominantly contained arabinose, xylose, galactose, and glucose, with small amounts of fucose and mannose and traces of rhamnose.

Linkages

Bacic et al. (1986) provided the first evidence for the glycosidic linkage analysis of maize root mucilage. This group identified 4 mol % of the sugars as terminal

galactopyranosyl residues, meaning a galactose residue linked only through the OH at C1 to some other sugar, thus forming the non-reducing end of a polymer chain or side chain. The total sugar residues were 2-linked galactopyranosyl (8%). In addition, they found some 3-linked galactosyl residues, plus galactosyl residues serving as branch points with linkages to 1, 2, and 3 positions or 1, 3, and 6 positions.

The fucosyl residues are mainly present as terminal residues (14.5 mol %). They also found 3-linked fucopyranosyl residues (8 mol%), and small amounts as 2-linked fucopyranosyl residue (1.5 mol%). The 2- and 3-linked fucopyranosyl residues are not normally seen in higher plant polysaccharides, but have been seen in heteropolymers isolated from diatoms and blue green alage. Fucosyl residues are often associated with high plant polysaccharides as terminal residues. They are found in pectin polysaccharides and xyloglucans.

Arabinose linkages were also detected. The linkages found were terminal arabinopyranosyl (1 mol %), terminal arabinofuranosyl (4 mol %), and 2-linked arabinofuranosyl (6 mol %). These data suggest that arabinose (with some galactose linked as terminal galactopyranosyl residues and 3-galactopyranosyl residues) is a side chain on the 3-linked galactoporanosyl backbone. The remaining sugars also contained three or four glycosidic linkages. It was concluded that there is a high diversity of linkages in maize mucilage. The high diversity indicates that mucilage is composed of several different heteropolysaccharides. It was concluded that these linkages (3,6- and 3-linked galactopyranosyl residues) are probably derived from a type II arabinogalactan (AG) or arabinogalactan protein (AGP). The terminal arabinopyranosyl residue has been seen in AGP. These data along with the evidence for terminal arabinofuranosyl residues

lend more credibility to the conclusion that mucilage is derived from AGP.

Cowpea and wheat mucilage were examined for linkage analysis (Moody et al., 1988). These analyses were compared with maize mucilage analysis. Moody et al (1988) found that cowpea and wheat contained only 3 mol% and 0.5 mol % of terminal fucosyl residues; whereas, maize contained 14.5 mol%. Cowpea contained 6 mol % of terminal galactopyranosyl residues and maize contained only 4 mol %. In either case, arabinose residues were present mainly in furanose form. In cowpea, arabinose residues showed 22 mol % as t-araf. Other arabinose linkages (3-linked araf; 5-linked araf ; 2,3,5 linked araf; 2-linked araf linked; and 3,5-linked residues) are less than 2 mol%. Arabinose in wheat mucilage also was present primarily as terminal arabinofuranosyl residues in smaller amounts were 2-t-araf, 3- t-araf, 5- t-araf and 2, 3, 5- t-araf linked residues. In addition, galactose is present in the pyranose form, primarily as 3-linked (cowpea, 10 mol%; wheat, 3 mol%), 6-linked (cowpea, 6 mol%; wheat, 2 mol%) and 3,6-linked (cowpea, 14%; wheat, 11 mol%) residues. There were other sugars found with glycosidic linkages. These data by Moody et al. (1988) indicated that both wheat and cowpea mucilage contained 3-, 6-, and 3, 6-linked galactopyranosyl residues, which are common linkages found in arabinogalactan proteins. Both contained a high diversity of linkages, as did maize mucilage.

Amino Acid Composition

Amino acid composition was done on cowpea, wheat, and maize mucilage. Cowpea and maize mucilage showed asparagine/aspartate, glutamine/glutamate, and glycine as the major amino acids. Serine, alanine and proline occurred in smaller

amounts. However, wheat showed greater amounts of glycine, alanine, leucine, valine, proline, glutamine/glutamate, serine, threonine, lysine, and asparagine/asparatate than maize or cowpea. All three contained low amounts of hydroxyproline.

Protein found in purified mucilage is only 5% (w/w) in maize, 5% (w/w) in wheat, and 3% (w/w) in cowpea. Some of this protein, characterized by the presence of hydroxproline, is probably associated with the AGPs (Bacic et al., 1986). The remaining protein maybe associated with enzymes that actively secrete mucilage(i.e. esterases, glycosidases, ATPases, acid phosphatase) or release root cap cells that are undergoing lysis (Rougier et al., 1979).

Arabinogalactan proteins

AGPs are high molecular weight polyglycans that are widely distributed throughout the plant kingdom. They are common components of higher plants and their exudates. AGPs contain 1-10% protein, which are rich in hydroxyproline/proline, alanine, serine, and threonine (Schultz et al, 1998). They are found predominately in the plasma membrane, cell wall and intercellular spaces (Ding et al., 1997). AGs are put into categories: Type I and Type II arabinogalactans.

Type I arabinogalactan structures contain a backbone of four to five residues (on average in a (1-4) linked β-D-Galp with two L-Araf units constituting short side chains at the O-3 position. Type II arabinogalactans are the larger group of the arabinogalactans. Type II are found in gymnosperms, angiosperms, particulary in gums, and in Bryophyta. The general structures of the Type II AGs are more complex than Type I AGs. Type II AG, they show more structural diversity. Methylation analysis has indicated that the

backbone consists of four to five residues of 1,3-β-D-Galp to which are attached side chains of 1,6-linked-β-D-Galp. This appears to be a common feature of all the Type II AGs. There are significant amounts of t-D-Galp Acid groups with traces of D-rhamnose. Some sidechains consist of L-Araf which are primarily 1,3-linked, however there are a few 1,5-linked.

AGPs have a highly complex structure and are thought to be functionally diverse. As of now AGPs' biological functions are uncertain. There is some evidence that AGPs play a messenger role in cell-cell interactions during cell differentiation, tissue development, and somatic embryogensis. The evidence for this comes from Casero et al. (1998), who used monoclonal antibodies that recognized two classes of developmentally regulated plant cell surface components, AGPs and extensins. The use of monoclonal antibodies to carbohydrate epitopes on AGPs demonstrated that AGPs can be expressed in an organ-, tissue-, and cell- specific manner, and the crosslinking of AGPs at the cell surface can lead to an inhibition of cell proliferation and root growth.

In a recent study, Shultz et al. (1998) reported that there is a glycosylphosphatidylinositol (GPI)-membrane anchor on AGP. The study revealed that the protein backbones are divided into two families of molecules that are grouped into two broad classes: classical and non-classical. It was shown that two classical AGPs contained the GPI-membrane anchor. They believe that these GPI-membrane anchor proteins maybe involved in signal transduction pathways in plants (Schultz et al., 1998). This discovery has opened a new door for AGP research and has indicated that AGPs are important protein to the plant cell.

Mucilage in the Rhizosphere

Mucilage is believed to have an influence on pathogenic microorganism colonization in the rhizosphere. The rhizosphere zone will vary in length, and there is a selective stimulation of certain bacteria. The bacteria which colonize roots' are predominately gram negative bacteria, such as *Rhizobium*. This is a good example of plant roots specificity on the selective stimulation of certain bacteria. This is shown by the symbiotic relationship between the root nodule bacterium and its legume host. This selective stimulation may be due to a direct effect of mucilage from the border cells. Hawes (1989) argues that one function of border cells is to provide the plant with a system for a "timed release" delivery of biologically active root exudates (mucilage) into the rhizosphere.

Hawes and Smith (1989) believed that recognition of border cells plays a role in colonization by bacteria. They used a microbial mutant. A Tn5 mutant of *A. tumefaciens* was used because it is "wound specific". This "wound specific" mut is only attracted to wounded roots, but not suspended the border cells. The roots were inoculated by directly stabbing with a scapel containing the bacteria; the mutant is fully virulent. When inoculating the soil, challenging the bacteria (contains mut) to finds a wounded root before healing occurs, the mutant is avirulent. These data indicated that loss of recognition of border cells impairs the ability of the bacterium to associate with plant roots (Hawes et al., 1989).

The understanding of "border cells" relationship with bacteria, and how mucilage is involved, is a new area of exploration for microbiology, biochemistry, and soil biology. In this recent study, we have examined the chemical structure of pea root mucilage and

the degradation of it by microbial organisms in the rhizosphere. We have observed that mucilage can be an excellent carbon source for microbial organisms. It was found that *R. leguminosarum* growth on pea root mucilage is dependent on the presence of the symbiotic plasmid that carries the genes required for host specific nodulation of pea. It is also believed that genes on the sym plasmid are involved in pea root mucilage degradation and utilization.

CHAPTER III

MATERIALS AND METHODS

Growing and Collection of Pea Root Mucilage

Pisum sativum L. (Little Marvel peas) were purchased from Burpee Seed Company. Mucilage was collected from pea seedlings growing in sand under sterile conditions in plastic containers. The sand was rinsed six times with tap water and three times with deionized water, then put into each plastic container and autoclaved for 30 minutes at 121^0 C. To sterilize the seeds, we treated them in 50% ethanol for 10 minutes, drained, and then added full strength bleach. We then shook the peas at room temperature in full strength bleach for 30 minutes. We rinsed the seeds with one liter of sterile water and put them back on the shaker for 30 minutes with 50% bleach. After 30 minutes, we rinsed the seeds ten times with sterile water. The seeds were put back on the shaker for two hours in 1 liter of sterile water. When two hours were complete, we washed the seeds ten times under a transfer hood. The seeds were drained well and poured onto the sterile sand in the plastic containers. The seeds were then covered with sterile sand and were enclosed in a plastic chamber. This chamber was covered with aluminum foil and put under the bench in the dark for seven days.

On the seventh day, the sand was shaken off the roots. The roots were submerged into 350 mL of sterile water and put on the shaker at room temperature for 2 hours per each chamber. Once the roots had soaked for 2 hours, they were removed and roots from the second chamber were added to the same water for 2 hours and so on. The 350 mL exudate was filtered through a nylon sheet to remove all the debris. Then the filtrate was

centrifuge at 10,000 rpm for 15 minutes. The supernatant (crude root exudate) was collected and then freeze dried. The crude root exudate was injected on a gel filtration column.

HPLC Gel Filtration Chromatography

The crude root exudate was separated on a TSK-Gel Toyo Pearl-Gel filtration column of HW 50 (S) using a Dionex Reagent Pump system for elution. The exudate was chromatographed on a 22.5 mm x 500 mm HW 50 (S) gel filtration column (fractionation range was 500-80,000) at a flow rate of approximately 2ml/min in a 50 mM ammonium acetate, pH 5.2 buffer. A SHODEX R1-71 refractive index detector monitored the separated exudate, and fractions were collected every minute on a Gilson Fraction collector. Certain fractions were collected from the above column, pooled and freeze dried.

The void volume from the above chromatography was chromatographed on a 10 mm x 500 mm Toyo Pearl HW 65 (s) gel filtration column (fractionation range was 41 x 10^5-1 x 10^7), using a Beckman 421 Controller, a Beckman 110B Solvent Delivery Module (pump), and a Beckman System Organizer for elution. The sample was pumped at a flow rate of 0.5ml/min in a 50 mM sodium carbonate with 25% formamide, pH 7.8 buffer. The void volume fraction was labeled with fluorescein isothiocyanate and monitored by a fluorescence detector FL-750BX HPLC-Plus Spectrofluorescence Detector (McPherson Instrument, Acton, MA, USA). The detector was equipped with 200 W Xenon-Mercury lamp with excitation wavelength set at 490 nm and the cut off filter at 495 nm. The sample was collected from the above column, pooled and freeze

dried.

The fragments that were generated from lithium wire in ethylenediamine were separated on a HW 40 (S) Toyo Pearl Column with a Dionex Reagent Pump system for elution. The fragments were chromatographed on a 22.5 mm x 500 mm HW 40 (S) gel filtration column (fractionation range was 100-10,000) at a flow rate of approximately 2ml/min in a 50 mM ammonium acetate, pH 5.2, buffer. A SHODEX R1-71 refractive index detector monitored the separated fragments, and fractions were collected every minute on a Gilson Fraction collector. Each fraction from the above was run through a HW 40 (s) column again to remove salts. Everything was the same except the flow rate was changed to 1 ml/min. Certain fractions were collected from the above column, pooled and freeze dried.

