Subunit Structure of Soybean Agglutinin*

(Received for publication, August 27, 1973)

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SUMMARY

Soybean agglutinin purified by affinity chromatography on Sepharose-γ-aminocaproyl-β-D-galactopyranosylamine was shown to be homogeneous on disc gel electrophoresis, ultracentrifugation, and gel filtration. Isoelectric focusing in a sucrose gradient gave a pI of 5.81. The extinction coefficient (ε_{1},155) was found to be 12.8 cm^{-1}. Measurement of the partial specific volume gave a value of 0.745 ml per g. The intrinsic sedimentation coefficient (s_{20,w}) was estimated as 6.0 ± 0.12 S, between pH 2.2 and 10.8, and as 2.15 ± 0.15 S in 0.1% sodium dodecyl sulfate, 8 M urea, and 6 M guanidine hydrochloride. The molecular weight of the agglutinin, determined by sedimentation equilibrium and by gel filtration, was found to be 122,000 ± 1,300 and 120,000 ± 10,000, respectively. Disc gel electrophoresis and gel filtration, both in the presence of sodium dodecyl sulfate, and sedimentation equilibrium in 6 M guanidine hydrochloride gave a subunit molecular weight of 30,000 ± 1,500 and 30,300 ± 400, respectively. Four alanine residues per 120,000 g were found by amino-terminal analysis. It is concluded that the agglutinin is a tetramer composed of identical subunits. Two binding sites for N-acetyl-D-galactosamine were found per 120,000 daltons by equilibrium dialysis and gel filtration, with an association constant K = 3.0 × 10^4 liter mole^{-1}.

Soybean agglutinin is one of a large group of plant agglutinins also known as plant lectins (1, 2). In addition to its ability to agglutinate erythrocytes (3, 4), it preferentially agglutinates malignant cells (5). These agglutination reactions are specifically inhibited by N-acetyl-D-galactosamine and to a lesser extent by N-galactosamine (6). SBA is a glycoprotein, containing 4.5% D-mannose and 1.5% N-acetyl-D-glucosamine (7). The carbohydrate moiety is bound to the protein by an N-acetyl-glucosaminyl linkage to the amide nitrogen of asparagine (8).

Previous reports suggested that SBA was composed of at least two polypeptide chains (9, 10). In the present study, evidence is presented indicating that SBA is a tetramer composed of identical subunits, molecular weight 30,300 ± 400, with two saccharide binding sites per 120,000.

EXPERIMENTAL PROCEDURES

Chemicals—Ultrapure grades of guanidine hydrochloride and urea (Mann) and specially pure sodium dodecyl sulfate (BDH) were used for the dissociation of SBA. N-[{}^{14}C]acetyl-D-galactosamine (334 mCi per mmole) was obtained from the Radiochemical Centre, Amersham. The proteins used for the calibration of the Sephadex columns and polyacrylamide gels were obtained from commercial sources and were chromatographically pure. All other chemicals were of reagent grade and the highest quality commercially available.

Soybean Agglutinin—SBA was purified by affinity chromatography on Sepharose-N-γ-aminocaproyl-β-D-galactopyranosylamine as previously described (11).

Assay of Hemagglutinating Activity—This was performed according to the photometric method of Lienar (12, 13).

Extinction Coefficient—Soybean agglutinin was dissolved in water, dialyzed extensively, and lyophilized. For determination of moisture 25 mg were dried to constant weight in a vacuum over P_{2}O_{5} at 100°. The moisture content was found to be 10.8%. Another sample (25 mg) was dissolved in 0.9% NaCl and diluted to afford a series of known concentrations of SBA. The absorbance was measured at 280 nm, and the nitrogen content was determined by a modification of the Kjeldahl method (14).

