Purification and Characterization of a Lectin from Wistaria floribunda Seeds

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TSUTOMU KUROKAWA, MASAO TSUDA, AND YUKIO SUGINO
From the Biological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-Ku, Osaka 532, Japan

A lectin from Wistaria floribunda seeds which specifically binds to N-acetyl-d-galactosamine was purified to homogeneity as judged by polyacrylamide gel electrophoresis. Its molecular weight was estimated to be 68,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. It dissociated into subunits on reduction with 2-mercaptoethanol with concomitant loss of hemagglutinating activity. On oxidation in air, the subunits reassociated into the lectin molecules with hemagglutinating activity. Carboxymethylation of the subunits with iodoacetic acid prevented their reassociation on oxidation in air. The molecular weight of the subunits was 32,000, which is about one-half that of the native lectin, suggesting that the lectin consists of two subunits. The results of total, NH₂-terminal, and COOH-terminal amino acid analyses, and mapping of the tryptic digest of the lectin indicated that these two subunits were indistinguishable and were probably identical, and that they were linked together covalently through a single disulfide bond. Equilibrium dialysis experiments show that the lectin and its subunit molecules are divalent and monovalent, respectively, with respect to sugar binding. The lectin is a glycoprotein, containing 3.2% carbohydrate. The carbohydrate moiety is composed of mannose, galactose, and glucosamine in a molar ratio of 1:2:1 and these sugars seem to be linked as a single oligosaccharide chain to each subunit of the protein.

We have purified several novel lectins from plant and microbial sources for use in studies on the cell surface and membranes of normal and transformed cells. As reported in this paper, we obtained an electrophoretically homogeneous lectin preparation (WFA) from Wistaria floribunda seeds. The hemagglutinating activity of extracts of Wistaria floribunda seeds was first reported by Boyd et al. (1), and recently Toyoshima et al. purified a phytomitogen and a lectin from these seeds (2, 3). However, the nature of these proteins was unknown, and in fact among the many lectins known, only concanavalin A has been fully characterized in terms of its structure and binding specificity (4-6). Detailed information on the structures and binding specificities of other lectins that are susceptible to chemical alteration would be of great value for studies on the cell surface and cell membrane. The lectin purified in this work from Wistaria floribunda seeds seems to differ from that purified by Toyoshima et al. from the same source and was shown to have a unique subunit composition. The present paper describes details of the purification and physicochemical and biochemical properties of this lectin and its subunits.

EXPERIMENTAL PROCEDURE

Materials

Wistaria floribunda seeds were a gift from the Takeda Pharmacognostic Research Laboratories. Carboxypeptidase A (EC 3.4.12.2, iPr₂P-F'-treated) was a product of Worthington. Trypsin (EC 3.4.21.4, twice crystallized) was purchased from Serva, Heidelberg. Pronase was a product of Kaken Chemical Co., Tokyo. Human erythrocytes were obtained from A, B, and H type donors, respectively. Pig erythrocytes were collected from mini-pigs, strain OHMINI 875, and mouse erythrocytes from C3H/He mice. Sheep erythrocytes were purchased from Biotest Research Co., Tokyo. Rabbit erythrocytes were obtained from commercially available rabbits. N-Acetyl-d-[1-¹³C]galactosamine was a product of the Radiochemical Centre, Amersham. ¹H₂O was purchased from New England Nuclear Corp., Boston. All other chemicals used were of reagent grade.

Analytical Methods

Protein was determined by the method of Lowry et al. (1) with bovine serum albumin as a standard. Neutral sugar was determined by the phenol method of Dubois et al. (8) with glucose as a standard. Sulfhydryl residues of protein were determined by Edman's method (9) using DTNB.

Disc Electrophoresis

Disc electrophoresis on polyacrylamide gel was carried out at pH 9.4, 8.0, and 4.0 in 7.5% gels as described by Ornstein and Davis (10). Polyacrylamide gel electrophoresis on gels containing SDS was carried out by the method of Weber and Osborn (11). Staining was performed with 0.2% Coomassie brilliant blue, R-250.

The abbreviations used were: iPr₂P-F, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; WFA, Wistaria floribunda lectin; RCM-WFA, reduced and carboxymethylated Wistaria floribunda lectin; PTH, phenylthiohydantoin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Isoelectric Focusing

Isoelectric points were determined by the electrofocusing technique using an LKB 8100-1 electrofocusing column. Carrier ampholine of pH 3 to 10 was used.

Molecular Weight Estimation

Molecular weights were estimated by SDS-polyacrylamide gel electrophoresis and gel filtration. SDS-polyacrylamide gel electrophoresis was carried out as described above and the molecular weight of the lectin was estimated by comparing its mobility with those of markers of known molecular weight. Gel filtration on a Sephadex G-100 column was performed as described by Andrews (12). The molecular weight of the lectin was estimated by comparison of its elution volume with those of molecular weight markers.