Gas Liquid Chromatography for Sugar Composition

The monosaccharide composition of mucilage was determined by GC analysis of the trimethylsilyl methyl glycoside derivatives. Methanolysis and derivatization were performed by a modification of the method of Chaplin (Chaplin, 1982). For the analysis of sugar composition, approximately 20 μg of each fraction were methanolyzed and trimethylsilylated. Each sample was weighed on a Cahn 29 electrobalance and placed in a 4 mL glass vial. Each vial had a screw a cap with teflon-lined lid. Along with sample, 100 nmoles of inositol was added for an internal standard. The samples were placed on a vacuum centrifuge to completely dry. To the dried samples, 200 μL of 1.5 M methanolic HCl and 50 μL of methyl acetate were added, then the tightly sealed vials were placed in the 80^0 C heat block overnight. The samples were removed from the heat block and

allowed to cool to room temperature. Once cooled, a few drops of t-butanol were added to each vial to remove the residual methanolic HCl. Each sample was evaporated to dryness under a stream of N_2. Fifty μL of a 1:3 Tri-Sil:Pyridine mixture (3 parts of anhydrous pyridine and 1 part of Tri-Sil Concentrate) were added; the reaction proceeded for 10 minutes. When the derivatization was complete, samples were dried under a stream of N_2 and then redissolved in 100 μL of isooctane. The samples were analyzed (1 μL of sample plus 1 μL isooctane) on a Varian 3300 Gas Liquid Chromatograph using a DB-1 fused silica capillary column (30 m X 0.25mm, 0.2 μm thickness). One μL of sample plus 1 μL of isooctane was injected at 105^0 C. After one minute, the temperature was raised at the rate of 10^0 C/min to 160^0 C. After a 4 minute hold, the temperature was raised at rate of 2^0C/min to 220^0 C. Then the temperature was raised again to 240^0 C at a rate of 10^0 C/min and was held there for 10 minutes to clean the column. The peaks were integrated on a Varian 4290 integrator.

Methylation Analysis

Methylation analysis was determined by GC-MS of the partially methylated alditol acetates performed mainly as described by An (An, 1991). Methylation was done in five steps.

Methylation:

About 300 μg of a sample was dissolved in 200 μL of DMSO overnight (or until completely dissolved) by using a glass syringe. Once the sample was dissolved, 300 μL of butyllithium (15 %) was added under N_2. After the reaction proceeded for 2 hours,

CH_3I was added and the reaction took place at room temperature for 30 minutes.

Extraction of Methylated sugars:

We extracted the methylated sugars on prewashed sep-pak C-18 cartridges (Waters Associates Inc., Milford, MA). The prewash (Mort et al., 1983) consisted of 5 ml of ethylacetate, 5 ml of methanol, and 15 ml (or more) of purified water. The methylated sugars were eluted by rinsing the cartridge with 3 ml of CH_3OH-$CHCl_3$ (1:1 V/V).

Hydrolysis:

For the hydrolysis reaction, 100 μL trifluoroacetic acid (TFA) was added, and the vial was heated at 121^0C for 1.5 hours. After hydrolysis was completed, 250 nmoles of an internal standard (inositol) was added. After adding a few drops of t-butanol, the samples were placed under N_2 until dried.

Reduction:

Reduction was done by adding 10 μL of 1 M NH_3OH and 100 μL of 0.3 M of KBH_4 in DMSO. The reaction was done at 40^0C for 1.5 hours. To decompose the excess KBH_4 we added 10 μL of glacial acetic acid (Harris et al., 1984).

Acetylation:

Adding 20 μL of 1-methylimidazole and 200 μL of acetic anhydride for 10 minutes acetylated the reduced sugars. To remove the excess acetic anhydride 5 ml water was added. The alditol acetates were extracted on a prewashed sep-pak C-18 cartridge

and eluted by 2-3 ml of methylene chloride. The top layer was removed and sodium sulfate was added to the bottom layer to remove the remaining water. The sample was dried under N_2 and was dissolved in ethy acetate.

Gas Liquid Chromatography:

This was used to analyze the methylated sugars. The methylated sugars were separated on a DB-1 fused silica capillary column (30 m x 0.25 mm, 0.2 μm thickness) installed in a Varian 3300 Gas Liquid Chromatograph equipped with an on-column injector and FID detector. One μL of sample plus μL of isooctane was injected at 105^0 C. After one 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 rate of 2^0 C/min to 220^0 C. Then the temperature was raised to 240^0C at a rate of 10^0C/min and held there for 10 minutes to clean the column. The peaks were integrated on a Varian 4290 integrator.

Gas Chromatography and Mass Spectrometry:

The partially methylated alditol acetate derivatives of sugars were analyzed on a GC-MS (HP 5989B Engine) to identify their glycosyl-linkage compositions. One to two μL of sample was injected on a DB225 fused silica, capillary flow column, with a capillary injection-system for on column injection. The analysis was done by GC-MS using EI modes. The program for the GC was set as followed: The injection was 80^0 C and held for 4 minutes at 160^0 C and then raised at a rate 2^0 C/min to 220^0 C, and was held there for 10 minutes to clean the column. The use of mass fragmentation patterns helped in determining the linkages of the sugars.

^{1}H-NMR Spectroscopy

^{1}H Spectra of samples were run at 30^0 C on a Varian Unity Inova 400 MHz NMR Spectrometer. The samples were dissolved in 800 µL D_2 and placed in 5 mm tubes. The probe used was a 5 mm ID Pulse Field Gradient (PFG) Indirect Detection Probe. ^{1}H spectra were also run on a Varian Unity Inova 600 MHz NMR Spectrometer. The samples were dissolved in 250 µL of D_2O and placed in 3 mm tubes. The probe used was a 3 mm ID-PFG Indirect Detection Probe. All chemical shifts were reported in PPM and were measured by reference to water (4.75 PPM).

Amino Acid Analysis by Capillary Zone Electrophoresis

The method used to determine amino acid composition was developed in our lab (Zhang et al., 1998). Only 50 µg of sample plus a crystal of phenol (roughly 1 mm) were hydrolyzed with 6.0 M HCl for 1.5 hours at 150^0 C. After the sample had cooled to room temperature 0.625 nmoles of ornithine was added and the sample was dried on the vacuum centrifuge until completely dried. The sample was resolubilized in 5 µL of 10 mM HCl, 25 µL of 0.2 M borate buffer, pH 8.8, and 5 µL of 10 mM derivatizing agent 6-aminoquinolyl-N-hyroxysuccinimidyl carbonate (AQC). The running buffer was prepared by mixing 3 ml of 0.1 M borate buffer, pH 9.0 (0.1M boric acid, pH adjusted with NaOH), 6 mL of 10 % SDS, and brought to final volume of 10 ml by adding 1 ml of dH_2O. The buffer was then filtered through a 0.45 µm nylon membrane and degassed with a water aspirator. The amino acid separation was run on a custom built capillary zone electrophoresis unit consisting of a Spellman model CZE 1000 R high voltage

supply and a linear UVIS 200 detector, equipped with a cell for on column capillary detection set at 254 nm. The sample was injected onto an 80 cm fused silica capillary by gravity-driven flow. The length of the capillary was 50 cm to the detection window. The electrophoresis was carried out at 18 kV with the positive electrode on the injection side. After each run the capillary was rinsed with purified water, 0.1 M NaOH, methanol, purified water, and running buffer.

Cleavage of Uronic Acids by Lithium Wire in Ethylenediamine

Lithium wire in ethylenediamine cleaves uronic acids residues in mucilage. Five mg of the sample was dissolved with 1 ml of ethylenediamine. Lithium wire was added to the mixture (after rinsing it with hexane). The sample turned blue and was allowed to remain blue for one hour while stirring with a magnetic stirring bar at room temperature. After one hour 500 µL of purified water was quickly added to quench the blue. A few drops of toluene were added and the samples were put on the vacuum centrifuge until completely dried. Once dried, 500 µL of purified water was added and the pH was adjusted to 5.2 by using acetic acid. The fragments were separated on a Toyo-pearl gel filtration HW 40-(S) column at a flow rate of 2 ml/min in 50 mM ammonium acetate, pH 5.2 buffer.

Desalting of Fragments Created by Lithium Wire in Ethylenediamine

Desalting resin:

Approximately 50 mL of a cation exchange resin (50X8-200, Dry Mesh 100-200; Sigma) were washed with 5% ammonium solution followed by several washings with 3

M ammonium acetate solution. After the wash, the resin was washed with purified water a few times. An equal amount of water was added to the resin and it was stored in the refrigerator (Korner et al., 1998).

Preparation of Minicolumn:

To prepare the minicolumn, 10 µL of the washed resin was added to a GELoader pipette tip (at the wide end). To the top of the resin, 10 µL of purified water was added. The column was packed and fitted with a plastic syringe filled with air, and the resin was pressed down to form the column in the narrow part of the GELoader pipette tip (Korner et al., 1998).

Preparation of Minicolumn:

The sample was loaded on the minicolumn by adding the sample with a GELoading tip to the top of the resin. Slowly the sample was forced through the column and collected in a small microfuge tube. To the eluted sample was added 0.5 mL of the resins (Korner et al., 1998).

Labeling of the Reducing End of Oligosaccharies with 8-Aminonaphthalene- 1, 3,6trisulphonate (ANTS) for Capillary Zone Electrophoresis

The mucilage was labeled with 8-aminonaphthalene- 1,3,6-trisulphonate (ANTS) and sodium cyanoborohydride (SCB) at their reducing end (Zhang et al., 1996). Approximately 300 µg of sample were weighed on a 29 electrobalance and placed in a microfuge tube. To each microfuge tube 100 µL of 50 mM NaOAc, pH 4.0 buffer and

0.4 μL of endo-arabinanase (0.012 Unit of enzyme, Megazyme, Ireland) were added. Each tube was incubated at 37^0C overnight. After the reaction was complete, only 1 μL of sample was added to 3 μL of ANTS (23 mM in 3% w/w CH_3CO_2H) and 0.5 μL of SCB (1M) in a small microfuge tube. They were put at 90^0 C for 1 hour. After cooling to room temperature, the sample was ready to be injected on CZE for analysis.

Calculation of enzyme amount

Three hundred μg of mucilage was prepared for digestion. We assumed that 100 μg were arabinan. Our goal was to calculate how much enzyme was needed to cleave 100% of arabinans in one hour. To estimate the moles of arabinose: 150 g/mol-18 H_2O assumed amount of arabinan (100 μg) divided by 132 g/mol (molecular weight of an arabinose residue); 1×10^{-4} g/ 132 g/mol $=7.57 \times 10^7$ mol. Converting mole to μmol (x 10^6 mol)=.757 μmol. To get units of enzyme, 0.757 μmol was divided by 60 minutes = 0.012 Unit of enzyme. To know amount of enzyme: 0.012 Unit/0.03 Unit/μL (concentration of enzyme)=0.4 μL of enzyme.

Capillary Zone Electrophoresis

The CZE unit consists of a custom built instrument, which includes a Spellman (Plainview, NY, USA) model CZE 1000R high voltage power supply with positive and negative polarity and a model FL-750BX fluorescence detector (McPherson Instrument, Acton, MA, USA). The detector was equipped with a cell for on column capillary detection. The excitation wavelength was set at 364 nm and a cut-off filter permitted

detection of emission beyond 440 nm. Some samples used a custom built laser induced fluorescence detector, which used a helium-cadmium laser for excitation and an intensified charge-coupled device camera for detection. A 520 nm bypass filter from Edmund Scientific (Barrington, NJ, USA) matching the emission maximum of the ANTS fluorescence was placed in the light path between capillary and the camera to attenuate scattered light (Mort, et al., 1998). A fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (335 μm OD) was used as the separation column. The capillaries were 60 or 46 cm in length, with 34 or 24 cm to detection window. Each new capillary was rinsed with 0.1 M phosphate, pH 2.5 running buffer overnight before use (Zhang et al., 1996). After each injection, the capillary was rinsed with the running buffer. The samples were injected by gravity driven flow, for 10-12 seconds. Electrophoresis was conducted at 18 kV with the negative electrode on the injection side.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

Preparation of Dihydroxybenzoic Acid (DHB) Matrix:

DHB was the matrix used for MALDI-MS. To prepare the matrix DHB was dissolved in 0.1% trifluoroacetic acid (TFA)-acetonitrile (70:30 V/V) to a concentration of 20 μg/μL. Only 0.5 μL of the matrix was added to the MALDI plate with 0.5 μL of 20 mM dibasic ammonium citrate and 0.5 μL sample solution (Korner et al., 1998). The sample was allowed to air dry. Once dried the sample plate was placed in the MALDI-MS for data analysis by mass spectrometry.

