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Structure of the Glycopeptide from Bovine Visual Pigment 500*

Joram Heller and Marianne A. Lawrence

ABSTRACT: A single glycopeptide containing nine amino acid residues was isolated from a peptic digest of the membrane protein bovine visual pigment$_{500}$ by chromatography on Dowex 50-X2. The carbohydrate composition of this peptide accounts for all the sugar present in visual pigment$_{500}$. A combination of chemical and enzymic methods established the sequence of the glycopeptide as Met-Asx(sugar)-Gly-Thr-Glu-Gly-Pro-Asn-Phe. All the carbohydrate was linked to Asp-2 through an alkali-stable bond, presumably an *N*-aspartylglycosylamine linkage. Digestion of the glycopeptide with β-acetylglucosaminase liberated two out of the three glucosamine residues as *N*-acetylglucosamine, leaving one glucosamine and all three mannose residues linked to the peptide. Digestion with α-mannosidase liberated 88% of the mannose residues present in the carbohydrate moiety as reducing sugar. It is concluded that bovine visual pigment$_{500}$ contains a single oligosaccharide moiety linked to the polypeptide chain through an *N*-aspartylglycosylamine linkage. Two of the *N*-acetylglucosamine residues and all the mannose residues are linked peripherally to the (*N*-acetyl)glucosamine that is linked to the asparagine residue. These experiments provide independent evidence that the molecular weight of visual pigment is approximately 28,000. It is suggested that the carbohydrate in visual pigment functions as a surface orientation marker assuring proper assembly of the molecule into the membrane structure. It is possible that the carbohydrate moieties which are often found in membrane proteins have a similar function.

Bovine visual pigment$_{500}$, a membrane protein which is a structural component of the rod outer segment disk system, was recently shown to be a glycoprotein containing three residues of glucosamine and three residues of neutral sugar per molecule (Heller, 1968). The present work attempts to define the type of linkage between the carbohydrate and the polypeptide chain and to determine the amino acid sequence around the linkage.

The experiments described in the present paper show that bovine visual pigment$_{500}$ contains a single oligosaccharide moiety linked to the polypeptide chain through an *N*-aspartylglycosylamine linkage.

Materials and Methods

Bovine visual pigment$_{500}$ was prepared and purified as previously described (Heller, 1968). Purified visual pigment, 5 μmoles, was dialyzed against deionized water to remove salts and was then denatured by adding four volumes of ethanol and incubating for 18 hr at 23°. The precipitated protein was collected by low-speed centrifugation and was then washed ten times with 80% aqueous ethanol at 23° over a period of 7

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days. The washed denatured protein was dissolved in 50% aqueous acetic acid and then adjusted to 10% aqueous acetic acid (pH 2.3). Twice-crystallized pepsin (Worthington, 5 mg, 1:27 w/w ratio of enzyme to substrate) dissolved in 10% aqueous acetic acid was added to the protein and the digestion was allowed to proceed for 24 hr at 30°. The digest was lyophilized and the dry powder was dissolved in 0.2 *M* pyridine–acetic acid buffer (pH 3.1). A precipitate was removed by low-speed centrifugation and the supernatant was collected. The precipitate was redissolved in 50% aqueous acetic acid and then adjusted to 10% aqueous acetic acid; a further 10 mg of pepsin was added and the protein was digested for a further 36 hr at 30°. After 20-hr digestion another 5 mg of pepsin was added. At the end of this period the digest was again lyophilized and the dry powder was dissolved in 0.2 M pyridine–acetic acid buffer (pH 3.1). A precipitate formed and was removed by slow-speed centrifugation, and the supernatant containing the soluble glycopeptide was collected. The two supernatants containing the glycopeptide in 0.2 M pyridine–acetic acid buffer were individually chromatographed on a 2.5 × 38 cm column of Dowex 50-X2 (200–400 mesh) equilibrated with the same buffer. The column temperature was 23° and the flow was 24 ml/hr. Fractions of 7 ml were collected.

An aliquot of 200 μl was removed from every other tube of the column fractions and analyzed by the ninhydrin method after alkaline hydrolysis according to Hirs *et al.* (1956), and a sample of 100 μl was removed from the same tube for the phenol–sulfuric acid method for neutral sugars according to Dubois *et al.* (1956) with all the components of the reaction reduced to half of their volume.