Amino Acid Analysis

Samples were hydrolyzed in redistilled HCl in evacuated, sealed tubes at 110° for 24, 48, 72 h. The amino acid contents of the hydrolysates were determined in a Hitachi amino acid analyzer (K1.A.3R) by the method of Spackman et al. (13). Cysteine was determined as cysteic acid by hydrolyzing the sample after performic acid oxidation (14). Tryptophan was determined by the spectrophotometric method of Goodwin and Morton (15).

Reduction and Carboxymethylation

Reduction of protein by 2-mercaptoethanol and subsequent carboxymethylation were carried out as described by Crestfield et al. (16). A mixture of 10 mg of protein, 4 ml of 1 M Tris/HCl buffer, pH 8.6, and 0.1 ml of 2-mercaptoethanol was sealed in a tube under nitrogen. The tube was kept for 4 h at room temperature and then a freshly prepared solution of 0.25 g of iodoacetic acid in 1 ml of 1 N NaOH was added and the mixture was kept in the dark for 20 min. Then the mixture was applied to a column of Sephadex G-25 (1.6 x 15 cm) previously equilibrated with 0.2 N acetic acid, and the column was eluted with the same solution. Fractions containing protein were pooled and dialyzed against 0.1 M Tris/HCl buffer, pH 8.5.

Oxidation of Reduced Protein

The protein was reduced as mentioned above. Then, 2-mercaptoethanol was removed from the reaction mixture by gel filtration on a Sephadex G-25 column (1.6 x 15 cm). The column was eluted with 0.1 M Tris/HCl buffer, pH 8.5. Fractions of eluate containing protein were pooled and air was bubbled through the solution. Then, the hemagglutinating activity of this oxidized protein was examined.

NH2-terminal Amino Acid Analysis

Edman Method—The Edman method modified by Blombäck and Yamashita (17) was applied to 6.8 mg samples. The main chromatographic systems used for identification of thePTH-derivatives have been described by Jeppsson and Sjöquist (18). The yields were calculated from the optical densities at 390 nm and the extinction coefficients reported by Sjöquist (19).

Danasylation Procedure—Danasylation of protein was performed as described by Gray (20). Dansylated protein (0.1 mg) was hydrolyzed and the dansylated amino acids were identified by two-dimensional thin layer chromatography (21).

COOH-terminal Amino Acid Analysis

Hydrazinolysis (22)—Samples (3.5 mg) were dissolved in 0.4 ml of anhydrous hydrazine and 50 mg of dry Amberlite CG-50 (H+ form) were added as catalyst. The mixtures were heated in evacuated, sealed tubes at 80° for 24 and 48 h. Then excess hydrazine was removed in vacuo over concentrated H2SO4. The residue containing the protein was suspended in a small volume of water and subjected to chromatography on a column (1 x 4 cm) of Amberlite CG-50 (H+ form). The fractions eluted first with 15 ml of water and then with 15 ml of 0.1 M ammonium acetate were each pooled and lyophilized and the residues were analyzed in an amino acid analyzer.

Selective 1H labeling—Selective 1H labeling was employed for the identification of the COOH-terminal amino acid (23). Samples (5 mg) were dissolved in 0.05 ml of H2O (200 mCi/ml). First 0.05 ml of pyridine and then 0.05 ml of acetic anhydride were added to the test solution and the mixture was held for 5 min at 0° and then for 15 min at room temperature. Then a further 0.05 ml of H2O and 0.1 ml each of pyridine and acetic anhydride were added and the mixture was incubated as before. The mixture was lyophilized and excess tritium was removed by lyophilization after dissolving the residue in 0.2 ml of 10% acetic acid. The dry residue was hydrolyzed in 0.5 ml of redistilled HCl at 110° for 24 h, and the hydrolysate was subjected to paper electrophoresis (Toyo No. 51 filter paper). Electrophoresis was carried out at 50 V/cm in pyridine/acetic buffer, pH 3.8. The electrophoretogram was cut into small pieces and the radioactivity of each piece was counted in toluene scintillator.

Carboxypeptidase A Digestion—Following the method of Ambrose (24), the sample (17.5 mg) was dissolved in 3.5 ml of 0.2 N ethylmorpholine acetate, pH 8.5, and then a carboxypeptidase A solution (ratio of protein/enzyme, 100/1, w/w) was added and the mixture was incubated at 22°. At appropriate intervals, 0.5-ml samples of the mixture were removed and trichloroacetic acid was added to a final concentration of 5% to stop the reaction. Insoluble materials were removed by centrifugation and the supernatant fluid was extracted with ether to remove trichloroacetic acid and dried. The residue containing released amino acids was analyzed in an amino acid analyzer. For determination of asparagine, the supernatant solutions of samples obtained at different intervals were hydrolyzed in 1 N HCl for 30 min at 100° and the hydrolysates were analyzed for aspartic acid in the amino acid analyzer.

