Physicochemical Studies on *Trichosanthes cucumerina* Seed Lectin

A Thesis
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DOCTOR OF PHILOSOPHY

By

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To

My loving parents, sisters and friends

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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 Dr. Musti J. Swamy.

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

Hyderabad
October 2002

ROOPA KENOTH

CERTIFICATE

Certified that the work embodied in this thesis entitled "**Physicochemical Studies on *Trichosanthes cucumerina* Seed Lectin**" has been carried out by **Ms. Roopa Kenoth**, under my supervsion and the same has not been submitted elsewhere for a degree.

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LIST OF ABREVIATIONS

BSA	bovine serum albumin
CD	circular dichroism
ConA	ConcanavalinA
CTAB	cetyltrimethylammonium bromide
CuTCPP	*meso*-tetra(4-carboxyphenyl)porphyrinato copper(II)
CuTMPyP	*meso*-tetra(4-methylpyridinium)porphyrinato copper (II)
CuTPPS	*Meso* -tetra(4-sulphonatophenyl) porphyrinato Cu(II)
DEPC	diethylpyrocarbonate
DSC	differential scaning calorimetry
DTNB	5, 5'- Dithiobis (2- nitrobenzoate)
EDTA	ethylenediaminetetraacetic acid
Gal	galactose
Galβ13GalNAc	3-*O*-*N*-acetylgalactopyranosyl-β-D-galactopyranoside
GalNAc	*N*-acetylgalactopyranoside
GNA	*Galanthus nivalis* agglutinin
H_2TMPyP	*meso*-tetra(4-methylpyridinium)porphyrine
IEF	isoelectric focusing
MCL	*Momordica charantia* lectin
MeβGal	methyl-β-galactopyranoside
MeαGal	methyl-α-galactopyranoside
MeUmbβGal	4-methylumbelliferyl-β-D-galactopyranoside
MeUmbαGal	methyl-umbelliferyl-α-D-galactopyranoside
NBS	*N*-bromosuccinimide

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PHA	phytohemagglutinin (previous nomenclature for red kidney bean isolectins)
RCA	*Ricinus cummunis* agglutinin
RIP	ribosome inactivating protein
SBA	soybean agglutinin
SDS	sodium dodecyl sulfate
SGSL	*Trichosanthes anguina* seed lectin
TCSL	*Trichosanthes cucumerina* seed lectin
TKL	*Trichosanthes kirilowii* lectin
TNBS	2,4,6-trinitrobenzyl sulphonate
Tris	tris(hydroxymethyl) aminomethane
Trp	tryptophan
WBA	winged bean agglutinin
WGA	wheat grem agglutinin
ZnTCPP	*meso*-tetra(4-carboxyphenyl)porphyrinato zinc(II)
ZnTMPyP	*meso*-tetra(4-methylpyridinium) porphyrinato zinc(II)
ZnTPPS	*Meso* -tetra(4-sulphonatophenyl) porphyrinato zinc(II)

Chapter I

INTRODUCTION

Lectins: Definition and historic developments

Lectins, also called agglutinins or hemagglutinins, have the longest history of plant proteins. The history of lectins started in the year 1888 when Herrmann Stillmark discovered the cell agglutinating properties of ricin, a toxic protein isolated from castor bean (*Ricinus communis*) extracts [cf. Kocourek, 1986]. Soon similar toxic proteins were identified from the seeds of *Croton tigliu* (croton) and *Abrus precatorius* (abrin) and from the bark of *Robinia psuedoacacia* (robin). The term **hemagglutinin** was introduced for the first time by Elfsrand [1898] for plant proteins that cause clumping of cells, due to the striking similarity of their activity to that of human and animal serum agglutinins. At that time toxicity was thought to be an intrinsic property of lectins. This idea was abandoned after the discovery of nontoxic lectins in seeds of legume such as *Phaseolus vulgaris*, *Pisum sativum, Lens culinaris* and *Vicia sativa* [Landsteiner & Raubitscheck, 1907]. Subsequently, a number of discoveries of new non-toxic plant lectins were reported. Though the majority of lectins represented plant kingdom, a number of lectins were reported from other organisms also; for example, in fungi, bacteria, viruses, invertebrates and vertebrates.