Mass Spectrometry:

The spectrum was obtained at Oklahoma University Health Science Center in the Department of Biochemistry, on a Perseptive Biosystems Voyager matrix-assisted laser desorption time-of-flight mass spectrometer in the positive ion mode.

Labeling of Oligosaccharide Hydroxyl Groups with Fluorescein Isothiocyanate (FITC)

FITC helped in determining the molecular weight of mucilage. We added 50 μg of sample to 200 μL of DMSO for 3 hours or until almost completely dissolved. The mucilage did not dissolve completely in the DMSO. After adding DMSO, 300 μg of FITC were added plus 0.4μL of dibutyltin dilaurate. The reaction was run for 2 hours in a heat block at 90^0 C (De Belder et al., 1975). Once the sample had cooled to room temperature 1 ml of 95 % ethanol was added to precipitate the labeled mucilage overnight. The top layer was removed, and 1 ml acetone was added to preciptitate, and centrifuged for 5 minutes to remove excess FITC. Washing with acetone was repeated until the sample looked clear. Once the excess labeling agent was removed, the sample was redissovled in 1 ml of purified water. Thirty μL of the sample was injected onto a HW 65 column.

Monosaccharide Analysis

Monosaccharide analysis was done to determine the monosaccharides in each fragment generated by lithium wire in ethylenediamine. Each reaction took place in a very small plastic cap that held only 2 μL total. The plastic caps were custom-made out

of Kel-F by the Oklahoma State University machine shop.

Hydrolysis, Derivatization, and CZE:

Approximately 2 nmoles of each fraction was treated with 2 M TFA at 121^0 C for 1 hour. Once hydrolysis was completed, 2 nmoles of an internal standard methylglucose were added. The sample was dried to completion. We added 2 μL of a dervitizing agent Anthanilic Acid. The agent was composed of 30 μg of anthanilic acid and 20 μg $NaCNBH_3$ dissolved in 1 ml of 2% borate, 4% NaOAc in methanol. A fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (335 μm OD) was used as the separation column. The capillaries were 80 cm in length, with 50 cm to detection window. Each new capillary and after every injection the capillary was rinsed with 0.1 M NaOH, purified water, and running buffer (200 mM borate buffer and 50 mM phosphate pH 7) (Sato et al., 1997). The samples were injected by gravity driven flow, for 5-10 seconds. Electrophoresis was conducted at 18 kV with the positive electrode on the injection side.

Data Collection for all experiments

The chromatographic data from all the above experiments were collected using a custom built data logger (Merz et al., 1992) and then downloaded into a Macintosh 8100/80 computer. The data were viewed and analyzed using a program called Kaleida Graph, which was developed by Abelbeck Software (distributed by Synergy Software, Reading, PA, USA).

CHAPTER IV

RESULTS AND DISCUSSION

Growing and the Collection of Pea Root Mucilage

The growing and isolation of pea root mucilage was done under sterile conditions. The sand was rinsed several times and autoclaved at 121^0 C for 30 minutes. The peas were rinsed several times with various solutions for sterilization. The seeds were drained well and then planted in the plastic container in a sterile transfer hood. On the average around 800 seeds were planted per chamber and grown in the dark for seven days. The filtrate was collected and freeze dried. The crude root exudate yielded around 20-40 mg.

We were concerned with the low yield of mucilage. Our first thoughts were the mucilage had moved from the roots and was in the sand, which the roots have been growing in. To test our theory, we washed the sand several times and ran the concentrated exudate through a Toyo Pearl HW 50 (S) column. The results showed that little or no mucilage remained in the sand. Secondly, we looked at the sand that was knocked off the roots. It showed similar results to the first test. Thirdly, we thought that mucilage was still bound to the root. We stained the roots with β-D-glucosyl Yariv Phenylglycoside (β-D-Glc) (Serpe, et al., 1993), which is a chromophoric molecule that selectively binds arabinogalactan-proteins (AGPs). If pea mucilage is an AGP then the Yariv antigen will selectively bind to pea mucilage that remained on the roots. When observing under the fluorescent microscope we saw that roots that had not been soaking in sterile water was very red. We believe the Yariv antigen did bind to the mucilage. The root that had been soaking in sterile water for 2 hours exhibited no red color. We let

the root set for several hours with the Yariv reagent and examined again. It again imparted no red color. In our second attempt to stain the roots with the Yariv antigen, we saw that the roots did not stain as they did before. We can not explain why it worked earlier and not later. We did notice that there was something staining. We set up a control and stained the mucilage by itself and we could not get pure mucilage to stain. We believe that these experiments indicated essentially that we were collecting all the mucilage that was produced by the roots.

Separation of the Exudate on HW 50 (S) TSK-Gel Toyo Pearl Size Exclusion Column

Around 20-40 mg of the crude root exudate was dissolved in 3 ml of 50 mM ammonium acetate, pH 5.2 buffer. A one mL sample of the exudate was injected onto the HW 50 (S) TSK-Gel Toyo Pearl Size Exclusion Column. Figure 5 illustrates fractionation of the crude root exudate on the TSK-Gel Toyo Pearl column. Above each fraction is shown the molecular size of the pullulan standards run on the same column. Fraction 1 eluted around 27-43 minutes and was a fairly high molecular weight fraction. The void volume was 20,000-200,000 molecular weight according to a standard run previously. Fraction 2 eluted directly after the void volume at 44-57 minutes and was not usually a peak, but as an area. Fractions 1 & 2 had sugar compositions that were quite similar, but fraction 2 contained less sugar. At 58-78 minutes fraction 3 eluted from the column. Fraction 3 had the greatest yield compared to the other fractions. Fractions 4, which eluted at 78-100 minutes, was very low molecular weight material and salts.

Table 1 shows a summary of the quantitative analysis of the crude root exudate.

Figure 5: Crude root exudate separated on HW 50 size exculsion column

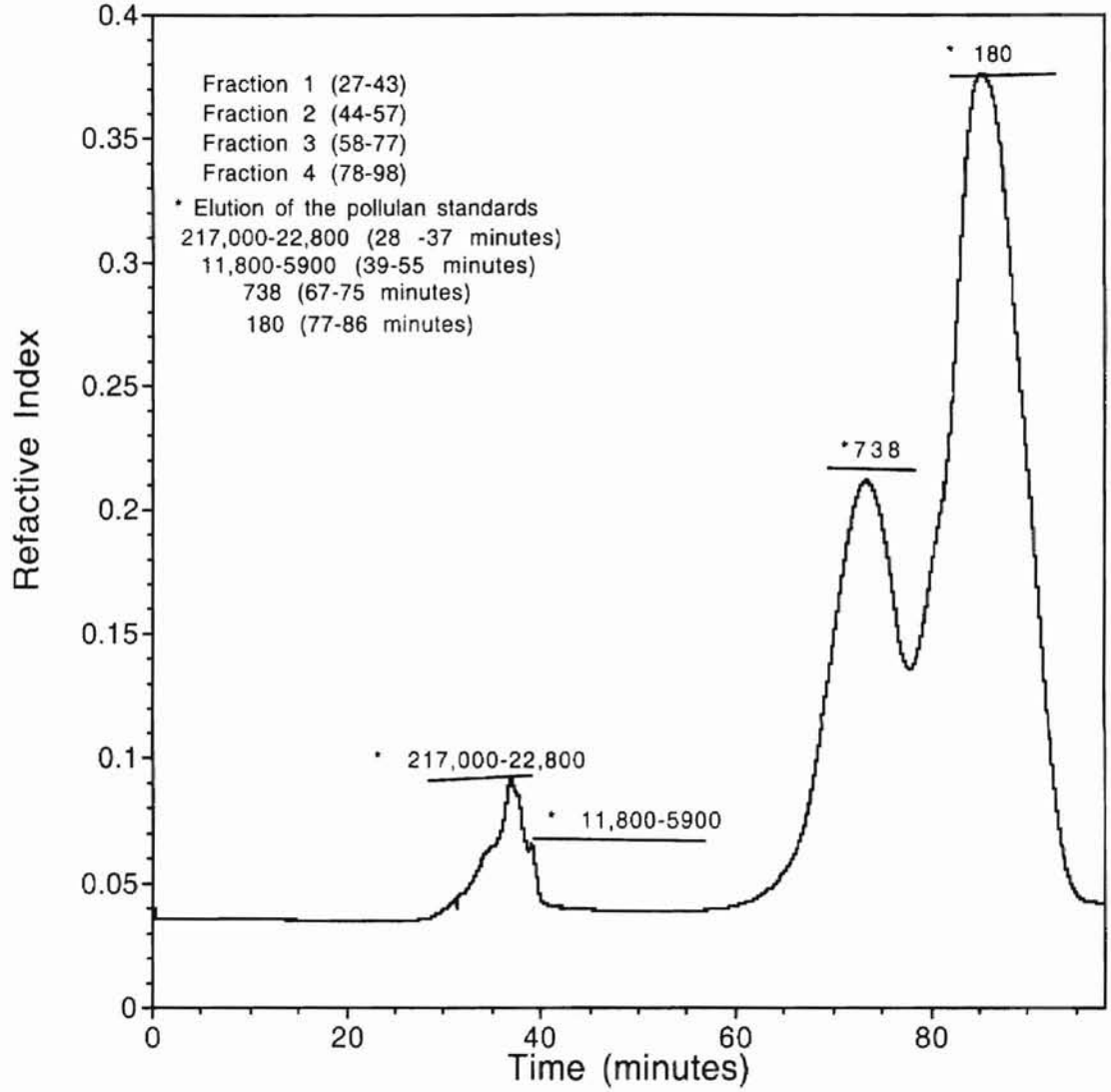


Table 1: Quantitative Analysis of Crude Root Exudate (28 mg) of each Peak from the HW 50(S)

Peak	% weight*	Weight (mg)
1	4	1.2
2	7	2
3	67	18
4	2.8	.79

*% weight of the starting material recovered in each fraction

When starting with 28.2 mg the yield was as follows: Fraction 1, the polymeric material, was 4% (1.16 mg), fraction 2 was 7% (2 mg), fraction 3 was 67% (18 mg), and fraction 4 was 2.8% (.790 mg). This amounts to a 23.2% loss during fractionation. The mucilage comes off in fraction 1 and usually yields 1-2 mg per 3 plastic containers, which gives us an average of around 1-2 μg per plant.

