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Purification, characterisation, and carbohydrate specificity of the lectin of *Ficus cunia*

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ABSTRACT

A lectin, isolated from the seeds of *Ficus cunia* and purified by affinity chromatography on fetuin–Sepharose, was homogeneous in PAGE, GPC, HPLC, and immunodiffusion, and had mol wt of 3200–3500. In SDS-PAGE and HPLC in the absence and presence of 2-mercaptoethanol, the lectin gave a single band or peak corresponding to M_r 3300–3500, thus indicating it to be a monomer. The lectin agglutinated human erythrocytes regardless of blood group, bound to Ehrlich ascites cells and to human rat spermatozoa, and was thermally stable; its activity was enhanced by Ca^{2+}. The lectin is a metalloprotein that was inactivated by dialysis with EDTA followed by acetic acid, but reactivated by the addition of Ca^{2+}. The lectin contained 2.0% of carbohydrates, large proportions of acidic amino acids, but little methionine. In hapten-inhibition assays, chitin oligosaccharides [(1 → 4)-linked β-GlcNAc] and *N*-acetyl-lactosamine were inhibitors of which *N*,*N*′,*N*″,*N*‴-tetra-acetylchitotetraose was the most potent. Among the macromolecules tested that contain either multiple *N*-acetyl-lactosamine and/or (1 → 4)/(1 → 6)-linked β-GlcNAc, asialofetuin glycopeptide was the most potent inhibitor. Thus, an *N*-acetyl group and substitution at C-1 of D-GlcN are necessary for binding.

INTRODUCTION

The main characteristics of lectins isolated from plants, bacteria, and animals are their ability to bind sugars and to participate in various biological events. Although lectins are classified on the basis of the monosaccharides that they bind, as determined by the inhibition of haemagglutination and/or precipitation, some preferentially bind di-, tri-, and tetra-saccharides. For example, the lectins of *Wistaria sinensis* and *Artocarpus integrifolia* (Jackfruit) show pronounced specificity for β-D-Gal-(1 → 4)-D-GlcNAc[1] and β-D-Gal-(1 → 3)-D-GalNAc[2–4], respectively. The lectins from *Solanum tuberosum* (potato) and *Luffa acutangula* (ridge gourd) preferentially bind to *N*,*N*′,*N*″,*N*‴-tetra-acetylchitotetraose [(1 → 4)-linked β-

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GlcNAc][5,6], whereas that of *Triticum vulgaris* (wheat germ) binds the corresponding trisaccharide[7]. In general, the lectins specific for chitin oligosaccharides[8] have been isolated mainly from Solanaceae [potato, *Datura stramonium*, *Lycopersicon esculentum* (tomato)], Gramineae [wheat germ, *Hordeum vulgare* (barley), *Secale cereale* (rye)], Leguminosae [(*Griffonia simplicifolia* II, *Cytisus sessilifolius*, *Ulex europeus* II)], and some fruit species that belong to the Cucurbitaceae [*Cucumis melo* (melon), *Cucumis sativa* (cucumber), *Cucurbita mazima* (pumkin), *Cucurbita pepo* (vegetable marrow), ridge gourd]. No such lectin has been isolated hitherto from the family Moraceae. We now report the purification and properties of such a lectin from *Ficus cunia* (Moraceae), and a comparison of its carbohydrate specificity with those of other GlcNAc-specific lectins.

EXPERIMENTAL

Materials.—*Ficus cunia* fruits were collected locally and the seeds were obtained from the receptacle. Fetuin, fetuin–Sepharose, β-D-Gal-(1 → 4)-D-GlcNAc, β-D-Gal-(1 → 6)-D-GlcNAc, β-D-Gal-(1 → 3)-D-GlcNAc, β-D-Gal-(1 → 3)-β-D-GlcNAc-$OC_6H_4NO_2$-*p*, β-D-Gal-(1 → 3)-β-D-GlcNAc-OCH_2Ph, acrylamide, chitin, and protein markers were obtained from Sigma (St. Louis, USA); *N*,*N*-methylenebisacrylamide, ammonium persulfate, and pronase P (*Streptomyces grieseus*) from Serva (Heidelberg, FRG); and neuraminidase from Behringwerke AG, (Marburg, FRG). All other chemicals and test substances used were of highest purified grade and were obtained from the sources reported earlier[4].

Bacterial strains.—The rfe-mutants of *E. coli* $O8^-$:$K\ 27^-$ strain F 1469 (ECA^-) and *E. coli* $O8^-$: K12 strain RK 21260 (ECA^-) were obtained through the courtesy of Dr. H. Mayer, (Max-Planck-Institut für Immunbiologie, FRG).

Cells.—Human and animal blood were obtained as described[1]. The erythrocytes were treated[9] with pronase P and neuraminidase. Ehrlich ascites carcinoma (EAC) cells were collected from Swiss albino mice. Human spermatozoa were obtained from healthy donors attending a local clinic for fertility tests. Rat spermatozoa were collected from cauda with a sterile needle and suspended in glycine–HCl (pH 3.9).

Methyl 2-acetamido-2-deoxy-α- and -β-D-glucopyranoside.—These methyl glycosides were prepared by treating 2-acetamido-2-deoxy-D-glucose with methanolic 2 M HCl boiling under reflux, and isolated by HPLC (Waters Associates, Model 440) on a μ-Bondapak column, using acetonitrile–water (9:1). The purity was judged by TLC (benzene–ether, 1:1) and $[\alpha]_D$ values (Perkin–Elmer, Spectropolarimeter 141).

Preparation of chitin oligosaccharides.—Chitin was hydrolysed partialy[10], the resulting oligosaccharides of D-GlcNAc were isolated using the HPLC system described above, and their purity was tested by TLC (EtOAc–acetic acid–MeOH–water, 12:3:3:2).