Tryptic Peptide Analysis

Protein was digested with trypsin as described by Joshi et al. (25). Samples (5 mg) were dissolved in 1 ml of 1% ammonium carbonate and digested by boiling for 10 min. Then, the hemagglutinin (ratio of protein/enzyme, 100/1, w/w) was added and the mixture was incubated at 37° for 24 h. Then, the same volume of trypsin as before was added and the mixture was incubated for another 24 h. The mixture was lyophilized and the residue was dissolved in dilute ammonia and spotted (about 1-μg portions) on Whatman 3MM paper (30 x 42 cm). Electrophoresis was conducted at pH 6.5 (v/v) solvent/pyridine, 100 ml; acetic acid, 4 ml; and water to 1000 ml) for 1 h at 20 V/cm. The paper was dried and chromatography was conducted in 1-butanol/acidic acid/water (4/1/5, by volume, upper phase) for 7 h. Tryptic peptide spots were examined by spraying the paper with 0.02% ninhydrin in acetone or 0.01% Fluram (26) in acetone. The periodate/benzidine reaction (27) was used to detect glycopeptides.

Carbohydrate Analysis

The carbohydrate composition of the lectin was determined by gas chromatography on an ECNSS-M column using the reduced and acetylated materials. Samples (2 to 5 mg) were hydrolyzed in 1 N HCl at 100° for 3, 5, and 7 h. The hydrolysates were reduced and acetylated as described by Yang and Hackett (28). The acetylated samples were developed on a column of 3% ECNSS-M coated on Gas-Chrom Q at 190° for 20 min and then programmed up to 230° (2°/min). The relative carbohydrate contents were estimated by cutting out carbohydrate peaks from the chart and weighing them.

Isolation of Glycopeptide

A glycopeptide was isolated after pronase digestion of the lectin by the method of Lie et al. (29). That is, the lectin sample (50 mg) was digested with pronase (ratio of protein/enzyme, 50/1, w/w) in 0.1 M phosphate buffer, pH 7.2, for 72 h at 37°. The digest was subjected to gel filtration on a column of Sephadex G-50 (1.5 x 70 cm) which had been equilibrated with 0.01 M Tris/HCl buffer, pH 8.0, and the column was eluted with the same buffer. The fractions containing carbohydrate were pooled and passed through to dryness. The residue was dissolved in a small volume of water, and passed through a column of Dowex 50-X8 (H+ form, 1 x 3 cm) to remove contaminating ninhydrin-positive materials. Ninhydrin-positive materials were removed more completely by successive paper electrophoresis and paper chromatography on Whatman 3MM paper employing the same system as that described in under “Tryptic Peptide Analysis.” The area corresponding to the periodate/benzidine-positive spot was cut out and the glycopeptide was eluted with water.

Titration and Inhibition Assay of Hemagglutination

Erythrocytes were washed three times with phosphate buffered saline and used for assay as a 2% suspension. Pig erythrocytes were used for all routine assays. Erythrocytes of other species were used for studies on species differences in hemagglutination specificity. For the
protein peak with 0.25 assay, 0.05-ml volumes of 2-fold serial dilutions of lectin solution were mixed with an equal volume of erythrocyte suspension and stood for 30 min at room temperature with occasional shaking. Then agglutination was examined and the end point of dilution (titer) showing positive hemagglutination was determined.

For assay of inhibition of hemagglutination by various sugars, 0.05-ml volumes of 2-fold serial dilutions of test sugar solutions were mixed with an equal volume of lectin solution at a concentration of 2.5 µg/ml. After 30 min, 0.1 ml of erythrocyte suspension was added and the mixtures were stood for another 30 min. Then their agglutination was examined and the minimal sugar concentrations showing hemagglutination inhibition were determined (30).

**Binding of N-Acetyl-d-galactosamine to Lectin and Its Carboxymethylated Subunits**

Binding of N-acetyl-d-galactosamine to lectin and its carboxymethylated subunits was examined by equilibrium dialysis, by placing 0.1 ml of a protein solution in one compartment and 0.1 ml of an N-acetyl-d-[1-14C]galactosamine (specific radioactivity, 1.9 × 10^6 cpm/µg) solution in the other compartment of the dialysis microcell. The membrane was made of Visking dialysis tubing. After shaking the dialysis cell for 5 h at room temperature, a 20-µl sample from each compartment of the dialysis cell was transferred to a vial and its radioactivity was counted in Bray's solution. The distribution of N-acetyl-d-[1-14C]galactosamine between the two compartments was estimated from the radioactivities. The results were plotted according to the equation (31),

\[ r/f = -Kr + NK \]

where \( r \) is the number of moles of sugar bound per g of protein, \( f \) is the molar concentration of free sugar, \( K \) is the intrinsic association constant for binding of sugar to protein, and \( N \) is the number of moles of sugar bound per g of protein at saturation.

