Isolation and Characterization of a Protein from Leaves and Stems of *Dolichos biflorus* That Cross Reacts with Antibodies to the Seed Lectin†

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ABSTRACT: The leaves and stems of the *Dolichos biflorus* plant contain a protein that cross reacts with antibodies to the seed lectin. This cross reactive material (CRM) has been isolated by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography on DEAE-cellulose and CM-cellulose. The isolated CRM forms a single diffuse band in polyacrylamide gel electrophoresis at pH 9.7 and gives a reaction of partial identity to the seed lectin when tested in immunodiffusion against antibodies to the seed lectin. The CRM has an amino acid composition similar to that of the seed lectin and has a molecular weight of 68 000–70 000 as estimated by measurements of retardation of electrophoretic mobility with increasing gel concentrations. Discontinuous polyacrylamide gel electrophoresis of the CRM in 0.1% sodium dodecyl sulfate and 8.0 M urea shows the CRM has a subunit identical in mobility with that of subunit IA of the seed lectin and a second subunit of higher molecular weight than subunit IA. Sequence determination of the first 13 NH_2-terminal residues of the CRM indicates that both subunits have the same NH_2-terminal sequence; this sequence is identical with the sequences of the seed lectin subunits with the exception of an aspartic acid in place of an asparagine at the second residue. Unlike the seed lectin, the CRM does not agglutinate nor inhibit the agglutination of type A erythrocytes, nor does it bind to polyleucyl hog blood group A + H substance. The relationship of the CRM to the seed lectin is discussed, including the possibility that the CRM may be a precursor to the active lectin.

The usefulness of plant lectins as tools for the study of cell surface architecture and complex carbohydrates has generated a large number of studies of lectins within recent years. At present much work has been done on the structures and specificities of many of these lectins (for reviews, see Liener, 1976; Lis & Sharon, 1977), but very little is known about their origin and function in the plants.

Although lectins have been reported to exist in a variety of plant tissues (Liener, 1976; Lis & Sharon, 1977; Dazzo & Brill, 1977), most of the lectins described to this date have been isolated from the seeds of leguminous plants where they constitute up to 10% of the nitrogen content of the seed extract. One such lectin is the *Dolichos biflorus* lectin which was first reported in the seeds of this plant by Bird (1951). This lectin reacts with blood group A substance (Boyd & Shapleigh, 1954; Bird, 1959) due to its specificity for terminal nonreducing α-*N*-acetyl-D-galactosamine residues (Etzler & Kabat, 1970); the specificity and structure of this lectin have been extensively characterized (Etzler & Kabat, 1970; Font et al., 1971; Etzler, 1972; Carter & Etzler, 1975a–c; Etzler et al., 1977; Hammarström et al., 1977). The lectin is a glycoprotein and exists in several forms due to microheterogeneity of the carbohydrate portion of the molecule (Carter & Etzler, 1975a). The predominant form of the lectin (form A) has a molecular weight of about 110 000 and is a tetramer consisting of two types of subunits, IA and IIA, that appear to differ from one another only at their COOH-terminal ends (Carter & Etzler, 1975b,c; Etzler et al., 1977).

In an attempt to gain some insight into the native role of this lectin, we recently conducted a developmental study of the lectin in various parts of the *Dolichos biflorus* plant during its life cycle (Talbot & Etzler, 1978). By the use of a radioimmunoassay, we detected small amounts of material in the leaves and stems of the plant that cross react with antibodies made against the seed lectin. In the present paper we report the isolation and characterization of this cross reactive material (CRM[1]) from the leaves and stems and discuss its possible relationship to the seed lectin.

Materials and Methods

Isolation of Seed Lectin. The lectin was isolated from extracts of *Dolichos biflorus* seeds as previously described (Etzler & Kabat, 1970; Etzler, 1972) by adsorption onto insoluble polyleucyl hog blood group A + H substance (Kaplan & Kabat, 1966) and specific elution from this immunoadsorbent with 0.01 M *N*-acetyl-D-galactosamine.

Analytical Methods. Protein concentration was measured by nitrogen determination using a modified ninhydrin method (Schiffman et al., 1964).

Amino acid analyses were performed on a Durrum Model D-500 amino acid analyzer after hydrolysis of the samples in constant boiling HCl for 24, 48, and 72 h at 110 °C.

