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DEGLYCOSYLATION OF ARABINOGALACTAN PROTEINS
FROM SUSPENSION-CULTURED SYCAMORE CELLS
VIA HYDROGEN FLUORIDE IN PYRIDINE

presented by

Yukio Akiyama

has been accepted towards fulfillment
of the requirements for

M.Sc. degree in Biochemistry

Deborah P. Delmer
Major professor

Date May 10, 1979

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DEGLYCOSYLATION OF ARABINOGALACTAN PROTEINS FROM SUSPENSION-CULTURED SYCAMORE CELLS VIA HYDROGEN FLUORIDE IN PYRIDINE

By

Yukio Akiyama

A THESIS

Submitted to
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1979

ABSTRACT

DEGLYCOSYLATION OF ARABINOGALACTAN PROTEINS FROM SUSPENSION-CULTURED SYCAMORE CELLS VIA HYDROGEN FLUORIDE IN PYRIDINE

By

Yukio Akiyama

In addition to the hydroxyproline in cell wall protein "extensin", cultured plant cells secrete soluble arabinogalactan proteins (AGPs), which also contain hydroxyproline. Because the high carbohydrate content of AGPs thwarts direct attempts at amino acid sequencing, they must be deglycosylated first.

Crude AGPs isolated from the medium of suspension-cultured sycamore cells were deglycosylated via 70% HF in pyridine (HF/pyr), which is easier to handle than liquid HF and found to be as efficient as HF. One hour HF/pyr treatment at room temperature removed over 90% of sugars from AGPs.

HF/pyr deglycosylated AGPs were partially purified by gel-filtration on Sephadex G-100 and by ion-exchange chromatography on SP-Sephadex C-50. The final fraction contained hydroxyproline, serine, alanine and aspartate as the predominant amino acids. This material was gel-electrophoresed. It stained poorly with coomassie blue, however, it was detected either by prelabelling the protein with fluorescein isothiocyanate or by labelling with ^{14}C-proline.

ACKNOWLEDGMENTS

The advice, assistance, encouragement and collaboration of Dr. U. V. Mani is most gratefully acknowledged. Thanks are also to Fumiko, Sharon, Joan, Barbara, Jim and to my committee members Dr. D. Delmer who is my academic adviser, Dr. P. Kindel and especially Dr. D. T. A. Lamport for many useful discussions and suggestions.

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LIST OF ABBREVIATIONS

AGPs, AGP(C), AGP(M)	arabinogalactan proteins, (C, from cytoplasm, M, from medium)
FITC	fluorescein isothiocyanate
HF	anhydrous hydrogen fluoride
HF/pyr	anhydrous hydrogen fluoride in pyridine
Hyp	hydroxyproline
Hyp-Arabs, Hyp-Ara_n (n;1-4)	a short chain of arabinose (1-4) on the hydroxyl group of Hyp
SDS	sodium dodecyl sulfate
SP-Sephadex C-50 (SP-C-50)	sulfopropyl Sephadex C-50
TCA	trichloroacetic acid

INTRODUCTION

Plant cells have a cell wall which is involved in several biological functions such as turgor and a resistance to pathogens. Thus the presence of wall is a primary barrier against disease and hence extensive research is being conducted in order to understand the complex cell wall network. Modern knowledge of the cell wall comes from chemical analysis studies, X-ray diffraction studies and work with the electron microscope. These studies led to the identification of cellulose, hemicellulose and pectic polysaccharides as the major component of the cell wall. Besides these components, Lamport (1) reported the presence of a hydroxyproline containing structural protein "extensin" in the cell wall. He suggested that extensin plays an important role in the structural rigidity of the cell wall and hence its role in the cellular extensive mechanism. By isolating and characterising hemicellulose and pectic polysaccharide fractions, Keegstra et al. (2) proposed a model for the structure of the primary cell wall of suspension-cultured sycamore cells. According to this somewhat hypothetical model, the cellulose microfibrils are cross-linked by the matrix polymers of the wall through a xyloglucan by means of hydrogen bonding. The xyloglucan is then linked at its reducing end to galactan which is attached to the rhamnogalacturonan of the wall. From the reducing end of rhamnogalacturonan is a 3-6 linked arabinogalactan which may be glycosidically linked to serine residues of the cell wall protein.

This model, which involves all but one of the wall polysaccharides in a covalently linked network (the exception being xyloglucan H-bonded to the cellulose microfibrils), is satisfyingly detailed except the link between extenisn and arabinogalactan. This linkage was hypothesised without direct evidence from studies on wall protein, but rather was derived from information concerning a soluble hydroxyproline-rich glycoprotein secreted into the medium. As will be discussed later, this soluble glycoprotein has been found to be an example of a new class of arabinogalactan proteins (3,4). Therefore, until more data are available from the work on the cell wall itself the method by which extensin is linked to wall polysaccharide must be regarded as undefined.

Steward (5) showed the presence of bound hydroxyproline in the hydrolysates of alcohol insoluble material in the tissue cultures of carrot or potato. Later, experiments of Lamport and Northcote (6) with sycamore cells and Dougall and Shimbayashi (7) with tobacco cells from the cultured medium showed that hydroxyproline is a major constituent in cell wall hydrolysates, accounting for about 30% of the total amino acids, the protein itself accounting for 2-10% of plant cell walls. Bound hydroxyproline was chemically characterised as trans-4-hydroxy-L-proline. The distribution of hydroxyproline has been studied in various parts of plants. Thus it has been shown to be present in tissues like cotyledon, hypocotyl, leaf, coleoptile, pericap and root (1). The presence of small amounts of material in the cytoplasm has also been reported. Bound hydroxyproline is also present in the cell wall of green algae, brown algae (traces!) and in the certain forms of fungi. The red algae did not contain any hydroxyproline (8).

The experiments of Lamport showed that wall protein is exceptionally

resistant to proteolytic enzymes (1). But after heating the wall for 1 hr at pH 1, he observed that chymotrypsin liberated 40%, and trypsin 26%, of amino acids from the sycamore cell walls. He showed that a crude mixture of enzymes posissing carbohydrase and protease activities released hydroxyproline glycopeptides from the cell wall of tomato (9, Table 1). These glycopeptides contained arabinose O-glycosidically attached to hydroxyproline which is stable to alkali. Therefore alkaline hydrolysis with saturated $Ba(OH)_2$ of the cell wall released a family of hydroxyproline arabinosides (Hyp-Arabs) (10, Figure 1). Most of the hydroxyproline residues in the tomato wall are O-substituted by tri- or tetra-saccharides of arabinose (11, Figure 2). The linkage was found to be $1\xrightarrow{\beta}3$ $1\xrightarrow{\beta}2$ $1\xrightarrow{\beta}2$ $1\xrightarrow{\beta}4$ Hyp by methylation analysis and pmr spectroscopy (12). The molecular models showed that β-linked hydroxyproline tri- and tetra-arabinosides will conform to the type polyproline II helix which favors the formation of 3 hydrogen bonds in the peptide backbone (13). β-Linked hydroxyproline tri- and tetra-arabinosides may provide a structural conformation to extensin what the triple helix does for collagen to produce a stable rod-like structure of high tensile strength.

Although extensin has not been solubilised in an intact form, Lamport obtained high and low molecular weight peptides from the acid treated cell walls with trypsin. The low molecular weight fraction accounts for about 1/3 of the wall bound hydroxyproline and accounts for 5 tryptic peptides (Table 2) whose sequence totals 48 residues (4). These peptides probably represent either the complete extensin sequence or about 1/3 of the sequence (Figure 3). The peptides represent the complete sequence if all the high molecular weight non-sequenced material has the same sequence as the low molecular weight, but has become

Table 1. Tomato Glycopeptides Obtained by Digestion of Tomato Cell Walls with "Cellulase".

	NH_2-Terminus
1. ara_{25} gal_6 hyp_{10} ser_3 tyr	SER
2. ara_{14} gal_3 hyp_{10} ser_3 lys_2 thr val	LYS
3. ara_{20} gal_4 hyp_9 ser_3 lys tyr	LYS
4. ara_{16} gal_4 hyp_9 ser_3 tyr	SER
5. ara_{16} gal_2 hyp_9 ser_3 lys_3 val tyr	LYS

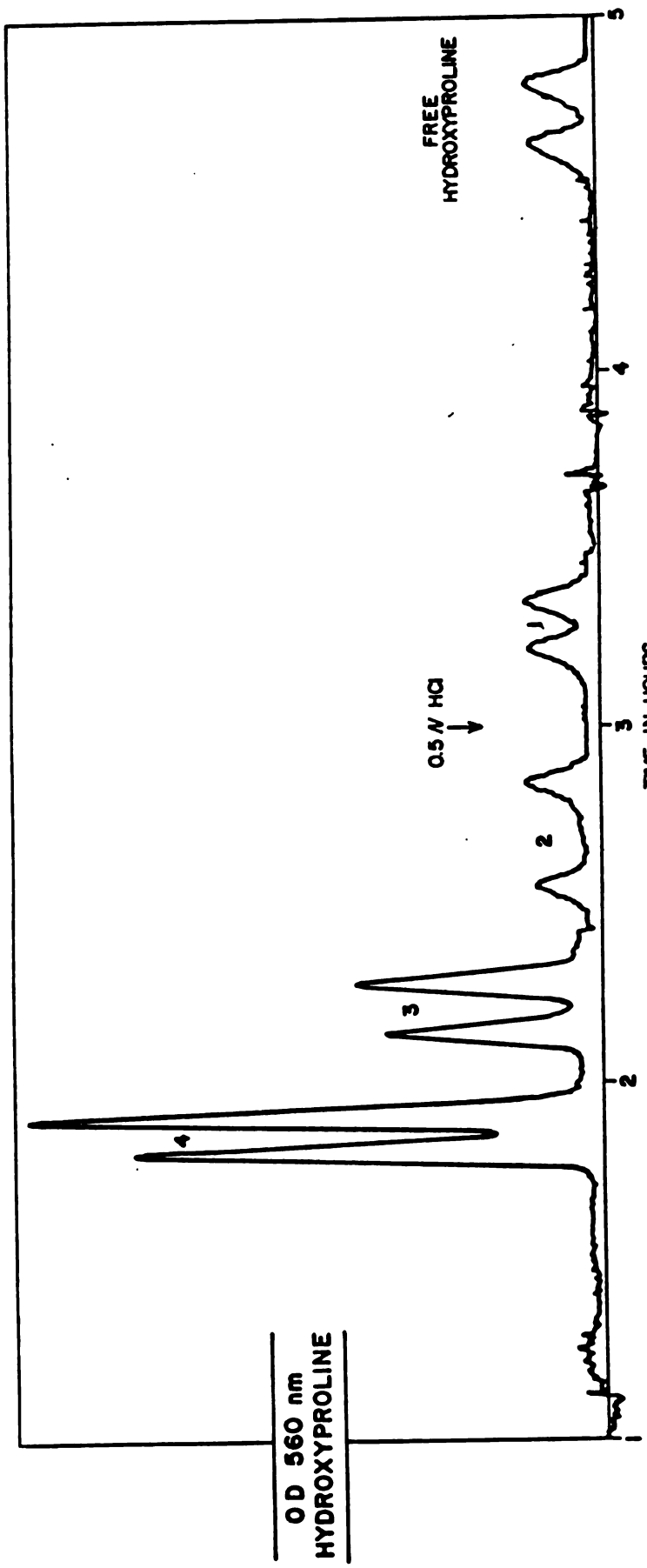


Figure 1. Separation of hydroxyproline-arabinosides by chromatography on chromobeads B.

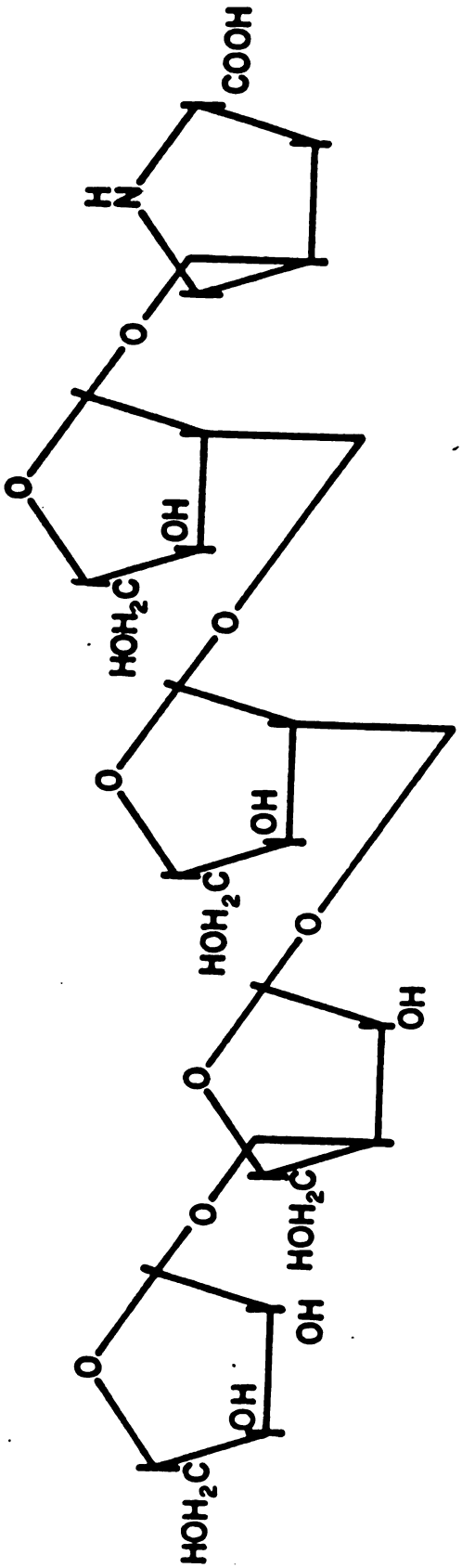


Figure 2. Hydroxyproline tetraarabinoside.

Table 2. Extensin Tryptides.

A. SER-HYP-HYP-HYP-HYP-SER-HYP-SER-HYP-HYP-HYP-HYP-("TYR"-TYR)-LYS Exists with 3, 2, or 1 galactose residures.

B. SER-HYP-HYP-HYP-HYP-SER-HYP-LYS

Exists with 2, 1, or 0 galactose residues.

C. SER-HYP-HYP-HYP-HYP-THR-HYP-VAL-TYR-LYS

Exists with 1 or 0 galactose residues.

D. SER-HYP-HYP-HYP-HYP-LYS

Exists with 1 or 0 galactose residues.

E. SER-HYP-HYP-HYP-HYP-VAL-"TYR"-LYS-LYS

Exists with 1 or 0 galactose residues.

