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Isolation and Characterization of Subunits from the Predominant Form of *Dolichos biflorus* Lectin†

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ABSTRACT: The subunits of the two molecular forms (A and B) of the *Dolichos biflorus* lectin were isolated by ion-exchange chromatography on DEAE-cellulose in 8.0 *M* urea. Subunits IA and IIA which comprise the predominant molecular form A of the lectin were found to have molecular weights of 27,700 and 27,300, respectively, as determined by sedimentation equilibrium studies in 8.0 *M* urea. These subunits have similar amino acid compositions and each have alanine at their amino-terminal ends. Comparison of the IA and IIA subunits by immunodiffusion against antisera to the seed extract as well as to subunits IA and IIA showed no antigenic differences between the two subunits. Carboxyl terminal analyses of subunits IA and IIA with carboxypeptidase A produced an essentially simultaneous release of both leucine and valine residues from subunit IA; no detectable amino acids were released from subunit IIA under identical conditions. The data suggest that the molecular form A of the lectin (molecular weight 113,000, Carter and Etzler, 1975) consists of four subunits with a possible stoichiometry of IA_2IIA_2. Other possible arrangements of the subunits are discussed.

The seeds of many plants contain proteins called lectins that have the ability to specifically agglutinate certain types of cells (for review see Lis and Sharon, 1973). A number of these lectins have specificities for some of the various blood group substances; among these lectins is the *Dolichos biflorus* lectin that was reported to agglutinate type A erythrocytes (Bird, 1951) and to precipitate blood group A substance (Boyd and Shapleigh, 1954; Bird, 1959). This lectin was isolated in a highly purified state by affinity chromatography on insoluble blood group A+H substance, and its blood group A specificity was found to be due to its ability to specifically combine with terminal nonreducing *N*-acetyl-α-D-galactosamine residues (Etzler and Kabat, 1970).

The isolated *Dolichos biflorus* lectin is a glycoprotein (Etzler and Kabat, 1970; Font et al., 1971) and has recently been fractionated into two electrophoretically distinguishable forms (A and B) by chromatography on concanavalin A-Sepharose. These two molecular forms of the lectin have differences in carbohydrate contents but have identical specificities and very similar amino acid compositions (Carter and Etzler, 1975).

The A and B forms of the lectin have molecular weights of 113,000 and 109,000, respectively, and are each dissociated into two types of subunits by discontinuous electrophoresis on sodium dodecyl sulfate–urea gels (Carter and Etzler, 1975).

In the present paper we report the isolation of the subunits of the *Dolichos biflorus* lectin and the characterization of the two types of subunits of the predominant form A of the lectin.

Materials and Methods

Isolation of Lectin. The *Dolichos biflorus* lectin was isolated from seed extracts as previously described (Etzler and Kabat, 1970; Etzler, 1972) by adsorption onto insoluble polyleucyl hog blood group A+H substance (Kaplan and Kabat, 1966) and specific elution from this immunoadsorbent with 0.01 *M* *N*-acetyl-D-galactosamine. After removal of the hapten by chromatography on Bio-Gel P-10, the lectin was concentrated by ultrafiltration in a Diaflo ultrafil-

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tration device using a XM-50 or PM-10 filter and stored at concentrations of 1–2 mg/ml in 0.01 *M* sodium phosphate buffer (pH 7.2) containing 0.9% NaCl and 0.02% sodium azide.

The isolated lectin, which will be called unfractionated lectin, was fractionated into forms A and B by chromatography on concanavalin A-Sepharose as previously described (Carter and Etzler, 1975). Form B of the lectin which represents less than 12% of the total lectin did not bind to the column whereas most of form A bound and was specifically eluted with methyl α-D-glucopyranoside.

Analytical Methods. Protein concentration was determined by a nitrogen determination using a modified ninhydrin method (Schiffman et al., 1964) or by the procedure of Lowry et al. (1951).

