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Isolation, Macromolecular Properties, and Combining Site of a Chito-oligosaccharide-specific Lectin from the Exudate of Ridge Gourd (*Luffa acutangula*)*

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A lectin specific for chito-oligosaccharides from the exudate of ridge gourd (*Luffa acutangula*) fruits has been purified to homogeneity by affinity chromatography. The lectin has a molecular weight of 48,000, an $s^0_{20,w}$ of 4.06 S and a Stokes radius of 2.9 nm. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a single band corresponding to M_r of 24,000 was observed both in the presence and absence of β-mercaptoethanol. The subunits in this dimeric lectin are, therefore, held together solely by noncovalent interactions. The lectin is not a glycoprotein, and secondary structure analysis by CD measurements showed 31% α-helix. The hemagglutinating activity of *L. acutangula* agglutinin was not inhibited by any of the monosaccharides tested. Among the disaccharides only di-*N*-acetylchitobiose was inhibitory. The inhibitory potency of chito-oligosaccharides increased dramatically with their size up to penta-*N*-acetylchitopentaose. The lectin has two binding sites for saccharides. The affinity of chito-oligosaccharides for *L. acutangula* lectin, as monitored by titrating the changes in the near UV-CD spectra and intrinsic fluorescence, increased strikingly with the number of GlcNAc units in them. The values of ΔG, ΔH, and ΔS for the binding process showed a pronounced dependence on the size of the chito-oligosaccharides, indicating that the binding of higher oligomers is progressively more favored thermodynamically than di-*N*-acetylchitobiose. The thermodynamic data are consistent with an extended binding site in this lectin, which accommodates a tetrasaccharide.

Lectins, a class of hemagglutinating proteins are ubiquitously distributed in nature (1–5). Because of the high degree of selectivity shown by the individual lectins for their interactions with glycoproteins and glycolipids (6–8), lectins are increasingly being employed as highly discriminating macromolecular probes in lymphocyte mitogenesis, in the purification of glycoproteins, in the study of cell surfaces of normal and cancerous cells, etc. (3, 4).

Lectins were initially discovered in plant seeds and therefore, these lectins have been most widely studied. Reports on their role in plants which relate their turnover in seeds may shed light on their functions in development and differentiation, and in root-bacterium symbiosis. Lectins, however, have also been found in other parts of the plant (2, 9). Currently, major efforts are being directed toward elucidating the relationship of lectins isolated from various parts of the plants and their overall function. In this regard, the discovery of a lectin from the phloem exudate of pumpkin (*Cucurbita maxima*) and some other cucurbits (10) provides an opportunity to investigate their functions in view of their unusual location in plants. The sugar specificity of these lectins was shown to be directed toward di-*N*-acetylchitobiose[1] (11), similar to the lectins from wheat germ (12), potato tubers (13), thorn apple (14), and tomato fruits (15, 16). In this study we report the isolation and characterization of a chito-oligosaccharide-binding lectin discovered in the exudate of ridge gourd (*Luffa acutangula*) fruits as a prelude to our studies on the structure-function relationship of exudate lectins. Lectins binding to GlcNAc or its oligomers have been described in the recent years; however, they appear to exhibit multiple specificities for binding to carbohydrate ligands (17–19). Therefore, the *L. acutangula* lectin should prove to be a valuable probe for studying the expression, biogenesis, and structure of chitobiosyl-containing glycoproteins. We show that this lectin possesses a rather highly extended binding site which accommodates a tetrasaccharide. This lectin, therefore, differs from all other GlcNAc-binding lectins including those from the other cucurbits.[2] The most likely arrangement of the GlcNAc units in the combining region of this lectin is also proposed.

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[1] The abbreviations used are: di-*N*-acetylchitobiose, *N,N'*-diacetylchitobiose; tri-*N*-acetylchitotriose, *N,N',N''*-triacetylchitotriose; tetra-*N*-acetylchitotetraose, *N,N',N'',N'''*-tetraacetylchitotetraose; penta-*N*-acetylchitopentaose, *N,N',N'',N''',N''''*-pentaacetylchitopentaose; ManNac, *N*-acetyl-D-mannosamine; LacNAc, *N*-acetyllactosasmine; MeαGlcNAc, methyl-2-acetamido-2-deoxy-α-D-glucopyranoside (MeβGlcNAc refers to β anomer); PBS, phosphate-buffered saline (0.02 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl); βME, β-mercaptoethanol; GuHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; ConA, concanavalin A; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

[2] Appeared as abstracts in the following: Anantharam, V., Patanjali, S. R., and Surolia, A. (1983) in the *Third Congress of the Federation of Asian and Oceanian Biochemists*, Bangkok, Thailand, Nov. 29–Dec. 2, 1983, p. 75; Surolia, A. (1984) in the *International Symposium on Biomolecular Structure and Interactions*, Bangalore, India, Dec. 17–22, 1984, p. 43.

EXPERIMENTAL PROCEDURES AND RESULTS[3]

DISCUSSION

This report describes the purification and characterization of a highly specific, chito-oligosaccharide-binding lectin from the exudate of *L. acutangula* fruits. A molecular weight of 48,000 for the native protein and 24,000 for the denatured protein obtained by several methods allowed us to conclude that the protein is composed of two identical subunits. Since the molecular weight of the native lectin as well as that of its subunits obtained by denaturation with sodium dodecyl sulfate or guanidine HCl did not vary on treatment with β-mercaptoethanol, disulfide bonds are apparently not involved in subunit association of this dimeric, cysteine-containing lectin. Its hydrodynamic properties are consistent with those expected for a globular protein. *L. acutangula* lectin is characterized by a high content of α-helix. The absence of β-pleated structure appears to be a distinct feature of this protein when compared with most other lectins (70–72).

