The glycosyl moeity of sainfoin lectin
by Anthony Eugene Namen

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biochemistry
Montana State University
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Abstract:
A lectin isolated from the seeds of sainfoin (Onobrychis vicii-folia, Scop, var Eski) was shown to be a glycoprotein containing 2.6% neutral carbohydrate. Gas-liquid chromatographic analysis of sugar alditol acetates and amino acid analysis indicated the presence of fucose, xylose, mannose, and glucosamine in molar ratio (to protein monomer) 1:1:2:2.

A glycopeptide accounting for 70% of the total carbohydrate was isolated from pronase digests of the lectin by gel filtration chromatography. The molar composition of the glycopeptide was determined to be: (Ser1Asx1Glx1Thr1ammonia1fucose1xylose1mannose2glucosamine2).

The proposed structure for the glycopeptide, based on sequence analysis, is: [Chemical Diagram not captured by OCR]

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Date 26 July, 1978

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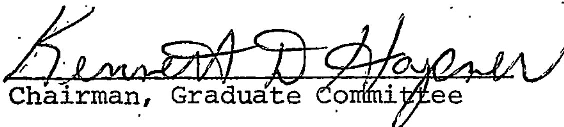
Approved: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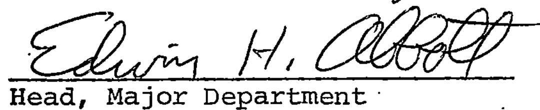
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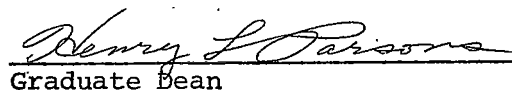
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TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	ix
INTRODUCTION	1
RESEARCH OBJECTIVES	9
EXPERIMENTAL PROCEDURE	10
Materials	10
Neutral Sugar Analysis	10
Thin Layer Chromatography	11
Amino Acid Analysis	11
Ninhydrin Assay	12
Isolation of Glycopeptide	12
Gas-Liquid Chromatography	14
β-Elimination Studies	16
Fucose Assay	16
Glycosidase Digestion of Glycopeptide	18
Nelson-Somogyi Reducing Sugar Assay	19
Carboxyl Terminal Analysis of Glycopeptide	20
Amino Terminal Analysis of Glycopeptide	20
Sequence Analysis of Glycopeptide	22
High Voltage Electrophoresis	23
RESULTS	24
Neutral Carbohydrate Analysis	24
Thin Layer Chromatography	24
Amino Acid and Amino Sugar Analysis	26
Isolation of Glycopeptide	29
Characterization of Glycopeptide	32
High Voltage Electrophoresis	37

Page

Neutral Sugar Analysis by Gas-Liquid Chromatography 39
Glycosidase Digestion of the Glycopeptide 43
Carboxyl Terminal Analysis of Glycopeptide 45
Amino Terminal Analysis of Glycopeptide 47
Sequence Analysis of Glycopeptide 48

DISCUSSION 51

Neutral Sugars 51
Amino Sugar 54
Carbohydrate-Peptide Linkage 56
Structural Aspects of the Carbohydrate Study 59
Amino Acid Sequence Analysis of the Glycopeptide 63

SUMMARY AND CONCLUSIONS 67

LITERATURE CITED 70

LIST OF TABLES

Table		Page
I.	Percentage Neutral Sugar of Sainfoin Lectin	24
II.	R_f Values of Neutral Sugars by TLC	26
III.	Amino Acid Content of Sainfoin Lectin	27
IV.	Amino Acid Analysis of Glycopeptide	35
V.	β-Elimination of the Glycopeptide in Mild Base	39
VI.	Neutral Sugar Analysis by Gas-Liquid Chromatography	40
VII.	Amino Acid Analysis of the Glycopeptide After Hydrazinolysis	47
VIII.	Amino Acid Analysis of the Alkylated Glycopeptide	48
IX.	Amino Acid Sequence Analysis of Glycopeptide	49
X.	Representative Glycolectins and Their Carbohydrate Content	53

LIST OF FIGURES

Figure | Page

1. Standard Curve relating the optical density at 490 nm to μg of neutral sugar analyzed by the phenol-sulfuric acid method 25

2. Time hydrolysis study relating the levels of glucosamine observed with varying times of hydrolysis on the intact lectin 28

3. Standard curve relating the level of available amino leucine nitrogen to optical density at 570 nm 30

4. Liberation of amino acids during pronase digestion 31

5. Gel filtration of pronase digest on Sephadex G-50 . . 33

6. Gel filtration profile of pronase digest on Sephadex G-25 34

7. Time-hydrolysis study of the purified glycopeptide relating the levels of glucosamine and ammonia released as a function of the time of hydrolysis . . . 36

8. High voltage electrophoresis of the purified glycopeptide 38

9. Standard curves for the various sugar alditol acetates, relating μg of carbohydrate analyzed to peak height 41

10. Gas chromatography tracings of a standard mixture analysis, and a purified glycopeptide analysis for neutral sugars 42

11. Standard curve relating μg fucose to optical density as determined by the Dische-Shettles cysteine-sulfuric acid method 44

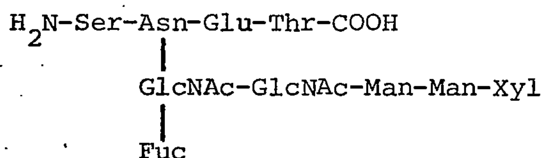
Figure		Page
12.	Standard curve relating μg reducing sugar to optical density at 520 nm, as determined by the Nelson-Somogyi method	46
13.	A) Schematic model demonstrating the H-bonding proposed by Marshall et al.	65
	B) Schematic model of the proposed hypothetical structure of the glycopeptide isolated from Sainfoin lectin	65

ABSTRACT

A lectin isolated from the seeds of sainfoin (Onobrychis viciifolia, Scop. var Eski) was shown to be a glycoprotein containing 2.6% neutral carbohydrate. Gas-liquid chromatographic analysis of sugar alditol acetates and amino acid analysis indicated the presence of fucose, xylose, mannose, and glucosamine in molar ratio (to protein monomer) 1:1:2:2.

A glycopeptide accounting for 70% of the total carbohydrate was isolated from pronase digests of the lectin by gel filtration chromatography. The molar composition of the glycopeptide was determined to be: $(Ser_1 Asx_1 Glx_1 Thr_1 ammonia_1 fucose_1 xylose_1 mannose_2 glucosamine_2)$.

The proposed structure for the glycopeptide, based on sequence analysis, is:



INTRODUCTION

In recent years many proteins have been shown to possess side chains of carbohydrate which are covalently bound to the protein, and numerous reviews have appeared dealing with the subject (1,2,3,4). Previously, carbohydrate found in association with most proteins was assumed to be an impurity, and measures were taken specifically to separate it from the protein. Improved methods of isolation, purification, and characterization have demonstrated that mixed polymers of sugars and amino acids are widely distributed in nature and participate in a wide variety of biological processes. In fact, it has become evident in the past ten years or so that the majority of proteins contain covalently bound carbohydrate and are classified as glycoproteins.

Glycoproteins can be simply defined as proteins containing covalently attached carbohydrate. Glycoproteins are widely distributed in nature, occurring not only in vertebrate and invertebrate systems, but also in plants, unicellular organisms, and even viruses.

The known or presumed functions of glycoproteins are diverse, spanning a wide range of vital biological activities (2). Almost all the proteins of plasma, with the notable exception of albumin, contain carbohydrate and fulfill such varied roles as transport, clotting, and antibody activity. Various hormones have been identified as glycoproteins (4). An increasing number of proteins with enzyme activity, including various hydrolases, oxidases, and transferases, have been

reported to contain covalently bound carbohydrate (5). They originate from a large variety of tissues and from organisms throughout the phylogenetic scale. The protective and lubricative roles of glycoproteins from epithelial secretions are well known. The members of the collagen family are glycoproteins, and they, along with the proteoglycans and various soluble glycoproteins, make up the bulk of the intercellular matrix which provides structural support to multicellular organisms.

But perhaps the most intriguing group of glycoproteins now being investigated are those found on the surfaces of cells. Although in mass they are only minor constituents of the plasma membrane, they are important because they are responsible, at least in part, for communicating the identify of the cell. Membrane glycoproteins can serve as antigenic determinants, virus receptors, markers of cellular identity, and appear to participate in cell-cell interactions.

Until recently, it was not appreciated that as a result of the possibility to form a large number of different structures from a small number of carbohydrate monomers, nature can use sugars for the synthesis of highly specific compounds that can act as carriers of biological information. It is well accepted that the carbohydrate moeities of membrane glycoproteins are ideally suited for the formation of specificity determinants that may be recognized by complementary structures on other cells or macromolecules.

It has been shown that the specificity of the major blood types are determined by the sugars present on the red cell surface (6). N-acetylgalactosamine is the immuno-determinant of blood type A, and galactose of blood type B. Interestingly, enzymic removal by specific glycosidases of N-acetylgalactosamine from type A erythrocytes, or of galactose from type B erythrocytes will convert both to type O erythrocytes.

Another interesting example of cell surface specificity is the so-called "homing of lymphocytes" (7). In this phenomenon, rat lymphocytes, injected into the rat through its tail, will migrate to the spleen. However, if prior to injection the lymphocytes were treated with a specific glycosidase to remove L-fucose from their surface, the lymphocytes migrated to the liver instead, as if the fucose on their surface dictated to the lymphocytes their destination.

An exciting development in the study of glycoproteins has been the discovery of the role of carbohydrates on the clearance and survival of glycoproteins in circulating blood, and their uptake by the liver (8). It has been shown that sialic acid is essential for the prolonged survival of most plasma glycoproteins in the circulation. Even partial removal of the sialic acid residues present results in prompt clearance of the glycoprotein by the liver. Galactose is very often the sugar pentultimate to the sialic acid residues at the non-reducing terminus. The rapid removal from circulation of the

asialoglycoproteins was shown to depend upon the presence of non-reducing unmodified terminal galactose residues. These observations show that intact terminal galactose residues are required for recognition by the plasma membrane of the liver cells.

It is now rather well accepted that alterations in sugar structure and architecture on cell surfaces are intimately connected with the process of malignant transformation. This has become an area of intense activity because of the widespread interest in the study of cancerous diseases. Some of the changes in cell surface structure can be detected and studied by the use of plant agglutinins, called lectins.

Lectins are the largest single group of plant glycoproteins known (9,10,11,12). They are predominantly glycoproteins, although there are a few notable exceptions, such as Concanavalin A and wheat germ agglutinin which are simple proteins. Lectins are distinguished as a class by their ability to preferentially bind certain specific sugars or carbohydrate structures. Since they characteristically contain multiple binding sites, due to their aggregated structure, they are capable of causing agglutination of cells containing the specific receptor carbohydrate. Lectins are predominantly plant glycoproteins, but they have now been shown to occur in snails and fish (13,14,15,16) and recently in mammalian liver (17,18). These cell agglutinating and sugar specific proteins have been known since the turn of the century but have only recently become the focus of

intense interest in a large number of laboratories. Lectins exhibit a host of interesting and unusual chemical and biological properties.

Lectins can agglutinate erythrocytes, in some cases with a very high specificity and are sometimes utilized in blood typing and study of the chemical structure of the blood group substances. They can specifically bind and precipitate polysaccharides and glycoproteins, and are finding wide applicability in affinity chromatography. Some lectins are mitogenic, that is, they stimulate the conversion of resting lymphocytes into actively growing and dividing blast-like cells. Because of these and other properties, lectins provide a useful tool for the study of specific binding sites on protein molecules. Because of their mitogenic activity, they are being used to study the biochemical events occurring in the initiation of cell division. Most important, however, is their ability to preferentially agglutinate malignant cells which has resulted in a growing use of lectins in investigating the architecture of cell surfaces and studying the changes which cells undergo upon transformation to malignancy.

The role of lectins, in nature, whether in plants or other organisms, is still unknown. It has been suggested that they function as antibodies to counteract soil bacteria (19,20,21); that they serve to protect plants against fungal attack by inhibiting fungal polysaccharidases (22); that because of their affinity for sugars they are involved in sugar transport and storage (9); that they serve for the

attachment of glycoprotein enzymes in organized multienzyme systems (9); that they are involved as attachment sites for symbiotic bacteria (23); or that they are involved in the transport of glycoproteins across membranes (24). Because of the mitogenic properties of lectins, some feel they may be involved in the control of cell division and seed germination in plants (9). Conclusive evidence is lacking either for or against any of these hypotheses.

Over a thousand different lectins have been isolated from various sources, and over fifty of them have been purified. Although lectins have been extensively studied during the last decade, little is known about their structure. Only for one lectin, concanavalin A, were both the sequence and the three-dimensional structure determined (25). Recently, the first 25 residues of the amino-terminal sequence of the β chains from lentil and pea lectins, of soybean and peanut agglutinins, and of the R & L subunits of phytohemagglutinin (PHA) were compared (26). Extensive homologies were found, ranging from near identity in the β chains of lentil and pea lectins, to 24% identity between soybean agglutinin and PHA.

Additional studies have compared the α and β chains of the lentil lectin to the amino acid sequence of concanavalin A (27). The α chain consists of 52 residues and the whole chain is homologous with the region between positions 72 and 121 from concanavalin A. The amino-terminal sequence of the β chain is homologous to another portion of

the concanavalin A molecule, between positions 123 and 165. This extensive homology suggests that the lentil α and β chains may be proteolytic fragments derived from a single polypeptide chain of the same length as concanavalin A. In addition, despite differing sugar binding specificities, a common ancestry for the genes coding for leguminous lectins appears likely.

In the few cases that have been carefully examined, such as concanavalin A (28), the pea lectin (29), and the lima bean lectin (30), lectins appear to require metals, particularly divalent cations for their activity. It has been suggested that all lectins are metalloproteins (31), but evidence for this is scanty at present.

Of the fifty odd lectins which have been purified, only a handful of them has been investigated with any detail as to the chemistry of their carbohydrate moeities. Little is known about the function of the carbohydrate moeities of lectins.