The next milestone in the history of plant lectins was the discovery of their ability to distinguish between different human blood groups specifically [Renkonen, 1948; Boyd & Reguera, 1949]. Based on the new interest initiated by this result, systematic screening of plant seeds for specific blood group activities were reported. Investigations on lectins as recognition molecules were undertaken. The term **lectin** was originated based on this observation ('legere' in Latin means 'to select').

The report by Sumner and Howell [1936] that sucrose inhibited the erythroagglutination activity of Concanavalin A (ConA), a lectin isolated from the seeds of *Canavalia ensiformis*, created a new interest in lectin research. A few years later it was proved that simple sugars are capable of inhibiting lectin activity and agglutination properties of lectins are based on specific sugar binding activity [Morgan & Watkins, 1953]. So lectins are considered as carbohydrate binding proteins thereafter. In 1960, Nowell observed the mitogenic properties of lectins from *Phaseolus vulgaris* [Nowell, 1960]. He observed that the lectin triggered the proliferation of quiescent, nondividing lymphocytes *in vitro*. The pioneering work by Aub and coworkers [1963, 1965a,b] and by Burger and Goldberg [1967] led to the discovery of the ability of lectins to preferentially recognize transformed cells.

Based on the above important observations lectins have been defined as "carbohydrate-binding proteins (glycoproteins) of non-immune origin which agglutinate cells and/or precipitate glycoconjugates" [Goldstein *et al.*, 1980]. Kocourek and Horejsi [1981] modified this definition to cover the monovalent sugar-binding proteins and the poorly agglutinating toxins. Later on lectins have been considered as carbohydrate binding proteins other than antibodies or enzymes [Barondes, 1988] because some lectins possess a second binding site that interacts with non-carbohydrate ligands. These definitions needed an update since molecular cloning of lectins and lectin related proteins has led to new insights. The finding that some plant enzymes (type 2 RIPs and class I chitinases) have carbohydrate as well as catalytic domains implies that definition of lectins cannot exclude all enzymes. Based on these considerations lectins have been defined recently as "proteins possessing at least one non-catalytic domain, which bind(s) reversibly to a specific mono- or oligosaccharide" [Peumans & Van Damme, 1995].

Occurrence

Lectins have now been isolated from a variety of species including bacteria, viruses, fungi, plants and animals. A number of them have been characterized in considerable detail with respect to macromolecular properties and carbohydrate binding specificity. Structural data of many lectins without and with bound carbohydrate is now available, making it possible to study the structural basis of the interactions that sustain these complexes. However, a discussion of lectins from all these sources is beyond the scope of this review, which will focus on the lectins from plants, mainly those that are isolated from seeds.

Reports of plant lectins purified to homogeneity far outnumber those from other sources. Their relative ease of availability and isolation could have contributed to this bias. Apart from seeds, lectins have been found in virtually all types of vegetative tissues. The concentration of lectins in seeds and vegetative tissues varies strongly. Seed lectins usually account for 1 to 10% of the total seed protein. In some species as high as 50% lectin content is reported whereas in others lectins are barely detectable with the techniques currently used. The same holds true for lectin concentration in vegetative tissues. Fruits, leaves, flowers, roots and other tissues usually contain low levels of lectin(s) [reviewed in Peumans & Van Damme, 1998]. The lectin content of both seed and vegetative tissues is often developmentally regulated. For example, many seed lectins accumulate during seed formation and disappear during germination. Similarly, most lectins from vegetative tissues behave as typical storage proteins and exhibit seasonal variations in their concentration [Peumans & Van Damme, 1995].

Physicochemical properties

A. Molecular structure

The amino acid sequences of several hundreds of lectins and three-dimensional structures of a few dozen of them have been elucidated and new sequences and structures are being added at an increasing rate. Some of the known plant lectin crystal structures are given in Fig. 1.1. At present over 100 members have been characterized from the largest and most thoroughly studied legume family alone [Goldstein *et al.*, 1997; Sharon & Lis, 1990; Konami *et al.*, 1995; Pusztai, 1991]. ConA was the first plant lectin to be purified and crystallized [Summer & Howell, 1936] and was also the first lectin whose amino acid sequence and three-dimensional structure were determined [Edelman *et al.*, 1972; Hardman & Ainsworth, 1972]. Typically legume lectins consist of two or four identical or almost identical subunits of 25-30 kDa each, with a single carbohydrate combining site with the same specificity. They also contain a tightly bound Ca^{2+} and a transition metal ion, predominantly Mn^{2+}, per subunit [Emmerich *et al.*, 1994]. In addition to their carbohydrate-binding site, several of the legume lectins possess a hydrophobic site that binds nonpolar compounds such as adenine and indoleacetic acid.