Unlike wheat germ agglutinin (WGA) (73), the agglutinating activity of *L. acutangula* lectin is not inhibited by GlcNAc or its methylglycosides. In this respect it differs from potato (72), thorn apple (17), and other cucurbits (11) as well, which are weakly inhibited by GlcNAc. The other *N*-acetylated hexosamines tested were also ineffective. This lectin is specifically inhibited by di-*N*-acetylchitobiose and higher oligomers of GlcNAc, and their potency increases strikingly with increase in their size. When compared with WGA, which is almost equally inhibited by tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetraose, and the *Cucurbita pepo* lectin where di-, tri-, and tetrasaccharides are equally potent inhibitors (11, 72), the *L. acutangula* lectin is notably better inhibited by tetra-*N*-acetylchitotetraose and penta-*N*-acetylchitopentaose, implying an even more extended combining site than in any lectin with related specificity. This lectin resembles marrow lectin and WGA in its ability to recognize the internal di-*N*-acetylchitobiosyl sequences in the *N*-linked glycopeptides from fetuin, ovalbumin, and soybean agglutinin (11, 35, 74, 75). Higher activities of the glycopeptides could be due to additional interactions with the sugar residues in the branching regions or due to a better orientation of the appropriate di-*N*-acetylchitobiosyl conformer in these complex carbohydrates. However, the possibility of multiple interactions, also termed as "cooperative effect" as observed for WGA (76, 77) may be ruled out to explain the stronger binding of *L. acutangula* agglutinin to these glycopeptides, as the soybean agglutinin glycopeptide, which is devoid of GlcNAc residues in the branching region, is an equally potent inhibitor when compared with the glycopeptides from fetuin and ovalbumin. The recognition of these glycopeptides by the lectin appears to be exclusively mediated by the core di-*N*-acetylchitobiosyl sequence in these glycopeptides. This is also consistent with our failure to observe the inhibition of agglutination by the oligosaccharide derived from endo-β-*N*-acetylglucosaminidase H treatment of soybean agglutinin glycopeptide. The residues on either side of the di-*N*-acetylchitobiosyl sequence by themselves do not, therefore, contribute appreciably to the binding process.[4] Better orientation of the di-*N*-acetylchitobiosyl sequence in the core region of these glycopeptides is, therefore presumably responsible for the higher potency of glycopeptides when compared to chito-oligosaccharides themselves.[5] Equilibrium dialysis data show that the stoichiometry for the lectin-sugar interaction is 1 per polypeptide chain. The near UV-CD spectrum of *L. acutangula* lectin shows considerable ligand concentration and size-dependent enhancements in the molar ellipticities. An increase in $\Delta\epsilon$ in the difference spectrum of the protein corresponding to the tryptophanyl side chains correlates qualitatively with the molar ellipticity changes observed in the near UV-CD spectrum, suggesting that most of these changes can be ascribed to perturbation of tryptophan absorbance in the protein.[6]

The enhancement in the lectin fluorescence, which is typical of tryptophan emission in proteins, and its blue shift on binding to chito-oligosaccharides are related to the number of GlcNAc units in these saccharides and reflect some unique spatial disposition of the bound saccharides with respect to a tryptophan residue in the binding site.[7] Increments in blue shift indicate a progressive increase in hydrophobicity of the environment around the tryptophan residue(s) with increase in the size of the saccharide.

The association constants (K_a) obtained by fluorescence as well as near UV-CD titrations differ greatly from one ligand to another. The binding affinities of *L. acutangula* lectin for various saccharides as obtained from fluorescence and CD spectroscopy show a more pronounced effect on ligand size than that observed for potato lectin and WGA (72, 79). The affinity of penta-*N*-acetylchitopentaose, tetra-*N*-acetylchitotetraose, and tri-*N*-acetylchitotriose is about 550, 78, and 9 times greater, respectively, than di-*N*-acetylchitobiose.

The increase in the values of association constants (K_a) with ligand size is apparently not related to a statistical increase of binding probability for the combining site accommodating a single sugar residue, as the magnitude of the affinities is much higher than what could be ascribed to statistical effects (80). Alternatively, this consistently large

[3] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–6, and Tables I–VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2245, cite the authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[4] We have also examined the binding of soybean agglutinin glycopeptide to the *L. acutangula* lectin by fluorescence spectroscopy where the extrapolated change in fluorescence intensity for totally bound glycopeptide [ΔF_a] amounts to 16% with a 2 nm blue shift in the emission maximum in the fluorescence of the lectin. The values of K_a at 23, 28, and 36 °C are 2.29×10^5 M^{-1}, 1.995×10^5 M^{-1}, and 1.349×10^5 M^{-1}, respectively. These values are quite close to the values obtained for penta-*N*-acetylchitopentaose. The values of $-\Delta H$ and $-\Delta S$ at 23 °C are 32.5 kJ mol^{-1} and 7.1 J mol^{-1} K^{-1}, respectively. A more favorable entropy when compared to the values obtained for chito-oligosaccharides is presumably responsible for the strong binding of the glycopeptide and may reflect a better juxtaposition of the glycopeptide determinants for the binding to the lectin. No enhancement was found on addition of soybean agglutinin AsN-oligosaccharide (5 μM) and ^{14}C-labeled Asn-GlcNAc (10 μM) at concentrations indicated in parentheses. However, since no thermodynamic results are available in the literature to compare the binding of glycopeptides to *L. acutangula* lectin, this phenomenon deserves greater attention.

[5] The fact that this lectin does not bind to *N*-1-[1-deoxy-*N*,*N*′-diacetylchitobiitol]-aminoethyl-Biogel P-300 (78), an affinity matrix with a high density of β-D-GlcNAc residues, also supports the conclusion that multiple interactions involving binding to monosaccharides alone are not sufficient to stabilize the complex.

[6] V. Anantharam, and A. Surolia, unpublished observations.

[7] Among the 3 tryptophan residues in the protein only one is accessible to *N*-bromosuccinimide when the reaction is carried out under native conditions. The modification of this tryptophan residue located on the surface of the lectin completely abrogates its carbohydrate-binding ability (V. Anantharam and A. Surolia, unpublished observations). The ligand-induced alterations in the emission spectra of the lectin can therefore be ascribed to the perturbation of the surface tryptophan residue which is also involved in its activity.

TABLE VII

Proposed arrangement of chito-oligosaccharides in various subsites of L. acutangula lectin and ΔG and ΔH contributions of each subsite for the ligand binding deduced from Table VI

These β(1→4)-linked saccharides are arranged in their respective subsites with the reducing end to the right. The contribution of each subsite to the association of an oligosaccharide with *L. acutangula* lectin was calculated by comparing ΔG and ΔH values for pairs of saccharides which differ by a single sugar residue. It is assumed that an additional sugar residue (italicized in the table) is bound at the subsite listed in the column with its contribution to ΔG and ΔH as determined by comparing the values for pairs of saccharides.

