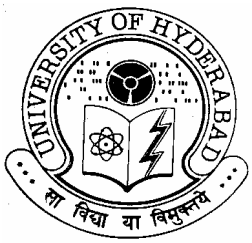
Physicochemical Studies on the Galactose-Specific Seed Lectins from *Momordica charantia* and *Trichosanthes dioica*

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Nabil Ali Mohammed Sultan



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STATEMENT

I hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the School of Chemistry, University of Hyderabad, Hyderabad, under the supervision of **Prof. Musti J. Swamy.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever the work described is based on the finding of other investigators.

Hyderabad

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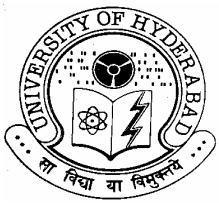

CERTIFICATE

Certified that the work embodied in this thesis entitled "**Physicochemical Studies on the Galactose-Specific Seed Lectins from *Momordica charantia* and *Trichosanthes dioica*"** has been carried out by **Mr. Nabil Ali Mohammed Sultan**, under my supervision and the same has not been submitted elsewhere for a degree.

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To my past and present lab mates, colleagues, friends and family I say thank you so much one and all.

Finally, the scholarship provided by Sana'a University, Republic of Yemen, is gratefully acknowledged.

Nabil Sultan

ABBREVIATIONS

APA	*Abrus precatorius* agglutinin
CBD	carbohydrate binding domain
Con A	concanavalin A
CGL	Cucurbitaceae galactose specific lectin
CRD	carbohydrate recognition domain
CuTMPyP	*meso*-tetra(4-methylpyridinium)porphyrinato Cu(II)
CuTPPC	*meso*-tetra(4-carboxyphenyl)porphyrinato Cu(II)
CuTPPS	*meso*-tetra(4-sulphonatophenyl)porphyrinato Cu(II)
DEPC	diethylpyrocarbonate
2-Deoxy Gal	2-deoxy-D-galactopyranoside
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
ECL	*Erythrina cristagalli* lectin
Fru	D-fructose
Fuc	D-fucose
Gal	D-galactose
Gal*N*Ac	*N*-acetyl-D-galactosamine
$GalNH_2$	D-galactosamine
Glc	D-glucose
Glc*N*Ac	*N*-acetyl-D-glucosamine
Gdn.Cl	guanidinum chloride
GNA	*Galanthus nivalis* (snow drop) agglutinin
H_2TMPyP	*meso*-tetra(4-methylpyridinium)porphyrin
H_2TPPC	*meso*-tetra(4-carboxyphenyl)porphyrin

H_2TPPS	*meso*-tetra(4-sulphonatophenyl)porphyrin
ITC	isothermal titration calorimetry
Man6P	mannose-6-phosphate
MBP	mannose-binding protein
MCL	*Momordica charantia* (bitter gourd) seed lectin
β-ME	β-mercaptoethanol
MeαGal	methyl-α-D-galactopyranoside
MeβGal	methyl-β-D-galactopyranoside
MeαGlc	methyl-α-D-glucopyranoside
MeβGlc	methyl-β-D-glucopyranoside
MeαMan	methyl-α-D-mannopyranoside
MeUmbαGal	4-methylumbelliferyl-α-D-galactopyranoside
MeUmbβGal	4-methylumbelliferyl-β-D-galactopyranoside
NBS	*N*-bromosuccinimide
Neu*N*Ac	*N*-acetyl neuraminic acid
PAGE	polyacrylamide gel electrophoresis
PBS	20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% sodium azide
PDB	protein data base
PHA	phytohaemagglutinin (previous nomenclature for red kidney bean) isolectins
PNA	peanut (*Arachis hypogaea*) agglutinin
*p*NPαGal	*p*-nitrophenyl-α-D-galactopyranoside
*p*NPβGal	*p*-nitrophenyl-β-D-galactopyranoside

*p*NPβthioGal	*p*-nitrophenyl-β-D-thiogalactopyranoside
RCA	*Ricinus communis* agglutinin
RIP	ribosome-inactivating protein
RNA	ribonucleic acid
SBA	soybean (*Glycine max*) agglutinin
SDS	sodium dodecyl sulphate
SGSL	snake gourd (*Trichosanthes anguina*) seed lectin
SNA	*Sambucus nigra* (elderberry tree) agglutinin
TCSL	*Tricosanthes cucumerina* seed lectin
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TxLC-I	first Tulipa hybrid lectin
UEA-I	*Ulex europeus* agglutinin I
UEA-II	*Ulex europeus* agglutinin II
WGA	wheat germ agglutinin
ZnTPPC	*meso*-tetra(4-carboxyphenyl)porphyrinato Zn(II)
ZnTPPS	*meso*-tetra(4-sulphonatophenyl)porphyrinato Zn(II)

CONTENTS

1. Lectins: Historical background and definitions

1.1. The discovery: The first observation of a lectin activity was made before 1860 by S. Weir Mitchell as a result of his studies on rattlesnake venom, and in 1886 he, with others, published an article about these studies, which contains a clear description of cell agglutination activity of rattlesnake venom [cf. Kilpatrick, 2002]. However, it is widely believed that the first discovery of lectins was made in 1888 when the toxicity of castor beans was attributed, by H. Stillmark, to the occurrence in the extract of these beans of a hemagglutinin (agent which cause clumping of erythrocytes), which he proved to be a protein and called it 'ricin' (from the scientific name of castor tree, *Ricinus communis*). Later it was shown that the potent cytotoxin ricin is a poor hemagglutinin and that the hemagglutination activity observed by Stillmark was mainly due to another nontoxic lectin of castor beans, *Ricinus communis* agglutinin (RCA). Soon after this discovery several similar plant hemagglutinins were identified, and in 1898 the term 'hemagglutinin' was introduced as a common name for all plant proteins that show hemagglutination activity.

1.2. Specific sugar binding activity: In 1936 it was observed by Sumner and Howell [1936] that cane sugar (sucrose) inhibits the hemagglutination activity of Con A. However, only in early 1950s it was realized that the molecular basis of this visible activity of lectins (i.e., cell agglutination) is based on a specific sugar binding activity of them. This led to a series of agglutination/agglutination inhibition assays employing sugars by Watkins and Morgan in the early 1950s [Watkins and Morgan, 1952; Morgan

and Watkins, 2000], on different lectins and red cell types, resulting in the following conclusions:

1. Lectins bind sugars specifically and reversibly. Thus lectins became distinguished from other protein groups by a clear functional criterion.
2. The ability of lectins to agglutinate cells, and the ability of specific saccharides to inhibit this agglutination, led to the discovery that cell surfaces carry sugar structures. Further, because lectins exhibited cell-type specific agglutination, it became clear that different types of cells express different carbohydrate structures on their surfaces. For example, some of the cell-surface carbohydrates on leucocytes are different from those expressed on erythrocytes. Also, normal cells and transformed cells have different sugar structures expressed on their surfaces.
3. α-Linked *N*-acetylgalactosamine and α-linked fucose are the molecular determinants of the type A and O blood-group antigens, respectively. That was the first determination of a sugar determinant of a blood group antigen. Since then many molecular determinants (sugars) of specific agglutination of cells by lectins were determined and now it is a fact that the specificity of lectin-sugar interaction is the basis for the specificity of lectin-cell interaction.

1.3. The terms 'lectin' vs 'agglutinin': Some lectins are blood group specific, i.e. they agglutinate erythrocytes of one or more, but not all, types within a particular blood group system (e.g., ABO system, [see Boyd, 1970]). The term 'lectins' (from Latin 'legere' which means 'to pick out or choose') was originally introduced to refer to the ability of these hemagglutinins to determine the blood group type of a given erythrocytes sample [Boyd and Shapleigh, 1954]. Since their discovery, lectins were

labeled with many other names. At present the term ‘lectin’ is the most commonly used name but the terms ‘agglutinin’ and 'hemagglutinin' still persist as synonyms. The name ‘lectin’, however is the appropriate one because the specific carbohydrate binding activity and not the agglutination activity is the functional criterion of these proteins (some lectins are monovalent, and just for this reason they can not agglutinate cells, and hence can not be called agglutinins. Yet, they can bind specific sugar structures in a selective manner). Thus, although the term ‘lectin’ was introduced to describe the blood group specific agglutination activity of some members of this class of proteins, it is much more appropriate to describe the specificity of their interaction with mono- and oligosaccharides. Moreover names like ‘(hem)agglutinin’ may cause some confusion with elements of other system, e.g., in the precipitation of antigens by their specific antibodies, the antibody is called 'agglutinin' in general and 'hemagglutinin' in case of erythrocyte antisera [Treffers, 1992].

1.4. Definition: The following definition of lectins is more flexible than all proposed before it and covers a broad range of proteins which behave differently with respect to their agglutination and precipitation properties: *lectins are proteins of nonimmune origin possessing at least one noncatalytic domain which binds reversibly to a specific mono- or oligosacchride.* The original definition [Peumans and Van Damme, 1995] does not include the condition ‘*of nonimmune origin*’ because it considers only plant lectins. This definition is applicable to the monovalent lectins (merolectins, see below) which can not agglutinate cells or precipitate glycoconjugates such as hevein, a small chitin binding lectin from the latex of rubber tree, *Hevea brasiliensis* [Van Parijs et al., 1991], and the monomeric mannose binding lectin from

Listera ovata [Van Damme et al., 1994]. This definition also includes some chimeric plant enzymes with genuine lectin domains (chimerolectins, see *1.5.3*) such as type 2 RIP [Fiorenzo et al., 1992] and class I chitinases, which are also monovalent lectins [Beintema, 1994].

1.5. Subdivision based on subunit composition: In terms of the number and activity of its subunits, a lectin can be classified into one or more of the following groups [Van Damme et al., 1998a].

1.5.1. Merolectins: These are small monomeric proteins with only a single carbohydrate binding domain (CBD). Therefore, they cannot agglutinate cells or precipitate glycoconjugates. The few merolectins discovered so far exist within the following plant lectins families: chitin binding lectins, monocot mannose binding lectins and type 2 RIP like lectins (discussed in *4.1*). Hevein is a chitin binding merolectin from the rubber tree latex [Van Parijs et al., 1991]. All chitin binding lectins are built of domains very similar to hevein (hevein like domains).

1.5.2. Hololectins: These lectins have at least two identical or very similar CBD with same specificity, i.e., they bind same or structurally related sugars, hence they are multivalent lectins and are therefore able to agglutinate cells and/or precipitate glycoconjugates. Most lectins are hololectins.

1.5.3. Chimerolectins: A chimerolectin is a fusion protein which consists of a lectin domain linked to some other unrelated domain independent in its action from the CBD(s) of the protein. Type 2 RIPs and class I chitinases are chimerolctins because they contain *N*-glycosidase or chitinase domains, respectively, linked to lectin domain(s). Ricin, a type 2 RIP, has two (or possibly three) [see Frankel et al., 1996]

galactose specific binding sites. Hence, it can also be considered as a hololectin, whereas class I chitinases have a single chitin binding site and hence they are merolectins.

Artificial chimerolectins with certain properties can be made by linking, in an appropriate way, the polypeptide domains of interest from natural sources [see, for example, Dose and Cornelissen, 1999]

1.5.4. Superlectins: These lectins possess more than one CBD but of different specificities. They are rare, and in plants they have been found so far only in some monocot species as 'monocot mannose binding lectins' (see *4.1.4.*) with one feature distinguishing them from the other family members – they have, in addition to the mannose binding site, another site which binds a carbohydrate structure other than mannose or oligomannose. So far only two such monocot mannose binding lectins have been studied in detail. The first is the tulip bulb lectin (TxLC-I), which exhibits dual specificity for mannose and Gal*N*Ac. Agglutination of rabbit erythrocytes by this lectin could be inhibited only by a mixture of these two sugars [Van Damme et al., 1996; Cammue et al., 1986]. And the second is the tuber lectin from *Xanthosoma sagittifolium*, which exhibits dual specificity for mannose and an *N*-linked oligosaccharide [Mo et al., 1999].

Very few animal and fungal lectins were found to be superlectins [see Sharon and Lis, 2003]. Of special interest is the homotetrameric anticancer lectin from the edible mushroom, *Agaricus bisporus*, which has been shown by crystallographic studies to posses two distinct binding sites (one each for Glc*N*Ac and Gal*N*Ac) on each of its single-domain subunits [Carrizo et al., 2004].

2. Carbohydrate binding

Study of specificity and affinity of the interaction of a lectin with sugars often starts via the semiquantitative method of hapten inhibition assay of the hemagglutination activity of the lectin by the sugars, which gives approximate affinities of the tested sugars to the lectin on a relative scale based on which the specificity of the lectin is assessed (see *2.1*). Chapter 5 contains detailed description of such an assay on the *Trichosanthes dioica* seed lectin [see also e.g., Lis et al., 1994]. Based on the results of such introductory studies, more accurate and sensitive physical techniques, like spectroscopy and calorimetry are employed to obtain the association constant (K_b), stoichiometry (n) and thermodynamic parameters (enthalpy, ΔH_b, and entropy, ΔS_b) of the lectin-sugar interaction. Chapters 3 and 4 present isothermal titration calorimetric and fluorescence spectroscopic investigations, respectively, on the interaction of *Momordica charantia* (bitter gourd) seed lectin with carbohydrates.

2.1. Specificity groups: Most lectins can be classified into few groups called 'specificity groups' according to the sugar to which they show the highest affinity, usually determined by hemagglutination inhibition assay. These groups are: mannose, mannose/glucose, mannose/maltose, galactose/Gal*N*Ac, Glc*N*Ac/(Glc*N*Ac)$_n$, fucose and sialic acid (the configuration is D for all sugars except for fucose, for which it is L) [Goldstein and Poretz, 1986; Van Damme et al., 1998b]. Lectins that bind monosaccharides other than those listed above are rare and are all from animal sources (e.g., P-type animal lectins, which recognize Man6P). The fact that these monosaccharides are the typical constituents of eukaryotic cell surface carbohydrates

reflect the role of lectins as recognition determinants in diverse biological processes [see, e.g., Sharon and Lis, 1989].

2.2. Unusual carbohydrate-specificities: Some lectins can not be considered strictly as members of one of the specificity groups mentioned above because of their unusual carbohydrate-specificities. These specificities are mentioned below briefly:

2.2.1. Complex specificity: Lectins with complex specificity are not inhibited by simple mono- or oligosaccharides because their binding site can be occupied only by a complex branched oligosaccharide. Most plant lectins with complex specificity are legume lectins such as PHA isolectins which are insensitive to any simple sugars but inhibited by very low concentrations of complex *N*- or *O*-glycan moieties of glycoproteins (or similar synthetic oligosaccharide) terminated with Galβ4Glc*N*Ac [see e.g., Kornfeld and Kornfeld, 1974].

2.2.2. Dual specificity: Superlectins (see *1.5.4*) are also described as lectins with dual specificity, i.e., they have more than one binding sites which recognize unrelated sugars so that they can be inhibited only by a mixture of these sugars.

2.2.3. Promiscuous specificity: The binding sites of lectins with this specificity recognize unrelated sugars (i.e., sugars belonging to different specificity groups), usually with significantly different affinities, which allows their classification into the group corresponding to the sugar with the higher affinity. WGA, for example, binds, in addition to Glc*N*Ac and its oligomers, Neu*N*Ac and even Gal*N*Ac [Monsigny et al., 1980], but is generally classified as Glc*N*Ac/$(Glc\mathit{N}Ac)_n$ specific. Several other plant lectins also have been shown to be *promiscuous*, such as the legume lectin UEA-II, which binds galactose and Glc*N*Ac [Loris et al., 2000], jacalin and artocarpin (see

4.1.7). Among animal lectins, several mannose binding lectins were found to bind other sugars like fucose and Glc*N*Ac.

Some of these promiscuous specificities can be rationalized by the topographical similarities between sugar pairs like mannose/fucose and Glc*N*Ac/Neu*N*Ac [Lis and Sharon, 1998].

***2.3. Anomeric preference*:** Certain lectins show anomeric specificity, i.e., within the same specificity group they bind one of the sugar anomers, α or β, stronger than the other. For example, the mannose/glucose specific legume lectins bind α-mannosides and α-glucosides with greater affinity than the corresponding β-anomers. Similarly, the galactose/Gal*N*Ac specific lectins from the seeds of Cucurbitaceae species prefer β-galactosides over α-galactosides [see e.g., Komath et al., 1996; chapter 5].

2.4. The aromatic effect: The nature of the aglycon of the glycoside ligand may affect the strength of the glycoside-lectin interaction. In particular, the binding of glycosides with aromatic aglycon to many lectins is stronger than that of the corresponding sugars with aliphatic aglycon, although opposite cases were also noticed, attesting to the presence of a hydrophobic pocket at or near the sugar binding site on the lectin. This aromatic (or hydrophobic) effect is sometimes so strong that it results in inverse anomeric specificity as is the case with *Griffonia simplicifolia* isolectin I-B4 which shows α or β anomeric preferential specificity with methyl or aromatic galactosides, respectively [De Boeck et al., 1981]. Aromatic substitutions at positions other than the anomeric one also leads to a much greater affinity of a lectin to its

specific sugars, e.g., the dansyl group in Gal*N*Dns, increases the affinity of soybean agglutinin to this sugar by about 160 fold over that observed for Gal*N*Ac [Swamy et al., 1986].

2.5. Ligand size effect: Although most lectins are inhibited by monosccharides, the inhibitory concentration is high compared to that of corresponding oligosaccharides which may have up to 10^3 fold higher association constant than the monosaccharides. Moreover, some lectins interact only very weakly or not at all with monosaccharides, e.g., chitin binding lectins with hevein domain (except those from Gramineae species) are not inhibited by Glc*N*Ac but need at least chitobiose, β1,4(Glc*N*Ac)$_2$, to be inhibited and are far more sensitive to the higher oligomers of Glc*N*Ac. This behavior, i.e., binding oligosaccharides stronger than monosaccharides, shown by most lectins, has been attributed, by results of X-ray crystallography studies, to extended carbohydrate binding sites on lectins that accommodate preferentially oligosaccharides. The functional significant of all these facts is that oligosccharides and not monosccharide are mostly the endogenous carbohyderate ligands of lectins [Lis and Sharon, 1998].

2.6. Quantitative study of lectin-carbohydrate interaction: Due to the crucial role of lectin-carbohydrate interaction in many biological recognition processes (see sections 5 and 6 below) it is important to determine the thermodynamic parameters associated with such interaction (n, K_b, ΔH_b and ΔS_b). These parameters are used generally to predict the extent of binding (affinity) in a given system under certain conditions. In case of lectin-carbohydrate interaction, the differences in binding

parameters of various sugars to a lectin help to predict the contribution of each substituent and functional group on the sugar to the overall binding process which provides information on the forces that govern the binding and the nature of binding site [see e.g., Schwarz et al., 1996].

Several physical methods can be used to obtain the binding parameters of a protein-ligand system. Among these, isothermal titration calorimetry (ITC) yields the most accurate information on the stoichiometry of binding and the thermodynamic parameters associated with the interaction, whereas fluorescence spectroscopy, due to its high sensitivity, is very widely used. In the studies reported in this thesis these two methods have been used for investigating carbohydrate binding to the *Momordica charantia* seed lectin (MCL).

In ITC the heat evolved (or absorbed) as the macromolecule is gradually saturated with the ligand is accurately measured, which allows simultaneous determination of all binding parameters (n, K_b, ΔH_b and ΔS_b) in a single run [Wiseman, 1989]. Additional useful parameters, heat capacity, ΔC_p, and van't Hoff enthalpy, $\Delta H_{b(v)}$, can also be obtained by repeating the ITC run at different temperature(s). The major part of Chapter 3 is on the energetics of sugar binding to MCL as determined by ITC.

Fluorescence spectroscopy is a sensitive and reliable method to study protein-ligand interactions. It is often the method of choice when ITC facility is not accessible or to overcome some technical difficulties (such as protein/ligand concentration required). In cases where ligand binding to a protein can significantly perturb the intrinsic fluorescence of the latter (usually when tryptophan/tyrosine exist in the binding site) this effect can be exploited to study the interaction [See e.g., Das et al.,

1981; Sastry and Surolia, 1986]. Otherwise, fluorescently-labeled sugar probes are required. 4-Methylumbelliferyl glycosides are among the most commonly used fluorescence probes for protein-carbohydrate interaction [see e.g., Loontiens et al., 1977; Khan et al., 1981; Gupta et al., 1992]. The emission intensity of the umbelliferyl moiety is very sensitive to its microenvironment and usually changes significantly upon binding to the lectin, thus providing a handle to follow the binding process. In Chapter 4, studies on the interaction of 4-methylumbelliferyl galactosides with MCL are reported [see also Sultan and Swamy, 2003]

3. Non-carbohydrate ligands

In addition to the sugar binding sites, many lectins possess also one or more binding site(s), for non-carbohydrate ligands, totally independent from the carbohydrate ligand binding sites [see e.g., Roberts and Goldstein, 1982, 1983a].

Porphyrins might be considered as a class of non-carbohydrate ligands of lectins since several plants lectins (Con A, pea lectin, jacalin, SGSL, TCSL and MCL) were found to bind some synthetic porphyrins with affinities comparable to that observed for the specific sugars. This binding takes place in the presence of saturating concentrations of a specific sugar as well as in the absence of any sugar, indicating that the interaction with porphyrins occurs at binding sites distinct from carbohydrate binding sites [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001; Chapter 2].

Relevant to the biological roles which plant lectins probably play in plants is that several of these lectins (mostly legume lectins) were found to strongly bind, *in vitro*, some hydrophobic biomolecules like adenine and adenine-based plant hormones

[see e.g., Roberts and Goldstein, 1983b; Maliarik and Goldstein, 1988; Gegg et al., 1992; Srinivas et al., 2000]. Equilibrium studies in solution showed that legume lectins bind adenine and related molecules in a stoichiometry of one (or two) per lectin molecule, however, the crystal structure of *Dolichos biflorus* seed lectin complex with adenine (perhaps the only one of its kind reported so far) shows two identical hydrophobic cavities within the lectin matrix, with each cavity accommodating two adenine molecules [Hamelryck et al., 1999].

Conservation of the binding site for adenine and related hydrophobic biomolecules among several legume lectins of different specificities and sources suggests that other lectins may also be able to bind such biomolecules. Binding of adenine and adenine-related phytohormones such as kinetin, zeatin, isopentyl adenine as well as abscisic and gibberellic acids to WGA has recently been reported [Bogoeva et al., 2004]. MCL was also found to bind adenine with moderate affinity, suggesting that it might be playing a role in adenine-based plant hormones transport within its natural source (unpublished observations from this lab).

4. Classification: lectin families

Lectins represent a heterogeneous group of proteins with wide diversity in size, structure, physicochemical properties, biological activities, sugars specificities and composition and architecture of binding sites. However, based on the sequence information and structural motifs, most lectins can be classified into a fairly small number of distinct families [Van Damme et al., 1998a; Drickamer, 1994]. This classification is dealt with very briefly below:

4.1. Plant lectins: The evolutionary relationships traced by similarities in the amino acid sequence and structural features among over 300 plant lectins known at present allow their classification into seven families: *1*. amaranthin lectins, *2*. (hevein-like) chitin binding lectins, *3*. Cucurbitaceae phloem lectins, *4*. monocot mannose binding lectins, *5*. type 2 RIP, *6*. legume lectins, *7*. jacalin-related lectins. Lectins that do not fall into any of the above families are referred to as unclassified lectins.

Three-dimensional structure and the binding site(s) fold of representative members of all these families, with the exception of Cucurbitaceae phloem lectins, are now available at atomic level and have been shown to be conserved within each family. This structural conservation is reflected in the fact that each one of the families mentioned above (except legume and jacalin-related lectins) shows very similar specificity among its members. Thus, each of these lectin families has its own typical fold and binding site motif and specificity [Van Damme et al., 1998a; Peumans et al., 2000], as shown below:

4.1.1. Amaranthin lectins: This family is a small group of Gal*N*Ac specific lectins. Members are approximately 66 kDa homodimeric unglycosylated proteins. They are found only in the seeds of the genus *Amaranthus* species. The prototype member of this lectin family is amaranthin, the lectin from *A. caudatus*, which binds preferentially to T-antigen disaccharide [Rinderle et al., 1989]. The crystal structure of amaranthin bound to benzylated T-antigen disaccharide has been solved at 2.2 Å resolution [Transue et al., 1997]. This structure shows that amaranthin monomer consists of two homologous *N*- and *C*-domains which are associated by extensive non-covalent forces in head-to-tail manner to form the dimer, resulting in the formation of a binding site at each interface between the *N*- and *C*-domains of the two facing

monomers (see Fig. 1A). The sugar binding activity of amaranthin depends on a complex network of H-bonds and some of them (those involve in binding of the non-reducing part of the disaccharide) are mediated by water. No hydrophobic contact is involved in the binding process.

4.1.2. Hevein-like chitin binding lectins: Not all chitin binding lectins belong to this family, but only those which have one or more hevein-domain(s) as the chitin binding domain. However, this family is widespread in many plant species of different taxonomy. Hevein-domain is a polypeptide structural unit of about forty amino acids. It binds chitin and lower oligomers of Glc*N*Ac specifically and exhibits sequence and structure similarities to hevein (a 43 amino acid chitin binding merolectin from the latex of rubber tree, *Hevea brasiliensis*). By definition, class I chitinases are chimerolectins belonging to this family because they have a single *N*-terminal hevein-domain linked to the chitinase domain [Beintema, 1994]. Most of this family members are hololectins such as WGA and the other lectins of Gramineae species which have essentially the same structure as that of WGA [Stinissen et al., 1983]. WGA was the first member of this family to be isolated and fully characterized. The monomer of WGA is built up of four identical hevein domains and two such monomers are non-covalently associated in a head-to-tail manner to form a dimer having four pairs of hevein domains (one from each monomer). Hence there are eight binding sites in

Fig. 1. (Opposite). Ribbon representation of crystal structures of representative lectins as mentioned in *4.1*. (A) amaranthin complexed with benzyl-α-T-antigen disaccharide [Transue et al., 1997], PDB code: 1JLX. (B) WGA complexed with Neu*N*Ac-α-(2,3)lactose [Wright, 1990], PDB code: 1WGC. (C) GNA complexed with MeαMan [Hester et al., 1995], PDB code 1MSA. (D) ricin complexed with lactose [Rutenber et al., 1991], PDB code: 2AAI. (E) Con A dimer complexed with MeαGlc [Harrop, 1996], PDB code: 1GIC. (F) jacalin complexed with MeαGal [Sankaranarayanan et al., 1996] PDB code 1JAC. All pictures were taken from **www.cermav.cnrs.fr/lectines**.

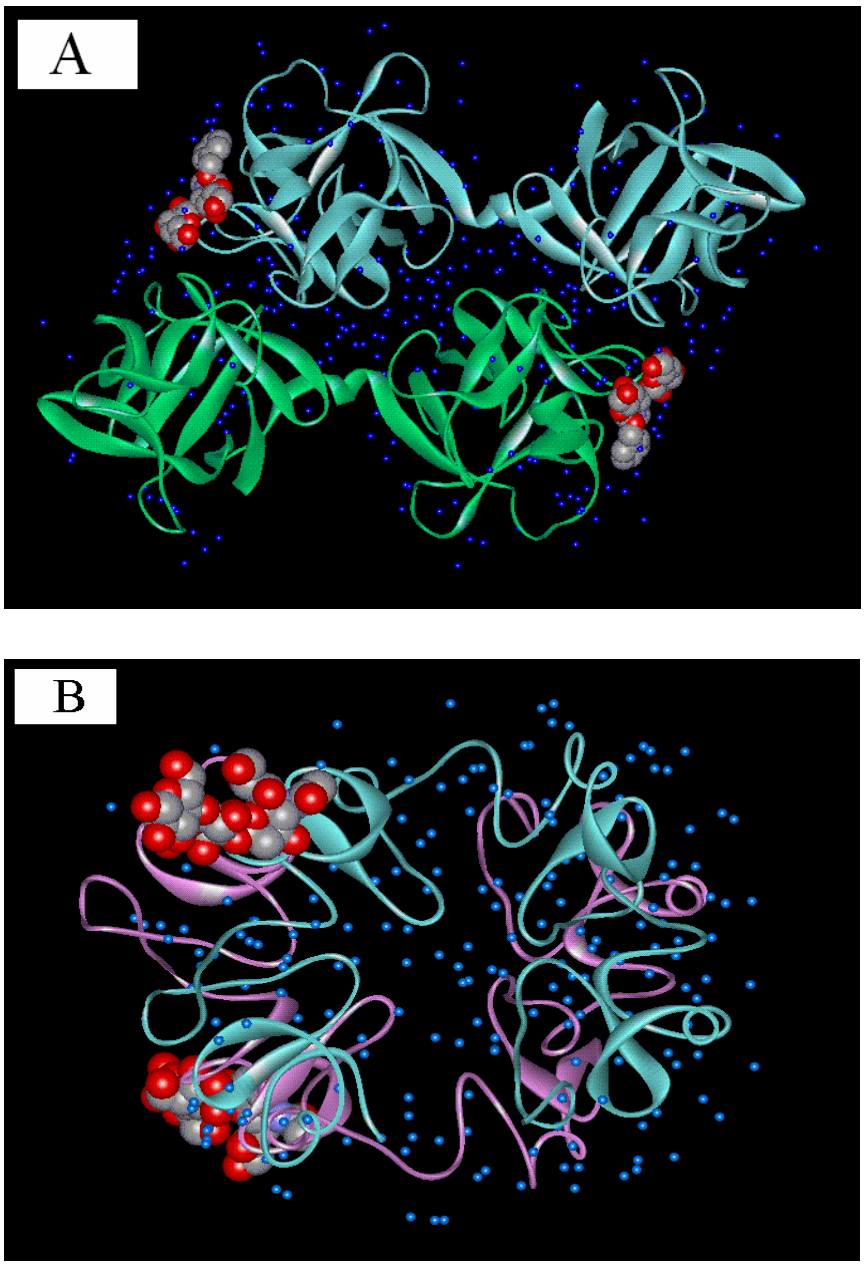

Fig. 1.

