CHANGES IN HOMOGALACTURONANS, POLYGALACTURONASE ACTIVITIES, AND CELL WALL LINKED PROTEINS DURING COTTON COTYLEDON EXPANSION

By

ZHIQUAN ZHANG

Bachelor of Science in Plant Biochemistry

Northeast Agricultural University

Harbin, China

1982

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1998

Thesis
1998D
Z63c

CHANGES IN HOMOGALACTURONANS, POLYGALACTURONASE ACTIVITIES, AND CELL WALL LINKED PROTEINS DURING COTTON COTYLEDON EXPANSION

Thesis Approved:

Andrew Mort
Thesis Adviser

Margaret L. Pierce

Richard C Essenberg

[illegible]

Robert L Matts

Wayne B. Powell
Dean of the Graduate College

ACKNOWLEDGMENT

I wish to express my sincere appreciation to my major adviser, Dr. Andrew Mort for his guidance, encouragement, patience, advice and friendship throughout my graduate program. I would like to thank Dr. Ziad El Rassi, Dr. Margaret Pierce, Dr. Robert Matts, and Dr. Richard Essenberg for serving on my graduate committee, and for their helpful suggestions throughout the study.

I wish to express my sincere gratitude to Dr. Jinhua An for his suggestion and friendship. I would like to thank Dr. Jerry Merz for his help with computer analysis of the Data. I thank all my colleagues in Dr. Mort's lab and all my friends for their cooperation and assistance.

I would like to thank my whole family for their affection, patience, support, and understanding throughout my study.

Finally, I would like to thank the Department of Biochemistry and Molecular Biology, Department of Energy, and Oklahoma Agricultural Experimental Station for this opportunity, and for my graduate assistantship.

TABLE OF CONTENTS

Chapter		Page
I	GENERAL INTRODUCTION	1
	Literature Cited	9
II	DETECTION AND DIFFERENTIATION OF PECTIC ENZYME ACTIVITY *IN VITRO* AND *IN VIVO* BY CAPILLARY ELECTROPHORESIS OF PRODUCTS FROM FLUORESCENT-LABELED SUBSTRATE	13
	Summary	13
	Introduction	14
	Materials and Methods	15
	Materials	15
	Plants	16
	Substrate preparation	16
	Oligomer derivatization	17
	Enzyme reaction	18
	Capillary electrophoresis	19
	Results and Discussion	20
	In vitro assay of enzyme activity	20
	In vivo assay of enzyme activity	26
	Concluding Remarks	28
	References	30
III	CHANGES IN HOMOGALACTURONANS AND ENZYMES DEGRADING THEM DURING COTTON COTYLEDON EXPANSION	31
	Abstract	31
	Introduction	32
	Materials and Methods	33
	Plant materials	33
	PG activity *in vivo*	34
	Standard Curve	35
	Cell wall preparation	36
	HF solvolysis and HG isolation	36
	HPLC gel filtration analysis	37
	Sugar composition analysis	38
	Deesterification of HGs	38
	Degree of esterification of HGs	39
	HG size distribution	39
	Results and Discussion	40
	Enzyme activities *in vivo*	41
	Cotyledon cell wall isolation	46

Chapter Page

Carbohydrate composition of cotyledon walls 46
HG molecular size distribution 51
Degree of esterification of HGs 55
Literature Cited 57

IV AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY OF THEIR 6-AMINOQUINOLYL-N-HYDROXYSUCCINIMIDYL CARBAMATE DERIVATIVES 60

Summary 60
Materials and Methods 61
Materials 61
Running buffer preparation...61
Standard amino acid derivatization 61
Protein hydrolyzation.. 62
Amino acid separation.. 62
Results and Discussion... 63
Separation mechanism... 63
Standard amino acid composition analysis............................ 66
Protein amino acid composition analysis.............................. 66
References 71

V GENERAL DISCUSSION.. 72
Literature Cited... 83

LIST OF TABLES

Table | Page

1. Weight of sugars (mg) in extracts from 500 mg 3-day-old cotton cotyledon cell walls.......... 48

2. Weight of sugars (mg) in extracts from 500 mg 5-day-old cotton cotyledon cell walls.......... 49

3. Weight of sugars (mg) in extracts from 500 mg 7-day-old cotton cotyledon cell walls.......... 50

4. Degree of esterafication of HGs from water extract and immidazole extract of different age cotyledons.......... 56

5. Reproducibility for retention time and peak response 67

6. Amino acid analysis of lysozyme protein hydrolysates 68

7. Amino acid analysis of presumed wir-1 protein hydrolysates using AQC-precolumn derivatization 69

8. Analyses of amino acid compositions of cell wall linked proteins during cotton cotyledon expansion.......... 81

LIST OF FIGURES

Figure Page

1. Chromatography on ion-exchange column of the 24% KOH extract followed EPG treatment of cell walls from 7 day old cotton cotyledons 3

2. Model of the pectin structure of plant primary cell walls 5

3. Electrophoretic behavior of GalA hexamer and products from the activity of *Aspergillus niger* endopolygalacturonase on the ANTS-labeled hexamer of GalA 21

4. Electrophoretic behavior of oligomers of GalA. A) Mixture of ANTS-labeled GalA oligomers. B) Same as A but treated with NaOH to remove secondary peaks 23

5. A) Electropherogram of products from ANTS-labeled hexamer of GalA digested to completion with endopolygalacturonase from *Erwinia carotovora.* B) Electropherogram of products from ANTS-labeled hexamer of GalA digested with pectolyase. "R" indicates reagent peak; peak numbering reflects degree of polymerization 25

6. *In vivo* pectic enzyme activity. Panels: A) Intercellular wash fluid (IWF) from cotyledons in which no ANTS-labeled substrate had been injected. B) IWF from cotyledons in which the rinsing of the intercellular spaces was started as soon as the labeled hexamer had been injected. C) IWF prepared after a 10-min incubation of labeled hexamer in the cotyledons. D) IWF after a 2-h incubation of labeled hexamer in the cotyledons 27

7. The areas of both the larger and smaller cotyledons on several cotton seedlings were measured with an area meter and plotted against the number of days after emergence from the soil 42

8. The mean and standard deviation of the enzyme activity detected in the intercellular spaces of cotyledons from three to nine days after emergence from the soil 43

9. The standard curve for polygalacturonase activity 45

10. Summary of method to extract cell walls from leaves 47

11. Toyopearl HW 50S molecular size profiles of homogalacturonans in water extract from (A). 3-day-old, (B). 5-day-old and (C)

Figure Page

7-day-old cotton cotyledon cell walls.. 52

12. PA 100 anion-exchange chromatography molecular size profiles of homogalacturonans in water extract from (A). 3-day-old, (B). 5-day-old and (C). 7-day-old cotton cotyledon walls. Panel D shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule in each fraction.. 53

13. PA 100 anion-exchange chromatography molecular size profiles of homogalacturonans in immidazole extract from (A). 3-day-old, (B). 5-day-old and (C). 7-day-old cotton cotyledon walls. Panel D shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule in each fraction 54

14. An electropherogram of the standard mixture of labeled amino acids using UV absorbance for detection 64

15. An electropherogram of the standard mixture of labeled amino acids using fluorescence for detection.. 65

16. The modes of polygalacturonase on ANTS labeled hexamer......................... 74

LIST OF ABBREVIATIONS

2-AP	2-aminopyridine
AGPs	arabinogalactan
Ala	alanine
ANTS	8-aminonaphthalene-1,3,6-trisulphonate
AQC	6-aminoquinolyl-N-hydroxysuccimidyl carbamate
Ara	arabinose
Arg	arginine
Asp	aspartic acid
Cys	cysteine
CZE	high performance capillary zone electrophoresis
DM	degree of methyl esterification
EPG	endopolygalacturonase
Gal	galactose
GalA	galacturonic acid
GC	gas chromatography
Glc	glucose
Glu	glutamic acid
Gly	glycine
GRPs	glycine-rich proteins
HG	homogalacturonan
His	histidine
HF	hydrogen fluoride

HPLC	high performance liquid chromatography
HRGPs	hydroxyproline-rich glycoproteins
Ile	isoleucine
Leu	leucine
IWF	intercellular wash fluid
Lys	lysine
Man	mannose
Met	methionine
MECC	micellar electrokinetic capillary chromatography
M.W.	molecular weight
NMR	nuclear magnetic resonance
PME	pectin methylesterase
PAW	phenol : acetic acid : water = 2 : 1 : 1 (v : v : v)
Phe	phenylalanine
PG	polygalacturonase
Pro	proline
PRPs	proline-rich proteins
Rha	rhamnose
RGaseA	rhamnogalacturonase A
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
Ser	serine
SCB	sodium cyanoborohydride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Thr	threonine

Trp	tryptophan
Tyr	tyrosine
Val	valine
Xyl	xylose

CHAPTER I

GENERAL INTRODUCTION

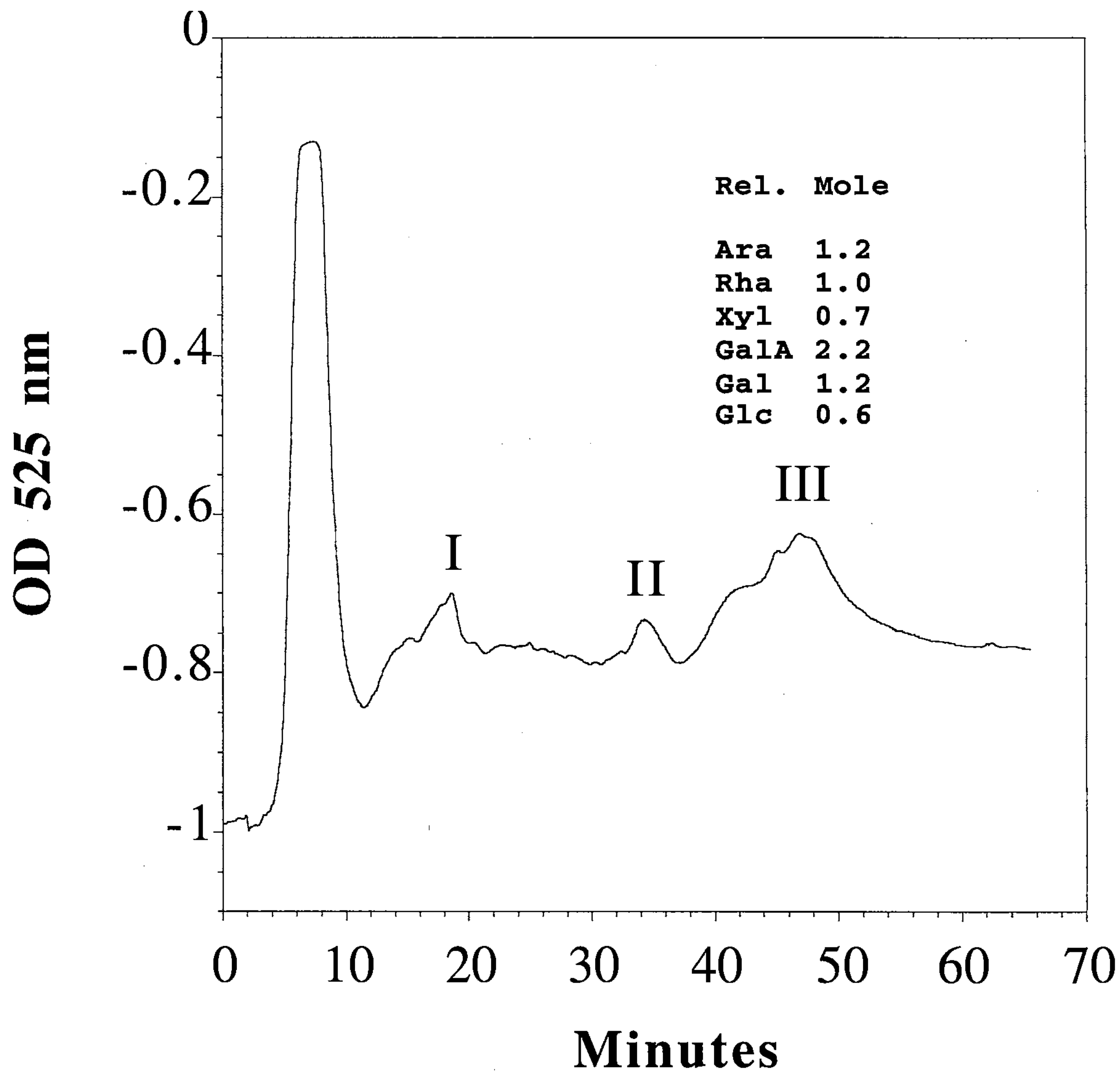
Plant cell walls are widely regarded as the major controlling factor in cell expansion. Cell walls influence cell growth by controlling cell size and shape. Cell walls do not normally get thinner during expansion. Thus, controlled, irreversible, turgor-driven extension of plant cells must involve the breakage and reformation of linkages within the cell walls (Taiz, 1984). When a cell grows, the bonds between existing wall polysaccharides are broken, and as the wall expands, newly synthesized wall polysaccharides are inserted between existing ones. In this way, cells can elongate many times their length without weakening the wall (Albersheim et al, 1996). To complete this complicated physiological process, degrading and reforming enzymes must be involved. Recently, the breakage of wall polysaccharides through disruption of cellulose-hemicellulose interactions by expansions (Cosgrove, 1993) and through cleavage of xyloglucans followed by relinkage by xyloglucan endotransglycosylase (Silva et al, 1994) has been extensively studied during plant growth and differentiations. However, little is known about breakage and reformation of the pectic polysaccharides during plant growth and development.

Plant cell walls form a single continuous extracellular matrix through the body of the plant and the walls of many cells together form the skeleton of plant tissues. The cell matrix consists of various types of polysaccharides, proteins, and lignins in varying amounts, organized in such a way that the cell wall is chemically rather stable and physically robust. The three major domains in plant cell walls were described in recent cell wall models (McCann and Roberts, 1991; Talbott and Ray, 1992; Carpita and Gibeaut,

1993). They are the cellulose-xyloglucan net-work, the pectic polysaccharides, and the structural proteins. These three domains are structurally independent and functionally interacting. This is in contrast with early models in which the matrix polymers were thought to be covalently crosslinked (Keegstra et al, 1973). Most recently, we found cross-linking between RGI and xyloglucan from cotton cotyledon cell walls (Zhang and Mort, unpublished). The 7 day old cotton cotyledon cell walls were treated with EPG and the cell wall polymers were solubilized with 24% KOH. The alkali extract was separated by ion-exchange chromatography. Sugar composition analysis indicated that there may be a RGI-Xyloglucan complex in expanding cotton cotyledon cell walls (Figure 1). Plant cell walls are divided into primary cell walls and secondary cell walls. The walls of growing plant cells are called primary cell walls consisting about 90% of the structural polysaccharides, such as cellulose, hemicellulose, and pectins. All secondary cell walls develop from primary cell walls. Cells no longer grow once lignin is added to their cell walls (Albersheim et al, 1996).

Pectin is one of the important constituents of plant cell walls. Pectic polymers contain a high proportion of galacturonic acid residues (Mort et al, 1998; Schols and Voragen, 1996). Three pectic polysaccharides in all primary cell walls have been extensively studied. They are homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). Recently another pectic polysaccharide based on an HG backbone has been discovered (Schols et al, 1995) and will probably become widely recognized as a component of cell walls. This is xylogalacturonan which consists of HG with single xylose residues linked directly to O-3 of many of the GalA residues. RGI is the major polysaccharide from suspension-cultured sycamore cell walls and found to represent 7-14% of the cell wall (McNeil et al, 1984). It has a backbone composed of as many as 100 repeats of the disaccharide [-2)-α-L-rhamnose-(1-4)-α-D-galacturonic acid-(1-]. Arabinosyl- and galactosyl-rich side chains are attached to *0* -4 of the rhamnosyl residues. (McNeil et al, 1980; Lau et al, 1985). RGI from cotton-suspension cells was obtained by

Figure 1. Chromatography on an ion-exchange column of the 24% KOH extract followed EPG treatment of cell walls from 7 day old cotton cotyledons. Sugar composition analysis of the fractions I, II and III indicated on the graph showed that fraction III was a complex of RGIs and Xyloglucans. The co-solubilization of RGIs and Xyloglucans means these two polysaccharides may link to each other within the plant cell walls.

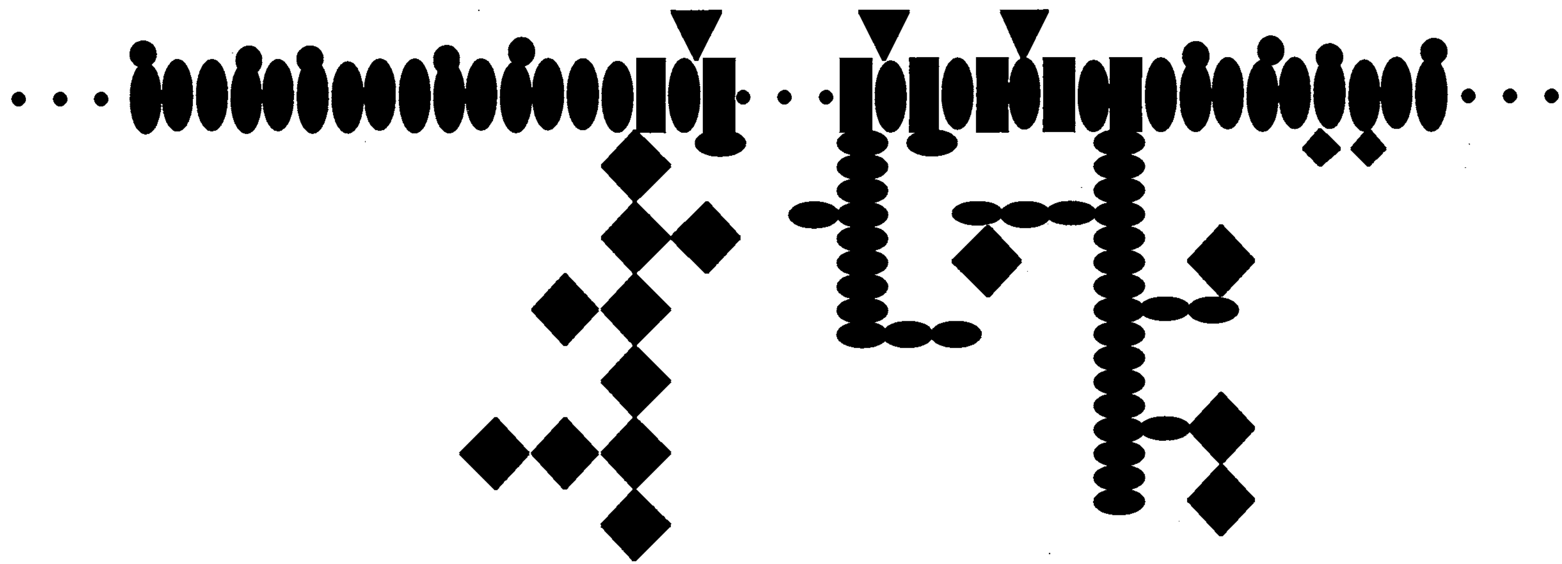

0
-0.2
-0.4
-0.6
-0.8
-1
OD 525 nm
0
10
20
30
40
50
60
70
Minutes
I
II
III
Rel. Mole
Ara 1.2
Rha 1.0
Xyl 0.7
GalA 2.2
Gal 1.2
Glc 0.6

anhydrous hydrogen fluoride (HF) treatment of cell walls at different temperatures, and then solubilized, purified and partially characterized (Mort et al, 1998; Mort et al, 1993; Mort et al, 1991; Mort, 1983; Mort and Bauer, 1982). RGII is a short section of HG with complex side chains of 7-9 residues attached via apiose, and shorter sidechains attached via ketodeoxyoctulosonic acid (McNeil et al, 1984; O' Neil et al, 1996). HG is a homopolymer of 1-4 linked α-D-galacturonic acid residues that may be esterified on galacturonic acid residues, and the extent to which different pectic fractions are esterified can vary greatly (McCann et al, 1994). Such extensive structural analysis of pectin polysaccharides in recent years has provided a detailed characterization of the linkage of HG and RGI. Both of which can be presented in a single pectin polymer (Figure 2).

In the food industry, pectins are commonly used in food additives as stabilizers, thickeners, and gel formers. Pectins are used in food nutrition because they are considered as dietary fiber. In health care, pectins and pectic polysaccharides are involved in several pharmacological activities, such as immunostimulating activity, anti-metastasis activity, anti-nephritis activity, hypoglycemic activity, and cholesterol decreasing effect. Pectins are also applicable for drug delivery and as a vaccine for typhoid fever (Yamada, 1996).

In biology, pectins are multifunctional compounds whose functions include structural, physiological, developmental and defensive roles. Pectins have a cohesive function between cells, because polyanionic galacturonan backbones are capable of binding calcium, resulting in their aggregation and the formation of gels (Jarvis, 1984). Oligomers of galacturonic acid of different lengths play different roles in plant physiological processes. Oligomers of lengths 2, 3, and 12 can induce protease inhibitors (Ryan, 1984), suppress lignification (Moerschbacher at al, 1990) and flowering and can elicit phytoalexin production (Albersheim et al, 1992; Northnagel et al, 1983). The most recent work has shown that the pectic oligosaccharides can promote ion pumping, production of active oxygen species, and phosphorylation of proteins. They were proposed to be components of pathways for transduction of plant hormone signals (Ryan and Farmer, 1991).

Figure 2. Model of the pectin structure of plant primary cell walls. It shows the linkage between homogalacturonan with some methyl and non methyl esterification and rhamnogalacturonan I with some complicated side chains.


Rha
Xyl
Acetyl
Gal
GalA
Ara
Methyl

The role of matrix pectic polysaccharides in growing cell walls, especially in fruit ripening has been extensively studied. Downshifts in pectin molecular size were observed during leaf aging (Arribas et al., 1991) and fruit ripening (Huber, 1983, 1992; Koch and Nevins, 1989; Huber and O'Donoghue, 1993). Changes in sugar composition of the cell walls along the axis of maize roots has been reported (Masuda and Pilet, 1983). Changes in RGI during fruit softening were reported (Fischer and Bennett, 1991; Nogata, 1996). Hegde found that there was an increase in a large apparent molecular size polymers, which was enriched with arabinose and rhamnose as fruits softened. (Hegde, 1995). McCann and Roberts found that a branched RG with a short side chain increases in amount during the time-course of differentiation of *Zinnia* mesophyll cells (McCann and Roberts, 1996). However, there are no reports on the changes in pectic polysaccharides during cotyledon expansion.

A number of pectin-degrading enzymes has been identified and their activities shown to correlate with plant growth and fruit ripening. Fruit ripening in many cases involves solubilization of pectin by the combined action of endopolygalacturonase (EPG) and pectin methylesterase (PME) (Crookes and Grierson, 1983). However, neither EPG nor PME-mediated pectin degradation alone accounts for the significant changes in fruit texture that accompany ripening. In transgenic tomato plants antisense inhibition of EPG activity to as little as 0.5% of wide-type levels does not prevent fruit softening (Smith et al, 1990). Similar results were obtained in tomato fruit in which PME was down-regulated using antisense RNA (Tieman et al, 1992). These results indicate that in addition to EPG and PME, other hydrolytic enzymes such as glycosidases (Gross et al 1995; Cheng and Huber 1997) and rhamnogalacturonase A (RGaseA) (Gross et al 1995) are likely to be involved in the textural changes associated with ripening.

