Phytohemagglutinin of the Lima Bean (*Phaseolus lunatus*). Isolation, Characterization, and Interaction with Type A Blood-Group Substance†

W. Galbraith and I. J. Goldstein*

ABSTRACT: The lima bean (*Phaseolus lunatus*) phytohemagglutinin was purified by specific absorption to insolubilized blood-group A substance followed by elution with 2-acetamido-2-deoxy-D-galactopyranose. Recycling Sephadex G-200 chromatography afforded two active lectin species: component II (mol wt 247,100) and component III (mol wt 124,400). Both components yield identical subunits of mol wt 31,000 upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. The lima bean lectins contain no methionine but possess two half-cystine residues per 31,000 molecular weight subunit. One of the sulfhydryl groups is free, the other being present as one-half of an interchain disulfide linkage between two subunits. Components II and III represent, therefore, tetramer and dimer of the 62,000 molecular weight disulfide-containing subunit. Once separated the two components do not appear to be interconvertible. A glycoprotein, the lima bean lectin, contains 6–7 moles of mannose, 2 moles of glucosamine, 1 mole of fucose, and less than stoichiometric amounts of pentose per 31,000 g of protein. The protein is strongly precipitated by concanavalin A which suggests nonreducing terminal α-D-mannopyranosyl residues. The purified lima bean lectin contains 0.052% Mn^{2+} and 0.166% Ca^{2+} with hemagglutinating and blood-group A precipitating activity dependent on the metal content. The lima bean lectin agglutinates type A human red blood cells very strongly, type B very slightly, and type O not at all. The specific hemagglutinating activity of component II is four times greater than component III. Both components precipitate type A human ovarian cyst blood-group substance or hog mucin type A substance but are less reactive with human type A_2 and B substances. Hog mucin type H(O) substance is not precipitated by the lectin. Components II and III give similar precipitin curves with type A substance. The best inhibitors of the lectin are 2-acetamido-2-deoxy-D-galactopyranose and its glycosides. Component III is inhibited three- to fourfold more readily than component II. More precipitation occurs at 37° than at 20° or at 4°. The lima bean lectin has a broad pH optimum (pH 4.5–8.5) with the activity strongly affected by the type of buffer used. EDTA at a concentration of 0.1 mM completely inhibits the precipitation reaction; this inhibition can be reversed by addition of divalent cations. Rabbit antisera to component III gives a reaction of complete identity with component II, and a slight cross-reaction with purified wax bean lectin.

In 1945 W. C. Boyd found that extracts from the lima bean (*Phaseolus lunatus*) specifically agglutinated type A human red blood cells (Boyd and Reguera, 1949). This was the first indication that plant agglutinins could be blood group specific. Since then a vast literature on phytohemagglutinins (lectins) has appeared (Boyd, 1963; Liener, 1964; Dechary, 1968). Several anti-A lectins have been purified to homogeneity and their physical and chemical properties reported (Hammarström and Kabat, 1969; Etzler and Kabat, 1970). They are all inhibited by *N*-acetyl-D-galactosamine, the immunodominant carbohydrate of type A blood-group substance (Kabat, 1956).

Between 1945 and 1970, investigators studying the lima bean lectin employed crude saline extracts or, at most, partially purified preparations (Boyd *et al.*, 1955; Bhatia *et al.*, 1968). After testing 62 varieties of lima bean for lectin activity (Boyd and Reguera, 1949), Boyd *et al.* (1955) achieved partial purification of the Sieva lima bean lectin by fractional alcohol precipitation.

In 1970 two groups (Gould and Scheinberg, 1970a; Galbraith and Goldstein, 1970) reported independently the purification of the lima bean lectin. The hemagglutinating activity of the lima bean lectin resides in two molecular species (component II, mol wt 247,100, and component III, mol wt 124,400), each with the same apparent amino acid and carbohydrate composition.

The present paper reports on some of the molecular properties of the lima bean lectin and the nature and specificity of the precipitin reaction which results when the lima bean lectins interact with type A blood-group substance. A preliminary note of some of these findings has already appeared (Galbraith and Goldstein, 1970).

Experimental Procedure

Materials. All chemicals used in this study were of reagent grade or the best quality available. Two preparations of human ovarian cyst type A blood-group substance were the gift of Dr. R. Poretz and Professor W. Watkins, Lister Institute, London. Dr. D. Aminoff, The University of Michigan, generously donated samples of hog mucin type A and H blood-group substances. Human ovarian cyst blood-group substances type B and A_2 were provided by Dr. E. A. Kabat, Columbia University, as were the disaccharide GalNAc-α-(1→3)Gal[1] and a sample of hog mucin A + H substance in-

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104. *Received January 17, 1972.* This research was supported by National Institutes of Health Research Grants AM-10171 and AM AI-10171 and Training Grant GM00187.

[1] Abbreviations used are: con A, concanavalin A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Gal, D-galactopyranose; GalN, 2-amino-2-deoxy-D-galactopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcN, 2-amino-2-deoxy-D-glucopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; ManNAc, 2-acetamido-2-deoxy-D-mannopyranose; PBS, phosphate-buffered saline.

solubilized with *N*-carboxyanhydroleucine. Dr. B. Weissmann, University of Illinois Medical School, kindly provided samples of phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside and a mixture of *o*- and *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranosides. Methyl 2-deoxy-2-(*p*-nitro- and *p*-aminobenzamido)-α-D-galactopyranosides were prepared by D. H. Buss in this laboratory. Methyl α-D-galactopyranoside, methyl 2-acetamido-2-deoxy-α-D-galactopyranoside, and rabbit antisera to con A were available in this laboratory. *N*-Acetylneuraminic acid was provided by Dr. G. W. Jourdian of this University. Human red blood cells (types A, B, and O) were obtained from the University of Michigan Hospital Blood Bank, courtesy of Dr. H. Oberman.