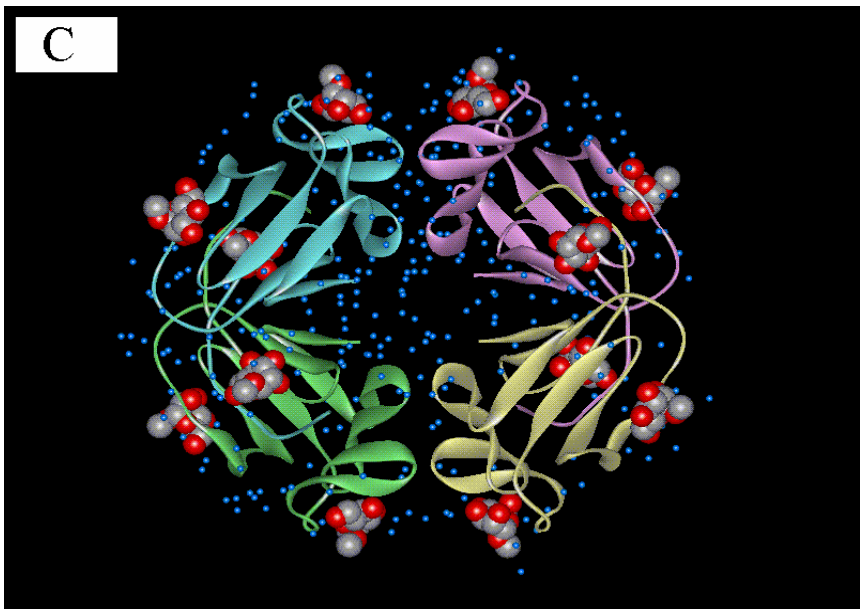

Fig. 1. (contd)

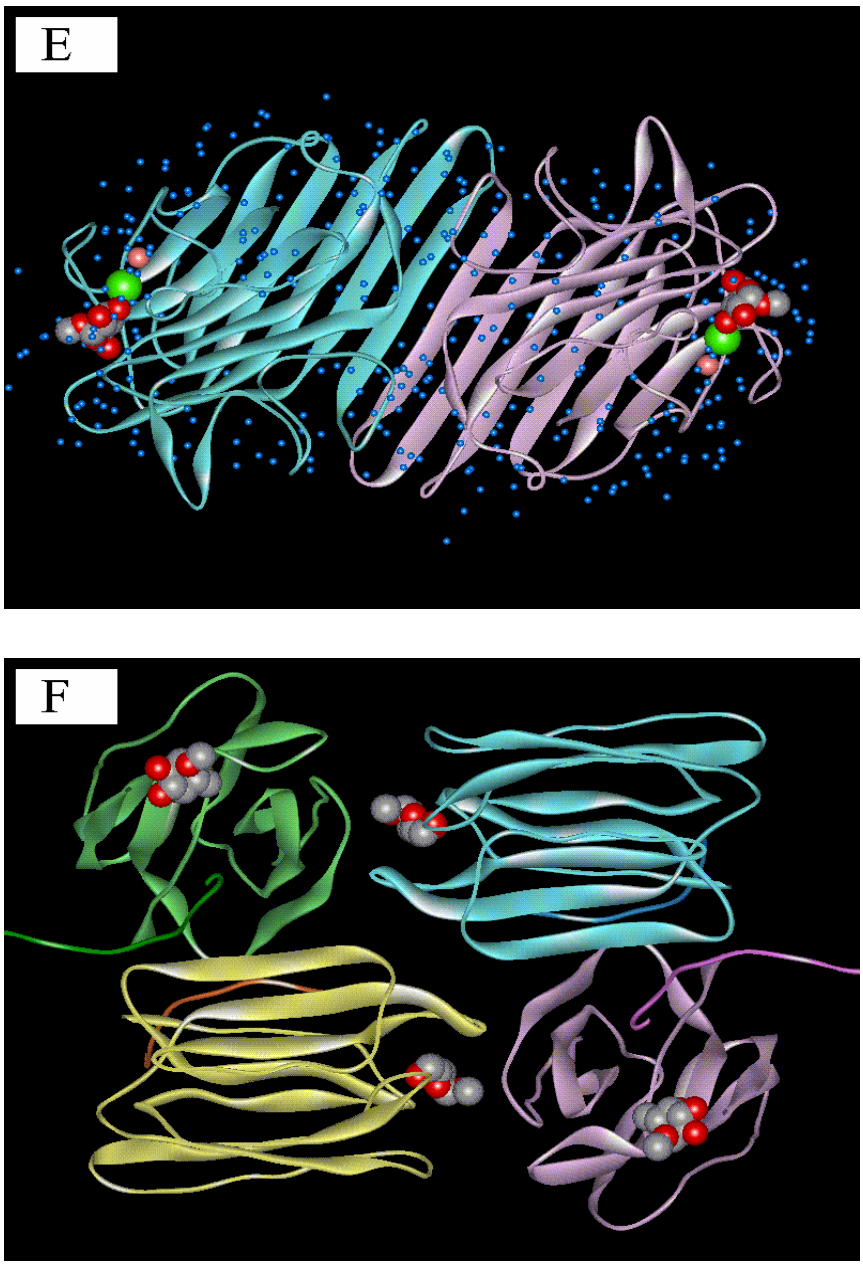

Fig. 1. (contd)

WGA; only four of them were shown by solution, crystal and modeling studies to be of high and roughly equivalent affinities whereas the remaining four are at least 2-fold weaker [Wright and Kellogg, 1996].

There are several reports of 3-D crystal structure of WGA and its complex with carbohydrate ligands. The 3-D structure of WGA complexed with *N*-acetylneuraminyllactose is shown in Fig. 1B [Wright, 1990]. Specificity studies showed that the binding site of this family of lectins is most complementary to $(GlcNAc)_4$ and in most cases the monomer is not inhibitory and at least the dimer is required for inhibition.

4.1.3. Cucurbitaceae phloem lectins: Members of this lectin family also bind chitin but their structure is not made of hevein domains as seen in the previous lectin family. So far they have been found only in the phloem exudates of Cucurbitaceae species. They are very different from galactose-specific lectins found in seeds and roots of the same plant family (see *4.1.8*). They are dimers composed of about 25 kDa unglycosylated subunits. Like most chitin binding lectins, they are inhibited only by oligomers of Glc*N*Ac [Sabnis and Hart, 1978; Anantharam et al., 1986; Sanadi and Surolia, 1994; Peumans et al., 2000].

4.1.4. Monocot mannose binding lectins: This family consists of a large number of lectins with restricted specificity to mannose and oligomannose, and found only in some of the monocot plant families. GNA (the mannose specific lectin from snowdrop bulbs) is the first member of this family to be isolated and characterized [Van Damme et al., 1987]. Detailed specificity, cloning and structural studies proved GNA to be different from any previously characterized lectin in its sequence, sugar binding

activity and 3-D structure [Shibuya et al., 1988; Van Damme et al., 1991; Hester et al., 1995]. Thus GNA became the first representative of a new lectin family which has grown rapidly since then by the isolation and characterization of similar lectins from several monocot plant families to form the superfamily of monocot mannose binding lectins [Barre et al., 1996]. The molecular structure of these proteins involves, in most cases including GNA, one, two or four non-covalently associated small protomers (11-14 kDa). The main secondary structural element of this protomer is β-sheet. It is built up of three tandemly arrayed domains and three mannose binding sites [Peumans et al., 2000]. The 3-D structure of GNA-MeαMan complex [Hester et al., 1995] is shown in Fig. 1C.

4.1.5. Type 2 RIPs: A typical type 2 RIP consists of a C-terminal domain (called B-chain) with a specific sugar binding activity (mostly to Gal/Gal*N*Ac) linked by a disulfide bridge to an *N*-terminal domain (called A-chain) with catalytic ribosome inactivation activity of eukaryotic ribosome. Thus, members of this family are chimeric proteins, i.e., they are built up of two structurally and functionally independent domains, and since one of these domains is a lectin, they are classified as chimerolectins (see *1.5.3*).

RIPs which are built up of only the A-chain are termed 'type 1 RIP'. Both type 1 RIP and A-chain of type 2 RIP share a highly similar sequence and once in the cytoplasm of an eukaryotic cell both can inactivate its ribosome by catalytic cleavage of the *N*-glycosidic bond of a single, highly conserved adenosine residue from the 28S subunit of ribosomal RNA (rRNA), resulting in the inhibition of protein synthesis, which leads to cell death. Most RIPs are also able to cleave such adenine *N*-glycosidic bonds at several positions on other polynucleotides as well [Barbieri et al., 1997].

Some type 2 RIPs, like ricin, are potent cytotoxic agents, where the B-chain (divalent lectin) serves to bind to the cell through the Gal/Gal*N*Ac moiety of the cell surface glycoconjugates. This binding facilitates the endocytosis of the toxin into the cytoplasm where the A-chain can start its role as mentioned above [Stirpe et al., 1992].

Type 2 RIPs have been found in the seeds and various vegetative tissues in at least ten plant families. The native type 2 RIPs consist of one, two or four similar protomers. The protomer consists of the A- and B-chain (usually both are glycosylated) linked by a disulfide bond as mentioned above and called A-s-s-B pair. The classical potent toxins, ricin from *Ricinus communis* seeds and abrin from *Abrus precatorius* seeds, are monomeric type 2 RIP i.e., they consist of a single A-s-s-B pair, whereas the lectins RCA and APA from the same sources, respectively and also the lectin from *Momordica charantia* seeds, MCL, are dimeric type 2 RIPs built up by noncovalent association of two A-s-s-B pairs [Van Damme et al., 1998a]. Inspite of the high sequence homogeneity between monomeric and dimeric type 2 RIPs, the later seem to have no or weak cytotoxicity and only two sugar binding sites instead of four.

Three-dimensional structure of several type 2 RIPs were solved. The crystal structures of ricin and abrin [Rutenber et al., 1991; Tahirov et al., 1995] show similar overall fold, where the B-chain is built up of tandem repeat of two similar domains and is devoid of extended regular secondary structures but stabilized by four intrachain disulfide bonds, unlike A-chain which consist of eight α-helices and six β-sheets. The 3-D structure of ricin complexed with lactose [Rutenber et al., 1991] is shown in Fig. 1D. Modeling studies, on the other hand, on type 2 RIPs from *Sambucus nigra* showed that the structure of their B-chain is very similar to that of ricin and abrin [Van Damme et al., 1998a].

4.1.6. Legume lectins: The most well-characterized lectins belong to this family of proteins. Therefore, it is the largest family of plant lectins comprising of proteins found only in legume species. It may be noted that not all lectins from legume plants belong to this family; for example, the type 2 RIPs abrin and APA, from the legume *Abrus precatorius*, and the class 1 chitinase from red kidney beans do not fall into this lectin family. All native legume lectins at physiological pH are tetramers (or dimers) of about 30 kDa protomers (mostly *N*-glycosylated) with one sugar binding site and one each for Mn^{2+} and Ca^{2+}, which are essential for sugar binding activity [Sharon and Lis, 2003]. Demetalisation of legume lectins leads to large changes in their structures and loss of the sugar binding activity. In most cases the protomers are identical or very similar; however, some legume species express two different protomers in their cells which may associate randomly to form different homogeneous and heterogeneous isolectins. The classical example for this is the PHA isolectins, E_4, E_3L_1, E_2L_2, E_1L_3 and L_4, from the common beans, *Phseolus vulgaris* [Feldsted et al., 1977].

In some lectins, which are called two-chain legume lectins, e.g., pea lectin [Trowbridge, 1974], the protomer polypeptide is fragmented unequally into light chain (α) and heavy chain (β).

The protomers in all legume lectins have very similar 3-D structure which is built up of two antiparallel β-sheets: one is a six-stranded flat sheet (back face) and the other is a seven-stranded concave sheet (front face). The β-sheets are interconnected by turns and loops to form a flattened dome. The β-folds of the seven-stranded sheet form the sugar and metals binding sites. The protomers associate noncovalently to form dimers or tetramers. The most common dimerization mode involves side-by-side

association of two monomers using their flat β-sheets in a two-fold symmetry plane to form a twelve-stranded β-sheet. As a result, the two binding sites are located at both ends of the dimer. This mode of dimerization in legume lectins is called the canonical dimerization. The 3-D structure of Con A dimer complexed with MeαGlc [Harrop, 1996], clearly depicting the canonical dimerization is shown in Fig. 1E. Some other modes of dimerization in legume lectins (called noncanonical dimerization) are also known [Elgavish and Shaanan, 2001]. All tetrameric lectins are formed by association of two dimers either by loop interactions using the central parts of the dimers, as in Con A, or by interactions between the outermost strands of the two twelve-stranded β-sheets creating a channel between them, as in SBA. PNA is unusual not only in its noncanonical dimer but also in being an asymmetric tetramer unlike any other homotetramer protein [Banerjee et al., 1994, 1996].

In spite of the high sequence homology and very similar 3-D structure among them, legume lectins cover almost all the monosaccharide specificities. This is because few changes in the amino acid sequence and lengths of loops involved in the sugar binding lead to binding sites of different conformations and hence of different specificities [Sharma et al., 1997].

4.1.7. Jacalin-related lectins: This family includes all lectins which show high similarity to jacalin, a lectin from jackfruit (*Artocarpus integrifolio*) seeds, in structure and sequence. The jackfruit seeds also contain another lectin very similar to jacalin (jacalin-related lectin) called artocarpin. Both lectins are homotetramers of very similar 3-D crystal structure in which the protomer has a novel fold (called β-prism fold), which consists of three four-stranded β-sheets in three-fold symmetry arrangement [Sankaranarayanan et al., 1996; Pratap et al., 2002]. The crystal structure

of jacalin-MeαGal complex is shown in Fig. 1F. Like jackfruit seeds, the black mulberry tree (*Morus nigra*) bark also contains two jacalin-related lectins: one is galactose specific (mornigaG) and the other is mannose specific (mornigaM) [Van Damme et al., 2002]. In fact, jacalin-related lectins are classified into two subfamilies: galactose specific lectins (like jacalin) which are found only in some Moraceae species, and mannose specific lectins (like artocarpin) which are widespread among flowering plants [Peumans et al., 2000].

Jacalin's monomer is synthesized as a preproprotein which, after removal of the signal peptide and a propeptide, is cleaved into two chains, a heavy (α) chain and a light (β) chain. Although it was suggested that carbohydrate specificity of jacalin is probably generated by the cleavage of the single chain polypeptide [Jeyaprakash et al., 2003], recent studies indicate that the carbohydrate specificity pattern of recombinant jacalin expressed in *E. coli*, which is not cleaved, is unaltered but its affinity is about 100-fold lower for a battery of sugars as compared to native jacalin [Sahasrabuddhe et al., 2004]. Therefore, it appears that post-translational cleavage of the jacalin polypeptide is required for the increase in the affinity for the saccharides that it binds. Artocarpin, the other lectin present in the jack fruit seeds, which is mannose-specific, is not cleaved.

Jacalin and artocarpin are *promiscuous* lectins (see *2.2.3*). Different agglutination/precipitation inhibition assays of jacalin by sugars showed that in addition to galactose and its derivatives, other unrelated sugars, especially mannose and MeαMan also inhibit the hemagglutination activity of this lectin [Dalmau and Freitas, 1989]. On the other hand, surface plasmon resonance hapten inhibition experiments

demonstrated that artocarpin interacts with a wide range of unrelated monosaccharides even though it displays a higher affinity for mannose [Barre et al., 2004].

Crystal structure of jacalin complexed with MeαMan at 2.0 Å resolution has been determined by Bourne et al. [2002], who attributed the ability of jacalin to bind monosaccharides with different hydroxyl conformations to its relatively large binding site and the flexibility of the β-prism fold.

4.1.8. Unclassified lectins: Lectins which can not be yet confidently considered to belong to any of the above mentioned families, due to the lack of structure and sequence information about them, are referred to as 'unclassified lectins'. Among those unclassified lectins, Cucurbitaceae galactose-specific lectins (CGL) are of special interest because all members studied so far among them resemble type 2 RIPs in that they are glycosylated, galactose/Gal*N*Ac specific lectins built up of one or more disulfide bridge-linked subunits (M_r 25-35 kDa) pairs, and also their amino acid composition, if known, is very similar to that of type 2 RIPs. The similarity between this group of lectins and type 2 RIPs is also shown by some structural studies:

1. Molecular replacement studies on crystals of *Trichosanthes kirilowii* lectin [Li et al., 2000, 2001] and *Trichosanthes anguina* lectin, SGSL [Manoj et al., 2001] have shown that these lectins are homologous to type 2 RIPs.
2. The various secondary structure element content of some of these lectins, determined by circular dichrosim spectroscopy (see chapters 2 and 5) is similar to that seen in crystal structure of some typical type 2 RIPs such as ricin and abrin (as described briefly in *4.1.5*).

For all these observations CGLs are speculated to be type 2 RIPs. In fact, the tetramer *Momordica charantia* seed lectin, MCL, was shown to be a ribosome

inactivating protein [Barbieri et al., 1980; Wang and Ng, 1998]. Only few members of this family have been purified and studied so far [see e.g., Chapter 5 and Mazumder et al., 1981; Falasca et al., 1989; Komath et al., 1996; Padma et al., 1999; Sultan et al., 2004b]. More investigations on existing and new members of this lectin family have to be done in order to classify these lectins as type 2 RIP (like) lectins or as a new distinct lectin family.

4.2. Animal lectins: In spite of the diversity of the overall structure of animal lectins, the carbohydrate binding activity of each lectin is ascribed to a limited portion of it called the carbohydrate recognition domain, CRD [Drickamer, 1988, 1994]. On the basis of the sequence motif of these CRDs, most animal lectins can be classified into a number of distinct types, the most notable of them are C-, S- and P-type lectins.

CDRs in each lectin type share invariant and highly conserved amino acids. In addition to the sequence similarity of their CRD, each animal lectin type shows the following properties among its member:

1. C-type lectins are multidomain cell surface and extracellular lectins with diverse specificities and require Ca^{2+} ions for sugar binding activity (hence the name C-type).
2. S-type lectins (better known nowadays as galactins) are both intra- and extracellular soluble lectins with specificity for β-galactosides (mainly lactose and *N*-acetylactosamine) and require cysteine residues with free -SH groups for sugar binding activity (hence the name S-type).

3. P-type lectins are mannose 6-phosphate recepters (hence the name P-type) of either high molecular weight (~300 kDa) and Ca^{2+}-independent activity or low molecular weight (~45 kDa) and Ca^{2+}-dependent activity.

5. Biological functions

Most biological functions of lectins are mediated by the ability of lectins to recognize specific carbohydrate structures, especially those that are present on cell surfaces [Sharon and Lis, 1989]. Some of these functions are described briefly here:

5.1. Plant lectins: Although plant lectins are known for a much longer time than other lectins, their biological functions in *vivo* are not well understood yet. However, there are many evidences, though not conclusive, that lectins play the following function in nature [Peumans and Van Damme 1995, Van Damme et al., 1998a]:

1. Recognition elements in symbiosis, mainly by legume lectins, between rhizobia (nitrogen-fixing bacteria) and leguminous plants.
2. Plant defense proteins, since many plant lectins have been shown to have one or more of these activities: antibacterial, antiviral, antifungal, antiinsect and antinutritional against plant-predatory and phytopathogenic organisms.
3. Storage proteins, since many plant lectins were found to be abundant in storage organs (seeds, tubers etc.) of developed plants and their level decreases dramatically during growth or seed germination.

5.2. Microbial lectins: The viral and bacterial surface lectins initiate infections by mediating the adhesion of these pathogenic agents to the target cells. This adhesion is a prerequisite for infections to occur and it is the result of specific binding of these lectins to the carbohydrate moieties on the target cells since it is inhibited (and hence infection prevented) by sugars to which these lectins are specific [Lis and Sharon, 1998]. This illustrate that carbohydrates can be used in prevention of infections caused by organisms which express surface lectins. This is the basis for development of *anti-adhesion therapy* [Ofek and Sharon, 2002]. Controversially, microbial lectins (mainly bacterial mannose specific lectins) may act against microbial infections via what is known as *lectinophagocytosis* [Sharon 1987; Ofek and Sharon, 1988] in which lectins on either the host's (man, mice) phagocytes or the infectious agent interact (in the absence of opsonins) with specific carbohydrate on the apposing cells, leading to the activation of phagocytes and ingestion and killing the infectious agent. The presence of the specific sugar (e.g. methyl α-mannoside in case of type 1 fimbriated *E. coli,* which express mannose binding domain on its surface) prevents the lectinophagocytosis of this bacteria.

5.3. Animal lectins: Animal lectins are the most understood lectins in terms of their biological functions. Some of these functions are:

1. *Traffic of lysosomal enzymes:* P-type lectins mediate the traffic of lysosmal enzymes from cell surface and golgi network to lysosomes. This traffic depends on binding of Man6P moieties of the oligomannose glycans of these enzymes to Man6P receptors (P-type lectins) [Drickamer, 1994].

2. *Clearance of glycoproteins:* Most mammalian serum glycoprotein glycans terminate in sialic acid. When these sialic acid residues are cleaved, the galactose residues become exposed and bind to hepatocyte asialoglycoprotein receptors (C-type galactose specific lectin). These glycoproteins are then rapidly internalized into hepatocytes and digested by lysosomes. It seems that desialylation of serum glycoproteins is a signal to indicate that these glycoproteins have become useless and must be cleared from circulation. Glycoprotein receptors which recognize terminal sugars other than galactose are also known [Drickamer, 1994].
3. *Defense:* Many animal lectins (mainly C-type lectins) are involved in immune response. In addition to the macrophage mannose receptor's role in lectinophagocytosis (see *5.2*), liver and serum MBPs bind in a carbohydrate-specific manner to several bacterial and fungal pathogens and act as opsonins, thus bypassing the need for antibody recognition step in the immune response aganist these pathogens [Weis et al., 1998].
4. *Cell adhesion and recognition:* Selectins, a class of C-type lectins, mediate the adhesion of circulating leukocytes to the endothelial cell of blood vessels. This adhesion is the result of selectin-carbohydrate interaction and is the first step in targeting leukocytes to lymphoid organs or to sites of inflammation [Vestweber and Blanks, 1999; McEver, 2002].

6. Applications

Based on their interaction with free and cell-surface carbohydrates and glycoconjugates, lectins, either native or, in most cases, labeled with fluorescent dyes, radioactive agents, antibodies, etc., serve as very useful tools in biochemistry, biology

and medicine [Sharon and Lis, 2003]. Most lectins in use are plant lectins. Some of their applications are mentioned here briefly:

6.1. Detection and purification of oligosaccharides and glycoconjugates: These molecules can be detected, even in complex mixtures, by Ouchterlony double-diffusion technique using the suitable lectins instead of antibodies [Carter and Sharon, 1977]. Lectins can be also used to stain glycopeptide bands after electrophoretic separation [Carlos et al., 2002]. Lectins are also used as histo- and cytochemicals for detection of glycoconjugates in tissues and membranes. For preparative purposes, purification of oligosaccharides and glycoconjugates by affinity chromatography on immobilized lectins is a very useful and well-established technique [see e.g., Osawa and Tsuji, 1987].

6.2. Cell differentiation: The ability of lectins to preferentially agglutinate certain cells, based on their surface sugars, is used to separate cells of interest from a mixture of cells. The agglutinated cells are then obtained intact for further studies by adding a specific sugar of the lectin. Some examples are: separation of immature thymocytes from the mature ones by PNA [Reisner and Sharon, 1980] and separation of T-lymphocytes from B-lymphocytes by SBA [Weppner and Adkison, 1980].

6.3. Bone marrow transplantation: SBA is used clinically to deplete T-cells (by agglutinating them) from donated bone marrow before transplanting it into infants suffering from severe *combined immunodeficiency*, a condition in which the host defense and immune response of the patient is severely affected which results in high

susceptibility to infectious diseases [Buckley et al., 1999]. The reason for removing T-cells from donated bone marrow is to prevent or minimize the risk of the life-threatening condition called '*graft versus host disease'* caused by the donor's T-cells.

SBA is also used in clinical trials to deplete cancer cells from bone marrow taken from cancer patients before retransplanting it again into them (autologous bone marrow transplantation) after they have received high doses of chemotherapy and/or radiation therapy treatment which cause sever damage to the bone marrow [Morecki et al., 1987].

Galectin1 (β-galactoside animal lectin) is known to modulate T-cell functions and apoptosis and was used to increase the survival (by about 70%) after transplantation of untreated bone marrow into mice [Baum et al., 2003].

6.4. Mitogenic stimulation of lymphocytes: Some lectins are mitogenic (see Table 1). Mitogenic lectins interact *in vitro* with lymphocytes (T and/or B cells) and stimulate them to undergo mitosis, i.e., growth and proliferation [Ashraf and Khan, 2003]. The initial step, but not the only requirement, leading to this stimulation is the binding of the mitogenic lectin to a surface sugar of the lymphocytes; hence sugars specific for mitogenic lectins inhibit their mitogenic activity. Mitogenic stimulation by lectins is used, among others, in assessment of several immunological phenomena [Lis and Sharon, 1986a,b].

6.5. Blood typing: Many lectins are blood group and subgroup specific, i.e., they agglutinate erythrocytes of one or few groups, within ABO and other systems, but do not or very weakly agglutinate erythrocytes of other blood groups. Some of these

lectins are commonly used in, among others uses, blood group typing, identification of secretors (individuals who secrete blood group substance in their saliva and other body fluids) and detection of polyagglutinability (a serious condition that results from any of several inherited or acquired abnormalities in the erythrocytes surface making them agglutinable by normal blood sera) [Khan et al., 2002]. Table 1 lists several blood group specific lectins.

Table 1. Some blood group specific and mitogenic lectins [Sharon and Lis, 2003]

Lectin name or source	Mitogenic activity		Blood group specificity
	T-cell	B-cell	
Artocarpin	+	+	
Con A	+		
ECL	+		
Hura crepitans	+		
Lens culinaris	+		
PHA	+		
Pisum sativum	+		
PNA	+		T
SBA	+		
Vicia sativa	+		
Phytolacca americana	+	+	
Wistaria floribunda	+		
Dolichos biflorus			A
G. simplicifolia (IA4)			A
Helix pomatia			A
Phaseolus lunatus			A
Vicia cracca			A
G. simplicifolia (IB4)			B
Marasmius oreades			B
Anguilla anguilla			O
Lotus tetragonolobus			O
UEA-I			O
Vicia graminea			N
Salvia sclarea			Tn

6.6. Drug targeting: Cancer cells differ from the corresponding normal cells in their cell surface sugar structure. Several lectins, such as WGA, Con A and SBA, can detect this difference and interact preferentially with cancer cells via their surface sugar. This fact led to attempts to use these lectins to target cytotoxic and chemotherapeutic agents (conjugated to lectin) selectively to cancer tissues [see e.g., Tsuruo et al., 1980; Wirth 1998; Minko, 2004].

Since some porphyrins are now used as photosensitizers in photodynamic therapy (PDT) [see e.g., Bonnett, 1995] and due to the ability of several lectins to bind porphyrins (see section 3 above), it is interesting to investigate the possibility of using lectins in PDT as carriers of porphyrins to target tissues which can be specifically recognized by lectins, thus improving tissue selectivity of the porphyrins in PDT.

7. Motivation for and focus of the present work

Since their identification, great strides have been made in lectin research and it is firmly established now that lectins are ubiquitous. In particular, plant lectins have been widely studied and among these the legume lectins have been extensively investigated due to their ease of availability and abundance. As a result of these extensive investigations, legume lectins have been very well characterized with respect to primary, secondary and 3-dimensional structures leading to the unraveling of evolutionary relationships among them. Lectins from some of the other plant families such as Moraceae, Euphorbiaceae and Amaryllidaceae have been investigated in lesser detail, whereas the lectins from Cucurbitaceae seeds have been much less studied. Therefore, in this study we have chosen to carry out investigations on Cucurbitaceae seed lectins.

Previous work from this laboratory was focused on lectins from the seeds of *Trichosanthes anguina* and *Trichosanthes cucumerina*. The present work deals with studies on two cucurbit seed lectins.

In the first part, detailed studies were carried out on the *Momordica charantia* (bitter gourd) seed lectin (MCL) by fluorescence spectroscopy, circular dichroism and isothermal titration calorimetry (ITC). Additionally, the binding of a variety of porphyrins to MCL was investigated by absorption spectroscopy.

