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STUDIES ON THE CHEMICAL MODIFICATION OF SOYBEAN AGGLUTININ*

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ABSTRACT

Soybean agglutinin was subjected to various chemical treatments in order to detect which amino acid residues are involved in its carbohydrate binding activity. Modification of tyrosine and histidine residues was demonstrated but had no effect on the haemagglutinating activity. Modification of amino groups with citraconic anhydride in the absence or presence of 0.1M D-galactose or 0.1M 2-acetamido-2-deoxy-D-galactose decreased the lectin activity, and treatment with cyclohexane-1,2-dione which modified arginyl groups had a similar effect. Reaction of carboxyl groups, activated by carbodiimide, with α-aminobutyric acid methyl ester led to the incorporation of ester groups giving an inactive material. Modification of tryptophan residues carried out in native and denaturing conditions with *N*-bromosuccinimide led to a complete loss of haemagglutinating activity. Treatment in the presence of the sugars partially protected the tryptophan residues from oxidation and some activity was retained. The results indicated that tryptophan groups are involved but that tyrosine and histidine groups are not involved in the binding site of the lectin. It is proposed that the side chains of lysine, arginine, glutamate, and aspartate are not involved in the active site, but that the charged groups are important for maintaining a fully active conformation. An alternative explanation is that these amino acid residues are in fairly close proximity to the active site.

INTRODUCTION

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin which agglutinate cells, precipitate glycoconjugates, or both. The properties and sources of lectins have been extensively reviewed[1,2]. Soybean agglutinin is a glycoprotein and has a molecular weight of 120 000 consisting of four identical subunits each of mol. wt. 30 000 (ref. 3). There are four binding sites per molecule[4] and each subunit carries an oligosaccharide chain $Man_9(GlcNAc)_2$. Soybean

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agglutinin interacts strongly with 2-acetamido-2-deoxy-D-galactose and to a lesser extent with D-galactose. Understanding the relationship between its structure and function would be facilitated by the study of the chemical nature of its combining site. Chemical modification of proteins serves as a tool to identify the amino acid residues involved in their binding activities. A detailed study of the chemical modification of various amino acid side-chains of soybean agglutinin was therefore carried out by observing the effect on the agglutinating activity and the degree of protection afforded by specific sugars.

EXPERIMENTAL

Protein concentration. — Protein concentration was determined by spectrophotometric measurement at 280 nm by use of an $E_{1\,cm}^{1\%}$ value of 12.8 as determined by Lotan *et al.*[3]

Amino acid analysis. — Amino acids were measured on a Locarte amino acid analyser after hydrolysis for 24 h in 3M toluene-4-sulphonic acid. Details of the buffer systems which were used are given elsewhere[5].

Analytical gel chromatography. — Comparative molecular size determinations were carried out on native and citraconylated soybean agglutinin on a prepacked Superose 12 column using a Pharmacia FPLC apparatus. Phosphate-buffered saline (pH 7.2) was the eluent and the flow rate was 0.5 mL/min. Protein elution was monitored by u.v. absorption.

Poly(acrylamide) gel electrophoresis (PAGE). — A Pharmacia "Phast" apparatus was used, 8–25% gradient gels were run in both native and sodium dodecyl sulphate systems and were stained with a silver stain according to the manufacturer's instructions.

Haemagglutinating activity. — Agglutination of trypsinised rabbit erythrocytes by native soybean agglutinin and modified lectin samples was measured in plastic microtitration trays. Lectin solutions were serially diluted with equal volumes of phosphate-buffered saline to give a final volume of 0.05 mL. To these dilutions was added a 2% (v/v) suspension of rabbit erythrocytes in phosphate-buffered saline (0.05 mL). The end point of the titration was estimated visually 2–4 h after addition of erythrocytes as the lowest dilution of lectin solution that showed agglutination. This was taken as one unit of haemagglutinating activity. Specific activities were calculated from this titre and the known starting concentration of lectin. Relative haemagglutinating activity of chemically modified lectin samples is expressed as percentage of the specific activity of native lectin.

Materials. — 2,4,6-Trinitrobenzenesulphonic acid, *N*-bromosuccinimide, α-aminobutyric acid methyl ester hydrochloride, 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride, ethoxyformic anhydride, trypsin, D-galactose, 2-acetamido-2-deoxy-D-galactose, and thioglycolate were from Sigma Chemical Co; cyclohexane-1,2-dione, and tetranitromethane were from Aldrich Chemical Co. All other chemicals were of analytical grade or the purest available.

Preparation of soybean agglutinin. — The agglutinin was prepared from soybean meal by affinity chromatography on 2-amino-2-deoxy-D-galactose–CH-Sepharose columns as described by Allen and Neuberger[6]. The purity of the preparations was determined by poly(acrylamide) gel electrophoresis both in the presence and absence of sodium dodecyl sulphate.

