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Chemical Modification Studies on *Ricinus communis* (Castor Bean) Agglutinin

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Ricinus communis agglutinin was subjected to various chemical treatments and the effect on its hemagglutinating and saccharide-binding properties was studied.

Acetylation, succinylation and citraconylation led to a complete loss in the activity of the agglutinin, whereas reductive methylation had no effect on the activity, showing that charged amino groups were involved in the hemagglutinating and saccharide-binding activity of *Ricinus* agglutinin.

Modification of tryptophyl, arginyl and carboxyl-group-containing residues did not lead to any loss in the activity of the agglutinin.

Acetylation of tyrosyl groups with *N*-acetylimidazole strongly reduced the hemagglutinating and saccharide-binding property of *Ricinus* agglutinin. The loss in activity was restored on deacetylation of the tyrosyl groups. Modification of tyrosyl residues also led to a change in the immunological properties of the agglutinin.

The initial rate of modification of tyrosyl and amino groups and the concomitant loss of activity was reduced in the presence of lactose.

Lectins, the carbohydrate-binding proteins, initiate several biological activities in cells, such as mitogenicity, preferential agglutination of tumor cells as compared to the normal cells [1–5]. The agglutinin from castor bean (*Ricinus communis* agglutinin) is a tetrameric glycoprotein with a molecular weight of 120000 and consists of two pairs of non-identical subunits, α_2 and β_2 [6]. The α subunits are linked to the β subunits through disulphide bonds. It has two binding sites for its specific sugar located on the β subunits [7]. It has been used to isolate galactose-containing biopolymers and for the resolution of microheterogeneity in glycoproteins [8].

Although the biological properties of *Ricinus* agglutinin has been studied extensively, the relationship between its structure and function has not been investigated. The identification of specific amino acid residues within the active sites of biopolymers is important for understanding the relationship between their biological activity and structure. Chemical modification are among the methods for identifying at least some of the essential residues [9]. In the present study we carried out chemical modifications of a number of amino acid residues of *Ricinus* agglutinin to determine the essential amino acid residues required for its sugar binding activity. The chemical modification studies indicate that at least two lysyl residues and one tyrosyl residue per average subunit are involved in the sugar-binding property of *Ricinus* agglutinin.

MATERIALS AND METHODS

Agglutinin and Anti-agglutinin

Ricinus communis agglutinin was prepared from locally available castor bean as described by Appukuttan et al. [10]. Antiserum against *Ricinus* agglutinin was obtained from rabbits immunized with formaldehyde-treated agglutinin as described for ricin [11]. Protein was estimated by precipitation with trichloroacetic acid as described earlier [12].

Chemicals

All reagents used were of analytical grade. Succinic anhydride, citraconic anhydride, *N*-acetylimidazole, 1,2-cyclohexanedione and 2-hydroxy-5-nitrobenzyl bromide were obtained from Pierce Chemical Co. [^{14}C]Acetic anhydride (88.5 mCi/mg) was obtained from Bhabha Atomic Research Centre, India. Sephadex G-100 and G-25 were purchased from Pharmacia (Sweden). Formaldehyde and sodium borohydride were obtained from British Drug Houses (England). [^{14}C]Glycine methyl ester (5 Ci/mol) was prepared according to Hassing et al. [13]. Biogel P-200 was purchased from Bio-Rad Laboratories.

Immunodiffusion

Ouchterlony plates were made up from 2% agarose in 0.14 M NaCl containing 20 mM sodium phosphate (pH 7.4), 20 mM lactose and 0.2% NaN_3. Lactose was added to avoid interaction between the lectin and serum glycoproteins containing terminal galactose [14] as well as agarose itself.

Measurement of Radioactivity

Radioactivity was measured in a Packard Tri-Carb C-2423 scintillation counter. Radioactivity of ^{14}C-labelled proteins was measured using toluene-based scintillation fluid and Triton X-100 in the ratio 2:1.

Hemagglutination Tests

The agglutinin preparations (0.1 mg/ml) were serially diluted in a microtitre plate and 0.1 ml of a 2% preparation of rabbit erythrocytes was added. After incubation at 25 °C for 2 h, the agglutination was measured visually. The percentage agglutination was calculated from the titre values obtained with the native lectin and by the modified lectin preparation.