Recently, we have established a new method to measure PG activities *in vivo* and *in vitro* (Zhang et al, 1996). HG oligomers, as an enzyme substrate, were obtained from autolysate of commercial pectic acid and the oligomers were labeled with ANTS. The

ANTS labeled oligomers were injected into the intercellular space of different age cotton cotyledons. After a certain time of incubation, the products were rinsed out by centrifugation from the whole cotton cotyledons and analyzed by capillary electrophoresis. Using this method, we simultaneously measured two PG activities and distinguished endo- and exo-enzyme activity and enzyme acting mode (Chapter 2) during cotton cotyledon expansion. Our preliminary results indicated that endo- and exo-PG may be involved in cotton cotyledon expansion (Zhang et al, 1996; Chapter 3).

Plant cell wall proteins such as extensins, the glycine-rich proteins (GRPs), the proline-rich proteins (PRPs), the solanaceous lectins, the arabinogalactan proteins (AGPs) and many functional enzyme proteins located in the cell walls have been extensively studied. Cell wall proteins account for about 10 percent of primary cell wall dry weight of dicotyledon plants. Extensins, a family of hydroxyproline-rich glycoproteins (HRGPs), for instance, are the best characterized and perhaps the most abundant structural proteins of dicot plant cell walls (Cooper et al 1987; Tierney and Varner, 1987; Cassab and Varner 1987; and Showalter, 1993). Extensins may be covalently cross-linked to some wall polysaccharides (Keegstra et al, 1973). The biochemical evidence for a covalent cross-link between extensin-pectin has been found by Qi and Mort (1995). Extensins may play important roles in plant growth (Lampport, 1980). However, little is known about the changes in wall proteins during plant growth and development.

Most recently, we have developed a sensitive method of amino acid analysis by capillary electrophoresis using 6-aminoquinolyl-N-hydroxysuccimidyl carbamate (AQC) precolumn derivatization (Zhang and Mort, 1997). Using this method we analyzed the cell wall proteins from cotton cotyledon of different ages. Our preliminary results indicated that dehydrated cotyledon primary cell walls were about 10% protein. There were no significant changes in amino acid composition but a decrease in the amount of cotyledon wall proteins as cotyledons expand (Chapter 5).

The techniques for isolation and characterization of plant cell wall pectins have been greatly developed. We can isolate, purify and characterize the pectin polysaccharides from plant tissues and suspension-cultured cells by using different strategies (Mort et al, in preparation) such as enzyme application, specific chemical agents, gas chromatography, HPLC, capillary electrophoresis, mass and NMR spectrometry. However, we do not know if there are any changes in HGs and the enzymes working on them during plant growth. Our work is intended to form the basis for identifying the factors responsible for plant growth and development.

In order to understand the architecture of plant cell walls and what function each component polymer plays during growth and differentiation of plants, one can compare the changes in enzyme activity levels and in the cell wall polymers during plant growth and development. The changes either in polymers or in enzymes may play an important role in plant growth. The most attention about models of cell growth has recently been paid on hemicellulose and cellulose-hemicellulose interactions, but pectin is one of the important components of all plant primary cell walls and the most abundant component in cell walls of most vegetative plant parts. Structurally, pectins form an important domain in the cell walls and may be cross-linked with hemicellulose or other wall polymers. Functionally, pectins are multifunctional compounds whose functions include structural, physiological, developmental and defensive roles. Evolutionarily, pectins have retained their structural complexity. All these characteristics of pectins suggest that they must have some functions in plant growth and development. Here, we focuses on the changes in homogalacturonans, polygalacturonase activity and wall proteins during cotton cotyledon expansion. We hope to be able to discern the role of pectins in plant growth.

LITERATURE CITED

Albersheim P, Darvill AG, Augar C, Cheong J-J, Eberhard S, Hahn MG, Marfa V, Mohnen D, O'Neill MA, Spiro MD, York WS (1992) Oligosaccharides: oligosaccharide molecules. Acct. Chem. Res. **25:** 77-83.

Albersheim, P, Darvill AG, O'Neill MA, Schols HA, Voragen AGJ (1996) In J. Visser and A.G.J. Voragen. An hypothesis: The same six polysaccharides are components of the primary cell walls of all higher plants. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., pp 47-55.

Arribas A, Revilla G, Zarra I, Lorences EP (1991) Changes in cell wall polysaccharides during the growth of *Phaseolus vulgris* leaves. J. Exp. Bot. **42:** 1181-1187.

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistencey of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1-30.

Cassab GI, Varner JE (1987) Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J. Cell Biol. **105:** 2581-2588.

Cheng GW, Huber DJ (1997) Carbohydrate solubilization of tomato locule tissue cell walls: Parallels with locule tissue liquefaction during ripening. Physiol. Plant. **101:** 51-58.

Cooper JB, Chen JA, Holst GJ, Varner JE (1987) Hydroxyproline-rich glycoproteins of plant cell walls. Trends Biochem. Sci. **12:** 24-27.

Cosgrove DJ (1993) How do plant cell walls extend? Plant Physiol. **102:** 1-6.

Crookes PR, Grierson D (1983) Ultrastructure of tomato fruit ripening and the role of polygalacturonase isozymes in cell wall degradation. Plant Physiol. **72:** 1088-1093.

Fischer RL Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. Ann. Rev. Plant Physiol. **42:** 675-703.

Gross KC, Starrett DA, Chen H-J (1995) Rhamnogalacturonase, α-galactosidase, and β-galactosidase: potential roles in fruit softening. Acta Hort. **398:** 121-129.

Hegde S (1995) Changes in cell wall polysaccharides during softening of 'Belle of Georgia' peaches. Ph D thesis, Oklahoma State University, Stillwater.

Huber DJ (1983) Polyuronide degradation and hemicellulose modification in ripening tomato fruit. J. Amer. Soc. Hort. Sci. **108:** 405-409.

Huber DJ (1992) The inactivation of pectin depolymerase associated with isolated tomato fruit cell wall: implications for the analysis of pectin solubility and molecular weight. Physiol. Plant. **86:** 25-32.

Huber DJ, O'Donoghue E (1993) Polyuronides in avocado (*Persea americana*) and tomato (*Lycoperiscon esculentum*) fruits exhibit markedly different patterns of molecular weight downshifts during ripening. Plant Physiol. **102:** 473-480.

Jarvis MC (1984) Structure and properties of pectin gels in plant cell walls. Plant Cell Environ. **7:** 153-164.

Keegstra K, Talmadge KW, Bauer WD, Albersheim P (1973) The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnection of the macromolecular components. Plant Physiol. **51:** 188-196.

Koch JL, Nevins DJ (1989) Tomato fruit cell wall. I. Use of purified tomato polygalacturonase and pectinmethylesterase to identify development changes in pectins. Plant Physiol. **91:** 816-822.

Lamport DTA (1980) Structure and function of plant glycoproteins. In P Stumpf, ed, The Biochemistry of Plants, **Vol 3.** Academic Press, New York, pp 501-541.

Lau JM, McNeil M, Darvill AG, Albershem P (1985) Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. Carbohydr. Res. **137:** 111-125.

Masuda Y, Pilet PE (1983) Mechanical properties and polysaccharide nature if the cell wall of maize root. Physiol Plant. **59:** 512-517.

McCann MC, Roberts K (1996) In J. Visser and A.G.J. Voragen Plant cell wall architecture: The role of pectins. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., pp 91-107.

McCann MC, Roberts K (1991) Architecture of the primary cell wall. In CW Lioyd, ed, The cytoskeletal basis of plant growth and form. Academic Press, New York,, pp 109-129.

McCann MC, Shi J, Roberts K, Carpita NC (1994) Changes in pectin structure and localization during the growth of unadapted and NaCI-adapted tobacco cells. Plant J. **5:** 773-785.

McNeil M, Darvill AG, Albersheim P (1980) Structure of plant cell walls. X. rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiol. **66:** 1128-1134.

McNeil M, Darvill AG, Fry SC, Albersheim P (1984) Structure and function of the primary cell walls of plants. Ann. Rev. Biochem. **53:** 625-663.

O'Neil MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill AG, Albersheim P (1996) Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. J. Biol. Biochem. **271:** 22923-22930.

Moerschbacher BM, Schrenk F, Graebner B, Noll U, Reisener HJ (1990) A wheat cell wall fragment suppresses elicitor-induced resistance responses and disturbs fungal development. J. Plant Physiol. **136:** 761-764.

Mort AJ (1983) An apparatus for safe and convenient handling of anhydrous liquid HF at controlled temperatures and reactions times. Application to the generation of oligosaccharides from polysaccharides. Carbohydr. Res. **122:** 351.

Mort AJ, An J, Qi X, Komalavilas P, Otika G, West P, Maness N (1998) Complete solubilization of pectins from cotton suspension culture cell wall with retention of most structural features: Implication for cell wall architecture. (in preparation)

Mort AJ, Bauer WD (1982) Application two new method for cleavage of polysaccharides into specific oligosaccharide fragments. Structure of the capsular and extracellular polysaccharides of *Rhizobium japonicum* that bind soybean lectin. J. Biol. Chem. **257:** 1870.

Mort AJ, Moerchbacher BM, Pierce ML, Maness NO (1991) Problems one may encounter during the extraction, purification and chromatography of pectin fragments and some solutions to them. Carbohydr. Res. **215:** 219-227

Mort AJ, Qiu F, Maness NO (1993) Determination of the Pattern of methyl esterification in pectins. Distribution of contiguous non-esterified residues. Carbohydr. Res. **247:** 21-35

Nogata Y, Yoza K, Kusumoto K, Ohta H In J .Visser and A.G.J. Voragen Changes in molecular weight and carbohydrate composition of cell wall polyuronide and hemicellulose during ripening in strawberry fruit. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., 1996, pp 591-596.

Northnagel EA, McNeil M, Albersheim P, Dell A (1983) Host-pathogen interactions. XXII. Agalacturonic acid olgosaccharide from plant cell walls elicits phytoalexins. Plant Physiol. **71:** 916-926.

Qi X, Behrens BX, West PR, Mort AJ (1995) solubilization and partial characterization of extensin fragments from cell walls of cotton suspansion cultures: evidence for a covalent cross-link between extensin and pectin. Plant Physiol. **108:** 1691-1701.

Ryan CA (1984) Pectic fragments regulate the expression of proteinase inhibitor genes in tomato plants. J. Cell Biochem. **8B:** 230.

Ryan CA, Farmer EE (1991) Oligosaccharide signals in plants: a current assessment. Annu. Rev. Plant Physiol. Mol. Bio. **42:** 651-674.

Schols HA, Voragen AGJ In J. Visser and A.G.J. Voragen. Complex pectins: Structure elucidation using enzymes. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., 1996, pp 3-19.

Schols HA, Bakx EJ, Schipper D, Voragen AGJ (1995) A xylogalacturonan subunit present in the modified hairy regions of apple pectin. Carbohydr. Res. **279:** 265-279.

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell **5:** 2-23.

Silva JD, Arrowsmith D, Hellyer A, Whiteman S, Robinson S (1994) Xyloglucan endotransglycosylase and plant growth. J. Experi. Botany. **45:** 1693-1701.

Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold C, Tucker GA, Schuch W, Harding S and others (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. Plant Mol Biol. **14:** 369.

Taiz L (1984) Plant cell expansion: regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. **35:** 585-657.

Talbott LD, Ray PM (1992) Changes in molecular size of previously deposited and newly synthesized pea cell wall matrix polysaccharides. Plant Physiol. **98:** 369-379.

Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. The Plant Cell, **4:** 667-679.

Tieney ML, Varner JE (1987) The extensins. Plant Physiol. **84:** 1-2.

Yamada H In J. Visser and A.G.J. Voragen. Contribution of pectin on health care. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., 1996, pp 173-190.

Zhang Z, Mort AJ. (1997) A sensitive method of amino acid analysis using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and capillary electrophoresis. Plant Phsiol. **114:** 89.

Zhang Z, Pierce ML, Mort AJ (1996) Detection and differentiation of pectic enzyme activity in vitro and in vivo by capillary electrophoresis of products from fluorescent-labeled substrate. Electrophoresis **17:** 372-378.

CHAPTER II

DETECTION AND DIFFERENTIATION OF PECTIC ENZYME ACTIVITY *IN VITRO* AND *IN VIVO* BY CAPILLARY ELECTROPHORESIS OF PRODUCTS FROM FLUORESCENT-LABELED SUBSTRATE

This chapter has been published in *Electrophoresis* **17:** 372-378 (1996) by the authors
Zhiquan Zhang, Margaret L. Pierce, and Andrew J. Mort.
Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK

SUMMARY

A sensitive assay is described for the detection of pectate-depolymerizing enzymes using a fluorescent end-labeled pectate oligomer. The labeled oligomer is allowed to react with the enzyme either *in vitro* or *in vivo*, such as inside the intercellular spaces of a cotton cotyledon, and after an appropriate incubation time the products are analyzed by capillary electrophoresis. The site and mode of action of the pectate-depolymerizing activity can be inferred from the products. Both endo- and exo-polygalacturonase activity, and lyase activity were distinguished. Since only the fluorescent oligomer and products from its labeled reducing end are detected, there is no interference from other compounds; only pectic enzyme activity is detected. By this type of analysis we can show that there is

considerable endo- and exopolygalacturonase activity in the intercellular spaces of cotton cotyledons.

---

1 INTRODUCTION

Most of the assays for endoglycanases rely on colorimetric determination of the reducing ends produced from a polymeric substrate. In some cases, this is not an ideal assay method because of the following reasons: 1) The actual mode of producing reducing ends is not directly determined. Thus, the enzyme may actually be hydrolyzing a different glycosidic linkage than expected. 2) There may be a large background of reducing groups in the enzyme preparation. 3) The enzyme may be ionically or covalently linked to an insoluble matrix, *e.g.* plant cell walls. Thus, one may not actually be able to get it into the assay mixture.

We desired to determine if there was endopolygalacturonase activity outside the plasma membranes of cells in cotton cotyledons. In this regard, we needed to be able either to rinse out the intercellular spaces within the cotyledons and assay the fluid we could recover for enzyme activity, or to introduce substrate into the intercellular spaces and, after recovering it, look for its degradation. Because of the abundance of monosaccharides in the extracellular water of plants [1] , the first approach gave a high background of reducing substances unless the fluid was dialyzed or treated in some other ways to remove monosaccharides. This was time consuming and could lead to loss of activity. In addition, any activity firmly bound to the cell walls would not be detected. The second approach would have more severe problems with background from reducing sugars unless a new assay could be devised.

During our ongoing work on determining the structures of plant cell wall pectins, we have been investigating the suitability of using 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) fluorescence labeling of galacturonic acid (GalA) for capillary electrophoresis. We

find that members of the homologous series of $GalA_n$ residues (where n is less than ~14) derived from pectic acid (a homopolymer of α-1-4 linked GalA residues) can be separated very efficiently using a non-coated capillary and 100 mM phosphate buffer (pH 2.5) as the electrolyte [2] . A pH of 2.5 is 0.8 units below the pKa of monomeric GalA. The apparent pKa of oligomeric GalA increases with the number of residues [3]. Hence, the ANTS label carries most of the charge on the GalA oligomers.

We reasoned that we could assay endopolygalacturonase activity with high sensitivity by using an ANTS-labeled oligomer containing a long enough chain of GalA residues for the enzyme to be able to act. Lee et al. [4] have already shown a similar strategy to work for studying the action pattern of chitinase. Since we expected little or no background fluorescence with the same excitation and emission characteristics as ANTS to be eluted from the plant, we injected labeled substrate into the cotyledons and eluted products from them.

There are several different classes of enzymes that degrade pectic acid [5] . They are divided into lyases, which cleave via a β-elimination mechanism, and hydrolases. Within each of these two classes there are both endo- and exo-acting enzymes. Action of each of these categories of enzymes on a reducing-end-labeled GalA oligomer gives rise to different initial fluorescent products. Thus, they all can be distinguished from each other.

2 MATERIALS AND METHODS

2.1 Materials

Di- and tri-galacturonic acids and pectolyase were purchased from Sigma (St. Louis, MO, USA); 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was from Molecular Probes, Inc. (Eugene, OR, USA); sodium cyanoborohydride and pectic acid were purchased from Aldrich (Milwaukee, WI, USA); HPLC-grade ammonium acetate was from Fisher (Fair Lawn, NJ, USA). DEAE HW65 was purchased from Supelco

(Bellefonte, PA, USA). All solvents were filtered through a 0.45-µm nylon filter and degassed in a sonication bath under vacuum. The endopolygalacturonase from *Aspergillus niger* was obtained from Megazyme Pty. Ltd. (Sydney, NSW, Australia), and the one from *Erwinia carotovora* was purified from an *E. coli* clone as described by Maness and Mort [6] . ANTS-labeled oligomers for electrophoretic standards were prepared as described by Mort and Chen [2] by a slight modification of the procedures of Jackson [7] and Stefansson and Novotny [8] .

2.2 Plants

The upland cotton (*Gossypium hirsutum* L.) line used was Ac44E, a single plant selection made by M. Essenberg of our department at Oklahoma State University (unpublished) from bacterial-blight susceptible cultivar Ac44 [9] . Plants were grown in flats or clay pots of Jiffy Mix-Plus in a Conviron E15 growth chamber as described previously [1] . Cotyledons, which in cotton are leaf like, were used for the experiments five days after seedling emergence.

2.3 Substrate preparation

2.3.1 Oligomer purification

A range of oligomers of GalA was generated by autoclave hydrolysis of pectic acid as described by Robertsen [10] . Oligomers of GalA in the range from two to eight residues were purified in a two-step process. First, the entire mixture was applied to a 22.5-mm x 250-mm stainless steel column (Alltech Associates, Inc., Deerfield, IL, USA) packed with DEAE toyopearl HW65 in 30 mM ammonium acetate buffer (pH 5.2) at 4 mL/min, and sample components were eluted after a 15-min lag period using buffer concentrations increased linearly in the following manner: 30 to 250 mM over 50 min, then to 380 mM over 60 min. The system was allowed to equilibrate at initial conditions for at

least 20 min prior to another injection. Approximately 95% of the effluent from the column went to a fraction collector, and the remaining 5% was split from the main stream and introduced into a post-column reaction system that was designed to use the bleaching of permanganate, as it oxidizes sugars, to show the concentration of sugars in a column effluent [11] . The fractions that corresponded to an oligomer (peak in chromatogram) were pooled. The individual oligomers were lyophilized and desalted by repeated lyophilization.

The second step in purification of the GalA oligomers was chromatography on a Dionex (Sunnyvale, CA, USA) BioLC HPLC System using a Dionex PA1 anion-exchange column (9 mm x 250 mm) with ammonium acetate buffer (pH 5.2) at a flow rate of 2.0 mL/min. A sample was injected with the system at equilibrium in 30 mM buffer. The concentration of the buffer was increased linearly from 30 mM to 280 mM over 10 min, then linearly to 400 mM over 5 min, to 650 mM over 30 min, and to 1000 mM over 15 min. The system was allowed to equilibrate at initial conditions for at least 20 min prior to next injection. Approximately 90% of the effluent from the column was collected in fractions, and the rest was used for post-column detection as described above. The fractions corresponding to each oligomer were pooled, lyophilized, and desalted by repeated lyophilization. The dry oligomers were stored at room temperature until use. Chromatographic data were collected using custom-built data loggers [12] and downloaded into a power Macintosh 8100/80 computer. Chromatographic data were viewed and quantitated by using the program Analog Connection Chrom (Strawberry Tree Computer, Inc., Sunnyvale, CA, USA) and KaleidaGraph, developed by Abelbeck Software (distributed by Synergy Software, Reading, PA, USA).

2.3.2 Oligomer derivatization

Labeling of GalA oligomers of five to seven residues, to be used as enzyme substrate, was performed by a slight modification of the procedures of Jackson [7] and

Stefansson and Novotny [8]. To about 1 mg of purified oligomer were added 0.9 mL of 23 mM ANTS, in 3% (w/w) acetic acid, and 0.1 mL of a 1 M solution of sodium cyanoborohydride in dimethylsulfoxide. The mixture was heated for 60 min at 90°C. Labling efficiency was not determined. Excess reagents were subsequently removed by dialysis to make the labeled oligomer suitable for enzyme digestion. Samples were dialyzed for more than 24 h in 1000 molecular weight cutoff tubing (Spectrum, Houston, TX, USA) against deionized water that was changed at least three times. GalA oligomers of the size used, or larger, do not pass through 1000-molecular weight cutoff tubing [13]. Dialyzed ANTS-labeled oligomers were lyophilized and stored in a freezer (–20°C) until use.

2.4 Enzyme reactions

2.4.1 Endopolygalacturonase activity *in vitro*

About 100 μg of ANTS-labeled oligomer of GalA was dissolved in 25 mM acetate buffer (pH 5.2) containing 0.2 U of endopolygalacturonase [EC 3.2.1.15]. Bacterial endopolygalacturonase from *Erwinia carotovora* or fungal endopolygalacturonase from *Aspergillus niger* were used. The reaction mixture was incubated at room temperature. Samples were taken from the solution at various times. The endopolygalacturonase activities were measured by using CZE as described below.

2.4.2 Lyase activity *in vitro*

ANTS-labeled hexamer (100 μg) was dissolved in 10 mM sodium carbonate buffer (pH 7.0) containing 1 U of pectolyase. The sample was incubated overnight at room temperature. Enzyme activities were measured using CZE.

2.4.3 Polygalacturonase activity *in vivo*

A pot containing six 5-day old cotton plants was taken from the growth chamber. Injection of cotyledons with the starting material (ANTS-labeled hexamer) was done in the middle of the photoperiod by the following procedure: About 20 μL (5μg/μL) of ANTS-labeled hexamer was injected into the intercellular space by a syringe that was designed for capillary GLC on-column injection (SGE, Inc., Austin, TX, USA) attached to a 0.17-mm OD fused-silica capillary (Alltech Associates, Inc., Deerfield, IL, USA) as a needle. The pot was put back in the growth chamber immediately after injection. Samples, consisting of entire cotyledons, were taken at various times after injection. At the indicated times, a cotyledon without its petiole was picked from the plant and placed in an Erlenmeyer flask (125 mL) containing about 30 mL of extracting solvent (25 mM sodium acetate buffer, pH 5.2). Vacuum was applied for 2 min through a water aspirator. Release of vacuum caused infiltration of the cotyledon's intercellular space by the extracting solvent. Infiltration of the cotyledon was completed by two or three subsequent 10-sec applications and release of the vacuum. The infiltrated cotyledon was removed from the Erlenmeyer flask with blunt tweezers, transferred to paper towels, and blotted with tissue. The cotyledon was rolled with a taper and put half way down into a 2-ml micro reaction vessel (Supelco, Inc., Bellefonte, PA, USA); this avoided contact between the cotyledon and the intercellular fluid that collected at the bottom of the vial during centrifugation. The cotyledon was centrifuged at 1500 x *g* in a bench-top centrifuge at room temperature for 30 min. About 0.3 mL of intercellular wash fluid (IWF) was collected from one cotyledon (5-days post-emergence) and lyophilized. Before analysis of enzyme activity, the freeze-dried IWF was dissolved in 3 μL of deionized water and centrifuged at 1500 x *g* for 5 min. Two μL of supernatant was taken for analysis. Control IWF from cotyledons that were not injected with substrate was prepared by the same procedure. Enzyme activities were measured using CZE.

