The Institute of Paper Chemistry

Appleton, Wisconsin

Doctor's Dissertation

The Isolation, Characterization, and Biological Testing of Xyloglucan From Suspension Cultured Loblolly Pine Cell Spent Medium

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June, 1987

THE ISOLATION, CHARACTERIZATION, AND BIOLOGICAL TESTING OF XYLOGLUCAN FROM SUSPENSION CULTURED LOBLOLLY PINE CELL SPENT MEDIUM

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in partial fulfillment of the requirements
of The Institute of Paper Chemistry
for the degree of Doctor of Philosophy
from Lawrence University
Appleton, Wisconsin

June, 1987

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ABSTRACT

A xyloglucan was isolated from spent loblolly pine (Pinus taeda) suspension culture medium. Isolation showed that the xyloglucan in the cell medium was complexed with an arabinogalactan and two types of protein. The arabinogalactan was not covalently bonded but was associated with the xyloglucan. Protein was strongly attached and could only be removed by protease treatment and by de-esterification. There appeared to be two types of protein, as neither a protease nor the breaking of ester bonds removed all of the protein from the xyloglucan. The molecular size of the isolated (> 90% carbohydrate) xyloglucan was determined to be ≈ 70,000 Daltons or over 300 sugar units in size.

The pine xyloglucan was derivatized and its permethylated alditol acetates analyzed by GC and GC/MS. A general structure of the pine xyloglucan was proposed. The pine xyloglucan contained 20% unbranched 1,4-β-glucan. The rest of the polymer was composed of a branched 1,4-β-glucan backbone with branches consisting of terminal xylose, terminal galactose, xylose-galactose-terminal fucose, galactose-terminal arabinose, and other combinations of these sugars. This xyloglucan from the spent pine medium was generally similar to other xyloglucans, except that it contained a substantial amount of terminal galactose, an uncommon configuration.

The pine xyloglucan was digested with a fungal cellulase. The resulting fragments were separated on a gel permeation chromatography column. The fragments appeared as a number of peaks ranging from perhaps deca- to monosaccharide in size. Large peaks that corresponded to a nonsaccharide, a heptasaccharide and a hexa- or pentasaccharide were dominant. The oligosaccharides represented by peaks were analyzed by GC and GC/MS. Although full structures were not determined, it was seen that the peaks contained specific sugars. The

heptasaccharide contained large amounts of xylose, whereas the other peaks seemed dominated by galactose. The abundance of terminal galactose may be a feature unique to conifer xyloglucans.

A number of biological tests were attempted with the xyloglucan, its fragments, and other polysaccharides in the medium. None of these tests showed any biological activity by pine polysaccharides. A test of the cellulase produced fragments in pea stem elongation showed no inhibitory activity.

INTRODUCTION

The primary cell wall of plants plays an important role in the development of the cells. Although mature cell walls and their major components, especially cellulose, have been the subjects of studies for nearly a century,[1] the primary cell wall was not an easy system to isolate and study before the advent of plant tissue culture. The young developing cell contains no secondary wall but is surrounded by the less rigid, primary cell wall. Tissue culture methods and the development of biochemical techniques have made possible the investigation of the dynamic, growing young cell and its primary cell wall.

Xyloglucans are some of the most interesting polysaccharides that have been isolated from the primary cell wall. These molecules have a cellulosic 1,4-β-glucan backbone and are highly branched. The branches consist of xylose units, some terminal and some further branched with additional galactose, arabinose, and fucose units. The composition of isolated xyloglucans varies with species and isolation method. The celluloselike backbone of this molecule makes hydrogen bonding to cellulose likely in the cell wall. The branches in the molecule may provide sites for forming covalent bonds with other cell wall components. These structural features are fairly common to many polysaccharides.

A unique feature of the xyloglucan seems to be its intimate involvement in cell elongation. Many researchers feel that the xyloglucan is covalently bonded to other cell wall constituents, and its hydrogen bonding ability makes it the most likely wall component to be adjacent to the cellulose. Because the xyloglucan is only bound to the cellulose by hydrogen bonding it is possible that these bonds are readily broken and reformed as the cell wall expands.

The exact nature of wall elongation is not known, but the xyloglucan is implicated. It is possible to rinse xyloglucan from elongating cells in amounts far greater than from static cells.[2,3] Xyloglucans have also been shown to be related to the enzymatic pathways of auxin stimulated cell elongation.[4] Fragments of both general cell wall polysaccharides and xyloglucans have been found to inhibit cell wall elongation.[5]

Cell wall fragments exhibit effects on organ development in tissue culture systems. Although xyloglucans have not been specifically implicated, it is interesting that some other polysaccharides seem to have regulatory functions. Tran Than Van and workers in France[6,7] have manipulated the development environments (e.g., pH, auxin, and cell wall fragment levels) of tobacco explants to produce many types of organs from a single type of tissue.

Recent work[8] on cellulose synthesis by Acetobacter xylinum has shown that molecules that sorb onto cellulose can interfere with cellulose crystallization and fibril formation. Because of this behavior, it can be speculated that the xyloglucan in an expanding cell wall could affect the deposition of cellulose.

Certain xyloglucans thus appear to be implicated as dynamic components of the primary cell walls. Cell elongation, cell morphology, cellulose deposition, and cell wall structure all may involve xyloglucans or fragments of xyloglucans. Although the primary cell wall is quantitatively insignificant in paper fibers, its role in the deposition of the cellulose and the development of cells make this investigation important. The xyloglucan may also play a role in the tissue culturing of conifers, an area of extreme interest at The Institute of Paper Chemistry. Thus, the study of the properties of the xyloglucan component of our major pulping species is a necessary step if further research is to produce

superior paper fibers. Xyloglucans of conifer species have not been studied extensively nor have they been compared with those others that have been studied. Therefore, this study of the xyloglucan from suspension cultured pine cells was undertaken.

LITERATURE REVIEW

XYLOGLUCANS

The cell walls of plants have long been the subject of chemical study, primarily because of the many uses of plant fibers. These studies focused on the secondary wall and its main component, cellulose. As techniques improved, other general groups of polysaccharides, i.e., hemicelluloses and pectins, were the subjects of research efforts.[9] Xyloglucanlike polymers were not isolated or named until Kooiman[10] discovered a polysaccharide in seeds (notably the tamarind[10,11]) that gave a positive iodine test as starch did but upon analysis proved not to be starch. Kooiman found similar amyloids in the seeds from hundreds of species. These xyloglucans were thought to be some kind of storage polysaccharide.

The isolation of fucoxyloglucan from the spent culture medium of sycamore cells (provided by Albersheim) was reported by Aspinall, Molloy, and Craig[12] in 1969. Earlier workers had suggested that xyloglucans were components of Englemann spruce bark and red spruce compression wood,[12] but it was not known if the xylose and glucose occurred in a distinct polymer. Aspinall was one of the first to describe a xyloglucan from a nonseed source, and it resembled the tamarind xyloglucan in its basic structure: a 1,4-β-glucan backbone (branched and unbranched), with xylose branches. Aspinall reported that the polymer he studied had terminal fucose units but no galactose.

Albersheim and his many co-workers undertook a study of the complete primary cell wall of suspension cultured sycamore (Acer pseudoplatanus) cells.[13-16] He employed new enzymatic extraction methods (which would influence most future work on xyloglucans) that had been used to study bacterial cell walls.[14] As a

result of this work, he proposed a general structure for the primary cell wall as well as structures for all of the wall components. The xyloglucan he found was proposed to be linked by hydrogen bonds to the cellulose and covalently to other wall polysaccharides. The structure of his xyloglucan was similar to Kooiman's except in the proportion of the less abundant sugars. It was much more similar to the seed amyloids than to Aspinall's polymer that did not contain galactose nor give a positive iodine test.

In parallel investigations Albersheim studied host-pathogen interactions,[17-19] specifically how fungal pathogens attack the cell walls in plants. From this extensive work Albersheim found that Colletotrichum lindmuthianum released two enzymes at different stages of cell culture. The first was an endopolygalacturonase, a pectinase that removed the charged, pectic polysaccharides from the wall. The second was an endoglucanase that fragmented polymers with a glucan backbone (a similar enzyme was isolated from Trichoderma viride, another fungal pathogen). These enzymes were purified extensively before use and enabled Albersheim to look at the primary cell wall in a new way. This alternative method substituted predictable cleavages for the more random bond cleavage that occurs with both acid and alkali extractions. In this manner Albersheim and co-workers were able to propose a complete primary cell wall structure, knowing more precisely which bonds in the wall had been cleaved. The endoglucanase also produced a fragmented xyloglucan.[15] These fragments, along with chemically extracted fragments, are of great importance and will be discussed later.

Albersheim's group looked at the xyloglucan isolated from the culture medium and the cell walls of the sycamore cells.[15] They found that the xyloglucan from the extracellular polysaccharides and its endoglucanase-released fragments were

similar to those fragments released from the wall by this enzyme. Albersheim made no mention of the need to remove proteins from the xyloglucan fraction of the extracellular polysaccharides. The molecular weight of extracellular xyloglucan was estimated at 10,000 Daltons, a figure close to earlier estimates for tamarind xyloglucan.[11]

Albersheim also studied the cell wall polysaccharides from monocots and Douglas-fir.[13] These were investigated to see if the cell wall polysaccharides were similar to those found in the sycamore. He found that the carbohydrates in the suspension cells of a number of grain species were all very similar to one another but were different from those in the sycamore cells. He did not feel that these species were diverse enough to be able to generalize this information for all monocots.[13] An arabinoxylan was found to be the main component. This polymer is not a major component in the cell walls of sycamore or other dicots that have been studied.[13] The monocots contained only trace amounts of xyloglucan. The Douglas-fir cells did resemble the sycamore cells more closely in make-up, with a xyloglucan being present in significant amounts. Further work with suspension cultured Douglas-fir cells has provided more specific information on the xyloglucan from this conifer species.[20]

Albersheim's group has provided the bulk of knowledge about xyloglucans from suspension cultured cells. His claim of biological activity of a xyloglucan fragment has provided the impetus for a large body of recent work (including this thesis) on xyloglucans throughout the world.[22-24]

XYLOGLUCANS FROM DIVERSE SOURCES

Research on xyloglucans has proceeded with both different sources and different isolation methods. Although enzymatic methods provide a unique view of

the cell wall components, other methods have shown the diverse properties of xyloglucans. By examining different sources, both of tissue type and species, an appreciation of the scope of xyloglucan composition (see Table 1) has been obtained.

Table 1. Features of xyloglucans.

A. Sugar configurations

Glc - glucose (D-p)
Gal - galactose (D-p)
Xyl - xylose (D-p)
Ara - arabinose (L-f)
Fuc - fucose (L-p)

B. Common linkages

$\underline{4}$ Glc $\underline{1}$ $\underline{4}$ Glc $\underline{1}$

Glc $\underline{6}$ $\underline{1}$ Xyl

C. Various linkages

Source	Term. Fuc	Term. Ara	Term. Gal	Gal - Gal	Active Nona-Saccha.	Unique Features
Sycamore[12]	x					No galactose
Sycamore[15]	x	x			x	
[a]h.w. camb.[33]	x	none	x	x		
Douglas-fir[20]	x	x	x	x		
Spinach[22]	x				x	*in vivo*
Apple[29]	x		x			Mannose
Potato[32]		x	x			Only term. Gal
Beans[24]	x	x	x	x		
Monocots[13]		Very little xyloglucan				Arabinoxylan

[a]Hardwood cambium.

Joseleau and Chambert[25,26] have explored the composition of the cell walls of *Rosa glauca* using a variety of solvent extractions. Suspension cultured

cells of Rosa glauca were extracted with alkali and a cellulose solvent. Multiple fractions from both of these extractions were analyzed, and it was concluded the "xyloglucan" in the cell wall is heterogeneous. Albersheim had postulated that the xyloglucan isolated by enzymatic means had a single structural role in the cell wall. Other work has indicated[26] that there exist several types of xyloglucans performing different functions within the cell wall. Although the primary cell wall xyloglucan (obtained from suspension cultured cells and their spent medium[27]) differed from those of more mature tissue, it was assumed that within the primary cell wall the xyloglucan was homogeneous. The extractions of the cell walls showed that some 50% of the xyloglucan was tenaciously connected to the cellulose, necessitating an acid hydrolysis to remove it. These workers found that although the accessible and nonaccessible fractions contained the same components, Ara, Fuc, Gal, Xyl, and Glc, the ratios of these sugars varied. Methylation data showed differences in their structure,[26] suggesting that xyloglucans may perform many diverse structural functions within the cell wall.

A number of xyloglucans have been isolated from the cell walls of more mature but nonseed tissue. These include xyloglucans from apples,[28,29] wheat bran,[30] rice bran,[31] potatoes,[32] Populus tremula and Tilia americana,[33] and french and runner beans.[34] Characteristics of these isolates relevant to this thesis follow. Simson and Timell[33] found that the xyloglucan from the cambial cells of two hardwoods were similar to others with 1,4-β-glucan backbones, linked at the 6 position to xylose branches. Some of the xylose branches were not terminal but had galactose, galactose-fucose, and possibly galactose-galactose branches, but no arabinose. Perhaps more importantly, Simson and Timell observed that the molecular weight of this polymer approached 100,000, some ten times the weight suggested for the sycamore[15] or tamarind[11] xyloglucans.

Work with the parenchymatous tissues of apple has provided information on xyloglucans from another source. The studies of Aspinall and Fanous[28] and Ruperez et al.[29] have shown that alkali extraction of these older cells yields different types of xyloglucans. Aspinall and Fanous found a fucoxyloglucan very similar to those found in other cells.[35,36] This polymer contained a 1,4-β-glucan backbone with xylose branches (6-position off backbone); some of these branches were more extensive, containing galactose and galactose-fucose branches attached at the 2-position to the xylose branches. No non-6-position branches were found.[28] Ruperez et al.[29] found, as in Rosa glauca,[25,26] that the apple polysaccharides extracted by differing concentrations of KOH contained two distinct xyloglucans. This was suspected because of differing xyloglucans observed in auxin-stimulated cell elongation.[3] Using an anion-exchange column, seven different xyloglucan rich fractions were isolated by Ruperez et al.[29] Most of the fractions were similar to other xyloglucans,[28,35] but some of the fractions contained large amounts of mannose. Although this mannose may be part of another polysaccharide, it is significant that a simple anion-exchange procedure could separate different "xyloglucan" fractions, a fact not previously reported.

Selvendran and co-workers have isolated xyloglucans from both potatoes[32] and beans.[24,34,35] In both cases nonsuspension cell walls were used. The general methods employed were alkali extractions and methylation analysis. The potato "arabinogalactoxyloglucan" isolated[32] resembled many other xyloglucans but contained no fucose. Fucose is a more common nonreducing end of the xylose branches, but arabinose is present in some other xyloglucans.[15,24] It has been speculated that the arabinose, when found, was a contaminating sugar and not part of the xyloglucan. Ring and Selvendran[32] showed that arabinose, a five

carbon sugar, can be present in the same position as the 6-deoxy hexose, fucose. The potato xyloglucan contained galactose only as a terminal sugar. In most of the other isolated xyloglucans, galactose was present both as a terminal nonreducing end of a branch and between the xylose and a fucose or arabinose terminal unit.

The work by O'Neil and Selvendran on dwarf french and runner beans is extensive. Using the techniques developed for the study of potato polysaccharides,[32] these workers have isolated and characterized a xyloglucan from runner bean cell wall material.[24,35] An alkaline extraction was used to isolate a xyloglucan similar to other xyloglucans.[35] This polymer contained both fucose and arabinose as terminal branch ends, and galactose as a terminal and nonterminal sugar. Partial acid hydrolysis and subsequent methylation showed a structure much like others that have been proposed.[15,26,28] A cellulase was used[24] to fragment the isolated xyloglucan into specific pieces much as Albersheim had done.[15] This enabled these workers to postulate an even more detailed structure (see Table 1). The molecular weight of this polymer was estimated at 100,000 Daltons.

These many examples of isolation techniques and overview of xyloglucan sources have shown: (1) The methods used affect the composition of the final isolated xyloglucan. (2) Different species contain xyloglucans of varying structure and composition.

AN OVERVIEW OF CHARACTERIZATION METHODS

When Kooiman first looked at the tamarind amyloid, he had to develop an iodine colorimetric test to rapidly quantify the polysaccharide found in seeds.[38] As we have seen there are now many different extraction and analysis techniques for cell wall polysaccharides. Perhaps the most important developments have

been in microanalysis techniques. Improvements in column chromatography[39] have made the isolation of very pure forms of both polysaccharides and enzymes possible. Many polysaccharides that vary only slightly in charge or size have been isolated.[13,29] This has enabled investigators to analyze complete cell walls and separate the different wall components using much less destructive methods than in the past.