**RESULTS**

**Extraction**—Wisteria floribunda seeds (100 g) were crushed and mixed with 1000 ml of 0.1 M Tris/HCl buffer, pH 7.5, at 4°C. The mixture was allowed to stand overnight and then centrifuged and the clear supernatant fluid (“crude extract”) was subjected to ammonium sulfate fractionation. The fractions precipitated by 0 to 0.4, 0.4 to 0.7, and 0.7 to 1.0 saturation of ammonium sulfate were each dissolved in 0.1 M Tris/HCl buffer, pH 7.5, and dialysed overnight against the same buffer. Almost 70% of the hemagglutinating activity was recovered in material precipitated at 0.4 to 0.7 saturation (“AS 0.4-0.7”).

**Gradient Solubilization of Ammonium Sulfate**—The AS 0.4-0.7 fraction was subjected to gradient solubilization with ammonium sulfate following the method of King (32). Ammonium sulfate was added to the AS 0.4-0.7 fraction to 0.8 saturation and the precipitated protein was mixed with Celite 545 and packed into a column. The column was eluted with a decreasing concentration of ammonium sulfate in buffer (Fig. 1). The hemagglutinating activity was eluted at 0.6 to 0.5 saturation of ammonium sulfate. Fractions with activity were pooled and dialyzed against 0.1 M Tris/HCl buffer, pH 8.5, and concentrated by Diaflo ultrafiltration (Amicon Co.) using a PM 10 membrane (“AS-Celite”).

**DEAE-Sephadex A-50 Column Chromatography**—The AS-Celite fraction was dialyzed against 0.1 M Tris/HCl buffer, pH 8.5, and applied to a column of DEAE-Sephadex A-50. The column was eluted with a linear gradient of 0 to 0.6 M NaCl (Fig. 2). The hemagglutinating activity was eluted in the main protein peak with 0.25 M NaCl. Fractions with activity were pooled and concentrated (“DEAE”).

**Sephadex G-200 Column Chromatography**—Further purification could be achieved by gel filtration on a column of Sephadex G-200. Fractions in the main peak, which had hemagglutinating activity, were pooled and concentrated (“G-200”).

The homogeneity of the G-200 fraction was confirmed by disc electrophoresis on polyacrylamide gel. A single band was obtained at either pH 9.4, 8.0, or 4.0 (Fig. 3A).

The purification of WFA is summarized in Table I. The overall purification was about 30-fold and the recovery of activity was 43%.

**Physical and Chemical Properties**

**Isoelectric Point**—The isoelectric point of WFA, estimated by isoelectric focusing, was 4.7.

**Molecular Weight**—The molecular weight was estimated by both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-100. On SDS-polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol in the gel system, the molecular weight of WFA was estimated to be 68,000 (Fig. 4). An approximate molecular weight of 75,000 was calculated from the elution volume on gel filtration on a Sephadex G-100 column (Fig. 5).

**Amino Acid Composition**—The amino acid composition of WFA is shown in Table II. WFA was rich in acidic and hydroxyamino acids and low in sulfur-containing amino acids. No methionine was detected. There were 2 half-cystine residues per WFA molecule.

**Subunit Structure**—WFA was treated with 6 M guanidine, 1 M propionic acid and subjected to gel filtration on Sephadex G-100 using the same denaturing solvent for elution. This condition is reported to dissociate noncovalently linked subunits of protein molecules (33). WFA was eluted as a single protein peak and its molecular weight was estimated to be about 70,000. This value was close to the value of 75,000 obtained using the same gel system without a denaturing solvent. When WFA was treated with 2-mercaptoethanol and then subjected to SDS-polyacrylamide gel electrophoresis it formed a single band at a position corresponding to a molecular weight...
Fig. 2. Chromatography of the AS-Celite fraction on DEAE-Sephadex A-50. The AS-Celite fraction (20 ml, 114 mg of protein) was dialyzed against 0.1 M Tris/HCl buffer, pH 8.5, and applied to a column of DEAE-Sephadex A-50 (2.5 × 36 cm) equilibrated with the same buffer. The column was eluted with 800 ml of a linear gradient of 0 to 0.6 M NaCl and fractions of 5 ml were collected at a flow rate of 40 ml/h. Indicated fractions were pooled.