This study is concerned with investigations on the carbohydrate moeity of a lectin isolated from the seeds of the legume, Sainfoin (Onobrychis viciifolia, Scop.), variety Eski. Sainfoin lectin has been established as a glycoprotein, but little work has been done concerning the quantitative and structural aspects of the carbohydrate portion. Studies involve primarily identification and quantitative estimation of the various neutral sugars and amino sugars present, as well as

elucidation of the nature of the unique structural aspect of glycoproteins, the covalent carbohydrate-peptide bond.

Hopefully, the future characterizations and experimentation with the glycosyl portion of lectins may lead to a better understanding of the role of both lectins and their carbohydrate moeities.

RESEARCH OBJECTIVES

Studies on the carbohydrate portions of glycoproteins can be divided into four major areas of study:

1. The identification and quantitative estimation of the various monosaccharides present.

2. Determination of the nature of the carbohydrate-peptide linkage.

3. Determination of the amino acid sequence on either side of the carbohydrate-peptide linkage site.

4. Determination of the sequence and chemical structure of the carbohydrate side chains.

The objectives of this study are to answer the questions posed by the first three major areas of study mentioned above.

EXPERIMENTAL PROCEDURE

Materials

Lectin used in these studies was purified from the seeds of Sainfoin (Onobrychis viciifolia, Scop.), variety Eski, by ammonium sulfate fractionation and affinity chromatography, according to the method of Hapner (32). Sephadex G-50 (Fine) and G-25 (Fine) were purchased from Pharmacia Fine Chemicals. Pronase-CB was a product of Calbiochem. The packing material (3% ECNSS-M on Gas Chrom Q) and glass columns for gas-liquid chromatography were purchased from Applied Science Laboratories, Inc. α-Mannosidase type III (Jack Bean), α-L-fucosidase from bovine epididymis, and β-N-acetylglucosaminidase (Jack Bean), were obtained from Sigma Chemical Company. Dansylated amino acid standards were obtained from Seikagaku Kogyo Company, Limited. Polyamide sheets for thin layer chromatography of the dansyl-amino acids were purchased from Gallard Schlesinger Chemical Corporation. All chemicals used were of reagent grade.

Neutral Sugar Analysis

For the colorimetric determination of neutral sugars, a modification of the phenol-sulfuric acid method of Dubois (33) was used. The procedure was modified to a microscale determination according to Misaki et al. (34), by reducing the volume in the original procedure as follows: 1 ml of an aqueous solution of carbohydrate containing 0.02 to 0.2 μMole carbohydrate, 0.5 ml of 5% phenol, and 3 ml of

concentrated sulfuric acid. After mixing, the samples were allowed to incubate for one hour and the absorbance determined at 490 nm. D-Mannose was used to prepare the standard curves.

Thin Layer Chromatography

Thin layer chromatography plates were prepared and developed according to Supelco bulletin, April, 1977 (55). The plates were prepared using Silica Gel H in 0.15 M KH_2PO_4. The solvent system used was n-butanol:acetone:water in the ratio (V/V) of 4:5:1. The staining solution used was diphenylamine-aniline in phosphoric acid, according to Brown (35).

Amino Acid Analysis

Samples for amino acid analysis were hydrolyzed for 22 hours at 110°C in constant boiling HCl in sealed, evacuated tubes. Samples for amino sugar analysis were hydrolyzed for 6 hours, 14 hours, and 22 hours in sealed evacuated tubes at 110°C with constant boiling HCl. The amino acid and amino sugar contents of the hydrolyzates were then determined with a Beckman Model 120C amino acid analyzer by the method of Spackman et al. (36).

Analyses for the presence of hydroxyproline were performed with a modification of the method of Spackman according to Miller and Piez (37). The long column buffer was titrated to pH 2.9 with 12M HCl.

With this buffer change, the hydroxyproline eluted at 70 minutes and aspartic acid eluted at 78 minutes.

Ninhydrin Assay

The levels of available amino nitrogen were determined in the pronase digestions with the ninhydrin reagent according to the method of Cocking and Yemm (38).

One ml of an amino acid solution containing 0.05-2.8 μg N (amino nitrogen) was mixed with 0.5 ml of 0.2 M sodium citrate buffer (pH 5.0). Ninhydrin reagent (0.2 ml) (5% ninhydrin in methyl cellosolve, W/V) was added to the sample followed by addition of 1 ml of a 2% solution of 0.01 M NaCN in methyl cellosolve, V/V. The samples were heated in a boiling water bath for 15 minutes, cooled to room temperature and the absorbance determined at 570 nm. Standard curves were prepared using leucine as standard.

Isolation of Glycopeptide

A glycopeptide was isolated after pronase digestion of the purified lectin by a modification of the method of Misaki and Goldstein (33). Purified lectin, 90-100 mg, was dissolved in 200 ml of 0.05 M phosphate buffer, pH 7.8, which also contained 2.5×10^{-4} M $CaCl_2$. A few drops of toluene were added to inhibit the growth of contaminating organisms. Two point five mg of Pronase-CB (Calbiochem Lot #530160,

86,400 proteolytic units Kaken/gram) was added to the solution, and incubated at 37°C in a shaker bath.

Monitoring the extent of digestion and determination of the amino acids released from the lectin were performed with the ninhydrin reagent according to the method of Cocking and Yemm (38). After 48 hours of incubation, an additional 2.5 mg of Pronase-CB was added and the incubation was carried on for an additional 48 hours. At the end of this period (96 hr), the solution was concentrated by flash evaporation to a total volume of 25 ml. Two point five mg of Pronase-CB was added again and allowed to incubate for an additional 48 hours, by which time the liberation of additional amino acids had ceased.

The digest was concentrated by flash evaporation to 5 ml, the pH adjusted to 3.5 with glacial acetic acid, and centrifuged for 15 minutes at 10,000 g. The clear supernatant was applied to a column of Sephadex G-50 Fine (2x88 cm) which had been previously equilibrated with 0.01 M acetic acid (pH 3.5), and eluted with the same buffer. The carbohydrate containing fractions, determined by the phenol-sulfuric acid method (33), were pooled, concentrated to 5 ml, and applied to the Sephadex G-50 column a second time. The carbohydrate containing portion was pooled, concentrated by flash evaporation to 3 ml and applied to a column of Sephadex G-25 Fine (2.5x97 cm) which had been previously equilibrated with 0.01 M acetic acid (pH 3.5) and eluted with the same buffer. The carbohydrate containing fractions

were pooled and used in subsequent glycopeptide studies for neutral sugars, amino sugars, and amino acids.

Gas-Liquid Chromatography (GLC)

A modification of the procedure of Albersheim (39), as per Griggs et al. (40), was used for the preparation of samples for gas-liquid chromatography.

One ml of 2 N trifluoracetic acid was added to the sample, which contained 50-100 µg of carbohydrate, as estimated by the phenol-sulfuric acid method. Prior to hydrolysis, 50 µg of inositol was added as an internal standard. The tubes were sealed and incubated at 120°C for one hour. The hydrolyzate was then flash evaporated to dryness at room temperature, to remove the residual trifluoracetic acid. The residue was redissolved in a small volume of water and passed over a small column (0.5 ml) of Dowex 50W-X2 (H^+ form), 200-400 mesh. The eluate was flash evaporated to dryness, and 1 ml of 1 N NH_4OH containing 1 mg of $NaBH_4$ was added. After incubation for one hour at room temperature, the solution was neutralized by the dropwise addition of glacial acetic acid until effervescence ceased, and evaporated to dryness. One ml of a methanol:benzene (5:1) mixture containing a drop of glacial acetic acid was added to the residue and allowed to incubate for 10 minutes at room temperature, and evaporated to dryness. The evaporation procedure was repeated four more times

using 1 ml of methanol, with a drop of glacial acetic acid added, each time. The evaporation procedure serves to remove the borate formed as the volatile methyl borate. One ml of acetic anhydride was added to the residue, the tube sealed, and incubated at 100°C for one hour. After acetylation the excess acetic anhydride was removed by flash evaporation at room temperature. The alditol acetates were then removed from the inorganic residue utilizing three extractions with 0.5 ml methylene chloride. The supernatants were taken to dryness with a stream of filtered air and resuspended in 50 µl of chloroform, of which 1 µl was then injected for GLC analysis.

Gas-liquid chromatography was performed on a Varian 3700 gas chromatograph, equipped with a hydrogen flame detector, and a Varian Model 9176 recorder. The samples were analyzed on a glass column (6'x2 mm) packed with 3% ECNSS-M on Gas Chrom Q (110 to 120 mesh, Applied Science). The samples were developed isothermally at 200°C, injector temperature 220°C, and attenuation of 4×10^{-10} amps/mV.

Standard curves were prepared for each monosaccharide-alditol acetate, relating µg of carbohydrate to peak height. Relative carbohydrate contents were subsequently obtained from the standard curves, and corrected for handling losses utilizing the inositol internal standard.

β-Elimination Studies

β-elimination studies were carried out by a modification of the method of Spiro (41). An aliquot of glycopeptide (0.087 μMole) was incubated for 14 hours at room temperature in 0.25 ml of 0.5 N NaOH. To an additional 0.087 μMole of glycopeptide, 0.25 ml of 0.5 N NaOH and 0.25 ml of 0.5 N HCl was added. The second (neutralized) aliquot was also incubated at room temperature for 14 hours, and serves as a control for non-specific destruction of serine or threonine.

After incubation, the alkaline sample was neutralized with 6 N HCl. Experimental and control samples were evaporated to dryness, and hydrolyzed in constant boiling HCl at 110°C for six hours. Following hydrolysis, the two samples were dried under high vacuum, and redissolved in 1 ml of 0.075 N sodium citrate buffer, pH 2.20, to give a final Na^+ concentration of 0.2 N. The amino acid content of the samples were determined according to the method of Spackman et al. (36).

Fucose Assay

Fucose levels were determined colorimetrically by the cysteine-sulfuric acid method of Dische and Shettles (42).

The assay is carried out on standards and unknowns containing 2-20 μg of fucose in one ml of water. A water blank is also analyzed. The samples and the sulfuric acid reagent are precooled in an ice bath before mixing. The sulfuric acid reagent is prepared by mixing six

volumes of conc H_2SO_4 and one volume of water. Four point five ml of the cold H_2SO_4 reagent is added to each sample. The samples are brought to room temperature, and subsequently immersed in a boiling water bath for exactly three minutes. After cooling to room temperature 0.1 ml of an aqueous 3% cysteine-HCl solution is added to each tube, and the absorbance read after 1-2 hours at 396 nm and a wavelength close to 427 nm.

Absorbance at 396 nm resulting from hexoses is corrected for by measuring the absorbance of the sample both at 396 nm and at a wavelength close to 427 nm where the hexose absorption due to the symmetrical shape of its spectrum, is equal to its absorption at 396 nm. The precise wavelength for the second reading is determined for each run by analyzing a 50 µg sample of mannose and determining the exact wavelength where its absorbance is equal to that at 396 nm. The difference in optical density between 396 nm and 427 nm is directly proportional to the fucose content of the sample.

To correct for any non-specific color development resulting from the action of the H_2SO_4 on the peptide portion of the sample, an aliquot of the sample is heated with the H_2SO_4, but no cysteine is added after heating. The absorption due to fucose in a given sample is then determined by subtracting the $OD_{396} - OD_{427}$ of the sample analyzed without cysteine, from the $OD_{396} - OD_{427}$ of the sample analyzed with cysteine.

Glycosidase Digestion of Glycopeptide

α-Mannosidase and β-N-acetylglucosaminidase digestions were carried out according to the procedure of Kawasaki and Ashwell (45). α-L-Fucosidase digestions were carried out as described by Misaki and Goldstein (34). An aliquot of glycopeptide (0.1 μMole) was flash evaporated to dryness in a 13x100 mm screw cap test tube. The glycopeptide was redissolved in 1 ml of 0.02 M sodium acetate buffer, pH 4.5. Finally, 1.0 unit of α-mannosidase was added, and the mixture incubated at 37°C for 24 hours in a shaker bath. One unit of α-mannosidase will hydrolyze 1 μMole of p-nitrophenyl-α-mannoside to p-nitrophenol and D-mannose/min at 37°C. Two controls were incubated along with each sample, one tube containing 0.1 μMole of glycopeptide in 1 ml of sodium acetate buffer, and the second tube containing 1 unit of enzyme in 1 ml of sodium acetate buffer. Following incubation, the tubes were assayed for the presence of reducing sugar by the Nelson-Somogyi method (46).

In the β-N-acetylglucosaminidase digestion, 0.1 μMole of glycopeptide was dissolved in 1 ml of 0.02 M sodium acetate buffer, pH 5.8, and one unit of β-N-acetylglucosaminidase was added. One unit of β-N-acetylglucosaminidase will hydrolyze 1.0 μMole of p-nitrophenyl-β-N-acetylglucosamine to p-nitrophenol and D-N-acetylglucosamine/min at 37°C. The mixture and controls were incubated for 24 hours at 37°C in a shaker bath, and assayed for the presence of reducing sugar.

α-L-Fucosidase digestions were carried out in 0.05 M sodium acetate buffer (pH 4.0). An aliquot of glycopeptide (0.1 μMole) was dissolved in 1 ml of sodium acetate buffer. After addition of 0.05 unit of α-L-fucosidase, the mixture and controls were incubated for 24 hours at 37°C in a shaker bath, and assayed for the presence of reducing sugar. One unit of α-L-fucosidase will hydrolyze 1 μMole of p-nitrophenyl-α-fucoside to p-nitrophenol and α-L-fucose/min at 37°C.

Nelson-Somogyi Reducing Sugar Assay

Assays for reducing sugar released after incubation of the glycopeptide with glycosidases was performed using the method of Nelson and Somogyi (46).

The reaction is carried out in 13x100 mm screw cap test tubes to minimize the surface area available for reoxidation. To samples containing 10-100 μg of reducing sugar in 1 ml of water, is added 1 ml of the copper reagent, as well as to a water blank. A mannose standard is also analyzed with each run. After mixing, the tubes are immersed in a boiling water bath for 30 minutes. After cooling to room temperature, 1 ml of the arsenomolybdate reagent is added to each tube. Finally, 3 ml of water is added to each tube, mixed well, and the absorbance determined at 520 nm. Standard curves were prepared using D-mannose.

