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Studies on Chemical Modification of *Tulipa gesneriana* Lectin

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Modification of lysine, tyrosine, histidine, aspartic acid and glutamic acid residues did not affect the agglutinating activity of the *Tulipa gesneriana* lectin (TGL). Modification of two arginine residues per subunit in the lectin with either 2,3-butanedione or phenylglyoxal led to an almost complete loss of activity. An inactive lectin modified with 2,3-butanedione recovered a full activity on dialysis against Tris–HCl buffer. The presence of 0.1 M (α-1→6) linked mannotriose, a potent inhibitor of the lectin, protected all the arginine residues from modification and the lectin was fully active. Circular dichroism spectroscopy showed that no significant conformational change of TGL occurred following arginine modification.

A treatment of the lectin solution with *N*-bromosuccinimide or 2-hydroxy-5-nitrobenzyl bromide, chemical reagents for tryptophan modification, caused turbidity of the solution, accompanied with complete loss of activity. The fluorescence emission spectrum of the lectin showed a characteristic tryptophan emission with a maximum centered at 336 nm. Upon addition of manno-oligosaccharides a decrease of the fluorescence intensity was observed, indicating that the environment of tryptophan residues altered.

These results suggest that arginine and tryptophan residues are importantly involved in the sugar binding of TGL.

Keywords chemical modification; amino acid residue; lectin; tulip bulb

Two lectins with different agglutinating activity exist in *Tulipa gesneriana* bulbs. One is the *T. gesneriana* lectin (TGL) which agglutinates yeasts[1] and the other is the *T. gesneriana* agglutinin (TGA) which agglutinates animal erythrocytes.[2] TGL consists of four identical subunits of M_r 17000. The lectin preferentially agglutinates or binds to yeast cells of *Saccharomyces* genus but not other yeast cells or animal erythrocytes. The sugar specificity of TGL is similar to those of typical manno-specific lectins such as concanavalin A, *Lens culinaris* lectin and *Pisum sativum* agglutinin, but TGL differs from these lectins in several respects. In particular, TGL can distinguish yeast mannans of *Saccharomyces* genus from those of *Candida* genus. As far as we know, no lectins except TGL have this ability.

Lectins bind carbohydrates at particular sites of the lectin molecules. In order to understand how lectins bind to carbohydrates, it is important to define the binding sites of the lectins. Therefore, we attempted to elucidate the amino acid residues required for carbohydrate-binding activity of TGL by chemical modification and spectrometric studies.

Experimental

Materials Phenylglyoxal monohydrate and tetranitromethane were purchased from Aldrich. Ethoxyformic anhydride and α-aminobutyric acid methyl ester were obtained from Sigma. Ethyl-3-(3-dimethylaminopropyl)carbodiimide and maltotriose were obtained from Peptide Institute Inc. and Seikagaku Kogyo (Japan), respectively. Sephadex G-25 was purchased from Pharmacia Fine Chemicals. Bio gel P-2 and P-60 were products of Bio-Rad Laboratories.

Preparation of TGL TGL was purified by using affinity chromatography on mannan-Sepharose 4B as described previously.[1] The purified lectin used in this study showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Preparation of Manno-oligosaccharides Mannan was isolated from baker's yeast by extraction with citrate buffer, pH 7.0, followed by copper complex formation.[3] A mixture of a series of linear oligosaccharides with (α-1→6)-linked mannose units was obtained from baker's yeast mannan according to the method of Lee and Ballou.[4] Mannobiose, -triose, and -tetraose were isolated from the mixture by gel filtration on a Sephadex G-25 (super fine) column.

Assay of Agglutinating Activity Agglutinating activity was determined using serial twofold dilutions of lectin solution in microtiter plates with a cell suspension of *Saccharomyces cerevisiae*.[1] Agglutination was observed 30 min later. The titer of TGL solution (1 mg/ml) was 64. When a modified lectin showed no agglutination even at a concentration of 1 mg/ml, we concluded that the modified lectin had completely lost the activity.

Amino Acid Analysis Samples were hydrolyzed for 24 h at 110 °C in 6 M HCl. Amino acid analysis was carried out with a Hitachi amino acid analyzer, model 835.

Determination of the N-Terminal Amino Acid The N-terminal amino acid of the lectin was determined by the dansyl chloride method[5] and (4-*N*,*N*-dimethylaminoazobenzene-4-isothiocyanate (DABITIC) method.[6]

Modification of Carboxyl Groups Modification of carboxyl groups in the lectin was performed using water-soluble carbodiimide according to the method of Carraway and Koshland.[7] An aliquot (100 μl) of 1 M ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) was added to 1 ml of a lectin soluion (2 mg/ml) in 1 M α-aminobutyric acid methyl ester, pH. 4.5. The mixture was stirred at room temperature and the pH was maintained at pH 4.75 by addition of HCl. After 6 h the solution was dialyzed exhaustively against distilled water and lyophilized. The lyophilized material was subjected to agglutinating assay and amino acid analysis. For determination of the total number of free carboxyl groups, the lectin was dissolved in 8 M urea containing 1 M α-aminobutyric acid methyl ester and was treated with EDC for 1 h as described above.