Appropriate fractions were pooled and taken to dryness in a rotary evaporator at 40°. The glycopeptide was further purified on a Sephadex G-25 (fine) column (2.5 × 37 cm) equilibrated with 1 M acetic acid at 23°. The column effluent was monitored as described above.

Appropriate fractions were pooled, taken to dryness in a rotary evaporator at 40°, transferred in deionized water to a vial, and stored frozen.

In another experiment 5 μmoles of visual pigment was digested with 5 mg of pepsin as described above. The digest was lyophilized, then suspended in water adjusted to pH 7 with trimethylamine and digested with 3 mg of pronase (Sigma) for 24 hr at 30°. At the end of this period the pronase was inactivated by boiling the digest for 3 min. The protein was then digested with 2 mg of subtilsin BPN′ for 48 hr at 30°. The digest was lyophilized and then dissolved in 5 ml of 0.2 M pyridine–acetic acid buffer (pH 3.1). An insoluble "core" was removed by centrifugation, and the soluble glycopeptide was isolated by ion-exchange chromatography on Dowex 50-X2 as described above for the peptic glycopeptide.

Two-dimensional peptide maps were performed on Whatman No. 3MM paper (55 × 45 cm). Electrophoresis was carried out at pH 6.5, 3000 V for 30 min, and descending chromatography with 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v) for 18 hr. Peptides were detected with the chlorination–KI procedure according to Mazur *et al.* (1962).

Analytical gel filtration of purified glycopeptide was performed on a column of Sephadex G-50 (0.9 × 58 cm). Glycopeptide 0.22 μmole in 0.2 ml (0.55% of bed volume) was applied and eluted with H_2O. Fractions of 0.5 ml were collected and assayed for neutral sugar and peptide as described above.

Amino acid analyses were performed with the Beckman Model 120C analyzer by the method of Spackman *et al.* (1958). Samples of 0.01–0.1 μmole of peptide in deionized water were mixed with an equal volume of concentrated HCl (analytical grade) and hydrolyzed at 110° in sealed, evacuated Pyrex tubes. Peptides were hydrolyzed for 24 hr. The hydrolysate was then evaporated over solid NaOH in an evacuated desiccator.

Hexosamines were determined on the short (0.9 × 12 cm) column of the analyzer eluted with 0.35 M sodium citrate buffer (pH 5.25). Samples of peptide (0.01–0.1 μmole) were hydrolyzed in 4 N HCl in sealed evacuated tubes for 6 and 12 hr at 100°. The hydrolysates were evaporated to dryness in a desiccator over NaOH pellets.

Neutral sugars were determined by the phenol–sulfuric acid method of Dubois *et al.* (1956) using a mixture of D-mannose and D-galactose (2:1) as standard. Neutral sugars were also determined by gas–liquid partition chromatography of the alditol acetate derivative as described by Kim *et al.* (1967) with *myo*-inositol as internal standard.

Subtractive Edman degradation was performed according to Konigsberg (1967) on 0.1–0.3 μmole of peptide.

Cleavage with cyanogen bromide was performed on 0.67 μmole of peptide according to Steers *et al.* (1965). The cleavage products were separated on a column (1.5 × 21 cm) of Sephadex G-25 (fine) equilibrated and eluted with 1% acetic acid at 23°. Fractions of 1.5 ml were collected and 0.15-ml aliquots were assayed with the ninhydrin reagent after alkaline hydrolysis (Hirs *et al.*, 1956).

Digestion with carboxypeptidase A (Worthington) was performed on 0.067 μmole of peptide at 30° in 0.03 M Tris buffer (pH 8) for 12 hr with an enzyme to substrate ratio of 1:20. The enzyme was solubilized as described by Potts *et al.* (1962). The digestion products were applied directly to the amino acid analyzer.

Complete hydrolysis of papain fragment was achieved by digesting 0.06 μmole of peptide with 0.05 mg of leucine aminopeptidase (Worthington) and with 0.003 μmole of carboxypeptidase A at pH 8.0, 37° for 36 hr. Amides were determined by amino acid analysis under appropriate conditions.