A	B	C	D	E	Contribution to $-\Delta G$	Contribution to $-\Delta H$
					kJ mol⁻¹	
GlcNAc						
GlcNAc-*GlcNAc*					17.0	41.0
GlcNAc-GlcNAc-*GlcNAc*					5.3	6.9
GlcNAc-GlcNAc-GlcNAc-*GlcNAc*					5.4	8.0
GlcNAc-GlcNAc-GlcNAc-GlcNAc-*GlcNAc*					5.1	0.1

increase in the K_a values for the binding of the lectin to saccharides with increase in their size can be explained if one assumes that the combining site of the lectin consists of a number of subsites, each of which accommodates one sugar unit, and that the association of a given subsite with a single sugar moiety is independent of other units in the saccharide. With the assumption that the $-\Delta G$ for any saccharide equals the sum of the free energies from each subsite filled, the contribution of $-\Delta G$ from each subsite in the lectin is obtained by comparing a pair of saccharides, and these values, together with the probable positions for the binding of saccharides, are listed in Table VII. B, C, and D are strong binding subsites, E is a moderate affinity subsite, and subsite A is unfavorable, since GlcNAc does not bind. The fact that there is a striking increase in $-\Delta G$ of binding as additional units of GlcNAc are added would support our view that B-C-D-E are contiguous favorable subsites.[8]

The thermodynamic parameters obtained from the temperature dependence of K_a values differ appreciably for oligosaccharides of different length (Table VI). Subsites C, D, and E contribute 6.9, 8.0, and 0.1 kJ mol^{-1}, respectively, to the binding enthalpy of the corresponding GlcNAc residues of the saccharides. In light of these data, we consider this lectin to possess an extended binding site which accommodates a tetrasaccharide. This is in marked contrast to concanavalin A, where the values of $-\Delta H$ remain constant with the increase in the chain length of α(1→2)-linked manno-oligosaccharides (81). In WGA, the values of $-\Delta H$ are ligand chain length-dependent up to the trisaccharide; however, the effect is larger for the disaccharide di-*N*-acetylchitobiose over that observed for GlcNAc (82). It is interesting to compare here the situation of *L. acutangula* lectin with lysozyme, where the bindings of GlcNAc, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose show a pronounced increase in $-\Delta H$ with increase in ligand chain length (83, 84). Likewise, a striking increase in the values of $-\Delta H$ would imply more extensive hydrogen bonding, and van der Waal's interactions in the binding site of *L. acutangula* lectin as the dimensions of the saccharide(s) are increased. Practically unchanged values of $-\Delta H$ for penta-*N*-acetylchitopentaose when compared with tetra-*N*-acetylchitotetraose indicate that contribution of subsite E to binding enthalpy is insignificant. However, a comparatively low value of $-\Delta S$ for penta-*N*-acetylchitopentaose suggests that the binding of this saccharide is highly favorable. The values of $-\Delta S$, which are larger than the entropies of mixing (51), also show a progressive increment as the number of GlcNAc units in the saccharides increase (up to the tetrasaccharide). These unfavorable changes in entropy conceivably reflect loss in translational entropy and/or changes in conformation(s) of the saccharides and/or the lectin. Nevertheless, the binding of saccharides to *L. acutangula* lectin is highly favorable because of an increase in the values of $-\Delta H$ which outweighs the unfavorable contribution of change in entropy.

[8] Schematization of the binding positions represented here may be an oversimplification of the binding process; however, the increase in the fluorescence intensity and the blue shift accompanying the ligand binding have allowed us to prefer the saccharide locations on various subsites as outlined in Table VII.

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Addendum—The values of the association constants for the binding of hexa-*N*-acetylchitohexaose (from Seikagaku Kogyo Co., Ltd., Tokyo, Japan) to *L. acutangula* lectin are 1.13×10^6 M^{-1}, 7.5×10^5 M^{-1}, 5.3×10^5 M^{-1}, and 2.75×10^5 at 15, 20, 25, and 36 °C, respectively. The values of $-\Delta G$, $-\Delta H$, and $-\Delta S$ correspond to 32.67 kJ mol^{-1}, 55 kJ mol^{-1}, and 75 J mol^{-1} K^{-1} at 25 °C, respectively.

A lack of increment in the value of $-\Delta H$ for the binding of hexa-*N*-acetylchitohexaose when compared with tetra-*N*-acetylchitotetraose is consistent with our proposal that the combining region of *L. acutangula* lectin is most complementary toward tetra-*N*-acetylchitotetraose.

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Supplement to Isolation, Macromolecular Properties and Combining Site of a Chito-oligosaccharide Specific Lectin from the Exudate of Ridge Gourd (*Luffa acutangula*).

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Experimental Procedures

Materials: Sepharose-6B, Sephadex G-100, G-50, G-25, G-15 and G-10 were products of Pharmacia, Uppsala, Sweden. Proteins used as molecular weight markers in gel chromatography, as well as SDS molecular weight markers, mannosamine hydrochloride, N-acetyllactosamine, chitin, fetuin, concanavalin A (Con A), wheat germ agglutinin [WGA], ovalbumin, neuraminidase, Dowex 50-X8,-X2, Dowex 1-X8 and Dansyl amino acids were products of Sigma Chemical Co., St. Louis, U.S.A. Endo-β-N-acetylglucosaminidase H was a product of Koch-Light Laboratories. Soybean agglutinin was prepared as described in (20). Con A and WGA were coupled to Sepharose-6B by the method of March et al. (21). Sodium boro [^{3}H] hydride, [^{125}I] NaI and [^{14}C] formaldehyde were products of Amersham Corporation, Illinois, U.S.A. Radioactive measurements were done using 1275 'Mini Gamma' gamma counter Model 43, LKB (Wallac) and on LKB [Wallac] 1217 Rack beta liquid-scintillation counter, with the aid of scintillation fluid, made up by dissolving 800 mg of diphenyloxazole and 20 mg of 1,4-bis [5-phenyloxazole-2-yl] benzene in 200 ml of toluene and 100 ml of Triton X-100. All other chemicals were commercial products of the highest quality available. Ridge gourd [*L.acutangula*] fruits were bought from local green grocers.

Methods

Preparation of N-acetylmannosamine, Methyl-2-acetamido-2-deoxy α and β-D-glucopyranosides: Mannosamine hydrochloride was N-acetylated as described in (22). Anomers of methyl GlcNAc were prepared as described in (23,24).

Preparation of chitin oligomers and manno-oligosaccharides: Chitin hydrolysate was prepared as described by Rupley (25). The chitin oligomers were separated on Biogel P-2 (26) and their purity was assessed by thin layer chromatography (27). α[1—>2] linked Manno-oligosaccharides were prepared as described in (28).

Analytical Methods

Neutral sugars were determined by phenol-sulfuric acid method (29). Mannose and equimolar mixture of mannose and galactose were used as standards for quantifying neutral sugars in SBA, fetuin and ovalbumin glycopeptides. Aminosugar content of the glycopeptides were determined by amino acid analyzer Model LKB 4400 after hydrolysis with 4N HCl at 100°C for 6h. Neutral sugar was also determined by hydrolyzing the glycopeptide in 1N HCl for 4h at 100°C in sealed and evacuated tubes. After the removal of acid, the hydrolysate was passed through coupled columns of Dowex 50-X8 (H^+ form) and Dowex 1-X8 (acetate form) (30). The neutral sugars thus eluted were detected and quantified by phenol-sulfuric acid method. Aspartic acid from glycopeptides in column chromatography experiments was detected by ninhydrin reaction (31). Paper chromatography for neutral sugars, hexosamines and oligosaccharides were carried out on Whatman No.1 paper by the descending technique and the solvent system used was pyridine-ethylacetate-water-acetic acid (5:5:3:1) with pyridine-ethylacetate-water (11:40:6) in the bottom of the chromatography chamber (32). Protein concentrations were determined by the dye-binding method of Bradford (33) or by their absorbance at 280 nm. Amino sugars were quantified as per the procedure given in (34).