Analytical Procedures

Amino Acid Analyses. Samples of purified lima bean lectin component II and component III were dialyzed exhaustively against 1 mM NaCl. Aliquots of these solutions were hydrolyzed in 6 N HCl at 110° for 14, 22, 30, and 58 hr for component II; 22, 48, and 72 hr for component III. An aliquot of component III was hydrolyzed for 44 hr in the presence of dimethyl sulfoxide to determine cysteic acid. Analyses of the hydrolyzed solutions were carried out by Dr. C. H. Williams and Mr. A. Collins on a Beckman Spinco amino acid analyzer Model 120B, according to the accelerated methods of Spackman (1967) for the acidic and neutral amino acids, and the general methods of Moore *et al.* (1958) and Spackman *et al.* (1958) for the basic column of the analyzer using the hydrolysis times of component II and extrapolating to zero time. The values for serine, threonine, and ammonia were corrected by extrapolation to zero time. Tryptophan was determined by the spectrophotometric method of Goodwin and Morton (1946).

Carbohydrate Analyses. The phenol–sulfuric acid method (Dubois *et al.*, 1956) was used for carbohydrate analyses with D-mannose as standard. Hexose was determined by a slight modification of the anthrone procedure (Iyer, 1968). Sialic acid was determined by the thiobarbituric acid method of Aminoff (1961) using *N*-acetylneuraminic acid as standard. The *O*-acetyl glycitol derivatives of the neutral sugars of the lima bean lectin were analyzed by the method of Lehnhardt and Winzler (1968) using methyl β-D-glucopyranoside as standard. Instead of the 1.3°/min increase from initial to final column temperatures, the following conditions were used: 1°/min, 160–182°; 2°/min, 182–210°. Resin-catalyzed acid hydrolysis as recommended by Lehnhardt and Winzler (1968) (0.01 N HCl in the presence of Dowex 50W-X2 (H^+) at 100° for 40 hr) gave incomplete hydrolysis of neutral sugars. Hydrolysis was also conducted using 1 N HCl at 100° for 2, 3, 4, and 5 hr, respectively. Hydrolysates were neutralized [Dowex 1-X8 (HCO_3^-)] and deionized [Dowex 50W-X2 (H^+)]. Following addition of methyl β-D-glucopyranoside, and evaporation to dryness, the neutral sugars were converted to alditol acetates by reduction with $NaBH_4$ followed by acetylation.

Metal Analyses. Analyses for protein-bound Mn^{2+} and Ca^{2+} were performed by atomic absorption spectroscopy on Beckman Model 440 and Instrumentation Laboratories Model 153 instruments using Mn_2Cl and $CaCl_2$ as standards.

Nitrogen Determination. The method described by So and Goldstein (1967) consisting of hydrolysis of the sample in 7 N H_2SO_4 followed by ninhydrin colorimetric determination of the resulting ammonium sulfate was used.

Protein Analyses. Protein in crude extracts was estimated by the Biuret method (Gornall *et al.*, 1949) and by absorbance at 280 nm. Purified lima bean lectin was determined by absorbance at 280 nm using $E_{1\,cm}^{1\%} = 12.3$ (Gould and Scheinberg, 1970a).

Molecular Weight. The sedimentation velocity of components II and III was determined using the Beckman Spinco Model E analytical ultracentrifuge equipped with ultraviolet and schlieren optics. Both AN-D and AN-E heads were used at a temperature of 20°. Sedimentation constants were determined at high speed (59,780 rpm) using a Kel-F cell and plain quartz windows. Schlieren photographs were measured using a Nikon shadowgraph projection microcomparator. Ultraviolet photographs taken at 280 nm were measured using a microdensitometer.

Molecular weight determinations by gel filtration were done by the method of Andrews (1964) using a 1.5 × 90 cm Pharmacia column packed with Sephadex G-200. Concentrated protein solution or protein in 10% sucrose solution was layered under the 0.15 M NaCl used to equilibrate the column. Standards were: xanthine oxidase, 280,000; catalase, 244,000; human γ-globulin fraction II (IgG), 150,000; bovine serum albumin, 67,000; egg albumin, 45,000.

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed at pH 9.5 and 4.3 (Reisfeld *et al.*, 1962) using Coomassie Blue stain (Chrambach *et al.*, 1967). Polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol was by the method of Weber and Osborn (1969) using as standards: bovine serum albumin, 67,000; egg albumin, 45,000; pepsin, 35,000; chymotrypsinogen, 25,700; and human hemoglobin, 15,500.

Assay Procedures

Hemagglutination assays were conducted by serial dilution of lectin in PBS (0.01 M sodium phosphate–0.15 M sodium chloride) (pH 7.0). The activity was expressed as titer, the reciprocal of the greatest dilution at which agglutination occurred. Specific activity was defined as titer per milligram of protein per milliliter. In preliminary experiments Na(EDTA) was added at low concentrations to the PBS to investigate its effect on the hemagglutination.

Precipitation Analyses. Quantitative precipitin analyses were performed in tapered 3-ml glass centrifuge tubes in a total volume of 200 μl. Incubation mixtures contained 2 μmoles of sodium phosphate buffer (pH 7.0), 28.5 μmoles of NaCl, and varying amounts of blood-group substance (10–90 μg). Unless indicated otherwise, incubations were conducted at 37° for 2 days in tubes capped with rubber stoppers. The tubes were centrifuged, and the washed precipitates were dissolved in 0.05 N NaOH (0.3–0.9 ml) and total protein was determined by a semimicro Lowry procedure (Mage and Dray, 1965).

For inhibition studies, the saccharide to be tested was dissolved in 0.15 M NaCl adjusted to pH 7.0 and an appropriate amount of the inhibitor solution added to the incubation mixture. The order of additions was: phosphate buffer, 0.15 M sodium chloride, type A blood-group substance (44.25 μg), inhibitor, and lastly lectin. The tubes were mixed thoroughly on a Vortex mixer before and after addition of the lectin.

Incubations with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were carried out in 0.15 M NaCl–0.01 M Tris buffer (pH 8.0) at 4°. Aliquots of 2 or 5 μl of a 0.01 M stock solution of DTNB in 0.2 M Tris were added to lectin, type A blood-group substance (44.25 μg) and Tris buffer in a total volume of 200 μl. Preincubation tubes refer to a 1-hr incubation of buffer, DTNB, and lectin at 4° prior to addition of cyst material. In normal tubes Tris, cyst blood-group substance, and lectin were mixed just prior to the addition of DTNB.

