Soybean Hemagglutinin, a Plant Glycoprotein

I. ISOLATION OF A GLYCOPEPTIDE*

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SUMMARY

1. A procedure is given for the preparation of purified soybean hemagglutinin from untoasted soybean flour. The final purification step involves column chromatography on calcium phosphate. The homogeneity of the purified protein was ascertained chromatographically and electrophoretically.

2. The purified soybean hemagglutinin was found to contain mannose (4.5%) and glucosamine (1%).

3. The carbohydrate moiety was released from the protein in the form of a glycopeptide on proteolysis of the hemagglutinin with Pronase. After purification on Sephadex and Dowex 50, the glycopeptide was found to contain aspartic acid in addition to essentially all the carbohydrate of soybean hemagglutinin.

4. The molecular weight of the glycopeptide as determined by equilibrium centrifugation (4,000) corresponds to that calculated from its aspartic acid, mannose, and glucosamine content (4,400), assuming a single aspartic acid residue per polysaccharide unit. From the size of the glycopeptide, the molecular weight of the soybean hemagglutinin (110,000), and its carbohydrate content it was concluded that the carbohydrate moiety in this glycoprotein is present as a single polysaccharide chain.

Considerable information has accumulated during the last decade on the structure and properties of glycoproteins isolated from animal tissues (1–3). Our knowledge concerning the occurrence and properties of plant glycoproteins is, however, still scanty. Although the presence of carbohydrates in various protein fractions obtained from plant material has been reported (4–10), very little information is available on the nature of the binding of the carbohydrate to the protein.

In the present article evidence is presented to show that soybean hemagglutinin is a glycoprotein resembling some animal glycoproteins, such as ovalbumin (11, 12), and ribonuclease B (13, 14), in its carbohydrate composition and type of carbohydrate-protein linkage. A procedure has been worked out for its chromatographic purification from crude soybean hemagglutinin prepared according to Liener (15). The chromatographically purified soybean hemagglutinin resembles the material purified by Wada, Pallansch, and Liener (16) by convection electrophoresis and was found to contain D-mannose in addition to D-glucosamine; the latter amino sugar was first found in soybean hemagglutinin by these authors (16). A Pronase digest of the purified soybean hemagglutinin yielded a glycopeptide containing mannose, glucosamine, and aspartic acid.

Preliminary reports on this work have appeared (17, 18).

EXPERIMENTAL PROCEDURE

Analytical Methods—Neutral sugar was determined by the phenol method of Dubois et al. (19) with mannose as standard. Amino sugar was determined by two different methods. (a) The Blix modification (20) of the Elson-Morgan procedure was the first method used; unless otherwise stated, hydrolysis for this assay was carried out with 2 N HCl for 14 hours at 100° in a sealed tube. (b) The ninhydrin method with the amino acid analyzer was also used; acid hydrolysis was carried out under the standard conditions employed for amino acid analysis (6 N HCl, 22 hours, 110°). For identification of carbohydrates, samples were hydrolyzed with 1 N H2SO4 at 100° for 6 hours, and the hydrolysates were neutralized with solid BaCO3; the BaSO4 precipitate was washed with water, and the supernatant, including the washings, was concentrated under reduced pressure. The residue was dissolved in water and the solution was chromatographed on Whatman No. 1 paper in the following solvents: (a) 1-butanol-acetic acid-water (5:1:4, v/v); (b) 1-butanol-ethanol-water (10:1:2, v/v); and (c) 1-butanol-pyridine-water (6:4:3, v/v). The spots were detected by the silver nitrate reagent (21).

Neutral sugars were also identified by column chromatography on Dowex 2 according to Hallen (22). The identity of the amino sugar component was ascertained by (a) chromatography on Dowex 50 in 0.3 M HCl according to Gardell (23); (b) degradation with ninhydrin and subsequent identification of the resulting pentoses according to the procedure of Stoffyn and Jeanloz (24); and (c) N-acetylation and chromatographic identification of the N-acetylhexosamine which was obtained (25). Sialic acid was assayed by the method of Werner and Odlin (26). Quantitative amino acid analyses were carried out on the Spinco amino acid analyzer, model 120, according to the method of Spackman, Stein, and Moore (27). Hydrolysis was routinely performed in evacuated sealed tubes for 22 hours at 110° at 6 M HCl. Tryptophan was determined on unhydrolyzed protein samples by the method of Spies and Chambers (28).