Binding to Guar Gum and Sepharose 6B

The binding of *Ricinus* agglutinin with guar gum was studied turbidimetrically as described earlier [15]. The percentage binding of the modified lectin preparations were obtained from the turbidity readings of the native and modified lectin. Lectin binding to Sepharose 6B was monitored by its retention on this affinity matrix, as described elsewhere [7].

Acetylation with [^{14}C]Acetic Anhydride

Acetylation of *Ricinus* agglutinin with [^{14}C]acetic anhydride was performed as described earlier [16]. To 1 ml *Ricinus* agglutinin (10 mg/ml) in saturated sodium acetate, was added five aliquots (8 µl each) of [^{14}C]acetic anhydride (1.77mCi/mg) over the course of 1 h. The number of acetyl groups incorporated was estimated from the radioactivity associated with the protein.

Reductive Methylation

Reductive methylation was carried out as described by Means and Feeney [17]. To 2 ml of a 3-mg/ml solution of *Ricinus* agglutinin in 0.2 M borate buffer (pH 9.0) at 0 °C was added 1 ml of a 0.5-mg/ml sodium borohydride solution followed by six aliquots (5 µl each) of 3.5% formaldehyde at intervals of 10 min. The procedure was repeated twice and excess reagent removed by dialysis against 20 mM sodium phosphate (pH 7.4) at 4 °C. The total number of free amino groups in the native protein was estimated to be 44 with reference to bovine serum albumin by trinitrobenzene sulfonic acid according to the method of Habeeb [18]. The number of amino groups modified was determined by estimation of remaining free amino groups by the same reagent.

Citraconylation and Decitraconylation

The amino groups of *Ricinus* agglutinin (5 mg/ml) were reversibly blocked by citraconic anhydride (50 mg/ml) at pH 8.0 according to the method of Dixon and Perham [19]. The number of amino groups modified was estimated as described for reductive methylation. The reaction was reversed by leaving the citraconylated protein overnight at 4 °C at pH 3.0.

Succinylation

Succinylation was carried out according to the method of Habeeb et al. [20]. *Ricinus* agglutinin (5 mg/ml) in saturated carbonate (pH 8.0) was incubated at 0 °C for 1 h with a 300-molar excess of succinic anhydride. The mixture was dialysed against 20 mM sodium phosphate (pH 7.4) and the number of modified groups was calculated as described earlier. The time-dependent succinylation of *Ricinus* agglutinin in the presence and absence of lactose was carried out similarly and aliquots were withdrawn at different time intervals. The excess reagent was separated by gel filtration over a column of Sephadex G-25 equilibrated with 20 mM sodium phosphate (pH 7.4).

Modification of Tryptophyl, Arginyl and Carboxyl Residues

Modification of tryptophyl, arginyl and carboxyl residues was carried by 2-hydroxy-5-nitrobenzyl bromide [21], 1,2-cyclohexanedione [22] and [^{14}C]glycine methyl ester [23], respectively.

Acetylation with N-*Acetylimidazole and Subsequent Deacetylation*

The acetylation was carried out as described by Riordan et al. [24]. To *Ricinus* agglutinin (3 mg/ml) in 0.01 M sodium phosphate (pH 7.5) a 1000-fold molar excess of *N*-acetylimidazole was added and kept at room temperature for 1 h.The *O*-acetyl groups introduced in the protein were removed by hydroxylamine as described earlier [23]. From the difference in absorbance of *N*,*O*-diacetylated protein and *N*-acetylated protein the number of tyrosyl groups modified was calculated [24]. The time-dependent acetylation of citraconylated agglutinin was carried out as described above. Aliquots were withdrawn from the reaction mixtures at different time intervals and desalted by gel filtration on Sephadex G-25 equilibrated with 20 mM sodium phosphate. The protein samples were decitraconylated, deacetylated and tested for activity. The time-dependent acetylation of reductively methylated protein in the presence of lactose was carried out similarly.

Molecular Weight Determination by Gel Filtration

Molecular weight of succinylated, reductively methylated, citraconylated and decitraconylated *Ricinus* agglutinin was determined on a Biogel P-200 column (2.0 × 120 cm) calibrated with the markers pepsin, ovalbumin, bovine serum albumin monomer and dimer, soybean agglutinin and immunoglobulin G.