The biggest steps in polysaccharide characterization have been the advent of complete, one step permethylation methods and advanced GC/MS and GC techniques. The Hakamori method,[40] with modifications, is now the most widely used methylation technique for polysaccharides. This is a vast improvement on most older methods.[41] Although the Hakamori method usually permethylates in one step, the preparation of the sodium or potassium methylsulfinylmethanide in dimethylsulfoxide (which forms a $CH_3SOCH_2^-$ anion) is tedious and difficult. In this thesis NaOH in DMSO, with MeI as a methylating agent, was used as the basic agent, as developed by Ciucano and Kerek.[42] They reported the NaOH hydroxide anion to ionize carbohydrate hydroxyls quicker and more completely than the dimsyl anion.

The methylated polysaccharide is then hydrolyzed, reduced and acetylated. These alditol acetates are then analyzed by GC and GC/MS. Almost all of the generated alditol acetates can be identified because of a large body of work that has been done on the methylated alditol acetates of sugars.[43,44,46] A number of different GC packings[45] can separate the sugars by retention times. Jansson et al.[46] have also recorded the mass spectra of most of the methylated alditol acetates of common sugars. The advent of fast atom bombardment mass spectrometry has made the identification of di-, tri-, and even tetrasaccharides possible.[24,47,48] This improvement in methods has made structural studies of polysaccharides far more accurate (even with μg quantities) and much simpler than previously possible.

ENZYMATIC METHODS

Enzymes that degrade polysaccharides have been studied extensively.[49] Most of the enzymes initially were of limited use in the study of cell walls. The cell wall material could not be degraded unless the cell walls were first chemically modified[18] or attacked with enzyme preparations containing a whole spectrum of enzymes active on a number of polysaccharide substrates. This made the removal of the different polysaccharides impossible except in one operation, resulting in the loss of information concerning the various cell wall components. Chemical modifications prior to attack with purer enzyme preparations could result in the breaking of labile bonds within the wall. Work by Selby and Maitland,[21,50] building on earlier work,[51-53] showed that two distinct enzymes were secreted by T. viride to solubilize unmodified cotton fibers. They were able to show that one of these enyzmes was unable to solubilize the cotton, whereas the other was unable to solubilize the unmodified cotton unless the former was present.[21]

Kerr and Albersheim[18] isolated a "wall modifying enzyme" from a commercial wall degrading enzyme mix. This enzyme was able to modify walls and make them open to maceration by a variety of known polysaccharide degrading enzymes.[18] This effect of "wall modification" had been observed earlier by Albersheim.[19] A similar enzyme was later isolated from C. lindemuthianum.[49] This enzyme is referred to as an endopolygalacturonase.[15] The purification of this enzyme made it possible for Albersheim to attack, selectively, the cell walls of suspension cultured sycamore cells without using any chemical means, giving him the ability to piece together all the components of the cell walls.[16] Another enzyme, a polyglucanase, was isolated from T. viride and used to fragment the xyloglucan isolated from the sycamore cell walls.[14-16]

BIOLOGICAL ACTIVITY OF POLYSACCHARIDES

The discovery of polysaccharides that act as regulatory molecules in plants is a fairly recent one. The five general types of plant regulatory molecules (auxins, abscisic acid, cytokinins, ethylene, and gibberellins) have been extensively studied. These molecules are rather general in their effects on plants. Each one can elicit several different responses.[6] Albersheim and his co-workers, often building on the observations of a multitude of other workers, have isolated a number of cell wall fragments that appear to have regulatory activity. Most of these discoveries were found as a result of Albersheim's studies of plant pathogens and the attacked plant cells. Later, he was able to relate this to his work on plant cell walls.

Albersheim and co-workers have isolated several different oligosaccharide chemical messengers, which he termed oligosaccharins. One of these is a glucan heptasaccharide that activates the cells to produce an antibiotic. It took his group 10 years to isolate the active fragment from tens of different heptasaccharides (1,3 and 1,6 bonded glucans) and hundreds of other oligosaccharides.[54] A portion of a pectic polysaccharide has been shown[55] to activate neighboring cells to produce antibiotics. West[56] has shown simple linear galacturonic acid oligosaccharide to have this effect in castor beans. Damage to cells can also cause the release of this oligosaccharide.[6] Ryan[57] has shown that polysaccharide fragments can even cause a system to defend itself against insects, by stimulating the synthesis of proteinase-inhibitors. Yamazaki[58] has shown that oligosaccharides can also trigger a mechanism called "hypersensitive death" in which neighboring cells kill themselves to delay attack of pathogens.[6] More generally, cell wall fragments have been shown to affect growing cell cultures.

Cell wall fragments (produced by a pectinase digestion of cell walls) from suspension-grown pear cells cause increased ethylene production in the pear cultures when added back.[59] A T. viride cellulase increased ethylene production in tobacco leaf disk.[60]

Perhaps the most startling effects of cell fragments on growing cells are the apparent developmental influences they have on tobacco epidermis cultures. For more than a decade Kiem Tran Than Van cultured thin layers of the floral branches of tobacco.[61-64] In some plant systems, pH and auxin to cytokinin ratio can influence how (morphogenetically) cultured cells develop.[65] Using the tobacco system and the expertise of Dr. Tran Than Van, Albersheim has investigated the effects of chemically and enzymatically produced cell wall fragments (sycamore) on developing tobacco explant cultures. Different pH's and regulator levels stimulate the explants to produce flowers, callus or vegetative buds. The addition of endopolygalacturonase-released cell wall fractions produced flowers and vegetative buds, vegetative buds, and again flowers and vegetative buds. Without the wall fragments, these cultures produced flowers, callus and vegetative buds, respectively. Alkali-extracted wall fragments induced the production of flowers, floral shoots and vegetative buds, roots and callus, and roots and callus, respectively.[7] Such ability to influence plant morphogenesis would have many potential applications. This work is extremely encouraging, but as detailed in this review, isolating specific active fragments (including non-polysaccharide fragments) can be extremely difficult.

BIOLOGICALLY ACTIVE XYLOGLUCANS

One of the mysteries of plant physiology is the expansion of the cell wall. Unlike animal cells, plant cells have a rigid cell wall that must somehow change

its physical characteristics to allow the cell to grow. Elongating cell walls have long been the subject of study.[66] The mechanism of auxin-induced cell elongation has also been studied.[2] Two phenomena have been investigated. The first involves the relationship between auxin and an increase in the activity of enzymes that cleave polysaccharides.[67,68] This enzymatic activity could be assumed to weaken the structure of the cell wall, thus allowing cell elongation. The second area is that of cell wall polysaccharide turnover (the simultaneous synthesis and release of cell material). Using radioisotopic and other analytical data, workers[51,69-71] have suggested that polysaccharides are turned over in elongating cell walls. Others have shown that auxin may increase cell wall polysaccharide turnover.[72,73]

Labavitch and Ray[2] found this previous work lacking in realistic growth conditions and reproducibility. Pea stem segments were fed ^{14}C-labeled glucose and then allowed to incorporate all of the label into the cell wall. Cell elongation was induced with the auxin, IAA, and then the cell walls were extracted and analyzed. These workers found that cell wall polysaccharide turnover affected specific cell wall components,[2] not the entire cell wall as reported earlier.[69,72] Most of the wall components were not affected, but there was significant turnover of pectinase-extractable galactose and a seeming conversion of a xyloglucan (or at least a release of xylose and glucose) from an insoluble form to a water soluble form.[74] This last observation was the subject of further work. Labavitch and Ray[3] found that the auxin, IAA, increased the amount of water-soluble xyloglucan fragments within minutes of treatment, coinciding with or perhaps before cell elongation.[73] This indicated a possible relationship between the xyloglucan release and changes in properties of the cell wall. They also found that other cell wall polysaccharides could be involved in the

xyloglucan release. This work spurred on many others. Bonner and Terry[75,76] developed a method of gentle centrifugation to remove water-soluble wall polysaccharides without excessive wash solution or contamination from nonextracellular elements. They too found auxin stimulating the release of a xyloglucan. Some xyloglucan changes have also been observed in suspension culture of carrots.[77]

Verma et al.[4] showed that auxin greatly increased the amount of endoglucanase produced by pea stem sections that were elongating. This was the same enzyme from T. viride that Albersheim[14-16] had used to isolate different size oligosaccharide fragments from sycamore xyloglucans. Other oligosaccharides have been shown to have biological activity,[6] so Albersheim's group attempted to see if xyloglucan fragments had any effect on auxin stimulated elongating pea stems. This system had yielded extractable xyloglucans[75] and a xyloglucan cleaving enzyme similar to that which produced specific xyloglucan fragments. The two main products of this endoglucanase activity are a nonasaccharide and a heptasaccharide, both consisting of four glucose units and three xylose branches, the nonasaccharide having a Gal-Fuc addition on one of the xylose branches.[15] These two molecules were added to elongating pea stems. The nonasaccharide inhibited the elongation significantly while the heptasaccharide had no effect. The nonasaccharide had this effect at very low concentrations (10^{-1}-10^{-2} μg/mL in the incubation medium), indicating that this molecule could indeed be a regulatory molecule.

Further work has shown that this nonasaccharide exists in vivo. Camirand and Maclachlan[78] isolated a fucose-containing nonasaccharide from pea microsomal membranes using radioactive labeling techniques. The fucose unit in the

nonasaccharide is of special significance because the xylose and glucose hepta-saccharide, lacking fucose, had no effect on the elongating pea stems. Other workers have focused on the xylose-glucose portion of the xyloglucan.[78] Fry[22] has also isolated a nonasaccharide from suspension cultured spinach cells. He found a number of fucose containing oligosaccharides, but the major one was a nonasaccharide. All of the sugars found in the spent culture medium were in very low concentrations. It is apparent, however, that the nonasaccharide found by York et al.[5] to be active in the pea stem system occurs in vivo.

XYLOGLUCANS IN THE CELL WALL

Maclachlan has made a major study of how xyloglucans interact with cellulose, and how they may affect the deposition of cellulose. Maclachlan has studied the role of endocellulases (an enzyme we have reviewed above) in cellulose biosynthesis.[79] To this end, Hayashi and Maclachan have used a variety of techniques to study xyloglucan and cellulose interactions in pea stems. They have isolated nona- and heptasaccharides and used lectins to locate the xyloglucans both on and between cellulose microfibrils.[80] They have seen that even though endoglucanase activity increases under auxin stimulation, these elongating cells deposit large amounts of xyloglucan.[81] They also concluded that the xyloglucan in the cell wall is the primary substrate for the endoglucanase.[81] They found that 2,4-D (an auxin) enhanced cellulose-xyloglucan deposition and ethylene inhibited deposition.[82] Finally, they noted that protoplasts from pea stems would form new walls more quickly when pea xyloglucan was added to the culture medium.[82] This showed that the xyloglucan polysaccharides and oligosaccharides are important in the deposition of the cell wall.

One of the properties of xyloglucans that makes them likely polysaccharides to influence cell wall deposition is their ability to sorb strongly onto cellulose. This property has been a boon for its purification.[12,15] Haigler[8,83] has studied how strongly sorbed molecules of varying size can influence the production and assembly of cellulose in A. xylinum. It is possible that the sorbing of xyloglucan and the actions of endoglucanases in the cell affect how the cellulose is deposited and ultimately how the cell wall is constructed.

OBJECTIVES

The objectives of this thesis were threefold: to isolate, to characterize and to biologically test the "xyloglucan" from a conifer source, Pinus taeda. When this study was proposed, there was already a fairly large body of work reported on xyloglucans, and during this investigation an even larger amount has been published. It was of special interest to the Tissue Culture Group at the Institute to investigate any effects that these xyloglucans might have on the morphogenic capacity of loblolly pine cultures. There is, however, a dearth of information on this subject in forest species, which is especially acute for conifers. This lack of information on this seemingly important primary cell wall component in forest species makes this study important to our industry.

It was hoped that more information about the xyloglucan in the suspension-cultured loblolly pine cell wall could be obtained in this thesis if special attention was paid to nondegradative isolation methods and the analysis of each product during isolation. Characterization of the polymer was to be based primarily on methylation data. Molecular size and additional characterization would also be attempted, including enzymatic fragmentation and methylation of these fragments, in order to gain insight into the fine structure of the molecule. The third phase of this research plan was to test the biological activity of the xyloglucan and fragments thereof obtained from various procedures. It was intended that the primary test would be to reintroduce xyloglucan into a growing cell culture and study its effects. A myriad of other tests of biological activity (such as protein synthesis or ethylene production for example) was available, and it was not determined at the outset which tests might prove best suited for this work.

The results of research based on these objectives were to be used to elucidate the structure of the xyloglucan and its associated polymers from loblolly pine, to check its variance from other reported polymers, and to assess the biological activity of the molecule and/or its fragments.

RESULTS AND DISCUSSION

ISOLATION OF THE XYLOGLUCAN

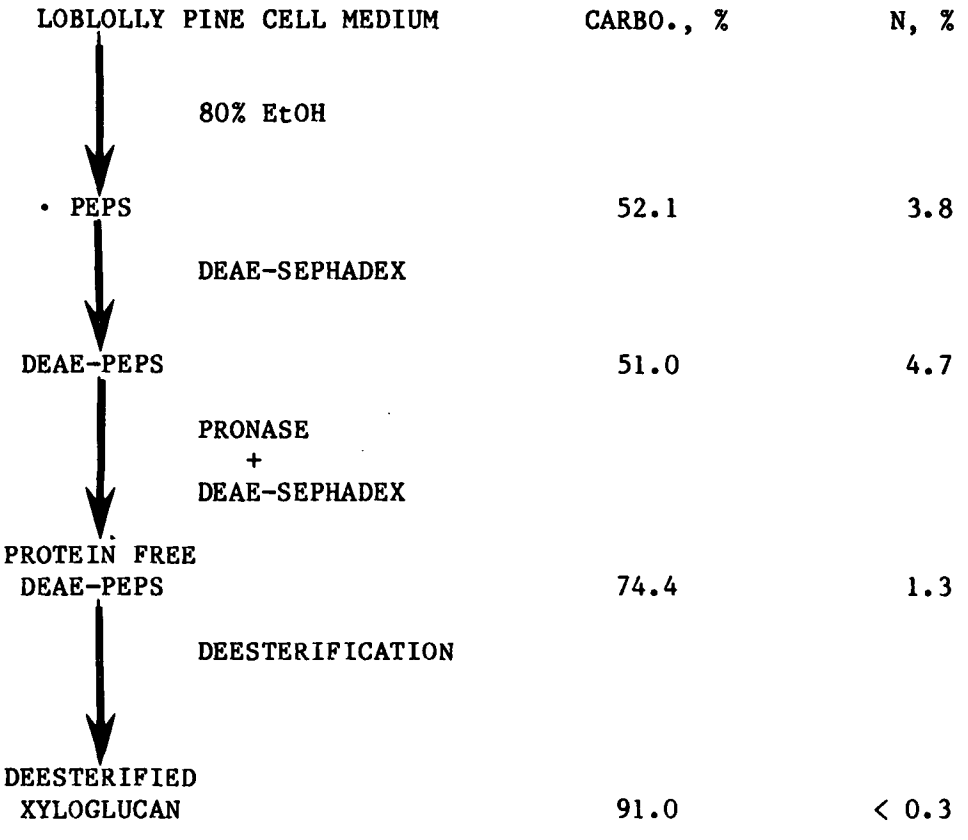
Loblolly pine cells (Pinus taeda) were suspension cultured in LM3 medium (see Appendix I). These cells were subcultured every 12-14 days. At this time the spent medium was also harvested. The cells were grown in large batches. After the spent medium was separated from the cells by filtration, the extracellular polysaccharides in up to 6 L of medium were immediately precipitated by adding four volumes of 95% ethanol. Any excess harvested medium was frozen and subjected to precipitation later. A liter of medium yielded 150 mg of pine extracellular polysaccharides (PEPS) on the average. This varied with the apparent health and growth of the cultured cells. One of the cell lines (3410) was judged to be less viable, as it produced lower yields of dark colored PEPS than the F-2 line. This line was abandoned as a PEPS source. PEPS was obtained by collecting the precipitated solids (via centrifugation) and redissolving the material in water. The majority of the ethanol precipitatable polysaccharides did not redissolve in water (0.35 g/L spent medium). This insoluble material was probably pectinaceous in nature. The redissolved polysaccharides were precipitated, centrifuged, and then redissolved again in water. Very little material did not redissolve in water the second time. The PEPS were then freeze-dried. The composition of the PEPS can be seen in Table 2.

The first step in the isolation of xyloglucan from PEPS (see Fig. 1) was the removal of an arabinose, galactose-rich polysaccharide. A column of DEAE (diethylaminoethyl)-Sephadex, a weakly anionic exchange chromatographic packing, was used to separate the arabinogalactan from the rest of the PEPS. This polymer could also be separated from the xyloglucan on a cellulose column where

the xyloglucan was hydrogen bonded to the cellulose and the arabinogalactan eluted with water. The arabinogalactan must have been associated only by hydrogen bonds, since the DEAE-Sephadex column was able to remove it from the xyloglucan. It appeared that the arabinogalactan was fairly pure, because its removal was not accompanied by the removal of a proportionate amount of protein or other unidentified material. This was the first clue that the xyloglucan was bonded strongly (at least more strongly than the arabinogalactan) to the proteinaceous portion of the extracellular polysaccharides.