Determination of Molecular Weight by Gel Filtration—Molecular weight determination was performed essentially as described by Andrews (15). The protein solution (1 ml, 3 to 5 mg per ml in 0.9% NaCl) was applied to a column (1.9 x 50 cm) of Sephadex G-150 (superfine), and the protein was eluted at 4° with 0.9% NaCl. Fractions (2 ml) were collected at a rate of 8 ml per hour. The column was calibrated with proteins of known molecular weight. The absorbance was measured at 230 nm and 280 nm, and elution volumes were determined from the position of the maxima of the elution profiles. Gel filtration in the presence of 0.1% sodium dodecyl sulfate was performed in a similar way, except that the column was operated at room temperature (23°). Before application, a 5-fold excess of sodium dodecyl sulfate relative to the weight of the protein was added to the protein solution which was then dialyzed extensively against 0.1% sodium dodecyl sulfate in 0.9% NaCl.
Disc Electrophoresis on Polyacrylamide Gels—Disc electrophoresis was performed essentially by the methods described by Ornstein (16) and Davis (17). Continuous gradients of acrylamide were prepared according to Kamm and Mes (18). Discontinuous buffer systems at pH 8.9 (17) and pH 4.5 (19) were used. Gel electrophoresis in the presence of sodium dodecyl sulfate was done according to the directions of Weber and Osborn (20) in 10% gels or in gradients of acrylamide (from 8 to 15%) containing 0.1% sodium dodecyl sulfate. Protein samples (50 to 100 μg) were treated with 2% mercaptoethanol and with sodium dodecyl sulfate which was added as solid (sodium dodecyl sulfate to protein ratio, 5:1 w/w) and dialyzed against 0.1% sodium dodecyl sulfate. The gels containing sodium dodecyl sulfate were calibrated with standard proteins for molecular weight estimation (20).

Gel electrophoresis in the presence of 8 M urea was performed in a continuous gradient of acrylamide from 4 to 8% with one of the buffers, pH 3.3, proposed by Avital and Elson (21). The 4% solution contained acrylamide (1.38 g), N,N'-methylene-bisacrylamide (3.7 mg), water (6.5 ml), DL-glutamic acid (0.32 g), and freshly deionized 10 M urea (28 ml). The mixture was stirred until the glutamic acid completely dissolved, then 0.08 ml of N,N'-tetramethylenebisacrylamide and 0.4 ml of 10 M freshly prepared ammonium persulfate were added. The 8% solution contained twice the amount of acrylamide and N,N'-methylenebisacrylamide and half the amount of N,N'-tetramethylethylenediamine. The electrode buffer for this system was prepared by dissolution of 3 g of DL-glutamic acid in 1.5 liters of water. The resulting pH was 3.3. The protein sample (100 μg) was applied in 8 M urea. Acrylamide and N,N'-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively (22). Proteins were detected by staining for 4 hours at 40° with 0.25% Coomassie brilliant blue R-250 in acetic acid-methanol-water (7:5:88 v/v/v) followed by destaining at 40°, first in the same solvent until bands could be observed, then with acetic acid-methanol-water (7:5:88 v/v/v). Gels were also stained for glycoproteins according to Zacharius et al. (23).

Measurements of mobilities of proteins in the calibrated sodium dodecyl sulfate gels were made from the top of the gel to the leading edge of the protein band.

Isoelectric Focusing—This was performed in a linear sucrose gradient containing 2% ampholine (LKB), pH 5 to 8, in the LKB model 8101 column (110 ml capacity) according to Vesterberg (24). SBA (30 mg) was dissolved in the “light solution” before it was poured into the gradient mixer. Electrofocusing was carried out at 4° and a final voltage of 500 volts for 60 hours. Fractions of 1 ml were collected at a rate of 1 ml per min; their pH was measured at 4° with a Radiometer 26 pH meter. The absorbance was read at 250 nm, and the hemaggulinating activity was determined after extensive dialysis of selected fractions against 0.9% NaCl.

Ultracentrifugal Analysis—The experiments were performed with a Spinco model E analytical ultracentrifuge equipped with an RTIC unit and schlieren phase plate optics. Sedimentation velocity measurements were performed at 20° with the use of an An-D rotor with single cells, each with a quartz window. Experiments were conducted at 59,780 rpm. The rate of boundary migration was determined by reading plates on a Nikon model 6C two-dimensional microcomparator. Apparent sedimentation coefficients were calculated by the least square method and corrected to standard conditions (v0, w0) (25, 26). The intrinsic diffusion coefficient (D0, w0) was determined with a synthetic boundary cell according to Ehrenberg (27).

