Studies on the chemical modification of potato (*Solanum tuberosum*) lectin and its effect on haemagglutinating activity

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1. Modification of potato (*Solanum tuberosum*) lectin with acetic anhydride blocked 5.1 amino and 2.7 tyrosyl groups per molecule of lectin and decreased the haem-agglutinating activity of the lectin. De-*O*-acetylation regenerated 2.0 of the tyrosyl groups and resulted in a recovery of activity. 2. Modification with citraconic anhydride or cyclohexane-1,2-dione did not greatly affect activity, although modification of amino and arginyl groups could be demonstrated. 3. Treatment with tetranitromethane nitrated 3.7 tyrosine residues per molecule of lectin with concomitant loss of activity. The presence of 0.1 M-*NN'**N''*-triacetylchitotriose (a potent inhibitor of the lectin) in the reaction medium protected all the tyrosyl residues from nitration and the lectin was fully active. 4. Modification of tryptophyl groups with 2-hydroxy-5-nitrobenzyl bromide and 2,3-dioxindoline-5-sulphonic acid modified 0.9 and 2.6 residues per molecule of lectin respectively with a loss of activity in each case. Reaction of potato lectin with 2,3-dioxindoline-5-sulphonic acid in the presence of inhibitor protected 2.4 residues of tryptophan from the reagent. Loss of haemagglutination activity was prevented under these conditions. 5. Reaction of carboxy groups, activated with carbodi-imide, with α -aminobutyric acid methyl ester led to the incorporation of 5.3 residues of the ester per molecule of lectin. Presence of inhibitor in this case, although protecting activity, did not prevent modification of carboxy groups; in fact an increase in the number of modified residues was seen. This effect could be imitated by performing the reaction in 8 M-urea. In both cases the number of carboxy groups modified was close to the total number of free carboxy groups as determined by the method of Hoare & Koshland [(1967) *J. Biol. Chem.* **242**, 2447–2453]. Guanidination of lysine residues after carboxy-group modification gave less homoarginine than did the unmodified lectin under the same conditions, suggesting the formation of intramolecular cross-links during carbodi-imide activation. 6. It is suggested from the results presented that amino, arginyl, methionyl, histidyl and carboxyl groups are not involved in the activity of the lectin and that tyrosyl and tryptophyl groups are very closely involved. These findings are similar to those reported for other proteins that bind *N*-acetylglucosamine oligomers and also fit the general trend in other lectins.

Lectins have been recently re-defined by Goldstein *et al.* (1980) as 'sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates'. The properties and sources of lectins have been extensively reviewed (Goldstein & Hayes, 1978; Lis & Sharon, 1980). The lectin from potato (*Solanum tuberosum*) tubers is an unusual glycoprotein that contains large amounts of 4-hydroxyproline and cystine and 47% by weight carbohydrate, composed mainly of arabinose (Allen & Neuberger,

1973). The molecule has at least two distinct domains, the larger one containing all the hydroxyproline and arabinose (mol.wt. 32 000–34 000), and at least one other that contains all the cystine and no carbohydrate (Allen *et al.*, 1978).

Lectins are similar to enzymes in that they exhibit a specific interaction with particular molecules, but are by definition restricted to interactions with sugars. The carbohydrate-binding site(s) of a lectin contains amino acids with side chains that contribute to the association of specific saccharide

groups with that lectin, in a manner similar to an enzyme-substrate association. An enzyme active site differs from a lectin active site in that the amino acid side chains, by their interactions with the substrate and other amino acid side chains, bring about the catalytic conversion of the substrate.

Chemical modifications of active-site amino acid side chains will alter their properties and bring about a change in the biological activity of the protein, if they contribute to it. This change in activity can be measured for a variety of modifications of different amino acid side chains, and thus an idea of those involved in the biological activity can be obtained.

An investigation of this sort on potato lectin would, it was hoped, enable us to compare the amino acids involved in binding with well-characterized proteins of similar specificity, e.g. the lectin wheat-germ agglutinin and the enzyme lysozyme. Both these molecules interact with oligosaccharides containing β -1 $\rightarrow$ 4-linked *N*-acetylglucosamine residues. It would also be interesting to compare the results obtained for potato lectin with those of other lectins, with different specificities, to see if there are common features in active sites that are necessary for sugar binding.

Experimental

Chemicals

2,4,6-Trinitrobenzenesulphonic acid, 2-hydroxy-5-nitrobenzyl bromide, α -aminobutyric acid methyl ester hydrochloride, 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide hydrochloride, 3-nitrotyrosine, citraconic anhydride and bromoethylamine hydrobromide were from Sigma (London) Chemical Co. (Poole, Dorset, U.K.); cyclohexane-1,2-dione, tetranitromethane and 2,3-dioxindoline-5-sulphonic acid (isatin-5-sulphonic acid) were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.); iodo[14 C]acetic acid was from The Radiochemical Centre (Amersham, Bucks., U.K.); *NN'*N"-tri-acetylchitotriose was purified from a partial acid hydrolysis of chitin by the method of Rupley (1964). All other chemicals were of AnalaR grade or the purest available.

Preparation of potato lectin

Potato lectin was purified by affinity chromatography on a *NN'*N"-tri-acetylchitotriosyl-Sepharose matrix as described by Desai & Allen (1979). The lectin gave an amino acid analysis very similar to that reported by Allen *et al.*, (1978), and this was used in calculating the number of residues of amino acids modified.

