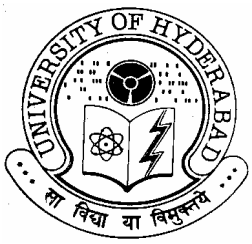
Physicochemical Studies on Galactose-Specific Seed Lectins from Cucurbitaceae

A thesis
Submitted for the degree of
DOCTOR OF PHILOSOPHY

By
M. Kavitha



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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. Any omission which might have occurred by oversight or error is regretted.

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CERTIFICATE

Certified that the work embodied in this thesis entitled **"Physicochemical studies on galactose-specific seed lectins from Cucurbitaceae"** has been carried out by **Ms. Kavitha M**. under my supervision and the same has not been submitted elsewhere for any degree.

Hyderabad

September 2008

Prof. Musti J. Swamy
(Thesis Supervisor)

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ABBREVIATIONS

ANS	8-anilinonaphthalene-1-sulfonate
BSA	bovine serum albumin
ΔC_p	change in excess heat capacity
CD	circular dichroism
Con A	Concanavalin A
CUTMPyP	*meso*-tetra-(4-methylpyridinium) porphyrinato copper(II)
DBL	*Dolichos biflorus* lectin
DSC	differential scanning calorimetry
2-dGal	2-deoxygalactose
Gal	Galactose
GalNAc	*N*-acetyl-D-galactosamine
$GalNH_2$	galactosamine
Gdn.HCl	guanidinine hydrochloride
ΔH_c	change in calorimetric enthalpy
ΔH_v	change in van't Hoff enthalpy
H_2TCCP	*meso*-tetra(4-carboxyphenyl) porphyrin
H_2TSPP	*meso*-tetra(4-sulfonatophenyl)porphyrin
MCL	*Momordica charantia* (bitter gourd) seed lectin
MeαGal	methyl-α-D-galactopyranoside
MeβGal	methyl-β-D-galactopyranoside
MeUmbβGlc	4-methylumbelliferyl-β-D-glucopyranoside
MeαGlc	methyl-α-D-glucopyranoside
MeαMan	methyl-α-D-mannopyranoside
MeβGlc	methyl-β-D-glucopyranoside
MeβSGal	methyl-β-D-thiogalactopyranoside

PAGE	polyacrylamide gel electrophoresis
PBS	20 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4
PDT	Photodynamic therapy
pNPαGlcNAc	p-nitrophenyl-α-*N*-acetyl-D-galactopyranoside
*p*NPβGal	*p*-nitrophenyl-β-D-galactopyranoside
PSL	Pea (*Pisum sativum*) seed lectin
RCA	*Ricinus cummunis* agglutinin
RIP	ribosome-inactivating protein
SBA	soybean (*Glycine max*) agglutinin
SDS	sodium dodecyl sulphate
SGSL	snake gourd (*Trichosanthes anguina*) seed lectin
TBS	10mM Tris-HCl buffer (0.15M NaCl +1mM $MnCl_2$+1 mM $CaCl_2$)
TCA	*Trichosanthes cordata* agglutinin
TCSL	*Tricosanthes cucumerina* seed lectin
TDSL	*Trichosanthes dioica* seed lectin
TKL-I	*Trichosanthes kirilowii* Maximowicz lectin
T_m	transition temperature
TNS	2,6-toluidinylnaphthalenesulfonate
Trp	tryptophan
WBA	winged bean agglutinin
WGA	wheat germ agglutinin
ZnTSPP	*meso*-tetra-(4-sulfonatophenyl)porphyrinato zinc(II)

Chapter 1

Introduction

Lectins

Definition and occurrence

Lectins are proteins of non-immune origin that bind to mono/oligosaccharides reversibly in a non-catalytic manner [Goldstein et al., 1980; Sharon & Lis, 2003]. Lectins are ubiquitously distributed in nature. They have been isolated from a variety of species including bacteria, viruses, fungi, plants and animals. The majority of these have been obtained from the seeds, where they accumulate during maturation and disappear upon germination. Many of the plant lectins are well characterized in considerable detail with respect to macromolecular properties and carbohydrate binding specificity [Lis & Sharon, 1986, 1998; Sharon & Lis, 2003]. Besides seeds, lectins have been found in virtually all types of vegetative tissue of plants [Peumans &Van Damme, 1998].

History

The earliest report of lectin activity, describing the cell agglutinating properties of ricin, a toxic protein isolated from castor bean (*Ricinus communis*) appeared in 1888 in the doctoral thesis of William Hermann Stillmark [Kocourek, 1986]. Soon similar toxic proteins were identified from the seeds of *Croton tigliu* (croton) and *Abrus precatorious* (abrin) and from the bark of *Robinia psuedoacacia* (robin). Non toxic lectins were discovered from the seeds of legume lectins, shattering the then prevalent notion that lectins were necessarily toxic proteins [Landsteiner & Raubitscheck, 1907].

The study of lectins picked up only in the late 1940's with the discovery of their ability to distinguish between different human blood groups [Renkonen, 1948; Boyd & Reguera, 1949]. Mitogenic stimulation of lymphocytes by phytohemagglutinin (or PHA) was discovered in 1960, by Peter C. Nowell, a

discovery that had a revolutionary impact on immunology, as at that time people thought that lymphocytes are dead end cells that could neither divide nor differentiate further [Nowell, 1977]. Affinity chromatography for the purification of lectins was introduced by Irwin J. Goldstein, who developed a simple and convenient technique for the direct isolation of the lectin from the crude extracts of jack bean meal by adsorption on a Sephadex column and elution with glucose [Agrawal & Goldstein, 1965; Goldstein, 1990].

Classification

Depending on the number and activity of subunits lectins are classified as merolectins, which contain a single carbohydrate binding domain (CBD), hololectins, that have at least two identical carbohydrate binding domains with the same specificity, and chimerolectins, which are fusion proteins, which consist of a lectin domain linked to some other domain independent in action from the CBD of the protein (e.g., Type II RIPs) and super lectins, which possess more than one CBD but with different specificities (Tulip bulb lectin) [van Damme et al., 1998a]. Most of the lectins known are hololectins.

Plant lectins have been classified into seven distinct families based on the sequence information and structural motifs [van Damme et al., 1998a, b]. Among these four families, namely the legume lectins (e.g., Concanavalin A, pea lectin and peanut lectin), lectins containing hevein domains (e.g., wheat germ agglutinin and *Hevea brasiliensis* lectin), monocot mannose binding lectins (e.g., garlic lectin and snow drop lectin) and type 2 ribosome inactivating proteins (e.g., ricin and abrin) comprise numerous number of lectins, and are considered to be large families. The jacalin related family (e.g., jacalin and artocarpin), Cucurbitaceae phloem lectins (e.g., *Cucurbita maxima* lectin, *Cucumis sativus* lectin and *Cucumis melo* lectin) and the amaranthin family lectins are at this moment only small protein families.

Carbohydrate binding

Ability of lectins to interact with carbohydrates specifically and reversibly has made them important tools in biochemical and immunochemical studies. To understand the function and properties of lectin, knowledge on sugar-lectin interaction is necessary. Lectins specific for monosaccharides for which they have the highest affinity are usually classified into five groups: mannose, galactose/*N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and *N*-acetylneuraminic acid [Lis & Sharon, 1998]. Oligosaccharides in general have higher affinity than monosaccharides, with association constants in the range of 10^5 to 10^7 M^{-1}; this is of special interest from the functional point of view as native ligands for lectins are oligosaccharides of glycoproteins or glycolipids. Association constants for lectin-monosaccharide interaction are typically in the range of 10^3 to 10^4 M^{-1}, showing that the interaction is weak. Despite such weak interaction lectins exhibit a high specificity for individual sugars [Goldstein & Poretz, 1986]. Apart from a few exceptions, galactose specific lectins do not react with glucose or mannose nor do those specific for mannose bind galactose. Certain lectins from the same specificity group possess anomeric specificity, i.e., they can distinguish between α or β- anomer of a sugar, with pronounced affinity for one of the anomers. For example, the lectins from *Griffinia simplicifolia* (B4) [Hayes & Goldstein, 1974] and lima bean lectin [Roberts & Goldstein, 1984] specifically bind to the α-anomer of galactose with a significantly higher avidity than the corresponding β-anomer.

Galactose specific lectins from the seeds of Cucurbitaceae preferentially bind to the β-anomer of galactose than the α-anomer [Komath et al., 1998; Sultan & Swamy, 2005a]. Many lectins tolerate variations at the C-2 position of pyranose ring to which they bind. Most lectins that bind galactose also interact with *N*-

acetylgalactosamine [Sharon & Lis, 2003]. Lectins tolerate very little variation at C-3 of the sugars they interact with although one or two exceptions are there. The hydroxyl group at the C4 position is important, because in general galactose specific lectins do not interact with glucose or mannose nor do those specific for mannose bind galactose. The nature of aglycon can influence lectin-glycoside interaction. Aromatic glycosides bind to lectins more strongly than aliphatic glycosides suggesting the presence of hydrophobic regions close to the carbohydrate binding site.

Carbohydrate binding sites appear as shallow depressions on the surface of proteins, and the sites appear to be preformed [Weis & Drickamer, 1996]. Lectins combine with carbohydrates *via* hydrogen bonds and hydrophobic interactions, van der Walls forces, and metal co-ordination bonds [Lis & Sharon, 1998; Weis & Drickamer, 1996]. Contacts between the sugar and lectin are often mediated by water molecules [Toone, 1994]. The importance of water in lectin sugar interactions has been demonstrated by isothermal titration calorimetry in the presence of increasing osmotic stress [Swaminathan et al., 1998].

Functions of lectins

Plant lectins

The functions of lectins are based on their ability to recognize specific carbohydrate structures of glycoconjugates that are present on cell surfaces. Although plant lectins are known for longer times, *in vivo* functions of the lectins in the plants remains obscure. They are thought to play a role in defence, as storage proteins and for the establishment of symbiosis between nitrogen-fixing bacteria, rhizobia and leguminous plants. In *Pisum sativum* (pea), apart from seeds, lectin is also present in small amounts in the tip of developing root hairs. The presence of lectin is

responsible for the specific association of bacteria and the root hair surface. This became known as the "lectin recognition hypothesis" [Kijne, 1996; Hirsch, 1999].

Toxicity of lectins for insects and higher animals, and their growth inhibitory effect on fungi are the basis for the assumption that these proteins may function in plant defence against predatory animals and different kinds of pathogens [Peumans & Van Damme, 1995; Pusztai & Bardocz, 1996]. Lectins which are present in the seeds and vegetative storage tissues exhibit a storage protein like behavior; it was proposed that these proteins function as storage proteins [Etzler, 1986; Van Damme et al., 1998a].

Viral and bacterial lectins

In viruses and bacteria cell surface lectins help in adhesion of the organisms to host cells. This is a prerequisite for infection to occur [Lis & Sharon, 2003]. This infection by bacteria can be prevented by pre-incubation of bacteria with specific sugars. Adhesion of lectin carrying bacteria to human polymorphonuclear cells is often followed by their ingestion and killing of bacteria, a process known as lectinophagocytosis. Lectins on bacterial surface are useful in the attachment of bacteria to primitive organisms and inanimate matter. For example, a galactose specific lectin present on the surface of the pathogen *Vibrio shiloi*, mediates the attachment of bacteria to the coral surface [Banin et al., 2001].

Animal lectins

A variety of functions have been clearly demonstrated for animal lectins. Galecitns are ubiquitous, soluble, animal lectins that are specific for β-galactosides such as lactose and *N*-acetyllactosamine. Galectins are thought to be important for the normal differentiation and growth of all multicellular animals [Perillo et al., 1998; Rabinovich, 1999; Leffler, 2001]. They have the ability to induce cell proliferation

and apoptosis, and have been implicated in tumor cell metastasis, leukocyte trafficking, organ morphogenesis, immune response and inflammation, as well as recognition of extracellular matrix.

Selectins belong to the C-type class of animal lectins and mediate selective adhesion of circulating leukocytes to the endothelial cells of blood vessels, a prerequisite for the removal of the leukocytes from the circulation and their migration into tissues. Certain selectins are expressed on the endothelial cell surface only in response to infections. Collectins are another group of C-type lectins, the name being derived from the presence of collagen like domains in these proteins [Epstein et al., 1996]. An important collectin is the serum mannose binding protein. The concentration of collectins increases during infection/inflammation. They function in innate immune system. They bind to the oligomannosides of microorganisms and cause lysis of the pathogens. P-type lectins or Mannose-6-phosphate receptors (MPRs) mediate the intracellular sorting and trafficking of lysosomal enzymes [Drickamer, 1994].

Applications of lectins

Sugar binding property of lectins has been employed in various medical and biotechnological uses. The earliest clinical application of lectins is blood typing [Kilpatrick & Green, 1992]. Lectins labeled with fluorescent dyes, gold particles, radioactive agents, antibodies, and enzymes are used as probes for cell-specific markers [Lis & Sharon, 1986]. Most of the lectins in use are derived from plants. Lectins are useful in isolation and characterization of glycoproteins, polysaccharides in solution and on cell surfaces. Separation of glycoproteins can be done by employing Ouchterlony double diffusion technique, in which lectins are employed in place of antibodies [Carter & Sharon, 1977]. The blotting technique is highly useful for the detection and identification of glycoproteins on cells, which

serve as receptors for bacterial cell surface lectins [Sharon & Ofek, 1995]. Dot-blotting technique is used to study changes in the glycosylation of glycoproteins, glycosylation of IgG occurring in patients with rheumatoid arthritis monitored with biotinylated RCA and GSL-II [Sumar et al., 1990]. Lectins are useful for the isolation and affinity purification of glycoconjugates like glycoproteins, glycopeptides, and oligosaccharides. ConA has been used for the separation of closely related glycopeptides which are found in proteolytic digests of glycoproteins [Baenziger & Fiete, 1979; Narasimhan et al., 1979].

Lectins are useful in cell fractionation, provided cells differ in their cell surface sugars [Sharon, 1983]. Agglutination is the most commonly used technique for cell separation by lectins; other methods include affinity chromatography of cells on immobilized columns or use of a fluorescence-activated cell sorter [Sharon & Lis, 2003]. PNA has proved to be an invaluable tool for the separation of human thymocytes [Sharon, 1983]. PNA is used for the fractionation of human or mouse thymocytes into two populations. Selective agglutination of SBA effectively separates mouse splenocytes into T- and B- cells [Reisner et al., 1976]. SBA has also been found to be useful for the purging of human bone marrow for transplantation [Reisner, 1983].

Certain lectins have the ability to induce mitogenic stimulation of lymphocytes. For example, ConA and pea lectin stimulate T cells whereas pokeweed lectin stimulates both T and B cells [Lis & Sharon, 1986]. Mitogenic stimulation by lectins is useful for studying the immunocompetence of patients suffering from a diversity of diseases including AIDS [Lis & Sharon, 1998]. Accumulation of glycoconjugates in the lysosomes during lysosomal disorders can be revealed by lectin staining [Alroy et al., 1994]. Difference between cell surface glycoproteins of tumor cells and normal cells has been detected with lectins. A few lectins are diagnostically useful for the detection of certain cancer cell lines. For example HPA was reported

to recognize a marker of breast cancer [Dwek et al., 2001]. PNA is useful in detecting cancers of breast and colon [Brinck et al., 1998].

Hydrophobic ligand binding

Although plant lectins are being widely used in a variety of applications in biochemical research and industry, as well as in medicine, the function of plant lectins remains obscure. Although carbohydrate binding property is a distinguishing feature of plant lectins, research done in the last two decades or so has shown that several lectins possess hydrophobic sites that binds small nonpolar compounds. For example, Concanavalin A binds to TNS and the phytoharmone indole acetic acid [Yang et al., 1974, Edelman & Wang, 1978]. Binding affinities for these ligands were in the range of 10^3-10^5 M^{-1}, which are comparable to binding affinities of specific carbohydrates to the lectins. The binding of non-carbohydrate ligands to lectins demonstrates the bifunctional nature of these proteins. Lectins have the ability to recognize the glycocode on different cell surfaces and distinguish between normal and diseased tissues, and these additional sites that bind to hydrophobic ligands may be viewed as potential drug carrying sites that could be exploited for drug delivery to sites of choice [Komath et al., 2006]. Porphyrins are biologically important molecules that have considerable hydrophobic character and lectin-porphyrin interaction has been investigated in considerable detail by Swamy and coworkers. The significant affinity of Porphyrin lectin complexes suggests that it may be possible to use lectins as carriers for targeting these porphyrins to tumor tissues as porphyrins are already under investigation in photodynamic therapy [Komath et al., 2006].

Binding of ANS and TNS to plant lectins

Several groups reported the interaction of hydrophobic fluorescent probes 1,8-anilinonaphthalenesulfonic acid (ANS) and 2,6-toluidinylnaphthalenesulfonic acid

(TNS) with legume lectins as well as non legume lectins [Roberts et al., 1983a,b; Yang et al., 1974; Houston, 1980]. Fluorescence quantum yields of ANS and TNS show marked enhancement upon binding to hydrophobic sites as compared to their fluorescence in aqueous solution. Titrations of these ligands measured by fluorescence enhancement indicate that the binding affinities were typically in the range of 10^3-10^4 M^{-1} [Roberts & Goldstein, 1983a]. Roberts and Goldstein also showed that addition of specific sugars did not inhibit binding of ANS, suggesting that the hydrophobic binding sites of lectins are independent of the carbohydrate binding sites.

Adenine and phytohormone binding to lectins

Binding of adenine and its derivatives as well as other hydrophobic molecules such as phytohormones by legume lectins was first reported by Roberts and Goldstein [1983b]. They identified high affinity adenine binding sites in lectins from soybean, *P. vulgaris,* and *D. biflorus***.** A single high affinity site for adenine and cytokinins has been identified on the lima bean lectin tetramer. Adenine derivatives possessing hydrophobic substituents bound to LBL with better affinities and this binding was in all probably mediated by the high affinity TNS binding site of the lectin [Roberts & Goldstein, 1982].

Photo affinity labeling studies by Goldstein's group on the adenine binding sites of lima bean lectin and kidney bean lectin yielded very interesting results [Maliarik & Goldstein, 1988]. They reported that adenine binding site is unique with respect to the other binding sites of lectins in that there is only one such site per tetramer. In Con A, adenine may bind at a site that is sandwiched between two ConA dimers. This is similar to that observed in pea lectin as well, although in pea lectin it does not make intermolecular contacts. Adenine binding sites of *Dolichos biflorus* lectin were characterized in detail by Marilyn Etzler's group [Gegg et al.,

1992; Gegg & Etzler, 1994]. They also suggested that perhaps cytokinins could serve as physiologically relevant ligands for lectins. They also demonstrated that adenine binding sites are distinct from ANS binding sites. Crystal structure of DBL complexed with adenine is shown in Fig. 1.1. From the figure it is seen that a total of four adenine molecules (two dimers) bind to the DBL tetramer [Hamelryck et al., 1999].

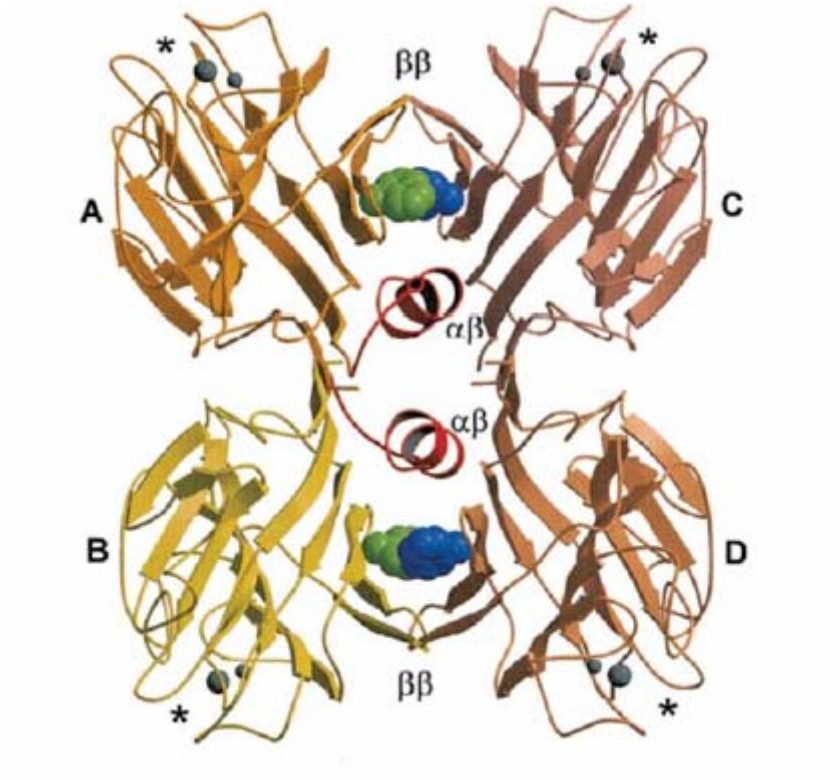

Fig. 1.1. Crystal structure of DBL in complex with adenine. Each subunit is shown in a different color. The four observed adenine molecules are shown as space-filling models in green and blue. The two types of dimer-dimer interfaces are indicated as αβ and ββ. The metal ions Ca^{+2} and Mn^{+2} are shown as large and small grey spheres. The locations of the four sugar-binding sites are indicated with an asterisk (*). Reproduced from Hamelryck et al., 1999 (Copyright (1999) Academic press).

Same type of interface is found in SBA as well as L-PHA, both of which are tetramers and binds adenine [Loris et al., 2000].

The interaction of TNS, adenine and phytohormones with wheat germ agglutinin has been investigated recently [Bogoeva et al., 2004]. Binding constants for adenine and adenine related phytoharmones such as kinetin, gibberillic acid, abscissic acid and zeatin were reported to be in the range 1.6-2.3×10^6 M^{-1}, which are higher than the affinity exhibited by this lectin towards different saccharides [Bogoeva et al., 2004]. WGA has two binding sites for TNS – a low affinity site and a high affinity site.

Porphyrin binding by lectins

Porphyrins are biologically important molecules. Many porphyrins occur in nature. They are bound to polypeptide chains, as seen in chlorophyll, hemoglobin, myoglobin and cytochrome *c*. Binding of synthetic porphyrins to proteins such as human serum albumin, BSA and low density lipoproteins has been investigated by several groups [Davilia & Harriman, 1990; Beaven et al., 1974; Reyftmann et al., 1984]. Porphyrins are also used as anti-microbial agents as they inhibit microbial growth in cell cultures [Bertoloni et al., 2000; Malik et al., 1990]. Porphyrins have been used as photosensitizers in photodynamic therapy (PDT), an approach to treat cancer [Kessel, 1986; Levy, 1995; Dougherty et al., 1998]. Porphyrins appear to be suitable for such an application, because in addition to being biocompatible, they have been found to exhibit a preferential localization in tumor tissue. In PDT when irradiated with light of appropriate wavelength, porphyrin photosensitizers interact with molecular oxygen and convert it into the corresponding singlet state, which then interacts with the surrounding tissue and leads to cell death. However, the selectivity of these sensitizers towards tumor cells as compared to normal cells has been reported to be about 2:1 in most cases [Klyashchitsky et al., 1994]. As some lectins are already under investigation for the targeted delivery of drugs, finding

lectins with high affinity for porphyrins may be useful in targeted delivery of porphyrins in PDT [Kitao & Hattori, 1977; Yamaguchi et al., 1979].

Interaction of porphyrins with Concanavalin A and pea lectin

Interaction of lectins with porphyrins has been characterized in significant detail by Swamy and colleagues [reviewed in Komath et al., 2006].

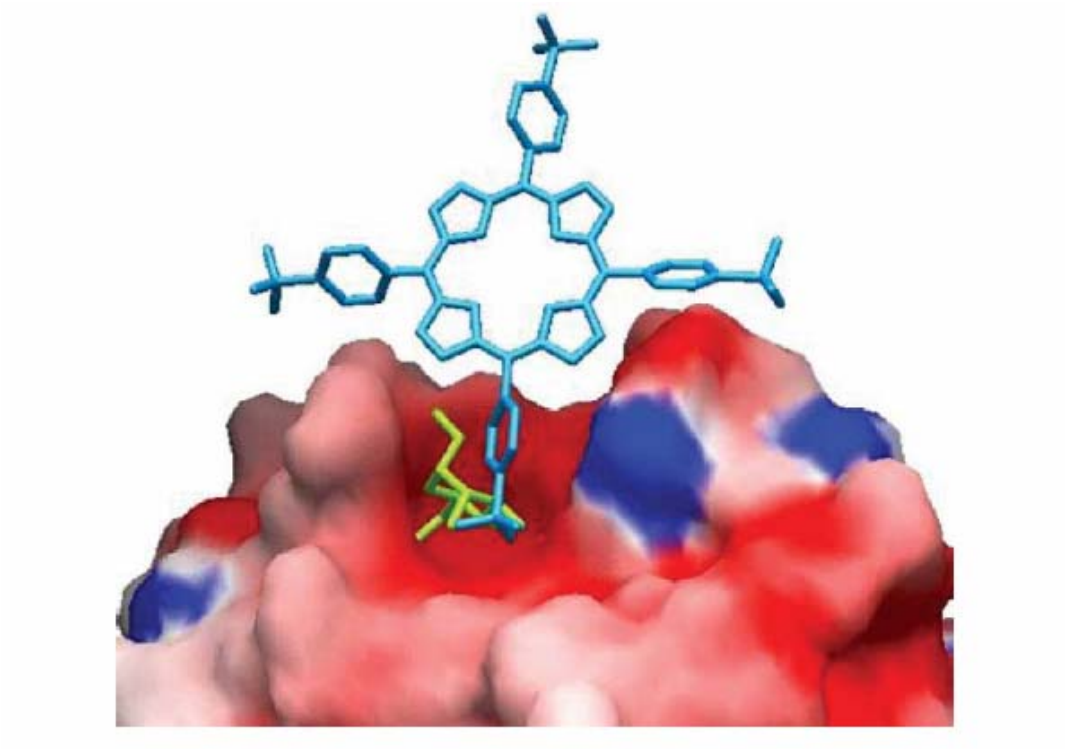

Fig. 1.2. Crystal structure of Con A–H_2TPPS complex. A superimposition of the ligand binding region of the complexes of the lectin with bound H_2TPPS and methyl-α-D-mannopyranoside is shown. Molecular surface of ConA is shown with color to indicate charge distribution (red, negative; blue, positive). From the picture it is clearly seen that H_2TPPS binds to ConA through the sulfonatophenyl group in a groove in which methyl-α-D- mannopyranoside is known to bind. Reproduced from Goel et al. [2001]. (Copyright (2001) American Society for Biochemistry and Molecular Biology, Inc.).

The very first studies on this aspect reported absorption and fluorescence spectral studies on the interaction of water soluble porphyrins, H_2TPPS and its metal derivative, ZnTPPS with Con A and pea lectin [Bhanu et al., 1997]. The association constants obtained were in the range of 1.2×10^4 M^{-1} - 6.3×10^4 M^{-1}. Each lectin subunit was found to bind one porphyrin molecule. Saccharide binding did not affect porphyrin binding, indicating that porphyrin binding takes place at a site different from the sugar binding site.

Crystal structure of Con A–H_2TPPS complex is shown in Fig. 1.2 [Goel et al., 2001]. This study has shown that sulfonatophenyl group of H_2TPPS occupies the sugar binding site. A pair of stacked porphyrins cross linked together molecules of Con A using two of their side groups each. The sulfonatophenyl group mimics seven of the eight hydrogen bonds that are involved in the interaction between Con A and MeαMan. The hydrogen bonds involving the C4 hydroxyl of the sugar are replaced by those involving a molecule of water.

Porphyrin binding to jacalin

Jacalin is a galactose specific homotetrameric lectin of Mr 66000 purified from the seeds of jack fruit (*Artocarpus integrifolia*) [Banerjee et al., 1991]. Jacalin binds the T-antigenic disaccharide, Galβ13GalNAc with a significantly higher affinity than other carbohydrate ligands. Crystal structure of jacalin complexed with methyl-α-D-galactopyranoside has been reported [Sankarnarayanan et al., 1996]. Porphyrin binding to jacalin was investigated at room temperature by absorption and fluorescence spectroscopy. The association constants obtained are in the range of 2.4×10^3 M^{-1}-1.3×10^5 M^{-1}[Komath et al., 2000a] which is comparable to the values obtained for other protein-porphyrin as well as lectin-porphyrin interaction. Binding affinities of jacalin for both positively charged and negatively charged porphyrins

were found to be similar, suggesting that jacalin-pophyrin interaction is mediated primarily by hydrophobic forces.

The crystal structure of (*meso*-tetrasulfanatophenylporphyrin)-jacalin complex, reported at 1.8Å resolution [Goel et al., 2004] is given in Fig. 1.3. In the jacalin-H_2TPPS complex a porphyrin pair is sandwiched between two symmetry related jacalin monomers leading to the cross linking of the lectin molecules in the crystal. Both stacking interactions and hydrogen bonds stabilize the lectin-porphyrin complex.

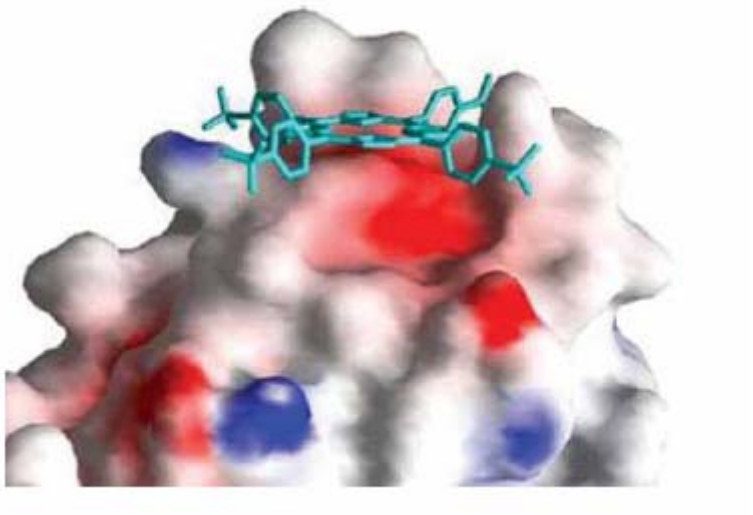

Fig. 1.3. Crystal structure of jacalin-H_2TPPS complex. The porphyrin molecule is shown in stick form and the ligand binding region of jacalin is shown in a molecular surface representation colored according to charge (red, negative; blue, positive). Reproduced from Goel et al. [2004]. (Copyright (2004) International Union of Crystallography).

Porphyrin binding to peanut agglutinin

Peanut agglutinin is a galactose specific, homotetrameric protein of M_r 110 kDa. The four subunits of PNA associate in an unusual manner giving rise to an open quaternary structure. PNA specifically recognizes the tumor-associated T-antigen

[Lotan et al., 1975] and its ability to differentiate between T-antigen and the more abundant cryptic T-and Tn antigens makes it a useful diagnostic tool [Swamy et al., 1991; Sharma et al., 1996]. Crystal structures of PNA with H_2TPPS as well as the ternary complex of PNA with lactose and H_2TPPS were reported [Goel et al., 2005].

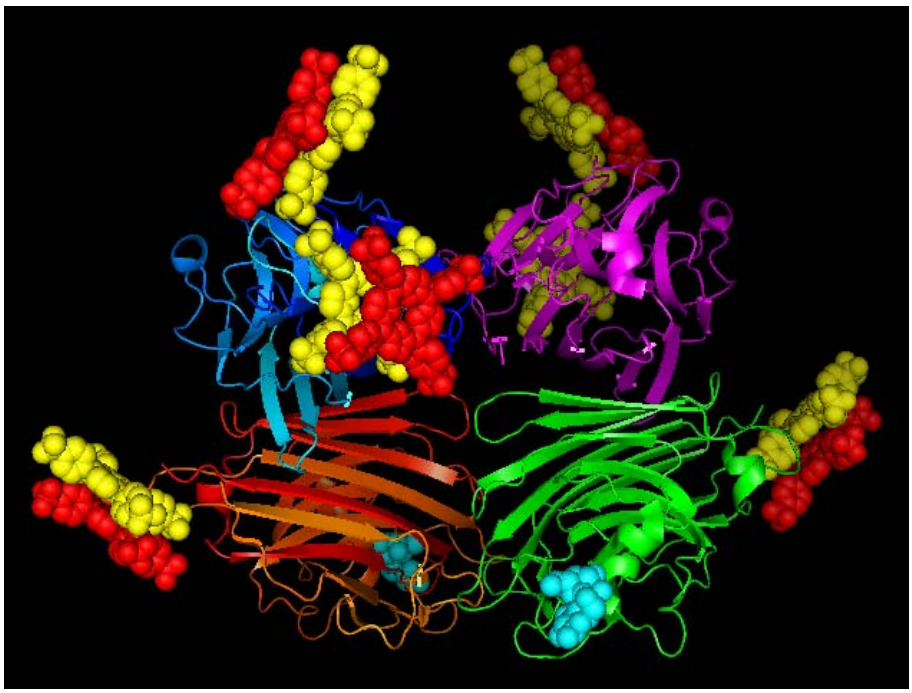

Fig. 1.4. Crystal structure of H_2TPPS-PNA-lactose ternary complex. Each subunit is shown in different colors (blue, subunit A; pink, subunit B; red subunit C; green, subunit D). Porphyrin molecules are shown as space-filling models in green and blue. Lactose (black) occupies the carbohydrate binding site in subunits C and D of the PNA ternary complex but not in subunits A and B. Reproduced and modified form Goel et al. [2005]. (Copy right (2005) American Chemical Society).