Sedimentation equilibrium studies were performed according to the meniscus depletion method of Yphantis (28) with the use of an An-F or An-J rotor with double-sector aluminium-filled Epon center ellipse and sapphire windows. Photographs were taken on Kodak spectroscopic plates type II-G. The data were processed with a computer program of Roark and Yphantis modified by Jhu and Pouyet, which was made available to us by Mr. M. Mevaroch from the Polymer Department, the Weizmann Institute of Science.

The partial specific volume was calculated on the basis of densities of SBA samples (2, 4, 8, and 10 mg per ml in 0.1 M sodium phosphate buffer, pH 7.2) measured at 20° ± 0.01°, with a digital precision density meter DMA 02C (produced by Anton Paar, K. G., Graz, Austria, according to the design of Stabinger et al. (29)). The calculation was according to the equation derived by Casassa and Eisenberg (30)

\[ \phi' = \frac{1}{\rho} \left( 1 - \frac{\rho - \rho_s}{\varepsilon} \right) \]

where \( \phi' \) the apparent specific volume, is equal to the partial specific volume (\( \bar{\rho} \)) for low protein concentrations such as those used in the present study. \( \rho \) and \( \rho_s \) are the densities of the protein solution and the buffer, respectively, and \( \varepsilon \) is the concentration of the protein solution in milligrams per ml.

NH2-terminal Analysis—SBA was dansylated according to the method of Gray (31) in 0.1 M sodium bicarbonate in 8 M urea, pH 8.5. The dansyl (5-dimethylaminonaphthalene-1-sulfonyl) derivative of the terminal amino acid was identified after acid hydrolysis of the dansylated protein by thin layer chromatography on polyamide layer sheets (15 × 15 cm, Cheng-Chin Trading Co. Ltd., Taiwan) according to the method of Woods and Wang (32).

Quantitative determination was based on the fluorodinitrobenzene method of Sanger (33) and was also performed in the presence of 8 M urea. Corrections for partial destruction of the terminal dinitrophenylamino acid were made by extrapolation to zero time of the amounts of terminal dinitrophenylamino acid recovered after various periods of hydrolysis.

Amino Acid Analysis Hydrolysis was performed with either constant boiling HCl (containing 0.5% phenol) or with methanesulfonic acid (for tryptophan estimation) in evacuated sealed tubes which were held for varying intervals (22, 48, or 72 hours) in an electrically heated aluminium block controlled at 110° ± 0.1° with a thermistor relay. Excess HCl was removed by repeated rotary evaporation under reduced pressure. The hydrolysates were analyzed by the method of Spackman et al. (34) on an amino acid analyzer with provision for automated sample injection and a photometric system optimally sensitive in the 0.05-μmole range (35). The values reported represent the average of at least two determinations with separate hydrolysates.

Methionine was determined as methionine sulfone after oxidation with performic acid (30).

Binding of N-Acetyl-d-galactosamine to Soybean Agglutinin—This was measured by equilibrium dialysis with the use of H-labeled N-acetyl-d-galactosamine. Dialysis membranes were cut from Visking (20/32) dialysis tubing, treated with three changes of boiling 1 mM EDTA, 5 min each time, stored in 0.1 mM EDTA at 4°, and washed with distilled water before use. In each experiment, 1 ml of SBA solution (4 to 7.5 mg per ml of 0.05 M phosphate buffer, pH 6.8) was pipetted into one compart-
ment and 1 ml of a solution containing varying amounts of labeled ligand (0.05 to 0.5 µmole, approximately 6 × 10^6 cpm per µmole) in the same buffer into the other compartment of the dialysis cell (model M-E, Technilab Instruments, Los Angeles, California). In some experiments the ligand and SBA were pipetted into the same compartment and dialyzed against buffer in the other compartment. The cells were gently rotated (1 rpm) for 16 hours at 4°C, and samples were then removed for assay. The concentration of the ligand on both sides of the membrane was determined by counting 2 to 3 aliquots (10 to 50 µl) in a Packard Tri-Carb liquid scintillation spectrometer in Bray's solution, and the concentration of SBA was determined spectrophotometrically.

The binding was also measured by gel filtration essentially as described by Hummel and Dreyer (37). A column (1.2 × 50 cm) of Sephadex G-25 (fine) was equilibrated at 4°C with 0.05 M phosphate buffer, pH 7.4, containing 0.1 mM N-[2H]acetyl-n-galactosamine (4.75 × 10^5 cpm per µmole). SBA (5 mg) was applied in 1 ml of the same buffer, which was also used for elution. Fractions of 1 ml were collected at a rate of 20 ml per hour. SBA was monitored by reading the absorbance at 280 nm, and N-acetyl-n-[^3]H]galactosamine was monitored by its radioactivity, measured as above.

