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FURTHER CHARACTERIZATION OF *Artocarpus lakoocha* LECTIN (ARTOCARPIN) PURIFIED USING RIVANOL*

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ABSTRACT

The α-D-galactosyl-binding lectin (artocarpin; mol. wt., 70,000) from *Artocarpus lakoocha* seeds has been purified using rivanol (6,9-diamino-2-ethoxyacridine lactate) and shown to be homogeneous by poly(acrylamide) disc gel electrophoresis and immunoelectrophoresis. It agglutinated human and several normal and enzyme-treated animal erythrocytes, and bound to rat lymphocytes and mouse ascites cells. The lectin is a metalloprotein with two sugar exothermic combining sites per lectin molecule of four subunits, and is inhibited by α-glycosides of D-Gal/D-GalNAc with the α-glycoside of β-D-Gal-(1→3)-D-GalNAc being the most potent.

INTRODUCTION

The omnipresence of lectins in plants, animals, and micro-organisms has been established by their capacity to agglutinate erythrocytes[1–4] and they can be purified readily using classical protein methods and by affinity chromatography on immobilized carbohydrates[5,6]. They are classified on the basis of the carbohydrates that inhibit the hemagglutination of erythrocytes and other cells or precipitation of carbohydrate-containing macromolecules[7]. Several lectins exhibit pronounced preferences for di-, tri-, and tetra-saccharides. For example, *Erythrina cristagalli* and peanut lectins show pronounced specificity for β-D-Gal-(1→4)-D-GlcNAc[8] and β-D-Gal-(1→3)-D-GalNAc[9].

In order to deploy lectins in studies of the structure and function of simple and complex carbohydrates in solution[10] and on cell surfaces[5,11], the identification and separation of cells[12], and the selection of lectin-resistant mutants of animal cells with altered glycosylation patterns[13,14], it is necessary to determine the specificities of their combining sites. *Maclura pomifera* lectin belonging to the family *Moraceae* is specific for α-D-galactopyranoside and 2-acetamido-2-deoxy-α-D-galactopyranoside residues[15]. Chuba and Kuhns reported[16] the relatively good in-

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hibitory activity of crude extracts of *M. pomifera* seeds by the antifreeze glycoprotein which possesses a large number of β-D-Gal-(1→3 or 4)-α-D-GalNAc-threonine moieties[17]. Similarly, β-D-Gal*p*-(1→3)-D-GalNAc inhibited the *M. pomifera*-induced agglutination of type O erythrocytes[18]. This result was confirmed by immunochemical studies[19] which showed that the combining size of the lectin was as large as a disaccharide and most complementary to β-D-Gal-(1→3)-D-GalNAc. The α-D-galactopyranosyl specificity of the lectins from two related species *Artocarpus integrifolia* and *A. lakoocha* (fam. *Moraceae*) has been demonstrated by Ouchterlony gel diffusion of partially purified lectin with several plant galactomannans[20], and the purification and characterization of the former lectin have been reported[21]. These two lectins have marked affinity for the Thomsen–Friedenreich (T) disaccharide, β-D-Gal-(1→3)-D-GalNAc, a well known receptor for peanut lectin[22]. β-D-Gal-(1→3)-D-GalNAc is the most specific inhibitor of *A. integrifolia* lectin, which suggests that this lectin has extended combining sites[23]. The lectin from *A. lakoocha* seeds has been purified by affinity chromatography on melibiose–agarose, and α-D-galactopyranosides and 2-acetamido-2-deoxy-α-D-galactopyranosides are potent inhibitors[24]. The purified lectin (artocarpin) has a mol. wt. of ~70,000, consists of four apparently identical subunits of mol. wt. 18,000, is a glycoprotein (11.7% of carbohydrate), and has a high content of aspartic acid, glutamic acid, and glycine with relatively very little cysteine and methionine[24]. Artocarpin is a potent and selective mitogen of T and B cells[25].

We now describe a method for rapid purification of artocarpin, its properties, and the specificity of the combining site.