Preparation of asialofetuin.—Fetuin (100 mg) was desialylated by treatment with 0.05 M H_2SO_4 at 80°C for 1 h to give asialofetuin (80 mg). The extent of desialylation was measured by the method of Aminoff[11].

Preparation of N-*linked glycopeptide.*—The glycopeptides from fetuin and asialofetuin were prepared as described[12]. Fetuin and asialofetuin (each 80 mg) were treated separately with 0.8 M $NaBH_4$ in 0.1 M NaOH at 37°C for 72 h. Each mixture was dialysed and concentrated, and the residue was digested with pronase (1%) in 0.15 M Tris-acetate buffer (pH 7.5) at 37°C for 24 h in the presence of a little toluene. More pronase (0.5%) was added, incubation was continued for 24 h, and the process was repeated twice more (total time of incubation, 96 h). Each incubate was subjected to chromatography on Sephadex G-50 and the fractions were monitored by the phenol–H_2SO_4 reaction according to Dubois et al.[13]. Each glycopeptide fraction was desalted on Sephadex G-25. The structures of the glycopeptides[14] are given in Table 1.

Soybean glycopeptide was prepared from soybean agglutinin purified on a column of hydroxyapatite[16].

Isolation of bacterial lipopolysaccharides (LPS) and their degraded products.—LPS were isolated from lyophilised bacterial cells by extraction[17] with phenol–$CHCl_3$–light petroleum. Each crude LPS was purified by repeated ultracentrifugation (40 000 rpm, 4 h), then treated with 0.25 M NaOH at 56°C for 1 h prior to Ouchterlony double-diffusion tests and inhibition of haemagglutination. The polysaccharide part (PS) of the LPS was isolated by degradation of the LPS with aq 1% acetic acid at 100°C for 2.5 h. The precipitated lipid A, isolated by centrifugation, was washed repeatedly with hot water and then with acetone. The supernatant solution was lyophilised to give the PS.

Preparation of lipid A backbone[18].—Briefly, the alkali-treated LPS was treated with 0.1 M HCl at 100°C for 30 min to yield lipid A-OH as a precipitate, part of which was hydrazinolysed and then *N*-acetylated to yield the lipid A backbone. The remainder was reduced with $NaBH_4$ in the presence of SDS, then hydrazinolysed, and *N*-acetylated to give the reduced lipid A backbone.

Purification of Ficus cunia *lectin.*—*F. cunia* seeds (50 g) were homogenised in a grinder with aq 0.85% NaCl (250 mL), the homogenate was stirred overnight at 4°C, then centrifuged (12 000 rpm, 40 min) in a refrigerated Sorvall RC-5B centrifuge, and the supernatant solution was applied to a column (1 × 10 cm) of fetuin–Sepharose, pre-equilibrated with 10 mM glycine–HCl buffer (pH 4.0) containing 20 mM $CaCl_2$. The unbound protein was eluted with the same buffer without $CaCl_2$, and the active protein was eluted from the column with PBS (10 mM phosphate containing 150 mM NaCl, pH 7.0). The fractions that showed haemagglutinating activity were combined, lyophilised, dialysed against 10 mM glycine–HCl (pH 4.0) containing 20 mM $CaCl_2$, and stored at −20°C.

Gel electrophoresis (PAGE).—The homogeneity of the lectin was assessed by electrophoresis on a 15% polyacrylamide gel at pH 4.3 in β-alanine–acetic acid buffer[19] and at pH 8.9 in Tris buffer[20]. Staining was performed with Amido Black

for the acidic gel and with Coomassie Brilliant Blue for the alkaline gel with destaining in aq 7% acetic acid.

Haemagglutination and haemagglutination-inhibition assays.—Haemagglutination assays were performed[9] in a Takatsy microtitre U-plate with two-fold serial dilution of the lectin in 10 mM glycine–HCl (pH 4.0). The haemagglutination-inhibition test was carried out as described[21].

Antiserum.—The crude lectin (2 mg/mL) was mixed with Freund's complete adjuvant (1:1) (Difco Laboratories) and injected (1.0 mL) intramuscularly into rabbits once a week for 3 weeks followed by a booster with Freund's incomplete adjuvant after 2 weeks. Blood was withdrawn by cardiac puncture in the following week for 2 consecutive days and the serum separated was preserved at −20°C.

Ouchterlony double diffusion.—The immunodiffusion was carried out on a 1% agar gel plate[22], using 100 μg of lectin in 10 μL of PBS and undiluted antiserum.

Determination of molecular weight.—The molecular weight of the lectin was determined by SDS-PAGE on a 10% gel[23] and GPC on a column (2 × 120 cm) of Sephadex G-50[24]. A solution (0.5 mL, 10 mg/mL) of the lectin was applied to the column and eluted with 0.01 M PBS at 9 mL/h (3-mL fractions). Dissociation and reduction of the protein were performed as described[1]. The molecular weight was also determined by comparing the mobility of the lectin with those of protein markers by HPLC on a Waters Associates protein analysis column I-125 (0.78 × 30 cm) of a solution (50 μL, 1 mg/mL) of the lectin, using (*a*) 10 mM PBS and (*b*) 10 mM glycine–HCl, and of a solution (100 μL, 1 mg/mL) of the lectin, using (*c*) 6 M urea and (*d*) 6 M urea containing 0.1% of 2-mercptoethanol at 48 mL/h. The protein peaks were assayed by UV absorbance at 280 nm. The protein markers were bovine serum albumin (M_r 68000), ovalbumin (45000), carbonic anhydrase (29000), trypsinogen (24000), lysozyme (14000), cytochrome C (12000), and glucagon (3500).

