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A chitotetrose specific lectin from *Luffa acutangula*: Physico-chemical properties and the assignment of orientation of sugars in the lectin binding site

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Abstract. A chitooligosaccharide specific lectin (*Luffa acutangula* agglutinin) has been purified from the exudate of ridge gourd fruits by affinity chromatography on soybean agglutinin-glycopeptides coupled to Sepharose-6B. The affinity purified lectin was found homogeneous by polyacrylamide gel electrophoresis, in sodium dodecyl sulphate-polyacrylamide gels, by gel filtration on Sephadex G-100 and by sedimentation velocity experiments. The relative molecular weight of this lectin is determined to be 48,000 ± 1,000 by gel chromatography and sedimentation equilibrium experiments. The sedimentation coefficient ($S_{20,\ w}$) was obtained to be 4·06 S. The Stokes' radius of the protein was found to be 2·9 nm by gel filtration. In sodium dodecyl sulphate-polyacrylamide gel electrophoresis the lectin gave a molecular weight of 24,000 in the presence as well as absence of 2-mercaptoethanol. The subunits in this dimeric lectin are therefore held by non-covalent interactions alone. The lectin is not a glycoprotein and circular dichroism spectral studies indicate that this lectin has 31% α-helix and no *β*-sheet. The lectin is found to bind specifically to chitooligosaccharides and the affinity of the lectin increases with increasing oligosaccharide chain length as monitored by near ultra-violet-circular dichroism and intrinsic fluorescence titration. The values of ΔG, ΔH and ΔS for the binding process showed a pronounced dependence on the size of the oligosaccharide. The values for both ΔH and ΔS show a significant increase with increase in the oligosaccharide chain length showing that the binding of higher oligomers is progressively more favoured thermodynamically than chitobiose itself. The thermodynamic data is consistent with an extended binding site in the lectin which accommodates a tetrasaccharide. Based on the thermodynamic data, blue shifts and fluorescence enhancement, spatial orientation of chitooligosaccharides in the combining site of the lectin is assigned.

Keywords. Chitotetrose; lectin; combining site; sugar orientation.

Introduction

Lectins are multivalent carbohydrate binding proteins (Goldstein and Hayes, 1978). When lectins which have multiple combining sites bind to the carbohydrates perched on the surface of the cells, they interconnect a large number of cells causing them to clump together or agglutinate. Lectins bind to sugars in a selective manner for example concanavalin-A binds to the *α*-D glucose or mannopyranosides whereas the Ricinus lectin (Podder *et al*., 1974) binds to galactopyranosides and glycoconjugates containing these sugars. Because of their sugar specificity their agglutination reaction is remarkably selective making lectins as unique probes for identifying and mapping

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Abrreviations used: SBA, Soybean agglutinin; SDS, sodium dodecyl sulphate; CD, circular dichroism.

sugars on the cell surface. For example, lectins can distinguish red cells of various blood groups and as they preferentially agglutinate malignant cells, they can readily distinguish such cells from the normal ones and reveal some of the changes cells undergo on malignant transformation. In addition they are increasingly being employed in studies of immune phenomena such as mitogenesis; cell–cell interactions, receptor–ligand interactions and in the purification of glycoconjugates (Surolia *et al*., 1975a, b). All of the lectins studied till recently have two or more combining sites which are complementary normally for a monosaccharide and infrequently for a disaccharide. Among the lectins the seed-lectins have been most widely studied as the seeds are rich in these proteins. The exact physiological roles of plant lectins are not known, though the seed-lectins have been speculated to be involved in root-bacterium symbiosis a prelude to nitrogen fixation and in plant development and differentiation (Lis and Sharon, 1973; Liener, 1979; Barondes, 1981). Since lectins have also been found in other parts of the plants, currently major efforts are being directed towards elucidating the relationship of lectins isolated from various parts of the plant and their function. In this regard, the recent discovery of lectins in the phloem exudates of plants by us as well as by others (Allen, 1979) provide a unique opportunity to investigate their biological roles. In addition, the binding of *L. acutangula* lectin subject of this article (Anantharam *et al*., 1983, 1984) is a pointer towards the possibility of discovering lectins with highly extended and complex carbohydrate binding subsites.