Determination of Sugar Composition by Gas Liquid Chromatography of Each Fraction

For analysis of sugar composition, approximately 20 μg of each fraction were methanolyzed and trimethylsilylated. They were analyzed on a Varian 3300 Gas Liquid Chromatograph using a DB- 1 fused silica capillary column and inositol was added as internal standard. Figure 6 shows a gas liquid chromatogram of mucilage, with the data reported in mole % (Table 2). In fraction 1 the dominating sugars are arabinose, 23.1% and galactose, 56.8%, followed by mannose (3.71%), xylose (3.6%), and glucuronic acid (6.0%). We see rhamnose (2.9%) and glucose (4.0%) in very small amounts. Fraction 2 showed arabinose (30.8%) and galactose (27.8%) are the dominating sugars. Glucose (20.4%) followed these two sugars in abundance. Rhamnose, xylose, glucuronic acid, and mannose are present in small amounts. Fraction 3 was mainly galactose (30%), glucose (28.7%), glucuronic acid (15.4 %) and with traces of other sugars. Fraction 4, the included volume, showed mainly glucose (75.6%), glucuronic acid (11.8%), and galactose (10%). The results are similar to those reported for mucilage of other plants. Table 3 shows the monosaccharide compositions of peas, cowpea, wheat, maize, and mucilage. Our results were similar to those of Bacic et al. (1986). Cowpea was the most

Figure 6: Gas Liquid Chromatogram of Pea Root Mucilage

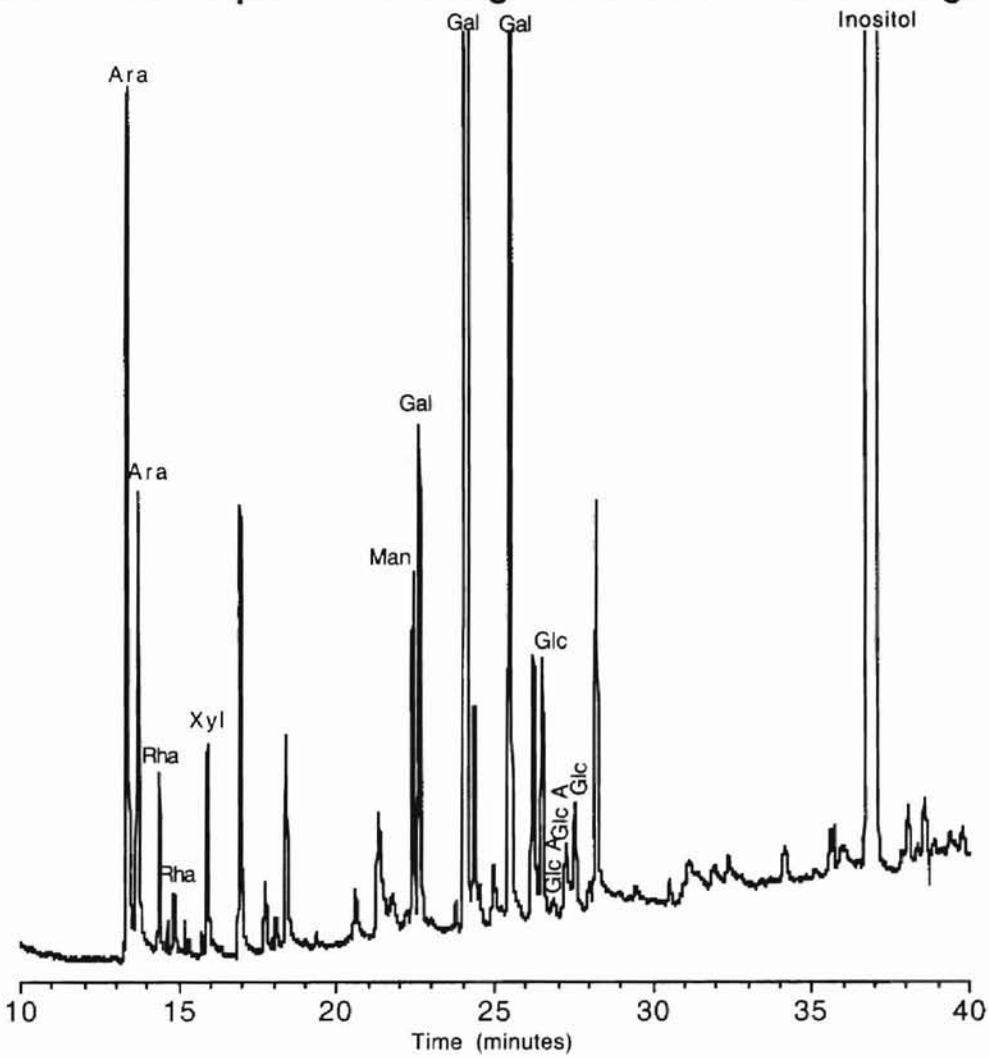


Table 2: Sugar Composition (Mole%) of Crude Root Extract Separated on Toyo Pearl Gel Exclusion HW 50 Column

Frac. #	Ara	Rha	Xyl	GlcA	Man	Gal	Glc
1	23	3	4	6	4	57	4
2	31	2	10	7	3	28	20
3†	13	3	Tr*	15	1	30	29
4†	Tr*	Tr*	0	12	Tr*	10	76

*Trace (Tr)

† Less than 10% of weight of these fractions were accounted for as sugars.

Table 3: Reported Compositions of exudate

Composition (weight%)	Cowpea[1]	Wheat[2]	Maize[3]	Rice[4]	Pea[5]
Protein %	3	5	6	Nd*	2-3
Carbohydrate %	97	95	94	Nd*	75

Monosaccharide	Cowpea	Wheat	Maize	Rice	Pea
Rha	2	Tr*	Nd*	Nd*	2.9
Fuc	9	3	21	5.2	Nd*
Ara	31	31	16	13.7	23.1
Xyl	7	33	14	18.3	3.6
Man	6	1.5	2	4.6	3.7
Gal	28	16.5	33.5	20.3	56.8
Glc	18.5	15	13	37.9	4
Uronic Acid	11.5	4	5	Nd*	6

Nd= not detected, Tr= trace

1. Moody et al., 1988
2. Moody et al., 1988
3. Bacic et al., 1980
4. Charboud, et al., 1984
5. This study

similar to pea in monosaccharide composition. They all contained arabinose and galactose in relatively high amounts along with other monosaccharides. In our monosaccharide analysis we did not detect fucose, whereas Bacic et al (1986) found a high fucose content in maize mucilage. Fucose in maize mucilage was linked at the 2- and 3- positions. In cowpea, wheat, and rice mucilages, fucose was also present, but as a minor constituent. The reason behind this might be due to certain studies that indicated fucosyl residues have been correlated with fungal propagules in the soil and pea roots could have less contact than wheat, cowpea, maize, and rice.

Methylation Analysis

The glycosyl-linkage composition of the mucilage was determined by GC-Mass Spectrometry of the partially methylated alditol acetate derivatives. The methylation analyses was done several times and averaged at Complex Carboydrate Research Center. As shown in Table 4, there was a wide variety of linkages.

Arabinose was present in the furanose form. The arabinofuranosyl residues were primarily terminal and 5-linked. In contrast to cowpea, arabinofuranosyl residues were primarily terminal with small amounts of 2-, 3-, 5- linked and 2,3,5-linked residues. Wheat showed results similar to our data with primarily arabinofuranosyl residues that were terminal, 3-, 5- and 2,3,5- linked. However, maize showed only small amounts of arabinofuranosyl residue.

Galactose, in the pyranose form, was predominantly linked, 3-, 6-, 3,6- and 3,4-linked. In small amounts, 3,4,6-galp and terminal galp was also reported (Bacic et al., 1986). In previous data Bacic found galactose was predominately in the pyranose form

Table 4: Methylation Analysis of Pea Root Mucilage

Sugar Residue	Percentage
t-araf	17
3-araf	1
5-araf	11
3,5-araf	1
2,5-araf	2
2,3,5-araf	2
t-gal	4
3galp	11
6-gal	5
3,4-gal	8
3,6-gal	10
3,4,6-gal	1
t-xylp	0
2 or 4-xyl	2
2,3-man	6
t-glc	8
4, 6-glc	2
2-hexp	2
4-hexp	2
3-hexp	3
2,4,6-hex	1
t-rha	1

and 3-, 6-, and 3,6-linked were the dominant linkages. We found small amounts of 4,6-linked glcp, which were the only glucose linkages detected. Similar results were seen for glucose in cowpea, wheat, and maize mucilage.

Mannose, found in the pyranose form, was 2,3-link, which is not often observed, but should not be too surprising, since Bacic et al. (1986) found maize contained mannose in the same linkage. However, cowpea mucilage showed terminal, 2-, and 2,3-linked residues.

From the presence of 3- and 3,6-linked galactose along with terminal arabino-furanose, it was likely that arabinogalactan or arabinogalactan proteins (AGP) were the major components of mucilage. The high amounts of 5-linked arabino-furanose indicated that pea arabinogalactan was quite different from that in most species in having an additional arabinose-containing polymer. Usually 5-linked ara is rhamnogalacturonan as side chains. The presence of the 2,3-linked mannose was not a surprise to us, because it is seen with linkages of type II arabinogalactans. Mannose 2,3-linked was reported in maize by Bacic et al. (1986).

^{1}H and ^{13}C-NMR

Each of the fractions from the TSK-Gel Toyo Pearl HW 50 (S) size exclusion column was analyzed by ^{1}H-Nuclear Magnetic Spectroscopy (figures 7-10). Each sample was dissolved in deuterium oxide and put into a magnetic field of 9.4 Telsa in a 51 mm bore Oxford super conducting magnet. The ^{1}H spectra showed signals between 3.5-5.5