Analytical procedures.—Protein contents were determined by the method of Lowry et al.[25], using bovine serum albumin as the standard, and total neutral sugar by the method of Dubois et al.[13], using D-glucose as the standard. The identification and quantitation of individual neutral and amino sugars were performed by GLC of the derived alditol acetates as described[26]. Amino acid analysis was performed after hydrolysis of the lectin (0.5 mg) with 6 M HCl at 110°C for 24 h followed by preparation of isothiocyanate derivatives and HPLC on a PICO TAG C_{18} reverse-phase column[27]. Tryptophan was determined spectrophotometrically[28].

Effect of pH.—Haemagglutination with the pure lectin was performed[29] in the pH range 3.5–8.0. Titration was effected in a citrate–phosphate buffer (10 mM, pH 3.5) and was continued by increasing the pH of the mixture by successive addition of satd aq $NaHCO_3$ up to pH 7.0. Haemagglutination was also recorded reversibly by lowering the pH with 0.1 M HCl. Haemagglutination was also performed in 0.05 M PBS at pH 8.0.

Effect of divalent cations.—The effect of divalent cations on the lectin was studied as described[29].

TABLE I

Structures of glycopeptides used for haemagglutination-inhibition

Glycopeptide	Structure
Soybean	α-Man-(1 → 2)-α-Man-(1 → 2)-α-Man-(1 → 3) α-Man-(1 → 2)-α-Man-(1 → 3) α-Man-(1 → 2)-α-Man-(1 → 6) α-Man-(1 → 6) β-Man-(1 → 4)-β-GlcNAc-(1 → 4)-β-GlcNAc-Asn
Fetuin	α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 4) α-NANA-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2) α-NANA-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6) α-Man-(1 → 3) β-Man-(1 → 4)-β-GlcNAc-(1 → 4)-β-GlcNAc-Asn
Asialofetuin	β-Gal-(1 → 4)-β-GlcNAc-(1 → 4) β-Gal-(1 → 4)-β-GlcNAc-(1 → 2) β-Gal-(1 → 4)-β-GlcNAc-(1 → 2)-α-Man-(1 → 6) α-Man-(1 → 3) β-Man-(1 → 4)-β-GlcNAc-(1 → 4)-β-GlcNAc-Asn

Metal analysis.—The metal content of the purified lectin (10.4 mg) was determined[21] and removal of the metal[30] was performed as described, and the metal content was analysed. The activity of the metal-free lectin was determined by haemagglutination with or without the addition of divalent cations.

Thermal stability.—A solution of the lectin (0.12 mg/mL) in glycine–HCl (pH 3.5)[31] was incubated for 30 min at temperatures in the range 25–100°C. Aliquots (0.05 mL) were withdrawn, cooled, and tested for haemagglutination activity at room temperature.

Interaction with cells.—The interaction of the lectin with Ehrlich ascites carcinoma (EAC) cells was performed by agglutination and the specificity of the reaction was determined by the agglutination-inhibition test using EAC instead of erythrocytes[21]. The interaction of lectin with human and rat spermatozoa was performed by agglutination, and its inhibition was tested with sugars using the cells ($\sim 10^6$ cells/mL) instead of erythrocytes.

UV difference spectroscopy[32].—The intensities of the UV difference absorption spectra of the lectin (80 μg/mL in glycine–HCl) and the lectin–sugar complex were determined as a function of increasing sugar concentration. The association constant (K_a) of the lectin–sugar complex was calculated[33] from the intercept on the ordinate of the plot $S/\Delta A$ vs. S, where S is the concentration of sugar and ΔA is the difference of UV absorptions of the lectin and the lectin–sugar complex.

Circular dichroism.—The CD spectra of the lectin were recorded with a Jasco J-500C spectropolarimeter. Solutions of the lectin (2 mL, 0.12 mg/mL) in 10 mM glycine–HCl (pH 3.0) and in 10 mM PBS (pH 7.0) were used. The CD spectra were recorded over the range 208–250 nm for each buffer. The data are expressed as mean residue ellipticities $[\theta]$ in deg.cm^2.dmol^{-1}, taking a mean residue weight of 110 for each amino acid in the polypeptide chain. All recordings were made in duplicate at 25 ± 1°C.

RESULTS

Purification of F. cunia *lectin.*—Elution of the extract of *F. cunia* seeds from a column of fetuin–Sepharose with 10 mM PBS (pH 7.0) followed by dialysis against

TABLE II

Purification of *Ficus cunia* lectin

Fraction	Protein (mg/mL)	Titre [a]	Specific [b] activity	Purification (-fold)
Crude extract	1.6	8	5	1
Affinity chromatography-purified lectin	0.06	128	2133	426

[a] Haemagglutination was performed with pronase-treated human B-erythrocytes.

[b] Expressed as titre per mg of protein per mL.