Carboxyl Terminal Analysis of Glycopeptide

Hydrazinolysis was used to determine the carboxyl terminal amino acid of the glycopeptide according to the method of Fraenkel-Conrat and Tsung (43).

An aliquot of the glycopeptide (0.1 μMole) was flash evaporated to dryness in an ignition tube. Hydrazine (0.2 ml) was added to the glycopeptide, and the tube sealed under vacuum. The mixture was then incubated at 80°C for 24 hours. After hydrazinolysis, the tube was opened and the hydrazine removed under high vacuum over concentrated H_2SO_4 and P_2O_5. The entire sample was analyzed on the amino acid analyzer according to the method of Spackman (36).

Amino Terminal Analysis of Glycopeptide

The amino terminus of the glycopeptide was investigated by two different methods. The first utilizes the dansyl chloride technique of Gray (44), and the second alkylation under alkaline conditions and amino acid analysis.

To 0.1 μMole of the glycopeptide in a small glass tube (6x50 mm), 15 μℓ of 0.2 N sodium bicarbonate was added, and the sample evaporated to dryness with a stream of filtered air. Fifteen μℓ of water and 15 μℓ of the dansyl-Cl reagent was added to the tube. The dansyl-Cl reagent is prepared by dissolving 2.5 μg of dansyl-Cl (dimethyl-aminonapthalenesulfonyl chloride) in 1 ml of acetone. The tube was

sealed with parafilm and incubated in the dark at 40°C for one hour. Following incubation, the solution was evaporated to dryness, and 0.1 ml of constant boiling HCl was added. The tube was sealed and incubated at 110°C for six hours. Following hydrolysis, the tube was opened and the solution taken to dryness under high vacuum. The residue was taken up in a drop of ethanol, and spotted on the corner of a 5x5 cm polyamide sheet.

The sheet is then developed in the first dimension with 1.5% (V/V) formic acid in water. After drying well, the sheet is developed in the second dimension in 9:1 (V/V) toluene:glacial acetic acid. Finally, the sheet is dried and again developed in the second dimension with ethyl acetate:methanol:glacial acetic acid 20:1:1 (V/V/V). The sheet is dried and examined under a UV lamp. The fluorescent dansylated amino derivatives are identified by comparison with known dansylated amino acids, which are run simultaneously on the opposite side of the polyamide sheet.

In the alkylation reaction, 10 nMole of glycopeptide was dissolved in 100 µℓ of 0.2 M sodium bicarbonate. One mg of iodoacetic acid dissolved in 20 µℓ of 0.1 N NaOH was added, the tube sealed with parafilm, and incubated at 50°C for 24 hours. The tube was evaporated to dryness with a stream of filtered air, and 0.2 ml of constant boiling HCl added. The tube was sealed and incubated for 6 hr at

110°C. After hydrolysis the mixture was analyzed on the amino acid analyzer according to Spackman et al. (36).

Sequence Analysis of Glycopeptide

Sequencing of the glycopeptide was carried out by a modification of the subtractive Edman degradation as described by Konigsberg (47). The glycopeptide is first coupled to phenyl isothiocyanate under alkaline conditions to form the N-substituted thiocarbamyl peptide. The coupling reaction is carried out by dissolving 30 n Mole of glycopeptide in 0.5 ml of redistilled pyridine, to which 50 µl of phenyl isothiocyanate is added. The mixture is incubated at 37°C under N_2 for two hours. After coupling, excess reagents and solvents are removed by evaporation under high vacuum. The derivitized glycopeptide is then cyclized by treatment with anhydrous triflouracetic acid. This results in cleavage of the amino terminal amino acid as the thiazolinone, and exposes the α-amino group of the pentultimate amino acid. Cyclization is carried out by adding 0.5 ml of anhydryous triflouracetic acid to the dried peptide, and incubating at 37°C, under N_2, for one hour. Following incubation, the triflouracetic acid is removed by evaporation under a stream of N_2. The thiazolinone is converted to the N-substituted thiohydantoin by dissolving the residue in 2 ml of 0.2 M glacial acetic acid and incubating at 60°C for 10 minutes. The mixture is then extracted with ethyl acetate to remove the thiohydantoin formed from the terminal amino acid. An aliquot of

the aqueous phase is dried, hydrolyzed, and analyzed for amino acid content, and a decrease in the level of an amino acid reveals its presence at the amino terminal. A second aliquot of the aqueous phase is alkylated as described earlier, hydrolyzed and examined for amino acid content. A decline in the level of a second amino acid identifies it as the pentultimate amino terminal residue. Finally, the remainder of the aqueous phase can then be subjected to another cycle of Edman degradation, followed by alkylation and amino acid analysis to identify the third amino acid from the amino terminal.

High Voltage Electrophoresis (HVE)

High voltage electrophoresis was carried out as described by Glazer et al. (48). HVE was performed with a Shandon Model L24. Electrophoresis was carried out at pH 6.5 using a buffer system consisting of 50 ml of pyridine, 2 ml of glacial acetic acid, and 900 ml of water. Samples containing from 20 to 100 nMoles of standards or peptides were spotted 9 inches from the positive electrode end of 57x20 cm sheets of Whatman 3 M paper. After electrophoresing for 1/2 hour at 4000 volts, the papers were dried and stained with a reagent consisting of 0.25% ninhydrin in n-butanol.

RESULTS

Neutral Carbohydrate Analysis

The standard curve for the microdetermination of neutral sugar by the phenol-sulfuric acid method is shown in Figure 1, and relates μg of neutral sugar (mannose used as standard) to optical density at 490 nm. The plot is linear within the range of 0-50 μg neutral sugar, and assays exhibited reproducible results. Analysis of the intact purified lectin for the presence of neutral sugar, established that sainfoin lectin was indeed a glycoprotein. The levels of carbohydrate detected with varying amounts of lectin are shown in Table I, and yield an average value of 2.6% neutral sugar by weight.

Table I. Percentage Neutral Sugar of Sainfoin Lectin

Lectin (mg)	Neutral Sugar (μg)	% Neutral Sugar
1.03	25.0	2.43
0.77	20.0	2.60
0.52	14.5	2.79
	Aver. =	2.60

Thin Layer Chromatography (TLC)

The R_f values determined by TLC for the neutral sugars typically found in glycoproteins are shown in Table II. Aliquots (1 mg) of the lectin were hydrolyzed for two hours in 2 N triflouracetic acid (TFA). The excess TFA was removed by flash evaporation, the hydrolyzate

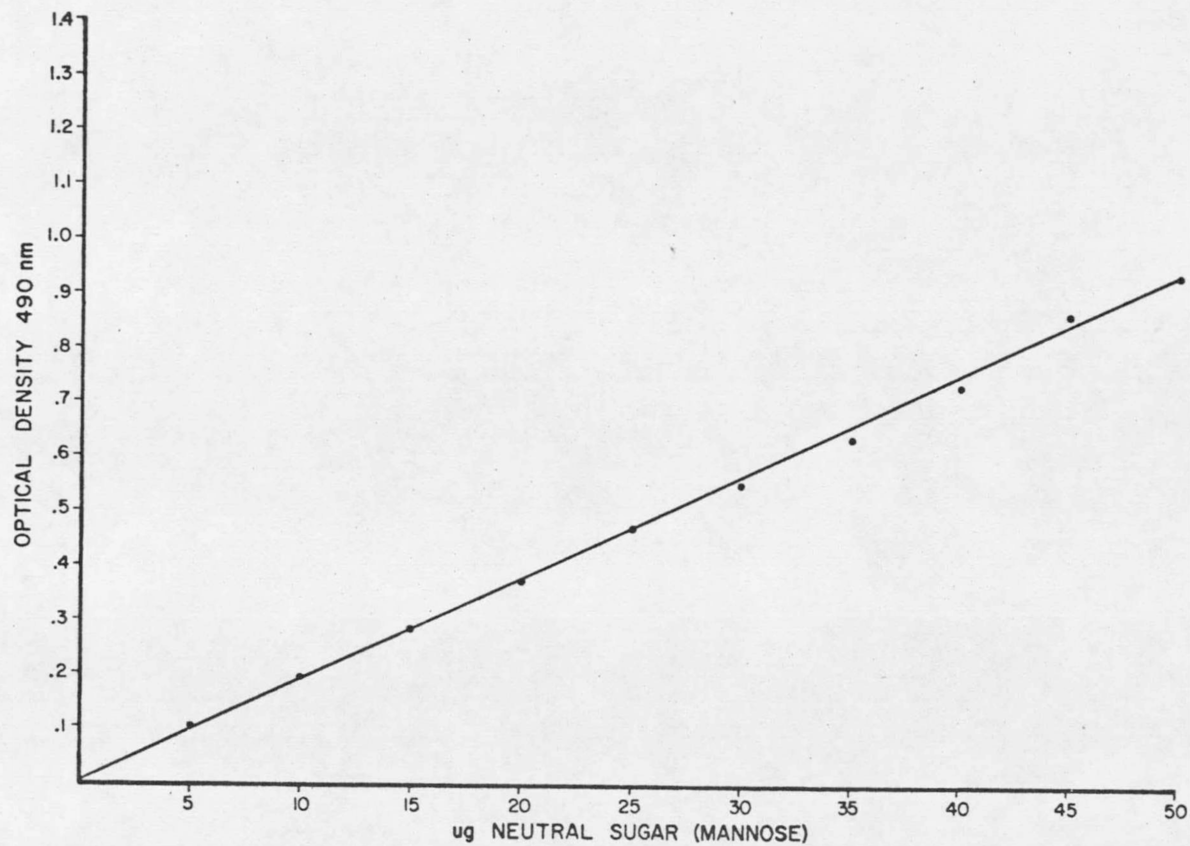


Fig. 1. Standard curve relating the optical density at 490 nm to μg of neutral sugar analyzed by the phenol-sulfuric acid method. D-Mannose was used as standard.

passed over a small column of Dowex 50-x-2 (H^+ form), and the eluate analyzed by TLC. Two spots were detected, one with an R_f value of 0.30, and one with an R_f value of 0.39. The spot with an R_f of 0.30 suggests the presence of mannose. The spot with an R_f of 0.39 could indicate either fucose or xylose, as resolution between the two was insufficient for positive identification.

Table II. R_f Values of Neutral Sugars by TLC

Neutral Sugar	R_f
Galactose	.23
Glucose	.28
Mannose	.31
Arabinose	.34
Fucose	.38
Xylose	.41

Amino Acid and Amino Sugar Analysis

Duplicate samples of the purified lectin were hydrolyzed and the amino acid content determined on the amino acid analyzer. The results expressed as µ Mole amino acid/µ Mole lectin, along with values previously obtained by Hapner (32) are shown in Table III.

A time hydrolysis study for the quantitative determination of amino sugars was performed. Samples of purified lectin were hydrolyzed in constant boiling HCl for 6, 14, and 22 hours, and the amino sugar

Table III. Amino Acid Content of Sainfoin Lectin

Amino Acid	Sample #1	Sample #2	Previous Results
Lysine	12.23	12.59	12.0
Histidine	4.00	4.04	4.0
Arginine	12.81	12.00	12.0
Aspartic Acid	35.84	35.50	34.0
Threonine	20.76	19.82	21.0
Serine	26.26	24.90	27.0
Glutamic Acid	19.76	19.20	19.0
Proline	11.46	11.00	11.0
Glycine	17.65	17.20	16.0
Alanine	13.11	12.30	12.0
1/2 Cysteine	0.0	0.0	0.0
Valine	18.58	17.95	18.0
Methionine	0.0	0.0	0.0
Isoleucine	13.11	12.83	13.0
Leucine	19.22	18.20	18.0
Tyrosine	7.51	7.29	7.0
Phenylalanine	16.27	15.88	17.0
Tryptophan	--	--	6.0

Values are expressed as μ Mole Amino Acid/μ Mole lectin, assuming a molecular weight of 27,000 as determined by Hapner (32).

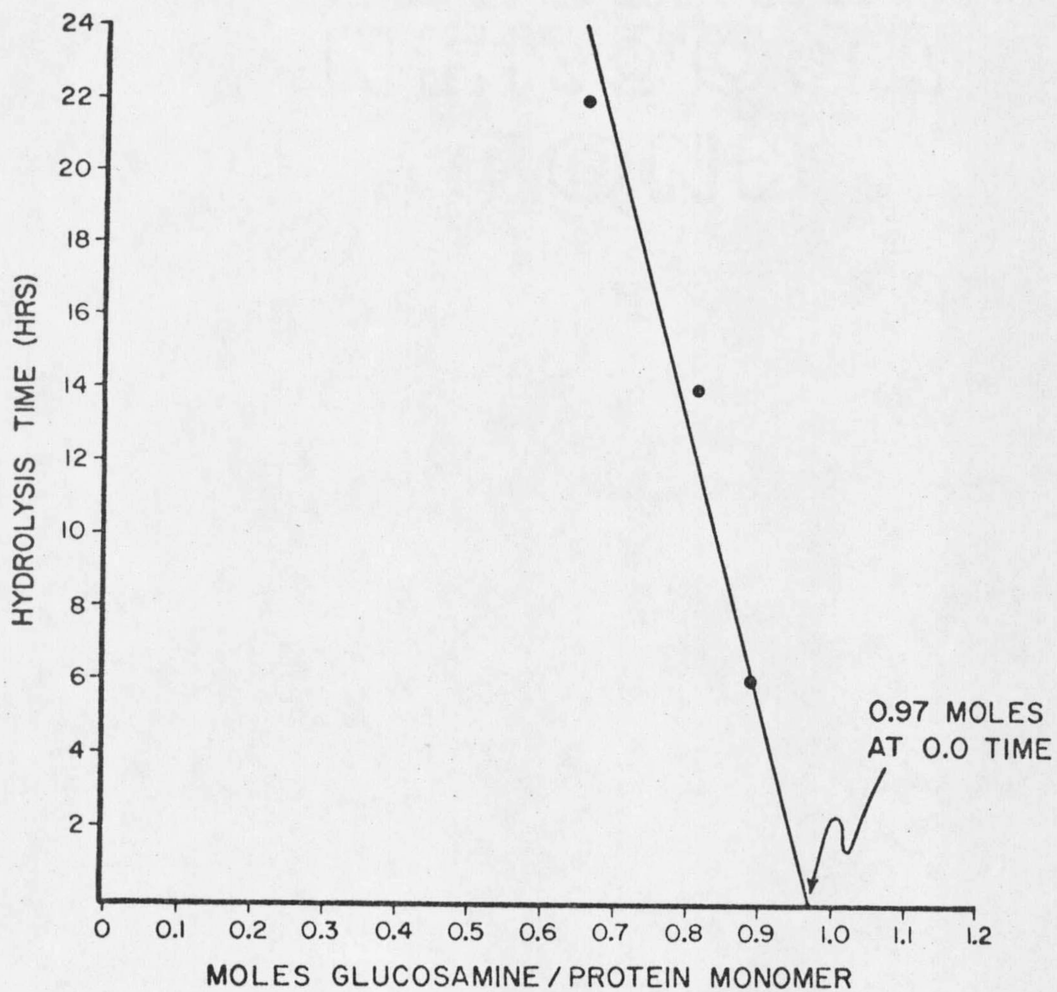


Fig. 2. Time hydrolysis study relating the levels of glucosamine observed with varying times of hydrolysis. Hydrolysis was carried out on the intact lectin in constant boiling HCl.

content determined on the amino acid analyzer. The only amino sugar detected was glucosamine, and the yield of glucosamine as a function of the time of hydrolysis is shown in Figure 2.