Samples were prepared for amino acid analyses as previously described (Carter and Etzler, 1975), and amino acid analyses were performed by Eldex Laboratories, Inc. (Menlo Park, CA), using a Durrum Model D-500 amino acid analyzer. Tryptophan was analyzed spectrophotometrically by the method of Goodwin and Morton (1946) in the presence of 0.1 *N* NaOH and 8.0 *M* urea as described by Wetlaufer (1962). Free sulfhydryl groups were determined using 5,5′-dithiobis(2-nitrobenzoic acid)[1] (Kastenschmidt et al., 1968).

Carboxyl terminal amino acid analyses utilizing carboxypeptidase A and amino-terminal amino acid analyses using 1-dimethylaminonaphthalene-5-sulfonyl chloride[2] were performed as previously described (Carter and Etzler, 1975).

Immunodiffusion was performed by the Ouchterlony (1948) method in 1% ionagar in 0.01 *M* sodium phosphate buffer (pH 7.2) containing 0.9% NaCl and 0.02% sodium azide. Antisera were produced in rabbits against a mixture of *Dolichos biflorus* seed extract with Freund's complete adjuvant.

The carbohydrate contents of the lectin subunits were determined by gas–liquid chromatography of the alditol acetate derivatives of sugars released after acid hydrolysis as previously described (Carter and Etzler, 1975).

Physicochemical Methods. Sedimentation equilibrium experiments were performed in a Spinco Model E analytical ultracentrifuge with Rayleigh interferometric optics at 25° according to the meniscus depletion method of Yphantis (1964). The lectin subunits were run in the presence of 0.1 *M* Tris-HCl buffer (pH 7.2) containing 0.2 *M* NaCl as a supporting electrolyte (Tanford, 1961) and 8.0 *M* urea as a dissociating agent (Kawahara et al., 1965; Hatefi and Hanstein, 1969). Solvent densities were calculated according to Kawahara and Tanford (1966). The partial specific volumes of the lectin subunits were calculated from their respective amino acid compositions (McMeekin et al., 1949). The interference fringe patterns were analyzed on a Nikon Model 6C comparator with a 50× objective and the resulting data evaluated with a least-squares fit computer program.[3] Equilibrium was assumed attained after a fringe count across the cell for two successive fringe patterns taken at 8-hr intervals was constant within experimental error (Van Holde, 1967).

Discontinuous polyacrylamide gel electrophoresis was carried out on an anionic pH 9.7 glycine system in the presence of 8.0 *M* urea and 0.1% sodium dodecyl sulfate as previously described (Carter and Etzler, 1975).

Results

Preliminary studies on the subunit composition of the *Dolichos biflorus* lectin showed the presence of four sharp bands when the lectin was subjected to discontinuous polyacrylamide gel electrophoresis at pH 9.7 in the presence of 8.0 *M* urea and 0.1% SDS. These four bands which do not represent purification artifacts (Carter and Etzler, 1975) were designated IA, IB, IIA, and IIB in order of increasing mobility (Figure 2) and previous work showed an increased proportion of subunits IA and IIA in form A of the lectin and subunits IB and IIB in form B (Carter and Etzler, 1975).

A number of methods of separation of the subunits were attempted utilizing their differences in charge to friction ratios. Of these methods, ion-exchange chromatography on DEAE-cellulose in 8.0 *M* urea was the most successful in terms of resolution and subunit recovery (Figure 1).

Electrophoretic analysis of peaks IA and IIA on sodium dodecyl sulfate–urea gels, pH 9.7, produced single bands which coelectrophorese with bands IA and IIA of whole lectin (Figure 2). Peaks IB and IIB were not electrophoretically homogeneous; they contained a small amount of material migrating in the IA and IIA regions, respectively. The other small peaks after IB and IIB did not have unique mobilities on the sodium dodecyl sulfate–urea gels and appear to represent charge modifications of the subunits due to reaction with spontaneously formed cyanate (Stark et al., 1960). These apparent charge modifications were confirmed by showing an increase in area of the spurious peaks after IB and IIB by a prolonged exposure of the lectin to urea at increased temperatures, conditions shown by Hagel et al. (1971) to increase cyanate formation in urea.