Isolation of soybean agglutinin glycopeptide (SBA-GP): SBA-GP was prepared as per the procedure of Lis and Sharon (35) with minor modifications as described below in brief. Soybean agglutinin (500 mg) was dissolved in 80 ml of 0.1 M HCl and its pH adjusted to 2. The protein was denatured by heating the solution for 15 min at 50°C, the pH was then brought to pH 8.5 with 1 M NaOH. To this solution, pronase (Calbiochem) (5 mg) in 5 ml of Tris buffer, pH 8.5 and 0.06 M in $CaCl_2$, was added and the mixture incubated for 72 h at 50°C. The digest was fractionated on Sephadex G-50 in 0.01 M acetic acid. The elution was monitored for neutral sugar and ninhydrin reaction. The fractions containing neutral sugars (eluting in the inner volume) were pooled, lyophilized and again digested with pronase and rechromatographed as stated above on the same column under similar conditions. The product thus purified was dissolved in 0.001 M pyridine acetate, pH 2.7 and chromatographed on Dowex 50-X2, in the same buffer. The major peak containing most of the glycopeptide was used for further studies. The structure of SBA glycopeptide (36) is given in Figure 1.

Preparation of asialofetuin and agalactofetuin: Fetuin was desialated by heating at 80°C in 0.05 N H_2SO_4. The extent of desialation was determined by thiobarbituric acid method (37). Asialofetuin (500 mg in 30 ml) was incubated with β-galactosidase from jackbean in 0.15 M sodium citrate buffer at pH 4.5 at 1 unit/ml concentration for 120 h at 37°C (38). The released galactose was quantified by neutral sugar content after its separation from reaction mixture using the coupled Dowex 50 and Dowex 1 column and charcoal-celite column.

Preparation of N-linked glycopeptides from fetuin, asialofetuin and agalactofetuin: N-linked glycopeptides were prepared by the procedure of Spiro and Bhoyroo (39). Fetuin (2 g), asialofetuin (2 g) and agalactofetuin (400 mg) were incubated separately with 0.8 M $NaBH_4$ in 0.1 M NaOH at 37°C for 72 h. The reaction mixture was brought to pH 5.0 by slow addition of 4N acetic acid. The sample was then thoroughly dialysed. Fetuin, asialofetuin and agalactofetuin were then subjected to pronase digestion at 37°C in 0.15 M Tris acetate buffer pH 7.8, containing 1.5 mM calcium acetate. Initially the 1% (w/w) of enzyme was added and again 0.5% (w/w) was added after 24 h and 48 h respectively. After 96 h, lyophilized pronase digest was dissolved in 0.1 M pyridine acetate buffer at pH 5.0 and was fractionated in this buffer on Sephadex G-50 to remove partially digested protein. The glycopeptides emerging in the inner volume of Sephadex G-50 column were detected by neutral sugar analysis. The glycopeptide containing fractions were pooled and again subjected to pronase digestion and rechromatographed on Sephadex G-50 as mentioned above. The glycopeptide thus obtained was purified on Sephadex G-25 and the peak eluting in the void volume was characterized for neutral sugar, amino sugar and amino acids as stated in the Analytical methods. The structure of the fetuin glycopeptide (40) is given in Figure 1.

Ovalbumin (100 mg/4 ml) was subjected to two cycles of pronase digestion in 0.15 M Tris acetate buffer pH 7.8 at 37°C. Initially 1% (w/w) of enzyme was added and again 0.5% (w/w) was added at 24 h and 48 h respectively. After 96 h, the lyophilized pronase digest was dissolved in 0.1 M pyridine acetate buffer at pH 5.0 and fractionated in the same buffer on Sephadex G-50. The glycopeptides eluted in the inner volume of Sephadex G-50 column were characterized for their chemical composition as above and the mixture of glycopeptides was used for further studies. The structure of the ovalbumin glycopeptides (Ov-GP) are given in (41-43).

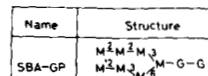
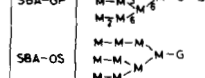
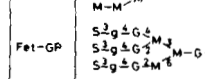
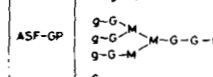
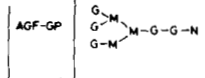
Name	Structure	S	Neutral sugar	Amino sugar	Amino acid
SBA-GP		–	8.6	2.2	1
SBA-OS		–	9.2	1.0	–
Fet-GP		2.83	5.8	4.6	1
ASF-GP		–	6.0	4.7	1
AGF-GP		–	2.8	5.2	1
Ov-GP*	——	–	5.7	3.9	1
Asn-GlcNAc	G-N	–	–	0.8	1

Fig.1: Structure and composition of glycopeptides and oligosaccharides used for hemagglutination studies. The following abbreviations are used: S, sialic acid; M, mannose; G, galactose; G, GlcNAc; N, asparagine; SBA-GP, soybean agglutinin glycopeptide; SBA-OS, SBA oligosaccharide; Fet-GP, fetuin glycopeptide; ASF-GP, asialofetuin glycopeptide; AGF-GP, agalactofetuin glycopeptide; Ov-GP, ovalbumin glycopeptide. In all cases the core structure is the following: Mα1, 6[Mα1,3] Mβ1, 4Gβ1, 4GβN. All peripheral gal and GlcNAc linkages are β, rest of the linkages are α. Arabic numerals indicate the linkage position to the underlying sugar when shown. For determining the ratios, amino acid and amino sugar were taken as reference for glycopeptides and oligosaccharides respectively. *In case of ovalbumin the mixture of glycopeptides were used without further purification.

Preparation of Soybean Agglutinin and Asialofetuin [^{14}C] Asn glycopeptides [SBA-GP and ASF-GP]: SBA-GP [1 mg as mannose] and ASF-GP [1.5 mg as neutral sugar] were labelled with [^{14}C] formaldehyde [3 μmol, 30 mCi/mol] independently by the method of Rice and Means (44). The labelled glycopeptides were purified by gel filtration on Sephadex G-10.