EXPERIMENTAL

Seeds of *A. lakoocha*, purchased locally, were ground finely, defatted with ether, and dried in air. All operations were carried out at 4°, unless otherwise stated.

β-D-Gal-(1→3)-D-GalNAc was synthesised[26] or purchased from Biocarb (Sweden), and desialylated glycophorin, bird'snest glycoprotein, and BSA-T antigen [β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_8$-CONH-]$_{30}$BSA were generously supplied by Professor G. Uhlenbruck (Medical University Clinic, Koln, F.R.G.).

Mouse Ehrlich ascites cells were grown and cultivated in the Department of Pharmacy (Jadavpur University) with the co-operation of Professor A. U. De. Blood was obtained from healthy humans by vein puncture and was collected in citrate–dextrose solution, from rat, mouse, and rabbit by ear vein or cardiac puncture, from duck and pigeon by sacrificing the animals, and from the other animals from the slaughter house.

Purification of A. lakoocha *lectin (artocarpin).* — A suspension of ground seeds of *A. lakoocha* (20 g) in aqueous 0.85% NaCl (saline, 100 mL) was stirred for 4 h at 4°, then centrifuged at 10,000*g* for 1 h in a refrigerated Sorvall RC-5B centrifuge. To the supernatant solution was added aqueous 0.4% rivanol (6,9-

diamino-2-ethoxyacridine lactate, 80 mL) with vigorous stirring. The precipitate was removed by centrifugation at 10,000*g* for 30 min. The excess of reagent in the supernatant solution was precipitated by the addition of solid potassium bromide and its complete removal was verified spectrophotometrically (300–400 nm). After stirring, the suspension was centrifuged at 20,000*g* for 1 h. The clear supernatant solution was dialyzed exhaustively against aqueous 0.85% NaCl followed by distilled water, and then lyophilized, and the artocarpin was stored at −20°.

Disc electrophoresis. — 7.5% Poly(acrylamide) gel was used at pH 4.3 in β-alanine–acetic acid buffer[27]. Discontinuous poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to Laemmli[28].

Preparation of antisera. — Rabbits were given three i.m. injections of purified artocarpin (~500 μg) in Freund's complete adjuvant (Difco Laboratories) at weekly intervals. A booster i.m. injection of an emulsion of artocarpin and Freund's incomplete adjuvant was given after an interval of 2 weeks. Blood was obtained by cardiac puncture in the following week for 2 consecutive days. After 1 month, the immunization and bleeding schedule was repeated and a second batch of antisera was obtained.

Immunoelectrophoresis. — A Desaphor HL electrophoresis apparatus (Desaga, Heidelberg, F.R.G.) with 1% agar in 0.05M sodium barbital buffer (pH 8.2) was used[29] at 5 volts/cm for 30 min.

Assay procedure. — Hemagglutination and hemagglutination-inhibition assays were performed as described[24], and protein was determined by the method of Lowry *et al.*[30], using bovine serum albumin as standard.

Interaction of artocarpin with rat lymphocytes. — Lymphocytes from CAP- rats were obtained from heparinized peripheral blood collected by cardiac puncture. Leucocyte-rich plasma was separated from erythrocytes by gravity sedimentation and layered on to a Ficoll–Hypaque gradient. Centrifugation was carried out for 15 min at 800*g*. The interphase, which contained lymphocytes of 95–98% purity and 100% viability, was removed, washed thrice with cold Eagle's medium, and diluted to the required concentration. The agglutination and inhibition of agglutination experiments were carried out as described[24] except that lymphocytes (5×10^3 cells/μL) were added instead of erythrocytes.

Interaction of artocarpin with Ehrlich ascites carcinoma cells. — Ehrlich ascites carcinoma (EAC) cells were collected from Swiss albino mice (body weight, 18–20 g) of either sex and suspended in sterile isotonic saline. A fixed number of viable cells (usually 2×10^6 cells/20 g body weight) were implanted i.p. in each mouse. After 7 days, the tumor cells were collected using a sterile needle and diluted with saline (cell count, 1.02×10^6). The interaction of artocarpin with EAC was observed by agglutination, and the specificity of the combining site was determined by the agglutination-inhibition test[24] using ascites cells instead of erythrocytes.