Rechromatography of peaks IA or IIA under the same conditions as above showed that most of the material was eluted in the IA or IIA region, respectively. However, a small percentage of each of the rechromatographed IA and IIA peaks was eluted in the IB or IIB region, respectively. Electrophoresis of this latter material showed it represented charge modification due to carbamylation of the IA or IIA subunits and not the production of new IB or IIB. These observations may account for the difficulty in obtaining homogeneous IB and IIB samples.

Preliminary renaturation studies, carried out by dialysis of the urea disrupted subunits against a number of buffers and solvents, failed to restore any of the original hemagglutinating activity of the lectin. The dialyzed subunit solutions also failed to inhibit hemagglutinating activity of nondisrupted lectin, thus indicating no binding activity of the urea disrupted subunit preparations.

The isolated IA and IIA subunits were subjected to sedimentation equilibrium at 25° and 44,000 rpm at respective concentrations of 0.388 and 0.836 A_{280} unit/ml in a solvent consisting of 8.0 *M* urea, 0.1 *M* Tris-HCl buffer (pH 7.3), and 0.2 *M* NaCl. The partial specific volumes as derived from the amino acid compositions were 0.730 ml/g for the IA subunit and 0.724 ml/g for the IIA subunit. Using these values, weight average molecular weights of the subunits were calculated to be 27,700 for the IA subunit and 27,300 for the IIA subunit. These values are in close agreement with molecular weights obtained on continuous pH sodium

[1] We would like to thank Dr. R. Rice for performing this analysis.

[2] Abbreviation used is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

[3] We would like to thank Edwina Beckman and David Bylund for their technical assistance in performing these molecular weight determinations.