Chemical modification of soybean lectin. — *Citraconylation.* The amino groups were modified by reaction with citraconic anhydride as described by Dixon and Perham[7]. Three portions (3 μL) of citraconic anhydride were added, with constant stirring, to soybean agglutinin (1–3 mg) in 0.1M Tris·HCl buffer, pH 8.0 (1 mL) at 20°, over the course of 30 min (final molarity of the reagent was 0.1M). The solution was then stirred for 1 h and dialysed exhaustively against 5mM Tris·HCl buffer, pH 8.6 at 0–4°. Decitraconylation was carried out by leaving the modified protein overnight at 4° at pH 3.0. The percentage modification in each of these reactions was determined by measuring unmodified amino groups with 2,4,6-trinitrobenzenesulphonic acid as described by Fields[8].

Treatment with tetranitromethane. The method of Riordan and Vallee[9] was used with a 10-fold molar excess of tetranitromethane over tyrosine. To the lectin (1 mg) in 0.05M Tris·HCl buffer, pH 8.0 (1 mL) containing M NaCl were added, at 20° and 15-min intervals with constant stirring, six 2-μL portions of 0.8M tetranitromethane. The mixture was stirred for 1 h, and the samples were either dialysed exhaustively against distilled water at 0–4° or filtered through a column (20 cm × 1 cm) of Sephadex G-25, equilibrated and irrigated with 0.05M Tris·HCl buffer, pH 8.0, containing M NaCl. Fractions were collected and those containing lectin were pooled.

Treatment with cyclohexane-1,2-dione. This was done by the method of Patthy and Smith[10]. Soybean agglutinin (3 mg) was dissolved in 0.2M sodium borate buffer, pH 9.0, containing 0.05M cyclohexane-1,2-dione (400 μL). The reaction vessel was flushed with N_2 and then incubated at 37°, in the dark, for 4 h. The reaction was terminated by the addition of 5% (v/v) acetic acid (1 mL) and then dialysed against 1% (v/v) acetic acid; the acid stabilises the product in the absence of borate. The reaction was reversed when the modified protein was dialysed exhaustively against 0.05M Tris·HCl buffer, pH 8.6, at 20°.

Reaction with ethoxyformic anhydride. Modification of histidine residues was carried out by the method of Melchior and Fahrney[11]. Soybean agglutinin was dissolved in 0.02M sodium phosphate buffer, pH 7.2, at a concentration of 1 mg/mL. A stock solution (25mM) of ethoxyformic anhydride was prepared in dry ethanol. Aliquots of this stock solution were added to the protein solution to a final concentration of 3-fold molar excess over the histidine content. The reaction was allowed to proceed with stirring at room temperature for appropriate time intervals and was terminated by adding an equal volume of 10mM cold histidine in phosphate buffer, pH 7.2. The mixture was then dialysed against 0.02M phosphate buffer, pH 7.2, at 4° to remove excess histidine and the reagent.

Decarbethoxylation. Aliquots of the reaction mixture and untreated control

were incubated with 0.05M $NH_2OH \cdot HCl$, pH 7.0, for 1 h at room temperature and then dialysed against 0.01M sodium phosphate buffer, pH 7.0.

Reaction with N-*bromosuccinimide.* Oxidation of soybean agglutinin with *N*-bromosuccinimide was carried out according to the method of Spande and Witkop[12] at room temperature. A solution of soybean agglutinin (2 mg) in either 0.1M sodium acetate buffer, pH 4.0 (2.0 mL), or in 0.1M acetic acid–8M urea (2.0 mL) was pipetted into a quartz cell. The cell was placed in a spectrophotometer, and the initial absorbance at 280 nm as well as the absorption spectrum were recorded against the reference cell containing the solvent only. An aliquot (10 μL) of 10mM *N*-bromosuccinimide in water was added with rapid mixing to the protein solution and to the reference cell. After 5 min, the absorbance at 280 nm and the spectrum were recorded. Addition of the reagent was continued in a stepwise manner until the absorbance readings at 280 nm no longer decreased. The number of tryptophan residues oxidised was calculated as described by Spande and Witkop[12]. The samples were dialysed exhaustively against phosphate-buffered saline to test for activity.

Carbodiimide activation followed by coupling with a nucleophile. Soybean agglutinin (5 mg) was dissolved in M α-aminobutyric acid methyl ester, pH 4.75 (1 mL). Two portions (50 μL) of 1.1M 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide were added at 30-min intervals. The mixture was stirred constantly for 5.5 h and then dialysed against distilled water at 0–4° before analysis.