Measurements of sedimentation velocity of the hemagglutinin...
were carried out in a Spinco model E ultracentrifuge equipped with schlieren phase plate optics. Diffusion coefficients were determined in a Spinco model E ultracentrifuge with a synthetic boundary cell according to Ehrenberg (29). The molecular weight of the glycopeptide in 0.1 M NaCl was determined by the sedimentation equilibrium method of Yphantis (30) with the use of a Spinco model E ultracentrifuge with a multichannel cell at 29,900 rpm.

Block electrophoresis (31) was carried out in 0.1 M acetic acid buffer, pH 4.2, with polyvinyl chloride particles (Pevikon, obtained from Fosfatbolaget, Stockholm) as support. A solution of 25 mg of protein in 0.5 ml of the same buffer was mixed with sufficient Pevikon particles to give a slurry which was then de-posit ed into a cut (0.5 cm wide) made in a block (0.5 × 0.8 × 30 cm) of the supporting material. A voltage of 300 volts was applied and the electrophoresis was run for 16 hours at 4°C. The block was sliced into 1-cm segments, each of which was eluted with 3 ml of water. Then the optical density at 280 nm and the neutral sugar content of the eluates were determined.

Discrete electrophoresis in polyacrylamide gels was carried out at pH 4.5 according to Reisfeld, Lewis, and Williams (32), and at pH 8.9 according to Ornstein and Davis (33). Staining was carried out with Amido black in 7% acetic acid, and destaining in an electric field with 7% acetic acid.

Chromatography on carboxymethyl cellulose was carried out in acetate buffer with increasing concentrations of sodium chloride. Carboxymethyl cellulose (Whatman No. 70) was equilibrated with 0.01 M acetate buffer, pH 4.0 (starting buffer); the suspension was poured into a column (1.1 × 32 cm), and allowed to settle under gravity. A solution of purified soybean hemagglutinin (30 mg in 6 ml of starting buffer) was applied to the column. The column was washed with 75 ml of starting buffer and then connected to a closed mixing chamber containing 200 ml of the same buffer. The following sequence of solutions was added gradually to the mixing chamber: (a) 200 ml of 0.15 M NaCl in starting buffer; (b) 200 ml of 0.5 M NaCl in starting buffer; (c) 70 ml of 1.0 M NaCl in starting buffer; and (d) 100 ml of 2.0 M NaCl in 0.67 M sodium acetate. Fractions of 4 ml were collected at a rate of 25 ml per hour. The chloride concentration in selected tubes was determined on the Aminco-Cotlove chloride titrator.

Hemagglutinating activity was assayed according to the method of Liener with trypsinized rabbit red blood cells (35).

Isolation of Soybean Hemagglutinin—Partially purified soybean hemagglutinin was prepared from untoasted soybean flour obtained from Central Soya, Chicago (Soyafuff 200W), in essentially the same manner as described by Liener (9). First precipitation of the hemagglutinin fraction from the acidified extract was effected between 0.40 and 0.70 saturation with ammonium sulfate, and not, as according to Liener, between 0.40 and 0.53. Final purification was achieved by chromatography on calcium phosphate prepared according to Tiselius, Hjertén, and Levin (36) as follows: 750 mg of partially purified hemagglutinin, obtained from 500 g of flour, were dissolved in 20 ml of 0.001 M phosphate buffer, pH 6.8, and the solution was applied to a column (2.8 × 15 cm). Elution was performed by increasing the concentration of the same buffer stepwise. Of the following concentrations, 150-ml volumes of each were used: 0.001 M, 0.05 M, 0.1 M, 0.2 M, and 0.5 M. Fractions of 10 ml were collected at a flow rate of 40 ml per hour and analyzed for absorbance at 280 μm, for neutral sugars, and for hemagglutinating activity. The fractions with hemagglutinating activity were pooled, dialyzed against distilled water at 4°C, and lyophilized.