Metal analysis. — To a solution of artocarpin (54.5 mg) in demineralized water (4 mL) was added 1:1 conc. HNO_3 and aqueous 70% $HClO_4$ (4 mL), the mixture was boiled under reflux for 30 min and then filtered, and the metal ions

were determined with a Perkin–Elmer model 2380 Atomic Absorption Spectrophotometer using an air–acetylene flame and a hollow cathode lamp. In order to prepare metal-free artocarpin, the lectin was exhaustively dialyzed against 0.1M EDTA, then M acetic acid[31], and finally aqueous 0.85% NaCl prepared from demineralized water.

Fluorescence quenching study. — To a cuvette containing 10^{-5}M 4-methylumbelliferyl α-D-galactopyranoside (2 mL) in saline were added increasing amounts of 1.42×10^{-4}M artocarpin (20–190 μL), and the fluorescence spectra were recorded with a Perkin–Elmer MPF-44A spectrophotometer (excitation at 318 nm and emission at >330 nm). A saline solution had no fluorescence between 330 and 450 nm. The association constant was measured from a Scatchard plot[32].

Equilibrium dialysis. — Perspex cells made of two chambers each of 1-mL capacity separated by a semipermeable membrane made from a dialysis bag were used. Aqueous artocarpin (0.9 mL, 2.35 mg/mL) and various concentrations of 4-methylumbelliferyl α-D-galactoside (0.9 mL, 50–180μM) were used. Dialysis was carried out for 48 h at 25° and the concentrations of fluorescent sugar in the protein-free chamber were determined[33] by absorbance at 318 nm. Equilibrium dialysis data were analyzed[32] by Scatchard plots.

Ouchterlony double diffusion[34]. — A 1% agar gel was used[24].

Quantitative precipitation and precipitation-inhibition assays. — Mixtures of fenugreek *(Trigonella foenum graecum)* galactomannan (5–150 μg) and artocarpin (100 μg) were made up to 0.3 mL with aqueous 0.85% NaCl, kept for 48 h at 0–4°, then centrifuged for 40 min at 2000*g* at 0–4°. Each precipitate was washed thrice with chilled aqueous 0.85% NaCl (1 mL) and then dissolved in 0.25M acetic acid (1 mL), and the absorbance at 280 nm was measured. The amount of nitrogen in each precipitate was calculated from a curve calibrated using bovine serum albumin after correction for the protein contribution of the lectin. For the quantitative precipitation-inhibition assay, the inhibitors (1–10 μmol) were added to aqueous artocarpin (0.05 mL, 1 mg/mL) and the mixtures were incubated at room temperature for 2 h. An optimum amount of galactomannan solution, needed to bring the system to equivalence, was then added to each mixture, the volume was made up to 0.2 mL with normal saline, and the mixtures were kept for 72 h at 0–4°. The amount of nitrogen in each precipitate was then assayed as described above. The appropriate blanks and controls were used in each assay.

RESULTS

Purification of artocarpin. — Addition of aqueous rivanol to an extract of *A. lakoocha* seeds removed the contaminating proteins, leaving the lectin in the supernatant solution. After removal of the excess of rivanol as the insoluble bromide, the lectin was isolated by dialysis and lyophilization (70 mg from 20 g of seeds). The purification achieved was 266-fold (see Table I).

Poly(acrylamide) disc gel electrophoresis at pH 4.3 gave a single sharp band

TABLE I

PURIFICATION OF ARTOCARPIN BY RIVANOL

Fraction	*Protein (mg/mL)*	*Titer*[a]	*Specific activity*[b]	*Purification (fold)*
Crude extract	5.8	64	11	1
Rivanol-purified lectin	0.35	1024	2924 (3277)[c]	266 (300)[c]

[a]Hemagglutination titer was determined with untreated human B erythrocytes. [b]Expressed as titer per mg of protein per mL. [c]The figures in brackets are of affinity column-purified lectins (see ref. 24).