Figure 7: ^{1}H-NMR Spectrum of Fraction 1 from HW 50 (S) column

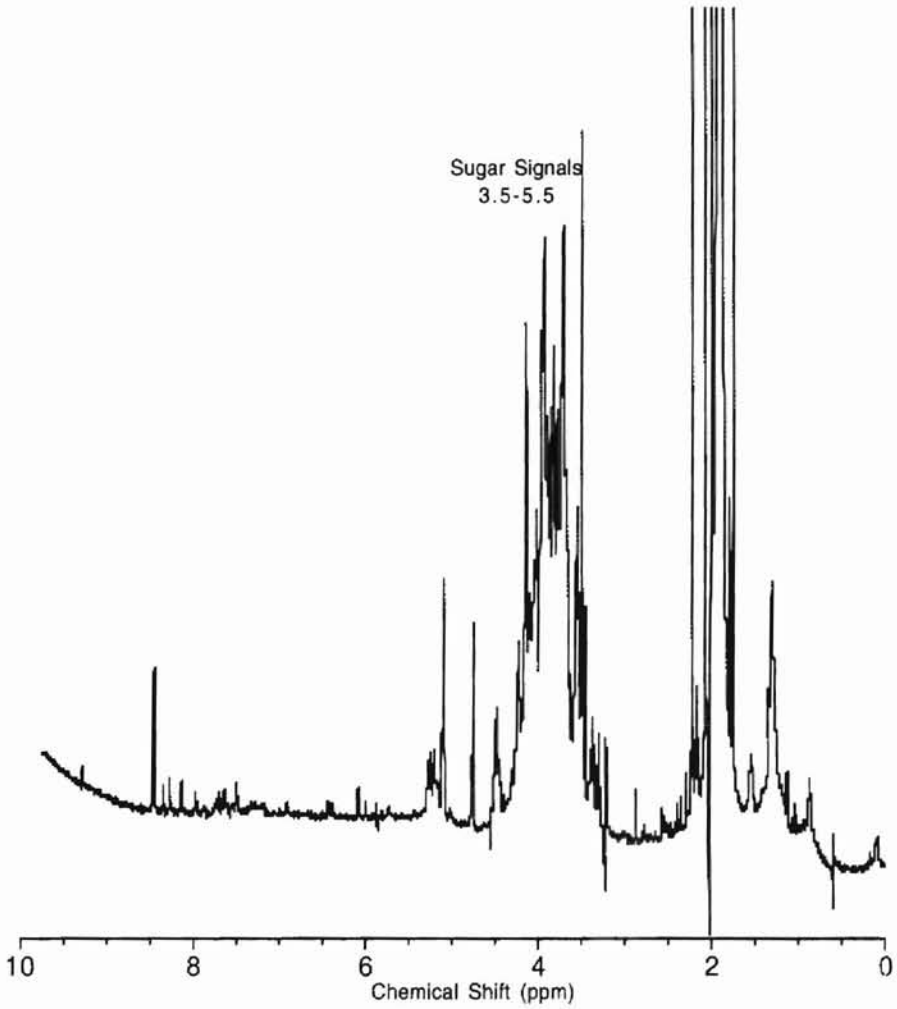


Figure 8: ^{1}H-NMR Spectrum of Fraction 2 from HW 50 (S) column

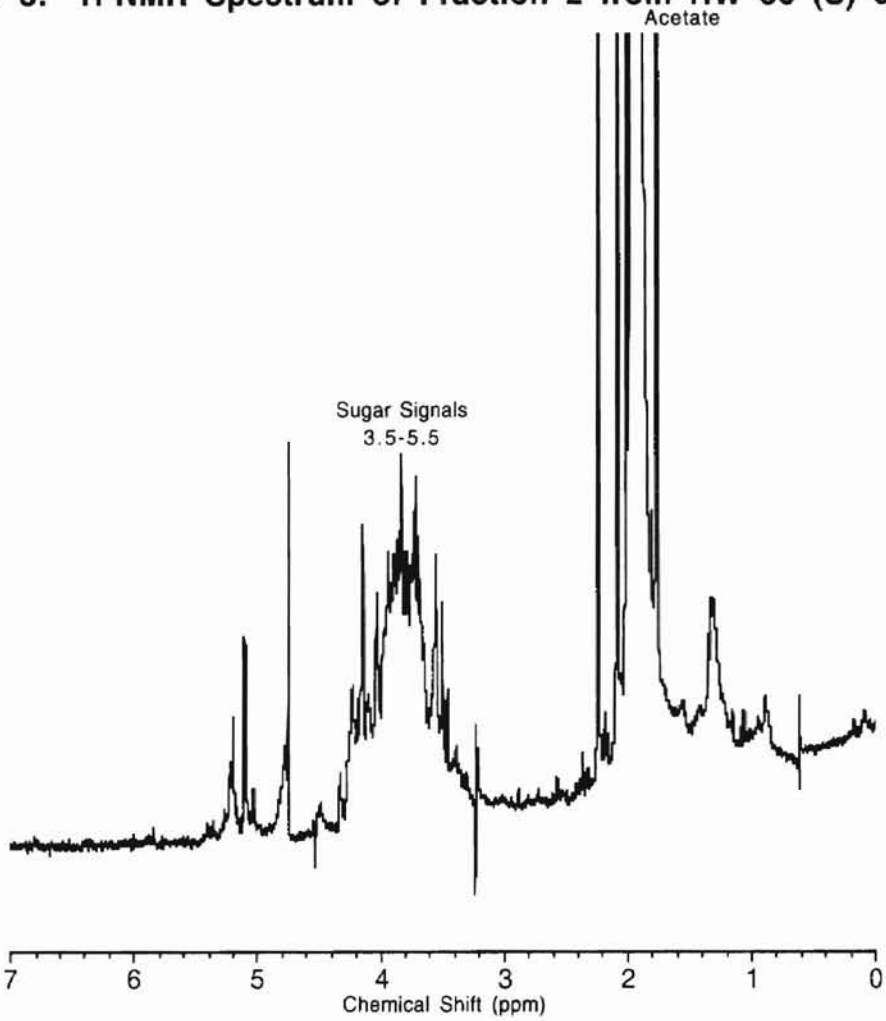


Figure 9: ^{1}H-NMR Spectrum of Fraction 3 from HW 50 (S) column

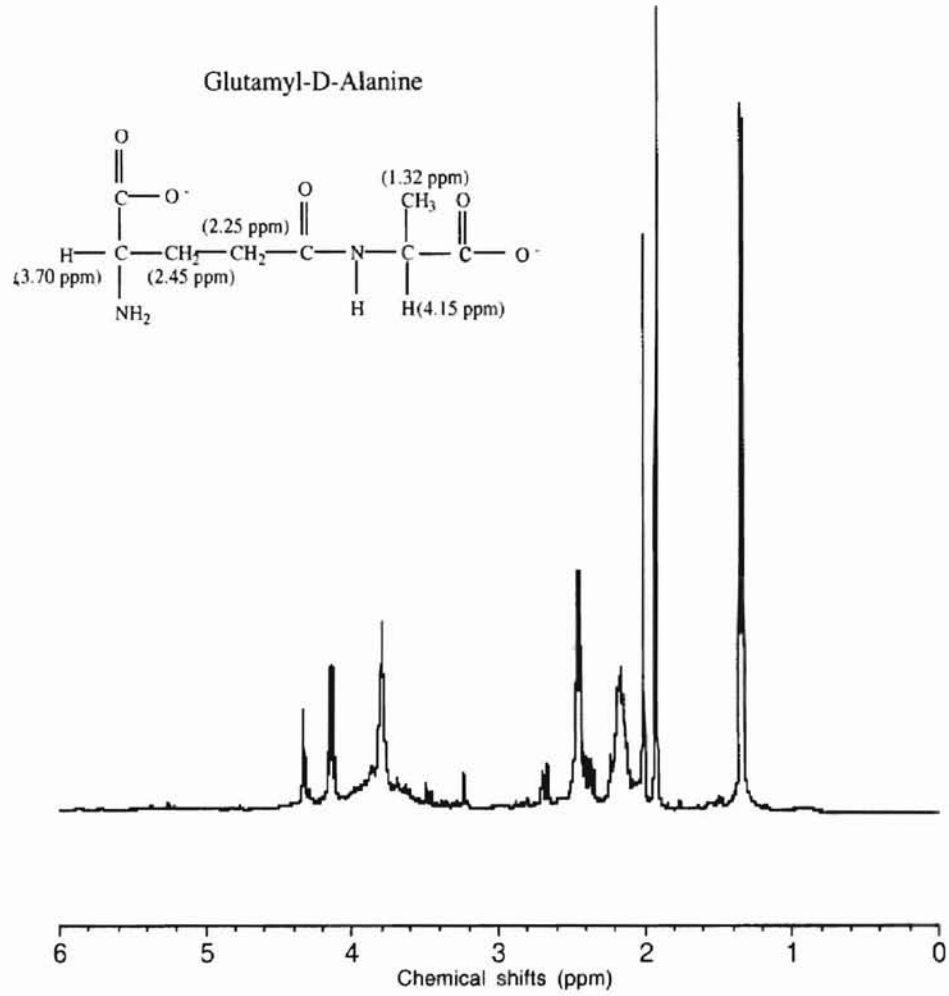
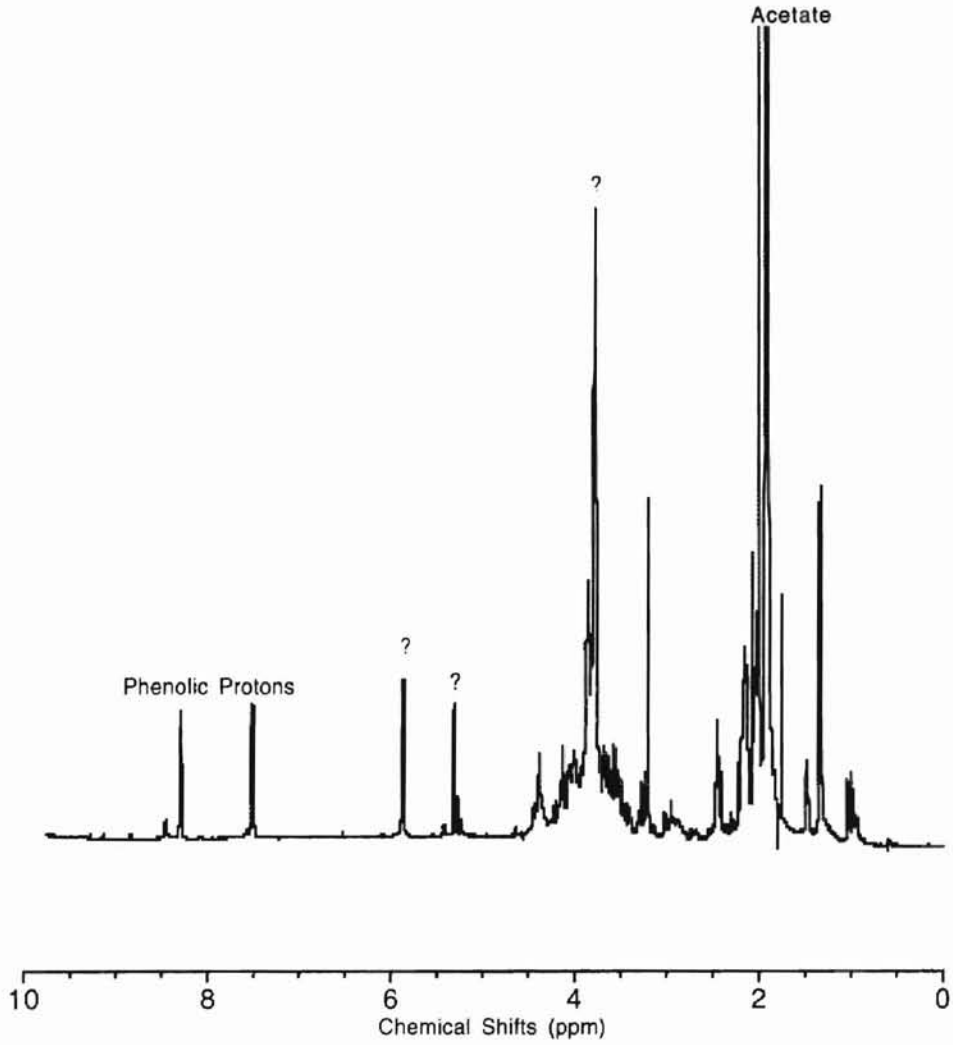


Figure 10: ^{1}H-NMR Spectrum of Fraction 4 HW 50 (S) Column



ppm and an acetate signal at 2.0 ppm. In fraction 4 (figure 10) at 7.5 and 8.3 ppm we observed the signals of phenolic protons. Phenolic signals should not be surprising, because they were also seen in maize and cowpea exudate (Bacic et al.,1983). By observing the NMR spectra, we can say that the carbohydrate material eluted mostly in fractions 1 & 2, whereas 3 & 4 contained little carbohydrate.

Fraction 3 contains very little signal from sugars, but its main component has an interesting feature. Table 1 shows fraction 3 contained 63% of the total weight of exudate. Judging from NMR data, fraction 3 contained mainly one compound. The structure of fraction 3 was determined using ^{1}H and ^{13}C-NMR Spectroscopy, FAB MS, and 2D-COSY-NMR. Figure 9 shows which proton signals were assigned to what carbons. On the alanine structure, two chemical shifts are shown. The first chemical shift was at 1.32 ppm, attributed to the CH_3 group protons. The second shift was at 4.15 ppm. The proton connected to the chiral carbon of alanine caused this chemical shift. In the glutamate structure, there were three sets of signals. At 2.25 ppm and 2.45 ppm were the signals of protons from the two CH_2 groups connected to alanine by a peptide bond. The final signal was at 3.70 ppm, which was due to a single proton connected to the chiral carbon of glutamyl. The ^{1}H-NMR spectrum along with the 2-D-NMR indicated that the structure was two amino acids: Alanine and Glutamate. They were linked to each other by a amide bond, through the R group of Glutamate to the amine group of the Alanine. The name of the structure was gamma-Alanyl-Glutamate (figure 9).

Labeling with Fluorescein Isothiocyanate (FITC)

Fluorescein Isothiocyanate (FITC) was used to randomly label the hydroxyl

groups of a polysaccharide under the influence of dibutyltin dilaurate. We estimated that the molecular weight ranges from 10,000 up to 1,000,000. This estimation was based on previous experiments we ran. In determining the molecular weight of pea mucilage we encountered some difficulties. When injecting mucilage onto the Toyo Pearl Gel Exclusion HW 65(s) column connected to a refractive index monitor, the mucilage started eluting in the void volume and did not end until the included volume. The results gave us a range of molecular weight. We tried the experiment again with the same column, but a different detector. In this experiment we used a $KMnO_4$ detector, because of the its increased selectivity of sugars (Thomas et al., 1994). The results gave us the range of molecular weight(10,000-1,000,000) as what was seen with refractive index monitor.