In the second part, a new galactose-specific lectin was purified from the seeds of *Trichosanthes dioica* by affinity chromatography and characterized with respect to macromolecular properties, secondary structure and carbohydrate specificity. The amino acid residues important for the sugar binding activity were identified by chemical modification studies using group-specific reagents. Finally, fluorescence quenching and time-resolved fluorescence studies were carried out to understand the environment of the tryptophan residues of this protein.

The results of these studies are expected to shed further light on our current understanding of Cucurbitaceae seed lectins.

1. Abstract

Owing to the use of porphyrins in photodynamic therapy for the treatment of malignant tumors, and the preferential interaction of lectins with tumor cells, studies on lectin-porphyrin interaction are of significant interest. In this study, the interaction of several free-base, and metalloporphyrins with *Momordica charantia* (bitter gourd) lectin (MCL) has been investigated by absorption spectroscopy. Difference absorption spectra revealed that significant changes occur in the *Soret* band region of the porphyrins upon binding to MCL and these changes have been monitored to obtain binding constants (K_b) of various porphyrins to MCL. The prophyin-binding affinity of MCL was not significantly affected by the presence of the specific sugar, lactose. In addition, the agglutination activity of MCL was also unaffected by the presence of the porphyrins used in this study, strongly suggesting that porphyrin and carbohydrate ligands bind at different sites. Both cationic and anionic porphyrins bind to the lectin with comparable affinity ($K_b = 10^3 - 10^5$ M^{-1}). The thermodynamic parameters associated with the interaction of several porphyrins, obtained from the temperature dependence of the K_b values, were found to be in the range: ΔH_b = -98.1 to -54.4 kJ. mol^{-1} and ΔS_b = -243.9 to -90.8 J. mol^{-1}. K^{-1}. These results indicate that porphyrin binding to MCL is governed by enthalpic forces and that the contribution from binding entropy is negative. Enthalpy-entropy compensation was observed in the interaction of different porphyrins with MCL, underscoring the role of water structure in the overall binding process. Analysis of CD spectra of MCL indicate that this protein contains about 13% α-helix, 36% β-sheet, 21% β-turn and rest unordered structures. Binding of porphyrins does not significantly alter the secondary and tertiary structures of MCL.

2. Introduction

Lectins are a class of structurally diverse proteins that are grouped together due to their carbohydrate binding property [see Chapter 1]. Although originally thought to be mediated primarily by hydrogen bonding between the hydroxyl groups of the sugars and the polar side chains of the lectins, structural studies during the last two decades have clearly shown that, in addition to hydrogen bonding, the binding of carbohydrates to lectins is mediated by van der Waals' forces, hydrophobic interactions and metal coordination bonds [Drickamer, 1997; Sharma and Surolia, 1997; Elgavish and Shaanan, 1998; Bouckaert et al., 1999]. Such diverse interactions are possible with carbohydrates because of their unique structural features characterized by both polar and non-polar surfaces.

Porphyrins are another class of biologically important molecules that possess both polar and non-polar features in their expansive structures. Although they are primarily hydrophobic and exhibit low solubility in aqueous media, porphyrins can exhibit interesting polar interactions under certain conditions making the study of their interaction with lectins possible.

In recent years, porphyrins are being used as photosensitizers in photodynamic therapy (PDT), a new modality for the treatment of malignant tumors [Kessel, 1986; Levy, 1995; Dougherty et at., 1998; Bonnett, 1995]. In PDT, the most likely process occurring is that the porphyrin, upon irradiation by light of suitable wavelength, is excited and transfers its excitation energy to molecular oxygen, converting it into the highly reactive singlet state. In this process porphyrin returns back to its ground state and becomes ready to repeat this cycle. The singlet oxygen thus formed reacts with the surrounding tissue, leading to cell necrosis [Bonnett, 1995]. Porphyrins have been found to be photosensitizers of choice in PDT because of their biocompatibility and their ability to preferentially localize in

tumor cells. However, in most cases the relative distribution of the photoactive porphyrin in the tumor tissue to that in the surrounding normal tissues is as low as 2:1 [Klyashchitsky et al., 1994], which is clearly not adequate for the therapeutic application. A possible approach to overcome this limitation is to conjugate the porphyrin to another agent that can direct it to the tumor tissue. In view of the known ability of certain lectins to preferentially bind tumor cells [Lis and Sharon, 1986b], it appeared that lectins may be used as specific targeting agents for porphyrin photosensitizers in PDT. Previous studies reporting the preparation and evaluation of the efficacy of some lectin-drug conjugates on tumor cells and animal models support the above idea [Kitao and Hattori, 1977; Wirth et al., 1998; Lazzaro et al., 1995]. Therefore, we have initiated a long-term program to investigate the interaction of several synthetic water-soluble porphyrins with lectins. In the initial studies, we have characterized the interaction of several free-base and metalloporphyrins with plant lectins such as Con A, pea lectin, jacalin, snake gourd (*Trichosanthes anguina*) seed lectin (SGSL) and *Trichosanthes cucumerina* seed lectin (TCSL) [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001].

Affinity purification and macromolecular characterization of *Momordica charantia* lectin (MCL) – a galactose-specific protein present in the seeds of bitter gourd – were reported about two decades ago. MCL is a tetrameric glycoprotein with a_2b_2-type subunit architecture and exhibits strong type-1 and weak type-2 ribosome inactivating protein activities as well as insulinomimetic activity* [Barbieri et al., 1979, 1980; Mazumder et al.,

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* *M. charantia* seeds extract has a high ribosome inactivation activity on cell-free systems. This activity is due, apart from MCL, to several type 1 RIPs. At least four of these RIPs, momordin (α-momorcharin), β-momorcharin [Fong et al., 1996], γ-momorcharin [Pu et al., 1996] and charantin [Parkash et al., 2002], have been isolated and characterized. Some *M. charantia* RIPs have been shown to have other biological activities as well, such as ribonuclase and anti-HIV activities [Mock et al., 1996; Fong et al., 2000; Lee-Huang et al., 1995], which makes this plant species very attractive for medical and biological research.

1981; Ng et al., 1986]. Chemical modification studies have shown that the side chains of tryptophan and tyrosine residues are important for the hemagglutinating and carbohydrate-binding activity of this lectin [Mazumder et al., 1981]. Steady-state and time-resolved fluorescence studies suggested that the tryptophan residues of MCL are in a heterogeneous environment, with at least two populations of tryptophan residues with different degrees of exposure being present in the protein [Padma et at., 1998]. In addition to hemagglutination-inhibition studies on MCL [Mazumder et al., 1981], the binding of a few saccharides has been investigated by monitoring saccharide-induced changes in the intrinsic fluorescence emission properties of the protein [Das et al., 1981] and the thermodynamic parameters associated with the binding of the fluorescently labeled saccharides, 4-methylumbelliferyl-*α*-D-galactopyranoside and 4-methylumbelliferyl-*β*-D-galactopyranoside to MCL were determined by fluorescence titrations [Khan et al., 1981; Chapter 4].

In the present study, investigations on the interaction of several synthetic water-soluble porphyrins with MCL were carried out. The thermodynamic forces governing the interaction of some of the porphyrins have been delineated from an analysis of the temperature dependence of the association constants. The results suggest that the interaction of porphyrins with MCL is governed by enthalpic forces, with the entropic contribution being negative. Additionally, CD spectral analysis showed that the secondary structure of this lectin consists of 13% α-helix, 36% β-sheet, 21% β-turns and rest unordered structure, which was unaffected by sugar or porphyrin binding.

3. Materials and Methods

3.1. Materials: Seeds of bitter gourd were purchased from local venders. Guar gum, lactose and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). All porphyrins used were synthesized and characterized as described earlier [Fleishcher et al., 1971; Kadish et al., 1989; Longo et al., 1969; Pasternack et al., 1972; Pasternack et al., 1973]. All other reagents were obtained from local suppliers and were of the highest purity available.

3.2. Momordica charantia lectin (MCL): MCL was purified by a combination of ammonium sulphate precipitation and affinity chromatography on cross-linked guar gum [Appukuttan et al., 1977], essentially as described earlier [Padma et al., 1998]. The affinity-purified protein yielded a single band on polyacrylamide gel electrophoresis [Laemmli, 1970], consistent with earlier reports [Mazumder et al., 1981; Khan et al., 1981; Das et al., 1981; Padma et al., 1998].

3.3. Activity assay: The activity of MCL was assessed by the agglutination and agglutination-inhibition assays using O(+) erythrocytes as described earlier for *T. cucumerina* seed lectin [Padma et al., 1999]. A 4 % suspension of the erythrocytes in PBS was mixed with serially diluted samples of the lectin in a 96-well ELISA plate and incubated at 4 °C for 90 min. The agglutination titer was then scored visually. In order to determine whether porphyrin binding alters the sugar-binding activity of the lectin, some of the agglutination experiments were conducted by pre-incubating the serially diluted

samples of the lectin with 25 mM CuTCPP, H_2TMPyP or H_2TPPS before adding the erythrocytes suspension.

3.4. Absorption spectroscopy: Absorption measurements were made on a Shimadzu model UV-3101PC UV-Vis-NIR double beam spectrophotometer using 1.0 cm path-length cells. Temperature was maintained constant (± 0.1 °C) by means of a Peltier device supplied by the manufacturer.

3.5. Concentration determination: Concentration of MCL was determined by Lowry's method [Lowry et al., 1951] using bovine serum albumin as the standard, and by recording A_{280} (1 mg/ml = 1.062 A_{280}). For calculation of K_b values (see *4.2*), MCL molar concentration, [*P*], was expressed in subunits assuming an average subunit M_r of 30 kDa (the tetramer Mr is 120 kDa [Mazumder et al., 1981]). Concentrations of porphyrins were determined spectrophotometrically using their molar absorptivities at the λ_{max} of the *Soret* band, as given in [Komath et al., 2000b].

3.6. Porphyrin binding: Porphyrin binding to MCL was investigated by the absorption titration method essentially as described earlier for SGSL [Komath et al., 2000b]. All titrations were performed in 10 mM sodium phosphate buffer containing 0.15 M NaCl and 0.02 % NaN_3, pH 7.4 (PBS). Porphyrin samples (2.4 ml of ~2.0 – 4.0 μM) were titrated by adding small aliquots of the lectin from a concentrated stock solution (ca. 30 mg/ml) using a Hamilton analytical micro syringe. An equal volume of the protein was added to the reference cell, to correct for any contribution to the absorption by the protein. UV-Vis spectra were recorded after an equilibration period of 2 minutes following

each addition. The spectra were multiplied by an appropriate factor in order to correct for dilution effects in the intensities resulting from the addition of the protein. In order to ensure reproducibility, all titrations were performed at least twice and average values are reported for the association constants.

3.7. Circular dichroism spectroscopy: CD spectra were recorded at 25 °C on a Jasco-J-810 spectropolarimeter available at the Central Instrumentation Laboratory, University of Hyderabad. Spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 1.5 nm. A cylindrical quartz cell of 1 mm path length was used for measurements in the 200-250 nm range while a cell of 10 mm path length was used for measurements in the 250-300 nm range. All measurements were made at a fixed lectin subunit concentration of 24.8 μM in the near UV region, which was diluted 10 times for measurements in the far UV region. Each spectrum reported is the average of four successive scans. Measurements were made in PBS and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis. Spectra were also recorded in the presence of a 25-fold molar excess of CuTCPP or CuTMPyP over MCL subunit concentration (resultant concentration of the porphyrin was 0.62 mM), in order to investigate the effect of porphyrin binding on the protein conformation. These spectra were also corrected by subtracting from them the spectrum of the buffer containing the same concentration of porphyrin.

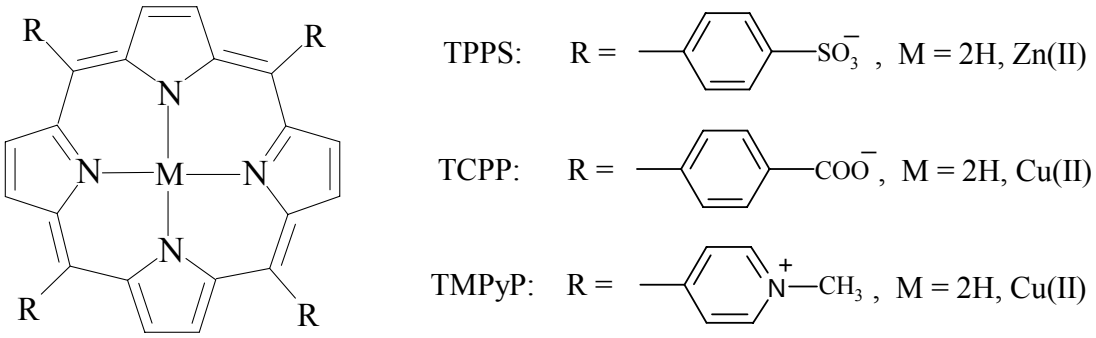
4. Results

A schematic diagram depicting the structure of various porphyrins used in this study is shown in Fig. 1 along with the corresponding λ_{max} and ε_{max} values for the *Soret* band. Some

of these values were taken from our earlier study [Komath et al., 2000b]. All porphyrins used in the present study obeyed Beer's law up to 5 μM, indicating that under the condition employed, the porphyrins were not aggregated [cf. Komath et al., 2000b].

4.1. Porphyrin binding to MCL – absorption and difference absorption spectra: Absorption spectra of CuTCPP (a tetraanionic porphyrin) in the *Soret* band region in the absence and in the presence of different concentrations of MCL, recorded at 20 °C, are shown in Fig. 2A. Spectrum 1 is that of CuTCPP alone and spectra 2-14 correspond to CuTCPP in the presence of increasing concentrations of MCL. From these spectra it is clear that the absorption maximum of the *Soret* band of the porphyrin, seen at 410.8 nm (spectrum 1), shifts to longer wavelengths with a concomitant decrease in the absorption intensity in the presence of added lectin. At the highest concentration of lectin, the absorption maximum is seen at around 415.4 nm (spectrum 14). Difference spectra obtained by subtracting the spectrum of porphyrin alone from the spectra obtained in the presence of different concentrations of the lectin are shown in Fig. 2B. These difference spectra are characterized by a minimum around 405 nm and a maximum around 422.4 nm. Titration of other anionic porphyrins, viz., H_2TCPP, H_2TPPS and ZnTPPS, also yielded absorption spectra and difference spectra with similar features (not shown).

Absorption spectra (*Soret* band region) of the tetracationic porphyrin, CuTMPyP, recorded in the absence (spectrum 1) and in the presence of increasing concentrations of MCL (spectra 2-14) are shown in Fig. 3A. The corresponding difference spectra are shown in Fig. 3B. The *Soret* band of CuTMPyP exhibits an absorption maximum around 424.8 nm, the intensity of which decreases significantly upon titrating with MCL.



Porphyrin	λ_{max} (nm)	$\varepsilon_{\lambda max} \times 10^{-5}$ (M^{-1} cm^{-1})
H_2TPPS	413.0	3.65
ZnTPPS	422.0	3.70
H_2TCPP	414.0	2.00
CuTCPP	410.8	1.53
ZnTCPP	422.0	1.62
H_2TMPyP	422.0	1.90
CuTMPyP	424.8	2.75

Fig. 1. Structures of the porphyrins investigated in this study and wavelengths of maximum absorption (λ_{max}) and molar extinction coefficient (ε) values for their *Soret* absorption bands.

However, the band position shifts only marginally and at the highest concentration of MCL (spectrum 14), it shifts to 426.2 nm. The difference spectra in turn show a single minimum around 420.6 nm (Fig. 3B). Titration of another cationic porphyrin, H_2TMPyP also yielded qualitatively similar absorption spectra and difference spectra in the *Soret* band region (not shown).

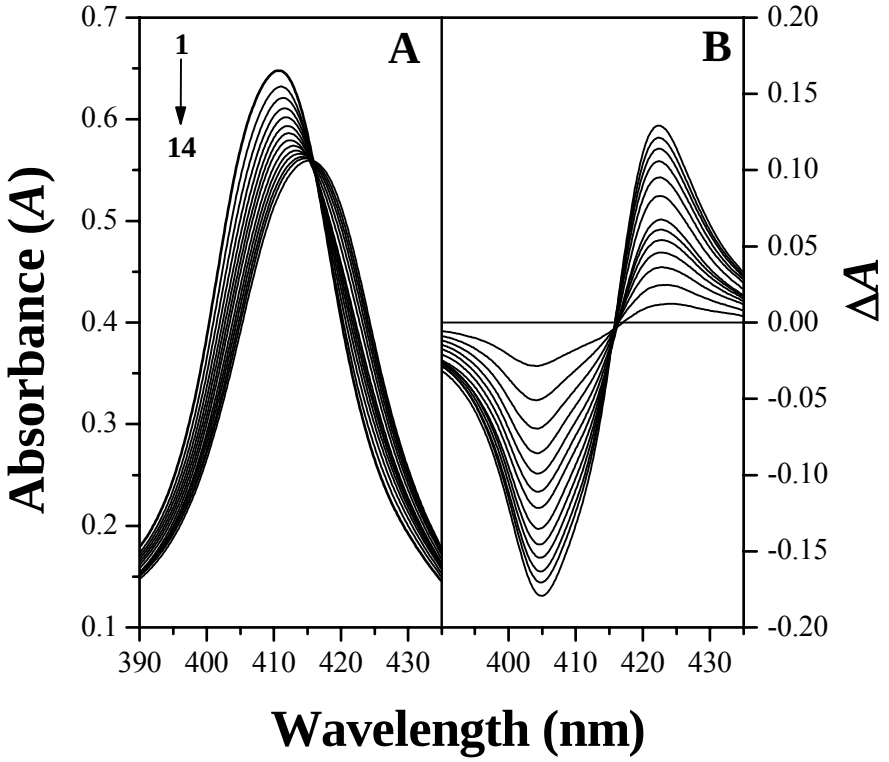


Fig. 2. (A) Absorption spectra of CuTCPP in the absence and in the presence of different concentrations of MCL. (B) Difference absorption spectra obtained by subtracting the spectrum of CuTCPP alone from the spectra obtained in the presence of different concentrations of MCL. Temperature = 20 °C.

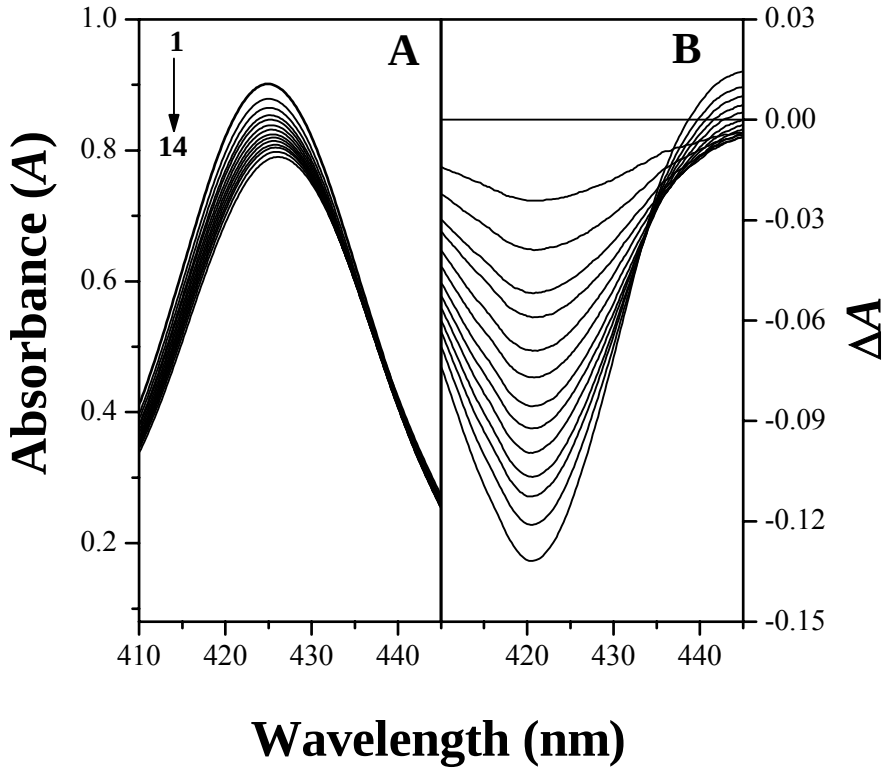


Fig. 3. (A) Absorption spectra of CuTMPyP in the absence and in the presence of different concentrations of MCL. (B) Difference absorption spectra obtained by subtracting the spectrum of CuTMPyP alone from the spectra obtained in the presence of different concentrations of MCL. Temperature = 20 °C.

4.2. Determination of association constants and thermodynamic parameters: A binding curve depicting progress of the titration of CuTCPP with MCL is shown in Fig. 4. Increasing the lectin concentration leads to an increase in the change in absorption intensity; however, the magnitude of the change decreases with increasing lectin concentration and thus displays saturation behavior. Inset of this Figure gives a plot of $1/\Delta A$ vs $1/[P]_t$ where ΔA is the change in absorbance at any point of the titration and $[P]_t$ is the corresponding total concentration of MCL in subunits. The Y-intercept of this plot yields the change in absorbance at infinite protein concentration, ΔA_∞. From this, the absorption intensity of the porphyrin when it is completely bound to the lectin, A_∞ can be determined. The titration data were analyzed according to Chipman method [Chipman et al., 1967], as described earlier for the binding of porphyrins to other lectins [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001]. From this analysis, the association constant, K_b, assuming a 1:1 lectin subunit to ligand stoichiometry raito, characterizing the porphyrin-MCL interaction was determined according to equation 1:

$$\log\{\Delta A/(A_c - A_\infty)\} = \log K_b + \log[P]_f \qquad (1)$$

where $[P]_f$, the free protein concentration, is given by

$$[P]_f = [P]_t - \{(\Delta A/\Delta A_\infty).[L]_t\} \qquad (2)$$

Where $[L]_t$ is the porphyrin concentration.

From equation 1 it is clear that the X-intercept of a plot of $\log\{\Delta A/(A_c - A_\infty)\}$ versus $\log[P]_f$ will yield pK_b for the lectin-porphyrin association. A representative plot of $\log\{\Delta A/(A_c - A_\infty)\}$ versus $\log[P]_f$ for the CuTCPP-MCL interaction at 20 °C is given in Fig. 5. This plot clearly shows that the data exhibit a linear dependence.

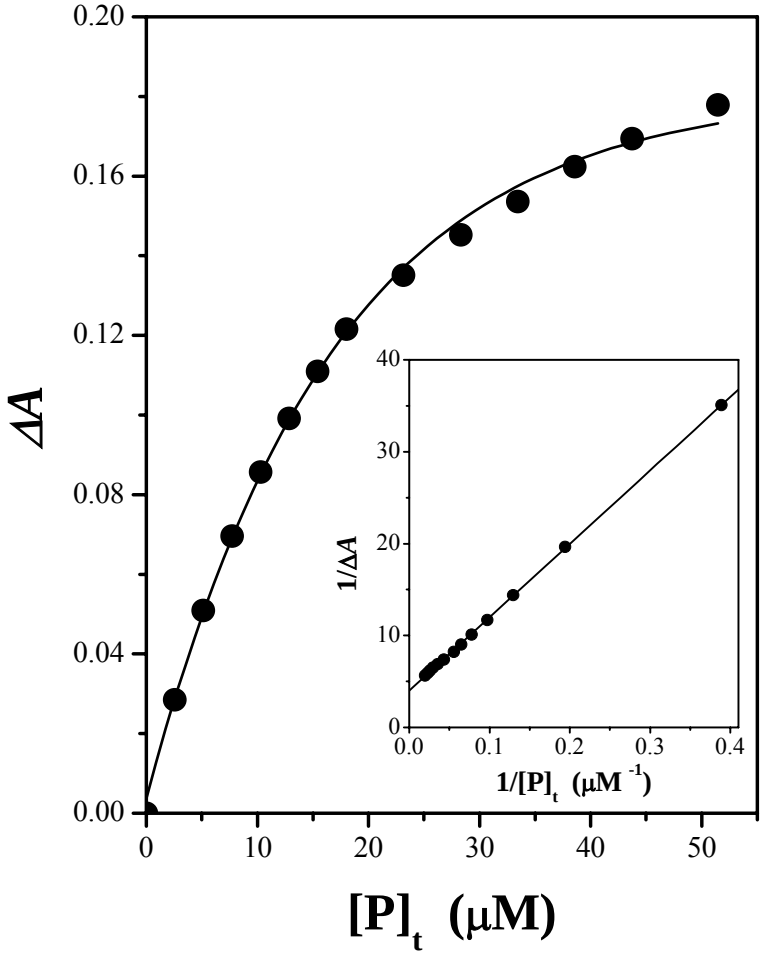


Fig. 4. Binding curve for the interaction of CuTCPP with MCL. The change in absorbance at 405 nm, resulting from the addition of MCL to the porphyrin at 20 °C is plotted as function of the total lectin concentration (in subunits). Inset gives a plot of $1/\Delta A$ as a function of the reciprocal of free protein concentration. Reciprocal of the Y-intercept of this plot gave the value of ΔA_{∞}, the change in absorbance intensity when all the porphyrin molecules are bound by the lectin.

The solid line represents a linear least squares fit of the data. From the X-intercept of this plot (Fig. 5) the K_b value for the CuTCPP-MCL interaction was determined as 5.85 $\times 10^4$ M^{-1}. Such plots for all porphyrins studied showed, within experimental error, slopes of unity supporting the assumption made above regarding the stoichiometry of binding. Following the same method, association constants of MCL interaction with this porphyrin as well as with H_2TPPS, CuTMPyP and H_2TMPyP have been determined at various temperatures. The K_b values obtained at 25 °C for all the porphyrins investigated

in this study, together with the corresponding values of ΔA_∞ and the slopes of linear double logarithmic plots, are listed in Table 1. The K_b values obtained at all temperatures studied for CuTCPP, H_2TPPS, CuTMPyP and H_2TMPyP are listed in Table 2.

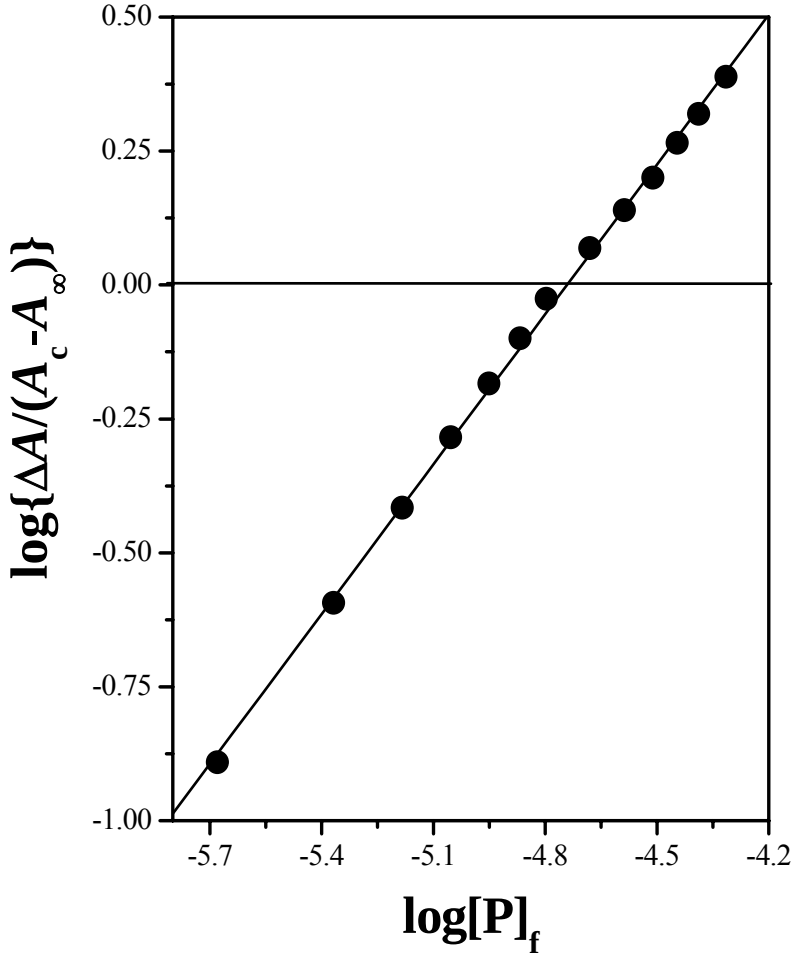


Fig. 5. Chipman plot for CuTCPP binding to MCL. The absorption titration data obtained at 20 °C for the CuTCPP-MCL interaction is analyzed according to [Chipman et al., 1967]. The X-intercept yielded the value of pK_b from which the association constant K_b was calculated.

From the association constants given in Table 1, the Gibbs free energies (ΔG^o) associated with the binding of different porphyrins to MCL were determined according to the expression:

$$\Delta G^o = -RT \ln K_b \qquad (3)$$

These ΔG^o values are also listed in Table 1.

Table 1: The maximal change in the porphyrin absorption (ΔA_∞) at infinite lectin concentration, the slopes of double logarithmic plots, the association constants (K_b), and the free energy of binding (ΔG^o) for MCL-porphyrin complexes at 25 °C. Average values from duplicate titrations are given.