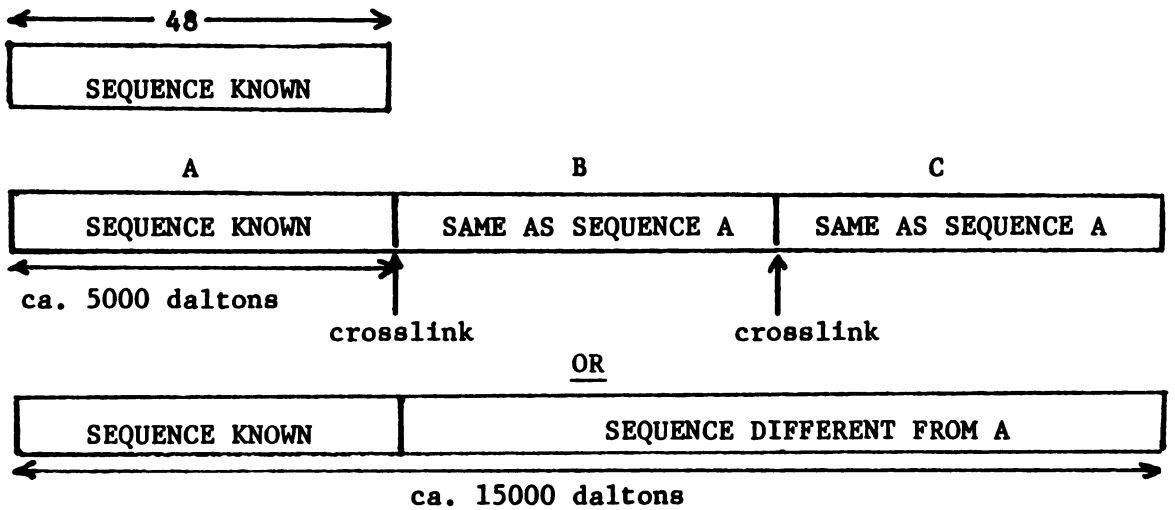
48 residues sequenced accounting for ca. 33% cell wall hydroxyproline

[SHHHHSHSHHHHXYK] [SHHHHSHK] [SHHHHTHVYK] [SHHHHK] [SHHHHVXKK]

Empirical Formula: HYP_{27} SER_8 LYS_6 VAL_2 TYR_2 X_2 THR_1

Possible interpretation--tryptic peptides represent 1/3 of the complete sequence

or the entire sequence:



Amino Acid Code: H = HYP Y = TYR
S = SER T = THR
K = LYS X = unknown tyrosine derivative
V = VAL

Figure 3. Possible amino acid sequences in extensin.

cross-linked during "maturation" similar to native cross-linked collagen or elastin. The most important notable findings in all of the extensin peptides is the occurrence of Ser-Hyp-Hyp-Hyp-Hyp (including sycamore-maple) and galactosylated serine residues (14). Lamport postulated that this is the region for the polysaccharide attachment of the cell wall. Later galactosyl serine was also found in the cell wall of carrot (15). Since methylation analysis of hydroxyproline-rich fracion of cell wall gave a lot of terminal arabinose residues, Keegstra et al. (2) thought arabinogalactan is attached to protein extensin via the Ser-O-Gal linkage. Extracting cell walls with $DMSO/H_2O/EtOH/NaBH_4/NaOH$, Lamport obtained some evidence for attachment of galactan (about 10 residues of galactose) to serine (4).

Besides the covalently bound cell wall glycoprotein, there are at least two other kinds of macromolecules containing hydroxyproline in the plant kingdom; one is a "classical" lectin, the other being arabino-galactan proteins (AGPs) also called "all β-lectins". ("Classical lectins have at least two carbohydrate-binding sites. "All β-lectins" may have only one carbohydrate-binding site.) The first hydroxyproline containing lectin was isolated by Allen et al. (16) from potato tubers. It is a glycoprotein consisting of 50% protein and 50% carbohydrate, the major sugar being arabinose (96%) with small amounts of galactose (4%). The molecular weight of the lectin was 50000. Hyp-Arabs and Ser-Gal linkages have been found in this glycoprotein and the linkage between hydroxyproline and arabinose was found to be β (17).

Another lectin containing hydroxyproline occurs in the seeds of Jimson weed (18). It is a glycoprotein consisting of 72% protein and 28% carbohydrate, the major sugar is arabinose with small amount of

galactose and glucosamine. The hydroxyproline content of this protein is 6.3%.

Besides these two lectins, the hydroxyproline containing protein isolated by Mani and Radhakrishnan from the leaves of sandal (19) is also a glycoprotein, consisting of 84% protein and 16% carbohydrate, the predominant sugars being arabinose with about 4% of galactose. The hydroxyproline content in this protein was 6%. A recent observation showed that sandal protein agglutinated the trypsinised human red blood cells (20). Hyp-Arabs have also been found in the sandal lectin.

Plants contain yet another type of macromolecule containing hydroxyproline, namely arabinogalactan proteins (AGPs) or so called "all β-lectins". The characteristics of AGPs are 1) the polymer is a glycoprotein with very high carbohydrate content (typically 80-95%), 2) the carbohydrate is an arabinogalactan (that's why they are called AGPs), 3) the protein portion is rich in hydroxyproline, serine, and alanine, and 4) the polymer precipitates with β-glucosyl Yariv antigen. AGPs have been found in various plants from medium of suspension-cultured cells (3, 21-23), cytoplasm of suspension-cultured cells (23-25), xylem sap (4), wheat endosperm (26), seeds (27), stigma exudates (28-30), and at the surface of plant plotoplasts (31). They are ineresting macromolecules because they are "glycoproteins in search of a function" and contenders for a role in the cell-cell recognition processes of plants. They are virtually ubiquitous in higher plants (27, 32).

Jermyn and Yeow (27) isolated "all β-lectins" (same as AGPs) from various seeds using Yariv antigen and they showed the presence of this macromolecule in a variety of plants. Anderson et al. (23) have isolated AGPs from suspension cultures of endosperm of Lolium multiflorum (rye

grass) by Yariv antigen precipitation. It has molecular weight 2.8 x 10^5 consisting of 84% carbohydrate with a small amount of protein (7%), rich in hydroxyproline, alanine and serine. The predominant sugars are galactose (64%) and arabinose (36%). The methylation analysis of this glycoprotein showed that branched 3-6 galactan is substituted by arabinofuranosyl residues and they also found homology in various arabinogalactan proteins from a variety of plants by methylation analysis. McNamara and Stone (33) examined the carbohydrate-peptide linkage in arabinogalactan peptide isolated from the wheat endosperm. The composition of this glycopeptide was 8% peptide which is rich in hydroxyproline and 92% carbohydrate. By successive treatment of arabinogalactan peptide with alkali, oxalic acid and enzymes, they obtained a small molecular weight compound which on further analysis was found to be 4-O-β-D-galactopyranosyl-oxy-L-proline.

AGPs were also shown to be present in the medium of suspension-cultured sycamore cells (3). Alkaline hydrolysis of crude AGPs from medium gave an interesting hydroxyproline-glycoside profile on Sephadex G-25. There was a hydroxyproline peak in the void volume as well as Hyp-Arabs in the inner volume. Further analysis of void volume fraction by gel-filtration and isoelectric focussing, followed by chemical analysis showed a singly residue of hydroxyproline with an arabinogalactan attached via the hydroxyl group. Further partial acid hydrolysis of this fraction with 0.1 N trifluoroacetic acid for 1 hr at 100°C yielded Hyp-Gal. Approxymately 50% of the hydroxyproline was found attached to arabinogalactan and 30% of hydroxyproline with Hyp-Arabs. The amino acid analysis of AGPs showed that hydroxyproline, alanine and serine are major amino acids. On this basis Lamport (4) suggested that the

polypeptide backbone of AGPs has a hydroxyproline-rich hydrophilic region and alanine-rich hydrophobic tain region. Jermyn and Yeow (27) observed high levels of hydroxyproline in all β-lectins after proteolytic digestion and they suggested that a fraction of the hydroxyproline exists in a "core" after removal of a hydroxyproline-poor "tail".

Even though both AGPs and extensin have a high hydroxyproline content, they differ in at least three ways; 1) hydroxyproline-glycoside profiles after alkaline hydrolysis; AGPs have hydroxyproline-arabino-galactan and Hyp-Arabs whereas extensin has only Hyp-Arabs, 2) alanine content; AGPs are alanine rich, however, extensin has little or no alanine, and 3) solubility; AGPs are extracted by aqueous solution, but it is hard to extract extensin from the cell wall. Despite these obvious differences there is the intriguing possibility of sequence homology between extensin and AGPs, for example, in the hypothetical hydroxyproline-rich region of AGPs.

Because of its very high carbohydrate content, it is difficult to sequence AGPs directly on the sequencer. Therefore it is necessary to deglycosylate AGPs before attempts at sequencing.

Mort and Lamport (34) used anhydrous HF for deglycosylation of glycoproteins without breaking the polypeptide chain. Anhydrous HF used routinely as a deprotecting reagent in protein chemistry, is an excellent protein solvent, and rapidly cleaves glycosidic linkages. For example, complete HF solvolysis of cellulose to glucose occurs within a minute at $0^{o}C$, under which conditions peptide bonds are quite stable. Anhydrous HF cleaved all the linkages of neutral and acidic sugars within 1 hr at $0^{o}C$, but the O-glycoside linkage of (N-acetylated) amino sugars require somewhat more severe conditions, 3 hrs at $23^{o}C$ (34).

However, HF did not cleave the N-glycosidic linkage between aspargine and N-acetyl glucosamine.

Because HF is relatively dangerous, it is difficult to handle and it requires special apparatus. Therefore HF in pyridine (HF/pyr), which is much easier to handle than liquid HF, has been used. The purpose of this work was to test the efficiency of HF/pyr for deglycosylation of AGPs and to obtain the peptides for further sequencing analysis. First the appropriate conditions of HF/pyr deglycosylation was determined. Second the efficiency of HF/pyr deglycosylation was compared to liquid HF deglycosylation. And finally the deglycosylated AGPs (from medium and cytoplasm) were partially purified.

MATERIALS AND METHODS

Materials.

All chemicals used were of analytical reagent or the best commercially available grade. HF/pyridine was bought from Pierce Chemical Company, Il. The HF apparatus was bought from Peninsula Lab., Ca. (34). Sephadex G-25 (fine), G-100, blue dextran 2000, SP-Sephadex C-50, ribonuclease, and apomyoglobin were purchased from Pharmacia, Sweden. Biogel P2 was purchased from Bio-Rad Lab., Ca. Radioactive ^{14}C-proline was bought from New England Nuclear Corp., England.

Crude AGPs Preparation.

From Culture Medium.

Sycamore-maple suspension-cultures (Acer pseudoplatanus L.) were grown in M6E medium. After 12 days of growth, cells were harvested by filtration on a coarse sintered funnel. The filtrate was centrifuged to remove any broken cells and debri at 8000 rpm for 20 min in Sorvall RC-2. Ethanol was added to make a 70% ethanolic solution which was then allowed to settle in the coldroom overnight. The pellet obtained after centrifugation at 10000 rpm for 30 min was freeze dried. This material was called AGP(M) and contained 3-4 µg of hydroxyproline per 1 mg of sample.

From Cytoplasm.

Cells were washed three times with growth medium salts and then resuspended in the medium (ca. 1:1 v/v) with 0.004 M $N_2S_2O_5$, and then

sonicated at 5°C for 4 min in a Bronwill Biosonik III sonicator. The homogenate was filtered through a 20 u nylon cloth in the coldroom. The filtrate was centrifuged at 9000 rpm for 10 min. To the supernatant solid TCA was added to 12.5% saturation and the solution was stirred in the coldroom till dissolved and allowed to stand overnight. The precipitate was removed by centrifugation (8000 rpm, 15 min) and the supernatant was dialysed against water for at least 2 days with 6 changes of distilled water. Dialysate was concentrated to small volume by evaporation, then dialysed again overnight with 2 changes of distilled water to remove any traces of TCA and then freeze dried. This freeze dried material was called AGP(C), and it contained about 9 µg of hydroxyproline per 1 mg of sample. The yields of AGP(M) and AGP(C) were 7 gm and 300 mg respectively from 1000 gm of cells (wet weight).

Twenty µCi of radioactive ^{14}C-proline (specific activity 250 mCi/nmole) was used in 600 ml culture medium for the preparation of radioactive AGPs. The samples (1 ml) were made up to 10 ml with Aquasol (New England Nuclear) and the radioactivity was measured in a Packard Tri-carb liquid scintillation counter.

Deglycosylation with HF/pyridine.

Completely dried sample (500 mg) was placed in a Kel-F vessel with a stirring bar and 2 ml of anhydrous methanol was added. Then 18 ml of HF/pyr was added and reaction vesell was capped and stirred for 1 hr at room temperature unless otherwise stated. After 1 hr 80 ml of cold water was added to quench the reaction and the solution was dialysed against water for 2 days with 6 changes of distilled water in the coldroom. After dialysis the solution was centrifuged (12000 rpm, 30 min) and the pellet and supernatant were freeze dried.

Deglycosylation with HF.

HF deglycosylation was performed as described by Mort and Lamport (34) using anhydrous methanol instead of anisole. After complete evaporation of HF, the deglycosylated sample was dissolved in 0.1 N NH_4OH and centrifuged to remove any insoluble material. The supernatant was loaded on a Sephadex G-100 column as described next.

Column Chromatography.

The gel-filtration experiment was performed in 0.1 N NH_4OH using a column of 500 ml volume (2.8 x 80 cm) with void volume of 154 ml (via blue dextran) unless otherwise stated. The sample volume used in gel-filtration was 5 ml. An aliquot of the fraction was used for hydroxyproline estimation by an automated hydroxyproline analyser (10) after prior hydrolysis of the sample in 5 N NaOH.

SP-Sephadex C-50 ion-exchange chromatography was performed as follows. The material to be fractionated was dissolved in 0.01 N HCl (pH 2) and centrifuged to remove any insoluble material. Then the sample was applied to a column (1.2 x 26 cm, bed volume 30 ml) and washed with 1.5 bed volume of 0.01 N HCl after which the column was eluted with a gradient between 0.01 N HCl and 1 M NaCl in 0.01 N HCl (5 bed volumes). An aliquot of the fraction was used for hydroxyproline determination.

Biogel P2 column (-400 mesh, 2 columns of 0.13 x 90 cm) was used for the separation of hydroxyproline-glycosides. The column was equilibrated and eluted with water containing 0.01% sodium azide. Column eluate was monitored continuously with the hydroxyproline analyser.

Disc-gel Electrophoresis of the Deglycosylated AGPs Fractions.

Deglycosylated AGPs fractions were electrophoresed in 12% polyacrylamide gels (35). Prior to electrophoresis the sample was prelabelled by the procedure of Muramoto et al. (36). About 500 ug of deglycosylated AGPs were dissolved in 30 ul of carbonate buffer (pH 9.5) and labelled with 5 ul of fluorescein isothiocyanate (FITC, 10 mg/ml solution in acetone) for 10 min at 50°C followed by gel-filtration on a Sephadex G-25 (fine) to remove excess FITC. The standard proteins used were ribonuclease, apomyoglobin, and a soluble hydroxyproline-containing lectin from leaves of Santalum album L. (19). After electrophoresis the sample was eluted from gel slices by a procedure of Drescher and Lee (37) modified by omitting fixation and staining. Three to 6 mm fluorescent bands were cut and placed in a 3 ml microflex tube. To this 0.2 ml of 1% SDS was added and homogenised gently for 5 min using a Kontes pestle. The vial was sealed and incubated at 40°C for overnight. Then 0.2 ml of 0.1% SDS was added to the mixture which was resuspended on a vortex mixer, then centrifuged for 5 min at 1000 x g. The supernatant was collected. The 0.1% SDS extraction of the pellet was repeated three more times. The volume of the pooled extracts was reduced to 100 ul by blowing down with nitrogen, and the extract applied to a Sephadex G-25 (fine) column (0.9 x 14 cm) equilibrated with water. The column was eluted with water. Using a long wave U. V. lamp, the separation of labelled protein from unreacted dye was observed. The fluorescent eluate which voided the column was collected. The material was freeze dried and analysed for amino acids either by conventional amino acid analyser or gas chromatography.

After electrophoresis of ^{14}C-labelled material, 2 mm gel slices starting from the origin were taken and transfered to a counting vial,

and then the Aquasol added, homogenised and counted in a scintillation counter.

Hydrolysis of Sample.