2.5 Capillary electrophoresis

A custom-built instrument for capillary electrophoresis included a Spellman (Plainview, NY, USA) Model CZE 1000 R high voltage power supply with positive and negative polarity and a Model FL-750BX fluorescence detector (McPherson Instruments, Acton, MA, USA). The detector was equipped with a cell for on-column capillary detection and a 200 W Xenon-Mercury lamp with the excitation wavelength set to 364 nm; a cut-off filter permitted detection of emission beyond 440 nm. Chromatographic data were collected, viewed, and analyzed as described above.

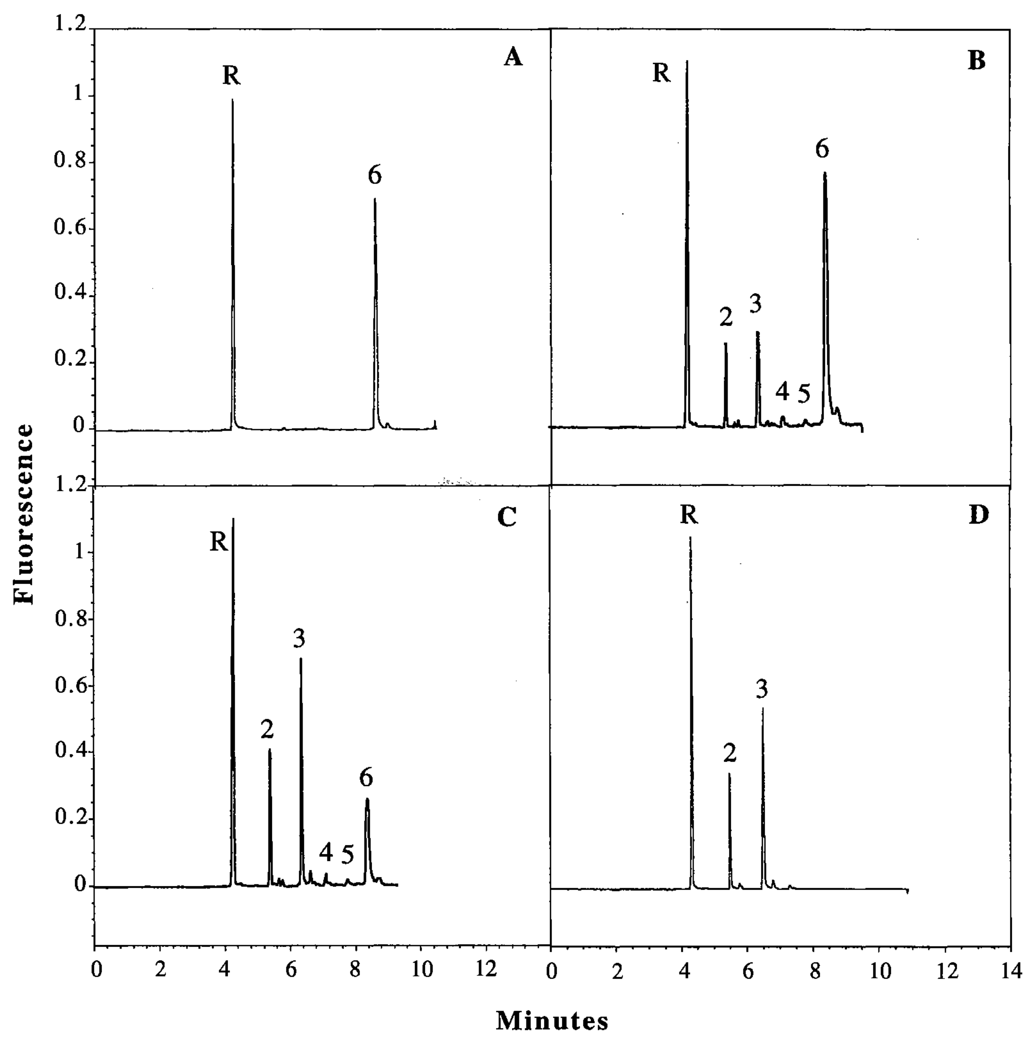
A 60-cm fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID (187 μm OD) was used as the separation column. The length was 34 cm to the detection window. New capillary was treated with running buffer (0.1 M phosphate buffer, pH 2.5) overnight before use. The capillary was rinsed with running buffer after each run and allowed to equilibrate for 10 min before each injection. Samples labeled directly before CZE rather than for use as substrate were derivatized as described above at 1/20 the scale, and the reaction mixture was directly injected. Other samples consisted of aliquots of enzyme reaction mixtures or reconstituted IWF at a concentration of about 1 μg/μL. Samples were introduced hydrodynamically, *i.e.*, by gravity-driven flow, for 5 or 7 sec, using an eight-cm hydrostatic head. Electrophoresis was conducted at 17 kV with the negative electrode on the injection side. The capillary was stored in deionized water for long-term storage.

3 RESULTS AND DISCUSSION

3.1 Suitability of ANTS-labeled hexamer of GalA as a substrate for pectate-degrading enzymes

Approximately 100 μg of ANTS-labeled hexamer was incubated overnight with endopolygalacturonase from the fungus *Aspergillus niger* and the products were directly analyzed by CZE. Figure 3 (A–D) shows the electrophoretic pattern of the substrate and

Figure 3. Electrophoretic behavior of GalA hexamer and products from the activity of *Aspergillus niger* endopolygalacturonase on the ANTS-labeled hexamer of GalA. Capillary, 60 cm total length (34 cm effective length)x50 µm ID. Running electrolyte: 0.1 M phosphate buffer, pH 2.5; applied voltage, -17 kV; detection, fluorescence at excitation= 364 nm and emission≥ 440 nm. Panels: A) ANTS-labeled hexamer. B) Products of endopolygalacturonase activity after 26 minutes of digestion. C) Products after 90 minutes of digestion. D) Products after a 3-h digestion."R" indicates reagent peak; peak numbering reflects degree of polymerization of the oligomers.



A
B
C
D
R
2
3
4 5
6
Fluorescence
Minutes
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14

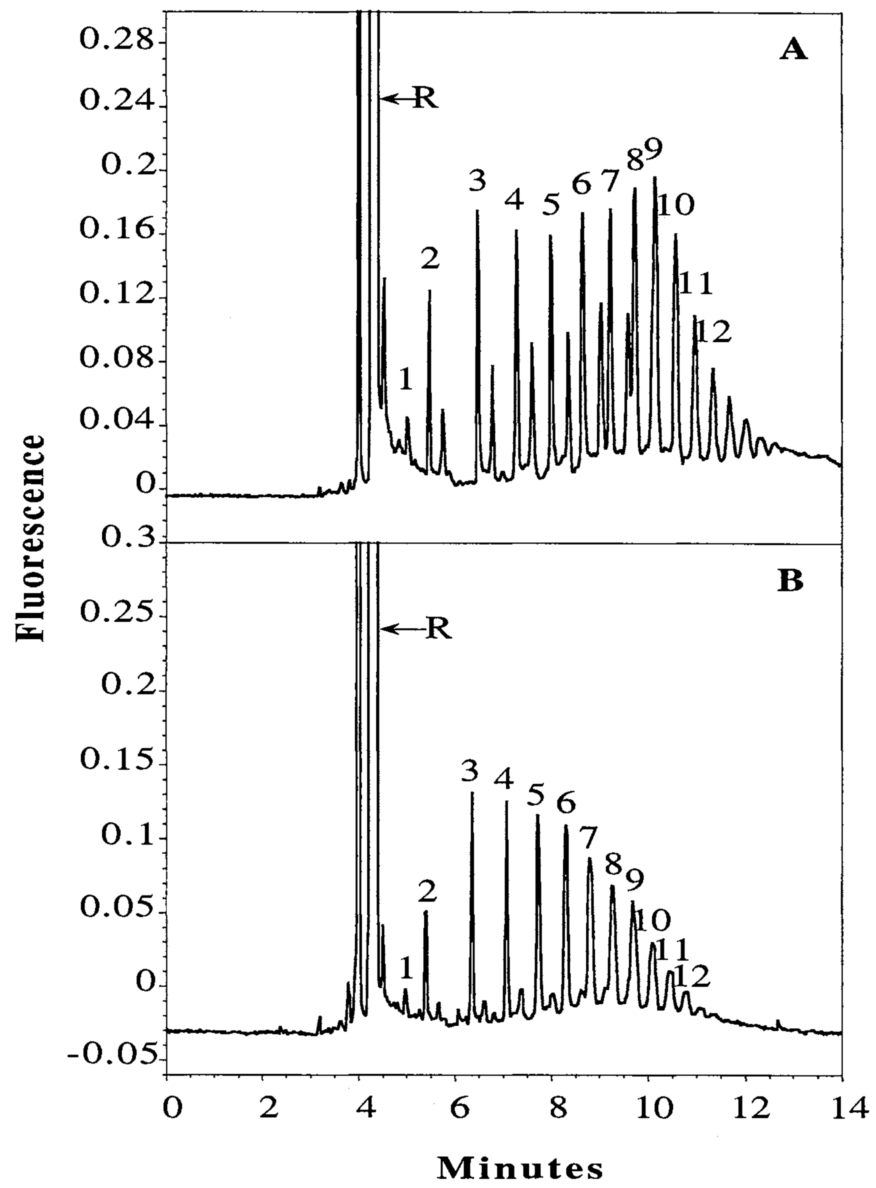
products after various periods of digestion. Clearly, the presence of the ANTS label at the reducing end of the oligomer did not prevent the endopolygalacturonase from acting on the oligomer. For identification of the various oligomers produced by the enzyme, a mixture of GalA oligomers and pure commercial monomer, dimer, and trimer of GalA were derivatized and electrophoresed under the same conditions. An electropherogram of the mixture is shown in Figure 4A.

In all of the electropherograms there is at least a trace of a secondary peak associated with each major peak. In some cases the secondary peaks are substantial, as shown in Figure 2A. We do not yet know what causes a secondary peak, but suggest that it is a complex of some kind between the oligomer and a divalent metal ion. The secondary peaks can be eliminated or at least greatly reduced by making the sample alkaline [13] by adding an equal volume of 0.1 M NaOH (Fig. 4B).

3.2 Substrate specificity revealed by CZE assay

From previous experiments with pectic acid, we know that the limit digest by the endopolygalacturonase from *Aspergillus* is the monomer, dimer, and trimer of GalA [2, 14] . It is not surprising, however, that the enzyme did not produce any labeled monomer from the ANTS-labeled substrate because what was the reducing end residue no longer resembles a reducing sugar. The reductive amination with ANTS causes the reducing terminal sugar to lose its ring structure and become part of a secondary amine. Interestingly, there was very little production of the labeled tetramer or pentamer at any time during the digestion. This can be interpreted as an inability of the enzyme to act as an exopolygalacturonase that would release either free GalA or the dimer of GalA from the nonreducing end of the hexamer. In other words, the active site of the enzyme needs at least three residues towards the nonreducing end from the position at which the hydrolysis occurs. Since both labeled dimer and trimer are produced from the hexamer, and the sixth residue is drastically modified by the labeling, it is likely that only a total of four residues is

Figure 4. Electrophoretic behavior of oligomers of GalA. A) Mixture of ANTS-labeled of GalA oligomers. B) Same as (A) but treated with NaOH to remove secondary peaks. Other details as in Figure 3.

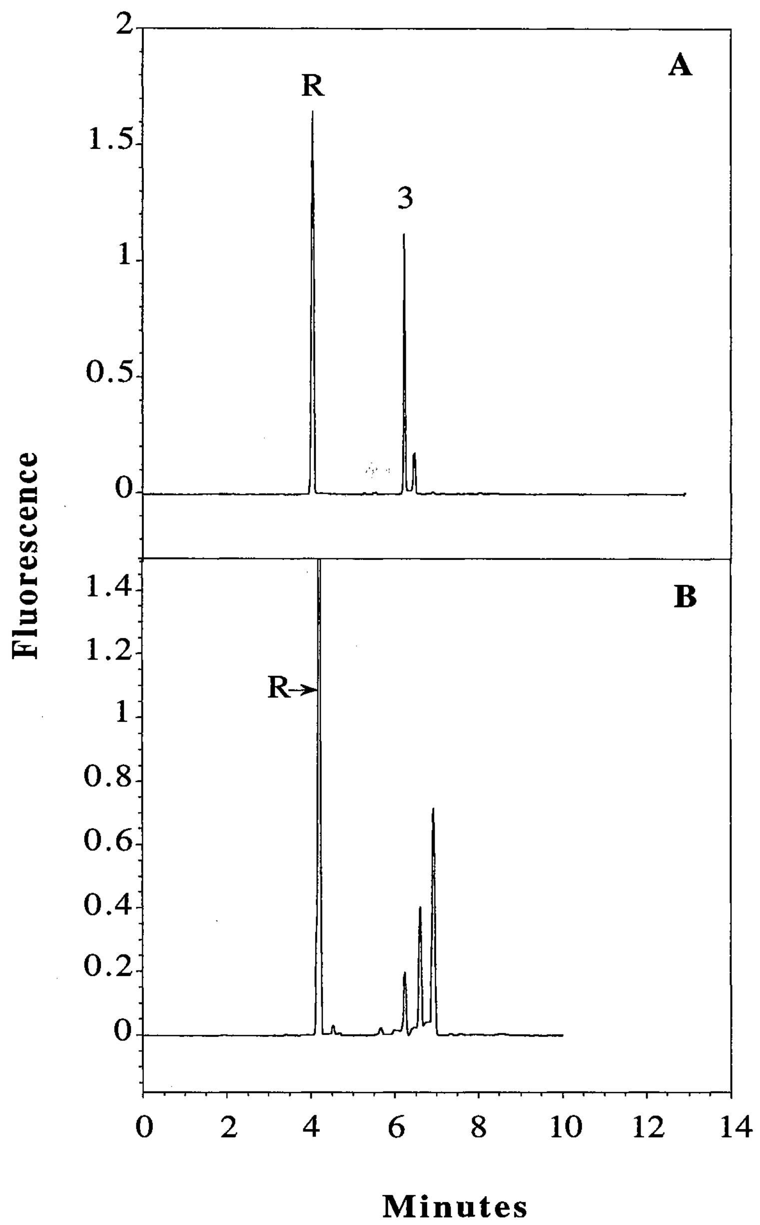

A
B
R
Fluorescence
Minutes
0.28
0.24
0.2
0.16
0.12
0.08
0.04
0
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11
12

actually needed for productive binding to the active site. These results are in good agreement with the observation of Koller and Neukom [15] that the pentamer and hexamer of GalA are digested by this enzyme to tri-, di-, and mono-GalA; whereas the tetramer (also investigated by Mill and Tuttobello [16]) gives rise to only trimer and monomer.

In contrast, when the endopolygalacturonase from *E. carotovora* was used, only the labeled trimer was formed from the hexamer (Fig. 5A), indicating that this bacterial enzyme recognizes five residues. A difference in product formation between the two enzymes was also observed if a labeled heptamer or pentamer was used as substrate. The fungal enzyme produced the labeled dimer, trimer, and tetramer from the heptamer; whereas the bacterial enzyme produced labeled trimer and tetramer. With the labeled pentamer as substrate, the fungal enzyme rapidly produced labeled dimer, but the *E. carotovora* enzyme was very slow to produce labeled dimer. Previous work on the activity of the endopolygalacturonase from *E. carotovora* [17] is consistent in part with our results. The enzyme was reported to produce exclusively di- and tri-GalA from the pentamer of GalA in its initial reaction and only slowly to give rise to monomer. No tetramer was observed at any time during the digestion. We suggest that the enzyme preparation used by Nasuno and Starr [17] was slightly contaminated with an exopolygalacturonase, which led to the slow production of monomer. Our *E. carotovora* endopolygalacturonase was synthesized in *E. coli*, which is not known to produce any pectin-degrading enzymes. Thus, contamination of our preparation with an exo- activity is unlikely.

In addition to producing polygalacturonases, plant pathogens, saprophytes, and perhaps also plants [18] produce pectate lyases, which cleave polypectate by an eliminative mechanism rather than by hydrolysis. The products of lyase activity contain a 4,5 unsaturation at their nonreducing ends. Figure 5B shows the products of digestion of the labeled hexamer with a crude commercial pectolyase. We did not have standards to allow precise identification of the lyase products. The trimer of GalA, if mixed with the lyase

Figure 5. A) Electropherogram of products from ANTS-labeled hexamer of GalA digested to completion with endopolygalacturonase from *Erwinia carotovora.* B) Electropherogram of products from ANTS-labeled hexamer of GalA digested with pectolyase. "R" indicates reagent peak; peak numbering reflects degree of polymerization. Other conditions as in Fig. 3.

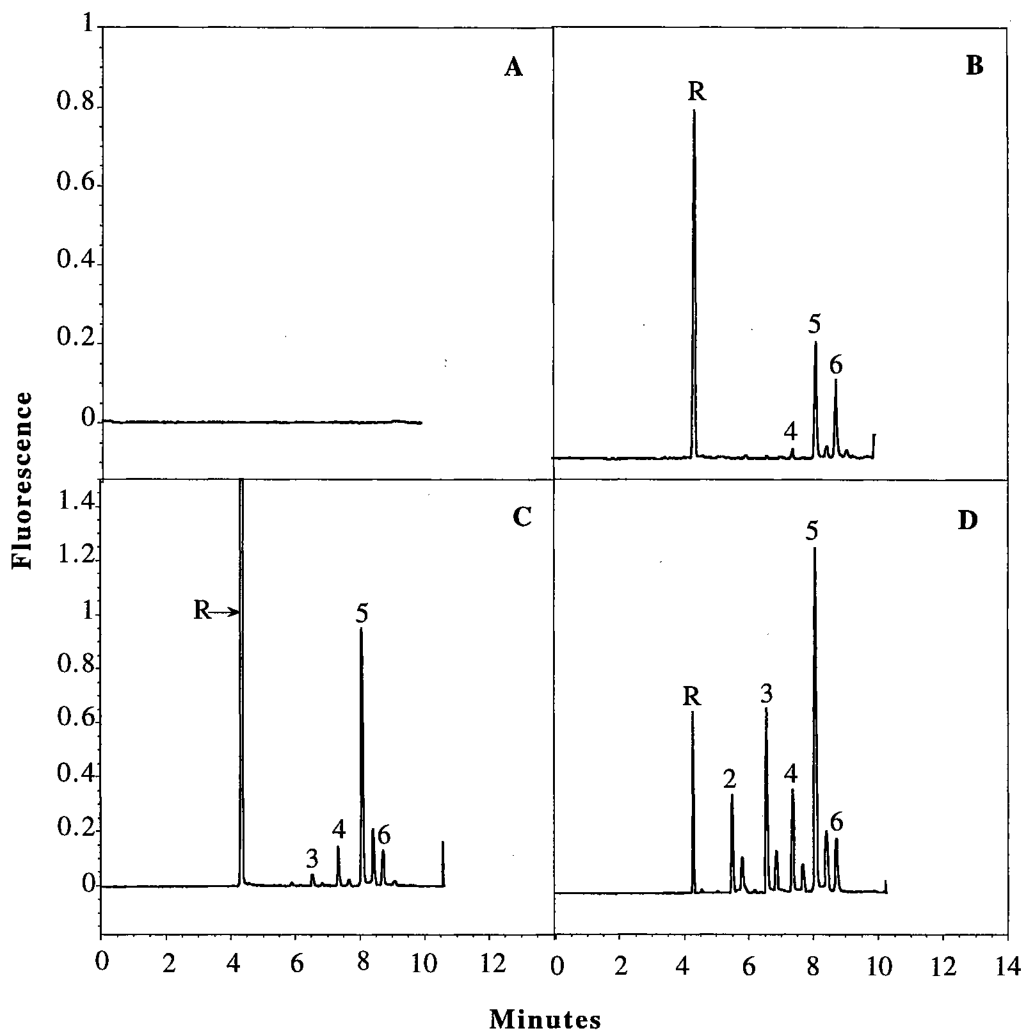

A
R
3
B
R
Fluorescence
Minutes
2
1.5
1
0.5
0
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14

products, migrates between the first and second peaks beyond the reagent peak in Figure 5B. The lyase unsaturated products have different migration times from the hydrolysis products. Thus, lyases could be readily differentiated from hydrolases by this assay method.

3.3 *In vivo* assay of enzyme activity

A small quantity (~100 μg) of labeled hexamer was injected into the intercellular spaces of a cotton cotyledon. With some practice this could be performed with no leakage of the solution out of the cotyledon. Injections of 10 μL caused water soaking of the cotyledon's intercellular spaces for a radius of about 0.5 cm around the injection point. Two injections were made per cotyledon. The total surface area of a cotyledon was approximately 15 cm^2. Thus, only approximately 10 percent of the cotyledon was exposed to the substrate. We do not know how far or how quickly the substrate or unbound reagent diffused during the incubation time. However, the entire intercellular space of the cotyledon was rinsed out for recovery of the labeled oligomer and its digestion products. Progress of the reaction can be seen by comparison of the proportion of the label in the reactant and the various products. If all oligomers are recovered from the cotyledon equally, the overall recovery of labeled oligomers does not need to be known to determine the extent of the reaction. Figure 6 shows a series of electropherograms of intercellular wash fluids. Panel A shows the complete lack of interference (at this sensitivity) in IWF from a cotyledon in which no labeled oligomer had been injected. It was not possible to obtain an electropherogram at zero time of incubation in the cotyledon because it takes at least 50 min to complete the infiltration with rinsing buffer, centrifugation, and freezing of the IWF, during which time the enzymes may be acting. The results obtained from starting the rinsing procedure immediately after the injection are shown in panel B. There had already been a high degree of conversion of the hexamer into pentamer, and even a small amount of tetramer had formed. This shows that there is a high degree of exogalacturonidase activity

Figure 6. *In vivo* pectic enzyme activity. Panels: A) Intercellular wash fluid (IWF) from cotyledons in which no ANTS-labeled substrate had been injected. B) IWF from cotyledons in which the rinsing of the intercellular spaces was started as soon as the labeled hexamer had been injected. C) IWF prepared after a 10-min incubation of labeled hexamer in the cotyledons. D) IWF after a 2-h incubation of labeled hexamer in the cotyledons.


A
B
C
D
R
R
R
2
3
3
4
4
4
5
5
5
6
6
6
Fluorescence
Minutes
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
2
4
6
8
10
12
14

in the intercellular spaces of the cotyledons. After increasing lengths of incubation of the cotyledons, the labeled oligomers that were recovered became shorter until they were exclusively the dimer and trimer, the same products as obtained by exhaustive digestion of the labeled hexamer by the *Aspergillus* endopolygalacturonase *in vitro*. During the progression of the digestion (Fig. 6, C and D), the distribution of products did not follow a smooth residue-by-residue loss, as one would predict for action of an exoglycanase. The pentamer accumulated rapidly with only slow accumulation of the tetramer. This characteristic of exogalacturonidases has been observed previously. Pressey and Avants [19] reported a decreasing activity of exogalacturonidase with decreasing length of substrate, and Garcia-Romera and Fry [20] noticed an accumulation of the pentamer of GalA when they incubated longer GalA oligomers with proteins obtained from Paul's Scarlet rose cell culture medium. In our experiments, at the longer times of incubation, but before all hydrolysis was completed, there was more trimer present than tetramer (Panel D), even when there was still more pentamer than tetramer. We take this to indicate that there is also endopolygalacturonase activity present which, if it has the same action pattern as the fungal endopolygalacturonase we used in our *in vitro* experiments, digests the hexamer to give only the labeled dimer and trimer.