Table 2. Composition of isolated neutral fractions.

Component	PEPS	DEAE-PEPS	Protease-Treated	Deesterified	Initially Deesterified
		Mole% of Carbohydrate Fraction			
Fucose	1.8	3.5	4.8	4.8	3.6
Arabinose	11.7	1.8	2.5	2.4	8.5
Xylose	5.7	15.9	21.2	30.4	13.6
Mannose	1.2	1.1	1.4	0.9	1.4
Galactose	22.1	5.1	8.6	8.1	15.1
Glucose	8.4	23.6	35.9	44.3	19.5
Total Carbohydrate (wt.%)	52.1	51.0	74.4	90.9	61.7
Nitrogen (wt.%)	3.8	4.7	1.3	< 0.3	2.9



• Pine Extracellular Polysaccharides

Figure 1. Pine xyloglucan isolation procedures.

At this point of the isolation, the carbohydrate portion of the neutral fraction appeared to be a xyloglucan. However, this fraction was only 50% carbohydrate. It was hoped that the proteinaceous portion could be removed in a "gentle" manner. To this end, isoelectric focusing was used to see if the protein was covalently attached to the xyloglucan. The carbohydrate migrated with the protein, indicating covalent bonding. The removal of the protein would have to be fairly "harsh" to break this covalent bond. A nonspecific protease (Pronase) was used to remove the protein. It was very surprising that a large amount (10%) of protein remained in the sample even after the Pronase treatment.

The carbohydrate portion increased to 73% and its composition remained similar to the xyloglucans of the literature.[15,24] Because of reports of acetyl groups[27] on xyloglucans, it was hoped that the pine xyloglucan could be isolated from the suspension medium without deesterification. This proved impossible.

After deesterification the solids collected by freeze-drying were over 90% carbohydate and contained a negligible protein component (see Table 2). The remaining portion of the xyloglucan fraction could not be specifically identified as any one organic material [in fact C,H,O analysis (see Table 3) showed a mere 2% noncarbohydrate organic material] such as lignin or tannins. No single inorganic element seemed to stand out as a contaminant either. This fraction was judged to be a sufficiently pure xyloglucan fragment and the rest of the work could proceed.

Table 3. Carbohydrate and elemental analysis of DEXG-3.[a]

Fuc, %	Ara, %	Xyl, %	Man, %	Gal, %	Glu, %	Total, %
4.5	2.2	29.6	0.8	8.1	44.7	89.9
5.1	2.6	31.2	1.1	8.1	43.9	92.0

C, %	H, %	O, %	N, %	Total, %
41.76	5.92	46.15	0.58	94.4
41.63	6.12	46.11	--	93.9

[a]Both tests run on duplicate samples.

Before turning to the methylation work, it is instructive to look at information derived from all of the isolation procedures that were attempted. These procedures suggest a number of things about the xyloglucan and its associated material in the medium. The first is that the xyloglucan isolated from

the spent cell medium probably originates from the remnants of cell walls that have sloughed off during cell growth and division and through agitation. It was unlikely that this large complex of xyloglucan and protein would be excreted by the cells. If this is true, the structure of the xyloglucan from the medium can still elucidate something of its situation in the cell wall.

The arabinogalactan that was removed by ion-exchange chromatography was not even necessarily associated with the xyloglucan, but may have simply precipitated with it from the medium. Although the arabinogalactan was not analyzed, a few facts can be deduced about its nature. It must contain some charged groups to be removed by anion exchange chromatography. It also has far less protein associated with it than the xyloglucan, as can be seen in Table 2.

Alternate procedures were attempted to bypass some of the isolation steps (Fig. 2). In the first, PEPS was treated with the protein cleaving enzyme before the arabinogalactan was removed. After anion-exchange chromatography, 3.9% of the sample was nitrogen, a much higher percentage than the 1.3% that remained after the Pronase and anion-exchange steps in the main isolation scheme. The arabinogalactan may interfere sterically with the Pronase, suggesting the arabinogalactan was in fact associated with the xyloglucan. The second alternate procedure (Fig. 2) involved the deesterification of the PEPS prior to anion-exchange chromatography. Although the new xyloglucan complex had less protein after deesterification and anion exchange chromatography, more of the arabinogalactan remained with the xyloglucan. This suggests that the deesterification removed some of the charged groups on the arabinogalactan that facilitated separation by anion-exchange.

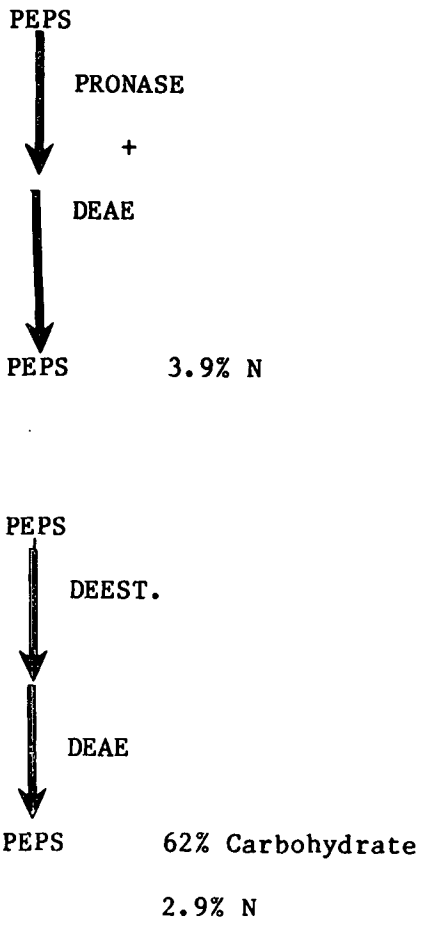


Figure 2. Alternate isolation procedures.

These data also pointed to the existence of two types of protein associated with the xyloglucan. Although both were strongly attached to the xyloglucan, one was removable by a protein cleaving enzyme while the other could be removed by deesterification and the probable breaking of an ester bond. Deesterification alone removed some protein but not all of it. This remaining protein in the directly deesterified fraction could be attached to the arabinogalactan that remained in the fractions, but it seems more likely from the data that the remaining protein was associated with the xyloglucan in a manner other than an ester bond. This protein was accessible to the protein cleaving enzyme unlike the ester-attached protein. The nature of the bonding of this second protein is unknown.

A second polysaccharide was isolated from the spent medium of the suspension cultured loblolly pine cells[84] by paper chromatography. Several liters of the medium-ethanol mixture were evaporated to a syrup after the PEPS was removed. From this syrup a small xylan oligosaccharide was isolated. This oligosaccharide is thought to be 5-8 xylose units long. This is an oligomer that has not been isolated before and will be discussed on page 51.

METHYLATION OF THE PINE XYLOGLUCAN

The majority of characterization work on the pine xyloglucan was done using methylation techniques. The basis of this characterization work was the Hakamori[40] method, which permethylates carbohydrates utilizing methyl iodide. A large volume of work by Björndal et al.[43,46] cataloging (gas chromatography/mass spectroscopy, GC/MS) the methylated alditol acetates of most common sugars makes identification of permethylated carbohydrates possible. As was discussed in the Introduction, methylation data can reveal the branching pattern of complex polysaccharides. This method, however, does not provide information on the specific structure (i.e., the relative position of branch points) of a polysaccharide.

The first attempts at methylation were performed on a commerical modified cellulose of fairly short length. The Hakamori method with the Sanford and Conrad modification[85] was used. This entailed the preparation of the dimsyl anion from sodium or potassium hydride and DMSO. Because this reaction is extremely water sensitive and generally tedious to perform, a simpler, more recent modification[42] was used. This method used the NaOH hydroxyl as the anion rather than the dimsyl. Although this method was water sensitive as well, it was simpler to prepare, and because the NaOH could be placed directly in a DMSO solution of polysaccharide and MeI, it was far easier to maintain a water-free

atmosphere. This reaction took only about 10 minutes. Methylation was only the first step in the derivatization of the polysaccharides for GC and GC/MS analysis. The extent of methylation was checked by infrared spectroscopy (IR), but the other steps were harder to monitor; in fact, it was only through the examination of the final products that deficient steps could be identified.

A sample called deesterified xyloglucan No. 2 (DEXG-2) was methylated with NaOH-MeI, extracted from the DMSO with chloroform, evaporated to dryness, hydrolyzed, reduced and acetylated. The logic behind this modification of the polysaccharides is to methylate the exposed hydroxyl groups. The oxygen atoms involved in inter- or intramolecular bonds are not methylated. Subsequent hydrolysis cleaves the glycosidic bonds and exposes the hydroxyl groups of the carbon atoms involved in intermolecular bonding. Reduction opens the ring and exposes the hydroxyl groups of the carbon atoms that participate in the intramolecular bond. These newly formed hydroxyls are acetylated. It is necessary to acetylate these methylated alditols to render them volatile enough to run with acceptably low retention times on the gas chromatograph. The resulting methylated alditol acetates have O-methyl groups on the unbranched positions of the sugars that make up the polysaccharide and O-acetyl groups on the branched positions. This procedure was performed on the DEXG-2; the total ion chromatogram can be seen in Fig. 3. This chromatogram was far cleaner and had far fewer peaks than earlier methylation attempts. There were, however, two problems with the methylated alditol acetates from DEXG-2. The first was the fact that the program that produced the well resolved chromatogram in Fig. 3 would not run known sugars in a predictable (as related to literature values[43,46]) manner. The second was the predominance of peaks in the early part of the chromatogram. This might have been caused by abnormally high response factors of

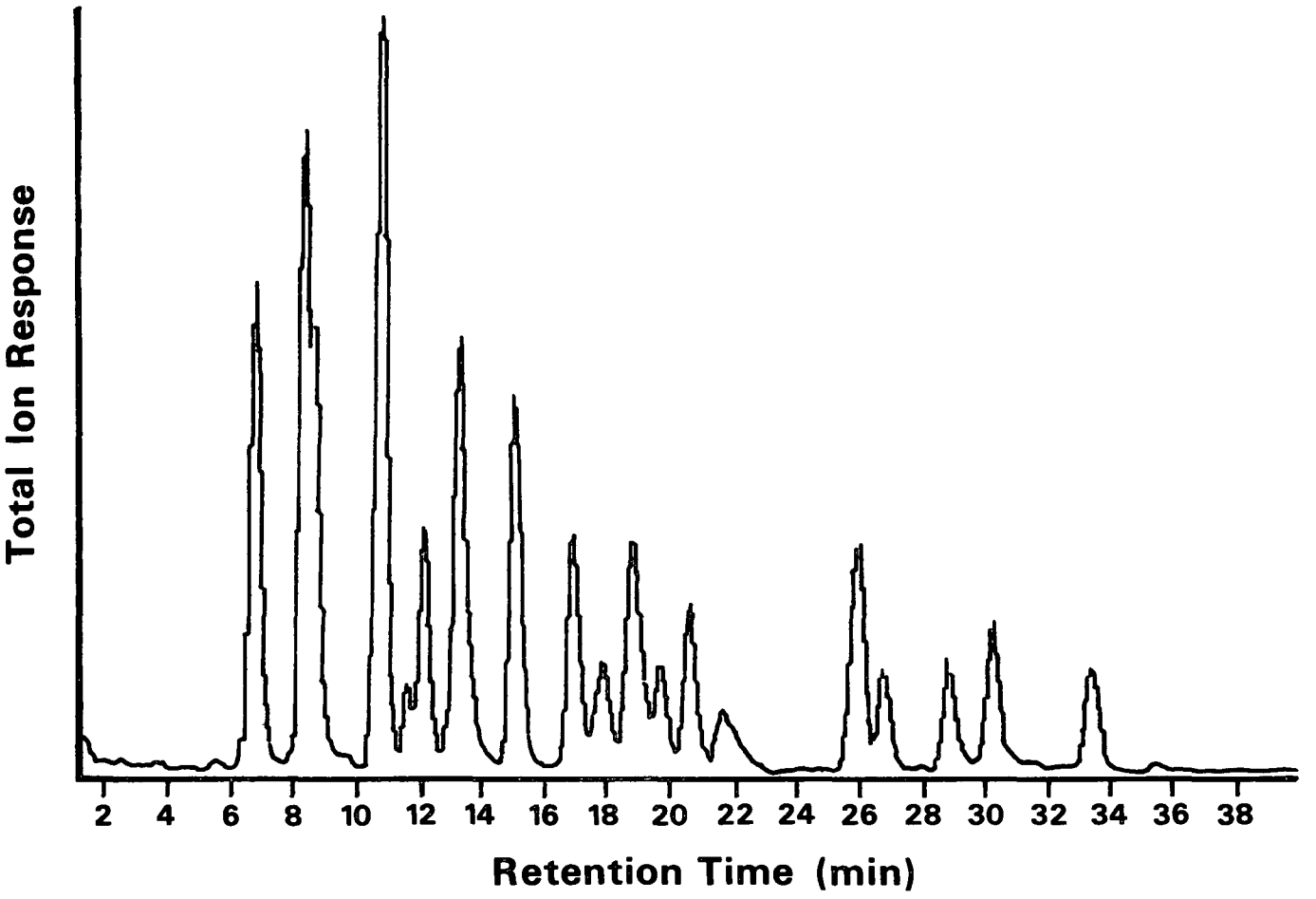


Figure 3. Total ion chromatogram of DEXG-2. Partially methylated alditol ran on SP-2340, 150-220°C at 2°/min.

the pentoses, deoxy hexoses or extensively methylated hexoses, or the loss of less extensively methylated hexoses. Since the response factors of all of these methylated alditol acetates have been shown to be similar[43] (within 5%), it was assumed that the derivatization procedures preferentially lost the less methylated hexoses. The most likely step for this loss was the acid hydrolysis. It is known[86] that mono- and di-O-methyl sugars can complex with barium salts. This can occur when the hydrolysis mixture is neutralized with barium carbonate. Upon filtration the complexed sugars cannot be washed from the barium carbonate and are lost. A third xyloglucan (DEXG-3) was methylated and hydrolyzed with 2N

trifluoroacetic acid (TFA).[87,88] Not only was this a simpler procedure than hydrolysis with 72% H_2SO_4, but no transfers or complexing occurred as the TFA was removed by evaporation. The total ion chromatogram of DEXG-3 in Fig. 4 shows a more probable distribution of xyloglucan components. Therefore, the problems with the methylation procedures were resolved. This provided a straightforward, workable means of producing analyzable derivatives.

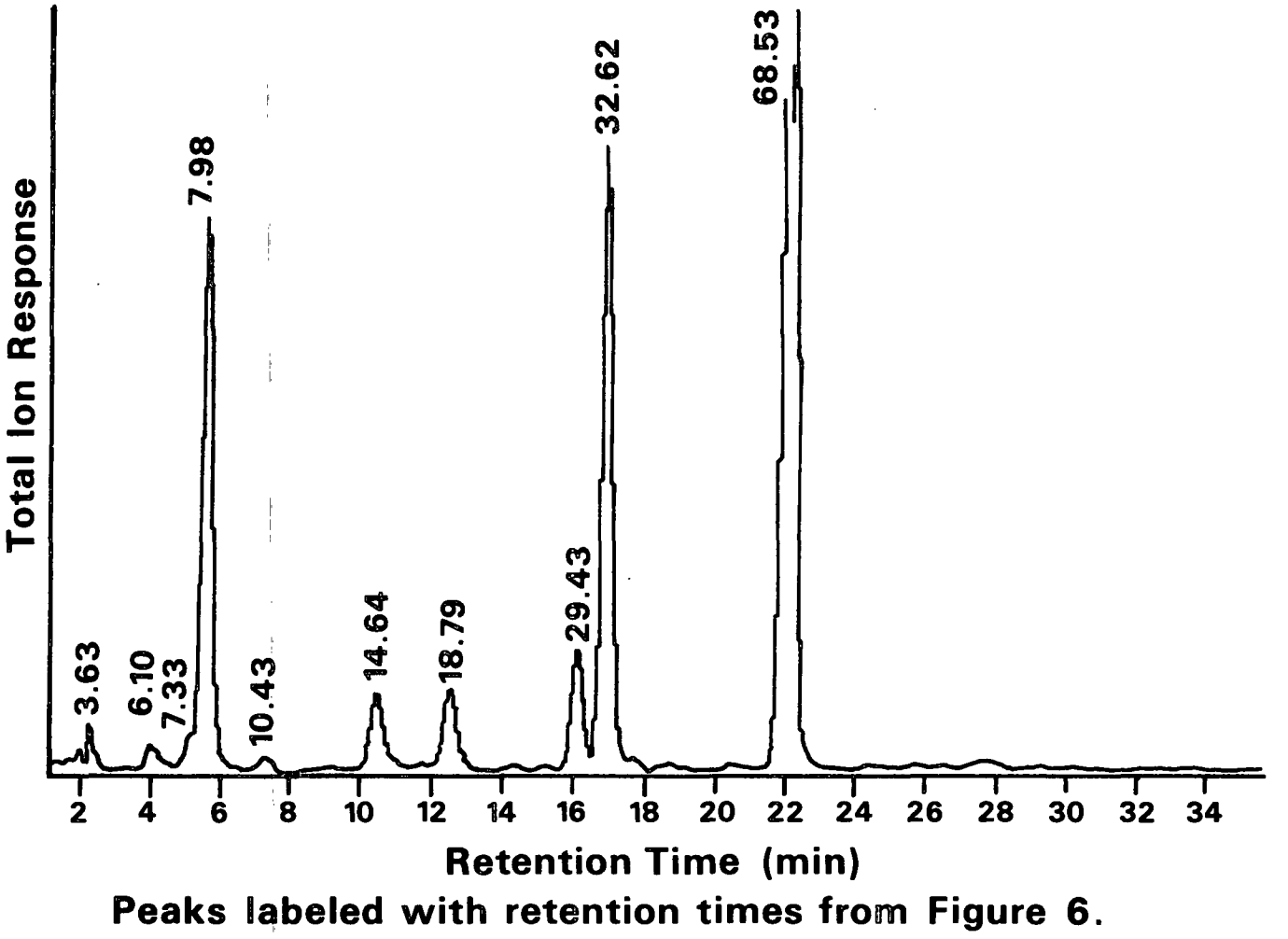


Figure 4. Total ion chromatogram of DEXG-3. Partially methylated alditol acetates run on SP-2340, 150-220°C at 2°/min.