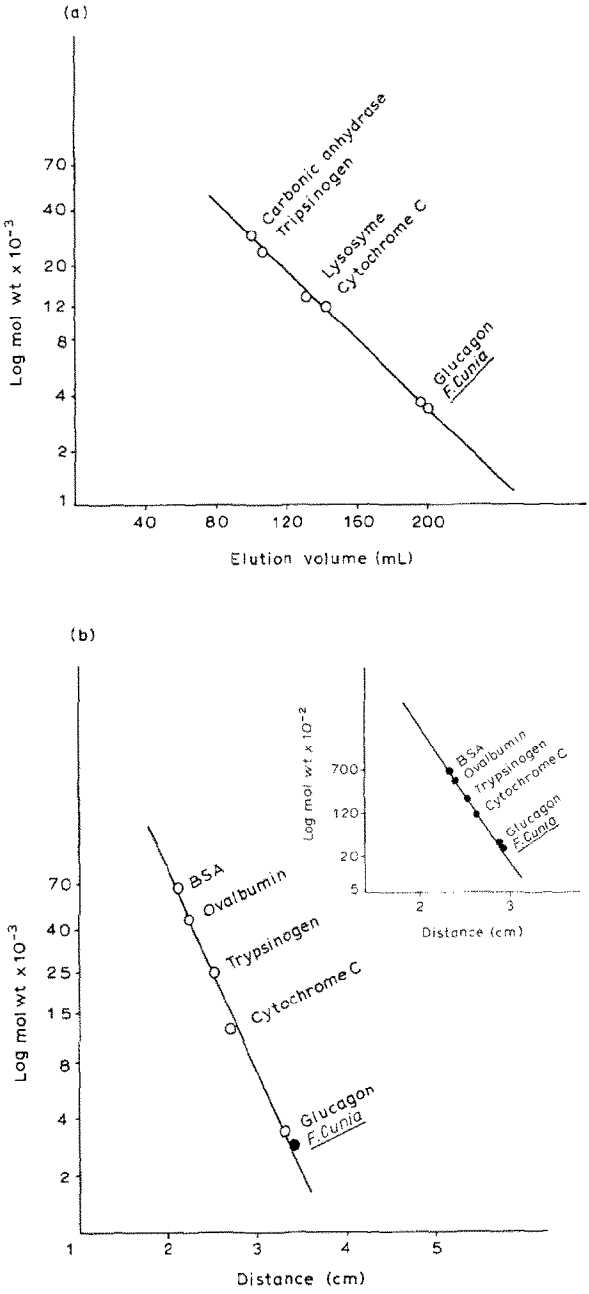

Fig. 1. Determination of the molecular weight of *F. cunia* lectin (see Experimental). (a) GPC on Sephadex G-50 by elution with 0.01 M PBS; (b) HPLC on I-125 by elution with 10 mM glycine–HCl (pH 3.5) and 10 mM PBS (inset); (c) HPLC on I-125 by elution with 6 M urea and 6 M urea containing 0.1% of 2-mercaptoethanol (inset).

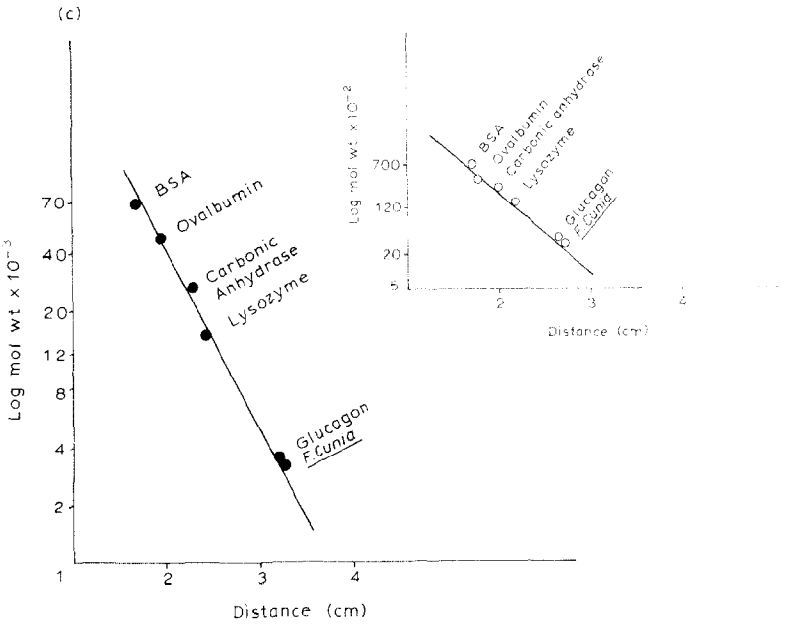

Fig. 1. (continued).

glycine–HCl buffer (pH 4.0) containing $CaCl_2$ yielded the lectin. Table II summarises the data on the purification (426-fold). The affinity column retained 0.3 mg of lectin per mL of gel, and the yield was 4.7 mg from 50 g of seeds.

Homogeneity.—PAGE of the lectin produced a single band at pH 4.3 and 8.9 indicative of homogeneity. The lectin yielded a single precipitin band with rabbit antiserum on immunodiffusion and gave a single peak in GPC and HPLC (see below).

Molecular weight.—The molecular weight of the lectin was estimated to be 3200 by GPC on Sephadex G-50 (Fig. 1a). HPLC on I-125 with glycine–HCl (pH 3.5), PBS (pH 6.8), 6 M urea, and 6 M urea containing 1% of 2-mercaptoethanol gave molecular weights in the range 3200–3500 (see Figs. 1b and 1c). SDS-PAGE, in the absence and presence of 2-mercaptoethanol, gave a single band corresponding to M_r 3500, thus showing the lectin to be a monomer.

Haemagglutination assays.—Table III summarises the results of haemagglutination tests with normal and enzyme-treated human and animal erythrocytes by the lectin. The lectin agglutinated human A, B, and O erythrocytes almost equally well, thus indicating it to be blood-group non-specific. The agglutinating activity of the lectin was enhanced by treatment of the cells with pronase and neuraminidase. The lectin moderately agglutinated sheep and chicken erythrocytes, but those of goat, rabbit, and rat were weakly agglutinated. Untreated erythrocytes of cow, buffalo, pig, and mouse were agglutinated only after treatment with pronase. Duck erythrocytes, either untreated or enzyme-treated, were not agglutinated.