Materials and methods

Sepharose-6B and Sephadex G-100 were products of Pharmacia, Uppsala, Sweden. Proteins used as molecular weight markers in gel chromatography and SDS-70 molecular weight kit were obtained from Sigma Chemical Co., St Louis, USA. Chitin, fetuin and all the monosaccharides and disaccharides used were also products of Sigma. Ridge gourd (*Luffa acutangula*) fruits were bought from local green grocers. Chitin oligomers were prepared as per the original procedure described by Rupley (1964). Soybean agglutinin (SBA) was prepared as described by Majumdar and Surolia (1978). N-linked glycopeptides from soybean agglutinin, fetuin, and ovalbumin glycopeptides were prepared as per the procedure of Spiro and Bhoyroo (1974). Soybean agglutinin glycopeptides were coupled to cyanogen bromide activated Sepharose-6B as described by March *et al.* (1974). Protein concentrations were determined by the dye binding method of Bradford (1976) or by their absorbance at 280 nm.

Purification of the lectin

Supernatant from 65 % ammonium sulphate fraction of phloem exudate was loaded on the soybean agglutinin glycopeptide-Sepharose column previously equilibriated with 0·02 M phosphate buffer pH 7·4 containing 0·15 M NaCl and 10 mM β-mercapto-ethanol (PBS-BME). The column was washed with the same buffer and protein was eluted with 0·1 M acetic acid.

Macromolecular properties

The molecular weight by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoreses, gel filtration and sedimentation equilibrium experiments were determined according to the method of Weber and Osborn (1969), Andrews (1970) and Yphantis (1964) respectively. The effective hydrodynamic radius (stokes radius) was determined from gel filtration (Ackers, 1967).

Haemogglutination inhibition

Serial dilution method of Lis *et al.* (1970) was used to determine 50 % inhibition values for various sugars. 4 % suspension of rabbit erythrocytes was used for the assay.

Circular dichroism spectra and titrations

Circular dichroism (CD) spectra were recorded with Jasco J-20 spectropolarimeter. The secondary structure from CD spectra was determined according to the method of Provencher and Glockner (1981). Titrations were performed at several temperatures by addition from a stock sugar solution, and the enhancement in the mean residue ellipticity in the aromatic region was monitored. The association constants (Ka) for protein-sugar interactions was determined according to the method of Chipman *et al.* (1967). ΔH was determined from Van't Hoff plot.

Fluorescence titrations

The intrinsic fluorescence of the protein was recorded with Perkin-Elmer MPF-44A spectrofluorimeter using a slit width of 5 nm on both monochromators. The lectin was executed at 295 nm and the emission spectra collected above 310 nm. Fluorimetric titrations were performed as described above.

Results and discussion

Ammonium sulphate fraction (65 %) of the phloem exudate from *L. acutangula* fruits was applied to the SBA glycopeptide–Sepharose. The bound lectin was eluted with 0·1 M acetic acid. The purified lectin is homogeneous by several criteria. These include a single band in polyacrylamide gel electrophoresis in acid as well as in alkaline pH and in the presence of SDS, a single symmetrical peak in gel chromatography on Sephadex G-100 and a single sedimenting boundary in sedimentation velocity experiments. The macromolecular properties of the lectin are listed in table 1. The sedimentation equilibrium studies and the analytical gel filtration have shown a molecular weight of 48,000 daltons for the native lectin both in the presence as well as in the absence of BME. The native lectin has a sedimentation coefficient of 4·06 S, whereas the value for the denatured protein is 2·15 S. In SDS-polyacrylamide gel electrophoresis, the lectin shows a molecular weight of 24,000 which does not change in the presence of BME. These data taken together indicate that the *L. acutangula* lectin is a dimer composed of 2 identical subunits of 24,000 daltons. Since the molecular weights of the native lectin as well as that of its subunits do not vary on treatment with BME, we

Table 1. Physical properties of *L. acutangula* lectin.

Parameter	Magnitude
Molecular weight[a] in PBS	48,000 ± 1000
Subunit molecular weight in SDS-PAGE	24,000 ± 1000
$s_{20\,w}$ in PBS	4·065
$s^{b}_{20\,w}$	2·155
$D_{20\,w} \times 10^{-7}$ ($cm^2 s^{-1}$)[c]	6·956
$\bar{v}$ ($mL g^{-1}$)[d]	0·71
Frictional ratio (f/f min)	1·02
Stokes radius (nm)[e]	2·85 ± 0·09
Secondary structure from CD	31 % helix
	69 % Random coil.