Modification of Lysine Residues TGL was modified with *O*-methylisourea following the method of Kimmel.[8] Lectin solution (2 mg/ml) in distilled water was mixed with an equal volume of 1.0 M *O*-methylisourea at pH 10.5 (adjusted with NaOH). The reaction was allowed to proceed for 4 d and 4 °C and stopped by dialysis against distilled water. The extent of modification was estimated from the homoarginine content of the modified lectin hydrolysate.

Modification of Histidine Residues TGL was reacted with ethoxyformic anhydride by a modification of the procedure of Rogers *et al.*[9] Ethoxyformic anhydride was added to a lectin solution (1 mg/ml, 0.1 M sodium phosphate buffer, pH 6.0) to give a final concentration of 3-fold molar excess over the histidine content. The reaction was allowed to proceed with stirring at room temperature and 50 μl aliquots were withdrawn from reaction mixture for agglutinating assay. The extent of modification was determined as described by Anderson and Ebner.[10]

Modification of Tyrosine Residues Modification of tyrosine residues was carried out by allowing of a 10-fold molar excess of 10% tetranitromethane in ethanol to react with a lectin solution (2 mg/ml) in 0.05 M Tris–HCl, pH 8.0 for 4 h at room temperature.[11] The reaction was terminated by passing the mixture through a Sephadex G-25 column equilibrated with 1% acetic acid. The degree of modification was determined spectrophotometrically and by amino acid analysis.

Modification of Tryptophan Residues Modifications of tryptophan residues of lectin were carried out at room temperature with *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide. Modification with *N*-

bromosuccinimide was performed following the method of Spande and Witkop[12]; 10 μl of 0.02 M *N*-bromosuccinimide solution in water was added to 1 ml of a lectin solution (2 mg/ml) in 0.1 M citrate buffer, pH 6.0. Modification with 2-hydroxy-5-nitrobenzyl bromide was carried out at room temperature according to the method of Barman and Koshland[13]; 100 μl of 2-hydroxy-5-nitrobenzyl bromide solution in dry acetone (50 mg/ml) was added to 1 ml of a lectin solution in 0.1M sodium acetate buffer, pH 5.0 (2 mg/ml).

Modification of Arginin Residues Modifications of arginine residues were carried out with phenylglyoxal and 2,3-butanedione. Modification with phenylglyoxal was performed following the method of Takahashi.[14] A freshly prepared phenylglyoxal solution in 0.1 M *N*-ethylmorpholine acetate buffer, pH 8.0, was added to 1 ml of a lectin solution (2 mg/ml) in the same buffer to give a final concentration of 20 mM. The mixture was incubated at room temperature in the dark and 50μl aliquots were pipetted out at given intervals for agglutination assay after addition of 5μl of 400 mM arginine solution. For the amino acid analysis, unreacted phenylglyoxal was removed from the reaction mixture by gel filtration on a Bio gel P-2 column (1 × 50 cm) equilibrated with 1 M acetic acid. After evaporation of acetic acid from the eluates, the residues were subjected to amino acid analysis to determine the extent of the modification. Modification of arginine residues with 2,3-butanedione was carried out according to the method of Riordan.[15] Reaction was carried out at room temperature by adding an aliquot of 1 M butanedione in 0.05 M borate buffer, pH 8.0, to a lectin solution (2 mg/ml) in the same buffer. Aliquots (50 μl) of the reaction mixture were removed at given intervals and assayed for agglutinating activity after addition of 5 μl of 400 mM arginine solution. The reaction mixture was desalted by passage through a Bio gel P-2 column (1 × 50 cm) equilibrated with 20% acetic acid and lyophilized after dialysis against 1% acetic acid at 4 °C. The lyophilized material was subjected to amino acid analysis to determine the extent of the modification. The reversibility of arginine modification with butanedione was examined. Lectin modified with butanedione was dialyzed against 0.05 M Tris–HCl buffer, pH 8.7, and further against distilled water at 4 °C to remove borate. A lectin control was run under the same conditions.

Protection by Mannotriose Arginine modification with 2,3-butanedione was carried out in the presence of 0.1 M (α-1→6)-mannotriose. After 1 h of incubation the reaction mixture was applied to a Bio gel P-2 column and was lyophilized after dialysis against 1% acetic acid and distilled water at 4 °C.

