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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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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Signature Anthony E. Nanen

Date 26 July, 1978

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Approved:

Kenneth D Hayner
Chairman, Graduate Committee

Edwin H. Abbott
Head, Major Department

Henry L Parsons
Graduate Dean

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This thesis is dedicated to my mother and father, whose encouragement and support has made this endeavor a reality.

TABLE OF CONTENTS

LIST OF TABLES

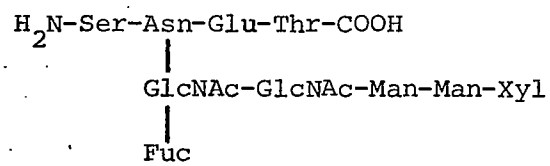
LIST OF FIGURES

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: ($Ser_1Asx_1Glx_1Thr_1ammonia_1fucose_1xylose_1mannose_2glucosamine_2$).

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

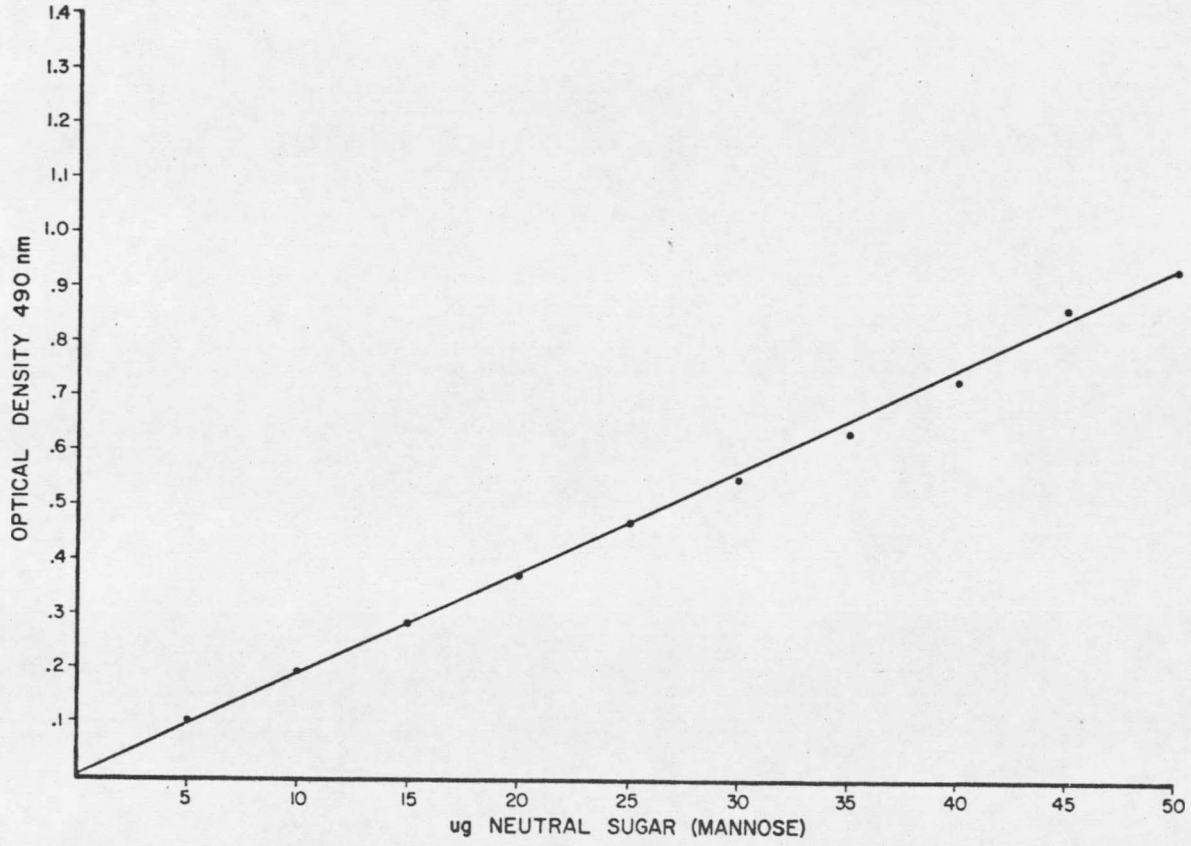


Fig. 1. Standard curve relating the optical density at 490 nm to µg of neutral sugar analyzed by the phenol-sulfuric acid method. D-Mannose was used as standard.

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