In both the complexes two porphyrin dimers bind at equivalent sites on subunits A and B, and two other porphyrin dimers bind at equivalent sites on C and D. However, binding sites on subunits A and D (or B and C) are not equivalent, which is in contrast to the crystal structure of ConA-H_2TPPS and jacalin-H_2TPPS

complexes where porphyrin dimers bind at equivalent sites on all four subunits of lectin tetramer. Additionally, a porphyrin trimer binds at a hydrophobic region located at the A-B interface. Thus in both H_2TPPS-PNA binary complex and H_2TPPS-PNA-lactose ternary complex four porphyrin dimers and one porphyrin trimer bind to the PNA tetramer at sites that are different from the carbohydrate binding sites.

Protein folding

Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three dimensional structure. Sanger determined the amino acid sequence of insulin, the first protein for which the amino acid sequence was determined [Sanger, 1952]. With the well known experiment of Anfinsen this area gained momentum where he has shown that a protein with broken disulfide bonds unfolded by urea can spontaneously refold, restoring its native disulfide bonds with full activity [Anfinsen, 1973]. This led him to propose that the three-dimensional structure is determined by the sequence of amino acids. The thermal unfolding of RNase A was shown to be a two-state process [Ginsburg & Carroll, 1965]. Unfolding of many small proteins was characterized by Tanford using the denaturant guanidine hydrochloride and it was observed that in each case the unfolding reaction was a two state process [Tanford, 1968,1970]. A two-state folding transition is explained simply as an equilibrium between a single folded conformation, and an unfolded state. The transition involves only these two states, with no accumulation of stable intermediates.

The Levinthal paradox [Levinthal, 1968] observes that if a protein folds by a complete search of all possible conformations, it would take an astronomical amount of time to do so, even if the molecule could try a new conformation every

10^{-13} second. This has led to believe that folding takes place through a series of intermediate states and pathways. Many models have been proposed to explain protein folding mechanism. Among these, the "frame work model" describes formation of secondary structures by local interactions formed according to the primary amino acid sequence but independent of tertiary structure; these secondary structures then come together to form tertiary structure [Kim & Baldwin, 1990; Dyson & Wright, 1993].

"The hydrophobic collapse model" proposes that native protein conformation forms by a rearrangement of a compact collapsed structure, with long range interactions occurring first, which is followed by the formation of secondary structures [Dill et al., 1995; Ptitsyn, 1996]. The ``nucleation model" proposes that tertiary structure forms immediately after the formation of secondary structure [Abkevich et al., 1994; Wetlaufer, 1973]. "Folding funnel theory" represents energy surface of a protein folding pathway as a funnel with a large number of unfolded conformations at the rim, and a single global minimum representing the native folded conformation and proposes that there is not just one but many possible paths from the denatured state to the folded state and each path leads downhill in energy [Leopold & Onuchic, 1992; Bryngelson, 1995].

Thermal unfolding studies on lectins by differential scanning calorimetry

Thermal unfolding processes of a number of legume lectins have been well characterized. Legume lectins are of interest to study as they have the remarkable characteristic of associating in different ways to form dimers and tetramers even though they have rather similar secondary and tertiary structures.

Detailed thermodynamic studies have been carried out on the thermal unfolding of Con A, pea lectin, lentil lectin, ECorL, WBA I and II, abrin and peanut lectin [Schwarz et al., 1993; Srinivas et al., 1998; Surolia et al., 1996; Schwarz et

al., 1991, Krupakar et al., 1999; Reddy et al., 1999]. Con A, pea lectin and lentil lectin unfold with a single irreversible transition. Unfolding temperature of Con A is 360-364K which is higher than pea lectin (343-347K) and lentil lectin (340-345K). Con A unfolds as $A_4 = 4U$, where A_4 is the folded tetramer, U is the unfolded state, while pea and lentil lectins unfold as $\alpha_2\beta_2 = 2U+2U'$, where U and U' are unfolded polypeptides. Con A, pea lectin and lentil lectin dimers unfold as single entities (ratio of calorimetric to van't Hoff enthalpy $\Delta H_c/\Delta H_v$ are close to one).

ECorL and WBA II lectins unfold irreversibly and the data could be best fitted to A↔2B two-state transition model [Surolia et al., 1996; Srinivas et al., 1998]. $\Delta H_c/\Delta H_v = 2$ indicates that the two domains unfold independently. DSC profile of WBA I dimer shows two peaks. Both transitions were irreversible. The unfolding pathway of peanut lectin is the most unusual [Reddy et al., 1999]. Thermal unfolding process of peanut agglutinin is reversible and could be best fitted to three-state model with two transitions occurring at around 331 and 336K. A $\Delta H_c/\Delta H_v = 4$ for first transition suggested that the dissociation of tetramer to monomeric units in the first step. For the second transition, the ratio of $\Delta H_c/\Delta H_v = 0.25$ suggested a non-dissociative second transition.

The DSC scan of abrin II shows two peaks (Fig. 1.5) which correspond to the B-subunit (transition temperature, (T_m) 319.2K) and the A-subunit (T_m 324.6K). Calorimetric data was fit to a two-state transition model for each of the two transition peaks [Krupakar et al., 1999]. $\Delta H_c/\Delta H_v = 2$ for the lower temperature transition, suggesting that it represents the unfolding of two domains at the denaturation temperature. Presence of lactose increases the T_m values as well as enthalpies of both transitions.

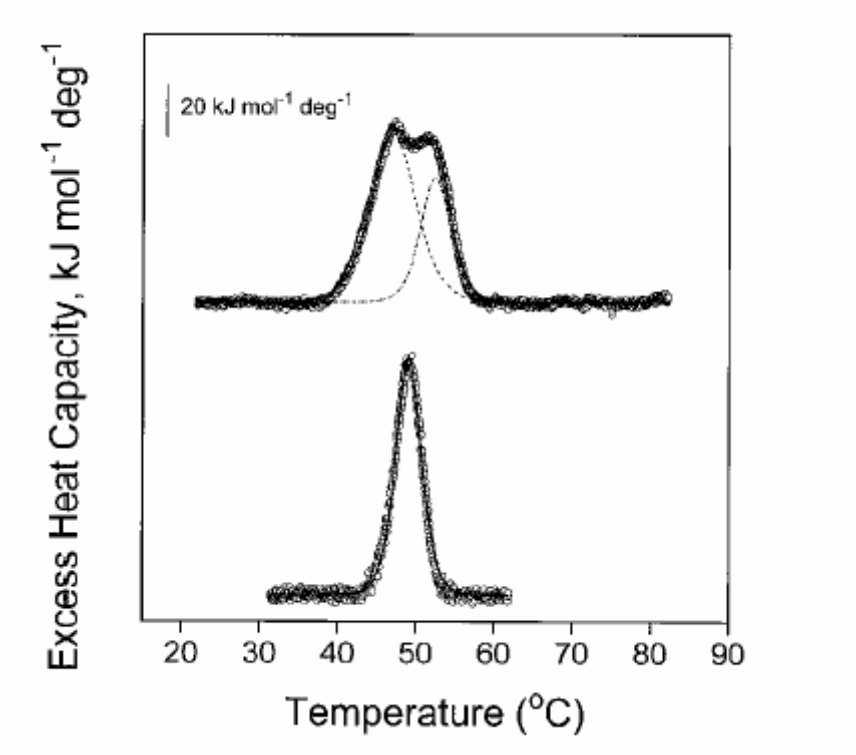

Fig. 1.5. DSC scan of Abrin II and its subunit. The DSC data for abrin II are resolved into two independent two-state curves. The data points are shown as open circles. The two-state fits of the data to lower- and higher-temperature transitions are shown as a dashed line and a dotted-dashed line, respectively. The sum of the contributions of the lower and higher-temperature components is shown as a solid line. Reproduced from Krupakar et al. [1999]. (Copyright (1999) Biochemical Society).

Cucurbitaceae seed lectins

Introduction

Lectins have been isolated from different vegetative tissues of cucurbits. Among them a few lectins isolated from the phloem exudate of cucurbits have been studied in some detail. For example chitooligosaccharide-specific lectins are reported from *Luffa acutangula, Coccinia indica, Cucurbita maxima, Cucumis sativus, Cucumis melo* have been isolated and characterized with respect to macromolecular and

carbohydrate binding properties [Anantharam et al., 1986; Sanadi & Surolia, 1994; Sabnis & Hart, 1978; Allen, 1979]. Along with phloem lectins, lectins have been reported from the pulp of *Cucumis sativus* [Skubatz & Kessler, 1988], and from root stocks of *Bryonia dioica* and *Marah macrocarpus* [Peumans et al., 1984, 1987].

Among the different plant lectins studied, legume lectins have been extensively studied. The amino acid sequences of many legume lectins have been determined and three-dimensional structures of a number of the have been elucidated by X-ray crystallography [Sharon & Lis, 2003]. Understanding of the relation between primary, secondary and tertiary structure of legume lectins resulted in the applications of these lectins. Studies on lectins from other families are very less and there is a need to characterize carbohydrate binding specificities and molecular properties of these lectins. The laboratory of M. J. Swamy (where the present work was carried out) has characterized lectins from *Tichosanthes anguina* (snake gourd), *Trichosanthes cucumerina*, *Trichosanthes dioica*, *Momordica charantia* (bitter gourd) seed lectins in considerable detail.

Isolation and physico chemical characterization

All the cucurbit seed lectins, isolated so far are galactose specific. A galactose-specific lectin has been purified from the seeds of *Trichosanthes kirilowii* Maximowicz. This lectin is a hetero dimer with an M_r of 57 kDa; the subunit masses are 37 and 27 kDa. It is a glycoprotein with an acidic pI [Falasca et al., 1989]. *Momordica charantia* (bitter gourd) seed lectin (MCL) is an $\alpha_2\beta_2$ type of tetramer with M_r of 120 kDa. It is a glycoprotein with about 10% of hexose sugars. MCL 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., 1981; Ng et al., 1986]. *Trichosanthes anguina* (snake gourd) seed lectin

(SGSL) is a heterodimer with an M_r of 55 kDa and subunits of M_r 32 kDa and 23 kDa. Snake gourd seed lectin is a glycoprotein with about 3.0% covalently bound sugar and a pI of 5.0 [Komath et al., 1996; Komath & Swamy 1998].

Trichosanthes cucumerina seed lectin (TCSL) is a glycoprotein of Mr 62 kDa that is made up of two nonidentical subunits of M_r 41 and 22 kDa. IEF experiments performed on TCSL gave three well separated peaks, indicating that the lectin may contain three isolectins with different pI [Padma et al., 1999]. *Trichosanthes dioica* seed lectin (TDSL) is a glycoprotein with 4.9% covalently bound sugar, with an M_r of 55 kDa, with subunits of M_r 37 and 24 kDa [Sultan et al., 2004b]. In SDS-PAGE, all these lectins moved as single bands in the absence of β-mercaptoethanol, whereas in the presence of β-mercaptoethanol two bands were observed suggesting that all of them are made up of two non identical subunits that are connected by one or more disulphide bridges [Komath et al., 1996; Padma et al., 1999; Sultan et al., 2004b].

Ouchterlony double-immunodiffusion experiments

Ouchterlony double-immunodiffusion experiments showed that anti-SGSL antiserum recognizes TCSL and vice versa, demonstrating that these two lectins are antigenically very similar [Padma et al., 1999]. This suggests that these two lectins are likely to be closely related in primary sequence as well as in the three-dimensional structure. On the other hand anti-TCSL anti serum did not recognize MCL and anti-MCL antiserum failed to cross-react with TCSL. These observations indicate that SGSL and TCSL are structurally different from MCL. It may also be noted that SGSL and TCSL are dimers, where as MCL is a tetramer [Komath et al., 1996; Padma et al., 1999].

Effect of pH and thermal stability

The hemagglutination activity of several cucurbitaceae seed lectins was found to depend on the pH of the medium. Agglutination activity of MCL and TCSL is maximum in the pH range 7.4-11.0 [Kenoth et al., 2003; Sultan et al., 2005]. For SGSL highest activity is seen in the pH range 6.0-10.0 [Komath et al., 2001]. At lower pH values all the three lectins show a steep decline in the activity. Lectin activity of MCL and SGSL is unaffected between 4 and 40 ^{o}C and decreases quite steeply between 50 and 60^{o} C. TCSL shows maximal activity between 4 and 60 ^{o}C, which decreases sharply at higher temperatures.

Chemical modification

Chemical modification studies indicate that tryptophan and tyrosine residues are important for the carbohydrate binding of *Momordica charantia* seed lectin (MCL) [Mazumder et al., 1981]. In the case of SGSL and TCSL imidazole side chains of histidine residues have been identified to be necessary for the carbohydrate binding and cell agglutinating activities [Komath et al., 1998, Kenoth et al., 2000]. Tyrosine residues are essential for the carbohydrate binding of *Trichosanthes dioica* seed lectin [Sultan et al., 2004b].

CD spectroscopy

Cucurbitaceae seed lectins are predominantly β-sheet proteins. Analysis of far UV CD spectra indicates that MCL is an α/β protein with predominant β-sheet content (36%) whereas the α-helical content is relatively much less (13%). Near UV CD spectrum of MCL is characterized by two prominent minima around 276 nm and 283 nm and a smaller minimum around 293 nm; these features have been assigned to the contributions from side chains of tryptophan and tyrosine residues [Sultan et al., 2004a]. Circular dichroism studies reveal that TCSL contains about 28.4% β-

sheet, 10.6% turns, while the α-helical content is negligible. The spectrum in the near UV region exhibits a minimum at 283 nm [Kenoth et al., 2003]. TDSL contains 13.3% α-helix, 36.7% β-sheet, 19.4% β-turns, and 31.6% unordered structure. The CD spectrum in the near UV region is characterized by a minimum around 283 nm and a shoulder at 293 nm [Sultan et al., 2004b].

Carbohydrate specificity and energetics of carbohydrate binding

Momordica charantia seed lectin is a galactose specific lectin. MCL has two identical carbohydrate binding sites. Thermodynamic parameters associated with the binding of fluorescently labeled sugars, 4-methylumbelliferyl-α-D-galactopyranoside (MeUmbαGal) and 4-methylumbelliferyl-β-D-galactopyranoside (MeUmbβGal) to MCL were determined by fluorescence spectroscopy [Sultan & Swamy, 2003]. Association constants obtained for β-anomers were in the range of 1.63-3.48 × 10^4 M^{-1} at different temperatures. K_a values obtained for the α-anomer were in the range of 0.99-1.69 × 10^4 M^{-1}. These values suggests that MCL prefers the β-anomer than the α-anomer. Thermodynamic parameters associated with the binding of underivatized sugars to MCL were investigated by isothermal titration calorimetry. Association constants obtained for different sugars were in the range of 0.56-1.31 × 10^4 M^{-1}. K_a values for *p*NPβGal and *p*NPβthioGal are even higher than for the umbelliferyl sugars, indicating that aromatic aglycon possibly exhibits some favorable interaction with the lectin combining site [Mazumdar et al., 1981; Sultan & Swamy, 2003,2005a]. Carbohydrate binding to MCL is primarily governed by enthalpic forces, with negative contribution from entropy. ΔC_p values obtained for the binding of different sugars to MCL are negative [Sultan & Swamy, 2005a]. Enthalpy-entropy compensation was observed for the binding of different sugars to MCL (Fig. 1.6). ΔC_p values for disaccharides were found to be nearly twice in magnitude as compared

with that of monosaccharides, which correlate well with changes in the solvent accessible surface areas [Sultan & Swamy, 2005a]. Thermodynamic parameters obtained for the interaction of MCL with different sugars are given in Table 1.1.

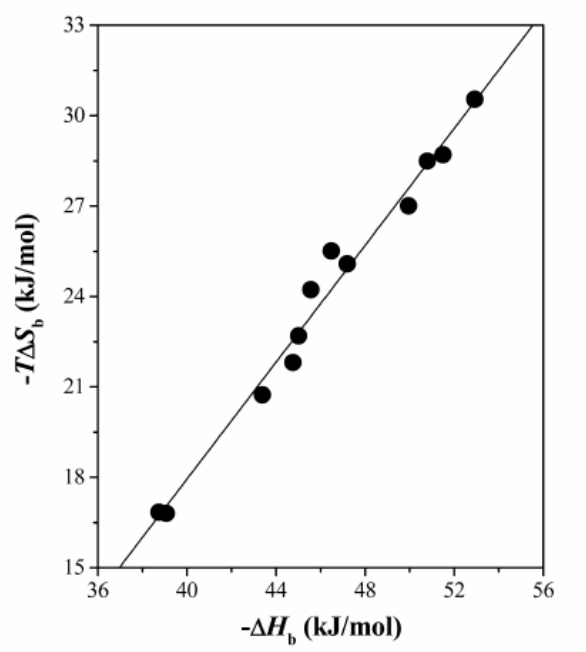

Fig. 1.6. Enthalpy-entropy compensation plot for MCL-saccharide interactions. The values of $-\Delta H_b$ and $-T\Delta S_b$ listed in Table 1 have been plotted. The straight line corresponds to a linear least squares fit of the data. Slope of the line is 0.965 and the correlation coefficient (R) is 0.991. Reproduced from Sultan & Swamy [2005]. (Copy right (2005) Elsevier Inc).

SGSL is also a galactose specific lectin with preference for the β-anomer. Binding of MeUmbβGal to SGSL resulted in a significant enhancement of fluorescence intensity of the sugar [Komath et al., 2001]. Each lectin subunit has one carbohydrate binding site. Carbohydrate binding of SGSL is enthalpy driven with negative contribution from entropy. Enthalpy-entropy compensation was observed for all the sugars.

Table 1.1. Association constants (K_b) and thermodynamic parameters for the binding of carbohydrates to *M. charantia* lectin. [a] Data obtained from a single titration experiment. [b] Data from three independent titrations. Reproduced from Sultan & Swamy [2005a] (Copy right (2005) Elsevier Inc).

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

Binding constants obtained for the interaction of lactulose were 2.7 times greater than that obtained for the interaction of lactose with SGSL. TCSL is yet another galactose-specific lectin with two sugar binding sites per dimer. Among the monosaccharides studied the lectin exhibits highest affinity for MeβGal, followed by MeαGal, demonstrating the preference of the lectin for the β-anomer. Galactose and Gal*N*Ac bind to TCSL with affinities that are 2 and 2.6 times weaker as compared to MeβGal. The association constant obtained for the interaction of MeUmbβGal with TCSL is 4.9×10^4 M^{-1} at 25°C. The association constants obtained with different saccharides are in the range of $4.9\text{-}0.94 \times 10^4$ M^{-1}.

Fluorescence quenching studies

Fluorescence emission maxima of MCL, SGSL, TCSL and TDSL are seen at 335 nm, 331 nm, 331 nm and 328 nm, respectively, indicating that tryptophan residues in these lectins are in a relatively hydrophobic environment. In all cases, quenching was highest with the neutral quencher, acrylamide, followed by succinimide, whereas extent of quenching with ionic quenchers, I^- and Cs^+ for all these lectins is significantly less [Padma et al., 1998; Komath & Swamy, 1999; Kenoth & Swamy, 2003; Sultan & Swamy, 2005b]. There is a large red shift in the emission maximum of the lectins upon denaturation by 6 M Gdn.HCl indicates greater exposure of the tryptophan residues to the aqueous environment. Sugar binding does not lead to any significant shift in the λ_{max} indicating that there are no major conformational changes which can lead to exposure of tryptophan residues to aqueous environment. However, in case of MCL extent of quenching is less in presence of lactose when compared to native lectin, suggesting that the sugar ligand provides a partial protection to the tryptophan residues [Padma et al., 1998]. Chemical modification studies on MCL indicate that tryptophan, and tyrosine residues are important for lactose binding. Even in case of TDSL, presence of lactose provided a partial

protection against quenching by I^-, Cs^+ and succinimide, but not acrylamide, where tyrosine residues are involved in the lectin activity [Sultan & Swamy, 2005b]. For SGSL and TCSL, presence of lactose did not significantly alter the extent of quenching [Komath & Swamy, 1999; Kenoth & Swamy, 2003].

Porphyrin binding to cucurbitaceae lectins

Thermodynamics of porphyrin binding in solution have been investigated for *Trichosanthes cucumerina* seed lectin (TCSL) and *Momordica charantia* (bitter guard) seed lectin (MCL). TCSL and MCL are β-galactose specific glycoproteins from the Cucurbitaceae family [Mazumdar et al., 1981; Padma et al., 1999; Kenoth et al., 2003]. Porphyrin binding to TCSL has been investigated by absorption spectroscopy at different temperatures. The association constants obtained for the TCSL-porphyrin complexes are in the range of 2×10^3 to 2×10^5 M^{-1} which are in the same range as the values obtained for porphyrin binding to other lectins and proteins [Kenoth et al., 2001]. Thermodynamic parameters obtained from van't Hoff analysis indicate that porphyrin interaction with TCSL is governed primarily by entropic forces, though there is some enthalpic contribution to the binding process. Stopped-flow studies carried out on the binding of CuTMPyP to TCSL indicate that porphyrin binding to TCSL is four times slower than diffusion controlled process and probably involves an intermediate. The dissociation rate constant of 0.29 s^{-1} obtained indicates that dissociation reaction is a slow process, which is comparable to the slow dissociation observed with fluorescently labeled saccharides bound to various lectins [Swamy et al., 1986; Puri et al., 1993]. Association constants for the interaction of different porphyrins with MCL vary between 5×10^3 to 1×10^5 M^{-1}. Association constants obtained in presence of lactose are comparable to those obtained in the absence of sugar, suggesting that porphyrin and carbohydrate ligands bind at different sites on the lectin. Presence of porphyrin

did not affect the secondary and tertiary structure of MCL [Sultan et al., 2004a]. Thermodynamic parameters obtained for MCL-porphyrin interaction suggest that the interaction is predominantly driven by stronger enthalpic contribution and that the entropic contribution is negative. Significant enthalpy-entropic compensation is observed, indicating the involvement of water molecules in the overall binding process.

Motivation and focus of the present work

The unusual ability of lectins to decode the information encoded in carbohydrates, and act as recognition determinants in a number of biological processes has made them important molecules to study. New lectins are being regularly discovered, and new properties of these lectins are being characterized by research groups all over the world. Among lectins, legume lectins are well studied and a great deal of information including primary structure and crystal structure of many of them is available. This has led to the application of these lectins in other areas of research as well.

A detailed knowledge of the properties of lectins is important in order to understand the structure-function relationships of these molecules. It is important to isolate new lectins and undertake physicochemical characterization of these lectins, which is expected to eventually lead to understanding and exploitation of lectins from other plant families as well. Since many species from Cucurbitaceae are cultivated for food in different parts of the world, it is of considerable interest to purify and characterize lectins from this family, and therefore in our laboratory we have undertaken a project on the purification and characterization of seed lectins from cucurbitaceae family. In previous work from our laboratory lectins from the seeds of *Trichosanthes anguina, Trichosanthes cucumerina,* and *Trichosanthes dioica* have been isolated and characterized.

In this study we have chosen to carry out further investigations on Cucurbitaceae seed lectins. In this direction, binding of hydrophobic ligands such as adenine, cytosine and uracil, as well as ANS to *Momordica charantia* (bitter gourd) seed lectin was studied by fluorescence spectroscopy. Thermal, chemical and acid unfolding of MCL was investigated by differential scanning calorimetry (DSC), fluorescence spectroscopy and circular dichroism spectroscopy. Detailed DSC, CD and fluorescence spectroscopic studies were carried out in order to investigate thermal and chemical unfolding of *Trichosanthes dioica* seed lectin.

A new galactose specific lectin from the seeds of *Trichosanthes cordata* has been purified by affinity chromatography and characterized with respect to macromolecular properties, carbohydrate specificity and secondary structure. Fluorescence quenching studies were carried out to understand the environment of tryptophan residues of this lectin. The results of the above studies are presented in chapters 2-5. In chapter 6 they are discussed and their relevance vis-à-vis other, similar studies. Thermodynamic studies on the interaction of porphyrins with pea lectin (*Pisum sativum*) are presented in an appendix to the main thesis.

Chapter 2

Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* (bitter gourd) seed lectin

Summary

The interaction of *Momordica charantia* (bitter gourd) seed lectin (MCL) with several nucleic acid bases has been investigated by monitoring changes induced in the protein fluorescence by ligand binding. Values of the binding constant, K_a were obtained as 1.1×10^4, 1.56×10^4 and 2.2×10^3 M^{-1} for adenine, cytosine and uracil, respectively. In addition, binding of 8-anilinonaphthalene 1-sulfonate (ANS) with MCL was investigated by fluorescence spectroscopy. Interaction with MCL at low pH results in a large enhancement of the fluorescence intensity of ANS with a concomitant blue shift in the emission λ_{max}, whereas at neutral and basic pH changes in both fluorescence intensity and emission maximum were very small, clearly suggesting that the MCL-ANS interaction is stronger at lower pH values. When excited at 295 nm in the presence of ANS, the protein fluorescence decreased with a concomitant increase in the emission intensity of ANS, suggesting resonance energy transfer from the tryptophan residues of MCL to ANS. Gel filtration profiles of MCL at pH values 2.0 and 7.4 are similar, indicating that the tetrameric nature of MCL is retained even at low pH. Addition of lactose or adenine to MCL-ANS mixture did not alter the change in ANS fluorescence suggesting that lactose, adenine and ANS bind to MCL at independent and non-interacting sites. These results are relevant to understanding the functional role of MCL in the parent tissue.

Introduction

Ability of lectins to bind carbohydrates specifically and reversibly has made them important tools for the identification and characterization of carbohydrate structures during cell growth, differentiation, malignancy etc [Lis & Sharon, 1998; Sharon & Lis, 2003]. In addition to the specific recognition of simple sugars and carbohydrate moieties of complex glycoconjugates, many plant lectins also interact with biologically relevant hydrophobic ligands such as adenine, cytokinin and indoleacetic acid, as well as hydrophobic probes, e.g., 1,8-anilinonaphthalene sulfonate (ANS) and 2,6-toluidinyl naphthalene sulfonate. Particularly, legume lectins were found to strongly bind, *in vitro*, some hydrophobic biomolecules like adenine and adenine-based plant hormones, suggesting that these may be physiologically relevant ligands for these proteins [Roberts & Goldstein, 1982, 1983; Loris et al., 1998; Gegg et al., 1992; Hamelryck et al., 1999; Srinivas et al., 2000; Komath et al., 2006]. More recent research indicates that some lectins also specifically recognize different water-soluble porphyrins with affinities comparable to those observed for the association of the lectins with specific sugars [Kavitha & Swamy, 2006; Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001; Komath et al., 2006]. In solution, porphyrin binding appears to take place at sites that are distinctly different from the sugar binding sites, although in some cases one of the porphyrins was found to bind at the sugar binding site in the crystalline complexes [Goel et al., 2001, 2004, 2005; Komath et al., 2006].

Conservation of the binding site for adenine and related hydrophobic biomolecules among several legume lectins of different specificities and sources [Hamelryck et al., 1999] suggests that other lectins may also be able to bind such biomolecules. In other studies, the interaction of hydrophobic ligands with two non-legume lectins, namely wheat germ agglutinin (WGA) and *Pseudomonas*

aeruginosa PA-I lectin, was investigated [Stoitsova et al., 2003; Bogoeva et al., 2004].

Momordica charantia lectin (MCL) is a galactose-specific lectin isolated from bitter gourd seeds. It is a tetrameric glycoprotein with $\alpha_2\beta_2$-type subunit architecture and exhibits strong type-1 and weak type-2 ribosome inactivating protein activities [Barbieri et al., 1979, 1980; Mazumder et al., 1981]. MCL preferentially recognizes the β-anomer of galactose over the α-anomer [Das et al., 1981; Sultan et al., 2003]. Interaction of MCL with different saccharides is well characterized by fluorescence spectroscopy and isothermal titration calorimetry [Sultan & Swamy, 2003, 2005a; Das et al., 1981]. Additionally, MCL also interacts with various free base and metallo-porphyrins and the binding is primarily governed by enthalpic forces [Sultan et al., 2004a]. In this study, the interaction of MCL with nucleic acid bases adenine, cytosine and uracil and a fluorescent hydrophobic probe, ANS has been investigated using fluorescence titrations. The results obtained indicate that carbohydrate ligands, nucleic acid bases and ANS bind to MCL at non-interacting sites and that the association constants for the latter two are comparable to those obtained for the interaction of various sugars with this lectin.

Materials and methods

Materials

Bitter gourd seeds were obtained from local seed suppliers. Guar gum, lactose, adenine, cytosine and uracil were obtained from Sigma (St. Louis, MO, USA). Sephadex G-100 was obtained from Pharmacia (Uppsala, Sweden). All other reagents were obtained from local suppliers and were of the highest purity available. For the experiments at different pH, the following buffers were used: 20

mM KCl-HCl (pH 1.0-2.0) 20 mM citrate-phosphate (3.0-5.0), 20 mM phosphate (pH 6.0-7.0), 20 mM Tris-HCl (pH 8), and 20 mM glycine/NaOH (pH 9.0-11.0). NaCl (150 mM) was included in all buffers to maintain a constant ionic strength.

Purification of *Momordica charantia* seed lectin

MCL was purified by a combination of ammonium sulphate precipitation and affinity chromatography on cross-linked guar gum [Sultan & Swamy, 2005; Appukuttan et al., 1977] as described earlier [Padma et al., 1998]. Concentration of purified MCL was determined by using ε_{280} value of 140,590 M^{-1}. cm^{-1}, calculated by the method of Edelhoch [Edelhock, 1967], as described in [Sultan & Swamy, 2005; Gill & Hippel, 1989]. Homogeneity of the affinity purified MCL was assessed by polyacrylamide gel electrophoresis, where it yielded a single band, consistent with earlier reports [Mazumder et al., 1981]. Desired concentrations of nucleic acid bases were obtained by dissolving accurately weighted amounts in an appropriate volume of the buffer. ANS concentration was determined by using its extinction coefficient of $E_{350}^{1\%} = 5000\ M^{-1}.cm^{-1}$ [Weber & Young, 1964].

Nucleic acid base binding to MCL

The interaction of nucleic acid bases, viz., adenine, cytosine and uracil with MCL was investigated by monitoring changes in the fluorescence intensity of the protein resulting from the binding of the ligand. Fluorescence spectra were recorded on a Spex Fluoromax-3 spectrofluorimeter using 2 and 8 nm slits on the excitation and emission monochromators, respectively. Titrations were carried out by adding small aliquots (5-20 μL) of the nucleic acid base stock (5-10 mM) in PBS to 2.0 mL of MCL (7.15 μM) in the same buffer. Samples were excited after an equilibration period of 90 seconds with 295 nm light and the emission spectra were recorded above 300 nm. Each spectrum was the average of two successive scans. All titrations were made at a constant room temperature of 27±1 ºC. Titrations were

performed in duplicate and the average K_a values are given. Spectra were corrected for inner filter effects and dilution effects.

ANS binding to MCL

Interaction of ANS with MCL was investigated by two different types of experiments. In the first type of experiment, carried out in PBS, small aliquots of ANS were added from a 1.0 mM stock solution to 2.0 mL of 2.3 μM MCL and after each addition, the fluorescence spectrum of the sample was recorded on a Spex Fluoromax-3 fluorescence spectrometer using 3 nm and 5 nm slits on the excitation and emission monochromators, respectively. Samples were excited at 365 nm, and the emission spectra were recorded between 400 and 600 nm. In another set of experiments, aimed at investigating the influence of pH on the MCL-ANS interaction, ANS samples (50 μM) in buffers of different pH with or without MCL (2.3 μM) were excited at 390 nm and the fluorescence spectrum was recorded above 400 nm. In another set of experiments MCL samples in the presence of different concentrations of ANS were excited at 295 nm, and the emission spectra were recorded above 300 nm. All fluorescence spectra were corrected for dilution effects, inner filter effects and for fluorescence of free ligand determined in parallel titrations without the protein.