RESULTS

Effect of Modification of Free Amino Groups

Acetic anhydride, succinic anhydride and citraconic anhydride modified 75–80% of the free amino groups of *Ricinus* agglutinin leading to a complete loss of its polysaccharide-binding and agglutinating activity (Table 1). However, reductive methylation of the free amino groups did not have much effect on the activity and the agglutinin retained 90% of its activity even when 83% of the amino groups were modified. The modification of the amino groups of the agglutinin did not lead to any change in the molecular weight (Fig. 1). Decitraconylation at pH 3.0 brought about nearly complete restoration of the polysaccharide binding and agglutinating activity of *Ricinus* agglutinin (Table 1). The time-dependent modification of the amino groups of *Ricinus* agglutinin by succinic anhydride showed that a nearly 50% loss in its activity took place when only four amino groups in the tetrameric protein were modified (Fig. 2). The presence of lactose decreased the rate of modification of these residues but did not provide complete protection against modification. The relative loss in the activity of the agglutinin with increasing number of amino groups modified indicated that at least two amino groups per average subunit were involved in polysaccharide binding and agglutinating activity (Fig. 3). Immunodiffusion studies showed that the amino group modification of the agglutinin by all the above reagents did not detectably influence the interaction of the agglutinin with its specific antibody (Table 1).

Effect of 2-Hydroxy-5-nitrobenzyl Bromide, 1,2-Cyclohexanedione and [^{14}C]Glycine Methyl Ester

Modification of tryptophyl residues by 2-hydroxy-5-nitrobenzyl bromide in the absence and presence of urea leads to

Table 1. *Effect of NH_2 group modification of* Ricinus *agglutinin*
The protein was treated with different reagents which modify the NH_2 groups and the number of residues modified was measured by determining the percentage of unmodified NH_2 groups by trinitrobenzene sulfonate. The indicated properties of the treated agglutinin were tested as described in the Materials and Methods. The activity is expressed as the percentage of chemically untreated agglutinin

Ricinus agglutinin	Residues modified	Immunochemical properties	Molecular weight	Hemagglutination	Binding to	
					guar gum	Sepharose
	mol/mol protein			%		
Acetylated	29	unchanged	unchanged	0	0	0
Succinylated	34.6	unchanged	unchanged	0	0	0
Citraconylated	35	unchanged	unchanged	0	0	0
Decitraconylated	0.5	unchanged	unchanged	88	70	93
Reductively methylated	29.6	unchanged	unchanged	85	87	95
Acetylated by *N*-acetylimidazole	19	minor change	unchanged	30	10	42

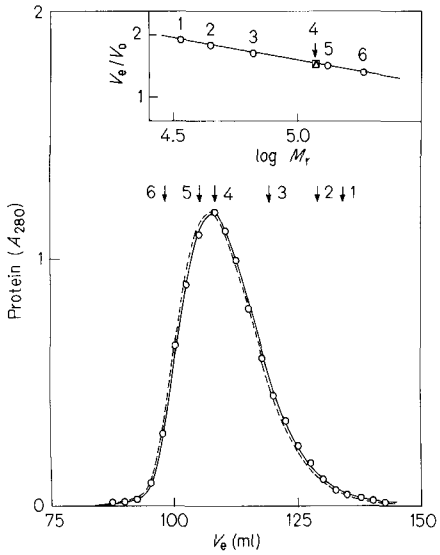

Fig. 1. *Gel filtration on Biogel P-200 column of native and chemically modified* Ricinus *agglutinin*. Elution profile of native (O——O) and succinylated (----) *Ricinus* agglutinin on Biogel P-200 column (2 × 120 cm). Inset shows the molecular weight of succinylated (△) and native (□) *Ricinus* agglutinin as determined by gel filtration. The columns were equilibrated and operated with 0.02 M phosphate buffer pH 7.4, containing 0.1 M NaCl at a flow rate of 25–30 ml/h. Fractions of 2.5 ml were collected. Molecular weight markers: 1, pepsin; 2 ovalbumin; 3, bovine serum albumin (monomer); 4, bovine serum albumin (dimer); 5, soybean agglutinin; 6, immunoglobulin G