For amino acid or quantitative hydroxyproline analysis, sample (1-5 mg) was hydrolysed with 200 ul of 6 N HCl in 1 ml microflex tube for 18 hrs at 110°C. After evaporation of HCl in a stream of nitrogen, 500 ul of 0.001 N HCl was added and an aliquot was used for analysis.

For qualitative hydroxyproline analysis of the column eluate, an aliquot (max. 200 µl, if the sample was more than 200 µl, first freeze dry then add NaOH) was hydrolysed with 800 µl of 5 N NaOH for 1 hr at 121°C in a polyethylene tube, then neutralised with 850 µl of 5 N HCl and subjected to automated hydroxyproline analyser.

For hydroxyproline-glycoside profile determination, the sample was hydrolysed with saturated (0.22 M) $Ba(OH)_2$ (10 mg/ml) in a 5 ml microflex vial for 18 hrs at 105°C, neutralised with concentrated H_2SO_4 (8 µl/ml $Ba(OH)_2$), and then centrifuged for 15 min at 10000 rpm. The supernatant was freeze dried and redissolved in 200 ul of water, then loaded on a Biogel P2 column as described earlier.

Sugar Analysis.

Sugar analysis was performed on the trimethyl silylated methyl glycosides as described by Bhatti et al. (38) using a Perkin-Elmer 900 gas chromatograph fitted with dual columns, with the output connected to a Spectra Physics System IV Autolab integrator (Figure 4). The support Gas-Chrom Q and stationary phases SE-30 and SP-2100 were bought from Supelco Co., Bellefonte, Pa.

Amino Acid Analysis.

Most amino acid analyses were performed by liquid chromatography

Figure 4. Gas liquid chromatography of sugars as their trimethylsilyl methyl glycosides.

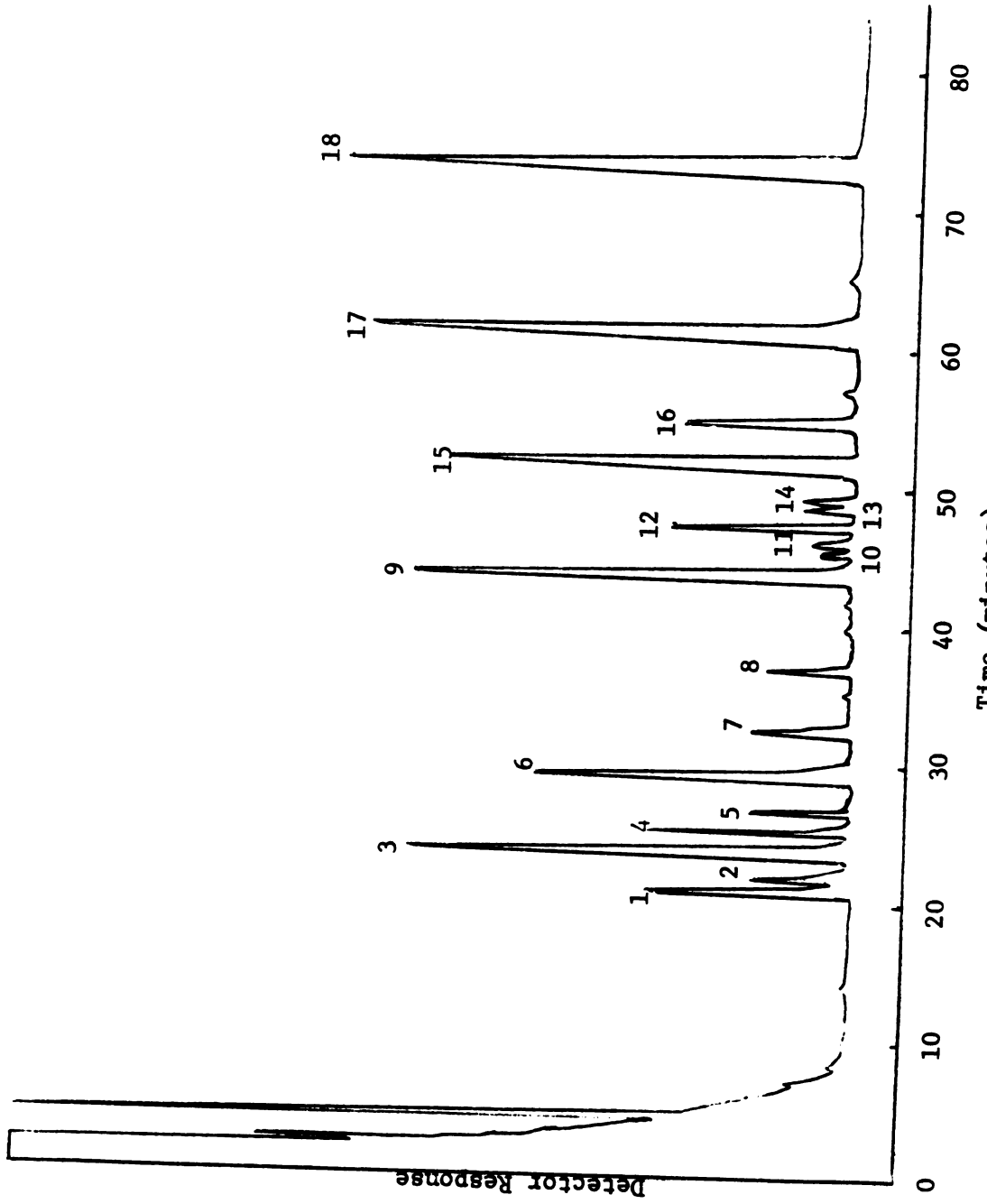
Twenty µg of each sugar (including inositol) plus 100 nanomoles of mannitol as internal standard were dried in a microflex tube in a vacuum desiccator over P_2O_5 at 50-60°C for at least 30 min.

Two hundred µl of dry 1.5 N methanolic HCl was added and dry nitrogen was blown over for 15 sec and then immediately sealed with cap and tufbond septum. The vial was placed in 95°C heating block for 90 min. The sample was dried via nitrogen evaporation at room temperature and placed in vacuum desiccator over P_2O_5 at room temperature for at least 1 hr, then it was trimethylsilylated by adding 50 µl of pyridine/trimethylchlorosilane/hexamethyldisilazane (5:1:1). Half an hour was allowed for the derivatization at room temperature.

Two µl of the solution was injected onto a 3% SP-2100 column (12' x 1/8") programmed from 120°C to 185°C at 1°C/min, with an initial hold of 4 min.

Identification of peaks:

1) Ara, 2) Ara, 3) Rha, 4) Fuc, 5) Fuc, 6) Xyl, 7) Xyl, 8) GalU, 9) Man, 10) Gal, 11) Man, 12) Gal, 13) GalU, 14) Gal, 15) Glc, 16) Glc, 17) Mannitol, 18) Inositol.


Detector Response
Time (minutes)
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15
16
17
18

Figure 4

(Figure 5), others were performed by gas liquid chromatography on the heptafluorobutyryl isobutyl ester derivatives as described by Mackenzie and Tenaschek (39, Figure 6).

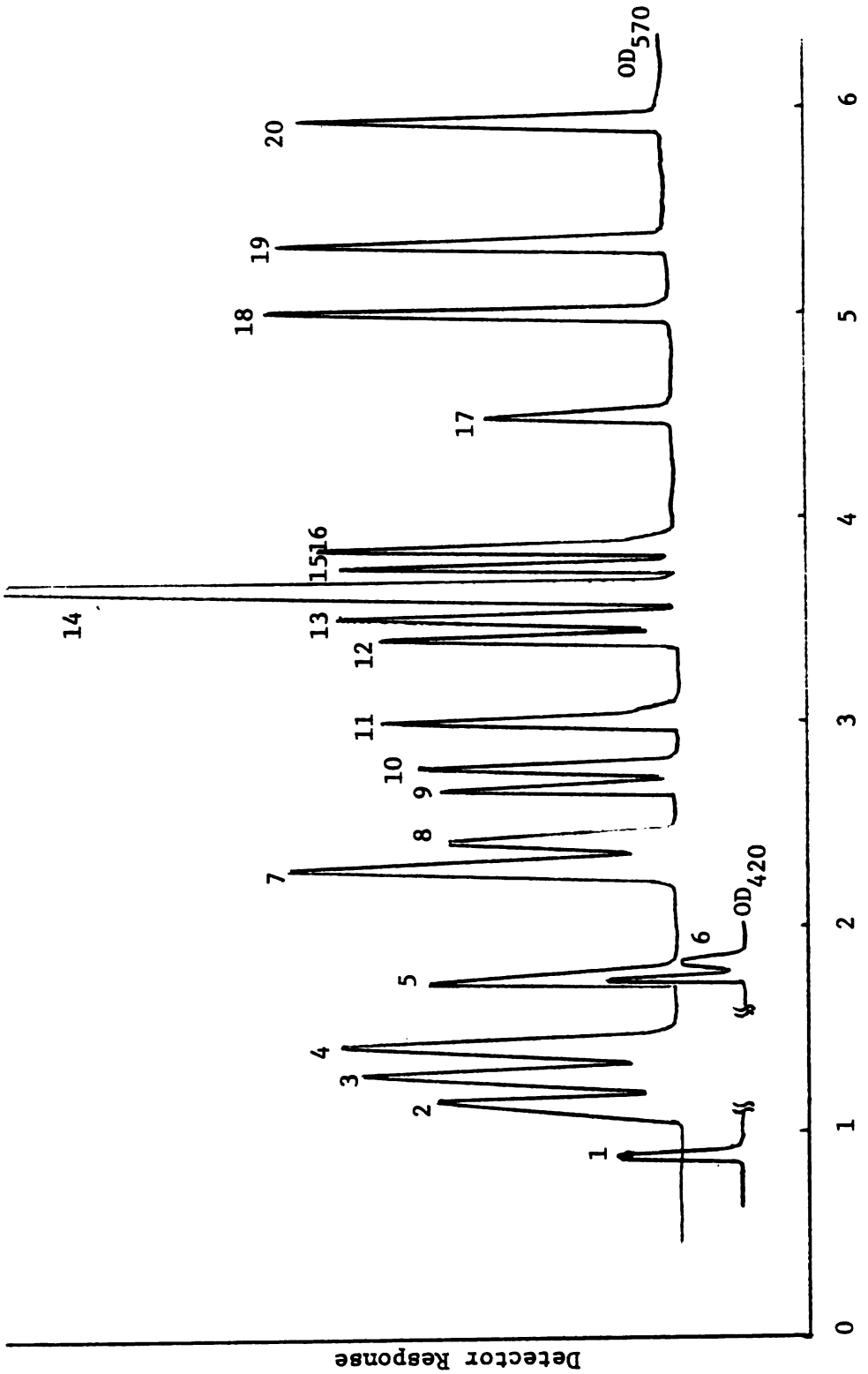
Hydroxyproline was determined on an automated hydroxyproline analyser (10) after hydrolysis of the sample.

Figure 5. Amino acid analysis by liquid chromatography.

Fifty nanomoles of each amino acid, 20 μg of hydroxyproline and 100 nanomoles of norleucine as internal standard were placed on top of Chromobeads C column and eluted with a combination pH, ionic strength gradient as described by Lamport (9). The eluent from the column was fed into an automated ninhydrin analyser and the peak areas were calculated by a Spectra Physics System IV Autolab integrator. Hydroxyproline and proline were detected by their absorbance at 420 nm, the other amino acids at 570 nm.

Identification of peaks:

1) Hyp, 2) Asp, 3) Thr, 4) Ser, 5) Glu, 6) Pro, 7) Gly, 8) Ala, 9) Val, 10) Cys, 11) Met, 12) Ile, 13) Leu, 14) Norleu, 15) Tyr, 16) Phe, 17) NH_4^+, 18) Lys, 19) His, 20) Arg.


Detector Response
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7
8
9
10
11
12
13
14
15
16
17
18
19
20
OD_{420}
OD_{570}

Figure 5

Figure 6. Gas liquid chromatography of amino acids as their heptafluorobutyryl isobutyl esters.

One hundred nanomoles of each amino acid plus 50 nanomoles of pipecolic acid as internal standard were dried in a 1 ml microflex vial under nitrogen stream at room temperature. Two hundred µl of methylene chloride were added and the sample was redried twice. One hundred µl of 3 N HCl in isobutanol were added and sonicated for 30 sec in sonic bath. The vial was placed in heating block at 120°C for 20 min and cooled to room temperature. After dried in nitrogen stream at room temperature, 50 µl of ethyl acetate and 20 µl of heptafluorobutyric anhydride were added and the vial was heated to 110°C for 10 min. Cooled to room temperature, the product was evaporated to incipient dryness in nitrogen stream at room temperature and dissolved in 20 µl of ethyl acetate/acetic anhydride (1:1, v/v). Two µl of this solution were injected onto same column as used for sugar analysis, programmed from 95°C to 250°C at 4°C/min, with an initial hold of 4 min.

Identification of peaks:

1) Ala, 2) Gly, 3) Val, 4) Thr, 5) Ser, 6) Leu, 7) Ile, 8) Pro, 9) Pipecolic acid, 10) Hyp, 11) Met, 12) Asp, 13) Phe, 14) Glu, 15) Lys, 16) Tyr, 17) Arg, 18) Cys.

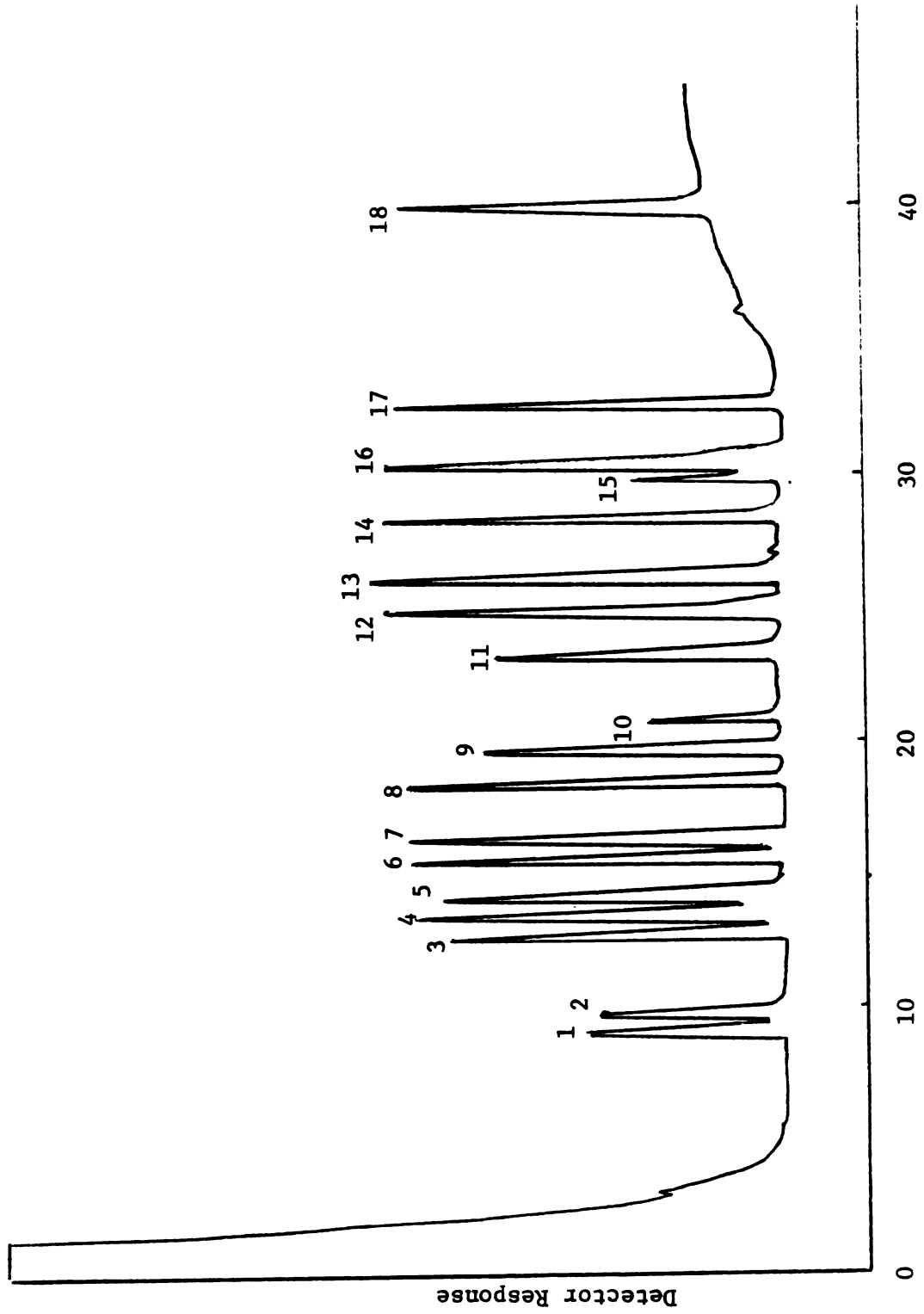

Detector Response
0
10
20
30
40
Time (minutes)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 6

RESULTS

Chemical Composition of Crude AGP(M) and AGP(C).