The next step was developing a system to identify the sugar derivatives. Methylated sugars that had been prepared up to 30 years before[84] were reduced and acetylated. The reduction was carried out to assure that the sugars were in an alditol form. This procedure, specifically the neutralization of the sodium borohydride with acetic acid, also provided sodium acetate, a catalyst necessary for acetylation with acetic anhydride. Most of the prepared sugars listed below were fairly pure.

2,3 di-O-methyl,1,4,5 tri-O-acetyl xylitol (23Me Xyl)

2-O-methyl,1,3,4,5,6 penta-O-acetyl galactitol (2Me Gal)

3-O-methyl 1,2,4,5,6 penta-O-acetyl glucitol (2Me Glc)

2,3,6 tri-O-methyl 1,4,5 tri-O-acetyl glucitol (236Me Glc)

2,4,6 tri-O-methyl 1,3,5 tri-O-acetyl glucitol (246Me Glc)

2,3,4,6 tetra-O-methyl 1,5 di-O-acetyl glucitol (2346Me Glc)

When there was contamination, the vast majority of the material was found by mass spectrometry to be the desired methylated alditol acetate. Without these standards, identification of the xyloglucan would have been impossible.

Identification of the xyloglucan components was achieved through the use of published retention times of methylated alditol acetates of sugars, since it was impossible to obtain all of the possible methylated sugars that could occur in the pine xyloglucan. It was first assumed that any conditions in the GC would produce retention times for the alditol acetates that correlated to the published values. The known sugars were run under the conditions that produced the best chromatograms of the DEXG-3. The retention times of the known sugars did not correlate to the published retention times[43,46] in any predictable manner. Finally, a separate GC was set up and a number of isothermal runs made

at temperatures ranging from 150 to 200°C. There was quite a difference in the retention times of the six methylated alditol acetates. The most extensively methylated (2346Me Glc) ran nearly ten times faster than the least methylated alditol acetate (3Me Glc). Between 170 and 190°C was a manageable range for these seven sugars on the ECNSS-M column. The methylated alditol acetates correlated well ($r > 0.999$) versus the literature[43] values (Fig. 5). The ECNSS-M column is commonly used for these sugar derivatives, but it was found to bleed extensively, especially in the programmed GC/MS runs. Another column, SP-2340 (Supelco) was tried and found to have little or no bleed while producing sharper peaks. On this column the standards were run isothermally. This column also correlated well ($r > 0.994$) to the literature values and was selected as the column of choice. The six alditol acetates provided enough information to identify the methylated alditol acetates obtained from the pine xyloglucan.

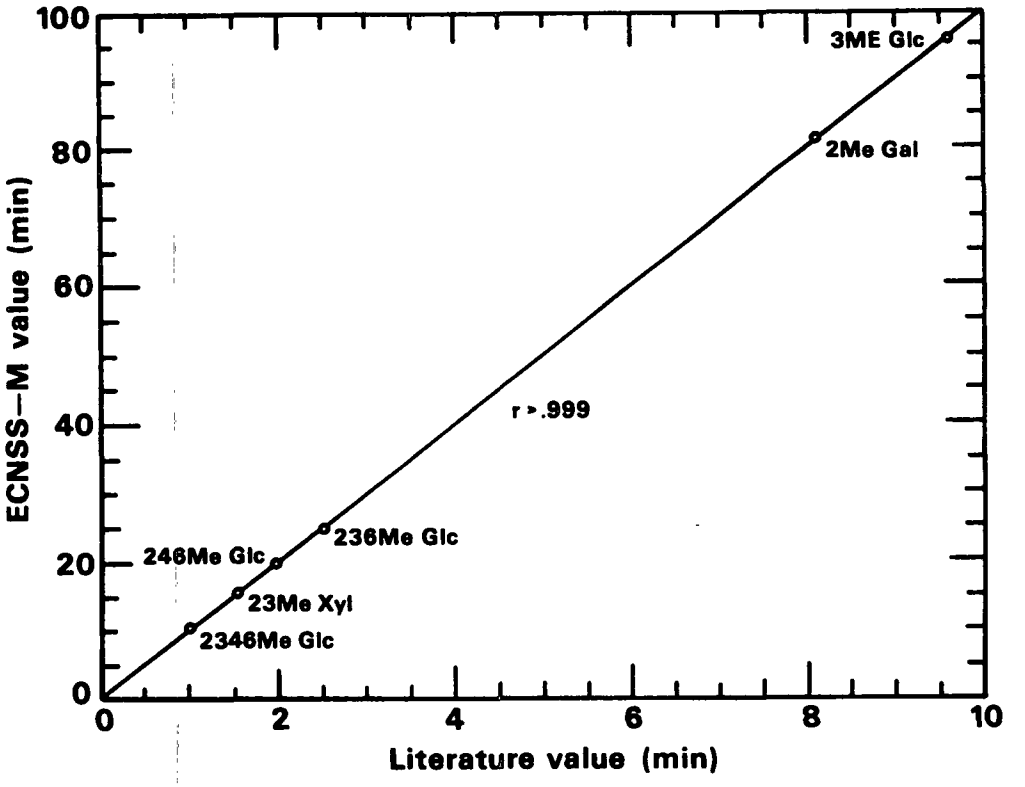


Figure 5. Prepared standard partially methylated alditol acetate retention times on ECNSS-M at 185°C vs. literature values.[43]

The gas chromatogram of DEXG-3 at 170°C on SP-2340 can be seen in Fig. 6. This isothermal run was found to be acceptable for the standards and the DEXG-3. At this lower temperature, the faster running alditol acetates separated well early in the chromatogram. The column was calibrated by running standards (usually 2346Me Glc and 236Me Glc). This was done to get a base for correlating the retention times of the DEXG-3 and the literature values. This became unnecessary when major peaks were identified and could then function as internal standards. Most of the large peaks were fairly easy to identify using retention times, knowledge of carbohydrate composition and the reported structures of other xyloglucans.[15,24] All of the peaks of the methylated alditol acetates obtained from DEXG-3 can be seen in Table 4. The peaks at 7.98, 32.62, and 68.52 minutes were easily identified by their size and retention times and were determined to be 234Me Xyl, 236Me Glc, and 23Me Glc, respectively. The other peaks had to be resolved using a combination of retention times and mass spectra.

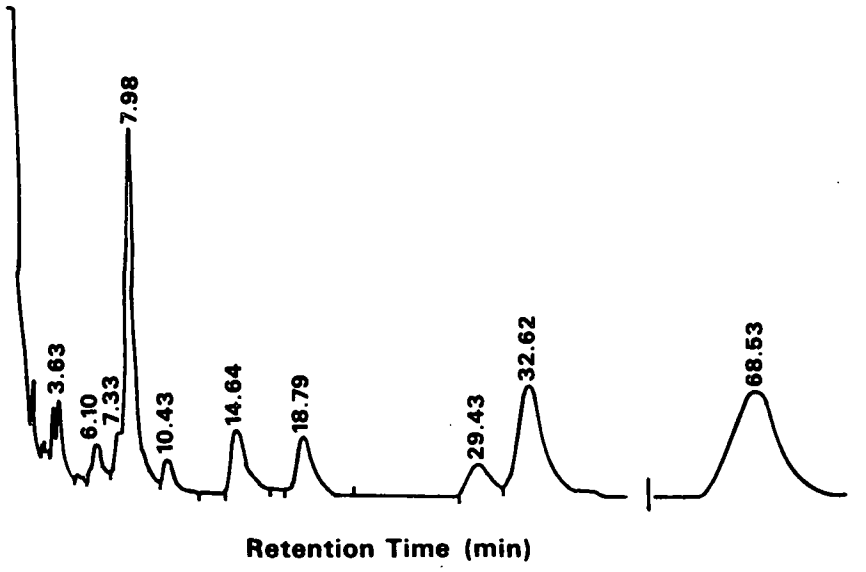


Figure 6. Gas chromatogram of DEXG-3. Partially methylated alditol acetates run on SP-2340 at 170°C.

Table 4. DEXG-3 methylation data.

GC data of TFA hydrolyzed, alditol acetates of methylated xyloglucan run isothermally at 170°C on SP-2340 and MS analysis.

RT, min	Area, %	Resolved Species
3.63	1.94	Unknown
6.10	2.12	235Me Ara
7.33	2.02	234Me Fuc
7.98	20.40	234Me Xyl
10.43	1.77	Unknown
14.64	6.20	2346Me Gal
18.79	6.46	23Me Xyl
29.43	4.66	346Me Gal
32.62	19.62	236Me Glc
68.53	32.36	23Me Glc

Since many of the methylated alditol acetates ran very close together (in respect to time) and most of the DEXG-3 products did not fall precisely on a specific literature value, mass spectrometry had to be employed to identify the smaller peaks. The mass spectra of methylated alditol acetates cannot distinguish between different hexoses or different members of other classes of sugars, but can determine the general class of the sugar and its methylation pattern. The data obtained from MS do not include a molecular ion, but using an available library of mass spectra from methylated alditol acetates,[46] the mass spectra can be a useful tool. The mass spectra of the DEXG-3 peaks can be seen in Appendix II. It was possible to program the column temperature for the DEXG-3 material on the GC/MS because the pattern of the peaks was sufficient to

relate them to the gas chromatogram that was run isothermally. This is why the patterns appear to be different for the GC and GC/MS chromatograms. This was done both to save time and produce sharper peaks. The resolution of the peaks after GC and GC/MS analysis can be seen in Table 4.

Over 96% of the DEXG-3 material in the gas chromatogram was identified. The remaining materials, contained in two peaks, were most probably degradation products of the derivatization procedures. The mass spectra of these peaks appear to be carbohydrate in nature but were unidentifiable. This is not uncommon in procedures such as these.[15,24]

This work provided enough information to propose a nonspecific structure for the pine xyloglucan. The sugar composition and proposed structure can be seen in Fig. 7. Some 20% of the polysaccharide is 1,4 bonded glucose units. These are assumed to be β bonds, which the optical rotation (≈ +20°) seemed to substantiate. The anomeric configurations have not been determined for any of these bonds, but because of the similarities of the xyloglucans, most workers have assumed anomeric configurations are common to all xyloglucans[28] (Table 1). It is not known whether these unbranched glucan sections occur in long stretches or a few at a time. The rest of the molecule has many features of common xyloglucans. Some of the interesting and unique features are the large number of terminal galactoses and the terminal arabinose and fucose units. These are not often seen together in one polymer. Overall the methylation data obtained meshed well with other workers' results and with the total carbohydrate analysis of the DEXG-3. Some of the more highly methylated pentoses and deoxy hexoses are volatile and might have been lost during the derivatization, but it appears they were not since the carbohydrate analysis and methylation data were very similar.

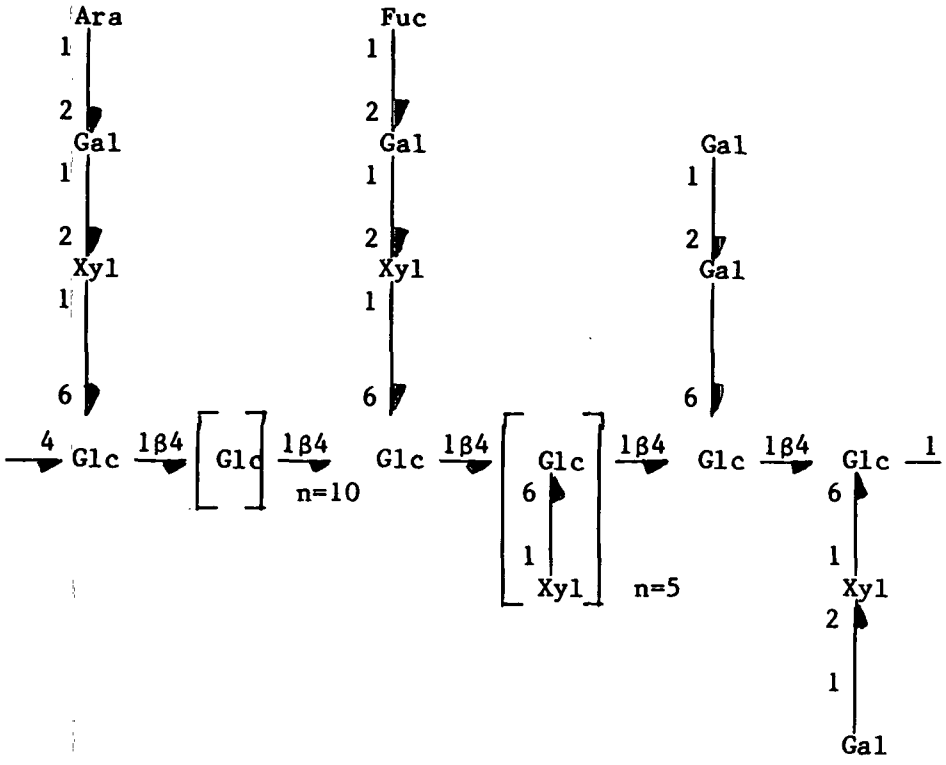


Figure 7. Proposed pine xyloglucan structure. Branches have been placed arbitrarily on the glucan backbone.

GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography was next used to separate enzymatically cleaved fragments of the deesterified xyloglucan. Fortunately, commercial grade fungal cellulases were available for this work; it would not have been within the scope of this thesis to grow the fungi and harvest the enzyme. The enzyme used was Cellulysin (Cal Biochem), which is isolated from T. viride. Albersheim[15] and other workers[24] have used an endoglucanase from T. viride to produce specific fragments for characterization and biological testing.[5]

Deesterified xyloglucan was enzymatically cleaved with Cellulysin. These fragments were then separated from the enzyme and the buffer solution via filtration and an ion-exchange resin. The salt-free solution was evaporated to a

few mL and placed on a Bio-Gel P-2 column. This gel permeation column excludes molecules above ≈ 1800 Daltons. The xyloglucan fragments were expected to be, at the largest, 15 sugar units in size or ≈ 2000 Daltons. Any uncleaved xyloglucan or other contaminants would then be excluded and pass quickly through the column. The Bio-Gel P-2 column was maintained at 55°C with a water jacket and a circulating pump. Extreme care had to be taken to degas the water eluent and the sample or air bubbles would form in the gel. The column was eluted at 5 mL/hr, a rate necessitated by the weak structure of the Bio-Gel packing. The fractions collected were tested with the phenol-sulfuric acid assay, a very sensitive test for total carbohydrate. This was chosen because of reproducibility problems with other tests such as the anthrone assay.

The initial elution pattern of a cellulase-fragmented deesterified xyloglucan can be seen in Fig. 8. Known linear oligosaccharides were run immediately after the xyloglucan fragments, and the elution volumes of glucose, cellobiose, stachyose, raffinose, and maltoheptose can also be seen in Fig. 8 (labeled G, C, S, R, and H). Comparison with the elution volumes of the knowns and the elution patterns of xyloglucans in the literature[15,24] showed the pine xyloglucan fragments to range in size from 11 or 12 sugar units to monosaccharides. Albersheim has reported that two major peaks are evident, the first a nonasaccharide, the second a heptasaccharide. The DEXG-3 fragments have two major peaks in their elution pattern that coincide with fragments of this size. The "C" and "D" peaks of the DEXG-3 fragments are most probably a nona- and a heptasaccharide, respectively. The remaining peaks comprise a significant portion of the enzymatically cleaved xyloglucan. The peaks D-G are made up of fragments ranging from 5 or 6 sugar units down to monosaccharides.