Digestion of 1.34 μmoles of glycopeptide with papain (Worthington) was performed as described by Smyth (1967) at pH 6.3, 37° for 24 hr with an enzyme to substrate molar ratio of 1:54. The digestion products were lyophilized and then applied in 0.2 M pyridine–acetic acid buffer pH 3.1 to a column (0.9 × 60 cm) of Dowex 50-X2 equilibrated with the same buffer at 42°. After 80 ml of the above buffer had passed through the column, a linear gradient was established between 200 ml of the above buffer and 200 ml of 2 M pyridine–acetic acid buffer (pH 5.0).

The alkali sensitivity of the oligosaccharide–polypeptide bond (Bray *et al.*, 1967) was probed by incubating alcohol-precipitated visual pigment in freshly prepared 0.2 N NaOH at 23° for 24 hr. The pH was then adjusted to 7.4 and the incubation mixture was dialyzed against water. The dialyzable and nondialyzable material was examined for neutral sugar by the phenol–sulfuric acid method and for glucosamine after acid hydrolysis. In addition, amino acid analyses were performed on aliquots of visual pigment before and after 24-hr incubation in NaOH.

The glycosidases β-*N*-acetyl-D-glucosaminase (EC 3.2.1.30) and α-D-mannosidase (EC 3.2.1.24) were purified from jack bean meal as described by Li (1966). The enzymes were

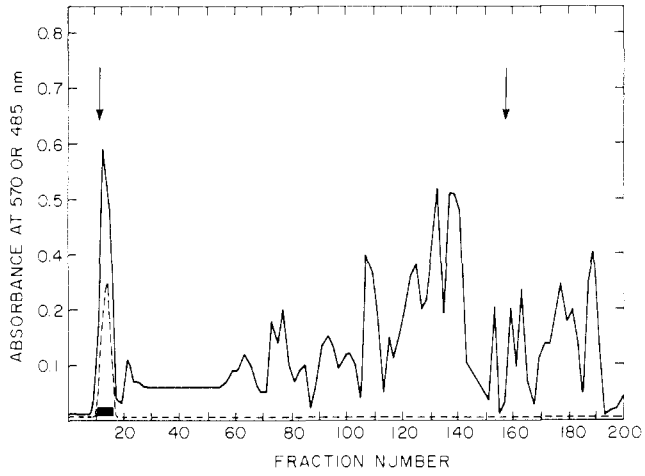

FIGURE 1: Chromatography of approximately 4.2 μmoles of peptic digest of visual pigment on a column of Dowex 50-X2 (2.5 × 38 cm) equilibrated with 0.2 M pyridine–acetic acid buffer (pH 3.1) at 23°. After eluting with the above buffer, a linear gradient elution (first arrow) between 500 ml of the above buffer and 500 ml of 2 M pyridine–acetic acid buffer (pH 5.0) was established. The column was finally washed with 8.5 M pyridine–acetic acid buffer (pH 5.6) (second arrow). Fractions of 7 ml were collected. Solid line: ninhydrin assay after alkaline hydrolysis; broken line: phenol–sulfuric acid assay. Bar represents pooled fraction.

assayed and the units of activity were defined as described by Bahl and Agrawal (1968). The purified α-mannosidase had an activity of 11.2 units/ml with 0.22 unit/ml (1.9%) of contaminating β-acetylglucosaminase activity. The purified β-acetylglucosaminase had an activity of 8.2 units/ml with no other glycosidase activity detectable. The peptic glycopeptide was digested with the purified glycosidases as described by Bahl (1969). Reducing activity released in the digestion mixture was determined by the ferricyanide method (Park and Johnson, 1949) and free *N*-acetylglucosamine was determined by the Morgan–Elson procedure as described by Neuberger and Marshall (1966). Glycopeptide (0.56 μmole) was digested with 3.4 units of α-mannosidase (including 0.066 unit of β-acetylglucosaminase as contaminant) for 42 hr, and in another experiment 0.2 μmole of glycopeptide was digested with 3.9 units of β-acetylglucosaminase for 46 hr. In some experiments the glycopeptide after glycosidase digestion was separated from the enzymes and liberated sugars by passage through a column (0.9 × 2 cm) of Dowex 50-X2 equilibrated with 0.2 M pyridine–acetic acid buffer (pH 3.1) and then on a column (0.9 × 10 cm) of Sephadex G-25 in H_2O. The sugars which remained attached to the peptide were then determined as described above.