As expected, the yield of glucosamine declines with extended hydrolysis times. If, however, the time vs. yield plot is extrapolated back to zero time, we obtain a value of 0.975 μ Mole glucosamine/μ Mole of lectin. It is assumed that the glucosamine detected is a breakdown product of the acid hydrolysis, and exists as N-acetylglucosamine in the intact lectin.

Hydroxyproline has been found to occur in several plant glycoproteins, and the lectin was analyzed for the presence of hydroxyproline. Amino acid analysis under conditions which allow the detection of hydroxyproline (Materials and Methods) showed the absence of detectable amounts of hydroxyproline.

Isolation of Glycopeptide

The purified lectin was digested extensively with Pronase-CB as described earlier. The progress of the digestion and the liberation of amino acids were monitored using the ninhydrin reagent. A standard curve relating the μg of available leucine amino nitrogen to optical density at 570 nm was constructed, and is shown in Figure 3. Figure 4 illustrates the progress of a typical digestion as a function of time. The level of amino acids liberated are plotted as percent of

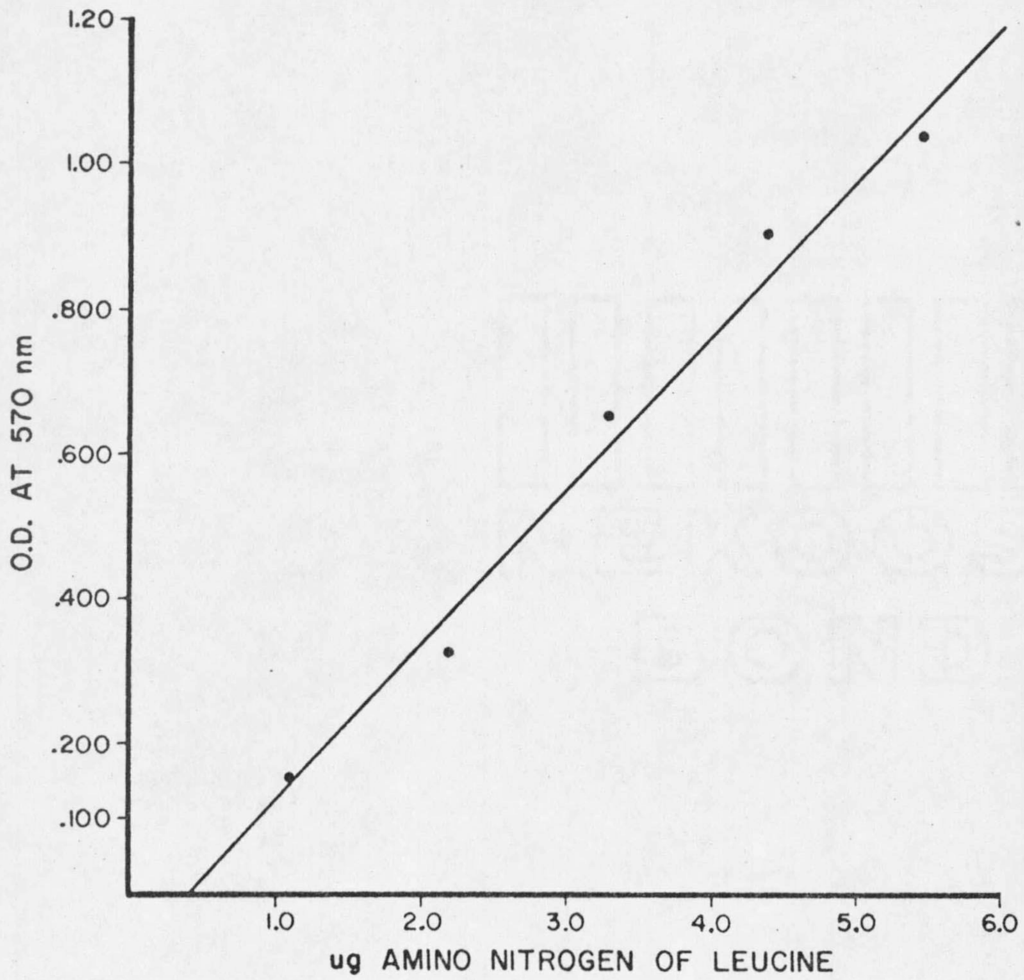


Fig. 3. Standard curve relating the level of available amino leucine nitrogen to optical density at 570 nm.

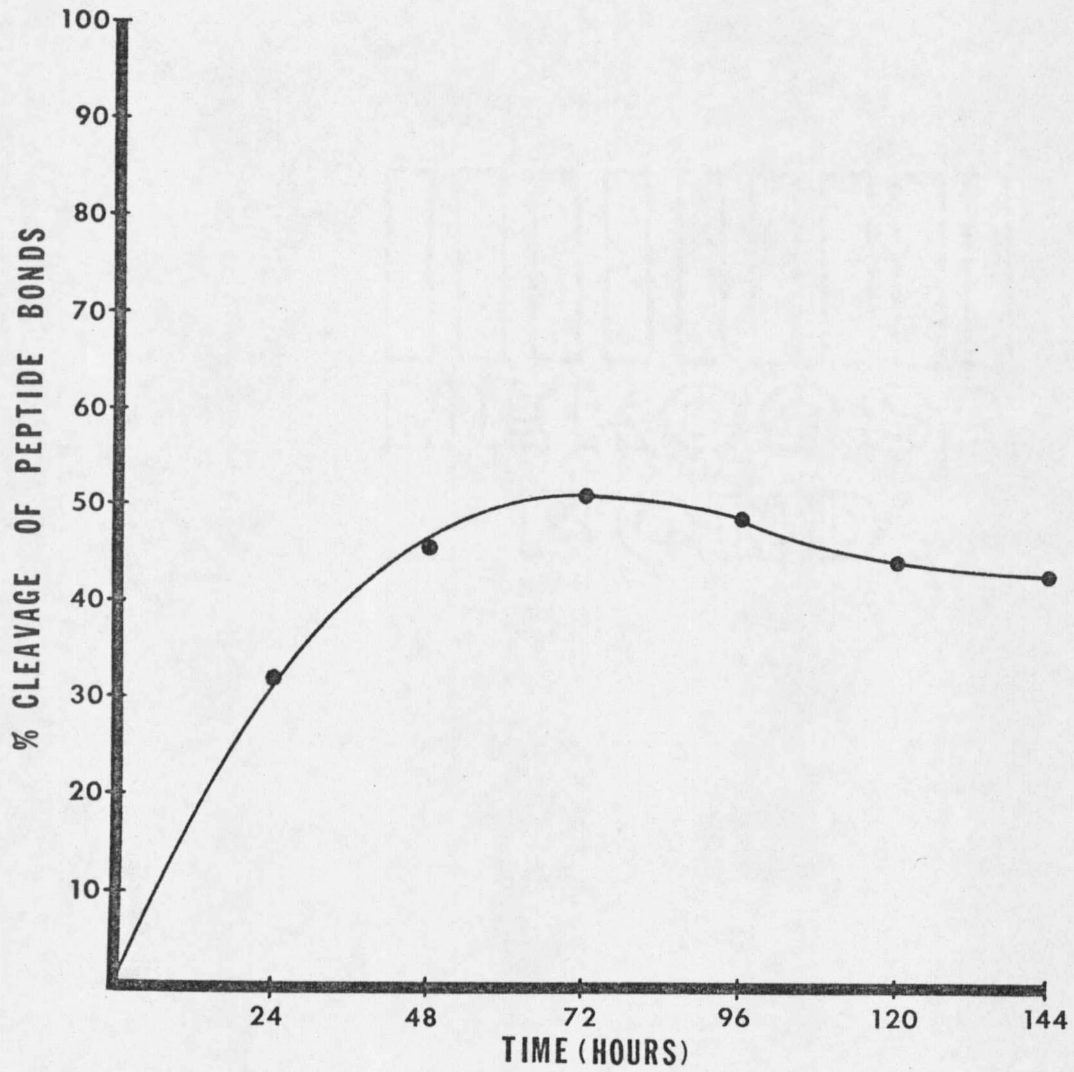


Fig. 4. Liberation of amino acids during a typical pronase digestion. The level of amino acids liberated are plotted as the percent of theoretical yield.

theoretical yield (complete digestion to single amino acids). The apparent percent cleavage declines after 72 hours of digestion. It was observed that a precipitate began to occur at that point, and visual accumulation of precipitate paralleled the decline in available amino nitrogen.

After the liberation of amino acids had ceased, the mixture was concentrated to a small volume (5 ml) and applied twice to a Sephadex G-50 column. The gel filtration profile of the pronase digest on the second Sephadex G-50 run is shown in Figure 5. Tube numbers 60-75 were pooled, concentrated to 3 ml and applied to a column of Sephadex G-25. The gel filtration profile of the digest on Sephadex G-25 is shown in Figure 6. Tube numbers 68-78 were pooled and used for subsequent glycopeptide characterization. A typical glycopeptide preparation contained between 60-70% of the original neutral sugar of the intact lectin, as determined by the phenol-sulfuric acid method.

Characterization of Glycopeptide

Aliquots of approximately 0.1 μ Mole of glycopeptide (calculated according to the amount of neutral sugar present) were hydrolyzed in constant boiling HCl for 6 hours and 22 hours at 110°C, and analyzed on the amino acid analyzer. Two separate glycopeptide preparations were analyzed and the results are shown in Table IV.

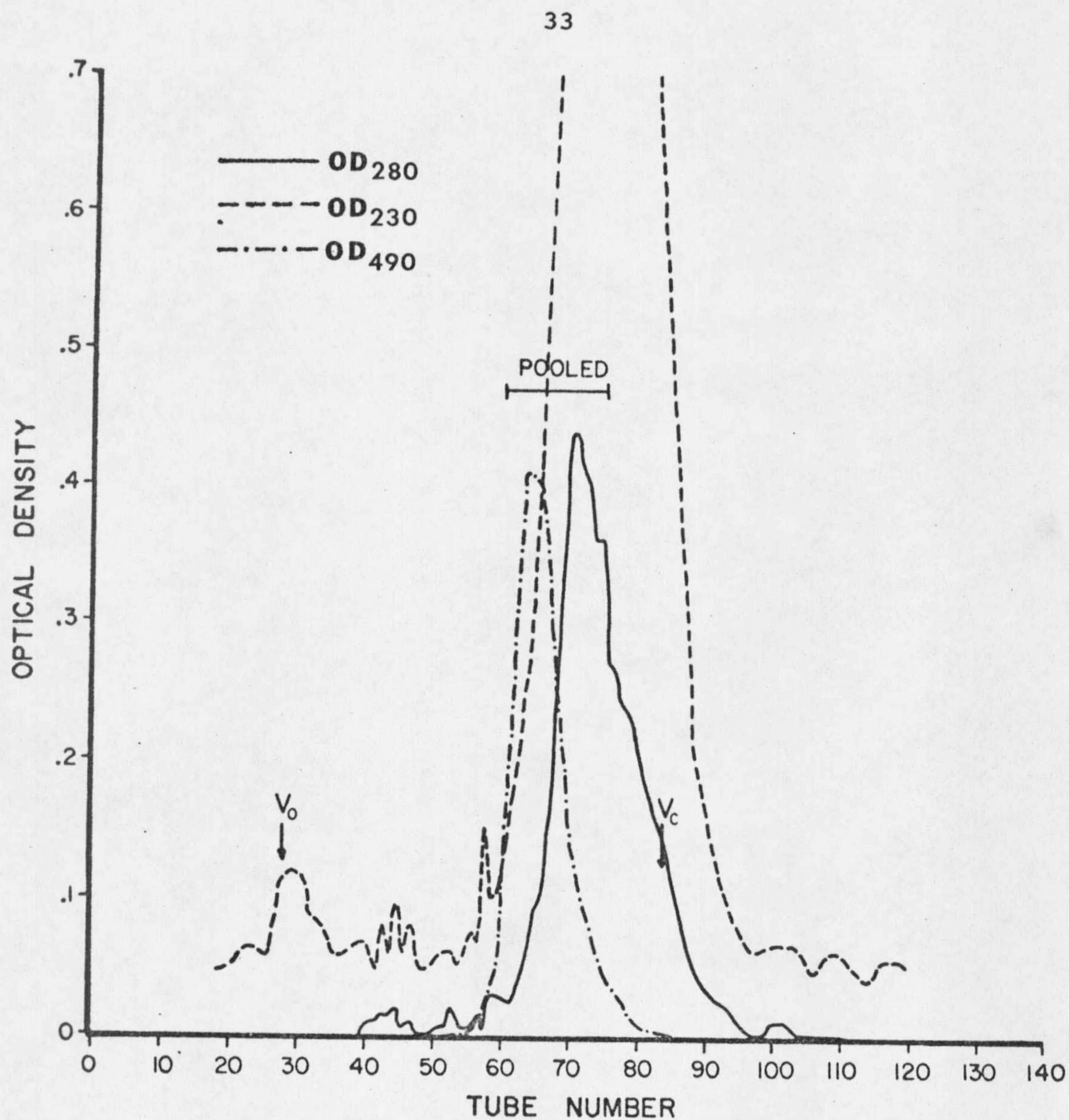


Fig. 5. Gel filtration of pronase digest on Sephadex G-50. The column (2x88 cm) was eluted with .01 M acetic acid at a flow rate of 26 ml/hr. Fractions of 2.6 ml were collected and analyzed for optical density at 230 nm and 280 nm, and optical density at 490 nm by the phenol-sulfuric acid method.