Primary structure

The first lectin for which the amino acid sequence was determined was ConA [Edelman *et al.*, 1972]. Subsequently, Cunningham *et al.* (1979) determined the primary structure of *Vicia faba* lectin (favin) and showed that the amino acid sequences of ConA and favin are related to each other in an unusual fashion. This study has stimulated interest for a detailed analysis of primary structure for several other legume lectins. So far, the primary structures of more than 40 legume lectins

Concanavalin A

Jacalin

Garlic lectin

wheat germ agglutinin

Fig.1.1. Three dimensional structure of some of the lectins obtained by X-ray diffraction.

have been established by chemical or molecular genetic techniques [Van Damme *et al.*, 1998]. They exhibit remarkable homologies, with 20% of the amino acid residues being invariant. The subunits of legume lectins are commonly made up of single polypeptide chains of about 250 amino acids that may carry one or two *N*-linked oligosaccharides [reviewed in Lis & Sharon, 1998]. Significant differences in function have been observed among legume lectins that share a high sequence homology. For example lectins from legume group Diocleinae show high sequence homology with ConA but are functionally different [Cavada *et al.*, 2001].

Secondary structure

At the earlier stage circular dichroic spectroscopy has given valuable information on the secondary structure of lectins in solution and the effect of saccharide binding on lectin conformation. Studies have shown that the lectin from red kidney bean [Jirgensons, 1979], peanut, lentil and soybean [Jirgensons, 1978] and *Dolichos biflorus* [Pere *et al.*, 1975] contain significant amounts of β- pleated sheet and no α -helix. Availability of high resolution X-ray diffraction technique in many laboratories led to the determination of the 3-dimensional structures of many plant lectins, yielding detailed information on their secondary and tertiary structures. It was established that β-pleated sheet is the predominant secondary structure among legume lectins and nearly 60% of their secondary structural elements comprise of β -strands [Prabu *et al.*, 1999; Vijayan & Chandra, 1999].

Three-dimensional structures

The three dimensional structure of more than twelve legume lectins, most of them in complex with mono- or oligosaccharides, has been elucidated by X-ray crystallography. The first reported structure is for ConA followed by pea, peanut, soybean etc. They are in the shape of a half-dome, with carbohydrate-binding sites forming a shallow depression at its apex. Metal ions are located close to the

combining site but do not bind directly to the carbohydrate. In each of the legume lectins, between 4 and 10 hydrogen bonds, some of them bridged by water molecules, as well as a few hydrophobic interactions, hold the monosaccharide in the combining site. A single carboxyl group of the sugar may be linked to the protein by more than one hydrogen bond [reviewed in Lis & Sharon, 1998; Sharon & Lis, 1995].

Being the oldest of all known lectins, the lectins of Euphorbiaceae family are very well characterized. Two structurally similar lectins have been characterized from the castor bean (*Ricinus communis*), one of which is a toxin (ricin) and the other an agglutinin [Olsnes, 1978]. Ricin is a heterodimeric toxic protein with a molecular weight of 60 kDa, made up of two polypeptide chains, A and B, that are linked by disulfide bonds. The B chain contains three carbohydrate-binding sites according to a recent report by Frankel and co-workers [1996]. The cytotoxic activity resides in the A chain which acts by enzymatically inactivating the RNA involved in protein synthesis. The B chain is made up of two globular domains, each of which comprises a link domain and three homologous 40-residue sub domains. The three-dimensional structure of ricin has been determined by X-ray crystallography [Rutenber *et al.*, 1991; Rutenber & Robertus, 1991]. The A-chain is a globular protein with extensive secondary structures, both β-pleated sheet and α-helix, and a reasonably prominent cleft, assumed to be the active site responsible for the toxic action of ricin. The complete amino acid sequences of both the chains have been reported. The B chain folds into two topologically similar domains, each binding lactose in a shallow cleft. RCA (*Ricinus communis* agglutinin) isolated from the same plant is a tetramer of the type $\alpha_2\beta_2$, which exist as two noncovalently associated heterodimers, each with a structure analogous to ricin but it is not toxic [Nicolson *et al.*, 1974; Olsnes *et al.*, 1974]. Preliminary

crystallographic characterization of RCA has shown that it forms an elongated molecule with two A chains at the center and a B chain at each end [Sweeney *et al.*, 1997]. The A chains are covalently associated, with a disulfide bridge between the chains.