Results

Isolation of Glycopeptide. Digestion of alcohol-denatured bovine visual pigment with pepsin in 10% acetic acid led to complete solubilization of the denatured protein. When the digest was lyophilized and then dissolved in 0.2 M pyridine–acetic acid buffer (pH 3.1), an insoluble "core" separated and was removed by centrifugation. Over 83% of the carbohydrate originally present in the molecule was found in the soluble fraction. The isolation of the peptic glycopeptide by ion-exchange chromatography on Dowex 50-X2 is shown in Figure 1. A single carbohydrate-containing peptide accounting for 74% of the sugar which was applied to the column was obtained. The glycopeptide was the most acidic peptide and appeared in the breakthrough column volume. A peptide map performed with 0.012 μmole showed a single acidic peptide while overloading the paper with ten times this amount still revealed only a single peptide. Analytical gel filtration of purified glycopeptide on Sephadex G-50 showed a single symmetrical peak in which the sugar and peptide were coincidental. The molar ratios of components of this glycopeptide were Asp, 2.00; Thr, 0.97; (Ser, 0.12); Glu, 1.04; Pro, 1.07; Gly, 2.00; (Ala, 0.26); Met, 0.94; Phe, 0.92; glucosamine, 2.70; and mannose, 3.13. Glucosamine and mannose were the only sugars found and they accounted for all the carbohydrate found in visual pigment. A previous identification of one residue of galactose in visual pigment (Heller, 1968) appears in the light of more sensitive methods to be in error. Another possibility is the presence of microheterogeneity in the neutral sugar composition (see, *e.g.*, Price *et al.*, 1969, and Catley *et al.*, 1969). Supporting this possibility was the finding of four to five neutral sugar residues in several samples of glycopeptide as determined by the phenol–sulfuric acid method. Unfortunately no gas chromatography determinations of neutral sugars were performed on these earlier samples.

After the removal of the glycopeptide obtained in the initial peptic digestion, an insoluble "core" was left. This "core" still contained some 15–17% of the original carbohydrate found in the molecule. When this "core" was redissolved in acetic acid and redigested with pepsin, a further 5–7% of soluble glycopeptide was obtained. When this material was chromatographed on Dowex 50-X2 the same acidic glycopeptide was obtained, and no other sugar-containing peptides were found.

When denatured visual pigment was digested with pepsin, subtilisin BPN′, and pronase, a glycopeptide containing four amino acid residues was obtained. This peptide chromatographed on Dowex 50-X2 at pH 3.1 as the most acidic peptide and appeared, just as the peptic glycopeptide, in the breakthrough volume. The molar ratios of the components of this peptide were Asp, 1.28; Thr, 1.00; (Glu, 0.20); Gly, 1.28; Met, 0.97; and glucosamine, 2.8; this peptide is thus derived from the peptic glycopeptide by further digestion (see below).

Amino Acid Sequence of Glycopeptide. The steps that were used in establishing the amino acid sequence are summarized in Table I. One-step Edman degradation performed on the whole glycopeptide showed the amino-terminal residue to be methionine. Cleavage of the glycopeptide with cyanogen bromine yielded two fragments, one of which was homoserine lactone and the other included the rest of the peptide with all the carbohydrate attached. When the peptic glycopeptide was digested with papain, two main fragments were isolated by chromatography on Dowex 50-X2 (H^+): an acidic dipeptide appearing in the breakthrough volume, composed of a methionyl and an aspartyl residue plus all the carbohydrate found in the peptic peptide, and a heptapeptide comprising the rest of the peptic peptide. A five-step Edman degradation established the sequence of this peptide as: Gly-Thr-Glu-Gly-Pro-Asn-Phe. The papain heptapeptide was an acidic peptide; the nature of the amide residue was established by a complete

enzymic hydrolysis with a combination of leucine aminopeptidase and carboxypeptidase. As mentioned above, a tetraglycopeptide (Met,Asx,Gly,Thr) was obtained from a combined digest of visual pigment by pepsin, subtilsin, and pronase. It is obvious that this tetrapeptide is derived from the amino-terminal end of the peptic glycopeptide and thus provides an overlap between papain peptides I and II.