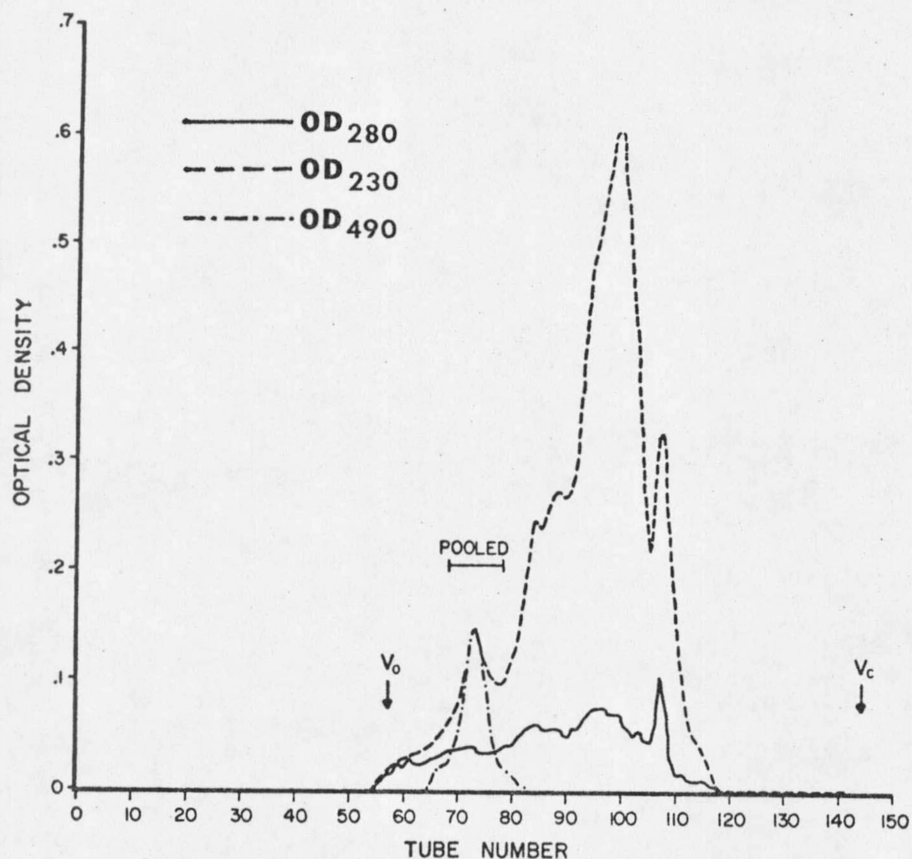


Fig. 6. Gel filtration of pronase digest on Sephadex G-25. The column (2.5x97 cm) was eluted with .01 M acetic acid at a flow rate of 26 ml/hr. Fractions of 2.6 ml were collected and analyzed for optical density at 230 nm and 280 nm, and for optical density at 490 nm by the phenol-sulfuric acid method.

Table IV. Amino Acid Analysis of Glycopeptide[a]

	Prep #I		Prep #II	
	6 hr hydrol	22 hr hydrol	6 hr hydrol	22 hr hydrol
Aspartic Acid	1.00	1.00	1.00	1.00
Threonine	0.83	0.84	0.89	0.91
Serine	0.82	0.82	0.92	0.83
Glutamic Acid	0.92	0.96	0.94	0.97
Glycine	Trace	Trace	Trace	Trace
Alanine	Trace	Trace	Trace	Trace
Glucosamine	1.03	0.98	1.11	1.05
Neutral Sugar[b] (μg)	607		630	

[a]Values are expressed as μ Mole of amino acid/μ Mole of Aspartic Acid.

[b]Neutral sugar was estimated by the phenol-sulfuric acid method and expressed as μg total neutral sugar/μ Mole Aspartic Acid.

The glycopeptide isolated from the pronase digestions consists of a tetrapeptide, containing one residue each of aspartic acid, threonine, serine, and glutamic acid, glucosamine, and trace amounts of glycine and alanine.

A time hydrolysis study was carried out to determine the amounts of glucosamine and ammonia liberated upon acid hydrolysis of the glycopeptide. Aliquots of the glycopeptide (10 nMole) were hydrolyzed in constant boiling HCl for varying periods of time, and the hydrolyzates analyzed for glucosamine and ammonia contents with the amino acid analyzer. The results are shown in Figure 7. Two residues of glucosamine and one residue of ammonia are released very rapidly from the

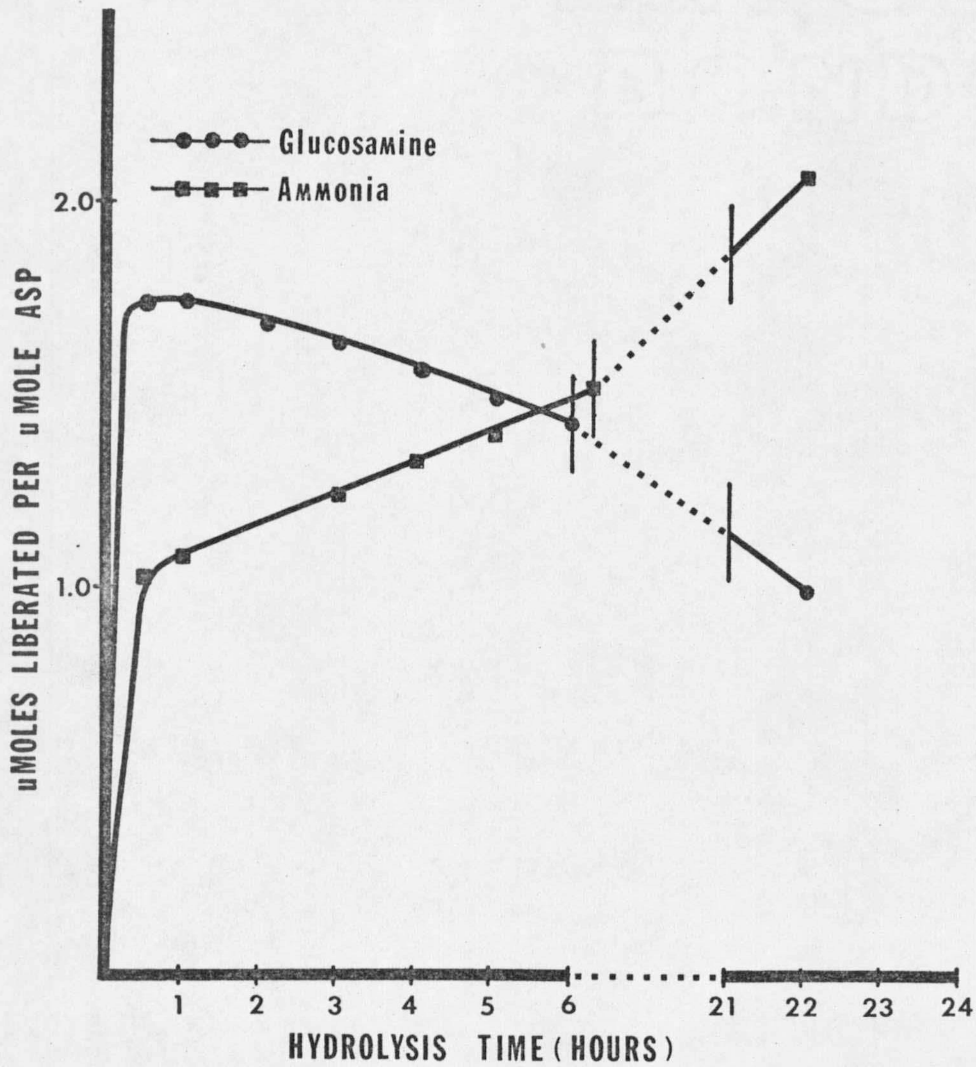


Fig. 7. Time-hydrolysis study of the purified glycopeptide relating the levels of glucosamine and ammonia released as a function of the time of hydrolysis. Hydrolysis of the glycopeptide was carried out in constant boiling HCl.

glycopeptide. Increasing hydrolysis times resulted in a gradual decline in glucosamine levels with a simultaneous rise in the observed levels of ammonia. The release of one residue of ammonia upon hydrolysis indicates the presence of one amide side chain in the glycopeptide.

The glycopeptide was subjected to treatment with mild base, conditions under which an O-glycosidic linkage to serine or threonine would undergo β-elimination. A decline of either amino acid upon subsequent amino acid analysis is indicative of an O-glycosidic type of linkage. The results of a β-elimination study of the glycopeptide is shown in Table V. Table V shows no evidence of a β-elimination reaction and indicates the absence of an O-glycosidic linkage to serine or threonine. The intact lectin was subjected to mild alkali in the presence of sodium sulfite, and failed to show any decline in the observed levels of serine or threonine or a rise in the levels of cysteic acid or α-amino-β-sulfonyl butyric acid.

High Voltage Electrophoresis

The glycopeptide was investigated by high voltage electrophoresis at pH 6.5. A schematic diagram of the migration pattern of the glycopeptide and standards is shown in Fig. 8. The migration pattern of the glycopeptide shows the presence of a single net negative charge at pH 6.5, as evidenced by its comigration with aspartic acid and glutamic acid. This is in agreement with the release of one residue of ammonia

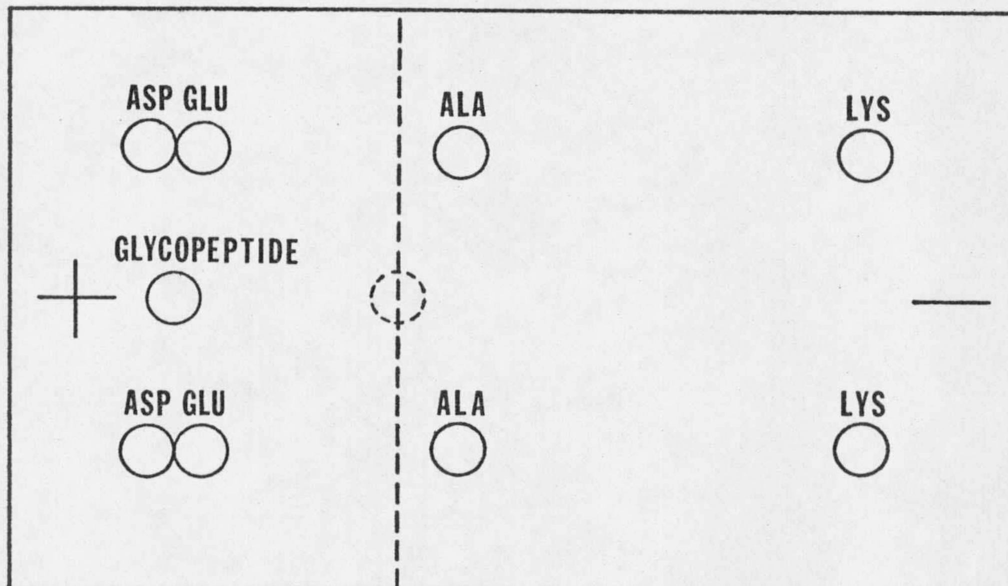


Fig. 8. High voltage electrophoresis of the purified glycopeptide. Samples were spotted on 57x20 cm sheets of Whatman 3 M paper. Electrophoresis was performed at pH 6.5 with pyridine:acetic acid:water (50:2:900) as solvent for 1/2 hour at 4000 volts. Major ninhydrin positive spots are enclosed by solid lines, and minor spots by dotted lines.

Table V. β-elimination of the Glycopeptide in Mild Base[a]

	Control[b]	NaOH
Aspartic Acid	1.00	1.00
Threonine	0.78	0.78
Serine	0.77	0.83
Glutamic Acid	0.84	0.87

[a]The results are expressed as μ Mole amino acid per μ Mole aspartic acid.

[b]The control sample contained glycopeptide, NaOH, and sufficient HCL to neutralize the NaOH.

upon acid hydrolysis, which indicates the presence of a single amide group in the intact glycopeptide. Presumably the net negative charge is due to the acidic side chain of glutamic acid.

Neutral Sugar Analysis by Gas-Liquid Chromatography

Initially, attempts to obtain a quantitative estimate of the various neutral sugars by gas-liquid chromatography (GLC) were directed at hydrolyzates of the native lectin. It is very difficult to determine conditions of hydrolysis for optimal release of carbohydrates from native glycoproteins, due to carbohydrate destruction in an acid environment and reaction of the liberated carbohydrate with liberated amino acids. Accordingly, it was decided to pursue quantitative carbohydrate determinations on the glycopeptide, which should offer superior results.

Standard solutions of the various alditol acetates were prepared and used to construct standard curves relating μg of carbohydrate to peak height for each monosaccharide. The standard curves are shown in Fig. 9.

Aliquots of two different glycopeptide preparations were subjected to acid hydrolysis and the released monosaccharides were reduced and acetylated as described earlier. Typical GLC chromatograms of a standard mixture and hydrolyzed and derivitized glycopeptide are illustrated in Fig. 10. The alditol acetates from the glycopeptide were identified by their characteristic retention time, and quantitated by relating μg to carbohydrate present to peak height. The results are shown in Table VI.

Table VI. Neutral Sugar Analysis by Gas-Liquid Chromatography[a]

	Glycopeptide I		
	A	B	Average
Fucose	0.59	0.48	0.53
Xylose	0.63	0.47	0.55
Mannose	1.66	1.94	1.80
	Glycopeptide II		
Fucose	0.64	0.68	0.66
Xylose	0.74	0.77	0.76
Mannose	2.28	1.43	1.86

[a]Values are expressed as μ Mole carbohydrate/μ Mole aspartic acid in the glycopeptide.

