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PURIFICATION AND CHARACTERIZATION OF AN α-D-GALACTOSYL-BINDING LECTIN FROM *Artocarpus lakoocha* SEEDS

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ABSTRACT

A lectin from *Artocarpus lakoocha* seeds has been purified by affinity chromatography on a melibiose–agarose column. The homogeneity of the purified lectin was tested by several criteria, *viz.*, poly(acrylamide)-gel electrophoresis, ultracentrifugal analysis, and gel filtration. The molecular weight of the lectin was estimated to be ~70,000 as determined by Sephadex gel filtration. SDS-poly-(acrylamide)-gel electrophoresis gave a single component of molecular weight 18,000, suggesting that the lectin is a tetramer composed of four apparently identical subunits. The lectin agglutinated human erythrocytes, regardless of blood group. *Artocarpus lakoocha* lectin is a glycoprotein, and contains 11.7% of carbohydrates, in which D-xylose (6%) is the main sugar, with smaller proportions of D-galactose, D-glucose, D-mannose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine. Amino acid analysis of the lectin revealed a high content of acidic and hydroxylic amino acids, a relatively low proportion of basic amino acids, and a trace of cysteine and methionine. In hapten-inhibition assays with simple sugars, glycosides of α-D-galactopyranose and *N*-acetyl-D-galactosamine were potent inhibitors of the purified lectin.

INTRODUCTION

Lectins constitute a class of proteins or glycoproteins having, for carbohydrates, a sharp binding-affinity which allows them to interact with glycoconjugates and polysaccharides. They occur in a wide variety of plants, in bacteria and other micro-organisms, in invertebrates, and in vertebrates. Some of these lectins have been purified and characterized in detail[1,2]. Because of their high degree of specificity, they have often been employed as highly discriminating agents in studies of normal and malignant cell membranes, in blood typing, in the purification of polysaccharides and glycoproteins, in the fractionation of cell populations and in mitogenesis of lymphocytes. Among the lectins showing α-D-galactosyl

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specificity that are described in the literature are those from *Bandeiraea simplicifolia* and *Maclura pomifera*[3,4]. Recently, we have purified and characterized an α-D-galactosyl-binding lectin from *Artocarpus integrifolia* (jackfruit) seeds[5,6]. It had already been reported by Chatterjee *et al.*[7] that the seeds of the related *Artocarpus lakoocha* contain a protein which specifically binds to (terminal) non-reducing α-D-galactosyl groups in the fenugreek galactomannan and which thus forms a precipitate.

With a view to isolating biopolymers containing α-D-galactopyranosyl end-groups by using an *A. lakoocha* lectin-immobilized affinity system, we have isolated this lectin by employing a melibiose–agarose column. We now describe the purification of *A. lakoocha* lectin and characterization of some of its serological, chemical, and physicochemical properties.

EXPERIMENTAL

The seeds of *A. lakoocha* were obtained from a local seed-supplier and were ground to a fine meal. All operations were carried out at 4°, unless otherwise stated.

Chemical compounds. — D-Galactose and TEMED were purchased from B.D.H. (U.K.). *N*-Acetyl-D-galactosamine, *o*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl α-D-galactopyranoside, methyl β-D-galactopyranoside, melibiose–agarose*, and acrylamide were products of Sigma Chemical Co. (St. Louis, U.S.A.). Methyl α-D-galactopyranoside was obtained from Calbiochem (U.S.A.). Methyl *N*-acetyl-α-D-galactosaminide was prepared according to Sarkar and Kabat[8]. Sephadex G-100 was purchased from Pharmacia (Uppsala, Sweden). *N,N'*-methylenebis(acrylamide), ammonium peroxysulfate, and pronase P were obtained from Serva (Heidelberg, F.R.G.). All other chemical compounds used were of analytical grade (from Merck and B.D.H.). Neuraminidase was obtained from Behringwerke AG (Marburg, F.R.G.).