TABLE III

Haemagglutination pattern of erythrocytes by *Ficus cunia* lectin [a]

Erythrocytes	Titre		
	Untreated	Pronase-treated	Neuraminidase-treated
Human			
A	16	128	128
B	16	256	128
O	16	64	64
AB	8	32	16
Sheep	8	16	32
Chicken	16	32	16
Goat	2	32	32
Rabbit	2	64	4
Rat	2	128	64

[a] At 60 μg/mL.

Carbohydrate and amino acid analyses.—Tables IV and V summarise the carbohydrate and amino acid analyses of the lectin which contained 2% of neutral carbohydrate, as determined by the phenol–H_2SO_4 reaction, a result in good agreement with the amount of neutral sugar (1.92%) and amino sugar (0.72%) determined by GLC of the lectin hydrolysates as the alditol acetates. The carbohydrate moiety contained xylose, mannose, galactose, glucose, 2-acetamido-2-deoxyglucose, and a trace of arabinose. The amino acid analysis revealed large proportions of glycine, alanine, serine, and glutamic acid.

Effect of pH.—The agglutination of erythrocytes by the lectin was decreased with increase of pH of the medium (Fig. 2) and became nil at pH 8 (not shown). The activity was regained by gradual decrease of pH of the medium.

Metal content and requirement for activity.—Atomic absorption spectroscopy of the lectin showed that Ca^{2+} was present in highest concentration (2.07 mol/mol of lectin). Mg^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and a trace of Mn^{2+} were also detected (Table VI). Exhaustive dialysis against EDTA and then against acetic acid removed 85% of the Cu^{2+} and 28% of the Ca^{2+} from the lectin, whereas there was no loss of Zn^{2+}, Fe^{2+}, and Mg^{2+}. This treatment inactivated the lectin in the haemagglutination assay. However, the addition of Ca^{2+} restored the activity (Fig. 3b). Addition

TABLE IV

Carbohydrate composition of *Ficus cunia* lectin

Sugar	g/100 g
Xyl	0.36
Man	0.66
Gal	0.28
Glc	0.61
GlcNAc	0.72

TABLE V

Amino acid composition of *Ficus cunia* lectin

Amino acid	g/100 g	Residue/mol [a]
Asp	5.8	2.0
Thr	2.5	1.0
Ser	10.5	4.0
Glu	15.0	4.0
Pro	2.5	1.0
Gly	16.2	8.0
Ala	11.5	5.0
Val	5.2	2.0
Met	2.8	1.0
Ile	2.5	1.0
Leu	2.6	1.0
Tyr	3.2	1.0
Phe	2.7	1.0
His	2.5	1.0
Lys	3.1	1.0
Trp	9.0	2.0

[a] Nearest integer.

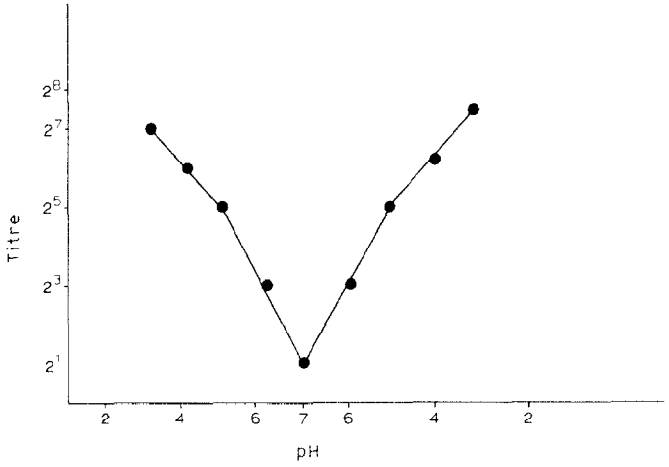

Fig. 2. Effect of pH on the agglutination of human-pronase-treated B-erythrocytes by *F. cunia* lectin.

TABLE VI

Metal content (mol/mol) of *Ficus cunia* lectin

Metal	Lectin	Dialysed lectin
Ca	2.07	1.5
Mg	0.50	0.48
Cu	0.25	0.04
Zn	0.19	0.17
Fe	0.28	0.26
Mn	trace	n.d. [a]

[a] Not determined.

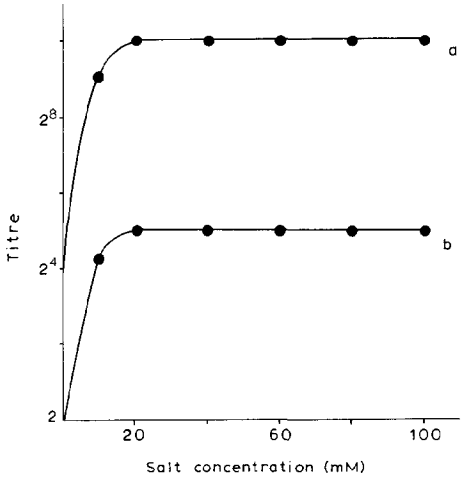

Fig. 3. Effect of Ca^{2+} on the agglutination of human-pronase-treated B-erythrocytes by (a) *F. cunia* lectin and (b) dialysed lectin.

of the above cations to the lectin also enhanced the haemagglutination titre (Fig. 3a).

Thermal stability.—The lectin was stable for a few days at room temperature. The haemagglutinating activity of the lectin decreased gradually with increase of temperature, but persisted even slightly above 95°C (Fig. 4).