We confirmed that fluoresceinisothiocyanate (FITC) reacts with sodium hyaluronate (a copolymer of 2-acetamido-2-deoxy-D-glucose and glucuronic acid) dissolved in formamide (Belder et al., 1975). FITC has been used to label many other polymers. FITC will randomly label a low proportion of hydroxyl groups of a polysaccharide. We used FITC to label 50 μg of mucilage. The labeled mucilage eluted in the fractionation volume of the HW 65 (s) column and the excess FITC eluted in the included volume (Figure 11). There were a few pullulan standards labeled (Figure 12) to generate a calibration curve to determine molecular weight. The results were as follows: molecular weight 788,000 eluted at 34 minutes, molecular weight 47,000 eluted at 49 minutes, molecular weight 22,000 eluted at 53minutes, and 1-aminopyrene 3,6,8-trisulfonic eluted at 60 minutes (included volume). Mucilage eluted around 48 minutes. Log of the molecular weight vs time of labeled standards were plotted on Kaleida Graph;

Figure 11: Mucilage Labeled with FITC run on HW 65 (s) column

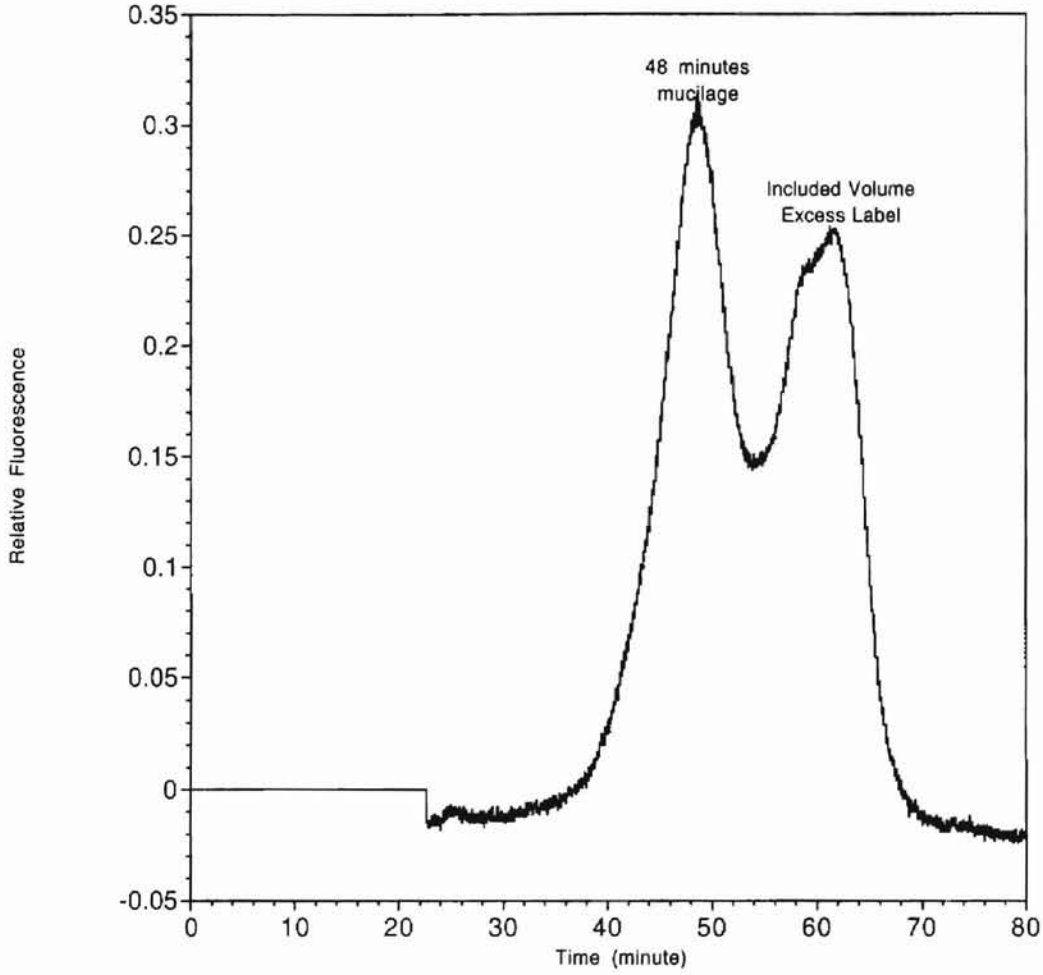
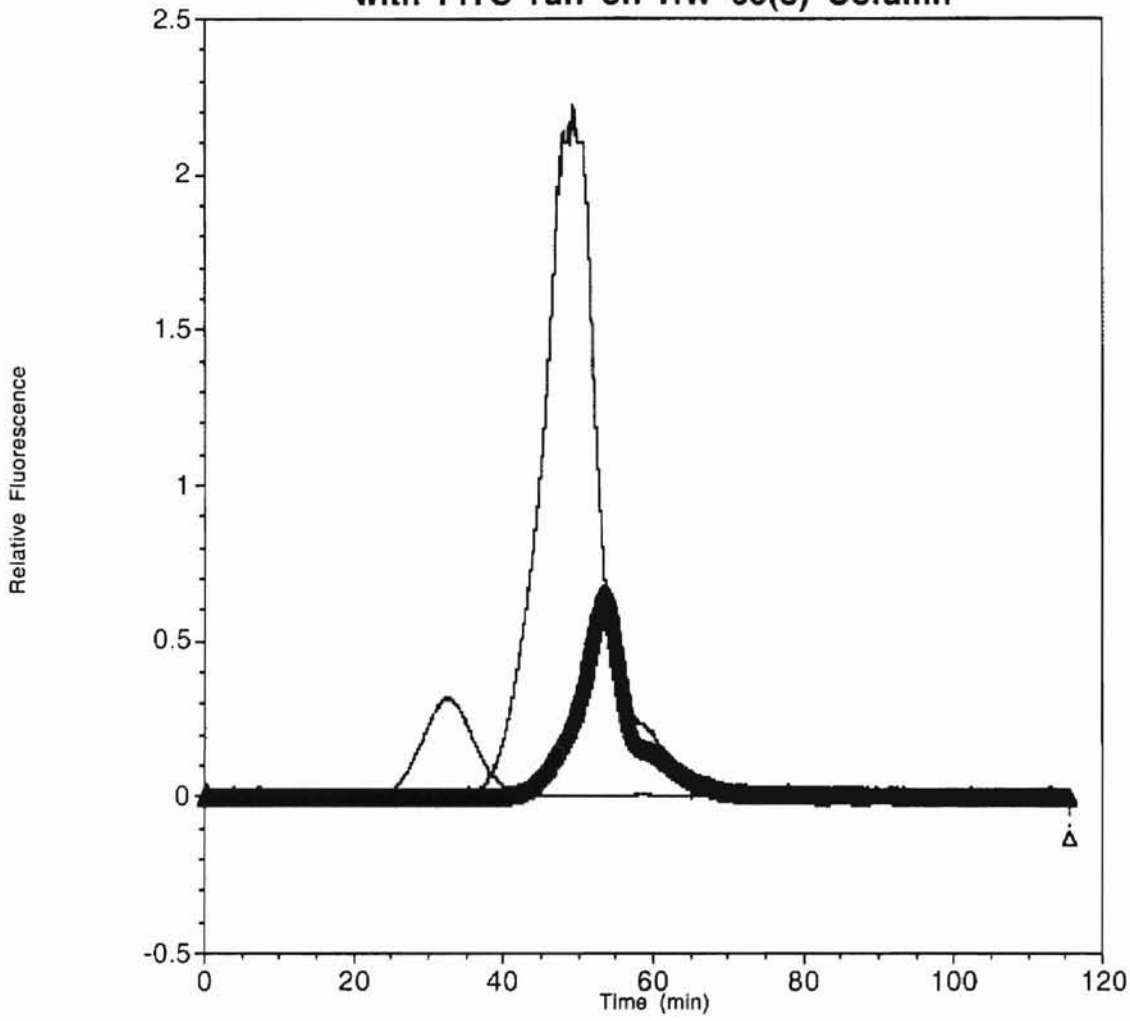


Figure 12: Pullulan Standards Labeled with FITC run on HW 65(s) Column



under these conditions mucilage has an approximate molecular weight of 56,000 (Figure 13).

Amino Acid Analysis

Amino Acid analyse were done to determine how much of the total molecular weight of mucilage contsisted of protein. By our calculations, 2-3 % of the total weight of mucilage was protein. The data were reported in mole % in table 5, which summarizes our data for amino acid analysis of pea root mucilage and compares it to what Moody et al. (1988) found in their study. We found that mucilage was rich in four amino acids: Hydroxproline (12.9%), serine (14.9 %), glycine (12.8 %), and glx (15.8 %). These results were similar to those of Moody et al. (1988) and also what has been found in AGPs (Schultz et al., 1998). They found the cowpea and maize protein moiety was characteristically rich in asparagine/asparatate, glutamate/glutamine, and glycine. In significant quantities serine, alanine, and hydroxyproline were present (Bacic et al., 1986). Our results showed proline hydroxlated, whereas, in the Moody et al. study proline was not hydroxylated.

Digestion with Endo-Arabinanase and Labeling of Oligomers with ANTS

Mucilage was digested with a commercial enzyme endo-arabinanase to fragment it into oligomers, to help determine some of its chemical structure. The reaction was allowed to go to completion. Only 1μL of sample was labeled with ANTS, because of the sensitivity of the detector. The data (figure 14) indicated that the enzyme did break up the mucilage and release oligomers. We have only seen these oligomers on the CZE, and they have not yet been identified. We also believed that this commercial enzyme

Figure 13: Log MW vs. Time on the HW 65 (s) of standards to determine MW of Mucilage