Spectroscopic Measurements Emission spectra of the lectin induced by excitation at 280 nm were recorded with a Hitachi F-3000 fluorescence spectrophotometer. The lectin was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl and was filtered on 0.45 μm filter (Toyo Roshi, Japan), The protein concentration was about 1 μM so that the absorbance at 280 nm was below 0.1. The solutions were contained in 1 cm quartz cells maintained at 25 °C. Emission spectra were corrected with rhodamine B before measurements.

Circular dichroism spectra were recorded on a Jasco J-500A spectropolarimeter between 200 and 320 nm. The path length of the cells was 1 cm. Native and modified lectins were dissolved in 50 mM borate buffer, pH 8.0.

Results and Discussion

N-Terminal Amino Acid Analysis No N-terminal amino acid was detected by dansyl chloride method or DABITIC method, suggesting a blocked N-terminus.

Modification of Carboxyl Groups, Histidine, Lysine and Tyrosine Residues Extents of modification of carboxyl groups, histidine, lysine and tyrosine residues by chemical treatments are shown in Table I. Chemical modification of carboxyl groups in the presence or absence of 8 M urea led to the incorporation of 6.5 and 5.9 α-aminobutyric acid methylester residues per subunits, respectively. The modified lectin showed the same agglutinating activity as that of the native lectin. The modifications of histidine, lysine and tyrosine residues also did not affect the agglutinating activity of the lectin, indicating that carboxyl groups and these residues did not play important roles in the sugar binding of the lectin.

Modification Tryptophan Residues TGL contains three tryptophan residues per subunits. Addition of *N*-bromosuccinimide or 2-hydroxy-5-nitrobenzyl bromide to a lectin solution caused turbidity of the solution and a complete loss of lectin activity within 5 min. It seemed that tryptophan residues were important to maintain the active structure of the lectin molecule.

Spectrofluorometric Studies On interaction with specific sugars several lectins exhibit a change of the fluorescence spectrum, which indicates an interaction between tryptophan residue(s) of the lectin molecule and the bound sugar.[16-18] We therefore attempted to examine the involvement of tryptophan residues in the sugar binding of TGL by spectrofluorometric studies. The fluorescence emission spectrum of the lectin showed a characteristic tryptophan emission with a maximum centered at 336 nm. Upon addition of (α-1→6)-linked mannotriose, a potent inhibitor of the lectin, a decrease of the fluorescence intensity was observed. Typical fluorescence spectra of the lectin in the presence of mannotriose are shown in Fig. 1. When the lectin was saturated with 20 mM mannotriose the maximum fluorescence intensity was decreased by 30%. Mannobiose and mannotetraose with α-1→6 linkages changed the fluorescence spectra of the lectin in a similar

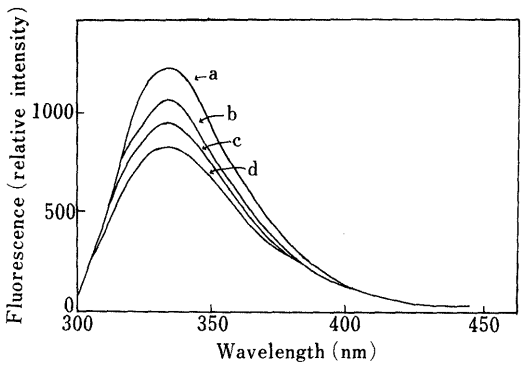

Fig. 1. Fluorescence Spectra of *T. gesneriana* Lectin in the Presence or Absence of Mannotriose

a, lectin only; b, lectin + 5 mM mannotriose; c, lectin + 10 mM mannotriose; d, lectin + 20 mM mannotriose.

TABLE I. Extent of Amino Acid Modification

Chemical reagents[a]	Residues modified	Number of residues per subunit of native lectin	Number of residues modified per subunit of modified lectin
O-Methylisourea	Lysine	8[b]	6.9
Tetranitromethane	Tyrosine	6[b]	4.4
Ethoxyformic anhydride	Histidine	2[b]	0.8
α-Aminobutyric acid	Aspartic acid and glutamic acid	6.5[c]	5.9

a) Chemical modifications were performed as described in the experimental section. *b*) The numbers of amino acid residues per subunit of native lectin are cited from ref. 1. *c*) Total number of residues per subunit of native lectin was determined in the presence of 8 M urea.

Table II. Effect of Modification of Arginine Residues on Agglutinating Activity of *Tulipa gesneriana* Lectin

Chemical treatment	Number of residues[a] modified per subunit	Agglutinating activity[b]
Phenylglyoxal	1.9	1/16
Butanedione	2.0	1/16
With 50 mM mannotriose	0.4	1
Dialysis against Tris–HCl buffer	0.8	1

a) The number of modified residues was determined by amino acid analysis as described in the experimental section. *b*) Agglutinating activity was compared with that of native lectin. The data represents the mean of three separate experiments.