Purification of A. lakoocha *lectin.* — Powdered *A. lakoocha* seeds (100 g) were stirred with 0.85% NaCl (1 L) overnight at 4°. The slurry was centrifuged in a refrigerated Sorvall RC-5B centrifuge at 15,000 r.p.m. for 40 min. The clear extract was fractionated between 0–40% and 40–80% saturation, respectively, by solid $(NH_4)_2SO_4$. The precipitate collected in each fraction by centrifugation was dissolved in the minimum quantity of PBS (10 mM phosphate containing 150mM NaCl, pH 7.0), and the solution dialyzed against PBS until free from NH_4^+ ions, centrifuged, and lyophilized. The most active (0–40%) hemagglutinin fraction (60 mg) was applied to a column (1 × 3.8 cm) of melibiose–agarose, pre-equilibrated with the same buffer, washed protein-free with the same buffer, and the active protein eluted from the column with PBS containing 0.2M D-galactose. The fractions showing hemagglutinating activity were pooled, mixed, concentrated by membrane ultrafiltration (PM-10, Amicon Corporation), dialyzed against PBS, and stored at −20° until used.

*Prepared by attaching melibiose to beaded agarose activated with divinyl sulfone.

Hemagglutination and hemagglutination-inhibition assay. — Hemagglutination assays were performed in a Takatsy microtiter plate *via* two-fold serial dilutions of the lectin in PBS, according to Chatterjee *et al.*[9]. The titer was expressed as the reciprocal of the highest dilution showing macroscopic agglutination; the specific activity was expressed as titer per mg of protein per mL. The hemagglutination-inhibition test was carried out as follows. To two-fold serial dilutions of test sugar solution (25 μL) was added an equal volume of two hemagglutinating doses of lectin. After incubation for 1 h at room temperature, 25 μL of a 2% suspension of erythrocytes was added, and the mixture was allowed to stand for 1 h more at room temperature. The degree of hemagglutination was examined, and the maximum dilution of sugar solution showing inhibition was recorded.

Disc electrophoresis. — Disc electrophoresis in 7.5% poly(acrylamide) gel was performed according to Reisfeld *et al.*[10] at pH 4.3 in β-alanine–acetic acid buffer, and at pH 8.9 in Tris–glycine buffer[11]. Staining was performed with Amido Black in 7% acetic acid, and destaining in 7% acetic acid in an electric field.

Analytical ultracentrifugation. — Sedimentation-velocity experiments were conducted according to Schachman[12], with a Beckman Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and using an AN-D-analytical rotor with standard aluminum cells. Photographs were taken before and after the rotor had attained a running speed of 56,000 r.p.m. at 4°.

Molecular-weight estimation. — Molecular weights were estimated by SDS-poly(acrylamide)-gel electrophoresis and gel filtration. SDS-poly(acrylamide)-gel electrophoresis was carried out on 10% poly(acrylamide) gel according to Laemmli[13]. Dissociation and reduction of protein were performed by heating for 5 min at 100° in 0.1% SDS, with or without 0.1% of 2-mercaptoethanol. Protein markers employed were bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,000), myoglobin (M_r 17,000), and cytochrome C (M_r 12,400). The molecular weight of the lectin was estimated by comparing its mobility with those of markers of known molecular weight. Staining for proteins was performed with 0.05% Coomassie Brilliant Blue; and destaining was conducted in 7.5% acetic acid containing 5% of methanol. Gel filtration on a column (90 × 1.6 cm) of Sephadex G-100 was performed as described by Andrews[14]. The molecular weight of the lectin was determined by comparison of its elution volume with those of molecular-weight markers.

Carbohydrate analysis. — The total neutral-carbohydrate content of the purified lectin was determined by the phenol–H_2SO_4 method of Dubois *et al.*[15]. For identification and estimation of individual neutral sugars, the lectin (1.0 mg) was hydrolyzed with 0.1M HCl for 48 h at 100° (with inositol as the standard). The hydrolyzate was made neutral with Ag_2CO_3, and the neutral sugars were analyzed by g.l.c. (Hewlett–Packard model 5730 A) as their alditol acetates, using 3% of ECNSS-M or 3% of OV-225 on Gas Chrom Q (100–120 mesh) as previously described[16]. For determination of the amino sugars, the lectin (0.9 mg) was hydrolyzed with 4M HCl for 6 h at 100°. The acid was removed by codistillation

with methanol, and finally over KOH pellets under vacuum. The hydrolyzate was analyzed in an amino acid analyzer.