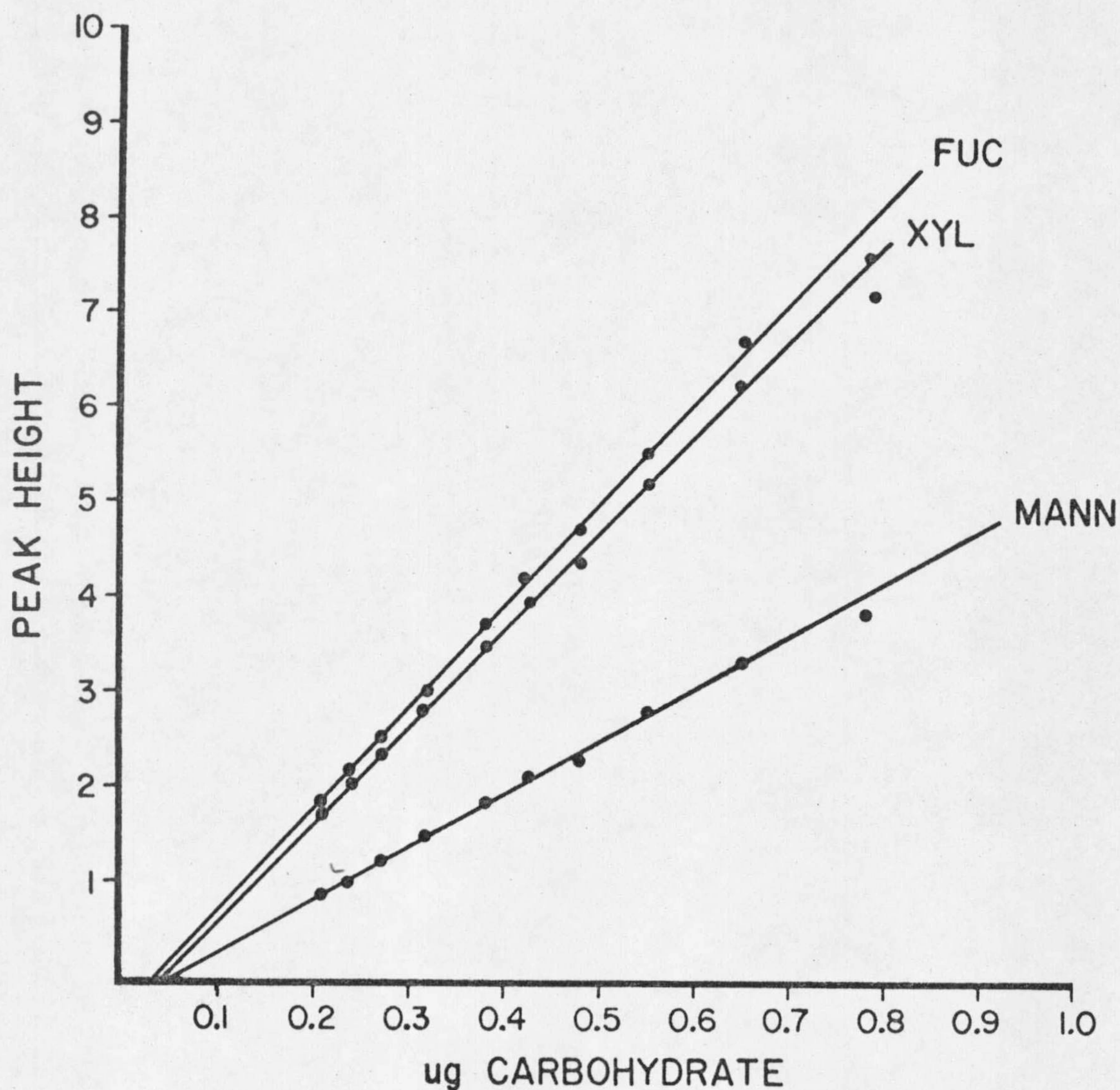


Fig. 9. Standard curves for the various sugar alditol acetates relating the μg of carbohydrate analyzed to peak height. Standard solutions of the monosaccharides were derivitized to the corresponding alditol acetates and quantitated by gas-liquid chromatography.

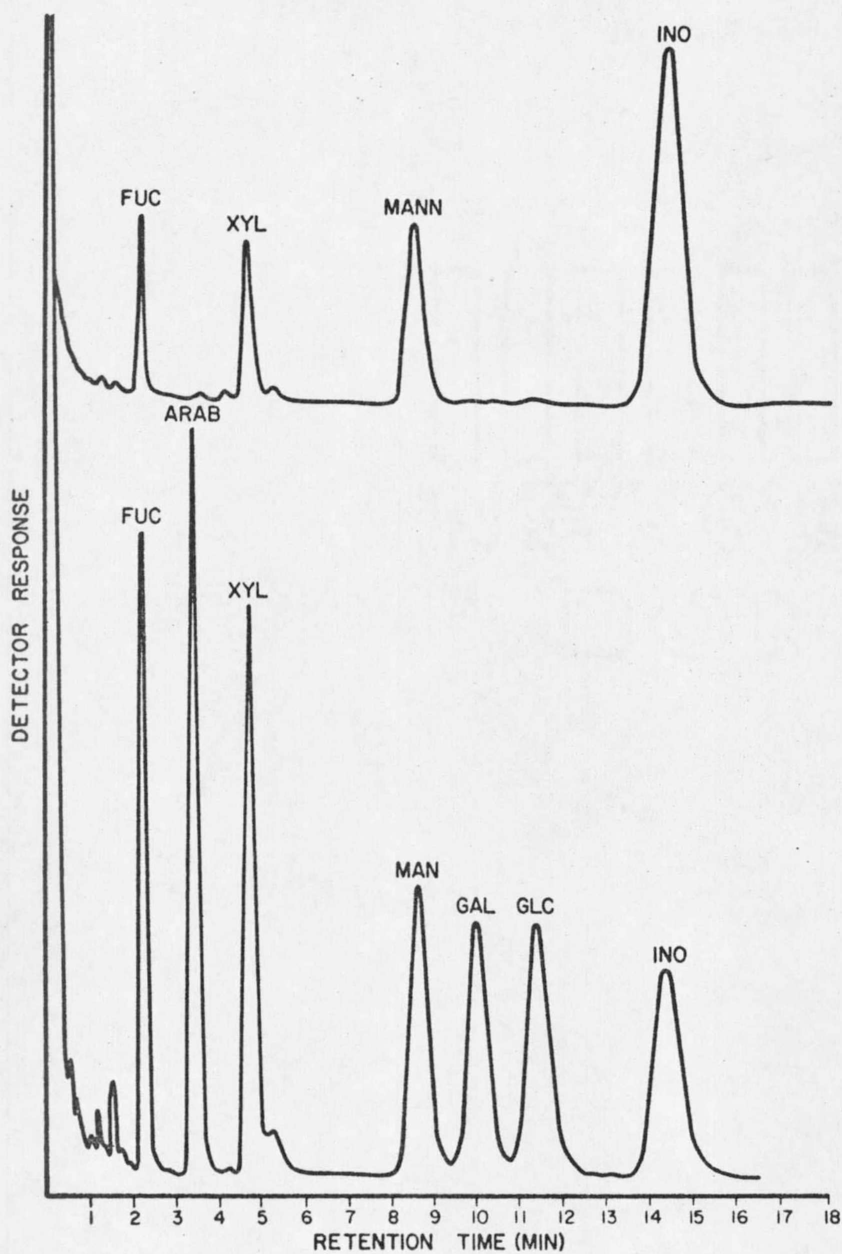


Fig. 10. Gas chromatography tracings of a standard mixture (lower tracing) and a typical analysis of the purified glycopeptide (upper tracing) after hydrolysis and derivitization.

The three neutral sugars fucose, xylose, and mannose appear to be present in the ratio of 1:1:2. The expected levels of total neutral sugar in the glycopeptide can be calculated to be 674 μg neutral sugar per μ Mole aspartic acid. This is in agreement with neutral sugar levels determined by the phenol-sulfuric acid method on duplicate glycopeptide preparations, 607 μg and 630 μg respectively, as shown in Table III.

The glycopeptide was analyzed for the presence of fucose by the colorimetric method of Dische and Shettles. A standard curve relating μg of fucose to optical density was constructed and is illustrated in Fig. 11. Replicate determinations on the glycopeptide yielded a value of 0.94 residues of fucose per μ Mole aspartic acid. This is in agreement with the GLC results suggesting the presence of one residue of fucose per mole of glycopeptide.

The glycosyl moeity of sainfoin lectin appears to be a single carbohydrate chain consisting of one residue each of fucose and xylose, and two residues each of glucosamine and mannose. It also appears likely that the carbohydrate-protein linkage is of the N-glycosidic type between N-acetylglucosamine and asparagine.

Glycosidase Digestion of the Glycopeptide

In an effort to gain some preliminary information regarding the sequence of the carbohydrate side chain, the glycopeptide was

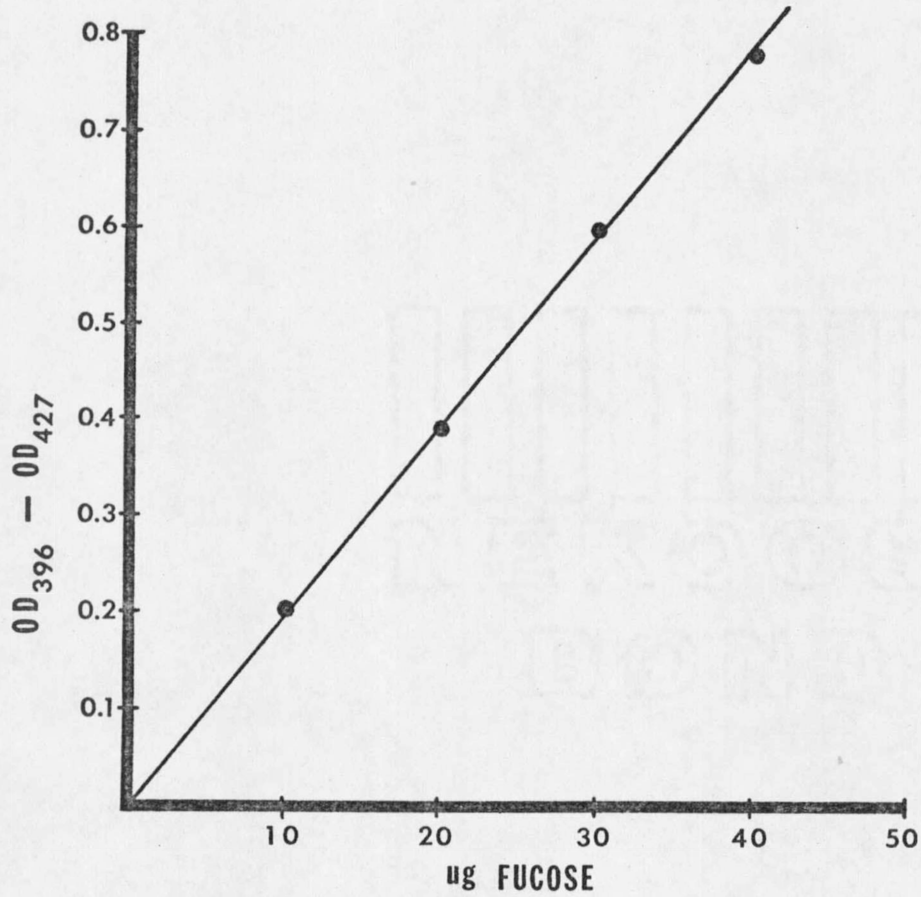


Fig. 11. Standard curve relating the μg fucose to optical density, as determined by the Dische-Shettles cysteine-sulfuric acid method.

incubated in the presence of various glycosidases. Sugars released by the glycosidases are detected as reducing sugar by the Nelson-Somogyi method (46). A standard curve for the Nelson-Somogyi method was constructed using mannose as standard and is shown in Fig. 12. The enzymes used are exoglycosidases and will remove their specific sugars from terminal non-reducing positions only. Incubation of the glycopeptide with various glycosidases revealed that the glycopeptide was resistant to digestion by α-mannosidase, β-N-acetylglucosaminidase, and α-L-fucosidase. It is not known whether the resistance to digestion is a result of the anomeric configuration of the glycosidic linkages, a result of xylose occupying the only terminal non-reducing position, or other structural or steric factors.

Carboxyl Terminal Analysis of the Glycopeptide

Hydrazinolysis of a protein ideally results in transamidation of all peptide bonds to the hydrazides, and only the carboxyl terminal amino acid is liberated as a free α-amino-α-carboxylic acid.

The glycopeptide (0.1 μ Mole) was subjected to hydrazinolysis and assayed for amino acid content. The results are shown in Table VII, indicating that threonine occupies the carboxyl terminal position of the glycopeptide.