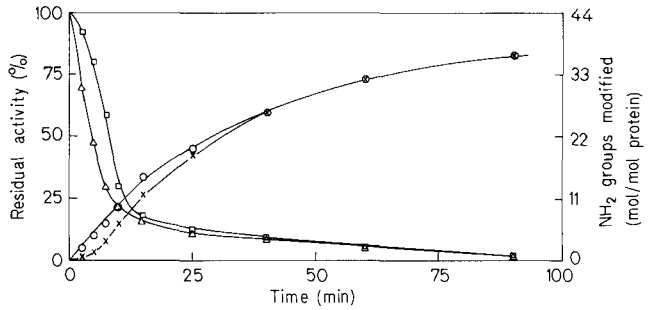

Fig. 2. *Changes in NH_2 group content and activity during succinylation of* Ricinus *agglutinin*. Succinylation was carried out as described in Materials and Methods. (△) Activity; (O) blocked NH_2 groups; (□) activity and (×) blocked NH_2 groups in the presence of 50 mM lactose

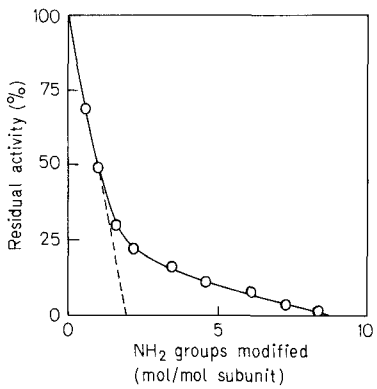

Fig. 3. *Changes in the activity of succinylated* Ricinus *agglutinin as a function of blocked NH_2 groups*. Succinylation of *Ricinus* agglutinin was carried out as described in Materials and Methods

modification of 6 and 16 residues respectively, but no loss in the polysaccharide-binding and agglutinating activity of *Ricinus* agglutinin takes place (Table 2). Even after modification of more than 60% of the arginyl residues by 1,2-cyclohexanedione and aspartyl and glutamyl residues by glycine methyl ester, the lectin retained its total activity as shown in Table 2.

Immunodiffusion test revealed that modification of tryptophyl, arginyl and the carboxyl groups of *Ricinus* agglutinin did not affect its interaction with specific antibody raised against it (Table 2).

Effect of Acetylation with N-*Acetylimidazole*

Acetylation of *Ricinus* agglutinin with *N*-acetylimidazole led to the modification of six tyrosyl residues and 19 amino groups. Deacetylation of *O*-acetyl-tyrosyl residues of the lectin showed only 10% recovery of its activity. The acetylation of citraconylated lectin showed that about six tyrosyl residues were acetylated per tetrameric protein molecule but complete de-*O*-acetylation did not lead to recovery of the sugar binding activity of the agglutinin. Deblocking of the amino groups of citraconylated and acetylated *Ricinus* agglutinin led to unmasking of all amino groups but no activity was regained. Deacetylation of the decitraconylated *Ricinus* agglutinin showed 95% recovery in the agglutinating and polysaccharide-binding activity of the lectin (Table 3). Acetylation of tyrosyl

Table 2. *Effect of chemical modification of* Ricinus communis *agglutinin*
The protein was treated with different chemical reagents, modifying different amino acid residues. Modification of tryptophyl residues of *Ricinus* agglutinin (2 mg/ml) with 2-hydroxy-5-nitrobenzyl bromide (25 mM) at pH 4.5 was carried out according to the method of Barman and Koshland [21]. The residues modified were determined spectrophotometrically as described there. Modification of arginyl residues of *Ricinus* agglutinin (2 mg/ml) by 1,2-cyclohexanedione (0.2 M) at pH 9.0 (0.2 M borate buffer) was carried out according to the method of Patthy and Smith [22]. The extent of modification was estimated as described there. Carboxyl group modification of *Ricinus* agglutinin (5 mg/ml) with [^{14}C]glycine methyl ester (200 mg/ml) was carried out according to the method of Rice and Etzler [23]. The number of carboxyl groups modified was determined by the radioactivity associated with the protein. The results are expressed as the percentage of chemically untreated agglutinin

Modifying agent	Residue modified	Residues modified	Immunochemical properties	Hemag-glutination	Binding to	
					guar gum	Sepharose
		mol/mol protein (%)		%		
[^{14}C]Glycine methyl ester	Glu + Asp	105 (60)	unchanged	100	98	100
1,2-Cyclohexadione	Arg	40 (64.5)	unchanged	100	100	100
2-Hydroxy-5-nitrobenzyl bromide	Trp	5.8	unchanged	96	95	98
2-Hydroxy-5-nitrobenzyl bromide in urea	Trp	15.7	unchanged	93	91	95