Porphyrin	ΔA_∞ (%)	Slope	$K_b \times 10^{-4}$ (M^{-1})	ΔG^o ($kJ.mol^{-1}$)
CuTMPyP	20.0	1.01	6.36	-27.40
H_2TMPyP	20.0	0.99	4.49	-26.55
CuTCPP	32.2	0.97	2.97	-25.53
H_2TCPP	48.2	1.03	2.84	-25.42
ZnTPPS	65.6	1.02	1.10	-23.07
H_2TPPS	34.2	1.05	0.58	-21.48

The thermodynamic parameters, enthalpy of binding (ΔH_b) and entropy of binding (ΔS_b) associated with the interaction of CuTCPP, H_2TPPS, CuTMPyP and H_2TMPyP were obtained by means of van't Hoff plots (see Fig. 6) according to the expression:

$$\ln K_b = - \Delta H_b/RT + \Delta S_b/R \qquad (4)$$

These parameters are also given in Table 2.

4.3. CD spectroscopy, secondary structure of MCL, and effect of porphyrin binding: Circular dichroic spectra of MCL, recorded in the far UV region and near UV region are given in Fig. 7A and 7B, respectively. Spectra obtained in the presence of 25-fold molar excess of CuTCPP and CuTMPyP are also shown in this Figure. Additionally,

Table 2: Association constants, K_b, obtained at different temperatures for the interaction of CuTCPP, CuTMPyP, H_2TMPyP and H_2TPPS with MCL and the corresponding thermodynamic parameters, ΔH_b and ΔS_b, obtained from the van't Hoff plots. Values shown in parentheses correspond to titrations performed in the presence of 0.1 M lactose.

Porphyrin	T (°C)	$K_b \times 10^{-4}$ (M^{-1})	ΔH_b (kJ.mol^{-1})	ΔS_b (J.mol^{-1}.K^{-1})
CuTMPyP	20	9.08		
	25	6.36	-54.4	-90.8
	25	(6.80)		
	30	4.35		
H_2TMPyP	20	6.60		
	25	4.49	-59.5	-110.8
	30	2.67		
	35	2.10		
	35	(2.15)		
CuTCPP	10	25.32		
	15	10.26		
	20	5.85		
	25	2.97	-98.1	-243.9
	25	(3.70)		
H_2TPPS	20	0.98		
	25	0.58	-85.3	-214.7
	30	0.31		

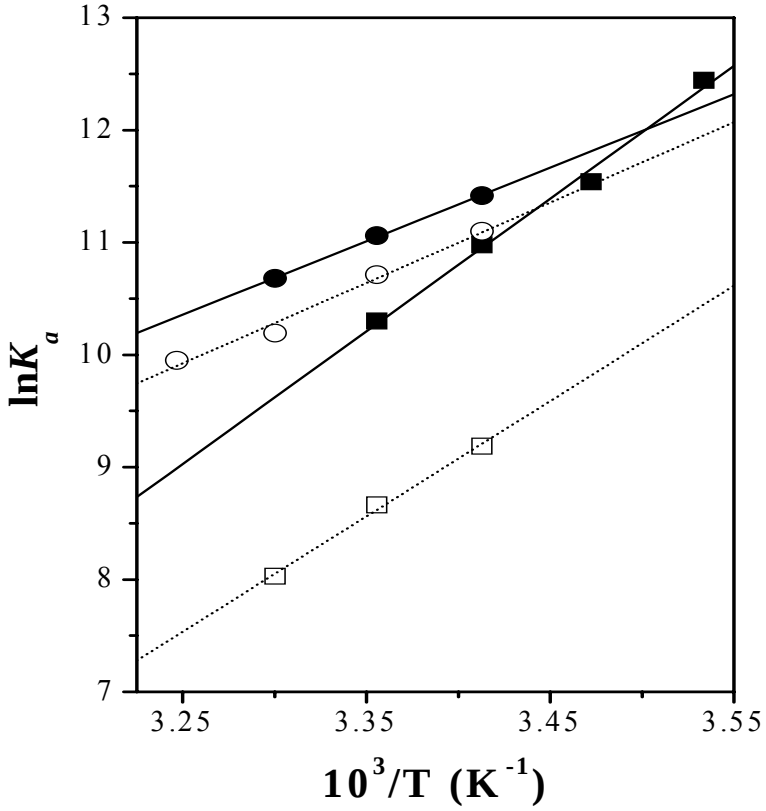


Fig. 6. Van't Hoff plots for the interaction of porphyrins with MCL. Closed squares, CuTCPP; open squares, H_2TPPS; closed circles, CuTMPyP; open circles, H_2TMPyP.

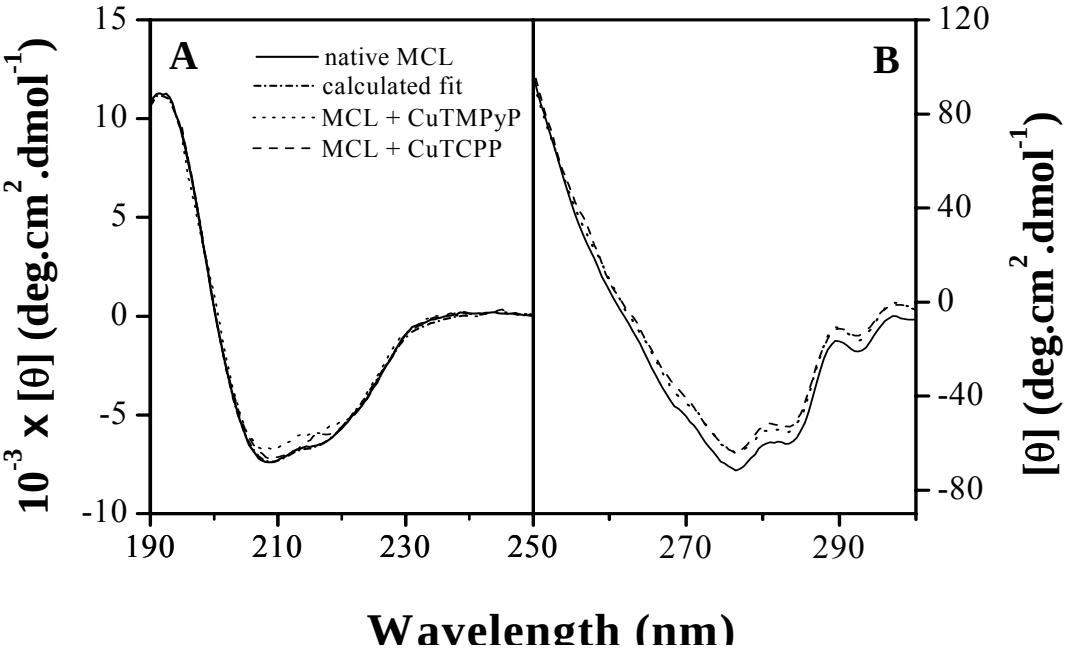


Fig. 7. Circular dichroic spectra of MCL alone and in the presence of porphyrins. The spectra were recorded at 25 °C. (A) Far UV region, (B) Near UV region. (——) native MCL (experimental), (-·-·-·-) native MCL (calculated fit), (·····) MCL + CuTMPyP, (– – –) MCL + CuTCPP. The porphyrins were present at 25-fold higher concentration over MCL (subunit concentration). See text for details.

a fit of the far-UV CD spectrum of native MCL, obtained by analysing the spectrum using the CDSSTR program is also given in Fig. 7A. The details of the spectral analysis are given below. The spectrum of MCL in the far UV region shows a minimum around 209 nm with a somewhat broad shoulder around 215-218 nm. These spectral features suggested the presence of both α-helix and β-sheet, but also indicated that the helix content is likely to be relatively low because the intensity around 222 nm (where α-helix exhibits a significant negative intensity) was low. In fact, the analysis of this spectrum shows that α-helix contributes only 13 % to the secondary structure of the lectin (see below).

The near UV spectrum has two prominent minima around 276 nm and 283 nm and a smaller minimum around 293 nm. These features can be correlated to the contributions from the side chains of tyrosine and tryptophan residues. The CD spectra obtained in the presence of CuTCPP or CuTMPyP indicate that binding of porphyrins to MCL leads to very marginal changes in the secondary and tertiary structures of MCL.

In order to obtain more quantitative information on the secondary structure of MCL and the effect of ligand binding on it, the far UV CD spectra of MCL in the native state as well as in the presence of CuTCPP and CuTMPyP were analysed by the CDSSTR program using the routines available in the website DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Compton and Johnson, 1986; Lobley and Wallace, 2001; Lobley et al., 2002]. Reference set 4 containing 43 proteins was used for fitting the experimental spectra. The results obtained from this analysis indicate that native MCL has 5% regular α-helix and 8% distorted α-helix which adds up to 13% of α-helical structures. Regular β-sheet structure was 23% and distorted β-sheet was 13%, yielding a total of 36% β-sheet. Of the remainder, β-turns account for 21% of the secondary structure of MCL,

while unordered structures comprise about 31%. Presence of either CuTCPP or CuTMPyP did not alter these values significantly.

5. Discussion

Considerable interest has been generated in recent years in the interaction of porphyrins with lectins with a view to use lectins as drug delivery agents for porphyrin-based sensitizers in PDT. Previous studies from our laboratory have shown that a variety of water-soluble porphyrins bind with considerable avidity to different plant seed lectins, viz., Con A, pea lectin, jacalin, *T. anguina* seed lectin and *T. cucumerina* seed lectin (TCSL) [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001]. The thermodynamic forces that stabilize the interaction of TCSL with a representative tetraanionic porphyrin (CuTPPS) and a representative tetracationic porphyrin (CuTMPyP) have also been delineated by variable temperature studies [Kenoth et al., 2001]. It has been found that the binding of these two porphyrins to TCSL is largely driven by favorable entropic forces and that the enthalphic contribution is considerably small. In contrast, results of the present study indicate that binding of porphyrins to MCL is enthalpically driven with the entropic contribution being negative.

The binding data presented in Table 1 indicate that association constants for the interaction of different porphyrins with MCL at 25 °C vary between 5×10^3 M^{-1} and 1×10^5 M^{-1}. Association constants for the binding of CuTCPP, CuTMPyP and H_2TMPyP determined in the presence of 0.1 M lactose are comparable to those obtained in the absence of any sugar (Table 2), clearly indicating that the porphyrin and sugar bind at different sites on the lectin surface. This is supported by hemagglutination experiments carried out in the presence of porphyrins, which indicated that the presence of CuTCPP,

H_2TMPyP or H_2TPPS at 25 mM concentration did not affect the cell agglutination activity of the lectin. Moreover, addition of 0.1 M lactose to CuTCPP-lectin complex did not reverse the changes induced by its binding to MCL in the absorption spectra of the porphyrin (not shown), further supporting the above interpretation. The range of K_b values obtained here for the interaction of different porphyrins with MCL is quite similar to that obtained for the interaction of the same porphyrins with the other Cucrbitaceae lectins, viz., SGSL and TCSL [Komath et al., 2000b; Kenoth et al., 2001] but, is somewhat higher than what was reported for the interaction of different mono- and disaccharides with MCL [Khan et al., 1981; Das et al., 1981; Chapter 4]. On the other hand, the binding of non-carbohydrate ligands which are primarily hydrophobic, such as adenine, 2,6-toludinylnaphthalene-sulphonic acid, auxins and cytokinins to a variety of plant lectins [Roberts and Goldstein, 1982, 1983a,b; Maliarik and Goldstein 1988; Gegg et al., 1992; Puri and Surolia, 1994] and that of H_2TPPS to human serum albumin and β-lactoglobulin at neutral pH [Andrade and Costa, 2002] are characterized by association constants in the range of $1 \times 10^5 – 6 \times 10^5$ M^{-1}. Interestingly, the fact that auxins and cytokinins mentioned above function as plant growth regulators [Roberts and Hooley, 1988] suggests that these molecules may act as endogenous ligands for the plant lectins.

The ability of tetracationic and tetranionic porphyrins to strongly bind lectins as reported here and also in our earlier studies thus indicates that, similar to auxins and cytokinins, porphyrins may also be considered as potential endogenous ligands for the plant lectins in their native tissues [Komath et al., 2000a,b; Kenoth et al., 2001].

The thermodynamic parameters, ΔH_b and ΔS_b, obtained from the van't Hoff analysis of the K_b values for CuTCPP, H_2TPPS, CuTMPyP and H_2TMPyP (Table 2), indicate that binding of these porphyrins to MCL is governed by enthalpic forces and that

the entropic contribution to the binding process is negative. The enthalpy and entropy of binding for the two tetracationic porphyrins, CuTMPyP and H_2TMPyP, are in the same range while the corresponding values for the tetraanionic porphyrins, CuTCPP and H_2TPPS are significantly different. This suggests that the specific interactions that mediate the binding of CuTMPyP and H_2TMPyP to the lectin are likely to be similar, while those that mediate the binding of CuTCPP and H_2TPPS to MCL could be different.

Although the values of ΔH_b associated with the binding of CuTCPP (-98.1 kJ.mol^{-1}) and H_2TPPS (-85.3 kJ.mol^{-1}) are significantly larger in magnitude than the corresponding values for CuTMPyP (-54.4 kJ.mol^{-1}) and H_2TMPyP (-59.5 kJ.mol^{-1}), they are compensated by negative contributions from the entropy of binding, resulting in weaker association constants for CuTCPP and H_2TPPS as compared to the two TMPyP derivatives.

A comparison of the thermodynamic parameters ΔH_b and ΔS_b associated with the binding of different porphyrins to MCL (Table 2) with the corresponding values obtained for the binding of CuTPPS (ΔH_b = -15.06 kJ.mol^{-1}; ΔS_b = 43.93 J.mol^{-1}.K^{-1}) and CuTMPyP (ΔH_b = -7.53 kJ.mol^{-1}; ΔS_b = 67.78 J.mol^{-1}.K^{-1}) to TCSL [Kenoth et al., 2001] reveals that the thermodynamic forces that stabilize the binding in the two cases are considerably different. While the binding of porphyrins to TCSL is associated with positive ΔS_b values, which favor the binding, interaction of porphyrins with MCL is predominantly driven by stronger enthalpic contribution and the entropic contribution is negative (Table 2). This suggests that while hydrophobic interactions such as stacking of aromatic side chains with the porphine core of the porphyrins, as observed in jacalin-H_2TPPS interaction, most likely favor the binding of porphyrins to TCSL, porphyrin association with MCL must have a

significant contribution from polar interactions such as hydrogen bonding, as observed in the Con A-H_2TPPS complex (see below).

A plot of ΔH_b versus $T\Delta S_b$ at 25 °C for the binding of CuTCPP, H_2TPPS, CuTMPyP and H_2TMPyP to MCL is shown in Fig. 8. From this plot it is seen that the data exhibit a linear dependence, clearly indicating that the binding of porphyrins to MCL is characterized by enthalpy-entropy compensation. Previously, enthalpy-entropy compensation has been observed in the interaction of carbohydrates with several lectins [cf. Toone, 1994; Schwartz et al., 1996; Komath et al., 2001]. This effect has been attributed to the crucial role played by water molecules, which are often involved in the

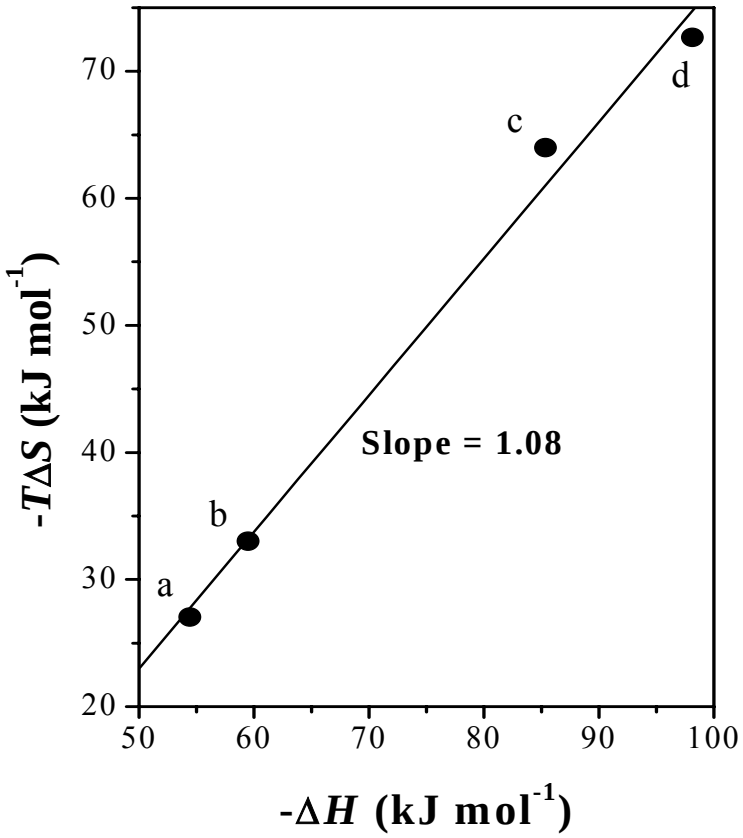


Fig. 8. Enthalpy-entropy compensation in porphyrin binding to MCL. The ΔH_b values for (a) CuTMPyP, (b) H_2TMPyP, (c) H_2TPPS and (d) CuTCPP were plotted as function of the $T\Delta S_b$ values (T = 298 K). The straight line represents a linear least squares fit of the data (slope = 1.08).

making and breaking of critical hydrogen bonds in the lectin-carbohydrate complexes [Lemieux, 1996]. It is also possible that conformational changes accompanying ligand binding lead to changes in the water structure. The thermodynamic studies presented here suggest that water molecules are likely to play a key role in the interaction of different porphyrins with MCL. Pertinently, single-crystal X-ray diffraction studies have shown that the binding of H_2TPPS to Con A is mediated exclusively by hydrogen bonds, some of which are water-mediated whereas the porphine core of the porphyrin exhibits no interaction with the protein [Goel et al., 2001]. On the other hand, the three-dimensional structure of H_2TPPS-jacalin complex shows that binding of the same porphyrin to jacalin is mediated by a combination of hydrogen bonding and nonpolar interactions, including aromatic stacking interactions between the phenyl rings of the porphyrin and Tyr78 and Tyr122 of the lectin [Goel et al., 2004].

The thermodynamic data presented here, as discussed above (i.e., favorable enthalpies and small negative entropies) with enthalpy-entropy compensation behavior suggest that water-mediated hydrogen bonds may play a significant role in the binding of porphyrins to MCL.

Analysis of the CD spectra (see Fig. 7) indicates that MCL is an α/β protein with larger β-sheet content (~36%) than α-helical content (13%). The observation that porphyrin binding does not result in significant changes in the secondary structure and tertiary structure of the protein, clearly indicates that the lectin does not undergo any detectable conformational changes upon binding of this ligand. X-ray diffraction studies indicate that binding of H_2TPPS to Con A does not lead to any detectable changes in the secondary and tertiary structures of the lectin [Goel et al., 2001], whereas considerable changes in the conformation of side chains, especially of aromatic residues such as tyrosine

have been observed when the same porphyrin binds to jacalin [Goel et al., 2004]. The CD studies presented here suggest that porphyrin binding to MCL is likely to be similar to porphyrin binding by Con A, and most likely involves very marginal or no conformational changes of the protein.

6. Conclusions

The interaction of several free-base and metalloporphyrins with the *Momordica charantia* seed lectin has been investigated in this study. Thermodynamic parameters associated with the binding of several porphyrins indicate that the MCL-porphyrin interaction is stabilized by enthalpic forces and that the entropic contribution is negative. CD spectral studies indicate that MCL is an α/β type protein with a higher fraction of β-sheet than α-helical content and that porphyrin binding does not significantly affect the secondary and tertiary structures of the protein. The significant binding strength exhibited by CuTCPP, H_2TMPyP and CuTMPyP in their interaction with MCL suggests that it may be possible to use MCL as a carrier for targeting these porphyrins to tumor tissues. Further studies with cultured cells and animal models will be necessary to investigate further the possible application of MCL in PDT.

1. Abstract

Physico-chemical and carbohydrate binding studies have been carried out on the *Momordica charantia* (bitter gourd) seed lectin (MCL). The hemagglutination activity of MCL is maximal in the pH range 7.4 – 11.0, but decreases steeply below pH 7.0. The lectin hemagglutination activity, assayed at 4 °C, is mostly unaffected by the thermal treatment in the temperature range 4–50 °C, but a sharp decrease in this activity is seen after treatment between 50 and 60 °C, which could be correlated to changes in the structure of the protein as seen by circular dichroism and fluorescence spectroscopy. Isothermal titration calorimetric studies show that the tetrameric MCL binds two sugar molecules and the binding constants (K_b), determined at 288.15 *K* for various saccharides were found to vary between 7.3×10^3 and 1.52×10^4 M^{-1}. The binding reactions for all the saccharides investigated were essentially enthalpy driven, with the binding enthalpies (ΔH_b) at 288.15 *K* being in the range of -50.99 and -43.39 $kJ.mol^{-1}$, whereas the contribution to the binding reaction from the entropy of binding was negative, with values of binding enthalpy (ΔS_b) ranging between -99.2 and -72.0 $J.mol^{-1}.K^{-1}$ at 288.15 *K*. Changes in heat capacity (ΔC_p) for the binding of disaccharides, lactose and lactulose were significantly larger in magnitude than those obtained for the monosaccharides, methyl-β-D-galactopyranoside and methyl-α-D-galactopyranoside, and could be correlated reasonably well with the surface areas of these ligands. Enthalpy–entropy compensation was observed for all the sugars studied, suggesting that water structure plays an important role in the overall binding reaction. CD spectroscopy indicates that carbohydrate binding does not lead to significant changes in the secondary and tertiary structures of MCL, suggesting that the carbohydrate binding sites on this lectin are mostly preformed.

2. Introduction

It is quite clear from Chapter 1 that lectins are ubiquitous in nature, and that they exhibit a variety of interesting properties such as the ability to distinguish between normal and malignant cells and specific recognition of different types of human blood groups. Lectins are also widely used in the purification and characterization of glycoconjugates [Peumans and Van Damme, 1998] and in the fractionation of cells for their use in bone marrow transplantation [Sharon and Lis, 2003] among many other uses.

In all the above processes involving biological recognition, binding of the lectins to carbohydrate ligands plays a crucial role. Therefore, the study of lectin-sugar interactions is very important in the characterization of any lectin. In addition to the empirical approach of hapten inhibition of the lectin activity by sugar, several quantitative physical techniques to study lectin-sugar interactions have been developed. In particular, isothermal titration calorimetry (ITC) directly yields valuable information on the thermodynamic forces that govern the binding of ligands to macromolecules and is being used currently for the study of carbohydrate-lectin interaction [Toone, 1994; Dam and Brewer, 2002].

A brief account of the work done on MCL in this laboratory and elsewhere is given in the Introduction of Chapter 2 (pp. 39-40). Although the thermodynamic parameters associated with the binding of the fluorescently labeled saccharides, 4-methylumbelliferyl-α-D-galactopyranoside and 4-methylumbelliferyl-β-D-galactopyranoside to MCL were determined by fluorescence titrations [Khan et al., 1981; Chapter 4], so far there have been no reports on the determination of thermodynamic parameters associated with the binding of underivatised sugars to MCL. In this study, MCL was further characterized with respect to its physico-chemical and carbohydrate-binding properties. The effect of pH and incubation at different temperatures on the hemagglutination activity of the lectin has been

investigated and thermodynamic parameters associated with the binding of a number of carbohydrate ligands to MCL have been elucidated by isothermal titration calorimetry. The results indicate that carbohydrate binding to MCL is governed primarily by enthalpic forces, with negative contribution from the entropy of binding. The ΔC_p values for the interaction of mono- and disaccharides were found to be negative and their magnitudes could be correlated with their size. Enthalpy-entropy compensation was observed for all the sugars investigated, emphasizing the role of water structure in the binding interaction.

3. Materials and Methods

3.1. Materials: Bitter gourd seeds were obtained from local seed shops. Guar gum, bovine serum albumin, lactose, lactulose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-thiogalactopyranoside and the reagents used in polyacrylamide gel electrophoresis were obtained from Sigma (St. Louis, MO, USA). All other reagents and chemicals used were obtained from local suppliers and were of the highest purity available.

3.2. Momordica charantia seed lectin: MCL was purified by affinity chromatography as mentioned in Chapter 2 (p 41). All purification steps were carried out in 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 0.02 % sodium azide (PBS) and the purified MCL was used in the same buffer for all further experiments unless otherwise indicated. Concentration of purified MCL was determined by using a ε_{280} value of 140,590 $M^{-1}.cm^{-1}$, calculated by the method of Edelhoch [Edelhoch, 1967] as described in [Gill and Hippel, 1989]. For this purpose, the number of tryptophan,

tyrosine and cystine residues were taken from [Mazumder at al., 1981]. It was assumed that all cysteine residues exist in the disulfide bridged form; however, because the extinction coeficient of cystine is very small compared to tryptophan and tyrosine, even if cystine contribution is ignored, the molar extinction coefficient of the protein would be affected by <0.5%, which is negligible.

3.3. Absorption spectroscopy: Absorption measurements were made on a Shimadzu UV-3101PC UV-Vis-NIR spectrometer using 1 cm pathlength cells.

3.4. Agglutination assay: Cell-agglutination activity of MCL was assayed by the hemagglutination technique as described in Chapter 2 (p 41).

3.5. pH dependence and thermal inactivation of MCL: The pH-dependence of agglutination activity was assessed by dialysing the lectin extensively against a buffer of desired pH, followed by the hemagglutination assay at 4 °C. The erythrocyte suspension used for the hemagglutination assay was also prepared in the same buffer. The following buffers were used: 30 mM acetate (pH 5.0), 30 mM phosphate (pH 6.0-7.0), 30 mM Tris/HCl (pH 8.0), 30 mM glycine/NaOH (pH 9.0-11.0). Sodium chloride (0.15 M) was included in all buffers in order to keep the ionic strength constant.

In the thermal inactivation experiments, samples of MCL in PBS were incubated at different temperatures for 60 min. The samples were then centrifuged and the clear supernatants tested for activity at 4 °C as described above. Also CD and fluorescence spectra of these MCL samples were recorded as mentioned below (*3.6* and *3.7*)

3.6. Circular dichroism spectroscopy: In order to investigate the effect of incubation at different temperatures on the structure of MCL, CD spectra in the far UV region, of native MCL as well as MCL samples incubated at different temperatures for 60 min, were recorded at 20 °C on a Jasco-J-810 spectropolarimeter. The details of measurements are similar to those described in Chapter 2 (p 43). Measurements were made in PBS and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

3.7. Fluorescence spectroscopy: Fluorescence spectra were recorded at room temperature on a Spex Fluoromax 3 fluorescence spectrometer from Jobin-Yvon. Samples of native MCL or MCL that was incubated at different temperatures for 60 minutes were placed in a 1 × 1 × 4.5 cm quartz cuvette for spectral measurement. Samples were excited at 280 nm and emission was recorded above 300 nm. Slit widths for the excitation and emission monochromators were maintained at 2 nm and 8 nm, respectively.

3.8. Modification of histidine residues of MCL: The imidazole side chains of histidine residues of MCL were modified by diethyl pyrocarbonate (DEPC) [Melchior and Fahrney, 1970]. DEPC was diluted 50-fold with absolute ethanol and the diluted reagent was used for the modification. The reagent was added to a ~1.0 mg/ml protein sample to give a final concentration of 2.5 mM. After a 2-hour incubation time at 20 °C, the reaction mixture was diluted 4-fold and the extent of modification was estimated by taking the ε_{250} value of ethoxyformyl histidine to be 1.6×10^3 $M^{-1}.cm^{-1}$ [Andersen and Ebner, 1979]. Agglutination assays with the modified lectin and native controls were done at 4 °C as described above.

3.9. Isothermal titration calorimetry: The calorimetric titrations were performed in the temperature range of 288.15 – 298.15 *K* with a VP-ITC isothermal titration calorimeter from Microcal (Northampton, MA, USA) as described in the manufacturer's instruction manual. Small aliquots (typically 5 μl) of the ligand solution (5.0 – 26.3 mM) were added from a rotating stirrer-syringe to a solution of MCL (1.445 ml, 0.225 – 0.402 mM of the dimer) in the calorimeter cell. Successive additions were separated by a 200-second interval to allow the exothermic peak resulting from the lectin-sugar interaction to return to the baseline. Usually the first injection was found to be inaccurate; therefore, a 1 or 2 μl injection was added first and the resultant point was deleted before the remaining data were analysed as described below.

For a system of one set of identical binding sites, the total heat evolved (or absorbed) during the binding process at the end of the *i*th injection, Q(*i*), is given by equation (1) [Wiseman et al., 1989]:

$$Q(i)= nP_t\Delta H_b V\{1 + X_t/nP_t + 1/nK_bP_t -[(1 + X_t/nP_t + 1/nK_bP_t)^2 - 4X_t/nP_t]^{½}\}/2 \qquad (1)$$

where n is the number of binding sites, P_t is the total protein concentration, X_t is the total ligand concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. Obviously the heat corresponding to the *i*th injection only, $\Delta Q(i)$, is equal to the difference between Q(*i*) and Q(*i-1*) and is given by the equation (2), which involves the necessary correction factor for the displaced volume (the injection volume dV_i)

$$\Delta Q(i) = Q(i) + dV_i/2V[Q(i) + Q(i\text{-}1)] - Q(i\text{-}1) \qquad (2)$$

The ITC unit measures $\Delta Q(i)$ value for every injection. These values are then fitted to equations (1) and (2) by a nonlinear least squares method using the data analysis program Origin® [MicroCal™, 2002]. The fit process involves initial guess of n, K_b and

ΔH_b which allows calculation of $\Delta Q(i)$ values as mentioned above for all injections and comparing them with the corresponding experimentally determined values. Based on this comparison the initial guess of n, K_b and ΔH_b is improved and the process is repeated till no further significant improvement in the fit can be obtained.