NH_2-terminal amino acids were determined by labeling the protein with DnsCl and subsequent acid hydrolysis (Zanetta et al., 1970). The Dns-amino acids were identified by cochromatography with standard Dns-amino acids on silica gel G thin-layer plates (Quanta 1 g Q 1; Quantum Industries, Fairfield, N.J.) in toluene–pyridine–acetic acid (150:50:3.5).

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[1] Abbreviations used: CRM, cross reactive material to antibodies against seed lectin; Dns-, 5-dimethylaminonaphthalene-1-sulfonyl; Pth, 3-phenyl-2-thiohydantoin; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate.

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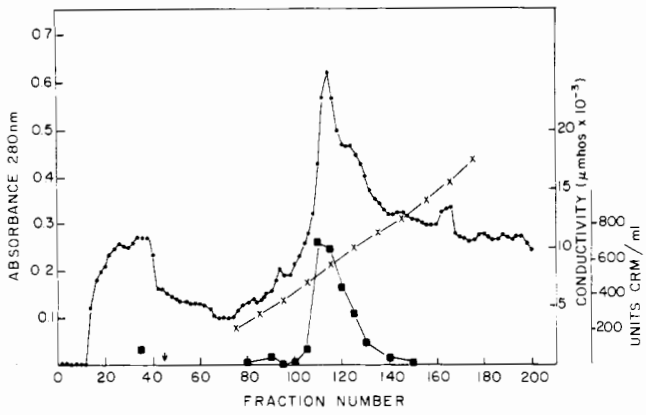

FIGURE 1: DEAE-cellulose chromatography of CRM from leaves and stems of *Dolichos biflorus*. The pellet obtained from precipitation of the plant extract with 40–80% $(NH_4)_2SO_4$ was dissolved in and dialyzed against 0.04 M Tris-HCl, pH 7.4, at 4 °C and applied to a 2.1 × 95 cm column of DEAE-cellulose (Whatman DE-52) at 4 °C; 16.5-mL fractions were collected. After washing the column with 1 bed volume of 0.04 M Tris-HCl, pH 7.4, a 4-L linear 0–0.4 M NaCl gradient in 0.04 M Tris-HCl, pH 7.4, was applied at tube 45 (↓). (●) Absorbance, 280 nm; (X) conductivity, × 10^{-3}; (■) units CRM/mL. Fractions 105–130 were pooled.

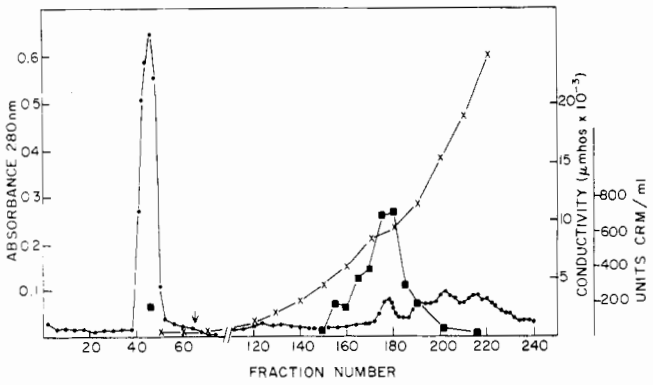

FIGURE 2: CM-cellulose chromatography of CRM from leaves and stems of *Dolichos biflorus*. The peak containing CRM from the DEAE-cellulose column (Figure 1, fractions 105–130) was dialyzed against 0.01 M Mes buffer, pH 6.0, and applied to a 2.5 × 40 cm column of CM-cellulose (Whatman CM-52) at 4 °C; 10.6-mL fractions were collected. The column was washed with 1 bed volume of 0.01 M Mes, pH 6.0. At tube 65 (↓) a 2-mL concave 0–1.0 M NaCl gradient in 0.01 M Mes, pH 6.0, was applied. (●) Absorbance, 280 nm; (X) conductivity, μmho × 10^{-3}; (■) units CRM/mL. Fractions 160–171, 172–182, 183–185, and 186–200 were pooled, dialyzed against H_2O, and lyophilized.