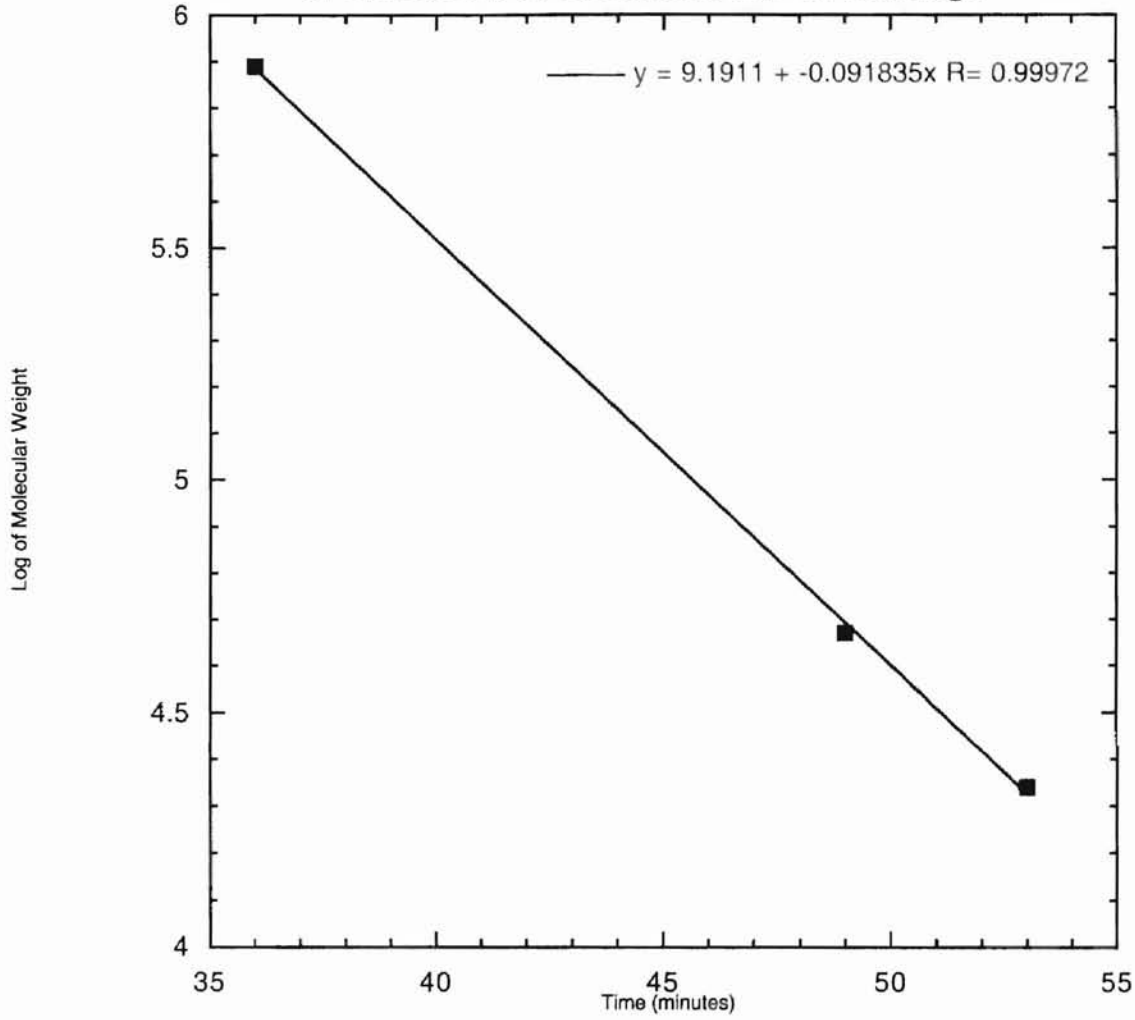
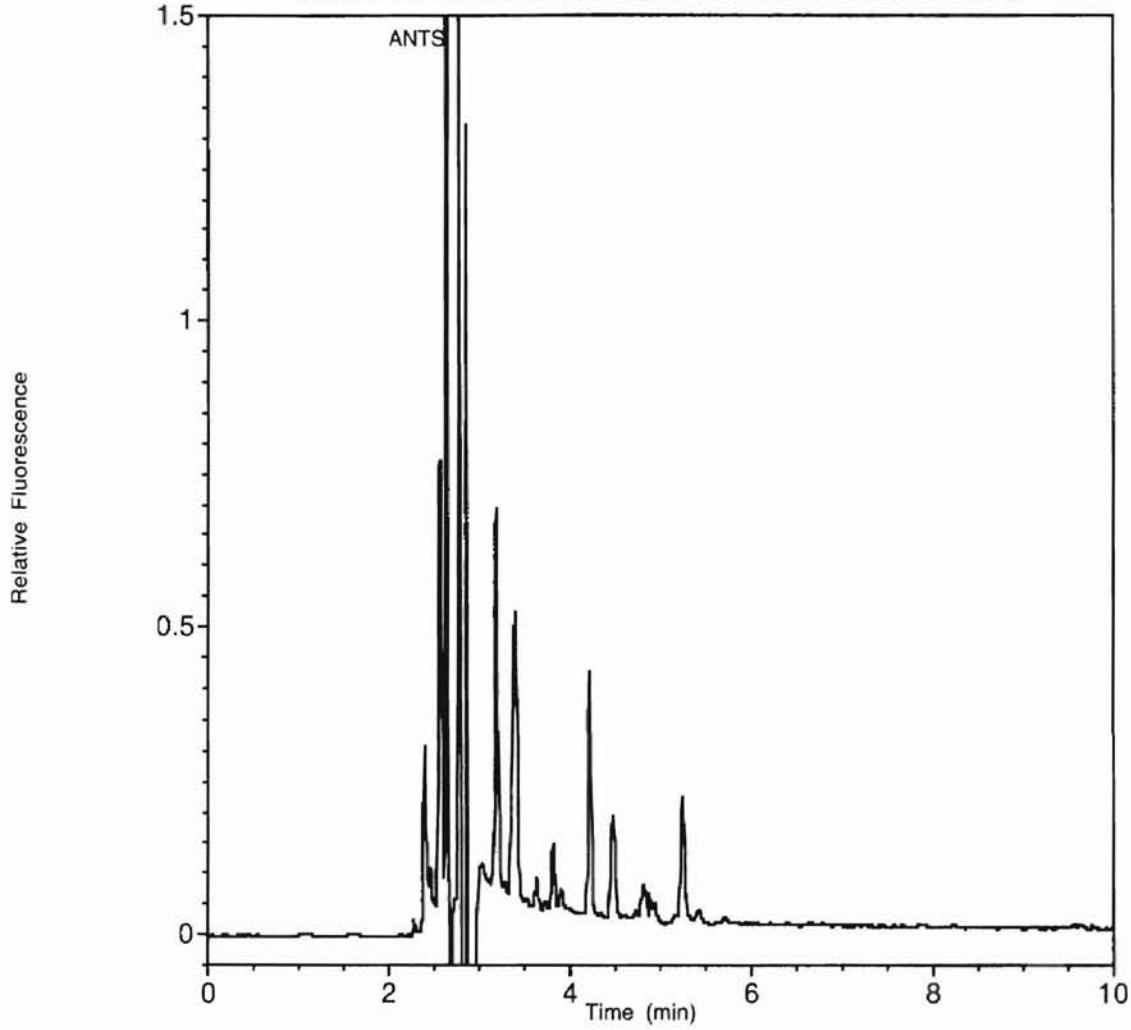


Table 5: Amino Acid Compositions of Reported Root Mucilage (mole %)

Amino Acid	Pea	Cowpea**	Wheat**	Maize**
Hyp	12.9	Tr*	0.7	0.7
Ser	14.9	7.4	8.6	6.1
Thr	9.5	5.1	7.3	5.6
Pro	6.4	5.9	6.2	8.2
Gly	12.8	13.1	10.1	13.8
Ala	8.1	7.2	9.3	8.1
His	0	0.9	1.9	1.5
Cys	0	0.4	1.1	Nd*
Glx	15.8	15.1	6.9	14.1
Asx	0.5	14.7	9.4	10.1
Tyr	4.7	1.6	3.0	1.4
Val	4.0	5.2	7.0	4.6
Met	0	0.8	1.8	1.0
Ile	2.6	3.4	4.4	2.7
Leu	2.3	5.1	7.7	4.4
Phe	2.4	2.9	3.9	2.3
Lys	1.9	2.0	5.6	1.8
Arg	1.2	2.0	3.6	2.6

*Tr=Trace, Nd=Not detected **Bacic et al., 1988

Figure 14: Mucilage digested with endo arabinanase and labeled with ANTS



was not 100 % pure.

Mucilage Cleaved with Lithium Wire in Ethylenediamine (EDA)

Lithium wire in ethylenediamine will cleave uronic acid residues. By thee monosaccharide analysis we knew that pea mucilage contained a small amount of uronic acid. We treated mucilage with EDA in the presence of small cut pieces of lithium wire to fragment the mucilage into oligomers. This was done to determine some information of the backbone of mucilage. Once the reaction was completed, we adjusted the pH and separated the products on a HW 40 (S) column. By comparing before and after lithium wire in EDA treatment, Figure 5 and Figure 15, we see that lithium wire in EDA cleaved some of the mucilage into fragments. Before treatment, the mucilage eluted in the void volume. After the treatment most of the mucilage eluted in the void volume, but after the void volume there were small fractions. We collected eight fractions. We did monosaccharide analysis and Gas Liquid Chromatography to determine the sugar composition. Our results are summarized in Table 6. Fractions 1-6 contained sugars. Fractions 7 and 8 were primarily salts from the lithium wire, but there could be very small fragments in these two fractions. According to a standard run on the HW 40 (S) column, we know that the fragments that eluted in peaks 7 and 8 had a molecular weight of approximately 500.

Mass Spectrometry

Matrix Assisted Laser Desorption/Ionization (MALDI)

The use of the MALDI-TOF mass spectrometry has been a powerful tool for

Figure 15: Mucilage treated with lithium wire in ethylenediamine HW 40 (S) column

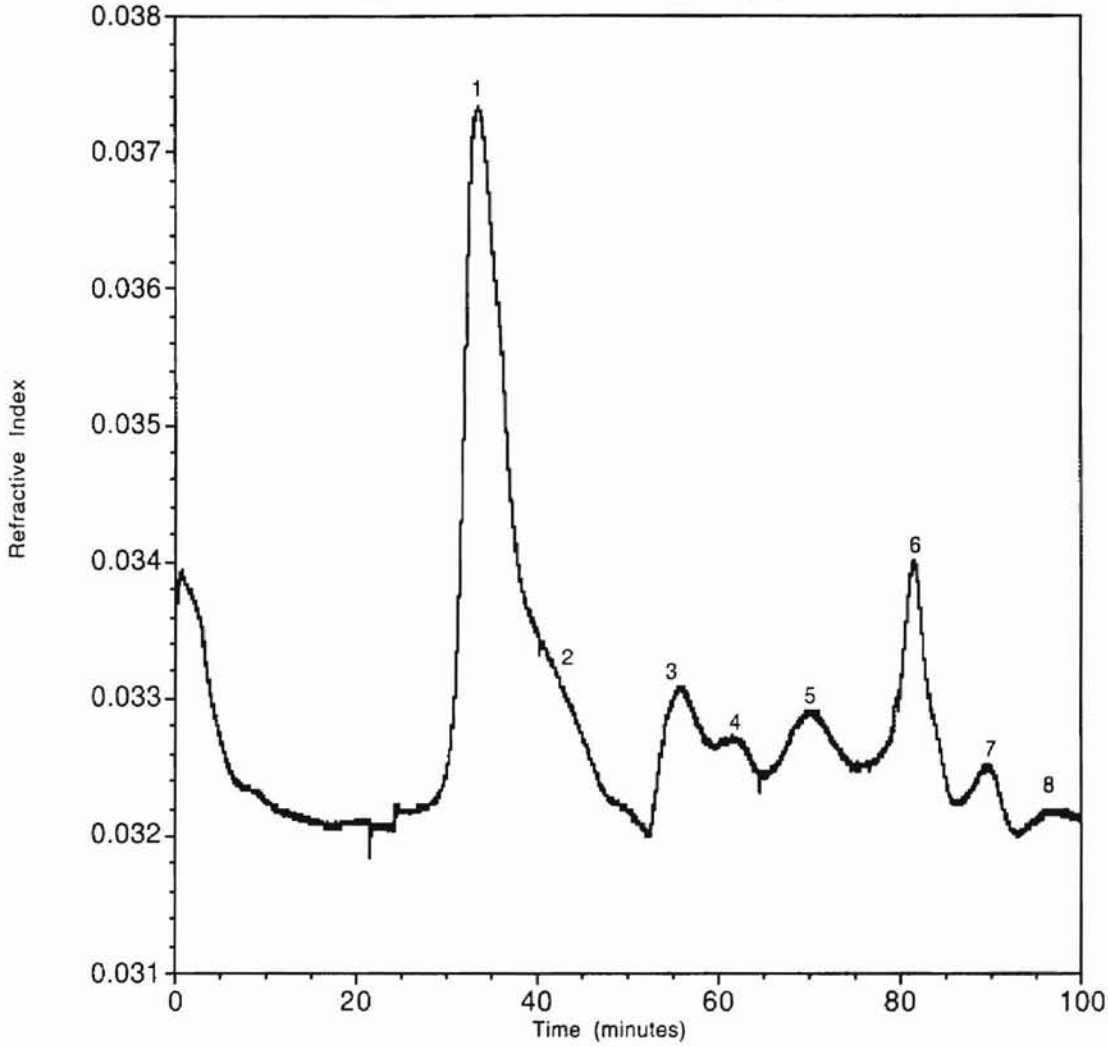


Table 6: Sugar Composition of Pea Root Mucilage of Each Fragment obtained by Lithium Wire in Ethylenediamine (mole %)