The thermodynamic parameters, ΔG^o_b and ΔS_b are calculated according to the basic thermodynamic equations (3) and (4):

$$\Delta G^o_b = -RT\ln K_b \qquad (3)$$

$$\Delta G^o_b = \Delta H_b - T\Delta S_b \qquad (4)$$

4. Results

4.1. pH optimum and thermal stability of MCL: In order to determine the pH dependence of the activity of *M. charantia* lectin, samples dialysed against appropriate buffers in the pH range 5.0 – 11.0 were tested for their ability to agglutinate human O (+) erythrocytes at 4 °C. Agglutination activity of MCL which is considerably low (ca. 43%) at pH 5.0, increases quite steeply to 86 % at pH 7.0 and 100 % at pH 7.4. It then remains unaltered up to pH 11.0 (Fig. 1A). The highest activity observed was taken as 100 %.

Thermal inactivation of MCL was studied by incubating the lectin at different temperatures for 60 minutes, followed by the hemagglutination assay at 4 °C for estimating the activity of the heat-treated protein. The results obtained are shown in Fig. 1B. It is seen that the lectin activity is not affected by incubation at temperatures up to about 40 °C and decreases only marginally upon incubation at temperatures up to 50 °C. Then the activity decreases quite steeply between 50 and 60 °C.

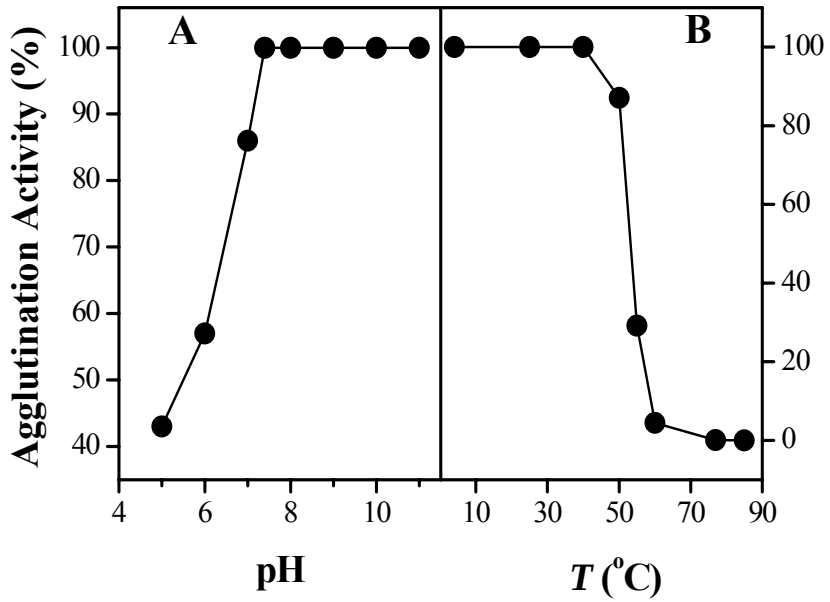


Fig. 1. Effect of (A) pH and (B) thermal inactivation on the agglutination activity of *M. charantia* lectin. To study the pH dependence, samples of MCL were first dialyzed thoroughly against buffers of desired pH. Their activity was then assessed by the hemagglutination technique at 4 °C. The percentage activity was calculated considering the maximum activity as 100%. To investigate the effect of temperature on the activity, the lectin samples taken from the same stock solution were incubated at desired temperatures for 60 minutes. They were then brought back to room temperature, centrifuged, and the clear supernatant tested for activity by the hemagglutination technique at 4 °C.

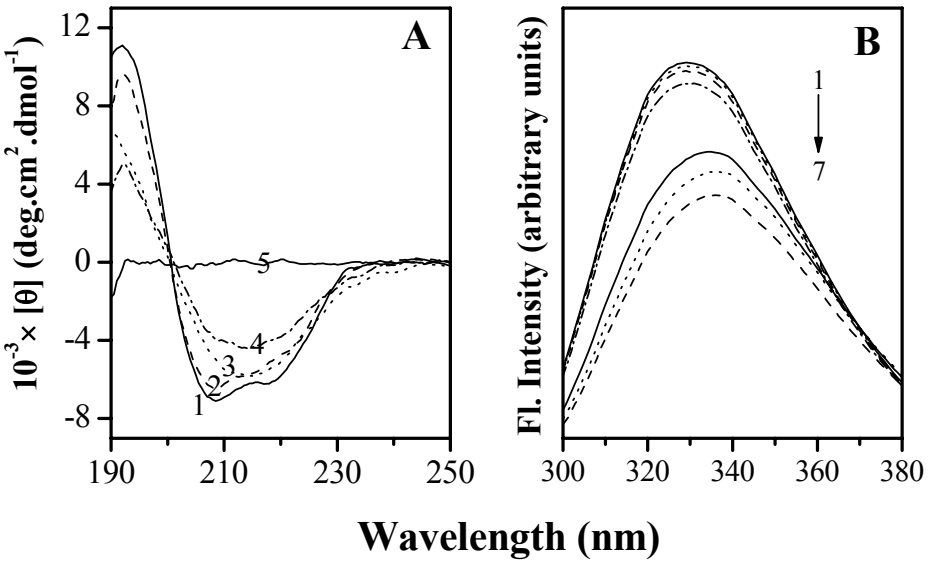


Fig. 2. Effect of thermal inactivation on the CD spectrum and fluorescence spectrum of MCL. The samples incubated at different temperatures were processed as described in the legend to Fig. 1. (**A**) CD spectra numbered 1-5 correspond to MCL samples that were incubated at 25, 50, 60, 77, and 85 °C, respectively. (**B**) Fluorescence spectra numbered 1-7 correspond to MCL samples incubated for 60 minutes at 4, 30, 40, 50, 60, 70, and 80 °C.

Incubation at temperatures above 70 °C led to a complete abrogation of the hemagglutination activity of MCL.

Far UV circular dichroism spectra and fluorescence spectra of native MCL and samples of MCL that were incubated at different temperatures are shown in Fig. 2A and Fig. 2B, respectively. The CD spectrum of MCL in the far UV region is consistent with the earlier reports and contains a minimum around 209 nm with a somewhat broad shoulder around 215-218 nm [Chapter 2]. The CD spectrum of MCL incubated at 50 °C changed only marginally as compared to the spectrum of the sample maintained at room temperature (Fig. 2A). While minor changes were seen in the band at ca. 208 nm and the shoulder at 215 nm upon incubation at 50 °C, the overall spectral shape is not significantly altered. However, marked changes are seen in the spectrum of the protein sample incubated at 60 °C. The spectral fine structure is lost and the band at 208 nm and the shoulder at 215 nm are no longer seen. Instead a less intense band centered around 214 nm is observed. The sample incubated at 77 °C also gave a similar spectrum, but with a further decrease in the intensity. Finally, the sample incubated at 85 °C does not exhibit any bands in the 200-250 nm region, indicating the loss of all secondary structure. These observations suggest a gradual loss in the secondary structure of the protein with increasing temperature in the range of ca. 60-85 °C.

The fluorescence spectra of MCL samples incubated at different temperatures exhibit somewhat parallel trends in the spectral properties (Fig. 2B). The emission intensity of the protein at 25 °C is centered around 329 nm, clearly indicating that the fluorescent tryptophan residues of MCL are in a hydrophobic environment. This suggests that they are buried in the hydrophobic core of the lectin. The emission maximum does not change in the temperature range between 4 and 40 °C (spectra 1-3), and the emission

intensity decreases very marginally with increase in temperature in this range. At 50 °C (spectrum 4), the emission maximum shifts to 331 nm and the emission intensity decreases slightly (by about 5%). These changes are indicative of minor alterations in the structure of the protein. The emission maximum shifts further to 334 nm at 60 °C (spectrum 5), with a significant and concomitant decrease in the emission intensity to about 79% of the original intensity. This trend continues with further increase in temperature; spectrum 6 shows that the emission maximum shifts to 336 nm at 70 °C with a further decrease in the emission intensity to 74.5 % of the original. At 80 °C the emission intensity decreases further to 69.1% of the intensity observed for native MCL although the emission maximum remains the same (spectrum 7).

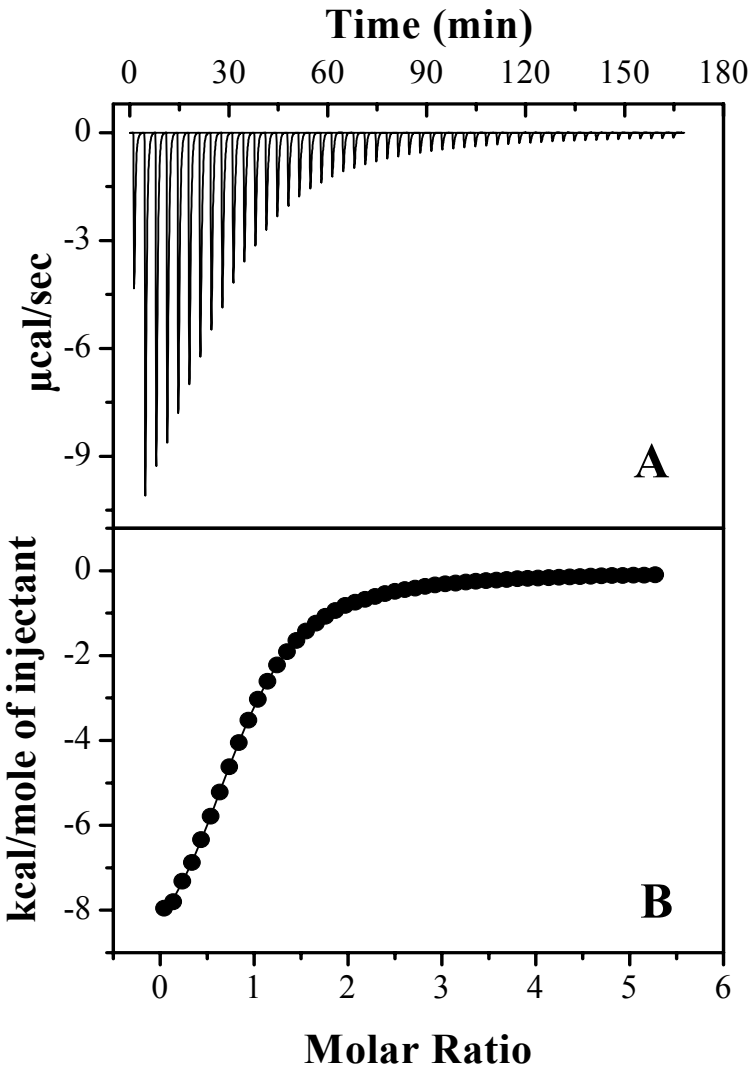
4.2. Histidine modification: Reaction of MCL with diethyl pyrocarbonate resulted in the modification of 7.2 (± 0.2) imidazole side chains of histidine residues/tetramer when the reaction was carried out for a period of 2 hours. Because amino acid analysis indicates that there are 11 histidine residues per MCL tetramer [Mazumder et al., 1981], the result of this experiment suggests that about 4 of these residues are inaccessible (buried) to the reagent in the native lectin. No loss of activity was detected for the histidine-modified MCL by hemagglutination assay, clearly indicating that the imidazole side chains of histidine residues are not involved in the carbohydrate-binding activity of the lectin.

4.3. Isothermal titration calorimetry and thermodynamics of carbohydrate binding: Results of a typical calorimetric titration experiment for the binding of lactulose to MCL at 288.15 *K* are shown in Fig. 3A. In this experiment 5 μl aliquots of a

9.74 mM solution of lactulose were added to a 1.445 ml sample of 0.343 mM MCL (dimer concentration) in the calorimeter cell at intervals of 200 seconds. From this figure it is seen that the exothermic heat of binding decreases monotonically with successive injections until saturation is achieved. A plot of the incremental heat released as a function of the lactulose/MCL dimer ratio is shown in Fig. 3B, together with a non-linear least squares fit of the data to equation 1.

MCL is an a_2b_2 type lectin and previous equilibrium dialysis and fluorescence spectroscopic studies indicated that the tetrameric lectin has two binding sites for the chromophoric and fluorescently labeled sugars, *p*-nitrophenyl-β-D-galactopyranoside (pNPβGal) and 4-methylumbelliferyl-β-D-galactopyranoside (MeUmbβGal) [Mazumder et al., 1981; Khan et al., 1981]. Therefore, the dimer concentration was used in the fits for the current titration data. The titration data could be fitted satisfactorily for a model with a single type of binding sites and the fit is shown as a solid line in Fig. 3B. This fit yielded the values of various parameters as: number of binding sites, n = 1.01 (± 0.002), binding constant, K_b = 1.3 (± 0.01) × 10^4 M^{-1}, enthalpy of binding, ΔH_b = -42.72 (± 0.12) kJ.mol^{-1}, entropy of binding, ΔS_b = -69.5 J.mol^{-1}.K^{-1}. Two independent titrations of the lectin with lactulose at 288.15 *K* yielded the average values for various parameters as: n = 1.03, K_b = 1.31 × 10^4 M^{-1}, ΔH_b = -43.39 kJ.mol^{-1}, ΔS_b = -72.0 J.mol^{-1}.K^{-1}. These values as well as the corresponding values obtained for the calorimetric titrations performed with different sugars at different temperatures are listed in Table 1. In addition, values of Gibbs free energy, ΔG^o_b, are also listed in this Table. For all ligands it has been observed that the titration data could be fit satisfactorily to a model with a single type of carbohydrate binding sites. The number of binding sites obtained from the different titrations was found to be in the range 0.88 – 1.03 per lectin dimer, validating the earlier

conclusions drawn from the fluorescence titrations and equilibrium dialysis measurements, that each dimer of the tetrameric lectin contains one carbohydrate-binding site [Mazumder et al., 1981; Khan et al., 1981].



Figu. 3. Calorimetric titration of MCL with lactulose. (**A**) Raw data obtained from 50 automatic injections of 5 µl aliquots of 9.74 mM lactulose into 0.343 mM MCL dimer at 288.15 *K*. (B) Non-linear least squares fit (——) of the incremental heat per mole of added ligand (●) for the titration in (A) against molar ratio to equations (1) and (2) in the text. The analysis yielded the following values: n = 1.01 (± 0.002), K_b = 1.3 (± 0.01) × 10^4 M^{-1}, ΔH_b = -42.72 (± 0.12) kJ.mol^{-1}, and ΔS_b = -69.5 J.mol^{-1}.K^{-1}.

In order to investigate the effect of temperature on the thermodynamic parameters associated with the binding, the ITC measurements were carried out at two or three

different temperatures for several of the saccharides. The data presented in Table 1 show that the binding enthalpies for different saccharides increase slightly with temperature. A plot of the binding enthalpy versus temperature for MeβGal and lactose, for which the

Table 1. Association constants (K_b) and thermodynamic parameters for the binding of carbohydrates to *M. charantia* lectin. Values shown are the average values from two independent titrations, unless otherwise indicated.

Sugar	T (K)	n	$10^{-3} \times K_b$ (M^{-1})	$-\Delta G^o_b$ ($kJ.mol^{-1}$)	$-\Delta H_b$ ($kJ.mol^{-1}$)	$-\Delta S_b$ ($J.mol^{-1}.K^{-1}$)
MeαGal	288.15	0.90	7.3	21.30	45.56	84.1
	293.15[a]	0.92	5.6	21.03	46.48	87.0
MeβGal	288.15[b]	0.90	10.6	22.19	50.99	100.1
	293.15	0.88	7.9	21.86	51.67	102.0
	298.15[a]	0.90	5.6	21.39	52.55	105.0
*p*NP-β-D-Gal	288.15[a]	0.94	14.7	22.98	44.64	75.3
*p*NP-β-D-thioGal	288.15[a]	1.00	15.2	23.05	44.77	75.7
Lactose	288.15	0.89	14.0	22.86	49.96	93.7
	293.15	0.95	11.7	22.82	51.51	97.9
	298.15[a]	0.94	8.4	22.38	52.93	102.5
Lactulose	288.15	1.03	13.1	22.70	43.39	72.0
	293.15[a]	1.00	10.6	22.58	45.02	77.4
Melibiose	288.15[a]	0.90	10.1	22.09	47.20	87.0

[a] Data obtained from a single titration experiment. [b] Data from three independent titrations.

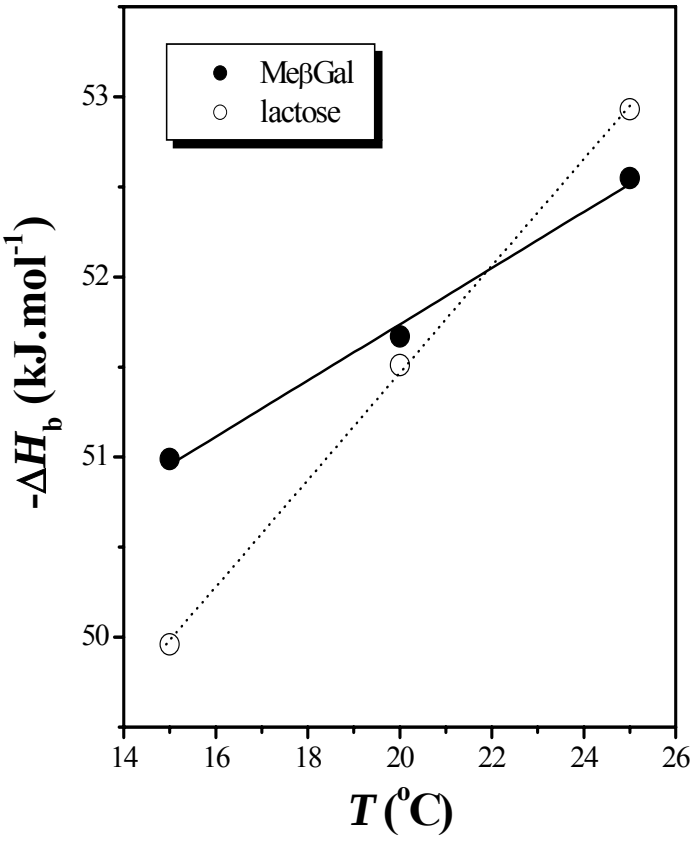


Fig. 4. Plot of -ΔH_b versus T for the binding of MeβGal (●) and lactose (○) to MCL. From the slope of the linear least squares fits, the ΔC_p values were obtained.

measurements were performed at three different temperatures, is given in Fig. 4.

It is seen that the ΔH_b values for both these sugars exhibit excellent linear dependence in the temperature range studied. Assuming ΔC_p to be temperature-independent, as clearly indicated by the linearity of ΔH_b-T plots in the studied temperature range (Fig. 4), ΔC_p values for MeβGal and lactose were estimated as -156 and -297 J.mol^{-1}.K^{-1}, respectively, from the slope of the corresponding line in Fig. 4. The ΔC_p values for MeαGal and lactulose were estimated as -184 and -326 J.mol^{-1}.K^{-1}, respectively, from the corresponding enthalpy values determined at two different temperatures (Table 1) using the experission ($\Delta C_p = \Delta\Delta H_b/\Delta T$). It is interesting to note

that the values obtained for the disaccharides, lactose and lactulose are nearly twice in magnitude as those obtained for the monosaccharides, MeβGal and MeαGal.

4.4. Effect of carbohydrate binding on the secondary structure of MCL – CD spectroscopy: The effect of carbohydrate binding on the secondary and tertiary structures of MCL was investigated by circular dichroic spectroscopy. CD spectra of MCL, recorded in the far UV region and near UV region are given in Fig. 5A and 5B, respectively.

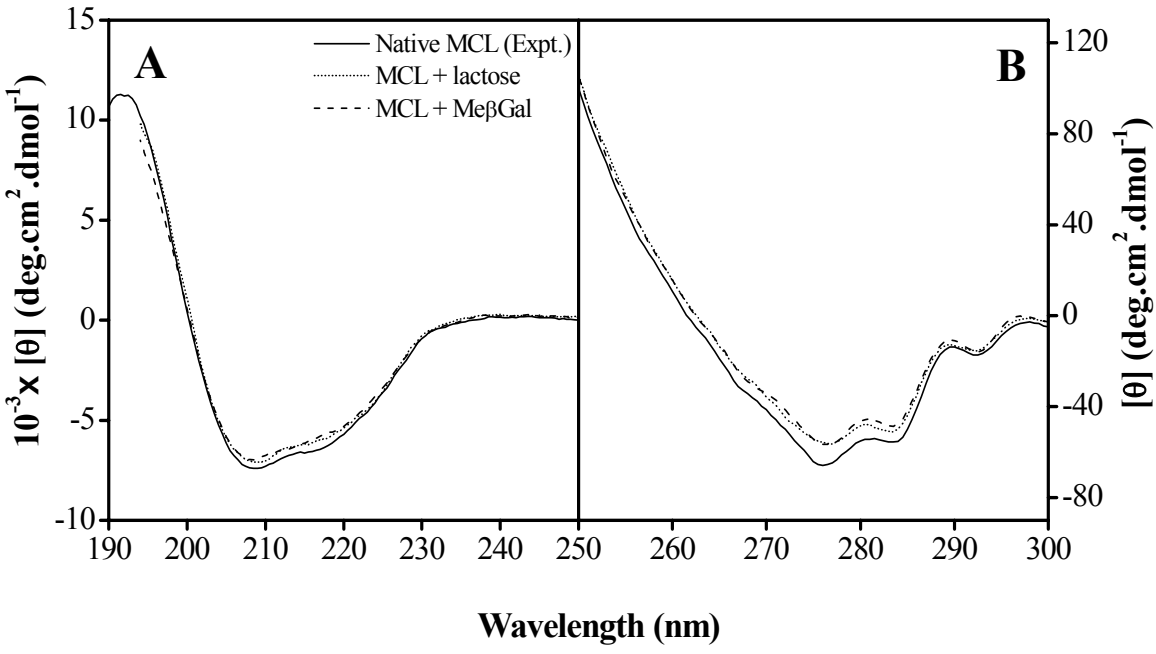


Fig. 5. Effect of carbohydrate binding on the circular dichroic spectra of *M. charantia* lectin. The spectra were recorded at 20 °C. (**A**) Far UV region. (**B**) Near UV region. (——) MCL alone, (·····) MCL + 100 mM lactose, (- - - -) MCL + 100 mM MeβGal.

Spectra recorded in the presence of 100 mM of MeβGal or 100 mM lactose are also shown in this Figure. The spectrum of MCL in the far UV region gave a minimum around 209 nm with a somewhat broad shoulder around 215-218 nm. The near UV

spectrum exhibits two major minima around 276 nm and 283 nm and a smaller minimum near 293 nm. These observations are consistent with those mentioned in Chapter 2 (p 53) [Sultan et al., 2004a].

The spectra presented in Fig. 5A show that binding of mono- and disaccharides such as MeβGal and lactose does not result in notable changes in the CD spectrum of MCL in the far UV region, clearly indicating that the secondary structure of this protein is not affected significantly by saccharide binding. Also, the changes in the near UV CD spectrum (Fig. 5B) are only minor in nature, suggesting that sugar binding does not significantly alter the orientation of the side chains of tyrosine and tryptophan residues of MCL, which are implicated in the sugar binding activity of the lectin as shown by chemical modification studies [Mazumder et al., 1981].

5. Discussion

MCL is a galactose-specific lectin that has been purified over two decades ago and characterized with respect to subunit composition, amino acid composition, molecular weight and *N*-terminal amino acid sequence [Mazumder et al., 1981; Wang and Ng, 1998]. It exhibits interesting biological properties such as RIP-like activity and insulinomimetic activity [Barbieri et al., 1979, 1980; Ng et al., 1986]. Although these activities are manifested through the carbohydrate binding property of this lectin, the thermodynamic forces that govern carbohydrate binding by this protein and the optimal pH and temperature for its activity have not yet been investigated in detail. In this study, the interaction of MCL with severl mono- and disaccharides was investigated by isothermal titration calorimetry. Further the dependence of activity and structure of MCL

on pH and thermal treatment were characterized. The results obtained are discussed below.

5.1. Thermal stability and pH-dependence of MCL: The agglutination activity of MCL is maximal in the pH range 7.4 – 11.0 and decreases rather steeply below pH 7.4. At pH 6.0 the activity is about 57%. It is pertinent here to compare the pH-activity profile of MCL with that of other lectins, especially other Cucurbitaceae seed lectins. In this respect, MCL is similar to another cucurbit seed lectin, TCSL, which requires histidine residues for its saccharide binding activity and also exhibits about 60 % activity at pH 6.0 [Kenoth et al., 2000, 2003]. SGSL, which is also from the Cucurbitaceae family and contains histidine residues in its active site [Komath et al., 1998], exhibits maximum activity in the pH range of 6.0 – 10.0, and the activity decreases steeply at lower pH [Komath et al., 2001]. For these two proteins, the steep decline in activity below pH 6.0 can be attributed to the change in protonation state of the histidine side chains that are required for the carbohydrate binding. Although chemical modification studies reported here clearly demonstrate that histidine residues are not directly involved in the sugar binding activity of MCL, it is interesting to note that the hemagglutination activity of MCL declines steeply below pH 7.4. Previous chemical modification studies have suggested that tyrosine and tryptophan residues are involved in the carbohydrate binding activity of MCL [Mazumder et al., 1981]. Because the tyrosine side chain undergoes protonation change near pH 10, this decrease in activity below pH 7.4 is unlikely to be due changes in the protonation of any of the active site amino acid residues, instead it might be due to structural changes.

The data presented in Fig. 1B clearly shows that the activity of MCL is unaffected up to 40 °C, with only a slight decrease at 50 °C. However, the activity decreases rather steeply between 50 and 60 °C. This is consistent with the results from CD spectroscopy which indicate significant irreversible alterations in the secondary structure of the protein at 60 °C and above, whereas the characteristic bands at 208 nm and 215 nm, observed in the spectrum of native MCL, are only marginally affected by heating at 50 °C. Changes in the fluorescence properties of MCL incubated at temperatures less than 50 °C are also minor in nature and suggest that the protein structure changes only marginally by heating at temperatures below 50 °C, whereas significant irreversible changes take place when the protein is incubated at 60 °C and above, as indicated by a considerable red shift in the emission maximum accompanied by a significant decrease in the emission intensity.

When compared with MCL, activity of TCSL was unaffected up to about 60 °C [Kenoth et al., 2003], clearly indicating that it is thermally more stable than MCL. However, MCL is more stable than SGSL which was rapidly inactivated at temperatures above 40 °C [Komath et al., 2001].

5.2. Isothermal titration calorimetry: Results of isothermal titration calorimetric studies presented in Table 1 clearly indicate that the tetrameric MCL has two identical binding sites for a variety of carbohydrate ligands. This is fully in agreement with the previous equilibrium dialysis results, which indicated that MCL tetramer binds to two molecules of MeUmbβGal or *p*NPβGal [Mazumder et al., 1981; Khan et al., 1981]. It is further seen that the association constant for MeβGal is approximately 1.5 times higher than that for MeαGal, clearly demonstrating that the lectin exhibits a preference for the β-anomeric derivatives of galactose. The association constants for *p*NPβGal and

*p*NPβthioGal are even higher than the value obtained for MeβGal, indicating that the aromatic aglycon possibly exhibits some favorable interaction with the lectin combining site. The association constants exhibited by the lectin for lactose (Galβ14Glc) and lactulose (Galβ14Fru) are comparable to each other, but are higher than the value obtained for MeβGal, whereas the K_b value for melibiose (Galα16Glc) is lower, suggesting that the second sugar moiety of these β-linked disaccharides may be involved in some additional interaction with the lectin combining site.

The thermodynamic data presented in Table 1 indicate that carbohydrate binding to MCL is governed primarily by enthalpic forces, with negative contribution from the entropy of binding for all the sugars investigated. In this respect the *M. charantia* lectin is similar to a number of other galactose-specific lectins such as winged bean basic lectin, *Ricinus communis* agglutinin and SGSL [Komath et al., 2001; Swaminathan et al., 1997; Sharma et al., 1998]. The enthalpically driven nature of the binding reactions suggests that the main factors that stabilize the interaction of carbohydrates with MCL are hydrogen bonding and van der Waals interactions.

Because the enthalpies of binding determined for the monosaccharides and disaccharides are in the same range, it is unlikely that the lectin has an extended combining site, which can accommodate a disaccharide or a larger structure. On the contrary, the present results suggest that the combining site of MCL accommodates only a monosaccharide (the galactose moiety) and the second sugar moiety would be involved in relatively weaker interaction with the lectin, possibly on the periphery of the sugar binding site. Further, CD spectra shown in Fig. 5 indicate that binding of both monosaccharides and disaccharides leads to comparable and marginal changes in the secondary and tertiary structures of the protein. This suggests that the carbohydrate

binding site of MCL is mostly preformed and the lectin does not require significant changes in its conformation to bind the sugar(s).