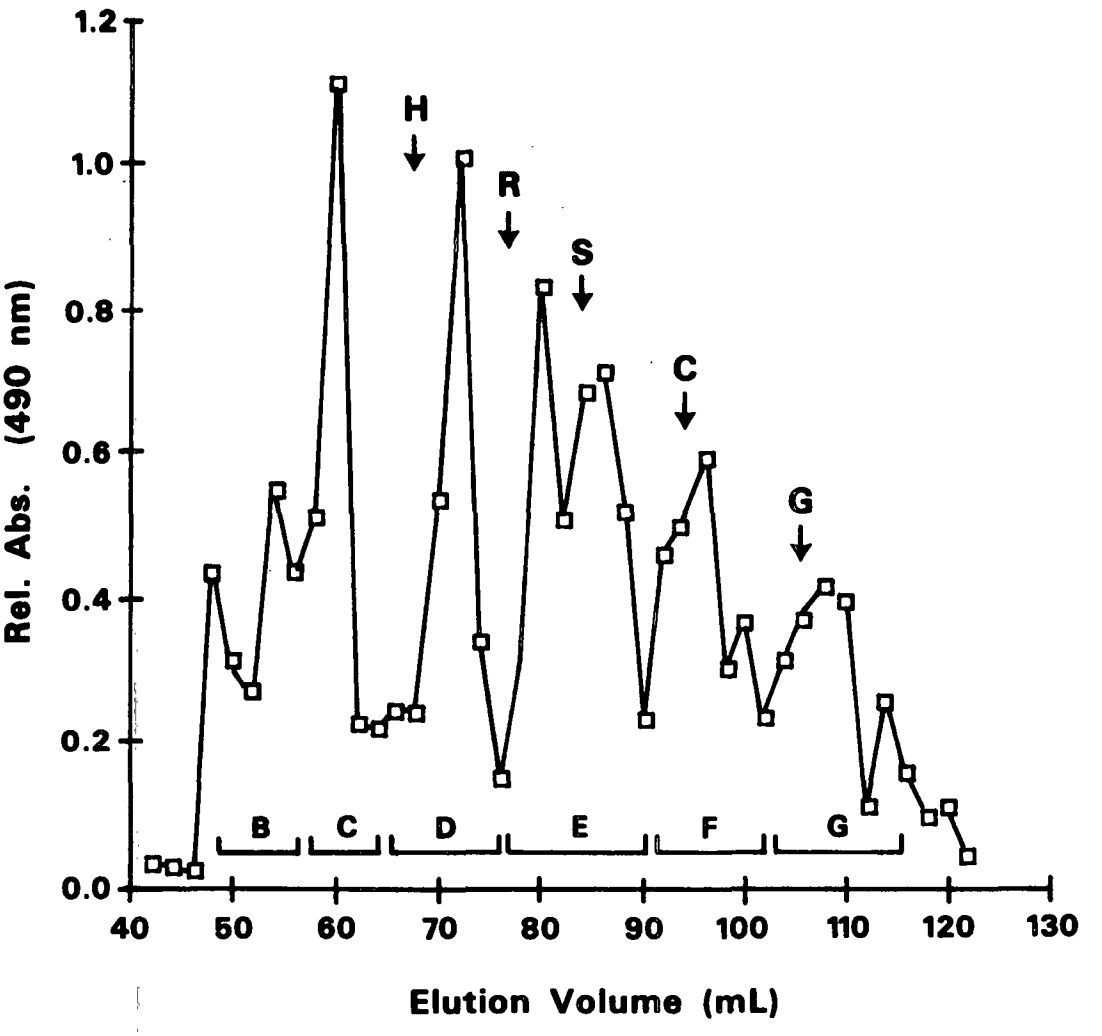


Figure 8. Elution pattern of cellulase-treated DEXG-3 on Bio-Gel P-2. Points labeled H, R, S, C, G are the elution volumes of maltoheptose, raffinose, stachyose, cellobiose, and glucose, respectively.

The pine xyloglucan produced far more of these smaller fragments than the sycamore xyloglucan.[15] In this aspect the pine xyloglucan more closely resembles the xyloglucan from runner beans.[24] The pine xyloglucan fragments were much broader in their range of size. If the cellulase consumes all of the unbranched glucose units except perhaps those that are the reducing end of branched fragments (as has been reported),[27] then pine xyloglucan unbranched glucose

probably occurs in short sections between small branched portions of the glucan backbone. The structure of the pine xyloglucan would then resemble the proposed runner bean xyloglucan more closely than some of the large blocks of branched sections proposed by Bauer[15] for sycamore.

The fractions comprising each peak eluted from the Bio-Gel P-2 column were placed on the column and eluted again. Most of the peaks (B-G) were fairly homogeneous in size but all were divided into smaller fractions. Appropriate fractions were pooled and modified for GC and GC/MS analysis. The procedure was the same as used for the xyloglucan polymer except that the fragments were prereduced with borodeuteride before methylation. It was hoped that the prereduced reducing end groups could be seen in the gas chromatogram and thus give an indication of the size of the fragments. To this end, a cellobiose sample was prereduced and derivatized to produce a standard of 12356 tetra-O-methyl 4-O-acetyl glucitol and 2346Me Glc. This standard was chosen because of the likelihood of unbranched glucose units being at the reducing terminus of the cellulase cleaved xyloglucan fragments. This was what had been reported earlier with sycamore xyloglucan.[15,27] A sample of tamarind xyloglucan was fragmented and one fragment analyzed with GC. This tamarind fragment had a reducing terminus of unbranched glucose which was easily seen in the gas chromatogram. This showed that the cellulase cleaved the tamarind xyloglucan in the same manner as the sycamore xyloglucan and that this method of end group analysis was valid.

The pine xyloglucan fragments did not produce such informative chromatograms. The first problem was one of lack of material. Neither the "B" nor "C" subfractions contained enough methylated alditol acetates to give chromatograms that contained an abundance of useful information. The "C" subfractions (4 of them) were later pooled and run again on the GC. The "E" fractions were also

pooled and rerun. These fragments, however, still did not yield significant specific structural information. Generally, xyloglucans are thought to be blocks of branched sections separated by unbranched glucan backbone. The size of these unbranched sections was unknown, but large unbranched sections were not likely to be present due to the small size of the fragments produced by cellulase cleavage. There was only enough unbranched glucose present in the polymer to separate branched sections with one or two glucose units. The composition of the branched blocks then revealed some of the "finer" structure of the xyloglucan.

One "B" subfraction and the pooled "C" subfractions yielded some information. One of the "B" fragments had one identifiable major peak. It was found to be 236Me Glc, indicating an unbranched glucose unit at the nonreducing end of the fragment. The pooled "C" peak provided more information. The major resolved peaks can be seen in Fig. 9. This octa- or nonasaccharide contains some unbranched and some branched glucose units at the reducing end, indicating a mixture of fragments in the peak. All of the peaks were probably mixtures of different fragments. This fraction also contained terminal galactose and glucose units, the former being one of the predominant peaks. This fragment did not contain any xylose or fucose, even though it was of a size similar to fucose containing xyloglucan fragments of other species.[15,22]

Major Species	Possible Nonreducing End Structures
2346Me Glc	Gal 1 | 6 | Glc
2346Me Gal	or
	Gal 1 | 6 | Glc $\xrightarrow{1\ \ 4}$ Glc

Figure 9. Major components of fraction "C" and a possible structure for the nonreducing end.

One of the "D" subfraction's gas chromatogram (Fig. 11) contained more information. It had several large, identifiable peaks. The methylated alditol acetates present in substantial amounts were 234Me Xyl, 1235Me Glc, and 346Me Gal. Lesser amounts of 2346Me Gal, 23Me Xyl, and 23Me Glc were found. This information suggested a fragment as seen in Fig. 10. The proposed fragment could be one of several in this peak. This was the only fraction that had a substantial amount of xylose. Although no fucose was evident in the chromatogram, the composition of this fragment resembled other fucose containing xyloglucan fragments.[15,24] There was, however, no reason to expect the loss of fucose.

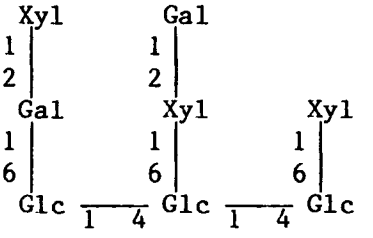
Major Species	Possible Structure
234Me Xyl	
1235Me Glc	
346Me Gal	
Minor Species	
2346Me Gal	
23Me Xyl	
23Me Glc	

Figure 10. "D" components and possible structure.

The pooled "E" fractions also provided enough data to ascertain the general structure of perhaps a portion of this oligosaccharide mixture. This hexa- or pentasaccharide contained a large amount of 2346Me Gal. These terminal units were presumed to be mostly bonded to the glucan backbone via a xylose unit. This fragment may have galactose units bonded directly to the backbone.

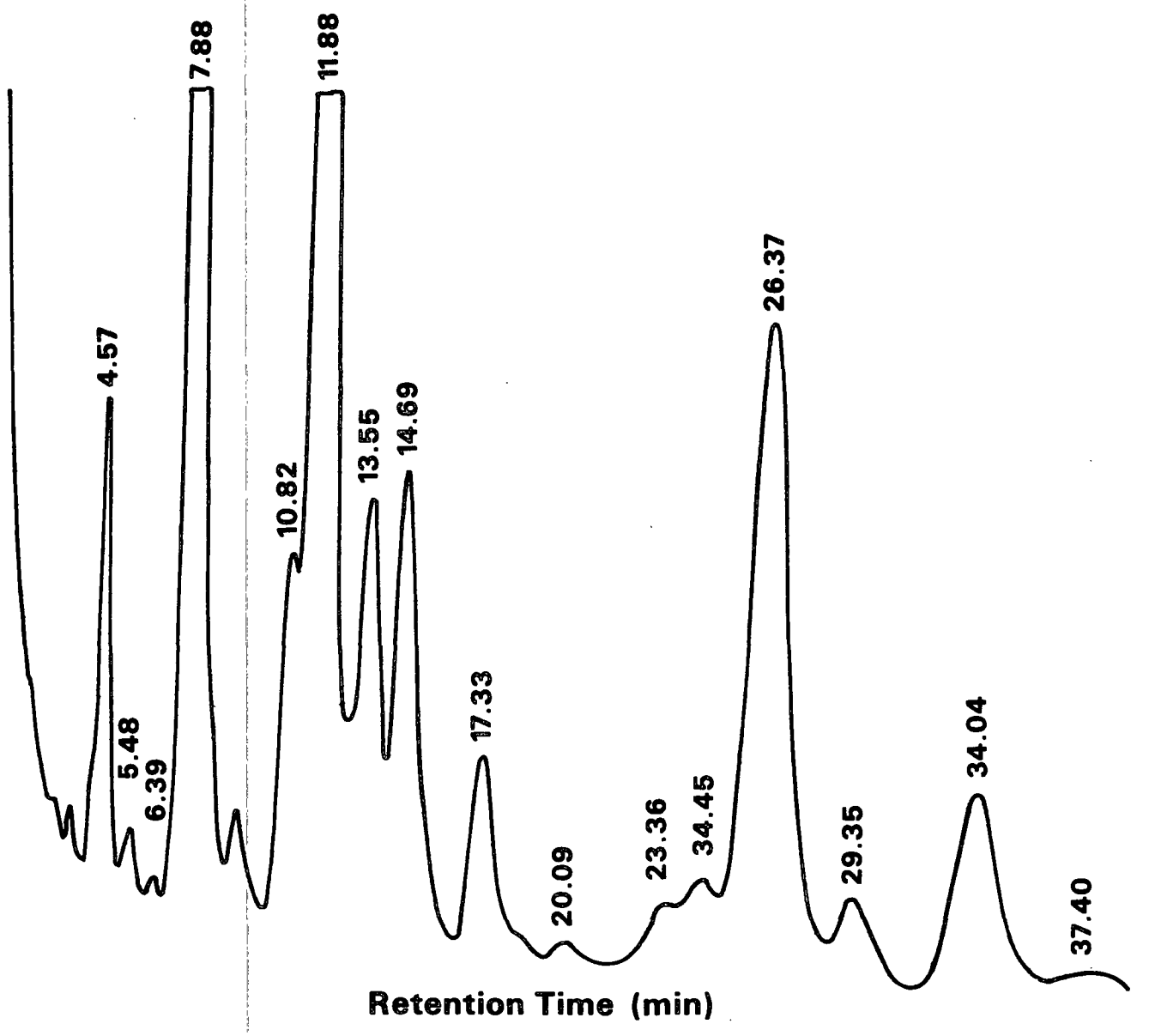


Figure 11. Gas chromatogram of "D" subfraction. Partially methylated alditol acetates run on SP-2340, 170°C.

The information from these fragments, although not complete, showed the pine xyloglucan to be different than other isolated xyloglucans. The xylose seemed to be contained in one set of fragments, while terminal galactose units were bunched in other pieces.

MOLECULAR SIZE

A sample of protein-free deesterified pine xyloglucan was run on a calibrated column of Sepharose CL-6B to determine its molecular size. The elution pattern of the xyloglucan was monitored using the phenol-sulfuric acid assay on 1 mL fractions. A graph of absorbance vs. fraction number can be seen in Fig. 12. One broad peak with a slight shoulder was found. The apex of the peak corresponds to a dextran molecular weight of ~ 70,000 Daltons or a xyloglucan containing more than 300 sugar units.[99] The calculated weight (Mw) and number (Mn) average molecular weights were 117,500 and 38,000, respectively. The pine xyloglucan from the suspension medium was fairly large (with respect to reported xyloglucan molecular weight)[11,15] and polydisperse.

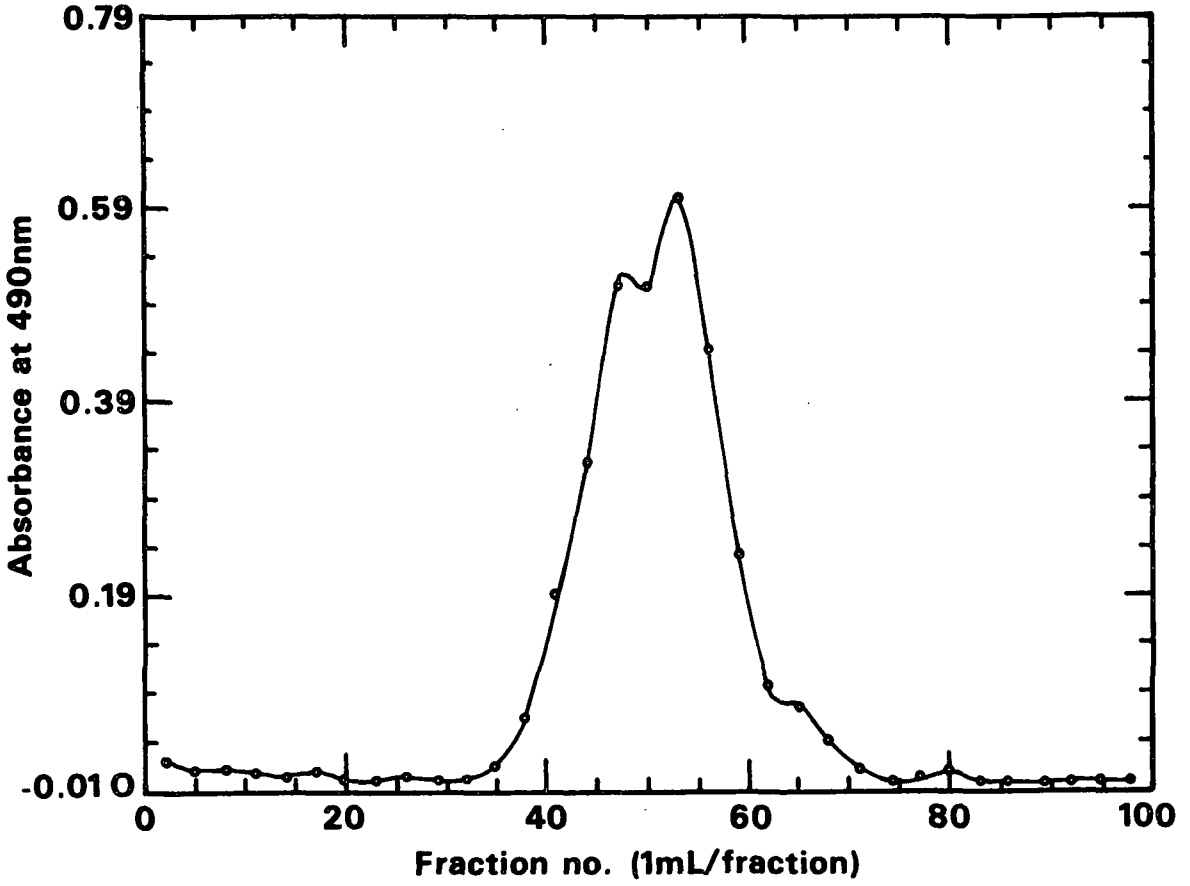


Figure 12. Elution pattern of DEXG-5 on Sepharose CL-6B.