The lectins isolated from cereals are rich in cysteine and consist mostly of two identical subunits. The wheat germ agglutinin, the only member of this family that has been characterized in detail, is a mixture of three isolectins that differ slightly in their amino acid composition. The isolectins have two identical subunits with four carbohydrate-binding sites located at the interface of subunits and are devoid of metals [Goldstein *et al.*, 1997; Emsley *et al.*, 1994]. Each subunit is made up of four homologous sub domains of 43 amino acids, held in a compact stable conformation by four interlocking disulfide bridges [Wright, 1984, 1987] that appear to be essential for the activity. There are altogether 16 disulfide bonds. The polypeptide chain of each domain is characterized by irregular folding, devoid of commonly occurring secondary structural elements such as α-helices or β-sheets. The specificity of cereal lectins is somewhat unusual, in that they interact with both sialic acid and *N*-acetylglucosamine. They are also unusual in having a very high content of half-cysteine residues and unlike many other lectins they do not have any covalently bound sugar [Allen *et al.*, 1973]. In the crystalline complex of WGA with sialyl-lactose, examined by X-rays at high resolution, the sialic acid is bound to lectin by a number of hydrogen bonds as well as by non-polar contacts with aromatic amino acids. The amino acids involved in the binding are not located in the same subunit, as found in other lectins, but belong to different subunits of the WGA dimer [Wright, 1990].

The lectins of amaryllis, orchid, and garlic families exhibit 80-90% sequence homology [Van Damme *et al.*, 1991, 1994]. These mannose specific

lectins are distinguished by their small monomer size, presence of 3-fold internal repeats of 36 amino acids, lack of metal ion requirement and weak affinity for the monosaccharide ligand [Chervanak & Toone, 1995]. The snowdrop (*Galanthus nivalis*) agglutinin (GNA) from this family whose three dimensional structure has been elucidated is tetrameric, each subunit is composed of three pseudo-symmetrically related β-sheet domains, with a conserved mannose-binding site. There is one inter-domain disulfide bond, between the second and third sub-domains, and the interior of the monomer is stabilized by conserved hydrophobic residues. The tetramer exists as two pairs of dimers. The lectin is unusual in that it has one carbohydrate-combining site per sub-domain i.e., the tetramer is dodecavalent [Hester *et al.*, 1995; Wright & Hester, 1996]. Crystal structures of monosaccharide and disaccharide complexes of GNA have revealed that all 12 binding sites of the tetramer are functional, and that the degree of occupancy is dependent on the availability of subsidiary interactions from neighboring subunits. Two unique mannopentose binding modes co-exist in the tetragonal structure (1 subunit/asymmetric unit) of the complex [Wright & Hester, 1996]. The two distinctly different binding modes observed show that the high affinity mannose binding occurs only at the two domain sites located near dimer interfaces.

The galactose specific lectin, Jacalin, represents Moraceae family. It is a homotetrameric, galactose-specific protein with a molecular weight of 66 kDa. Each subunit of this lectin has a carbohydrate-binding site that recognizes the α-anomer of galactose or *N*-acetylgalactosamine [Gupta *et al.*, 1992]. Jacalin has attracted a great deal of attention due to its specific recognition of the tumor associated T-antigenic disaccharide, Galβ13GalNAcα [Sastry *et al.*, 1986; Mahanta *et al.*, 1990]. Each of its subunits consists of a heavy chain (α) of 133 amino acids and light chain (β) of 20 residues [Kabir & Daar, 1994]. X-ray crystallographic