Table 3. *Effect of acetylation of* Ricinus *agglutinin*
The proteins were treated with *N*-acetylimidazole and the number of tyrosyl residues modified was determined spectrophotometrically. The indicated properties of the agglutinin were studied as described in the Materials and Methods and the results are expressed as the percentage of the chemically untreated agglutinin

Agglutinin	NH_2OH added	Tyrosyl residues modified	Immunochemical properties	Hemag-glutination	Binding to	
					guar gum	Sepharose
		mol/mol protein		%		
Native	–	5.9	changed	0.5	0	4
	+	0.05	unchanged	10	10	15
Citraconylated	–	6.1	changed	0.8	0	5
	+	0.08	unchanged	0.6	0	4
Decitraconylated	–	6.3	changed	0.6	0	6
	+	0.06	unchanged	95	95	96

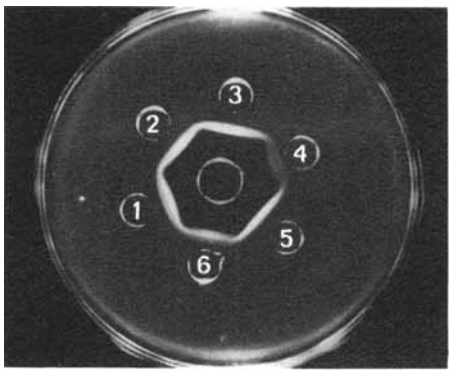

Fig. 4. *Immunodiffusion test of methylated and acetylated* Ricinus *agglutinin.* Center well, anti-(*Ricinus* agglutinin); wells 1, 3 and 5, native *Ricinus* agglutinin; well 2, reductively methylated *Ricinus* agglutinin; well 4, acettylated *Ricinus* agglutinin; well 6, deactylated *Ricinus* agglutinin. Each well contained 0.1 mg protein

residues of *Ricinus* agglutinin affected the immunological properties of the lectin. Contiguous and fused precipitin bands in the immunodiffusion test (Fig. 4) indicate retention of immunological identity for modified forms of the agglutinin except for some inhomogeneity after tyrosyl modification.

Acetylation of *Ricinus* agglutinin as a function of time was carried out after blocking of the amino groups with citraconic anhydride. Acetylation of the tyrosyl residues showed that nearly 55% loss in the sugar-binding activity of *Ricinus* agglutinin occurred with modification of only two tyrosyl residues (Fig. 5) Lactose decreased the rate of acetylation. A plot of relative loss in activity against number of tyrosyl residues modified per average subunit indicated that one tyrosyl residue per subunit was involved in the sugar-binding activity of the lectin (Fig. 6). Since reductively methylated agglutinin retains its activity but blocks the lysyl residues from being acetylated by *N*-acetylimidazole, this modified protein was used to study the effect of presence of lactose on acetylation of tyrosyl residues. Here lactose also decreased the initial rate of modification.

DISCUSSION

An extensive acetylation of the lysyl residues of *Ricinus* agglutinin with acetic anhydride led to a total loss in its sugar-binding activity. Since acetic anhydride is not specific for lysyl residues but also modifies tyrosyl residues, succinic anhydride and citraconic anhydride (which show preference for the amino groups [19, 25]) were used to modify the lysyl residues of *Ricinus* agglutinin. Succinylation and citra-

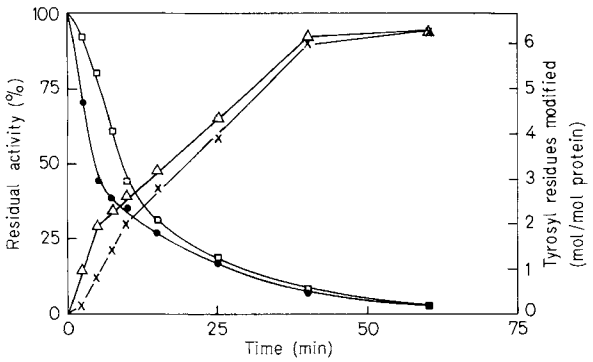

Fig. 5. *Changes in tyrosine content and activity during acetylation of citraconylated* Ricinus *agglutinin.* Acetylation with *N*-acetylimidazole was carried out as described in Materials and Methods. (●) Activity; (△) blocked tyrosyl residues; (□) activity and (×) blocked tyrosyl residues in the presence of 50 ml lactose