Isolation of Glycopeptide from Soybean Hemagglutinin—Purified hemagglutinin was digested with Pronase in 0.05 M phosphate buffer, pH 7.4, for 72 hours at 37°C; the reaction mixture contained 30 mg of substrate and 1 mg of enzyme per ml. The digest (15 ml) was then subjected to gel filtration on a column (2.4 × 30 cm) of Sephadex G-50, previously equilibrated with 0.01 M Tris buffer, pH 8.5. Elution was carried out with the same buffer at a rate of 24 ml per hour; 6-ml fractions were collected. The fractions were analyzed for absorbance at 280 μm, for neutral sugars, and for amino groups by the ninhydrin reagent (37). The fractions containing carbohydrate were pooled and lyophilized. The dry residue was dissolved in a small volume of water (5 to 10 ml), and the bulk of the contaminating ninhydrin-positive compounds were removed from the concentrated solution by passing through a column (0.9 × 5 cm) of Dowex 50-X8, 200 to 400 mesh, in H⁺ form. The resin was washed until the effluent gave a negative phenol test. Further removal of ninhydrin-reacting material could be achieved by chromatography on Sephadex G-25. For this purpose the effluent obtained from the Dowex column was lyophilized, dissolved in 5 ml of 0.01 M acetic acid, and applied to a column (1.4 × 50 cm) of Sephadex G-25, previously equilibrated with 0.01 M acetic acid. The carbohydrate-containing material emerged with the void volume of the column as a single sharp peak.

RESULTS

The partially purified hemagglutinin obtained from soybean meal had a hemagglutinating activity of 1600 units per mg and contained 7 to 8% neutral sugars (w/w). Chromatography of this preparation on calcium phosphate yielded five fractions, all of which contained both protein and carbohydrate in varying proportions (see Fig. 1 and Table I). Hemagglutinating ac-

![Fig. 1. Chromatography of crude soybean hemagglutinin on calcium phosphate. Crude hemagglutinin (750 mg) dissolved in 20 ml of 0.001 M phosphate buffer, pH 6.8, was applied to a column (2.8 × 15 cm). Elution was performed by a stepwise increase of the concentration of phosphate buffer, pH 6.8. The arrows indicate the points of change and the concentration of the buffer. Fractions of 10 ml were collected at a flow rate of 40 ml per hour. I, optical density at 280 μm; II, neutral sugars; III, hemagglutinating activity.](image-url)
TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Applied protein</th>
<th>Applied carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3-5</td>
<td>13-11</td>
</tr>
<tr>
<td>II</td>
<td>30-52</td>
<td>50-55</td>
</tr>
<tr>
<td>III</td>
<td>20-22</td>
<td>5-7</td>
</tr>
<tr>
<td>IV</td>
<td>28-30</td>
<td>22-25</td>
</tr>
<tr>
<td>V</td>
<td>1-2</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2. Rechromatography of purified soybean hemagglutinin on calcium phosphate. Purified hemagglutinin (200 mg) was dissolved in 20 ml of 0.001 M phosphate buffer, pH 6.8, and applied to a column (2.5 x 1.5 cm). Elution was as described in the legend to Fig. 1.

Fig. 3. Chromatography of purified soybean hemagglutinin on carboxymethyl cellulose. Purified hemagglutinin (30 mg) was dissolved in 6 ml of 0.01 M acetate buffer, pH 4.2, and applied to a column (1 x 30 cm) of carboxymethyl cellulose in equilibrium with the same buffer. The mixing chamber contained 200 ml of starting buffer. The reservoir contained (a) 75 ml of starting buffer; (b) 200 ml of 0.15 M NaCl in starting buffer; (c) 200 ml of 0.5 M NaCl in starting buffer; (d) 70 ml of 1.0 M NaCl in starting buffer; and (e) 100 ml of 2.0 M NaCl in 0.67 M sodium acetate. Fractions of 1 ml were collected at a rate of 25 ml per hour. ---, optical density at 280 nm; --, neutral sugars; ----, molarity of NaCl concentration.

Fig. 4. Block electrophoresis of purified soybean hemagglutinin on polyvinyl chloride particles (Pevikon) in 0.1 M acetate buffer, pH 4.2. ---, optical density at 280 nm; --, neutral sugars.

Fig. 5. Activity of purified soybean hemagglutinin in Fraction IV was found in only one fraction, Fraction IV, which had been eluted with 0.2 M phosphate buffer, pH 6.8. The specific activity of the material in this fraction was 5400 units per mg. A purification of 3.5-fold had thus been achieved. The recovery of the active material was 90%. The purified soybean hemagglutinin (Fraction IV) was found to contain 4.5% neutral sugars (w/w).

The homogeneity of the purified hemagglutinin was tested by chromatography on calcium phosphate, on carboxymethyl cellulose, and on Sephadex G-50, and by ultracentrifugation and electrophoresis. The results of rechromatography on calcium phosphate, given in Fig. 2, show that the purified hemagglutinin emerges practically as a single peak at the same position as in the original chromatogram (cf. Fig. 1). The chromatographic behavior of purified soybean hemagglutinin on carboxymethyl cellulose (Fig. 3) shows that the protein and carbohydrate are eluted together as a single peak. A single peak containing the same relative amounts of protein and carbohydrate was also obtained when the purified hemagglutinin was chromatographed on Sephadex G-50 in 0.01 M Tris buffer, pH 8.5, in either the absence or presence of guanidine hydrochloride. In the latter experiment the buffer solution was 4.0 M in guanidine hydrochloride and the Sephadex column was pre-equilibrated with the same solution.