XYLAN OLIGOSACCHARIDE

A small xylan oligosaccharide was isolated from the ethanol solution remaining after precipitation of the extracellular polysaccharides. Large quantities of this ethanol/medium solution were evaporated to a syrup. Paper chromatography was used to separate the xylan from the sucrose and other medium components. Paper chromatography also showed this xylan to be 5-8 xylose units in size and lacking any uronic acids.[84] Xylans are a major hemicellulose component of secondary walls[9] but are not often found in primary walls and have not been isolated from suspension culture medium.

This xylan oligosaccharide was modified for GC analysis and the chromatogram obtained. The only identifiable peak was that of a di-O-methyl xylan (23 or 34), indicating a 1,2 or 1,4 linked unbranched xylan.

BIOLOGICAL TESTING

A number of biological assays were used to test for the possible activity of a number of different fractions isolated from the spent suspension medium of the pine cells. Various assays have been used by other workers[5,7,59] to test the biological activity of xyloglucans and other polymer fragments from primary cell walls and suspension media. Most of these have been discussed in the Introduction, and will not be reviewed here.

The first assay used was the culturing of tobacco explants. Tran Than Van[7] has found that pH and auxin levels can influence the organs that develop from thin strips of tobacco floral branches. Cell wall fragments of sycamore supplied by Albersheim[6] have also been shown to have an effect on organ development. Tobacco plants of two cultivars were grown. The first was a commercial

variety whose seeds were supplied by the University of Wisconsin - Madison. The second was samsun, a laboratory cultivar that grew quickly to flowering. Because the samsun matured quickly, took up less space, and is the cultivar used by Tran Than Van, the growing of the commercial variety was abandoned. The samsun was harvested at a time when the terminal flower had become a large (2 cm) fruit. Thin strips of the floral branches were skinned off and floated on liquid media varying in pH, auxin level and pine medium polysaccharide fractions. A number of trials were prepared, but the controls only produced a hard, green callus. Since organs like those produced in Tran Than Van's laboratory were not induced even in the controls, these experiments were abandoned. This is a very sensitive and difficult technique. It is not surprising that in the short time available it could not be mastered. Although disappointing, there were a number of other assays to be attempted.

The next experiment used to try to establish biological activity of the pine media polysaccharides was one that measured the amount of protein synthesized by Norway spruce callus. Clumps of white (embryogenic) callus were placed in media containing ^{14}C-labeled leucine and various levels of pine polysaccharides. After incubating for 12 hours, the cells were ground and the soluble proteins collected. The sample was divided. One portion was assayed for protein while the other was counted for radioactivity. The counts per mg protein varied widely; in fact, some of the standard deviations were larger than the mean values. This method of measuring cell growth, especially with callus cells, is variable and will only show effects that differ by perhaps an order of magnitude. Another biological test was then attempted.

The testing of xyloglucan fragments for activity in the Norway spruce embryogenic system was performed. The tissue culture team at the Institute has developed a protocol to produce plantlets from Norway spruce callus.[91] This procedure was followed until the development stage. At this point control callus was taken from an activated charcoal-agar medium without any growth regulators and placed on an IBA-ABA medium. To administer the treatment, the callus pieces were removed from the charcoal medium and placed on glass pads that were in a liquid IBA, ABA medium with various concentrations of cellulase-produced xyloglucan fragments. After two weeks, callus samples were weighed and dispersed, and embryos were counted. Although initially it appeared that the 0.01 mg/mL xyloglucan treatment might inhibit embryo development (see Table 5), these results were not reproducible. In other trials, different concentrations of fragments appeared to stimulate, retard, or have no effect on embryo development. In general, with hindsight, the floating glass pads appeared to be detrimental to embryo development. However, when designing the tests it was feared that the agar might hinder movement of the polysaccharides enough to create gradients, so the glass pad-liquid medium system was developed.

A xyloglucan fragment has only been shown to have biological activity in one biological assay, namely, auxin-stimulated pea stem elongation.[5] This assay method was discussed earlier in relation to xyloglucan turnover in elongating cell walls. Wall extracts containing many polysaccharide types have been shown to affect leucine uptake and incorporation into protein,[58] tobacco morphogenesis[7] and ethylene production.[59] York *et al.*[5] are the only workers to report specific xyloglucan activity. They found that fucose-containing nonasaccharides inhibited pea stem elongation. The concentration at which this fragment had an effect was low (≈ 100 ng/mL), suggesting it acts as a regulatory molecule. Using a

mixture of pine xyloglucan fragments (Fig. 8) produced by cellulase digestion, the procedures of York et al.[5] were followed. Usually 14 vials containing 6 pea stem sections each were put out at one time. The vials were duplicates of seven types of media: a plus 2,4-D, a minus 2,4-D and five vials of plus 2,4-D with levels of xyloglucan fragments ranging from 10 to 0.001 μg/mL of medium in steps of ten. The pea segments were excised from the third internode, 4 mm below the apical hook. It was found that if only one section was taken from the same portion of each plant, the segments elongated much more uniformly. Special efforts were also made to keep the stems floating on the top of the media, as ones that sank usually failed to grow.

Table 5. Embryo development assay.

	XG Concentration (mg/mL)			
Sample	0.0	0.01	0.1	1.0
	Embryos/mg			
1A	0.3677	0.1531	0.2203	0.2398
1B	0.4444	0.1952	0.2320	0.3938
2A	0.1542	0.1990	0.1157	0.4042
2B	0.1793	0.2345	0.2806	0.3624
3A	0.1449	0.0752		0.0802
3B	0.1479	0.1910		0.0775
4A	0.2836	0.1457		0.2438
4B	0.1043	0.1930		0.1984

Treatment	N	Mean	Standard Deviation	Duncan's New Multiple Range Test $\alpha = 0.05$
0.00	8	0.2283	0.1230	A
0.01	8	0.1633	0.0506	A
0.10	4	0.2122	0.0694	A
1.00	8	0.2500	0.1301	A

A number of runs of this size were made. All but one resulted in the xyloglucan fragments showing no significant inhibitory activity. One run showed that at a concentration of 0.1 µg/mL the fragments had a ≈ 20% inhibitory effect. This set of data contained some spread (Table 6) that might explain these results. The results of other runs can be seen in Appendix III. Overall, the amount of elongation was fairly consistent run to run, and the fragments had no effect on the elongation. This testing was comprehensive, and it can be said that the xyloglucan fragments produced by enzymatic degradation with Cellulysin (Cal-Biochem) did not affect pea stem elongation.

Table 6. Pea stem elongation test.

Trial 4b	Mean	s.d.	Duncan's New Multiple Range Test $\alpha = 0.05$
+ 2,4-D (0.9 µM)			
0.00 XG frag (µg/mL)	93.0	4.7	A
0.001 XG frag (µg/mL)	87.0	7.3	AB
0.01 XG frag (µg/mL)	87.8	7.7	AB
0.10 XG frag (µg/mL)	84.8	4.9	B
10.0 XG frag (µg/mL)	89.7	6.2	AB
- 2,4-D			
0.00 XG frag (µg/mL)	67.8	1.1	C

This is not necessarily surprising, as the size chromatography data, although appearing to indicate a nonasaccharide fragment, did not show a major fucose-containing fragment that would relate to the active fragment reported by York *et al.*[5] This specific fragment may not exist in the pine xyloglucan nor be released by Cellulysin.

CONCLUSIONS

The conclusions of this thesis focus in three areas: the isolation, characterization, and limited biological testing of a pine xyloglucan.

A xyloglucan was isolated from the spent suspension culture medium of loblolly pine cells. The xyloglucan in the medium was loosely associated with an arabinogalactan but covalently attached to protein. One portion of the protein was readily accessible to a general protease, whereas the other was removed only upon the deesterification of the xyloglucan. This second type of protein was most likely linked to the xyloglucan via ester linkages with the polysaccharide. The xyloglucan polymer was estimated to be over 300 sugar units in size. This was larger than some reported xyloglucans,[11,15] and suggests either that other extraction methods cleaved the polysaccharide or that the pine xyloglucan is larger than others.

Methylation data provided information on the general structure of the pine xyloglucan (Fig. 7). The proposed structure has many features of reported xyloglucans from many species (Table 1). The isolated pine xyloglucan had a 1,4-β-glucan backbone which had branched and unbranched sections. The branches were Xyl, Xyl-Gal, and Xyl-Gal branches with terminal Ara or Fuc units. The pine xyloglucan contained a number of terminal galactose units. This is not a common structure for xyloglucans. A relative abundance of terminal galactose has also been recently reported in a Douglas-fir xyloglucan.[20] This feature may be one that is common to conifer xyloglucans.

The fragments produced from the pine xyloglucan by digestion with a fungal cellulase were analyzed by gel filtration chromatography and by GC and GC/MS. The results showed the xyloglucan had been highly fragmented, the fragments

ranging in size from 10-11 sugar units to monosaccharides. The most numerous fragments were proposed to be a nonasaccharide, a heptasaccharide and a hexa- or pentasaccharide. Some of these fragments contained large amounts of xylose, while others had none. Some fragments were rich in terminal galactose. This information suggested that the structure of the pine xyloglucan was specific in nature. Blocks of differing branches were separated by unbranched glucan and the composition of these branches is specific.

Finally, biological testing was conducted using various fractions from the spent medium. Many different biological tests were performed, none of which showed any pine medium components to be active. The most conclusive test was one which measured the effects of cellulase-produced pine xyloglucan fragments on auxin-induced pea stem elongation. No inhibitory effects were seen. A sycamore nonasaccharide containing fucose has been shown to inhibit auxin-stimulated pea stem elongation. This nonasaccharide is probably not present in the pine xyloglucan fragments.

EXPERIMENTAL

GROWING CELLS AND HARVESTING SPENT MEDIUM

LM3 medium (Appendix I) with 2.5 mg/L 2,4-D was prepared in batches of 4 liters, brought to a pH of 5.8, and autoclaved. Medium (400 mL) was placed in each 2 or 3 liter sterilized flask. Flasks were inoculated with 3410 or F-2 strains of loblolly pine cells (pooled sources initiated from immature embryos on LM medium with 2,4-D) in a 1:10 ratio, i.e., 40 mL of growing 10-12 day old cells in their medium were put into every 400 mL (1 flask) of fresh medium. The flasks were then placed in the dark on a shaker operated at 46 rpm. The cells were harvested after 12-14 days. The cells were removed by filtration on sintered glass and discarded. The medium solids in the filtrate were precipitated as below or the medium frozen for future precipitation.

ETHANOL PRECIPITATION OF PEPS

Pine cell culture medium was put into 4 volumes of 95% ethanol. Solids produced were allowed to settle overnight or longer. The great majority (80%) of the solution was siphoned off and the remainder centrifuged. The pellet was redissolved in water (50 mL/liter of original medium) and placed again in four volumes of 95% ethanol. These newly precipitated solids were collected by centrifugation, redissolved in a small amount of water, and freeze-dried.

CELLULOSE COLUMN LIQUID CHROMATOGRAPHY

Whatman CC-31 powdered cellulose (10 g) was slurried with distilled water and left overnight. This slurry was then poured into a 15 mm diameter column to a height of 200 mm. A liter of water was eluted to settle the packing. The PEPS was dissolved in ~ 50 mL of water and put on the column. The column was

eluted successively with 100 mL each of water, 0.5 M NaOH and 7 M urea. In between these elutions the column was rinsed with distilled water. The fractions collected were dialyzed exhaustively (48 hr) against running tap water. They were then freeze dried.

CARBOHYDRATE ANALYSIS

The TAPPI method[92] was performed by A. Webb of the Analytical Department.

ELEMENTAL ANALYSIS

Micro-Tech Laboratories, Inc. (Skokie, Illinois) conducted the C, H, O, and N analysis.

ISOELECTRIC FOCUSING

Isoelectric focusing was performed on an LKB 2117 Multiphor, with a flat bed of polyacrylamide gel. Samples (20 μL) of a 5 mg/mL solution of crude polysaccharides were placed on the gel. After focusing, the gel was halved. One-half was stained for carbohydrates,[93] the other half for proteins with Coomassie blue.[94]

PROTEASE TREATMENT

Up to 200 mg of polysaccharides were dissolved in 2-3 mL of water and treated with Pronase (Calbiochem) a protease from Streptomyces griseus, (1:5, Pronase:polysaccharide, w:w). This was put on a shaker for a few minutes and then placed in a water bath at 32°C for 4-5 hours. This Pronase treated PEPS was further resolved on a DEAE-Sephadex column.

DEAE SEPHADEX CHROMATOGRAPHY

Two grams of DEAE Sephadex, A-25-120, (Sigma Chemical Co.), was slurried in 50 mL of 10 mM potassium phosphate buffer, pH 6.5. After this was allowed to stand overnight, the fines were decanted off and the DEAE Sephadex was washed several times. The slurry was then poured into a 9 mm column to a final height of 150 mm. PEPS (up to 300 mg) was dissolved in a few mL of water and placed on the column. The column was then eluted with the phosphate buffer until the eluent no longer gave a positive iodine test. This xyloglucan-rich fraction (50 mL) was allowed to stand over a mixed bed resin (Baker, M-614) for 12 hours to remove the buffer, and then freeze dried.

PREPARATION FOR GC AND GC/MS

The lyophilized polysaccharide sample (≈ 200 mg) was dried over P_2O_5. Dry DMSO (20 mL) was added to the sample under N_2 and sealed in an 18 mL vial. This was shaken for 12 hours or until the sample had dissolved. Under an N_2 atmosphere, the dissolved sample, 1 mL of MeI, 1 g of ground NaOH, and a stir bar were sealed in an 18 mL vial. This mixture was stirred rapidly for 12 minutes. The mixture was then poured into 300 mL of water and washed thrice with chloroform (100 mL). The chloroform portion was then evaporated to dryness.

To the dry methylated sample was added 2 mL of 72% sulfuric acid. After the sample had dissolved it was diluted with water to 35 mL, placed in a sealed vial, and kept at 120°C for 1 hour. This solution was then diluted to 70 mL, neutralized with barium carbonate, and left to stand overnight. The solution was filtered on a cellulose pad and placed over Dowex 50W-X8 cation exchange resin to remove residual barium salts. This solution was evaporated to dryness and placed in a sealed vial with 100 mg of sodium borohydride and 20 mL of water

and left overnight. The borohydride was quenched with acetic acid. Methanol (50 mL) was added and the solution was evaporated until the remaining solution would not evaporate at 40°C. Methanol was added and evaporated again. This was continued until all odor of acetic acid was gone. All of the evaporations after hydrolysis were done below 40°C to minimize the loss of the volatile sugars.

To the vial containing the sample was added ≈ 4 mL of acetic anhydride. The vial was sealed and mixed; then heated for 3 hours at 120°C. The vial was allowed to cool and then was diluted with water and extracted with chloroform to separate the sample from the sodium salts. The sample was evaporated to dryness and rewet with methanol and evaporated until the acetic anhydride was gone. The sample was placed in a desiccator over KOH to remove any residual acetic anhydride.

Finally the sample was dissolved in dichloromethane for the GC/MS analysis.

$NaBD_4$ REDUCTION

A small quantity of degraded xyloglucan in 10 mL of water was reduced with $NaBD_4$ (10 mg, overnight). This solution was desalted with Dowex AG50W-X8 (H^+) resin. The boric acid was removed by addition of methanol and evaporated as trimethyl boron. The sample was dried over P_2O_5 and methylated.

GC/MS AND GC

The GC/MS analyses were conducted on a Hewlett-Packard 5985 system using a 2 mm x 6 ft glass column packed with SP-2340 (Supelco Inc.). Runs were made with a temperature program to hold at 170°C for 13 minutes and then increase at 3°C/minute to 220°C. The GC analyses were done on a Packard Model 417 with a

SP-2340 packing in a 2 mm x 6 ft glass column and run isothermally at various temperatures ranging from 150-195°C.

TRIFLUOROACETIC ACID HYDROLYSIS

The methylated sample (≈ 80 mg) and 15 mL of 2N trifluoroacetic acid (TFA) were sealed in a 25 mL bottle with a septum. This was heated for 1 hour at 121°C. The TFA was then evaporated on a rotary evaporator at 40°C. Methanol was added to increase the evaporation rate.

ENZYMATIC DEGRADATION

Deesterified pine xyloglucan (200 mg) was dissolved in 20 mL of 50 mM sodium acetate buffer (pH 5.0). Cellulase (100 units) (Cellulysin, Calbiochem, from T. viride) and 0.01% (w/w) sodium azide were added and the solution shaken for 48 hours at 40°C. One unit of enzyme liberated 1 mmole of glucose from cellulose in 1 hour at pH 5.0 and 37°C.