Fig. 3. Polyacrylamide gel electrophoresis of the purified WFA and RCW. WFA (A) and RCW (B) were run on 7.5% gels in (a) Tris/glycine buffer, pH 9.4; (b) Tris/diethylbarbituric acid buffer, pH 8.0; and (c) alanine/acetic acid buffer, pH 4.0.

Fig. 4. Estimation of the molecular weight of WFA by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out with 7.5% gels in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS. Samples were heated at 100°C for 1 min in a, 0.01 M sodium phosphate buffer, pH 7.2, containing 1% SDS, 10% glycerol, and 0.001% bromophenol blue; b, the same solution as a supplemented with 1% 2-mercaptoethanol. The mobility of proteins was measured and the relationship of the logarithm of the molecular weights of several marker proteins to their mobilities was plotted. Molecular weight markers (molecular weights shown in parentheses) used were: 1, myoglobin (16,000); 2, chymotrypsinogen A (25,000); 3, ovalbumin (45,000); 4, albumin (67,000); and 5, immunoglobulin G (160,000).

Fig. 5. Estimation of the molecular weight of WFA by gel filtration on Sephadex G-100. Gel filtration was performed as described in the text. The relationship of the logarithms of the molecular weights of several marker proteins to their elution volumes was plotted. The markers used were the same as those in Fig. 4.

TABLE I

<table>
<thead>
<tr>
<th>Total protein</th>
<th>Hemagglutination titer (per mg protein)</th>
<th>Activity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>9.7</td>
<td>32</td>
</tr>
<tr>
<td>AS 0.4-0.7</td>
<td>1.68</td>
<td>128</td>
</tr>
<tr>
<td>AS-Celite</td>
<td>0.36</td>
<td>256</td>
</tr>
<tr>
<td>DEAE</td>
<td>0.25</td>
<td>512</td>
</tr>
<tr>
<td>G-200</td>
<td>0.13</td>
<td>1024</td>
</tr>
</tbody>
</table>

of 32,000, which was about half that of WFA (Fig. 4). This indicated that WFA was dissociated into subunits of about half the size of the native molecule by reduction with 2-mercaptoethanol. WFA contained 2 half-cystine residues per molecule, and no free sulfhydryl residues were detected by quantitative analysis using Ellman's technique with DTNB. Thus the two subunits in the intact WFA molecule were linked by a single disulfide bond between 2 half-cystine residues.

WFA was reduced with 2-mercaptoethanol and then carboxymethylated with iodoacetic acid. This reduced and S-carboxymethylated preparation (RCW) migrated to the same position as that of the subunits on SDS-polyacrylamide gel electrophoresis. The RCW preparation gave a single band on polyacrylamide gel electrophoresis at either pH 9.4, 8.0, or 4.0 (Fig. 3B).

End Group Analysis—The only NH₂-terminal amino acid of WFA detected by the Edman method or the dansyl technique was lysine. Quantitative analysis by the Edman method showed that 1.5 mol of PTH-lysine was released per mol of WFA. Thus assuming that there are 2 NH₂-terminal lysine residues in WFA, the recovery of PTH-lysine was estimated to be 75%.

The COOH-terminal amino acid was studied by three different methods: hydrazinolysis, selective ¹H labeling, and carboxypeptidase A digestion. After hydrazinolysis, the material was passed through a column of Amberlite CG-50 to remove the hydrazides. No free
amino acids could be detected in the eluate of the column with an amino acid analyzer.

The COOH-terminal residue of WFA was labeled using the selective $^3$H labeling method. After acid hydrolysis of the labeled protein, appreciable radioactivity was found only in the region of aspartic acid on the electrophoretogram (Fig. 6).

On treatment of WFA with carboxypeptidase A, an appreciable amount of an amino acid which eluted in the position of serine on amino acid analysis was liberated in the initial stage of digestion. In the system used for amino acids analysis (13), serine and asparagine are reported to be eluted in the same position. To distinguish between serine and asparagine, amino acids liberated by the enzyme were subjected to amino acid analysis after acid hydrolysis. Most of the amino acid was eluted as aspartic acid; little remained in the position of serine (Fig. 7), indicating that the amino acid liberated with carboxypeptidase A was asparagine.

From these results using three different methods, the COOH-terminal amino acid of WFA was concluded to be asparagine. On hydrazinolysis, the COOH-terminal asparagine must have been converted to asparagine-$\beta$-hydrazide, so that it could not be detected in an amino acid analyzer.

Tryptic Peptide Analysis—Peptide maps were made of the products of tryptic digestion. From the results of amino acid analysis, 40 tryptic peptide fragments should be formed (20 lysine and 10 arginine). However only 24 ninhydrin-positive spots were seen (Fig. 8), which is about half the expected number.