Amino acid analysis. — The purified lectin (450 μg) was hydrolyzed with 6M HCl in a sealed tube under nitrogen for 24 h at 110°. The amino acid content of the hydrolyzate was determined in an amino acid analyzer (Beckman) according to the method of Spackman *et al.*[17]. Tryptophan was determined on an unhydrolyzed protein sample by the spectrophotometric method of Goodwin and Morton[18].

Protein analysis. — The protein content of different fractions was measured by the method of Lowry *et al.*[19], using bovine serum albumin as the standard; and the protein in column-eluate fractions was also monitored spectrophotometrically, at 280 nm.

Oxidation of lectin by metaperiodate. — Purified *A. lakoocha* lectin (1 mg/mL) in 50mM acetate buffer (pH 5.1) was mixed with an equal volume of 250mM sodium metaperiodate dissolved in the same buffer. The mixture was incubated at 0–4° in the dark. Aliquots (0.2 mL) were taken at regular intervals of time and dialyzed against PBS; the hemagglutination assay was performed as described before.

Ouchterlony double diffusion. — Ouchterlony plates were prepared as described by Chatterjee *et al.*[7]. The wells were filled with 20 μL test-samples (5 mg/mL), and the plates were developed overnight at 25°.

Physicochemical studies. — Hemagglutination of *A. lakoocha* lectin was performed (*a*) in the pH range 4–10, (*b*) in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+} ions in the concentration range 5–100 mM, and (*c*) at temperatures lying between 25 and 95°, as described[20].

RESULTS

Purification of A. lakoocha *lectin.* — The hemagglutinin from *A. lakoocha* was partially purified by ammonium sulfate fractionation. Upon addition to a melibiose–agarose column and subsequent elution by 0.2M D-galactose in PBS, the most active fraction, 0–40% (see Table I) yielded a major product (see Fig. 1). The purification achieved was 300-fold. Table I summarizes the purification scheme for *A. lakoocha* lectin. The affinity column retained 4 mg of lectin per mL of gel, and the yield was 12 mg.

Homogeneity. — The homogeneity of *A. lakoocha* lectin was tested by poly(acrylamide) disc-gel electrophoresis, analytical ultracentrifugation, and gel filtration. Poly(acrylamide) disc-gel electrophoresis at pH 4.3 produced a single sharp band, but at pH 8.9 the lectin produced a single diffused band (see Fig. 2). The lectin sedimented as a symmetrical peak at pH 7.0 by ultracentrifugal analysis. A single protein peak was obtained upon chromatography on a column of Sephadex G-100.

Hemagglutination assays. — *A. lakoocha* lectin agglutinated human O, A, and B erythrocytes, but the agglutination titer with blood-group B erythrocytes was

TABLE I

PURIFICATION OF *Artocarpus lakoocha* LECTIN

Fraction	*Protein (mg/mL)*	*Titer, B cell*	*Specific activity*[a]	*Purification (fold)*
Crude extract	2.9	32	11	1
Ammonium sulfate fractionation				
(a) 0–40% saturation	2.4	1024	426.6	40
(b) 40–80% saturation	1.9	64	33.6	3
Affinity-chromatography-purified lectin	1.25	4096	3277	300

[a]Specific activity is expressed as titer per mg of protein per mL.

slightly higher compared to those for other blood groups. The minimum amount of purified lectin that was needed to agglutinate B erythrocytes was 0.25 μg per mL.

Molecular weight. — The total molecular weight of native *A. lakoocha* lectin was estimated to be ~70,000 (see Fig. 3) by gel filtration on a column of Sephadex G-100. However, SDS-poly(acrylamide)-gel electrophoresis, with or without added 2-mercaptoethanol, showed a single band corresponding to a molecular weight of 18,000 (see Figs. 2 and 4). Thus, the lectin is a tetramer composed of four apparently identical sub-units joined noncovalently in the same molecule.