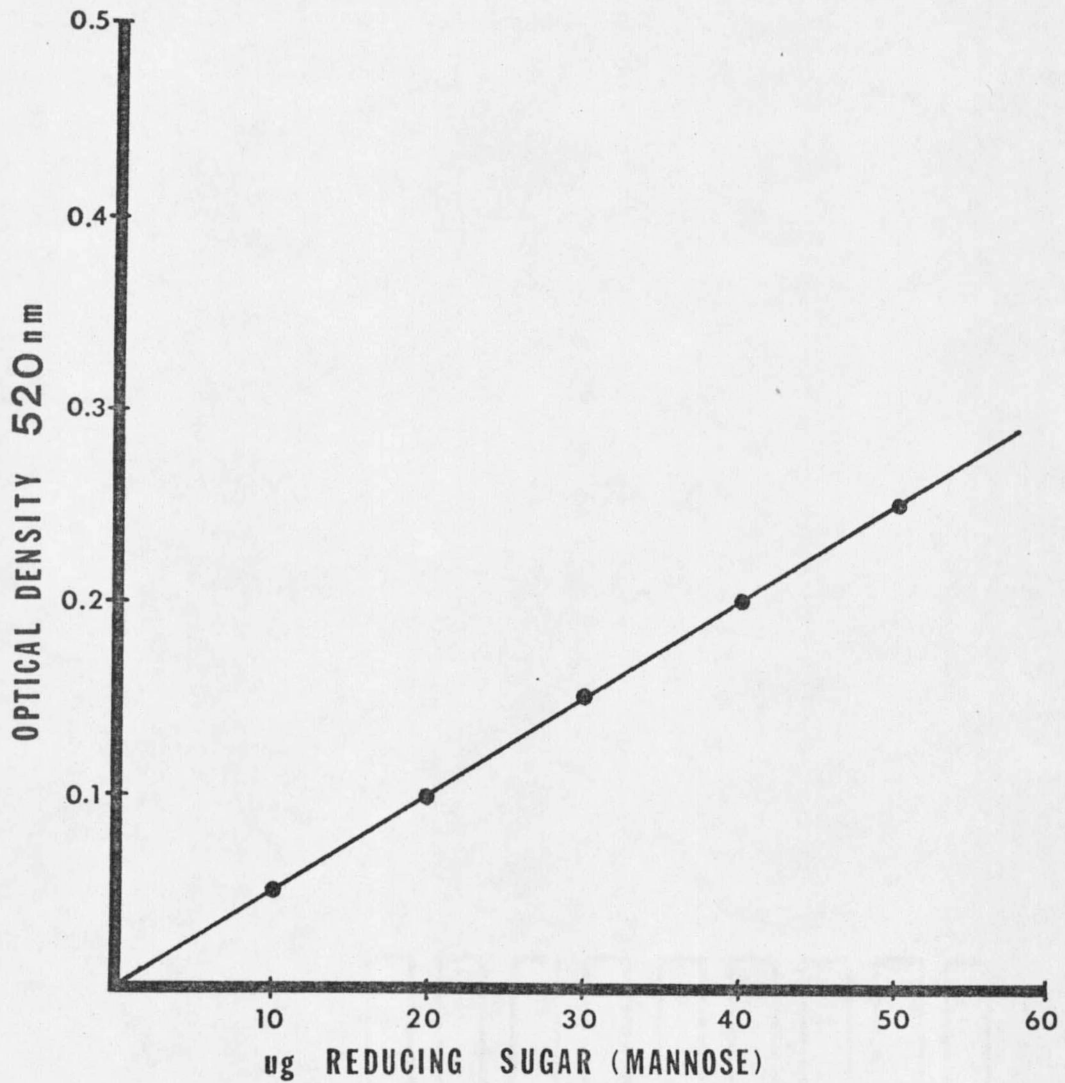


Fig. 12. Standard curve relating the µg reducing sugar to optical density at 520 nm, as determined by the Nelson-Somogyi method. Mannose was used as standard.

Table VII. Amino Acid Analysis of the Glycopeptide After Hydrazinolysis[a]

Amino Acid	Post Hydrazinolysis
Aspartic Acid	2.6
Threonine	31.0
Serine	7.0
Glutamic Acid	2.4

[a]Results are expressed as percentage of theoretical yield.

Amino Terminal Analysis of the Glycopeptide

Initial investigations of the amino terminus of the glycopeptide were carried out using the dansyl chloride technique of Gray. Very poor yields were obtained, and the amino terminus amino acid could not be identified with certainty. However, a weak spot was obtained, which migrated in the position of serine.

The glycopeptide was subsequently subjected to alkylation with iodoacetic acid. After alkylation and acid hydrolysis, the mixture was examined on the amino acid analyzer. The results are shown in Table VIII. Only three amino acid peaks were detected, eluting in the positions of aspartic acid, threonine, and glutamic acid. The serine peak was effectively eliminated upon alkylation, and indicates that serine is the amino terminal amino acid. In addition, the levels of glucosamine detected before and after alkylation were unchanged. If the amino sugar was present in the glycopeptide as glucosamine, the free amino group would also undergo alkylation, and the glucosamine

peak should decline on amino acid analysis. Absence of any decline in the glucosamine agrees with the contention that the amino sugar exists as N-acetylglucosamine in the unhydrolyzed glycopeptide.

Table VIII. Amino Acid Analysis of the Alkylated Glycopeptide[a]

Amino Acid	Pre-Alkylation	Post-Alkylation
Aspartic Acid	1.0	1.0
Threonine	0.89	0.92
Serine	0.92	0.20
Glutamic Acid	0.94	1.15
Glucosamine	1.05	1.01

[a]Results expressed as μ Mole amino acid/μ Mole aspartic acid.

Sequence Analysis of the Glycopeptide

The determination of the amino acid sequence of the glycopeptide was accomplished by a combination of the subtractive Edman degradation and alkylation. The amino terminal amino acid was identified by subjecting the glycopeptide to either alkylation or one cycle of Edman degradation and observing which amino acid level declined upon amino acid analysis. The amino acid pentultimate to the amino terminus was identified by declining levels of a second amino acid after one cycle of Edman degradation followed by alkylation. The third amino acid in the sequence was evidenced by a decline in the levels of a third amino acid after two cycles of Edman degradation and alkylation. The results are displayed in Table IX.

Table IX. Amino Acid Sequence Analysis of Glycopeptide[a]

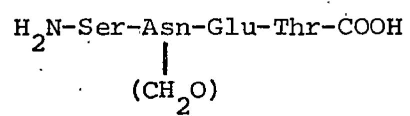
Amino Acid	Intact	Alkylated	1 Edman	1 Edman + Alkyl	2 Edman + Alkyl
Asp	1.12	1.09	1.09	<u>0.36</u>[b]	<u>0.38</u>
Thr	1.00	1.00	1.00	1.00	1.00
Ser	1.03	<u>0.22</u>	<u>0.37</u>	<u>0.26</u>	<u>0.0</u>
Glu	1.06	1.30	1.18	1.14	<u>0.34</u>[c]

[a]Values are expressed as μ Mole amino acid/μ Mole Threonine.

[b]Value was adjusted to account for the amount of aspartic acid unavailable for alkylation due to incomplete removal of serine in the preceding Edman reaction.

[c]Value was adjusted to account for the amount of glutamic acid unavailable for alkylation due to incomplete removal of aspartic acid in the preceding Edman reaction.

According to the results in Table IX, both direct alkylation and subtractive Edman degradation identify the amino terminal amino acid as serine. The decline in aspartic acid levels after one cycle of Edman degradation and alkylation, identify aspartic acid (asparagine) as occupying the position pentultimate to the amino terminal. Finally, the decline of glutamic acid after two cycles of Edman degradation and alkylation, identifies glutamic acid as the third amino acid in the sequence from the amino terminal. Threonine would necessarily occupy the fourth position, or carboxyl terminal. This is in agreement with the hydrazinolysis data, which also indicated threonine was the carboxyl terminal amino acid. The sequence of the glycopeptide is confirmed as:


H2N-Ser-Asn-Glu-Thr-COOH
(CH2O)

DISCUSSION

Neutral Sugars

Colorimetric determination of the total neutral sugar present indicated sainfoin lectin contains 2.6% neutral sugar by weight (Table I). Assuming a subunit molecular weight of 26,000 daltons, this translates to 674 gms of neutral sugar per mole lectin monomer. If an average value of 180 gm per mole of carbohydrate is assumed, the lectin should contain 674/180 or 3.76 residues of neutral sugar per lectin monomer. At least according to colorimetric methods, the lectin appears to contain four residues of neutral sugar. Colorimetric assay of the glycopeptide isolated from the pronase digestion agrees reasonably well with these results, namely an average value of 618 gm per mole of glycopeptide (Table III). The fact that each mole of glycopeptide contains four residues of neutral sugar indicates that all of the neutral sugar present in the intact lectin is contained in a single oligosaccharide chain.

Gas-liquid chromatography of the glycopeptide confirmed the presence of four residues of neutral sugar per mole of glycopeptide (Table VI). Additionally, the four residues of neutral sugar consist of one residue each of fucose and xylose, and two residues of mannose. Thin layer chromatography had earlier indicated the presence of mannose, as well as a spot which could have been either fucose or xylose. Specific colorimetric assays for the presence of fucose confirmed the existence

of one residue of fucose per mole of glycopeptide.

Most of the plant lectins which have been isolated in pure form are glycoproteins, and exhibit a wide diversity as to their carbohydrate content and distribution, as indicated in Table X. The carbohydrate content ranges from a low of 2% in the lentil lectin, to over 50% in the potato lectin. Most, however, contain less than 10% carbohydrate. The composition of the monosaccharides also varies widely; the lectin from the yellow wax bean contains all the monosaccharides commonly occurring in plant glycoproteins, while the potato lectin contains only a single monosaccharide, arabinose. N-acetylglucosamine (GlcNAc) is the most commonly occurring monosaccharide, being present in all the lectins shown in Table X, with the exception of the potato lectin. Mannose is also very common, occurring in all the lectins shown except the potato lectin and the lentil lectin. The remaining monosaccharides occur with varying frequencies and amounts.

The lectin from Robinia pseudoacacia (49) has been reported to contain the same monosaccharides as observed in sainfoin lectin. However, the monosaccharides of the Robinia lectin were only determined qualitatively, and quantitative information is not available at present. In addition, a number of the reports concerning lectins indicate the presence of fucose, xylose, and arabinose in small quantities. In many cases it is not clear whether the sugars actually have any structural significance, or originate from a contaminating

Table X. Representative Glycolectins and their Carbohydrate Content

Source	% Sugar	Gal	Glc	Man	Fuc	Arab	Xyl	GlcNAc
Leguminosae								
Bauhinia purpura	11.0	+	+	+	+		+	+
Dolichos biflorus (Horse gram)	3.5			+				+
Glycine Max (Soybean)	5.7			+				+
Lens culinaris (Lentil)	3.0		+					+
Phasoleus lunatus (Lima bean)	4.0			+	+			+
Phasoleus vulgaris (Black kidney bean)	5.7			+			+	+
Phasoleus vulgaris (Red kidney bean)	8.9			+	+	+	+	+
Phasoleus vulgaris (Yellow wax bean)	10.0	+	+	+	+	+	+	+
Robinia pseudoacacia (Black locust)	10.7			+	+		+	+
Ulex Europeus (Gorse)	21.7	+		+		+		+
Other Plants								
Phytolacca americana (Pokeweed)	5%		+	+	+			+
Solanum tuberosum (Potato)	52.0					+		
Wisteria floribunda	11.4			+	+	+	+	+

For data source see Sharon, Ref. #24.

hemicellulose. In the glycosyl moeity of sainfoin lectin, good evidence has been presented for the presence of one residue each of fucose and xylose, and two residues of mannose.

Amino Sugar

Amino acid analysis of the intact lectin after hydrolysis in constant boiling HCl for 6, 14, and 22 hours showed the presence of only a single amino sugar, glucosamine. Furthermore, a time-hydrolysis study followed by amino acid analysis (Fig. 2) indicated the presence of a single residue of glucosamine per lectin monomer. At this point it appeared that the oligosaccharide chain consisted of fucose, xylose, mannose and one residue of N-acetylglucosamine (GlcNAc) per lectin monomer.

Amino acid analysis of the purified glycopeptide was performed after hydrolysis with constant boiling HCl for 6 and 22 hours (Table IV). Slightly more than one residue of glucosamine was observed at six hours and close to one residue observed at 22 hours. This is not in agreement with earlier estimates obtained on the intact lectin. According to Glazer et al. (50), yields on the glucosamine contents of glycoproteins after hydrolysis for 22 hours in 6 N HCl typically approach 50%. To resolve this conflict, the time-release of glucosamine in 6 N HCl was determined on the glycopeptide (Fig. 7). The maximum yield of glucosamine was 1.75 residues per mole of glycopeptide, obtained after 1/2 hour of hydrolysis at 110°C. Longer

hydrolysis times resulted in the gradual decline of glucosamine yields to one residue at 22 hours, agreeing very well with the 50% yield value as expressed by Glazer. This explains the previous yields of glucosamine obtained from the glycopeptide. The results obtained on the intact lectin are less clear, but could be due to increased levels of destruction of glucosamine in the presence of large amounts of liberated amino acids.

Hydrolysis of both the intact lectin and the glycopeptide were carried out in constant boiling HCl. I would not anticipate that rates of destruction of the glucosamine due to the acid environment could account for the difference in the results. It seems more likely that the low yields from the intact lectin may be due to interactions of the glucosamine with amino acids liberated during the hydrolysis. There is also the less likely possibility that the low yields from the intact lectin is caused by resistance to hydrolytic release of the glucosamine. It is, however, difficult to imagine the maintenance of structural integrity in an environment of 6 N HCl.

In summary, the evidence indicates that the carbohydrate moeity of the lectin consists of a single oligosaccharide chain containing one residue each of fucose and xylose, and two residues each of mannose and glucosamine. It is likely that the hydrolysis product, glucosamine, actually exists as N-acetylglucosamine in the intact lectin and glycopeptide. This has been shown to be the case in those plant

glycoproteins extensively studied to date. In addition, after alkylation (carboxymethylation) of the glycopeptide, no decreases were observed in the levels of glucosamine obtained upon amino acid analysis. If the amino group on C-2 of the glucosamine existed as the free amino group, alkylation should occur, resulting in a decline in the observed glucosamine levels. Additionally, the high voltage electrophoresis results are incompatible with non-acetylated glucosamine.

Carbohydrate-Peptide Linkage

The glycopeptide isolated from the pronase digestions consists of a tetrapeptide, containing one residue each of aspartic acid, threonine, serine, and glutamic acid, and two residues of N-acetylglucosamine (GlcNAc). Of the four amino acids present, three have been found to occur in carbohydrate-protein linkages.

The hydroxy amino acids, serine and threonine, occur in O-glycosidic linkage in carbohydrate bonds, which may undergo β-elimination in mild base. The unsaturated amino acid products of the β-elimination reaction are unstable to acid hydrolysis, and a decline should occur in serine or threonine levels upon amino acid analysis of the alkali treated glycopeptide if an O-glycosidic type of bond is present. Asparagine occurs in N-glycosidic linkage to GlcNAc, which is stable to mild alkali.