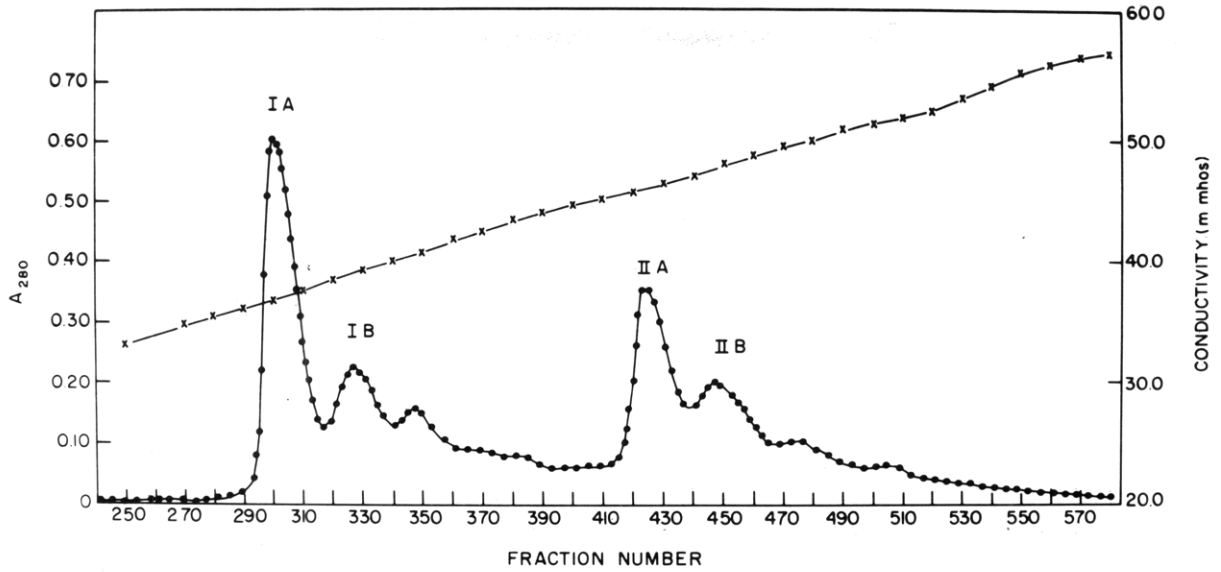

FIGURE 1: Ion exchange chromatography of *Dolichos biflorus* lectin on DEAE-cellulose in 8.0 *M* urea. Ion exchange chromatography was carried out on DEAE-cellulose (Whatman DE52) columns, 1.7 cm × 120.0 cm, in the presence of 8.0 *M* cyanate free urea–0.04 *M* Tris-HCl buffer (pH 7.3) at room temperature. The 150-mg sample was applied to the column in 10.0 *M* urea–0.04 *M* Tris-HCl (pH 7.3) at a concentration of approximately 2.5 A_{280} units/ml. The column was then washed with one bed volume of 8.0 *M* urea–0.04 *M* Tris-HCl (pH 7.3) and then a 2.0-l. linear 0–0.075 *M* NaCl gradient in 0.04 *M* Tris-HCl (pH 7.3)–8.0 *M* urea was applied. The column was then washed with two bed volumes of 2.0 *M* NaCl in 0.04 *M* Tris-HCl (pH 7.3)–8.0 *M* urea. Salt gradients were analyzed using a conductivity meter. The linear NaCl salt gradient was applied to the column and collected starting at fraction 1. No significant quantity of A_{280} absorbing material was eluted prior to fraction 250 or after fraction 520. (O) A_{280}, (x) conductivity. The pooled fractions were extensively dialyzed against 0.04 *M* Tris-HCl (pH 7.3) and 0.02% NaN_3 at 4°. The samples were then dialyzed against 0.1 *M* $(NH_4)_2CO_3$ buffer (pH 8.5); 70–80% of the protein was recovered. After a few days the protein would slowly precipitate out of the latter buffer.

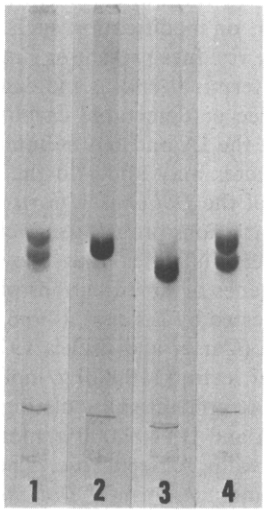

FIGURE 2: Discontinuous polyacrylamide gel electrophoresis of isolated IA and IIA *Dolichos biflorus* subunits on pH 9.7 glycine gels in the presence of 0.1% sodium dodecyl sulfate and 8.0 *M* urea. (1) Unfractionated lectin, (2) peak IA, (3) peak IIA, (4) recombined peak IA plus peak IIA.

dodecyl sulfate gels (Carter and Etzler, 1975). The sedimentation equilibrium data plots are linear indicating that the IA and IIA subunits are homogeneous and not self-aggregating under the experimental conditions used.

Immunodiffusion of subunits IA and IIA against rabbit antisera prepared against the crude seed extract and against the isolated IA and IIA subunits failed to detect any antigenic differences between the two subunits.

The IA and IIA subunits have very similar amino acid compositions (Table I). The amino acid analyses showed no detectable homocitrulline which would appear as a result of lysine reaction with cyanate in the urea (Stark et al., 1960).

Amino terminal amino acid analyses were performed on the isolated IA and IIA subunits. After labeling with dansyl chloride and subsequent acid hydrolysis, dansyl-alanine was found to cochromatograph with the dansylated amino terminal residue of each of the subunits in five solvent systems. These data indicate that the only detectable or free amino acid terminus of subunits IA and IIA is alanine.

Subunit carboxyl terminal amino acids were determined by enzymatic hydrolysis with carboxypeptidase A. A kinetic study of the released carboxyl terminal amino acids indicated an essentially simultaneous release of both leucine and valine residues from subunit IA. Quantitation of the released carboxyl terminal amino acids by amino acid analyses showed at least 1 mol of both leucine and valine released per 1 mol of subunit IA. Carboxypeptidase A treatment of subunit IIA under the same conditions failed to release any significant quantities of any amino acid. The hydrolysis conditions used in the subunit carboxyl terminal amino acid analyses were essentially the same as those used for carboxyl terminal analyses of the A and B forms of the intact *Dolichos biflorus* lectin (Carter and Etzler, 1975). The quantity and rate of release of carboxyl terminal leucine and valine residues from subunit IA can account for the leucine and valine released from the intact A form assuming there are two IA subunits per form A molecule.

The carbohydrate contents of subunits IA and IIA were similar both in mannose and *N*-acetylglucosamine composition (Table II).