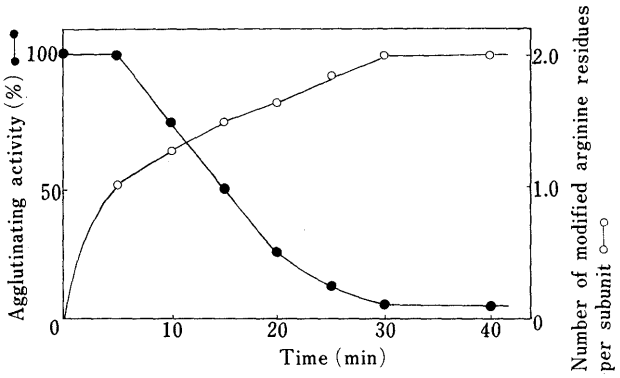

Fig. 2. Inactivation of *T. gesneriana* Lectin by 20 mM Butanedione

The lectin (2 mg/ml) in 50 mM borate buffer, pH 8.0, was incubated with 20 mM 2,3-butanedione at room temperature, and 50 μl aliquots were withdrawn at given intervals for the agglutination assay. The extent of modification was determined by amino acid analysis as described in the experimental sections. Each point represents the mean of three separate experiments.

manner to mannotriose, but D-mannose caused no significant change of fluorescence spectrum even at a concentration of 100 mM. There was no clear relationship between capacity to decrease the maximum fluorescence intensity of the lectin and inhibitory capacity toward the lectin activity of these manno-oligosaccharides. However, maltotriose, which is not an inhibitor of the lectin, caused no significant change of the fluorescence spectrum of the lectin even at a concentration of 50 mM. These results indicated that the environment of tryptophan residues was altered by manno-oligosaccharides, that is, tryptophan residues seemed to be involved in the sugar binding of TGL.

Modification of Arginine Residues Reaction of the lectin with 20 mM phenylglyoxal or butanedione modified two of four arginine residues per subunit and was accompanied with an almost complete loss of activity (Table II). The time course of activity during modification with butanedione is shown in Fig. 2. Agglutinating activity was lost progressively, but it was not abolished completely even after 40 min. Complete inactivation and further modification were not achieved even when the modification reaction was carried out for 2 h with 40 mM butanedione.

Amino acid analyses showed that no residues other than arginine were modified by these reagents. In order to examine whether the inactivation of the lectin by the modification was due to a dissociation of the lectin to its subunits, the modified lectin was subjected to gel filtration. The results confirmed that the lectin was not dissociated to its subunits by the modification with butanedione or phenylglyoxal (data not shown).

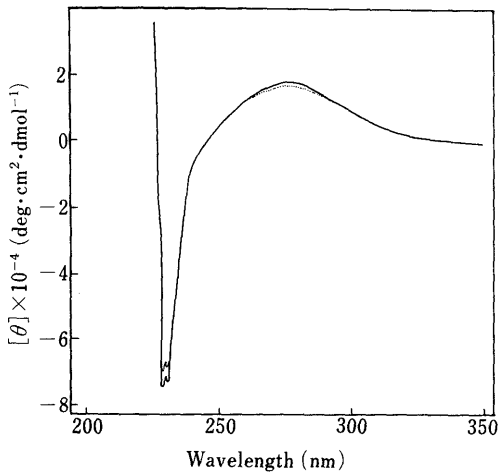

Fig. 3. Circular Dichroism Spectra of Native and Arginine Modified Lectin

——, native lectin; ------, arginine modified lectin.

The circular dichroism spectrum of the modified lectin was essentially identical with that of native lectin, suggesting that no gross conformational change occurred as a result of the modification of two arginine residues per subunit in the lectin (Fig. 3).

In the presence of a potent inhibitor, (α-1→6) mannotriose, arginine residues of the lectin were protected from modification by butanedione and the lectin retained full activity (Table II). Modification of arginine residues in protein by butanedione is known to be reversible on removal of excess butanedione and borate.[15] Therefore, reactivation experiments were performed to demonstrate that inactivation of lectin was not due to denaturation. Dialysis of the modified lectin against Tris–HCl buffer restored the full activity, but only one of the two modified arginine residues per subunit was reappeared (Table II). These results suggested that two arginine residues in the lectin subunit were exposed on the protein surface, and one of the two arginine residues was involved in the sugar binding of TGL.

Several amino acids residues such as cystein,[19] histidine,[20] tryptophan,[16,21,22] tyrosine,[23-25] aspartic acid and glutamic acid[24,26,27] have been reported to be involved in the sugar binding of lectins. Little information on the role of arginine residue in the sugar binding of lectins has been obtained,[28] though arginine residue are known to be essential for the biological activities of many enzymes and hormones. This study shows that arginine and tryptophan residues play important roles in the agglutinating activity (sugar binding) of TGL.

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