studies have shown that subunits of Jacalin are made up of three four-stranded antiparallel β sheets, arranged like the faces of a triangular prism, with loops connecting the strands in the sheets. It is stabilized by hydrophobic interactions in the core of subunit and a small number of hydrogen bonds involving the main chain, as well as side chain atoms. This kind of arrangement called β-prism fold is not found in any other lectin [Sankaranarayan *et al.*, 1996]. Apart from Moraceae family lectins, several lectins evolutionary related to Jacalin have been found in taxonomically distant species. For example, rhizomes of hedge bindweed (*Calystegia sepium*, Convolvulacea) contain a lectin called calsepa that shares sequence similarity with Jacalin [Van Damme *et al.*, 1996]. These lectins do not recognize Gal, GalNAc or the T-antigen, but exhibits a clear preference for mannose/maltose [Peumans *et al.*, 1997].

A high content of L-arabiose and also the presence of rare amino acid hydroxyproline are the characteristics of Solanaceae lectins (eg. thorn apple, tomato and potato) [Allen *et al.*, 1978; Nachbar *et al.*, 1980; Crowley & Goldstein, 1982; Desai *et al.*, 1981; Ashford *et al.*, 1982]. Most of these lectins are glycoproteins composed of equal amounts of protein and carbohydrates, the latter consisting of 85% L-arabinose and 15% galactose. The lectins are specific for chitin oligosaccharides and exist as dimers of two identical subunits. Each subunit consists of two domains, a carbohydrate-binding region fused to a hydroxyproline rich, highly glycosylated module. The former shares sequence similarity with other chitin-binding plant proteins and also with platelet-aggregation inhibitors from snake venoms [Kieliszewski *et al.*, 1994]. The hydroxyproline-rich domain, in turn, is similar to extensins, a family of glycoproteins that are components of plant cell walls. Three-dimensional structure of none of these lectins is known. Potato (*Solanum tuberosum*) lectin, a chimeric chitin-binding protein from this family, is

comprised of a lectin domain fused to a hydroxyproline-rich glycoprotein domain. Accurate determination of the molecular weight of the lectin by MALDI mass spectrometry has shown that the subunit molecular weight is 65,500 (± 1100) and that of a totally deglycosylated sample is 31,250 (± 30) [Allen *et al.*, 1996]. This indicates that the lectin contains 52.3 (± 1)% carbohydrate, with a considerable number of glycoforms being present. Partial sequences and other analyses confirm the existence of three distinct domains in the lectin. These are: (1) an *N*-terminal region which is rich in proline but poor in hydroxyproline; (2) a glycosylated region with a glycosylated molecular weight of 45,300 (± 1100) and a deglycosylated molecular weight of 11,050 (± 50) which is extremely rich in glycosylated hydroxyproline residues with a sequence similar to extensins; and (3) a cysteine-rich domain which has the sugar binding site that shows partial conservation of a repeated motif common to many chitin-binding proteins of the hevein family including wheat-germ agglutinin. Sequence similarities identify potato lectin as a member of both the hevein and extensin families of plant proteins.

Lectins from Cucurbitaceae family

Lectins have been isolated from the different vegetative tissues of cucurbits. The most important among them are the Cucurbitaceae phloem lectins. They are a small family of chitin-binding agglutinins that are found exclusively in the phloem exudate of cucurbit species. These lectins have been shown to be present in species belonging to the genera *Citrullus*, *Coccinia*, *Cucumis*, *Cucurbita*, *Luffa* and *Sechium*. For example lectins have been reported from *Cucurbita maxima*, *Cucumis sativus* and *Cucumis melo* [Sabnis & Hart, 1978; Allen, 1979]. Chitooligosaccharide-specific lectins from the exudate of ridge gourd (*Luffa acutangula*) and *Coccinia indica* have also been purified and characterized in considerable detail [Anantharam *et al.*, 1985,1986; Sanadi & Surolia, 1994]. All

cucurbitaceae phloem lectins in general are dimeric proteins built up of unglycosylated subunits of about 25 kDa. They also exhibit an exclusive specificity towards oligomers of GlcNAc.