Circular Dichroism spectroscopy

CD spectra were recorded on a Jasco-J-810 spectropolarimeter at a scan speed of 50 nm/min with a response time of 4 s and a slit width of 1.5 nm. A cylindrical quartz cell of 1mm path length was used. MCL concentration was 1.3 μM in tetramer for all the measurements. To investigate the effect of ANS, CD spectra were recorded for MCL samples incubated with 20 μl of 5 mM ANS. Each spectrum recorded is

the average of four successive scans. Buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

Results and discussion

Nucleic acid base binding to MCL

Association constants for the binding of nucleic acid bases to MCL were determined by monitoring ligand-induced changes in the fluorescence properties of the lectin. Fig. 2.1A shows the fluorescence titration of MCL with cytosine where spectrum 1 corresponds to MCL alone and spectra 2-10 correspond to MCL in the presence of increasing concentrations of cytosine. It is clearly seen from this figure that upon excitation at 295 nm, MCL yields an emission spectrum centred at 332 nm (spectrum 1). Addition of cytosine resulted in a decrease in the emission intensity of MCL without any noticeable change in the emission maximum (spectra 2-10). Similar spectra were obtained for the titration of MCL with adenine and uracil (not shown). A plot of the change in fluorescence intensity (ΔF) as a function of added cytosine concentration yields the binding curve for the MCL-cytosine titration (Fig. 2.1B), which shows the gradual saturation of MCL binding sites with increasing concentrations of cytosine. The above fluorescence data were analysed in the following manner in order to obtain the association constants, K_a. First a plot of $1/\Delta F$ *vs* $1/[L]$ was obtained from the fluorescence spectra corresponding to the MCL-cytosine titration (see Fig. 2.2A). Here ΔF ($=F_o-F$) is the change in fluorescence intensity at any point in the titration, and F_o and F are the fluorescence intensities of MCL alone and with added ligand, respectively, and $[L]$ is the ligand concentration.

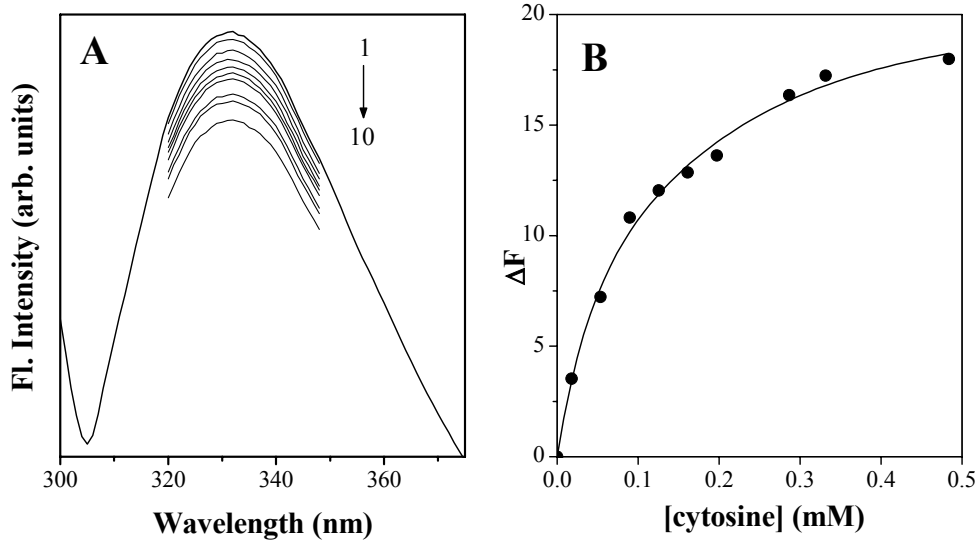

Fig. 2.1 A) Emission spectra of MCL recorded alone and after adding increasing aliquots of cytosine to 2.0 mL of MCL in PBS. Spectrum 1 corresponds to MCL alone and spectra 2-10 correspond to MCL in the presence of increasing concentrations of cytosine. B) Binding curve obtained by plotting the change in fluorescence as a function of cytosine concentration.

From the intercept of the plot the fluorescence intensity of the sample at infinite concentration, F_∞ was calculated. Using the F_∞ value obtained, the fluorescence data was further analysed according to the Chipman equation, which is given by [Chipman et al., 1967]:

$$\text{Log } \{\Delta F/(F-F_\infty)\} = \log K_a + \log [L]_f \qquad (2.1)$$

where $[L]_f$ is the free ligand concentration, and is given by:

$$[L]_f = [L]_t - \{(\Delta F/ \Delta F_\infty).[P]\} \qquad (2.2)$$

Eq. 1 yields the pK_a of the interaction from the X-intercept of a plot of log $\{\Delta F/(F-F_\infty)\}$ versus log $[L]_f$. Such a plot for the MCL-cytosine titration is given in Fig. 2.2 B, the X-intercept of which yields the pK_a value as -4.09. From this value the association constant, K_a was obtained as 1.56×10^4 M^{-1}. The slope of the plot was

close to unity indicating that each MCL subunit binds one molecule of the nucleic acid base. The fluorescence titration data obtained for the interaction of adenine and uracil with MCL was also analysed in a similar manner and the corresponding association constants were determined as 1.1×10^4 and 2.2×10^3 M^{-1} for adenine and uracil, respectively. These association constants are in the same range as those estimated earlier for the binding of carbohydrates to MCL [Das et al., 1981; Sultan & Swamy 2003, 2005a], suggesting that nucleic acid base binding also may have a physiological role like carbohydrate binding.

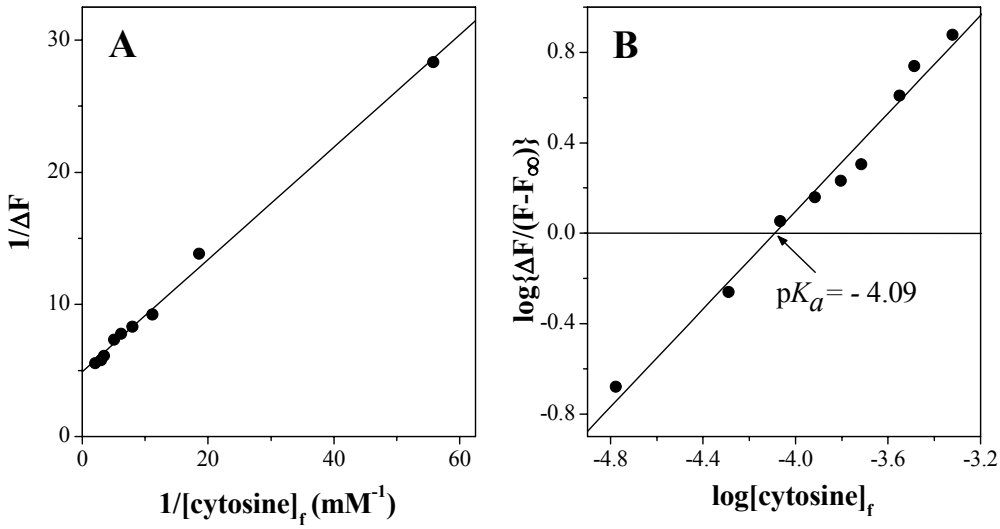

Fig. 2.2. A) A double reciprocal plot of 1/Δ*F* versus 1/[cytosine]. From the Y-intercept the fluorescence change, ΔF_∞ at saturation binding is obtained. B) A double-log plot of the binding of cytosine to MCL. X-intercept gives the pK_a.

In order to investigate the effect of carbohydrate binding on the MCL-nucleic acid base binding, we have carried out fluorescence titrations for the binding of adenine in the presence of 0.1 M lactose. These titrations yielded a K_a value of 1.06×10^4 M^{-1} for the MCL-adenine interaction, which is in excellent agreement with the value of 1.1×10^4 M^{-1} obtained in the absence of lactose, indicating that adenine

and lactose bind at independent sites on the protein. Previous work from this laboratory has demonstrated that several water-soluble porphyrins that are predominantly hydrophobic in nature also interact with MCL at a site that is distinct from the carbohydrate binding site [Sultan et al., 2004a]. In this respect MCL is similar to most legume lectins in having binding sites for non-carbohydrate ligands that are distinctly different from the sugar binding sites. For example, radioligand binding experiments demonstrated that presence of Gal*N*Ac had no effect on the binding of adenine to *Dolichos biflorus* lectin [Gegg et al., 1992]. Additionally, the three dimensional structure of DBL in complex with adenine, obtained from single-crystal diffraction studies, has shown that adenine binds to DBL at cavities formed by dimer-dimer interfaces whereas carbohydrate ligands bind at shallow depressions on the surface of the protein, which are well removed from the dimer interfaces [Gegg et al., 1992; Hamelryck et al., 2006]. Adenine binding to WBA I was also not influenced by the presence of sugar ligand, methyl-α-D-galactopyranoside [Puri & Surolia, 1994].

ANS binding to MCL

Fluorescence titrations of ANS binding to MCL were carried out at pH 2 and pH 7. Spectra corresponding to a titration performed at pH 2.0 are given in Fig. 2.3A. It can be seen from this figure that the fluorescence intensity of ANS is enhanced upon binding to MCL. Enhancement in the fluorescence intensity of ANS was also observed for the binding of ANS to a number of other lectins [Roberts & Goldstein, 1982, 1983]. The binding curve given in Fig. 2.3B shows that increasing ANS concentration leads to an increase in the change in fluorescence intensity, although the magnitude of the change decreases with increasing ANS concentration, depicting saturation behaviour. From the titration data the affinity constant for the binding of ANS to MCL at pH 2.0 was estimated by Scatchard analysis as

described in [Roberts & Goldstein, 1982]. Lectin concentration used was very low in order to maintain $[ANS]_{bound} << [ANS]_{total}$. In order to analyse the data further $[ANS]_{total}$ was used as a good approximation of free ligand concentration [Roberts & Goldstein, 1982].

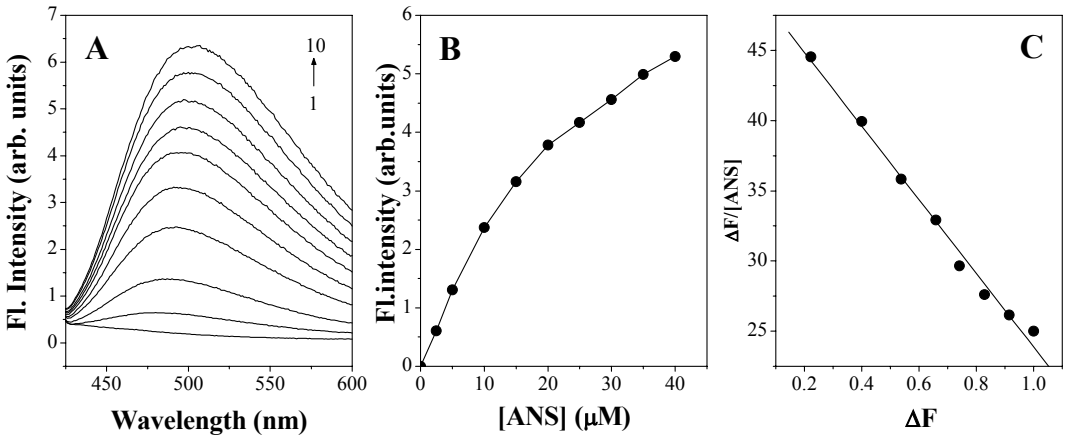

Fig. 2.3. A) Fluorescence spectra of ANS binding to MCL. ANS was excited at 365nm. Spectrum 1 corresponds to MCL alone in the absence of ANS, and spectra 2-8 were recorded in the presence of increasing concentrations of ANS. Net fluorescence enhancement was determined by subtraction of free ANS fluorescence determined in a parallel titration. (B) Binding curve for the interaction of ANS with MCL at pH 2 obtained by plotting fluorescence intensity as function of ANS concentration. C) Linear Scatchard plot (ΔF / [ANS] *vs* ΔF) of the binding data. The slope of this plot gives association constant for the interaction.

The corresponding Scatchard plot is given in Fig.2.3 C. From the slope of this plot the association constant, K_a was calculated as 2.6×10^4 M^{-1}. Very small changes were observed in the fluorescence intensity of ANS in the titrations carried out at pH 7.0, suggesting a rather weak association between MCL and ANS. The magnitude of the changes was too small for estimating an association constant from the titration data. Fluorescence spectra of ANS recorded in presence of MCL at different pH are shown in Fig. 2.4.

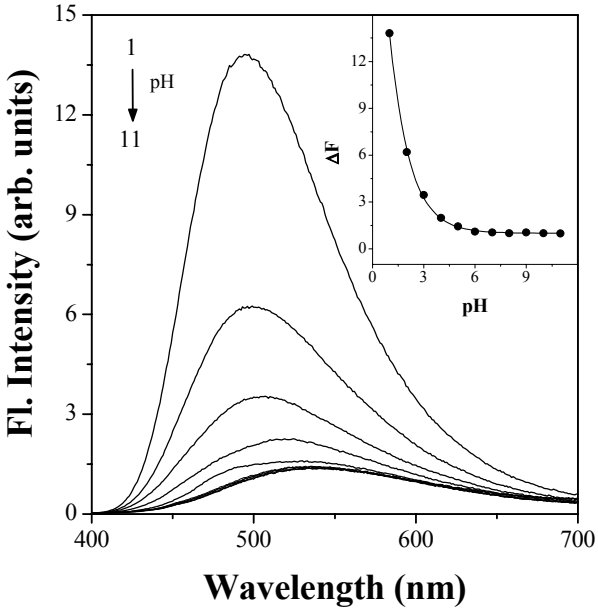

Fig. 2.4. Fluorescence spectra of ANS in the presence of MCL at different pH. Spectra were recorded from pH 1 to pH 10 at fixed ANS concentration. ANS was excited at 390 nm. The inset gives the fluorescence intensity difference between free and MCL-bound ANS at 494 nm as a function of pH.

These spectra show that the fluorescence enhancement is maximum at pH 1 and decreases up to pH 6 asymptotically and then remains constant with increasing pH. Inset of the figure shows enhancement of ANS fluorescence upon interaction with MCL as a function of pH at 494 nm. Enhancement is also accompanied by a blue shift with the emission λ_{max} of 533 nm at pH 7.0 shifting to 518 nm at pH 4.0 and to 496 nm at pH 1.0. Enhancement of ANS fluorescence intensity and associated blue shift of emission λ_{max} is generally attributed to hydrophobicity of the binding site [Stryer, 1965]. The emission λ_{max} of MCL at pH 2 is seen at 332 nm, suggesting that the Trp residues of the protein are in a relatively hydrophobic environment, i.e., buried inside the protein matrix. In addition, the far UV CD spectrum of MCL recorded at pH 2.0 is very similar to that obtained at pH 7.0, indicating that the

secondary structure of MCL at pH 2.0 is essentially identical to that at neutral pH (Fig. 2.5).

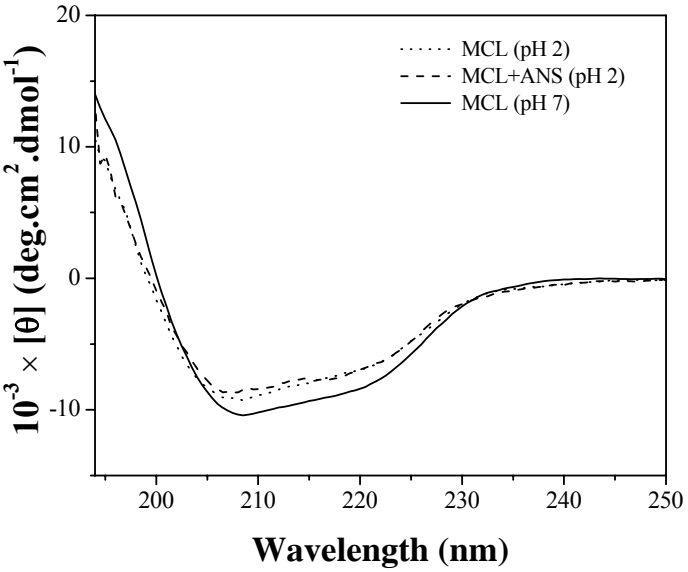

Fig. 2.5. Far UV CD spectra of MCL. (——) MCL at pH 7.0, (-----) MCL + ANS at pH 7.0, (·····) MCL at pH 2.0.

In addition, the far UV CD spectrum of MCL recorded in presence of ANS at pH 2.0 is identical to the spectrum recorded in the absence of ANS, suggesting that ANS binding did not lead to any alteration in the secondary structure of MCL. This is consistent with earlier studies where it was observed that binding of porphyrins did not lead to any changes in the secondary structure of MCL [Sultan et al., 2004a]. Finally, gel filtration profiles obtained at pH 2.0 and 7.0 are nearly identical indicating that MCL exists as a tetramer at pH 2.0 (data not shown). However, since ANS binds to MCL very weakly at pH 7.0, whereas a significantly stronger binding is seen at pH 2.0, some changes must take place at the tertiary structure level, resulting in the exposure of at least some hydrophobic residues in MCL. It is very likely that MCL exists in a molten globule-like state at low pH

with some hydrophobic patches exposed to the solvent since it has been shown for a number of proteins that formation of molten globule structure is associated with an increase in the fluorescence emission intensity of ANS [Hawe, 2008].

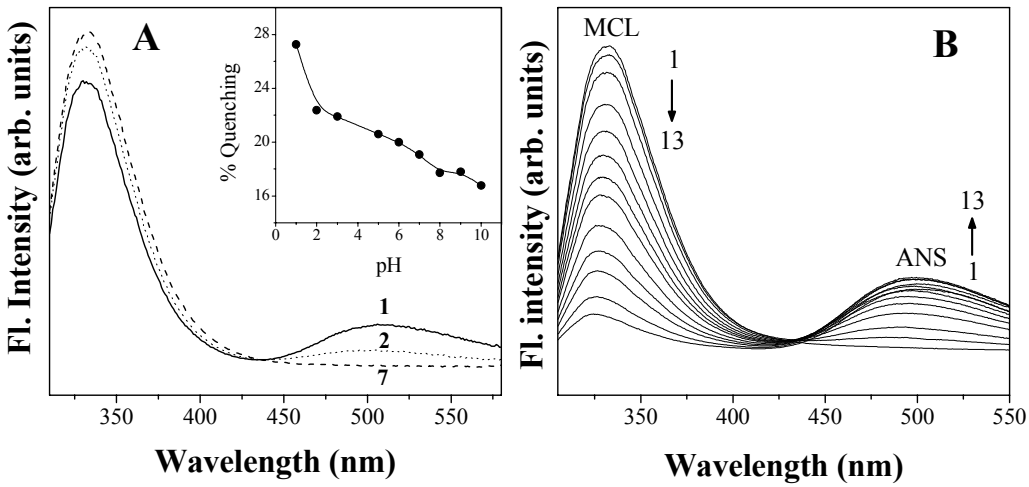

Fig. 2.6. A) Fluorescence quenching of MCL by ANS at pH 1 (solid line), pH 2 (dotted line), pH 7 (dashed line), this figure also shows energy transfer in MCL-ANS interaction at lower pH. Excitation of MCL is at 295 nm. Inset of the figure shows % quenching of MCL fluorescence by ANS at different pH. B) Fluorescence quenching of MCL in presence of different concentrations of MCL at pH 2. Spectrum 1 corresponds to MCL alone and spectra 2-13 are in presence of increasing concentrations of ANS. As MCL fluorescence intensity decreases, ANS fluorescence intensity increases. Free ANS fluorescence determined in a parallel titration was subtracted.

Fluorescence emission spectra of MCL in the presence of ANS at pH 1, 2 and 7 are shown in Fig. 2.6A. The protein was excited at 295 nm in each case. It is clearly seen from these spectra that the emission intensity of MCL is quenched considerably by ANS at low pH whereas at higher pH the extent of quenching is lower. Percentage quenching at different pH in presence of ANS is given in the inset to Fig. 2.6A. This figure shows that percentage quenching decreases with increase in pH. In addition, at low pH another peak is seen in the 450-500 nm region, which corresponds to ANS emission. This strongly suggests that there is energy transfer between the Trp residues of MCL and the bound ANS. Titrations

carried out at pH 2.0 indicate that increasing the concentration of ANS while keeping the MCL concentration fixed results in a decrease in MCL fluorescence intensity whereas the emission due to ANS increases, with an isosbestic point at ca. 435 nm (Fig. 2.6B). These observations are consistent with the interaction of ANS with hydrophobic regions on the protein surface and resonance energy transfer from the Trp residues of MCL and the bound ANS. Besides the hydrophobic interaction, electrostatic forces may also contribute to the overall association since at low pH ANS carries a negative charge whereas the protein will be positively charged. Thus both hydrophobic and electrostatic interactions are likely to play a role in the binding of ANS to MCL.

In an earlier report from this laboratory the binding of porphyrins to MCL was characterized [Sultan et al., 2004a]. In the present study the ability of MCL to interact with several nucleic acid bases such as adenine, cytosine and uracil, which are also predominantly hydrophobic in nature, is investigated. In addition, it is shown that the hydrophobic fluorescent probe, ANS binds to MCL at low pH and that this binding is associated with a significant enhancement in its emission intensity with a concomitant blue shift in the emission maximum. Taken together these observations suggest that MCL is capable of recognizing a number of hydrophobic ligands which differ significantly in size and structure, i.e., MCL exhibits considerable tolerance to the chemical structure and size of the ligand. Previously, it has been reported that WGA exhibits considerable tolerance with regard to the size and structure of the hydrophobic ligands that it interacts with [Bogoeva et al., 2004]. Therefore, it appears that MCL is similar to WGA in this respect.

The affinity exhibited by MCL for the nucleic acid bases is comparable to the affinities exhibited by this protein for mono- and disaccharides [Das et al., 1981; Sultan & Swamy, 2003, 2005] as well as different porphyrins [Sultan et al.,

2004a]. This suggests that the hydrophobic ligand binding by MCL may have biological significance, especially in view of the fact that both nucleic acid bases and porphyrins are present in biological systems in nature. Especially, adenine derivatives are known to function as phytohormones and the ability of MCL to recognize adenine and other nucleic acid bases suggests that lectins may bind to phytohormones in the parent tissue and may be involved in their transport or in modulating their free concentrations, which may be relevant to regulation of plant growth. Since adenine binding site and carbohydrate binding are independent of each other, it is possible that MCL can bind to the hydrophobic ligand and carry it to a site bearing a specific carbohydrate structure that is recognized by the carbohydrate binding site.

In summary, in the present study the association constants determined by monitoring ligand-induced changes in the protein fluorescence indicate that nucleic acid bases such as adenine and cytosine bind to MCL with affinities that are comparable to those obtained with various mono and disaccharides. Titrations performed at different pH values indicate that binding of ANS to MCL is stronger at lower pH. Presence of the specific saccharide, lactose did not affect the binding of adenine or ANS, and addition of lactose or adenine to MCL-ANS mixture did not alter the change in ANS fluorescence induced by its binding to MCL suggesting that lactose, adenine and ANS bind to MCL at independent and non-interacting sites. Gel filtration profiles of MCL at pH values 2 and 7.4 are similar indicating that the tetrameric nature of MCL is retained even at low pH.

Chapter 3

Chemical, acidic and thermal denaturation studies on *Momordica charantia* (bitter gourd) seed lectin

Summary

Thermal stability of *Momordica charantia* seed lectin (MCL) was studied as a function of pH, scan rate, and at different ligand concentrations by using high-sensitivity differential scanning calorimetry. The DSC endotherm obtained at pH 7.4 consists of two entities with different melting temperatures, with transition temperatures at ca. 333.7 K, and 338 K. The unfolding process is irreversible and could be described by a three-state model. For MCL tetramer $\Delta H_c/\Delta H_v$ ratio is close to 4 for the first transition and ~2 for the second transition, suggesting that four and two cooperative units are involved in the first and second transitions, respectively. In the presence of lactose both the low-temperature transition and high-temperature transition shifted to higher temperatures, suggesting that ligand binding stabilizes the native conformation of MCL. Endotherms recorded as a function of pH indicate that MCL has more stability at lower pH. Unfolding of MCL induced by Gdn.HCl was investigated by monitoring the intrinsic fluorescence properties of the protein. The results obtained indicated that chemical denaturation of MCL can also be described by a three-state process, involving an intermediate populated at ~3 M Gdn.HCl. These observations suggest that the chemical and thermal unfolding processes are similar in that both of them proceed via an intermediate. The far UV and near UV CD spectra of MCL were nearly identical at different pH values and indicate that secondary and tertiary structure of MCL do not change with pH, suggesting that the structure of MCL is stable over a wide pH range.

Introduction

Lectins are proteins of non-immune origin that occur ubiquitously in nature. Lectins bind carbohydrates reversibly and with a high degree of specificity. Carbohydrate binding property of lectins has made them important tools in a number of areas of biological research such as isolation and purification of glycoconjugates, mitogenic stimulation of lymphocytes as well as in clinical and biomedical applications such as blood typing, mapping of neuronal path ways etc [Lis & Sharon, 1998; Sharon & Lis, 2003].

Three-dimensional structure of proteins is maintained by various secondary forces such as hydrophobic interactions, hydrogen bonding, van der Waals' interactions, electrostatic forces and disulfide bonds. Physical and biological properties of a protein can be altered by conditions that affect its native conformation. The conformational stability is the free energy difference between the native folded state and unfolded state under physiological conditions**.** Determination of conformational stability of proteins is critical for a knowledge of the physical interactions that stabilize the protein. The stability of a protein is generally estimated based on the analysis of unfolding transitions induced by denaturants, such as urea or Gdn.HCl, or by changes in the pH, ionic strength, or temperature, measured either spectroscopically or calorimetrically [Agashe & Udgaonkar, 1995; Nicholson & Scholtz, 1996; Johnson et al., 1995].

Momordica charantia seed lectin (MCL) is a galactose specific tetrameric glycoprotein with $\alpha_2\beta_2$ – type subunit architecture. Its macromolecular properties and carbohydrate-binding specificity towards monosaccharides and disaccharides has been studied [Sultan & Swamy, 2003; Mazumder et al., 1981; Khan et al., 1981; Das et al., 1981; Padma et al., 1998]. MCL exhibits strong type-1 and weak type-2 ribosome inactivating protein activities as well as insulinomimetic activity

[Barbieri et al., 1979, 1980; Ng et al., 1986]. Circular dichroism studies reveal that MCL contains 36% β-sheet, 21% β-turns, 13% α-helix and rest unordered structure [Sultan et al., 2004a]. Thermodynamic studies on porphyrin binding to MCL indicate that the interaction is governed primarily by enthalpic forces [Sultan et al., 2004a]. Effect of pH and incubation at different temperatures on the hemaagglutination activity has been studied and thermodynamic parameters associated with the binding of different carbohydrates to MCL have been investigated in detail using isothermal titration calorimetry [Sultan & Swamy, 2005a]. ITC results indicate that carbohydrate binding to MCL is governed primarily by enthalpic forces, and the ΔC_p values for the interaction were found to be negative.

To obtain better understanding of structural and functional properties of MCL we have carried out differential scanning calorimetric studies on this protein at different pH, at different ligand concentrations, and at different scan rates. The effect of pH on the secondary and tertiary structure of MCL has been investigated by CD spectroscopy. Chemical unfolding of MCL by Gdn.HCl was investigated by monitoring changes in the fluorescence characteristics of the protein. DSC measurements provide information regarding transition temperature (T_m) for the unfolding of MCL and calorimetric enthalpy (ΔH_c), van't Hoff enthalpy (ΔH_v), and the changes in excess heat capacity (ΔC_p).

Materials and Methods

Materials

Bitter gourd seeds were obtained from local seed shops. Guar gum, lactose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside and *N*-acetyl-D-

galactosamine were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade were obtained from local suppliers.

Methods

Purification of *Momardica charantia* seed lectin

MCL was purified by a combination of ammonium sulfate precipitation and affinity chromatography on guar gum as described previously [Sultan & Swamy, 2005a]. Purity of the eluted protein was assessed by PAGE where it gave a single band, consistent with earlier reports [Mazumder et al., 1981; Padma et al., 1998]. Concentration of MCL was determined by using its extinction coefficient of ε_{280} = 140,590 $M^{-1}.cm^{-1}$, calculated by the method of Edelhock [Edelhoch, 1967] as described in [Gill & Hippel, 1989].

Differential scanning calorimetry

DSC measurements were performed on a MicroCal VP DSC ultra sensitive differential scanning micro calorimeter (MicroCal LLC, Northampton, MA, USA). Experiments were carried out as a function of pH, scan rate, and at different ligand concentrations. For the experiments at different pH samples were dialyzed extensively against large volumes of the desired buffer. Samples were degassed before loading in to cells. For experiments at different pH, the following buffers were used: 20 mM KCl-HCl (pH 1.0-2.0) 20 mM citrate-phosphate (3.0-5.0), 20 mM phosphate (pH 6.0-7.0), 20 mM Tris-HCl (pH 8), and 20 mM glycine/NaOH (pH 9.0-11.0). NaCl (150 mM) was included in all buffers to maintain a constant ionic strength. Buffer scans were subtracted from the thermograms corresponding to the lectin samples before further analysis. Data were analyzed by Origin software provided by the DSC manufacturer.

Circular dichroism spectroscopy

CD spectra of MCL samples that were dialyzed against buffers of different pH were recorded at room temperature on a Jasco J-810 spectropolarimeter at a scan speed of 50 nm/min using 10 mm path length quartz cells. Data were collected with a response time of 4 s and a slit width of 2 nm. Far UV CD spectra were recorded at a scan speed of 50 nm/min using 1 mm path length quartz cell. A slit width of 1.5 nm, and a response time of 4 s were used for data collection. Each spectrum was the average of three successive scans. Measurements in the near UV region were performed with ca. 5.9 μM MCL tetramer and for measurements in the far UV region the concentration used was 0.7 μM in lectin tetramer. Buffer scans, recorded under identical conditions, were subtracted from the spectra of the lectin before further analysis.

Fluorescence spectroscopy

Unfolding of MCL as a function of Gdn.HCl concentration was monitored by fluorescence spectroscopy. Fluorescence measurements were performed on a Spex Fluoromax-3 spectrofluorimeter (Jobin Yvon Ltd, Edison, NJ, USA, website: www.jobinyvon.com). Lectin samples (~0.7 μM) were incubated with increasing concentrations of Gdn.HCl overnight before the measurements were performed. Samples were excited at 280 nm and emission spectra were recorded above 300 nm. The slit widths used were 3 and 5 nm, respectively for excitation and emission monochromators.

Results and discussion

Differential Scanning Calorimetry

In this study, the thermal stability of *Momordica charantia* seed lectin has been investigated under different conditions by using high-sensitivity differential

scanning calorimetric measurements. Fig. 3.1 shows a typical DSC thermogram of MCL corrected for buffer base line at pH 7.2 and at a scan rate of 30^{o}C/hr along with the fit of the transition data to a non-two-state model. The calorimetric scan consists of two entities melting at different temperatures. The lower transition peak has a T_m of 333.7 K with a calorimetric enthalpy (ΔH_c) of 1157 kJ.mol^{-1}, while the higher transition peak has a T_m of 338 K with a ΔH_c of 1084 kJ.mol^{-1} (Table 3.1). Thermal denaturation of MCL was completely irreversible, since no transition could be seen when the sample was subjected to a second heating scan after it was cooled from the first run. Similar irreversible behavior has been observed with abrin II. The DSC scan of abrin II also exhibited two transition peaks which correspond to its two subunits, A and B [Krupakar et al., 1999]. In order to investigate whether the above unfolding transitions of MCL are kinetically controlled or thermodynamically controlled, DSC scans were performed at different scan rates. The results obtained, summarized in Table 3.1, indicate that the thermodynamic quantities ΔH_c and ΔH_v are independent of scan rate although a slight increase in T_m was observed when the scan rate was increased by more than a factor of 3; this increase could be accounted for by differences in the instrumental response at different scan rates. Therefore equilibrium thermodynamic model is applied instead of irreversible model [Sanchez-Ruiz et al., 1988]. The equilibrium two-state thermodynamic transition model was applied earlier to thermal transitions of winged bean (*Psophocarpus tetragonolobus*) acidic lectin [Srinivas et al., 1998] and abrin II [Krupakar et al., 1999].

The ratio of calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) for the 1st transition is greater than 3, suggesting that four entities are involved in the first transition. For the 2nd transition $\Delta H_c/\Delta H_v$ ratio is close to 2 and suggests that two

entities are involved in the second transition. The two transitions during thermal unfolding can be analyzed by two possible models as summarized below.

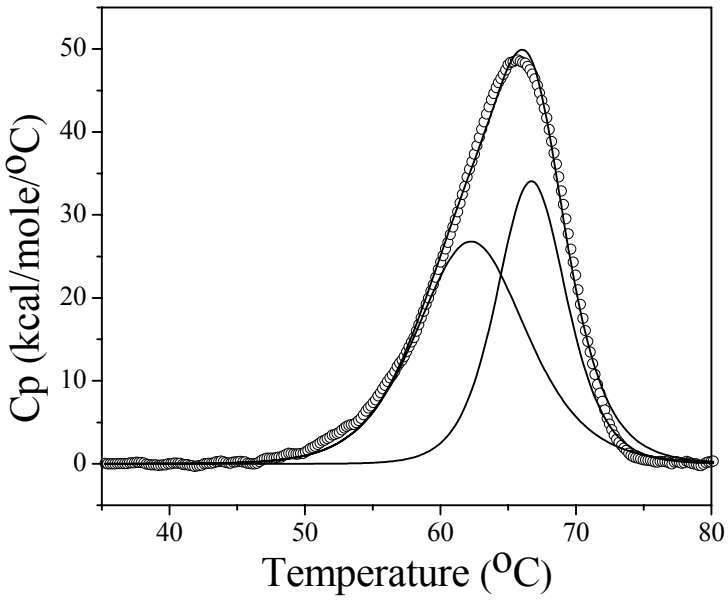

Fig. 3.1. DSC scan of *Momordica charantia* seed lectin in 20 mM phosphate buffer containing 150 mM NaCl at pH 7.2 at a scan rate of 40 k h^{-1}. The data points are shown as open circles, and the solid lines are the best fits of the DSC data to the non-two-state transition model. Concentration of MCL tetramer is 0.0035 mM.