Mode of Linkage between Peptide and Oligosaccharide. Incubation of visual pigment with 0.2 N NaOH for 24 hr led to the loss of only 2% of neutral sugar as dialyzable material and to the complete recovery of glucosamine in the nondialyzable fraction. Interestingly enough, the same procedure led to the loss of exactly two threonine and one serine residues. In an attempt to clarify this point, proteins known to be free of carbohydrate, namely, cytochrome *c*, trypsin, and chymotrypsin, were incubated with 0.2 N NaOH for 24 hr at 23°. In each case there was a loss of between 5 and 15% of hydroxyamino acids. It is clear, thus, that the mere loss of a serine or threonine residue after alkali treatment in a protein relatively rich in hydroxy residues cannot serve as an indication as to the type of linkage between the sugar and the protein.

The amino acid sequence shows that all the carbohydrate is bound to the aspartyl residue at position 2. Since with the use of glycosidases (see below) it is possible to remove all the mannose residues and two out of three glucosamine residues, the linkage between the aspartyl residue and the carbohydrate moiety has to be through the third glucosamine residue, presumably the *N*-acetyl form. Since the only known linkage between a glucosamine and an aspartyl residue is the *N*-acetylglycosylamine linkage involving the amide nitrogen of asparagine (Neuberger *et al.*, 1966), it is assumed that the same type of linkage takes place in visual pigment.

Structure of Oligosaccharide. Digestion of the glycopeptide with β-acetylglucosaminase liberated free *N*-acetylglucosamine, as detected with the Morgan–Elson reagent. When the peptide was purified after digestion, 1.25 residues of glucosamine out of 2.7 (3.0) and all the mannose were still linked to the peptide. On the other hand, when the glycopeptide was digested with α-mannosidase containing low levels of β-acetylglucosaminase activity, 2.75 out of 3.13 residues of mannose were liberated as reducing sugar and only 1.25 residues of glucosamine remained linked to the peptide. These experiments are interpreted to show that two residues of *N*-acetylglucosamine are peripheral and are probably linked to mannose. The linkage is of the β configuration. Moreover, the three mannose residues are peripheral to the one glucosamine residue which constitutes the linkage with the asparagine residue in the peptide and are linked in the α configuration. The amino group of the glucosamine which is bound to the asparagine residue is most probably N acetylated.

TABLE I: Amino Acid Sequence of Peptic Glycopeptide from Visual Pigment.[a]

Sequence Met-Asx(CH_2O)-Gly-Thr-Glu-Gly-Pro-Asn-Phe	
Edman degradation	Asp, 2.00; **$MetSO_2$, 0.36;** Thr, 0.98; Glu, 1.05; Pro, 1.03; Gly, 2.08; Phe, 0.97
Carboxypeptidase A	Phe, 0.89
Cyanogen bromide	
Fragment I	Homoserine
Fragment II	Asp, Thr, Glu, Pro, Gly, Phe + all the sugar
Papain	
Peptide I	Met, 0.93; Asp, 1.00 + all the sugar
Peptide II	Asp, 1.00; Thr, 0.94; Glu, 1.08; Pro, 0.94; Gly, 1.97; Phe, 0.94
Edman degradation of papain peptide II	
Step 1	Asp, 1.00; Thr, 0.91; Glu, 1.07; Pro, 0.93; **Gly, 1.09;** Phe, 0.94
Step 2	Asp, 1.00; **Thr, 0.39;** Glu, 1.08; Pro, 1.03; Gly, 1.04; Phe, 0.91
Step 3	Asp, 1.00; Thr, 0.33; **Glu, 0.65;** Pro, 1.00; Gly, 0.99; Phe, 1.01
Step 4	Asp, 1.00; Thr, 0.32; Glu, 0.55; Pro, 0.96; **Gly, 0.67;** Phe, 1.00
Step 5	Asp, 1.00; Thr, 0.34; Glu, 0.62; **Pro, 0.63;** Gly, 0.78; Phe, 0.98
Aminopeptidase + carboxypeptidase A of papain peptide II	Asp, 0.15; Thr, 1.12; Glu, 1.09; Pro, 0.90; Gly, 2.00; Asn, 1.02

[a] Numbers are residues per molecule of glycopeptide. Symbols in boldface represent the residue removed by Edman degradation.