Ultracentrifugation of a 1% solution of purified soybean hemagglutinin in 0.1 M phosphate buffer, pH 7.4, yielded a single symmetrical peak during the entire course of the run, corresponding to a sedimentation constant of $s_{20,w} = 6.08$. The diffusion coefficient, $D_{20,w}$, was found to be $5.0 \times 10^{-7} \text{ cm}^2 \text{ per sec}$. From these data a molecular weight of 110,000 was calculated, assuming a partial specific volume of 0.75 (38).

Electrophoresis of the purified hemagglutinin on Pevikon gave a single symmetrical peak as shown in Fig. 4. The neutral sugars migrated together with the protein, and each of the fractions collected contained a constant percentage of neutral sugars. The electrophoretic homogeneity of the purified soybean hemag-
TABLE II
Composition of purified soybean hemagglutinin

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Residues per 100,000 g, dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>97</td>
</tr>
<tr>
<td>Threonine</td>
<td>50</td>
</tr>
<tr>
<td>Serine</td>
<td>76</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>52</td>
</tr>
<tr>
<td>Proline</td>
<td>47</td>
</tr>
<tr>
<td>Glycine</td>
<td>40</td>
</tr>
<tr>
<td>Alanine</td>
<td>40</td>
</tr>
<tr>
<td>Valine</td>
<td>45</td>
</tr>
<tr>
<td>Methionine</td>
<td>16</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16</td>
</tr>
<tr>
<td>Leucine</td>
<td>18</td>
</tr>
<tr>
<td>Phenylnalanine</td>
<td>14</td>
</tr>
<tr>
<td>Histidine</td>
<td>45</td>
</tr>
<tr>
<td>Lysine</td>
<td>40</td>
</tr>
<tr>
<td>Arginine</td>
<td>18</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>18</td>
</tr>
<tr>
<td>Cystine</td>
<td>Traces</td>
</tr>
<tr>
<td>Mannose</td>
<td>25</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>3 to 5 residues/100,000 g</td>
</tr>
<tr>
<td>Nitrogen (Kjeldahl)</td>
<td>13.2%</td>
</tr>
<tr>
<td>Nitrogen accounted for</td>
<td>98%</td>
</tr>
</tbody>
</table>

* Obtained by linear extrapolation to zero time of the results obtained after hydrolysis for 22, 48, and 72 hours.

a Determined as cysteic acid after oxidation with performic acid.
b Determined by the phenol method.
c Estimated by the ninhydrin method with the use of the amino acid analyzer after hydrolysis with 6 N HCl at 110°C for 22 hours.
d Assayed by the Blix (20) modification of the Elson-Morgan procedure after hydrolysis with 2 N HCl at 100°C for 14 hours.

glutinin was confirmed by gel electrophoresis on polyacrylamide. A single band was obtained at pH 4.5 as well as at pH 8.9.

The amino acid and carbohydrate compositions of soybean hemagglutinin are given in Table II. This hemagglutinin is rich in the acidic amino acids and is low in sulfur-containing amino acids. Special mention should be made of the rather high content of serine and threonine as well as of proline and glycine.

Mannose and glucosamine were the only carbohydrates found to be present as constituents of soybean hemagglutinin. The exact assay of the glucosamine content is rather difficult in view of the problems involved in the quantitative liberation of the hexosamine from its glycosidic linkage and in view of the interference which has been observed when neutral sugars and amino acids are heated together (40); it is not surprising, therefore, that variations in the determination of glucosamine were observed.

The sedimentation constant of the purified hemagglutinin described here, as well as its amino acid composition, resemble closely that recorded by Wada et al. (18) on their purified soybean hemagglutinin, which had been isolated from crude hemagglutinin by convection electrophoresis. Marked differences were observed only in the content of serine, leucine, and lysine.