In the first model the MCL tetramer dissociates in to dimers A_2 and B_2 in the first transition. In the second transition, the dimers dissociate into the constituent monomers which simultaneously unfold as described by the following equation:

$$A_2B_2 \Leftrightarrow A_2+B_2 \Leftrightarrow 2A_u+2B_u \qquad (3.1)$$

In the second model the tetramer dissociates into two AB dimers in the first transition. The dimers then dissociate into the constituent monomers while simultaneously unfolding as described by the following equation:

$$A_2B_2 \Leftrightarrow 2AB \Leftrightarrow 2A_u+2B_u \qquad (3.2)$$

Here A_u and B_u are the unfolded forms of A and B.

Among these two models, the second model describes the most likely mode of unfolding of MCL because according to the first model the dimers A_2 and B_2 will have the same unfolding temperature which is rather unlikely.

Table 3.1. Dependence of DSC transition quantities of MCL at pH 7.2 on scan rate. Concentration of MCL tetramer is 0.0035 mM.

Scan rate (°C/ hr)	T_{m1} (K)	ΔH_{c1} (kJ.mol^{-1})	ΔH_{v1} (kJ.mol^{-1})	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} (kJ.Mol^{-1})	ΔH_{v2} (kJ.Mol^{-1})	$\Delta H_{c2}/\Delta H_{v2}$
20	332.5	1168	344	3.39	337	977	547	1.77
30	333.7	1157	332	3.48	338	1084	546	1.98
40	335.3	1187	348	3.41	339	975	555	1.75
60	336.6	1161	336	3.45	341	1064	535	1.98

Unfolding parameters of MCL obtained from DSC scans performed in the presence of saturating amounts of different ligands are given in Table 3.2. In the presence of carbohydrate ligands, transition temperatures, calorimetric enthalpies and van't Hoff enthalpies increase, suggesting that carbohydrate binding stabilizes the native conformation of MCL. Stabilization of native structure of protein by carbohydrate binding has been observed for many lectins, including abrin II, peanut lectin, winged bean acidic lectin and *Erythrina corallodendron* lectin [Krupakar et al., 1999; Reddy et al., 1999; Surolia et al., 1996; Srinivas et al., 1998].

At saturating concentrations of lactose, the T_m value increased by 8 K for both the transitions. At constant lectin concentration the denaturation transition in the presence of bound ligand can be expressed as

$$\ln [L] = -\Delta H_v(L)/ [RT_p m] + \text{constant} \quad (3.3)$$

Table. 3.2. Thermodynamic parameters obtained from DSC measurements on MCL in presence of different concentrations of lactose. Scan rate was 30 $K.h^{-1}$. Concentration of MCL used was 0.00497 mM in dimer. The errors were less than 0.02% for T_m, and less than 10% for ΔH_c and ΔH_v.

Lactose (mM)	T_{m1} (K)	ΔH_{c1} ($kJ.Mol^{-1}$)	ΔH_{v1} ($kJ.Mol^{-1}$)	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} ($kJ.Mol^{-1}$)	ΔH_{v2} ($kJ.Mol^{-1}$)	$\Delta H_{c2}/\Delta H_{v2}$
0	333.7	1157	332	3.48	338	1084	546	1.98
5	337.9	1369	400	3.422	341.8	1214	656	1.85
10	339	1279	416	3.07	343	1208	708	1.706
20	339.7	1411	376	3.75	343.9	1335	631	2.11
30	340.1	1567	373	4.2	344.5	1418	640	2.21
50	342.2	1396	460	3.03	345.8	933	744	1.25

A plot of ln [L] vs $1/T_p$ (Fig. 3.2) yielded the van't Hoff enthalpy, where L is the concentration of ligand and T_p is the temperature at which transition peak is maximum. From this plot the van't Hoff enthalpy was calculated as 1107 kJ. mol^{-1} for the lower-temperature transition with the m value of 2. The m value was known from ITC binding studies, which showed that MCL tetramer binds to two lactose molecules [Sultan & Swamy, 2005a]. For the higher-transition peak the calculated van't Hoff enthalpy value is 597 $kJ.mol^{-1}$ which is close to the van't Hoff enthalpy values determined by non-two-state fit.

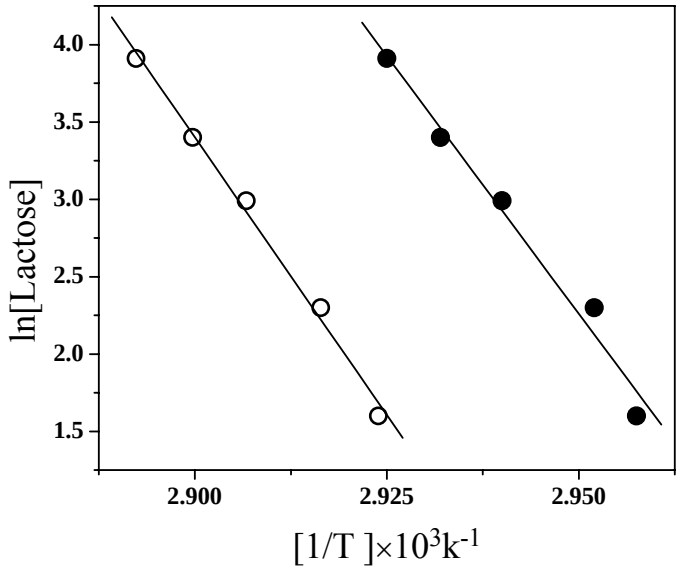

Fig. 3.2. Plots of ln[Lactose] vs 1/T_p for MCL. The lines are best linear least-square fits of ln [Lactose] against 1/Tp, for the lower temperature transition peak (•) and higher temperature transition peak (o). Scan rate was 30 ^{o}C per hour. The correlation coefficients are 0.99 for the lower-temperature and higher-temperature transition peaks.

Effect of pH

Effect of pH on the unfolding transition of MCL was studied by performing DSC scans on the MCL samples that were dialyzed against buffers of different pH in the range 2-10. The thermodynamic data obtained from these scans is given in Table 3.3. Data obtained with samples in the pH range 4-6 were unsatisfactory and could not be fit due to precipitation of the samples. It has been observed that the T_m values increase with decrease in pH between 10 and 5. A shift of about 12 K was observed when the pH was lowered from 10 to 5, indicating that MCL is more stable at lower pH. When the pH was decreased further from 4 to 2, the T_m decreased indicating decreased stability of the the lectin at very low pH (see Table 3.3).

Table 3.3. Effect of pH on the thermal transitions of MCL. *At pH 4, 5 and 6 the data could not be fit due to sample precipitation.

pH	T_{m1} (K)	ΔH_{c1} (kJ.mol^{-1})	ΔH_{v1} (kJ.mol^{-1})	ΔH_{c1} /ΔH_{v1}	T_{m2} (K)	ΔH_{c2} (kJ.Mol^{-1})	ΔH_{v2} (kJ.Mol^{-1})	ΔH_{c2} /ΔH_{v2}
2	333.4	1028	374	2.7	337	841	647	1.3
3	337.9	1010	398	2.53	342	827	668	1.23
4*	346							
5*	346.7							
6*	344							
7	335.7	1045	332.3	3.14	340.4	1024	550	1.86
8	335.9	1032	320.6	3.21	340.3	823.5	526	1.56
9	334.5	1124	355	3.16	338.5	748	581	1.28
10	334	1187	357	3.32	338	1011	530	1.9

pH dependent denaturation

Fluorescence spectra of MCL at different pH are shown in Fig. 3.3 A. The emission λ_{max} of MCL at pH 7.2 is seen at 332 nm, which is in agreement with the results reported in Chapter 2 and clearly indicates that tryptophan residues are predominantly buried in the hydrophobic interior of the protein. Fluorescence spectra of MCL samples of different pH did not show any shift in the λ_{max}, indicating that MCL is relatively stable over a wide pH range and does not unfold even at pH 1.0. The emission intensity remained almost constant in the pH range 1-8, but decreased at higher pH. The maximum decrease in fluorescence intensity was 24% at pH 2.

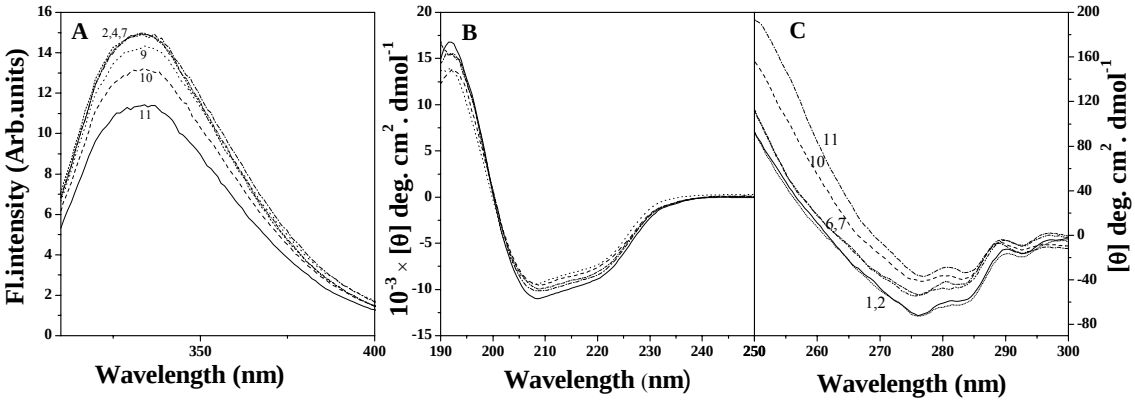

Fig. 3.3. pH-dependent denaturation of MCL lectin. A) Fluorescence spectra at different pH, The pH at which each spectrum was recorded is indicated. B) Far UV CD spectra of MCL at different pH. The symbols used are: pH 2 (.....), pH 5 (----), pH 7 (—), pH 9 (—.—.—), pH 11(-.-.-.-). C) Near UV CD spectra of MCL. The pH at which each spectrum was recorded is indicated. Inset of C shows pH vs CD signal intensity.

Figs. 3.3B and 3.3C show the near and far UV CD spectra of MCL recorded at different pH. The near UV spectrum exhibits two major minima around 276 and 283 nm and a smaller minimum near 293 nm. The far UV spectrum exhibits a minimum around 209 nm, and a broad shoulder around 215-218 nm. Both these observations are consistent with earlier observations from this laboratory [Sultan & Swamy, 2004a]. There is no change in the near UV CD spectrum of MCL when the pH of the protein is varied between 1 and 11, except small differences in intensity. These observations indicate that tertiary structure of MCL does not change when the pH is varied and show that MCL is stable over a wide range of pH. Changes observed in the Far UV CD spectrum of MCL when the pH is altered are again minor (Fig. 3.3C) and reflect that the secondary structure of MCL also does not change when the pH is altered. Gel filtration profiles obtained at pH 2 and pH 7 are very similar and show a single peak, which indicates that the tetrameric lectin does

not undergo dissociation or aggregation (not shown). This also suggests that even at low pH, MCL exists as tetramer.

Denaturation of MCL by Gdn.HCl

Chemical denaturation of MCL by Gdn.HCl was studied by monitoring changes in the intrinsic fluorescence properties of the protein when the concentration of Gdn.HCl was varied. Fluorescence emission spectra recorded in presence of different concentrations of Gdn.HCl are shown in Fig. 3.4A. The emission λ_{max} of

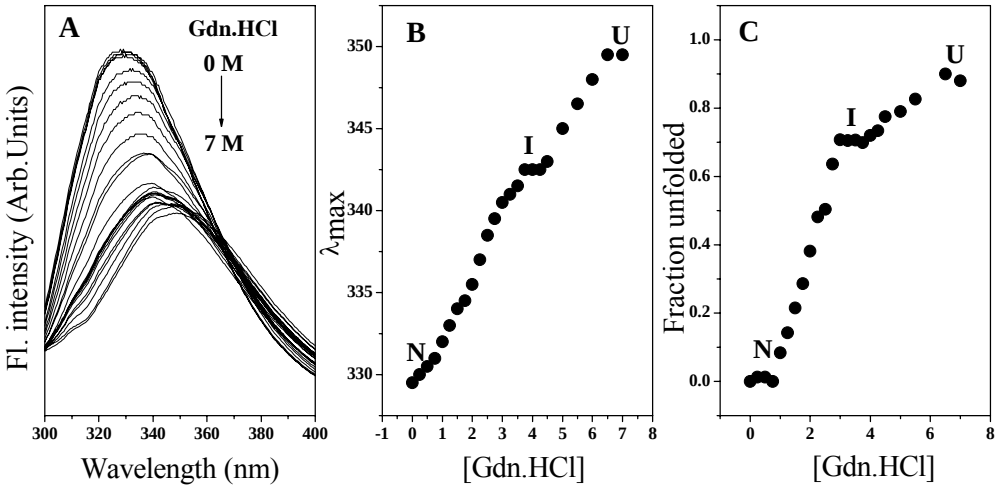

Fig. 3.4. Equilibrium unfolding of MCL in presence of Gdn.HCl. (A) Fluorescence spectra of the TDSL in presence of increasing concentrations of Gdn.HCl. Spectrum 1 corresponds to native lectin and spectra 2-21 correspond to the lectin in presence of increasing concentrations of Gdn.HCl. (B) Plot of emission λ_{max} as a function of Gdn.HCl concentration. (C) Unfolding curve for Gdn.HCl induced unfolding, N, I, and U denote native, intermediate and unfolded states of the protein.

MCL seen at 332 nm under native conditions (spectrum 1) exhibits a red shift as the concentration of Gdn.HCl is increased. This is accompanied by a pronounced decrease in the fluorescence intensity. The emission maximum increases with increase in the concentration of the denaturant up to 3.0 M, with a clear plateau

being seen between 3.0 and 4.0 M Gdn.HCl (Fig. 3.4B). At 6.5 M concentration of the denaturant 35% quenching was observed and which was accompanied by a red shift in the λ_{max} to 350 nm. This indicates complete exposure of tryptophan residues to solvent and is fully in agreement with complete unfolding of the protein. From the fluorescence emission spectra the fraction unfolded (f_U) was calculated according to the expression:

$$f_U = (F_F - F_o)/(F_F\text{-}F_U) \qquad (3.4)$$

where F_F is the fluorescence intensity of the fully folded protein, F_o is the fluorescence intensity at any point of denaturant concentration and F_U is the fluorescence intensity of the fully unfolded protein. A plot of f_U as a function of the denaturant concentration describes the denaturation profile of the protein. A denaturation curve obtained for the unfolding of MCL is shown in Fig. 3.4C. From the curve it is seen that unfolding is a three-state-process, which involves an intermediate that is well populated at 3.0-4.0 M Gdn.HCl. Thus the chaotrope-induced denaturation of MCL is consistent with the DSC data.

In summary, unfolding of MCL was studied by differential scanning calorimetry and fluorescence spectroscopy. The results indicate that unfolding of MCL is a three-state process, involving an intermediate structure.

Chapter 4

Physicochemical and differential scanning calorimetric studies on *Trichosanthes dioica* seed lectin

Summary

Physico-chemical and unfolding studies have been carried out on *Trichosanthes dioica* seed lectin (TDSL). The lectin exhibited maximum activity between pH 7.0 and 10.0, which decreased steeply at lower pH. The hemagglutination activity of TDSL was unaffected in the temperature range 4 – 50 ^{o}C, but decreased rapidly at higher temperatures. Differential scanning calorimetric studies indicate that thermal unfolding of TDSL is an irreversible process, which could be described by a three-state model. The calorimetric scan recorded at pH 7.0 consists of two transitions, occurring at around 338.6 K, and 342.8 K. In the presence of carbohydrate ligands both these transitions shifted to higher temperatures, suggesting that ligand binding stabilizes the native conformation of the protein. The unfolding temperature was highest at pH 5.0 indicating that TDSL is more stable at acidic pH. Gdn.HCl induced unfolding, monitored by following changes in the intrinsic fluorescence properties of the protein, was also observed to be a three-state process involving an intermediate. CD spectroscopy indicates that the secondary and tertiary structure of TDSL are rather similar at different pH values, indicating that the lectin is stable over a wide range of pH.

Introduction

Lectins are proteins of non-immune origin that bind carbohydrates reversibly with a high degree of specificity in a non-catalytic manner. The unusual ability of lectins to specifically recognize carbohydrate structures has made them important tools to study cell surface glycans, glycoconjugates in solution, mitogenic stimulation of lymphocytes as well as in clinical and biomedical applications such as blood typing, enzyme replacement therapy for treatment of Gaucher disease etc [Sharon & Lis, 2003; Lis & Sharon, 1998].

Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three dimensional structure. The three dimensional structure of a protein is maintained by covalent and non-covalent interactions. Protein physical and biological properties can be altered by conditions that affect the native conformation of the protein. The conformational stability of proteins is defined as the difference in free energy change between the folded state and unfolded state under physiological conditions. The conformational stability of protein is determined by analysis of denaturant-induced or thermal unfolding of proteins determined either by spectroscopy or calorimetry [Agashe & Udgaonkar, 1995; Nicholson & Scholtz, 1996; Johnson et al., 1995].

Trichosanthes dioica seed lectin (TDSL) is a galactose specific protein. It is a heterodimer with an M_r of 55 kDa. TDSL is a glycoprotein with about 4.9% covalently bound sugar. CD spectroscopic studies have shown that the secondary structure of TDSL contains 13.3% α-helix, 36.7% β-sheet, 19.4% β-turns and 31.6% unordered structure. Chemical modification studies have implicated tyrosine residues in the carbohydrate binding activity of the lectin [Sultan et al., 2004b]. Additionally, fluorescence quenching studies employing neutral and charged quenchers suggested that the tryptophan residues are largely buried in the hydrophobic core of the protein

and that at least one Trp residue has positively charged residue(s) in close proximity [Sultan & Swamy, 2005b].

In the present study, with the aim of understanding the stability of TDSL to thermal and chemical denaturation processes, we have carried out differential scanning calorimetric studies at different pH and also investigated the unfolding of the protein by Gdn.HCl. Additionally, the effect of pH and temperature on the hemagglutination activity of TDSL has been investigated. Thermodynamic parameters characterizing the unfolding transition(s) such as transition temperature (T_m), calorimetric enthalpy (ΔH_c), van't Hoff enthalpy (ΔH_v), and the changes in excess heat capacity (ΔC_p) were obtained from the DSC measurements, whereas changes in the secondary and tertiary structures at different temperatures were investigated by CD spectroscopy. Chemical unfolding by Gdn.HCl was studied by fluorescence spectroscopy monitoring changes in the protein intrinsic fluorescence emission characteristics.

Materials and Methods

Materials

Trichosanthes dioica seeds were obtained from local vendors. Guar gum, lactose, lactulose, galactose, GalNAc, MeαGal and MeβGal were purchased from Sigma (St. Louis, MO, USA). All the other reagents used were of highest purity available and obtained from local suppliers.

Purification of *Trichosanthes dioica* seed lectin

TDSL was purified by a combination of ammonium sulfate precipitation and affinity chromatography on cross-linked guar gum as described earlier [Sultan et al., 2004b]. Purity of the affinity eluted protein was assessed by polyacrylamide gel electrophoresis where it moved as a single band [Laemmli, 1970]. Concentration of TDSL was determined by Lowry assay [Lowry et al., 1951]. For experiments at different pH, the

following buffers were used: 20 mM KCl-HCl (pH 1.0-2.0), 20 mM citrate-phosphate (3.0-5.0), 20 mM phosphate (pH 6.0-7.0), 20 mM Tris-HCl (pH 8), and 20 mM glycine/NaOH (pH 9.0-11.0). NaCl (150 mM) was included in all buffers to maintain a constant ionic strength. Protein samples were dialyzed against the buffer of appropriate pH before further experimentation.

Agglutination assay

Agglutination activity of TDSL was assayed by the hemagglutination technique as described previously [Sultan & Swamy, 2005a]. A 4% suspension of human O (+) erythrocytes in 20 mM buffer containing 150 mM NaCl was mixed with serially diluted samples of the lectin in a 96-well ELISA plate and incubated at 4 ^{o}C for 1 h. The agglutination titer was scored visually.

pH dependence and thermal inactivation of TDSL

Agglutination activity of TDSL at different pH was assessed by dialyzing the lectin samples extensively against a buffer of desired pH, followed by the hemagglutination assay at 4 ^{o}C. The erythrocyte suspension used for the hemagglutination assay was also prepared in the same buffer.

Effect of temperature on the agglutination activity was investigated by incubating lectin samples at the desired temperature for 30 min. The samples were then allowed to cool to room temperature, clarified by centrifugation and the clear supernatants were tested for hemagglutination activity as described above.

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco-J-810 spectropolarimeter equipped with a Peltier thermostat supplied by the manufacturer. Protein samples were taken in a 2 mm path length rectangular quartz cuvette. Spectra were recorded at a scan rate of 20 nm/min with a response time of 4 sec and a band width of 1.5 nm. Lectin concentrations used

were 2.8 μM and 16 μM, respectively, for measurements in the far UV and near UV regions. Each spectrum was the average of 4 accumulations. CD spectra were recorded at different temperatures in the near UV region and far UV region in order to investigate the effect of temperature on the secondary and tertiary structure of TDSL. Buffer scans recorded under the same conditions were subtracted before further analysis.

Differential scanning calorimetry (DSC)

DSC measurements were made on a MicroCal VP-DSC differential scanning calorimeter (MicroCal LLC, Northampton, MA, USA) equipped with two fixed cells, a reference cell and a sample cell. DSC experiments were carried out as a function of pH, scan rate, and at different ligand concentrations. Samples were dialyzed extensively against buffers of desired pH before recording the thermograms. Buffer solutions and protein solutions were degassed before loading. All the data were analyzed by using the Origin DSC software provided by the manufacturer.

Fluorescence spectroscopy

Unfolding of TDSL induced by Gdn.HCl was monitored by fluorescence spectroscopy. Fluorescence measurements were performed on a Spex Fluoromax-3 spectrofluorimeter (Jobin Yvon Ltd, Edison, NJ, USA, website: www.jobinyvon.com). Lectin samples were incubated with different concentrations of Gdn.HCl overnight before the measurements were taken. Lectin concentration used for fluorescence measurements was 1.8 μM. Samples were excited at 280 nm and emission spectra were recorded above 300 nm. The slit widths used were 3 and 5 nm, respectively, for excitation and emission monochromators.

Results

pH optimum and thermal stability of TDSL

Activity of the TDSL samples dialyzed against appropriate buffers in the pH range 3-10 were tested by haemagglutination assay and the relative activities observed were plotted as function of the sample pH (Fig. 4.1A).

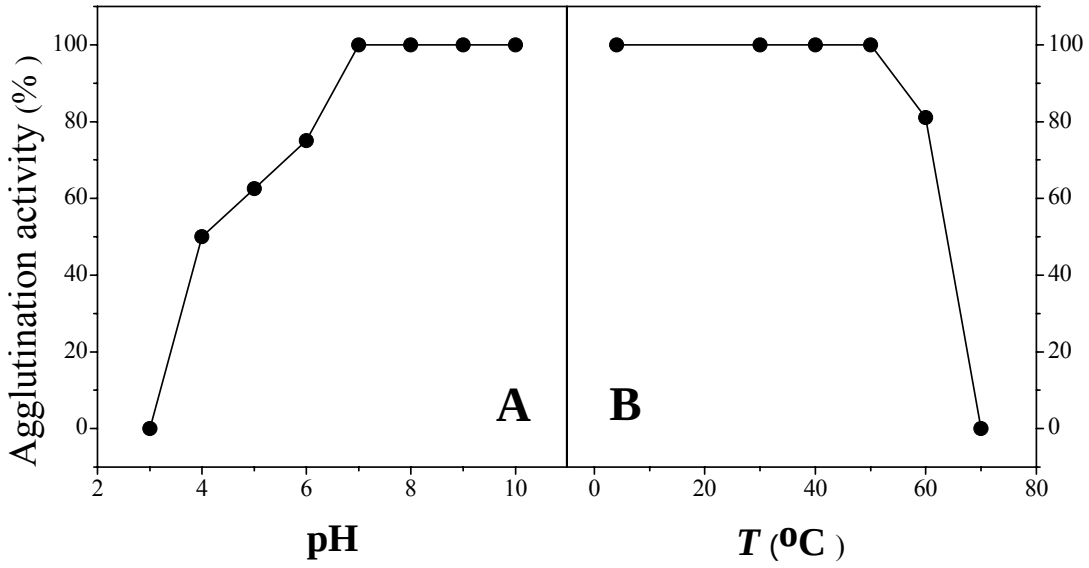

Fig. 4.1. Effect of (A) pH and (B) thermal inactivation on the agglutination activity of *T. dioica lectin.* To study pH dependence TDSL samples were dialyzed against buffers of desired pH. Activity was assessed by hemagglutination technique at 4 ^{o}C. The percentage activity was calculated assuming the maximum activity as 100%. Effect of temperature was investigated by incubating samples at desired temperature for 30 min. They were then cooled to room temperature, centrifuged and the clear supernatant was tested for agglutination activity.

The results indicate that only 50% relative activity is observed at pH 4, which increases to 75% at pH 6, and to 100% at pH 7. Hemagglutination activity remains unaltered with further increase in pH up to 10 (Fig. 4.1A).

Thermal inactivation of TDSL was investigated by incubating lectin samples at different temperatures for 30 min followed by the hemagglutination assay to check lectin activity of the heat-treated samples. The results obtained are given in Fig. 4.1B. It is seen from the figure that lectin activity is unaffected up to 50 ^{o}C, while at higher temperatures activity decreases sharply. Incubation at 60 ^{o}C reduced the activity to 81% as compared to the native protein, whereas incubation at 70 ^{o}C led to a complete loss in the hemagglutination activity of TDSL.

Far UV and near UV circular dichroism spectra of TDSL recorded at different temperatures are given in Fig. 4.2A and 4.2B, respectively. The far UV CD spectrum of TDSL shows two minima around 208 nm and 222 nm, which is consistent with earlier reports [Sultan & Swamy, 2004b]. It is seen from this figure that the CD spectrum of TDSL incubated at 50 ^{o}C (spectrum 2) is essentially identical to the spectrum recorded at room temperature (spectrum 1). Significantly larger changes were observed in the CD spectra recorded at 65 ^{o}C and 70 ^{o}C, suggesting the occurrence of a thermally-induced unfolding phase transition in this temperature range. This is clearly seen from the Fig. 2C, which gives a plot of ellipticity at 206 nm as a function of temperature.

The near UV CD spectrum of TDSL is characterized by a minimum around 283 nm and a shoulder around 293 nm (Fig. 4.2B). All the CD spectra of TDSL samples incubated between 30 and 60 ^{o}C are very similar, indicating that the tertiary structure of the protein does not change when it is heated up to 60 ^{o}C, whereas incubation at 70 ^{o}C led to large changes in the spectral features, indicating a significant loss in the tertiary structure. In order to monitor this more closely, intensity of the CD signal at 283 nm (corresponding to the minimum in the near UV spectrum) was monitored as a function of temperature (Fig. 4.2D).

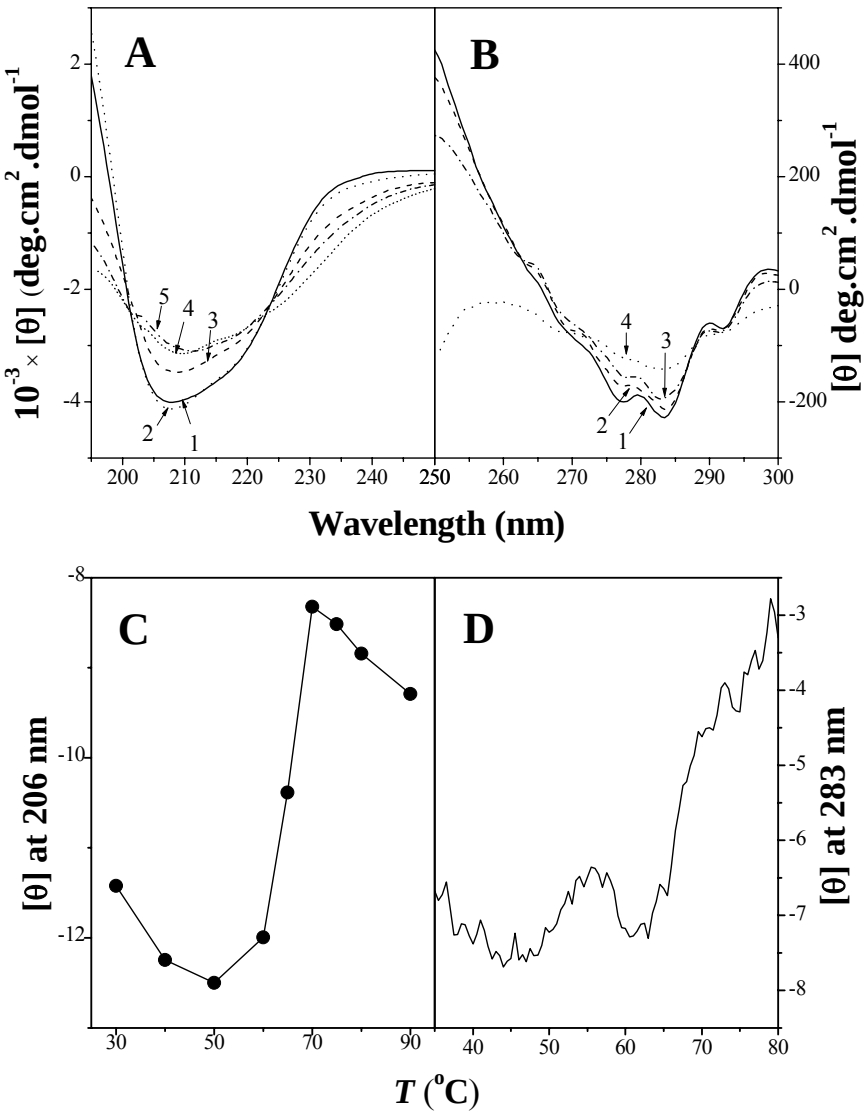

Fig. 4.2. Effect of thermal inactivation on the CD spectrum of TDSL. (A) Far UV CD spectra of TDSL. Samples that were incubated at 30, 50, 65, 70, and 90 °C were numbered as 1-5 respectively. (B) Near UV CD spectra of TDSL samples incubated at 30, 50, 60 and 70 °C were numbered as 1-4 respectively. (C) A plot of elipticity (Θ) at 206nm vs temperature. (D) The elipticity (Θ) in the near UV region of the TDSL was monitored at 283 nm as a function of temperature.

In this experiment lectin sample was heated at a scan rate of 1^{o} per minute in the temperature range 30 to 90 ^{o}C. While only marginal changes are observed in the signal intensity between 30 and 63 ^{o}C, a steep increase is observed in the signal intensity between 65 ^{o}C and 70 ^{o}C, clearly indicating that a major transition occurs at ca. 67-68 ^{o}C in the protein tertiary structure. When the heating scan was completed and the sample was cooled to room temperature, a white precipitate was observed in the sample cell, indicating irreversible thermal denaturation of the lectin.

Far UV and near UV CD spectra of TDSL at different pH are shown in the Fig. 4.3A and 4.3B, respectively. Only marginal changes are seen in the CD spectra between pH 2 and pH 10, suggesting that the lectin structure is quite stable in this pH range.