For detection of glycopeptides, maps were stained with periodate/benzidine. Only one spot gave a positive reaction for carbohydrate and this spot was also ninhydrin-positive.

Carbohydrate Composition—WFA is a glycoprotein. Its neutral sugar content was determined to be 2.4% by the phenol/sulfuric acid method. Its carbohydrate composition, determined by gas chromatography, is shown in Table III: the constituent carbohydrates were mannose, galactose, and gluco-

![Fig. 6. Radioactive amino acids in the hydrolysate of WFA after $^3$H labeling. Electrophoresis was carried out at 50 V/cm in pyridine/acetic acid buffer, pH 3.5. The electrophoretogram shows the positions of authentic amino acids.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>g/100 g protein</th>
<th>residues/molecule$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>5.5</td>
<td>29.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.6</td>
<td>17.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>15.9</td>
<td>80.9</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.3</td>
<td>49.1</td>
</tr>
<tr>
<td>Serine</td>
<td>7.0</td>
<td>54.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.5</td>
<td>39.5</td>
</tr>
<tr>
<td>Proline</td>
<td>4.8</td>
<td>33.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.6</td>
<td>31.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.1</td>
<td>39.2</td>
</tr>
<tr>
<td>Cysteine$^b$</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Valine</td>
<td>7.3</td>
<td>50.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.3</td>
<td>19.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.2</td>
<td>49.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.1</td>
<td>17.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.9</td>
<td>27.3</td>
</tr>
<tr>
<td>Tryptophan$^c$</td>
<td>2.6</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Total 92.3

$^a$The molecular weight was assumed to be 68,000.

$^b$Estimated as cysteic acid by performic acid oxidation.

$^c$Estimated spectrophotometrically.

![Fig. 7. Release of COOH-terminal amino acids by digestion of WFA with carboxypeptidase A. Digestion of WFA with carboxypeptidase A was carried out as described in the text. Liberated amino acids were hydrolyzed with 1 N HCl at 100°C for 30 min and then determined in an amino acid analyzer. O, aspartic acid; ●, serine.](http://www.jbc.org/)

![Fig. 8. Tryptic peptide map of WFA. Trypsin digestion and mapping of peptides were carried out as described in the text. 1st dimension, electrophoresis; 2nd dimension, chromatography. The ninhydrin-positive spots are enclosed by solid lines. P, position of periodate/benzidine-positive spot.](http://www.jbc.org/)

Table II

<table>
<thead>
<tr>
<th>Amino acid composition of WFA</th>
</tr>
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<tr>
<td>Values are the means of those obtained with three different digestion times (24, 48, and 72 h).</td>
</tr>
</tbody>
</table>

Table II
saccharoamine, in a molar ratio of about 1:2:1. The carbohydrate content of WFA, including amino sugar, was calculated to be 3.2%. Only one peptide containing carbohydrate was detected on tryptic peptide maps (Fig. 8), as mentioned above.

Isolation of Glycopeptide—A glycopeptide was isolated after digestion of WFA with pronase. For this, the digest was subjected to Sephadex G-50 column chromatography, and the low molecular weight fractions containing about 90% of the carbohydrates of WFA were pooled. To remove contaminating ninhydrin-positive materials, the solution was passed through a column of Dowex 50-X8 and the unadsorbed material was further purified by paper electrophoresis and paper chromatography, as shown in Fig. 9. Throughout these purification procedures, glycopeptide was detected as a single spot. The area corresponding to the periodate/phenazine-positive spot was cut out and the glycopeptide was eluted with water.

The composition of the purified glycopeptide is given in Table IV. It contained aspartic acid in an equimolar amount to glucosamine and no other amino acid was detected. The molar ratio of glucosamine to neutral sugar was about 1:3, which was practically equal to the value determined with undigested WFA.

**Biological and Biochemical Properties**

Specificity of Hemagglutination—As shown in Table V, WFA agglutinated all human A, B, and H type erythrocytes, having the highest agglutination titer for the A type. Among the erythrocytes of various mammalian species examined, it showed the highest agglutination titer with pig erythrocytes. The minimal concentration of WFA for agglutination of pig erythrocytes was 1 μg/ml.

In inhibition assays with various simple sugars, N-acetyl-D-galactosamine was found to be the most potent inhibitor (Table VI). Galactose was weakly inhibitory. The minimal concentration needed for hemagglutination inhibition was 20-fold higher than that of N-acetyl-D-galactosamine. From comparison of the inhibitory activities of lactose and melibiose, and of p-nitrophenyl-α- and β-D-galactopyranoside, it is clear that WFA shows preference for the β-glycosidic linkage rather than the α-glycosidic linkage.