Potato lectin concentration

The concentration of potato lectin was determined either by spectrophotometric measurement at

205nm by using an $A_{1\text{cm}}^{1\%}$ value of 302.8 or by measuring optical rotation at 365nm and by using an $[\alpha]_{365}$ value of $+111^\circ$ (*c* 0.2 in water).

Reduction and alkylation of potato lectin

Lectin was reduced by dithiothreitol in 6M-guanidinium chloride in 0.1M-Tris/HCl buffer, pH 8.6, and then alkylated with either iodoacetic acid (Königsberg, 1972) or bromoethylamine hydrobromide (Cole, 1967). **Special care was taken in the use of the bromo compound in view of its mutagenic properties.**

Haemagglutinating activity

Agglutination of rabbit erythrocytes by native potato lectin and modified lectin samples was measured in plastic microtitration trays. Lectin solutions were serially diluted with equal volumes of iso-osmotic phosphate-buffered saline, pH 7.2 (Allen *et al.*, 1976), to give a final volume of 0.2 ml. To these dilutions was added 0.1 ml of a 3% (v/v) suspension of rabbit erythrocytes in phosphate-buffered saline, pH 7.2, to give a final erythrocyte suspension of 1% (v/v). 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 a percentage of the specific activity of native potato lectin.

Amino acid analysis

Amino acids were measured on a Locarte Mini amino acid analyser by reaction with ninhydrin. Samples were hydrolysed in 3M-toluene-4-sulphonic acid for 24 h at 110°C *in vacuo*. After hydrolysis the samples were two-thirds neutralized with NaOH. These were then loaded directly on to the analyser. Basic amino acids were separated on a column (15 cm $\times$ 0.9 cm) by successive elution with sodium citrate buffers: pH 5.28 (0.35M) for 90 min, and pH 6.65 (1M) for 70 min, at 60°C. Neutral and acidic amino acids were separated on a column (23 cm $\times$ 0.9 cm) by successive elution with 0.2M-sodium citrate buffers: pH 3.15 for 35 min, pH 3.30 for 70 min and pH 4.25 for 80 min. The column was run at 38°C for the first buffer and subsequently at 60°C. This system separates hydroxyproline from aspartic acid and α -aminobutyric acid from alanine.

Immunodiffusion

Modified lectin samples were tested by double immunodiffusion in 1% agarose in 0.15M-NaCl for their reactivity with antisera directed against both the whole lectin and the non-glycosylated region.

The latter was produced by precipitating the antibodies directed against the glycosylated domain with the complete glycopeptide (mol.wt. 34 000) produced by Pronase digestion of potato lectin (Allen *et al.*, 1978). The precipitate was removed by centrifugation and the supernatant was used directly (Ashford *et al.*, 1980).

Chemical modification of potato lectin

All the treatments were performed under mild conditions and with reagents that had a high specificity for particular amino acid side chains. The reagents were also chosen because the reaction products could be determined quantitatively either directly or indirectly after a second treatment. Also, where possible, reagents were chosen where the modification produced could be reversed, regenerating the original amino acid residue. Thus, if the lectin lost activity and the modification were reversible, and on reversal activity was regained, extra weight could be added to the involvement of that amino acid in the biological activity rather than the effect being due to a side reaction.

(a) *Acetylation*. The method was based on that employed by Rice & Etzler (1975). Four 1 μ l portions of acetic anhydride were added, with constant stirring, to 5 mg of lectin in 1 ml of 50% saturated sodium acetate, pH 8.3, at 0°C, over the course of 1 h. The pH was maintained at 8.3 by addition of NaOH. After this treatment the sample was dialysed exhaustively against distilled water at 0–4°C.

(b) *Citraconylation*. The method of Dixon & Perham (1968) was used. Three 3 μ l portions of citraconic anhydride were added, with constant stirring, to 3 mg of potato lectin in 1 ml of 0.1 M-Tris/HCl buffer, pH 8.0, at 20°C, over the course of 30 min. The pH was maintained between 8 and 9 by addition of NaOH. After 1 h the solution was dialysed exhaustively against 5 mM-Tris/HCl buffer, pH 8.6, at 0–4°C.

(c) *De-O-acylation*. Samples of acylated lectin were made to 1 M with respect to hydroxylamine by addition of an equal volume of 2 M-hydroxylamine hydrochloride adjusted to pH 7.5 with NaOH. The solutions were mixed and left with occasional agitation for 10 min at 20°C. The samples were then dialysed exhaustively against distilled water at 0–4°C.

(d) *Acid treatment*. Lectin samples after treatments (b) and (c) were dialysed against 0.1 M-acetic acid at 20°C for 18 h and then exhaustively against distilled water at 0–4°C.

(e) *Determination of amino groups with 2,4,6-trinitrobenzenesulphonic acid* (Fields, 1972). To 1 ml of lectin solution in 0.05 M-Na₂B₄O₇ in 0.05 M-NaOH was added 0.02 ml of 1.1 M-2,4,6-trinitrobenzenesulphonic acid. After 5 min the reaction was

terminated by the addition of 2 ml of 0.1 M-NaH₂PO₄ containing 1.5 mM-Na₂SO₄.