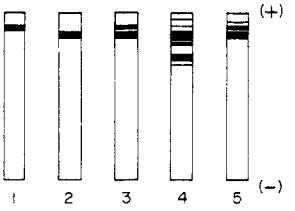

FIGURE 1: Disc gel electrophoresis of lima bean lectins. 6 mA/gel for 2 hr in pH 4.3, 7.5% gel. (1) 34 μg of component II; (2) 30 μg of component III; (3) 40 μg of insolubilized type A column eluent; (4) 25 μg of active ammonium sulfate fraction; (5) 40 μg of aged eluent.

For the solubility experiments, aliquots of PBS (pH 7.0) were added to the standard mixture (44.25 μg of blood-group substance) to provide the additional volume. The buffer solutions used to determine the pH dependence of lectin–blood-group substance interaction were made 0.01 M in buffer, 0.15 M NaCl, and adjusted to the desired pH after mixing buffer and salt.

Quantitative precipitation with con A was performed as described by So and Goldstein (1967), but using one-fourth scale and determining protein in the washed precipitates by the micro-Lowry method as above. Con A was prepared according to Agrawal and Goldstein (1967) and stored in 1.0 M NaCl at 4°.

Lima Bean Lectin. Purified lima bean lectin was prepared by specific adsorption to a column of type A human ovarian cyst blood-group substance insolubilized by treatment with *N*-carboxyanhydroleucine (Etzler and Kabat, 1970) followed by elution with *N*-acetyl-D-galactosamine as described by Galbraith and Goldstein (1970). The lectin was dialyzed against 0.15 M NaCl to remove GalNAc. Resolution of the two components was achieved by recycling chromatography at 20° on a column (2.5 × 95 cm) of Sephadex G-200. The protein was also prepared by the procedure of Gould and Scheinberg (1970a).

Antibody to Component III. Component III (2 mg in 1 ml of 0.15 M NaCl) was emulsified with 1 ml of Freund's complete adjuvant (Difco) in a Virtis homogenizer. Each of two New Zealand white rabbits received 0.5 ml of thee mulsion (0.5 mg of component III), 0.1 ml in each of four footpads and 0.1 ml subcutaneously in the back. This immunization procedure was conducted three times at weekly intervals. Three weeks after the last injection the rabbits were bled from the ear and bled weekly thereafter. Agar gel double diffusion was carried out as described by Goldstein and So (1965).

Results

Purity and Molecular Weight of Components II and III. Lima bean lectin components II and III were shown to be homogeneous by the following criteria: disc gel electrophoresis at two pH values, polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol, analytical ultracentrifuge patterns, and chromatography on Sephadex G-200. Polyacrylamide disc gel electrophoresis at pH 4.3 was selected as the standard method to evaluate purity. Disc gel patterns obtained during various stages in the purification of the lima bean lectins are shown in Figure 1. At pH 9.0 both components migrated as a single diffuse band whereas at pH 4.3 components II and III separated into two discrete bands, component III moving ahead of component II.

Upon storage at 4° of purified component II or III at concentrations greater than 0.2%, an inactive precipitate slowly formed accompanied by a second band which moved more slowly than either component II or III upon disc gel electrophoresis at pH 4.3. There was, however, no indication of the transformation of component II into component III or the reverse, even after storage at 4° for several months.

TABLE I: Amino Acid Analysis of Components II and III.

Amino Acid	Moles/31,000 g of Protein	
	Component II	Component III
Lysine	11.2	13.4
Histidine	3.3	4.0
Ammonia	26.5	21.8
Arginine	1.55	1.91
Aspartic acid	24.3	30.0
Threonine	14.0	18.0
Serine	27.0	34.1
Glutamic acid	13.8	17.2
Proline	10.4	11.5
Glycine	15.3	18.7
Alanine	17.8	21.8
Half-cystine		2.0
Valine	16.5	20.9
Methionine	0	0
Isoleucine	10.9	13.9
Leucine	23.2	28.5
Tyrosine	4.1	4.76
Phenylalanine	12.7	15.6
Tryptophan	4.8	5.1
Glucosamine	1.7	0.98

Polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol of component II, III, or a mixture of the two components gave a single discrete band, an indication of both the purity and relationship of the two proteins. The estimated maximum molecular weight of the polypeptide chain was approximately 31,000 (*cf.* Gould and Scheinberg, 1970a).

Components II and III migrated as single peaks in the ultracentrifuge, there being little variation in sedimentation coefficient over a wide range of protein concentrations (0.6–3.4 mg/ml, component II; 0.7–10 mg/ml, component III). The sedimentation constants ($s_{20,w}$) for components II and III were calculated to be 9.603 and 6.078, respectively, making the usual corrections for concentration and solvent effects. Using the intrinsic viscosity, 0.052, and partial specific volume, 0.73, reported by Gould and Scheinberg (1970a), the calculated molecular weights are component II, 247,100 and component III, 124,400, this assuming the molecules do not deviate significantly from rigid spheres (Schachman, 1957).

In agreement with previous results (Galbraith and Goldstein, 1970), gel filtration of purified, isolated components II and III on a standardized Sephadex G-200 column gave molecular weights of 195,000 and 108,000–112,000, respectively, somewhat below those obtained in the ultracentrifuge.

Amino Acid Analyses. Results of the amino acid analyses of the lima bean lectin components II and III are shown in Table I. Whereas the number of residues in the subunit of each component differed, the ratio of amino acids in each component, standardized to the six most stable residues, was identical.

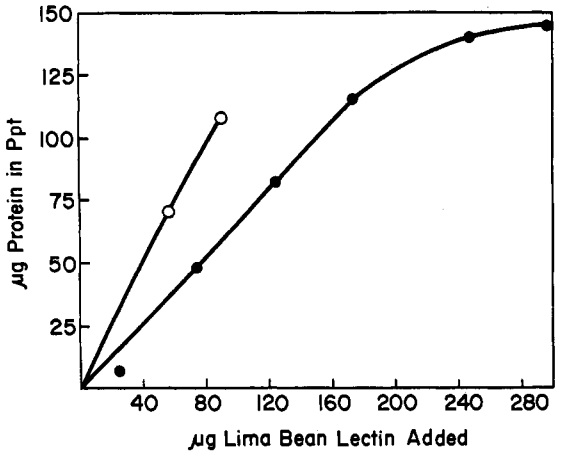

FIGURE 2: Quantitative precipitation of con A with lima bean lectin components II and III. 99 μg of con A/tube. (○) Component II; (●) component III.