GEL FILTRATION OF DEGRADED MATERIAL

The above cellulase-treated material was desalted on Dowex AG50W-X8 (H^+) resin, evaporated to a few mL, filtered, and placed on a Bio-Gel P-2 (200-400 mesh) (Bio-Rad) column. The column was 1 x 90 cm and was kept at 55°C. In the initial separation, 3 mL fractions were collected at a flow rate of 5 mL/hour. The material from the main peaks of this collection was placed on the column again and 1 mL fractions collected. One hundred microliter samples of the fractions were assayed for total carbohydrate with the phenol-sulfuric acid test.[89]

PHENOL-SULFURIC ACID ASSAY OF TOTAL CARBOHYDRATE[89]

One hundred μL of sample, 100 μL 5% phenol (w/v), and 500 μL of concentrated sulfuric acid were placed in a 1 mL cuvette. After mixing the mixture was let stand for 30 minutes. Relative absorbance at 490 nm was read on a Perkin-Elmer 576 spectrophotometer *versus* a distilled water, phenol, sulfuric acid reference.

TOBACCO CULTURING

Tobacco plants, *Nicotiana tabacum L.* c.v. samsun and Havana 501, were grown from seed in the greenhouse. After 12-15 weeks flowers appeared. The flowers were cut when the cyme contained 30-40 buds and ≈ 30% of the flowers were open. Explants were taken from the basal parts of the floral branches that had been surface sterilized (7% household bleach v/v for 15 minutes) and rinsed thrice with sterile water. These explants were 1 x 10 mm and contained 1 epidermal layer, 1 subepidermal layer, and a few layers of parenchyma cells. These strips were floated on liquid media (20 strips/10 cm Petri dish with 20 mL medium) made of MS macro and micro elements, *myo*-inositol (100 mg/L), thiamine-HCl (0.4 mg/L), glucose (30 g/L), and kinetin (5×10^{-7} *M*). Two levels of IBA were added, 5×10^{-7} *M* in medium I and 3×10^{-6} *M* in medium II. The media was filter sterilized through a 0.20 micron filter. The explants were placed in media I at pH 3.8 and media II at pH 5.0 and 6.2. These dishes were placed in an incubator at 25°C with 24 hours light. The explants were examined for callus and organ growth after incubating for 30 days.[7]

^{14}C-LEUCINE INCORPORATION

Small (15-100 mg) pieces of embryogenic Norway spruce callus were placed in small test tubes containing 200 μL of basal medium.[95] In each of these tubes

was 1 μCi ^{14}C-leucine (Amersham, 348 mCi/mmol) and xyloglucan fragments at concentrations of 0, 0.01, 0.1, and 1.0 mg/mL. These samples were allowed to incubate for 12 hours. The tissue was then filtered, washed with water, and weighed. The tissue was then ground in 50 mM Hepes (Sigma Chemical) buffer at pH 7.5 with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical) at a ratio of 1 mL buffer per 100 mg tissue. This was centrifuged and the supernate saved. A 100 μL aliquot was removed for a Bradford protein assay.[95] The remaining sample was removed and precipitated with an equal amount of 20% TCA. The precipitated protein was collected on a glass pad. The pad was then counted in a Beckman LS 3801 scintillation counter using solvent-free cocktail (Isolab). Each run consisted of six samples of each polysaccharide level.

EMBRYO DEVELOPMENT ASSAY

The protocol for embryo development and counting was per the procedures of Becwar, Noland, and Wann[91] except that during the two week development stage, the callus was grown on a glass pad that rested on a screen in liquid medium containing IBA, ABA, and xyloglucan fragments. The liquid medium (12 mL) was in a Petri dish situated on a rotating shaker at 60 rpm. Two samples of approximately 100 mg each of callus were dispersed for counting after the two weeks. The remaining embryogenic callus was placed on solid medium, without xyloglucan fragments, to develop further. Each Petri dish contained two callus pieces that yielded two samples for counting apiece. The mean of three observers' embryo total was used as the number of embryos per plate. Five runs of this experiment were performed.

PEA STEM ELONGATION

The procedures described by York et al.[5] were followed except only one segment was excised from each stem.

ISOLATION OF NONPRECIPITATABLE MEDIUM COMPONENTS

The ethanol-medium mixture remaining after the PEPS had been collected was let stand in a hood until most of the ethanol had evaporated. The remaining liquid was evaporated to a syrup. This syrup was placed on chromatographic paper and eluted with a 6:3:4 (v:v:v) mixture of ethyl acetate:acetic acid:water. Chromatograms were stained with silver nitrate and p-anisidine to detect organic material and reducing groups.

DETERMINATION OF MOLECULAR SIZE

A column (1 x 120 cm) was packed with Sepharose CL-6B (Pharmacia Fine Chemicals AB.). The column was calibrated with four dextrans of known size and a blue dextran to determine void volume. All of these dextrans were purchased from Pharmacia Fine Chemicals AB. A small sample (5 mg) of pine xyloglucan was dissolved in 0.5 mL of 0.1N NaOH. This was placed on the column and eluted with 0.1N NaOH at a rate of 8 mL/hour. Fractions (1 mL) were collected and analyzed with the phenol-sulfuric acid assay.

ACKNOWLEDGMENTS

I would like to first of all acknowledge and thank The Institute of Paper Chemistry for the educational opportunity provided.

Special thanks are in order to L. Borchardt, M. Bovee, A. Webb, L. Kroll, S. Verhagen, J. Carlson, G. Wyckoff, J. Reed, J. Wyckoff, D. Hanson, W. Molinarolo, and others that I have forgotten, for their help, without which this could not have happened.

To my committee, Drs. M. R. Becwar, D. B. Easty, cochairmen M. A. Johnson and N. S. Thompson, I would like to express my most sincere thanks for the guidance and especially the patience they showed throughout this thesis.

Finally, thanks to all of those people who took care of the rest of my mind for these six years: Mom, Dad, Paul, Ken, Hugh, Len, Sue, Pam, Karen, Erin, Sib, and Paul.

Gee Norm, this is almost starting to be fun.

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APPENDIX I

LM3 GROWTH MEDIUM

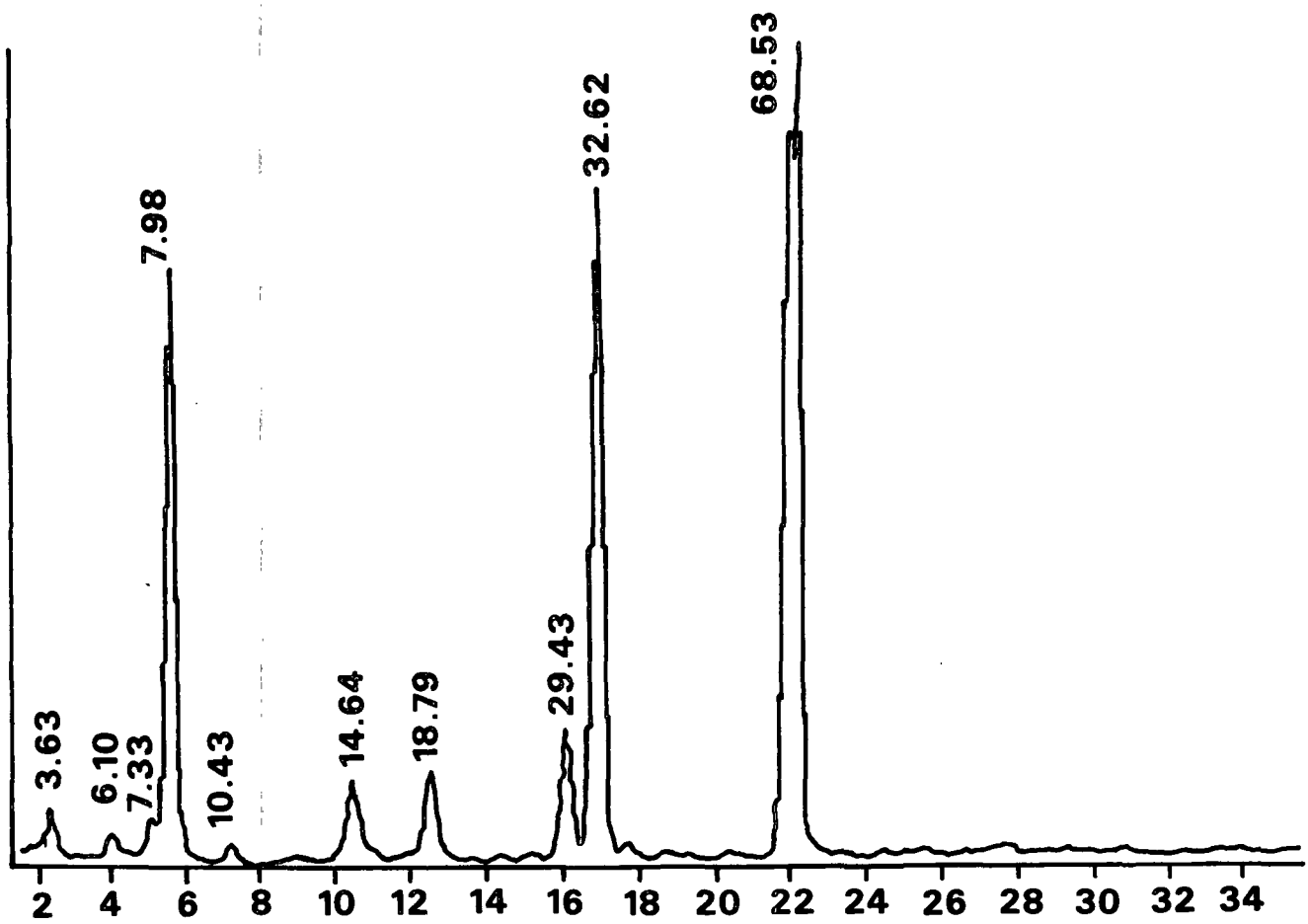
Component	Level[a]
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	340
$CaCl_2$	22
$MgSO_4 \cdot 7H_2O$	370
$Mg(NO_3)_2 \cdot 6H_2O$	1538
H_3BO_3	31.0
KI	4.15
$MnSO_4 \cdot H_2O$	21.0
$ZnSO_4 \cdot 7H_2O$	43.0
$Na_2MoO_4 \cdot 2H_2O$	1.25
$CuSO_4 \cdot 5H_2O$	0.5
$CoCl_2 \cdot 6H_2O$	0.13
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
inositol	250
nicotinic acid	0.5
pyridoxine-HCl	0.1
thiamine-HCl	0.1
sucrose	30,000
pH	5.8

[a] mg/L (ppm).

APPENDIX II

MASS SPECTRA OF DEXG-3

Mass spectra of alditol acetates of methylated DEXG-3 are seen on the following pages. The peaks are labeled with retention times from Fig. 6, as seen in the total ion chromatogram of DEXG-3 seen below. Resolution of the GC/MS of the DEXG-3 peaks may be seen in Table 4.

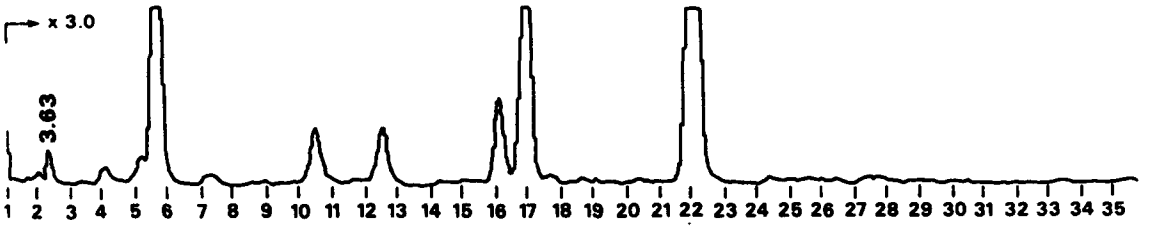


FRN 14121, GRN 17

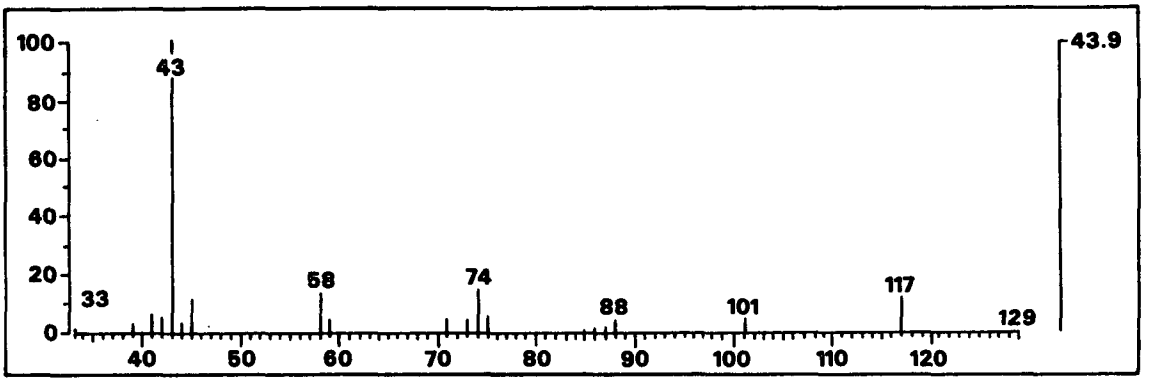
DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



AVERAGED SPECTRUM • BASE PK/ABUND: 43.2/32000. + 27 + 27 -24 -34

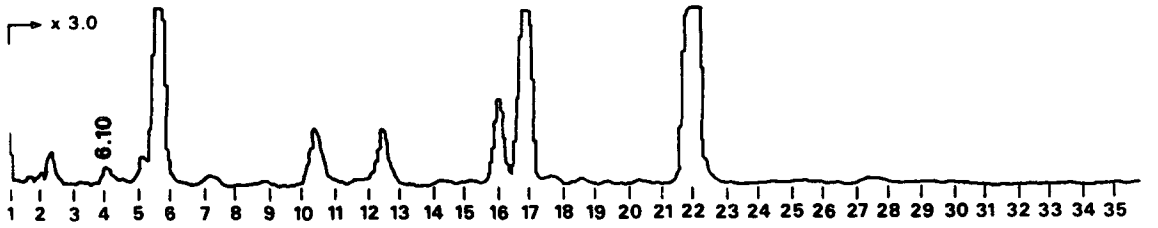


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



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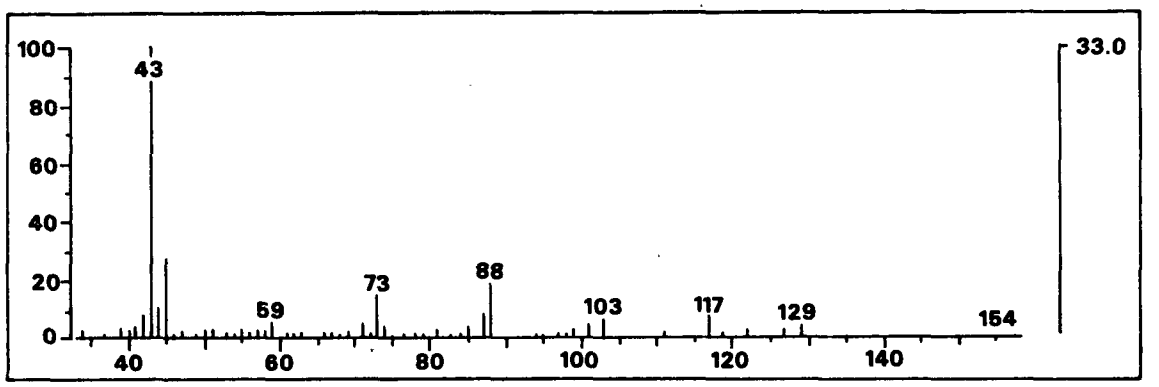
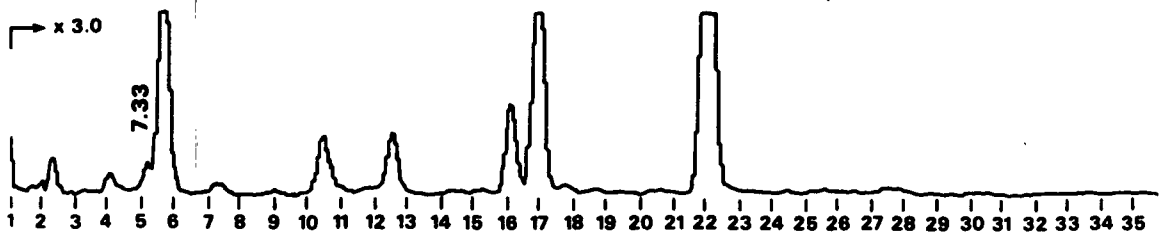