[a] Determined from gel filtration on Sephadex G 100, sedimentation equilibrium and from $S_{20\,w}$, ν and stokes radius.
[b] In presence of 6 M guanidine hydrochloride.
[c] Calculated from stokes radius, $s_{20\,w}$ and mol. wt.
[d] Calculated from aminoacid composition.
[e] Calculated from $S_{20\,w}$, ν and mol. wt. and from gel filtration.

reasoned that the disulphide bonding is not involved in subunit association of this dimeric lectin. Its hydrodynamic properties such as the sedimentation coefficient, diffusion coefficient, the stokes radius, the frictional ratio, etc. are consistent with those expected for a globular protein. *L. acutangula* lectin contains 31 % α-helix and no β-sheet. A predominant α-helical content and a total absence of β-pleated sheet in *L. acutangula* lectin appears to be a distinct feature of this protein when compared with other plant lectins (Jirgensons, 1978).

Haemagglutination inhibition studies revealed that none of the acetylated monosac charides and several disaccharides were ineffective even at very high concentrations. However, there is a striking inhibition by chitobiose. This inhibitory effect is even more pronounced as the chain length of the chitooligosaccharides is increased. Thus chitotriose, chitotetrose and chitopentose are 32, 266 and 400 times more potent inhibitors than chitobiose respectively. A notably better inhibition by higher oligomers of chitooligosaccharides for this lectin when compared with other N-acetylglucosamine specific lectins such as wheat germ, (Allen, 1973) *Datura stramonium* and *Solanum tuberosum* (Desai *et al.,* 1981) prompted us to suggest that the combining site of *L. acutangula* lectin is much larger than any of these lectins and probably encompasses a pentasaccharide (table 2). N-linked glycopeptides from fetuin, soybean agglutinin and ovalbumin are even more potent inhibitors than chitooligosaccharides. These glycopeptides have a core structure: Manβ(1→4) GlcNAc β(1→4) GlcNAc β-AsN. The higher activities of glycopeptides could be either due to additional interactions of flanking residues on either side of the chitobiosyl sequences in the combining site or due to a better orientation of the suitable chitobiosyl conformer in these complex carbohydrates for binding to *L. acutangula* lectin. This lectin, unlike wheat germ agglutinin is absolutely specific for chitooligosaccharides alone as the inhibitory potency of N-linked glycopeptides from fetuin, asialofetuin and agalactofetuin is identical. Bovine submaxillary mucin or its asialo derivative is ineffective unlike in

Table 2. Inhibitory power of various sugars[a] on the haemagglutination by *L. acutangula* lectin.

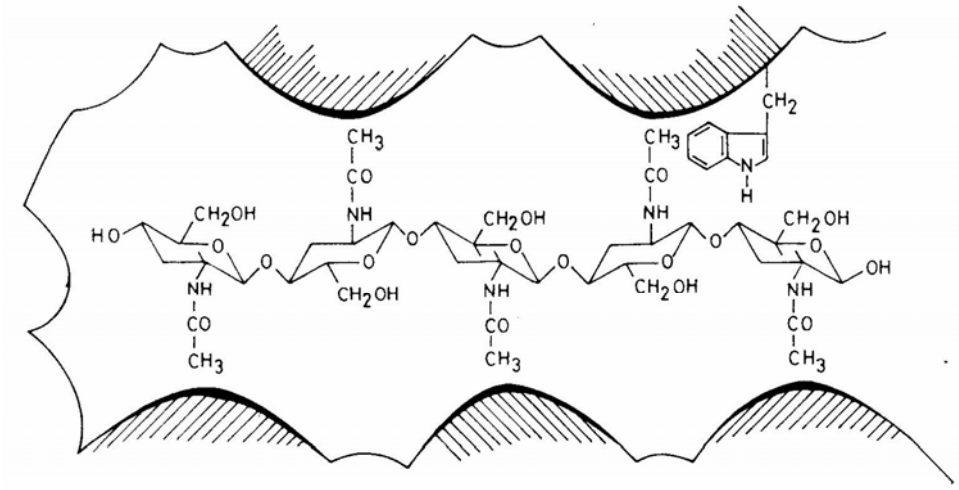
Sugar	Concention giving 50% inhibition (mM)	Relative[b] inhibitory power
N,N′-Diacetylchitobiose	4	1
N,N′,N″-Triacetylchitotriose	0·125	32
N,N′,N″,N‴-Tetraacetylchitotetrose	0·015	266·7
N,N′,N″,N‴,N⁗-Pentaacetylchitopentose	0·01	400
Soybean agglutinin glycopeptide	0·0014	2,860
Fetuin glycopeptide	0·005	800
Asialofetuin glycopeptide	0·0055	727
Agalactofetuin glycopeptide	0·005	800
Ovalbumin glycopeptide	0·004	1,000
Bovine submaxillary mucin	5 mgs	—
Asialobovine submaxillary mucin	5 mgs	—
N-Acetyllactosamine	156	0.026