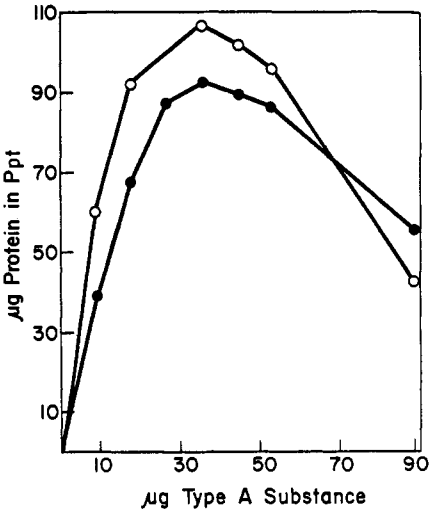

FIGURE 3: Quantitative precipitation curves of lima bean lectins with type A blood-group substance. (○) 119 μg of component II per tube; (●) 110 μg of component III per tube.

The difference in the absolute number of amino acid residues found in Table I reflects disparities in the amount of the protein used for the analyses.

The molecular weight of the subunit, calculated from the data in Table I, corrected to 2 moles of arginine/mole of subunit and including 4% neutral sugar, is 30,800. The disparity in the absolute number of residues present in the subunit of components II and III also arose in the results published by Gould and Scheinberg (1970a). Molecular weight calculation by summation of their amino acid analyses gave 21,600 and 20,900, respectively, for components II and III. Although Gould and Scheinberg reported a smaller number of residues for each amino acid, the ratio of the number of amino acids in the protein agrees with those presented in Table I. The subunit molecular weight of 31,000 was used as a basis for these calculations since the same subunit was found for both components II and III.

Carbohydrate Content. Phenol–H_2SO_4 and anthrone analysis of lima bean lectin components II and III gave 3–5% carbohydrate and 4.5% hexose, respectively. Gas–liquid chromatographic analysis of the carbohydrate residue of component III and the natural mixture of components II and III eluted from the insolubilized type A column (freed of noncovalently bound sugar by passage over a Bio-Gel P-60 column) gave 6–7 moles of mannose and 1 mole of fucose per 31,000 g of protein. Trace amounts of pentose were also found, xylose at levels less than 0.5 mole/31,000 g of protein, and even lower amounts of arabinose. The trace of glucose reported earlier (Galbraith and Goldstein, 1970) was removed by filtration on Bio-Gel. Amino acid analysis (Table I) of component II gave approximately 2 moles of glucosamine/subunit of protein, this value being obtained by extrapolation to zero time of a series of times controls. Lower recoveries and more extended hydrolysis times gave approximately 1 mole of glucosamine for component III. No sialic acid was found in either native or hydrolyzed lectin.

Treatment of the lima bean lectin (components II plus III) with an alkaline solution (0.05 N NaOH) of $NaBH_4$ did not liberate any dialyzable carbohydrate (Iyer and Carlson, 1971; Mayo and Carlson, 1970), suggesting that the carbohydrate moiety of the lima bean lectin is not glycosidically bound to seryl or threonyl residues. Further information on the nature of the carbohydrate moiety of the lima bean lectin was obtained from the precipitin curves of con A with components II and III as shown in Figure 2. These curves are similar to those obtained by Goldstein *et al.* (1969) for the interaction of con A with other glycoproteins, including the lectins of the wax bean (*Phaseolus vulgaris*) and the soybean (*Glycine max*). Methyl α-D-mannopyranoside inhibited the precipitation reaction between con A and lima bean lectin, demonstrating its specificity.

Absorption Spectra. The ultraviolet (uv) absorption spectra recorded for lima bean lectin components II and III were identical, neither component exhibiting an absorbance between 320 nm–700 nm. The spectra are very similar to that of con A (Agrawal and Goldstein, 1967).

Specificity of Lima Bean Lectin. The specific activity of lima bean lectin components II and III toward type A human erythrocytes was 5100 and 1300, respectively, and toward type B erythrocytes 20 and 5.1, respectively. Neither component reacted with type O human red blood cells or native or trypsinized (Liener, 1955) rabbit erythrocytes.

The same specificity pattern was shown in Ouchterlony double diffusion plates against blood-group specific substance. Both components II and III reacted strongly with type A human ovarian cyst blood-group substance and hog mucin type A substance. Reaction of lima bean lectin with type A_2 human ovarian cyst blood-group material was not as strong as with A. Components II and III reacted differentially toward blood-group B substance. After 24 hours, component II had reacted with type B blood-group substance whereas component III had not. After further incubation, component III formed a slight precipitin band with type B blood-group substance. Neither component reacted with type H hog submaxillary mucin.

Typical precipitin curves between components II and III and type A blood-group substance are shown in Figure 3. Component II reached equivalence earlier and had a broader equivalence zone than component III. About 80–90% of the protein added was found in the precipitate. The type A blood-group substance used as antigen has a very low tyrosine and tryptophan content and thus makes an insignificant contribution to the Lowry analysis for the amount of protein in the precipitate (100 μg of type A blood-group substance gave a Lowry value equivalent to 4.2 μg of protein).

The precipitation of blood-group substance types A_2 and B

TABLE II: Inhibition of Lima Bean Lectin by Saccharides.

Saccharide	μmoles of Inhibitor for 50% Inhibn	
	Component III	Component II
Methyl 2-deoxy-2-(*p*-nitrobenzamido)-α-D-galactopyranoside	0.8	
Methyl 2-deoxy-2-(*p*-aminobenzamido)-α-D-galactopyranoside	0.9	
3-*O*-α-(2-Acetamido-2-deoxy-D-galactopyranosyl)-D-galactose	52% at 2.1[a]	
Phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	3.7	
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	5	8
p- and *o*-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	5.2	
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside	26% at 5[a]	
N-Acetyl-D-galactosamine	14	27
D-Galactosamine	25	
D-Glucosamine	31	
N-Acetyl-D-glucosamine	59	150
Methyl α-D-galactopyranoside	72	42% at 200[a]
Methyl β-D-galactopyranoside	230	
Melibiose	72	30% at 125[a]
Lactose	72	
D-Galactose	95	27% at 300[a]
D-Fucose	110	
N-Acetyl-D-mannosamine	15% at 150[a]	

[a] Indicates the percentage inhibition for the micromoles of inhibitor noted.

by both components gave specificity data parallel to those already described. Under conditions where type A substance precipitated 90% of the lectin, types A_2 and B precipitated 66 and 13%, respectively, of component II (95 μg) and 21 and 0% of component III (94 μg) added to the reaction mixture.