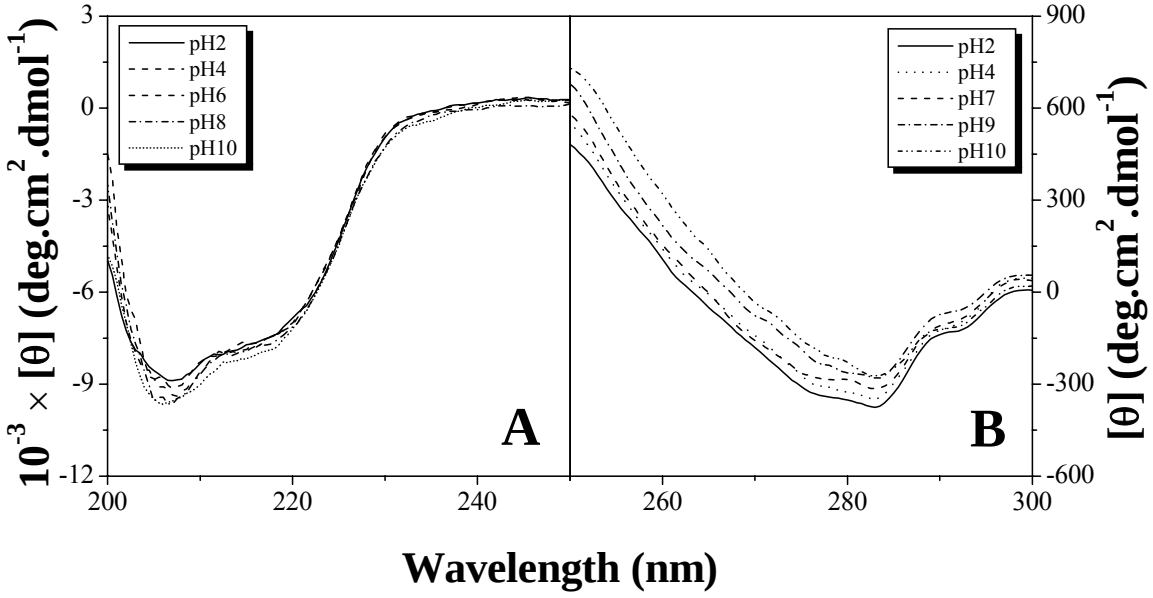

Fig. 4.3. Effect of pH on the CD spectrum of TDSL. (A) Far UV CD spectrum (B) Near UV CD spectrum. The pH at which each spectrum was recorded is indicated in the figure.

Differential scanning calorimetry

A representative DSC thermogram of the *T. dioica* lectin along with the fit of the single transition peak data to a non-two-state transition model is shown in Fig. 4.4. Both two-state method and non-two-state method use the Levenberg-Marquardt non-linear least square method. Two-state model gives calorimetric enthalpy (ΔH_c) change and the thermal mid-point of transition (T_m), whereas non-two-state model gives van't Hoff enthalpy (ΔH_v) in addition to calorimetric enthalpy and transition mid-temperature. The transition peak consists of two non-two-state transitions melting at different temperatures. The lower-temperature transition has a T_m of 338.42 K with

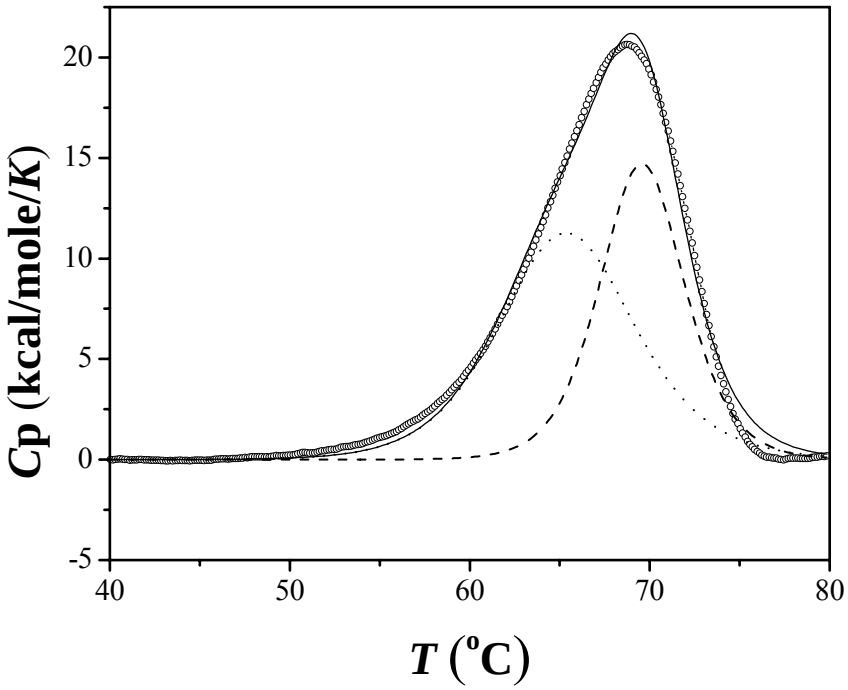

Fig. 4.4. DSC scan of *Trichosanthes dioica* seed lectin in 20 mM phosphate buffer containing 150 mM NaCl at pH 7.4 at a scan rate of 30 °C. The data points are shown as open circles, and the solid lines are the best fits of the DSC data to the non-two-state transition model. Concentration of *T.dioica* used was 0.0163 mM

a ΔH_m value of 474 kJ. mol^{-1}, while the higher-temperature transition is characterized by a T_m of 342.6 K with ΔH_m = 385 kJ. mol^{-1}. A white precipitate was observed in the cell after the scan, and the peak did not reappear upon a rescan of the sample suggesting that the transition is irreversible. To find out any kinetic effects on the thermal unfolding of TDSL, the data were collected at different scan rates. No change was seen in the T_m values of the lower and higher transition peaks at different scan rates, when the scan rate was increased by a factor of 4. Additionally, calorimetric and van't Hoff enthalpies did not change when scan rate was varied suggesting that equilibrium thermodynamics can be applied to evaluate the thermodynamic parameters [Manly et al., 1985; Hinz & Schwarz, 2001]. Ratio of calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) for the first transition peak is considerably greater than 1 for TDSL dimer indicating that the two subunits of the protein unfold as a single entity during denaturation. For the second transition the ratio $\Delta H_c/\Delta H_v$ is about half the value observed for the first transition, which implies that intermediate transitions are observed in the transition profile [Hinz & Schwarz, 2001].

Effect of ligand

In the presence of saturating amounts of ligands also TDSL exhibits two transition peaks. These peaks are again fitted to a non-two-state transition model. DSC data obtained in the presence of different ligands is shown in Table 4.1. Both the lower-temperature transition and the higher-temperature transition shift to higher temperatures in presence of different carbohydrate ligands, suggesting that ligand binding stabilizes the native conformation of TDSL. Among all the carbohydrate ligands used, binding of lactose induced the maximum shift of 5.38 K in the T_m. Shifts induced in the T_m of TDSL by other sugars are: lactulose (4.8 K), MeβGal (4.11 K), MeαGal (3.18 K) and GalNAc (3.53 K). From the ligand-induced shifts in the T_m, the association constants at

the denaturation temperature [$K_b(T_c)$] were calculated according to equation (4.1) [Schellman, 1975]:

$$K_b(T_c) = \{\exp[(T_c - T_m)\Delta H_c / nRT_cT_m] - 1\}/[L] \quad (4.1)$$

Table 4.1. Thermodynamic parameters from DSC measurements on the thermal transition of TDSL in presence of ligands. DSC scan rate was 30 $K.h^{-1}$. Concentration of TDSL used is 0.0163 mM per dimer. Ligand concentration used for ligands except lactose is 0.1 M. The errors were less than 0.02% for T_m, less than 10% for ΔH_c and ΔH_v respectively.

Ligand	T_{m1} (K)	ΔH_{c1} ($kJ.mol^{-1}$)	ΔH_{v1} ($kJ.mol^{-1}$)	$\Delta H_{c1}/\Delta H_{v1}$	T_{m2} (K)	ΔH_{c2} ($kJ.mol^{-1}$)	ΔH_{v2} ($kJ.mol^{-1}$)	$\Delta H_{c2}/\Delta H_{v2}$
-	338.4	474	378	1.25	342.6	385	618	0.62
MeβGal	343.3	577	456	1.26	347	373.5	727	0.51
MeαGal	341.9	527	418	1.26	345.9	420	664	0.63
GalNAc	342.6	568	426	1.3	346.4	439	651	0.67
Lactulose	344.8	392	576	0.68	347.9	209	900	0.23
Lactose								
10 mM	340.8	564	481	1.17	344.7	457	687	0.66
20 mM	341	683	428	1.59	345.2	574	675	0.84
30 mM	342.5	713	463	1.5	346.2	522	717	0.72
50 mM	344.2	614	560	1.1	347.6	351	836	0.42
100 mM	344.9	518	493	1.05	348.4	296	819	0.36

where T_c and T_m are denaturation temperatures in the presence and absence of lactose, respectively, ΔH_c is the calorimetric enthalpy of the carbohydrate ligand-protein

complex, [L] is the ligand concentration and n is the number of binding sites on the dimeric protein.

From the above analysis, values of the binding constant for various ligands calculated at denaturation temperature are 188, 133, 87.5, 60 and 48 M^{-1} for lactose, lactulose, MeβGal, GalNAc and MeαGal, respectively. The ratio of $\Delta H_c/\Delta H_v$ in presence of different ligands is similar to that obtained with the native lectin for both transitions indicating that the unfolding behavior of both the liganded form and unliganded forms of TDSL are similar. The denaturation transition in presence of bound ligand can be written as follows [Fukuda et al., 1983]:

$$\ln [L] = -\Delta H_v [L]/[RT_p m] + \text{constant} \qquad (4.2)$$

where T_p is the temperature where transition temperature is maximum, [L] is the ligand concentration, and m is the number of ligand molecules.

A plot of ln [L] versus $1/T_p$ for lactose is shown in Fig. 4.5. From the slope of this plot using the value of m as 2, van't Hoff enthalpy for the lower-temperature transition was estimated as 768 ± 140 kJ. mol^{-1}, whereas the ΔH_v value for the higher-temperature transition was calculated as 954 ± 118 kJ. mol^{-1}. These values are close to the values obtained by non-two-state fit of the transition data in presence of lactose. Since T_p is not determined from the fits of non-two-state method, the agreement between the values ΔH_v and $-\Delta H_v [L]$ is an independent conformation. Therefore, it can be concluded that two lactose molecules bind to each TDSL dimer.

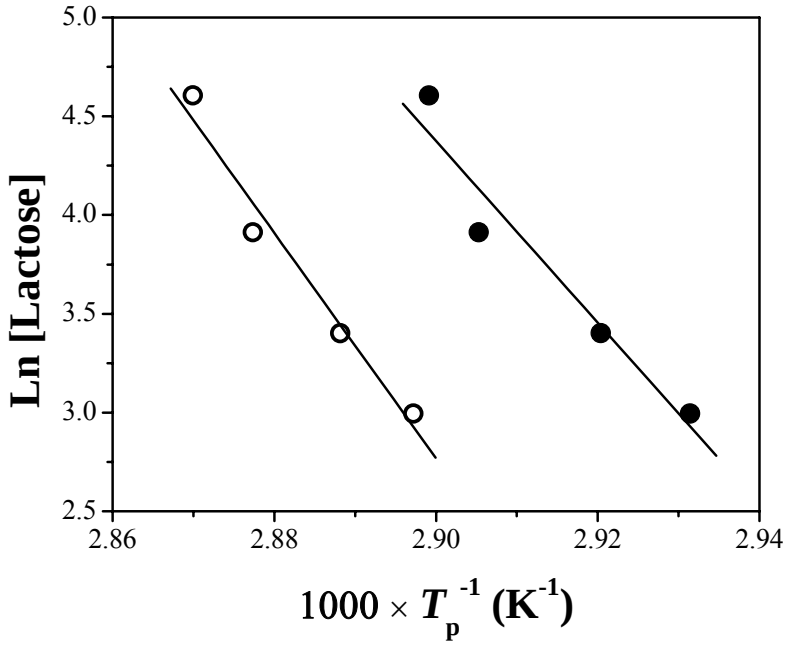

Fig. 4.5. Plots of ln[Lactose] vs 1/T_p for TDSL. The lines are best linear least-square fits of ln [Lactose] against 1/Tp, for the lower temperature transition peak (•) and higher temperature transition peak (o). Scan rate was 30 °C per hour. The correlation coefficients are 0.96 and 0.98 for the lower temperature and higher temperature transition peaks respectively.

Effect of pH

DSC data obtained for the thermal transition of TDSL at different pH (2-10) is presented in Table 4.2. CD studies in this pH range (Fig. 4.3) show that the secondary and tertiary structures of TDSL are very similar, indicating that TDSL is stable over this wide range of pH. At all pH the calorimetric data could be satisfactorily fit by a non-two-state, two-peak model. From the data presented in Table 4.2 it is seen that at pH 2 the T_m is 326.5 K which increases with pH up to pH 5. Thermal stability of TDSL is more at pH 4 and pH 5. Ratio of $\Delta H_c/\Delta H_v$ increases along with pH. Between pH 7 and 10 both T_{m1} and T_{m2} remain nearly constant, suggesting that the lectin is stable in the wide range of pH.

Table 4.2. Thermodynamic quantities from DSC measurements on the thermal transition of *Trichosanthes dioica* seed lectin as a function of pH**.** T_{m1}, ΔH_{c1}, ΔH_{v1} are the thermodynamic parameters corresponding to lower-temperature transition, where as T_{m2}, ΔH_{c2}, ΔH_{v2} corresponds to higher-temperature transition. Concentration of TDSL used was 0.0163 mM in dimer. DSC scan rate was 30 $K.h^{-1}$. The errors were less than 0.05% for T_m less than 10% for ΔH_c and ΔH_v, respectively.

pH	T_{m1} (K)	ΔH_{c1} ($kJ.mol^{-1}$)	ΔH_{v1} ($kJ.mol^{-1}$)	ΔH_{c1} /ΔH_{v1}	T_{m2} (K)	ΔH_{c2} ($kJ.mol^{-1}$)	ΔH_{v2} ($kJ.mol^{-1}$)	ΔH_{c2} /ΔH_{v2}
2	326.5	461	444	1.04	330	348	706	0.49
3	333.66	465	420	1.1	337	402	669	0.6
4	340	647	638	1.01	343	501	973	0.51
5	340.5	526	555	0.947	344	310	1049	0.3
6	339.6	785	547	1.4	343	668	869	0.76
7	338.6	610	379	1.6	342.8	568	601	0.9
8	338.4	756	434	1.74	343.15	501	693	0.72
9	338.8	598	412	1.45	343	447	664	0.67
10	338.6	706	361	1.95	343	606	610	0.99

Chemical denaturation

Denaturation of TDSL with Gdn.HCl was monitored by following changes in the intrinsic fluorescence characteristics of the protein when the concentration of the denaturant was varied. Tryptophan fluorescence emission λ_{max} of native TDSL is seen at 328 nm (Fig. 4.6A), which is in agreement with previously published results and suggests that the tryptophan residues are well buried in the interior of the protein [Sultan & Swamy, 2005b; Lackowicz, 1989]. The emission maximum increases with increase in the concentration of the denaturant up to 2.0 M, with a clear plateau being seen between 2.0 and 2.5 M Gdn.HCl, where the λ_{max} remains constant at 340.5 nm

(Fig. 4.6B). As the concentration of the denaturant is increased further the emission λ_{max} again increases gradually and reaches a maximum of 349 nm at 4.5 M Gdn.HCl and remains constant thereafter. The emission λ_{max} of 349 nm indicates complete exposure of the Trp residues to the aqueous medium (solvent) and indicates complete unfolding of the protein. The unfolding of TDSL is also associated with a quenching of the fluorescence intensity (Fig. 4.6A).

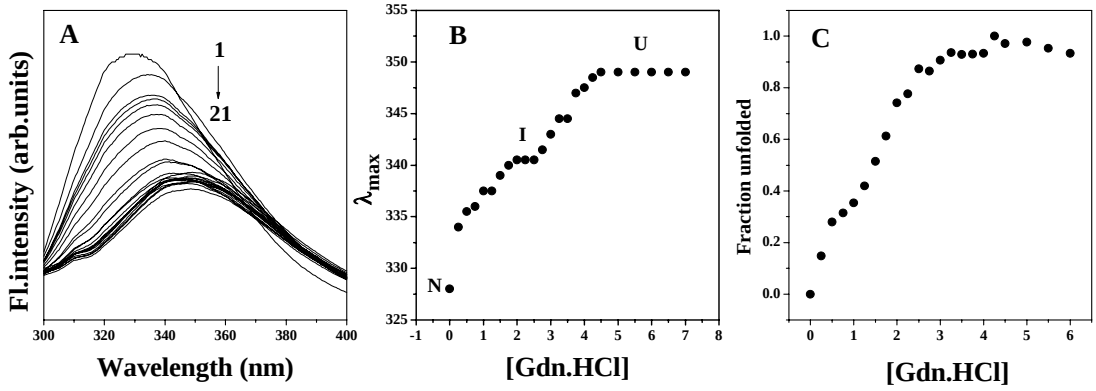

Fig. 4.6. Equilibrium unfolding of TDSL in presence of GdnHCl. (A) Fluorescence spectra of the TDSL in presence of increasing concentrations of Gdn.HCl. Spectrum 1 corresponds to native lectin, 2-21 corresponds to spectra in presence of increasing concentrations of GdnHCl. (B) Plot of emission λ_{max} as a function of Gdn.HCl concentration. N, I, and U denote native, intermediate and unfolded states of the protein. (C) Unfolding curve for Gdn.HCl induced denaturation of TDSL.

A maximum of 48.2% quenching was observed around 5 M Gdn.HCl. The observation of a plateau in the emission λ_{max}, as alluded to above, is consistent with the presence of a stable intermediate structure in the unfolding process at 2.0 – 2.5 M of the denaturant. At higher concentrations of Gdn.HCl, a shoulder is observed in the fluorescence spectrum near 307 nm which may be attributed to the tyrosine residues (Fig. 4.6A). This shoulder is not due to the solvent Raman band as the spectra were corrected by subtracting buffer blanks recorded under identical conditions. Since resonance energy

transfer between Tyr and Trp residues is not expected to occur in the denatured protein this observation is consistent with complete unfolding of TDSL [Khan et al., 2007].

From the fluorescence emission spectra the fraction unfolded (f_U) was calculated according to the expression:

$$f_U = (F_F - F_o)/(F_F\text{-}F_U) \qquad (4.3)$$

where F_F is the fluorescence intensity of the fully folded protein, F_o is the fluorescence intensity at any point of denaturant concentration and F_U is the fluorescence intensity of the fully unfolded protein. A plot of f_U as a function of the denaturant concentration describes the denaturation profile of the protein. A denaturation curve obtained for the unfolding of TDSL is shown in Fig. 4.6C. From the curve it is seen that unfolding follows a three-state mechanism with an intermediate. Gdn.HCl induced denaturation of TDSL is qualitatively consistent with DSC data.

Discussion

Trichosanthes dioica seed lectin is a galactose-specific protein that has been isolated recently in our laboratory. In a previous publication we reported its isolation, and characterization with respect to macromolecular properties, saccharide specificity, and the amino acid side chains involved in its carbohydrate binding activity and also investigated the exposure and accessibility of the tryptophan residues of this protein [Sultan et al., 2004b; Sultan & Swamy. 2005b]. In the present study this lectin has been further characterized with respect to its activity at different pH as well as incubation at different temperatures. The thermal, chemical, and acid-induced unfolding of TDSL has been investigated by differential scanning calorimetry, fluorescence spectroscopy and CD spectroscopy. The results obtained are discussed here.

Thermal stability and pH-dependence of TDSL

The hemagglutination activity of TDSL is unchanged in the pH range 7.0-11.0. At lower pH the activity decreases steeply. This is somewhat similar to the activity of other Cucurbitaceae seed lectins. TCSL and MCL exhibit maximum activity in the pH range from 8.0-11.0 [Sultan & Swamy, 2005a; Kenoth et al., 2003] where as another cucurbit seed lectin SGSL exhibits maximum activity in the pH range of 6.0-10.0 [Komath et al., 2001]. The data presented in Fig. 1B clearly shows that the hemagglutination activity of TDSL was unaffected by increase in the temperature up to 50 ^{o}C, with close to 20% decrease at 60 ^{o}C, whereas incubation at 70 ^{o}C resulted in a total loss of the activity. In comparison, the activity of TCSL was unaffected up to about 60 ^{o}C, and decreased to 50% at 70 ^{o}C, indicating it is thermally more stable than TDSL. However, TDSL appear to be more stable than MCL and SGSL, which were inactivated by incubation above 50 ^{o}C and 40 ^{o}C, respectively [Sultan & Swamy, 2005a; Kenoth et al., 2003; Komath et al., 2001]. These observations are broadly in agreement with the results from CD spectroscopy in which significant alterations were observed in the secondary structure of TDSL at 65 ^{o}C, with the lectin losing its complete tertiary structure at 70 ^{o}C, and DSC studies, which showed that the unfolding transition of TDSL occurs at 69 ^{o}C.

Differential scanning calorimetry

The DSC scan of MCL shows two peaks suggesting that the lectin contains two entities melting at different temperatures. This is similar to the ribosome inactivating protein abrin II and winged bean basic lectin which also unfold as two entities [Krupakar et al., 1999; Schwarz et al., 1991]. $\Delta H_c/\Delta H_v$ ratio for the lower-temperature transition is ~1.0 at lower pH (Table 4.1) and as pH increases the ratio also increases and becomes close to 2.0 at pH 10.0, the ratios greater than one may indicate presence of intermediate state. Gdn.HCl denaturation studies on TDSL by fluorescence spectroscopy also show

that unfolding of TDSL is a biphasic process. Pea nut agglutinin also shows biphasic denaturation [Reddy et al., 1999]. The ratio of 1 shows unfolding of lower-temperature transition is more cooperative, indicating that two domains interact more strongly with each other at lower pH. $\Delta H_c/\Delta H_v$ ratio is greater than 1 for unfolding of ConA tetramer, indicating the presence of an intermediate state [Schwarz et al., 1993]. Binding of carbohydrate ligands to TDSL increase the thermal stability of the protein as indicated by increased T_m values for both transitions, suggesting that ligand binding occurs preferentially to the folded state of the lectin. Shifts of T_m in presence of lactose and lactulose are higher when compared to MeβGal and GalNAc, suggesting that the second sugar moiety of these β-linked disaccharides may be involved in some additional interaction with the lectin combining site, which is similar to another cucurbit seed lectin, MCL. Shift of T_m is higher for MeβGal when compared to MeαGal, which is in agreement with the preference of the lectin for the β-anomeric derivatives of galactose.

Binding constant values determined at denaturation temperature with different carbohydrate ligands show that the disaccharides, lactose and lactulose bind with higher affinity than the monosaccharides. Further, the preference of TDSL for the β-linked sugars is reflected in the higher affinity exhibited by the lectin for MeβGal as compared to MeαGal. These results are in agreement with the relative inhibitory potencies determined by hemagglutination-inhibition assays [Sultan et al., 2004b]. Another cucurbit seed lectin, MCL exhibits a similar trend in its recognition of various mono and disaccharides [Sultan & Swamy, 2005a]. The binding of two lactose molecules to one molecule of TDSL dimer was concluded from the plot of ln [lactose] against $1/T_p$ for both lower and higher transitions. Ratio of $\Delta H_c/\Delta H_v$ remains unaltered in presence of ligand suggesting that the mechanism of unfolding pattern is not influenced by ligand binding. DSC studies at different pH suggest that TDSL is more stable at pH 4 and 5,

and that the secondary and tertiary structure of the protein do not change when the pH is varied, which shows that TDSL is stable over a wide range of pH. Gdn.HCl induced denaturation studies of TDSL by fluorescence spectroscopy also show that unfolding of TDSL is also a three-state process with an intermediate.

In summary, these results indicate that TDSL is maximally active in the pH range 7.0-10.0 and is stable up to 60 ^{o}C. Thermal unfolding of TDSL is an irreversible, three-state process. Chemical denaturation also follows a three-state unfolding mechanism. TDSL is more stable at lower pH. Binding of carbohydrate ligands stabilizes the native conformation of the protein and increases the denaturation temperature of TDSL. Binding constants calculated for different carbohydrate ligands at denaturation temperature indicate that TDSL preferentially bind to β-linked galactopyranosides.

Chapter 5

Purification and Physicochemical Characterization of two Galactose-specific Isolectins from the seeds of *Trichosanthes cordata*

Summary

A galactose-specific lectin has been purified from the seeds of *Trichosanthes cordata* by affinity chromatography on cross-linked guar gum. The affinity purified *T. cordata* agglutinin (TCA) yielded a single diffuse band in polyacrylamide gel electrophoresis under native conditions, whereas in SDS-PAGE in the absence of β-mercaptoethanol it gave two closely-spaced bands. The affinity-eluted lectin could be resolved into two isolectins, TCA-I and TCA-II by ion-exchange chromatography on DEAE cellulose. The molecular weights of the isolectins were determined as 59 and 52 kDa by SDS-PAGE. TCA-I is a heterodimer in which the two subunits with masses of 32 and 27 kDa, are covalently connected by one or more disulfide bonds. TCA-I and TCA-II are glycoproteins with 6.2% and 6.8% covalently bound neutral sugar, respectively and are very similar in secondary structure as determined by CD spectroscopy. These isolectins have similar carbohydrate specificities revealed by hemagglutination-inhibition assays. The agglutination activity of TCA-I was found to be highest in the pH range 7.0-8.0. The lectin activity was unaffected between 0 and 40 °C, but decreased dramatically above 40 °C. Association constant for the interaction of TCA-I with lactose was determined as 7.42×10^3 M^{-1} at 25 °C. The exposure and accessibility of the tryptophan residues of TCA-I and the effect of ligand binding on them have been probed by quenching studies employing neutral and ionic quenchers.

Introduction

Lectins are structurally diverse oligomeric proteins composed of subunits, one or more of which carry a sugar-binding site [Lis & Sharon, 1998]. Lectins are ubiquitous in nature and are found in plants, animals, microorganisms including fungi and bacteria [Lis & Sharon, 1986; Peumans & Van Damme, 1998]. The functions of lectins are extremely diverse and all are based on the ability to bind or recognize the carbohydrate moieties of glycoconjugates. Lectins are useful as molecular tools for isolating glycoconjugates, for typing blood, mitogenic stimulation of lymphocytes, cell fractionation, bone marrow transplantation and preferential agglutination of tumor cells [Goldstein & Poretz, 1986; Sharon & Lis, 2003]. Some plant lectins play a role in defense mechanisms of the plant. In some legumes, lectins have been shown to mediate plant symbiosis with nitrogen fixing bacteria [Sharon & Lis, 2003].

Legume lectins are the most thoroughly studied family of proteins due to their presence in large quantities in seeds [Sharon & Lis, 2003], and have been characterized well with respect to macromolecular properties and carbohydrate binding specificity. Numerous legume lectins have been sequenced completely and their three-dimensional structures have been elucidated [Rini, 1995; Sharon & Lis, 2003; Strosberg et al., 1986; Rougé et al., 1991; Van Damme et al., 1998]. The structural studies on legume lectins led to a deeper understanding of the evolutionary relationships among legume lectins and provided many details about the interaction of proteins and sugars and hydrophobic ligands to these lectins [Komath et al., 2006; Sinha et al., 2007].

Studies on lectins from other plant families are fewer, and therefore there is a need to investigate them and characterize their physico-chemical and carbohydrate binding properties in detail. Since many species from Cucurbitaceae

are cultivated for food in different countries, it is of considerable interest to purify and characterize lectins from this family and lectins have been isolated from the seeds and the phloem exudate of several cucurbit species [Barbieri et al., 1979; Read & Narthcote, 1983; Ananthram et al., 1986; Falasca et al., 1989]. Cucurbit seed lectins are of particular interest since many of them show structural homology to type-II ribosome inactivating proteins. Yet they do not activate ribosomes or do so only weakly. However, only two seed lectins from this family (*Momordica charantia* lectin, and *Trichosanthes kirilowii* seed lectin) were characterized in detail with respect to carbohydrate binding and macromolecular properties [Barbieri et al., 1979; Falasca et al., 1989; Barbieri et al., 1980; Das et al., 1981; Khan et al., 1981; Mazumder et al., 1981; Ng et al., 1986; Padma et al., 1998; Sultan & Swamy, 2003; Sultan et al., 2004a; Sultan & Swamy, 2005a]. In order to fill this lacuna, this laboratory took up a long-term research program to investigate and characterize cucurbit seed lectins and reported the high yield purification and characterization of three galactose-specific lectins from the seeds of *Trichosanthes anguina*, *Trichosanthes cucumerina* and *Trichosanthes dioica* [Komath et al., 1996; Komath & Swamy, 1998; Komath et al., 1998; Komath & Swamy, 1999; Padma et al., 1999; Komath et al., 2000 a,b; Kenoth et al., 2000; Komath et al., 2001; Kenoth et al., 2001; Manoj et al., 2001; Kenoth et al., 2003; Kenoth & Swamy, 2003; Sultan et al., 2004b; Sultan & Swamy, 2005b].

In the present study, a galactose specific lectin has been purified in high yield from the seeds of *Trichosanthes cordata* by affinity chromatography on cross-linked guar gum. The *T. cordata* agglutinin (TCA) could be resolved into two isolectins (TCA-I and TCA-II) by ion exchange chromatography on DEAE-cellulose. The two isolectins were characterized with respect to carbohydrate specificity, glycoprotein nature and secondary structure as determined by CD spectroscopy. In addition, fluorescence spectroscopic studies were carried out to

determine the association constant for the binding of lactose to TCA-I and to probe the environment of tryptophan residues in it.

Materials and methods

Materials

Trichosanthes cordata seeds were obtained from a local seed vendor. Guar gum, acrylamide, bis-acrylamide, TEMED, lactose, galactose, MeαGal, MeβGal, 2-dGal, $GalNH_2$.HCl, GalNAc, fucose, L-arabinose, glucose, MeαGlc, MeβGlc, GlcNAc, mannose, MeαMan, MeβSGal, *p*NPβGal, MeUmbβGlc, pNPαGlcNAc, lactulose, melibiose, cellobiose, maltose, DEAE-Cellulose and Tris base were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other reagents used were of the highest purity available.

Purification of *Trichosanthes cordata* lectin

About 70 grams of *T. cordata* seeds were homogenized in a kitchen blender and defatted thrice with 300 ml of acetone. The defatted seed meal was air dried and mixed with 500 ml of 1M NaCl containing 0.03% sodium azide and kept under constant stirring overnight at 4 ^{o}C. 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 sulphate precipitation (85% saturation) and the precipitate obtained was dissolved in a small volume of 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.03% sodium azide (PBS) and dialyzed extensively against the same buffer. The dialyzed sample was then centrifuged at 4500 rpm for 30 min and the clear supernatant was collected. The supernatant obtained in the previous step was applied to a column of guar gum (2.5 × 20.0 cm) [Appukuttan et al., 1977] at a flow rate of ~20 ml/hr. The break-through 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 0.02. The bound lectin was then eluted with 0.2 M lactose in PBS. Twelve mL fractions were collected and elution was monitored by A_{280nm}.

Hemagglutination and hemagglutination-inhibition assays

The lectin activity of crude seed extracts of *T. cordata* and purified TCA was assayed by the hemagglutination technique as described previously [Sultan et al., 2004b]. Fifty microliters of a 4% suspension of human O(+) erythrocytes in 10 mM PBS was added to an equal volume of serially 2-fold diluted samples of the lectin in a 96-well ELISA plate and incubated at 4 °C for 1 hour. The agglutination titer was scored visually. Hemagglutination-inhibition assays with the purified TCA were done according to the following procedure. In the first well of the microtiter plate 40 µl of the sugar solution was placed and serially 2-fold diluted. To each well 10 µl of lectin solution (1.4 mg/ml) was added. After incubating for 10 minutes at 4 ^{o}C, 50 µl of a 4% erythrocyte suspension was added and incubated at 4 °C for 1 hour and then the agglutination titer was scored visually.

Effect of pH and thermal inactivation of TCA-I

The dependence of agglutination activity of TCA-I on the pH of the medium was investigated by dialyzing the lectin with buffer of desired pH and then assaying for the hemagglutination activity. The buffers used for different pH values are: 30 mM citrate (pH 5), 30 mM phosphate (pH 6-7), 30 mM Tris-HCl (pH 8) and 30 mM glycine-NaOH (pH 9-11). All buffers contained 0.15 M NaCl.

To investigate the effect of temperature on the activity of TCA-I, protein samples were incubated at different temperatures for 30 min and cooled to room temperature. The samples were then centrifuged and the clear supernatants obtained were assayed for agglutination activity as described above.

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-denaturing 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.

Gel filtration

Gel filtration experiments were performed using a column of Superose-12 (30 × 1 cm) on a Pharmacia FPLC. About 1 mg of the protein in 1 ml of PBS containing 0.1 M lactose was applied to the column, which was pre-equilibrated with the same buffer. Chromatograms were run at a flow rate of 30 ml/hour and the elution was monitored by $A_{280\ nm}$. Elution volumes were determined from the retention times obtained from the chromatographic traces. The column was calibrated with bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), soybean trypsin inhibitor (Mr 21,500), lysozyme (Mr 14,300) and tendamistat (Mr 9,000) as standards.

Ion-exchange chromatography

In order to separate the isolectins of TCA, the affinity purified lectin (~ 11 mg) was dialyzed against 20 mM Tris-HCl buffer (pH 8) and then loaded on a DEAE-Cellulose column (~1×15 cm, 15 ml bed volume), pre-equilibrated with the same buffer. Most of the protein sample was bound to the column under these conditions. After washing the column with one bed volume of the same buffer, elution was carried out successively with one bed volume each of 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the same buffer.