(Fig. 1a), and a single arc was obtained on immunoelectrophoresis with its rabbit antiserum (Fig. 1b).

Hemagglutination assay. — The results using various normal and enzyme-treated red cells of different origin are given in Table II. Artocarpin proved to be a strong agglutinin. It agglutinated human erythrocytes irrespective of the blood group and those of other animals (except cow and pig) very strongly. The erythrocytes of chicken and goat were agglutinated only after treatment with pronase or neuraminidase.

Interaction with rat lymphocytes. — Lymphocytes isolated from peripheral blood of CAP-rats were agglutinated by artocarpin and the minimum concentration required was 1 μg/mL, four times that needed to agglutinate human erythrocytes. Of the carbohydrates tested, methyl α-D-galactopyranoside was the most potent inhibitor (0.78mM completely inhibited two agglutination doses of lectin) and melibiose [α-D-Gal-(1→6)-D-Glc] was less effective (1.56mM). Methyl β-D-galactopyranoside and lactose did not inhibit even at 200mM. The results confirm the specificity of *A. lakoocha* lectin[24] for α-D-galactopyranoside residues.

Interaction with mouse ascites cells. — Artocarpin agglutinated ascites cells isolated from Swiss albino mice. The minimum concentration (0.28 mg/mL) was somewhat higher than that (0.25 μg/mL) required to agglutinate erythrocytes. The

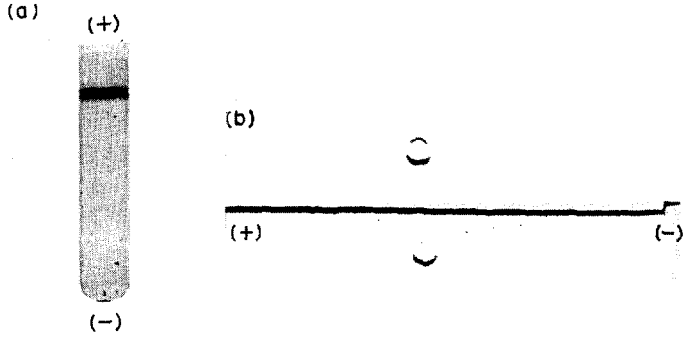

Fig. 1. (a) Poly(acrylamide) gel electrophoresis of artocarpin (60 μg, pH 4.3) for 3 h at 3 mA; (b) immunoelectrophoresis of artocarpin. Upper and lower wells were filled with rivanol-purified lectin and the trough was filled with rabbit antiserum to the purified lectin.

TABLE II

HEMAGGLUTINATION PATTERN OF HUMAN AND ANIMAL ERYTHROCYTES BY ARTOCARPIN[a]

Erythrocytes	*Titer*		
	Untreated	*Pronase-treated*	*Neuraminidase-treated*
Human[b]	32	512	1024
Goat	0	32	256
Pig	16	2048	256
Buffalo	64	4096	512
Cow	4	2048	n.d.[c]
Pigeon	128	2048	2048
Chicken	0	2	512
Sheep	32	4096	1024
Rat	4096	4096	n.d.
Mouse	256	4096	n.d.
Duck	64	2048	512
Rabbit	256	2048	2048

[a]At 50 μg/mL. [b]Irrespective of A, B, or O blood group. [c]Not determined.

inhibition of agglutination by mono- and oligo-saccharides (Table III) was similar to that of hemagglutination-inhibition. Artocarpin had the strongest binding affinity for a synthetic derivative of T-disaccharide, namely, β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-$NHCO(CH_2)_7$-$COOCH_3$.