The sugar composition of crude AGP(M) and AGP(C) is in Table 3 and the amino acid analysis is in Table 4. The major sugars are Ara, Xyl, Gal, and Glc and the predominant amino acids are Hyp, Ser, Ala, Glu, Val, and Lys. From the amino acid and sugar analysis, AGP(M) consists of 90% carbohydrate and 10% protein and AGP(C) consists of 80% carbohydrate and 20% protein.

Deglycosylation Time Course of AGP(M) with HF/pyridine.

In order to find the optimum time required to remove most of the sugars from AGPs, 100 mg of AGP(M) was treated at different time intervals (0 to 240 min) with anhydrous HF in pyridine (9 ml) in the presence of anhydrous methanol (1 ml) at room temperature and the reaction was quenched by the addition of 40 ml of water. Then the solution was dialysed against water for 2 days in the coldroom and the dialysate was freeze dried. Figure 7 shows the kinetics of removal of sugars at different time intervals. It is clear from the Table 5 that there was an appreciable decrease in the total weight after deglycosylation with a complete recovery of hydroxyproline at different time intervals. Table 6 shows mole % of sugar and moles of sugar per mole of hydroxyproline remaining after the treatment of AGP(M) with HF/pyr at different time intervals. More than 90% of sugars were removed after 45 min treatment with HF/pyr (140 moles to 14 moles sugar

Table 3. Sugar Analysis of Crude AGP(M) and AGP(C)[a]

	AGP(M)	AGP(C)
Ara	23.4 (32.8)	41.5 (13.2)
Rha	2.7 (3.9)	5.2 (1.7)
Fuc	5.4 (7.6)	2.3 (0.7)
Xyl	19.2 (26.8)	5.2 (1.6)
GalU	8.2 (11.5)	2.2 (0.7)
Man	1.1 (1.5)	5.1 (1.6)
Gal	25.4 (35.6)	35.4 (11.2)
Glc	14.6 (20.4)	3.0 (1.0)

[a]Data expressed as mole % of total sugars.
Numbers in parenthesis indicates the moles of sugar per 1 mole of Hyp.

Table 4. Amino Acid Analysis of Crude AGP(M) and AGP(C)[a]

	AGP(M)	AGP(C)
Hyp	6.1	9.1
Asp	10.6	13.2
Thr	5.8	7.3
Ser	7.8	10.7
Glu	9.2	14.3
Pro	n.d.	n.d.
Gly	8.3	8.3
Ala	7.1	11.3
Val	7.4	6.3
Cys	0.2	0.6
Met	1.2	0.6
Ile	3.0	2.6
Leu	5.3	4.3
Tyr	2.2	0.8
Phe	4.1	1.5
Lys	6.6	6.3
His	4.1	1.2
Arg	2.3	1.4

[a]Data expressed as mole % of total amino acids.
n.d. not determined.

Figure 7. The kinetics of removal of sugars from AGP(M) at different time intervals.

One hundred mg of AGP(M) were treated with 9 ml HF/pyr and 1 ml MeOH at different time intervals at room temperature. After adding water to quench the reaction, the solution was dialysed and freeze dried. One to 2 mg of each material were analysed for sugar.

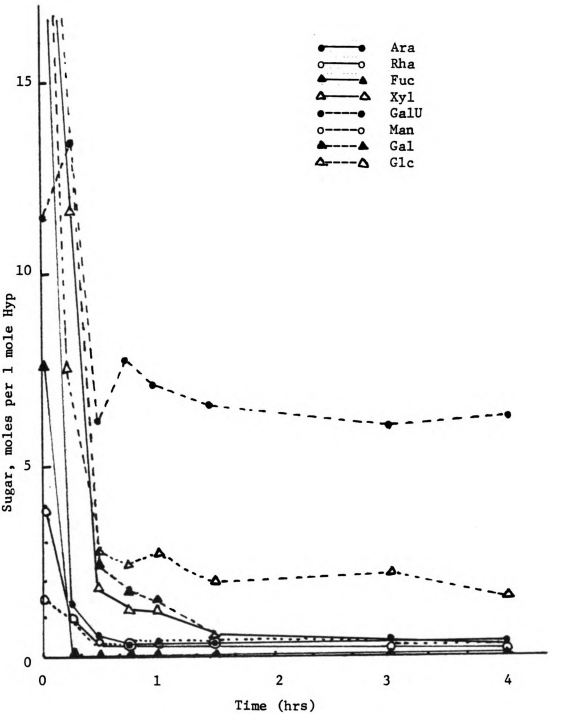

Ara
Rha
Fuc
Xyl
GalU
Man
Gal
Glc
15
10
5
0
Sugar, moles per 1 mole Hyp
0
1
2
3
4
Time (hrs)

Figure 7

Table 5. Hydroxyproline Recovery after Deglycosylation of AGP(M) at Different Time Intervals using anhydrous HF/pyr plus 10% Methanol at Room Temperature

Time (min)	0	15	30	45	60	90	180	240
Weight of the Deglycosylated AGPs (mg)	100	56.7	49.8	43.7	40.5	36.6	36.7	34.8
% Removed by HF/pyr Treatment	0	43.3	50.2	56.3	59.5	63.4	63.3	65.2
Hydroxyproline (Total, μg)	330	271	392	331	262	286	345	310
% Recovered	100	82	119	100	79	87	105	94

Table 6. Sugar Analysis of AGP(M) after Treatment with HF/pyr at Different Time Intervals

Time Course of HF/pyr Treatment (min)	0	15	30	45	60	90	180	240
				Mole % of Total Sugar				
Ara	23.4	2.5	3.1	2.2	3.0	2.4	2.5	1.9
Rha	2.7	1.9	1.9	2.0	2.4	1.9	1.3	1.0
Fuc	5.4	0	0.6	0	0	0	0	0
Xyl	19.2	22.0	13.0	8.4	8.6	4.0	2.9	2.2
GalU	8.2	25.0	43.4	55.0	50.5	62.2	60.0	67.5
Man	1.1	2.3	2.1	3.4	5.0	5.8	6.3	6.3
Gal	25.4	31.9	16.4	11.7	10.7	4.4	4.2	3.2
Glc	14.6	14.3	19.4	17.3	19.8	19.3	22.7	17.8
				Moles per 1 Mole of Hyp				
Ara	32.8	1.3	0.5	0.3	0.4	0.3	0.3	0.2
Rha	3.9	1.0	0.3	0.3	0.3	0.2	0.1	0.1
Fuc	7.6	0	0.1	0	0	0	0	0
Xyl	26.8	11.7	1.9	1.2	1.2	0.4	0.3	0.2
GalU	11.5	13.3	6.2	7.8	7.1	6.5	6.0	6.2
Man	1.5	1.2	0.3	0.5	0.7	0.6	0.6	0.6
Gal	35.6	17.0	2.4	1.7	1.5	0.5	0.4	0.3
Glc	20.4	7.6	2.8	2.4	2.8	2.0	2.3	1.6
Total	140	53	15	14	14	11	10	9

per mole of hydroxyproline). Galacturonic acid was the major sugar remaining after HF/pyr treatment (6-7 moles per mole of hydroxyproline).

Figure 8 shows the hydroxyproline-glycoside profile of deglycosylated AGP(M) at different time intervals on Biogel P2 column. Six peaks corresponding to Hyp-arabinogalactan (void), Hyp-Ara_4, Hyp-Ara_3, Hyp-Ara_2, Hyp-Ara_1 and free Hyp were observed with AGP(M) after alkaline hydrolysis with $Ba(OH)_2$. There was a decrease in the amount of Hyp-arabinogalactan and Hyp-Arabs whereas free Hyp increased as the time of HF/pyr treatment increases. However, there was a peak at the Hyp-Ara_1 region (before free Hyp) appearing after 15 min of treatment of AGP(M) with HF/pyr and was at a maximum at 45 min after treatment. In 90 min deglycosylated AGP(M), there was 16% of Hyp in the Hyp-Ara_1 region and 84% was in the free Hyp region. The peak in the Hyp-Ara_1 position did not contain any arabinose and therefore it is not Hyp-Ara_1. But, galactose and glucose were found in HF/pyr "resistant" fraction. The molar ratio of total sugar to Hyp was about 1. These results suggest the possibility of Hyp-Gal and Hyp-Glc in the HF/pyr "resistant" fraction. This corroborated well with earlier results from this laboratory (3) which raised the possibility of glucosyl hydroxyproline.

Effect of Temperature on HF/pyr Treatment.

Mort and Lamport (34) observed that the efficiency of HF deglycosylation is different at $0^{\circ}C$ and at room temperature. Therefore deglycosylation of AGP(M) with HF/pyr was performed at $0^{\circ}C$ and at room temperature for 1 hr. After treatment and dialysis, the dialysate was centrifuged at 12000 rpm for 20 min to separate the soluble and insoluble material. Amino acid and sugar analyses of these fractions are given in Table 7 and 8. The efficiency of deglycosylation was 1.4

Figure 8. The hydroxyproline-glycoside profile of deglycosylated AGP(M) at different time intervals on Biogel P2 column.

About 20 mg of deglycosylated AGP(M) at different time intervals were treated with 2 ml of 0.22 M $Ba(OH)_2$ in a 5 ml microflex vial for 18 hrs at 105°C. Then it was neutralised with concentrated H_2SO_4, and then centrifuged. The supernatant was freeze dried and redissolved in 200 µl of water, then loaded on a Biogel P2 column.

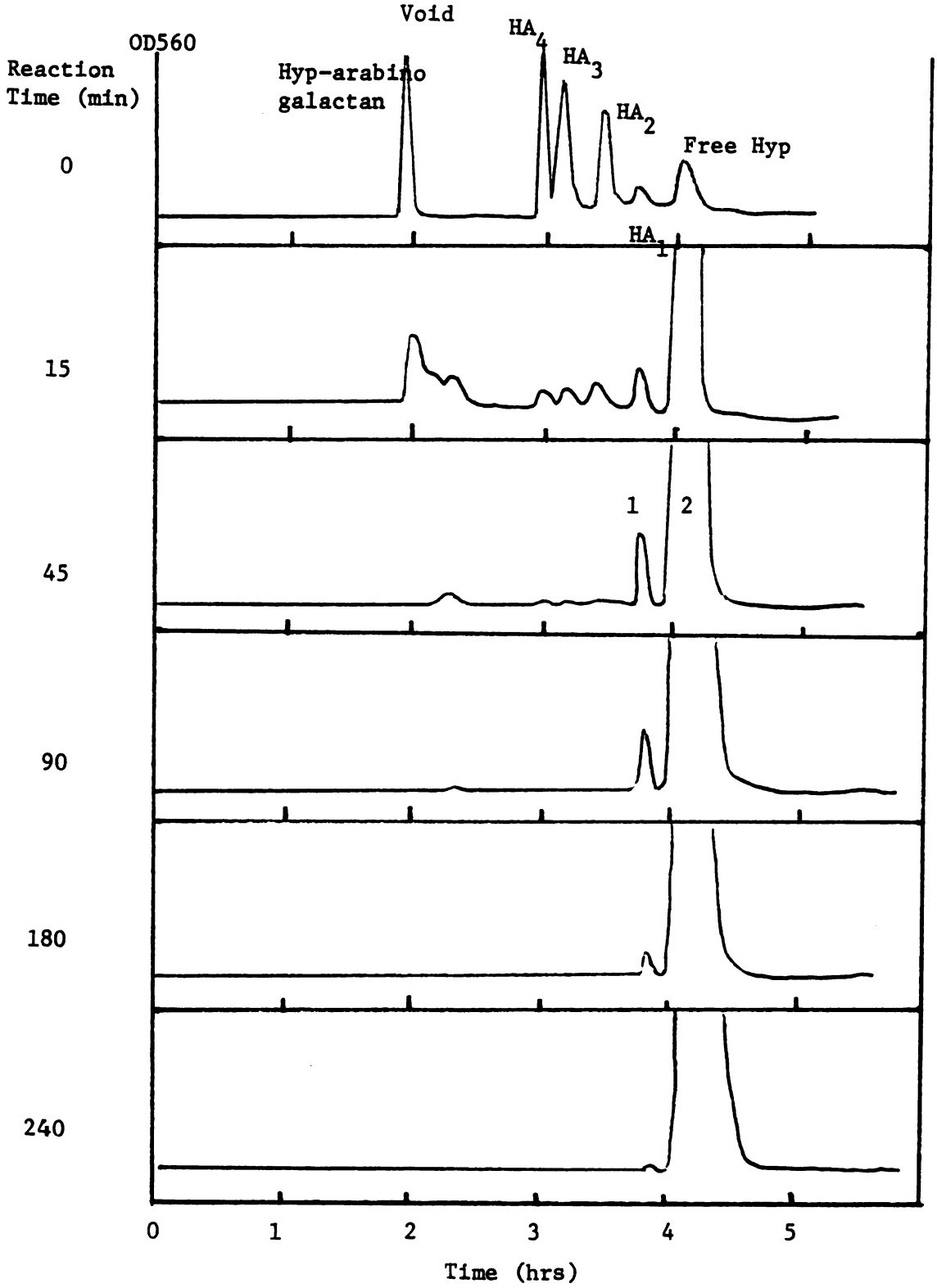

OD560
Reaction Time (min)
Void
Hyp-arabino galactan
HA4
HA3
HA2
Free Hyp
HA1
0
15
45
90
180
240
1
2
0
1
2
3
4
5
Time (hrs)

Figure 8

Table 7. Sugar Analysis of the HF/pyr Treated AGP(M) at 0°C and at Room Temperature (RT) for 1 hr

Fraction	Soluble after HF/pyr		Insoluble after HF/pyr	
Temperature	RT	0°C	RT	0°C
Hyp (mole)	1	1	1	1
Sugar (moles per 1 mole of Hyp)				
Ara	0.4	1.9	0.1	0
Rha	0.2	0.7	0	0
Fuc	0	0.1	0	0
Xyl	0.4	10.3	0.2	1.0
GalU	22.6	19.5	5.8	8.7
Man	0.8	0.7	0.2	0.6
Gal	1.2	13.1	0.3	1.8
Glc	17.0	5.5	0.8	0.8
Total	43	52	7	13

Table 8. Amino Acid Analysis of the HF/pyr Treated AGP(M) at 0°C and at Room Temperature (RT)

Fraction	Soluble after HF/pyr		Insoluble after HF/pyr	
Temperature	RT	0°C	RT	0°C
Amino Acids (mole %)				
Hyp	11.5	7.1	6.1	8.6
Asp	12.0	12.8	11.0	10.9
Thr	7.3	8.3	6.2	5.8
Ser	11.7	12.7	7.8	7.9
Glu	9.9	10.0	8.8	9.6
Pro	n.d.[a]	n.d.	8.3	9.3
Gly	8.0	8.7	7.4	7.3
Ala	11.4	15.2	6.4	6.9
Val	5.0	7.4	6.3	5.9
Cys	0	0	1.3	0.7
Met	1.7	0	1.0	1.1
Ile	3.0	3.1	2.9	3.0
Leu	5.0	4.2	5.2	4.9
Tyr	1.4	0.7	1.9	2.0
Phe	2.9	0.9	4.3	3.4
Lys	4.8	5.3	7.5	6.4
His	3.5	1.7	4.9	4.0
Arg	1.0	1.9	2.5	2.3

[a] n.d. not determined.

times greater at room temperature than at 0°C. Therefore the HF/pyr deglycosylation has been performed routinely at room temperature for 1 hr.