The ΔC_p values obtained for the binding of different sugars to MCL are negative. Negative values of ΔC_p have also been reported for the association of H-antigenic trisaccharide derivatives with winged bean acidic lectin (-585 to -1632 $J.mol^{-1}.K^{-1}$) and for the binding of chitobiose (-347 $J.mol^{-1}.K^{-1}$) and chitotriose (-498 $J.mol^{-1}.K^{-1}$) to lysozyme [Srinivas et al., 1999; García-Hernández et al., 2003]. In the absence of any protonation/deprotonation taking place during the binding process, the ΔC_p are generally attributed to hydration and dehydration of polar and apolar groups, which correlate well with changes in the solvent-accessible surface areas [Gómez et al., 1995].

Conformational changes resulting from the binding reaction may also be responsible for a change in the heat capacity upon binding [Baxa et al., 2001]. The ΔC_p value obtained for the binding of disaccharides, lactose (-297 $J.mol^{-1}.K^{-1}$) and lactulose (-326 $J.mol^{-1}.K^{-1}$) to MCL are comparable to the ΔC_p value observed for the binding of the disaccharide chitobiose to lysozyme [Gómez et al., 1995] and are significantly larger in magnitude than those obtained for the binding of monosaccharides, MeβGal (-156 $J.mol^{-1}.K^{-1}$) and MeαGal (-184 $J.mol^{-1}.K^{-1}$).

The surface areas of the minimum-energy conformations of the different sugars estimated using *Accelrys MS modeling 3.0.1* (http://www.accelrys.com) molecular modeling package are: MeβGal (215 Å), MeαGal (210 Å), lactose (328 Å) and lactulose (346 Å). A comparison of the ΔC_p values and molecular surface areas for the monosaccharides and disaccharides indicates that the ΔC_p values obtained for the interaction of different sugars to MCL can be correlated reasonably well to their surface areas. In view of the fact that the ITC experiments reported in this study were carried out

at pH 7.4 and because the hemagglutination activity of the protein was not affected by the modification of imidazole side chains of histidine residues – which have the highest probability to change their protonation state near this pH – it is unlikely that protonation changes would be involved in saccharide binding to MCL. Also, because the CD spectra shown in Fig. 5 indicate that the secondary structure of MCL does not change upon binding of MeβGal or lactose and the minor changes seen in the tertiary structure region are almost identical for the binding of these two ligands, it appears that the changes in heat capacity associated with the binding of different sugars are not due to conformational changes in the protein either. Therefore, it is most likely that the entire change in heat capacity associated with the binding process results from hydration changes at the protein binding site and the saccharide surfaces.

The thermodynamic parameters associated with the binding of various saccharides to MCL, determined at different temperatures (Table 1), indicate that the changes in enthalpy and entropy are compensatory. This is clearly seen in Fig. 6, where the values of $-\Delta H_b$ are plotted against the corresponding $-T\Delta S_b$ values for the various sugars and temperatures stusied. The slope of the plot is 0.97 with the correlation coefficient, R = 0.991. This shows that within the temperature range where the calorimetric measurements have been made in the present study (288.15 – 298.15 *K*), the binding of different sugars follows enthalpy-entropy compensation. Enthalpy-entropy compensation has been observed in a number of protein-ligand systems including lectin-carbohydrate interactions where the experimental conditions are the same and the ligand is altered [cf. Chapter 2; Komath et al., 2001; Swaminathan et al., 1997; Lumry and Rajender, 1970; Belleau and Lavoie, 1968; Schwarz et al., 1993; Gupta et al., 1996; Surolia et al., 1996]. One explanation that has been given for this phenomenon is that as

the ligand becomes more tightly bound to the protein, leading to larger decrease in ΔH_b, the rotational and vibrational degrees of freedom decrease, resulting in a decrease in the entropy [Leffler and Grunwald, 1963].

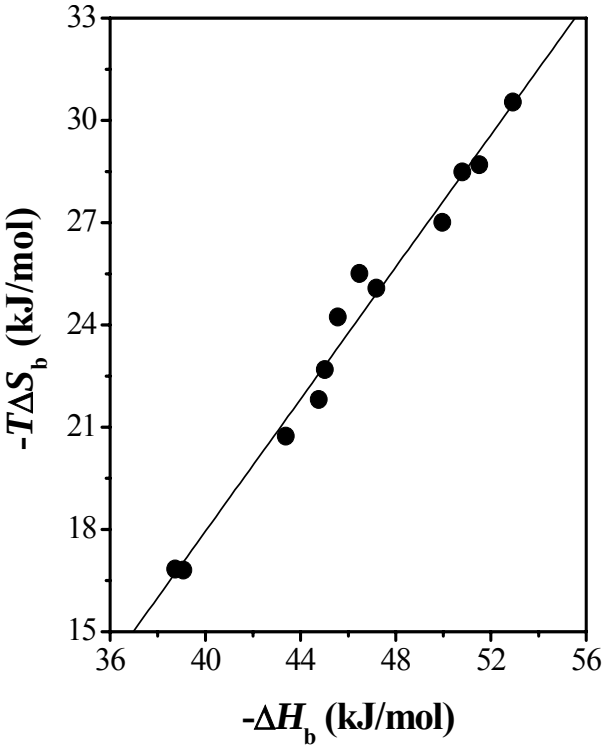


Fig. 6. Enthalpy-entropy compensation plot for MCL-saccharide interactions. The values of $-\Delta H_b$ and $-T\Delta S_b$ listed in Table 1 have been plotted. The straight line corresponds to a linear least squares fit of the data. Slope of the line is 0.965 and the correlation coefficient (R) is 0.991.

It may be expected that in such a case, ligand binding would result in changes in the protein conformation/structure, which are expected to be different for different ligands that have different ΔH_b values associated with their binding. However, from Fig. 5 it can be seen that ligand-induced changes in the CD spectrum of MCL are minor in nature and that binding of either MeβGal or lactose, results in nearly identical changes in the CD spectrum of the protein, both in the near UV region and in the far UV region. Therefore, the above model does not satisfactorily explain the enthalpy-entropy

compensation observed in MCL-carbohydrate interaction. An alternative explanation proposed for enthalpy-entropy compensation in protein-ligand interactions is related to the reorganization of water structure around the protein binding site and the carbohydrate ligand [Spohr et al., 1992; Lemieux, 1996]. Removal of water from the interacting surfaces increases the binding energy, ΔG^{o}_{b}, due to an increase in the entropy, whereas a net inclusion of water during binding should be unfavorable to the binding process due to negative entropic contribution. In the latter case, binding implies that the loss in entropy due to ordering of water molecules must be compensated by a favorable binding enthalpy [Srinivas et al., 1999]. Studies on many lectins emphasize the role of water in mediating protein-carbohydrate interactions and in a recent study, direct evidence was provided for the involvement of water in the binding of mannooligosaccharides to Con A [Swaminathan et al., 1998]. These observations suggest that the enthalpy-entropy compensation observed here in MCL-carbohydrate interaction may be explained in terms of changes in water structure associated with saccharide binding.

If the enthalpy is exactly compensated by entropy, then the ΔH_b versus $T\Delta S_b$ plot should have a slope close to one [Eads et al., 1998], whereas deviation from this value suggests different energetic mechanisms to the binding process [Srinivas et al., 1999; Munske et al., 1984; Sigurskjold and Bundle, 1992; Dam et al., 1998]. The slope of 0.97 obtained for the enthalpy-entropy compensation plot (see Fig. 6), clearly shows that in MCL-sugar interaction enthalpy of binding and entropy of binding are almost exactly compensated.

In summary, in this study the physicochemical and carbohydrate binding properties of the *Momordica charantia* lectin have been investigated by a variety of approaches. These studies reveal that MCL is optimally active in the pH range 7.4 – 11.0

and is stable up to ca. 50 °C. Circular dichroism and fluorescence spectra recorded after treating the lectin at different temperatures show that significant irreversible changes occur in the protein structure between 50 and 60 °C (and above), which are consistent with a significant unfolding of the protein in this temperature range.

Isothermal titration calorimetric studies clearly demonstrate the preferential affinity of MCL to β-linked galactopyranosides over α-linked ones. Changes in heat capacity for the binding of disaccharides were found to be nearly twice in magnitude as compared to those obtained for monosaccharides. Enthalpy-entropy compensation is observed for the binding of different saccharides to this lectin, highlighting the role of water structure in the overall binding process.

1. Abstract

Binding of 4-methylumbelliferyl-α-D-galactopyranoside (MeUmbαGal) and the corresponding β-anomer (MeUmbβGal) to the *Momordica charantia* (bitter gourd) lectin (MCL) has been investigated by fluorescence spectroscopy. Binding of MeUmbαGal to MCL resulted in a decrease in the fluorescence intensity of the ligand. Saturation binding at 25 °C resulted in a 17.8 % decrease in the fluorescence intensity of the ligand. Quenching of the ligand fluorescence intensity was temperature dependent and decreased with increase in the temperature. Addition of lactose reversed the quenching due to the binding, indicating that the decrease in the fluorescence intensity of MeUmbαGal is due to the interaction of its carbohydrate moiety with the lectin. The changes in the fluorescence intensity of MeUmbαGal, resulting from the binding were analysed to obtain the association constants for the binding process at different temperatures. At 25 °C, the binding constant, K_b, was determined to be 1.14×10^4 M^{-1} and from the temperature dependence of the K_b values the enthalpy and entropy of binding were estimated as ΔH_b = -25.9 KJ.mol^{-1} and ΔS_b = -9.1 J.mol^{-1}.K^{-1}. A comparison of these values with the ΔH_b and ΔS_b values obtained in the same way for the binding of MeUmbβGal (-36.3 KJ.mol^{-1} and -39.3 J.mol^{-1}.K^{-1}, respectively) revealed that the higher affinity of the β-anomer is due to a larger enthalpy of binding, which overrides a larger negative entropy of binding for the latter.

2. Introduction

4-Methylumbelliferylglycosides are useful florescent probes to investigate sugar-binding properties of lectins [Decastel et al., 1984]. Usually the fluorescence intensity of the methyumbelliferyl moiety is quenched upon specific binding of these sugars by lectins. Some examples are: binding of MeUmbαMan by Con A [Dean and Homer, 1973], binding of MeUmbβGal by MCL and cold agglutinin [Khan et al., 1981; Mitra and Sarkar, 1989] and binding of MeUmb(α,β)Gal by jacalin [Gupta et al., 1992]. In all these cases the binding results in complete quenching of methyumbelliferyl moiety (i.e the quantum yield of lectin-bound fluorophore is zero). Lectins may behave differently towards α and β isomers of these fluorescently labeled sugars. For example, the basic winged bean agglutinin is essentially an α-galactoside specific lectin, however, it binds MeUmbβGal even stronger than the MeUmbαGal resulting in about 70 % fluorescence enhancement of the former sugar versus about 24 % fluorescence quenching of the later sugar [Puri et al., 1993]. A similar situation was found in the interaction of PNA with these sugars [Decastel et al., 1982]. Some lectins, ricin and RCA, show no effect at all on the fluorescence intensity of methylumbelliferyl moiety, but in such cases the binding can still be studied by monitoring changes in the fluorescence polarization or anisotropy [Khan et al., 1980; Houston and Dooley, 1982].

The fluorescence quenching of 4-MeUmb aglycone upon specific binding of these glycosides to different lectins was attributed to a decrease in the polarity of the fluorophore environment [Dean and Homer, 1973] or to the interaction of the fluorophore with aromatic and sulfur containing residues in the lectin binding site [Midoux et al., 1983]. The fluorescence enhancement, on the other hand was correlated to an increase in the medium viscosity [Decastel et al., 1984].

Among the Cucurbitaceae seed lectins, SGSL and TCSL were found to significantly enhance the fluorescence intensity of MeUmbβGal but only slightly quench the corresponding α-anomer [Komath et al., 2001; Kenoth et al., 2003], whereas MCL completely quenches the former sugar [Khan et al., 1981] and, as shown in this study, partially quenches the fluorescence of the α-anomer. The binding of MeUmbβGal to MCL was investigated at different temperatures by monitoring the changes in the fluorescence intensity of the ligand upon titration with the lectin in order to determine the thermodynamic parameters associated with the binding [Khan et al., 1981], whereas the binding of MeUmbαGal, the corresponding α-anomer was not studied. In this study the interaction of MeUmbαGal (and also MeUmbβGal for the sake of comparison) with MCL was investigated at different temperatures in order to delineate the thermodynamic basis for the higher affinity of the protein for the β-linked galactose moiety.

3. Materials and Methods

3.1. Materials and MCL: 4-methylumbelliferyl-α- and β-D-galactopyranosides and lactose were obtained from Sigma (St. Louis, MO, USA). Concentrations of the umbelliferyl sugars were determined by absorption spectroscopy using their molar absorptivity, $\varepsilon_{318} = 1.36 \times 10^4$ [Decastel et al., 1984]. MCL was purified and characterized as mentioned in Chapter 2 (p 41). For Chipman analysis of binding data (p 93), MCL concentration was expressed in dimers since ITC studies have shown that each MCL tetramer binds two sugar molecules (see p 73).

3.2. Fluorescence spectroscopy: Fluorescence measurements were performed on a Hitachi F-3010 fluorescence spectrometer, equipped with a water-jacketed cuvette holder that was maintained at constant temperature by means of a circulatory water bath. Titrations were performed by the addition of small aliquots of the lectin from a concentrated stock solution (ca. 30 mg/ml) to 1.0 mL of a 5 μM solution of the fluorescent sugar. A two-minute equilibration time was given after addition of each MCL aliquot, then sugar sample was excited at 318 nm. Emission spectra were recorded in the wavelength range of 330-450 nm. Slits of 5 nm were used for both excitation and emission monochromators. All the binding data reported here correspond to the average values obtained from two different titrations.

4. Results and Discussion

Fluorescence spectra of MeUmbαGal alone and in the presence of different concentrations of MCL, recorded at 25 °C, are shown in Fig. 1. From this Figure it is evident that addition of the lectin decreases the fluorescence intensity of MeUmbαGal. Further, the changes in the fluorescence intensity resulting from protein addition could be reversed by the addition of lactose or galactose, demonstrating that the binding is mediated through the saccharide moiety of the labeled sugar. Similar results were reported for the titration of MeUmbβGal with MCL [Khan et al., 1981].

Fluorescence intensities at λ_{max} (obtained from Fig. 1) were corrected for dilution by added lectin before further analysis. A binding curve, depicting the change in the fluorescence intensity of the ligand ΔF ($\Delta F = F_o - F$, where F_o is the initial intensity before adding any protein) as a function of the added protein concentration, $[P]_t$, is shown in Fig. 2. Here, it is seen that the binding curve displays saturation

behavior, clearly indicating that the binding occurs at specific binding sites on the protein.

A plot of $1/\Delta F$ as a function of $1/[P]_t$, is given in the inset to Fig. 2. The data yielded a linear fit. Clearly, the Y-intercept of this plot is equal to $1/\Delta F_\infty$ (ΔF_∞ is the change in fluorescence intensity at infinite protein concentration), from which ΔF_∞ and F_∞ can be obtained.

It was observed that ΔF_∞ value was temperature-dependent and decreased with increase in temperature. At 15 °C, the fluorescence intensity of MeUmbαGal decreased by 25 %, whereas at 30 °C, the decrease was only 15.2 % (Table 1). Thus the quantum yield of methylumbelliferyl moiety in MCL-MeUmbαGal compex is greater than zero ($F_\infty > 0$) and increases with temperature unlike the case in MCL-MeUmbβGal complex where the fluorescence quantum yield was found to be zero ($F_\infty = 0$) [Khan et al., 1981].

The titration data were analysed according to Equation 1 in order to obtain the association constants, K_b [Chipman et al., 1967].

$$\log \{\Delta F / (F-F_\infty)\} = \log K_b + \log [P]_f \qquad (1)$$

where $[P]_f$ is the free protein concentration at any point during the titration and was obtained according to Equation (2) in which $[L]_t$ is the sugar concentration [Komath et al., 2001].

$$[P]_f = [P]_t - \{(\Delta F/\Delta F_\infty)\,[L]_t\} \qquad (2)$$

Association constants for the binding of the umbelliferyl sugar were obtained from the abscissa (as pK_b) of the plot of $\log \{\Delta F / (F-F_\infty)\}$ *vs* $\log [P]_f$. A representative plot for the titration data obtained at 25 °C is given in Fig. 3, from which the K_b value was

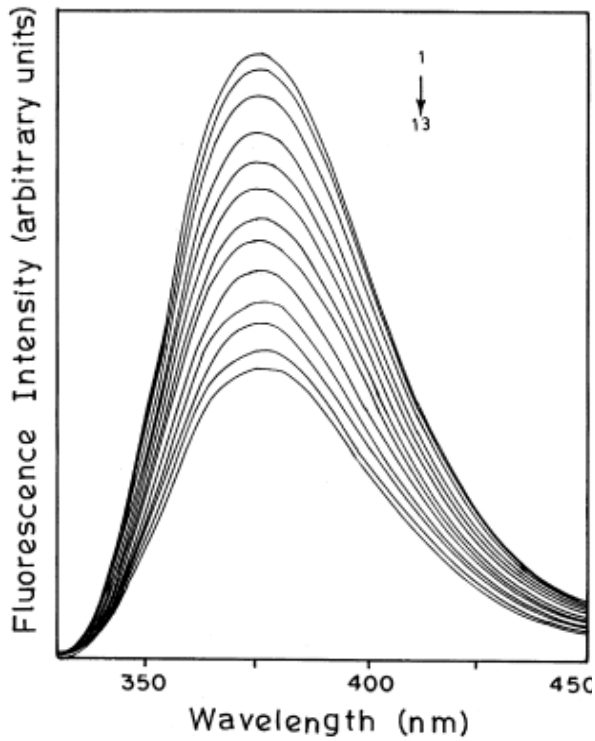


Fig. 1. Fluorescence spectra of MeUmbαGal in the absence and in the presence of *M. charantia* lectin. Spectrum 1 is that of MeUmbαGal alone and spectra 2 to 13 were recorded after the addition of increasing concentrations of MCL. T = 25 °C.

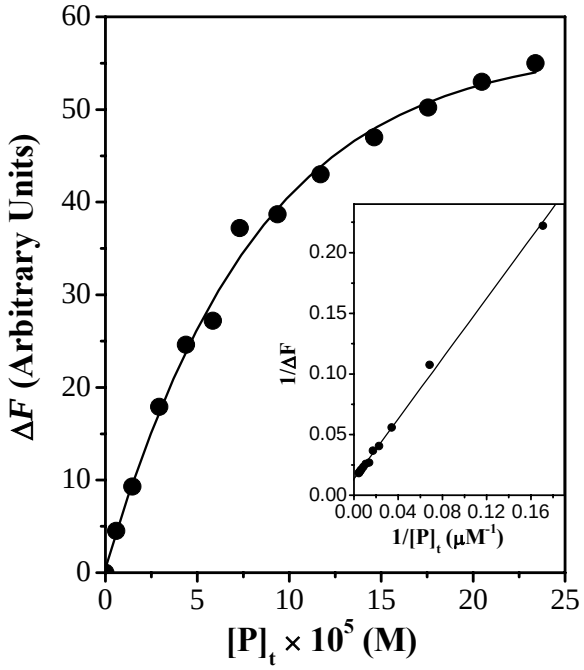


Fig. 2. Binding curve for the titration of MeUmbαGal by MCL at 25 °C. The change in fluorescence intensity at 376 nm (Δ*F*) was plotted as a function of the added protein concentration. Inset gives a plot of (1/Δ*F*) as a function of the reciprocal protein concentration. From the Y-intercept of this plot fluorescence intensity of the ligand at saturation binding was determined. See text for further details.

estimated as 1.14×10^4 M^{-1}. Association constants for the MeUmbαGal - MCL interaction thus obtained at all temperature studied are listed in Table 1.

As mentioned above, the binding of MeUmbβGal to MCL was investigated earlier by Khan et al. [1981]. However, in order to compare the data obtained for both α- and β-anomeric derivatives of the sugar under similar conditions and with the same lot of the purified protein, the binding of MeUmbβGal has also been investigated in this study. Experiments were performed essentially as described above for MeUmbαGal and it has been observed that the fluorescence intensity of the ligand is totally quenched at saturation binding ($F_\infty = 0$) at all temperatures investigated, an observation consistent with the results of Khan et al. [1981].

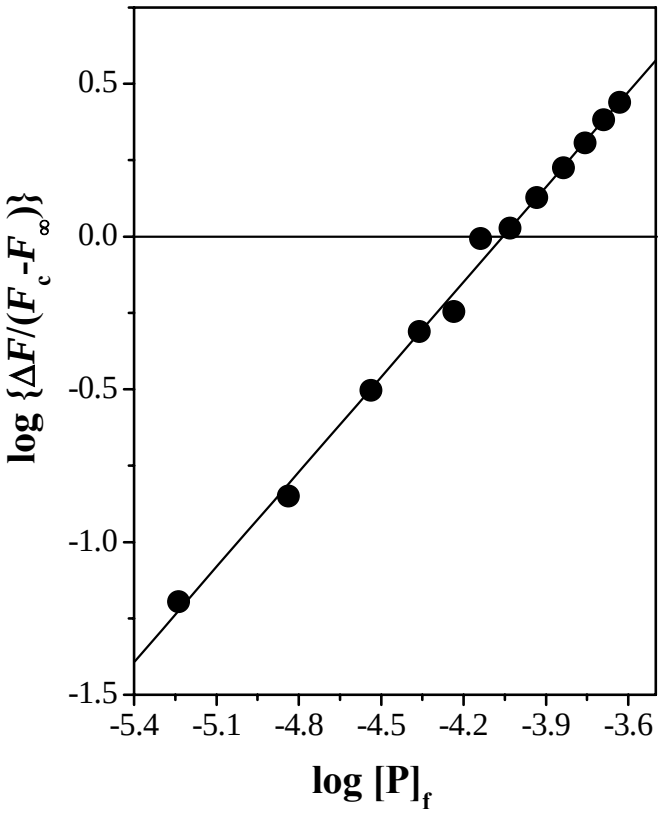


Fig. 3. Chipman plot for the binding of MeUmbαGal to MCL. The abscissa of the plot yielded pK_b, from which the association constant, K_b was determined. T = 25 °C

The titration data were analysed as described above for the MeUmbαGal-MCL interaction, in order to determine the K_b values for the binding of MeUmbβGal to MCL. At 25 °C, K_b was obtained as 2.12×10^4 M^{-1}, which is in excellent agreement with the value of 2.1×10^4 M^{-1} obtained from equilibrium dialysis measurements and also compares well with the value of 1.98×10^4 M^{-1}, obtained from fluorescence titrations by Khan et al. [1981]. The K_b values obtained at all temperatures studied are also listed in Table 1.

From the K_b values obtained at different temperatures it is clear that the interaction of MeUmbβGal with MCL is characterized by a stronger affinity than that of MeUmbαGal, the K_b values for the former sugar being 1.6 to 2.0 times higher than the latter (Table 1). These results are consistent with the earlier observations that MeβGal is 1.5 times more potent than MeαGal in its ability to inhibit the hemagglutination activity by MCL [Mazumder et al., 1981].

The temperature dependence of the association constants for MeUmbαGal and MeUmbβGal has been analysed by van't Hoff plots of ln K_b *vs* 1/T (see Fig. 4). Linear fits were obtained in each case. From the slope and intercept of these plots enthalpy of binding (ΔH_b) and entropy of binding (ΔS_b) have been determined according to Equation (3):

$$\ln K_b = - \Delta H_b/RT + \Delta S_b/R \qquad (3)$$

The van't Hoff's enthalpy and entropy of binding for MeUmbαGal-MCL interaction were obtained as ΔH_b = -25.9 kJ.mol^{-1} and ΔS_b = -9.1 J.mol^{-1}.K^{-1}, whereas the corresponding values for the binding of MeUmbβGal to MCL have been determined to be ΔH_b = -36.3 kJ.mol^{-1} and ΔS_b = -39.3 J.mol^{-1}.K^{-1} as shown in Table 1.

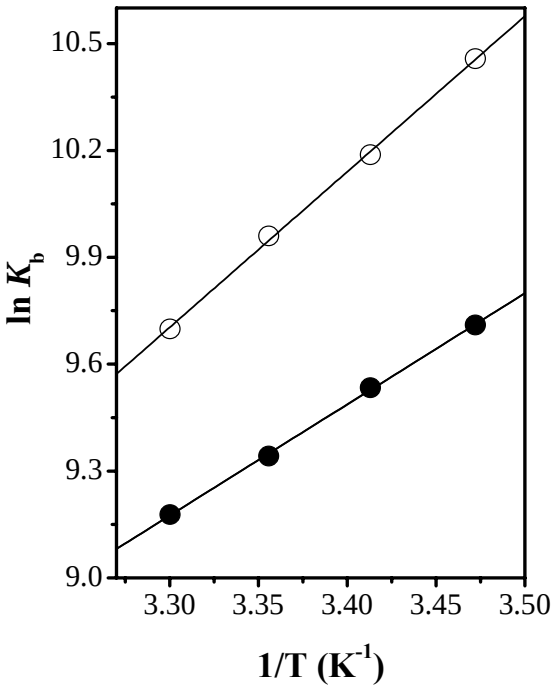


Fig. 4. Van't Hoff plot for the binding of MeUmbαGal (●) and MeUmbβGal (○) to MCL. The thermodynamic parameters, enthalpy of binding (ΔH_b) and entropy of binding (ΔS_b) were determined from the slope and intercept, respectively, of this plot according to Equation 3. See text for further details.

Table 1. Maximum changes in fluorescence intensity (ΔF_∞), association constants (K_b) and thermodynamic parameters (ΔH_b, ΔS_b) associated with the binding of MeUmbαGal and MeUmbβGal to *Momordica charantia* lectin, determined from the fluorescence spectral titrations.

T (°C)	ΔF_∞ (%)	$K_b \times 10^{-3}$ (M^{-1})	ΔH_b (kJ.mol^{-1})	ΔS_b (J.mol^{-1}.K^{-1})
MecumαGal				
15	25.0	16.9	-25.9	-9.1
20	19.2	14.1		
25	17.8	11.7		
30	15.2	9.9		
MeUmbβGal				
15	100	34.8	-36.3	-39.3
20	100	26.6		
25	100	21.2		
30	100	16.3		

These thermodynamic parameters clearly indicate that binding of the MeUmbβGal is favored by a considerably larger enthalpy contribution than that of α-anomer. In addition, binding of the α-anomer is associated with a smaller negative entropy as compared to the β-anomer. Therefore, the larger ΔH_b value associated with the binding of MeUmbβGal more than compensates for the larger negative contribution from the entropy of binding, leading to a larger K_b value for the binding of MeUmbβGal than for MeUmbαGal. It is possible that binding of MeUmbβGal is associated with additional hydrogen bonding interactions between the anomeric oxygen atom and the combining site of the lectin, which are absent in the binding of MeUmbαGal. While this leads to an increase in the binding enthalpy, the additional ordering could lead to more decrease in the entropy of the system. The thermodynamic data presented in Table 1 are consistent with this model.

1. Abstract

A new galactose-specific lectin has been purified from the extracts of *Trichosanthes dioica* seeds by affinity chromatography on cross-linked guar gum. The purified lectin (*Trichosanthes dioica* seed lectin, TDSL) moved as a single symmetrical peak on gel filtration on Superose-12 in the presence of 0.1 M lactose with a M_r of 55 kDa. In the absence of ligand (lactose) the movement was retarded, indicating a possible interaction of the lectin with the column matrix. In SDS-PAGE, in the presence of β-mercaptoethanol, two non-identical bands of M_r 24 kDa and 37 kDa were observed, whereas in the absence of β-mercaptoethanol the lectin yielded a single band corresponding to ~55,000 Daltons, indicating that the two subunits of TDSL are connected by one or more disulfide bridges. TDSL is a glycoprotein with about 4.9% covalently bound neutral sugar. Analysis of far-UV CD spectrum by three different methods (CDSSTR, CONTINLL and SELCON3) shows that the secondary structure content of TDSL is 13.3% α-helix, 36.7% β-sheet, 19.4% β-turns and 31.6% unordered structure. Among a battery of sugars investigated, TDSL was inhibited strongly by β-D-galactopyranosides, with 4-methylumbelliferyl-β-D-galactopyranoside being the best ligand. Chemical modification studies indicate that tyrosine residues are important for the carbohydrate-binding and hemagglutinating activities of the lectin. A partial protection was observed when the tyrosine modification was performed in the presence of 0.2 M lactose. The tryptophan residues of TDSL appear to be buried in the protein interior as they could not be modified under native conditions, whereas upon denaturation with 8M urea two tryptophan residues could be selectively modified by *N*-bromosuccinimide. The subunit composition and size, secondary structure content and

sugar specificity of this lectin are similar to those of type-2 ribosome inactivating proteins, suggesting that TDSL may belong to this lectin family.

2. Introduction

Lectins have been the subject of intense investigation over the last few decades owing to the variety of interesting biological properties exhibited by them making them very useful tools in biochemistry, biology and medicine (see Chapter 1).

Among the different lectins studied so far, those isolated from the legume seeds are the most widely studied as these seeds have been found to be very rich in the lectin content [Sharon and Lis, 2003]. The amino acid sequences and 3-dimensional structures of many legume lectins have been determined, resulting in a detailed understanding of the evolutionary relationships in the primary, secondary, tertiary and quaternary structure among these proteins [Sharon and Lis, 2003; Strosberg et al., 1986; Rougé et al., 1991; Rini 1995; Van Damme et al., 1998a]. Additionally these studies have resulted in the development of a variety of applications for the legume lectins [Sharon and Lis, 2003]. However, lectins from the other plant families have not been studied in such detail and there is a need to purify and characterize the molecular properties and sugar-binding specificity of lectins from other plant families also.