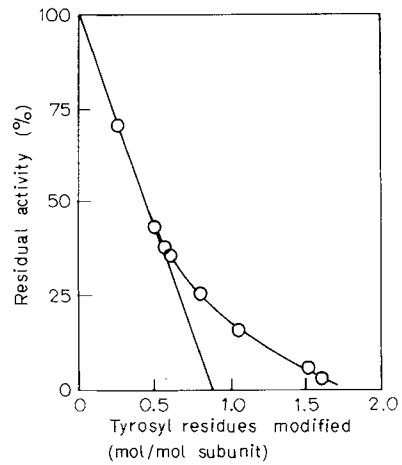

Fig. 6. *Changes in the activity upon O-acetylation of citraconylated* Ricinus *agglutinin as function of blocked tyrosyl residues.* Acetylation of *Ricinus* agglutinin was carried out with *N*-acetylimidazole as described in Materials and Methods

conylation of the lectin also resulted in a complete loss of its sugar-binding property. Decitraconylation led to the recovery of the sugar-binding and cell-agglutinating activity of the lectin. Conversion of the lysyl residues to *N*-monomethyl and *N*,*N*-dimethyl lysine did not alter its activity when more than 80% of the lysyl residues were reductively methylated [17].

Succinylation converts cationic amino groups to anionic residues, often resulting in unfolding and or dissociation of proteins into subunits [20,26]. However, the molecular weight of succinylated *Ricinus* agglutinin, as determined by gel filtration, showed no significant change, excluding the possibility that the loss in its activity was due to dissociation. Similarly no change in molecular size of acetylated, citraconylated and reductively methylated *Ricinus* agglutinin in comparison to the native lectin was found. Reductive methylation does not lead to a drastic change in the pK_a of the ε-amino groups of lysine and the effects of these modifications on *Ricinus* agglutinin indicate that the cationic charge contributed by the lysyl residues is essential for its sugar-binding activity. Similar effects have been found in the sweet-tasting protein thaumatin I, where acetylation of four lysyl residues led to a complete loss in its biological activity whereas reductive methylation did not impair [27]. The maleyl derivative of lentil lectin [28] and succinyl or acetyl derivatives of pea lectin [29] do not dissociate but lose their heamagglutinating activity, as found here for *Ricinus* agglutinin. However, they differ in the respect that lysyl-group modification of lentil and pea lectin does not impair their sugar-binding activity whereas it affects the sugar-binding activity of *Ricinus* agglutinin.

Succinylation of lysyl residues of *Ricinus* agglutinin as a function of time indicated that there were two types of amino groups: the essential lysyl residues, required for the sugar-binding activity of the agglutinin, reacted faster than the non-essential residues. Relative loss in activity with increase in the number of lysyl residues modified indicated that two lysyl residues per average subunit were involved in the sugar-binding activity of the lectin.

Acetylation by *N*-acetylimidazole led to a concomitant modification of lysyl residues of *Ricinus* agglutinin. The number of lysyl residues modified was 19 and the modification is as extensive as that of lentil lectin by this reagent [30]. Acetylation of hydroxyl groups of tyrosine of *Ricinus* agglutinin resulted in complete loss of the activity of the agglutinin although only 45% of the lysyl residues were modified. On the other hand, as shown in Fig. 2, the modification of 45% of lysyl residues by succinic anhydride leads to nearly 90% loss of its activity, suggesting that tyrosyl residues might also be involved in the sugar-binding activity of *Ricinus* agglutinin.

The loss in activity of *Ricinus* agglutinin by acetylation of tyrosine by *N*-acetylimidazole is reversible by hydroxylamine treatment, although only 10% of the activity is regained. The regained activity after deacetylation is in accordance with the activity remaining on 45% substitution of lysyl residues with succinic anhydride. Similarly, reversible loss of agglutinating activity has been found after acetylation of lentil lectin [30] and one form of wheat-germ agglutinin [23]. The requirement of tyrosyl residues for the sugar-binding activity of *Ricinus* agglutinin was further substantiated by the results obtained by acetylation of the agglutinin when the lysyl residues were reversibly blocked by citraconic anhydride. The time-dependent acetylation of tyrosyl residues revealed that at least one tyrosyl residue per average subunit was involved in the agglutinating and saccharide-binding activity of *Ricinus* agglutinin.

Modification studies carried out on *Ricinus* agglutinin in the presence of its ligand, lactose, showed that it decreased the initial rate of modification and loss of activity. Although this decrease indicates the involvement of lysyl and tyrosyl residues in the sugar-binding activity of this lectin, a lack of total protection has been shown previously [31].