Interaction with cells.—The lectin agglutinated EAC, human, and rat spermatozoa in an acidic medium. The minimum concentrations of the lectin required to

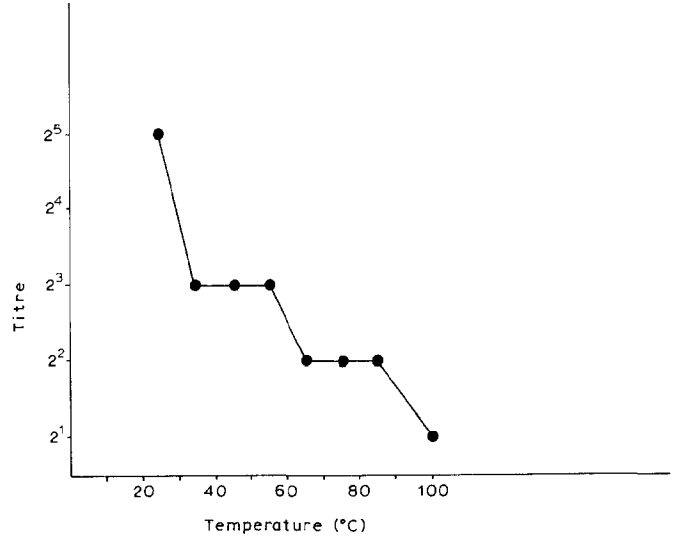

Fig. 4. Thermal stability of *F. cunia* lectin as indicated by haemagglutinating activity towards human-pronase-treated B-erythrocytes.

TABLE VII

Haemagglutination-inhibition assay of *Ficus cunia* lectin by carbohydrates

Inhibitor	Minimum inhibitory concentration [a] (mM)
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	25.0
Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside	12.5
β-D-Gal-(1 → 4)-D-Glc	200.0
β-D-Gal-(1 → 4)-D-GlcNAc	3.12
N,N'-Diacetylchitobiose	6.25
N,N',N''-Triacetylchitotriose	3.12
N,N',N'',N'''-Tetra-acetylchitotetraose	0.78
β-D-GlcNAc-(1 → 6)-D-GlcNAc (lipid A backbone)	2.74
β-D-GlcNAc-(1 → 6)-D-GlcNAc-OH (lipid A backbone reduced)	2.74
Fetuin glycopeptide	0.20
Asialofetuin glycopeptide	0.075
Soybean glycopeptide	0.285

[a] Required for complete inhibition of two haemagglutinating doses of lectin: D-GlcN (400 mM), D-GlcNAc (400 mM), D-Glc (200 mM), α-D-Glc-OMe (400 mM), β-D-Glc-OMe (400 mM), D-Man (200 mM), α-D-Man-OMe (200 mM), D-Gal (200 mM), α-D-Gal-OMe (200 mM), β-D-Gal-OMe (200 mM), L-Fuc (200 mM), β-D-Glc-(1 → 4)-D-Glc (400 mM), α-D-Glc-(1 → 4)-D-Glc (400 mM), β-D-Gal-(1 → 6)-D-GlcNAc (100 mM), β-D-Gal-(1 → 3)-D-GlcNAc (100 mM), β-D-Gal-(1 → 3)-β-D-GlcNAc-$OPhNO_2$-*p* (100 mM), β-D-Gal-(1 → 3)-β-D-GlcNAc-OCH_2Ph (100 mM). Melibiose (400 mM) and raffinose (200 mM) were non-inhibitory.

agglutinate EAC and human spermatozoa were 12.5 and 6.25 μg/mL, respectively, whereas that for rat spermatozoa was 1.56 μg/mL. The respective values were 27, 13.3, and 3.4 times higher than the value (0.46 μg/mL) for human erythrocytes. Each agglutination was inhibited strongly by *N,N',N'',N'''*-tetra-acetylchitotetraose (2.25 mM), fetuin (0.6 mM), and asialofetuin glycopeptide (0.25 mM), suggesting that the interaction was carbohydrate specific.

Inhibition assays.—Tables VII and VIII show the results of a haemagglutination-inhibition study of the lectin with carbohydrates and glycoconjugates. It was evident from the structures in Table I that the macromolecules containing either multiple *N*-acetyl-lactosylamine and/or *N,N'*-diacetylchitobiose moieties were

TABLE VIII

Haemagglutination-inhibition assay of *Ficus cunia* lectin by macromolecules

Inhibitor	Minimum inhibitory concentration (mg/mL) [a]
Fetuin	0.75
Asialofetuin	0.35
Horse erythrocyte mucoid	0.625
Birds nest glycoprotein	1.25
Human chorionic gonadotropin	0.93
E. coli RK 21260 LPS	0.185
E. coli F 1469 LPS	0.185

[a] Required for complete inhibition of two haemagglutinating doses of lectin.

inhibitors of the lectin. Sialic acid was non-inhibitory. Furthermore, removal of sialic acid from either glycoproteins or glycopeptides facilitated the inhibition. (Table VII). The glycopeptide of asialofetuin at only 0.075 mM inhibited two haemagglutinating doses of lectin and was three and four times more potent than those of fetuin and soybean, which required 0.20 and 0.285 mM, respectively. Similarly, asialofetuin (0.35 mM) was twice as effective as fetuin (0.75 mM). The haemagglutinating activity of the lectin was inhibited by LPS preparations from two strains of *E. coli*. The polysaccharide part of these bacterial LPS did not inhibit, although the lipid A backbone and its reduced form did. These results show that the lectin can also recognise the β-GlcNAc-(1 → 6)-GlcNAc moiety in the backbone of lipid A. However, this backbone and its reduced form had less affinity for the lectin than the glycopeptides of asialofetuin, fetuin, and soybean which, respectively, were ~ 36, 14, and 10 times more inhibitory. Among the macromolecules tested, the *E. coli* LPS were the most effective (0.185 mg/mL). Horse erythrocyte mucoid, like fetuin, was a good inhibitor, whereas human chorionic gonadotropin and birds-nest glycoprotein were moderate inhibitors.