4 CONCLUDING REMARKS

We have shown that oligomers of GalA labeled at their reducing end with a fluorophore can be very useful for detection and rapid characterization of various pectic enzymes both *in vitro* and *in vivo* by CZE. It should be possible to extend the methods described here to studies of any other glycanase, so long as a suitable substrate can be prepared and labeled and the products can be readily separated by CZE.

By knowing the exact nature of the labeled substrate and products from the action of the enzyme, one can infer considerable detail about the number of sugar residues involved in binding the substrate to the active site of the enzyme and the location of the

cleavage site within the bound segment of the polymer. This type of information could be very helpful, in conjunction with X-ray crystallographic results, in understanding the exact mechanisms of polysaccharide-degrading enzymes.

The ability to detect and characterize glycanases in living tissues or other complex matrices should lead to a better understanding of these ezymes in growth and differentiation.

5 ACKNOWLEDGMENTS

This research was supported in part by DOE grant DE-FG02-93ER20102 and has been approved for publication by the director of the Oklahoma Agricultural Experiment Station.

6 REFERENCES

[1] Pierce, M. L., Essenberg, M., Mort, A., *Phytopathology*. 1993, *83*, 344-349.

[2] Mort, A. J., Chen, E. M. W., *Electrophoresis*. 1996, *17*, 379-383.

[3] Ravant, G., Ranaudo, M., *Biopolymers* 1980, *19*, 2209-2222.

[4] Lee, K-B., Kim, Y-S., Linhardt, R. J., *Electropphoresis* 1991, *12*, 636-640. Maness, N. O., Mort, A. J., *Anal. Biochem.* 1989, *178*, 248-254.

[5] Sakai, T., Sakamoto, T., Hallaert, J., Vandamme, E. J., *Adv. Appl. Microbiol.* 1993, *39*, 213-294.

[6] Maness, N. O., Mort, A. J., *Anal. Biochem.*, 1989, *178*, 248-354.

[7] Jackson, P., *Biochem. J.* 1990, *270*, 705-713.

[8] Stefansson, M., Novotny, M., *Carbohydr. Res.* 1994, *258*, 1-9.

[9] Brinkerhoff, L. A., *Okla. Agric. Exp. Stn. Tech. Bull.* 1963, *T98*, 1-95.

[10] Robertsen, B., *Physiol. Plant Pathol.* 1986, *28*, 137-148.

[11] Thomas, J., Mort, A. J., *Anal. Biochem.* 1994, *223*, 99-104.

[12] Merz, J. M., Mort, A. J., *Anal. Biochem.* 1992, *207*, 351-353.

[13] Mort, A. J., Moerschbacher, B. M., Pierce, M. L., Maness, N. O., *Carbohydr. Res.* 1991, *215*, 219-227.

[14] Rexova-Benkova, L., Markovic, O., *Adv. Carbohydr. Chem. Biochem.* 1976, *33*, 323-385.

[15] Koller, A., Neukom, H., *Eur. J. Biochem.* 1969, *7*, 485-489.

[16] Mill, P. J., Tuttobello, R., *Biochem. J.* 1961, *79*, 57-64.

[17] Nasuno, S., Starr, M. P., *J. Biol. Chem.* 1966, *241*, 5298-5306.

[18] Wing, R. A., Yamaguchi, J., Larabell, S., K., Ursin, V. M., S., M., *Plant Mol. Biol.* 1989, *14*, 17-28.

[19] Pressey, R., Avants, J. K., *Phytochemistry*. 1975, *14*, 957-961.

[20] Garcia-Romera, I., Fry, S. C., *Phytochemistry*. 1994, *35*, 67-72.

CHAPTER III

CHANGES
IN HOMOGALACTURONANS
AND ENZYMES DEGRADING THEM
DURING COTTON COTYLEDON EXPANSION

This chapter will be submitted to Plant Physiology by the authors Zhiquan Zhang and Andrew J. Mort.

Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078-3035 USA

Changes in homogalacturonans (HGs) and enzymes degrading them have been investigated during cotton cotyledon expansion. Cell walls were prepared from 3, 5 and 7-day-old cotton cotyledons and treated with liquid HF at -23°C. This treatment cleaves the glycosidic linkages of most neutral sugar linkages in the walls without degrading HGs. HGs with a relatively high degree of esterification can then be solubilized with water, and those with low esterification with concentrated imidazole buffer. The majority of HGs were obtained in the water extracts. The degrees of esterification (DM) were 57, 47, and 47% in water extracts and 34, 25, and 27% in imidazole extracts, in 3, 5 and 7-day-old cotton cotyledons, respectively. Molecular size analysis of HGs in the water extract by gel filtration chromatography indicated that average molecular size of the HG increased during cotton cotyledon expansion. Using a PA100 ion-exchange column, we can separate the

members of a GalA homologous series up to approximately 70 residues. The results from HG molecular length distribution analysis indicated that the HG at 3 days was more highly enzyme degraded than that in the older cotyledons in both water extract and imidazole extract. Using a newly developed *in vivo* assay for pectin degrading enzymes that involves fluorescent labeled oligomers of GalA as substrate and capillary electrophoresis for product analysis, we found that endo- and exogalacturonidases are present in the cotyledon extracellular spaces and there is a dramatic change in the levels of exo- and endo-PG activity as the cotyledons change their rate of expansion. The EPG activity was highest during the initial stages of the cotyledon expansion. However, the exo-PG activity was highest in the later stages of the cotyledon expansion.

Understanding the biochemistry of plant cell wall polymers and wall enzymes is important because they may play crucial roles during plant cell expansion. Plant cells may expand tremendously in volume before reaching maturity (Cosgrove, 1997). The cell wall can accommodate this enormous expansion without loosing mechanical integrity and generally without getting thinner (Cosgrove, 1997). Thus, the extension of plant cells must involve the breakage and reformation of the linkages within cell walls (Taiz, 1984; Albersheim et al, 1996) by enzymes which degrade and reform wall polymers (Fry, 1995).

Homogalacturonan (HG) is one of the components of pectic polysaccharides of the intercellular matrix in all plant primary cell walls. The structures and the functions of the plant pectins have been extensively studied in recent years (Mort et al, 1998, 1993, 1991; Schols and Voragen, 1996; Carpita and Gibeaut, 1993). HG is a homopolymer of 1-4 linked α-D-galacturonic acid residues that may be esterified with methyl or non-methyl groups (Kim and Carpita, 1992; McCann et al, 1994) and the extent to which different pectic fractions are esterified can vary greatly (McCann et al, 1990). The matrix homogalacturonans play an important role in growing cell walls, especially in fruit

ripening. Downshifts in pectin molecular size have been reported during leaf aging (Arribas et al., 1991) and fruit ripening (Huber, 1983, 1992; Koch and Nevins, 1989; Huber and O'Donoghue, 1993). In expanding vegetative organs, pectic polymers crosslinked via calcium bridges are believed to contribute to wall strength and regulation of growth (Knox et al, 1990; Yamaoka and Chiba, 1983). Changes in sugar composition of the cell walls along the axis of elongating maize roots has been reported (Masuda and Pilet, 1983).

Recently, more and more attention has been paid to the pectin-degrading enzymes, whose activities have been shown to correlate with plant growth and fruit ripening. Polygalacturonase has been identified in tomato abscission zones (Tucker et al, 1984) and in pollen grains of diverse plants, including *Oenothera* and maiz (Brown and Crouch, 1990; Pressey and Reger, 1989). In many cases Polygalacturonase (PG) and pectin methylesterase (PME) may be involved in fruit softening (Crookes and Grierson 1983). Rhamnogalacturonase A (RGaseA) activity was detected in ripening tomato (Gross et al 1995) and in expanding cotton cotyledons (Zhang et al, 1998).

No study has been published on the biochemical basis of the pectic polysaccharides, pectin-degrading enzyme activities and their implication in cotyledon expansion. The objective of this study was to extract pectic polysaccharides, to characterize the changes in the carbohydrate composition, esterification, and molecular weight/size distribution and to detect endo- and exo-polygalacturonase activities during cotton cotyledon expansion.

MATERIALS AND METHODS

Plant Materials

The upland cotton (*Gossypium hirsutum* L.) line used was Ac44E, a single plant selection made by M. Essenberg of our department at Oklahoma State University (unpublished) from bacterial-blight susceptible cultivar Ac44. Plants were grown in flats or clay pots of Jiffy Mix-Plus in a Conviron E15 growth chamber as described by Pierce et al

(Pierce et al 1993). Cotyledons, which in cotton are leaf like, were used for the experiments. Cotyledon areas were measured by a leaf area meter (LAMBDA Instruments Corporation, USA). The relative growth rate was calculated as 100 times the average increase in area per hour over a 24 hour period divided by the area at the beginning of the time period.

PG Activity *in vivo*

PG activities *in vivo* were measured as described by Zhang et al (1996) with a slight modification. The injection of starting materials into different age cotton cotyledons was carried out in the growth chamber in the middle of the photoperiod by the follows: about 20 µl (5µg/µl) of ANTS labeled hexamer of GalA was injected into the intercellular space by using a 10 µl syringe fitted with a needle made of a 15 cm section of a 0.17 mm o.d. fused silica capillary (Alltech Associates, Inc., Deerfield, IL, USA). At a certain time, cotyledons were excised from the plant and placed in an Erlenmeyer flask (125 ml) containing about 30 ml of the ice-cold extracting solvent (25 mM sodium acetate buffer, pH 5.2). The following steps were carried out at 4°C in a cold room. Vacuum was applied for 2 min from a water aspirator. Release of vacuum caused infiltration of the cotyledon's intercellular space by the extracting solvent. Infiltration of the cotyledon was completed by a 10-sec application and then release of the vacuum 2-3 times. The infiltrated cotyledon was removed from Erlenmeyer flask with the large tweezers, transferred to paper towels, and blotted with Kimwipes. Then, the cotyledon was rolled with a taper and put into a 1 ml Reacti-Vial (Supelco, Inc., Bellefonte, PA, USA) reaching only half way to the bottom to avoid the contact of cotyledon with the intercellular wash fluids (IWF) during the centrifugation. The cotyledon was centrifuged at 4000 rpm in a Safety-Head Centrifuge (Clay-Adams Co., Inc., New York) for 15 min. About 0.3 ml of IWF per cotyledon was collected and lyophilized. Before the enzyme activity measurement, the freeze-dried IWF of

the cotyledon was dissolved in 3 μl of NP-water and centrifuged at 4000 rpm for 10 min. 2 μl of supernatant was taken. Enzyme activities were measured by using CZE.

A 60 cm fused silica capillary (Polymicro Technologies, Phoenix, AZ) of 50 μm i. d. (187 μm o.d.) was used as the separation column. The length was 34 cm from injection end to the detection window. The new capillary was treated with running buffer (0.1 M phosphate buffer, pH 2.5) overnight. The capillary was rinsed with running buffer after each run and allowed to equilibrate for 2 min before each injection. Samples were introduced hydrodynamically and electrophoresis was conducted at 17 kV with the negative electrode on the injection side. The capillary was stored in NP-water for long-term storage.

The home-built instrumental setup for capillary electrophoresis was comprised of a Spellman Model CZE 1000 R high voltage power supply (Plainview, NY, USA) with positive and negative polarity and a Model FL-750 HPLC Plus Spectrofluorescence Detector (Mcpherson Instrument, Acton, MA, USA) equipped with a cell for on-column capillary detection and a 200 W Xenon-Mercury lamp with the excitation wavelength set to 364 nm; a cut-off filter permitted detection of emission beyond 440 nm. Chromatographic data were collected using custom built data loggers (Merz and Mort, 1992) and downloaded into a Power Macintosh 8100/80 computer. Chromatographic data were viewed and quantitated using the program Analog Connection Chrom (Strawberry Tree Computer, Inc.) and KaleidaGraph (Abelbeck Software).

Enzyme activity was calculated by peak area ratio per unit time. The peak area ratio was calculated by dividing the sum of the areas of the peaks representing the products of a particular enzyme activity by the sum of all peak areas. The enzyme activity can be estimated from the peak area ratio by comparing with the standard curve as following description, which linear between EPG unit and peak area ratio.

Enzyme Activity Standard Curve

Aliquots of 10 μg of ANTS labeled oligomers in 2 μl of 25 mM sodium acetate buffer, pH 4.0 were incubated with 2 μl of different numbers of units of EPG enzyme dilution at 40°C for 5 min, respectively. The enzyme products were analyzed by capillary electrophoresis as described above. The standard curve was plotted as EPG unit vs the peak area ratio, which was calculated by dividing the sum of the area of the peaks representing the products of a particular enzyme activity by the sum of all peak areas.

Cell Wall Preparation

The cotyledons from Acala 44 cotton were excised, cleaned and the main veins were removed. The cotyledons were immediately immersed in liquid nitrogen and crushed into a fine powder by using pestle and motor. The frozen powders were then homogenized with PAW buffer (phenol : acetic acid : water = 2 : 1 : 1) by using the polytron (Brinkmann Instruments, Inc., Westbury, NY, USA) for 9 min with three or four intervals at high speed. The cells were viewed under microscope to check for complete rupture. The mixture was placed in 10 vol of PAW buffer at 4°C overnight. The mixture was then centrifuged at 4000 g for 30 min, and the supernatant was discarded. The residues were washed three times with NP-water and were placed in 10 vol of methyl sulfoxide in the cold room overnight to remove the starch. The mixture was filtered on nylon cloth (Small Parts Inc., Miami Lakes, FL, USA). The residue was washed twice with methyl sulfoxide and three times with 5 vol of choloroform : methanol (1 : 1); the organic solvent was removed by a gentle suction to a coarse sintered-glass funnel and the cell walls were washed with 5 vol of acetone at least three times, air-dried, and stored in room temperature until use.

HF Solvolysis and HG Isolation

The cell walls were treated with anhydrous liquid HF as described by Mort (1983) with the modifications described by Qi et al (1993). In the experiment, 500 mg of dry cell walls were placed in a Teflon reaction vessel. The vessel was incubated in -23°C cooled 95% ethanol at least 20 min. The cooling bath temperature was maintained by an immersion cooler (Neslab Instruments Inc., Newington, NH, USA) and a temperature regulator (FTS System, Inc., NY, USA). The cooled cell walls were treated with about 20 ml of HF at - 23°C for 30 min. To stop the reaction, the reaction vessel was immersed in liquid nitrogen for a while and then cold ether (cooled by adding liquid nitrogen to the ether) was added to the reaction mixture. The quenched reaction mixture was allowed to come to room temperature and then was filtered on a Teflon filter. The HF-ether in the filtrate (Ether Extract) was evaporated under vacuum through a liquid nitrogen trap. The residue was placed in NP-water at 4°C overnight. The mixture was filtered with a Teflon filter and the residue was washed with water and filtered at least two times. The filtrates were combined and freeze-dried (Water Extract). The residue was placed in 0.5 M imidazole buffer, pH 7.0, at 4°C overnight. The suspension was filtered and then washed with the same buffer two times. The filtrates were combined and freeze-dried (Imidazole Extract). The residue was washed with water and freeze-dried (Residue).

HPLC Gel Filtration Analysis

The HGs in the water extract described above were further fractionated on Toyopearl HW 50 (S) from Supelco Inc. (Bellefonte, PA, USA), packed in a stainless steel column (50 x 2 cm) from Alltech Associates, Inc. (Deerfield, IL, USA). The column was equilibrated with 50 mM ammonium acetate buffer, pH 5.2, with a flow rate of 2 ml/min. The sugars were detected using a refractive index monitor (Shodex Denko K.K., Japan) and collected in 2-ml fractions. The eluent was pumped with a Dionex Reagent Pump

(Sunnyvale, CA, USA) and the samples were injected via a Valco N60 6-port injector (Houston, TX, USA). Where appropriate, depending on the shape of the peaks, selected fractions were pooled, freeze-dried, desalted by repeated freeze-drying for at least three times, and analyzed for their sugar composition and molecular size.

Sugar Composition Analysis

Carbohydrate compositions were determined by gas chromatographic analysis of the trimethylsilyl methyl glycosides. Methanolysis and derivatization were carried out by a modification of the protocol of Chaplin (1982). Briefly, about 50 μg of dry sugars were weighed on a CAHN 29 electrobalance (Instrument Group Walnut Creek Division, Walnut Creek, CA, USA) and placed in screw-cap glass vials fitted with Teflon-lined lids containing 100 nmoles of inositol as an internal standard. Two hundred μl of 1.5 M methanolic HCl and 100 μl of methylacetate were added and incubated in a 80°C heating block overnight. The vials were removed and cooled down to room temperature. After adding 5 to 7 drops of t-butanol, the samples were evaporated to dryness under a stream of nitrogen gas. Fifty μl of trimethylsilylating reagent, which was prepared fresh in an exhaust hood by mixing 1 part of Tri-Sil (Pierce Chemical Company, Rockford, IL, USA) with 3 parts of dry pyridine (Pierce Chemical Company, Rockford, IL, USA), was applied and allowed to react at room temperature for at least 15 min. The derivatized samples were evaporated slowly under a stream of nitrogen just to dryness, and redissolved in 50 to 100 μl isooctane. A 1 μl aliquot was injected into a fused silica capillary column installed in a Varian 3300 gas-liquid chromatograph. Peak integration was performed using a Varian 4290 integrator.

Deesterification of HGs

HGs was dissolved with 0.1 M NaOH. The pH was monitored with pH paper and maintained around 12 by adding 0.1 M NaOH. The mixture was incubated at room temperature for 15 min and the pH was then adjusted to 6-7 with 0.1 M acetic acid. The de-esterified HGs were dialyzed against water with 1000 MW cutoff dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA, USA) overnight and freeze-dried.

Degree of Esterification (DM) of HGs

The degrees of esterification of HGs were determined by the method described by Maness et al (1990). Briefly, around 100 μg of HGs was suspended in 40 μl of 1.0 M imidazole-HCl buffer, pH 7.0 and cooled on ice. About 4 mg of sodium borohydride was added and the sample vials were loosely capped and placed on ice at least 1 h. About 20 μl of glacial acetic acid was then slowly added to decompose the excess borohydride. An equal volume of distilled water was added and the reduced HGs were obtained by precipitation with 4 volume of 95% ethanol. The sample was resuspended in water and then precipitated with ethanol two times for removing the salts, and then dried with a speed vacuum. The glycosyl compositions of both the reduced HG and the non-reduced HG were determined by GC analysis. DM was calculated by:

$$DM = \frac{\text{galactose (R) - galactose (N)}}{\text{galactose (R) - galactose (N) + galacturonic acid (R)}} \times 100$$

where galactose (R), galactose (N), and galacturonic acid (R) represent reduced galactose, non-reduced galactose, and reduced galacturonic acid, respectively.

HG Size Analysis by Ion-Exchange Chromatography

HG molecular size distribution was determined by ion-exchange chromatography as described by Maness et al. (1991). HGs were labeled with 2-aminopyridine (2-AP) at their reducing end by a condensation reaction. The 2-AP labeled HGs were

chromatographed on a CarboPac PA100 HPLC anion-exchange column (4 x 250 mm) using a Dionex Bio-LC Carbohydrate System (Dionex Corporation, Sunneyvale, CA) at an eluent flow rate of 1.0 ml/min. The 2-AP labeled HGs were detected by a Shimadzu RF 535 fluorescence detector (Shimadzu, Kyoto, Japan), with the exciting wavelength set at 290 nm and emission wavelength at 350 nm. The separation was carried out by using a gradient consisting of solvent 1 (water) and solvent 2 (1.0 M Phosphate buffer, pH 7.0, containing 0.25 M NaCl). Sample components were injected into the system equilibrated with 50 mM phosphate buffer and eluted after a 3 min lag period using a linear gradient of phosphate buffer from 50 mM to 270 mM over 47 min, to 350 mM over 40 min, followed by an increase to 430 mM over another 65 min, then to 500 mM over 5 min, with a final 5 min hold at 500 mM to wash the column. The system was allowed to equilibrate at initial conditions for at least 10 min prior to another injection. Chromatographic data were collected by using custom built data loggers as described above.

RESULTS AND DISCUSSION

Cotton Cotyledon Growth Rate

Each cotton plant has a smaller size and a larger size cotyledon. The areas of the smaller size and larger size cotyledons were measured using an area meter and plotted separately. The time course and relative growth rate of cotton cotyledons are shown in Figure 7. The relative growth rate ($cm^2\ cm^{-2}\ h^{-1}$) was calculated with respect to the leaf area at the start of each 24-h period based on the larger size cotyledon areas, e.g. the average increase in area per hour over 24 hour period divided by the area at the beginning of the time period. This method of presenting the data highlights the much more rapid expansion rate of the younger cotyledons (Van Volkenburgh et al, 1985). The relative growth rate of the cotyledon dramatically decreased until day 5 and then remained low until the cotyledon reached mature size on day 9. However, the growth of the cotyledon did not completely cease until day 10.

Enzyme Activities *in vivo*

PG activities were determined by an *in vivo* measurement of degradation of a fluorescent tagged oligomer of pectic acid (Zhang et al 1996). About 100 μg (5μg/μl) of labeled GalA hexamer, as a substrate, was injected into the intercellular spaces of the larger cotyledons in the different aged cotton plants. Two injections were made per cotyledon to avoid leakage of the solution out of the cotyledon. About 1 cm^2 of soaked area of the cotyledon's intercellular spaces was caused by injection of 10 μl labeled hexamer. The total surface area of the larger size cotyledons was approximately 7 to 18 cm^2 from 3 to 9 days (Figure 7). Thus only approximately 11-30% of the tissue in the cotyledons from 3 to 9-days was exposed to the substrate. Figure 8 shows the relative amounts of exo- and endo-polygalacturonase activity throughout the cotton cotyledon expansion. Determining the amount of enzyme activity in a living cotyledon is not a highly controlled experiment. We do not know what volume of intercellular space was infiltrated with substrate, or how that changes as the cotyledon expands. We do not know the pH of the extracellular space, nor whether the enzyme activities are fixed in the walls or mobile. A potential problem is that there could be more efficient rinsing out of the cotyledons of small oligomers compared to larger ones. Despite all of the unknowns, we obtained consistent results using the following procedure. Incubation times with the substrate were adjusted empirically (5 minutes to 3 hours between the injection time and immersion of the cotyledon in cold infiltration buffer) to allow the reaction to proceed only until about 75% of the fluorescent labeled substrate had been converted to products. Rather than assuming complete recovery of reactants and products, we calculated the percent of the reactant which had been converted to product by dividing the sum of the areas of the peaks representing products of a particular enzyme activity by the sum of all peak areas. Activity was expressed as percent conversion per unit of time. Then, we can convert the peak area ratio to the enzyme activity

Figure 7. The areas of both the larger and smaller cotyledons on several cotton seedlings were measured with an area meter and plotted against the number of days after emergence from the soil. Before three days the cotyledons were often still trapped within the seed coats, so their area could not easily be measured. Relative growth rate was calculated as 100 times the average increase in area per hour over a 24 hour period divided by the area at the beginning of the time period based on larger cotyledon areas.