Hemagglutinating Activity of Reduced and Reoxidized WFA—WFA was dissociated into two subunits by reduction with 2-mercaptoethanol. The alkylated subunits (RCM-WFA) had no detectable hemagglutinating activity. In the presence of 1% 2-mercaptoethanol in the assay mixture, WFA showed no hemagglutinating activity. When this preparation was reoxidized, with air, after removal of 2-mercaptoethanol by gel filtration, the activity reappeared.

**Table IV**

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<thead>
<tr>
<th>Carbohydrate composition of WFA</th>
<th>% by weight</th>
<th>residues/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose</td>
<td>0.77</td>
<td>2.91</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.63</td>
<td>6.16</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.85</td>
<td>2.46</td>
</tr>
</tbody>
</table>

*Values were calculated from the total neutral sugar content and the relative amount of each sugar.

*The molecular weight was assumed to be 65,000.

**Table V**

<table>
<thead>
<tr>
<th>Specificity of WFA in agglutination of erythrocytes of various mammals</th>
<th>Titer (per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>19</td>
</tr>
<tr>
<td>A</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
</tr>
<tr>
<td>Pig</td>
<td>1024</td>
</tr>
<tr>
<td>Rabbit</td>
<td>256</td>
</tr>
<tr>
<td>Mouse</td>
<td>Trace</td>
</tr>
<tr>
<td>Sheep</td>
<td>Trace</td>
</tr>
</tbody>
</table>

*Estimated by the phenol method.

**Table VI**

<table>
<thead>
<tr>
<th>Inhibition of hemagglutination of WFA by simple sugars</th>
<th>Minimal inhibitory concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl-D-galactosamine</td>
<td>0.663</td>
</tr>
<tr>
<td>Lactose</td>
<td>1</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-galactopyranoside</td>
<td>1</td>
</tr>
<tr>
<td>p-Nitrophenyl-β-D-N-acetylgalactosaminide</td>
<td>0.663</td>
</tr>
</tbody>
</table>
The number of NH₂-terminal lysine molecules per molecule of WFA was approximately 2. (e) Only asparagine could be detected as COOH-terminal amino acid. (f) The number of peptide spots found on fractionating the tryptic digest was about half the value expected from the basic amino acid content.

WFA contained a carbohydrate moiety which constituted 3.2% of the total weight and consisted of mannose, galactose, and glucosamine. These carbohydrates seemed to exist as an oligosaccharide chain of a single kind, linked to the protein molecule in the ratio of one chain per subunit. The glycopeptide isolated contained aspartic acid and the carbohydrate moiety, indicating that the carbohydrate chain was attached to this aspartic acid residue by a covalent linkage.

The fact that WFA was dissociated into subunits by reduction with 2-mercaptoethanol, but not by treatment with a denaturing solvent strongly supports the role of the disulfide bond in association of the subunits. The dissociated subunits showed no hemagglutinating activity.

Recently, we have re-examined the molecular weights of WFA and RCM-WFA at several pH values by polyacrylamide gel electrophoresis according to Hedrick and Smith (34), without SDS in the system. Under neutral and acidic conditions, the molecular weight values were almost equal to those obtained by procedures described under "Experimental Procedure." However, under alkaline conditions (pH 9.4 or higher), molecular weights of WFA and RCM-WFA were estimated to be 125,000 and 67,000, respectively. This indicates that these protein molecules tend to associate to form dimers under alkaline conditions. Similar protein-protein association under alkaline conditions were also reported for concanavalin A (35).

The difference between the molecular weight of 68,000 and 75,000 estimated by SDS-polyacrylamide gel electrophoresis and gel filtration, respectively, might be due to a conformational difference in the protein molecules under the conditions employed in these procedures. The more exact value could be estimated using other methods, such as equilibrium sedimentation, but in this paper, 68,000 is tentatively adopted as the molecular weight of WFA.

On tryptic peptide analysis, the number of observed spots was slightly more than half that expected from the results of amino acid analysis. This slightly higher value might be due to the activity of trace amount of chymotrypsin contaminating trypsin preparation used.

The values for carbohydrate given in Table V do not represent even numbers per WFA molecule. This may be due to the fact that these values were calculated from the ratio of each sugar and the total neutral sugar content. Quantitative measurement of each carbohydrate is necessary to estimate the value accurately. From analytical results on the isolated glycopeptide, there should be 2 mannose and 4 galactose residues per WFA molecule, assuming that there are 2 glucosamine residues.

Toyoshima and Osawa have recently reported the purification and characterization of a lectin from Wistaria floribunda seeds (3). Their lectin (WFH) was also a glycoprotein, which was similar to WFA in chromatographic behavior and sugar specificity, but differed in molecular weight, subunit structure, and carbohydrate composition. WFH was reported to be a tetramer composed of four subunits, each having a molecular weight of 35,000, whereas on gel filtration under conditions which did not seem to denature proteins WFA appeared to be dimer composed of two identical subunits. These two lectins are different in carbohydrate content.