Treatment of [^{14}C] labelled SBA-GP and ASF-GP with endo-β-N-acetylglucosaminidase H: [^{14}C] labelled SBA-GP [300 μgs as mannose, containing 2.2 x 10^5 cpm] was mixed with an equal amount of unlabelled SBA-GP in 1 ml of 0.1 M acetate buffer pH 5.5. This was then incubated with 0.2 units of endo-β-N-acetylglucosaminidase H for 20 h at 37°C (45). The reaction was stopped by heating for 1 min in a boiling water bath and the reaction mixture was fractionated on Sephadex G-15 in 0.01 M acetic acid, where 2 peaks containing radioactivity were observed. Higher molecular weight [peak 1] which had 19% radioactivity of the starting material was pooled and chromatographed on Dowex 50-X2 equilibrated with 1 mM pyridine acetate buffer, pH 2.7, and the peak containing neutral sugar which was devoid of radioactivity was pooled. Lack of radioactivity in the neutral sugar containing fractions indicated that all of the undigested glycopeptide and GlcNAc-AsN have been separated from the oligosaccharide fraction.

[^{14}C] labelled ASF-GP [500 μg, as neutral sugar, 1.9 x 10^5 cpm] was also treated with 0.15 unit of endo-β-N-acetylglucosaminidase H and chromatographed on a Sephadex G-15 column where a single peak containing radioactivity was obtained. The only fraction obtained was eluted again on Dowex 50-X2 where the fractions containing radioactivity and neutral sugar were coincident. Based on these we presume that ASF-GP has not been cleaved by endo-β-N-acetylglucosaminidase H (46).

Preparation of affinity support: Soybean agglutinin glycopeptide (200 mg) was coupled to cyanogen bromide activated Sepharose-6B (125 ml) in 0.2 M sodium bicarbonate buffer pH 9.0 for 16 h at 4°C (21). After blocking the unreacted groups from the gel and exhaustive washing, the amount of glycopeptide coupled was estimated to be 350 μg/ml gel.

Purification of ridge gourd lectin: The buffer used in all experiments was 0.02 M sodium phosphate, pH 7.4, containing 0.15 M NaCl and 10 mM β-mercaptoethanol (PBS-βME). All the procedures were carried out at 4°C. Fruits were bled (47) and exudate was collected into oxygen free buffer. The buffer containing the exudate was then centrifuged at 9000 x g for 10 min and the protein in the supernatant was precipitated by 65% ammonium sulfate. The precipitate was then dialysed against PBS-βME.

Affinity chromatography: The clear supernatant from the previous step was loaded on soybean agglutinin glycopeptide-Sepharose-6B column equilibrated with PBS-βME. The column was washed with the same buffer till no more protein could be detected in the washings (A_{280} < 0.005). The bound protein was eluted with 0.1 M acetic acid and its elution profile was monitored by absorbance at 280 nm.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was carried out at pH 4.5 (48) and at pH 8.0 (49). SDS gel electrophoresis in 10% polyacrylamide gels was performed according to Weber and Osborn (50) in the presence and absence of 1.0% βME.

Sedimentation velocity and equilibrium measurements: Sedimentation velocity measurements were performed at 41000 rpm with Beckman L8-70 ultracentrifuge equipped with a photoelectric scanner. The experiments were carried out at 20°C in presence and absence of 1% βME and 6 M guanidine hydrochloride (GuHCl). Protein concentrations in the range of 0.6 to 1.0 mg/ml were used. The sedimentation coefficients ($s^0_{20,w}$) were calculated as described in van Holde (51). Sedimentation equilibrium analysis was performed with Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Low speed (11,270 rpm) equilibrium sedimentation was carried out at 20°C. For these studies native lectin (A_{280} = 1.0) was dissolved in PBS with and without 1% βME dialyzed extensively against the same buffer to be used as reference. The molecular weight was determined as described by Yphantis (52).

Gel filtration: Gel filtration was performed on Sephadex G-100 in the presence and absence of 1% βME in PBS. The column was calibrated with bovine serum albumin [Mr 67,000 ; R 3.61 nm], ovalbumin [45,000 ; 2.9], β-lactoglobulin [35,000 ; 2.66], chymotrypsinogen [23,000 ; 2.25] and ribonuclease [13,000 ; 1.9]. The molecular weight and the Stokes' radius of the lectin were determined as described in (53-55). The Stokes' radius of the markers were taken from Tanford (56).

NH_2-terminal end group analysis: Qualitative NH_2-terminal end group analysis was carried out by the dansylation method modified for proteins as described by Gray (57). NH_2-terminal amino acid was identified by chromatography on polyamide layers (58) using Dansyl amino acids as standard.

Amino acid composition: Protein samples for amino acid analysis were concentrated in vacuo, hydrolysed in 6.0 N HCl at 110°C for 24 h under nitrogen and analysed on a Beckman 120C amino acid analyser. Amino acid analyses were carried out using the single-column, three buffer system (Durham Chemical Corporation). The lectin was also hydrolysed in the presence of dimethylsulfoxide to determine cysteine residues (59). The amino acid composition was determined using a computer program of Hoy et al. (60). Tryptophan content was determined according to the method of Barman and Koshland (61) both in the presence and absence of 8.0 M urea. Total tryptophan was also determined according to the method of Edelhoch (62). The partial specific volume ($\bar{v}$) was calculated according to Schachman (63). $\bar{v}$ in buffer containing 6 M GuHCl was assumed to be 0.69 ml g^{-1}, as a difference of 0.02 is expected in that solvent (64).

Hemagglutination assay: The agglutination activity was assayed according to the photometric method of Lis and Sharon (65). The lectin was serially diluted in 0.5 ml of saline in 10 x 75 mm test tubes. To each tube was added 0.5 ml of standard rabbit erythrocyte suspension so that an absorbance of 1 at 620 nm was obtained. After 2 h the absorbance at 620 nm was recorded. One hemagglutination unit [HU] is defined as the amount of the lectin [1 μg] which is required to cause a 50% decrease in the absorbance of erythrocyte suspension in 2 h. For 100% agglutination [A_{620} < 0.07] the amount of the lectin required in the assay in 2 μg/ml.

For inhibition studies, the lectin [8 μg/ml] was incubated with varying concentrations of sugars in 0.5 ml for 2 h at 25°C and mixed with an equal volume of standard erythrocyte suspension. Excess of the lectin [4 hemagglutination units in final assay volume] was used in the hemagglutination inhibition assay to ensure that agglutination is complete in the absence of inhibitor. After a 2 h incubation, the absorbance was measured at 620 nm. Percentage inhibition was calculated from the ratio of the absorbance of the tubes containing a mixture of the lectin and the inhibitor, to those containing cell suspension alone. The amount of inhibitor giving the same absorbance as that of the control [without lectin] was taken as the value for 100% inhibition.