RESULTS AND DISCUSSION

Homogeneity of Soybean Agglutinin—Disc electrophoresis at acidic and alkaline pH of SBA obtained by affinity chromatography revealed a single protein band (Fig. 1, A, B, and C) even when high loads of SBA (300 µg per gel) were applied. In sedimentation velocity studies, SBA afforded a single symmetrical peak in buffers ranging in pH from 2.2 to 10.8, indicating neither apparent heterogeneity nor association-dissociation phenomena. There was no significant effect of protein concentration on the sedimentation coefficient in the range of 3 to 10 mg of SBA per ml. The same value of $\tilde{s}_{20\text{,w}} = 6.0 \pm 0.125$ S was obtained throughout the pH and concentration ranges mentioned above. Gel filtration on Sephadex G-150 (superfine) gave a symmetrical protein peak that coincided with the peak of activity, giving a constant specific activity (6,500 to 7,000 hemagglutinating units per mg) throughout the peak. There was no effect of SBA concentration in the range 0.5 to 10 mg of protein per ml on the position of the elution peak and thus on the molecular size of SBA. Isodiclectric focusing in a sucrose gradient in the range pH 5 to 8 yielded a single symmetrical protein and activity peak with a maximum at pH 5.81 (Fig. 2).

Extinction Coefficient—The extinction coefficient on a moisture-free basis was found to be $A_{280\text{,nm}} = 12.8\text{ cm}^{-1}$ and the nitrogen content 15.5%.

Molecular Weight—Gel filtration on Sephadex G-150 (superfine) gave a molecular weight of 120,000 ± 10,000 for SBA, relative to bovine γ-globulin (mol wt 150,000), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 45,000), and chymotrypsinogen (mol wt 25,000) (Fig. 3).

Sedimentation equilibrium measurements performed in 0.1 M sodium phosphate buffer, pH 7.2, gave linear Yphantis plots for the protein concentrations used (0.2 to 0.6 mg of SBA per ml) (Fig. 4). The molecular weight was calculated to be 122,000 ± 1,300 employing the experimentally determined value of 0.745 ml per g for the partial specific volume. This molecular weight is in good agreement with the value of 110,000 which has been determined by sedimentation and diffusion for SBA purified by conventional methods (7).

**Fig. 1.** Disc electrophoresis of SBA on polyacrylamide gels. The direction of migration is from the top. Electrophoresis was performed with 100 µg of SBA at pH 4.5 in 7.5% acrylamide gel at 2 mA for 4½ hours (A); the same conditions, but stained for glycoproteins (B); at pH 8.9 in 7.5% acrylamide gel at 1 mA for 5 hours (C); according to Weber and Osborn (20) in 10% acrylamide gel containing 0.1% sodium dodecyl sulfate (D); and at pH 3.3 in the presence of 8 M urea in a gradient of acrylamide from 4% (top) to 8% (bottom) at 2 mA for 2 hours (E). Gels A, C, D, and E were stained for protein.

**Fig. 2.** Isoelectric focusing of SBA in a linear sucrose gradient. The details of the experiment are given under Experimental Procedures.

**Fig. 3.** Determination of the molecular weight of SBA by gel filtration. A column (1.9 × 50 cm) of Sephadex G-150 (superfine) was used with 0.9% NaCl as eluent ( ), or with 0.1% sodium dodecyl sulfate in 0.9% NaCl as eluent ( ). For details see text.
Fig. 4. Sedimentation equilibrium measurement of SBA. Centrifugation was performed at 16,000 rpm for 19 hours. The sample, at a concentration of 0.4 mg per ml, had been previously dialyzed against 0.1 M sodium phosphate buffer, pH 7.2. The plot is of the fringe displacement $f$, in micrometers, with respect to the radial distance in centimeters squared. Inset, weight average molecular weight as a function of protein concentration (represented as fringe displacement).