Discussion

The results obtained show that all the carbohydrate in bovine visual $pigment_{500}$ is present as a single oligosaccharide chain attached to an aspartyl residue. A unique glycopeptide, whose sugar composition accounted for all the carbohydrate present in visual pigment, was obtained in an overall yield of better than 60% from proteolytic digests performed under various conditions. Purification of the glycopeptide proved to be particularly easy since it was the most acidic peptide and, as such, was not retained by columns of the acidic resin Dowex 50-X2 (H^+). The fact that the glycopeptide is not retained by the column is probably due to the steric interference by the carbohydrate moiety of the interaction between the methionylamino group and the acidic resin.

Determination of the amino acid sequence of this peptide clearly established that the carbohydrate is attached to the aspartyl residue at position 2. This is shown by the fact that cyanogen bromide cleavage of the glycopeptide resulted in the formation of free homoserine and a fragment that contained the rest of the glycopeptide, including all the sugar. On the other hand, digestion with papain resulted in the formation of the dipeptide Met-Asp containing all the sugar and a heptapeptide accounting for the rest of the peptide. In this connection, it is interesting to note the specificity of papain in cleaving the peptide bond at the carboxyl side of the carbohydrate-linking aspartyl residue. A similar specificity was reported by Rosevear and Smith (1961).

The studies with glycosidases show that glucosamine (most probably *N*-acetyl) is the sugar residue linked to the aspartyl residue since it is possible to remove all the mannose and two out of three glucosamine residues, leaving a single glucosamine residue linked to the peptide. The glycosidase studies cannot distinguish whether the two peripheral *N*-acetylglucosamine residues are linked to the central glucosamine or to the mannose residues (although the latter possibility seems more plausible). Nor can these studies tell whether the mannose residues form a linear or a branched structure. It is possible though to specify the type of linkage between the mannose residues and their reducing end neighbor as an α configuration while the linkage between the *N*-acetylglucosamine residue and their reducing end neighbor is a β configuration.

The sequence -Asp(CH_2O)-X-Thr- has been found in glycoproteins before, and its frequency has been commented upon (Eylar, 1965; Wagh *et al.*, 1969; Catley *et al.*, 1969). The only other glycoprotein with a similar sequence to the one reported here, namely -Asp(CH_2O)-Gly-Thr-, is α_1 -acid glycoprotein from human plasma (Satake *et al.*, 1965).

The number of glucosamine residues found after acid hydrolysis was always 2.7–2.8. The actual number in the glycopeptide is probably 3.0. The lower figure obtained is due to initial deacylation of the *N*-acetylglucosamine making the glycosidic bond relatively resistant to hydrolysis (Gottschalk and Ada, 1956; Catley *et al.*, 1969).

Molecular Weight of Visual Pigment. The number of glucosamine residues in intact visual pigment was found to be three per apoprotein molecule of mol wt 26,400. The molecular weight of visual pigment was estimated from data obtained by gel filtration on calibrated columns and then from the minimal molecular weight found by amino acid analysis (Heller, 1968). The results reported in this paper showing that the visual pigment glycopeptide contains three residues of glucosamine were calculated from the internal evidence supplied by the peptide composition. Since these two values for the number of glucosamine residues in the intact protein and in the glycopeptide are independent of each other and since they agree, this is taken as additional supportive evidence that the molecular weight of bovine visual pigment apoprotein is indeed 26,000–27,000.

Possible Function of Carbohydrate Moiety in Visual Pigment. Visual pigment is a membrane protein and a structural component of the retinal rod outer segment disk membrane system. It has recently been shown that the visual pigment apoprotein is synthesized in the photoreceptor inner segment and is then transported to the photoreceptor outer segment where it is continuously assembled into new disk membranes (Young, 1967; Hall *et al.*, 1969). It seems attractive to hypothesize that the function of the hydrophilic oligosaccharide moiety on the surface of the visual pigment molecule is to serve as a vectorial orientation marker during the assembly of the molecule into the membrane mosaic. Thus, the sugar might assure a proper approach and alignment of the newly added molecule onto the already-existing membrane. It is assumed here that the interactions of the newly arrived visual pigment surface with the other visual pigment molecules (and/or other components) to form the membrane aggregate is mainly through hydrophobic forces. The hydrophilic surface formed by the sugar moiety would tend, thus, to orient itself toward the aqueous phase. This, in turn, will tend to bring the hydrophobic part of the membrane protein surface in proper orientation with the growing membrane. In this sense, then, the carbohydrate moiety acts as a surface marker. It is interesting in this connection to note that many membrane proteins are known to have a sugar moiety, and it is possible that in all these proteins one of the functions of the carbohydrate is to be a surface marker (see, *e.g.*, Cook, 1968, Bakeman and Wasemiller, 1967, and Rosenberg and Guidotti, 1968).