[a] Oligosaccharides contained $\beta(1 \rightarrow 4)$ linkages.
[b] Relative inhibitory power is with respect to N,N'Diacetylchitobiose.

wheat germ agglutinin. In contrast to wheat germ agglutinin where it is observed that a homopolymer of sialic acid is a poorer inhibitor than the monomer itself, in *L. acutangula* lectin, homopolymers of N-acetylglucosamine are potent inhibitors than N-acetyl-glucosamine to which it does not bind even at very high concentrations. Wheat germ agglutinin binds very well to glycopeptides through their sialic acid residues if they are clustered and the ligand complex with lectin is stabilized due to multiple interactions (Bhavanandan and Katlie, 1979; Monsigny *et al*., 1981). This multivalent interaction or 'cooperative effect' is completely ruled out in case of *L. acutangula* lectin as it was observed that it fails to bind to *ß*-D-GlcNAc Synsorb or N-1-(1-deoxydi-N-acetyl chitobiitol) aminoethyl-Biogel P-150 (Baues and Gray, 1977), affinity matrices containing high density of *ß*-D-GlcNAc residues.

In order to further characterize quantitatively the nature of combining sites, we have carried out extensive thermodynamic studies on binding of chitooligosaccharides to the lectin. The values of the association constant as determined by ligand dependant perturbation of the CD spectra in the near UV region and intrinsic fluorescence for the binding of lectin to chitobiose, chitotriose, chitotetrose and chitopentose were strongly related to the size of the oligosaccharide. The affinity of chitopentose, chitotetrose and chitotriose is about 558, 78 and 9 times greater respectively than for chitobiose.

This increase in the Ka values with the size of the chitooligosaccharides can arise from a statistical increase of binding probability for a combining site that accomodates a single sugar residue. However, the increase in the affinities for oligosaccharides is much higher than what could be attributed to statistical effects. The statistical effect is totally ruled out especially in the light of undetectable binding for N-acetylglucosamine. Alternatively, large and consistent increase in the values of Ka and ΔG (figure 1) for the binding of lectin to oligosaccharides of increasing dimensions may be explained if one assumes that the combining site of the lectin consists of a number of



Parameter	A	B	C	D	E
$-\Delta G$ Contribution	0	17	5.3	5.4	5.1
$-\Delta H$ Contribution	0	41	6.9	8.0	0.1
$-\Delta S$ Contribution	0	80.6	5.3	8.8	−16.7
% Fluorescence enhancement	0	5	14.5	22	29
Blue shift contribution	0	0	2	4	4

Figure 1. Schematic representation of the binding site of *L. acutangula* lectin. Various subsites are marked A to E and the ΔG, ΔH and ΔS contribution of the various subsites for binding to the complementary sugars along with the overall changes in the protein intrinsic fluorescence parameters are also listed.

subsites, each of which accomodates one sugar unit, and that the association of a given subsite with a single sugar moiety is additive with respect to the other units of the saccharides. The ΔG contribution of various subsites is obtained by comparing the ΔG values of a pair of oligosaccharides. The location of subsites on protein molecule with their respective free energies of association is shown in figure 1. B, C and D are strong subsites whereas A is unfavourable *i.e.*, it contributes a positive term for the overall ΔG of binding and E is a moderate binding subsite. The observation that GlcNAc does not bind to the protein supports our view that the subsite A is unfavourable. The fact that there is a pronounced increase in the free energy of binding as another unit of GlcNAc is added, is in accordance with our assumption that B is a strong binding subsite. Likewise, a consistently large increase in ΔG for chitotriose, tetrose and pentose would support our suggestion that B, C, D and E are the contiguous favourable subsites.