Reduction of tri-N-acetyl chitotriose: To dried tri-N-acetyl chitotriose (10.0 mg) in PBS, sodium boro [^{3}H] hydride (4.0 mg, 100 mCi/mmol) in 50 μl of 0.05 N sodium hydroxide solution was added and the reaction mixture was incubated at 50°C for 30 min. Unlabelled sodium borohydride (10.0 mg) was subsequently added, and the reaction was continued for an additional 30 min. The reaction was terminated by neutralization with 0.75 N sulphuric acid. The reaction mixture was then loaded on a Biogel P-2 column equilibrated in water and aliquots of each fraction eluted were counted on a scintillation counter. The peak fractions were pooled and the purity was confirmed by thin layer chromatography using pyridine/ethylacetate/acetic acid/water (5:3:3:1).

Equilibrium dialysis: Binding of radioactive tri-N-acetyl chitotriose (0.7 mCi/mmol) obtained from the previous step to L.acutangula lectin was measured by equilibrium dialysis. A sample of 200 μl of the lectin (13.12μM) in PBS-βME was introduced into one compartment, and a solution of 200 μl of various amounts of radioactive ligand (0.012 to 1.2 mM) was introduced into the other compartment of the dialysis cell. The cells were shaken for 60 h at 5°C and samples were removed and counted on a scintillation counter and the binding data analysed according to Scatchard (66).

Fluorescence spectra: The intrinsic fluorescence of the protein was recorded with a Perkin-Elmer MPF-44A spectrofluorimeter using a slit width of 5 nm on both monochromators. The lectin in PBSβME was excited at 295 nm. The fluorimetric titrations were performed by addition of aliquots from a stock sugar solution. When the fluorescence of the lectin was measured in the presence of a saccharide, two control solutions with buffer alone and saccharide alone were also measured. The fluorescence measurements were performed at 20°C.

CD spectra: The spectra were recorded with a Jasco J-20 spectropolarimeter in a 1.0 mm cell for the region 200-250 nm and a 5.0 mm cell for the region 250-300 nm. The data are expressed as mean residue ellipticities (Θ) in deg cm^2/dmol, the mean residue weight being taken as 110. The percentage of secondary structure of L.acutangula agglutinin was determined according to the method of Provencher and Glockner (67) using a program written by Provencher (68). Titrations were performed by addition of aliquots from stock sugar solution and the enhancement in the mean residue ellipticity in the aromatic region was monitored. The cuvettes were filled with 1.0 ml of protein solution (35 μM) in PBS-βME and mounted in a thermostatic (t ± 0.5°C) copper holder. The titrations were performed at 15°, 20°, 25° and 36°C.

Results

Purification of L.acutangula agglutinin: Most of the agglutinating activity present in the phloem exudate of L.acutangula fruits was recovered in the 65% ammonium sulfate fraction. Subsequent purification was accomplished using soybean agglutinin glycopeptide-Sepharose-6B column, as the corresponding glycopeptide was a very good inhibitor of hemagglutination caused by the lectin. The lectin bound to the affinity adsorbant was eluted as a single peak with 0.1 M acetic acid, and dialysed against PBS-βME to remove acetic acid. Usually 20-22 mgs of lectin was obtained from 4.5 ml of exudate extracted from 5 kg fruits. This amounts to an overall recovery of 70-75% from the crude extract with a specific hemagglutination activity of 1000 (Table 1). An estimate of the active protein yield indicates that 8-10% of the total protein in exudate constitutes the lectin.

Table I: Purification of L.acutangula agglutinin.

Fraction	Volume (ml)	Total Protein (mg)	Specific[a] activity (units/mg protein)	Total activity x 10^{-3}	% Recovery
9000 x g supernatant	24.5	210.0	118	25.0	100.0
65% $(NH_4)_2SO_4$ fraction	38.0	125.4	154	19.0	76.0
Purified lectin	25.0	18.0	1000	18.0	72.0

[a] One unit of activity is defined as that amount of lectin which gives a 50% agglutination of erythrocytes in the hemagglutination assay.

Purity and macromolecular properties: Purified L.acutangula agglutinin was found homogeneous by polyacrylamide gel electrophoresis in SDS, acid and alkaline pH and also by gel filtration (Fig.2A). The homogeneity of the lectin was further confirmed by sedimentation velocity and equilibrium experiments. Polyacrylamide gel electrophoresis in the presence of SDS yielded an M_r of 24000 ± 1000 (Fig.2B). Gel-filtration of the lectin on Sephadex G-100, gave a single symmetrical peak with a molecular weight of 47000 ± 1000 in presence and absence of βME (Fig.2B) and the elution profile monitored by absorbance at 280 nm was coincident with the hemagglutinating activity of the lectin. These values remain unaltered with βME. The Stokes' radius of the native protein was determined to be 2.9 nm from the gel chromatography experiments. In sedimentation velocity experiments, the lectin gave a single sedimenting boundary with a $s^o_{20,w}$ value of 4.06 S. The value of M_r 49500 ± 1000 was obtained using a partial specific volume ($\bar{v}$) of 0.71 ml/g, a Stokes' radius of 2.9 nm and a sedimentation coefficient of 4.06 S. For a molecular mass of 48000, a $s^o_{20,w}$ of 4.06 S and $\bar{v}$ of 0.71 ml/g, the estimated f/f_{min} of 1.02 imply that L.acutangula lectin is a globular protein. The size homogeneity was further confirmed by sedimentation equilibrium experiments where a M_r of 48000 was obtained (Fig.2C). On dissociation with 6 M GuHCl in the presence and absence of βME, an $s^o_{20,w}$ of 2.15 S was obtained indicating that the lectin dissociated into a species which is half its native size. A single amino acid glycine was found at the amino terminal end further substantiating the homogeneity of the protein. The macromolecular properties of the lectin are listed in Table II.

Amino acid and carbohydrate analyses: No neutral sugar (< 0.1%) could be detected by phenol-sulphuric acid method and no amino sugar was detected during amino acid analysis. The purified lectin did not bind to Con A-Sepharose or to WGA-Sepharose. These data taken together indicate that this lectin is not a glycoprotein. The amino acid composition of the agglutinin is given in Table III. This protein contains high amounts of aspartic acid and glutamic acid residues and/or their amides in addition to leucine, valine serine and glycine. Hydroxyproline is absent. It contains two free half cysteines which are not critical for its activity. On storage the protein tends to precipitate in the absence of βME and, therefore, it is convenient to use buffer containing βME during storage.

Hemagglutination activity: All the monosaccharides tested fail to inhibit the activity of L.acutangula agglutinin even at very high concentrations (Table IV). The disaccharides cellobiose, maltose, melibiose and lactose were also ineffective. The lectin activity was inhibited by β(1—>4) linked oligomers of GlcNAc and their inhibitory potency increased dramatically with increase in chain length. N-linked glycopeptides of soybean agglutinin, fetuin and ovalbumin were potent inhibitors of lectin activity. N-linked glycopeptides of fetuin, asialofetuin and agalactofetuin were almost equally effective. N-acetyllactosamine showed very weak inhibition.