In addition to phloem lectins, GalNAc-specific lectins consisting of two different disulfide bridge-linked subunits have been reported from root stocks of *Bryonia dioica* and *Marah macrocarpus* [Peumans *et al.*, 1984, 1987]. Lectins have also been reported from the pulp of *Cucumis sativus* [Skubatz & Kessler, 1988]. Three isolectins are isolated from the tuber of *Trichosanthes kirilowii maxim* [Yeung *et al.*, 1986]. All three isolectins are composed of two polypeptide chains, both with a molecular weight of roughly 30 kDa, and have an acidic pI. They can bind specifically to galactose and GalNAc. Preliminary X-ray characterization has been done for the *T. kirilowii* lectin1 (TKL-1). Spatial arrangement of the two chains of TKL-1 is found to be identical to that of type 2 RIPs. It also has immunological properties similar to some RIPs [Li *et al.*, 2000].

A galactose-specific lectin has been isolated from the seeds of *Trichosanthes Kirilowii* Maximowicz [Falasca *et al.*, 1989]. This lectin has an acidic pI and a molecular wt. of 57 kDa with subunits of mol. wt. 37 and 27 kDa. It does not exhibit any blood group specificity. Amongst the other seed lectins, the lectin from *Momordica charantia* (MCL) and snake gourd seed lectin (SGSL) have been extensively characterized. The *M. charantia* seed lectin is a $\alpha_2\beta_2$ type of tetramer with M_r 116,000. It shows no blood group specificity but exhibits a preference for β- galactopyranosides over the α- anomers [Mazumder *et al.*, 1981]. It has two sugar-binding sites per molecule and requires a tryptophan residue for its activity [Khan *et al.*, 1981; Mazumder *et al.*, 1981]. Partial (N-terminal) sequencing of this lectin indicates that it shows a certain extent of sequence similarity to β momorcharin, a ribosome inactivating protein (RIP) from the same

seed, and other RIPs, and some degree of homology in sequence to the lectins from *Cucurbita maxima*, *Cucurbita argyrosperma*, *Sambucus nigra* and *Ricinus communis* [Wang & Ng, 1998]. There has also been a report of on the isolation of a toxin (momordin) and a lectin (*Momordica* agglutinin) from the fruit itself [Li, 1980; Lin *et al.*, 1978]. They are shown to be single polypeptide chains with molecular weight of 23,678 and 31,762 kDa respectively. The *T. anguina* seed lectin is glycosylated and has an acidic pI of 5.0. The lectin shows a preference for the β-anomer of galactose. It contains two subunits of M_r, 32 and 23 kDa, which are linked by disulfide bridges [Komath *et al.*, 1996; Komath & Swamy, 1998a]. Chemical modification studies indicated the presence of histidine residues in the sugar-binding site of SGSL [Komath *et al.*, 1998b]. It has two sugar-binding sites per molecule [Komath *et al.*, 2001]. Hydrophobic binding of the lectin with porphyrins shows that the lectin has two binding sites for porphyrins that appear to be different from those for carbohydrates [Komath *et al.*, 2000]. Preliminary X-ray characterization using molecular replacement method indicates that the lectin is homologous to type 2 RIPs [Manoj *et al.*, 2001]. A seed lectin has been reported from *Trichosanthes cucumerina* also. The *T. cucumerina* seed lectin (TCSL) is very similar to SGSL in many ways. It is also a heterodimer with subunits of molecular weight 42 and 23 kDa and is specific for the β-anomer of galactose over the corresponding α-anomer [Padma *et al.*, 1999]. Although the Cucurbitaceae seed lectins seem to be structurally homologous to type 2 RIPs, they do not inactivate ribosomes or do so only weakly [Wang & Ng, 1998; Li *et al.*, 2000; Manoj *et al.*, 2001]. Complete amino acid sequence data is necessary for a detailed structural analysis leading to structure-function relationship of these lectins and RIPs.

B. Carbohydrate binding specificity

It is of vital importance to establish the carbohydrate-binding specificity of a lectin in order to make use of it as a tool in biochemical and immunochemical studies. Sugar-lectin complementarity is determined most generally by hapten-inhibition technique.