The values of the association constants of chitooligosaccharides-lectin complexes

decrease with increasing temperature. The temperature dependent Ka values can be utilized to evaluate Van't Hoff enthalpies and a marked increase in the values of ΔH and ΔS with increase in the N-acetylglucosamine units in the oligosaccharide was also observed. This striking increase in the values of ΔH on increasing the dimensions of the ligand is consistent with an elongated combining site in the lectin but also imply more extensive hydrogen bonding and hydrophobic interactions in the binding site. The ΔH contribution which were obtained by comparing the ΔH values for a pair of oligosaccharides and these are given in figure 1. The increase in ΔH between chitobiose and chititriose equals 6·9 KJ mol^{-1} which might occur due to the contribution to the binding enthalphy by N-acetylglucosamine residue in subsite C. Likewise the contribution of subsites D and E to this interaction amounts to 8 and 0·1 KJ mol^{-1} respectively. This is in marked contrast with Con A (Van Landschoot *et al*., 1978) where an extended binding site has been ruled out as values of ΔH remain constant with an increase in ligand chain length. For lysozyme on the other hand, which has an extended binding site, the values of ΔH are strongly related to the size of the oligosaccharide.

The values for changes in entropy are also shown in figure 1. The values of entropy thus obtained vary significantly and again correlate well with the dimensions of the ligand. The observed increase in ΔS values are larger than the entropy of mixing. An unfavourable increase in the value of ΔS with the length of oligosaccharides could presumably be due to a loss in translational entropy and a change in the conformation of the saccharides or the lectin or both which accompany this association. Irrespective of the factors causing this increase in the magnitude of ΔS a correspondingly greater increase in the ΔH value outweighs the contribution of ΔS and thereby making favourable the overall process of the recognition of chitooligosaccarides by *L. acutangula* lectin.

Fluorescence spectrum of this protein shows an emission, maxima centred at 336 nm which is characteristic of typtophan emission in proteins. The enhancement of the protein fluorescence intensity and the blue shift are strongly related to the number of N-acetylglucosamine units in the inhibitory oligosaccharide (figure 1). This blue shift indicates that in lectin-sugar complexes some tryptophan residues are in more hydrophobic environment than in the free lectin. The ligand induced fluorescence emission characteristics of the lectin reflects some unique spatial orientation of various saccharides with respect to one or more indole moieties of tryptophan side chains in the combining region of the lectin. Based on the data a model for the lectin subsite is shown in figure 1. This model is deduced from the observation that chitobiose does not perturb the emission of tryptophan side chain in the combining site of the lectin and hence its subsites (subsites A and B) should be located distant from this tryptophan residues. Moreover, the distinct perturbation of its fluorescence by tri, tetra and pentasaccharides has allowed us to assign the binding of 3rd, 4th and 5th residue of the oligosaccharide at subsites, C, D and E respectively. Since maximal enhancement of protein fluorescence and blue shift occur on binding of chitotetrose and chitopentose, we have assigned the position of tryptophan between the subsites D and E.

Since the characterization of *L. acutangula* lectin has been accomplished, we are now directing out attention towards its biological role. In the light of the available information, the lectin can be postulated to have the following physiological function in the plant:

(i) It may be serving a structural role. In the exudate the lectin is found to be crosslinked by disulphide bond to another major protein of 80,000 daltons which is filamentous in nature. The lectin, therefore, could then anchor the filamentous protein to glycoproteins of the sievetube plasma membrane as it binds very tightly to chitobiosyl sequences which occur commonly in the core regions of many plant glycoproteins.
(ii) It may be serving an antiparasitic function. Since this lectin binds to chitooligosaccharides and chitin being the principle component of fungal cell walls it could inhibit chitin synthesis and thereby inhibit the growth of fungal hyphae and thus help in the defence of plant against infection.
(iii) It may be involved in the wound sealing mechanism. Since the exudate forms gel on exposure to air, the lectin along with other proteins, forms extensive disulphide cross links resulting in the formation of a plug *i.e.* wound-sealing, analogous to the blood clotting phenomenon in higher vertebrates. Subsequent to wound-sealing the lectin by virtue of its being there might protect against any further infection.

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