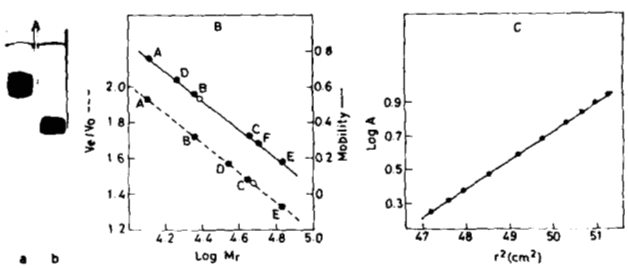


Fig.2(A). Polyacrylamide gel electrophoresis of L.acutangula lectin. Protein loaded on the gels was 40 µgs. (a) at pH 8.3 in 7.5% acrylamide gels. (b) SDS gel electrophoresis in 10% gels. 2(B). Molecular weight determination of L.acutangula lectin by Sephadex G-100 chromatography (o - - o) and SDS gel electrophoresis (o—o). The reference proteins are A, ribonuclease (M_r 13000); B, chymotrypsinogen (23,000); C, ovalbumin (45,000); D, β-lactoglobulin (18,400, in gel filtration it moves as a dimer, 35,000); E, bovine serum albumin (67,000); F, human I_gG heavy chain (50,000). The positions of the lectin are indicated by open circles. 2(C). Sedimentation equilibrium analysis of L.acutangula lectin. A linear plot of the logarithm of the equilibrium protein absorbance versus the square of the radial distance to the centre of the rotor is indicative of the size homogeneity of the L.acutangula lectin.

Table II: Physical properties of L.acutangula agglutinin

Parameter	Magnitude	Parameter	Magnitude
$s^o_{20,w}$	4.06 S[a]	$D_{20,w}$x10^{-7} (cm^2s^{-1})	
in GuHCl	2.15 S[a]	from R	6.877
Molecular weight		from s & M_r	7.083
from SE[a,b]	48000±1000	Frictional ratio (f/f_{min})	1.02
from s[c] & R[d]	49500	R(nm)	
from gel filtration[a]	47500±2000	from s, $\bar{v}$ & M_r	2.82
from SDS-PAGE[a]	24000±1000	from gel filtration	2.9
$\bar{v}$ (ml g^{-1})	0.71		

[a] Values unaffected by the presence or absence of β-mercaptoethanol.
[b] SE sedimentation equilibrium. [c] $s^o_{20,w}$ sedimentation coefficient.
[d] R Stokes' radius. Density of 1.005 g ml^{-1} and 1.15 used for calculations were for phosphate buffer and buffer containing 6 M GuHCl respectively.

Table III: Amino acid composition of L.acutangula lectin[a].

Amino acid	Content	Amino acid	Content (mol/mol)
Ala	30	Leu	44
Cys[b]	4	Met	8
Asp	44	Pro	18
Glu	36	Arg	22
Phe	20	Ser	32
Gly	38	Thr	18
His	10	Val	52
Ile	24	Tyr	14
Lys	24	Trp[c]	6
			444

[a] M_r taken to be 48,000. [b] Cysteine determined as cysteic acid (59).
[c] Tryptophan was determined as per procedures in (61,62).

Table IV: Inhibitory power of various sugars on the hemagglutination by L.acutangula lectin.

Sugar	Concentration giving 100% inhibition (mM)	Relative[b] inhibitory power
$(GlcNAc)_2$[a]	6.0	1
$(GlcNAc)_3$	0.3	20
$(GlcNAc)_4$	0.0275	218
$(GlcNAc)_5$	0.02	300
SBA-GP	0.006	1000
Fet-GP	0.01	600
ASF-GP	0.008	750
ASF-GP	0.008	750
Ov-GP	0.007	857
SBA-OS	>0.06*	-
LacNAc	160	0.038
Mannobiose[c]	>75	-
Mannotriose	>60	-
GlcNAc	>500	-
MeαGlcNAc	>50	-
MeβGlcNAc	>50	-

[a] $(GlcNAc)_{2,3,4,5}$ are β(1—>4) linked di, tri, tetra and pentasaccharides of GlcNAc respectively. [b] Relative inhibitory power is with respect to $(GlcNAc)_2$. GalNAc (500 mM), ManNAc (500 mM), Lactose (400 mM), Cellobiose (200 mM), Melibiose (400 mM), Maltose (400 mM), Mannose (200 mM) and β-D-GlcNAc-(1—>N)AsN(25µM) were not inhibitory at concentrations indicated in parentheses. *Higher concentrations could not be used as this saccharide was limiting.
[c] Mannobiose and mannotriose are α(1—>2) linked di- and trisaccharides of mannose, respectively.

Equilibrium dialysis: The results of a typical equilibrium dialysis experiment are expressed in Fig.3. The results, plotted according to Scatchard (66), on extrapolation at ordinate yielded a value of 2.06, indicating two binding sites for tri-N-acetylchitotriose per molecule of L.acutangula lectin. The binding constant was calculated to be 2.75 x 10^4 M^{-1}.

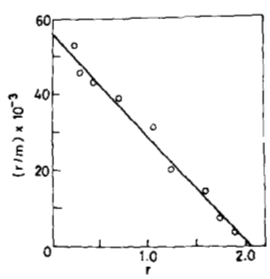


Fig.3. Equilibrium dialysis data for the binding of radiolabelled tri-N-acetylchitotriose to L.acutangula lectin, plotted according to Scatchard.

Intrinsic fluorescence studies: The fluorescence emission spectrum of L.acutangula lectin given in Fig.4(A) shows an emission maximum at 336 nm. On addition to the chito-oligosaccharides, the fluorescence is enhanced with a blue shift in the emission maximum towards 332 nm. Fig.4(B) shows the change in fluorescence intensity as a function of tri-N-acetylchitotriose concentration together with a representative graphical determination (inset) of the association constant (K_a), according to the method of Chipman et al. (69), yielding a value of 1.26 x 10^4 M^{-1}. The stoichiometry of lectin-sugar binding for the protein with an equivalent weight of M_r 24000 corresponds to 1. The thermodynamic parameters for binding of various saccharides determined by this method along with the fluorescence emission characteristics of the lectin saturated with them are listed in Table V.