Each of the three indices of lima bean lectin activity that were used, hemagglutination, Ouchterlony double diffusion, and precipitation analysis, gave the same results: both components are very reactive with type A, less reactive with type A_2, slightly reactive with type B and inactive with type O blood-group substances. Moreover, component II was consistently more reactive than component III especially with regard to the less reactive types A_2 and B substrates.

Hapten Inhibition Studies. The specificity of the lima bean lectin binding site was probed by inhibition of the precipitation reaction. The inhibitory effect of various saccharides on the lectin–blood group substance interaction is listed in Table II. It is readily apparent that the GalNAc glycosides were the best inhibitors tested. There appears to be little difference between the three α-glycosides of *N*-acetyl-D-galactosamine and its anomeric β-glycoside. They each require about the same number of μmoles to give 50% inhibition of precipitation.

The type A active disaccharide [3-*O*-α-(2-acetamido-2-deoxy)-D-galactopyranosyl-D-galactose)] which gave 52% inhibition at a level of 2.1 μmoles was one of the best inhibitors available, although only one inhibition point could be determined due to lack of material.

Interestingly, methyl 2-deoxy-2-(*p*-nitrobenzamido and *p*-aminobenzamido)-α-D-galactopyranosides were the most potent inhibitors tested. These data suggest that there may be a region on the protein which interacts specifically with the aromatic moiety at the C-2 position of D-galactosamine (*cf.* Poretz and Goldstein, 1971).

Free GalNAc was the next best inhibitor of component III–type A blood-group substance interaction requiring 14 μmoles for 50% inhibition. GlcNAc which differs from GalNAc only in the configuration of the C-4 hydroxyl group was four times less effective as an inhibitor than GalNAc. ManNAc differing from GlcNAc in the configuration of the acetamido group at the C-2 position gave virtually no inhibition (15% inhibition for 150 μmoles). Several nonamino sugars were found to be better inhibitors than ManNAc including D-galactose, D-fucose, and several galactosides. Methyl α-D-galactopyranoside and melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) were equivalent in inhibitory power and slightly better than D-galactose whereas methyl β-D-galactopyranoside was about three times poorer than the α-glycoside. GalNAc is about seven times better than galactose as an inhibitor. D-Fucose (6-deoxy-D-galactose) was less inhibitory than D-galactose requiring almost 8-fold the quantity of GalNAc to achieve 50% inhibition of the component III–type A blood-group substance precipitation reaction. GalN and GlcN, the unacetylated amino sugars, are special cases which do not seem to fit the pattern established by the other inhibitors. Whereas GalN is less inhibitory than GalNAc, GlcN is better than GlcNAc and at high concentrations is almost as good as GalN. It might have been expected that GlcN would have been less inhibitory than GlcNAc following the pattern for the more inhibitory GalNAc and GalN.

The more complete inhibition studies were conducted on component III since it was available in greater quantity and required less inhibitor to achieve a given inhibition. As shown in Table II, component II required twice as much Me-α-GalNAc or GalNAc to give 50% inhibition as did an equal weight of component III.

Metal Content and Requirement for Activity. Inhibition of both hemagglutination and precipitin activities of the lima bean lectin by EDTA suggested the presence of protein-bound metal. Earlier studies (Galbraith and Goldstein, 1970) reported the presence of Mn^{2+} and Ca^{2+} in the lectins from lima bean, wax bean, and soybean and established the requirement of metal ion for their activity. Atomic absorption measurements revealed about 1 (0.7–1.1) mole of Mn^{2+}/mole of component III and 1.2 moles of Mn^{2+}/mole of component II. The latter value is low compared to the expected 1.4–2.2 moles of Mn^{2+}/mole of component II if component II were indeed a dimer of component III. The Ca^{2+} content was considerably higher among the various preparations tested: 4–7 moles of Ca^{2+}/mole of component III and 17 moles of Ca^{2+}/mole of component II. It is interesting that the lowest Ca^{2+} value obtained was 4 moles of Ca^{2+}/mole of component III which is the number of subunits in component III.

Figure 4 demonstrates the effect of adding EDTA directly to the incubation mixture. As little as 0.1 mM EDTA completely inhibited the precipitin reaction. Parallel tubes which contained 0.1 mM EDTA and 0.4 mM Mn^{2+} or citrate gave virtually control amounts of precipitate. Each of the following

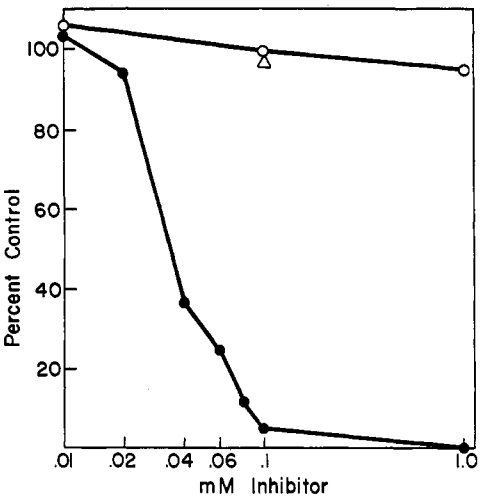

FIGURE 4: Effect of metal chelators on component III–type A substance interaction. 95 μg of component III; 44.25 μg of type A substance. (●) EDTA; (○) citrate; (△) EDTA plus 80 nmoles of $MnCl_2$.