Determination of the extent of modification. The extent of citraconylation was determined by measuring the remaining amino groups by the reaction with 2,4,6-trinitrobenzenesulphonic acid and comparing the values obtained with native lectin. Incorporation of 2,4,6-trinitrophenyl groups was measured spectrophotometrically at 420 nm by use of an E value of 192 000 $L \cdot mol^{-1} \cdot cm^{-1}$. Nitration of phenolic hydroxy groups was measured by amino acid analysis after acid hydrolysis. The extent of arginine modification was determined by amino acid analysis after hydrolysis in 6M HCl containing thioglycollate[13] (20 μL) for 24 h at 110°. The extent of ethoxyformylhistidine formation was determined spectrophotometrically at 242 nm (E_{242} 3200 $L \cdot mol^{-1} \cdot cm^{-1}$) or preferably at 250 nm (E_{250} 1600 $L \cdot mol^{-1} \cdot cm^{-1}$) which corresponds to a minimum in the absorption spectrum of soybean agglutinin. The number of oxidised tryptophan residues was calculated by the decrease in absorbance at 280 nm as described by Spande and Witkop[12]. Incorporation of α-aminobutyric acid methyl ester into the lectin was determined by measurement of α-aminobutyric acid by amino acid analysis after acid hydrolysis.

Protection by D-*galactose and 2-acetamido-2-deoxy*-D-*galactose.* — Parallel experiments were performed for each of the chemical reactions in which the lectin was preincubated for 30 min with 0.1M D-galactose or 0.1M 2-acetamido-2-deoxy-D-galactose before the addition of the chemical reagent to the lectin.

RESULTS

Substitution or modification of an amino acid residue of a lectin which

destroys its agglutinating activity might be taken to imply that the residue was involved in the active site. This explanation need not necessarily be correct as the modification could have caused a conformational change in the protein to an inactive form which is not capable of binding sugars. Another possible effect of modifications is that, since lectins consists of subunits, a modification could cause dissociation of subunits which would mean that the lectins would not cause agglutination without the binding site being affected. In this study, we have used reagents with a high degree of specificity for particular side-chains. Reagents were also chosen so that the reaction products could be determined quantitatively and preferably capable of reversal to regenerate the original amino acids. In all cases, the reactions were carried out in both the presence and absence of the specific hapten sugars D-galactose and 2-acetamido-2-deoxy-D-galactose (see Table I).

Treatment with citraconic anhydride. — Treatment with citraconic anhydride

TABLE I

EFFECT OF CHEMICAL MODIFICATIONS ON HAEMAGGLUTINATING ACTIVITY OF SOYBEAN AGGLUTININ

Treatment of agglutinin and residued modified	*Percentage modification*	*Haemagglutinating activity (%)*[a]
Citraconylation (Lys)	95	0
+ D-Galactose	88	0
+ 2-Acetamido-2-deoxy-D-galactose	86	0
Citraconylation then *N*-decitraconylation	13	90
Nitration (Tyr)	15	90
+ D-Galactose	14	70
+ 2-Acetamido-2-deoxy-D-galactose	14	70
Cyclohexane-1,2-dione (Arg)	51	6
+ D-Galactose	40	6
+ 2-Acetamido-2-deoxy-D-galactose	44	6
Cyclohexane-1,2-dione reversed with Tris	10	20
Ethoxyformic anhydride (His)	41	100
Ethoxyformic anhydride reversed with hydroxylamine	10	100
Activation with carbodiimide then reaction with α-aminobutyric acid methyl ester (Asp + Glu)	48	0
+ D-Galactose	44	0
+ 2-Acetamido-2-deoxy-D-galactose	38	0
N-Bromosuccinimide (Trp)	58	0
+ D-Galactose	43	5
+ 2-Acetamido-2-deoxy-D-galactose	47	5
+ 8.0M Urea	92	0

[a]The haemagglutinating activity is expressed as a percentage of that given by native, unmodified lectin under the same conditions. Due to the variability of the assay, significant inactivation was considered to have taken place only if the modified derivative showed an activity of 25% (*i.e.*, two serial dilutions) or less than that of the native lectin.

blocked 13.6 amino groups per subunit, whereas reaction in the presence of 0.1M D-galactose or 0.1M 2-acetamido-2-deoxy-D-galactose blocked 12.3 and 12.0 amino groups, respectively. The haemagglutinating activity was destroyed by this treatment. Citraconyl groups are labile to acid, and when the citraconylated lectin was dialysed against 0.1M acetic acid, almost complete reversal of citraconylation was observed (Table I) and the activity was restored. The loss of activity could be due to modification of lysine residues in or near the binding site, or to a conformational change due to the change in charge of the derivative. Citraconylation did not cause dissociation of the subunits since gel chromatography in non-dissociating conditions showed that the citraconylated derivative was eluted in the same position as the native lectin, which is eluted as a tetramer (mol. wt. 120 000).