(f) *Nitration*. The method of Riordan & Vallee (1972) was used with a 10-fold molar excess of tetranitromethane over tyrosine. To 1 mg of lectin in 1 ml of 0.05 M-Tris/HCl buffer, pH 8.0, at 20°C, three 2 μ l portions of 0.84 M-tetranitromethane were added at 15 min intervals with constant stirring. After 1 h the samples were either dialysed exhaustively against distilled water at 0–4°C or passed through a column (15 cm $\times$ 1 cm) of Sephadex G-25, equilibrated and run with 0.05 M-Tris/HCl buffer, pH 8.0. Fractions were collected and those containing lectin were pooled.

(g) *Treatment with 2-hydroxy-5-nitrobenzyl bromide*. The procedure of Horton & Koshland (1972) was used. To 2 mg of lectin dissolved in 1 ml of 0.18 M-acetic acid, pH 2.7, five 20 μ l portions of 2-hydroxy-5-nitrobenzyl bromide solution (50 mg/ml in dry acetone) were added. The pH was maintained below 3.0 by addition of acetic acid. After 1 h, modified lectin was separated from reaction products by gel filtration on a column (15 cm $\times$ 1 cm) of Sephadex G-25 equilibrated and run in 0.18 M-acetic acid. The fractions containing lectin were pooled and dialysed exhaustively against distilled water at 0–4°C.

(h) *Treatment with 2,3-dioxindoline-5-sulphonic acid*. This reagent was used at 50-fold molar excess over tryptophan as described by Atassi & Zablocki (1976). Lectin (1 mg) was added to 1 ml of 0.1 M-acetic acid containing 0.25 mg of 2,3-dioxindoline-5-sulphonic acid, and the solution was thoroughly mixed and then stirred for 16 h at 20°C. The sample was then dialysed exhaustively against distilled water at 0–4°C.

(i) *Modification with cyclohexane-1,2-dione*. The method used was that described by Patthy & Smith (1975). Lectin (2 mg) was dissolved in 200 μ l of 0.2 M-sodium borate buffer, pH 9.0, containing 0.05 M-cyclohexane-1,2-dione. The reaction vessel was flushed with N₂ and then incubated at 37°C, in the dark, for 4 h. The reaction was terminated by the addition of 1 ml of 5% (v/v) acetic acid. For amino acid analysis the mixture was then dialysed for 16 h against 2 litres of 5% (v/v) acetic acid, and then exhaustively against 1% (v/v) acetic acid; the acid stabilizes the interaction in the absence of borate.

(j) *Reversal of cyclohexane-1,2-dione modification*. (1) Modified lectin was incubated at 37°C for 4 h in 1 M-hydroxylamine, pH 7.5, under N₂, and then dialysed exhaustively against distilled water at 0–4°C. (2) Modified lectin was dialysed exhaustively against 0.05 M-Tris/HCl buffer, pH 8.6, at 20°C.

(k) *Carbodi-imide activation followed by coupling with a nucleophile*. Samples of lectin were dissolved in 1 ml of 1 M- α -aminobutyric acid methyl

ester, pH 4.75. Two 50 μ l portions of 1.1 M-1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide were added at 30 min intervals. The mixture was stirred constantly and the pH was maintained at 4.75 by addition of HCl. After 5 $\frac{1}{2}$ h the samples were dialysed exhaustively against distilled water at 0–4°C. A period of 5 $\frac{1}{2}$ h was found to be necessary for full activation in non-denaturing systems (Carraway & Koshland, 1972).

(I) *Guanidination of lysine groups.* Lectin samples were dissolved in 0.2 M-glycine/NaOH buffer, pH 10.5, to give an approximate concentration of 1 mg/ml. An equal volume of 0.6 M-*O*-methylisourea adjusted to pH 10.5 with NaOH was added. The resulting solutions were then kept at 5°C for 24 h. The reagents were removed from the modified lectin by exhaustive dialysis against distilled water at 0–4°C.

Determination of the extent of modification

The extent of acetylation and citraconylation was determined by measuring the remaining amino and phenolic hydroxy groups by reaction with 2,4,6-trinitrobenzenesulphonic acid and tetranitromethane respectively and comparing the values obtained with those of native lectin. Incorporation of trinitrophenyl groups was measured spectrophotometrically at 420 nm by using an ϵ value of 19200 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. Nitration of phenolic hydroxy groups was measured by amino acid analysis after hydrolysis. 2-Hydroxy-5-nitrobenzyl bromide and 2,3-dioxindoline-5-sulphonic acid reaction products were measured spectrophotometrically at 367 nm for 2,3-dioxindoline-5-sulphonic acid-modified protein by using $\epsilon = 7680$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ and at 410 nm for 2-hydroxy-5-nitrobenzyl bromide-treated protein, after adjustment to pH 12.0 with NaOH by using $\epsilon = 18450$ litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. 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. The arginine-cyclohexane-1,2-dione complex has been reported to be destroyed, with some regeneration of arginine, by hydrolysis with 6 M-HCl for 24 h at 110°C (Patthy & Smith, 1975). No regeneration of arginine from the complex was found under our hydrolysis conditions. Therefore the extent of modification of arginine by cyclohexane-1,2-dione was determined by amino acid analysis from the loss of arginine on acid hydrolysis. Guanidination of lysine was determined by measurement of homoarginine by amino acid analysis, after acid hydrolysis.