Deglycosylation of AGP(M) via HF/pyridine.

Partial Purification of Deglycosylated Hydroxyprolin-containing Material.

Figure 9 shows the flow sheet for the partial purification of deglycosylated hydroxyproline-containing material. Most of the deglycosylated material (ca. 75%) remained insoluble after dialysis against water. Therefore the solution was centrifuged and the further fractionation was done with the supernatant fraction. This material was freeze dried and loaded on a Sephadex G-100 column (500 ml bed volume) with 0.1 N NH_4OH (64% of the hydroxyproline dessolved). There were four peaks of hydroxyproline-containing material (Figure 10). Peak I (void) contained 10% of the total hydroxyproline, while the percentage of hydroxyproline in peaks II, III and IV is 25, 40 and 25, respectively. Sugar analyses of these four fractions are in Table 9. Peak III was chosen for further purification. This fraction was freeze dried and dissolved in 0.01 N HCl and loaded on an SP-Sephadex C-50 column. The hydroxyproline-containing material was retarded and appeared in the gradient elution (Figure 11). The amino acid analysis and sugar analysis of this fraction are given in Table 10. The hydroxyproline content of this fraction was about 17 mole %. In this fraction the ratio of sugars to protein (w/w) was 15:85. Peak II was also purified in the same way. The final fraction was obtained from SP-Sephadex C-50 at the 0.35 M NaCl region. The amino acid and sugar composition of this fraction is given in Table 10. The ratio of sugars to protein in this fraction (w/w)

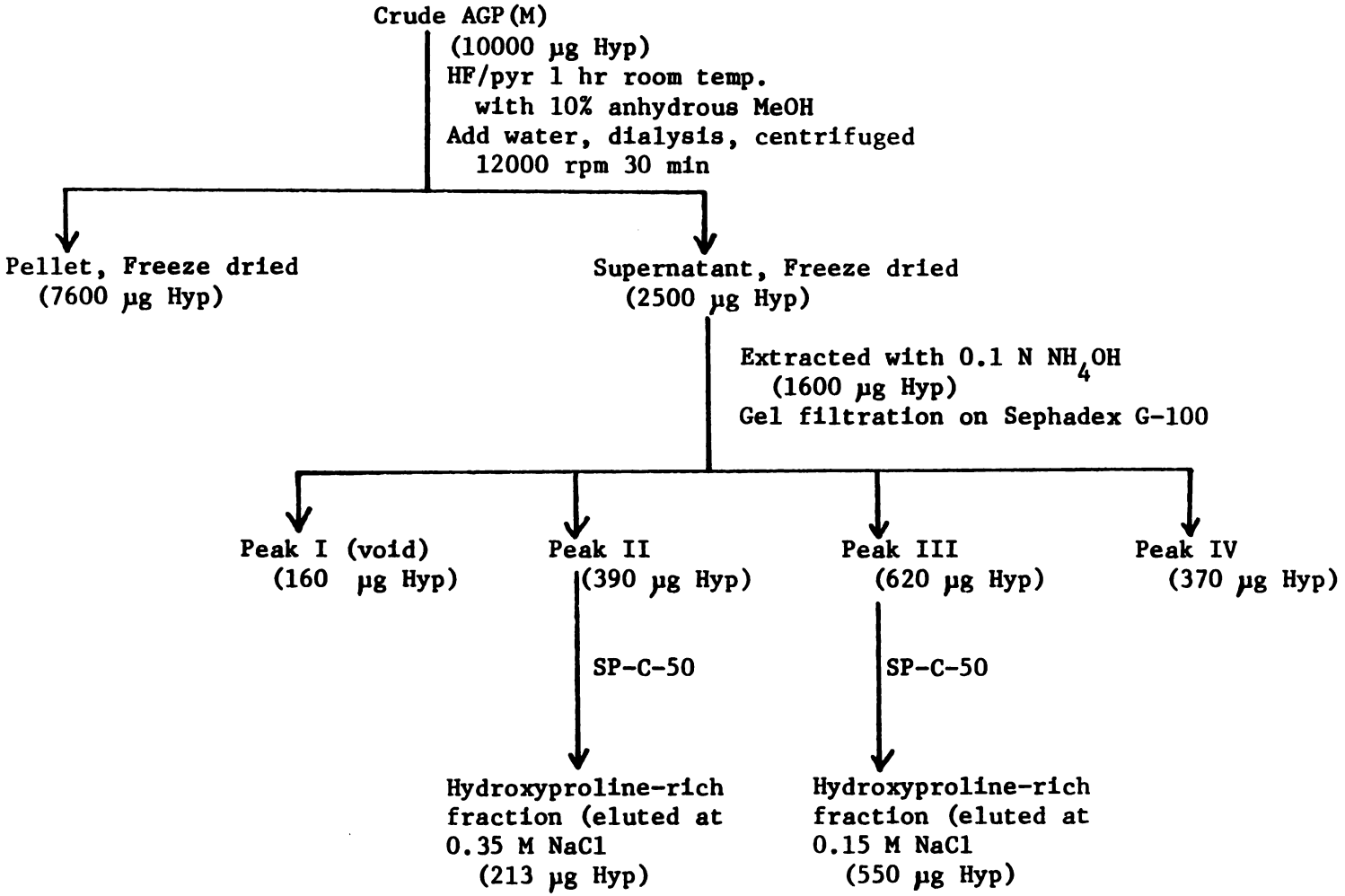


Figure 9. The flow sheet for the partial purification of hydroxyproline-rich material from AGP(M).

Figure 10. Gel filtration of deglycosylated AGP(M) with HF/pyr on a Sephadex G-100.

Five hundred mg of AGP(M) were treated with 18 ml HF/pyr plus 2 ml MeOH for 1 hr at room temperature. After adding 80 ml water to quench the reaction, the solution was dialysed and centrifuged. The supernatant was freeze dried (150 mg). This material was extracted with 5 ml 0.1 N NH_4OH, centrifuged and the supernatant was loaded on a Sephadex G-100 column (80 x 2.8 cm). The column was equilibrated and eluted with 0.1 N NH_4OH. Ten ml fractions were collected and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

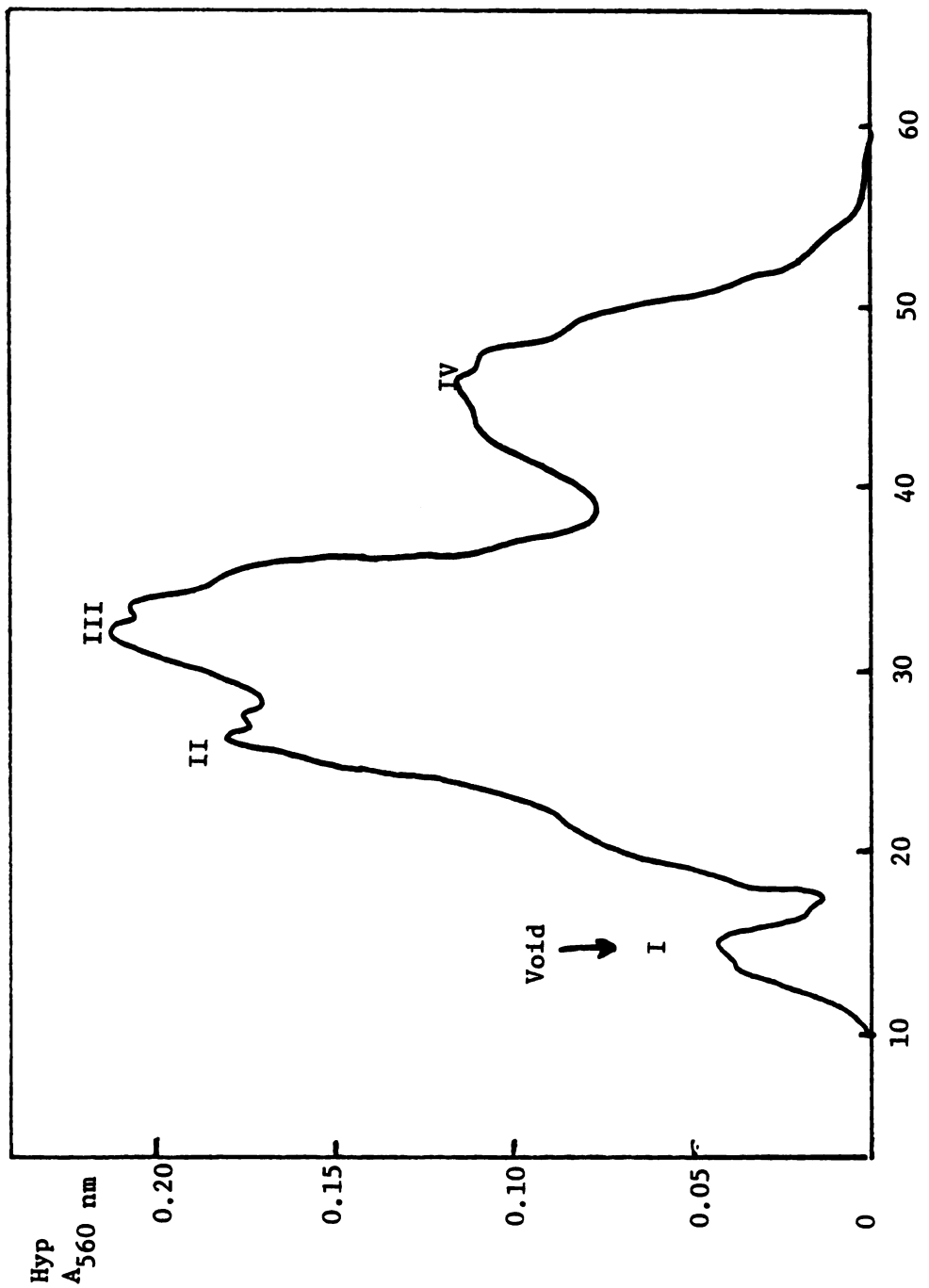

Hyp
$A_{560\ nm}$
0.20
0.15
0.10
0.05
0
10
20
30
40
50
60
Fraction Number (10 ml)
Void
I
II
III
IV

Figure 10

Table 9. Sugar Composition of Sephadex G-100 Fractions of AGP(M)/HF/pyr/Super

Fractions	I	II	III	IV
	Mole % of Total Sugar			
Ara	1.2	0	0	4.5
Rha	0	0	0	2.8
Fuc	0	0	0	0
Xyl	0	0	0	6.4
GalU	87.0	89.4	82.9	36.6
Man	4.9	3.9	3.9	2.0
Gal	0.9	1.4	1.8	11.7
Glc	6.0	5.4	11.4	36.0
	Moles per 1 Mole of Hyp			
Ara	1.96	0	0	1.79
Rha	0	0	0	1.11
Fuc	0	0	0	0
Xyl	0	0	0	2.54
GalU	142	33.1	20.5	14.6
Man	7.95	1.43	0.95	0.82
Gal	1.50	0.50	0.45	4.66
Glc	9.78	2.01	2.80	14.3

Figure 11. SP-Sephadex C-50 column chromatography of deglycosylated sycamore-maple AGP(M) with HF/pyr, G-100 peak III fraction.

Sephadex G-100 peak III (see Fig. 10) was pooled and freeze dried. This material was dissolved in 0.01 N HCl and centrifuged. The supernatant was applied to an SP-Sephadex C-50 column. Five ml fraction was collected and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

The elution schedule was as follows: fractions 1 to 10, with 0.01 N HCl; fractions 11 to 41, with gradient between 0.01 N HCl, and 0.01 N HCl containing 1 M NaCl.

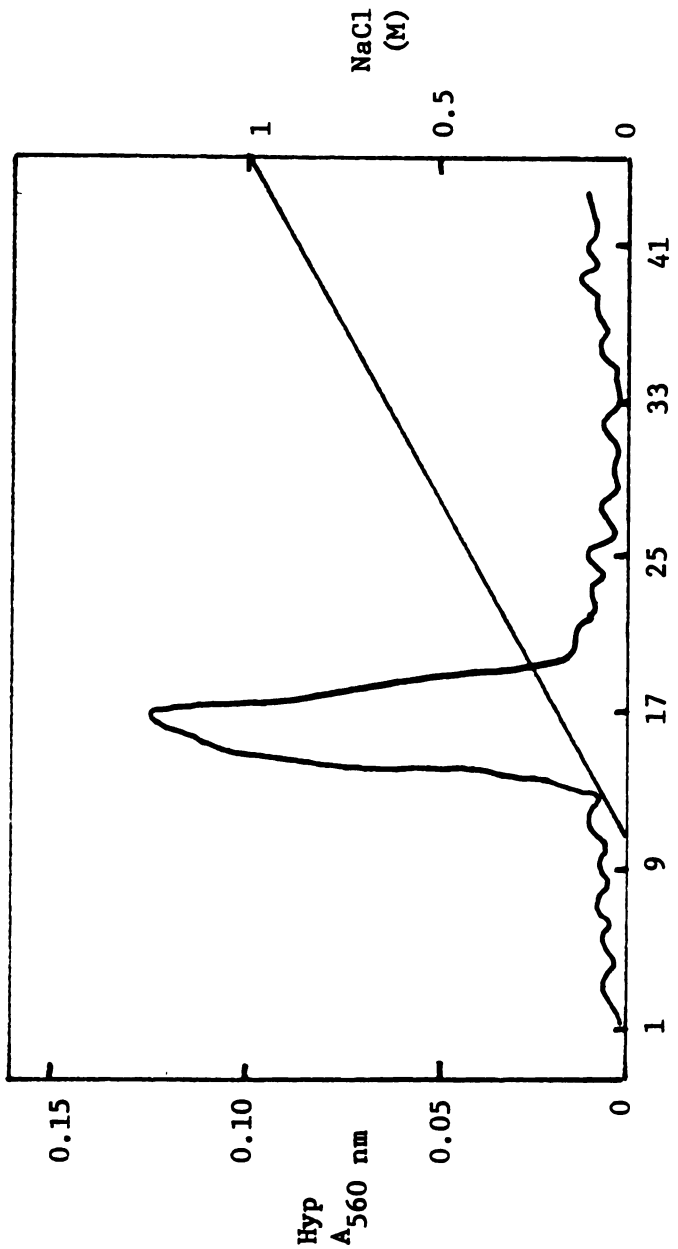

Hyp
A_{560} nm
0.15
0.10
0.05
0
NaCl
(M)
1
0.5
0
1
9
17
25
33
41
Fraction Number (5 ml)

Figure 11

Table 10. A Comparison of the Chemical Compositions of Partially Purified Hydroxyproline-containing Fractions from Deglycosylated AGP(M)

Fraction	HF/pyr/G-100 II/ SP-C-50	HF/pyr/G-100 III/ SP-C-50	HF/G-100 II/ SP-C-50
Concentration of NaCl (M)	0.35	0.15	0.28
Final Yield (%, Hyp basis)	2	5	6
	Sugar, mole % (moles per 1 mole Hyp)		
Ara	4.6(0.08)	0 (0)	14.9(0.05)
Rha	0 (0)	0 (0)	0 (0)
Fuc	0 (0)	0 (0)	0 (0)
Xyl	7.0(0.05)	6.4(0.03)	19.1(0.07)
GalU	0 (0)	0 (0)	0 (0)
Man	10.7(0.15)	18.7(0.11)	0 (0)
Gal	50.0(0.24)	47.1(0.26)	8.7(0.04)
Glc	27.6(0.31)	27.9(0.15)	46.7(0.20)
	Amino Acids, mole %		
Hyp	22.8	17.4	17.7
Asp	6.9	11.5	11.1
Thr	9.6	8.3	9.5
Ser	15.0	16.3	14.1
Glu	4.4	6.9	6.8
Pro	2.1	n.d.	n.d.
Gly	5.6	5.0	5.8
Ala	15.3	11.9	14.8
Val	6.2	5.9	6.7
Cys	0	0.6	0
Met	0	1.4	0
Ile	2.0	5.0	2.8
Leu	3.4	2.9	4.2
Tyr	0	0.4	0
Phe	1.5	1.9	1.4
Lys	3.3	3.1	3.3
His	0.7	0.6	0.6
Arg	1.2	1.0	1.1

n.d. not determined.

was 13:87.