Binding of monosacharides

The carbohydrate specificity of lectins varies greatly with respect to the binding of simple saccharides. The affinity of lectins for monosaccharides is usually weak, with association constants in the millimolar range, yet it is highly selective. Lectins exhibit a wide range of variations with respect to the configuration and substitution patterns at different carbon atoms of monosaccharides. Lectins are often classified into five groups based on the specificity for simple sugars: mannose binding lectins, galactose/*N*-acetylgalactosamine binding lectins, *N*-acetylglucosamine binding lectins, fucose binding lectins and *N*-acetylneuraminic acid binding lectins [Lis & Sharon, 1998]. Certain lectins belonging to the same specificity group combine preferentially, sometimes almost exclusively, either with the α- or β-glycosides of the respective monosaccharide, where as others lack anomeric specificity. While the lectin from *Griffonia simplicifolia* (B4) [Hayes & Goldstein, 1974] and lima bean lectin [Roberts & Goldstein, 1984] are specific for the α-anomer of galactose, snake gourd lectin [Komath *et al.*, 1998] and *P. vulgaris* lectin [Kornfeld & Kornfeld, 1969,1970,1974] are specific for the β-anomer of galactose. On the other hand, lectins from soybean, *M. charantia* and castor bean are almost indifferent in their anomeric specificity.

Many lectins tolerate some variations at the C-2 of the sugar to which they bind. Lectins, that show a primary specificity for mannose such as ConA

[Goldstein *et al.*, 1965; So & Goldstein, 1967], pea lectin [Van Wauwe *et al.*, 1975] and lentil lectin [Young *et al.*, 1971; Toyoshima *et al.*, 1970] also bind to glucose, and a lesser extent, to *N*-acetylglucosamine. Similarly, several lectins that are specific for *N*-acetylgalactosamine also bind to galactose. Soybean agglutinin [Pereira *et al.*, 1974] and lima bean lectin [Galbraith & Goldstein, 1972] are examples of this. Lectins tolerate very little variation at C-3 of the sugars they bind though one or two exceptions are there. The C-4 hydroxyl group of carbohydrates is critically involved in lectin binding. Mannose/glucose binding lectins do not interact with galactose and vice versa. Similarly, *N*-acetylglucosamine binding lectins do not interact with *N*-acetylgalactosamine binding lectins [Lis & Sharon, 1984].

The properties of aglycon may markedly influence interaction of a glycoside with a lectin. Aromatic glycosides bind to many lectins much more strongly than aliphatic ones, attesting to the presence of hydrophobic region close to the carbohydrate-binding site. The hydrophobic effect is at times so strong that lectins that show a marked preference for methyl α-glycosides over the corresponding β anomers exhibit an inverse specificity when tested with corresponding p-nitrophenyl glycosides [Lis & Sharon, 1984]. The lectins within each group may also differ markedly in their affinity for other derivatives. Polar interactions between carbohydrate hydroxyl groups and polar side chains of amino acid residues within a lectin's hydrophobic binding site appear to be ideal model for specific carbohydrate-lectin interactions [Poretz & Goldstein, 1970].

Binding of oligosaccharides

Lectins often exhibit an exquisite specificity for di-, tri- and tetra saccharides with association constants up to 1000 fold higher as compared with monosaccharides. Certain lectins interact only with oligosaccharides. *Datura stramonium* lectin, for

example, is inhibited only by the oligosaccharides of GlcNAc [Horejsi & Kocourk, 1978; Kilpatrick & Yeoman, 1978]. The binding of oligosaccharides is of special significance as they are most likely the natural ligands of lectins. The selectivity of lectins towards their natural targets, usually oligosaccharides, is assumed to be achieved through multiple binding by mechanisms of additional binding in subsites (or extended sites) and subunit multivalency. The usually low affinity for monosaccharides is elicited during this process [reviewed in Elgavish & Shaanan, 1997]. In subsite binding, one monosaccharide, usually the terminal one, is bound at the primary binding site of the lectin. Additional monosaccharides along the carbohydrate chain are bound to secondary subsites on the lectin. This kind of selectivity enhancement is demonstrated in the binding of the Glc/Man-specific *Lathyrus ochrus* lectin (LOL) to a series of mannose containing oligosaccharides [Bourne *et al.*, 1992] and that of cholera toxin to GM1 ganglioside through the terminal sialic acid and galactose [Merritt *et al.*, 1994]. Subunit multivalency is exhibited when several units of the same lectin bind to different extensions of a branched oligosaccharide as in case of asialoglycoprotein receptor [Rice *et al.*, 1990] or to separate carbohydrate chains as in case of the trimeric mannose-binding protein [Sheriff *et al.*, 1994; Weis & Drickamer, 1994]. Oligosaccharides are flexible molecules with considerable freedom of rotation around glycosidic bonds connecting the individual monosaccharide constituents. Because of their flexibility, oligosaccharides that differ in their chemical structure may have substantial topographic features in common and, as a result of this similarity, will bind to the same lectin [e.g., Lewis^b and Lewis^y blood group determinants bind to *Griffonia simplicifolia* lectin, Spohr *et al.*, 1985]. On the other hand, different lectins specific for the same oligosaccharide may recognize different regions on its surface.