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Purification and Characterization of a Lectin (Plant Hemagglutinin) with Blood Group A Specificity from *Dolichos biflorus**

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ABSTRACT: The blood group A specific lectin from *Dolichos biflorus* seeds was purified by adsorption on to insoluble polyleucyl blood group A substance and subsequent elution with 2-deoxy-β-D-galactopyranoside (D-GalNAc). The purified lectin was homogeneous by ultracentrifugation, gave one diffuse band on acrylamide gel electrophoresis under acid and alkaline conditions, formed one line in immunodiffusion and immunoelectrophoresis against rabbit antisera to the crude seed extract, and was totally precipitated by human blood group A substance; it had a tendency to aggregate in solution. Amino acid analyses of the purified lectin showed a large amount of aspartic acid and serine but no cysteine or methionine. The lectin contains about 2% hexose and has a molecular weight of 140,000 and an isoelectric point of pH 4.5. The lectin precipitated with blood group A_1 and A_2 substances as well as with the streptococcal group C polysaccharide. The reactivity of each of these polysaccharides is ascribed to terminal nonreducing α-linked D-GalNAc residues. The lectin did not precipitate with blood group B or H substances, with an ovarian cyst substance lacking A, B, H, Le^a, or Le^b activity, with group A streptococcal polysaccharide, with teichoic acids or with the periodate degradation stages of a blood group H substance. Inhibition of precipitation with various monosaccharides, glycosides, and oligosaccharides indicates that the combining site of the lectin is specific for terminal α-linked D-GalNAc. There is some uncertainty as to the size of the combining site. Although the A-active di- and trisaccharides were equal in inhibiting power to methyl 2-acetamido-2-deoxy-α-D-galactoside, the A-active-reduced pentasaccharide required only about six-tenths the molar concentration for comparable degrees of inhibition. No heterogeneity in the combining site was detected among various lectin fractions differentially eluted from the immunoadsorbent.

Many plants contain agglutinins capable of combining specifically with animal erythrocytes and other cells (for reviews, see Krüpe, 1956, Mäkelä, 1957, and Bird, 1959). These hemagglutinins, called lectins (Boyd and Shapleigh, 1954b), are most frequently found in the seeds of leguminous plants and differ from one another in their specificities, certain of which are directed toward the blood group ABH substances (*cf.* Mäkelä, 1957).

The many similarities in specificity of these plant hemagglutinins to antibodies to blood group substances (Kaplan and Kabat, 1966; Moreno and Kabat, 1969) make it desirable to obtain them in highly purified form, to establish whether they are homogeneous or heterogeneous with respect to their combining sites, to obtain information as to the dimensions of their complementary regions, and ultimately to elucidate the three-dimensional structure of their specific sites.

The seeds of *Dolichos biflorus* contain lectin-agglutinating type A_1 red blood cells (Bird, 1951, 1952a,b) and specifically precipitating with blood group A substance (Boyd and Shapleigh, 1954a; Bird, 1959). Several workers have attempted to purify this lectin by alcohol precipitation (Bird, 1959) and by fractionation procedures based on charge (Kocourek and Jamieson, 1967). The present study describes the purification of the *D. biflorus* lectin by the use of the specific insoluble immunoadsorbent, polyleucyl hog blood group A + H substance (Kaplan and Kabat, 1966; Moreno and Kabat, 1969; Hammarström and Kabat, 1969), its characterization and a study of its combining site.

Materials

D. biflorus seeds were a gift from Col. G. W. G. Bird. The various blood group substances and blood group oligosaccharides used in this study are preparations previously prepared and described in this laboratory (*cf.* Kabat, 1956; Allen and Kabat, 1959; Schiffman *et al.*, 1962, 1964; Lloyd

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