The glycopeptide was completely resistant to β-elimination in mild alkali. The intact lectin was also subjected to mild alkali, in the

presence of sodium sulfite, and failed to show any decline in the observed levels of serine or threonine or a rise in the observed levels of cysteic acid or α-amino-β-sulfonylbutyric acid. This suggests the presence of an N-glycosidic linkage between GlcNAc and asparagine.

When the glycopeptide was assayed for the release of ammonia with varying times (Fig. 7), it was found that one residue of ammonia and 1.75 residues of glucosamine were rapidly released from the glycopeptide. As the glucosamine levels declined to one residue at 22 hours, the ammonia levels slowly rose to two residues at 22 hours.

The rapid appearance of glucosamine and simultaneous appearance of one residue of ammonia likely results from hydrolysis of the N-glycosidic bond, by which equimolar amounts of aspartic acid, ammonia, and glucosamine are liberated. This reaction characteristically proceeds with a half life of 17 minutes in 2 N HCl at 100°C (51). This correlates with the time release data for glucosamine and ammonia, as maximum release of glucosamine (1.75 residues) and release of one residue of amide nitrogen as ammonia is completed in one half hour in 6 N HCl. A greater than equimolar amount of glucosamine is anticipated due to the presence of the second GlcNAc in the oligosaccharide. This is consistent with an argument for the presence of aspartic acid in the glycopeptide as asparagine. The second residue of ammonia appearing on extended hydrolysis times is apparently due to acid

catalyzed destruction of glucosamine, which disappears proportionately to the appearance of ammonia.

High voltage electrophoresis of the glycopeptide at pH 6.5 revealed a migration pattern indicating the presence of a single net negative charge. This is compatible with the presence of only one amide group in the glycopeptide. If the amide group is associated with asparagine, presumably the net negative charge exhibited is a result of the acidic side chain of glutamic acid.

In all the plant glycolectins in which the glycopeptide linkage has been established, the linkage was almost unanimously found to consist of GlcNAc-Asn, the only exception to date containing an arabinose-hydroxyproline linkage. Sainfoin lectin and the isolated glycopeptide was shown to be devoid of detectable amounts of hydroxyproline, and it is concluded that the carbohydrate is attached by a N-glycosidic GlcNAc-Asn linkage. Definitive evidence for the presence of the GlcNAc-Asn linkage would involve isolation of a glycopeptide containing only glucosamine and asparagine. The marked resistance of the glycopeptide to further enzymatic digestion by pronase and carboxypeptidase precluded an easy isolation of the asparagine-carbohydrate linkage in this study.

Structural Aspects of the Carbohydrate Moeity

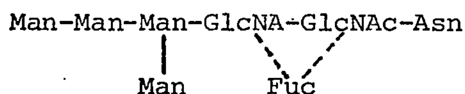
Of the many glycoprotein lectins, few have been studied in any detail as to the structural chemistry of their carbohydrate moeities. One of the first plant glycoproteins to be recognized and studied was soybean agglutinin (SBA). Characterization of SBA has been carried out primarily through the efforts of Sharon and Lis (12). SBA was shown to contain 4.5% mannose and 1.5% GlcNAc. The carbohydrate-peptide linking group was isolated and shown to be identical in its composition and behavior on paper chromatography and electrophoresis with N-acetyl-D-glucosaminyl-β-L-asparagine. Studies still in progress have led to the assignment of the following tentative structure for the carbohydrate moeity of SBA.

```
                  Man-α(1-6)
(Man-α(1-2))  <              > Man-β-(1-4)-GlcNAc-β(1-4)--------
            6     Man-α(1-3)

------ GlcNAc - (1-N)-Asn
```

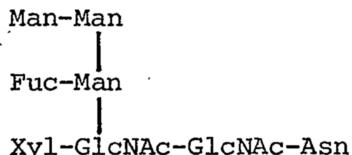
Similar structures appear to be present in other glycolectins such as phytohemagglutinin (52) and the castor bean agglutinin (53) in which asparaginyl-linked carbohydrate units consist of $(Man)n(GlcNAc)_2$ where n = 4 to 7 units.

Misaki et al. (34) has recently presented evidence for the structure of a glycopeptide isolated from the lima bean lectin, containing $Fuc_1Man_4GlcNAc_2$-Asn, and is shown below.



It is interesting to observe that the fucose residue is most probably linked to one of the internal GlcNAc residues.

A glycopeptide isolated from the plant proteolytic enzyme, bromelain, shows a marked similarity to sainfoin lectin in carbohydrate composition (54). The glycopeptide was composed of GlcNAc, mannose, xylose, and fucose in the molar ratios of 2:3:1. From detailed chemical and enzymatic studies, a structure was proposed for the carbohydrate moeity of bromelain, and is shown below.



Recently the first mammalian lectin was isolated in pure form from rabbit liver, and two different glycopeptides were isolated from pronase digests of the lectin (45). One of the glycopeptides was very similar in structure to the glycopeptide isolated from SBA. The second glycopeptide is much more complex and contains GlcNAc, mannose, galactose, and sialic acid, a monosaccharide usually found in animal glycoproteins but not in plant glycoproteins.

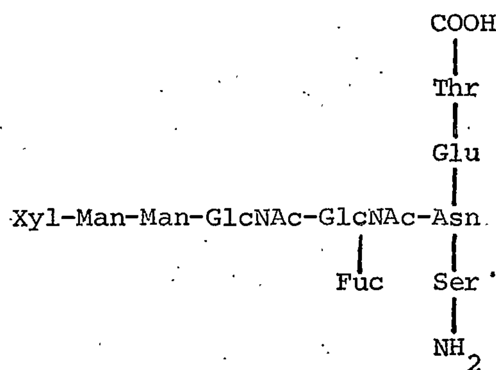
Several generalities of structure have begun to emerge for the majority of lectins. The glycopeptide linkage most commonly consists

of a N,N'-diacetylchitobiose unit (GlcNAc-GlcNAc) covalently linked to asparagine. Also, a residue of mannose is usually linked to the diacetylchitobiose unit through a β-linkage. Additional mannose residues are usually linked to the oligosaccharide chain through α-linkages. α-L-Fucose is usually found to occur in a terminal non-reducing position and may be linked to interior residues of the oligosaccharide chain.

From the presently known carbohydrate structures, it appears very likely that sainfoin lectin contains the commonly found N,N'-diacetylchitobiose unit, which is linked N-glycosidically to asparagine providing the carbohydrate-peptide linkage. In an effort to gain some structural information concerning the oligosaccharide side chain, the glycopeptide was incubated in the presence of several commercially available glycosidases. Incubations in the presence of α-mannosidase, α-L-fucosidase, and β-N-acetylglucosaminidase all failed to show the release of detectable levels of carbohydrate. The absence of release of carbohydrate in the presence of β-N-acetylglucosaminidase is not surprising, as the various glycosidases are active on only terminal non-reducing sugars and it is believed that the GlcNAc present is in an internal position. The resistance of the glycopeptide to α-mannosidase is also not wholly unexpected. Typically, one mannose is linked to the diacetylchitobiose through a β-linkage which is resistant to α-mannosidase) and usually occupies an internal position. The second residue of mannose present is most likely linked to the

first mannose residue. The linkage could be of the β-configuration or interior to either fucose or xylose, both of which typically occupy terminal non-reducing positions.

Since fucose is predominantly found to occur in a terminal non-reducing position, the absence of release of fucose by α-L-fucosidase is surprising. It is not known whether the resistance to digestion is a result of the anomeric configuration of the glycosidic linkages, a result of xylose occupying the only terminal non-reducing position, or other unknown structural or steric factors. However, a reasonable hypothetical structure can be proposed, based upon the current results of other investigations and the glycosidase digestion data, and is shown below.



Xylose occupying the proposed non-reducing position could account for the resistance to α-mannosidase and β-N-acetylglucosaminidase. If fucose is linked to an interior GlcNAc, as found in the lima bean lectin, molecular interaction with, or a steric hindrance by the peptide portion, may be responsible for the resistance to

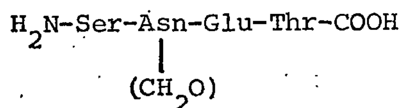
α-L-fucosidase. Resistance to fucosidase could also conceivably be a result of an unreactive anomeric configuration.

Unfortunately, β-D-xylosidase is commercially unavailable. It would be of great interest to expose the glycopeptide to xylosidase and establish if xylose does indeed occupy the proposed terminal position. If this were the case, sequential digestion with mannosidase, fucosidase, and β-N-acetylglucosaminidase following xylosidase digestion, could yield extensive information on the oligosaccharide structure and sequence.

Further elucidation of the structure of the oligosaccharide chain is beyond the tenure of this present study. Additional techniques such as methylation or periodate oxidation, as well as a more complete selection of glycosidases will lead to a future determination of the carbohydrate sequence and structure.

Amino Acid Sequence Analysis of the Glycopeptide

The amino acid sequence of the glycopeptide was determined by a combination of subtractive Edman degradation and alkylation with iodoacetic acid (carboxy methylation) under alkaline conditions. The sequence was established as



Examination of a large number of glycopeptides with the GlcNAc-Asn glycosidic linkage has revealed a common amino acid sequence near the Asn (3). This sequence is either Asn-X-Ser or Asn-X-Thr, with the carbohydrate linked to the Asn. Marshall (3) suggested that in the sequence Asn-X-Thr(Ser), a hydrogen bond is formed between the side chain carbonyl of asparagine and the hydroxyl group of the hydroxy amino acid, as shown in Figure 13A.

The glycopeptide isolated in this study contains a sequence which is consistent with this common structure. A space filling model of the glycopeptide incorporating the H-bond between Asn and Thr was constructed. The resulting three-dimensional structure suggested it was just as likely that a H-bond should also exist between the free amino group of serine and the carboxyl group of the glutamic acid side chain. This proposed structure is shown schematically in Figure 13B.

The proposed structure could be expected to account for the glycopeptide's resistance toward further enzymatic degradation by aminopeptidases (in pronase) and carboxypeptidase. Both terminal amino acids are involved in hydrogen bonded structures which could limit their availability to the proteolytic enzymes. Additionally, the bulky carbohydrate portion, per se, may limit proteolytic hydrolysis. Feasibility of this model is enhanced by the observation that dansyl chloride failed to react with the serine amino group in significant yield, wherease ninhydrin, iodoacetic acid, and phenylisothiocynate

Fig. 13. A) Schematic model demonstrating the H-bonding present in GlcNAc-Asn linkages, as proposed by Marshall et al., Ref. #3.

B) Schematic model proposed to demonstrate H-bonding present in a glycopeptide isolated from Sainfoin lectin.

did. Involvement of the serine amino group in an ionic bond with the glutamyl carboxyl group would lower its pKa and thus limit reaction with short half-life reagents such as dansyl chloride. The nearness of the glutamyl carboxyl group to the threonine residue could also contribute to the observed carboxypeptidase resistance.

The study of lectins and plant glycoproteins is still in its infancy. Some structural features of these compounds are beginning to emerge, but many more glycoproteins (both plant and animal) need to be isolated and characterized before any firm generalizations can be made as to their chemical constitution. At present, next to nothing is known about the function in nature of most plant glycoproteins, be they lectins, toxins or other proteins, and no clear role for their carbohydrate side chains has yet emerged.

The renewed interest in lectins and plant biochemistry in general will hopefully lead to systematic studies of plant glycoproteins. Such studies will provide much needed insight into the chemistry and physiological functions of these molecules and may also contribute to the improved utilization of plants by man.

SUMMARY AND CONCLUSIONS

A lectin isolated from the seeds of sainfoin (Onobrychis viciifolia, Scop. var Eski) was shown to be a glycoprotein. Colorimetric analysis by the phenol-sulfuric acid method indicated the lectin contained 2.6% neutral sugar by weight.

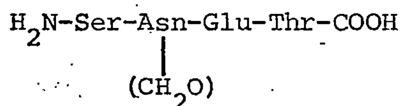
A glycopeptide accounting for 70% of the total carbohydrate was isolated from pronase digests of the lectin by gel filtration chromatography. Amino acid analysis of the glycopeptide demonstrated the presence of two residues of glucosamine per protein monomer, corresponding to 1.6% amino sugar by weight. Gas-liquid chromatography analysis after hydrolysis and derivitization to the corresponding alditol acetates showed the presence of fucose, xylose, and mannose in a molar ratio of 1:1:2.

Amino acid analysis also demonstrated the presence of equimolar quantities of aspartic acid, threonine, serine, and glutamic acid. In addition, one residue of ammonia per mole of glycopeptide was liberated on amino acid analysis, suggesting the presence of one amide group in the glycopeptide. High voltage electrophoresis of the glycopeptide at pH 6.5 indicted the presence of a single net negative charge. Presumably, the amide group is present as asparagine and the net negative charge is related to the acidic side chain of glutamic acid.

Treatment of both the intact lectin and the purified glycopeptide with mild base failed to show any evidence of β-elimination, suggesting

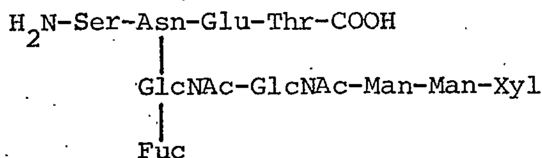
an N-glycosidic N-acetylglucosamine-asparaginyl glycopeptide linkage.

Hydrazinolysis and amino acid analysis of the glycopeptide indicated that threonine was the carboxyl terminal amino acid. Alkylation combined with subtractive Edman degradation established the amino acid sequence as,



confirming the hydrazinolysis results.

Incubation of the glycopeptide with α-mannosidase, β-N-acetylglucosaminidase, and α-L-fucosidase showed that the carbohydrate moeity of the lectin was resistant to all three of the glycosidases. Resistance to glycosidase digestion and correlation with the current literature led to the proposal of a likely structure for the glycopeptide, and is shown below:



Future study is necessary for complete characterization of the glycopeptide. Conditions to allow more complete proteolysis of the glycopeptide must be determined, as definitive evidence for the GlcNAc-Asn linkage would involve isolation of a glycopeptide with only asparagine present. In addition, techniques such as methylation and Smith

degradation, as well as a more complete assortment of glycosidases will allow the determination of the sequence and structure of the carbohydrate moeity.

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