Nitration of tyrosine. — Tetranitromethane, which specifically converts tyrosine residues to 3-nitrotyrosyl derivatives, only converted one out of six residues either in the presence or absence of hapten sugars. There was no loss of activity following this treatment, nor any change in subunit interaction. This indicated that tyrosine residues are either not involved in the binding site or that most of them were not accessible to the reagent.

Modification of arginyl groups. — The arginyl group is specifically modified by cyclohexane-1,2-dione, and modification of ~3.5 of the seven residues was achieved with the reagent with a loss of more than 90% of the haemagglutinating activity. The hapten sugars slightly reduced the extent of modification of the residues but did not protect the haemagglutinating activity. The haemagglutinating activity was not restored on reversal of the arginine modification, which indicated that the initial reaction had caused a severe disruption of the structure of the lectin and that this, rather than a modification of the binding site, is the reason for the effect of this reagent.

Modification of histidyl groups. — Ethoxyformic anhydride was shown to have modified 2.2 of the five histidine residues, but this modification (which was reversible) had no significant effect on the haemagglutinating activity, showing that these histidyl residues are not involved in the lectin activity.

Reaction of carboxyl groups with carbodiimide followed by coupling with α-aminobutyric acid methyl ester. — The carbodiimide was used to activate the carboxyl groups of the lectin in the presence of α-aminobutyric acid methyl ester, and the extent of the modification could be measured (by amino acid analyses) by the number of α-aminobutyric residues incorporated into the protein. About 50% of the carboxyl groups were modified and there was almost complete inactivation of the lectin activity. Although the presence of hapten sugars slightly reduced the percentage of modification, there was no protection of lectin activity. This partial protection may indicate that one or more carboxyl groups are near the binding site, and that the substitution causes a steric hindrance. It is also possible that modification of 12 or 13 residues resulting in a loss of negative charge had caused a conformational change that had affected the binding site.

Tryptophan modification by N-*bromosuccinimide.* — Oxidation of the tryp-

tophan residues was investigated under both denaturing and non-denaturing conditions. In the presence of 8.0M urea, 5.5 out of six residues of tryptophan could be modified by the reagent, which resulted in a complete loss of activity for dialysed preparations (controls treated with urea alone showed a restoration of activity after dialysis). Treatment of native lectin with *N*-bromosuccinimide also produced an inactive preparation, although only 3.5 residues per subunit were oxidised. In the presence of the sugar haptens, there was a small but significant protection of the haemagglutinating activity. In different experiments, the range of protection with 2-acetamido-2-deoxy-D-galactose was between 5 and 25%, but the former figure correlated with the figure in Table I for the percentage modification. Since the modified lectin had a mol. wt. of ~120 000 when electrophoresed under non-denaturing conditions, the effect of the modifications was therefore probably on the binding site of the lectin rather than on subunit interactions.

DISCUSSION

In this work, we have been able to demonstrate that modification of several amino acids, namely, tryptophan, arginine, lysine, aspartate, and glutamate, has the effect of reducing or completely blocking the haemagglutinating activity of soybean agglutinin. The use of the haptens D-galactose and 2-acetamido-2-deoxy-D-galactose has shown that, although one or more residues of tryptophan, lysine, aspartate, glutamate, and arginine are protected from modification by the presence of these sugars, only in the case of tryptophan is the activity partially protected. We could therefore, conclude that one or more tryptophan residues are present in the binding site. This conclusion is in agreement with the circular dichroism studies of Thomas *et al.*[15], and is similar to that made for a number of other lectins, such as concanavalin A[16], wheat germ agglutinin[17], potato lectin[6], pea[18], and the D-galactose-binding lectin from rabbit liver[19]. This indicated the importance of hydrophobic interactions in the binding sites of soybean and other lectins.

The charged amino acids are not so clearly involved in the binding site as is tryptophan. Histidine residues reacted with the reagent, but the modification did not affect the lectin activity. Lysine, arginine, aspartate, and glutamate modifications did cause inactivation, and although the hapten sugars reduced the extent of modification, there was no protection of lectin activity. This suggested that, although some of these charged residues may be adjacent to the binding site, the most likely effect of the modification is that the unmodified charged side-chains are important for maintaining the lectin in a fully active conformation.

The lack of reactivity of the tyrosine residues, only one of six being reactive with tetranitromethane, was unexpected in the light of a previous report[20] of the essential nature of tyrosine in soybean agglutinin and the indications from circular dichroism[15]. Concanavalin A, which shows considerable sequence homology with soybean agglutinin (see refs. 14 and 21), does have tyrosine residues in areas which are presumptive carbohydrate-binding sites but, in the analogous positions of

soybean agglutinin, these are replaced by phenylalanine (position 10 of Con A) and aspartate (position 100 of Con A). From the analyses of Hemperly and Cunningham[21], it would seem that the regions that are in the active site of the legume lectins are more variable than other regions.

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