Estimation of protein and sugar

Protein concentrations were estimated by the method of Lowry et al. [1951] with bovine serum albumin as the standard. Neutral sugar was estimated according to Dubois et al. [1956] with D-galactose as the standard.

Circular dichroism spectroscopy

CD spectra of TCA-I were recorded on a Jasco-J810 spectropolarimeter at a scan speed of 20 nm/min with a response time of 4 s and slit width of 1.5 nm. A cylindrical quartz cuvette of 0.2 mm path length was used in the far UV region while the cell pathlength was 10 mm for measurements in the near UV region. All measurements were made at a lectin concentration of 0.16 mg/ml. Each spectrum was the average of five consecutive scans. Buffer scans recorded under similar conditions were subtracted from protein spectra before further analysis. In order to investigate the effect of carbohydrate binding on the protein secondary and tertiary structure, spectra were also recorded in the presence of 75 mM lactose. The content of different secondary structure elements of the two isolectins were estimated by the analysis of their CD spectra in the wavelength range 240-190 nm using the CDSSTR program [Compton & Johnson, 1986; Sreerama & Woody, 2000] and the routines available in the website DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Li et al., 2000; Lobley et al., 2002].

Absorption spectroscopy and fluorescence spectroscopy

Absorption measurements were carried out on a Shimadzu UV-PC 3101 UV-Vis-NIR spectrophotometer using 1-cm pathlength cells. Fluorescence spectra were recorded on a Spex Fluoromax 3 fluorescence spectrometer. Slit widths of 3 and 6 nm were used on the excitation and emission monochromators for measurements with the native protein, whereas the corresponding slit widths used for the denatured protein were 4 and 10 nm, respectively.

Fluorescence quenching studies

For fluorescence spectroscopy TCA-I samples with $A_{280} \leq 0.1$ were irradiated with 295 nm light to selectively excite tryptophan residues of the protein. Quenching studies were carried out by the adding small aliquots of the quencher from a 5 M stock solution to the protein samples. Fluorescence spectra were recorded after each addition. For quenching with iodide ion, 0.2 mM sodium thiosulphate was added to the quencher stock solution to prevent the formation of triiodide ion (I_3^-). All measurements were performed at 25 °C. Experiments were performed in duplicate and average results were reported. Fluorescence intensities were corrected for volume changes and for inner filter effects before further analysis.

Lactose binding

Binding of lactose to TCA-I was investigated by monitoring changes in the fluorescence intensity of the protein induced by sugar binding. Samples were excited at 280 nm and emission spectra were recorded between 300 and 400 nm. Titrations were carried out by adding small aliquots of sugar from a 7-15 mM stock solution in PBS to 2.5 ml of the lectin with $A_{280} \leq 0.1$ in the same buffer. Fluorescence spectra were recorded for the protein alone and after each addition of the ligand. An equilibration period of 2 minutes was given after the addition of each aliquot. All spectra were corrected for volume changes and for inner filter effects before further analysis.

Results and Discussion

The seed extract of *Trichosanthes cordata* exhibited agglutination activity which could be inhibited by galactose and its derivatives. In view of this observation, we developed a purification procedure based on affinity chromatography on cross-linked guar gum as the principal step. The affinity purified *Trichosanthes cordata*

agglutinin (TCA) was further resolved into two isolectins (TCA-I and TCA-II) by ion exchange chromatography on DEAE-cellulose. TCA-I and TCA-II have been characterized in considerable detail with respect to macromolecular and saccharide binding properties. Finally, fluorescence quenching studies were carried out to probe the accessibility and exposure of the Trp residues in TCA-I and the binding of lactose to the protein was investigated by monitoring ligand-induced changes in the protein intrinsic fluorescence. The results obtained from these studies are discussed here.

Purification and macromolecular properties of *T. cordata* seed lectin

The *T. cordata* lectin was purified by ammonium sulphate precipitation followed by affinity chromatography on cross-linked guar gum. The lectin eluted as a single peak from the affinity column when 0.2 M lactose was used as the eluant (Fig. 5.1A). About 47 mg of purified lectin was obtained from 70 grams of whole *T. cordata* seeds. In non-reducing SDS-PAGE the affinity purified lectin yielded two closely spaced bands (Fig. 5.2A). In order to verify whether these two bands correspond to different isolectins, the affinity purified protein was subjected to ion exchange chromatography on DEAE-cellulose, which resulted in the protein being resolved into two components (Fig. 5.1B), indicating that TCA contains two isolectins. The two isolectins were designated TCA-I and TCA-II and each of them gave a single band in non-reducing SDS-PAGE (Fig. 5.2B). By comparing the mobility of these two bands with that of standard proteins the molecular weights of TCA-I and TCA-II were estimated as 59 and 52 kDa, respectively (Fig. 5.2D).

In reducing SDS-PAGE, TCA-I showed two bands (Fig. 5.2C) indicating that it is composed of two disulfide-linked subunits. By comparison with the mobility of standard proteins, the masses of the two subunits were estimated as 32 and 27 kDa. In this regard TCA-I is similar to several other galactose specific seed

lectins, e.g., those from *T. kirilowii*, *T. anguina*, *T. cucumerina* and *T. dioica*, all of which are also made up of two non-identical subunits of comparable size and are linked by disulfide bonds [Falasca et al., 1989; Komath et al., 1996; Padma et al., 1999; Sultan et al., 2004b]. Therefore, it appears that covalent association of subunits is a common feature among the heterodimeric lectins from cucurbit seeds. It is interesting to note that type 2 RIPs are also galactose specific proteins that are made up of heterodimers which are covalently- linked by disulphide bond(s). Additionally, the secondary structure of all these proteins is made up of a combination of α-helix, β-sheet and unordered structures [Van Damme et al., 1998]. Thus, sugar specificity, subunit size and composition and secondary structure of TCA-I appear to be similar to type-2 RIPs. Single crystal X-ray crystallographic studies on other cucurbit seed lectins, namely *Trichosanthes kirilowii* seed lectin and SGSL, suggest that these lectins exhibit structural homology with type-2 RIPs and immunodiffusion experiments have shown that TCSL is structurally similar to SGSL [Padma et al., 1999; Manoj et al., 2001; Li et al., 2000]. From the above observations we may conclude that the galactose specific, hetero-dimeric cucurbit seed lectins which are covalently linked by disulphide bonds may be structurally similar to type-2 RIPs. TCA-II, on the other hand, gave several bands (at least three) in reducing SDS-PAGE (Fig. 5.2C). In gel filtration in the absence of lactose TCA-I yielded a very broad peak whereas in the presence of 0.1 M lactose a single, sharp peak was observed (Fig. 5.1C). This observation suggested that the lectin most likely interacts with the column matrix (Superose). By comparing with elution profiles of standard proteins the molecular weight of TCA-I was estimated to be 49 kDa (Fig. 5.1D). This value is somewhat lower than the M_r estimated from SDS-PAGE and indicates that despite the presence of lactose, the lectin may still be retarded somewhat due to interaction with the column matrix.

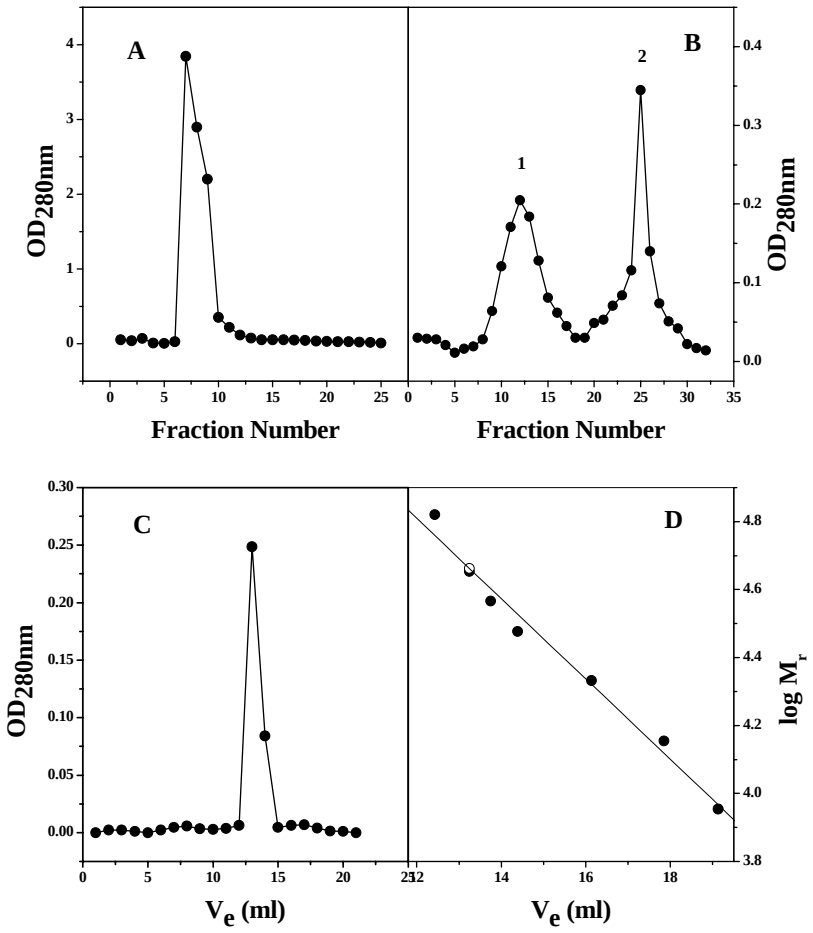

Fig. 5.1. A) Affinity chromatography of *T. cordata* lectin on cross-linked guar gum. B) Separation of TCA isolectins by ion-exchange chromatography. The affinity purified TCA (10.8 mg) in Tris/HCl buffer, pH 8.0 was applied to a DEAE Cellulose column (15 × 1 cm), pre-equilibrated with the same buffer. The column was washed with 15 ml of the same buffer and then eluted successively with TBS containing 0, 100, 200, 300, 400, and 500 mM NaCl (15 ml each). Fractions (3 ml) were collected and elution was monitored by A_{280nm}. Fractions corresponding to the horizontal bars under the two peaks were pooled separately and designated TCA-I and TCA-II. C) Gel filtration of TCA-I on Superose-12. The absorbance at 280 nm is plotted as a function of the elution volume (V_e). D) A plot of log M_r versus elution volume (V_e). Closed circles correspond to standard proteins: 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), soybean trypsin inhibitor (M_r 21,500), lysozyme (M_r 14,300), tendamistat (M_r 9,000). Open circle corresponds to TCA.

Table 5.1. Purification of *Trichosanthes cordata* seed lectin (from 70 g of seeds).

Sample	Total activity (Agglutination units)	Total protein (mg)	Specific activity (units/mg)	Percent recovery	Purification fold
Aqueous Extract	320000	8860	36.1	100	1.0
Ammonium sulphate precipitation (85%)	194304	3624	53.6	61	1.5
Affinity purified protein	177664	47	3780.1	56	105

Glycoprotein nature of TCA-I and TCA-II

Estimation of carbohydrate was done by phenol sulphuric acid assay. This assay has shown that TCA-I contains 6.2% neutral sugar and TCA-II contains 6.8% neutral sugar, indicating that both TCA-I and TCA-II are glycoproteins. In this respect they are similar to other cucurbit seed lectins MCL, SGSL, TCSL and TDSL, which are all glycoproteins with neutral carbohydrate content ranging from 3-10%.

Hemagglutination activity and its inhibition by sugars

Hemagglutination-inhibition experiments were carried out on TCA-I to probe its carbohydrate specificity and the results were summarised in Table 5.2. The results show that agglutination activity of this lectin is inhibited strongly by galactose and its derivatives. The inhibitory potency of MeβGal is about 2.5 fold higher than that of MeαGal, indicating that equatorial configuration at the anomeric position is better recognised. Both 2-dGal and $GalNH_2$ are approximately 3-fold weaker in their inhibitory potency as compared to Gal, clearly demonstrating that the

equatorial hydroxyl group at C2 in galactose is an important locus for the interaction of carbohydrates with *T. cordata* lectin. On the other hand GalNAc

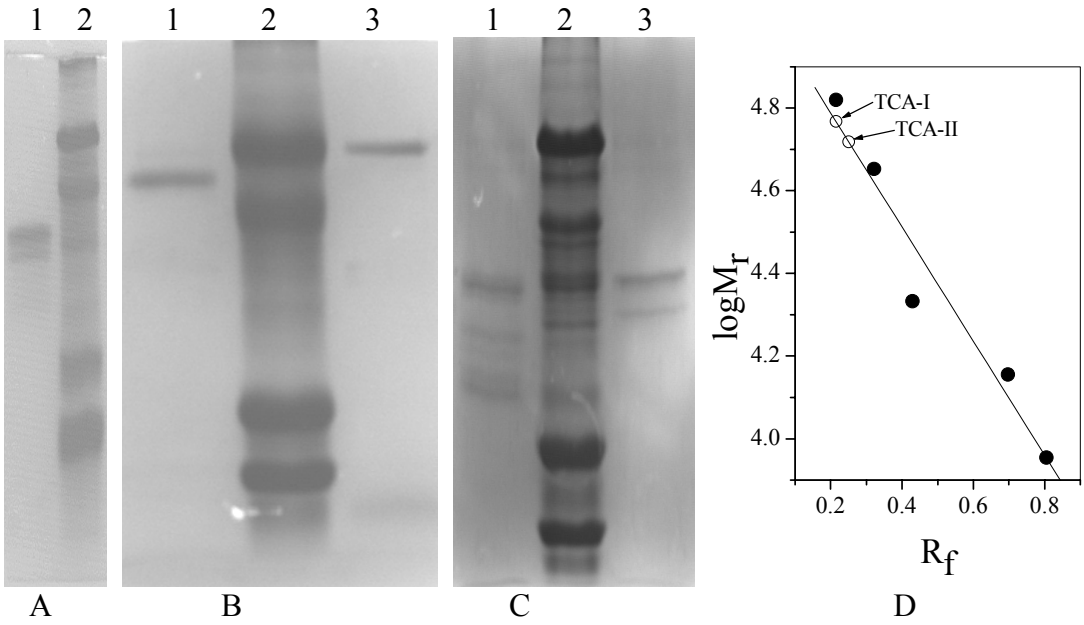

Fig. 5.2. Polyacrylamide gel electrophoresis of TCA. A) SDS-PAGE of affinity purified TCA: Lane 1) affinity purified TCA, Lane 2) protein molecular weight markers. B) SDS-PAGE of TCA isolectins separated by ion exchange chromatography: Lane 1) TCA II, Lane 2) Markers, Lane 3) TCA I. C) SDS-PAGE under reducing conditions (in presence of β-mercaptoethanol): Lane 1) TCA II, Lane 2) Markers, Lane 3) TCA I. D) Plot of relative mobilities (R_f) *vs* log M_r. Solid circles are for protein marks whereas open circles are for TCA isoforms. Protein molecular weight markers used are: a) bovine serum albumin (66 kDa), b) ovalbumin (45 kDa), c) soybean trypsin inhibitor (20.1 kDa), d) lysozyme (13.4 kDa), and e) tendamistat (9 kDa).

is about three times stronger than galactose, suggesting that the acetamido moiety of this sugar makes a positive contribution towards binding. Overall, these results indicate that *T. cordata* lectin is Gal/GalNAc specific with higher affinity towards GalNAc. In this respect TCA is similar to TDSL, another cucurbit seed lectin but differs from two other cucurbit seed lectins, namely SGSL and TCSL [Sultan et al., 2004b; Komath & Swamy, 1999; Kenoth & Swamy, 2003].

Among the various mono and disaccharides investigated, *p*NPβGal exhibited the strongest affinity for TCA-I, which appears to be most likely due to additional interactions of the aromatic aglycon with a hydrophobic pocket in the neighbourhood of the binding site. Hydrophobic aglycons or substituents on sugars have been reported to stabilize their interaction with a number of lectins [Komath et al., 2006]. Fucose is only half as potent as galactose, clearly showing that the hydroxyl group on the C6 of galactose makes a positive contribution in the binding of carbohydrates to lectin. However, fucose is twice as effective as L-arabinose, suggesting that the C-6 methyl group of fucose exhibits a favourable interaction with the combining site of lectin, although the contribution from this interaction is weaker than the interaction of the C-6 hydroxymethyl group in galactose. Glucose, mannose and their methyl glycosides did not show any inhibition, suggesting that equatorial hydroxyl at C-2 and an axial hydroxyl group at C-4 are not only important loci for the lectin but also reversal of configuration at these positions might lead to unfavourable interaction of the ligand with the lectin combining site, which results in their non-recognition by the lectin. The disaccharides lactose (Galβ14Glc) and lactulose (Galβ14Fru) exhibit 2-fold higher affinity than galactose and these are comparable to MeβGal, where as melibiose (Galα16Glc) is 2-fold weaker than galactose. These results suggest that TCA-I prefers the β-anomers of galactose. Other disaccharides, maltose and cellobiose did not inhibit the hemagglutination activity of the lectin indicating that TCA-I does not recognize dimers of glucose also.

Thermal stability and pH dependence of TCA-I

Samples of TCA-I dialyzed against appropriate buffers in the pH range 4.0-12.0 were tested for agglutination activity at 4 ^{o}C. The relative activity of the lectin was less than 20% at pH 5.0, but increases steeply and reaches 100% at pH 7.0. The

activity decreases only marginally at pH 10.0 (~95%), but decreases to about 80% at pH 11.0 (Fig. 5.3A). These results indicate that agglutination activity of TCA-I

Table 5.2. Inhibition of agglutination activity of *T. cordata* seed lectin by various saccharides. Protein concentration was 150 μg/ml (after all reagents were mixed).

Sugar[1]	Minimum Concentration for inhibition[2]	Relative inhibitory potency (galactose = 1.0)
Gal	25.2	1.00
2-deoxyGal	71.8	0.35
$GalNH_2$.HCl	81.6	0.31
GalNAc	8.7	2.90
MeαGal	26.0	0.97
MeβGal	11.2	2.25
Fucose	42.6	0.59
MeβthioGal	13.7	1.84
*p*NPβGal	7.6	3.32
L-Arabinose	83.9	0.30
Lactose	8.8	2.86
Lactulose	10.2	2.47
Melibiose	38.2	0.66

[1]All sugars are of D configuration except noted otherwise.
[2]The following sugars did not inhibit the hemagglutination activity of TCA up to the resultant concentrations indicated in the parentheses: Glucose, MeαGlc, MeβGlc, GlcNAc, mannose, MeαMan, cellobiose, maltose (all 100 mM), MeUmbβGlc (16 mM), pNPαGlcNAc (5 mM).

is maximal in the pH range 7.0-10.0. In this respect TCA-I is rather similar to several other Cucurbitaceae seed lectins, namely MCL, SGSL and TCSL [Sultan & Swamy, 2005a; Komath et al., 2001; Kenoth & Swamy, 2003]. MCL and TCSL are active in the pH range 7.0-11.0 whereas SGSL exhibits maximum activity in the pH range 6.0-10.0.

Thermal inactivation of TCA-I was investigated by incubating lectin samples at different temperatures for 30 minutes followed by assaying for agglutination activity. The lectin activity was unaffected between 4 and 40 °C, but decreases above 40 °C (Fig. 5.3B). When compared with TCA-I, activity of TCSL was unaffected up to 60 °C, whereas activity of MCL was stable up to about 50 °C. This shows that TCSL and MCL are more thermostable than TCA-I [Sultan & Swamy, 2005a; Kenoth et al., 2003]. However, another cucurbit seed lectin, SGSL is similar to TCA-I as it was rapidly inactivated at temperatures above 40 °C [Komath et al., 2001].

Fluorescence spectra of TCA-I samples that were incubated at 5 and 70 °C are shown in Fig. 5.3C. From this Figure it can be seen that the fluorescence λ_{max} does not change during the heating, although the fluorescence intensity decreased at higher temperature. In fact, the emission λ_{max} of spectra recorded at different temperatures between 5 and 70 ^{o}C was seen at the same wavelength. The emission intensity, however, decreased linearly with increase in temperature as shown in the inset of Fig. 5.3C. These results indicate that tryptophan residues of TCA-I are largely buried in hydrophobic core of the protein and do not experience any increase in solvent accessibility due to the heating, suggesting that this thermal treatment does not induce any significant unfolding. MCL, another cucurbit seed lectin undergoes unfolding above 50 ^{o}C, where significant changes were observed

in the fluorescence spectrum with a considerable red shift in the emission maximum [Sultan & Swamy, 2005a].

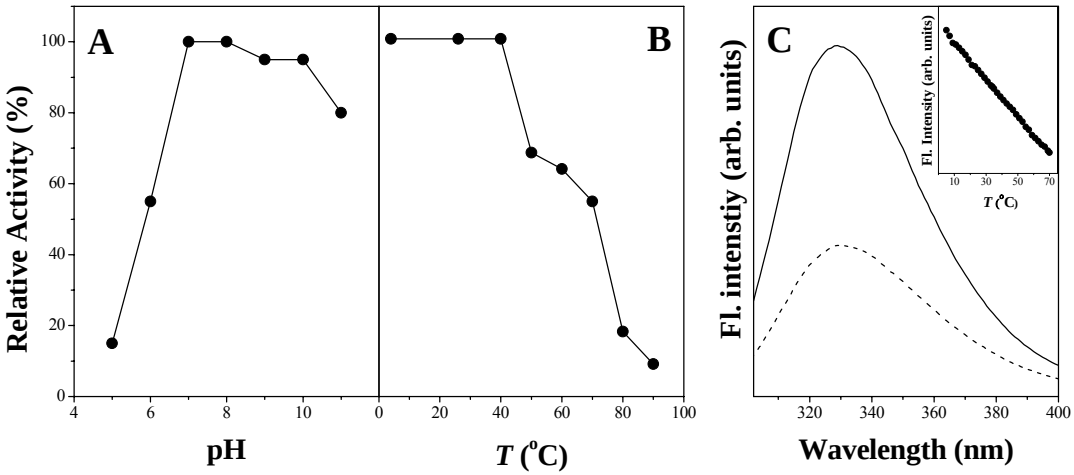

Fig. 5.3. Effect of pH (A) and temperature (B) on the agglutination activity of *T. cordata* lectin. (C) Fluorescence spectra of TCA-I: (—) 5 °C and (----) 70 °C. *Inset* shows the emission intensity at 329 nm as a function of temperature.

CD spectroscopy and secondary structure

CD spectra of TCA-I under native conditions in the absence of any ligand and in the presence 75 mM lactose are shown in Fig. 5.4. The far UV CD spectrum is characterized by minima at ca. 208 and 217 nm, which suggest the presence of both α-helical and β-sheet conformations. However, the absence of a clearly identifiable band around 222 nm indicated that the α-helical content must be relatively low. In order to draw conclusions on the relative content of different secondary structural elements in the protein, the far UV CD spectrum of TCA-I was analyzed by the CDSSTR program [Compton & Johnson, 1986; Sreerama & Woody, 2000] using the software routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Lobley & Wallace, 2001; Lobley et al., 2002]. The values of various secondary structure elements in TCA-I obtained by this analysis are: 8% α-helix (2% regular and 6% distorted), 38% β-sheet (25% regular

and 13% distorted), 20% turns and 33% unordered structures. The calculated fit obtained from this analysis is shown as a dotted line in Fig. 5.4. From this figure it is seen that the calculated fit is in excellent agreement with the experimentally obtained spectrum. CD spectra that are very similar to those of TCA-I both in the near UV and far UV regions were obtained for TCA-II (not shown) and their analysis indicated that it is made up of 10% α-helix (3% regular and 7% distorted), 37% β-sheet (24% regular and 13% distorted), 20% turns and 32% unordered structures. Two other Cucurbitaceae seed lectins that are galactose-specific, namely MCL and TDSL were reported to contain very similar secondary structure [Sultan et al., 2004a, b]. The far UV CD spectrum recorded in the presence of lactose is nearly identical to the spectrum obtained in the absence of sugar, suggesting that carbohydrate binding does not alter the secondary structure of the lectin.

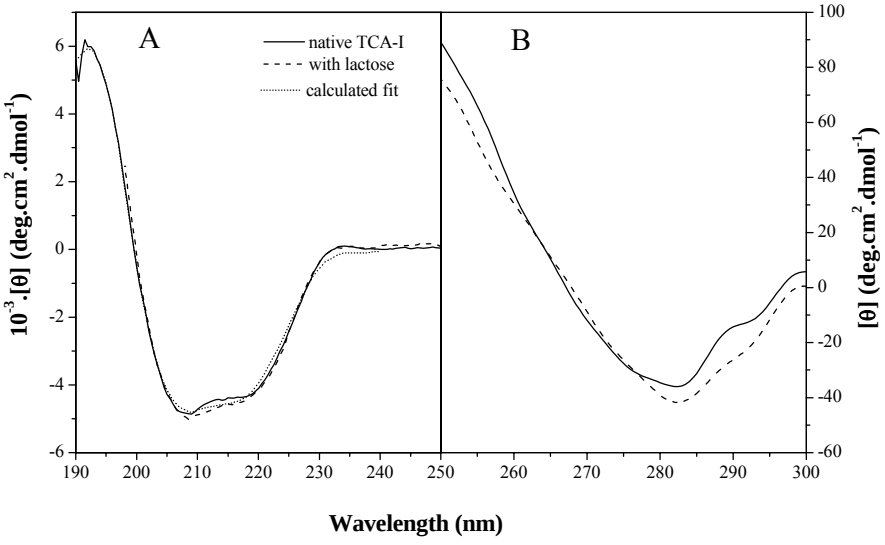

Fig. 5.4. CD spectra of TCA-I in the absence and in the presence of lactose and analysis by CDSSTR method. A) far UV region, B) near UV region. (————) experimental spectrum, (·····) calculated fit by CDSSTR, (- - - - - -) with 0.1 M lactose.

The near UV CD spectrum of TCA-I is characterized by a minimum at 282.5 nm and a shoulder around 292 nm. These bands can be assigned to the side chains of Tyr and Trp residues, respectively. In the presence of near saturating concentrations of lactose (75 mM), the intensity of both the bands increased noticeably, suggesting perturbation of both Tyr and Trp residues upon ligand binding.

Lactose binding to TCA-I

Upon excitation at 280 nm TCA-I gave an emission spectrum centered at 329 nm. Titration of the protein with lactose resulted in a decrease in the emission intensity (spectra not shown). The fluorescence intensity at saturation binding was determined from the Y-intercept of a plot of $F_o/\Delta F$ versus $1/[L]_t$, where F_o is the initial fluorescence intensity, F is the fluorescence intensity at any point during the titration, ΔF (= $F_o - F$) is the change in fluorescence intensity at any point of the titration and $[L]_t$ is the total concentration of the ligand (lactose). Such an analysis indicated that the intrinsic fluorescence intensity of the protein decreases by about 5% at saturation binding. The fluorescence titration data was then analyzed by the method of Chipman et al. [1967] according to the equation:

$$\log \{\Delta F/(F-F_\infty)\} = \log K_a + \log [L]_f \qquad (5.1)$$

where F_∞ is the fluorescence intensity of the protein at infinite concentration of the ligand, K_b is the association constant, and $[L]_f$ is the free ligand concentration at each point of the titration and was obtained from the following Equation:

$$[L]_f = [L]_t - \{(\Delta F/\Delta F_\infty)\,[P]_t\} \qquad (5.2)$$

where ΔF_∞ (= $F_o - F_\infty$) is the change in fluorescence intensity at saturation binding and $[P]_t$ is the total protein concentration. A double-logarithmic plot for the binding of lactose to TCA-I is shown in Fig. 5.5. The X-intercept of the plot

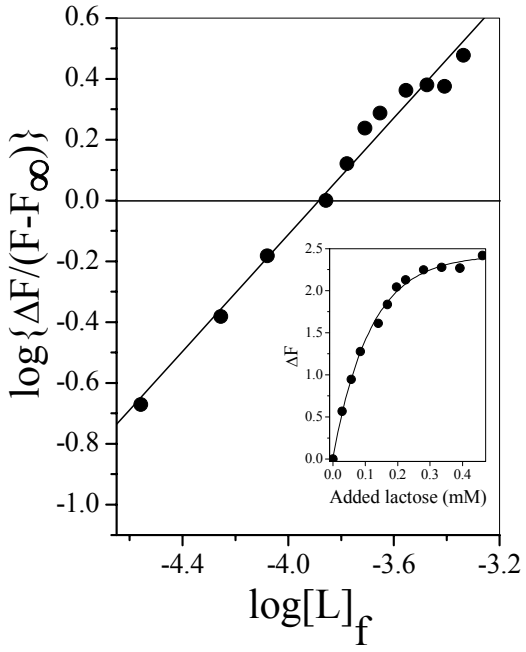

Fig. 5.5. Chipman plot for fluorescence titration of TCA-I with lactose. Inset shows the binding curve.

Yielded the pK_a value of the interaction from which the K_a value was estimated as 7.64×10^3 M^{-1}. Two independent titrations yielded an average value of 7.42×10^3 M^{-1}.

Quenching of the intrinsic fluorescence of TCA-I

Fluorescence quenching studies were carried out on TCA-I in order to investigate the microenvironment of the indole side chains of Trp residues of the lectin. Acrylamide, which is neutral and iodide, an anion have been employed as quenchers. It is well known that the neutral acrylamide can penetrate partially into the folded protein matrix and hence quench the fluorescence of even partially buried Trp residues whereas iodide ion, being charged can quench only surface exposed Trp residues [Eftink & Ghiron, 1976, 1981].

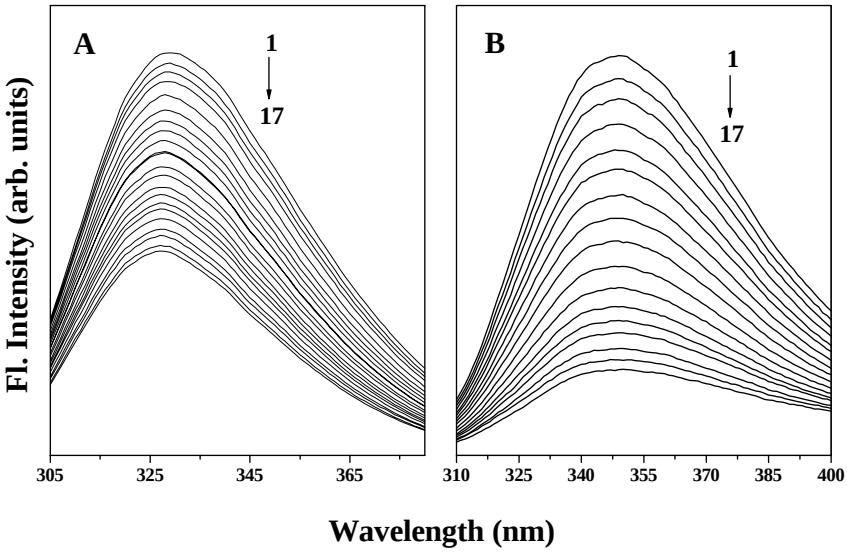

Fig. 5.6. Fluorescence spectra of TCA-I in the absence and in the presence of acrylamide. (A) Under native conditions, B) in the presence of 6 M Gdn.HCl. Spectrum 1 corresponds to TCA-I alone and the remaining spectra correspond to TCA-I in the presence of increasing concentrations of acrylamide (indicated by the arrow).

Fluorescence spectra of TCA-I in the native state and upon denaturation with 6 M Gdn.HCl, recorded in the absence and in the presence of different concentrations of acrylamide and iodide are shown in Fig. 5.6A and Fig 5.6B, respectively. The native lectin exhibits an emission maximum at 329 nm, which is red shifted to 349 nm upon denaturation. From this figure it is seen that quenching by acrylamide is higher with the denatured lectin than for the native lectin, suggesting that accessibility of tryptophan residues to the quencher increases when the lectin is unfolded. Quenching by iodide also increases in presence of Gdn.HCl. These observations indicate that the indole side chains of tryptophan residues in TCA-I become significantly more exposed to the bulk aqueous medium upon denaturation. The extent of quenching achieved at a resultant concentration of 0.5 M of each quencher under different conditions is given in Table 5.3.

Table 5.3. Summary of parameters obtained from intrinsic fluorescence quenching of TCA-I.