Determination of the total number of free carboxy groups (Hoare & Koshland, 1967)

S-Aminoethylcysteinyl potato lectin (1 mg) was dissolved in 8 M-urea containing 1 M- α -aminobutyric

acid methyl ester, pH 4.75. After 30 min at 20°C the sample was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide as described above. After 1 h of reaction the sample was dialysed exhaustively against distilled water at 0–4°C. The amount of α -aminobutyric acid present after acid hydrolysis was measured by amino acid analysis.

Measurement of thiol, methionyl and histidyl groups

Samples of potato lectin (1 mg) were treated with 5 μ Ci of iodo[¹⁴C]acetic acid (51 mCi/mmol), in the absence of denaturant and without prior reduction, in either (a) 0.1 M-acetic acid, (b) 0.2 M-sodium acetate buffer, pH 4.5, or (c) 0.1 M-Tris/HCl buffer, pH 8.6. The reaction was allowed to proceed for 2 h at 37°C. Samples were then dialysed exhaustively against distilled water at 0–4°C. Radioactivity of the samples was measured in a Searle Analytic Mark 3 liquid-scintillation counter in mixtures of PPO (2,5-diphenyloxazole) (2 g) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] (0.05 g) dissolved in toluene (500 ml) plus Triton X-100 (250 ml).

Results and discussion

Proteins exist in solution in various conformations. In the case of an enzyme or a lectin the different conformers may be associated with various degrees of activity, and some conformers may be inactive. If substitutions or other modifications of amino acid residues destroy lectin activity, the most obvious explanation of such a finding is that the residues are part of the active site and involved in the binding of the ligand. It is, however, possible that the residues in question are not part of the active site but are required for the maintenance of the active conformation.

If the presence of a saturating concentration of an inhibitor or pseudo-substrate prevents the attacking reagent from modifying amino acid residues (with a retention of lectin activity, where modification in their absence led to a loss of activity) the assumption that these residues are part of the active site is greatly strengthened. However, it is not impossible that the combination of the lectin with the inhibitor, as well as protecting the active centre, brings about a conformational change that in turn makes amino acid residues distant from the active site unavailable to the attacking reagent.

To agglutinate cells a lectin must be multivalent. Potato lectin exists in solution as a mixture of monomer and dimer (30% dimer at 1 mg/ml) and may require dimerization to exhibit haemagglutinating activity (Allen *et al.*, 1978). If this is indeed the case then the considerations above will also apply to this situation. Chemical modification of

amino acids could possibly prevent them from fulfilling their role in dimerization.

The carbohydrate-binding site of potato lectin is able to interact with oligosaccharides containing β -1 $\rightarrow$ 4 linked *N*-acetylglucosamine residues. The maximum complementarity seems to be with the tetrasaccharide (Allen & Neuberger, 1973). An extended cleft was proposed for the binding site similar to that suggested by Allen *et al.* (1973) for wheat-germ agglutinin, a lectin with a similar specificity. It is assumed for the present study that the active centre of potato lectin is open to the aqueous environment and thus amino acids within the centre should be readily modified by reaction under relatively mild aqueous conditions. Reagents were chosen that were both highly specific and where their reaction could be performed in non-denaturing media. This approach should also prevent any gross structural deformation of the lectin and allow conclusions to be drawn concerning the available groups that may be modified, with respect to their involvement in binding and thus in the haemagglutinating activity of the lectin.

The active site(s) in potato lectin was reasoned to lie in the non-glycosylated domain(s) of the molecule by Allen *et al.* (1978). Evidence in favour of this was obtained when it was found that the glycopeptide produced by proteolysis from potato lectin was not capable of causing agglutination nor was it able to inhibit agglutination mediated by the intact lectin.

The non-glycosylated portion of potato lectin is highly cross-linked by disulphide bridges and these are assumed to be essential in maintaining the structure of the binding site (Allen *et al.*, 1978). In support of this assumption we have found that neither *S*-carboxymethylcysteinyl- nor *S*-aminoethylcysteinyl-potato lectin are able to agglutinate rabbit erythrocytes. Allen & Neuberger (1973) stated that there are no free thiol groups in potato lectin; this is confirmed by the low incorporation of radioactive label from iodo[14 C]acetic acid into the unreduced lectin at pH 8.6, equivalent to 0.25 carboxymethyl group per molecule. Even lower incorporation of carboxymethyl groups into the lectin was seen at pH 4.5 (0.16) and pH 2.9 (0.10), indicating that histidyl and methionyl groups are also unavailable to the reagent and are therefore unlikely to play a part in the haemagglutinating activity of the lectin. Histidine is not present in all preparations of the lectin and thus can also be excluded on these grounds, as it would be expected that active-site amino acids would be a constant feature of the primary sequence.

Modification of potato lectin with acetic anhydride

On reaction of potato lectin with acetic anhydride, an average of 5.1 amino groups were

modified (see Table 1) out of a total of ten amino groups per molecule of lectin, including the terminal α -amino group. This reagent can also modify phenolic hydroxy groups under the conditions used, and indeed 2.7 tyrosyl groups out of a total of eight per molecule of lectin were found to be unavailable to tetranitromethane after acetic anhydride treatment, indicating that they had been acetylated. After incubation in 1M-hydroxylamine, pH 7.5, which removes *O*-acyl but not *N*-acyl groups, the number of tyrosyl groups unavailable to tetranitromethane in the lectin decreased to 0.7 per molecule.