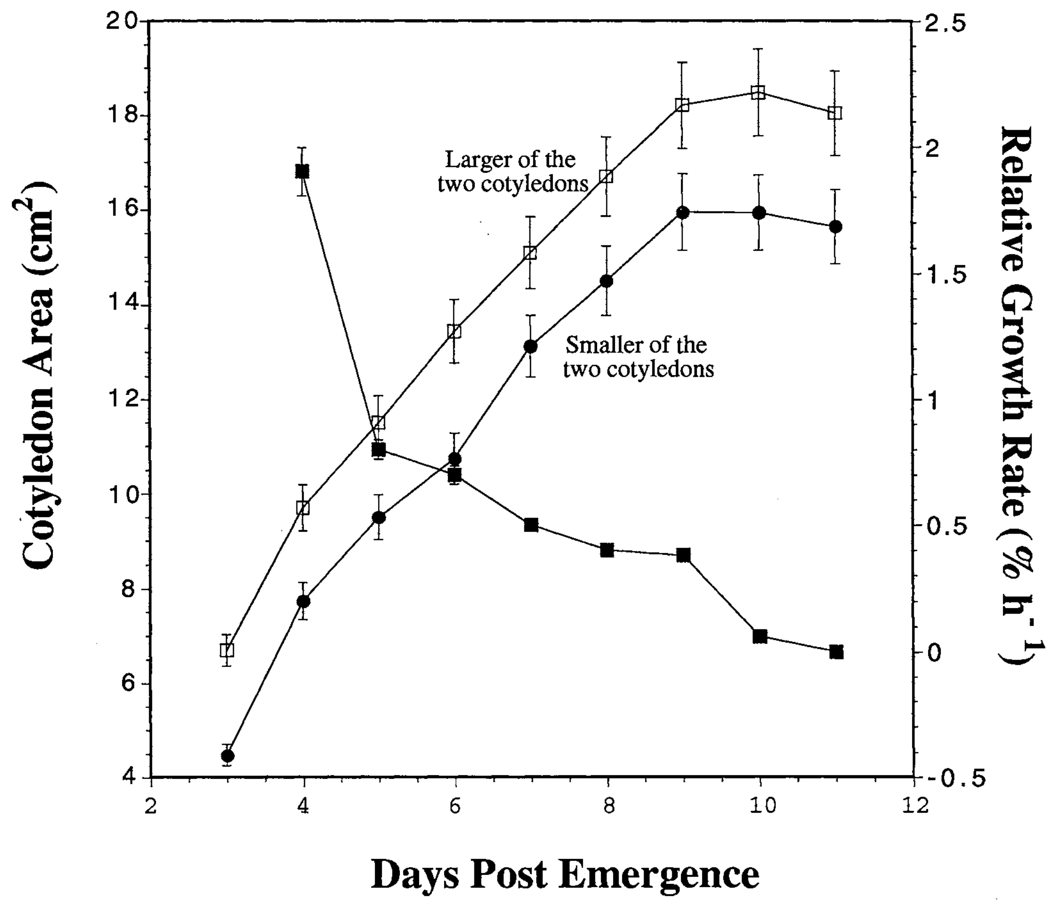
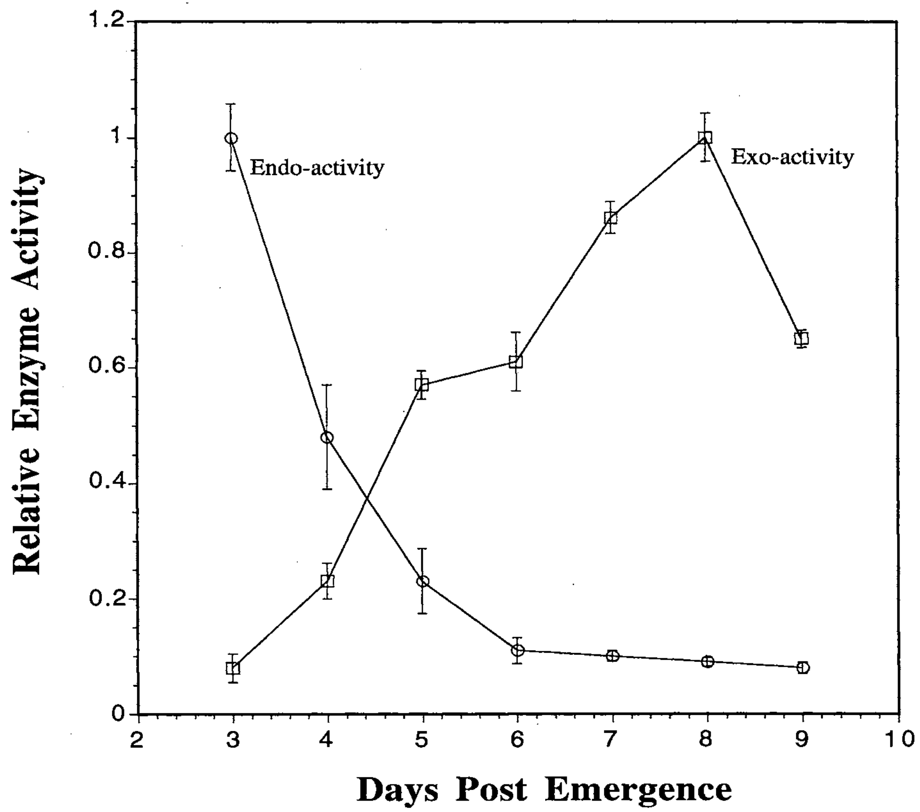

Larger of the two cotyledons
Smaller of the two cotyledons
Cotyledon Area (cm2)
Relative Growth Rate (% h-1)
Days Post Emergence
20
18
16
14
12
10
8
6
4
2.5
2
1.5
1
0.5
0
-0.5
2
4
6
8
10
12

Figure 8. The mean and standard deviation of the enzyme activity detected in the intercellular spaces of cotyledons from three to nine days after emergence from the soil.


1.2
1
0.8
0.6
0.4
0.2
0
Relative Enzyme Activity
Endo-activity
Exo-activity
2
3
4
5
6
7
8
9
10
Days Post Emergence

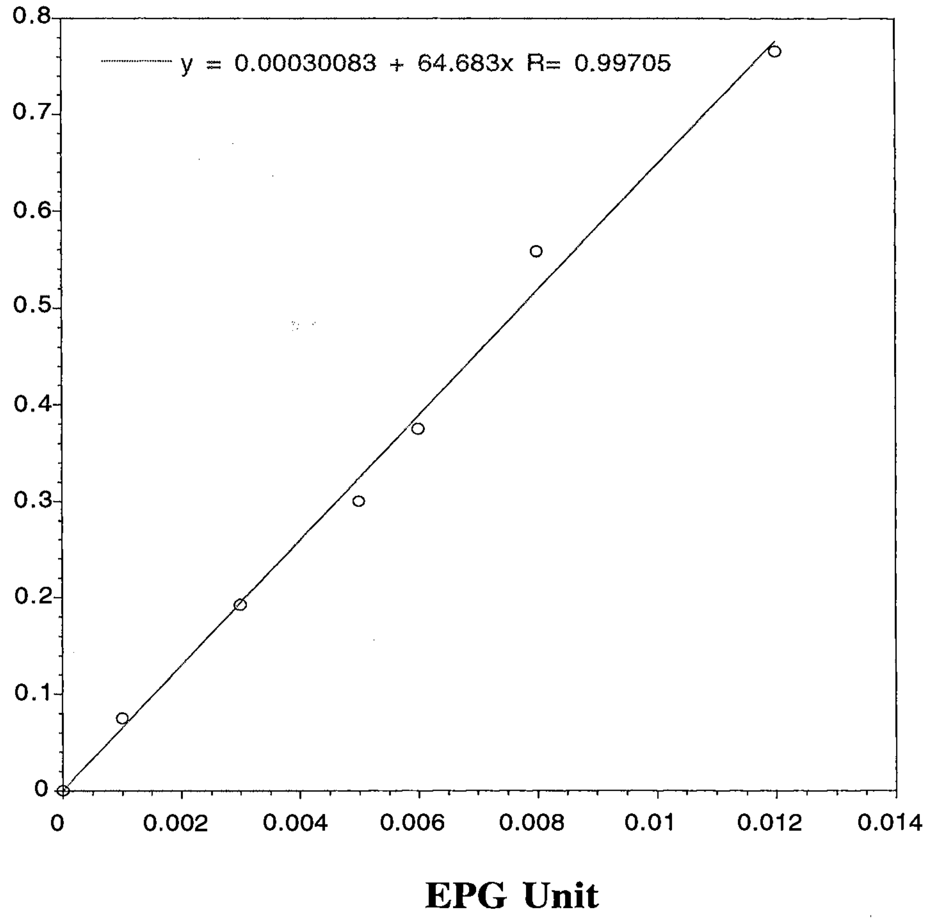
by comparing with the standard curve (Figure 9).

The results indicated that there is a dramatic change in the levels of exo- and endo-PG activity as the cotyledons change their rate of expansion. It was estimated that about 700 μg of HGs cm^{-2} h^{-1} could be digested to monomer by EPG in 3-day old cotyledons, but only about 60 μg of HGs cm^{-2} h^{-1} in 9-day-old cotton cotyledons. From sugar and cell wall dry weight (~ 6 mg/cm^2 of 3-day-old cotyledons) analysis, we estimated an average of 1 mg of HGs cm^{-2} in expanding cotton cotyledons. The EPG activity was highest during the initial stages of the expansion and showed a very good correlation with relative growth rate (Figure 7 compared to Figure 8). This may be because the pectin needs to be degraded to allow expansion, or perhaps the middle lamella needs to be weakened to allow cell separation for the production of the airspaces needed for gas diffusion. The exopolygalacturonase activity may be needed for salvage of the pieces of polygalacturonic acid released during the expansion phase. It may be important for the plant not to accumulate pieces of polygalacturonic acid approx. twelve residues in length because these are known to induce disease resistance responses.

Cotyledon Cell Wall Isolation

The final procedure developed for isolation of walls from cotton cotyledon is shown in Figure 10. The main veins of cotyledons were removed before homogenization. Liquid nitrogen was used not only to make cotyledons into fine powders, but also to fix cell wall metabolism by stopping wall enzyme activities. PAW (Phenol:Acetic acid:Water=2:2:1) buffer was used to remove cytoplasmic proteins, to inactivate wall-linked enzymes that might degrade constituent polysaccharides, and to reduce depolymeration. DMSO was used to remove starch that might alter sugar composition analysis. The last step in the purification procedure involved treatment of the wall preparation with chloroform : methanol (1:1 v:v) to remove lipids and to dislodge

Figure 9. The standard curve for polygalacturonase activity. ANTS labeled hexamer in 25 mM acetate buffer, pH 4.0 was incubated with different levels of EPG from fungus (Megazyme) at 40°C for 5 min. The enzyme products were analyzed by capillary electrophoresis. The plot presents the linear relationship between EPG Unit and the peak area ratio. One Unit of activity is defined as the amount of enzyme required to release one micromole of reducing-end equivalents from the substrate employed at pH 4 and 40°C.



Peak Area Ratio
(peak area of dimer and trimer/all peak area)
y = 0.00030083 + 64.683x R= 0.99705
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.002
0.004
0.006
0.008
0.01
0.012
0.014
EPG Unit

membrane-bound proteins that might be associated with the wall fragments. To facilitate storage, the final wall preparations were dried by solvent exchange through acetone. Yield of the dried cell walls from cotton cotyledon was about 3.0 g 100 g^{-1} fresh weight. Iodine staining indicated that the wall preparations were essentially free of adherent starch (data not shown).

Carbohydrate Composition of Cotyledon Walls

To know whether there were changes in sugar composition of HGs and cell walls during cotyledon expasion, the sugar content of HGs and cell walls from cotyledons of different ages during their expansion are shown in Table 1, 2, and 3. From Table 1, 2, and 3 we can see that about 20% of cotyledon wall sugars is GalA. During cotton cotyledon expansion there was a general increase in GalA and Rha and a decrease in Ara and Xyl, but no uniform change in neutral sugars Gal and Glc. The sugar contents are expressed as sugar weight from 500 mg cotyledon walls. Only about 40% of the sugars from cell walls was recovered by GC analysis. We did not know what the remaining of 60% of cell walls was. However, treated HGs in water extract with EPG before sugar analysis, The recovery was largely increased (Table 1, 2, and 3).

Molecular Size of HGs

Gel filtration profiles of the HGs from water extraction of cotyledons of different ages are shown in Figure 11. From the chromatograms, it is clear that the HG molecular size shifts towards a higher average molecular weight and the amount of the large size HGs was increased during cotyledon expansion. In the younger cotyledons a smaller molecular weight range was observed, however, in older cotyledons the peak was shifted towards two directions, higher molecular weight and low molecular weight. This indicates a concomitant change in EPG and exogalacturonase activities during cotyledon expansion

Figure 10. The summary of the extraction procedure of cell walls from cotton cotyledons.

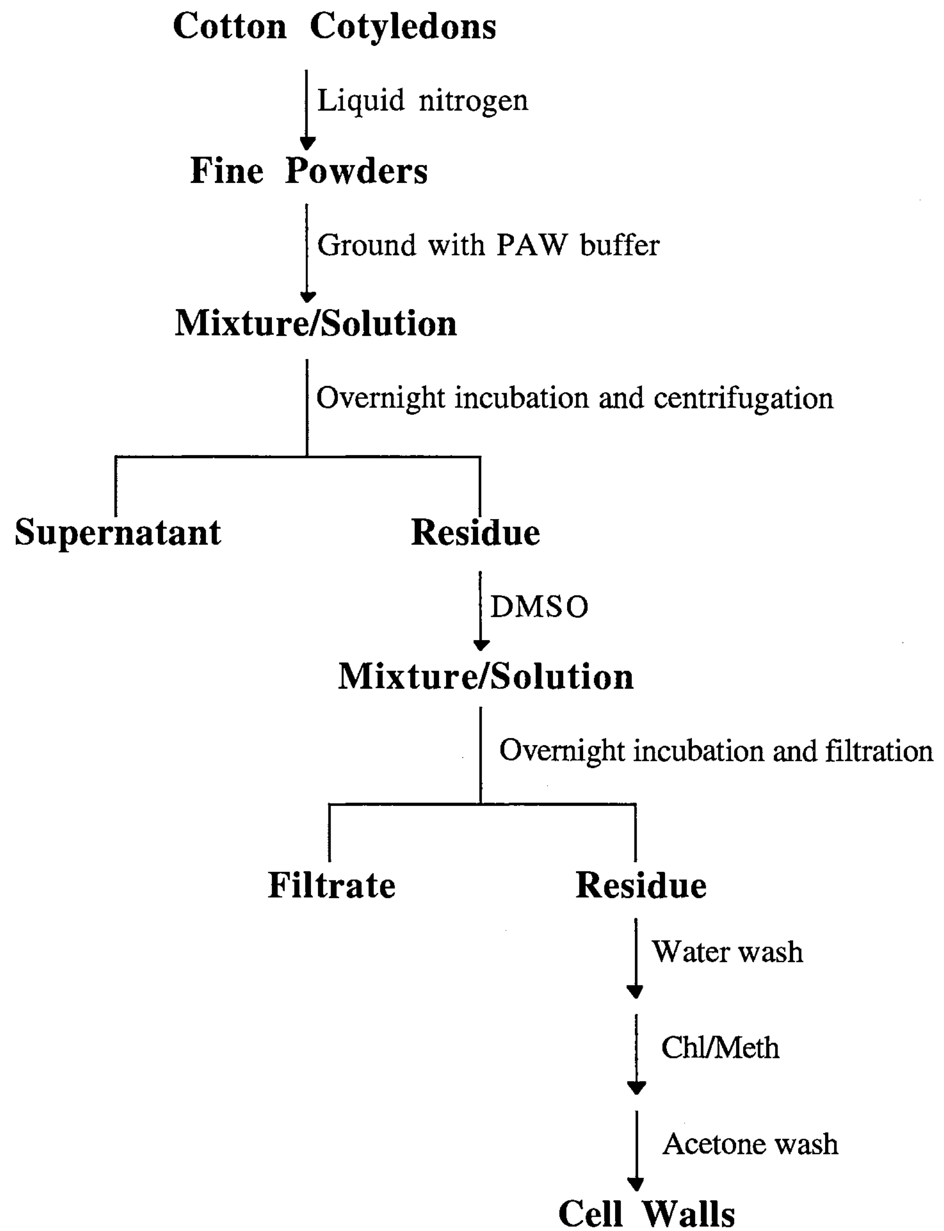

Cotton Cotyledons
Liquid nitrogen
Fine Powders
Ground with PAW buffer
Mixture/Solution
Overnight incubation and centrifugation
Supernatant
Residue
DMSO
Mixture/Solution
Overnight incubation and filtration
Filtrate
Residue
Water wash
Chl/Meth
Acetone wash
Cell Walls

Table 1. Weight of Sugars (mg) in Extracts from 500 mg 3-d Cotton Cotyledon Cell Walls (n=5)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact Walls	27 ± 1	10 ± 3	20 ± 3	95 ± 8	21 ± 1	9 ± 2	182
Ether	20 ± 3	4 ± 1	10 ± 2	2 ± 1	4 ± 1	2 ± 1	42
Water	4 ± 1	5 ± 1	4 ± 1	55 ± 1	15 ± 1	5 ± 1	88
Water*	5 ± 1	6 ± 1	5 ± 1	72 ± 1	19 ± 1	4 ± 1	111
Imidazole	0 ± 0	0 ± 0	1 ± 0	23 ± 4	0 ± 0	0 ± 0	24
Residue	1 ±	0 ± 0	4 ± 0	4 ± 2	1 ± 0	1 ± 1	11
Recovery (%)	94	100	92	88	88	88	91
Recovery*(%)	98	100	100	100	100	85	100

* The HGs were treated with EPG before sugar analyses.

Table 2. Weight of Sugars (mg) in Extracts from 500 mg 5-d Cotton cotyledon Cell Walls (n=5)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact Wall	25 ± 6	12 ± 2	16 ± 2	97 ± 6	20 ± 2	10 ± 3	180
Ether	19 ± 2	6 ± 1	10 ± 1	3 ± 0	5 ± 0	1 ± 1	44
Water	4 ± 1	5 ± 1	4 ± 0	43 ± 3	8 ± 0	1 ± 1	65
Water*	2 ± 1	3 ± 1	5 ± 1	82 ± 5	11 ± 0	3 ± 0	106
Imidazole	0 ± 0	0 ± 0	1 ± 0	17 ± 1	0 ± 0	1 ± 0	19
Residue	0 ± 0	0 ± 0	2 ± 0	4 ± 1	1 ± 0	7 ± 0	14
Recovery (%)	91	89	100	69	67	97	79
Recovery* (%)	84	76	100	100	80	100	100

* The HGs were treated with EPG before sugar analysis.

Table 3. Weight of Sugars (mg) in Extracts from 500 mg 7-d Cotton Cotyledon Cell Walls (n=5)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact wall	24 ± 4	14 ± 1	15 ± 1	102 ± 6	21 ± 1	13 ± 3	189
Ether	21 ± 2	5 ± 1	9 ± 2	4 ± 1	6 ± 1	6 ± 1	51
Water	1 ± 1	2 ± 1	3 ± 1	46 ± 3	8 ± 1	5 ± 1	65
Water*	2 ± 0	4 ± 1	2 ± 1	76 ± 3	10 ± 1	4 ± 0	98
Imidazole	0 ± 0	0 ± 0	0 ± 0	12 ± 3	0 ± 0	0 ± 0	12
Residue	1 ± 1	0 ± 0	2 ± 0	4 ± 1	1 ± 0	8 ± 0	16
Recovery (%)	99	58	89	64	67	100	76
Recovery* (%)	100	68	89	93	77	100	94

* The HGs were treated with EPG before sugar analysis

(Figure 8). EPG may degrade the large existing HG molecules into a smaller ones in the younger cotyledons. The newly synthesized large HG molecules were not degraded as much in the older cotyledons because there was very low EPG activity in the older cotyledons. The peak area was wider in older cotyledons than that in younger cotyledons (Figure 11). This may be caused by exogalacturonase activity. Exogalacturonase activity may cause the accumulation of the oligomers of less than five residues. Previous investigations of plant exogalacturonases have shown that they have low activity on oligomers of less than five residues (Pressey and Avants, 1975; Garcia-Romera and Fry, 1994). As exogalacturonase activity increased during cotyledon expansion, the accumulation of the small oligomers of GalA could occur.

The length of the HG backbone both in the water extract and in the imidazole extract from HF treated cotyledon walls can be estimated by an anion-exchange chromatography (Figure 12 and 13). The HGs from cotyledons of different ages and from commercial pectic acid, as a standard, were labeled with 2-aminopyridine and separated using a PA100 anion-exchange column with fluorescence detection. The chromatogram resulting from commercial pectic acid showed that we can separate the pectic oligomers up to about 70 GalA residues. The peak identification of short oligomers was carried out by co-chromatography with commercial standards (e.g. dimer and trimer) and the identity of the longer ones inferred by assuming that the commercial pectic acid contained only a homologous series of GalA oligomers. The peaks on HG chromatograms were identified by comparison with those for commercial pectic acid. Comparing the HGs from water extract (Figure 12) and imdazole extract (Figure 13) among 3, 5, and 7 day old cotyledons, respectively, we can see that HGs from younger cotyledons appears to be somewhat more degraded in both extracts. Since the HF treatments we used to solubilize HGs are the same in all cases, the changes in HG size may be caused by endopolygalacturonase degradation (Figure 8).

Figure 11. Toyopearl HW 50S molecular size profiles of homogalacturonans in water extract from (A). 3-day-old, (B). 5-day-old and (C). 7-day-old cotton cotyledon cell walls. The column was equilibrated with 50 mM ammonium acetate buffer, pH 5.2, with a flow rate of 2 ml/min. The sugars were detected by refractive index monitor and collected in 2 ml per fraction.