Dissociation into Subunits—Disc gel electrophoresis in the presence of either 0.1% sodium dodecyl sulfate or 8 M urea yielded a single protein band (Fig. 1, D and E). From electrophoresis in the presence of 0.1% sodium dodecyl sulfate the molecular weight of this protein was calculated to be 30,000 ± 1,500, relative to bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 45,000), glyceraldehyde phosphate dehydrogenase (mol wt 36,000), chymotrypsinogen (mol wt 25,000), and lysozyme (mol wt 14,400) (Fig. 5). Gel filtration on Sephadex G-150 (superfine) in the presence of 0.1% sodium dodecyl sulfate afforded a single peak of protein which emerged from the column in a position corresponding to mol wt 30,000 ± 3,000, relative to catalase (mol wt 67,000), ovalbumin (mol wt 45,000), chymotrypsinogen (mol wt 25,000), and cytochrome c (mol wt 12,400) (Fig. 3). Ultracentrifugation of SBA (5, 10, and 15 mg per ml) in 0.1% sodium dodecyl sulfate gave a single peak with an intrinsic sedimentation coefficient of 2.15 ± 0.05 S. Ultracentrifugation of SBA (at the same concentrations as above) in 8 M guanidine hydrochloride afforded a single peak with $s_{20,w}^2 = 1.98 ± 0.075 S$ and $s_{20,w}^2 = 2.34 ± 0.05 S$, respectively.

Sedimentation equilibrium in 6 M guanidine hydrochloride of SBA (0.3 to 0.9 mg per ml) gave a linear Yphantis plot (Fig. 6), from which a molecular weight of 30,300 ± 400 was calculated by the use of the value of 0.735 ml per g for the partial specific volume, assuming a decrease of 0.01 ml per g in the unfolded subunit as suggested by Castellino and Barker (38).

A single spot corresponding to dansylalanine was obtained by thin layer chromatography of a hydrolysate of 10 to 50 amino acids of dansylated SBA on polyamide sheets. This result confirms previous findings reported by Wada et al. (9). Quantitative determination of NH$_2$ termini performed by the fluorodinitrobenzene method in the presence of 8 M urea gave 1 mole of dinitrophenylalanine per 30,000 g of SBA.

These results clearly show that native SBA (mol wt 120,000) is a tetramer comprised of four identical subunits (mol wt 30,000). Oligomeric structure seems to be a characteristic of many of the lectins studied to date (1). Some physical and chemical properties of SBA and of its subunit are summarized in Table 1. Amino Acid Analysis—The results of amino acid analyses of SBA, reported as the number of residues per subunit, are given...
in Table II. The value chosen for the molecular weight of the monomer is 28,000 on the assumption that there is a carbohydrate side chain of 2,000 in each subunit. In agreement with earlier results (7, 9), SBA is devoid of cysteine, low in methionine, but rich in acidic and hydroxyamino acids. For most amino acids, however, the values are higher than those previously reported. This is most probably due to the greater purity of the SBA preparation used and to improvements in techniques of amino acid analysis.

Binding of N-Acetyl-D-galactosamine to Soybean Agglutinin—The results of a typical equilibrium dialysis experiment are represented in Fig. 7. The results were plotted according to the following equations:

\[ A. \quad \frac{r}{c} = nK - rK \]
\[ B. \quad \frac{1}{r} = \frac{1}{nK} + \frac{1}{n} \]
\[ C. \quad \log \frac{r}{n-r} = a \log K + a \log c \]

in which \( r \) is the number of moles of N-acetyl-D-galactosamine bound per mole of SBA, \( c \) is the concentration (moles per liter) of free sugar, \( n \) is the maximal value for \( r \), \( K \) is the association constant, and \( a \) is the index for the heterogeneity of binding sites with respect to \( K \).