Haemagglutination by the lectin was inhibited by methyl 2-acetamido-2-deoxy-α- and -β-D-glucopyranoside (25 and 12.5 mM, respectively), whereas the methyl glycosides of α- and β-D-glucopyranose, α- and β-D-galactopyranose, and α-D-mannopyranose were inactive at 200 mM. 2-Acetamido-2-deoxy-D-glucose was a

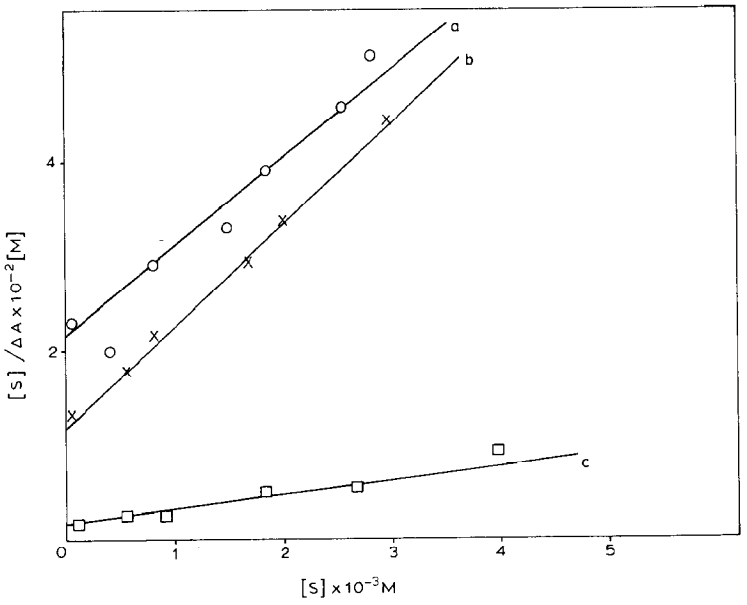

Fig. 5. Determination of the association constant (K_a) by Scatchard plot. *F. cunia* lectin (0.08 mg/mL) was titrated with various amounts (10–80 μL) of (a) 30 mM *N,N'*-diacetylchitobiose, (b) 20 mM *N,N',N''*-triacetylchitotriose, and (c) 10 mM *N,N',N'',N'''*-tetra-acetylchitotetraose at 22°C in 10 mM glycine–HCl (pH 3.5).

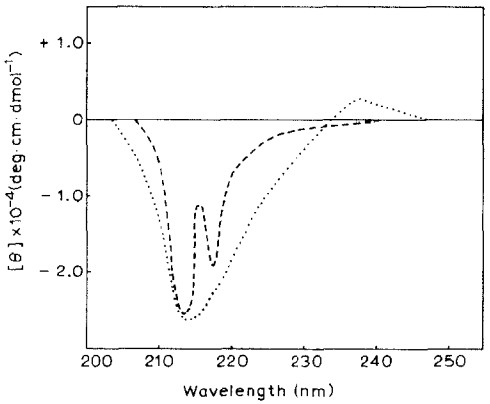

Fig. 6. CD spectrum of *F. cunia* lectin in the region 200–250 nm in 10 mM glycine–HCl (pH 3.0) (— — —) and 10 mM phosphate buffer (pH 7.0) (·····). The light path was 1 mm and the protein concentration was 0.12 mg/mL.

non-inhibitor. *N*,*N*′,*N*″,*N*‴-Tetra-acetylchitotetraose was a more potent inhibitor (0.78 mM) than *N*,*N*′,*N*″-triacetylchitotriose (3.12 mM) and *N*,*N*′-diacetylchitobiose (6.25 mM), and was 3.5 times more inhibitory than β-D-GlcNAc-(1 → 6)-D-GlcNAc and its reduced form, which, in turn, were about 2.3 times more effective than *N*,*N*′-diacetylchitobiose. Lactose was a poor inhibitor (200 mM), whereas *N*-acetyl-lactosamine was a good inhibitor (3.12 mM).

UV difference spectroscopy.—Fig. 5 represents the plot of $S/\Delta A$ vs. S, obtained by UV difference spectroscopy for the binding of the lectins to *N*,*N*′,*N*″,*N*‴-tetra-acetylchitotetraose, *N*,*N*′,*N*″-triacetylchitotriose, and *N*,*N*′-diacetylchitobiose. The association constant (K_a), calculated from the intercept, revealed that *N*,*N*′,*N*″,*N*‴-tetra-acetylchitotetraose has a higher binding capacity (1.33×10^{3} M^{-1}) than *N*,*N*′,*N*″-triacetylchitotriose (8.77×10^{2} M^{-1}) and *N*,*N*′-diacetylchitobiose (6.26×10^{2} M^{-1}).

Circular dichroism.—The CD spectra of the lectin at pH 3.0 and 7.0 in the range 200–250 nm are shown in Fig. 6. At pH 3.0, two negative extrema appear at 213 and 218 nm, whereas, at pH 7.0, the curve has a negative extremum at 216 nm and a small positive extremum at 238 nm, which probably reflect different conformations.

DISCUSSION

The agglutinating activity of purified *F. cunia* lectin is strongly pH-dependent. If this phenomenon is due to changes in conformation, then alteration of the pH of the eluent in affinity chromatography should affect desorption. The change in conformation of the lectin at acidic and neutral pH is also substantiated by CD (Fig. 6). Since the β-conformation of a protein is usually characterised by a negative extremum at 216 nm and by a small positive extremum at 238 nm, it is

concluded that the lectin has mostly the β-conformation at pH 7.0, whereas, at pH 3.0, the negative extrema at 213 and 218 nm indicate contributions from other conformations. The desorption of the lectin in affinity chromatography was accomplished by raising the pH of the buffer from 4.0 to 7.0.