For radioactive AGP(M), the purification procedure described in the flow sheet (Figure 9) was used for the fractionation. The radioactivity present in the SP-Sephadex C-50 fraction (peak III) was 45000 cpm.

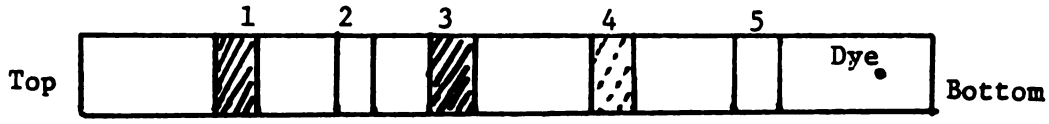
SDS Disc-gel Electrophoresis of Hydroxyproline-rich Fractions.

So far there are no reports of the gel-electrophoresis of AGPs either intact or deglycosylated. This may be due to their poor reactivity with dyes such as coomassie blue. For example, the major fraction of the deglycosylated sycamore-maple AGPs stained very poorly, if at all, with coomassie blue R250 (40). Therefore the combined coomassie blue G250 staining/fixation method of Blakesly and Boezi (41) was tried to avoid the possibility that a hydroxyproline-rich protein might be eluted from the gel during the lengthy fixation/destaining procedures commonly in use. However, the deglycosylated AGPs fractions again stained poorly. Therefore AGPs were prelabelled by two methods, first by growth in ^{14}C-proline and second by reaction with FITC. The prelabelled fractions were subjected to polyacrylamide gel-electrophoresis and appropriate gel slices were eluted, desalted, and analysed for amino acids via gas chromatography and conventional amino acid analyser, all as described in Materials and Methods. Electrophoresis of the (FITC-labelled) major fraction of deglycosylated AGPs showed one minor and two major but somewhat diffuse fluorescent bands which were eluted and analysed (Table 11). After electrophoresis of the (^{14}C-labelled) major fraction of deglycosylated AGPs, 2 mm gel slices were prepared for ^{14}C assay in a liquid scintillation spectrometer. Most of the ^{14}C-labelled material was present in slice 9, 16-18 mm from the

Table 11. Amino Acid Composition of Sycamore AGP(M) Peak III/SP-C-50 before and after Electrophoresis (mole %)

		Slice 1		Slice 2		Slice 3	
	AA1	GC	AA	GC	AA	GC	AA
Hyp	17.4	19.9	26.5	19.0	15.7	5.0	n.d.
Asp	11.5	4.5	5.8	9.9	10.8	16.3	23.5
Thr	8.3	6.8	8.2	9.5	8.0	10.7	13.7
Ser	16.3	19.0	21.7	20.5	17.9	14.7	17.1
Glu	6.9	6.7	7.5	3.0	7.4	9.1	9.2
Pro	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gly	5.0	17.0	7.8	n.d.	7.0	n.d.	3.5
Ala	11.9	11.7	15.4	12.2	14.7	12.0	n.d.
Val	5.9	2.4	n.d.	5.7	4.7	7.4	10.9
Cys	0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Met	1.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ile	2.9	3.0	n.d.	5.3	3.4	5.8	7.3
Leu	5.0	1.8	n.d.	6.4	5.8	8.6	14.2
Tyr	0.4	0.8	2.9	n.d.	n.d.	1.8	n.d.
Phe	1.9	2.4	n.d.	4.1	2.2	5.4	n.d.
Lys	2.1	1.5	3.8	n.d.	2.5	2.7	n.d.
His	0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arg	1.0	1.8	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. not determined.



AA1 Data obtained from amino acid analyser before labelling with FITC (i.e. starting material before electrophoresis).

GC Data obtained from gas chromatography after labelling with FITC.

AA Data obtained from amino acid analyser after labelling with FITC.

Six hundred µg of protein (100 µg hydroxyproline) was labelled with FITC amd applied to gel. The regions with intense fluorescent bands (slice 1 and 3), faint fluorescent band (slice 4), fluorescent smear (slice 2) and no fluorescent region (slice 5, control) were cut from the top of the gel and the material was eluted as described under methods. The amino acid analysis was performed with this material after hydrolysis. Slices 4 and 5 gave no amino acids. The hydroxyproline recovery from the gel was 60%.

Figure 12. Amount of radioactivity in gel slices.

Radioactive AGP(M) was deglycosylated with HF/pyr and the deglycosylated AGPs were purified according to Figure 9. The final fraction after AP-C-50 was gel-electrophoresed and 2 mm gel slices were prepared for ^{14}C assay in a liquid scintillation spectrometer.

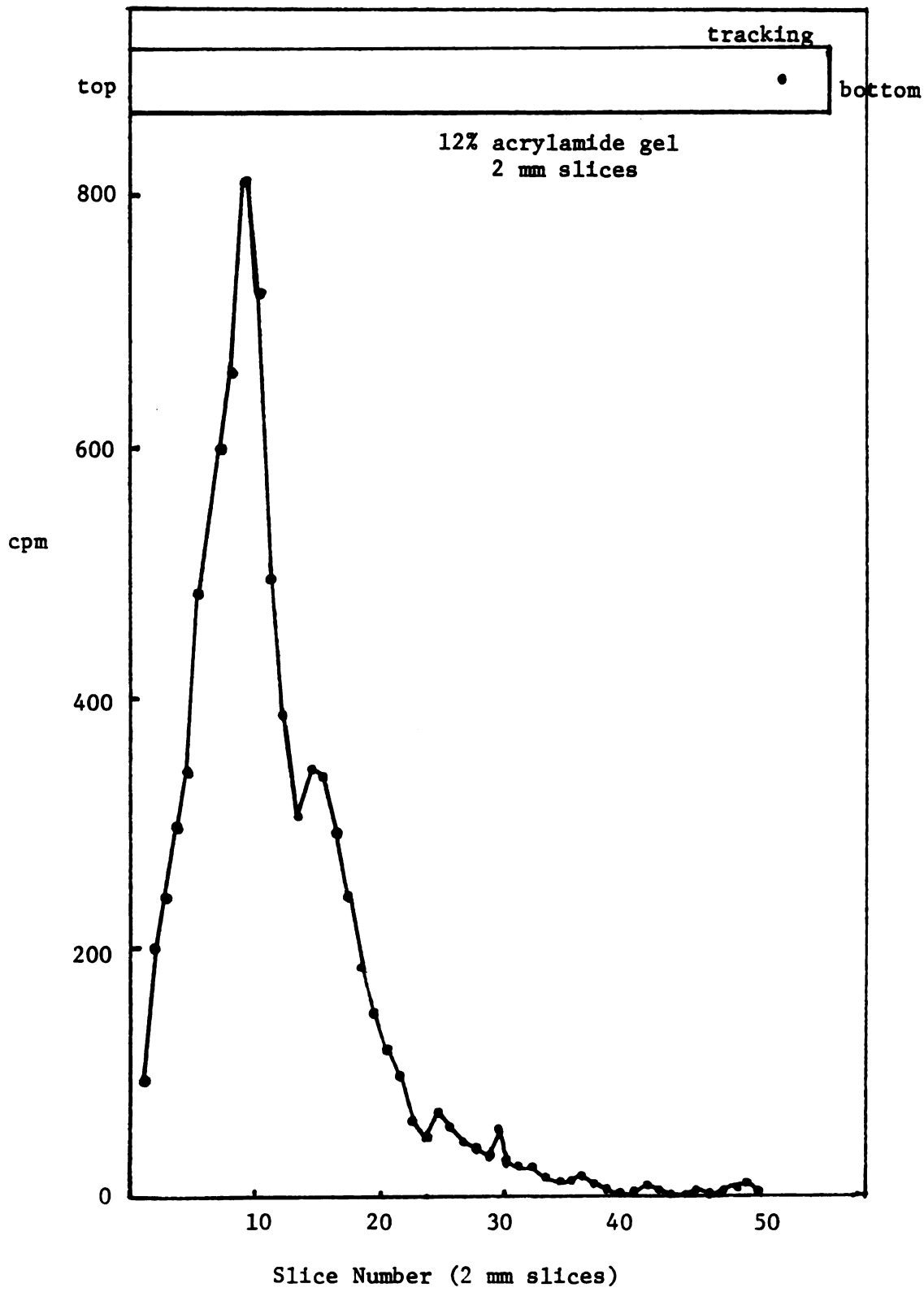

tracking
top
bottom
12% acrylamide gel
2 mm slices
800
600
400
200
0
cpm
10
20
30
40
50
Slice Number (2 mm slices)

Figure 12

origin (Figure 12). In a separate experiment, a portion of the major ^{14}C-labelled fraction was hydrolysed with 6 N HCl and the hydrolysate was separated on an amino acid analyser, and successive 1 ml fractions were assayed for ^{14}C-label in a liquid scintillation spectrometer. The results showed an exclusive distribution of label between hydroxyproline (80%) and proline (20%). As a check on the methodology used above, control proteins were also analysed (ribonuclease, apomyoglobin, and the soluble hydroxyproline-rich glycoprotein from Santalum album). Table 12 shows the reasonably good agreement for the amino acid analysis (by gas chromatography and conventional amino acid analyser) of control proteins before and after SDS gel-electrophoresis.

HF Control Experiment.

To compare the efficiency of HF/pyr with anhydrous HF, the control experiment with HF was performed. Five hundred mg of AGP(M) was deglycosylated with 18 ml of HF and 2 ml of anhydrous methanol for 1 hr at room temperature. Further fractionation was as for the HF/pyr experiment. After evaporation of HF, the deglycosylated material was extracted with 0.1 N NH_4OH and the extract was loaded on a Sephadex G-100 column (bed volumn 500 ml). Figure 13 shows the elution profile, which differs slightly from the HF/pyr experiment (see Figure 10): there were only three peaks containing hydroxyproline. The major peak (II) was further purified with SP-Sephadex C-50 column chromatography. A single hydroxyproline peak appeared in a gradient region (Figure 14). The chemical composition of this material and the final yield in terms of hydroxyproline were compared with the results obtained from HF/pyr experiment (Table 10). The amino acid compositions of the major peaks using HF/pyr (peak III/SP-C-50) and HF (peak II/SP-C-50)

Table 12. Amino Acid Composition of Standard Proteins (mole %)

	Ribonuclease			Apomyoglobin			Sandal Protein	
	a	b	c	a	b	c	a'	c
Hyp	0	0	0	0	0	0	5.7	9.0
Asp	12.1	19.6	10.5	5.2	6.5	7.5	9.0	6.7
Thr	8.1	9.4	17.9	3.3	3.8	4.5	5.0	5.0
Ser	12.1	12.7	13.6	3.9	4.0	4.1	5.9	8.6
Glu	9.7	13.4	3.5	12.5	14.6	16.7	9.4	7.9
Pro	3.2	3.1	n.d.	2.6	3.3	5.9	6.5	4.0
Gly	2.4	n.d.	n.d.	7.2	6.3	n.d.	14.5	16.3
Ala	9.7	7.2	12.9	11.9	11.6	14.1	9.2	10.6
Val	7.3	6.1	8.5	5.2	5.2	5.4	4.0	3.3
Cys	6.5	n.d.	n.d.	n.d.	n.d.	n.d.	2.6	n.d.
Met	3.2	8.1	1.9	1.3	0.8	n.d.	1.9	n.d.
Ile	2.4	1.3	1.4	5.9	6.5	8.7	3.3	3.5
Leu	1.6	1.4	1.8	12.4	13.2	15.3	6.3	7.2
Tyr	4.8	6.6	4.9	2.0	4.6	10.6	5.2	1.1
Phe	2.4	3.2	3.2	3.9	5.4	7.3	4.8	4.2
Lys	8.1	6.5	11.4	11.8	9.4	n.d.	1.9	5.3
His	3.2	n.d.	4.3	7.8	n.d.	n.d.	1.1	n.d.
Arg	3.2	1.3	4.3	2.6	4.8	n.d.	3.6	6.6

n.d. not determined.

Ribonuclease (200 μg), apomyoglobin (200 μg), and sandal protein (120 μg) were labelled with FITC and whole material was applied on the gel. In the case of sandal protein, there was 70% recovery of hydroxyproline from the gel.

a Data taken from the book "Handbook of Biochemistry".
a' Data obtained from amino acid analyser.
b Amino acid composition obtained from gas chromatography before electrophoresis.
c Gas chromatographic analysis of the fluorescent material eluted from the gel after electrophoresis.

Figure 13. Gel filtration of HF-deglycosylated AGP(M) on Sephadex G-100.