Inhibition assays. — The results of hemagglutination inhibition tests of *A. lakoocha* lectin with haptenic sugars are given in Table II, from which it is evident that the lectin displays high specificity for α-D-galactopyranosyl groups.

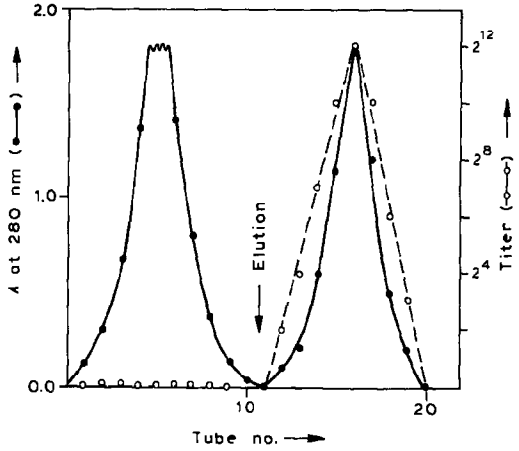

Fig. 1. Affinity chromatography of *A. lakoocha* agglutinin on a melibiose–agarose column. The ammonium sulfate (0–40%) fraction (60 mg) obtained from an extract of 10 g of pulverized seeds was applied to a column (1 × 3.8 cm) at 4°, and, after washing out the unbound proteins with 44 mL of PBS (4-mL fractions), the lectin was eluted (3-mL fractions) with 0.2M D-galactose in PBS. The arrow indicates the elution of the column by 0.2M D-galactose solution.

TABLE II

HEMAGGLUTINATION-INHIBITION ASSAY[a] OF *Artocarpus lakoocha* LECTIN BY CARBOHYDRATES

Carbohydrate	*Minimum inhibitory concentration (mM)*
D-Galactose	200
Methyl α-D-galactopyranoside	3.12
o-Nitrophenyl α-D-galactopyranoside	6.25
p-Nitrophenyl α-D-galactopyranoside	6.25
N-Acetyl-D-galactosamine	200
Methyl *N*-acetyl-α-D-galactosaminide	12.5
Melibiose	12.5
Raffinose	25

[a]Inhibitory activity of the carbohydrate is expressed as the minimum concentration (mM) required for complete inhibition of two hemagglutinating doses of lectin. Tests were performed with human erythrocytes of blood group B. D-Glucose, D-mannose, D-fucose, L-fucose, L-arabinose, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxy-D-glucose, methyl β-D-galactoside, *p*-nitrophenyl β-D-galactoside, D-galacturonic acid, methyl α-D-glucoside, and lactose were noninhibitory up to 200mM concentration.

Thus, methyl α-D-galactopyranoside is twice as potent as *o*- and *p*-nitrophenyl-α-D-galactopyranoside, which are twice as effective as melibiose and four times as effective as raffinose. D-Galactose and *N*-acetyl-D-galactosamine were found to be poor inhibitors, requiring a 200mM concentration in order to prevent hemagglutina-

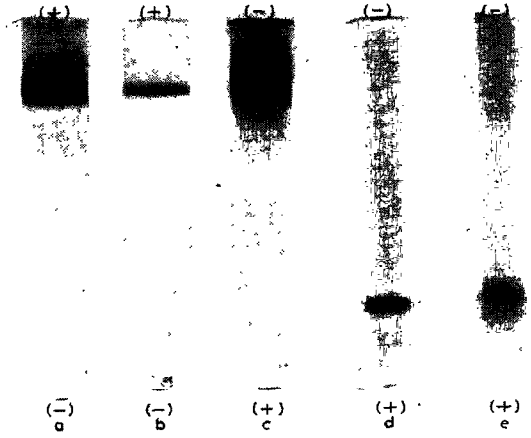

Fig. 2. Poly(acrylamide) disc-gel electrophoresis of *A. lakoocha* agglutinin. Electrophoresis was performed in 7.5% acrylamide gels with 50–100 μg of lectin: (*a*) crude extract at pH 4.3 at 3 mA for 4 h; (*b*) purified lectin at pH 4.3 at 3 mA for 4 h; (*c*) purified lectin at pH 8.9 at 5 mA for 5 h, in 10% gel; (*d*) in the presence of 0.1% SDS at 8 mA for 4 h; (*e*) in the presence of 0.1% SDS and 0.1% 2-mercaptoethanol at 8 mA for 4 h. The migration of protein was from the top. The gels were stained for protein with Coomassie Brilliant Blue R-250 or Amido Black.