The results of this work indicate that tyrosyl and lysyl residues are involved in the carbohydrate-binding activity of *Ricinus* agglutinin and are also involved in its agglutinating activity. Their possible presence at the carbohydrate-binding site is in accordance with its polar nature [32].

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REFERENCES

1. Goldstein, I.J. & Hayes, C.E. (1978 *Adv. Carbohydr. Chem. Biochem. 35*, 127–340.
2. Lis, H. & Sharon, N. (1973) *Annu. Rev. Biochem. 42*, 541–547.
3. Novogrodosky, A. & Katchalski, E. (1971) *Biochim. Biophys. Acta, 228*, 579–583.

4. Cuatrecasas, P. & Tell, G. P. E. (1973) *Proc. Natl Acad. Sci. USA, 70*, 485–489.
5. Moscona, A. A. (1971) *Science (Wash. DC) 171*, 905–907.
6. Nicolson, G. L., Blaustein, J. & Etzler, M. (1974) *Biochemistry, 13*, 543–547.
7. Surolia, A., Bachhawat, B. K., Vithyathil, P. J. & Podder, S. K. (1978) *Indian J. Biochem. Biophys. 15*, 248–250.
8. Surolia, A., Ahmed, A. & Bachhawat, B. K. (1974) *Biochim. Biophys. Acta, 371*, 491–500.
9. Horiike, K. & McCormick, D. B. (1979) *J. Theor. Biol. 79*, 403–414.
10. Appukuttan, P. S., Surolia, A. & Bachhawat, B. K. (1979) *Indian J. Biochem. Biophys. 14*, 382–384.
11. Olsnes, S. & Saltvedt, E. (1975) *J. Immunol. 114*, 1743–1748.
12. Layne, E. (1957) *Methods Enzymol. 3*, 447–454.
13. Hassing, G. S., Goldstein, I. J. & Marini, M. (1971) *Biochim. Biophys. Acta, 243*, 90–97.
14. Olsnes, S., Refsness, K., Christensen, T. B. & Pihl, A. (1975) *Biochim. Biophys. Acta, 405*, 1–10.
15. Van Wauwe, J. P., Loontiens, F. G. & De Bruyne, C. K. (1973) *Biochim. Biophys. Acta, 313*, 99–105.
16. Fraenkel-Conrat, H. (1957) *Methods Enzymol. 4*, 247–269.
17. Means, G. E. & Feeney, R. E. (1968) *Biochemistry, 7*, 2192–2201.
18. Habeeb, A. F. S. A. (1967) *Arch. Biochem. Biophys. 119*, 264–268.
19. Dixon, H. B. F. & Perham, R. N. (1968) *Biochem. J. 109*, 312–314.
20. Habeeb, A. F. S. A., Cassidy, H. G. & Singer, S. J. (1958) *Biochim. Biophys. Acta, 29*, 587–593.
21. Barman, T. E. & Koshland, D. E., Jr (1967) *J. Biol. Chem. 22*, 1084–1091.
22. Patthy, L. & Smith, E. L. (1975) *J. Biol. Chem. 250*, 557–564.
23. Rice, R. H. & Etzler, M. E. (1975) *Biochemistry, 14*, 4093–4099.
24. Riordan, J. F., Wacker, W. E. C. & Vallee, B. L. (1965) *Biochemistry, 4*, 1758–1765.
25. Klotz, M. I. (1967) *Methods Enzymol. 11*, 576–580.
26. Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A. & Edelman, G. M. (1973) *Proc. Natl Acad. Sci. USA, 70*, 1012–1016.
27. Vander Wel, H. & Bel, W. J. (1976) *Chem. Senses Flavor. 2*, 211–218.
28. Vancurova, D., Ticha, M. & Kocourek, J. (1976) *Biochim. Biophys. Acta, 453*, 301–310.
29. Trowbridge, I. S. (1973) *Proc. Natl Acad. Sci. USA*, 70, 3650–3654.
30. Young, N. M. (1974) *Biochim. Biophys. Acta, 366*, 46–52.
31. Privat, J. P., Lotan, R., Boucherd, P., Sharon, N. & Monsigny, M. (1976) *Eur. J. Biochem. 68*, 563–572.
32. Khan, M. I., Mathew, M. K., Balaram, P. & Surolia, A. (1980) *Biochem. J. 191*, 395–600.

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