Five hundred mg of AGP(M) were treated with 18 ml HF plus 2 ml MeOH for 1 hr at room temperature. After evaporating HF, the material was extracted with 0.1 N NH_4OH. The extract was centrifuged and the supernatant (about 10 ml) was fractionated with two separate applications on an 80 x 2.8 cm Sephadex G-100 column equilibrated with 0.1 N NH_4OH. Fraction volume was 10 ml and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

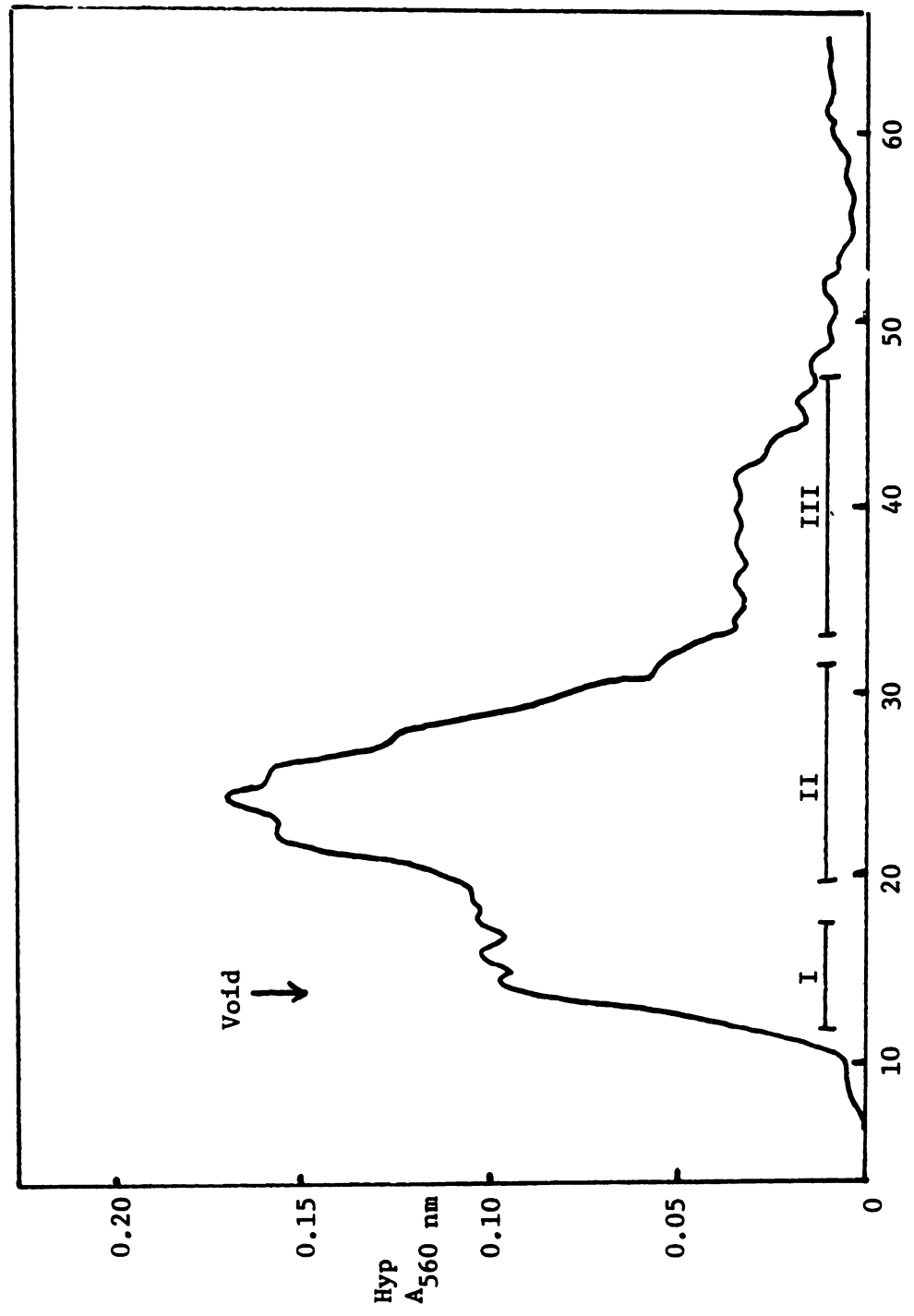

0.20
0.15
0.10
0.05
0
Hyp
$A_{560\ nm}$
Void
I
II
III
10
20
30
40
50
60
Fraction Number

Figure 13

Figure 14. SP-Sephadex C-50 column chromatography of HF-deglycosylated AGP(M), G-100 peak II fraction.

Sephadex G-100 peak II (see Fig. 13) was pooled and freeze dried. This material was dissolved in 0.01 N HCl and centrifuged. The supernatant was applied on an SP-C-50 column. Fraction volume was 2 ml and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

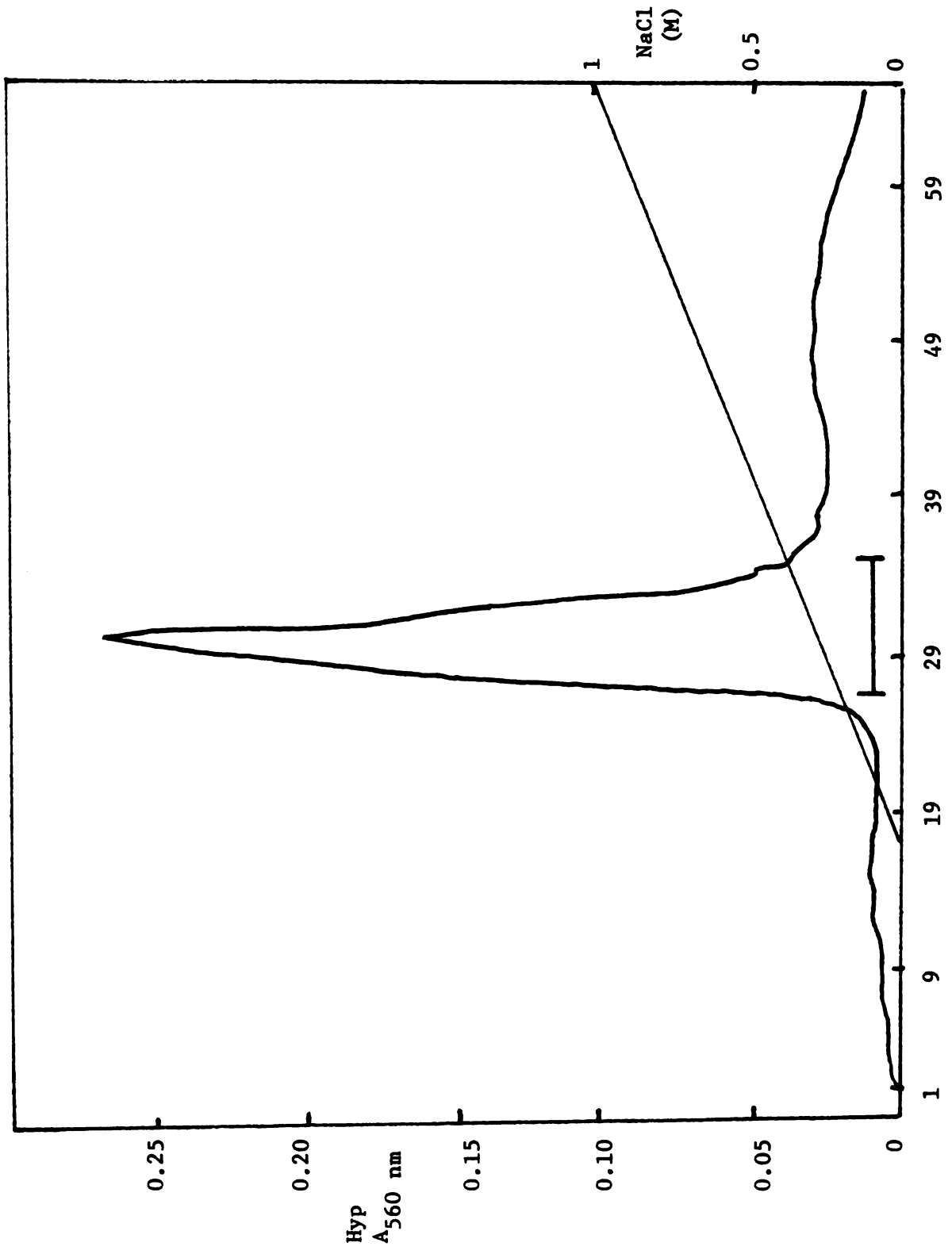

Hyp
$A_{560\ nm}$
0.25
0.20
0.15
0.10
0.05
0
1
9
19
29
39
49
59
Fraction Number
NaCl
(M)
1
0.5
0

Figure 14

were similar. The major difference was that there was more glucose left in the HF experiment than HF/pyr, whereas there was more galactose in HF/pyr than in HF. The efficiency of deglycosylation of other sugars by both reagents was almost same. The final yield of SP-C-50 fractions was also almost same (Table 10). So HF/pyr is thought to be as efficient as anhydrous HF for the deglycosylation of AGP(M).

Further Fractionation of HF/pyr Insoluble Fraction.

After HF/pyr treatment of AGP(M) and dialysis, there was insoluble material which was freeze dried, and attempts made to extract hydroxyproline because it had a lot of hydroxyproline (75% of original AGP(M), see Figure 9). Some solvents (water, 0.01 N HCl, 0.5 M NaCl and 0.1 N NH_4OH) were tried to extract hydroxyproline. As 0.5 M NaCl gave the most hydroxyproline extraction (60% extracted), the insoluble material was extracted with 0.5 M NaCl twice (10 ml/gm). After centrifuging to remove the insoluble material, the supernatant was gel-filtered on Sephadex G-100 (2.6 x 80 cm, bed volume 400 ml) equilibrated with 0.5 M NaCl. The elution profile is given in Figure 15. Three peaks contained hydroxyproline. One of these three peaks (III) was chosen for further fractionation. This fraction was dialysed against 0.01 N HCl for 2 days in the coldroom with 6 changes of 0.01 N HCl, the dialysate was loaded on SP-Sephadex C-50 column as described earlier. Three peaks were obtained, one at washing, and two within the gradient (0.2 M and 0.5 M NaCl region). The hydroxyproline distribution in these three peaks were 18%, 33% and 49%. The two major peaks which appeared at 0.2 M and 0.5 M NaCl were analysed for sugar and amino acid (Table 13). Amino acid analyses suggested that first peak in the gradient may come from AGPs because of its high alanine content and the second peak may come

Figure 15. Gel filtration of HF/pyr insoluble material after extracting with 0.5 M NaCl.

HF/pyr insoluble material from AGP(M) (1 gm) was extracted with 10 ml of 0.5 M NaCl twice. The extract (20 ml) was fractionated by four separate applications on an 80 x 2.6 cm Sephadex G-100 column equilibrated with 0.5 M NaCl. Five ml fractions were collected and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

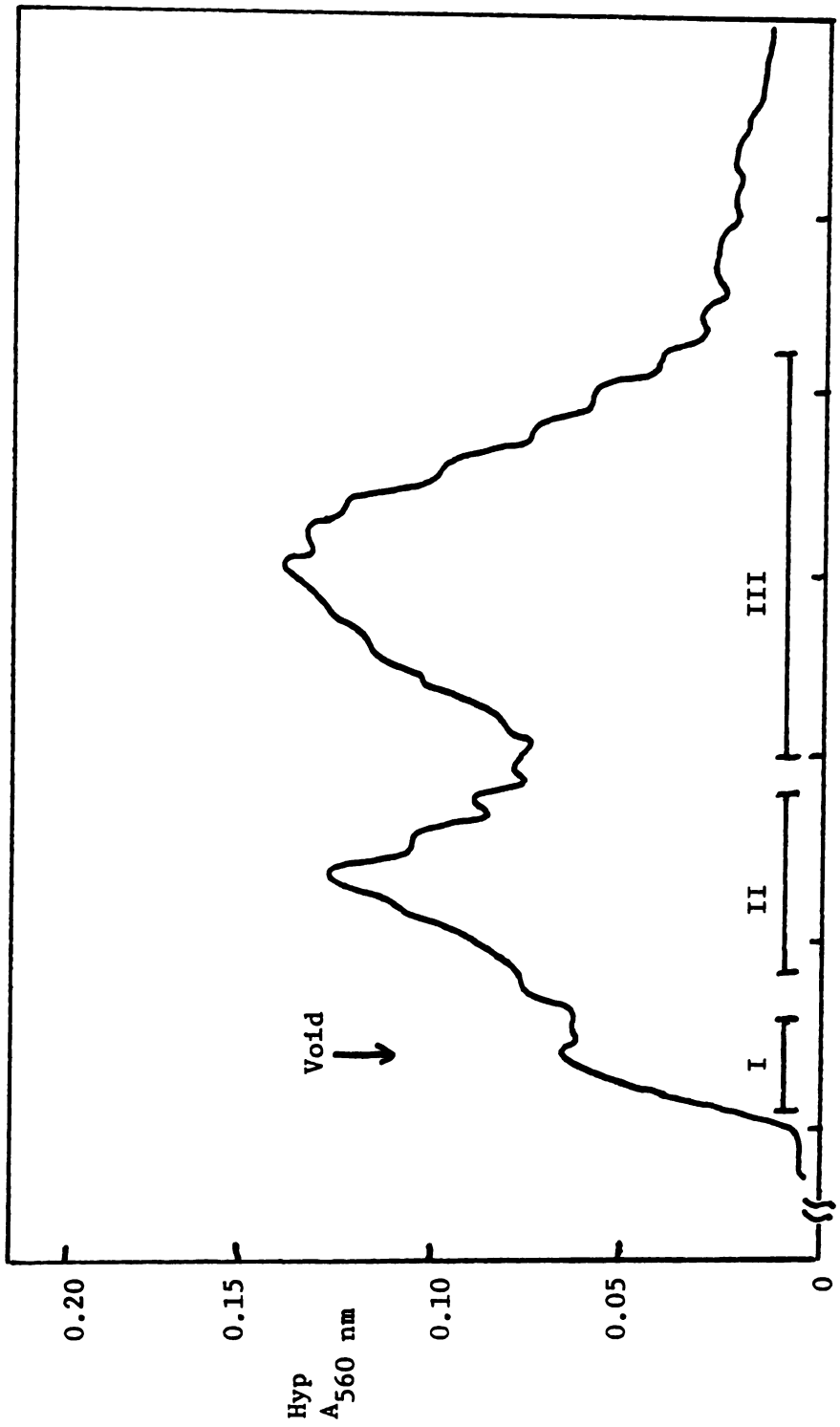

0.20
0.15
0.10
0.05
0
Hyp
$A_{560\ nm}$
Void
I
II
III
20
30
40
50
60
70
Fraction Number

Figure 15

Table 13. Chemical Composition of AGP(M)/HF/pyr/Insoluble/NaCl Extract/G-100 III/ Fractionated on SP-C-50

Fraction	Gradient I	Gradient II	Sandal Lectin[a]
NaCl Concentration (M)	0.2	0.5	-
	Sugar, mole % (moles per 1 mole Hyp)		
Ara	0 (0)	0 (0)	
Rha	0 (0)	0 (0)	
Fuc	0 (0)	0 (0)	
Xyl	0 (0)	0 (0)	
GalU	0 (0)	0 (0)	
Man	49.1 (1.54)	0 (0)	
Gal	29.7 (0.93)	82.1 (0.17)	
Glc	21.2 (0.66)	17.9 (0.04)	
	Amino Acids, mole %		
Hyp	9.0	7.2	5.9
Asp	13.0	10.0	10.6
Thr	8.7	4.7	4.8
Ser	14.6	5.9	5.9
Glu	5.9	11.0	12.1
Pro	n.d.	6.6	6.3
Gly	7.4	13.4	13.4
Ala	12.2	5.6	8.6
Val	5.8	3.9	4.1
Cys	4.3	0.3	0.9
Met	0	2.0	0.7
Ile	3.3	1.7	3.3
Leu	7.0	3.4	6.1
Tyr	0.8	1.5	3.7
Phe	1.9	4.5	3.5
Lys	1.9	8.6	4.3
His	0.6	7.6	1.3
Arg	1.6	2.1	5.8

n.d. not determined.

[a]Taken from ref. (19).

from a lectin-like protein because the amino acid composition (especially acidic amino acids) were similar to those from hydroxyproline-containing sandal lectin (Table 13).

AGP(C) Deglycosylation via HF/pyridine.

Jermyn and Yeow (27) suggested that secreted AGPs into the medium of suspension-cultured cells would have been exposed to the full effect of any degradation enzymes present. If so, AGP(C), which could be the precursor of AGP(M), may have less hydroxyproline than AGP(M), if enzyme preferentially degraded the hydroxyproline poor region of AGPs.