NH_2-terminal sequence analysis was performed by automated Edman degradations in dimethylallylamine buffer using a Beckman Model 890c sequencer with program 102974. The sample was added to the reaction cup in 100% formic acid, and the cleaved residues were mixed with Pth-norleucine as an internal standard and converted to their Pth derivatives as described by Niall (1973). The residues were identified by gas–liquid chromatography of the Pth derivatives before and/or after trimethylsilylation (Pisano & Bronzert, 1969; Klebe et al., 1966). Identification was confirmed by thin-layer chromatography on polyamide sheets (Kulbe, 1974). The hydrophobic amino acids were quantitated by measurement of peak height obtained upon gas–liquid chromatography of the Pth derivatives.

CNBr cleavage was performed as previously described (Carter & Etzler, 1975c) following the general method of Gross (1967). The lyophilized protein (2 mg) was dissolved in 1 mL of 90% formic acid, reacted with 15 mg of CNBr at room temperature for 24 h, and then dialyzed and lyophilized.

Immunochemical Methods. Hemagglutination was performed with a Takatsy microtitrator using 0.025-mL loops and a 2% suspension of type A_1 erythrocytes.

Immunodiffusion was performed by the Ouchterlony (1948) method using 1% ion agar in 0.01 M phosphate buffer, pH 7.2, containing 0.9% NaCl and 0.02% NaN_3.

The radioimmunoassay used to detect the CRM is a competitive assay based on the ability of the CRM to inhibit the precipitation of ^{14}C-labeled *Dolichos biflorus* lectin by antibodies to the lectin. The radioimmunoassay was performed as previously described (Talbot & Etzler, 1978). One unit of CRM is defined as the amount of CRM equivalent to 1 ng of seed lectin in its ability to inhibit precipitation of the lectin–antibody complex.

Physicochemical Methods. Discontinuous electrophoresis was run using 10% polyacrylamide gels in an anionic pH 9.7 glycine gel system (Wu & Bruening, 1971) as previously described (Carter & Etzler, 1975a). For subunit characterization, 8.0 M urea and 0.1% sodium dodecyl sulfate were included in the above system using 7% polyacrylamide gels, and the samples were heated in the sample buffer at 65 °C for 20 min before electrophoresis (Carter & Etzler, 1975a). The gels were stained with Coomassie Brilliant Blue (Weber & Osborne, 1969) or by the periodic acid–Schiff reaction (Segrest & Jackson, 1972).

The molecular weight of the CRM was determined electrophoretically by measurements of mobility retardation with increasing gel concentrations as described by Hedrick & Smith (1968).

Plants. Dolichos biflorus seeds (F. W. Schumacher, Sandwich, Mass.) were planted in UC Mix 1, Fertilizer II(c) (Baker, 1957) and grown in a greenhouse.

Results

Purification of CRM from Stems and Leaves of Dolichos biflorus. Preliminary experiments established that the CRM from stems and leaves cannot be isolated by the affinity chromatographic procedure used for the purification of the seed lectin. It was thus elected to employ classical fractionation procedures for the isolation of the CRM as described below.

The stems and leaves were removed from 5–6 week old *Dolichos biflorus* plants and immediately frozen in liquid nitrogen. The frozen tissue was ground to a powder in a Waring Blendor and, while still frozen, was added to a fivefold excess (w/v) of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.15 M isoascorbic acid and 0.002 M thioglycolic acid as recommended by Loomis (1974) to prevent modification due to phenolic compounds in the plant tissue. After stirring for 30 min, the particulate matter was removed by centrifugation, and the supernatant was fractionated with $(NH_4)_2SO_4$.

The pellet obtained with 40–80% $(NH_4)_2SO_4$ contained most of the CRM. This pellet was dialyzed at 4 °C against 0.04 M Tris-HCl, pH 7.4, and chromatographed on DEAE-cellulose (Figure 1). The fractions containing the CRM were pooled, dialyzed against 0.01 M Mes, pH 6.0, and chromatographed on CM-cellulose (Figure 2). The CRM was eluted as a single asymmetrical peak; portions of this peak were pooled as indicated in the figure legend, dialyzed against H_2O, and lyophilized. Approximately 9 mg of CRM was obtained from 995 g of plant tissue. Each of the pooled fractions showed similar electrophoretic patterns when run in $NaDodSO_4$–urea gels except that a small amount of extraneous material was found in the fractions from the leading and trailing edges of the peak. The material obtained in the central portion of the peak from this column (fractions 172–182) and from subsequent preparations was characterized as described below.