Metal analysis. — Artocarpin is a metalloprotein, and atomic absorption spectroscopy showed that 1 mol of the lectin contained 2.1 mol of Mg^{2+}, 1.8 mol of Ca^{2+}, 0.92 mol of Fe^{2+}/Fe^{3+}, 0.4 mol of Zn^{2+}, 0.06 mol of Cr^{3+}, and 0.01 mol of Mn^{2+}. Dialysis of artocarpin against EDTA followed by treatment with acetic acid did not remove any of these metal ions or affect the hemagglutinating activity, or enhance by the incorporation of Ca^{2+} and/or other bivalent ions in saline (aqueous

TABLE III

INHIBITION OF ARTOCARPIN–ASCITES CELLS AGGLUTINATION BY CARBOHYDRATES

Carbohydrate	*Concentration*[a]
D-Galactose	300
Methyl α-D-galactopyranoside	3.1
Methyl β-D-galactopyranoside	n.i.[b]
2-Acetamido-2-deoxy-D-galactose	200
o-Nitrophenyl α-D-galactopyranoside	6.2
Lactose	n.i.
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	6.2
β-Gal-(1→3)-α-GalNAc-O-$(CH_2)_2$-$NHCO(CH_2)_7$-$COOCH_3$	0.78
Melibiose	12.5
Raffinose	25

[a]Minimal concentration of sugar (mM) required to inhibit two agglutinating doses of lectin. [b]No inhibition up to 200mM.

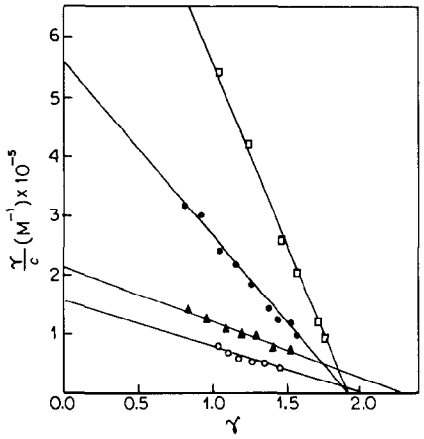

Fig. 2. Scatchard plot for the binding of 4-methylumbelliferyl α-D-galactopyranoside to artocarpin. The values of the binding sites (valency) were obtained from the intercept on the x-axis, and association constants were determined from the Scatchard equation $1/r = 1/nK_a \times 1/c + 1/n$, where n = number of binding sites, K_a = association constant, c = concentration of free sugar, and r = ratio of moles of sugar bound per mole of lectin; 5° (□), 15° (●), 25° (▲), 30° (○).

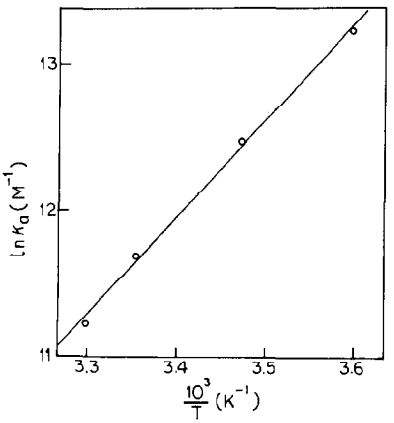

Fig. 3. van't Hoff plots for the binding of 4-methylumbelliferyl α-D-galactopyranoside to artocarpin.

0.85% NaCl). Thus, the role of metal ions in the hemagglutinating property of the lectin could not be ascertained by the above experiment.

Fluorescence quenching and equilibrium dialysis. — The fluorescence of 4-methylumbelliferyl α-D-galactopyranoside at 374 nm was quenched by artocarpin. Scatchard plots of data obtained at 5°, 15°, 25°, and 30° showed that artocarpin has 2 binding sites (see Fig. 2) with association constants (× 10^5) of 5.55, 2.65, 1.2, and 0.76M^{-1}, respectively. The ΔH, ΔG, and ΔS values (see Fig. 3) are given in Table IV. A Scatchard plot on the results of equilibrium dialysis using the 4-methylumbelliferyl α-D-galactopyranoside also revealed 2 binding sites per mol of