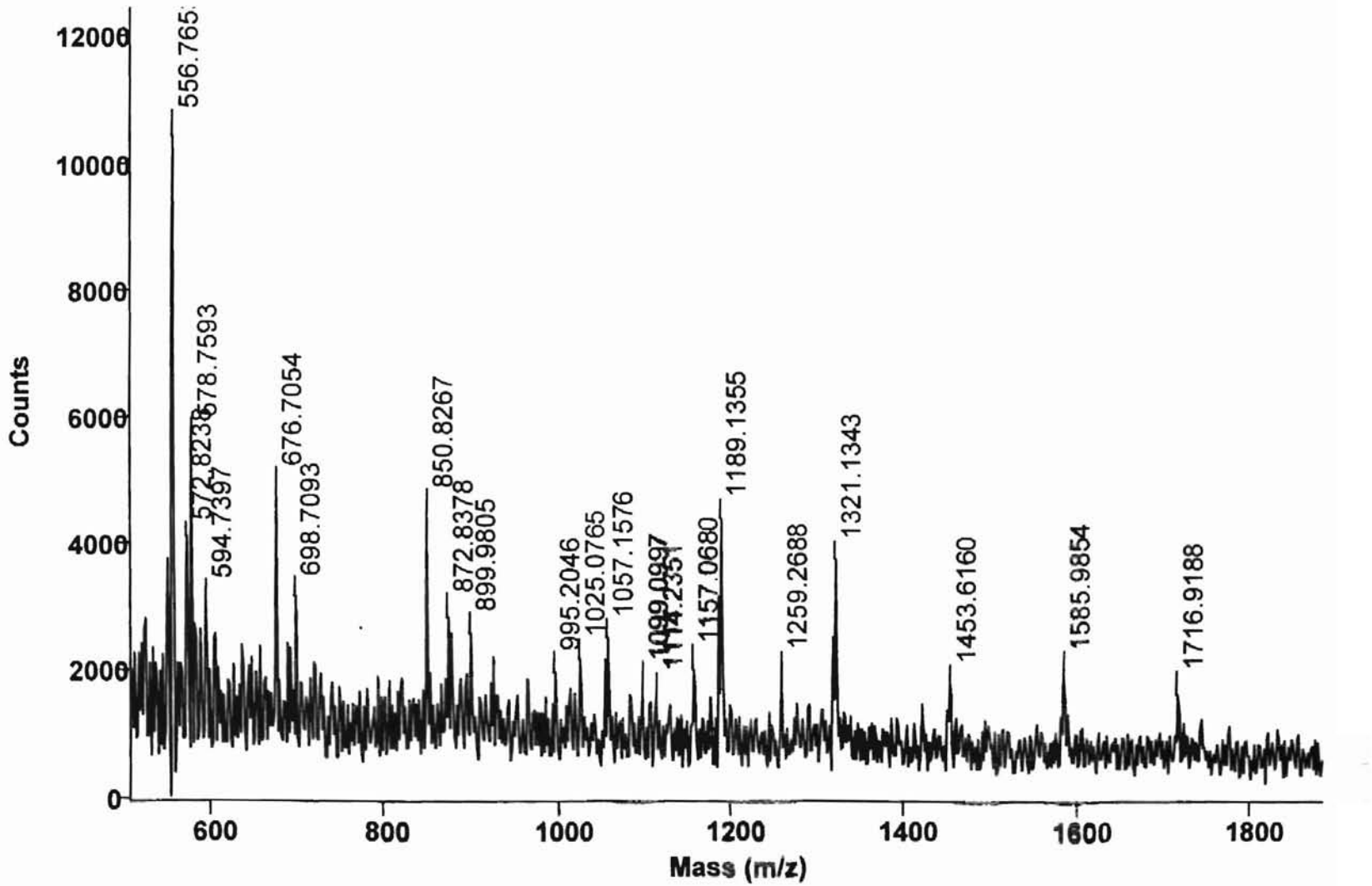
Peak	Ara	Rha	Xyl	GlcU	Man	Gal	Glc
1	35	1.2	2.2	Tr*	6.5	47	6.5
2	36.5	0	0	0	0	63.4	0
3	0	0	0	0	0	100	0
4	37	Tr*	0	0	6.67	49.1	7.23
5	33.9	Tr*	0	0	7	58.9	0
6	37.7	Tr*	0	0	6.5	55.7	0
7	0	Tr*	0	0	0	0	0
8	0	0	0	0	0	0	0

*Tr=Trace

looking at masses of oligosaccharides. We used MALDI to identify the olgiosaccharides that were created by the EDA with lithium wire treatment. All the peaks that eluted from the HW 40(S) column were examined with MALDI. Various technical problems were encountered with this experiment. First, we had the problem of desalting, the sample amount was very small. In desalting, we used a method by Korner et al. (1998). The low sample amount should not matter, because the MALDI was known for its high sensitivity. From our results, fractions 1-3, 5, and 6 that eluted off the HW 40 (S) column did not give us any mass ranges corresponds to where they eluted from the HW 40(S) column. Fraction 4 showed an arabinose series staring at 1717.25 mass (m/z) (figure 16). At 1717.25 mass (m/z) down to 1057.1576 mass (m/z), we see a difference in size of 132, which is the size of an arabinose residue. Fractions 7 and 8 showed nothing, because these fractions eluted next to the included volume. The included volume was where most of the salts eluted.

^{1}H-NMR was used to examine fractions to verify if we had sugar present in our sample. We used the 600 MHz NMR with the 3 mm probe, because of the high sensitivity. All the fractions except fraction 8 showed signals with chemical shifts (ppm) between 3.5-5.5. This convinced us that we were not removing all of the salts. If this were true, that would explain why we saw no signals with the MALDI, which is very sensitive to salts. The next method helped in concluding that fractions 1-7 contained sugar residues.

Figure 16: Mass Spectrum of Fraction 4 from the Lithium Wire in EDA run on HW 40 (S) Column.



Monosaccharide Analysis and Gas Liquid Chromatography for Sugar Composition of Mucilage Treated with Lithium Wire in Ethylenediamine.

Monosaccharide analysis and gas liquid chromatgraphy were used to determine the sugar composition of each fraction from the Toyo Pearl Gel Exclusion HW 40(S) column. Judging from the mass spectral and NMR data that the EDA with lithium wire did cleave the mucilage into fragments, we needed to consider the sugar compositions. The data are summarized in table 6. The sugar composition of all peaks indicated that the lithium wire in EDA destroyed almost all of the glucuronic acid residues. Most of the peaks contained arabinose and galactose as the dominating sugars, followed by mannose and glucose, with traces of rhamnose residues.

CHAPTER V

SUMMARY

Mucilage has been described on the root tip surface of a variety of higher plants. Many functions of mucilage have been associated with roots, soil, and bacteria that live in the soil. It is an essential polysaccharide for the root tip and in the rhizosphere. In the rhizosphere, there are various intense activities in the soil with biological, chemical, and physical implications. Mucilage in the rhizosphere was seen to come in contact with certain bacteria, such as rhizobia. Rhizobia can degrade mucilage and use it as a carbon source for its own metabolism.

There has been little work done on the chemical structure of the polymers of mucilage due to the difficulties in obtaining mucilage. For example, out of 800 seeds planted, we obtained 1 mg-2 mg per three plastic containers, which gives us an average of only 1-2 μg per plant. From our experiments with rinsing the sand and with the Yariv antigen we are convinced that roots do not produce much mucilage under the conditions used.

Despite obtaining small amounts of mucilage, we were still able to obtain some information on its chemical structure. We can conclude that mucilage was a high molecular weight polysaccharide that elutes in the void volume of a HW 50(S) column. By combining these data with the data from labeling with FITC, we can estimate mucilage molecular weight to be approximately 56,000.

The composition of pea root mucilage was determined by variety of experiments. Mucilage was composed primarily of carbohydrates (95% w/w) and a small amount of

protein (2-3 % w/w). Cowpea, wheat, and maize root mucilages have also been shown to be primarily carbohydrates and a small amount of protein. For pea, the sugar composition was predominately arabinose and galactose followed by mannose, xylose, and glucuronic acid. Rhamnose, galacturonic acid, and glucose were detected in small amounts. In comparing pea mucilage sugar composition with other species, we found some differences. Other mucilage was shown to have fucose present in its composition, especially in maize, in which fucose was a predominate monosaccharide. Pea mucilage was unique in the fact that fucose was not present.

The linkages found in pea mucilage compared to other mucilage were similar. Methylation analysis showed there were a variety of linkages. The high amount of 3,6-linked galactopyranose and terminal arabinofuranose combined with the monosaccharide analysis, that pea root mucilage was predominately an arabinogalactan/protein mixed with other cell wall like polysaccharides. The high amount of 5-linked arabinose was surprising, because it is usually associated with rhamnogalacturon an as a side chain, which is not seen in arabinogalactans. This indicated that pea arabinogalactan was quite different from that in most species or that there was an additional arabinose containing polymer. The alpha-1, 5-linked arabinofuran in the mucilage was confirmed by our finding that endo-alpha-1, 5-arabinofuranase released oligosaccharides from it. These released oligomers have been only seen on capillary zone electrophoresis and have not been identified.

The protein composition was rich in hydroxyproline, serine, glycine, and glutamine/glutamate. This was another indication that mucilage could be derived from arabinogalactan protein. Hydroxyproline was only detected in small amounts in root

mucilages from other plant species.

The experiments using lithium wire in ethylenediamine has not been done with other mucilage species; we had no other data with which to compare our results. We feel confident that treating the mucilage with lithium wire in ethylenediamine, we did succeed in fragmenting it. The lithium wire in ethylenediamine destroyed almost all of the glucuronic acid residues. We believe that we have identified several fragments. The mass spectrum of fraction 4 contained series of peaks starting at 1057.15 m/z increasing m/z by 132. This data is consistant with the fragments containing six hexose, one hex uronic acid, and one to five pentose residues. In the monosaccharide analysis, the void volume contained almost the same sugar composition as the intact mucilage. Arabinose and galactose were the dominant sugars, and mannose, xylose and some remaining glucuronic acid followed in abundance. Throughout the remaining fractions, we saw almost the same number of arbinoses to galactoses except for fraction 3. Fraction 3 contained 100% galactose. Combining these data with the results from the mass spectra, we saw almost the same number of arbinoses to galactoses except for fraction 3. Fraction 3 contained 100% galactose. I propose that there are two possibilities for structure. The first possibility is that pea mucilage contains a galp backbone with arabinose as a side chain and with glcA residues dispersed throughout the backbone and/or throughout the arabinose side chains. The second possibility is a small backbone that consists of alternating with glcA residue and a mannose residue with complex side chains. For example, the main side chain would consist of galp residues with arabinose residues as a side chain to the main side chain. If we conclude the first possibility, then pea root mucilage could be derived from an arabinogalactan/protein, if so then it is unique

arabinogalactan/protein. However, if the second possibility is true then root mucilage looks like gum ghatti. Gum ghatti is a plant gum that are substitutes of Type II arabinogalactans.

Other evidence for arabinogalactan/protein is that arabinogalactan proteins have a high water binding capacity and can form gels, which may serve physiologically as anti-desiccants and gelling agents. The ability of mucilage to act as a lubricant and anti-desiccant is likely related to its ability to form gels. Its sugar compositions and linkages 3-, 6-, and 3,6-linked galactopyransoe were found in our mucilage and arabinogalactan type II and or arabinogalactan proteins in plant polysaccharides. Every experiment adds up to mucilage could be derived from arabinogalactan/protein, except two. First, the 5-linked arabinofuranose is not often observed with arabinogalactans, but with rhamnogalacturonans. Second, the monosaccharide analysis and the mass spectra indicated that it was a polymer with two possible structures. From these observations, we can not conclude what the exact answer of pea root mucilage chemical structure. It is hard to saw which possibility is right, because of the small amount of mucilage we had to work with. Both possibilities are link to arabinogalactan/protein. Whatever the possibility of structure is, we can say that either one was derived from an arabinogalactan/protein. We also can say that mucilage is unique from all the other arabinogalactan species, or that there was an additional arabinose-containing polymer. The mechanism to make such a unique polysaccharide has to involve something more sophisticated than for most arabinogalactan/proteins species. This unique organization of these polymers could explain the specificity that mucilage has on certain ions and microorganisms.

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VITA

Angela Renee Jones

Candidate for the degree of Masters of Science

Thesis: PARTIAL CHARACTERIZATION OF PEA ROOT MUCILAGE

Major Field: Biochemistry and Molecular Biology

Biographical:

Education: Received Bachelor of Science degree in Cellular and Molecular Biology at Oklahoma State University, Stillwater, OK, USA in May 1996. Completed requirements for Master of Science Degree at Oklahoma State University, Stillwater, OK USA in May 1999.

Professional Experience: Teaching Assistant in Department of Chemistry, Oklahoma State University, Stillwater, OK, USA, Jan. 1999, to present. Research Assistant, Department of Biochemistry, Oklahoma State University, Stillwater, OK USA, April 1997-Dec. 1998. Teaching Oklahoma Principle of Science Scholar for minorities in Graduate College, Oklahoma State University, Stillwater, OK, USA, Summer 1997 and 1998. Research Assistant in Department of Biochemistry, Oklahoma State University, Stillwater, OK, USA, Oct. 1995 to April 1997.

Professional Memberships: American Association for the Advancement of Science American Society of Plant Physiologist, Phi Lambda Upsilon Honorary Chemical Society.