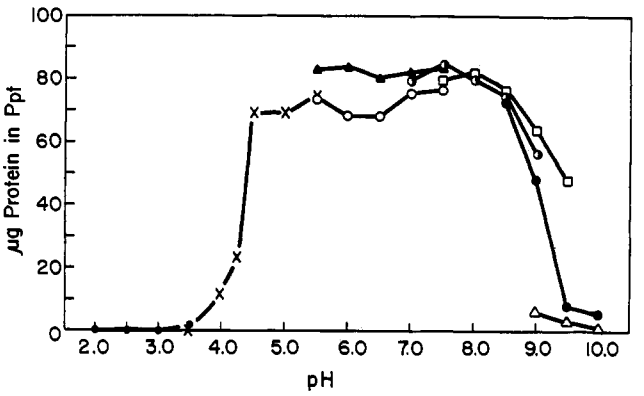

FIGURE 5: Effect of pH on component III–type A substance interaction. 95 μg of component III; 44.25 μg of type A substance. (●) Glycine, (×) acetate, (○) imidazole, (▲) phosphate, (○) Tris, (□) Veronal, and (△) carbonate.

divalent cations, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cr^{2+}, and Fe^{2+}, was tested for activation of component III at a level of 5×10^{-5} M but gave no increase in precipitating activity whereas Cd^{2+} and Cu^{2+} inhibited precipitation by 18 and 80%, respectively.

Investigation of the effect of Cu^{2+} revealed that 10^{-4}, 10^{-5}, and 10^{-6} M Cu^{2+} led to 98%, 14% and 8% inhibitions of precipitin activity, respectively. The lectin used for these experiments contained about 1.5×10^{-5} M free sulfhydryl groups.

Additional studies on the metal involvement in the activity of the lima bean lectin were carried out using metal free component III (MF-III) and component III which was dialyzed against EDTA followed by dialysis to remove the EDTA. MF-III contained 10% of the Mn^{2+} of native III and reduced Ca^{2+}. Disc gel electrophoresis comparing native component III and MF-III gave the same single band indicating that loss of metal did not cause dissociation into subunits. In a precipitin curve 66% of the MF-III precipitated with type A blood-group substance and the curve was shifted to the left, that is, antigen excess occurred earlier. MF-III could be brought to normal precipitation activity by addition of 1.25×10^{-7} M Mn^{2+} or any of the following divalent cations to the incubation mixture: Mg^{2+}, Ca^{2+}, Sr^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}. No other metal chelator was as effective as EDTA as an inhibitor of lima bean lectin activity. Citrate, oxalate, *o*-phenanthroline, and 8-hydroxyquinoline gave no inhibition at concentrations where EDTA gave complete inhibition of precipitation.

Reaction with DTNB. Incubation of lima bean lectin with DTNB at 4° indicates that both components II and III are totally inhibited by 0.1 mM reagent. Prior addition of blood-group A substance to the lectin affords slight protection of the lectin's sulfhydryl groups, but increasing the DTNB concentration from 0.1 to 0.25 mM overcame the protective influence of the blood-group substance. Gould and Scheinberg (1970b) demonstrated that GalNAc partially protected the lima bean lectin against DTNB inactivation.

Effect of pH on Precipitin Reaction. The pH-dependence curve of the lima bean lectin–type A blood-group substance interaction (Figure 5) is quite broad, extending from pH 4.5 to 8.5. The curve rises rather sharply in the acidic region and falls off more gradually in the alkaline region. The latter region is buffer dependent. The observed diminished activity of the lectin at high pH values is probably due to the instability of the protein and the metal-chelating properties of the buffer used. For example, Veronal buffer, which is not considered to be a metal chelator, afforded greater lectin activity than carbonate which is a good metal chelator.

Effect of Ionic Strength. The lima bean lectin proved to be relatively insensitive to the ionic strength of the medium as shown in Figure 6. Thus, the amount of precipitate formed between lima bean lectin and blood-group A substance varied only slightly between 0.15 and 2.5 M NaCl or KCl. The same amount of protein was precipitated even at NaCl concentrations as low as 0.05 M. Of the halides, chloride and bromide gave about the same quantity of precipitate, but iodide inhibited the reaction even at 0.15 M. Isothiocyanate also proved to be an effective inhibitor although not, on a molar basis, as effective as iodide.

Effect of Time, Volume, and Temperature. At 37° maximum precipitation required 2 days whereas at 22° maximum precipitation occurred only after 7 days. We therefore chose 2 days at 37° as the standard incubation time. Increasing the volume of the reaction mixture resulted in less precipitate being formed as shown in Figure 7. At 37° the solubility for lima bean lectin component III–blood-group A substance was 38 μg of protein/ml and for component II, 36 μg of protein/ml.

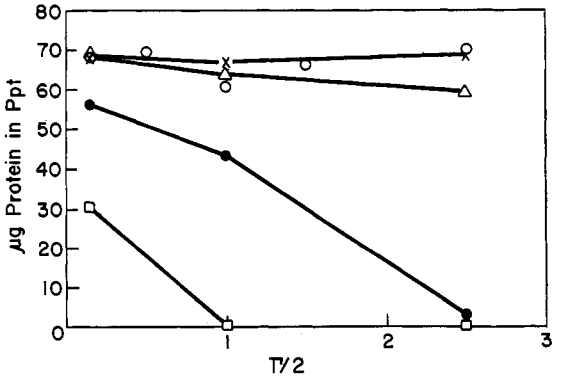

FIGURE 6: Effect of ionic strength on component III–type A substance interaction. 95 μg of component III, 44.25 μg of type A substance. (○) NaCl, (×) KCl, (△) KBr, (●) KSCN, and (□) KI.

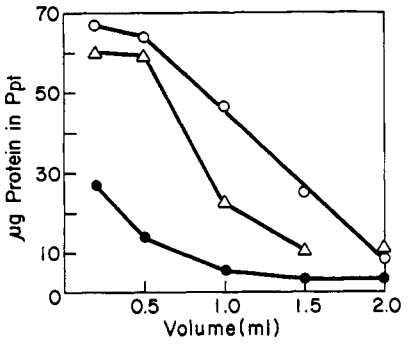

FIGURE 7: Effect of reaction volume and temperature on component III–type A substance precipitation. 95 μg of component III, 44.25 μg of type A substance. (○) 37°, (△) 22° and (●) 4°.