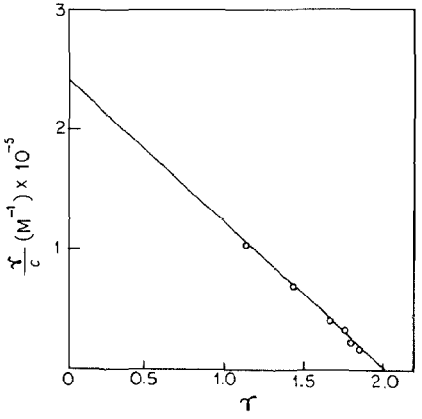

Fig. 4. Scatchard plot for the binding of 4-methylumbelliferyl α-D-galactopyranoside to artocarpin. Equilibrium was attained in aqueous 0.85% NaCl for 48 h at 25°, using 2.35 mg/mL lectin. The number of binding sites and the association constant were determined as described in Fig. 2.

artocarpin with an association constant of $1.3 \times 10^5 M^{-1}$ (see Fig. 4).

Haemagglutination-inhibition assay. — Table V shows the results of the inhibition of artocarpin-induced hemagglutination by various carbohydrates. Of the compounds studied, the lectin had the strongest affinity (0.78mM) for β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOH_3$. The affinity for β-D-Gal-(1→3)-D-GalNAc was 1.56mM. The other affinities were much lower. β-D-Galactopyranose and its glycosides were non-inhibitors.

Quantitative precipitation and precipitation inhibition assays. — The quantitative precipitin reaction of artocarpin with fenugreek *(Trigonella foenum graecum)* seed galactomannan is shown in Fig. 5. The amount of nitrogen in the precipitate increased to a maximum of 69%. The abilities of various low-molecular-weight carbohydrates to cause 50% inhibition of precipitation are listed in Table VI. The data were obtained from inhibition curves. Of the inhibitors tested, β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$ was the most potent (2.2μM). Methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-galactopyranoside, lactose, and D-fucose were almost inactive.

TABLE IV

TEMPERATURE PARAMETERS FOR THE BINDING OF 4-METHYLUMBELLIFERYL α-D-GALACTOPYRANOSIDE BY ARTOCARPIN

Temperature (°)	*Association constant* (K_a) (M^{-1})	$-\Delta G$ ($kJ \cdot mol^{-1}$)	$-\Delta H$ ($kJ \cdot mol^{-1}$)	$-\Delta S$ ($J \cdot mol^{-1} \cdot K^{-1}$)
5	5.55×10^5	30.63		90.07
15	2.65×10^5	29.73		90.08
			55.67	
25	1.20×10^5	28.74		90.40
30	0.76×10^5	28.32		90.28

TABLE V

HEMAGGLUTINATION-INHIBITION[a] OF ARTOCARPIN BY CARBOHYDRATES

Carbohydrate	*Minimum inhibitory concentration (mM)*
D-Galactose[b]	200
Methyl α-D-galactopyranoside[b]	3.1
o-Nitrophenyl α-D-galactopyranoside[b]	6.25
p-Nitrophenyl α-D-galactopyranoside[b]	6.25
2-Acetamido-2-deoxy-D-galactose[b]	200
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside[b]	6.25
o-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	3.1
p-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	3.1
β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$	0.78
β-D-Gal-(1→3)-D-GalNAc	1.56
Melibiose[b]	12.5
Raffinose[b]	25

[a]Expressed as the minimum concentration (mM) required for complete inhibition of two hemagglutinating doses of lectin. [b]Taken from ref. 24.

TABLE VI

INHIBITION OF ARTOCARPIN-GALACTOMANNAN PRECIPITATION

Inhibitor	*Quantity giving 50% inhibition (μM)*
β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$	2.2
Methyl α-D-galactopyranoside	5.2
Methyl β-D-galactopyranoside	>100.0 (33%)[a]
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside	7.0
o-Nitrophenyl α-D-galactopyranoside	7.4
p-Nitrophenyl α-D-galactopyranoside	7.7
o-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	3.6
p-Nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside	4.1
Melibiose	12.4
Lactose	>100.0 (33%)
Raffinose	34.0
D-Galactose	100.0
2-Acetamido-2-deoxy-D-galactose	110.0
D-Fucose	>100.0 (30%)
Methyl α-D-glucopyranoside	>100.0 (21%)
Methyl α-D-mannopyranoside	>100.0 (21%)

[a]The figures in brackets are the percentage inhibitions at 100μM.