Quencher and Condition	ΔF (%)	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	f_a (%)	K_a (M^{-1})
Acrylamide					
native	34.0	1.90	1.09	0.48	5.21
+ 0.1 M lactose	36.1	1.71	1.05	0.53	4.33
+ 0.1 M MeβGal	34.6	1.65	1.09	0.49	4.89
+ 6 M Gdn.HCl	74.7	5.62*	------	0.93	6.37
Iodide					
native	6.8	0.36	0.07	0.08	9.85
+ 0.1 M lactose	3.2	0.12	0.02	0.04	5.49
+ 6 M Gdn.HCl	60.1	1.70	------	0.81	2.53

While acrylamide quenched about 34% of the total fluorescence intensity of the protein, the ionic quencher iodide was able to quench only about 6.8% of the protein intrinsic fluorescence, clearly indicating that the Trp residues of the protein are predominantly buried in the hydrophobic core of the protein matrix and are therefore inaccessible to the charged iodide ion. This is also consistent with the emission maximum of the protein at 329 nm, which indicates that Trp residues are in a significantly nonpolar environment. Presence of 0.1 M lactose, the sugar that is specifically recognized by the *T. cordata* lectin, had only marginal effect on the

extent of quenching with acrylamide (36.0%) but decreased the quenching by iodide to about one half (3.2%). Decrease in quenching by iodide in presence of ligand most likely indicates a modest compaction of the protein, resulting in a general decrease in the accessibility of the Trp residues to iodide without altering their exposure to acrylamide. Such an interpretation is consistent with the results of CD spectroscopic studies, which suggested that ligand binding does not lead to any changes in the secondary structure of the protein whereas minor changes are expected in the tertiary structure. The weak effect of bound sugar on lectin fluorescence quenching by acrylamide was also observed for another Cucurbit seed lectin, namely TDSL [Sultan & Swamy, 2005b]. Denaturation with 6 M Gdn.HCl results in an increase in the observed quenching to 74.7% and 60.1% with acrylamide and iodide, respectively.

The steady-state fluorescence quenching data obtained with acrylamide and iodide were analysed by the Stern-Volmer and modified Stern-Volmer equations 5.3 and 5.4, respectively [Lehrer, 1971]:

$$F_o/F_c = 1 + K_{SV}[Q] \qquad (5.3)$$

$$F_o/\Delta F = f_a^{-1} + 1/(K_a f_a [Q]) \qquad (5.4)$$

where F_o and F_c are respective fluorescence intensities, corrected for dilution, in the absence and in the presence of quencher, [Q] is the resultant quencher concentration, K_{SV} is Stern–Volmer quenching constant, ΔF (=F_o- F_c) is the change in fluorescence intensity at any point in the quenching titration, f_a is the fraction of the total fluorophores accessible to the quencher and K_a is the corresponding Stern–Volmer association constant for the fraction of the fluorophores. The Stern–Volmer plots obtained with both acrylamide and iodide were biphasic (Fig. 5.7), clearly indicating that there are at least two sets of fluorescent Trp residues in TCA-I that differ in the accessibility to both neutral and ionic quenchers. Quenching

measurements were also carried out in the presence of saturating concentrations of MeβGal and lactose, which also yielded biphasic Stern-Volmer plots (not shown). Theses observations are consistent with the results expected from dynamic fluorescence quenching of proteins with two or more Trp residues in different microenvironments with different accessibilities to the quenchers. From the slopes of the two phases of these plots the corresponding Stern–Volmer constants (K_{SV1} and K_{SV2}) were obtained and listed in Table 5.3. The K_{SV} values obtained indicate that acrylamide is significantly more efficient than I^- in quenching the intrinsic fluorescence of TCA-I. Binding of the monosaccharide and disaccharide ligands (MeβGal and lactose) decreases the K_{SV1} value for acrylamide quenching but does not significantly affect K_{SV2}, indicating that the more accessible fluorophores of the native protein are partially shielded by ligand binding whereas the less accessible fluorophores of TCA-I are not affected. On the other hand, for quenching by iodide ion both types of fluorophores become less accessible. Most likely ligand binding results in an overall compaction of the protein leading to a general decrease in the accessibility of the fluorophores to the quenchers.

Under denaturing conditions Stern–Volmer plot of quenching with iodide ion became linear indicating that the iodide ion now has equal accessibilities to each tryptophan residue in the protein. On the other hand, Stern–Volmer plot for acrylamide was linear at low quencher concentrations, but showed upward curvature at higher concentrations of the quencher (Fig. 5.7), indicating that the quenching process occurs via both dynamic and static mechanisms.

Similar results were also obtained with another Cucurbitaceae seed lectin, TCSL [Kenoth & Swamy, 2003]. The dynamic and static components can be separated using a plot of $(F_o/F - 1)/[Q]$ *versus* [Q], whose slope (S) gives the

product K_DK_S whereas the Y-intercept (I) yields (K_D + K_S). Value of the static quenching constant K_S can then be obtained by incorporating the values of S and I

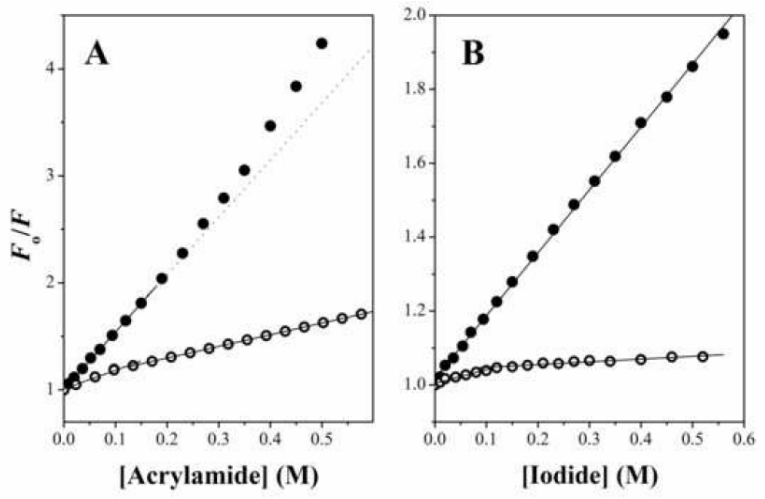

Fig. 5.7. Stern-Volmer plots for fluorescence quenching of TCA-I by (A) acrylamide and (B) iodide ion; (•) native, (o) denatured. Linear least square fits for different components are shown as solid lines. The dashed line in (A) shows an extrapolation of the least squares fit of the initial linear part of the data.

in the quadratic equation [Lacowicz, 1999]:

$$K_S^2 - K_SI + S = 0 \qquad (5.5)$$

The plot of (F_o/F – 1)/[Q] *versus* [Q] gave the values of slope (S) and intercept (I) as 1.542 M^{-2} and 5.425 M^{-1}, respectively. Incorporating these values in eq. 5.5 yielded the two values of K_S as 5.125 M^{-1}or 0.3 M^{-1}. If K_S is 5.125 M^{-1} then K_D will be 0.3 M^{-1}, (since K_S + K_D = 5.425 M^{-1}), whereas if K_S is 0.3 M^{-1}, then K_D will be 5.125 M^{-1}.

From the initial linear portion of the Stern-Volmer plot the Stern-Volmer quenching constant (K_D) was estimated as 5.62 M^{-1}. This is likely to yield a slight overestimate of the K_{SV} value because of the upward curving nature of the plot. Therefore, it would be appropriate to assign the value of 5.125 M^{-1} (which is

slightly lower than the above value) to the dynamic quenching constant (K_D) and the value of 0.3 M^{-1} to the static quenching constant (K_S).

Modified Stern-Volmer plots for TCA-I with both acrylamide and iodide under different conditions yielded linear fits (not shown). From the Y-intercepts of these plots, f_a, fraction of the total fluorescence that is accessible to the quencher, and the corresponding Stern-Volmer quenching constants (K_a) were estimated and the values obtained are presented in Table 5.3. It is clearly seen from the data given that in native TCA-I about 48% of the fluorescence is accessible to acrylamide, whereas iodide ion could access only 8% of the total fluorescence intensity. Denaturation with 6 M Gdn.HCl led to an increase in the accessibility to 93% for acrylamide whereas for the ionic quencher, I^-, fraction accessible increased to 81%. This reflects the large increase in the solvent accessibility of tryptopan residues in the unfolded protein. Even in the presence of 6 M Gdn.HCl, the neutral quencher acrylamide could not achieve 100% accessibility, suggesting the presence of some local order due to the presence of disulphide bonds in the protein polypeptide chain. The lower accessibility observed with iodide must be due to the presence of negatively charged residues in the vicinity of some of the tryptophan residues. In the presence of specific ligands, lactose and MeβGal, the accessible fraction of fluorescence intensity did not change significantly (within 10% of the accessibility without sugar), whereas for iodide ion quenching accessibility decreased to 50% of the value in the absence of sugar.

In summary a new galactose-specific lectin has been isolated and purified from the seed extract of *Trichosanthes cordata* by affinity chromatography on cross-linked guar gum. The *T. cordata* agglutinin (TCA) has been resolved into two isolectins (TCA-I and TCA-II) by ion-exchange chromatography. Both TCA-I and TCA-II are similar in their carbohydrate specificity and have similar secondary

structure, which consists of predominantly β-sheet and β-turns and very little α-helix. Fluorescence titrations yielded an association constant of 7.42×10^{3} M^{-1} for the interaction of lactose with TCA-I. Fluorescence quenching studies indicate that the tryptophan residues of TCA-I are mostly buried in the hydrophobic core of the folded protein.

Chapter 6

General Discussion and Conclusions

General discussion and conclusions

In the studies reported in this thesis, physicochemical studies were carried out on three galactose specific seed lectins from Cucurbitaceae, namely *Momordica charantia* seed lectin, *Trichosanthes dioica* seed lectin and *Trichosanthes cordata* seed lectin. The *M. charantia* lectin was investigated in considerable detail earlier by others as well as us with respect to macromolecular properties, binding of carbohydrate and porphyrin ligands. In studies reported in Chapter 2 the interaction of MCL with several nucleic acid bases and the hydrophobic base ANS have been studied using fluorescence spectroscopy. In the studies presented in Chapter 3 thermal, chemical and acid-induced unfolding of MCL was investigated by differential scanning calorimetry, fluorescence and circular dichroism spectroscopy. The *Trichosanthes dioica* seed lectin has been purified in this laboratory and characterized in some detail with respect to macromolecular properties and tryptophan exposure and accessibility using fluorescence spectroscopy. In Chapter 4, thermal, chemical and acid-induced unfolding of TDSL has been investigated by differential scanning calorimetry, fluorescence and circular dichroism spectroscopy. In Chapter 5, a new galactose specific lectin was isolated from the seeds of *Trichosanthes cordata* and characterized with respect to physicochemical properties, carbohydrate binding, and fluorescence quenching. The results obtained from the above studies are summarized below.

The interaction of *Momordica charantia* seed lectin with several nucleic acid bases and ANS was investigated by fluorescence spectroscopy. The association constants obtained for MCL-nucleic acid base interaction were comparable to the affinity constants obtained for the interaction of MCL with different mono- and disaccharides as well as with different water soluble porphyrins, suggesting that the nucleic acid base binding may also have a

physiological role like carbohydrate binding. The slope of the plot for the interaction of MCL-nucleic acid base was close to unity, indicating that each MCL subunit binds one molecule of the nucleic acid base. Titrations carried out in presence of 0.1 M lactose yielded an association constant which is close to the value obtained in the absence of lactose, indicating that adenine and lactose bind at independent sites on the protein. Previous work from this laboratory has demonstrated that several water-soluble porphyrins that are predominantly hydrophobic in nature also interact with MCL at a site that is distinct from the carbohydrate binding site. In this respect MCL is similar to most legume lectins in having binding sites for non-carbohydrate ligands that are distinctly different from the sugar binding sites. Fluorescence intensity of ANS is enhanced upon binding to MCL. Enhancement in the fluorescence intensity of ANS was also observed for the binding of ANS to a number of other lectins. The fluorescence enhancement is maximum at pH 2 with a concomitant blue shift in the emission λ_{max}, whereas at neutral and basic pH changes in both fluorescence intensity and emission maximum were very small, clearly suggesting that the MCL-ANS interaction is stronger at lower pH values. Enhancement of ANS fluorescence intensity and associated blue shift of emission λ_{max} is generally attributed to hydrophobicity of the binding site.

Far UV CD spectrum of MCL recorded in presence of ANS at pH 2.0 was nearly identical to the spectrum recorded in the absence of ANS, suggesting that ANS binding did not lead to any alteration in the secondary structure of MCL. When excited at 295 nm in the presence of ANS, the protein fluorescence decreased with a concomitant increase in the emission intensity of ANS, suggesting resonance energy transfer from the tryptophan residues of MCL to ANS. Presence of specific saccharide, lactose did not affect binding of adenine or ANS, and addition of lactose or adenine to MCL-ANS mixture did not alter the change in ANS

fluorescence induced by its binding to MCL suggesting that lactose, adenine and ANS bind to MCL at independent and non-interacting sites.

Thermal stability of MCL was studied as a function of pH, scan rate, and at different ligand concentrations by using high-sensitivity differential scanning calorimetry. The calorimetric scan consisted of two entities melting at different temperatures. The lower transition peak has a T_m of 333.7 K with ΔH_c of 1157 kJ.mol^{-1}, while the higher transition peak has a T_m of 338 K with a ΔH_c of 1084 kJ.mol^{-1}. DSC scan of abrin II also consists of two transition peaks which correspond to subunit A and B. The unfolding process is irreversible and could be described by a three-state model. The results at higher scan rates indicate that the thermodynamic quantities ΔH_c and ΔH_v are independent of scan rate although there is a slight increase in T_m when the scan rate was increased over 3-fold and this increase may be accounted for by differences in instrumental response. Therefore equilibrium thermodynamic model is applied instead of irreversible model. For MCL tetramer, the $\Delta H_c/\Delta H_v$ ratio is close to 4 for the first transition and ~2 for the second transition, suggesting that four and two cooperative units are involved in the first and second transitions, respectively. In the presence of lactose both the low-temperature transition and high-temperature transition shifted to higher values, suggesting that ligand binding stabilizes the native conformation of MCL.

Effect of pH on the unfolding transition of MCL was studied by DSC measurements of MCL samples as a function of pH in the range 2-10. Endotherms recorded as a function of pH indicate that MCL is most stable at pH 5.0. The near UV and far UV CD spectra of MCL were rather similar in the pH range of 2.0 – 10.0, indicating that the secondary and tertiary structure of MCL did not change in this pH range, and attest to the stability of the protein over a broad pH range. Gel filtration profiles at pH 2 and pH 7 are similar, indicating that even at low pH MCL exists as tetramer.

Chemical unfolding of MCL induced by Gdn.HCl was monitored by fluorescence spectroscopy. These studies revealed that chemical unfolding of MCL is also a three-state process, which involves an intermediate that is well populated at a Gdn.HCl concentration of 3 M. These observations suggest that both chemical and thermal unfolding mechanisms are similar in that both of them proceed via an intermediate.

Physico-chemical and unfolding studies have been carried out on *Trichosanthes dioica* seed lectin (TDSL). The lectin activity was found to be maximum in the pH range 7-10 and decreased quite rapidly below pH 7.0. The lectin activity was unaffected in the temperature range 4 – 50 ^{o}C, while at higher temperatures a sharp decrease in the activity was noticed. DSC results indicate that unfolding of TDSL is an irreversible process and could be described by a three-state model. At pH 7 the calorimetric scan consists of two transitions, occurring at 338.6 K, and 342.8 K.

Ratio of calorimetric enthalpy to van't Hoff enthalpy ($\Delta H_c/\Delta H_v$) for the first transition peak is greater than 1 for TDSL dimer indicating that two subunits unfold as a single entity during denaturation. For the second transition $\Delta H_c/\Delta H_v = 0.5$ implies intermediate transitions are observed in the transition profile. TDSL is more stable at pH 4 and 5. In presence of lactose both lower-temperature transition and higher-temperature transition shifted to higher values suggesting that ligand binding stabilizes the native conformation of TDSL. Among several carbohydrate ligands used, lactose maximum shift in the T_m, which is consistent with the strong affinity of the lectin for this disaccharide. Binding constants calculated for different carbohydrate ligands at denaturation temperature indicate that TDSL preferentially binds to β-linked galactopyranosides.

Far and near UV circular dichroism spectra of TDSL recorded at different temperatures up to 65 ^{o}C were very similar, but significant changes were seen in the spectrum recorded at 70 ^{o}C, suggesting thermal unfolding of the protein between these two temperatures, which is consistent with the above DSC results. The near and far UV CD spectra of *T. dioica* lectin recorded at different pH values were rather similar, indicating that the lectin is stable over a wide pH range. Denaturation of TDSL induced by Gdn.HCl, monitored by following changes in the intrinsic fluorescence properties of the protein, indicated that the chemical unfolding of this lectin is also a three-state process, which proceeds through an intermediate. These studies show that Gdn.HCl induced denaturation of TDSL is qualitatively similar to the thermal unfolding of the lectin as deduced from DSC studies.

The seed extracts of *Trichosanthes cordata* exhibited strong agglutination activity, which could be inhibited by galactose and its derivatives. Based on this observation, a galactose-specific lectin has been purified from the *T. cordata* seed extract by affinity chromatography on cross-linked guar gum. The affinity purified *T. cordata* agglutinin (TCA) yielded a diffuse single band in polyacrylamide gel electrophoresis under native conditions, whereas in SDS-PAGE in the absence of β-mercaptoethanol it gave two closely-spaced bands. The affinity-eluted lectin could be resolved into two isolectins, TCA-I and TCA-II by ion-exchange chromatography on DEAE cellulose. These two isolectins moved as single bands in SDS-PAGE in the absence of β-mercaptoethanol. TCA-I and TCA-II are glycoproteins with 6.2% and 6.8% covalently bound neutral sugar, respectively.

In reducing SDS-PAGE, TCA-I showed two bands indicating that it is composed of two disulfide-linked subunits whose masses were estimated as 32 and 27 kDa, whereas TCA-II yielded several bands. Therefore, all further studies were

conducted with TCA-I. TCA-I eluted as a single symmetrical peak on gel filtration on Superose-12 in the presence of 0.1 M lactose with an M_r of 49.0 kDa. In the absence of ligand the movement was retarded, indicating a possible interaction of the lectin with the column matrix. Analysis of far UV CD spectrum showed that TCA-I contains 8% α-helix, 38% β-sheet, 20% β-turns and 33% unordered structures.

Agglutination activity of TCA-I was inhibited strongly by galactose and its derivatives. β-Galactosides were generally better inhibitors than α-galactosides with *p*-nitrophenyl-β-D-galactopyranoside being the strongest ligand among a battery of sugars investigated. The agglutination activity of TCA-I was found to be highest in the pH range 7.0-8.0. The lectin activity was unaffected between 0 and 40 ^oC, but the activity decreased steeply at higher temperatures. Association constant for the interaction of TCA-I with lactose was determined as 7.42×10^3 M^{-1} at 25 ^oC by fluorescence spectroscopy. The exposure and accessibility of the tryptophan residues of TCA-I and the effect of ligand binding on them have been probed by fluorescence quenching studies employing a neutral quencher, acrylamide and an anionic quencher, iodide ion. These studies revealed that the tryptophan residues of TCA are buried in the hydrophobic core of the folded polypeptide chain.

From the above studies it can be concluded that hydrophobic ligand properties of MCL were further elaborated, and it can be seen from these studies that adenine binding site and carbohydrate binding are independent of each other, it is possible that MCL can bind to the hydrophobic ligand and carry it to a site bearing a specific carbohydrate structure that is recognized by the carbohydrate binding site. Unfolding studies were carried out on MCL and TDSL lectins to understand the mechanism of their unfolding. In addition, a new galactose specific lectin has been purified from the seeds of *Trichosanthes cordata*, which belongs to Cucurbitaceae and characterized in considerable detail.

Appendix

Thermodynamic studies on the interaction of water-soluble porphyrins with the glucose/mannose-specific lectin from garden pea (*Pisum sativum*)

Summary

Due to the application of porphyrins as photosensitizers in photodynamic therapy to treat cancer, and the ability of some lectins to preferentially recognize tumor cells, studies on the interaction of porphyrins with lectins are of considerable interest. In this study thermodynamic studies were carried out on the interaction of several free-base and metallo-porphyrins with pea (*Pisum sativum*) lectin (PSL). Association constants (K_a) were obtained by absorption titrations by monitoring changes in the *Soret* band of the porphyrins and the K_a values obtained for various porphyrins at different temperatures are in the range of 1.0×10^4 to 8.0×10^4 M^{-1}. Both cationic and anionic porphyrins were found to bind to PSL with comparable affinity. Presence of 0.1 M methyl-α-D-mannopyranoside – a carbohydrate ligand that is specifically recognised by PSL – did not affect the binding significantly, suggesting that porphyrin and sugar bind at different sites on the lectin. From the temperature dependence of the K_a values, the thermodynamic parameters, change in enthalpy and change in entropy associated with the binding process were estimated. These values were found to be in the range: ΔH^o = -95.4 to -33.9 kJ.mol^{-1} and ΔS^o = -237.2 to -32.2 J. mol^{-1}.K^{-1}, indicating that porphyrin binding to pea lectin is driven largely by enthalpic forces with the entropic contribution being negative. Enthalpy-entropy compensation was observed in the interaction of different porphyrins to PSL, with the exception of *meso*-tetra-(4-sulfonatophenyl)porphyrinato zinc(II), emphasizing the role of water structure in the overall binding process. Circular dichroism and differential scanning calorimetric studies indicate that while porphyrin binding does not induce significant changes in the lectin structure and thermal stability, carbohydrate binding induces moderate changes in the tertiary structure of the protein and also increases its thermal unfolding temperature and the enthalpy of the unfolding transition.

Introduction

Carbohydrate recognition of lectins has made them important tools in a number of areas of biological research such as isolation and purification of glycoconjugates, mitogenic stimulation of lymphocytes as well as in clinical/biomedical applications such as blood typing and fractionation of cells for use in bone marrow transplantation [Lis & Sharon, 1998; Sharon & Lis, 2003; Ambrosi et al., 2005]. In some legume plants, lectins mediate the interaction between root nodules and rhizobia. Although carbohydrate binding as alluded to above has been the main focus of research on lectins for several decades, there have been a few reports indicating that ligands other than carbohydrates are also recognised by some lectins [Komath et al., 2006]. Especially, it has been shown that several lectins bind small, hydrophobic molecules such as ANS, TNS, adenine and cytokinin. The earliest example of this type is the observation that several small, hydrophobic compounds bind to Concanavalin A [Hardmann & Ainsworth, 1973]. Subsequently, it has been shown that lima bean lectin binds to adenine and several of its derivatives with cytokinin activity, indicating that lectins may have a role to play in the regulation of their activity [Roberts & Goldstien, 1982, 1983; Maliarik & Goldstein, 1988; Gegg et al., 1992].

Porphyrins are a group of biologically important hydrophobic molecules. Although porphyrins are being used as photosensitizers in photodynamic therapy (PDT) for the treatment of cancer [Kessel, 1986; Bonnet, 1995; Levy, 1995; Dougherty et al., 1988], the selectivity of porphyrins for tumor cells is generally not adequate to ensure their selective killing by the photodynamic action [Klyashchitsky et al., 1994]. Because some lectins preferentially bind to tumor cells, lectins could potentially be used as targeting agents for porphyrin photosensitizers in PDT. Therefore, it was considered interesting to investigate the interaction of porphyrins with lectins. In earlier studies, we characterized the interaction of several free-base and metallo-porphyrins with some of

the well-characterized plant lectins such as Con A, pea lectin, jacalin, snake gourd (*Trichosanthes anguina*) seed lectin (SGSL), *Trichosanthes cucumerina* seed lectin (TCSL), and *Momordica charantia* seed lectin (MCL) by absorption and fluorescence spectral titrations and also determined the 3-dimensional structures of porphyrin complexes of Con A, jacalin and peanut agglutinin [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001; Sultan et al., 2004; Goel et al., 2001, 2004, 2005]. Among these, thermodynamic forces that govern porphyrin binding have been characterized only for TCSL and MCL, which showed that the interaction of porphyrins with TCSL is stabilized by positive entropic contribution, whereas porphyrin binding to MCL is driven primarily by enthalpic forces. In order to understand the forces that mediate the interaction of different porphyrins with different lectins, it is necessary to carry out similar studies with other lectins as well.

Pea lectin is a homodimeric, glucose/mannose-specific protein isolated from the seeds of garden pea (*Pisum sativum*). The protein is a dimer with an M_r of 49 kDa, with each subunit being made up of two noncovalently associated polypeptides of M_r 7 kDa and 17 kDa [Trowbridge, 1974]. Pea lectin is a metalloprotein and contains one Mn^{+2} ion and two Ca^{+2} ions per monomer [Bhattacharya et al., 1985]. The metal ions are necessary to maintain the lectin structure in a functionally active form. In this report we have investigated the interaction of pea lectin with several water soluble free-base and metallo-porphyrins. Binding experiments were performed at different temperatures in order to delineate the thermodynamic forces that govern the interaction. The results show that pea lectin-porphyrin interaction is governed by enthalpic forces, with the entropic contribution being negative. Additionally, circular dichroism and differential scanning calorimetric studies show that porphyrin binding does not significantly affect the protein structure and stability whereas sugar binding has a stabilizing effect on the protein structure.

Materials and Methods

Materials

Pea seeds were obtained from local vendors. Sephadex G-100 was purchased from pharmacia, Uppsala, Sweden. Methyl-α-D-mannopyranoside, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), and the reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from Sigma (St. Louis. MO, USA). All Porphyrins used in this study were synthesized and characterized as described previously [Fleishcher et al., 1971; Kadish et al., 1989; Longo et al., 1969; Pasternack et al., 1972, 1973]. All the other reagents were purchased from local suppliers and were of the highest purity available.

Purification of *Pisum sativum* (pea) lectin

Pea lectin was purified by affinity chromatography on Sephadex G-100 as described earlier [Trowbridge, 1974]. The entire purification was done in 10 mM Tris-HCl buffer containing 0.15 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.2 (TBS) and the purified lectin was maintained in the same buffer. Purity of the affinity eluted protein was checked by SDS-PAGE where the lectin gave two bands, consistent with literature reports [Trowbridge, 1974]. Concentration of the purified lectin was estimated spectrophotometrically by using an $A_{280nm}^{1\%,1cm}$ value of 15.5 [Bhattacharya et al., 1985] and was expressed in subunits corresponding to 24.5 kDa, unless specified otherwise.

Porphyrin binding

Porphyrin binding to pea lectin was investigated at different temperatures by absorption spectroscopy as described earlier [Sultan et al., 2004a]. Absorption measurements were performed on a Shimadzu model UV3101PC UV-Vis-NIR double-beam spectrophotometer using 1.0-cm path-length cells. Temperature was maintained constant (± 0.1^{o}C) by means of a Peltier device supplied by the manufacturer. Titrations were

performed by adding small aliquots of the lectin from a concentrated stock solution (>20 mg/mL) in TBS to 2.0 mL of a 2.0-3.0 μM solution of the porphyrin in the same buffer taken in a spectrophotometer cuvette. Absorption spectra were recorded after an equilibration period of 2 minutes. The spectra were corrected for dilution effects resulting from the increase in sample volume due to addition of the protein. All titrations were performed three to five times and the average values are reported.

CD spectroscopy

CD studies were performed on a Jasco-J-810 spectropolarimeter. Samples dissolved in TBS were placed in a rectangular quartz cuvette of 2-mm path-length and spectra were recorded at a scan speed of 20 nm/min with a response time of 2 seconds and a slit width of 1 nm. Measurements in the near UV region were performed with ca. 35 μM lectin dimer and for measurements in the far UV region the concentration used was 1.3 μM in lectin dimer. CD spectra of PSL were also recorded in the presence of 0.2 M MeαMan (both near UV and far UV regions) or 0.4 mg/ml (0.32 mM) H_2TSPP in the near UV region and 0.1 mM in the far UV region, in order to investigate the effect of porphyrins/carbohydrate binding on the protein structure. Buffer scans, recorded under identical conditions, were subtracted from the experimental spectra before further analysis.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) studies were performed on a MicroCal VP differential scanning calorimeter with a scan speed of 20°/hr (Celsius scale). Thermograms were recorded with lectin samples of 36 μM concentration in TBS at pH 7.2. Porphyrin concentration used was 0.32 mM. Pea lectin was dialysed extensively against the buffer and degassed prior to loading into the cell. Buffer scans were

subtracted from the thermograms corresponding to the lectin samples for analysis. Data were analysed by Origin software supplied by the manufacturer.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Jobin-Yvon Spex Fluoromax 3 fluorescence spectrometer. Slit widths of 3 and 5 nm were used on the excitation and emission monochromators, respectively. Samples of PSL (≤ 0.1 OD) were irradiated with 280 nm light and emission spectra were recorded above 300 nm. In order to investigate the effect of MeαMan binding on the protein fluorescence, a 2.0 mL solution of PSL was titrated by adding small aliquots of the sugar from a 50 mM stock solution. Emission spectra were recorded after each addition and the emission spectra were corrected for dilution effects before further analysis.

Results

Porphyrin binding to pea lectin: Absorption and difference absorption spectroscopy

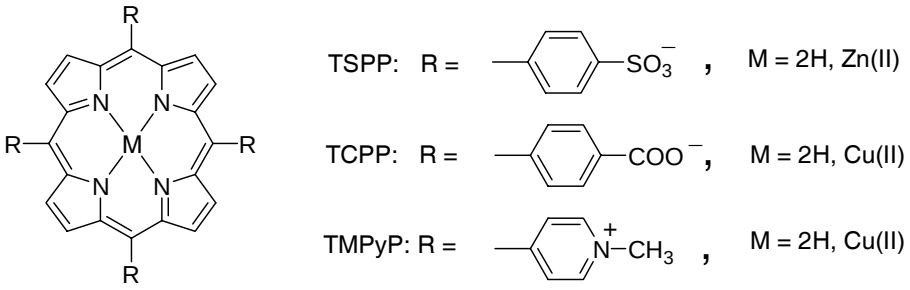

Fig. A.1. Structures of the porphyrins used in this study

A schematic diagram representing the structure of various porphyrins used in this study is shown in Fig. A.1. The λ_{max} and ε_{max} values for the *Soret* band of these porphyrins were taken from our earlier studies and were used for estimating their concentrations [Komath et al., 2000; Sultan et al., 2004a]. All the porphyrins used in this study obeyed

Beer's law up to 5 μM concentration, suggesting that under the experimental conditions they were not aggregated.

Absorption spectra in the *Soret* band region for CuTMPyP, a tetra-cationic porphyrin, and for H_2TCPP, a tetra-anionic porphyrin, in the absence and presence of pea lectin, are shown in Fig. A.2 A and B. In each figure, spectrum 1 corresponds to the porphyrin alone and spectra 2-16 correspond to those obtained in the presence of increasing concentrations of pea lectin. From these spectra it is seen that the λ_{max} of the *Soret* band occurs at 414.5 nm and 425 nm for H_2TCPP and CuTMPyP, respectively. The intensity of the *Soret* band decreases upon addition of pea lectin, accompanied by a marginal shift in the band position; at the highest concentration of pea lectin employed in these titrations the λ_{max} is observed at 415 nm and 426 nm for H_2TCPP and CuTMPyP, respectively.

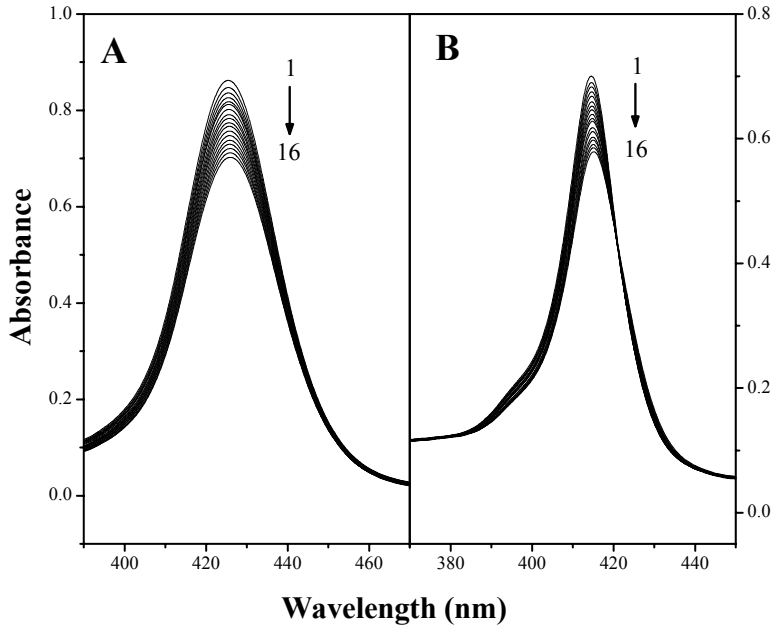

Fig. A.2. Absorption spectra of porphyrins in the absence and in the presence of pea lectin. A) CuTMPyP, B) H_2TCPP. In each panel, spectrum 1 corresponds to the porphyrin alone and spectra 2-16 are those that are recorded in the presence of increasing concentrations of pea lectin.

Difference spectra obtained by subtracting the porphyrin spectrum in buffer from those of the porphyrins in the presence of different concentrations of pea lectin are shown in Fig. A.3 A and B, respectively, for the titrations of CuTMPyP and H_2TCPP with pea lectin. Titration of other anionic porphyrins, namely H_2TSPP and ZnTSPP, with pea lectin yielded absorption and difference absorption spectra that are qualitatively similar to those obtained with H_2TCPP in the *Soret* band region.