From the Cucurbitaceae species, the lectin from the seeds of bitter gourd (*Momordica charantia*) has been studied in considerable detail [Barbieri et al., 1979; Barbieri et al., 1980; Das et al., 1981; Mazumder et al., 1981; Khan et al., 1981; Ng et al., 1986; Padma et al., 1998; Chapters 2, 3 and 4] and *Trichosanthes kirilowii* seed lectin was also investigated in some detail [Falasca et al., 1989; Li et al., 2000, 2001].

Interestingly, they show significant similarity to type-2 ribosome inactivating proteins (RIPs) in several aspects (see Chapter 1, p 25).

During the last decade our laboratory has been investigating the lectin activities in cucurbit seeds and reported on the high-yield purification and characterization of two galactose-specific lectins from the seeds of *Trichosanthes anguina* and *Trichosanthes cucumerina* [Komath et al., 1996, 1998, 2000b and 2001; Komath and Swamy, 1998 1and 1999; Padma et al., 1999; Kenoth et al., 2000, 2001 and 2003; Kenoth and Swamy, 2003; Manoj et al., 2001]. In this study, we report the purification, macromolecular characterization and the saccharide specificity of a new galactose-specific lectin from the seeds of *Trichosanthes dioica*. In addition, the amino acid side chains that are involved in its saccharide-binding and hemagglutinating activities were studied by chemical modification. The results obtained indicate that the *Trichosanthes dioica* seed lectin (TDSL) is a heterodimer of M_r 55 kDa, with subunit masses of 24 and 37 kDa and that tyrosine residues are important for the sugar-binding activity of the lectin.

3. Material and Methods

3.1. Materials: *T. dioica* seeds were obtained from the University of Agricultural Sciences, Rajendra Nagar (Hyderabad, India). 2,4,6-Trinitrobenzenesulfonate (TNBS) was purchased from Fluka (Buchs, Switzerland) and 5,5'-dithiobis(2-nitrobenzoate) (DTNB) was a product of Serva. Diethyl pyrocarbonate (ethoxyformic anhydride), citraconic anhydride, *N*-acetylimidazole, sodium dodecyl sulfate, 2-mercaptoethanol, acrylamide, bisacrylamide, TEMED, galactose, 2-

deoxygalactose, galactosamine, *N*-acetylgalactosamine, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, D-fucose, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, arabinose, lactose, lactulose, melibiose and guar gum were obtained from Sigma (St. Louis, MO, USA). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden). *N*-Bromosuccinimide was purchased from Sisco Research Laboratories (Mumbai, India) and recrystallized prior to use. All other chemicals used were of the highest purity available.

3.2. Absorbance measurements: All absorbance measurements were performed on a Shimadzu UV3101PC UV-Vis-NIR spectrophotometer using 1-cm pathlength cells.

3.3. Purification of Trichosanthes dioica seed lectin: About 10 g of deshelled *T. dioica* seeds were homogenized in a kitchen blender and defatted with 3 × 100 ml of distilled acetone. The defatted seed meal was air-dried, mixed with 200 ml of 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.02 % sodium azide (PBS), and stirred at 4 ^{o}C for 12 h. This suspension was then filtered and the filtrate was centrifuged at 4500 rpm for 30 min in a Remi C23 centrifuge. The supernatant was subjected to ammonium sulfate precipitation (80% saturation) and the precipitate obtained was dissolved in a small volume of PBS and dialyzed extensively against the same buffer. The dialyzed sample was centrifuged at 4500 rpm for 30 minutes and the clear supernatant was collected. The supernatant obtained in the previous step was loaded on a column (2.0 × 10.0 cm) of cross-linked guar gum [Appukuttan et al., 1977]

at a flow rate of ~20 ml/hr. The breakthrough was reloaded in order to ensure complete binding of the lectin to the matrix. The column was then washed with PBS until the absorbance of the sample at 280 nm fell below 0.03. The bound lectin was then eluted with 0.2 M lactose in PBS. Ten ml fractions were collected and elution was monitored by A_{280nm}. Fractions with $A_{280nm} > 0.1$ were pooled and dialyzed thoroughly against PBS.

3.4. Hemagglutination and hemagglutination-inhibition assays: Hemagglutination assays were carried out using normal human A(+) or O(+) erythrocytes in 96-well microtiter plates. All solutions were made in PBS and all dilutions were also carried out using PBS. Seed extract or the purified protein solution (50 μl) was placed in the first well and serially diluted (2-fold dilution into successive wells). Then 50 μl of 4 % erythrocyte suspension was added and after incubating the plate for 1 h at 4 ^{o}C, the hemagglutination titer was scored visually. Hemagglutination-inhibition assays with purified TDSL were done according to the following procedure.

In the first well of microtiter plate 40 μl of the sugar solution was placed and serially 2-fold diluted. Then 10 μl of the protein solution (0.6 mg/ml) was added to each well. After incubating the plate at 4 ^{o}C for 10 minutes, 50 μl of a 4 % erythrocyte suspension was added to each well. The plate was then incubated at 4 ^{o}C for another 1 hour after which the titer was scored visually.

3.5. Gel electrophoresis: SDS-PAGE was carried out according to Laemmli [1970] on slab gels with 5 % and 10 % acrylamide in stacking and resolving gels, respectively. PAGE under non-danaturing conditions was performed in 7.5 % slab gels

using Tris-glycine buffer at basic pH (8.3) as described in [Laemmli, 1970]. The gels were stained with Coomassie Brilliant Blue R-250.

3.6. Gel filtration: Gel filtration was performed using a Pharmacia FPLC on a column of Superose-12 (30 × 1 cm). About 1 mg of the protein in 1 ml of PBS containing 0.1 M lactose was loaded onto the column pre-equilibrated with the same buffer. Chromatograms were run at a flow rate of 30 ml/hour and the elution was monitored by A_{280}. Elution volumes were determined from the retention times obtained from the chromatographic traces. For determination of TDSL M_r, the column was calibrated with bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), β-lactoglobulin (M_r 36,000) and carbonic anhydrase (M_r 30,000) as standards.

3.7. Circular dichroic spectroscopy: CD spectra were recorded at 25 °C on a Jasco-J-810 spectropolarimeter available at the Central Instrumentation Laboratory, University of Hyderabad. Spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 1.5 nm. A cylindrical quartz cell of 1 mm or 2 mm path length was used for measurements in the 200-250 nm range while the cell path length was 10 mm for measurements in the 250-300 nm range. All measurements were made at a lectin concentration of 0.16 mg/ml. Each spectrum was the average of 5 successive scans. Measurements were made in PBS and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

3.8. Modification of free amino groups: The ε-amino side chains of lysine residues and the free *N*-terminal amino groups of TDSL were modified by citraconic anhydride [Dixon and Perham, 1968] essentially as described previously [Komath et

al., 1998; Kenoth et al., 2000]. The extent of modification was estimated using 2,4,6-trinitrobenzenesulfonate according to Habeeb [1966], using bovine serum albumin and ovalbumin as standards and the average values obtained were reported.

3.9. Modification of tyrosine residues: The phenoxy side chains of tyrosine residues were modified using *N*-acetylimidazole [Riordan, 1965]. A 2.5 mg/ml concentration lectin sample in PBS was incubated with a 60-fold molar excess of the reagent for 1 hour at room temperature, under constant stirring. The reaction was also carried out in the presence of 0.2 M lactose in order to check whether the sugar could protect the tyrosine residues from modification. In each case, the modified protein was freed from the excess reagent by gel filtration on a column of Sephadex G-50 (1.2 × 40 cm) and the peak fraction was tested for activity (after removing lactose, if any, by dialysis against PBS). In some experiments the excess reagent was removed by dialysis against PBS. The extent of modification was estimated as described in [Riordan, 1965].

Tyrosine modification was reversed by treatment with 0.5 M hydroxylamine for 2 hours and then the protein was freed of the reagents by dialysis against PBS. The samples were then tested for activity by the hemagglutination assay.

3.10. Modification of cysteine residues: The thiol side chains of cysteine residues of TDSL were modified with Ellman's reagent (DTNB) under denaturing conditions [Glazer, 1976]. Modification of the native protein was done according to Janatova et al. [1968]. Modification with iodoacetamide was done after reducing the disulfide bonds with β-mercaptoethanol. Similar to the snake gourd seed lectin [Komath et al., 1998] and *T. cucumerina* seed lectin [Kenoth et al., 2000], the disulfide

bond(s) in TDSL also could not be reduced under native conditions. Therefore, the lectin was denatured with 8 M urea and the disulfide bonds reduced with β-ME. The free thiol groups were then modified with iodoacetamide by incubating the denatured and reduced lectin (0.18 mg/ml in 8M urea) with 50 mM iodoacetamide for 60 min at room temperature. The modified sample was dialyzed extensively against PBS and the lectin activity was examined by the hemagglutination assay.

3.11. Modification of tryptophan residues: Tryptophan modification was done in citrate buffer, pH 4.0, with *N*-bromosuccinimide [Spande and Witkop, 1967]. The modification reaction was done in a spectrophotometer cuvette on a ~1.0 mg/ml concentration protein sample. Small aliquots of the reagent from a 10 mM stock solution in water were added from a freshly prepared aqueous solution to the sample cuvette as well as the reference cell. After each addition a two-minute time interval was given and the absorption spectrum was recorded between 250-310 nm. Modification was monitored by following changes in absorption at 278 nm. Addition of the reagent was stopped when further addition led to deviation from isosbestic point at 265 nm. Since it was observed that the tryptophan residues of TDSL could not be modified selectively under native condition, modification was done on the protein denatured by 8 M urea in the same buffer in order to estimate the total number of tryptophan residues present in the protein.

3.12. Histidine modification: The imidazole side chains of histidine residues of TDSL were modified at room temperature by diethyl pyrocarbonate, DEPC [Melchior and Fahrney, 1970]. Ten microliters of DEPC was diluted 20-fold with absolute ethanol and the diluted reagent was used for the modification reaction. 5-15 μl aliquots of the

reagent were added to a 2.0 mg/ml protein sample such that the final concentration of the reagent did not exceed 3 mM and the reaction was allowed to proceed for 2 hours.

Aliquots of the reaction mixture were withdrawn at different time intervals and mixed with an equal volume of 10 mM histidine to quench the reaction at those time intervals. After removing the excess reagent by dialysis or gel filtration, the extent of modification was estimated by taking the ε_{250} value of ethoxyformyl histidine to be 1.6 $\times 10^3$ $M^{-1}.cm^{-1}$. The modification was reversed by treating the modified lectin with 50 mM hydroxylamine at room temperature for 10 minutes, or by incubating the modified samples at 37 °C for 2 hours [Anderson and Ebner, 1979].

3.13. Estimation of protein and carbohydrate: Protein was estimated by the method of Lowry et al. [1951] using bovine serum albumin as standard or by monitoring absorbance at 280 nm. Neutral sugar was estimated by the phenol-sulfuric acid assay [Dubois et al., 1956], using galactose as the standard.

4. Results and Discussion

Hemagglutination assays indicated that aqueous extracts of *Trichosanthes dioica* seeds contain a lectin activity, which could be inhibited by galactose and its derivatives. Based on this observation, we designed a purification procedure employing affinity chromatography on cross-linked guar gum as the key purification step. The affinity-purified lectin has been characterized with respect to macromolecular and carbohydrate binding properties. Finally, chemical modification studies were performed in order to identify the amino acid side chains important for the activity of the lectin. The results of these studies are discussed here.

4.1. Purification, molecular weight and subunit composition of T. dioica seed lectin: The *Trichosanthes dioica* seed lectin (TDSL) was purified by a combination of ammonium sulfate precipitation and affinity chromatography on cross-linked guar gum. The lectin eluted as a single peak from the affinity column when 0.2 M lactose in PBS was used as eluent (Fig. 1). About 20 mg of lectin was obtained from 10 grams of th deshelled seeds and the overall yield of lectin activity was about 30 %.

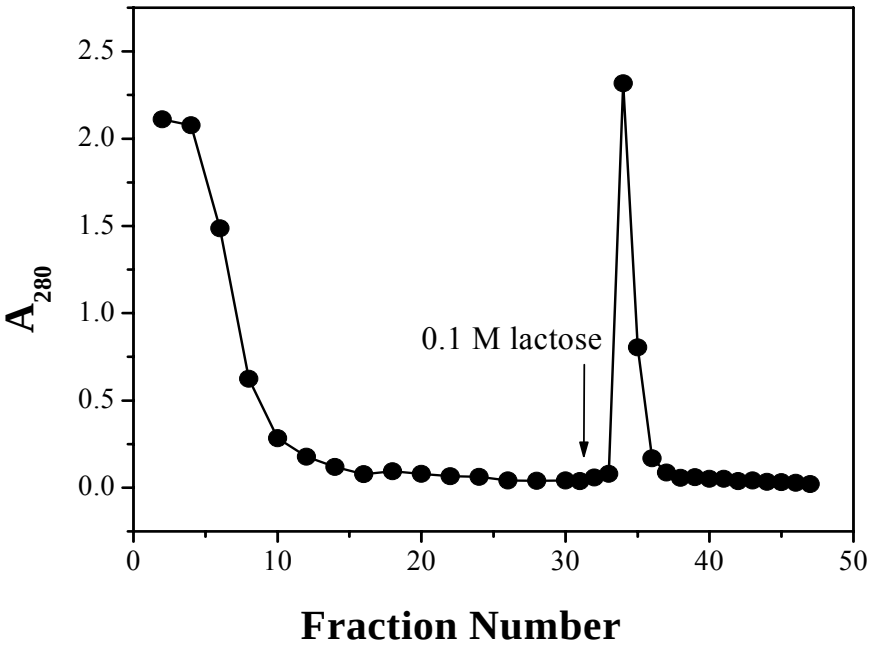


Fig. 1. Purification of *T. dioica* seed lectin by affinity chromatography. The absorbance of the column effluent at 280 nm was plotted as a function of the fraction number. Fraction size was 10 ml.

The *T. dioica* lectin gave a single band in polyacrylamide gel electrophoresis under native conditions (Fig. 2A), indicating that the affinity chromatographic purification yielded a homogenous lectin sample. In SDS- PAGE, in the absence of any reducing agent the lectin moved as a single band (Fig. 2B) whereas in the presence of β-mercaptoethanol it yielded two bands (Fig. 2C). This suggests that the two subunits of

TDSL are covalently connected by one or more disulfide bonds. By comparing the mobility of the two bands of TDSL with the mobility of standard proteins (Fig. 2C), the molecular weights of the two subunits have been estimated to be 37 kDa and 24 kDa, respectively (Fig. 2D). The galactose-specific seed lectins from *T. kirilowii, T. anguina* (snake gourd) and *T. cucumerina* also are made up of two non-identical subunits of similar size that are covalently linked by disulfide bonds [Falasca al., 1989; Komath et al., 1996; Padma et al., 1999]. Thus, covalent association of the two subunits appears to be a common feature among the heterodimeric lectins from cucurbit seeds. It is interesting to note here that type 2 RIPs are also made up of two non-identical subunits (ca. 30-35 kDa and 20-25 kDa) that are covalently connected by disulfide bond(s). Additionally, they are also galactose-specfic proteins and their secondary structure is made up of a combination of α-helix, β-sheet and unordered structures [Van Damme et al., 1998a]. Thus TDSL appears to be similar to type 2 RIPs in its subunit size and composition, subunit association by disulfide bond(s), sugar specificity and secondary structure (see below), suggesting that it may belong to the same structural class. Some of the other cucurbit seed lectins such as TKL-1, SGSL and TCSL may also belong to the type-2 RIP-like protein class. Indeed, single crystal X-ray crystallographic studies indicate that two other cucurbit seed lectins, namely TKL-1 and SGSL, exhibit structural homology to type-2 RIPs [Li et al., 2000, 2001; Manoj et al., 2001]. TCSL, another cucurbit seed lectin, was shown to exhibit immunological cross-reactivity to SGSL [Padmaet al., 1999]. All these observations, taken together, suggest that the galactose-specific, hetero-dimeric cucurbit seed lectins in which the two subunits are linked by disulfide bond(s) may be structurally similar to the type-2 RIPs.

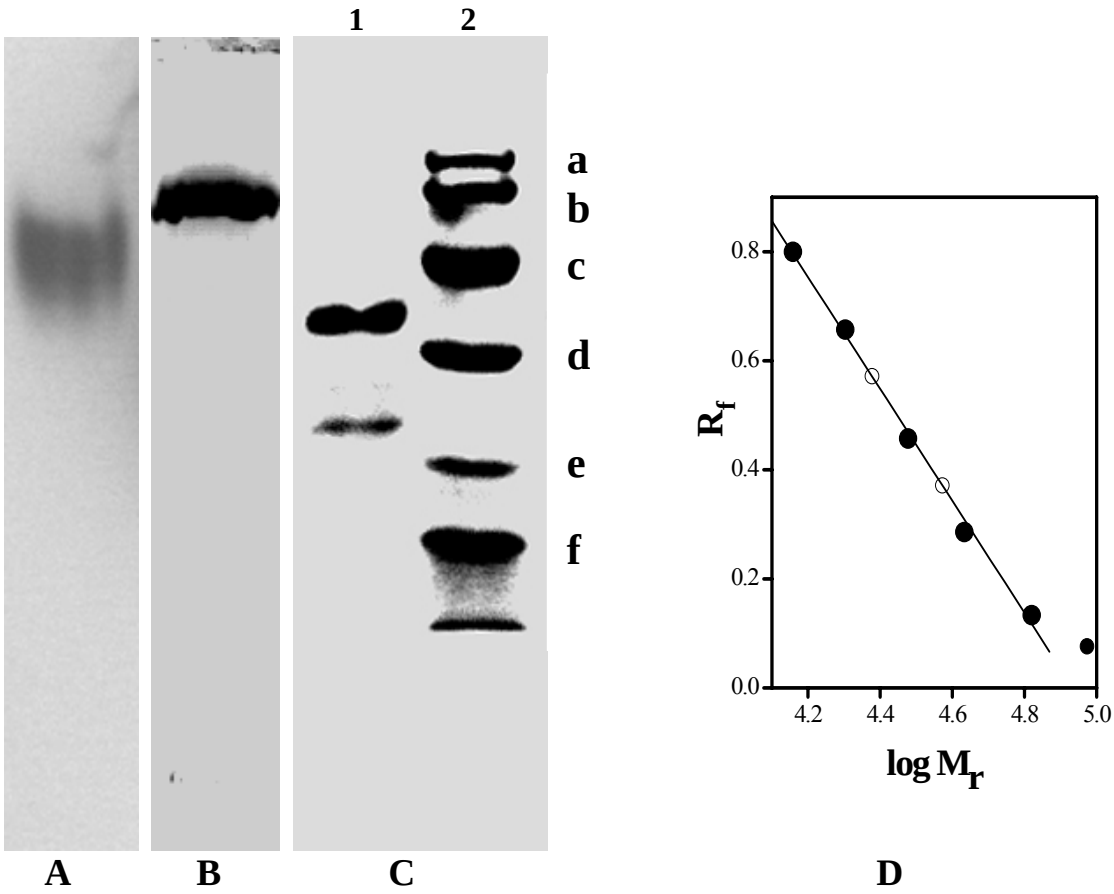


Fig. 2. Polyacrylamide gel electrophoresis of *T. dioica* seed lectin. (A) native PAGE, (B) SDS-PAGE in the absence of β-mercaptoethanol, (C) SDS-PAGE in the presence of β-mercaptoethanol. Lane 1 is for TDSL and lane 2 is for molecular weight markers: a. phosphorylase b (94 kDa), b. bovine serum albumin (66 kDa), c. ovalbumin (45 kDa), d. carbonic anhydrase (30 kDa), e. soybean trypsin inhibitor (20.1 kDa) and f. α-lactalbumin (14.4 kDa). The bottom band in lane 2 of C is due to the tracking dye. (D) Estimation of the molecular weights of TDSL subunits. The relative mobilities (R_f) of different proteins used as markers in SDS-PAGE (shown in C) are plotted as a function of log M_r (closed circles). The data points for the two subunits of TDSL are shown as open circles. The point corresponding to phosphorylase b was not used in the linear fit. From this plot the masses of the two subunits of TDSL have been estimated to be 37 kDa and 24 kDa.

In gel filtration experiments on Superose-12, the native *T. dioica* seed lectin yielded a very broad peak in the absence of any ligand, suggesting that the lectin possibly interacts with the column matrix. In order to verify this the gel filtration experiments were carried out in the presence of 0.1 M lactose, where TDSL eluted as a single, symmetrical peak of a significantly reduced width (Fig. 3). By comparing with

elution profiles of standard proteins its molecular weight was estimated to be 55,000 (Fig. 3, inset).

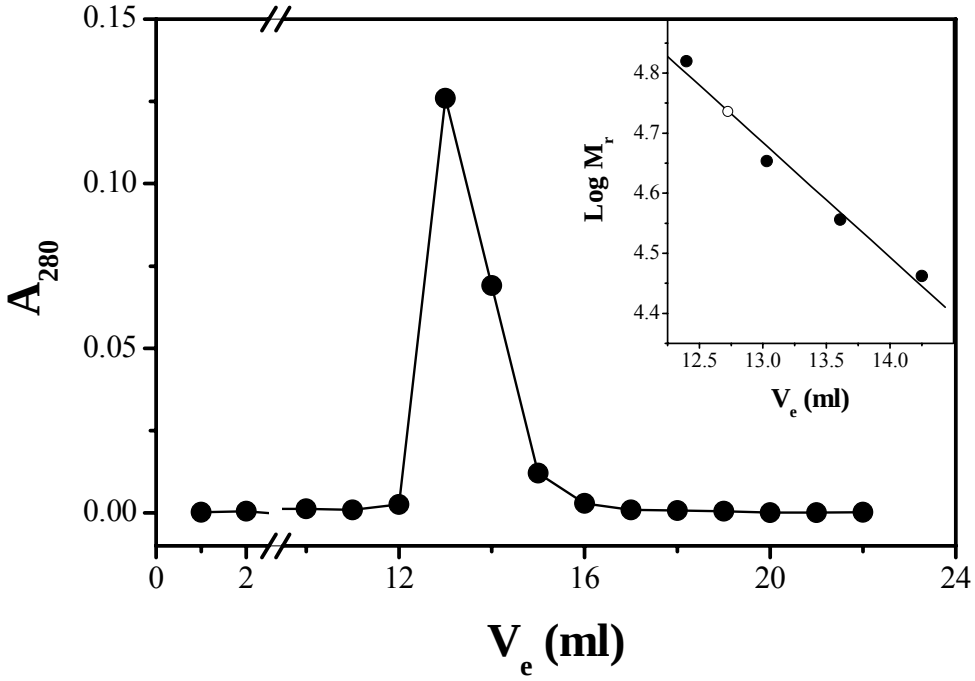


Fig. 3. Gel filtration of TDSL on Superose-12. The absorbance at 280 nm is plotted as a function of elution volume, V_e. *Inset* shows a plot of log M_r versus elution volume (V_e), from which the molecular weight of native TDSL was estimated. Closed circles correspond to standard proteins: bovine serum albumin (66 kDa), ovalbumin (45 kDa), β-lactoglobulin (36 kDa) and carbonic anhydrase (30 kDa). Open circle corresponds to TDSL.

4.2. Glycoprotein nature of TDSL: Carbohydrate estimation by the phenol-sulfuric acid assay has shown that TDSL contains about 4.9 % neutral sugar, clearly indicating that it is a glycoprotein. In this respect TDSL is similar to the other seed lectins from the the Cucurbitaceae such as MCL, SGSL and TCSL which are all glycoproteins with neutral sugar contents ranging between 3.0 and 10 % [Mazumder et al., 1981; Komath et al., 1996; Padma et al., 1999].

4.3. Carbohydrate specificity

4.3.1. Binding of monosaccharides: The carbohydrate binding specificity of the *T. dioica* seed lectin was probed by the hemagglutination-inhibition method. Whereas the lectin activity was inhibited by galactose and its derivatives such as *N*-acetylgalactosamine, MeαGal, MeβGal etc., glucose, mannose and their methyl glycosides did not inhibit the lectin up to 100 mM concentration. Minimum concentrations of different saccharides required for complete inhibition and their relative inhibitory potencies, calculated with respect to galactose as the reference, are given in Table 1.

From this data it can be seen that 2-deoxygalactose is about 8-fold weaker compared to galactose, clearly indicating that the equatorial hydroxy group on C-2 of galactose is an important locus for carbohydrate binding to the lectin. The inhibitory potency of MeβGal is 4 times better when compared to the corresponding α-anomer, indicating that equatorial configuration at the anomeric position is better recognized. Galactosamine is as weak an inhibitor as 2-deoxygalactose, indicating that the amino group can not contribute to the binding interaction with the lectin. However, Gal*N*Ac, which is the *N*-acetyl derivative of galactosamine is a better inhibitor of the agglutination activity of TDSL than the parent sugar, galactose. These results suggest that TDSL is a Gal/Gal*N*Ac specific lectin, with a somewhat stronger affinity for Gal*N*Ac than Gal. In contrast, other cucurbit seed lectins such as SGSL, TCSL and MCL recognize Gal better than Gal*N*Ac [Mazumder et al., 1981; Komath et al., 1996; Padma et al., 1999]. In this respect the *T. dioica* lectin appears to be similar to the lectins from *Bauhinia purpurea alba* and *Maclura pomifera,* which show a 3-5 fold higher affinity towards GalNAc over Gal [Wu et al., 1980; Sarkar et al., 1980]. D-

Fucose is only half as potent as galactose, suggesting that the C-6 hydroxy group also makes a positive contribution to the binding of the sugar in the lectin combining site. Arabinose binds with a two-fold higher affinity as compared to D-fucose, indicating that the C-6 methyl group of D-fucose probably encounters some unfavorable interaction with the combining site of the lectin. A hydrophobic moiety attached at the anomeric position seems to make a favorable contribution as both the α- and β- *p*-nitrophenyl derivatives of galactose bind to the lectin with an affinity that is twice that of the corresponding methyl glycosides. This suggests the presence of a hydrophobic pocket near the sugar binding site, which can interact favorably with the hydrophobic moiety connected at the anomeric position. Similar enhancement in the binding affinity of saccharides bearing hydrophobic aglycons has been reported for several other lectins such as soybean agglutinin, jacalin, and winged bean basic lectin, suggesting the presence of hydrophobic pockets near their saccharide binding sites [Swamy et al., 1986; Gupta et al., 1992; Puri et al., 1993]. In contrast, binding affinity of peanut lectin to 4-methylumbelliferyl glycosides is comparable to its affinity towards the corresponding underivatized sugar, clearly indicating that the non-polar aglycon does not interact with the lectin [Loontiens, 1983].

Non-binding of glucose, mannose and their methyl glycosides shows that an equatorial hydroxyl at C-2 and an axial hydroxyl group at C-4 are not only important binding loci for the lectin, but that reversal of configuration at these positions might lead to unfavorable interaction of the ligand with the lectin combining site, resulting in their non-recognition by the lectin.

Table 1. Inhibition of the agglutination activity of *T. dioica* seed lectin by various saccharides. Protein concentration was 60 μg/ml (after all reagents were mixed).

Sugar[a]	Minimum concentration for inhibition[b] (mM)	Relative inhibitory potency (galactose = 1.0)
Gal	3.13	1.00
2-deoxyGal	25.00	0.13
$GalNH_2.HCl$	25.00	0.13
Gal*N*Ac	1.56	2.00
MeαGal	6.25	0.50
MeβGal	1.56	2.00
Fucose	12.50	0.25
MeUmbβGal	0.50	6.20
*p*NPαGal	3.70	0.84
*p*NPβGal	0.80	3.88
Arabinose	6.25	0.50
Lactose	1.56	2.00
Lactulose	1.56	2.00
Melibiose	12.50	0.25

[a]All sugars are of D configuration.
[b]The following sugars did not inhibit the hemagglutination activity of TDSL at upto 100 mM resultant sugar concentration: Glucose, mannose, MeαGlc, MeβGlc, MeαMan, cellobiose, maltose.

4.3.2. Binding of disaccharides: Among the disaccharides investigated, both lactose (Galβ14Glc) and lactulose (Galβ14Fru) are twice as potent as galactose in inhibiting the agglutination activity of the *T. dioica* lectin, and are comparable to MeβGal, whereas melibiose (Galα16Glc) is 4-fold weaker as compared to galactose.

These results are consistent with the preference of TDSL for the β-anomers of Gal. Additionally, these galactose-containing disaccharides are broadly comparable to monosaccharides in their inhibitory potency, suggesting that the lectin combining site most likely accommodates only a monosaccharide unit. These results also indicate that the lectin does not exhibit significant additional interactions with the second sugar of the above disaccharides.

Finally, the inability of cellobiose and maltose to inhibit the hemagglutination activity of the lectin indicates that TDSL does not recognize dimers of glucose also, and that the observed inhibition by the other disacchrides lactose, lactulose and melibiose is essentially due to their galactose moiety.