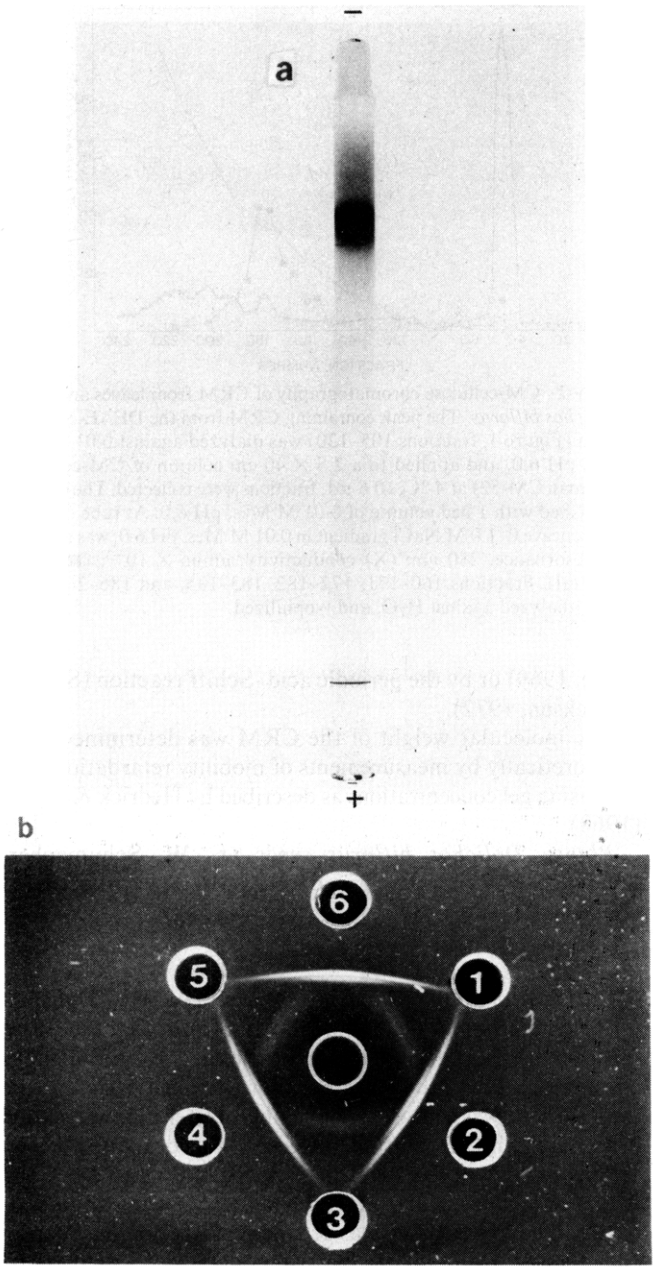

FIGURE 3: (a) Discontinuous polyacrylamide gel electrophoresis of isolated CRM on pH 9.7 glycine gels. The gel was stained with Coomassie Brilliant Blue. Migration is from top (−) to bottom (+). (b) Immunodiffusion of CRM and seed lectin against antiserum to seed lectin. Wells 1, 3, and 5 contain CRM; wells 2, 4, and 6 contain seed lectin.

Characterization of CRM. The isolated CRM forms a single diffuse band with a different mobility than that of the seed lectin upon discontinuous polyacrylamide gel electrophoresis in a pH 9.7 glycine system (Figure 3a). When tested in immunodiffusion against antiserum to the seed lectin, the CRM shows a reaction of partial identity to the seed lectin (Figure 3b). Both the CRM and the seed lectin form two closely spaced bands in immunodiffusion.

The CRM does not agglutinate type A_1 erythrocytes nor does it inhibit the agglutination of type A_1 erythrocytes by the seed lectin when tested at concentrations as high as 2 mg/mL. The CRM also does not bind to polyleucyl hog blood group A + H substance, the affinity resin used to purify the seed lectin (Etzler & Kabat, 1970). In an attempt to determine whether the inactivity of the CRM is due to modification caused by the extraction procedure, seed lectin was extracted by the above procedure used for isolation of the CRM. The seed lectin extracted in this manner was active, and no difference in hemagglutination titer was found between seed lectin extracted in the presence or in the absence of the leaf and stem tissue. These results suggest that the inability of the CRM to combine with blood group A substance may be a characteristic of the native molecule rather than due to modification imposed by the isolation procedure.