Discussion

The subunit structures of a number of lectins have recently been described. Gould and Scheinberg (1970) showed that the 138,000-g/mol form of the *Phaseolus lunatus* (lima bean) lectin is composed of four 31,000-g/mol subunits. The *Phaseolus vulgaris* lectin (red kidney bean)

Table I: Amino Acid Composition of Subunit IA, Subunit IIA, and Unfractionated Lectin.

Amino Acid	Unfractionated Lectin[a] (Residues/ 110,800 Mol Wt)	Subunit IA[a] (Residues/ 27,700 Mol Wt)	Subunit IIA[a] (Residues/27,300 Mol Wt)
Asp	140.6	32.1	33.7
Thr[b]	75.7	17.6	19.3
Ser[b]	180.5	43.8	43.4
Glu	78.1	17.9	19.6
Pro	44.7	13.1	11.5
Gly	78.1	18.0	21.1
Ala	109.1	25.8	26.2
Val	88.5	22.0	21.0
Met	5.1	1.3	1.4
Ile	60.9	16.3	15.3
Leu	83.4	21.1	20.1
Tyr	28.6	8.5	7.2
Phe	45.2	12.0	11.7
His	10.3	2.8	2.5
Lys	37.4	10.1	8.7
Arg	24.2	6.0	5.4
Trp[c]	17.9	4.5	4.3
Cys[d]	0		

[a] The number of residues was calculated by averaging the values obtained from acid hydrolysis in 6 *N* constant boiling HCl for 24, 48, and 72 hr. [b] The serine and threonine values were obtained by linear extrapolation to zero hydrolysis time. [c] Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (1946). [d] Determined using 5, 5′-dithiobis(2-nitrobenzoic acid).

Table II: Carbohydrate Content of Isolated Subunits IA and IIA.[a]

Subunit	nmoles of Mannose/ nmoles of Subunit	nmoles of *N*-Acetyl-glucosamine/ nmoles of Subunit	% Carbohydrate	
			Mannose	*N*-Acetyl-glucosamine
IA	5.1	1.1	3.3	0.9
IIA	7.8	1.5	5.2	1.2

[a] Carbohydrate analyses of subunits IA and IIA of the predominant A form of *Dolichos biflorus* lectin. Alditol acetate derivatives of sugars released by acid hydrolysis of isolated lectin subunits were compared with the alditol acetate derivatives of standard sugars (inositol, xylose, mannose, *N*-acetylglucosamine, and rhamnose) by gas–liquid chromatography. All carbohydrate content values were calculated assuming molecular weights of 27,700 for subunit IA and 27,300 for subunit IIA.

was found to consist of two major glycoprotein subunit types (Allan and Crumpton, 1971; Miller et al., 1973). The studies of Edmundson et al. (1971), and Cunningham et al. (1972), indicate a subunit molecular weight of 27,000 for concanavalin A, and the soybean agglutinin (*Glycine max*) has been described as a tetramer of identical subunits of 30,000 molecular weight (Lotan et al., 1974). A tetrameric structure composed of four apparently identical subunits has also been proposed for the lectin from *Bandeiraea simplicifolia* (Hayes and Goldstein, 1974).

The *Dolichos biflorus* lectin has been fractionated into two molecular forms (A and B) each of which can be dissociated into two types of subunits (Carter and Etzler, 1975). In the present study these subunits were isolated by ion-exchange chromatography on DEAE-cellulose in 8.0 *M* urea. The IA and IIA subunits which constitute the predominant form A of the lectin were found to have molecular weights of 27,700 and 27,300, respectively. These values are in agreement with the molecular weights estimated by electrophoresis on continuous pH sodium dodecyl sulfate gels (Carter and Etzler, 1975). Amino acid and carbohydrate analyses of the IA and IIA subunits showed a high degree of similarity between them; both subunits were also found to have alanine at their amino terminal end. The similarities between subunits IA and IIA in molecular weight, composition, and amino terminal amino acid may account for the failure of Pere et al. (1974), to detect more than one type of subunit in the lectin.