Treatment of potato lectin with citraconic anhydride

Treatment with citraconic anhydride as described by Dixon & Perham (1968) blocked 5.7 amino groups per molecule of lectin; subsequent treatment with 1M-hydroxylamine, pH 7.5, gave no change in the extent of modification. Tyrosine was not modified by this procedure. Citraconyl groups are labile to acid, and when citraconylated lectin was dialysed against 0.1M-acetic acid for 18h, the number of amino acids modified decreased as expected, but full reversal was not achieved (see Table 1).

Nitration of tyrosine

Tetranitromethane is specific for tyrosyl groups; when the lectin was treated with excess tetranitromethane, 3.7 out of eight tyrosyl groups per molecule of lectin were converted into nitrotyrosine (see Table 1). When nitration with tetranitromethane was repeated in the presence of 0.1M-*NN'*-triacetylchitotriose, to saturate the carbohydrate-binding site, no nitrotyrosine residues were detectable on subsequent amino acid analysis.

Modification of arginyl groups

The arginine guanido group is specifically modified by cyclohexane-1,2-dione, and the resulting complex can be stabilized by borate ions (Patthy & Smith, 1975). Blocking of two out of the three arginyl groups in the lectin was achieved by this procedure. Reversal of this modification can be accomplished by either nucleophilic attack or removal of borate by dialysis. Treatment with 1M-hydroxylamine, pH 7.5, regenerated one of the two modified groups. Exhaustive dialysis with the additional measure of the presence of a nucleophile (in this case Tris) gave complete reversal.

Modification of tryptophyl groups

2-Hydroxy-5-nitrobenzyl bromide (Koshland's reagent) was found to modify a single tryptophan residue, of the eight in potato lectin, when used at 60-fold molar excess over tryptophan (see Table 1). 2-Hydroxy-5-nitrobenzyl bromide is a highly reactive molecule and reacts with water to give the corresponding alcohol; it must therefore be made up

Table 1. *Extent of amino acid modification by chemical treatments*

Details of the chemical treatments and determination of the degree of modifications are given in the Experimental section. The numbers of amino acids per molecule of lectin are from Allen *et al.* (1978). The numbers of amino acid groups modified are expressed as the mean result for two or three separate experiments, except where otherwise indicated. The numbers in parentheses are the range of values obtained.

Chemical treatment of potato lectin	Type of groups modified	No. of groups per molecule of native lectin	No. of groups modified per molecule of lectin by treatment
Acetic anhydride	Lys ϵ -amino	9	5.1 (4.6–5.6)
	N-Terminal α -amino	1	
	Tyr hydroxy	8	
Citraconic anhydride	Lys ϵ -amino	9	5.7 (5.5–5.9)
	N-Terminal α -amino	1	
	Lys ϵ -amino	9	
Acetic anhydride followed by hydroxylamine, pH 7.5	N-Terminal α -amino	1	6.2, $n = 1$
	Tyr hydroxy	8	
	Lys ϵ -amino	9	
Citraconic anhydride followed by hydroxylamine, pH 7.5	N-Terminal α -amino	1	5.5 (5.3–5.5)
	Lys ϵ -amino	9	
	N-Terminal α -amino	1	
Citraconic anhydride followed by hydroxylamine, pH 7.5, then 0.1 M-acetic acid	Tyr hydroxy	8	1.7 (1.3–2.0)
	Lys ϵ -amino	9	
	N-Terminal α -amino	1	
Tetranitromethane	Tyr hydroxy	8	3.7 (3.2–4.1)
Tetranitromethane in presence of inhibitor*	Tyr hydroxy	8	0
Cyclohexane-1,2-dione	Arg guanido	3	2.0 (1.8–2.2)
Cyclohexane-1,2-dione followed by hydroxylamine, pH 7.5	Arg guanido	3	1.0 (0.9–1.0)
Cyclohexane-1,2-dione followed by 5 mM-Tris/HCl, pH 8.6	Arg guanido	3	0.2 (0.1–0.5)
2-Hydroxy-5-nitrobenzyl bromide	Trp indole ring	8	0.9 (0.9–1.0)
2,3-Dioxindoline-5-sulphonic acid	Trp indole ring	8	2.6 (2.5–2.6)
2,3-Dioxindoline-5-sulphonic acid in the presence of inhibitor*	Trp indole ring	8	0.2 (0.2–0.3)
Carbodi-imide, then α -aminobutyric acid methyl ester	Glu γ -carboxy	17†	5.3 (4.6–6.6)
	Asp β -carboxy	12†	
	C-Terminal α -carboxy	1†	
Carbodi-imide, then α -aminobutyric acid methyl ester in presence of inhibitor*	Glu γ -carboxy	17†	7.8 (7.4–8.6)
	Asp β -carboxy	12†	
	C-Terminal α -carboxy	1†	
Carbodi-imide, then α -aminobutyric acid methyl ester in 8 M-urea	Glu γ -carboxy	17†	7.1 (6.5–7.6)
	Asp β -carboxy	12†	
	C-Terminal α -carboxy	1†	
Carbodi-imide, then α -aminobutyric acid methyl ester followed by guanidination with <i>O</i> -methylisourea	Glu γ -carboxy	17†	4.9, $n = 1$
	Asp β -carboxy	12†	
	C-Terminal α -carboxy	1†	
Guanidination with <i>O</i> -methylisourea	Lys ϵ -amino	9	1.8, $n = 1$
	Lys ϵ -amino	9	4.1, $n = 1$

* *NN'N''*-Triacetylchitotriose.