TABLE I: Amino Acid Composition of Lectin Isolated from Seeds and CRM Isolated from Leaves and Stems of *Dolichos biflorus*.[a]

	Seed lectin (μmol of AA/mg of protein)	CRM from leaves and stems (μmol of AA/mg of protein)
Asp	0.890	0.921
Thr	0.544	0.639
Ser	1.134	1.234
Glu	0.532	0.466
Pro	0.400	0.320
Gly	0.518	0.499
Ala	0.743	0.699
Val	0.593	0.534
Met	0.039	0.040
Ile	0.512	0.486
Leu	0.660	0.621
Tyr	O.280	0.251
Phe	0.382	0.431
His	0.097	0.096
Lys	0.316	0.292
Arg	0.205	0.237

[a] The data are the average of values obtained after hydrolysis for: 24, 48, and 72 h in 6 N constant boiling HCl for the seed lectin; and 48 and 72 h under the same conditions for the CRM. The serine and threonine values were obtained by extrapolation to zero hydrolysis time. Cysteine and tryptophan were not determined. The mg of protein values were calculated from nitrogen determination, assuming 15% nitrogen.

The CRM has an estimated molecular weight of 68 000–70 000 as determined from measurements of retardation of CRM mobility with increasing polyacrylamide gel concentrations according to the method of Hedrick & Smith (1968). Amino acid analyses of the CRM show that it is very similar to the seed lectin in amino acid composition (Table I).

Subunit Structure of CRM. Discontinuous polyacrylamide gel electrophoresis of the CRM at pH 9.7 in the presence of 8.0 M urea and 0.1% $NaDodSO_4$ shows the presence of two bands (Figure 4). One of these bands has a mobility identical with that of subunit IA of the seed lectin, and the second band has a slower mobility than subunit IA, thus indicating it has a higher molecular weight than subunit IA. No band was seen corresponding to subunit IIA of the seed lectin. Both of the subunits of the CRM stained with the periodic acid–Schiff reaction, thus indicating that, as in the case of the seed lectin (Carter & Etzler, 1975a), the CRM subunits contain carbohydrate.

The CRM was reacted with Dns-Cl, and the Dns subunits were separated by discontinuous polyacrylamide gel electrophoresis at pH 9.7 in the presence of 8.0 M urea and 0.1% $NaDodSO_4$. The subunits were visualized at 365 nm, excised, and eluted from the polyacrylamide by the method of Weber & Osborne (1969) with the inclusion of 8 M urea and 0.1% $NaDodSO_4$ in the eluting buffer. The eluted subunits were dialyzed successively against 80% acetone containing 0.01 M NaCl, H_2O, 80% acetone, and H_2O to remove the $NaDodSO_4$ and urea (Rice, 1974) and then lyophilized. After acid hydrolysis, the Dns-amino-terminal residue of each subunit was

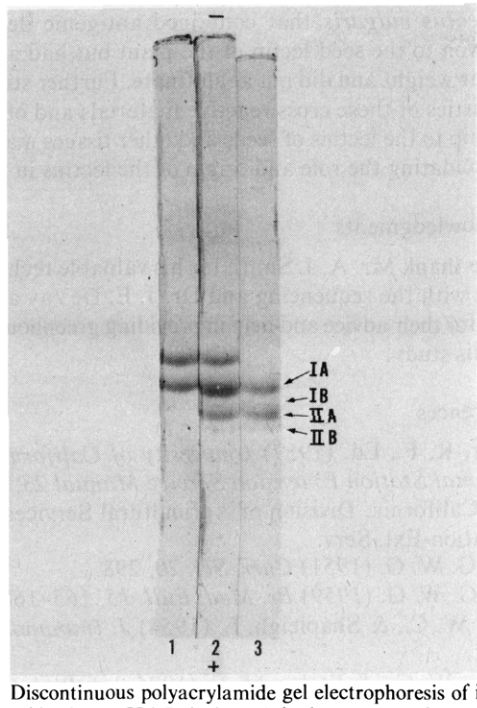

FIGURE 4: Discontinuous polyacrylamide gel electrophoresis of isolated CRM and seed lectin on pH 9.7 glycine gels in the presence of 0.1% sodium dodecyl sulfate and 8.0 M urea. (1) CRM; (2) CRM + seed lectin; (3) seed lectin. Bands IA and IIA of the seed lectin are the subunits from form A and bands IB and IIB are subunits from form B (Carter & Etzler, 1975a). Gels are stained with Coomassie Brilliant Blue. Migration is from top (−) to bottom (+).