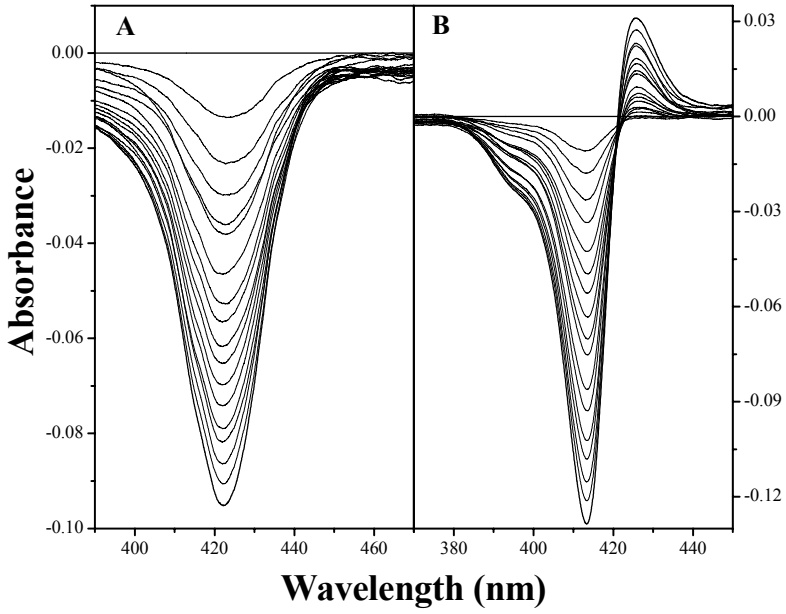

Fig. A.3. Difference absorption spectra for porphyrin binding to pea lectin. A) CuTMPyP, B) H_2TCPP. The difference spectra were obtained by subtracting the spectrum of the porphyrin alone from those obtained in the presence of different concentrations of pea lectin, shown in the corresponding panels in Fig. A.2.

Determination of association constants and thermodynamic parameters

A binding curve obtained by plotting change in the absorbance maximum (ΔA_{max}) as a function of added lectin concentration for pea lectin-H_2TCPP titration, carried out at 30 ^{o}C, is given in Fig. A.4 A. From this figure it can be seen that the absorption intensity of

the porphyrin changes sharply at the initial stages whereas at higher concentrations the magnitude of the change decreases indicating that binding of H_2TCPP to pea lectin displays saturation behaviour. A plot of $A_o/\Delta A$ *versus* $1/[P]_t$, where A_o refers to the absorbance of the sample in the absence of protein, ΔA is the change in absorbance at each point of the titration and $[P]_t$ is the corresponding concentration of PSL, yielded a straight line for the titration of H_2TCPP with pea lectin (see inset to Fig. A.4 A). The ordinate of this plot gives ΔA_∞, the change in absorption intensity when all the porphyrin molecules are completely bound to pea lectin. From this, the absorption intensity of the porphyrin when it is completely bound to PSL was calculated. The titration data were further analyzed according to the model of Chipman et al. [1967] as described previously for the binding of porphyrins to other lectins in order to determine the association

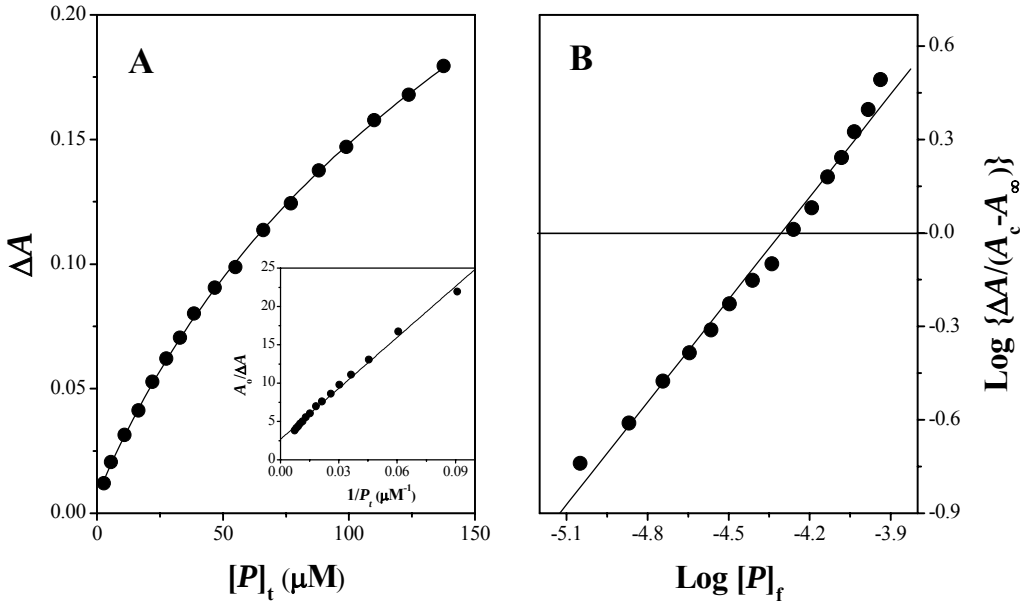

Fig. A.4. Analysis of the binding data for the titration of H_2TCPP with pea lectin. A) Binding curve obtained by plotting the change in absorbance (ΔA) at 413.5 nm as a function of the added lectin subunit concentration. The inset shows a plot of $A_o/\Delta A$ *vs* $1/[P]_t$. From the Y-intercept of this plot, A_∞, the absorbance of the sample at infinite concentration of the lectin was calculated. B) Chipman plot for the determination of the association constant, K_a. The X-

intercept of the plot gives the pK_a value of the binding reaction, from which the K_a value is obtained. Temperature = 30°C.

constants for PSL-porphyrin interaction [Bhanu et al., 1997; Komath et al., 2000a,b; Kenoth et al., 2001; Sultan et al., 2004a]. Plots of log $[P]_f$ *versus* log $[(\Delta A/(A_c - A_\infty)]$ were obtained for each titration and from the X-intercepts of these plots, association constants for the lectin-porphyrin interaction were estimated graphically according to the equation:

$$\log[(\Delta A/(A_c - A_\infty)] = \log K_a + \log [P]_f \quad \text{(A.1)}$$

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

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

where A_c is the absorption intensity of the porphyrin at any point of the titration, ΔA_∞ is the change in absorption intensity at saturation binding, $[L]_t$ is the total concentration of porphyrin, and $[P]_f$ is the free protein concentration. A representative plot for the interaction of H_2TCPP with pea lectin is given in Fig. A.4 B. The slope of this plot is found to be ~1.0, indicating that each lectin subunit binds one porphyrin molecule. From the abscissa of this plot, the association constant, K_a, for the pea lectin-H_2TCPP interaction at 30 °C was obtained as 2.1×10^4 M^{-1}. Two independent measurements yielded an average value of 2.16×10^4 M^{-1}. This value along with the association constants obtained for the binding of H_2TCCP, H_2TSPP, CuTMPyP and ZnTSPP to pea lectin at different temperatures are given in Table A.1. From the association constants presented in Table A.1, the Gibb's free energies for pea lectin-porphyrin interaction were calculated using the expression:

$$\Delta G = -RT \ln K_a \quad \text{(A.3)}$$

The thermodynamic parameters, change in enthalpy (ΔH^o) and change in entropy (ΔS^o), were calculated from van't Hoff plots ($\ln K_a$ versus $1/T$) according to the equation:

$$\ln K_a = -\Delta H°/RT + \Delta S°/R \quad \text{(A.4)}$$

The van't Hoff plots obtained for pea lectin-porphyrin interaction are given in Fig.A.5 and the values of change in enthalpy entropy obtained from it are presented in Table A.1.

Table A.1. Association constants, K_a, obtained at different temperatures for the interaction of various porphyrins with pea lectin and the corresponding thermodynamic parameters, ΔH^o and ΔS^o, obtained from van't Hoff plots.

Porphyrin	Temperature (°C)	$K_a \times 10^{-4}$ (M^{-1})	$\Delta H°$ (kJ. mol^{-1})	$\Delta S°$ (J. mol^{-1}. K^{-1})
CuTMPyP	15	2.94 (±0.8)		
	20	2.32 (±0.4)		
	20	5.20 (±1.4)*	-33.9	-32.2
	25	1.79 (±0.3)		
	30	1.48 (±0.13)		
H_2TCPP	15	4.19 (±1.2)		
	20	3.44 (±2.0)		
	20	3.39(±0.12)*		
	25	2.45 (±0.8)	-35.7	-35.4
	30	2.16 (±0.4)		
H_2TSPP	20	3.72 (±0.7)		
	20	3.68 (±2.0)*		
	25	2.90 (±1.5)	-58.6	-112.0
	30	1.65 (±1.0)		
	35	1.22 (±0.1)		
ZnTSPP	15	7.97 (±0.72)		
	20	4.22 (±1.3)	-95.4	-237.2
	20	1.07 (±0.2)*		
	25	2.20 (±0.7)		

*Values obtained from titrations performed in the presence of 0.1 M MeαMan

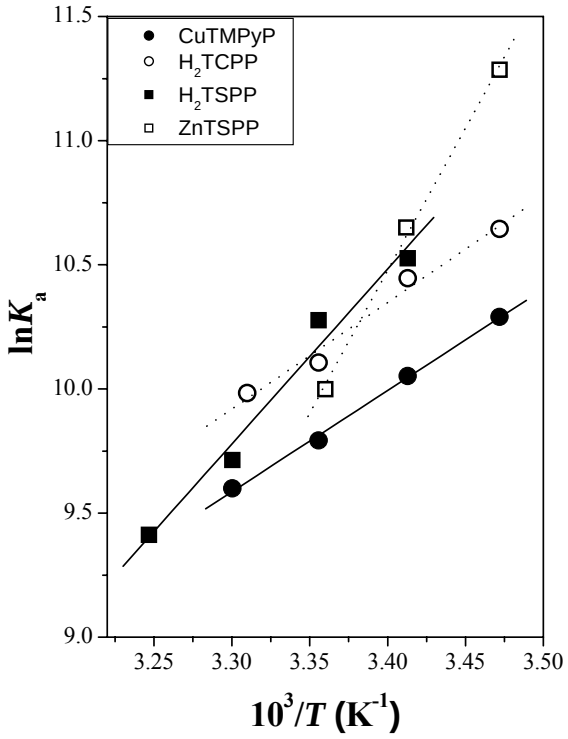

Fig. A.5. van't Hoff plots for the interaction of porphyrins with pea lectin. (O) H_2TCPP, (●) CuTMPyP, (■) H_2TSPP, (□) ZnTSPP

Binding of MeαMan to pea lectin: Fluorescence spectroscopy

The fluorescence spectrum of pea lectin is characterized by an emission maximum at 321 nm, indicating that the indole side chains of the tryptophan residues in this protein are in a hydrophobic environment. Titration of the protein with MeαMan resulted in a small, but reproducible decrease in the fluorescence intensity without any change in the emission λ_{max}. At saturation binding the maximum change observed was in the range of 7-8% of the initial fluorescence intensity of the protein. Analysis of the changes in the fluorescence intensity according to Chipman et al. [1967] as described earlier for the interaction of galactose-containing sugars with jack fruit (*Artocarpus integrifolia*) seed lectin [Sastry & Surolia, 1986] yielded an association constant of 1.38×10^4 M^{-1} at 25°C.

This value is in good agreement with the value of 1.65×10^4 M^{-1} obtained from titration calorimetry measurements performed at 25.3 ^oC and pH 7.4 [Schwarz et al., 1993].

Effect of porphyrin and sugar binding on the secondary structure of pea lectin: CD spectroscopy

The effect of porphyrin binding and sugar binding on the structure of pea lectin has been investigated by CD spectroscopy. Spectra of PSL alone and in the presence of H_2TSPP and MeαMan are shown in Fig. A.6. The CD spectrum of pea lectin alone in the far UV region is characterized by a strong negative band with a minimum around 230 nm, and is in good agreement with earlier reports [Herrmann et al., 1978]. However, since the fraction of individual secondary structural elements such as α-helix and β-sheet have not been analyzed in the earlier study, we have determined the content of different types of secondary structures in pea lectin using the K2D program using the routines available at the website DICHROWEB (http://www.cryst.bbk.ac.uk/cdweb/html/) [Lobley & Wallace, 2001; Lobley et al., 2002]. This analysis yielded the following results for the secondary structure of pea lectin: 2% α-helix, 51% β-sheet and 47% random coil. These results are consistent with the notion that pea lectin is a predominantly β-sheet protein with negligible α-helical content in solution [Bureš et al., 1972; Herrmann et al., 1978]. From the crystal structure of PSL [Einspahr et al., 1986], it is seen that the protein contains about 51.1% β-sheet and rest unordered structure and our results from the CD spectroscopy are in excellent agreement with this. The near UV CD spectrum of PSL exhibits a maximum around 271 nm and a minimum at 284 nm (Fig. A.6 B). Since the CD spectrum in near UV region was not reported earlier, this is a new observation. Since the effect of carbohydrate binding on the secondary structure of pea lectin has not been investigated earlier by CD spectroscopy, we considered it interesting to investigate this aspect in this study and to compare the results with the effects observed upon porphyrin binding to this lectin.

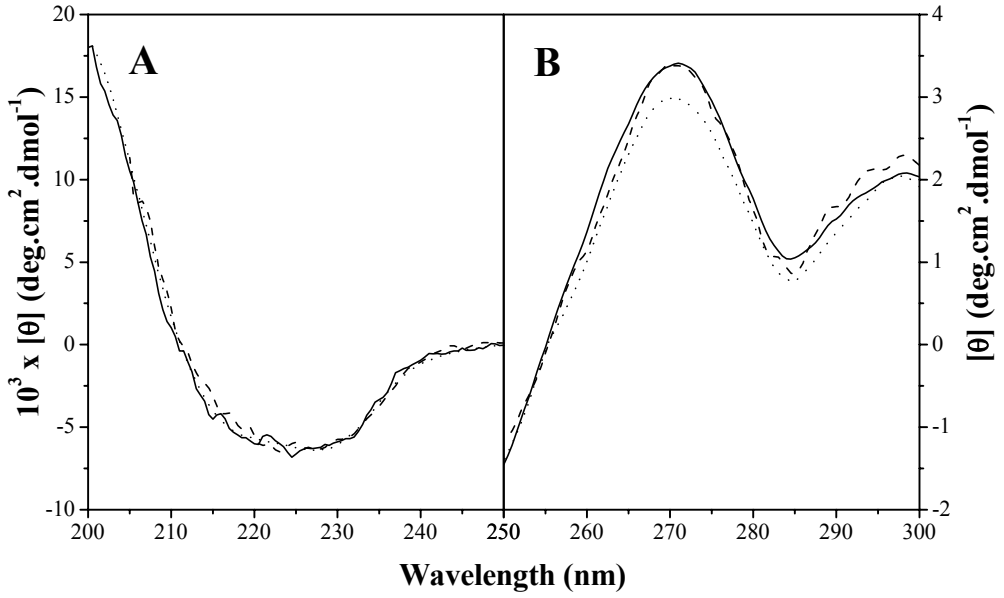

Fig. A.6. Effect of sugar and porphyrin on the CD spectra of pea lectin. A) Near UV region, B) Far UV region. (—) Native pea lectin, (------) pea lectin + H_2TSPP, (·····) pea lectin + MeαMan. The spectrum of pea lectin obtained in the presence of both MeαMan and H_2TSPP (not shown) was essentially identical to the spectrum obtained in the presence of MeαMan alone. See text for details.

While the CD spectra of pea lectin in the absence and in the presence of saturating concentrations of MeαMan are nearly identical in the far UV region (Fig. A.6 A, dotted lines), the near UV spectrum obtained in the presence of MeαMan exhibits a decreased intensity with maximum change being seen around 271 nm (Fig. A.6 B, dotted lines). On the other hand, porphyrin binding does not seem to affect the secondary and tertiary structures of PSL as the CD spectra of pea lectin alone and in the presence of H_2TCPP are essentially identical in the far UV region and the differences in the near UV region are relatively smaller in nature (Fig. A.6, dashed lines).

Effect of porphyrin binding on the thermal stability of pea lectin: Differential scanning calorimetry.

Although sugar binding in general has been shown to result in a stabilization of the lectin structure, as seen from an increase in the thermal unfolding temperature, the effect of porphyrin binding on the thermal stability of lectins has not been investigated before.

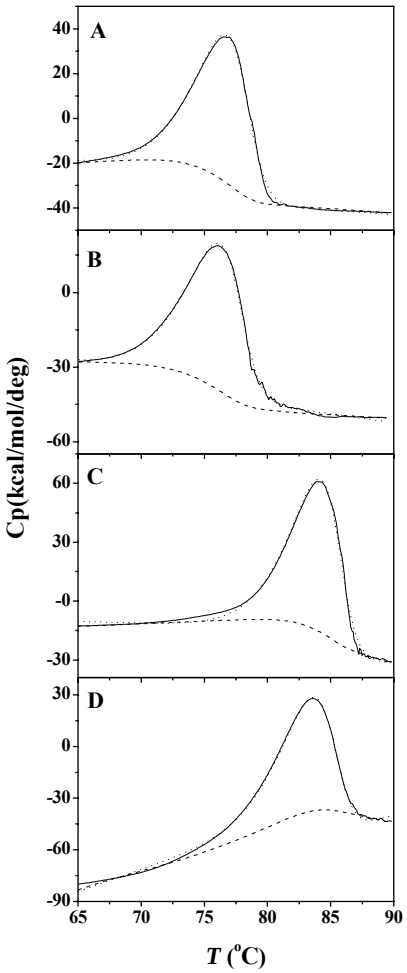

Fig. A.7. Differential scanning calorimetric thermograms of pea lectin. A) Pea lectin alone, B) pea lectin + 0.32 mM H_2TSPP, C) pea lectin + 0.2 M MeαMan, D) pea lectin + 0.32 mM H_2TSPP + 0.2 M MeαMan.

Therefore, DSC studies were carried out in the present study to investigate if porphyrin binding has any effect on the stability of pea lectin. Experiments were also carried out in the presence of saturating concentrations of MeαMan in order to compare the effect of sugar binding and porphyrin binding under identical conditions. Thermograms of PSL in the native state and in the presence of 0.2 M MeαMan or 12-fold excess of H_2TSPP are shown in Fig. A.7. Higher concentrations of the porphyrin could not be used due the low solubility of the porphyrin in aqueous media. Thermogram **A**, which corresponds to pea lectin alone, exhibits an unfolding transition centred at 76.6 ^{o}C, with an enthalpy value of 1360 kJ/mol.

Presence of porphyrin does not seem to affect the phase transition temperature (76.2 ^{o}C) although the transition enthalpy is marginally increased to 1420 (±7) kJ/mol as seen from thermogram **B**. On the other hand, binding of MeαMan leads to an increase in the thermal unfolding temperature of PSL to 84.1 ^{o}C, with a concomitant increase in the enthalpy of the transition to 1672 (± 44) kJ/mol (thermogram **C**). In the presence of both H_2TSPP and MeαMan pea lectin yields a thermogram with a transition centred at 83.7 ^{o}C with a transition enthalpy of 1502 (± 15) kJ/mol (thermogram **D**).

Discussion

Studies carried out in the last decade indicate that porphyrins bind to different plant lectins with considerable affinity [Bhanu et al., 1997; Komath et al., 2000 a, b, 2006; Kenoth et al., 2001; Sultan et al., 2004a]. Detailed thermodynamic analysis of porphyrin binding to two Cucurbitaceae seed lectins, namely TCSL and MCL indicated that the TCSL-porphyrin interaction is primarily stabilized by entropic factors [Kenoth et al., 2001], where as binding of porphyrins to MCL is mediated by strong enthalpic forces, with negative contribution from entropy of binding [Sultan et al., 2004a]. Single-crystal X-ray diffraction studies on the interaction of H_2TSPP with different lectins, viz., Con A,

jacalin and peanut agglutinin (PNA) have revealed distinctly different modes of interaction between the lectin and the porphyrin [Goel et al., 2001, 2004, 2005; Komath et al., 2006]. These observations, taken together, have indicated that the mode of interaction of and thermodynamic forces stabilizing the binding for different lectin-porphyrin combinations could be quite different. Therefore, it was considered important to investigate the thermodynamic forces characterizing the interaction of different lectins with different porphyrins. In this study, the interaction of several water-soluble porphyrins with pea lectin has been investigated by difference absorption spectroscopy, circular dichroism and differential scanning calorimetry and the results obtained are discussed below.

Values of the association constants presented in Table 1 show that the binding affinities characterizing the interaction of different porphyrins with PSL are in the range of $1.0 \times 10^4 - 8.0 \times 10^4$ M^{-1}, which are broadly in the same range as those observed for the other lectin-porphyrin systems studied earlier as well as with the affinities that characterize the binding of simple mono- and disaccharides to the plant lectins investigated in these studies [Sastry et al., 1986; Schwarz et al., 1993, 1996; Bhanu et al., 1997, Komath et al., 2000 a, b; Komath et al., 2001; Kenoth et al, 2001, 2003; Sultan et al., 2004a; Sultan & Swamy, 2005a]. Moreover, the binding constants, K_a, obtained in presence of MeαMan, a monosaccharide that is specifically recognized by pea lectin, are comparable to the values obtained in the absence of sugar, suggesting that porphyrin and sugar bind at different locations on the lectin surface. Further, these results indicate that binding of porphyrin or sugar does not result in any significant alterations in the lectin structure that can lead to major changes in the binding affinity of the other ligand. Results of CD spectral studies (see Fig. A.6), are also in good agreement with this interpretation. Binding of small, hydrophobic molecules such as adenine, auxins, cytokinins, ANS and TNS to different plant lectins is characterized by association

constants in the range of $1 \times 10^3 – 2.3 \times 10^6$ M^{-1} [Hardmann & Ainsworth 1973; Roberts & Goldstien, 1982, 1983; Maliarik & Goldstein, 1988; Gegg et al., 1994; Komath et al., 2006]. In view of the fact that auxins, cytokinins and certain derivatives of adenine act as plant growth regulators [Roberts & Hooley, 1988], it is attractive to postulate that they may act as endogenous ligands to lectins. Since porphyrins bind to lectins with affinities in the same range as those for the above hydrophobic ligands, as reported in this study as well in earlier studies from this laboratory [Bhanu et al., 1997; Komath et al., 2000a, 2000b, 2006; Kenoth et al., 2001; Sultan et al., 2004a], porphyrins may also be considered as potential endogenous ligands for plant lectins.

It is interesting to note that both CuTMPyP, which is cationic and the three anionic porphyrins, namely H_2TSPP, H_2TCPP and ZnTSPP bind to pea lectin with broadly comparable affinities. Although it is difficult to rationalize this observation in the absence of structural information on the complexes between PSL and different porphyrins, it is likely that the mode of binding of cationic and anionic porphyrins would be different. It has also been observed in earlier studies that both cationic and anionic porphyrins bind to different lectins with rather similar affinities. It is necessary to investigate the 3-dimensional structures of different lectin-porphyrin complexes in order to understand the underlying structural principles that stabilize the complexes between different porphyrins and lectins.

From the association constants presented in Table 1 two distinct features can be noted. First, the association constant for ZnTSPP at low temperature (15^oC) is significantly higher than those obtained at the same temperature for the other porphyrins. Second, the K_a values for ZnTSPP exhibit a steeper temperature gradient. This is clearly evident in the van't Hoff plots shown in Fig. A.5. The stronger affinity of ZnTSPP may depend, at least in part, on the preference of Zn(II) ion to interact with additional ligands besides the four coordinations that it forms with the porphine core in the square planar

geometry, in order to attain hexacoordination leading to octahedral geometry. Interaction of the Zn(II) ion with functional groups on the protein surface may satisfy these additional coordination requirements and increase the binding affinity. Modulation of these additional interactions by change in temperature may result in the steeper temperature gradient observed for ZnTSPP as compared to the free base porphyrins, namely H_2TSPP and H_2TCPP. Although the Cu(II) ion in CuTMPyP may also form such additional interactions, in view of its cationic nature its mode of interaction with PSL is likely to be different from that of the above anionic porphyrins and hence it is not possible to compare its binding with the binding of the other anionic porphyrins.

The thermodynamic parameters ($\Delta H°$ and $\Delta S°$) obtained for pea lectin-porphyrin interaction (Table 1) suggest that the overall binding process is governed by enthalpic forces with the entropic contribution being negative. However, the $\Delta H°$ values obtained for the binding of different porphyrins vary significantly. Thus the magnitude of the change in enthalpy for CuTMPyP (-33.9 kJ.mol^{-1}) and H_2TCPP (-35.7 kJ.mol^{-1}) are significantly smaller than the value obtained for the binding of H_2TSPP (-58.6 kJ.mol^{-1}), which in turn is considerably smaller than the value of -95.4 kJ.mol^{-1} obtained for the pea lectin-ZnTSPP interaction. Interestingly, the negative enthalpy of binding for different porphyrins was compensated by proportionately large negative entropy ($\Delta S°$ = -32.2 J.mol^{-1}.K^{-1}, -35.4 J.mol^{-1}.K^{-1}, -112.1 J.mol^{-1} K^{-1} and -237.2 J.mol^{-1}.K^{-1}, respectively, for CuTMPyP, H_2TCPP, H_2TSPP, and ZnTSPP). These results suggest that porphyrin binding to PSL is likely to involve polar interactions such as hydrogen bonding, as observed in the ConA-H_2TPPS complex [Goel et al., 2001].

A plot of $\Delta H°$ *versus* $T\Delta S°$, shown in Fig. A.8, yielded a straight line with the exception of ZnTSPP. This indicates compensatory nature of changes in enthalpy and entropy, associated with the binding of the remaining three porphyrins, namely CuTMPyP, H_2TCPP and H_2TSPP, to pea lectin. A slope of 1.0 for such a plot indicates

that enthalpy is exactly compensated by entropy [Eads et al., 1998], whereas deviation from this value suggests dominance of either enthalpy or entropy in the binding mechanism. The slope of the linear fit shown in Fig. A.8 (0.961) is very close to unity, and thus indicates nearly exact match between enthalpy and entropy of binding. The fact that ZnTSPP does not fit in this line indicates that the nature of the forces that govern its interaction with the pea lectin are somewhat different from those that are responsible for the interaction of the other porphyrins with the lectin. This is consistent with the possibility of the Zn(II) ion forming additional coordination interactions with the protein surface, as alluded to above.

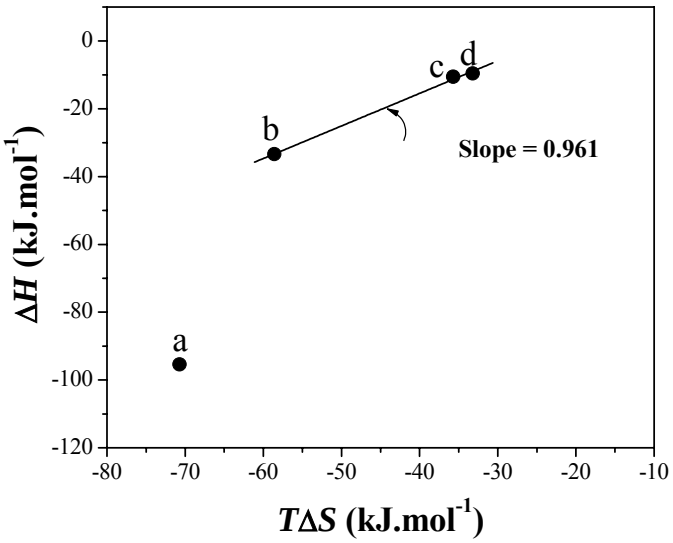

Fig. A.8. Enthalpy-entropy compensation in porphyrin binding to pea lectin. a) ZnTSPP, b) H_2TSPP, c) H_2TCPP, d) CuTMPyP. The straight line represents a linear least squares fit of the data (slope = 0.961). The data for ZnTSPP was not included in the fit.

Enthalpy-entropy compensation was observed earlier for the interaction of various porphyrins to MCL [Sultan et al., 2004a] as well as in the interaction of carbohydrates with several lectins [Sultan & Swamy, 2005a; Schwarz et al., 1993; Ramkumar et al., 1995; Lemieux, 1996; Schwarz et al., 1996; Surolia et al., 1996; Srinivas et al., 1999;

Komath et al., 2001]. Monosaccharide binding to PSL was also accompanied by enthalpy-entropy compensation [Schwarz et al., 1993, 1996]. Enthalpy-entropy compensation has been attributed to the solvent reorganisation during binding process [Lemieux, 1996]. In this context, it is interesting to note that in the crystal structure of Con A-H_2TSPP complex, binding of H_2TSPP to Con A is mediated by hydrogen bonds with bridging water molecules in between [Goel et al., 2001]. It is possible that porphyrin binding to pea lectin may be similar to the binding of porphyrins to Con A and involve water molecules in the overall binding process. Results of differential scanning calorimetric studies on pea lectin in the absence and in the presence of porphyrin and MeαMan gave interesting results (see Fig. A.7). While porphyrin binding did not lead to any noticeable change in the unfolding temperature and had only a marginal effect on the enthalpy of the unfolding transition, in the presence of MeαMan the unfolding temperature increased from 76.6 ^{o}C to 84.1 ^{o}C accompanied by nearly 18% increase in the ΔH value. The present DSC results on PSL under native conditions and in the presence of MeαMan are in good agreement with the earlier results of Schwarz et al. [1993]. These observations are also consistent with the results obtained from CD spectroscopy, which indicated that porphyrin binding does not alter the secondary and tertiary structures of pea lectin, whereas carbohydrate binding led to moderate changes in the tertiary structure of the protein (Fig. A.6). It is pertinent to note here that CD spectroscopy shows that binding of porphyrins to MCL also does not result in significant changes in the secondary and tertiary structures of the protein [Sultan et al., 2004].

In summary, in the present study the interaction of several water-soluble free-base and metallo-porphyrins with the glucose/mannose specific lectin from garden pea (*Pisum sativum*) has been investigated. ZnTSPP exhibited a stronger affinity to the lectin as compared to the other porphyrins, which could be attributed to additional interactions of the Zn(II) ion with the protein surface. Thermodynamic parameters

obtained from van't Hoff analysis of the binding constants indicate that porphyrin binding to PSL is predominantly enthalpic in nature with negative contribution from the entropic factors. CD studies demonstrate that porphyrin binding does not alter the secondary and tertiary structures of the protein, whereas binding of MeαMan leads to small, but distinct changes in the tertiary structure. These results are further corroborated by differential scanning calorimetry, which showed that while porphyrin binding did not have any effect on the thermal unfolding temperature of pea lectin, binding of MeαMan increased both the unfolding temperature as well as the enthalpy of the unfolding transition considerably.

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List of Publications

1. **Kavitha, M**., and Swamy, M.J. (2006) Thermodynamic studies on the interaction of water-soluble porphyrins with the glucose/mannose-specific lectin from garden pea (*Pisum sativum*). *IUBMB Life*. **58**, 720-730.
2. Komath, S.S., **Kavitha, M**., and Swamy, M.J. (2006) Beyond carbohydrate binding: new directions in plant lectin research. *Org. Biomol. Chem*. **4**, 973-988.
3. **Kavitha, M.,** Sultan, N.A.M., and Swamy, M.J. (2008) Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* (bitter gourd) seed lectin (*J. Photochem. Photobiol B: Biol.* (under revision))
4. Sultan, N.A.M., **Kavitha, M.**, and Swamy, M.J. (2008) Purification and physicochemical characterization of two galactose-specific isolectins from the seeds of *Trichosanthes cordata* (Communicated).
5. **Kavitha, M.**, and Swamy, M.J. (2008) Thermal, chemical and acid unfolding studies on *Momordica charantia* (bitter gourd) seed lectin (To be communicated).
6. **Kavitha, M.**, and Swamy, M.J. (2008) Physicochemical characterization and unfolding studies on *Trichosanthes dioica* seed lectin (manuscript under preparation).

Participation in Symposia, Workshop etc.

1. Presented a poster in the Glycans 2006 Symposium, held during February 22 -26, 2006 at Indian institute of science (I. I. Sc.) Bangalore: Title: Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* seed lectin.

2. Participated in the work shop on Molecular characterization of glycoproteins and glycolipids and their interactions with lectins and receptors held during February 20- 28, 2006 at Indian institute of science (I. I. Sc.) Bangalore.
3. Presented a poster in the In house symposium ***Chemfest* 2005** held at School of chemistry, University of Hyderabad, India**.** Title: Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* seed lectin
4. Presented a poster in the In house symposium ***Chemfest* 2006** held at School of chemistry, University of Hyderabad, India**.** Title: Thermodynamic analysis of porphyrin binding *Pisum sativum* (Pea) seed lectin.
5. Presented a poster in the In house symposium ***Chemfest* 2007** held at School of chemistry, University of Hyderabad, India**.** Gave an oral presentation and also presented a poster on Purification of two galactose-specific isolectins from the seeds of *Trichosanthes cordata* and Thermodynamic studies on the interaction of water-soluble porphyrins with pea (*Pisum sativum*) lectin.