**DISCUSSION**

_Wistaria floribunda_ lectin purified as described in this paper was essentially homogeneous. The homogeneity of the purified WFA was confirmed by polyacrylamide gel electrophoresis at several pH values.

The present results show that the WFA molecule is composed of two identical subunits, each with a molecular weight of approximately 32,000. These two subunits are linked through a single disulfide bond of the 2 half-cystine residues present in the WFA molecule. The intact WFA molecule and two subunits are di- and monovalent, respectively, with respect to sugar binding.

The conclusion that the two subunits are identical is based on the following findings. (a) The molecular weight of the subunits was about half that of WFA. (b) Reduction and carboxymethylation of WFA yielded subunits that formed a single band on polyacrylamide gel electrophoresis. (c) Only lysine could be detected as NH₂-terminal amino acid. (d) The number of NH₂-terminal lysine molecules per molecule of WFA was approximately 2. (e) Only asparagine could be detected as COOH-terminal amino acid. (f) The number of

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**FIG. 10.** Scatchard plot of data on binding of N-acetyl-N-galactosamine. Experimental details are given in the text. O, WFA; ●, RCM-WFA.

_Binding of N-Acetyl-N-galactosamine to WFA and RCM-WFA._ From the subunit structure of the WFA molecule and the reversible inactivation of the hemagglutinating activity of WFA by 2-mercaptoethanol, it seemed very likely that WFA and RCM-WFA would be divalent and monovalent, respectively, with respect to sugar binding. The following experiments showed that this was the case. The bindings of N-acetyl-N-galactosamine, a specific inhibitor of WFA agglutination, to WFA and RCM-WFA were examined by the equilibrium dialysis method. A Scatchard plot of the results of binding experiments is shown in Fig. 10. N-Acetyl-N-galactosamine bound equally well to WFA and RCM-WFA, indicating that the binding was not affected by reduction and alkylation. The following experiments showed that this was the case. The bindings of N-acetyl-N-galactosamine, a specific inhibitor of WFA agglutination, to WFA and RCM-WFA were examined by the equilibrium dialysis method. A Scatchard plot of the results of binding experiments is shown in Fig. 10. N-Acetyl-N-galactosamine bound equally well to WFA and RCM-WFA, indicating that the binding was not affected by reduction and alkylation. The fact that the experimental points fell on a straight line showed that the binding sites were equivalent and independent. A slope of $K = 1.28 \times 10^5$ M⁻¹ and an x intercept of $N = 2.5 \times 10^4$ mol of sites/g were obtained for both proteins, and the numbers of binding sites per molecule of WFA and RCM-WFA were calculated to be 1.70 and 0.85, respectively.

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*a T. Kurokawa, M. Tsuda, and Y. Sugino, unpublished observations.*
also differ in amino acid composition. WFA contained methionine and lacked cysteine, whereas WFA contained 2 half-cystine residues and lacked methionine. As discussed above, the half-cystine residues of WFA were essential for linkage of the two subunits through a disulfide bond. Thus, WFA and WFH may be distinct proteins, although their characters are somewhat similar and they were purified from the same source. The relationship, if any, between WFA and WFH requires further detailed comparative study.

WFA bound specifically to N-acetyl-d-galactosamine residues, especially to those with a β-glycosidic linkage. There are few reports on the specific binding of lectins to N-acetyl-d-galactosamine, except for those on human type A erythrocyte-specific lectins. All the type A-specific lectins reported so far show little or no agglutinating activity against cultured mammalian cells (36). WFA agglutinated mammalian cells and so could be utilized as a marker of cell transformation. Results obtained in our laboratory indicated that both the agglutinability and the numbers of binding sites of WFA of transformed cells may be greater than those of normal cells (37). These characters of WFA differ from those of other lectins used previously as markers of cell transformation, such as concanavalin A and wheat germ agglutinin.

As mentioned several times under “Results” and “Discussion,” the reduced and carboxymethylated subunit (RCM-WFA) was a monovalent lectin which retained the ability to bind to sugar haptons but had lost agglutinating activity. Unlike multivalent lectins, monovalent lectin does not form cross-linkages between sugar chains located on the cell surface. Due to this property and its unique specificity towards the β-glycosidic linkage of N-acetyl-d-galactosamine, WFA will be useful in studies of various glycosylated materials and of the cell surface structure.

Acknowledgment—We would like to thank Dr. Sadaaki Iwanaga, Institute for Protein Research, Osaka University, Osaka, for his helpful suggestion on the end group analyses.

REFERENCES

Purification and characterization of a lectin from Wistaria floribunda seeds.
T Kurokawa, M Tsuda and Y Sugino


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