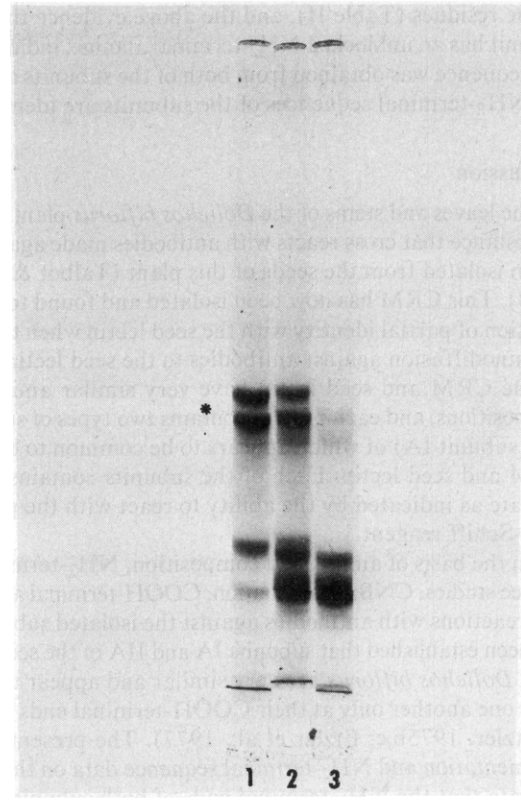

FIGURE 5: Discontinuous polyacrylamide gel electrophoresis of CNBr cleaved CRM and seed lectin on pH 9.7 glycine gels in the presence of 0.1% sodium dodecyl sulfate and 8.0 M urea. (1) CNBr treated CRM; (2) CNBr treated CRM + CNBr treated seed lectin; (3) CNBr treated seed lectin. (*) The upper bands on each gel are the uncleaved subunits (see Figure 4). Although both samples were treated under the same conditions, the CRM was less susceptible to CNBr cleavage than the seed lectin.

found to cochromatograph with Dns-alanine. These data indicate that alanine is the NH_2-terminal amino acid residue of each of the CRM subunits; alanine is the NH_2-terminal residue of subunits IA and IIA of the seed lectin (Carter & Etzler, 1975b; Etzler et al., 1977).

CNBr Cleavage and NH_2-Terminal Sequence of CRM. Subunits IA and IIA of the seed lectin each have 1 methionine residue located in the central portion of the polypeptide chain (Carter & Etzler, 1975c). The NH_2-terminal fragments of these subunits, isolated after CNBr cleavage of the lectin, have identical electrophoretic mobilities in NaDodSO$_4$–urea gels whereas the COOH-terminal fragments of the subunits differ from one another in electrophoretic mobility (Carter & Etzler, 1975c). The identity of the NH_2-terminal ends of these subunits has been confirmed by showing that they have identical NH_2-terminal amino acid sequences (Etzler et al., 1977). In an effort to establish whether a similar relationship exists between the subunits of the CRM and to establish the relationship of these subunits with those of the seed lectin, the CRM was subjected to CNBr cleavage and to NH_2-terminal sequence analysis.

CNBr cleavage of the CRM, followed by electrophoresis of the fragments in NaDodSO$_4$–urea gels, shows a heavy band with a slightly lower mobility than the NH_2-terminal fragment produced by CNBr cleavage of the seed lectin, and a lighter band with a mobility similar to the COOH-terminal fragments of the seed lectin (Figure 5). Although such an electrophoretic pattern would be expected if one of the CRM subunits had a COOH-terminal fragment of approximately equal size to that of the NH_2-terminal fragment, verification of this interpretation must await isolation of sufficient quantities of the CRM subunits so that CNBr cleavage can be performed on each individual subunit.

As shown in Table II, the sequence of the 13 NH_2-terminal residues of the CRM subunits is identical with the sequences of subunits IA and IIA of the seed lectin with the exception of an aspartic acid at position 2 of the CRM instead of the asparagine present at the same position in the seed lectin subunits. Although the NH_2-terminal sequence of the CRM was done on a mixture of both subunits due to lack of sufficient quantities of isolated subunits, only one sequence of amino acids was detected with a repetitive yield of 93%. The yields

TABLE II: NH_2-Terminal Amino Acid Sequence of CRM from *Dolichos biflorus* and Its Comparison with the Sequence of the Seed Lectin.