† No allowance for the extent of amidation of these amino acid carboxy groups is made. See the text for full discussion.

in dry solvents and added repeatedly to the reaction mixture as it is soon consumed. Atassi & Zablocki (1976) described the use of a water-soluble reagent, 2,3-dioxindoline-5-sulphonic acid, that reacts specifically with tryptophan residues. This reagent when tested modified 2.6 of the eight tryptophan residues present in the lectin. Reaction with 2,3-dioxindoline-5-sulphonic acid, with 0.1 M-*NN'N''*-triacetylchitotriose present in the reaction mixture gave only a small extent of modification (see Table 1).

The reactivities of the amino acids concerned with the reagents described above can give indications of their location in the lectin molecule. For instance, only 50–60% of the amino groups in the lectin are freely reactive with the acid anhydrides, suggesting that the remainder are internal or hindered in some other way from reacting. Under the conditions used, all available amino groups should be modified. Acetic anhydride also modifies almost three out of eight tyrosine residues, which is in fairly close agreement with the value of approximately four

obtained by nitration. The fact that all of these tyrosine residues are protected from reacting with tetranitromethane by the presence of a specific inhibitor of the lectin seems to indicate that these tyrosine residues are either in the binding site of the lectin or are protected by a conformation change masking them from the reactive environment. The same argument can be applied in the case of modification of tryptophan residues. Koshland's reagent, perhaps because of its high reactivity with water, modified a single residue, whereas 2,3-dioxoindoline-5-sulphonic acid modified 2.6 residues per molecule of lectin. Almost complete protection was seen with the latter reagent when inhibitor was present. Arginyl groups appear to be distributed in a similar way to lysine groups, with two-thirds of the residues reacting; the reaction conditions in this case are such that a complex is formed that is easily disrupted if borate is removed, although the acid conditions of dialysis are supposed to maintain the complex in a form that allows quantification by amino acid analysis.

Reaction of carboxy groups with carbodi-imide, followed by coupling with α -aminobutyric acid methyl ester

1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide was used to activate free carboxy groups, in potato lectin, in the presence of 1M- α -aminobutyric acid methyl ester. The extent of the reaction can be determined by the number of non-dialysable α -aminobutyric acid methyl ester groups introduced into the molecule. The treatment led to the incorporation of an average of 5.3 α -aminobutyric acid methyl ester residues per molecule of lectin in five separate experiments. Attempts at protection from this modification led to an unexpected increase in the number of modified carboxy groups (see Table 1). This increase in the number of available carboxy groups could be mimicked by performing the modification reaction in 8M-urea. The small extent of modification of dicarboxylic amino acids by this method, considering that on amino acid analysis a total of 29 aspartic acid/asparagine + glutamic acid/glutamine residues were found, which with the C-terminal amino acid gives a possible 30 carboxy groups per molecule of lectin, suggests that the majority of the carboxy groups are present as amides. This was confirmed by the finding that only an average of 6.7 α -aminobutyric acid methyl ester residues (see Table 1) were incorporated per molecule of reduced, S-aminoethylated lectin under the conditions used by Hoare & Koshland (1967) for determination of total number of free carboxy groups. This is a similar number to that introduced into the unreduced lectin in 8M-urea and only slightly less than that seen with 0.1M-NN'N''-triacetylchitotriose (see Table 1). The degree of

amidation of carboxy groups is consistent with the observation of Allen *et al.* (1978) that potato lectin is a very basic protein with isoelectins with pI values varying from 9.4 to 9.6. A likely explanation of the discrepancy between apparent numbers of free carboxy groups under native and denaturing conditions, considering the nature of the reaction, is as follows: in the native situation several amino and carboxy groups in the protein are in close proximity to each other and perhaps involved in electrostatic interactions. Activation of the carboxy groups with carbodi-imide in these cases leads to reaction with the protein amino group rather than the amino group of α -aminobutyric acid methyl ester, resulting in intramolecular amide cross-linking. Denatured lectin, reduced or unreduced, undergoes a change in structure such that the reacting amino and carboxy groups are too far apart for the amide linkage to form, resulting in an increased incorporation of α -aminobutyric acid methyl ester residues, indicating that there are more free carboxy groups. Some weight can be added to this theory by the results of a single experiment where after modification of carboxy groups the modified lectin was treated with O-methylisourea to guanidinate lysine side chains to homoarginine (see Table 1). A value of 1.8 homoarginine residues per molecule was found, which if compared with the value of 4.1 residues of homoarginine per molecule obtained by guanidination of native, unmodified lectin, indicates that two lysine ϵ -amino groups per molecule of lectin are unavailable in the modified lectin. This in turn suggests that there are two intramolecular amides formed, if the previous postulate holds. This difference of two is also seen between the numbers of α -aminobutyric acid methyl ester residues introduced into the native and denatured lectins (see Table 1). From the above explanation and results it can be implied that the effect seen when the reaction with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide and α -aminobutyric acid methyl ester is performed in the presence of inhibitor, i.e. an increase in free carboxy groups, is due to a conformational change in the lectin on binding to substrate and that this also disrupts the close association of paired amino and carboxy groups.