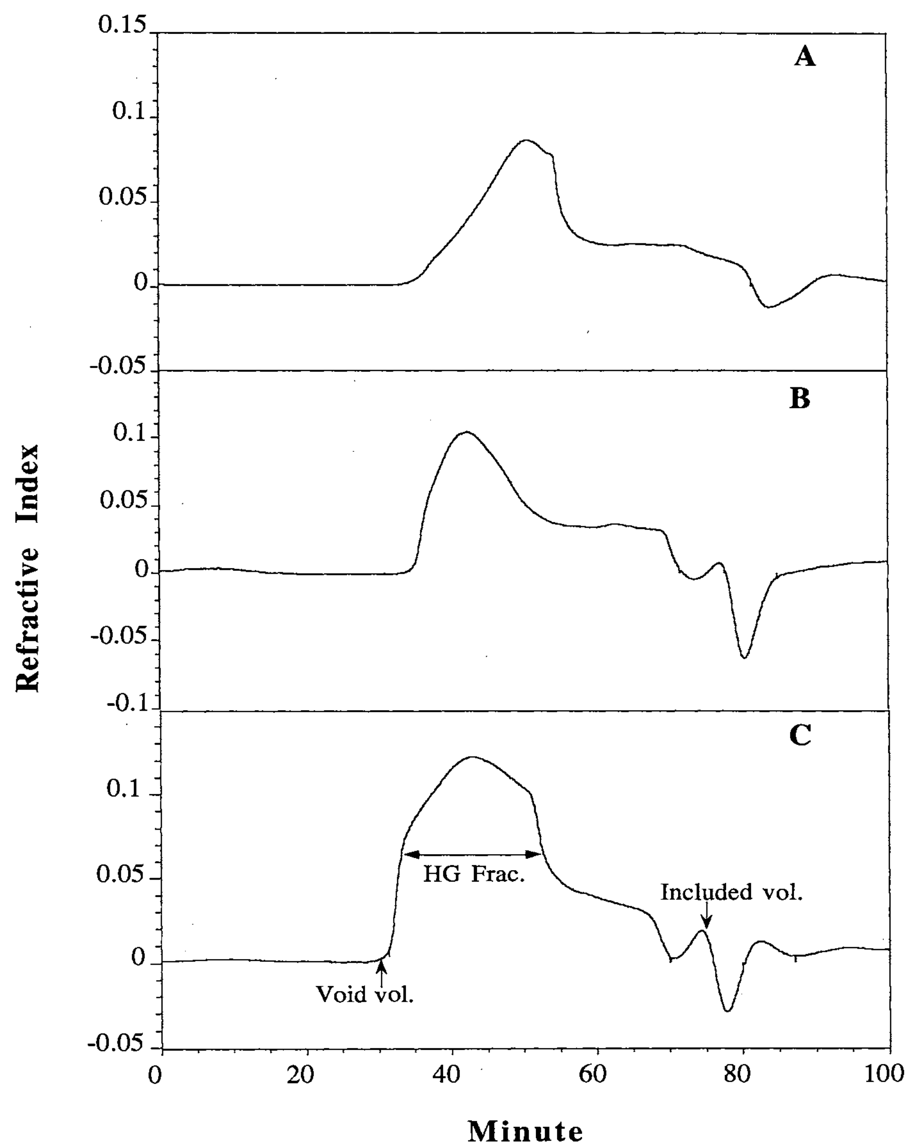
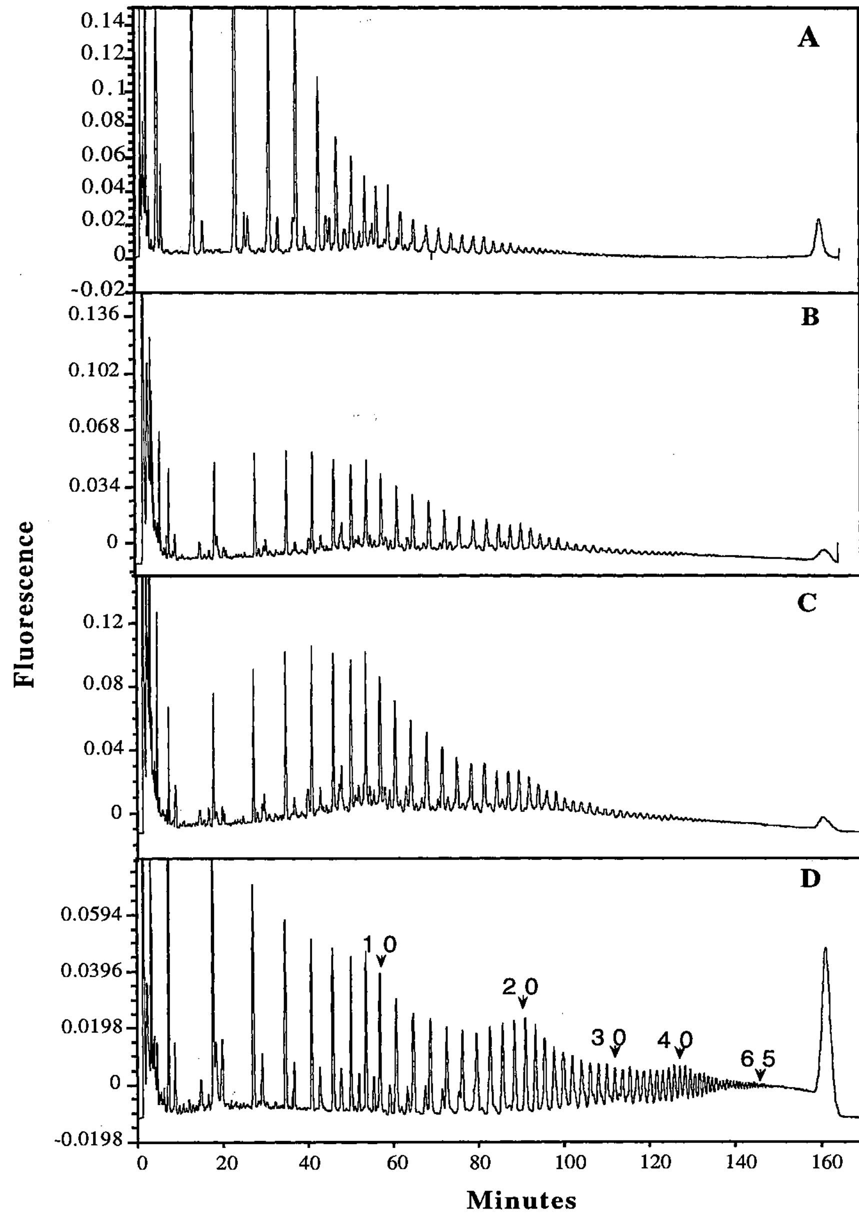
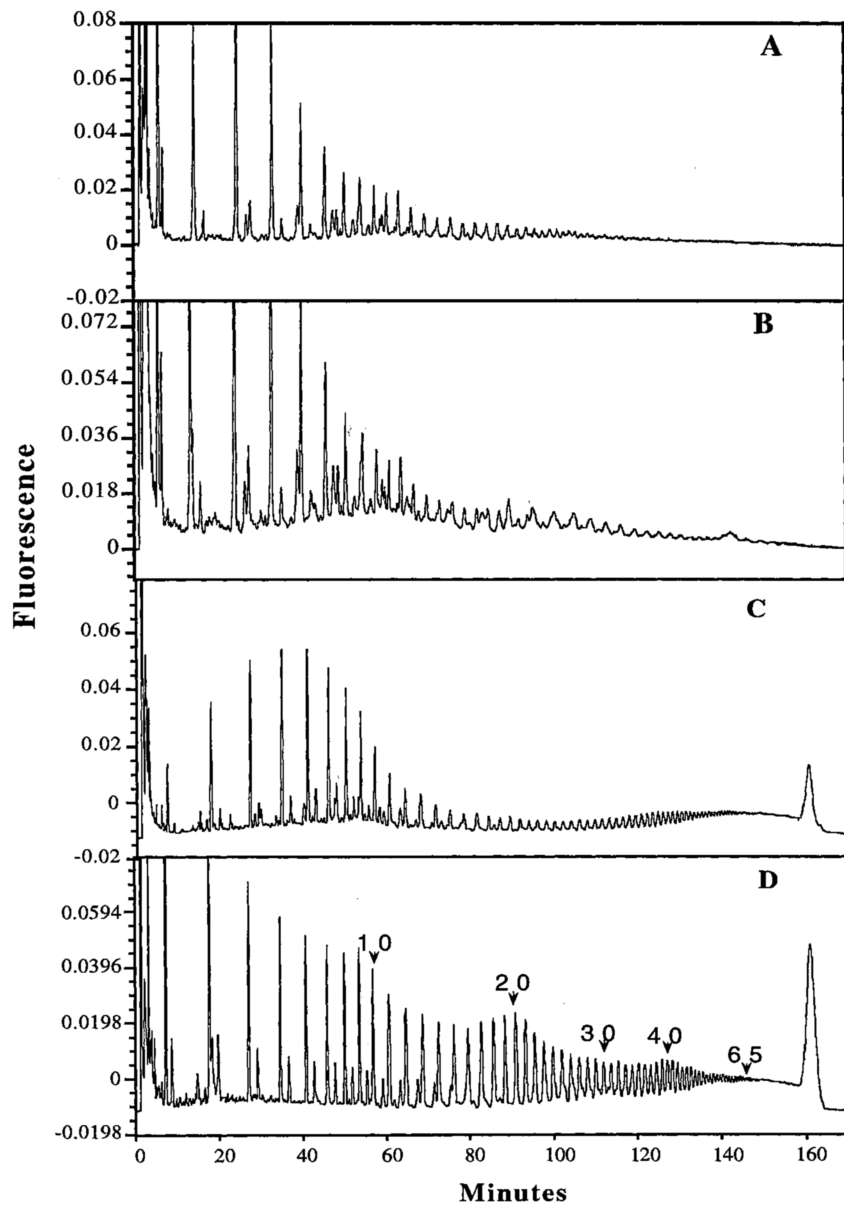

A
B
C
0.15
0.1
0.05
0
-0.05
-0.1
Refractive Index
HG Frac.
Included vol.
Void vol.
0
20
40
60
80
100
Minute

Figure 12. PA100 anion-exchange chromatography molecular size profiles of homogalacturonans in water extract from (A). 3-day-old, (B). 5-day-old and (C). 7-day-old cotton cotyledon walls. Panel D shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule in each fraction. An eluent flow was 1.0 ml/min. The 2-AP labeled HGs were detected by a Shimadzu RF 535 fluorescence detector with exciting wavelength set at 290 nm and emission wavelength at 350 nm. The gradient consisted of solvent 1 (water), solvent 2 (1.0 M phosphate buffer, pH 7.0, containing 0.25 M NaCl). Sample components were injected into the system equilibrated with 50 mM phosphate buffer and eluted after 3 min lag period using a linear gradient of phosphate buffer from 50 mM to 270 mM over 47 min, to 350 mM over the next 40 min, then an increase to 430 mM over another 65 min, then to 500 mM over 5 min, with a final 5 min hold at 500 mM to wash the column.



A
B
C
D
Fluorescence
Minutes
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.02
0.136
0.102
0.068
0.034
0
0.12
0.08
0.04
0
0.0594
0.0396
0.0198
0
-0.0198
0
20
40
60
80
100
120
140
160
10
20
30
40
65

Figure 13. PA100 anion-exchange chromatography molecular size profiles of homogalacturonans in imidazole extract from (A). 3-day-old, (B). 5-day-old and (C). 7-day-old cotton cotyledon walls. Panel D shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule in each fraction. An eluent flow was 1.0 ml/min. The 2-AP labeled HGs were detected by a Shimadzu RF 535 fluorescence detector with exciting wavelength set at 290 nm and emission wavelength at 350 nm. The gradient consisted of solvent 1 (water), solvent 2 (1.0 M phosphate buffer, pH 7.0, containing 0.25 M NaCl). Sample components were injected into the system equilibrated with 50 mM phosphate buffer and eluted after 3 min lag period using a linear gradient of phosphate buffer from 50 mM to 270 mM over 47 min, to 350 mM over the next 40 min, then an increase to 430 mM over another 65 min, then to 500 mM over 5 min, with a final 5 min hold at 500 mM to wash the column.


Fluorescence
Minutes
A
B
C
D
0.08
0.06
0.04
0.02
0
-0.02
0.072
0.054
0.036
0.018
0.0594
0.0396
0.0198
-0.0198
0
20
40
60
80
100
120
140
160
10
20
30
40
65

Degree of Esterification (DM) of Hgs

The DM of HGs from either the water extract or the imidazole extract from cotyledons different ages was measured based on the GalA content of the fractions. These values are reported in Table 4. The degrees of esterification (DM) were 57, 47, and 47% in water extracts and 34, 25, and 27% in imidazole extracts, in 3, 5, 7-day-old cotton cotyledons, respectively. Obviously, the DM of HGs in the water-soluble extract is higher than that in the imidazole-soluble extract. There is no noticeable change to the DM between 5 and 7 day old cotyledons, not only in imidazole-soluble extract but also in water-soluble extract. The DM of HGs of 3-day-old cotyledons is dramatically higher than those in older cotyledons (Table 4). A high level of DM prevents the pectic polymers from associating with divalent calcium ions and may cause the lower cohesion of middle lamenar pectins. The structural change of the middle lamenar pectins may be more favorable for enzyme digestion.

Table 4. Degree of Esterification of HGs from Water Extract and Imidazole Extract during Cotton Cotyledon Expansion (n=5)

Fraction \ DM / Day	DM (%)		
	3-d	5-d	7-d
Imidazole Extract	34 ± 2	25 ± 1	27 ± 1
Water Extract	57 ± 2	47 ± 0	47 ± 2

LITERATURE CITED

Albersheim, P, Darvill AG, O'Neill MA, Schols HA, Voragen AGJ (1996) In J. Visser and A.G.J. Voragen. An hypothesis: The same six polysaccharides are components of the primary cell walls of all higher plants. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., pp 47-55.

Arribas A, Revilla G, Zarra I, Lorences EP (1991) Changes in cell wall polysaccharides during the growth of *Phaseolus vulgris* leaves. J. Exp. Bot. **42:** 1181-1187.

Brown SM, Crouch ML (1990) Characterization of a gene family abundantly expressed in *Oenothra organensis* pollen that shows sequence similarity to polygalacturonase. Plant Cell **2:** 263-274.

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistencey of molecular structure with the physical properties of the walls during growth. Plant J. **3:** 1-30.

Chaplin MF (1982) A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-lquid chromatography. Anal. Biochem. **123:** 334-341.

Cosgrove DJ (1997) Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. Plant Cell. **9:** 1031-1041.

Crookes PR, Grierson D (1983) Ultrastructure of tomato fruit ripening and the role of polygalacturonase isozymes in cell wall degradation . Plant Physiol. **72:** 1088-

Fry S (1995) Polysaccharide-modifying enzymes in the plant cell wall. Ann. Rev. Plant Physiol. Plant Mol. Biol. **46:** 497-520

Garcia-Romera I, Fry SC (1994) Absence of transglycosylation with oligogalacturonides in plant cells. *Phytochemistry* **35:** 67-72.

Gross KC, Starrett DA, Chen HJ (1995) Rhamnogalacturonase, α-galactosidase, and β-galactosidase: potential roles in fruit softening. Acta Hort. **398:** 121-129.

Huber DJ (1983) Polyuronide degradation and hemicellulose modification in ripening tomato fruit. J. Amer. Soc. Hort. Sci. **108:** 405-409.

Huber DJ (1992) The inactivation of pectin depolymerase associated with isolated tomato fruit cell wall: implications for the analysis of pectin solubility and molecular weight. Physiol. Plant. **86:** 25-32.

Huber DJ, O'Donoghue E (1993) Polyuronides in avocado (*Persea americana*) and tomato (*Lycoperiscon esculentum*) fruits exhibit markedly different patterns of molecular weight downshifts during ripening. Plant Physiol. **102:** 473-480.

Kim JB, Carpita NC (1992) Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. Plant Physiol. **98:** 646-653.

Knox JP, Linstead PJ, King J, Cooper C, Roberts K (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. Planta **181:** 512-521.

Koch JL, Nevins DJ (1989) Tomato fruit cell wall. I. Use of purified tomato polygalacturonase and pectinmethylesterase to identify development changes in pectins. Plant Physiol. **91:** 816-822.

Mannes NO, Miranda ET, Mort AJ (1991) Recovery of sugar derivatives from 2-aminopyridine labeling mixture for high-performance liquid chromatography using UV or fluorescent detection. J. Chromatogr. **587:** 177-183.

Mannes NO, Ryan JD, Mort AJ (1990) Determination of the degree of methyl esterification of pectins in small samples by selective reduction of esterified galacturonic acid to galactose. Anal. Biochem. **185:** 346-352.

Masuda Y, Pilet PE (1983) Mechanical properties and polysaccharide nature if the cell wall of maize root. Physiol Plant. **59:** 512-517.

McCann MC, Shi J, Roberts K, Carpita NC (1994) Changes in pectin structure and localization during the growth of unadapted and NaCl-adapted tobacco cells. Plant J. **5(6):** 773-785.

McCann MC, Wells B, Roberts K (1990) Direct visualization of cross-links in the primary plant cell wall. J. Cell Sci. **96:** 323-334.

Merz J, Mort AJ (1992) The construction and use of an inexpensive data collection system for high-resolution chromatography. Anal. Biochem. **207:** 351-353.

Mort AJ (1983) An apparatus for safe and convenient handling of anhydrous liquid HF at controlled temperatures and reaction times. Application to the generation of oligosaccharides from polysaccharides. Carbohydr. Res. **122:** 315-321.

Mort AJ, An J, Qi X, Komalavilas P, Otika G, West P, and Maness N (1998) Complete solubilization of pectins from cotton suspension culture cell wall with retention of most structural features: Implication for cell wall architecture. (in preparation)

Mort AJ, Moerchbacher BM, Pierce ML, Maness NO (1991) Problems one may encounter during the extraction, purification and chromatography of pectin fragments and some solutions to them. Carbohydr. Res. **215:** 219-227

Mort AJ, Qiu F, Maness NO (1993) Determination of the Pattern of methyl esterification in pectins. Distribution of contiguous non-esterified residues. Carbohydr. Res. **247:** 21-35

Pierce ML, Essenberg M, Mort AJ (1993) A comparison of the quantities of exopolysaccharide produced by *Xanthomonas campestris* PV. *malvacearum* in susceptible and resistant cotton cotyledons during early stages of infection. Phytopahtology **83:** 344-349.

Pressey R, Avants JK (1975) Modes of action of carrot and peach exopolygalacturonases. *Phytochemistry* **14:** 957-961.

Pressey R, and Reger BJ (1989) Polygalacturonase in pollen from corn and other grasses. Plant Sci. **59:** 57-62.

Qi X, Behrens BA, Mort AJ (1993) Highly selective cleavage of furnanosyl linkages in liquid hydrogen fluoride. Carbohydr. Res. **249:** 265-274.

Schols HA, Voragen AGJ In J. Visser and A.G.J. Voragen. Complex pectins: Structure elucidation using enzymes. (Eds) *Pectins and Pectinases* : Vol. 14, *progress in Biotechnology* , Elsevier Sci. B.V., 1996, pp 3-19.

Taiz L (1984) Plant cell expansion: regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. **35:** 585-657.

Tucker GA, Schindler CB, Roberts JA (1984) Flower abscission in mutant tomato plants. Planta **160:** 164-167.

Van Volkenburgh E, Schmidt MG, Cleland RE (1985) Loss of capacity for acid-induced wall loosing as the principal cause of the cessation of cell enlargement in light-grown bean leaves. Planta **163:** 500-505.

Yamaoka T, Chiba N (1983) Changes in the coagulating ability of pectin during the growth of soybean hypocotyls. Plant Cell Physiol. **24:** 1281-1290.

Zhang Z, Pierce M, Mort AJ (1996) Detection and differentiation of pectic enzyme activity *in vitro* and *in vivo* by capillary electrophoresis of products from fluorescent-labeled substrate. Electrophoresis 17: 372-378.

Zhang Z, Zhan D, Mort AJ (1998) *In vivo* and *in vitro* measurements of rhmnogalacturonase activity by capillary electrophoresis. Abstract for ASPP Meeting.

CHAPTER IV

AMINO ACID ANALYSIS OF PROTEIN HYDROLYSATES BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY OF THEIR 6-AMINOQUINOLYL-N-HYDROXYSUCCINIMIDYL CARBAMATE DERIVATIVES

This chapter has been submitted to Analytical Biochemistry by the authors Zhiquan Zhang, Bing Xie Behrens[1], B. Anthony Behrens[2], Henrike Boermans[3] and Andrew Mort[4]

Department of Biochemistry and Molecular Biology, Oklahoma State university, Stillwater, OK 74078-3035

[1] Present address: UAMS Medical Center, Department of Neurology, College of Medicine, Slot 500, 4301 W. Markham St, Little Rock, AK 72205-7199

[2] Present address: Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman OK 73019-0370

[3] Present address: WWU Münster, Institut für Biochemie und Biotechnologie der Pflanzen, Hindenburgplatz 55, D-48143 Münster, Germany

---

Use of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization of amino acids for analysis of protein hydrolysates (1) is becoming quite popular. These derivatives have some advantages over previously used precolumn derivatives in that they are very stable (2) and are formed rapidly and almost quantitatively over a considerable range of reaction conditions (1). The derivatives can be detected by both UV absorption and fluorescence. In all previous reports the derivatives were separated on reverse phase columns using gradients of acetonitrile and aqueous buffer. Here we report that the derivatives can be separated rapidly using micellar electrokinetic capillary chromatography

(MECC). The advantage of this method is the extremely small volume of reagents used, including only microliters of acetonitrile, per sample. So long as the volumes used for manipulations of the sample are kept to a minimum, submicrogram amounts of protein can be analysed since only nanoliters of sample are injected into the capillary. In preliminary experiments, proteins blotted onto PVDF membranes from SDS gels could be analysed.

MATERIALS AND METHODS.

Materials: Boric acid was purchased from Mallinckrodt (St. Louis, MO, USA); SDS, L-ornithine, hydroxyproline and lysozyme were from Sigma (St. Louis, MO, USA); Amino acid standard H and 6 N HCl was from Pierce (Rockford, IL, USA); the fused-silica capillary was purchased from Polymicro Technology (Phoenix, AZ, USA). AQC was synthesized as previously described (1).

Preparation of running buffer (30 mM borate, pH 9.0, containing 6% SDS): The running buffer was made with 3 ml of 100 mM borate buffer, pH 9.0 (100 mM boric acid, pH adjusted with NaOH), 6 ml of 10% SDS, and the final volume was brought to 10 ml with NanoPure deionized water (NP-water). The solution was filtered through a 0.45 μm nylon membrane (Alltech, Deerfield, IL, USA) and degassed with a water aspirator. This buffer was made fresh daily and treated gently, avoiding SDS bubbles.

10 μl of a 0.0625 nmoles/μl dilution of amino acid standard H in 0.1 N HCl , 10 μl of 0.0625 nmoles/μl hydroxyproline in 0.1 N HCl and 10 μl of 0.0625 nmoles/μl orithine in 0.1 N HCl, as an internal satndard, were dried completely in a 500 μL microcentrifuge tube in a speed vacuum. The dried amino acids were resolubilized in 5 μl of 0.01N HCl, the volume was brought to 25 μl with 0.2 M borate buffer, pH 8.8, and the derivatives

formed via the addition of 5 μl of 10 mM AQC (3 mg/ml) in dry acetonitrile (1). Samples could be stored for several months in a -20 C freezer without noticeable degradation.

Proteins and peptides (up to 1 μg plus a crystal of phenol (roughly 1 mm^3, J.T. Baker Chemical CO., Phillipsburg, NJ, USA) were hydrolyzed with 0.2 ml 6 N HCl in a Reacti-vial for 1.5 h at 150 C (3). After the hydrolysis 0.625 nmoles of ornithine was added. Insoluble materials were removed by centrifugation (4000 g, 10 min). The supernatent was transferred to a 500 μl microcentrifuge tube, dried in a speed vacuum, and then derivatized as described above for the standards.

A cell wall protein extract from primary wheat leaves infected with Erysiphe graminis f. sp. hordei (4) was separated by SDS-PAGE, blotted onto PVDF membrane and stained using Coomassie Brillant Blue R250. The lane was cut in half and one portion used to identify the WIR-1 protein immunologically. The band of WIR-1 protein was cut from the other half of the strip and hydrolysed for the analysis. The Coomassie-stained WIR-1 protein formed a barely visible band on the blotting menbrane.