The *F. cunia* lectin is a human-blood-group non-specific erythro-agglutinin and also agglutinated animal erythrocytes. It behaved like an incomplete non-agglutinating lectin for cow, buffalo, pig, and mouse erythrocytes, and the receptor was detectable serologically only after treatment with pronase, which removes sterically hindering glycoproteins, and neuraminidase which reduces the zeta potential[34].

Although most of the plant lectins have molecular weights in the range 30 000–140 000, *F. cunia* lectin consists of a single polypeptide chain with M_r 3 500 that contains little carbohydrate, which is comparable to the chitin oligosaccharide-specific monomeric lectin of M_r 5 000 isolated from the wood tissue of *Robinia pseudoacacia*[35]. A blood-group-A-specific lectin from the seeds of *Crotalaria striata* has been reported[29], which has a molecular weight of 3 400–3 500.

Like the lectins from *A. integrifolia*[4], *Artocarpus lakoocha*[31], *Erythrina indica*[36], and *C. striata*[29], *F. cunia* lectin is remarkably stable to temperature. Similar to Con A and lectins from *Lens culinaris*, *Griffonia simplicifolia* I (GS I), *A. integrifolia*[4], and *A. lakoocha*[21], *F. cunia* lectin is a metalloprotein that contains large proportions of Ca^{2+} and Mg^{2+} by which its activity is markedly enhanced, a phenomenon generally observed for lectins. Removal of the cations from the lectin by dialysis against EDTA and then against acetic acid impaired the activity, although there was no significant loss in the metal content. The maximum activity of the lectin was at pH 3.5 and was lost above pH 7.5. Such pH-dependent activity was also observed in the ability of the lectin to bind EAC and human and rat spermatozoa. The agglutination of EAC by several lectins[4,21,37–39] is well known. Inhibition of agglutination of the above-mentioned cells by chitin oligosaccharides and glycopeptides containing GlcNAc residues gave insight into the presence of such oligosaccharides as receptors.

As shown by Table VII, haemagglutination by *F. cunia* lectin was inhibited only by the methyl α- and β-glycosides of D-GlcNAc and not by D-GlcNAc. In contrast, potato, tomato, and *Datura* lectins bind neither to D-GlcNAc nor its methyl α- and β-glycosides[8]. The lectins from cereal grains, e.g., wheat germ, barley, rye, rice, and *G. simplicifolia* II, bind to the above glycosides and the parent sugar, thus showing an inhibition pattern that is subtly different to that of *F. cunia* lectin. Thus, the *N*-acetyl group and the 1-substituent in D-GlcNAc-OMe are necessary for binding.

The absolute requirement of the *N*-acetyl group was demonstrated by the fact that D-glucose, maltose, and cellobiose did not bind to the *F. cunia* lectin. 2-Acetamido-2-deoxy-D-glucose with a 4-*O*-β-D-galactopyranosyl substituent, i.e., *N*-acetyl-lactosamine, inhibited the binding, whereas the 3- and 6-linked analogues and the *p*-nitrophenyl and benzyl glycosides of β-D-Gal-(1 → 3)-β-D-GlcNAc had

no effect (Table VII). Although *N*-acetyl-lactosamine was not an inhibitor of wheat germ and potato lectins[5], S-XIV capsular polysaccharide with internal 4- and 6-substituted GlcNAc residues interacted with *Datura*[40] and wheat germ[41] lectins. *F. cunia* lectin, unlike wheat germ[42] and rice[43] lectins but like potato, tomato, and *Datura* lectins, binds N,N',N'',N'''-tetra-acetylchitotetraose more strongly than the tri- and di-saccharide analogues. Inhibition by (1 → 4)-linked oligosaccharides of β-D-GlcNAc indicates that HO-4 must be substituted for effective binding to the lectin. However, the lipid A backbone, which contains β-D-GlcNAc-(1 → 6)-D-GlcNAc as an epitope in LPS for *F. cunia* lectin, inhibited the haemagglutination-inhibition reaction, as did reduced lipid A backbone, which indicates that the terminal β-D-GlcNAc-(1 → 6) moiety is the minimum requirement for binding to the lectin. The higher inhibitory potency of β-D-GlcNAc-(1 → 6)-D-GlcNAc compared to that of the 4-linked analogue is probably due to the greater flexibility of the molecule because of the presence of an extra bond.

The binding affinity of *F. cunia* lectin for chitin oligosaccharides was also demonstrated by the fact that the lectin-induced agglutination was inhibited by fetuin[14], asialofetuin, which contains multiple N,N'-diacetylchitobiosyl residues, and human chorionic gonadotropin (HCG), which has multiple β-Gal-(1 → ?)-GlcNAc units[44]. The inhibition of agglutination by birds nest glycoprotein[45] and horse erythrocyte mucoid[34] is presumably due to the presence of *N*-acetyl-lactosamine residues. The highest inhibitory potency exhibited by *E. coli* LPS is due not only to (1 → 6)-linked β-GlcNAc units but also to hydrophobic interaction of the fatty acid chains.

The marked affinity of *F. cunia* lectin for chitin oligosaccharides was also demonstrated by UV difference spectroscopy. If tryptophan or tyrosine chromophores are involved in a conformational change associated with the interaction of the sugar and the lectin, a change in the UV spectrum should occur. By the addition of various concentrations of sugars and taking λ_{max} in the difference spectrum as a measure of the formation of the lectin–sugar complex, the binding constant K_a was calculated, which was a maximum for N,N',N'',N'''-tetra-acetylchitotetraose (1.33×10^3 M^{-1}), a value comparable to that (3.75×10^3 M^{-1}) for the *Datura* lectin–N,N',N''-triacetylchitotriose interaction[8].

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