Effect of chemical modifications on haemagglutinating activity of potato lectin

The assay of haemagglutinating activity relies on serial 2-fold dilutions of the lectin, and, because of this, a change of a single dilution represents a 2-fold increase or decrease in activity. To avoid ambiguities arising from dilution errors, significant inactivation was only considered to have taken place if the modified lectin had a haemagglutination titre that had decreased by three or more dilutions compared with the titre of the native lectin (i.e. a relative

activity of 12.5% or less). Table 2 lists the effects of the treatments on the ability of the lectin to agglutinate erythrocytes. As can be seen, a marked decrease in activity resulted from acetylation of the lectin with acetic anhydride, which could be reversed by treatment with 1M-hydroxylamine, pH 7.5, to de-*O*-acetylate modified tyrosine residues. This suggests that tyrosyl groups are important for haemagglutinating activity and that amino groups are probably not. Further evidence for the latter was obtained when citraconylation of amino groups gave no significant inactivation of the lectin (75% active). Nitration of tyrosine with tetranitromethane led to a loss of activity (<10% active), thus supporting the data obtained by de-*O*-acetylation of acetic anhydride-treated lectin. Yet more support was given to this by performing the nitration with a saturating concentration of inhibitor in the reaction mixture (see Table 3). None of the previous modification of tyrosine residues was seen and the lectin remained fully active. These results not only strongly implicate the involvement of tyrosine residues in the haemagglutinating activity of the lectin but also suggest that some, if not all, the tyrosyl groups previously available to the reagent are possibly involved at the binding site. Modification of arginyl groups has no significant effect on the activity of the lectin (>55% active). Modification of tryptophan residues, however, has a marked effect. With 2-hydroxy-5-nitrobenzyl bromide a single tryptophan residue of the eight in the lectin is modified and the specific activity of the modified lectin is

decreased to 6% of that of native lectin. Treatment with 2,3-dioxindoline-5-sulphonic acid modifies 2.6 tryptophan residues and has a similar inactivating effect. Protection from modification was seen with 2,3-dioxindoline-5-sulphonic acid in the presence of specific inhibitor (see Table 3) and the lectin retained activity. Again this implies that not only are the tryptophyl groups, which are modified, involved in activity, but that the two groups protected by inhibitor may lie in the binding site. The data from the blocking of carboxy groups present a less clear picture. Haemagglutinating activity was greatly decreased by treatment with carbodi-imide and α -aminobutyric acid methyl ester and protection experiments showed that inhibitor prevented this reduction. However, treatment in the presence of inhibitor led to an increase in the number of modified carboxyl residues (see Table 1). As discussed above, it is possible that there is formation of intramolecular amide links by the carbodi-imide activation of carboxy groups. This could effectively lock the lectin into an inactive conformation and prevent any conformation changes (necessary to or brought about by binding of substrate) that stabilize the interaction, thus decreasing the haemagglutinating activity of the lectin. Pretreatment with inhibitor could prevent this inactivation, not by protecting groups from modification, but by bringing about the conformational change that breaks the envisaged interaction, thus preventing the cross-linkages being formed. Further evidence for this is provided by the observations that a known denaturant, 8M-urea, has

Table 2. *Effect of chemical modifications on haemagglutinating activity of potato lectin*

Details of the chemical treatments and the determination of haemagglutinating activity are given in the Experimental section. Haemagglutinating activity is expressed as a percentage of that given by native, unmodified lectin under the same conditions. Due to the nature of the assay, significant inactivation was considered to have taken place only when the modified lectin showed an activity of 12.5% or less than that of native lectin. Activities given as greater than (>) and less than (<) the given values are the lowest and highest activities obtained from repeated assays of these samples respectively.

Treatment to lectin	Haemagglutinating activity (%)
Reduction and carboxymethylation	0
Reduction and aminoethylation	0
Acetylation	4
Citraconylation	75
Acetylation then de- <i>O</i> -acetylation	>55
Citraconylation then de- <i>N</i> -citraconylation	100
Nitration	<10
Cyclohexane-1,2-dione	>55
Cyclohexane-1,2-dione reversed with hydroxylamine	>52
Cyclohexane-1,2-dione reversed with Tris	100
2-Hydroxy-5-nitrobenzyl bromide	6
2,3-Dioxindoline-5-sulphonic acid	4
Activation with carbodi-imide then reaction with α -aminobutyric acid methyl ester	2
Activation with carbodi-imide and then reaction with α -aminobutyric acid methyl ester in 8M-urea	20

Table 3. *Effect of NN'N''-triacetylchitotriose on the extent of modification and inactivation of potato lectin*

Potato lectin was treated as described in the Experimental section but with the inclusion of 0.1 M-NN'N''-triacetylchitotriose in the reaction mixture. Results are expressed as in Tables 1 and 2. Inhibitor was removed by dialysis against 1% (v/v) acetic acid before determination of haemagglutinating activity. A negative value (*) indicates an increase in amino acid modification under these conditions. See the text for full discussion.