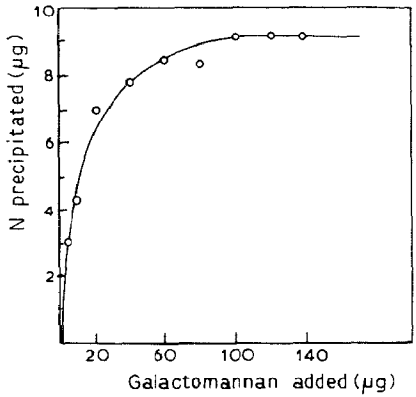

Fig. 5. Quantitative precipitin curve of artocarpin (13.3 μg of N) with fenugreek galactomannan; total volume, 300 μL.

DISCUSSION

The simple procedure described for the purification of artocarpin involves precipitation of the non-agglutinable proteins from a crude extract of *A. lakoocha* seeds by rivanol. The lectin purified by this method had physical and chemical properties similar to those of the lectin purified[24] by affinity chromatography on melibiose–agarose.

Peanut agglutinin (PNA) has been purified by rivanol precipitation[35], and IgG can be separated from mixtures with other immunoglobulins in human serum by treatment with rivanol which precipitates all the other immunoglobulins. Artocarpin antiserum cross-precipitated human IgG in agar gel diffusion[36], thereby facilitating the purification of artocarpin. An anti-T-like lectin, isolated from jackfruit *(A. integrifolia)*, which is specific for β-D-Gal-(1→3)-D-GalNAc[22,23] and has immunological properties[37,38] similar to those[39] of artocarpin, was also purified using rivanol[40]. Although the purification (266-fold) is slightly less than that (300-fold) achieved by affinity chromatography[24], the method adopted in the present study is amenable to large-scale production, is cheaper, and less time-consuming.

Artocarpin strongly agglutinated several vertebrate erythrocytes including human, and chicken and goat erythrocytes after treatment with neuraminidase or pronase. The lectin was shown not to be adsorbed on to the erythrocytes of these last two species, which is remarkable because normally treatment with pronase does not uncover receptors, but removes sterically hindering glycoproteins, whereas neuraminidase reduces the zeta potential[41].

Evidence has been obtained[42,43] that the interaction of lymphocytes and lectins involves binding to specific carbohydrates on the lymphocyte surface. Similar to the interaction of ConA with rat lymphocytes through methyl α-D-mannopyranoside[44], artocarpin bound with cells specifically through an α-D-galactopyranoside as evidenced from inhibition results. Whether this binding increases the metabolic activity of the cells could not be determined.

The strong interaction of Ehrlich ascites tumor cells with plant-seed lectins, such as those from *R. communis*, *P. vulgaris*, and *B. simplicifolia*, is well established[45–47], and weaker agglutination has been observed with lectins from wheat germ, lentil, pea, broad bean, soybean, and potato[47]. Artocarpin also agglutinated Ehrlich tumor cells, presumably due to binding with α-D-Gal residues present on the EAC-plasma membrane glycopeptide (1→3)- and (1→6)-linked to the subterminal Gal units[48]. The lectin–ascites agglutination is carbohydrate-specific as shown by inhibition studies (Table III).

Artocarpin, like many other purified lectins, is a metalloprotein and its reactivity like those of the lectins from *E. indica*[49], *M. pomifera*[19], and *A. integrifolia*[40] was not affected by dialysis against EDTA followed by acetic acid. The activity of the lectins from *B. simplicifolia*[50], ConA[51], soybean, waxbean, lima bean[52], lentil, and garden pea[53] was affected by the above treatment, since they required either Ca^{2+} or both Ca^{2+} and Mn^{2+} for their activity.