Residue no.	CRM	% yield[a] of CRM	Subunit I and II[b] of seed lectin
1	Ala	93	Ala
2	Asp		Asn
3	Ile	77	Ile
4	Gln		Gln
5	Ser		Ser
6	Phe	61	Phe
7	Ser		Ser
8	Phe	55	Phe
9	Lys		Lys
10	Asn		Asn
11	Phe	33	Phe
12	Asn[c]		Asn
13	Ser		Ser

[a] The yield is based on the assumption that each nmol of CRM contains 2 nmol of subunit. [b] Subunits I and II of the seed lectin have identical NH_2-terminal amino acid sequences (Etzler et al., 1977). [c] Positively distinguished from aspartic acid only by thin-layer chromatography.

of the residues (Table II), and the above evidence that each subunit has an unblocked NH_2-terminal alanine, indicate that the sequence was obtained from both of the subunits and that the NH_2-terminal sequences of the subunits are identical.

Discussion

The leaves and stems of the *Dolichos biflorus* plant contain a substance that cross reacts with antibodies made against the lectin isolated from the seeds of this plant (Talbot & Etzler, 1978). This CRM has now been isolated and found to form a reaction of partial identity with the seed lectin when tested in immunodiffusion against antibodies to the seed lectin.

The CRM and seed lectin have very similar amino acid compositions, and each protein contains two types of subunits, one (subunit IA) of which appears to be common to both the CRM and seed lectin. Each of the subunits contains carbohydrate as indicated by the ability to react with the periodic acid–Schiff reagent.

On the basis of amino acid composition, NH_2-terminal sequence studies, CNBr fractionation, COOH-terminal analyses, and reactions with antibodies against the isolated subunits, it has been established that subunits IA and IIA of the seed lectin from *Dolichos biflorus* are very similar and appear to differ from one another only at their COOH-terminal ends (Carter & Etzler, 1975b,c; Etzler et al., 1977). The present CNBr fragmentation and NH_2-terminal sequence data on the CRM indicate that the NH_2-terminal ends of both subunits of the CRM are identical with one another and may also be identical with the seed lectin subunits with the exception of an amidation of the aspartic acid at position 2 in the subunits of the seed lectin. The similarities in NH_2-terminal sequences raise the possibility that all of the subunits of the CRM and the seed lectin may represent different stages of completion or proteolytic modification of the COOH-terminal end of a common polypeptide chain.

The isolated CRM appears to be a dimer with a molecular weight of 68 000–70 000 in contrast to the tetrameric 110 000 molecular weight form of the seed lectin (Carter & Etzler, 1975a). The inability of the CRM to react with blood group A substance indicates that the CRM is either inactive or has a specificity different from that of the seed lectin. The combination experiments reported above suggest that the failure of the CRM to combine with blood group A substance is probably not due to any modification that occurs during the extraction procedure.

The inability of the CRM to combine with blood group A substance and the relationship of its subunits to the subunits of the seed lectin suggest the possibility that the CRM may be a precursor to the active lectin. Activation might proceed by a proteolytic conversion of the heavy subunit of the CRM to subunit IIA of the active lectin followed by an association of two dimers to form a tetramer. Such an activation might account for the rapid increase of active lectin during maturation of the seed (Talbot & Etzler, 1978). The formation of active proteins by proteolytic cleavage of precursors is a well-established phenomenon in biological systems (Neurath & Walsh, 1976). It must be recognized, however, that any precursor–product relationship between the CRM and the active lectin would have to be reconciled with the difference in distribution of these components in the plant.

Although the function of the CRM is not known at present, there is an indication that the existence of such a component in the leaves and stems is not unique to the *Dolichos biflorus* plant. On the basis of immunodiffusion data, Mialonier et al. (1973) reported the presence of a substance in the leaves of *Phaseolus vulgaris* that contained antigenic determinants common to the seed lectin of the plant but had a lower molecular weight and did not agglutinate. Further studies of the properties of these cross reactive materials and of their relationship to the lectins of seeds and other tissues may be useful in elucidating the role and origin of the lectins in the plants.