The amino acid separations were carried out using a custom-built instrument for capillary electrophoresis consisting of a Spellman (Plainview, NY, USA) Model CZE 1000 R high voltage power supply and a LINEAR UVIS 200 detector (Fisher, Fairlawn, NJ, USA). equipped with a cell for on-column capillary detection set at 254 nm or a Model FL-750BX fluorescence detector (McPherson Instruments, Acton, MA, USA). The fluorescence detector was equipped with a cell for on-column capillary detection and a 200 W Xenon-Mercury lamp with the excitation wavelength set to 245 nm; a cut-off filter permitted detection of emission beyond 380 nm. Chromatographic data were collected using custom-built data logger (5) and downloaded into a power Macintosh 8100/80 computer. Chromatographic data were viewed and analysed using the programs Analog Connection Chrom (Strawberry Tree Computer, Inc., Sunnyvale, CA, USA) and KaleidaGraph, developed by Abelbeck Software (distributed by Synergy Software,

Reading, PA, USA). An 80 cm long fused-silica capillary of 50 μm ID was used as the separation column. The length was 50 cm to the detection window. New capillaries were conditioned by rinsing with 50 μl of 0.1 M NaOH, followed with NP-water and then running buffer. The capillary was rinsed sequentially with NP-water, 0.1 M NaOH, methanol, NP-water and running buffer after each run and allowed to equilibrate with the current on for one min before each injection. Samples were introduced by gravity-driven flow (~10 cm height differential between inlet and outlet) for 10-15 s and electrophoresis was conducted at 18 KV with the positive electrode on the injection side. After several runs, the buffer of the electrode reservoirs was replaced for reproducible results. For short-term storage, the capillary was rinsed sequentially with NP-water, NaOH, and NP-water and stored in NP-water. However, for a long-term storage, the capillary was flushed with 0.1 M NaOH, rinsed with NP-water and methanol, and dried with nitrogen gas.

RESULTS AND DISCUSSION

Several previous reports have described separation of AQC-labeled amino acids by reverse phase HPLC. In capillary electrophoresis an analogous type of separation can be attained by introducing charged micelles into the running buffer. Above the critical micelle concentration, SDS forms negatively charged micelles which move electrophoretically towards the anode. However, in a plain fused silica capillary, the presence of mobile, cationic counterions to the fixed negative charges on the surface of the capillary causes an electroosmotic flow of the electrolyte buffer toward the cathode. At pH 9 the net result is that even the SDS micelles move toward the cathode. The net migration rate of an AQC-labeled amino acid in this system is the result of two factors: (1) The rate of electoosmotic flow of the electrolyte and (2) the proportion of the time the amino acid is partitioned into the SDS micelles. The overriding effect allowing the separation is the partitioning into the micelles. Those labeled amino acids which are hydrophobic (e.g. lysine, which has two

Figure 14. An electropherogram of the standard mixture of labeled amino acids using UV absorbance for detection. The separation of AQC labeled amino acids was on an 80 cm long fused-silica capillary (50 μm i.d.). The length was 50 cm to the detection window. Electrophoresis was conducted at 18 KV with the positive electrode on the injection side and 30 mM Borate, pH 9.0 containing 6% SDS as a running buffer. (R= escess reagent AQC)

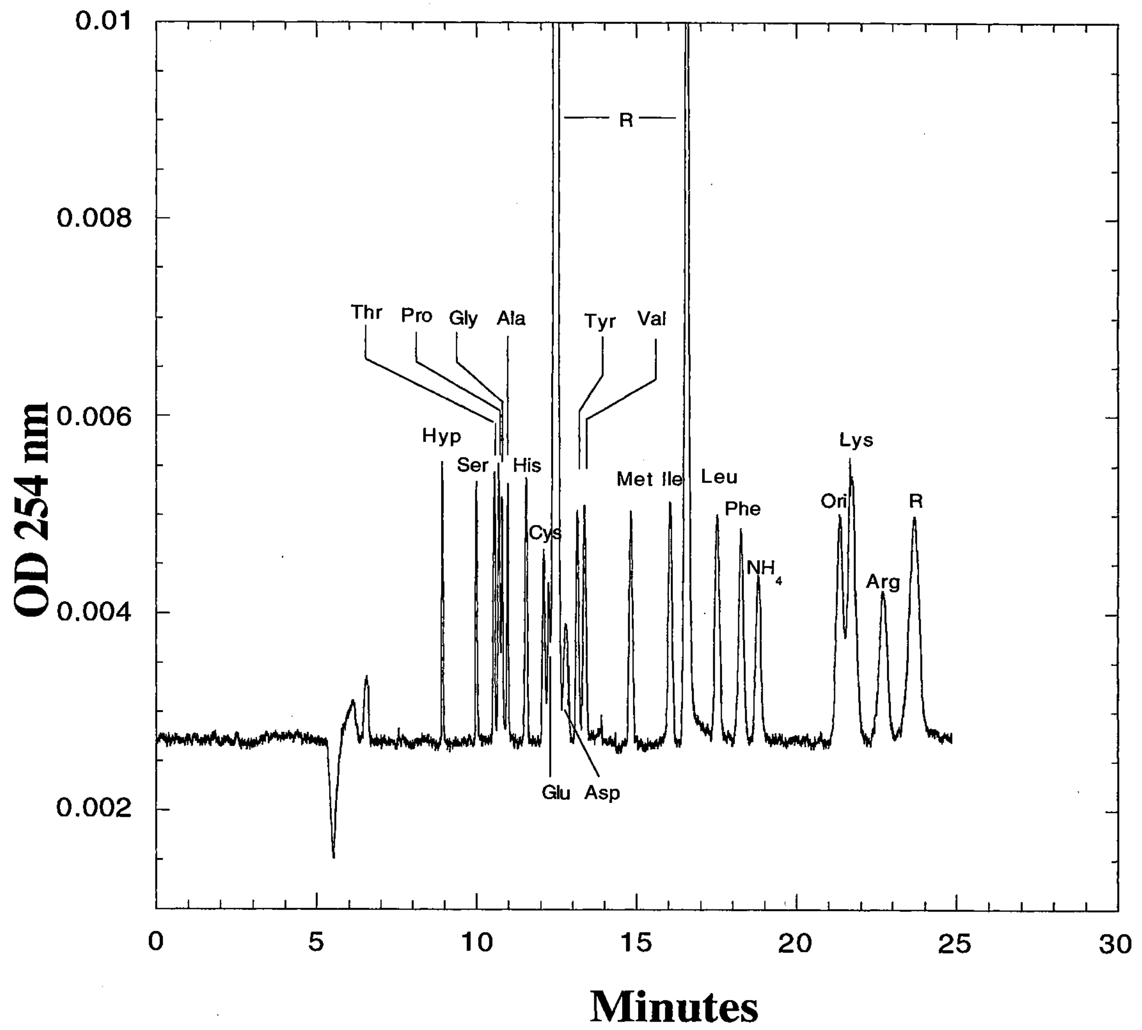
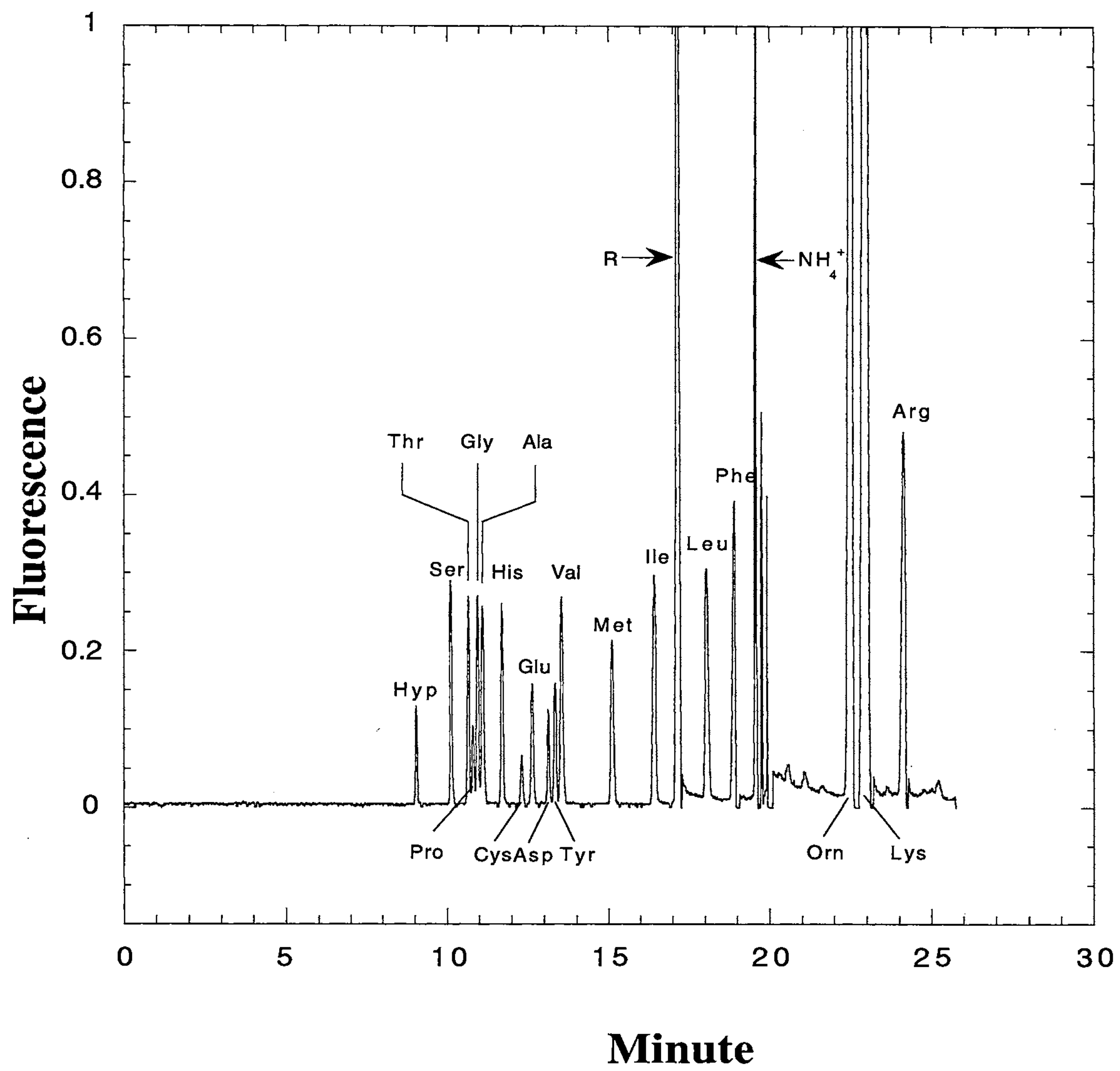

0.01
0.008
0.006
0.004
0.002
OD 254 nm
0
5
10
15
20
25
30
Minutes
R
Thr
Pro
Gly
Ala
Tyr
Val
Hyp
Ser
His
Met
Ile
Leu
Lys
Phe
Orn
R
Cys
NH4
Arg
Glu
Asp

Figure 15. An electropherogram of the standard mixture of labeled amino acids using fluorescence for detection. The separation of AQC labeled amino acids was on an 80 cm long fused-silica capillary (50 μm i.d.). The length was 50 cm to the detection window. Electrophoresis was conducted at +18 KV with the positive electrode on the injection side and 30 mM Borate, pH 9.0 containing 6% SDS as a running buffer. (R= escess reagent AQC)



Fluorescence
Minute
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
R
NH4+
Thr
Gly
Ala
Ser
His
Val
Hyp
Glu
Met
Ile
Leu
Phe
Arg
Pro
Cys
Asp
Tyr
Orn
Lys

aminoquinoline groups attached) partition strongly into the micelles. Those amino acids which partition little into the micelles are carried along by the electroosmotic flow and reach the detector quickly. The electropherogram in Figure 14 shows separation of an amino acid standard mixture using UV detection at 254 nm. Ornithine was used as an internal standard for quantitative analysis. Hydroxyproline was added to the standard mixture because of our interest in plant cell wall hydroxyproline-rich glycoproteins.

Figure 15 shows separation of the amino acid standard mixture with detection by fluorescence, with the excitation wavelength set to 245 nm and a cut-off filter passing emission beyond 380 nm. Table 5 gives the relative responses of the various amino acids relative to ornithine, and shows the reproducibility of migration times that can be attained. A large increase in the relative responses of the amino acids as they migrate more slowly is apparent using UV detection. In contrast the relative responses of the amino acids in HPLC separations are almost constant except for the cases in which there are two amino groups per molecule. Our explanation of this is that, as the molecules migrate more slowly, they spend more time in the detection window and are effectively "counted" more times in the detector. In HPLC separations, all molecules move through the detector window at the same rate. The increase in relative response is much greater using fluorescence. We hypothesize that this is caused by the combined effects of the slower migration of the later peaks and the increase in quantum efficiency of fluorescence as the molecules partition more into the non-polar centers of the SDS micelles.

To test the suitability of the method for quantitative analysis of amino acid hydrolysates of proteins, one microgram aliquots of lysozyme were analysed. Table 6 shows how well the analyses fit to the predicted amino acid molar ratios deduced from the amino acid sequence.

In order to test whether the method was applicable to a real-life sample, a protein weakly visible on a Coomassie-stained PVDF membrane (we estimate ~ 100 ng) was analysed, after hydrolysis of the band on the membrane. Destaining of the membrane

Table 5. Reproducibility for Retention Time and Peak Response (n=10)

Amino Acid	Relative Retention[a] Time	Relative Response[b] (by UV)	Relative Response[b] (by FL)
Hyp	0.418 ± 0.001	0.225 ± 0.002	0.023 ± 0.002
Ser	0.468 ± 0.001	0.276 ± 0.003	0.081 ± 0.004
Thr	0.494 ± 0.001	0.305 ± 0.003	0.077 ± 0.002
Pro	0.501 ± 0.001	0.303 ± 0.003	0.025 ± 0.001
Gly	0.506 ± 0.001	0.303 ± 0.003	0.089 ± 0.002
Ala	0.514 ± 0.001	0.268 ± 0.001	0.081 ± 0.005
His	0.534 ± 0.008	0.315 ± 0.001	0.071 ± 0.002
Cys	0.567 ± 0.001	0.279 ± 0.001	0.018 ± 0.001
Glu	0.575 ± 0.001	0.275 ± 0.002	0.049 ± 0.001
Asp	0.598 ± 0.001	0.314 ± 0.001	0.037 ± 0.001
Tyr	0.601 ± 0.003	0.463 ± 0.006	0.039 ± 0.003
Val	0.615 ± 0.002	0.490 ± 0.004	0.092 ± 0.019
Met	0.625 ± 0.003	0.428 ± 0.003	0.054 ± 0.003
Ile	0.695 ± 0.002	0.563 ± 0.010	0.112 ± 0.002
Leu	0.753 ± 0.003	0.853 ± 0.003	0.133 ± 0.001
Phe	0.822 ± 0.003	0.800 ± 0.005	0.179 ± 0.006
Orn	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000
Lys	1.017 ± 0.001	1.150 ± 0.033	1.466 ± 0.027
Arg	1.063 ± 0.002	0.816 ± 0.006	0.281 ± 0.002

[a] Retention time of amino acid/Retention time of internal standard (Orn) ± standard deviation

[b] Peak area of amino acid/Peak area of internal satndard (Orn) ± standard deviation

Table 6. Amino Acid Analysis of Lysozyme Protein Hydrolysate

Amino Acid	Lysozyme Protein of HEW (n = 3)		
	UV Detection	Fluorescent Detection	From Seq.
Ser	10.19 ± 0.23[a]	9.98 ± 0.15	10
Thr	6.89 ± 0.46	7.13 ± 0.23	7
Pro	2.04 ± 0.17	2.02 ± 0.08	2
Gly	12.12 ± 0.25	12.04 ± 0.25	12
Ala	12.29 ± 0.15	12.09 ± 0.16	12
His	1.01 ± 0.36	1.07 ± 0.13	1
Cys	8.11 ± 0.27	8.06 ± 0.27	8
Glu	5.10 ± 0.15	5.10 ± 0.36	5
Asp	21.18 ± 0.28	20.96 ± 0.46	21
Tyr	3.08 ± 0.13	3.07 ± 0.12	3
Val	6.05 ± 0.12	5.99 ± 0.17	6
Met	1.99 ± 0.35	2.02 ± 0.14	2
Ile	6.47 ± 0.42	5.97 ± 0.07	6
Leu	7.75 ± 0.56	8.01 ± 0.25	8
Phe	3.23 ± 0.42	3.12 ± 0.23	3
Lys	6.16 ± 0.10	6.26 ± 0.43	6
Arg	11.65 ± 0.63	11.12 ± 0.12	11

[a] number of residues ± standard deviation, optimized empirically for the best fit to the known amino acid sequense.

Table 7. Amino Acid Analysis of Presumed WIR-1 Protein Hydrolysate Using AQC-Precolumn Derivatization[b]

Amino acid	presumed WIR-1 protein (n=12)	
	UV detection	from sequence
Ser	7.55 ± 0.86[a]	6
Thr	7.06 ± 1.42	6
Pro	7.02 ± 0.87	9
Gly	14.00 ±	14
Ala	11.85 ± 0.77	14
His	0.00 ± 0.00	1
Cys	3.82 ± 0.61	3
Glx	8.69 ± 1.04	3
Asx	8.45 ± 0.94	4
Tyr	1.77 ± 0.85	2
Val	8.19 ± 1.24	9
Met	0.00 ± 0.00	1
Ile	4.68 ± 1.18	4
Leu	9.01 ± 2.21	6
Phe	5.56 ± 1.74	1
Lys	4.14 ± 1.46	0
Arg	6.00 ± 1.40	5

[a] numbers of residues ± standard deviation, normalized to 14 glycine residues.
[b] attibution to Ms Henrike Boermans.

proved not to be necessary. Table 7 compares the amino acid composition determined by CZE with that derived from the gene sequence. The amino acid composition obtained is highly reproducible and corresponds quite well with the expected composition. The few differences observed could be explained by the the actual protein analyzed being from a different member of a small gene family than the gene sequenced (4).

Several precautions must be taken to be assured of obtaining reproducible and reliable results: 1) the molar ratio of AQC reagent to total amino groups in the sample should be around 4:1. If too little labeling reagent is present, there is competition for labeling between amino acids. Some amino acids label more rapidly than others, skewing the deduced composition. If too much labeling reagent is present the peaks for Glu and Asp may be obscured by a reagent peak. 2) the rinsing of the capillary between runs is necessary to maintain constant migration times. We think that SDS builds up on the walls of the capillary over time, causing an increase in migration time and broadening of the peaks. Returning the capillary to its initial condition by the sequence of rinsings obviates this problem. Use of an automated CZE instrument makes the rinsings trivial.

Using the method for amino acid analysis that we present here, one can analyse a sample within three to five hours including the hydrolysis time. Because the derivatives are very stable and only nanoliters are used for the analysis, standards need only be prepared infrequently and samples can be injected multiple times.

ACKNOWLEDGMENTS

This research was supported, in part, by Department of Energy grant DE-FG03-96ER20215 and a travel grant to HB from the German Research Council.

REFERENCES

1. Cohen, S. A., and Michaud, D. P. (1993) *Anal. Biochem.* **211,** 279-287.
2. Strydom, D. J., and Cohen, S. A. (1994) *Anal. Biochem.* **222,** 19-28.
3. Palace, G. P., and C. H. Phoebe, J. (1997) *Anal. Biochem.* **244,** 393-403.
4. Bull, J., Mauch, Hertig, C., Rebmann, G., and Dudler, R. (1992) *Mol.Plant-Microbe Interact.* **5,** 516-519.
5. Merz, J. M., and Mort, A. J. (1992) *Anal. Biochem.* **207,** 351-353.

CHAPTER V

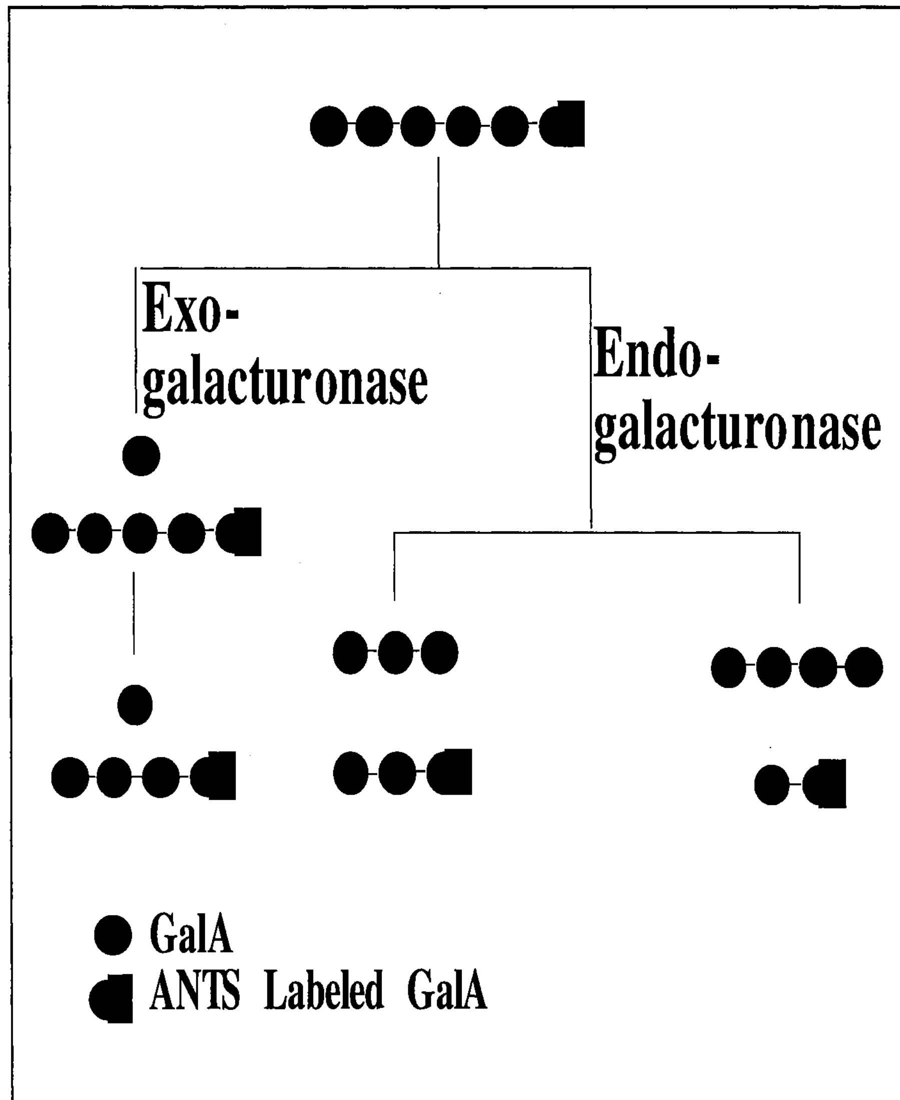
GENERAL DISCUSSION

Recently, plant cell wall polysaccharides, wall proteins and enzymes have been paid more and more attention during plant growth and development (Cosgrove, 1997; Zhang and Mort, 1996; Fry, 1995). The concept has been well established that the primary cell wall is a metabolically active compartment of the cell (Hoson, 1993). The wall enzymes play important roles for degrading and reforming the linkages within the cell wall (Taiz 1984). This can allow cell walls to extend in size without getting thinner (Cosgrove 1997). Perhaps the most direct evidence for wall polysaccharide changes occurring in the walls of living cells is obtained by microscopy (Sheldrake, 1970). It was observed that all structural wall material vanishes from quite a large area during the differentiation of xylem vessel elements, which means the cellulose has been degraded by cellulase and cellobiohydrolase. Extensive wall lysis can also be seen in the thick walls of certain seeds during and after germination (Reis et al, 1987; Edwards et al, 1986; Halmer, 1989; Leung and Bewley, 1983; McCleary, 1983; Aspinall et al 1967). It is assumed that the degradation products are used by the seedling as a carbon source. Other evidence for wall reactions *in vivo* comes from analysis of wall polysaccharides. Auxin- and H^+ - treated plant tissues often exhibit a rapid fall in the mean molecular weight of wall xyloglucan (Lorences and Zarra 1987; Nishitani and Masuda, 1982; Talbott and Ray, 1992). Changes in the degree of esterification and molecular weight and size of pectic polysaccharides have been claimed (Yamaoka and Chiva, 1983; McCann and Roberts 1994; Talbott and Ray, 1992; Zhang and Mort 1996; Tanimoto and Huber, 1997).