Quenching of the fluorescent glycoside 4-methylumbelliferyl α-D-galactopyranoside occurred on binding to artocarpin, which could be due to the presence of nonpolar regions in or around the sugar-binding site of the lectin. Similar quenching has been observed for wheat-germ agglutinin[33], ConA[54], *M. charantia*[55], rice[56], and *P. aeruginosa* lectin[57]. The value of the association constant (K_a) for 4-methylumbelliferyl α-D-galactopyranoside and artocarpin (Table IV) decreased with increasing temperature, indicating an exothermic process. Similar observations have been noted for lectins from wheat germ[33], ConA[54], *M. charantia*[55], rice[56], and two other α-D-galactosyl-specific lectins from *B. simplicifolia* (BS I)[58] and *A. integrifolia*[59]. The thermodynamic parameters ΔG, ΔH, and ΔS were only slightly affected by temperature changes (Table IV). The association constant (K_a), calculated from equilibrium dialysis at 25°, corresponded well with that from fluorescence quenching studies.

The quantitative precipitin and precipitin-inhibition studies of artocarpin provide some information on the combining site. The maximum nitrogen content of the material precipitated with fenugreek galactomannan was 69%, thus confirming the result of an agar-gel diffusion experiment[20]. The combining site of artocarpin is specific for D-Gal or D-GalNAc and is blocked by glycosides of α-D-Gal or α-D-GalNAc. The β-glycosides are either substantially very less active (Table VI) or without effect (Table V), except for the T-disaccharide, β-D-Gal-(1→3)-D-GalNAc or its conjugate, β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$.

The better inhibition by the *o*- and *p*-nitrophenyl glycopyranosides of D-Gal or D-GalNAc for the lectins of *M. pomifera*[19], ConA[60], *S. japonica*[61], peanut[62], *Axinella polypoides*[63], and *Aaptos papillata*[64] was due to hydrophobic interaction of the aglyconic part of the sugar and the combining site of the lectin. The inhibition studies with artocarpin (Tables V and VI) do not indicate whether hydrophobic interaction plays a role in lectin binding. This is also true for β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$ and β-D-Gal-(1→3)-D-GalNAc. D-

Fucose was either inactive (Table V) or had very little effect (Table VI), indicating that the presence of HO-6 is necessary for binding. Similar results were obtained with methyl α-D-gluco- and -manno-pyranoside, the 4- and 2-epimers of methyl α-D-galactopyranoside. The very slight binding may be due to α-orientation of the methyl group. Such phenomena were also observed with *M. pomifera* lectin[15]. The findings of the earlier hemagglutination-inhibition studies[24] together with present results (Table V) accord with those of the precipitation inhibition studies. The order of decreasing potency for both was β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$ > β-D-Gal-(1→3)-D-GalNAc > *o*- and *p*-NO_2Ph-α-D-GalNAc > methyl α-D-Gal > *o*- and *p*-NO_2Ph-α-D-Gal = methyl α-D-GalNAc > melibiose > raffinose > D-Gal = D-GalNAc. From Table VI, it is evident that β-D-Gal, lactose, and D-fucose were poor inhibitors. Thus, it can be concluded that the size of the combining site of artocarpin is most complementary to the disaccharide, β-D-Gal-(1→3)-D-GalNAc. This conclusion was substantiated by the precipitation by artocarpin of BSA-T antigen, [β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_8$-$CONH]_{30}$-BSA, desialylated glycophorin, and bird's nest glycoprotein (data not shown). The Ouchterlony gel precipitin bands were presumably due to the binding of the lectin with the β-D-Gal-(1→3)-D-GalNAc residue, the presence of which in them was already established by chemical analyses[65,66].

These investigations for the specificity of artocarpin are of interest since the so-called peanut lectin receptor, the immunodominant group of the T-antigen, serves as a marker for various tumor tissues[67,68]. Thus, artocarpin may be of use for the investigation of tumor-associated carbohydrate receptors of this type.

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