The solubility appeared to increase at volumes greater than 0.5 ml for the incubations at 22 and at 37°. There was not a substantial difference in the amount of reaction at 37° or at 22°, but at 4° much less precipitation occurred. Component II gave the same type of solubility data as that shown in Figure 7 for component III.

Antibody to Component III. The immunization protocol described in the Experimental Section induced antibody to component III in each of the rabbits injected. Antisera from both of the rabbits reacted strongly with component III. Each antisera also reacted strongly with component II and a reaction of identity was observed between component III and component II. Anti-component III gives a slight cross-reaction with purified wax bean lectin and with Difco PHA-P and PHA-M preparations but not with soybean lectin or con A. Both the wax bean and the Difco lectins are purified from *Phaseolus vulgaris* which belongs to the same genus as the lima bean, *Phaseolus lunatus*, whereas the soybean and jack bean are more distantly related. Perhaps the cross-reaction indicates a phylogenetic relationship between the lectins of the wax bean and the lima bean as revealed by their antigenic structure. Anti-component III gave precipitation reactions in Ouchterlony plates with the lima bean extracts at all stages of the purification procedure.

The total cross-reaction between anti-component III and component II is a further indication of the close relationship between the two lima bean lectin components. All the antigenic determinants on component III also appear to be present on component II.

Discussion

The lima bean lectin, isolated by specific adsorption to insolubilized type A substance followed by elution with GalNAc (Galbraith and Goldstein, 1970), was separated by recycling gel chromatography into two active components, each of which was homogeneous by several criteria and had the same disc gel electrophoretic mobility as components II and III prepared by the Gould and Scheinberg procedure (Gould and Scheinberg, 1970a).

Molecular weight estimation of the two lima bean lectins by sedimentation velocity and Sephadex gel filtration gave divergent results: 124,400 and 247,100 in the ultracentrifuge and 108,000–112,000 and 195,000 by gel filtration. Gould and Scheinberg (1970a) also reported difficulty in determining the molecular weight of the lima bean lectins by gel filtration and finally resorted to the use of acrylamide gel electrophoresis. Similar difficulties have been encountered in determining the molecular weight of other lectins (Howard and Sage, 1969; Ticha *et al.*, 1970; Toyoshima *et al.*, 1970).

The subunit structure of the lima bean lectins has been investigated thoroughly by Gould and Scheinberg (1970a,b). The similarity of the subunits from both components II and III was an essential link in the evidence indicating that components II and III are probably the same protein in two different states of aggregation.

With respect to its amino acid composition, the lima bean lectin appears to be similar to most other purified phytohemagglutinins, being rich in acidic and hydroxylic and poor in the sulfur-containing amino acids.

With but one exception [concanavalin A (Agrawal and Goldstein, 1968)], all purified lectins have been shown to contain carbohydrate, at least by the phenol–H_2SO_4 test. The carbohydrate composition of the lima bean lectin is similar to that of other lectins containing principally mannose (6–7 moles), fucose (1 mole), and glucosamine (2 moles) per subunit of 31,000 molecular weight. The absence of galactosamine as detected by the amino acid analyzer indicated that dialysis had removed this amino sugar which was used to elute the lectin from the adsorption column. The failure of the lima bean lectin to yield dialyzable carbohydrate upon treatment with alkaline borohydride suggests that the carbohydrate residue may be bound to the protein by means of the Asn-GlcNAc linkage which was determined to be present in the phytohemagglutinin of the soybean (Lis *et al.*, 1969). This argument is strengthened by the identification of glucosamine as one of the component sugars of the lima bean phytohemagglutinin.

The two lima bean lectins, similar in their behavior to the wax bean and soybean lectins, gave rise to precipitin curves with con A (Goldstein *et al.*, 1969) (Figure 2) and it is suggested that the basis for these interactions may be α-D-mannopyranosyl termini.

Lima bean lectin was shown to contain Mn^{2+} and Ca^{2+} and the Mn^{2+} was found to be essential for the activity of the lectin (Galbraith and Goldstein, 1970). The function of the Mn^{2+} and the Ca^{2+} in the lima bean lectin remains unclear. Whereas the Mn^{2+} was removed by dialysis against EDTA, the Ca^{2+} remained tightly bound, and metal-free component III retained two-thirds of its activity. Nevertheless, low concentrations of EDTA (10^{-4} M) in the incubation mixture totally inhibited the lectin. These results may indicate that although EDTA can bind to the protein-bound metal and interfere with saccharide binding it cannot remove the metal from the lectin. Possibly the EDTA replaces a ligand of the metal and in so doing disrupts the lectin's saccharide-binding site. It was interesting to note that virtually any divalent cation, independent of specific size and electronic configuration, can restore activity to metal-free component III.

From the limited amount of information available the simplest model would be one in which a single Ca^{2+} ion is bound by a subunit of mol wt 31,000 and one Mn^{2+} ion is associated with the four subunits that comprise component III, perhaps maintaining the tertiary or quaternary structure of the lectin.

Inhibition studies on the precipitation reaction between lima bean lectin and human ovarian cyst blood-group A substance (Table II) showed that glycosides of *N*-acetyl-D-galactosamine were most complementary to the lima bean binding sites. The proper orientation of the C-4 hydroxyl group is forthcoming from the fact that *N*-acetyl-D-galactosamine inhibits the precipitation reaction four times better than *N*-acetyl-D-glucosamine. The importance of the configuration of the acetamido group on C-2 of the pyranose ring is evident

from the fact that *N*-acetyl-D-mannosamine is a poorer inhibitor (15% inhibition for 150 μmoles) than *N*-acetyl-D-glucosamine, which gave about 25% of the inhibition of *N*-acetyl-D-galactosamine. D-Fucose (6-deoxy-D-galactose) was somewhat less inhibitory than D-galactose suggesting a possible role for the primary hydroxyl group at C-6. The specificity at the glycosidic linkage was not distinct for the α- and β-glycosides of *N*-acetyl-D-galactosamine that were tested; however, methyl α-D-galactopyranoside was three times more effective an inhibitor than the corresponding methyl β-galactoside. The free amino sugars GalN and GlcN obviously inhibit differently than the N-acetylated amino sugars. In addition to fitting into the saccharide binding site the free amino groups may be interacting with a corresponding charged site on the lectin or with the lectin-bound metal.