4.4. Circular dichroism studies and secondary structure of TDSL: Circular dichroic spectra of TDSL in the native state and in the presence of 0.1 M lactose are shown in Fig. 4. The far UV CD spectrum (solid line, Fig. 4A) of the native protein is characterized by two minima around 208 nm and 222 nm, suggesting the presence of α-helical conformation. However, the spectral band at 208 nm is more intense than that at 222 nm, indicating that the CD spectrum contains a significant contribution from other structures as well. In order to derive quantitative information on the content of different secondary structural elements in this protein, the CD spectrum was analysed by three different methods, namely CDSSTR [Compton and Johnson, 1986; Sreerama and Woody, 2000], CONTINLL [Provencher and Glockner, 1981; Stokkum et al., 1990] and SELCON3 [Sreerema and Woody, 1993; Sreerema et al, 1999] employing the software routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Lobley and Wallace, 2001; Lobley et al., 2002]. A basis set containing 43 proteins

was used as reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 2. Among the three methods, the best fit was obtained with CDSSTR and the resulting fitted spectrum using this method is shown in Fig. 4A (dotted line) and it is seen that the fit is in good agreement with the experimental spectrum (solid line). The content of various secondary structures obtained for native TDSL by this method are: 3% regular α-helix, 7% distorted α-helix (adding to a total of 10% α-helical structures), 23% regular β-sheet, 14% distorted β-sheet (which add up to a total of 37% β-sheet structures), 21% β-turn and 32% unordered structures. Average values for the three methods used are: 13.3 (±5.9) % α-helix, 36.7 (±2.5) % β-sheet, 19.4 (±4.9) % turns and 31.6 (±1.5) % unordered structures. These average values are in good agreement with the values obtained by CDSSTR, which yielded the best fit

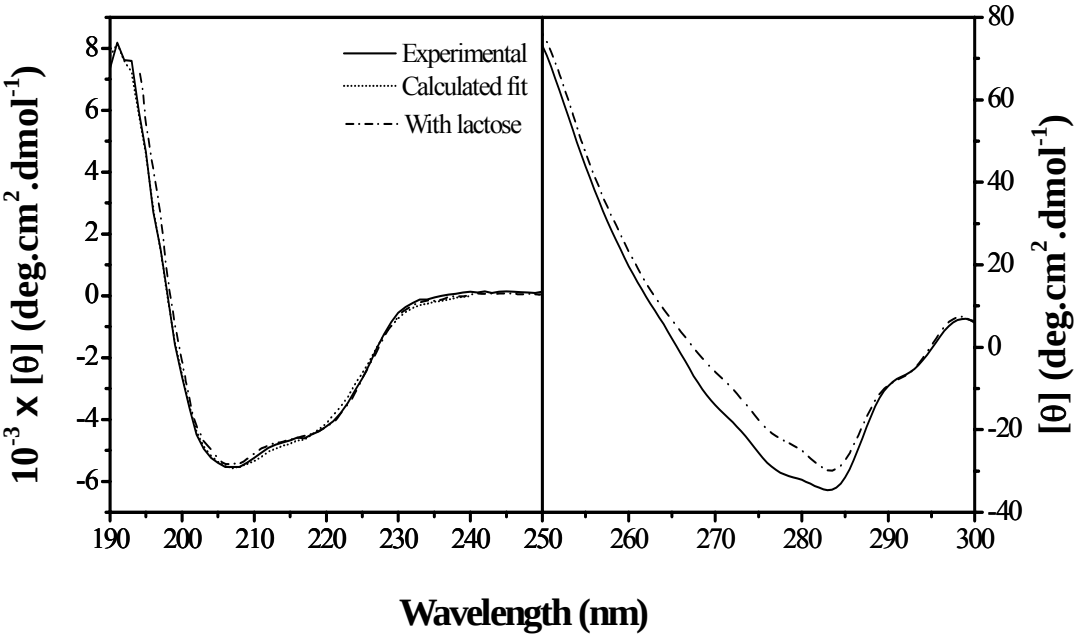


Fig. 4. CD spectra of TDSL in the absence and in the presence of lactose and analysis by CDSSTR method. (A) far UV region, (B) near UV region. (——) spectrum of TDSL, (·····) calculated fit by CDSSTR, (-·-·-·-) spectrum of TDSL in the presence of 0.1 M lactose. See text for details.

Table 2. Results of CD spectral analysis. The far-UV CD spectrum of native TDSL was analysed by three different methods, namely CDSSTR, SELCON3 and CONTINLL, using the software routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) and the fractions of different secondary structures obtained are presented here[a]. The normalized root mean square deviation (NRMSD), a measure for the deviation of the calculated spectrum from the experimental one was between 0.033 for CDSSTR, and 0.282 for CONTINLL.

Method	α_R	α_D	Total α	β_R	β_D	Total β	turn	U
CDSSTR	0.030	0.070	0.100	0.230	0.140	0.370	0.210	0.320
SELCON3	0.071	0.130	0.201	0.241	0.149	0.390	0.139	0.300
CONTINLL	0.036	0.061	0.097	0.215	0.126	0.341	0.234	0.329
Average			0.133 (±0.059)			0.367 (±0.025)	0.194 (±0.049)	0.316 (±0.015)

[a]α_R and α_D correspond to regular and distorted α helical structures, β_R and β_D correspond to regular and distorted β-sheet structures and U corresponds to unordered structure.

values and indicate that TDSL is a predominantly β-sheet protein with a relatively small α-helical content. Fairly similar values for the secondary structure elements were obtained for the other cucurbit lectin studied in this project, MCL (see Chapter 2).

The CD spectrum in the far UV region, recorded in the presence of 0.1 M lactose, is nearly identical to the spectrum of the native TDSL, suggesting that the ligand binding does not lead to any detectable changes in the secondary structure of the protein.

In the near UV region (250-300 nm), the CD spectrum is characterized by a minimum around 283 nm with a shoulder around 293 nm (Fig. 4B). The band centered around 283 nm and the shoulder at 293 nm can be assigned to the side chains of tyrosine and tryptophan residues, respectively. Presence of lactose at near-saturating concentration (0.1 M) led to a decrease in the intensity of the negative band in the region ca. 265-285 nm, suggesting that the tyrosine side chains are perturbed by

saccharide binding. This is consistent with the chemical modification studies, which implicated tyrosine side chains in the carbohydrate binding by TDSL (see below). On the other hand, the intensity of the shoulder at 293 nm is unaltered in the presence of lactose, indicating that the side chains of tryptophan residues are not affected by ligand binding. This observation is again consistent with the results from chemical modification experiments (see below).

4.5. Chemical modification studies: Reaction of TDSL with 2,4,6-trinitrobenzenesulfonate (TNBS) resulted in the modification of about 23 amino groups per molecule of the lectin. About 22 of these residues could be modified by citraconic anhydride (Table 3). Since there are two polypeptide chains in TDSL, this suggests that there are about 20-21 accessible lysine residues in the protein, with the other two reactive amino groups most likely being from the *N*-terminal amino acids of the two polypeptide chains. Neither TNBS modification nor citraconylation altered the hemagglutination activity of the lectin.

Reaction of the lectin with DTNB under native conditions resulted in the modification of only 0.2 thiol residues per molecule. Denaturation of the lectin with 8M urea also led to the modification of only 0.2 thiol groups, indicating that there are no free thiol groups in this protein. This modification did not result in any changes in the activity of the lectin.

Attempts to selectively modify the tryptophan residues in native TDSL with *N*-bromosuccinimide did not succeed. No isosbestic point was observed in the 260-265 nm region in the absorption spectra when the protein was treated with NBS and the protein samples became turbid, suggesting that the protein was getting fragmented.

This suggested that the tryptophan residues are not accessible to the reagent in the native lectin and that NBS was reacting with other residues of the protein leading, most likely, to cleavage of the polypeptide. When the reaction was carried out in the presence of 8M urea, a distinct isosbestic point was observed at ~265 nm (Fig. 5), suggesting that under these conditions reaction of NBS was restricted to the tryptophan residues only [cf. Patanjali et al., 1984]. A total of 2 tryptophan residues per TDSL dimer could be modified under this condition (Table 3). When the modified lectin was brought back to the native conditions by dialysis against PBS and tested for activity by the hemagglutination assay, its activity was found to be unchanged compared to a control TDSL sample treated similarly but without modification. The tryptophan residues of SGSL and TCSL were also found to be inaccessible to NBS in the native condition and could be modified only upon denaturation [Komath et al., 1998; Komath and Swamy, 1999; Kenoth et al., 2000].

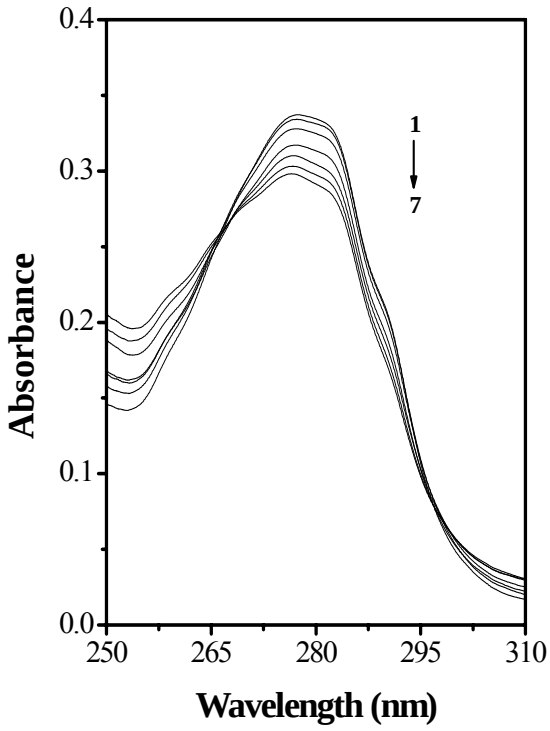


Fig. 5. Modification of tryptophan residues of TDSL with N-bromosuccinimide. Spectrum 1 corresponds to TDSL alone and spectra 2-7 correspond to TDSL upon reaction with increasing concentrations of NBS.

Table 3. Summary of results obtained from the chemical modification studies on *Trichosanthes dioica* seed lectin.

Reagent	Residue modified	Number of residues modified/molecule or percent modification	Agglutination activity after modification
Diethyl pyrocarbonate			
Without denaturant	Histidine	8.1	unchanged[a]
With 8M urea	Histidine	17.7	no activity[a]
2,4,6-trinitrobenzene			
Sulforic acid[b]	Lysine	22.8	unchanged
Citraconic anhydride[c,b]	Lysine	21.7 (95 %)	unchanged
N-acetylimidazole			
Without denaturant	Tyrosine	6.9	no activity
With 6M Gdn.HCl	Tyrosine	19.3	no activity
5,5'-Dithiobis(2-nitrobenzoic acid)			
Without urea	Cysteine	0.21	unchanged
With 10 mM β-ME but without urea	Cysteine	n.d.	unchanged
With 8M urea	Cysteine	0.19	unchanged
N-bromosuccinimide			
With 8M urea	Tryptophan	2.0	unchanged

[a]See the text.

[b]The % modification value was arrived at based on the fact that subsequent to the reaction with citraconic anhydride, only about 1.2 free amino groups were available for modification with TNBS.

[c]These reagents also react with the *N*-terminal amino groups in addition to the ε-amino groups of lysine residues. Therefore, the number of residues modified includes the *N*-terminal amino groups as well.
n.d.= not determined.

Modification under native conditions with *N*-acetylimidazole resulted in the modification of about 7 tyrosine residues, whereas modification after denaturing the lectin with 8 M urea led to the modification of 19 tyrosine residues per molecule of TDSL. Modification under both conditions led to a complete loss of the hemagglutination activity of the lectin (Table 3). When the hemagglutination activity was monitored as a function of the extent of modification, it was observed that the modification of one tyrosine residue/lectin dimer did not alter the activity of the lectin, but thereafter the activity decreased with increasing modification (Fig. 6). Modification of a second Tyr residue resulted in a 25 % decrease in the agglutination activity. Modification of 3.1, 4.2 and 5.1 tyrosine residues decreased the hemagglutination activity of the lectin by 50, 69 and 91%, respectively. Modification of 6.9 tyrosine residues led to the complete abrogation of the hemagglutination activity of the *T. dioica* lectin. Treatment of the tyrosine-modified lectin (6.9 residues/molecule) with 0.5 M of hydroxylamine for 2 hours, which reverses the modification, led to a 65 % recovery of the hemagglutinating activity, clearly indicating that loss of activity is due to the modification of tyrosine residues alone and not due to any structural/conformational changes of the protein.

In order to investigate whether the presence of the specific sugar has any effect on the tyrosine modification, the modification reaction was carried out in the presence of 0.2 M lactose. The results obtained are presented in Fig. 7. It is clear from this figure that reactivity of the tyrosine residues toward the modifying reagent is significantly reduced when the ligand is bound to the protein. Whereas 7 tyrosine residues could be modified in the native lectin in 130 minutes, only 3 tyrosine residues could be modified in the same period of time when the reaction was carried out in the

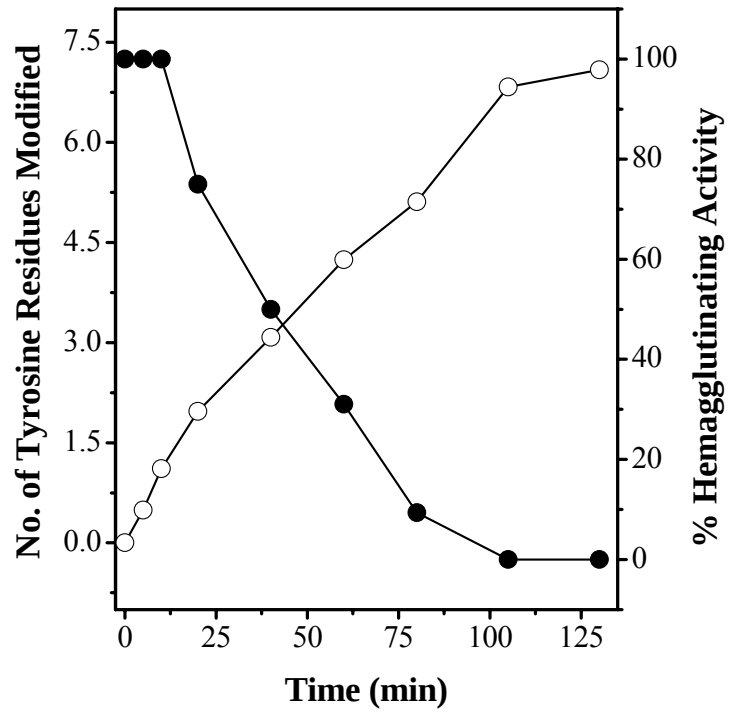


Fig. 6. Kinetics of tyrosine modification and the effect of modification on the hemagglutinating activity of *T. dioica* seed lectin. The extent of modification and the hemagglutination activity of the modified samples are plotted as a function of the reaction time. (○) number of tyrosines modified, (●) hemagglutinating activity.

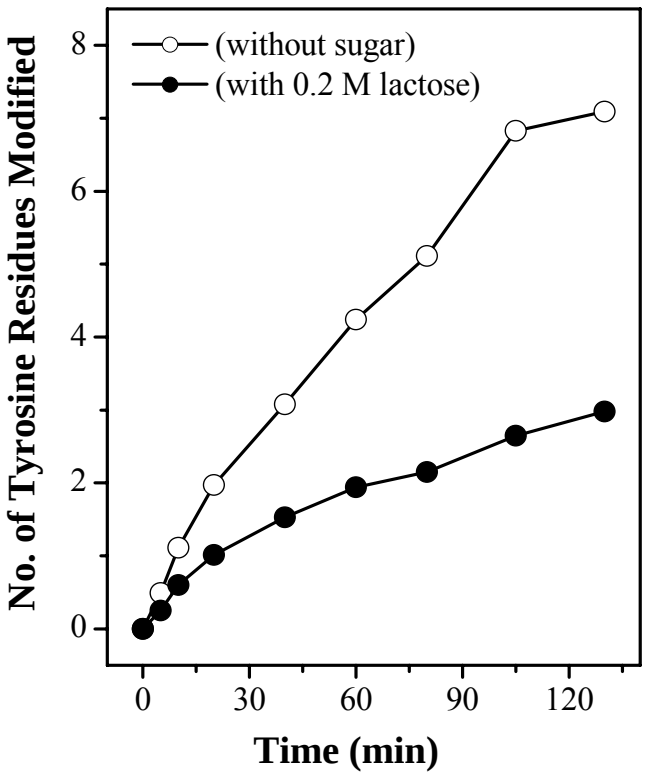


Fig. 7. Protection of tyrosine modification of *T. dioica* seed lectin by lactose. See the text for experimental details.

presence of 0.2 M lactose, with all other conditions being identical. Thus sugar binding results in a partial protection of the tryptophan residues of TDSL from their reaction with *N*-acetylimidazole.

Modification of TDSL with diethyl pyrocarbonate under native conditions led to the modification of 8.1 histidine residues per molecule. This did not result in any alteration in the activity of the lectin. When the modification reaction was carried out on the protein denatured with 8M urea, a total of 17.7 histidine residues were modified. The modified lectin did not show any activity when brought back to native conditions. Reversal of histidine modification by treating with 50 mM hydroxylamine for 10 minutes or by incubating the modified lectin at 37 °C for 2 hours did not result in any recovery of the activity. These observations suggest that treatment with diethyl pyrocarbonate under denaturing conditions led to the modification of other residues besides histidine. When the diethyl pyrocarbonate-modified lectin was treated with 0.5 M hydroxylamine for 2 hours some of the hmagglutinating activity could be recovered. Because this treatment reverses tyrosine modification by diethyl pyrocarbonate, it appears that the loss in activity resulting from modification by diethyl pyrocarbonate is most likely due to its reaction with tyrosine residues rather than due to the modification of the histidine residues. These observations are consistent with the results of tyrosine modification with *N*-acetylimidazole, presented above.

4.6. Effect of modification on protein secondary structure: The CD spectrum of tyrosine-modified TDSL in the far UV region (spectrum not shown) is nearly identical to that of native TDSL (see Fig. 4). This clearly indicates that modification of tyrosine residues does not affect the secondary structure of the lectin and suggests that the loss

in sugar-binding and hemagglutination activities of the protein is due to the modification alone and not due to any changes in the protein conformation. This in turn suggests that the side chains of one or more tyrosine residues are directly involved in the saccharide binding activity of the lectin.

5. Conclusions

In this study, the galactose-specific seed lectin from *Trichosanthes dioica* has been purified to homogeneity and characterized with respect to macromolecular properties, saccharide specificity and the amino acid side chains involved in its carbohydrate binding activity. The lectin resembles several other cucurbit seed lectins such as SGSL, TCSL and TKL-1 in the subunit composition and sugar specificity [Falasca et al., 1989; Komath et al., 1996; Komath and Swamy, 1998; Padma et al., 1999]. The two subunits of the heterodimeric lectin are linked by disulfide bond(s) and CD spectroscopy indicates that its secondary structure consists of α-helix, β-sheet and unordered structures. Comparison of the subunit composition and size, association of subunits by disulfide bond(s), secondary structure and carbohydrate specificity of TDSL with those of type 2 RIPs suggests that TDSL may be a type 2 RIP like protein.

General discussion and conclusions

Physico-chemical and carbohydrate-binding studies were carried out on the galactose-specific seed lectins from *Momordica charantia* (bitter gourd) and *Trichosanthes dioica*. The former, namely *Momordica charantia* seed lectin (MCL) has been studied in some detail in the 1970s and 1980s, but no further investigations were carried out on the physico-chemical properties and ligand binding characteristics of this protein during a period of about 15 years before the current investigations were taken up. Therefore, further studies on the ligand binding properties of MCL were chosen to be carried out. In the studies reported in this thesis, three different approaches were used to investigate the ligand-binding properties of MCL (Chapters 2, 3, and 4). Firstly, the binding of several free-base and metalloporphyrins to MCL was investigated by different absorption spectroscopy, in view of the recent observations from this laboratory that a number of plant lectins bind porphyrins. In a second study, binding of a number of mono- and disaccharides to MCL was investigated by isothermal titration calorimetry (ITC), in order to obtain accurate information on the binding of various sugars to this protein. Finally, the interaction of two fluorescently labeled sugars, namely 4-methylumbelliferyl-α- and β-D-galactopyranosides to MCL was investigated by fluorescence spectroscopy. In each study, the enthalpy and entropy associated with the binding process were determined in order understand the thermodynamic forces that govern the lectin-ligand interaction.

Studies reported in the second part of the thesis (Chapters 5 and 6) deal with the purification, physico-chemical characterization, and carbohydrate specificity of a new lectin from the seeds of another Cucurbitaceae species, namely *Trichosanthes dioica*. In addition, the amino acid residues that are important for the sugar-binding activity of

this lectin were identified by chemical modification studies employing group-specific reagents. Finally, fluorescence quenching and time-resolved fluorescence studies were carried out in order to characterize the environment of the indole side chains of the tryptophan residues in this protein. The results of these studies are briefly summarized below.

The interaction of several free-base and metalloporphyrins with MCL was investigated by absorption spectroscopy. The association constants of the interaction were found comparable to that of lectin-sugar interactions and were not affected significantly by the presence of lactose in the interaction medium. Additionally, presence of porphyrins in the medium did not affect the hemagglutination activity of the lectin, indicating that both sugars and porphyrins have distinct binding sites on MCL, i.e., the lectin is capable of binding porphyrins and cells simultaneously. Similar results were obtained for several other plant lectins in this laboratory. Unlike the *Trichosanthes cucumerina* seed lectin (TCSL), the interaction of MCL with porphyrins is enthalpically driven with negative net contribution from entropy. The interaction is also characterized by enthalpy-entropy compensation. These results suggest that the mode of MCL-porphyrin interaction is most likely similar to that observed in the crystal structure of Con A-H_2TPPS complex in which the interaction is mediated by an extensive network of H-bonds, some of which are water-mediated whereas no hydrophobic interaction is observed.

Isothermal titration calorimetry (ITC) is a powerful technique to obtain accurate thermodynamic data on protein-ligand interactions. Therefore, this technique was chosen for investigating the binding of carbohydrates to MCL. These studies showed that each lectin tetramer (120 kDa) has two saccharide binding sites. Other seed lectins

from Cucurbitaceae such as SGSL, TCSL and TDSL, which are dimeric can be represented as ab pair of subunits with at least two binding sites per dimer. On the other hand, MCL can be regarded as a dimer of this pair, i.e., a_2b_2 or $(ab)_2$, however it has only two active binding sites instead of four. In this regard MCL is similar to APA and RCA which are believed to be dimers of abrin and ricin, respectively, but have only two active binding sites per tetramer. This is an another aspect of the similarity between Cucurbitaceae galactose-binding lectins (CGLs) and type 2 RIPs.

Thermodynamic parameters obtained from the ITC studies indicate that MCL-sugar interaction is enthalpically driven with negative contribution from binding entropy. Enthalpy-entropy compensation and small negative heat capacity change were also observed for MCL-sugar interactions, phenomena which are usually attributed to participation of water molecules in the binding process and/or changes in water structure around ligand and binding site.

CD measurements show no significant changes in the secondary and tertiary structures of MCL upon binding the sugar or porphyrin ligand suggesting that binding sites of these ligands on MCL are preset and the binding process involves very marginal or no conformational changes of the lectin.

Binding of 4-methylumbelliferyl α- and β-D-galactopyranosides to MCL was investigated by fluorescence spectroscopy. Binding of these fluorescent sugars to this lectin results in a quenching of their fluorescence intensity. In comparison, SGSL and TDSL enhance the β-anomer fluorescence but only slightly quench the fluorescence of the α-anomer. The interaction of MCL with these sugars was studied by monitoring the quenching of their fluorescence induced by binding to the lectin at different temperatures to obtain the binding constants and thermodynamic parameters (K_b, ΔH_b,

ΔS_b) associated with the binding process. Analysis of these parameters indicated that the higher affinity of the lectin for the β-anomer is due to larger enthalpy of binding, which overrides a larger negative entropy of binding associated with the binding of the corresponding β-anomer.

Trichosanthes dioica seed lectin, TDSL, was purified by affinity chromatography on cross-linked guar gum matrix. Its homogeneity was verified by gel filtration and polyacrylamide gel electrophoresis. These techniques were also used to determine its relative molecular weight, M_r. On Superose-12, in the presence of 0.1 M lactose, TDSL moved as a single peak with an M_r of 55 kDa. In SDS-PAGE, in non-reducing condition, a single band was observed while in presence of β-mercaptoethanol, two non-identical bands of M_r 24 kDa and 37 kDa were observed indicating that the two subunits of TDSL are connected by one or more disulfide bridges. Like other Cucurbitaceae galactose specific lectins, such as SGSL, TCSL and MCL, it is also a glycoprotein with about 5 % neutral sugar. Its secondary structure was found, by CD spectroscopy, to be built of 13.3 % α-helix, 36.7 % β-sheet, 19.4 % β-turns and 31.6 % unordered structure. The content of these elements in the secondary structure of MCL, determined by the same technique was also very similar.

All these findings, and several other observations discussed in Chapters 1 and 5, suggest that Cucurbitaceae galactose specific lectins are closely related proteins and show significant similarity to type 2 RIPs. These similarities appear to be strong enough to suggest a close structural similarity between CGLs and type 2 RIPs. However, further investigations on these proteins with regard to amino acid sequence, 3-dimensional structure and RIP activity assays need to be carried out on these lectins to confirm this hypothesis.

Hapten inhibition assays of the hemagglutination activity of TDSL by sugars showed that it is a galactose-specific lectin with preferential affinity to β-galacotosides over the α-isomers, a property shown also by the other well-characterized CGLs, namely MCL, SGSL and TCSL. However, in contrast to these CGLs, TDSL binds Gal*N*Ac stronger than galactose itself. Another noteworthy point is that, like MCL and SGSL (and unlike TCSL), TDSL binds galactosides with bulky aromatic aglycon stronger than galactose or aliphatic galactosides. Therefore, although there is a gross similarity in sugar binding properties among these lectins, there are subtle differences in the fine sugar specificity of the CGLs. In the light of this observation, it would be interesting to investigate the binding of complex oligosaccharides bearing galactose and Gal*N*Ac as terminal sugars with the CGLs and compare the binding profiles for the various lectins.

Chemical modification experiments showed that tyrosine is a key residue in the sugar binding site of TDSL. About 7 tyrosine residues could be acetylated by *N*-acetylimidazole under native condition (see Chapter 5), resulting in total loss of agglutination and sugar-binding activities of the lectin. The degree of activity loss was proportional to the number of tyrosine residues modified. The presence of lactose partially protected tyrosine residues from acetylation conforming the involvement of tyrosine in the sugar-binding activity. No changes in the secondary structure of the modified lectin could be observed by CD measurements indicating that the activity loss is due to the acetylation of tyrosine residues alone. No free sulfhydryl could be detected and attempt to reduce the disulfide bond(s) under native condition failed as was the case with SGSL and TCSL. No loss of activity could be detected upon chemical modification of lysine, histidine and tryptophan residues. Similar studies on SGSL and

TCSL showed histidine residues and not tyrosine residues to be essential for their sugar binding activity while in MCL tyrosine and also tryptophan residues were found essential. Therefore one can conclude from these observations that Cucurbitaceae galactose specific lectins show close saccharide specificity inspite of presence of some differences at least in the amino acid building the binding site. Legume lectins on the other hand have highly conserved binding sites both in fold and amino acid composition, but show diverse specificities. Lectins therefore appear to adopt different mechanisms for similar recognition processes.

In chemical modification studies the tryptophan residues of TDSL could not be oxidized selectively with *N*-bromosuccinimide under native condition, indicating that they are not accessible to the modifying reagent but buried in the hydrophobic matrix of the protein. This was supported strongly by fluorescence experiments carried out on the lectin since the fluorescence maximum (excited at 295 nm) was red shifted from 328 to 343 nm with a concomitant large decrease in the intensity upon incubating the lectin in 6 M Gdn.HCl. In order to obtain more detailed information, fluorescence quenching experiments with two neutral quenchers (acrylamide and succinimide) and two ionic quenchers (I^- and Cs^+) were carried out on TDSL. Under native conditions, all these quenchers experienced only partial accessibility to tryptophan residues. The highest degree of quenching was observed for acrylamide followed by the bulkier molecule succinimide whereas the ionic species I^- and Cs^+ exhibited only weak quenching. Upon denaturation, acrylamide showed complete accessibility, closely followed by succinimide (91 % accessibility) and I^- (77 % accessibility), whereas the accessibility was only 41 % for Cs^+, indicating the presence of positively charged amino acid side chain(s) in the vicinity of at least one tryptophan residue in the denatured lectin. The

presence of lactose partially protected tryptophan residues from quenching by succinimide, I^- and Cs^+. However, it did not result in any significant changes in emission λ_{max}, fluorescence decay average life time or CD spectrum, indicating that sugar binding by TDSL doesn't perturb the lectin conformation.

In summary, the studies reported in this thesis lead to a further understanding of the physico-chemical and carbohydrate binding properties of *Momordica charantia* (bitter gourd) lectin (MCL). In addition, it has been shown that MCL binds with considerable affinity, a variety of cationic and anionic porphyrins. This suggests that MCL may find some application in photodynamic therapy. However, additional work is necessary to explore this possibility further. Besides the studies on MCL, a new Gal/GalNAc specific lectin from the seeds of another Cucurbitaceae species, namely *Trichosanthes dioica* has been purified and characterized in considerable detail. The properties of this lectin suggest that, like other CGLs it may belong to the class of type 2 RIP lectins.

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LIST OF PUBLICATIONS

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