In plots \( A \) and \( B \) (Fig. 7) extrapolation yields a value of \( n = 1.92 \) and \( n = 2.0 \), respectively, indicating that there are two binding sites for N-acetyl-D-galactosamine per molecule of SBA. From the slope of the linear plot \( A \), the binding constant was

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**Table I**

Properties of soybean agglutinin and its subunit

| Property                        | Value                      |
|---------------------------------|----------------------------|
| Extinction coefficient (\( A_{\text{ext}} \)) | 12.8 cm\(^{-1} \)         |
| Isoelectric point (pI)         | 5.81                       |
| Diffusion coefficient (\( D_{\text{diff}} \)) | 5.0 \( \times \) 10\(^{-7} \) cm\(^2\) s\(^{-1} \) |
| Sedimentation coefficient (\( s_{\text{sed}} \)) | 0. S \( (\text{in } 6 \text{ M GuHCl)} \) |
| Partial specific volume (\( \overline{P} \)) | 0.745 ml g\(^{-1} \)     |
| Molecular weight (M)            | 120,000 - 10,000 (gel filtration) |
|                                | 30,000 - 1,500 (gel filtration in 0.1% SDS) |
|                                | 122,300 (sedimentation diffusion) |
|                                | 30,000 - 1,500 (gel electrophoresis in 0.1% SDS) |
|                                | 122,000 \( \pm \) 1,300 (sedimentation equilibrium) |
| Frictional ratio (\( f/f_0 \)) | 1.3                       |
| NH\(_2\)-terminal residue*      | 4 alanines                 |

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**Table II**

Amino acid composition of soybean agglutinin

| Amino acid        | Micrograms per mg* | Micromoles per mg | Residues per subunit^* |
|-------------------|--------------------|------------------|------------------------|
| Lysine            | 55.56              | 0.422            | 13                     |
| Histidine         | 28.80              | 0.106            | 6                      |
| Arginine          | 33.72              | 0.215            | 7                      |
| Aspartic acid     | 140.27             | 1.219            | 38                     |
| Threonine\(^a\)   | 56.94              | 0.554            | 17                     |
| Serine\(^a\)      | 68.27              | 0.783            | 24                     |
| Glutamic acid     | 71.55              | 0.552            | 17                     |
| Proline           | 59.57              | 0.613            | 19                     |
| Glycine           | 27.72              | 0.485            | 13                     |
| Alanine           | 55.21              | 0.748            | 23                     |
| Half-cystine      | 0.0                | 0.0              | 0                      |
| Valine            | 63.91              | 0.638            | 29                     |
| Methionine\(^a\)  | 4.87               | 0.036            | 1                      |
| Isoleucine        | 58.34              | 0.515            | 16                     |
| Leucine           | 94.76              | 0.837            | 26                     |
| Tyrosine          | 28.58              | 0.174            | 5                      |
| Phenylalanine     | 72.93              | 0.494            | 15                     |
| Tryptophane\(^a\) | 41.23              | 0.207            | 6                      |
| Total             | 855.23             | 8.698            | 270                    |

\* Corrected for 10.8\% moisture.
\^ Subunit molecular weight 28,000 on the assumption that there is one carbohydrate side chain of 2,000 per subunit (see Footnote 2 in the text).
\(^a\) Determined as methionine sulfoxide after performic acid oxidation.
\(^*\) Determined in a sample hydrolyzed in methanesulfonic acid.
FIG. 8. Measurement of the binding of \( N-{\text{3H}} \)acetyl-d-galactosamine to SBA by gel filtration. A column (1.2 X 50 cm) of Sephadex G-25 (fine) was used; other details are given in the text.

calculated to be \( K = 3.0 \times 10^4 \) liter mole\(^{-1}\). In plot C, the slope (a) is equal to 1, indicating that both sites are identical.

A similar value (2 to 2.4 moles of N-acetyl-d-galactosamine bound per mole of SBA) was obtained by gel filtration on a Sephadex G-25 column equilibrated with radioactive ligand (Fig. 8).

For the agglutination of cells, a lectin molecule should possess at least two binding sites, as is found in antibodies. Concanavalin A usually occurs as a dimer with two binding sites (42); two binding sites for methyl \( \alpha \)-d-glucopyranoside were found in lentil lectin (43); two of four sites for \( \alpha \)-fucose were found in the lectin from \textit{Lotus tetrugondobus} (44). Usually the number of binding sites is equal to the number of subunits. SBA seems to be an exception, since the number of saccharide-binding sites is half the number of subunits. “Half-of-the-sites” reactivity has been observed in several oligomeric enzymes (45), but there is no accepted explanation for this phenomenon.

Acknowledgment—We thank Mr. N. Alonzo for performing the amino acid analyses.

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Subunit Structure of Soybean Agglutinin
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J. Biol. Chem. 1974, 249:1219-1224.

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