Differences were found between subunits IA and IIA at the carboxyl terminal ends of these subunits. Subunit IA was found to have leucine and valine at its carboxyl terminal. The rapid cleavage of both of these amino acids by carboxypeptidase A makes it difficult to determine which of these two amino acids is at the end. Identical treatment of subunit IIA with carboxypeptidase A released no significant quantities of any amino acid thus suggesting that the carboxyl end of this subunit differed from that of subunit IA and was either blocked or had an amino acid not susceptible to carboxypeptidase A.

The similarities observed in molecular weight, amino terminal amino acid residues, amino acid and carbohydrate compositions, and antigenic determinants between subunits IA and IIA of the predominant A form of *Dolichos biflorus* lectin lend support to the idea that subunits IA and IIA represent slight alterations of one type of subunit. These alterations may take the form of carbohydrate variation, amino acid substitution, or modification such as variation in the number of amide residues present or simply as modification at the carboxyl terminal end as indicated by the apparent differences in free or detectable carboxyl terminal amino acid residues for the IA and IIA subunits. These variations in subunit character may apply to the differences seen in forms A and B of the *Dolichos biflorus* lectin as well. The fact that these variations probably do not come about as purification artifacts indicates that they may result from closely related genes or modifications of the lectin prior to isolation as suggested by Lis et al. (1966).

Previous work (Carter and Etzler, 1975) showed that the *Dolichos biflorus* lectin (110,800 g/mol) can be fractionated into two electrophoretically distinguishable forms, A (113,000 g/mol) and B (109,000 g/mol), by chromatography on concanavalin A-Sepharose. The above data show that the predominant A form of *Dolichos biflorus* lectin is composed of four similar but not identical subunits. These subunits can be separated into two predominant classes, IA (27,700 g/mol) and IIA (27,300 g/mol), on the basis of charge variation. The stoichiometry of the subunit arrangement in the native lectin has not yet been determined, leaving three possible arrangements. (1) The A form may consist of a mixture of two molecules consisting of IA_4 and IIA_4 subunit arrangements. The B form could likewise have the conformation of IB_4 and IIB_4. (2) Another possible arrangement is a continuously variable mixture of IA, IIA, IB, and IIB subunits ranging from IA_4 and IIA_4 at one extreme representing the A form through intermediates such as IAIIAIBIIB to the B form extreme of IB_4 and IIB_4. A similar type of model has been proposed by Miller et al. (1973), for the subunit structure of the *Phaseolus vulgaris* (red kidney bean) lectin. (3) The last possibility and the most simplistic defines the A form as IA_2IIA_2 and the B form as a IB_2IIB_2 arrangement.

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Resonance Raman Studies of Hemerythrin–Ligand Complexes†

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ABSTRACT: Resonance Raman spectroscopy has been used as a probe of the structure of ligands at the active site of hemerythrin. Molecularly revealing insights have been obtained with oxyhemerythrin and with metazidohemerythrin. This spectroscopic technique has also facilitated a comparison of oxygen carrier within erythrocytes with that in solution. The electronic state of the bound O_2 is the same in the natural environment as in the artificial one.

In hemerythrin, the non-heme oxygen-carrying pigment of several invertebrate phyla, two Fe atoms bind one molecule of O_2 (Klotz and Klotz, 1955; Klotz et al., 1957; Boeri and Ghiretti-Magaldi, 1957). In view of this stoichiometry it has been assumed that O_2 bridges the two Fe atoms. On the basis of chemical and spectroscopic evidence originally (Klotz, 1955) and Mössbauer, optical and magnetic studies more recently (Okamura et al., 1969; Garbett et al., 1969, 1971; Dawson et al., 1972), the state of the active site has been represented as

$$Fe^{III}\text{-}O_2^{2-}\text{-}Fe^{III} \quad (1)$$

The techniques mentioned examine the state of the Fe atoms in hemerythrin, and the oxidation state of the oxygen, O_2^{2-}, has been assigned largely indirectly. Recently we have demonstrated that resonance Raman spectroscopy can scrutinize the vibrational modes of the oxygen at the active site of hemerythrin (Dunn et al., 1973). This probe has now been extended to hemerythrin within erythrocytes and studies have been expanded to ligands other than molecular oxygen.

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