Chemical treatment	Type of amino acid modified	No. of amino acids modified per molecule of lectin	No. of amino acids protected by chitotriose	Haemagglutinating activity (%)
2,3-Dioxindoline-5-sulphonic acid	Trp	0.2 (0.2–0.3)	2.4	>48
Tetranitromethane	Tyr	0	3.7	100
Activation with carbodi-imide then reaction with α -aminobutyric acid methyl ester	Glu and Asp	7.8 (7.4–8.6)	–2.5*	>54

a slight protective effect against inactivation by this treatment (see Table 2) and that, in addition, the number of α -aminobutyric acid methyl ester groups incorporated into the lectin is increased compared with the identical modification reaction performed on the lectin in the absence of denaturant. The data presented for modification of carboxy groups do not seem to imply the essential nature of free carboxy groups to the activity of the lectin, as would be expected if they were involved in the binding site.

Interaction of modified lectin with anti-(potato lectin) serum

Samples of lectins from each of the modification procedures were tested against anti-(potato lectin) serum by double immunodiffusion. All solutions of modified lectin gave a positive interaction with the antiserum to the whole lectin. Reduction and alkylation of disulphide bridges renders the lectin inactive and disrupts the secondary structure. *S*-Aminoethylcysteinyl- and *S*-carboxymethylcysteinyl-potato lectin can therefore no longer interact with antisera that have been precipitated with potato-lectin glycopeptide. All other lectin derivatives were still able to give a precipitation line with the antiserum to the non-glycosylated portion of potato lectin. Therefore no structural changes sufficient to disrupt the antigenic sites were brought about by the modification methods employed.

Comparison of the results obtained by chemical modification of potato lectin with those of other carbohydrate-binding proteins

Krop-Watorek *et al.* (1980), in their study of the chemical modification of potato lectin, suggest the involvement of amino groups and tyrosyl groups in lectin activity. The results obtained in our own study also point to the involvement of tyrosine, but not of amino groups. In addition, our results implicate tryptophan in the carbohydrate-binding site of the lectin. The data presented here also provide evi-

dence for the non-involvement of arginyl, methionyl, histidyl and carboxy groups in binding, although the last do seem to be involved in a structural or conformational role through intramolecular electrostatic interactions with lysine amino groups. Comparison of our data with those published for other proteins that bind oligomers of *N*-acetylglucosamine show some similarities and some marked differences. Study of wheat-germ agglutinin by Rice & Etzler (1975) showed that amino groups did not seem to be essential to activity, but that modification of tyrosyl and carboxy groups led to a decrease in haemagglutination titre. The latter results were complicated by a dissociation of the lectin dimer, which could account for the loss in activity seen, especially as Wright (1980) has indicated that the binding sites of wheat-germ agglutinin are dependent on co-operation between protomers.

Privat *et al.* (1976) studied the effect of *N*-bromosuccinimide on wheat-germ agglutinin and found that modification of one tryptophan residue per subunit caused a loss in haemagglutinating activity and a decrease in the affinity for NN'N''-triacetylchitotriose. Treatment of the agglutinin in the presence of specific oligosaccharide inhibitors required higher concentrations of *N*-bromosuccinimide to achieve the same degree of modification as in their absence. This suggests at least a partial protective effect of oligosaccharide inhibitors towards tryptophan, in turn supporting suggested involvement in the lectin binding site. A review of the results of chemical modification of constituent amino acids in vertebrate lysozymes (Imoto *et al.*, 1972) reported that amino, arginyl and histidyl groups did not seem to be required for binding of oligosaccharides of the enzyme. Tryptophyl and carboxy groups, however, were found to be essential for binding and thus the activity of the enzyme. The involvement of these groups has also been shown by X-ray-crystallographic techniques. Both types of

amino acids were found to be in intimate contact with the substrate (Blake *et al.*, 1967).

Thus of these three proteins that possess similar specificities and have been studied in depth, all have in common the involvement of tryptophan; potato lectin and wheat-germ agglutinin have tyrosine implicated and lysozyme and wheat-germ agglutinin both have carboxy groups involved in their respective activities. It might be safe to speculate that similarities in the modes of binding of *N*-acetylglucosamine oligomers to these proteins may be found when full sequence and X-ray-crystallographic studies are performed on the lectins to complement what is already established for lysozyme.

A variety of other lectins have been studied and the involvement has been suggested, in binding of carbohydrate and haemagglutinating activity, of: carboxy groups in concanavalin A (Hassing *et al.*, 1971) and *Bandeiria simplicifolia* lectin (Lönngren & Goldstein, 1976); tyrosine in soya-bean (*Glycine max*) agglutinin (Liener & Wada, 1956), lentil (*Lens esculenta*) lectin (Vančurová *et al.*, 1976) and the lectin from *Viscum album* (mistletoe) (Ziska *et al.*, 1979); tryptophan in concanavalin A (Hassing & Goldstein, 1972) and *Pisum sativum* (pea) lectin (Čermáková *et al.*, 1976). From this it would seem that only a limited number of types of amino acids have been found to be involved in the active sites of carbohydrate-binding proteins. It is not surprising that these amino acids (tryptophan, tyrosine, aspartic acid and glutamic acid) are those that are capable of participating in hydrogen-bonding with hydroxy and acetamido groups in sugars. The present results show that the potato lectin active-site amino acids fit into this general trend for carbohydrate-binding proteins and are also very similar to those of other proteins with a similar specificity.

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