The lima bean lectin was found to be active over a wide pH range (Figure 6). Precipitating activity ceased rather abruptly between pH 4.0 and 4.5, compared to a more gradual loss of activity at alkaline pH values. In comparison, the pH optimum for the precipitation of dextrans by con A was 5.7–7.5 (So and Goldstein, 1967) although considerable binding of monosaccharides was found even at pH 2.4 (Hassing and Goldstein, 1970). In alkaline buffers some of the loss in activity of the lima bean lectin may well be due to the metal-chelating properties of the carbonate ion.

Ionic strength appeared to have little effect on the precipitation of the lima bean lectin by type A blood-group substance providing a minimal amount of salt (0.05 M NaCl) was present. NaCl, KCl, and KBr all gave maximal precipitation from 0.15 to 2.5 M. However, KI and KCNS both inhibited the reaction. These same two salts inhibited con A–dextran precipitation (So and Goldstein, 1967) and antibody–antigen interaction (Kleinschmidt and Boyer, 1952).

Increasing the volume of the reaction mixture resulted in decreased precipitation of the blood-group substance by lima bean lectin. Similar solubility properties were demonstrated for the lectins of the jack bean (So and Goldstein, 1967) and *Dolichos biflorus* (Etzler and Kabat, 1970). The precipitation reaction was also shown to be temperature dependent, more precipitation occurring at 37° than at 25 or 4° (*cf.* So and Goldstein, 1967).

The immunological reaction of identity between rabbit anti-lima bean component III and components II and III provides strong evidence that components II and III are related molecular species. The slight cross-reaction between anti-component II and purified wax bean lectin and with Difco PHA-P and PHA-M preparations is interesting. Considering the high degree of specificity of the immune response it is anticipated that certain surface portions of these two lectins should be similar.

All of the chemical, biological, and immunological data indicate that components II and III represent the same protein in two different states of aggregation. In view of the disulfide-linked subunit of molecular weight 62,000 noted by Gould and Scheinberg (1970b), it is believed that components II and III represent tetramer and dimer, respectively. Multiple hemagglutinating molecular species from one plant source are not unique, but the dimer–tetramer system described for the lima bean is the only one reported thus far. The soybean hemagglutinating activity was found to reside in four, very similar but distinct, molecules (Lis *et al.*, 1966) which had approximately the same molecular weights but varied in amino acid composition. A similar situation was found for the hemagglutinins of *Lotus tetragonolobus* where three different L-fucose binding proteins were reported (Kalb, 1968). Two different hemagglutinating proteins also have been found in both the lentil (Ticha *et al.*, 1970) and the pea (Entlicher *et al.*, 1970). As further phytohemagglutinins are analyzed it appears that a great proportion are being found to exist in multiple forms. We propose the term "isolectins" to describe this phenomenon.

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Immunochemistry of Sperm-Whale Myoglobin. Conformation and Immunochemistry of Derivative Reduced at Some Carboxyl Groups by Diborane†

M. Z. Atassi* and M. T. Perlstein

ABSTRACT: A homogeneous derivative was prepared by reduction of apomyoglobin (ApoMb) with diborane at −10° for 4 hr. In the derivative, glutamic acid residues at positions 83 and 85 were reduced to their corresponding hydroxy acid (*i.e.*, 2-amino-5-hydroxyvaleric acid). Recombination of the reduced ApoMb with unmodified ferriheme yielded the corresponding reduced myoglobin (R-Mb) derivative. In starch gel electrophoresis, R-Mb migrated as a single band with a mobility of 0.90 relative to native myoglobin (Mb). Absorption spectra in the ultraviolet–visible range, optical rotatory dispersion and circular dichroism parameters of R-Mb and Mb were quantitatively identical. Immunochemical studies showed that R-Mb and Mb had equal reactivity both with antisera to Mb and with antisera to R-Mb. The results show that no conformational changes take place upon reduction of the γ-carboxyl groups of glutamic acid residues 83 and 85. Also these two amino acids are not located in an antigenic reactive region of Mb. From these findings and other already published results, it was possible to narrow down further the size of a previously located antigenic reactive region in Mb so that it will now fall within, but may not include all of, the sequence 86–102.

Information concerning the antigenic structure of sperm-whale myoglobin has been reported in several previous publications from this laboratory. Discrete antigenic reactive regions have been exhaustively delineated by studying the specific immunochemical interactions of a variety of overlapping peptides obtained by chemical and enzymic cleavage procedures and by specific chemical modification of various amino acids in protein and immunochemically reactive peptides. The reactive regions occupy four corners between helices on the surface of the molecule. Interactions between adjacent *reactive regions* and their incorporation into *reactive sites* are difficult to investigate, but are important for understanding the three-dimensional nature of the reactive site. This may be approached by studying the conformation and immunochemistry of specifically modified derivatives which will also be helpful in further narrowing down of antigenic reactive regions. Previously, valuable information has been obtained concerning the contribution to antigenic structure of the methionines (Atassi, 1967a, 1969), the tryptophans (Atassi and Caruso, 1968), the tyrosines (Atassi, 1968), and the arginines (Atassi and Thomas, 1969). We wish now to report on the role of two glutamic acid residues.

Carboxyl groups can be modified by esterification but these reactions lack specificity. Reaction with carbodiimides yields derivatives that are not easy to characterize (Sheehan and Hlavka, 1956; Franzblau *et al.*, 1963; Goodfriend *et al.*, 1964; Riehm and Scheraga, 1966) and this complication is avoided when activation with carbodiimides is followed by coupling with amino acid or peptide esters (Hoare and Koshland, 1966; Wilchek *et al.*, 1967). Carboxyl groups can also be modified by reaction with isoxalium salts (Bodlaender *et al.*, 1969), giving an enol ester. The reactive enol ester may be coupled with nucleophiles. In these procedures, where the carboxyl group is activated, reaction may give rise to intramolecular (and intermolecular) cross-linking with favorably placed amino groups. Carbodiimides have been shown to react with sulfhydryl groups in proteins (Carraway and Triplett, 1970). Also, the modification is not permanent and will be removed by acid or alkaline hydrolysis and cannot be used to identify the residue modified (*i.e.*, aspartic, glutamic, or C terminus). Recently, it was reported that car-

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