The hexosamine content of soybean hemagglutinin as determined by us (3 to 5 residues/100,000 g) was found to be markedly smaller than that reported by Wada et al. (16) (30 to 55 residues/100,000 g). In this connection it is pertinent to note that analysis in our laboratory of a sample of purified soybean hemagglutinin kindly supplied by Dr. Liener yielded a value of 3 to 5 residues/100,000 g. This sample, when chromatographed by us on a calcium phosphate column, was eluted from the column at the same concentration of phosphate buffer as the hemagglutinin purified by us. Furthermore, the hemagglutinating activity of Dr. Liener's preparation, 5,700 units per mg, was the same as that of our purified preparation when both preparations were tested simultaneously under identical conditions.

Digestion of the purified hemagglutinin with Pronase and chromatography of the digest on Sephadex G-50 (Fig. 5) yielded a low molecular weight fraction, F-I, which contained about 95% of the carbohydrates of soybean hemagglutinin. This fraction also contained ninhydrin-positive material which could be re

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Chromatography of purified soybean hemagglutinin on Sephadex G-50 with 0.01 M Tris buffer, pH 8.7, before and after proteolytic digestion. A, untreated; B, digested with Pronase (1 mg of enzyme per 30 mg of hemagglutinin in 1 ml of 0.05 M phosphate buffer, pH 7.4, incubated for 72 hours at 37°C). The digest (15 ml) was applied to a column (2.4 x 30 cm); fractions of 6 ml collected. —, optical density at 280 nm; ---, neutral sugars; ---, α-amino groups (ninhydrin reaction).
Soybean Hemagglutinin, a Plant Glycoprotein. I

Mannose was assayed by the phenol method (19). Glucosamine was determined according to Blix (20). The content of glucosamine in soybean hemagglutinin purified by a different technique was reported originally by Wada et al. (16). The content of the amino sugar reported by these authors was, however, considerably higher than that found by us. The reason for this discrepancy is not clear.

A variety of techniques failed to dissociate the carbohydrate from the protein; these techniques included chromatography on Sephadex in the presence of 4 M guanidine hydrochloride as well as ion exchange, calcium phosphate chromatography, and electrophoresis. The carbohydrate moiety was released from the protein in the form of a glycopeptide on proteolytic degradation of the hemagglutinin with Pronase. After purification on Sephadex and Dowex 50, the glycopeptide was found to contain aspartic acid in addition to essentially all the carbohydrate of soybean hemagglutinin. Its molecular weight, as estimated by equilibrium centrifugation, corresponds to that calculated from its chemical composition. The purified protein contains 4.6% mannose and 1% glucosamine. The presence of glucosamine in soybean hemagglutinin purified by a different technique was reported by Edsall (28). The molecular weight value is also in agreement with the calculated molecular weight of the glycopeptide based on the analytical data given in Table III.

DISCUSSION

In the present study a new procedure for the purification of soybean hemagglutinin is described. This procedure made use of chromatography on calcium phosphate. The homogeneity of the hemagglutinin was ascertained by various physicochemical techniques. The purified protein contains 4.5% mannose and 1% glucosamine. The presence of glucosamine in soybean hemagglutinin, 110,000, and its carbohydrate content, 4.5%, it may be concluded that the carbohydrate moiety is present in this hemagglutinin as a single polysaccharide unit.

Comparison of the results described here for soybean hemagglutinin with those reported for some animal glycoproteins reveals a number of interesting similarities. (a) The sugar constituents of the carbohydrate moiety of soybean hemagglutinin, mannose and glucosamine, are the same as those found in a number of different animal glycoproteins (3, 13). (b) The structure of the carbohydrate unit appears to be that of a heteropolysaccharide chain of moderate size. This type of structure has been found in ovalbumin (11), fetuin (41), α1-acid glycoprotein (49), and other glycoproteins (3). (c) The presence of aspartic acid in the glycopeptide together with the carbohydrate moiety indicates that the carbohydrate chain is attached to this amino acid by a covalent link. Work on ovalbumin (12), γ-globulin (43), and α1-acid glycoprotein (44) had indicated that aspartic acid is involved in the glycopeptide linkage in these proteins.
glucosamine. Our results are consistent with the possibility of such a linkage.

Our findings show clearly that glycoproteins similar to those found in animal tissues do also exist in plants. Indeed, while this paper was in preparation, Pusztai (45) reported the isolation of a glycoprotein containing mannose and glucosamine from kidney beans.

Acknowledgments—We wish to thank Dr. E. Meyer of Central Soya, Chicago, for a generous gift of soybean meal, and Dr. I. E. Liener for a sample of purified soybean hemagglutinin. Thanks are due to Mrs. H. Latter for skillful technical assistance, to Mr. A. Lustig for the ultracentrifuge analyses, and to Mrs. J. Conu for the amino acid analyses.

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