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



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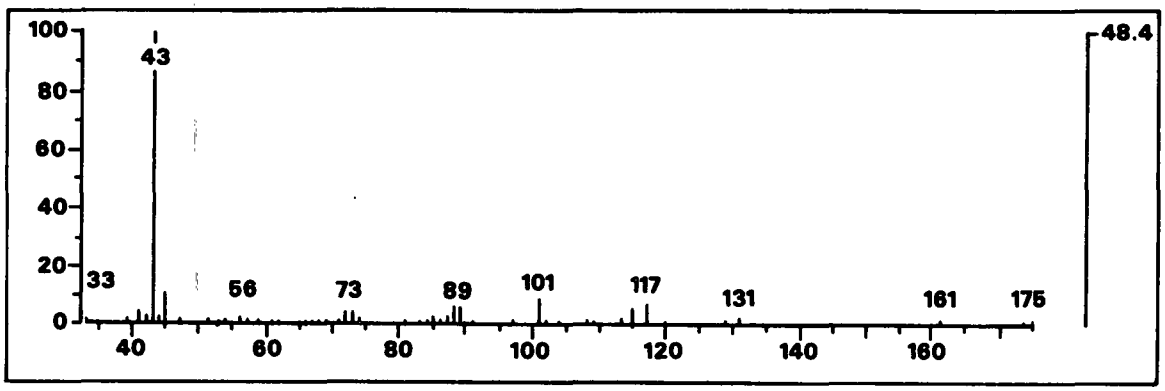
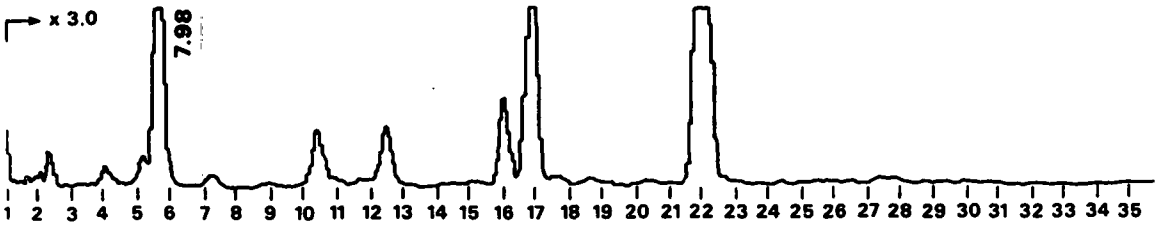


FRN 14121, GRN 17

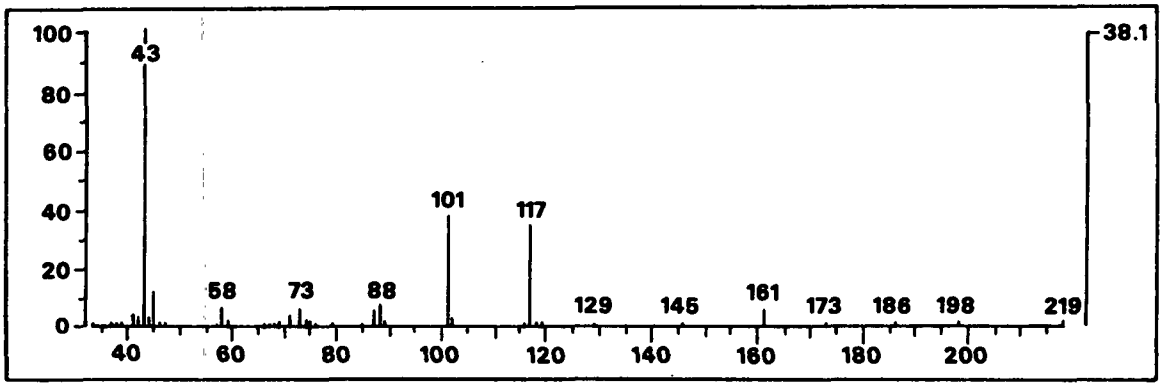
DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



AVERAGED SPECTRUM • BASE PK/ABUND: 43.1/32000. + 97 -111

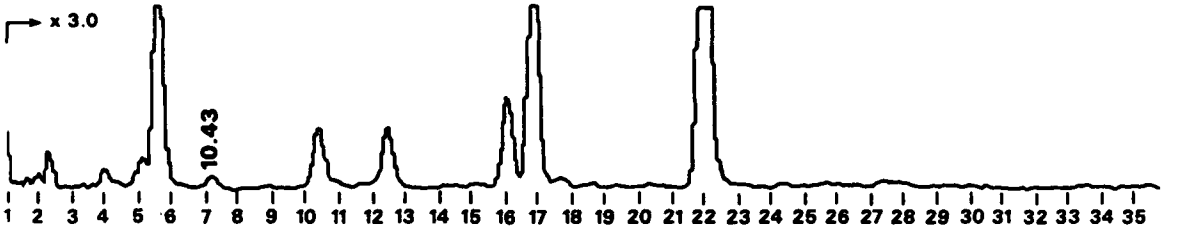


FRN 14121, GRN 17

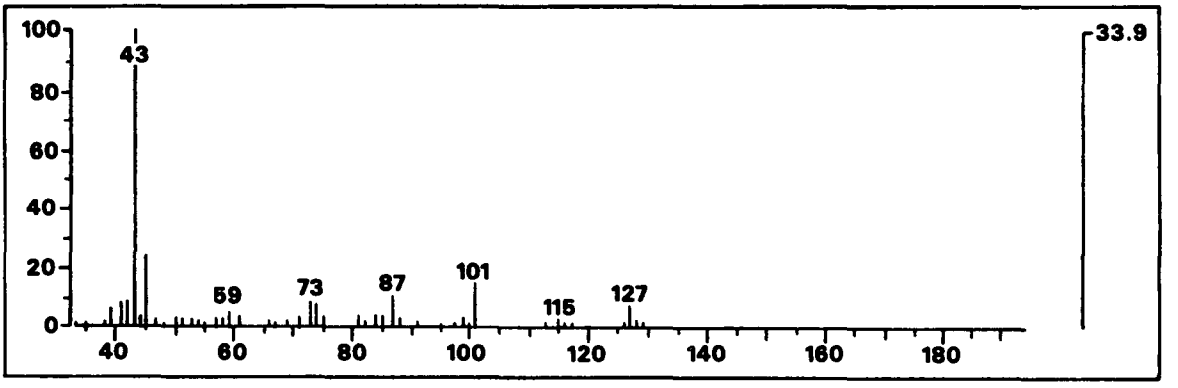
DEXG3, 170–13MIN–3DEG/MIN

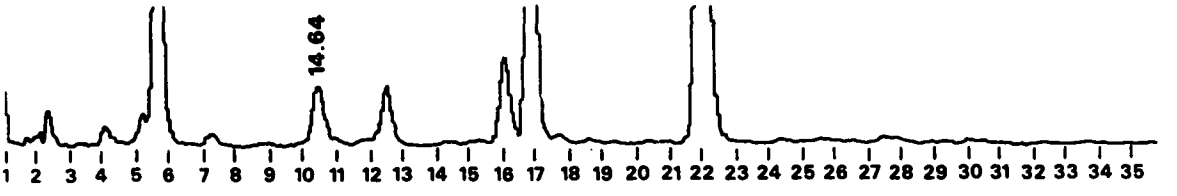
744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



AVERAGED SPECTRUM • BASE PK/ABUND: 43.1/32000. +133 -146





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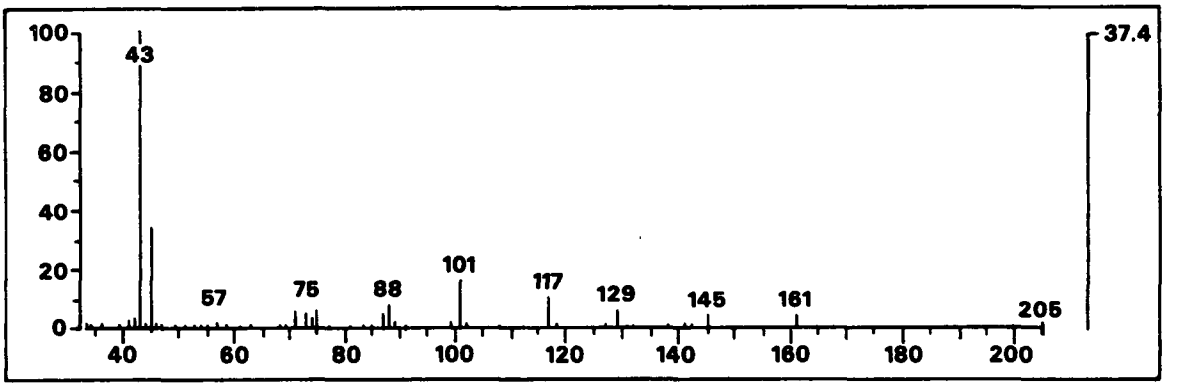
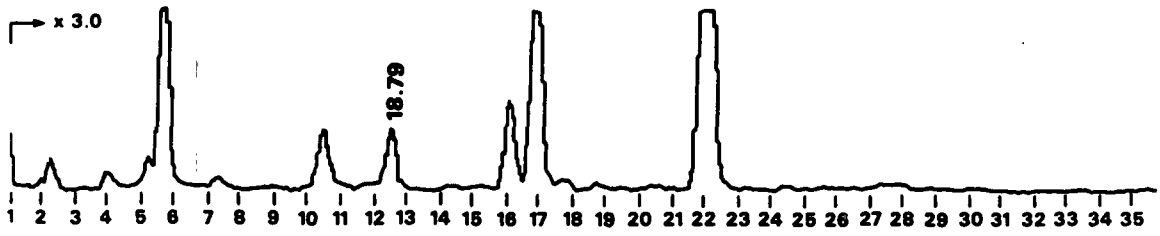


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



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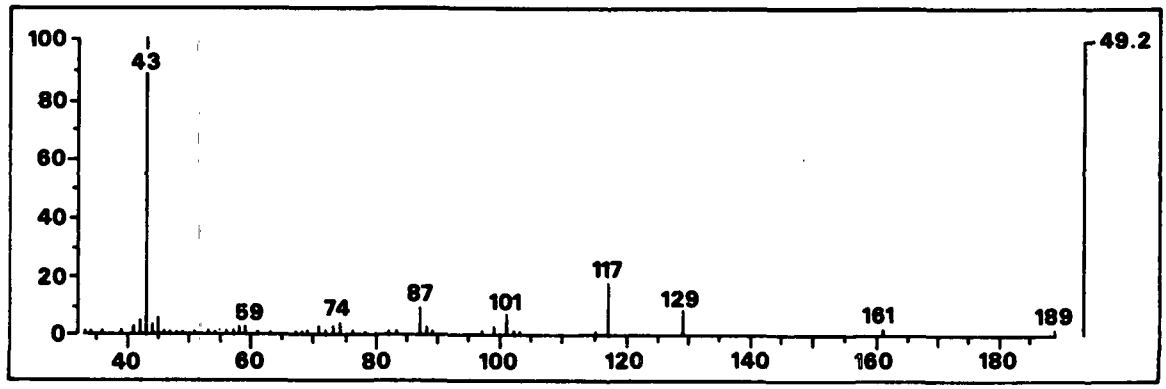
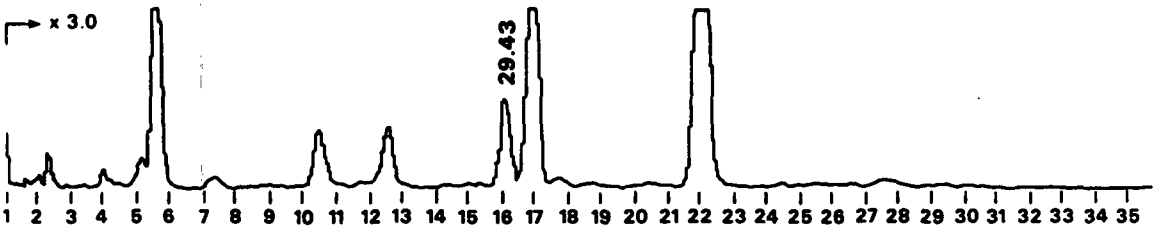


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



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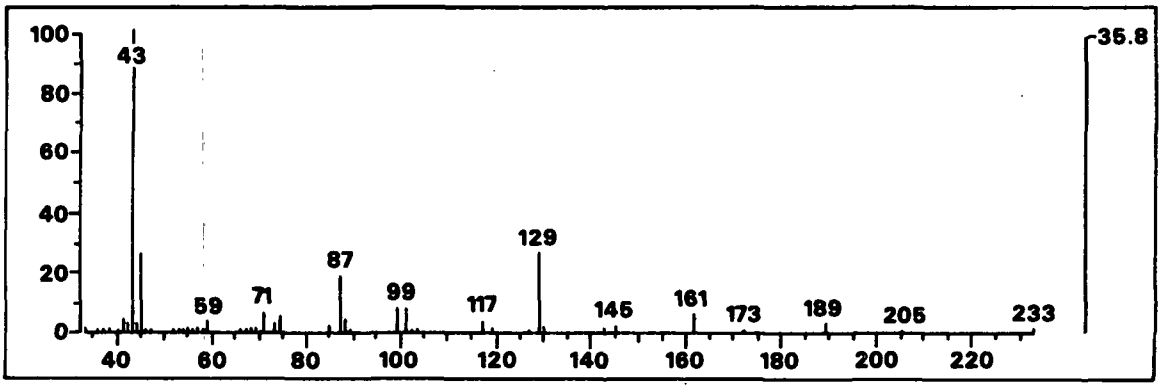
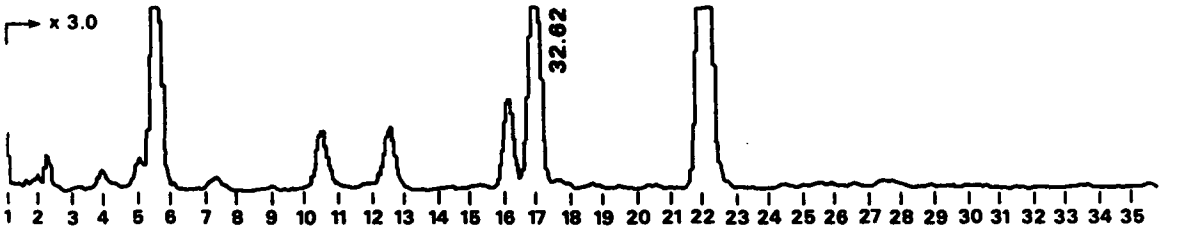


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



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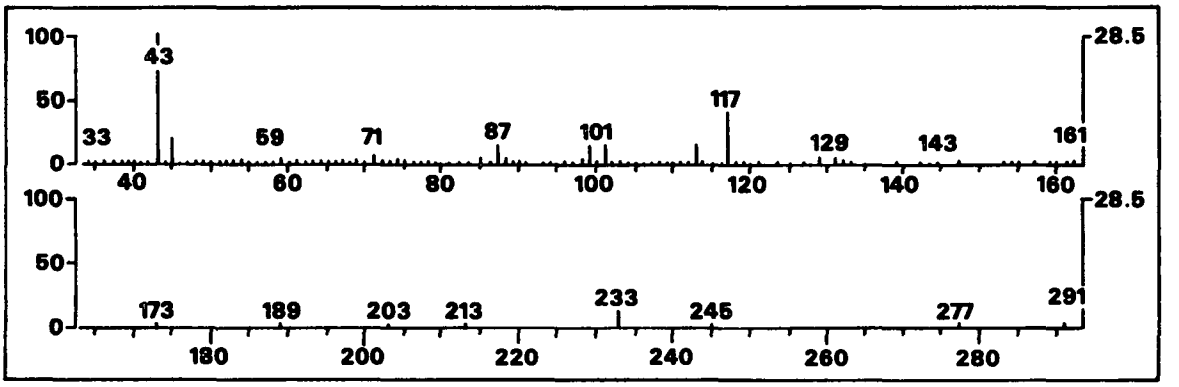
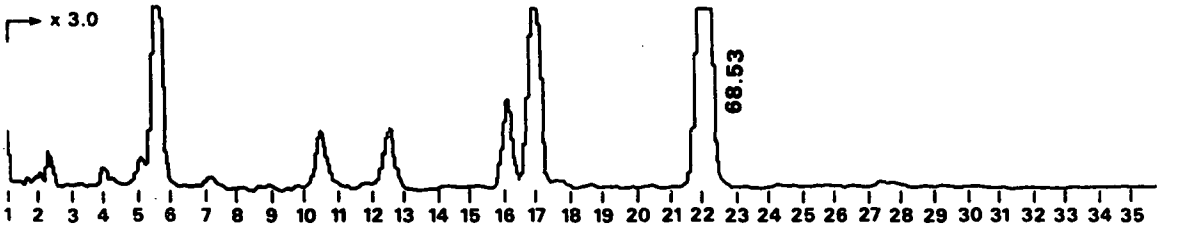


FRN 14121, GRN 17

DEXG3, 170–13MIN–3DEG/MIN

744 SCANS (744 SCANS, 34.67 MINS)

MASS RANGE: 32.0, 694.4 TOTAL ABUND = 2317493.



AVERAGED SPECTRUM • BASE PK/ABUND: 43.1/32000. -434 + 446