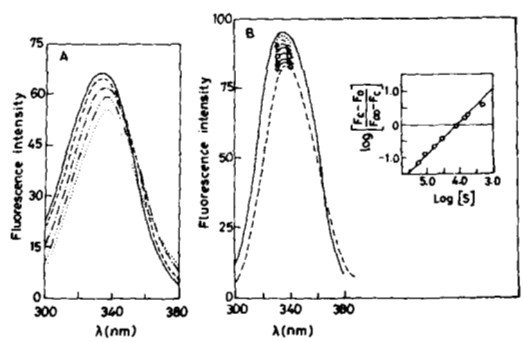


Fig.4. Fluorescence spectra of L.acutangula lectin (50 µg/ml) and its complexes with saccharides. (A) (....) lectin spectrum; (-...-) lectin with 1 mM di-N-acetylchitobiose; (-.-.-) lectin with 150 µM tri-N-acetylchitotriose; (—) lectin with 24 µM tetra-N-acetylchitotetraose; (——) lectin with 13 µM penta-N-acetylchitopentaose. (B) Fluorescence spectra of L.acutangula lectin as enhanced by chitotriose. The fluorescence spectra were recorded in the absence (——) and after addition of defined aliquots from a stock of 10 mM tri-N-acetylchitotriose to 1 ml of 2µM solution of lectin. (Δ-Δ-Δ) 1 µl, (∆-∆-∆) 2 µl, (∂-∂-∂) 3 µl, (o-o-o) 5 µl, (x-x-x) 12.0 ml, (-.-.-) 28 µl, (....) 48 µl and (——) 123 µl. After each addition of the sugar, part of the spectrum was recorded and the total spectrum is given after the final addition. A plot for the determination of association constant from data shown in B (Inset).

Table V: Fluorescence emission characteristics, association constants and free energy of association of saccharides with L.acutangula lectin at 20°C.

Saccharide	K_a^a (M^{-1} x 10^{-3})	$-\Delta G$ (kJ mol^{-1})	% enhancement	Blue shift (nm)
$(GlcNAc)_2$	1.4 (±0.1)	17.65 (±0.17)	5	-
$(GlcNAc)_3$	12.6 (±1.0)	23.0 (±0.18)	14.5	2
$(GlcNAc)_4$	97.0 (± 7)	28.0 (±0.20)	22	4
$(GlcNAc)_5$	650.0 (±55)	32.6 (±0.20)	29	4

[a] K_a and the % enhancement in fluorescence for the binding of various saccharides were calculated at the respective shifted fluorescence emission maxima for these ligands. Values in parentheses indicate standard deviation.

Far UV-CD spectrum: Analysis of the CD spectrum of L.acutangula lectin gave 31% helix content (Fig.5A(a)).

Near UV-CD spectrum: Near UV-CD bands show an increase in the molar ellipticities on titration with chito-oligosaccharides (Fig.5A(b)). The change in the molar ellipticity as a function of the ligand concentration (Fig.5B) allowed the determination of K_a and the stoichiometry of the reaction by plotting $\log[(\Theta_c - \Theta_o)/(\Theta_\alpha - \Theta_c)]$ versus $\log[S]$, where Θ_o, Θ_c and Θ_α are initial ellipticity, ellipticity after addition of the sugar, and ellipticity value where all the binding sites of the lectin are saturated with sugar, respectively. The value of K_a and the slope of this representative plot of titration at 20°C are 1.1 x 10^4 M^{-1} and 1.0 respectively (Fig.5C). Values of K_a for various oligosaccharides thus determined at several temperatures were utilized for evaluating ΔH by van't Hoff plots (Fig.6) and the resulting thermodynamic parameters are given in Table VI.

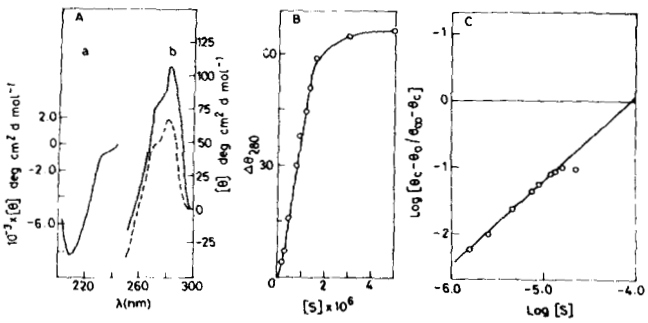


Fig.5. (A). CD spectra of L.acutangula lectin. (a) The concentration of the lectin in the far UV region (200-250 nm) was 0.02% in PBS containing 2 mM dithiothreitol and the near UV region (250-300 nm) was 0.9% (b) CD enhancement spectra of L.acutangula lectin by tri-N-acetylchitotriose in the near UV region. Lower curve represents spectrum of the protein alone and the upper curve represents the final spectrum after addition of saturating concentrations of tri-N-acetylchitotriose. (B). Curve of the molar ellipticity enhancement of L.acutangula lectin by tri-N-acetylchitotriose. [S] represents the sugar concentration and $\Delta\Theta_{280}$ is the sugar induced difference in molar ellipticity at 280 nm. (C). The association constant from data in (B).

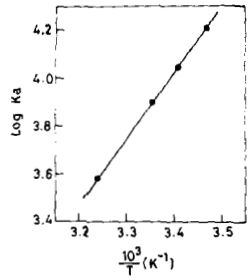


Fig.6: van't Hoff plot for the binding of L.acutangula lectin to tri-N-acetylchitotriose. K_a values for the lectin-sugar interaction were determined by near UV-CD titrations.

Table VI: Association constants and thermodynamic properties of L.acutangula lectin-saccharide complexes as determined from CD measurements.

Saccharide	K_a (M^{-1} x 10^{-3})				at 25°C		
	15°C	20°C	25°C	36°C	$-\Delta G$	$-\Delta H^a$	$-\Delta S^b$
					kJ mol^{-1}		J mol^{-1} K^{-1}
$(GlcNAc)_2$	1.6 (±0.03)	1.2 (±0.04)	0.8 (±0.04)	0.5 (±0.07)	16.56 (±0.13)	41.0 (±1.9)	81.0 (±4.0)
$(GlcNAc)_3$	15.9 (±0.5)	11.2 (±0.3)	8 (±0.6)	4 (±0.7)	22.27 (±0.20)	47.9 (±2.1)	86 (±6.0)
$(GlcNAc)_4$	148 (±8)	100 (±9)	72 (±7)	35 (±9)	27.7 (±0.25)	55.9 (±2.4)	95 (±9.0)
$(GlcNAc)_5$	1120 (±50)	700 (±60)	525 (±40)	271 (±43)	32.63 (±0.19)	56.0 (±2.4)	78 (±5.0)

[a] ΔH was obtained from van't Hoff plots. ΔS was calculated using the equation $\Delta G = \Delta H - T\Delta S$. Values in parentheses indicate standard deviation.