A 280 mg of AGP(C) (2550 µg Hyp) was deglycosylated with 4.5 ml of HF/pyr and 0.5 ml of anhydrous methanol at room temperature for 1 hr. Further fractionation was according to Figure 9. The supernatant fraction (2400 µg Hyp) was freeze dried and extracted with 0.1 N NH_4OH (1480 µg Hyp extracted) and loaded on Sephadex G-100 column (bed volume 500 ml). Figure 16 shows the elution profile. The major peak (II, 830 µg Hyp) was loaded on SP-Sephadex C-50 column (30 ml bed volume). The single hydroxyproline peak appeared at 0.39 M NaCl (Figure 17). This fraction (660 µg Hyp) was analysed for sugar and amino acid composition and the result is shown in Table 14 together with AGP(M)/HF/pyr/Super,/G-100 III/SP-C-50. Both sugar and amino acid compositions were similar in these two fractions, suggesting that AGP(M) would not be an extensive degradation product of AGP(C) in sycamore suspension cultures.

Figure 16. Gel filtration of deglycosylated AGP(C) with HF/pyr on a Sephadex G-100.

AGP(C) (280 mg) was treated with HF/pyr (4.5 ml) and MeOH (0.5 ml) for 1 hr at room temperature. After adding water to quench the reaction, the solution was dialysed and centrifuged. The supernatant was freeze dried (55 mg). This material was extracted with 0.1 N NH_4OH and the extract (2 ml) was loaded on a Sephadex G-100 column (70 x 1.4 cm) equilibrated with 0.1 N NH_4OH. Two ml fractions were collected and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

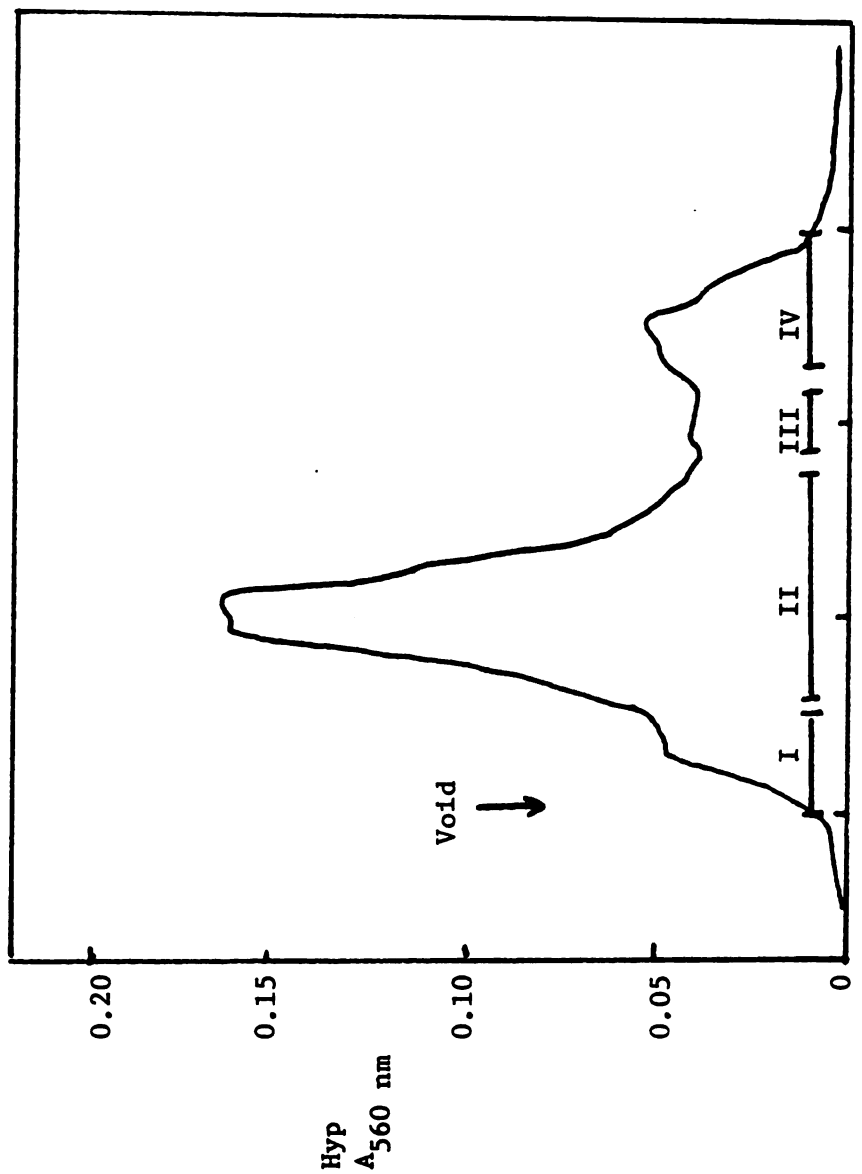

0.20
0.15
0.10
0.05
0
Hyp
$A_{560\ nm}$
Void
I
II
III
IV
15
25
35
45
Fraction Number

Figure 16

Figure 17. SP-Sephadex C-50 column chromatography of deglycosylated AGP(C) with HF/pyr, G-100 peak II fraction.

Sephadex G-100 peak II (see Fig. 16) was pooled and freeze dried, and extracted with 0.01 N HCl. The extract was applied on an SP-C-50 column. Two ml fractions were collected and 200 µl aliquots of alternate fractions were assayed for hydroxyproline.

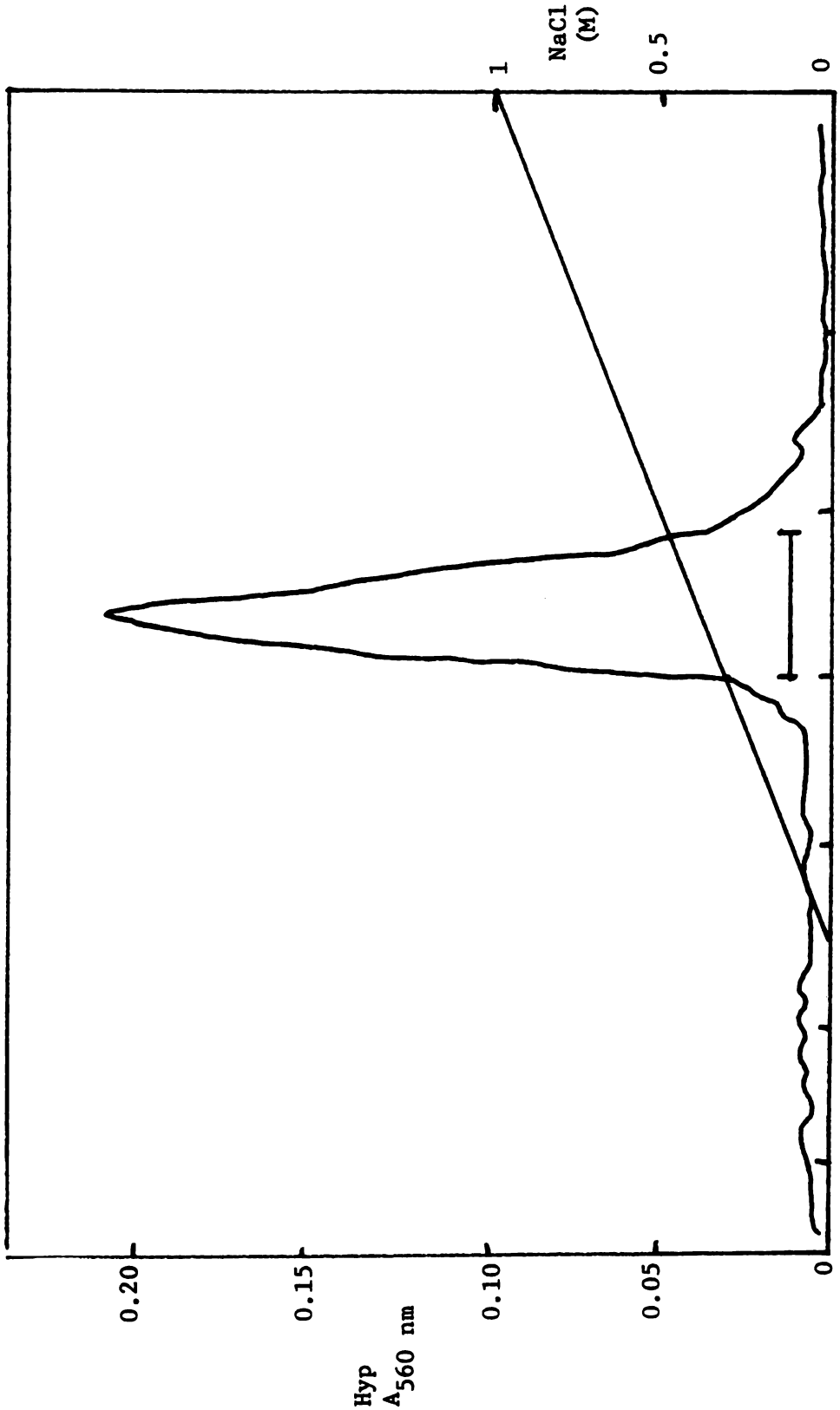

Hyp
$A_{560\ nm}$
0.20
0.15
0.10
0.05
0
5
15
25
35
45
55
Fraction Number
NaCl
(M)
1
0.5
0

Figure 17

Table 14. Chemical Composition of Major Peaks of HF/pyr Deglycosylated AGP(C) and AGP(M)

Fraction	AGP(C)/HF/pyr/Super/ G-100 II/SP-C-50	AGP(M)/HF/pyr/Super/ G-100 III/SP-C-50
Concentration of NaCl (M)	0.39	0.15
	Sugar, mole % (moles per 1 mole Hyp)	
Ara	10.2 (0.12)	0 (0)
Rha	0 (0)	0 (0)
Fuc	0 (0)	0 (0)
Xyl	5.8 (0.07)	6.4 (0.03)
GalU	0 (0)	0 (0)
Man	18.5 (0.23)	18.7 (0.11)
Gal	28.5 (0.36)	47.1 (0.26)
Glc	37.0 (0.47)	27.9 (0.15)
	Amino Acids, mole %	
Hyp	14.0	17.4
Asp	13.4	11.5
Thr	7.6	8.3
Ser	11.0	16.3
Glu	10.8	6.9
Pro	5.0	n.d.
Gly	5.7	5.0
Ala	12.7	11.9
Val	7.1	5.9
Cys	0	0.6
Met	0	1.4
Ile	2.1	5.0
Leu	3.6	2.9
Tyr	0.5	0.4
Phe	0.7	1.9
Lys	4.4	3.1
His	0.6	0.6
Arg	0.9	1.0

n.d. not determined.

DISCUSSIONS

The major conclusions in this work are as follows.

1. HF in pyridine deglycosylates arabinogalactan proteins (AGPs, from the medium of suspension-cultured sycamore cells) as efficiently as does anhydrous HF. Considering the ease of handling, HF/pyr is a better reagent for the deglycosylation of AGPs than anhydrous HF.
2. The efficiency of deglycosylation of HF/pyr depends on the temperature and reaction time. The best result was obtained by treating for 1.5 hrs at room temperature (more than 90% of sugars were removed). One hr solvolysis at room temperature was almost as efficient as that of 1.5 hrs at room temperature.
3. The major sugars which remained undialysable after HF/pyr deglycosylation were galacturonic acid and glucose which were almost completely removed from the protein portion after gel-filtration on Sephadex G-100 and ion-exchange chromatography on SP-Sephadex C-50.
4. After SP-Sephadex C-50 column chromatography, the partially purified AGPs contained less than 1 residue of total sugars per 1 mole of hydroxyproline. The amino acid analysis of the final fraction showed that hydroxyproline, serine, alanine and aspartic acid are predominant amino acids. These four accounted for 57% of the total amino acids.
5. This final material entered the polyacrylamide gel but gave a rather diffuse band. This was confirmed by prelabelling the protein with FITC and ^{14}C-proline and by eluting from the gel followed by

amino acid analysis.

6. Arabinogalactan proteins isolated from the cytoplasm of sycamore cells were also deglycosylated with HF/pyr. The final fraction after SP-C-50 showed almost the same chemical composition as those of AGPs from the culture medium, which suggested that they may be the same and the AGPs of the culture medium was not an extensive degradation product of cytoplasmic AGPs.

7. Hydroxyproline-glucose may be present in AGPs. This was obtained as an HF/pyr "semi-resistant" hydroxyproline-glycoside together with hydroxyproline-galactose by Biogel P2 column chromatography after $Ba(OH)_2$ hydrolysis of partially deglycosylated AGPs. The identification of Hyp-Glc is in progress.

In order to examine the protein portion of AGPs, they need to be deglycosylated first because of their high carbohydrate content. Chemical deglycosylation was tried by using anhydrous HF, which is examined by Mort and Lamport (34), but here it was modified by the use of 70% HF in pyridine.

First the optimum condition was determined. Time course experiment (Table 6) and temperature experiment (Table 7) showed that HF/pyr treatment for 1 hr at room temperature removed over 90% of sugars from AGPs. The control experiment by use of anhydrous HF showed no significant difference from the use of HF/pyr. At room temperature HF/pyr removed almost all of Ara, Rha, Fuc and Man from AGPs within 30 min, and Xyl and Gal were removed within 90 min almost completely. Certain amounts of GalU and Glc were present undialysable even after 4 hrs HF/pyr treatment. But the polyuronide was completely removed from the protein by SP-C-50 column chromatography, indicating the

polyuronide does not bound covalently to the protein portion. Almost all of glucose was removed from the protein after partial purification by Sephadex G-100 and SP-C-50. At this stage more than 99% of sugars were removed.

The great advantage of HF/pyr is that it is easier to handle than liquid HF and it does not require special apparatus. If HF/pyr deglycosylates other glycoprotein as it does for AGPs, it will become a good method for chemical deglycosylation. The only problem will arise if the polyuronide is going to be deglycosylated. In this case anhydrous HF will be better than HF/pyr.

The deglycosylated AGPs were partially purified. The final fraction contained less than 1 redidue of sugar per 1 mole of hydroxyproline. Cytoplasmic AGPs were also isolated and deglycosylated. The partially purified fraction showed the similar chemical compositions to those from medium AGPs. Both AGPs may be the same and the medium AGPs would not be an extensive degradation product of cytoplasmic AGPs. The sequencing analysis of this partially purified deglycosylated AGPs is now in progress.

There were HF/pyr "resistant" hydroxyproline-glycosides in AGPs. They were obtained by gel-filtration on Biogel P2 column and appeared at the region of Hyp-Ara_1 after HF/pyr treatment of AGPs followed by $Ba(OH)_2$ hydrolysis. Sugar analysis of this fraction showed the presence of galactose (80%) and glucose (20%), suggesting the possibility of Hyp-Glc as well as Hyp-Gal as HF/pyr "resistant" hydroxyproline-glycosides. If Hyp-Glc is identified, that will be the first finding of this linkage in plant.

What is the function of AGPs? There are at least three possibilities.

First is that it would be the precursor of cell wall protein "extensin". If an enzyme removes hydroxyproline-poor alanine-rich tail portion from AGPs, the hydroxyproline-rich region could become extensin. Amino acid sequence of AGPs will give some information. Second is that it could play a role in the cell-cell recognition processed in plants, because they are extracellular macromolecules, they are glycoproteins, and they are ubiquitous in higher plants. Third possibility is that they could be the plasticiser in cell wall network to cross-link polysaccharides. In any case the further structural anaysis of AGPs and extensin will give more information about their functions in plants.

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