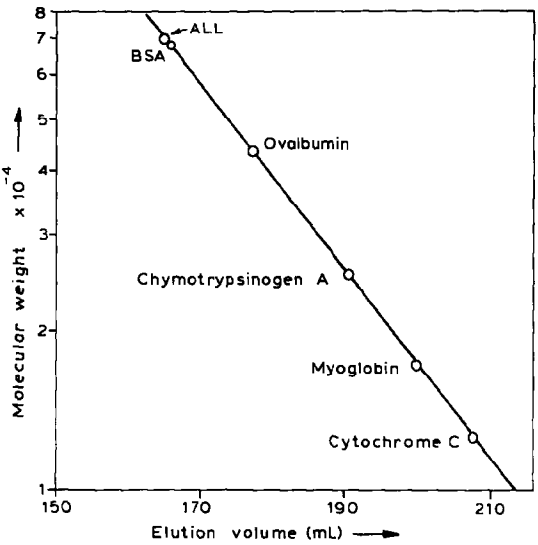

Fig. 3. Determination of the molecular weight of *A. lakoocha* lectin by gel filtration. A column (1.6 × 90 cm) of Sephadex G-100 was used with PBS as the eluant at 4°. The protein solution (1 mL, 10 mg/mL in eluting buffer) was applied to the column, and 5-mL fractions were collected at the rate of 10 mL/h. Elution volumes were determined from the position of the maxima of the elution profiles.

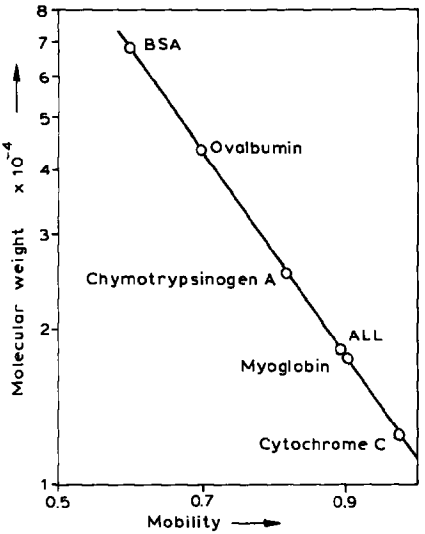

Fig. 4. Determination of the subunit molecular weight of *A. lakoocha* lectin by poly(acrylamide)-gel (10%) electrophoresis in the presence of 0.1% SDS. Standard protein samples together with *A. lakoocha* lectin (50 μg each) were applied, and, after staining with Coomassie Brilliant Blue R-250, their mobility was measured from the top of the gel to the leading edge of the protein band.

TABLE III

(a) AMINO ACID COMPOSITION OF PURIFIED *Artocarpus lakoocha* LECTIN

Amino acid	*g/100 g*	*Mol/18,000 g*
Asp	7.72	10.52
Thr	5.29	7.80
Ser	5.30	9.08
Glu	7.61	10.54
Pro	—	—
Gly	5.55	13.32
Ala	2.76	5.58
Cys	0.34	0.50
Val	4.98	7.66
Met	0.17	0.20
Ile	4.81	6.61
Leu	5.12	7.03
Tyr	4.35	4.32
Phe	6.74	7.35
His	1.14	1.32
Lys	4.81	5.93
Arg	2.46	2.54
Trp	6.38	5.63

(b) CARBOHYDRATE COMPOSITION OF PURIFIED *Artocarpus lakoocha* LECTIN

Sugar	*g/100 g*	*Mol/18,000 g*
Xyl	6.0	7.2
Man	1.0	1.0
Gal	1.4	1.4
Glc	2.0	2.0
GlcN	0.9	0.9
ManN	0.4	0.4

tion, whereas the methyl α-glycoside of *N*-acetyl-D-galactosamine required a 12.5-mM concentration for the same, and was thus one-fourth as potent as methyl α-D-galactopyranoside. Methyl β-D-galactopyranoside and lactose were noninhibitors, even at a concentration of >200mM.