In order to directly detect wall enzyme activity *in vivo*, we have recently established a new method, which allows us to measure pectin-degrading enzyme activity as the cell is growing (Zhang et al, 1996). The method is very easy to conduct, including: injection of ANTS-labeled hexamer of GalA into the intercellular spaces of cotyledons, incubation for a certain time, centrifugation of enzyme products from whole cotyledon and analysis by capillary electrophoresis. The enzyme activity was calculated from the peak area ratio, which was calculated by dividing the sum of the area of the peaks representing products of a particular enzyme activity by the sum of all peak areas. We can convert the peak ratio to enzyme activity by comparing with a standard curve (Chapter 3).

For the *in vitro* measurement, this is a very good method for detecting degrading enzymes. Our results indicated that fluorescent labeled oligomers of GalA at their reducing end could be very useful not only for pectin degrading enzyme activity detection, but also for rapid characterization of enzyme action modes by capillary electrophoresis (Figure 16). Comparing the nature of the labeled starting material with that of the labeled products, we can distinguish endo- and exo- enzyme activities and infer of the size enzyme binding and cutting site. EPG needs at least four adjacent nonesterified GalA residues to bind in a pectin and cleave the linkage between the third and fourth residues of GalA from nonreducing terminus (Koller and Neukom, 1969; Mort and Chen, 1996; Zhang et al, 1996). So we can obtain ANTS-labeled dimer and trimer from the hexamer after EPG digestion (Zhang et al, 1996). This method could be extended to detection of any other glycanase, as long as a suitable substrate can be prepared, labeled and the products can be separated by capillary electrophoresis. Also, we can imagine that we could use labeled oligomer to detect synthetic enzyme activity during plant cell growth. The labeled oligomer substrate can be incubated with labeled or unlabeled oligomers containing corresponding synthetic enzyme. If we can detect any larger size product oligomer than that of any reactants after a certain time of incubation, it means that there are some synthetic enzyme activities there, which catalyzed the polymerization of reactant oligomers. If we can label the oligomer substrates

Figure 16. The modes of polygalacturonase activity on ANTS labeled hexamer of GalA. Endopolygalacturonase needs at least four adjacent nonesterified GalA residues to be able to act in a pectin and cleave the linkage between the third and fourth residues from the non-reducing end. Only ANTS labeled dimer and trimer can be produced from ANTS-labeled hexamer by endopolygalacturonase. Exopolygalacturonase can cleave one residue at a time from the non-reducing end but is almost inactivated by oligomers of less than five residues.


Exo-
galacturonase
Endo-
galacturonase
GalA
ANTS Labeled GalA

at their nonreducing end and do the same investigation, we could obtain some very useful information about enzyme action mechanisms.

In vivo measurements are much more complicated than *in vitro* measurements. *In vivo* measurement is not an entirely controllable experiment because we have many unknowns such as the amount and nature of the enzymes, the pH, the injected substrate distribution and diffusion in cotyledon intercellular spaces. Ignoring these uncertainties, we successfully obtained very consistent results using the procedure as described in Chapter 3 during cotton cotyledon expansion (Figure 8 in Chapter 3). The key parameter for *in vivo* measurement is whether enzyme products can be equally rinsed out of the cotyledons or not. As long as enzyme products can be equally centrifuged out of cotyledons, we do not need to know how much labeled substrate was injected into the cotyledons and how much enzyme product was rinsed out from the cotyledons because the peak area ratio must be independent of those factors. Our reproducible results from repeated experiments using cotyledons of the same age in different cotton plants (only larger cotyledons were used for enzyme detection) indicated that enzyme products could be reproducibly rinsed out of cotyledons because there was not a large standard deviation among our measurements (see Figure 8 in Chapter 3). Despite all of these uncertainties and unknowns, we did find there were large changes in endo- and exo-PG activities during cotton cotyledon expansion (Chapter 3). The EPG activity was highest during the initial stages of the expansion and showed a very good correlation with relative growth rate and HG size distribution (Chapter 3). This may be because turnover of the pectins was involved in loosening the cell walls during cotyledon expansion. The rapid expansion of the younger cotyledons may need the fast turnover of the pectins. To complete this process, the high enzyme activity would be needed. *In vivo* measurements of enzyme activities provide direct evidence that the growing walls are an active compartment in plant cells. The ability to detect and characterize enzymes in living tissues allows us to be better understand plant growth and development.

Most recently, we have adapted the method described in Chapter 2 to detect rhamnogalacturonase (RGase) activities *in vivo* and *in vitro* (Zhang et al, 1998). Oligomers of RGI backbone were generated and purified by the procedure as described by Zhan et al (1998). The oligomers of RGI backbone with 16 sugar residues were labeled with ANTS. The labeled oligomers were used as an enzyme substrate to detect RGase enzyme activities *in vivo* and *in vitro* as described for EPG. Our preliminary results indicated that there were significant changes in RGase activities among cotton cotyledons of different ages (Zhang and Mort, unpublished).

From our recent results with Exo-PG, EPG and RGase and the diversity of theories and experimental results of previous researchers we believe that plant cell expansion may be a very complicated process and may involve the combined action of many enzymes to complete this physiological process. Thus, surveying as many enzyme activities as possible in one expanding tissue must help to define the complexity of the system. From this point we will then be able to think about the future experiments that should be designed to test the importance of individual enzymes by using antisense suppression or chemical inhibitors of particular enzymes.

We chose cotton cotyledons as a experimental materials for the following reasons. 1) In cotton upon germination of the seed they expand into a leaflike organ. 2) They seem to be the least complex organ, containing only a few different cell types, veins and a large intercellular spaces so fluids can be easily injected and rinsed out. 3) They expand over a period of 10 days after emergence from the soil, and so, are a model for expansion studies, and do not require a long growth period before they are ready for harvest. 4) During the development of the cotyledons the plants are small, and so do not take very much space in a growth chamber. 5) Cotton is one of the most important economical crops all over the world.

Plant growth and development are tremendously influenced by seed quality and environmental conditions such as water, light, air, and soil. To obtain homogenous cotton

cotyledons for expansion studies, we selected high quality cotton seeds and only the cotyledons that came out of the soil the same day were involved in our studies. The same soil mixture (Jiffy Mix-Plus) was used throughout the experiment, and watering time and light intensity were controlled very well in the growth chamber (Pierce et al 1993). The time course of cotyledon expansion was shown in Chapter 3. We consistently found a larger and smaller cotyledon on each plant, and so the area of each was plotted separately. Following the example of Van Volkenburgh et al (1985), we also plotted the relative growth rate of the larger cotyledon, that is, the rate of growth of the cotyledon over a 24 hour time period divided by the area of the cotyledon at the beginning of the 24 hour time period. This method of presenting the data highlights the much more rapid expansion rate of the younger cotyledons. We did not obtain the data for expansion and enzyme activity from 1 and 2 day old cotyledons, because 1 day old and even some of 2 day old cotyledons are still inside the seed coat or not completely emerged from it.

To better understand the characteristics of pectic polymers of growing plant cell walls, we expect to obtain the polymers as close their native form as possible. Thus, our approach was designed to minimize enzyme digestion of the pectic polymers during the initial stages of extraction. The cell walls from cotton cotyledons of the different ages were prepared at low temperature (4°C) and acidic buffer condition. The low temperature can stop or inactivate wall enzymes that might degrade the polymers (Chapter 3) and the acidic conditions can prevent β-elimination of the wall polymers. The walls were then treated with liquid HF at -23°C. This treatment cleaves the glycosidic linkages of most neutral sugar linkages and every rhamnosyl linkage in the walls without degrading HGs. HGs with a relatively high degree of esterification can then be solubilized with water, and those with low esterification with concentrated imidazole buffer.

We expected that the length of the polymer chains should represent the length of the uninterrupted poly GalA stretches in the intact cell wall because the HF -23°C treatment we

used to solubilize the HG would cleave Rha linkages. We can obtain an unambiguous measure of the distribution of lengths of pure HG regions by labeling their reducing ends with the fluorescent label 2-aminopyridine (Mannes et al, 1991) and then using a very high resolution ion exchange column to separate each individual member of the $GalA_n$ homologous series (Chapter 3). Unfortunately, when HF cleaves the pectin chain at a Rha residue it causes the Rha to glycosidically link itself to the 2-position of the GalA residue immediately previous to it in the chain (Komalavilas and Mort, 1989). This eliminates the reducing terminus of the chain and prevents labeling by 2-aminopyridine. This problem can be overcome, but with some loss of accuracy in determining the original chain length, by treating the pectin with HF at -12°C to selectively remove the terminal Rha residue (Oiu and Mort, unpublished). If the imidazole-solubilised HG from suspension culture walls is given this second HF treatment, we can see a wide range of HG segments of lengths at least up to 60 residues. The water soluble material shows somewhat shorter segments. However, in both cases without the second treatment we get very little labeling (Qiu and Mort, unpublished). With the cotyledons we found good labeling without the second HF treatment (Chapter 3). This puzzled us for a while, until we realized that during expansion of cotyledons there may be turnover of pectins involved in loosening the walls, and that if the turnover involved endopolygalacturonase digestion of the pectins that GalA reducing ends would be present.

Comparing the water-soluble (methyl esterified) and imidazole soluble (slightly esterified) HGs among 3, 5, and 7-day-old cotyledons, respectively, we can see that HGs from younger cotyledons appear to be somewhat more degraded in either extract. These correlated well with the levels of endopolygalacturonase activity (Chapter 3). The results indicated that polygalacturonases may be involved in cotton cotyledon expansion.

Another parameter that we need to characterize is the degree of esterification (DM) of HGs. The role of the DM during plant growth and development has often been questioned since it is not clear whether an increase or a decrease can best explain pectin

solubilization. However, A high level of DM causes weakened ionic binding of the pectic polymers with divalent calcium ions and would thus lower cohesion of the middle lamella pectin and lead the structure of polygalacturonic acid junction zone to change (Carpita and Gibeaut 1993). The structure change and lower cohesion of middle lamella pectins may favor the effectiveness of the pectin degrading enzymes. Our results of DMs from cotyledons of the different ages provided direct evidence that there may be a relationship between EPG activity and DM of HGs (Chapter 3). The highest EPG activity was found in the youngest cotyledons containing HGs with the highest DM among our observations. It is clear that EPG needs at least four adjacent unesterified GalA residues to cleave pectins (Mort and Chen 1996; Zhang et al 1996). However, there is no difficulty for EPG to find four adjacent unesterified GalA residues on the HGs with about 50% DM if the esterification is randomly distributed (Mort et al, 1993). High DM HGs may just allow EPG to cleave the specific sites on HGs, in other words, to make a certain length of HG which is needed in the physiological process in that stage. Measuring pectin methylesterase (PME) activities during cotyledon expansion might be helpful to obtain conclusive links among DM, EPG, PME and cotyledon expansion.

Carbohydrate composition analysis of walls and HGs form cotyledons of the different ages indicated that around 20% of cotyledon wall sugars is GalA. However, there was no trend towards a big change in sugar composition during cotton cotyledon expansion, even though there was a slight increase in GalA and Rha, and a decrease in Ara and Xyl (Chapter 3). Cotton cotyledon wall HGs apparently changed their molecular weight/size and DM rather than sugar composition during their expansion.

As I described in Chapter 1, the growing plant cell walls contain some structural proteins (Showalter 1993; Carpita and Gibeaut, 1993) and enzyme proteins (Cosgrove, 1997; Fry, 1995). The wall structural proteins vary greatly in their abundance, depending on cell type, maturation, and environmental stimulation. To know whether there are changes in proteins during cotton cotyledon expansion, most recently we developed a

sensitive method for analysis of amino acid composition of hydrolyzed proteins (Zhang and Mort, 1997; Chapter 4). We separated AQC derivatized amino acids by micellar electrokinetic capillary chromatography (MECC) instead of reverse phase HPLC as in previous reports (Weiss et al, 1997; Strydom and Cohen,1994; Palace and Phoebe, 1997; Cohen and Michaud, 1993). The derivatives can be detected by both absorbance and fluorescence (Chapter 4). Since only nanoliters of samples are needed for the injection into the capillary, we need only prepare a very small volume of samples with submicrogram amounts of protein and extremely small volume of reagents. The other big advantage of this method is that we can directly inject derivatives into the capillary without precleaning the excess reagents because of the very small injection volume used. Using this method we can analyze a sample within three to five hours including the protein hydrolysis time (Chapter 4).

We analyzed amino acid compositions of wall proteins from 3, 5 and 7-day-old cotton cotyledons. Our results indicated that there were no significant changes in amino acid composittion, but changes in amount of proteins during cotton cotyledon expansion (Table 8). Also, this method has been used to analyze the free amino acids during loblolly pine seed germination (Wu et al, 1998 in preparation) and the wheat leaf cell wall proteins (Chapter 4).

Many wall enzymes and structural proteins have been identified and their biological roles correlated with plant growth and development. The structures and functions of wall polysaccharides such as cellulose fibril, hemicellulose, and pectins have been extensively studied. Wall polymer metabolism during plant growth has been paid more and more attention, but we have far to go in determining the nature and rate of the *in vivo* reactions catalyzed by wall enzymes and in testing their proposed roles. We have far to go in determining the factors involved in plant cell expansion, growth and development. The new techniques can help us to know more details about cell wall components during plant growth. The ability to control wall extension may not only uncover further mechanisms of

Table 8. Analyses of Amino Acid Compositions of Cell Wall Linked Proteins During Cotton Cotyledon Expansion

Amino Acid	Weight % * (n=3)		
	3-day-old	5-day-old	7-day-old
Hyp	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ser	0.57 ± 0.09	0.59 ± 0.08	0.45 ± 0.03
Thr	0.62 ± 0.13	0.63 ± 0.10	0.45 ± 0.03
Pro	0.54 ± 0.05	0.53 ± 0.06	0.48 ± 0.01
Gly	0.68 ± 0.08	0.75 ± 0.03	0.63 ± .010
Ala	0.62 ± 0.11	0.56 ± 0.12	0.50 ± 0.03
His	0.41 ± 0.04	0.23 ± 0.05	0.22 ± 0.01
Cys	0.03 ± 0.04	0.03 ± 0.05	0.03 ± 0.04
Glx	1.12 ± 0.08	1.15 ± 0.51	0.94 ± 0.05
Asx	0.85 ± 0.11	0.88 ± 0.40	0.87 ± 0.36
Tyr	0.49 ± 0.13	0.44 ± 0.10	0.30 ± 0.05
Val	0.64 ± 0.06	0.59 ± 0.03	0.48 ± 0.08
Met	0.26 ± 0.12	0.16 ± 0.12	0.26 ± 0.15
Ile	0.58 ± 0.06	0.51 ± 0.02	0.44 ± 0.03
Leu	0.72 ± 0.05	0.64 ± 0.06	0.56 ± 0.02
Phe	0.60 ± 0.13	0.51 ± 0.02	0.41 ± 0.03
Lys	0.61 ± 0.06	0.62 ± 0.11	0.56 ± 0.03
Arg	0.80 ± 0.19	0.67 ± 0.07	0.60 ± 0.11
Total Weight %	10.32 ± 0.53	9.49 ± 1.20	8.18 ± 0.37

* weight percent ± standard deviation.

wall enlargement, but may have great significance to increase the yields of agricultural crops.

LITERATURE CITED

Aspinall GO, Begbie R, Hamilton A, Whyte JNC (1967) Polysaccharides of soybeans. III. Extraction and fractionation of polysaccharides from cotyledon meal. J. Chem. Soc. C. **1967:** 1065-1070.

Carpita NC, Gibeaut DM (1993) Structureal models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1-30.

Cohen SA, Michaud DP (1993) Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hedroxysuccinimidyl carbamate, and its application for the analysis of hedrolysate amino acids via high-performance liquid chromatography. Anal. Biochem. **211:** 279-287.

Cosgrove DJ (1997) Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. Plant Cell. **9:** 1031-1041

Edwards M, Dea ICM, Bulpin PV, Reid JSG (1986) Purification and properties of a novel xyloglucan-specific endo-(1→4)-β-D-glucanase from germinated nasturtium seeds (*Tropaeolum majus* L.). J. Biol. Chem. **261:** 9489-9494.

Fry S (1995) Polysaccharide-modifying enzymes in the plant cell wall. Ann. Rev. Plant Physiol. Plant Mol. Biol. **46:** 497-520

Halmer P (1989) *De novo* synthesis of mannanase by the endosperm of *Lactuca sativa.* Phytochemistry **28:** 371-378.

Hoson T (1993) Regulation of polysaccharide breakdown during auxin-induced cell wall loosening. J. Plant Res. **106:** 369-381.

Komalavilas P, Mort AJ (1989) The acetylation at O-3 of galacturonic acid in the rhamnose-rich portion of pectins. Carbohydr. Res. **189:** 261-272.

Koller A, Neukom H (1969) Untersuchungen Ÿber den abbaumechanismus einer gereinigten polygalakturonase aus *Aspergillus niger* . Eur. J. Biochem. **7:** 485-489

Leung DWM, Bewley JD (1983) A role for α-galactosidase in the degradation of the endosperm cell walls of lettuce seeds cv Grand Rapids. Planta **157:** 274-277.

Lorences EP, Zarra I (1987) Auxin-induced growth in hypocotyl segments of *Pinus pinaster* Aiton. Changes in molecular weight distribution of hemicellulosic polysaccharides. J. Exp. Bot. **38:** 960-967.

Mannes NO, Miranda ET, Mort AJ (1991) Recovery of sugar derivatives from 2-aminopyridine labeling mixture for high-performance liquid chromatography using UV or fluorescent detection. J. Chromatogr. **587:** 177-183.

McCann MC, Roberts K (1994) Changes in cell walll architecture during cell elongation. J. Exp. Botany **45:** 1683-1691.

McCleary BV (1983) Enzymic interactions in the hydrolysis of galactomannan in germination guar: the role of exo-β-mannase. Phytochemistry **22:** 649-658.

Mort AJ, Chen EMW (1996) Separation of 8-aminonaphthalene-1,3,6-trisulfonate (ANTS)-labeled oligomers containing galacturonic acid by capillary electrophoresis: Application to determining the substrate specificity of endopolygalacturonases. Electrophoresis **17:** 379-383.

Mort AJ, Qiu F, Maness NO (1993) Determination of the Pattern of methyl esterification in pectins. Distribution of contiguous non-esterified residues. Carbohydr. Res. **247:** 21-35

Nishitani K, Masuda Y (1982) Acid pH-induced structural changes in cell wall xyloglucans in *Vigna angularis* epicotyl segments. Plant Sci. Lett. **28:** 87-94.

Palace GP, Phoebe CH (1997) Quantitative determination of amino acid levels in neutral and glucosamine-containing carbohydrate polymers. **244:** 393-403.

Pierce ML, Essenberg M, Mort AJ (1993) A comparison of the quantities of exopolysaccharide produced by *Xanthomonas campestris* pv. *malvacearum* in susceptible and resistant cotton cotyledons during early stages of infection. Phytopahtology **83:** 344-349.

Reis D, Vian B, Derzens D, Roland, JC (1987) Sequential patterns of intramural digestion of galactoxyloglucan in tamarind seedlings. Planta **170:** 60-73.

Sheldrake AR (1970) Cellulase and cell differentiation in *Acer pseudoplatanus*. Planta **95:** 167-178.

Showalter AM (1993) Structure and Function of plant cell wall proteins. Plant Cell **5:** 9-23.

Strydom DJ, Cohen SA (1994) Comparison of amino acid analyses by phenylisothiocyanate and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate precolumn derivatization. Anal. Biochem. **222:** 19-28.

Talbott LD, Ray PM (1992) Changes in size of previously deposited and newly synthesized pea cell wall matrix polysaccharides. Effects of auxin and turgor. Plant Physiol. **98:** 369-379.

Taiz L (1984) Plant cell expansion: regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. **35:** 585-657.

Tanimoto E, Huber DJ (1997) Effect of the molecular mass of polyuronides in the cell walls of Alaska pea roots. Plant Cell Physiol. **38:** 25-35.

Van Volkenburgh E, Schmidt MG, Cleland RE (1985) Loss of capacity for acid-induced wall looseing as the principal cause of the cessation of cell enlargement in light-grown bean leaves. Planta **163:** 500-505.

Weiss T, Bernhardt G, Buschauer A, Jauch K, Zirgibl H. (1997) High-resolution reversed-phase high-performance liquid chromatography analysis of polyamines and their monoacetyl conjugates by fluorescence detection after derivatization with N-hydroxysuccinimidyl 6-quinoliny carbamate. Anal. Biochem. **247:** 294-304.

Wu L, Hallgren SW, Zhang Z, Ferris DM, Mort AJ (1998) Effects of solid matrix priming on free amino acid accumulation and water status in loblolly pine *Pinus taeda* L. seeds (in preparation)

Yamaoka T, Chiva N (1983) Changes in the coagulating ability of pectin during the growth of soybean hypocotyls. Plant Cell Physiol. **24:** 1281-1290.

Zhan DF , Janssen P, Mort AJ (1998) Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. (accepted by Carbohydrate Research)

Zhang Z, Pierce M, Mort AJ (1996) Detection and differentiation of pectic enzyme activity *in vitro* and *in vivo* by capillary electrophoresis of products from fluorescent-labeled substrate. Electrophoresis 17: 372-378.

Zhang Z, Zhan D, Mort AJ (1998) *In vivo* and *in vitro* measurements of rhmnogalacturonase activity by capillary electrophoresis. Abstract for ASPP Meeting.

Zhang Z, Mort AJ (1997) A sensitive method of amino acid analysis using precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and capillary electrophoresis. Plant Physiol. **114:** 89.

Zhang Z, Mort AJ (1996) Changes in homogalacturonans and degrading enzymes during leafy cotton cotyledon expansion. Plant Physiol. **111:** 147.

VITA

Zhiquan Zhang

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHANGES IN HOMOGALACTURONANS, POLYGALACTURONASE ACTIVITIES AND CELL WALL LINKED PROTEINS DURING COTTON COTYLEDON EXPANSION

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Hulan, Heilongjiang, China, April 4, 1957, the son of Jinglan Shen and Yuzhong Zhang.

Education: Graduated from First High School, Hulan, Heilongjiang, China, in July 1974; received Bachelor of Science Degree in Plant Biochemistry and Physiology fro Northeast Agricultural University, Harbin, China, in 1982; completed requirements for the Doctor of Philosophy Degree at Oklahoma State University in July, 1998.

Professional Experience: Research Associate in the Department of Plant Biochemistry and Physiology, Beijing Agricultural University, Beijing, China Aug. 1982 to Aug. 1984; Teaching and research Assistant in the Department of Bioengineering, Northeast Agricultural University, Harbin, China. Aug. 1984 to Aug. 1987; Visiting Scholar in the Department of Biochemistry and Center for Biological Resource Recovery, University of Georgia, Athens, GA Sep. 1990 to Aug. 1992; Research Assistant in the Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK, Aug. 1992 to Present.

Honors and Organizations Member of American Society of Plant Physiologists. Member of American Association for the Advancement of Science.