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Thermodynamics of Protein Cross-Links†

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ABSTRACT: The thermal transitions of native lysozyme and a well-characterized cross-linked derivative of lysozyme [Imoto, T., and Rupley, J. A. (1973), *J. Mol. Biol. 80,* 657] have been studied in 1.94 M guanidine hydrochloride at pH 2. The observed increase in the melting temperature from 32.4 °C for native lysozyme to 61.8 °C for the cross-linked derivative corresponds to a calculated 5.2 kcal/mol increase in the free energy of denaturation. This free-energy change is attributed to the decreased entropy of the unfolded polypeptide chain following introduction of a cross-link and is shown to compare well with theoretical predictions. The possibility that an introduction of a cross-link could also affect the enthalpy of an unfolded protein was investigated. The heats of reduction of bovine serum albumin and lysozyme by dithioerythritol in 6 M guanidine hydrochloride were determined and compared to that for the model peptide, oxidized glutathione. The near identity of the observed heats was taken as evidence that the introduction of cross-links into a random-coil protein does not, in general, introduce strain.

Many proteins contain cross-links, the function of which presumably is to stabilize the native conformation. Particular attention has been paid to intrachain disulfide cross-links, commonly found in extracellular proteins. Discussions of this topic usually emphasize the loss of entropy when a random coil is cross-linked, thus destabilizing the unfolded state. This entropy loss has not been measured experimentally, although estimates of it can be made using the models of Flory (1956), Schellman (1955), or Poland and Scheraga (1965). Also, there are few data available on the thermodynamics of disulfide bond formation for either folded or unfolded forms of proteins (Lapanje and Rupley, 1973; Creighton, 1975), though there are somewhat more data for model compounds (Sunner, 1955; Cleland, 1964; Gorin and Doughty, 1968). There have been many reports on the kinetics of disulfide reactions of proteins and small compounds (e.g., Sears et al., 1977; Creighton, 1975; Weber and Hartter, 1974).

The goals of this paper are twofold: (1) to describe a system in which the "destabilizing" effect of a cross-link in a random coil can be measured and to compare the experimental free-energy change to theoretical predictions and (2) to measure the enthalpy of disulfide bond reduction for unfolded BSA,[1] amplifying previous data obtained for lysozyme (Lapanje and Rupley, 1973).

It is helpful to use the reactions of the cycle of Figure 1 in discussing the thermodynamics of the introduction of cross-links into a single-chain macromolecule. Reactions 2 and 3 are the unfolding of the cross-linked and uncross-linked molecules, respectively. Reaction 4 is the introduction of a single cross-link into the unfolded molecule, which is assumed to be random coil whether cross-linked or not. We ignore the chemistry of the establishing of the covalent cross-link, i.e., the free energy of reaction 4 reflects only the configurational effects associated with cross-linking. These configurational effects comprise an entropy contribution to the free energy of reaction 4 and perhaps an enthalpy contribution. Reaction 1 is the introduction of the cross-link into the folded molecule. We ignore as for reaction 4 the covalent chemistry of establishing the cross-link; i.e., we assume that it is the same for reactions 1 and 4.

The thermodynamic parameters of reaction 4 are of interest for comparing with theoretical predictions and for assessing the contribution of cross-links to conformational stability. There is no straightforward way of studying reaction 4, but the thermodynamics of reactions 2 and 3 can be determined through experiment, allowing calculation of the difference in thermodynamic parameters for reactions 1 and 4. Then, if the thermodynamics of reaction 1 can be determined, those of reaction 4 can be evaluated.

It is possible to carry out the above measurements using lysozyme and a lysozyme derivative. Inactivation of lysozyme by iodine results in an ester bond being formed between the side-chain carboxylate of Glu-35 and the enol form of oxindolealanine-108 (Fraenkel-Conrat, 1950; Hartdegen and Rupley, 1973; Imoto and Rupley, 1973). This particular cross-link is appropriate to study because the effects of its introduction on the conformation of lysozyme (i.e., reaction 1) have been investigated by x-ray crystallographic (Beddell et al., 1975), ^{13}C NMR (Norton and Allerhand, 1976), and equilibrium binding studies (Imoto and Rupley, 1973).

The thermodynamic parameters for the unfolding of cross-linked and native lysozyme (reactions 2 and 3) must be determined for the same experimental conditions. This, in principle, can be done by standard techniques, but there are experimental difficulties because the melting temperatures differ by nearly 30 °C. Thus, under experimental conditions for which the cross-linked derivative is half unfolded, an im-

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[1] Abbreviations used are: Gdn-HCl, guanidine hydrochloride; BSA, bovine serum albumin; DTE, dithioerythritol.

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