Carbohydrate and amino acid analyses. — Tables III a and b summarize the results of carbohydrate and amino acid analyses of *A. lakoocha* lectin. The carbohydrate analysis of the lectin revealed the presence of xylose, galactose, mannose, glucose, glucosamine, and a trace of mannosamine. The lectin contains 11% of neutral carbohydrate, as determined by the phenol–H_2SO_4 reaction, a result in agreement with the proportions of neutral sugar (10.4%) and amino sugar (1.3%) determined by g.l.c. and amino acid analyzer, respectively. The amino acid analysis of the lectin is similar to that of a number of other purified lectins, characterized by a high content of acidic amino acids, a relatively low proportion of basic amino acids, and very little cysteine and methionine. Remarkable is the absence of proline

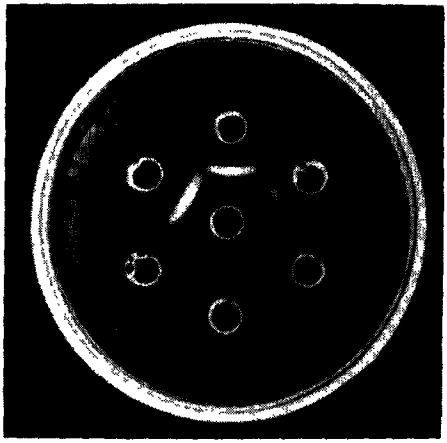

Fig. 5. Precipitin reaction of *A. lakoocha* lectin (in middle well) with various polysaccharides: (1) fenugreek galactomannan; (2) guar galactomannan; (3) *Achatina fulica* snail galactan; (4) *Lymnaea stagnalis* snail galactan; (5) bovine-lung galactan, and (6) *Palmyra palm* galactomannan. The wells are numbered clockwise from the top (Well 1). All wells were filled with 20 μL of test substances (5 mg/mL).

in the hydrolyzate of the lectin. The high amount of ammonia liberated indicates that most of the aspartic and glutamic acid residues are present in the amide form (not presented in Table III).

Ouchterlony double diffusion. — On an Ouchterlony agar-gel plate (see Fig. 5), the *A. lakoocha* lectin precipitated the galactomannans of fenugreek, *Palmyra palm,* and guar, but not the galactan from the albumin gland of the snails *Achatina fulica* and *Lymnaea stagnalis,* and bovine-lung galactan.

TABLE IV

THERMAL STABILITY[a] OF *Artocarpus lakoocha* LECTIN

Temperature (°C)	*Titer*
25	4096
35	4096
45	1024
55	1024
65	256
75	64
85	16
95	4

[a] *A. lakoocha* lectin (1 mg/mL) in phosphate-buffered saline was incubated for 30 min over a range of 25–95°. After cooling, aliquots (25 μL) were taken, and a hemagglutination assay was performed, using human B erythrocytes.

Physicochemical properties. — The *A. lakoocha* lectin was stable in the pH range 5.0–10.0, but was very labile at pH 4.0–4.6. The hemagglutination titer was 4096 between pH 5 and 7.5, and gradually fell to 16 at pH 10. The activity of *A. lakoocha* lectin remained unchanged in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+} ions (5mM–100mM). The thermal stability of *A. lakoocha* is given in Table IV, which shows that the lectin is thermally stable, and, even after incubation for 30 min at 95°, the protein agglutinated human erythrocytes. *A. lakoocha* lectin lost its agglutinating activity by the action of sodium metaperiodate.

DISCUSSION

The purification procedure described herein produced a protein homogeneous by several standard physical methods. The purified *A. lakoocha* lectin is similar in its physical and chemical properties to many other purified lectins[1]. The molecular weight of the lectin (70,000) and its subunits (18,000) is nearly twice those of *A. integrifolia* (42,000 and 11,400), respectively[5,6]. Moreover, the lectin from *Artocarpus lakoocha* is composed of four apparently identical subunits joined noncovalently in the molecule, and it thus resembles those of *A. integrifolia* or *Arachis hypogaea*[21], although the molecular weight (110,000) of the latter and its subunits (27,500) is much higher than those of the lectins of both species of *Artocarpus*. Like many other lectins, it contains high proportions of acidic and hydroxylic amino acids and a paucity of sulfur-containing amino acids. It contains 11.7% of carbohydrates and, except for *Arachis* and wheat-germ agglutinin, resembles other lectins.

In the hapten-inhibition assay (see Table II), *A. lakoocha* lectin shows, in sugar binding, very close similarity to *A. integrifolia*[5,6]. Among the inhibitors tested, methyl α-D-galactopyranoside was the most potent. By comparing methyl α-D-galactopyranoside with methyl β-D-galactopyranoside, *p*-nitrophenyl α-D-galactopyranoside with *p*-nitrophenyl β-D-galactopyranoside, and melibiose with lactose, it was evident that the α-anomeric configuration is very important in binding, as the methoxyl group in the α position contributes positively in stabilizing the protein–sugar complex, whereas a β-methoxyl group has a negative effect. The aglycon part of the glycosides exhibits no influence in sugar binding, as both *o*- and *p*-nitrophenyl α-D-galactopyranoside show good inhibition, due to their α-glycosidic linkages, whereas their β-anomeric form, like β-D-galactosides, did not inhibit at all. D-Galactose and *N*-acetyl-D-galactosamine were poor inhibitors, and about one sixty-fifth as effective as methyl α-D-galactopyranoside, whereas the methyl α-glycoside of *N*-acetyl-D-galactosamine showed good inhibition. This finding was expected, in view of the ability of the lectin to agglutinate type A erythrocytes, in addition to those of type B.

N-Acetylation of galactosamine is evidently necessary for binding to the lectin, as the unsubstituted sugar was not an inhibitor. A 6-(hydroxymethyl) group is essential for binding to the lectin. D-Fucose, L-arabinose, and D-galacturonic

acid, which are identical with D-galactose at C-2, C-3, and C-4 but lack a 6-hydroxyl group, were noninhibitory. The configuration of C-4 also appears to be important. Methyl α-D-glucopyranoside, which is the C-4 epimer of methyl α-D-galactopyranoside, did not inhibit the hemagglutination reaction. Other D-galactosyl-specific lectins, such as those from *Ricinus communis, Abrus precatorious,* and *Viscum album*[1,22], exhibited a preference for β-D-galactosides. Of the oligosaccharides tested, melibiose and raffinose, in which terminal D-galactose is linked α-glycosidically to the penultimate sugar, gave good inhibition, whereas lactose, in which terminal galactose is bound by a β-glycosidic linkage to D-glucose, did not inhibit. The hapten-inhibition assay supports the conclusion that the lectin from *A. lakoocha* binds sugars exclusively in the α-D-galactopyranose configuration.

The specificity of *A. lakoocha* lectin as determined by hapten inhibition study has been substantiated by a precipitation study on Ouchterlony gel plates. The *A. lakoocha* lectin gave a strong precipitin reaction with plant galactomannans[23], in which nonreducing (terminal) D-galactosyl groups are α-glycosidically linked to the D-mannose backbone, and failed to precipitate those galactans of both vertebrate and invertebrate origin which are composed of β-(1→3)- and -(1→6)-linked D-galactosyl units[24].

Unlike the lectin con A, the loss of hemagglutinating activity of *A. lakoocha* on oxidation with sodium metaperiodate is presumably due to cleavage of bonds between carbon atoms (bearing hydroxyl groups) of the sugar units in the lectin. The sugar moiety of a lectin may play an important supporting role in the maintenance of an appropriate conformation of the lectin for the interaction with the receptors, although the possibilities of oxidation of certain amino acids, *e.g.*, tryptophan or cysteine, or both, cannot be precluded. On prolonged storage in lyophilized form in a refrigerator, the lectin tends to aggregate to give material having a molecular weight of 120,000–140,000. Such a phenomenon was observed for lectins of *Maclura pomifera*[4] and *A. integrifolia*[5].

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