C. Blood group specificity

The studies by Boyd [1947] and Renkonen [1948] on phytohaemagglutinins can be considered as the first approach to investigate the blood group specificity of plant lectins. Boyd observed that saline extract prepared from dried lima beans (*Phaseolus lunatus*) agglutinated erythrocytes of some human individuals specifically and not those of others. He found that the differences could be correlated with blood groups. Following their studies several lectins with blood group specificity were discovered. For example, *Dolichos biflorus* lectin specifically agglutinates A blood group erythrocytes and *Griffonia simplicifolia* I (B_4) is specific for B blood group, whereas the lectins from *Lotus tetragonolobus* and *Ulex europeus* are specific for the O blood group [Lis & Sharon, 1986].

D. Hydrophobic binding

Many lectins bind hydrophobic sugar derivatives more strongly than the analogous non-hydrophobic derivatives. This suggests the existence of hydrophobic regions near the carbohydrate binding sites of lectins. In addition, several lectins bind hydrophobic compounds devoid of sugar moieties. Such binding is not inhibited by specific sugars indicating that hydrophobic ligands bind to lectins at sites distinct from the carbohydrate binding sites. Some of the hydrophobic compounds that interact with lectins are indoleacetic acid, 1,8-anilinonaphthalenesulfonic acid, 2,6-toluidinosulfonic acid, adenine and adenine-derived plant hormones, i.e., cytokinins [Roberts & Goldstein, 1982,1983; Maliarik & Goldstein, 1988; Gegg *et al.*, 1992; Puri & Surolia, 1994]. The binding affinity of some of the lectins for these ligands is sufficiently high that they may be compared with carbohydrates in their affinity (in the range 10^3 - 10^6 M^{-1}). The exact function of this binding is unknown, but

adenine/cytokinins-binding lectins may be involved in the storage/transport of phytohormones or in plant growth regulation. It has been suggested that lectins may function not only by virtue of their ability to bind carbohydrates but also by serving as binding proteins for biologically active hydrophobic ligands. The fact that for most of the lectins, particularly those of plant origin, a physiologically relevant carbohydrate ligand has not been found to date adds credence to this belief. Though hydrophobic contacts are known to be very important in stabilizing protein-protein and protein-membrane interactions, only recently such contacts are shown to be present in lectins also. For example, the cryoprotective galactose-specific lectins from mistletoe bind to head groups of digalactolipids in thylakoid membranes and efficiency of this binding depends on hydrophobicity [Hincha *et al.*, 1997]. A number of animal lectins have multifunctional domains and some of these are endogenously involved in hydrophobic contacts with receptors/ligands [Asperg *et al.*, 1997; Barondes, 1981; Hinek *et al.*, 1988; Kuroki *et al.*, 1997].

Biological properties

Agglutination activity

Though lectins are discovered without agglutination activity also majority of them show agglutination activity. The observation that some lectins preferentially agglutinate tumor cells as compared to normal cells has stimulated much interest in this particular property of lectins. Some Gal/GalNAc specific lectins recognize the T-antigenic disaccharide, Galβ13GalNAc [for example, Puri *et al.*, 1992; Sastry *et al.*, 1986]. The tumor selectivity of lectins has resulted in attempts to use lectins for targeted drug delivery in chemotherapy [Gabius & Gabius, 1991].

Mitogenic stimulation of lymphocytes

Certain lectins can stimulate the triggering of quiescent, nondividing lymphocytes into a state of growth and proliferation. The first mitogenic lectin to be identified was PHA, the lectin from red kidney bean [Nowell, 1960]. Now many lectins have been recognized to have mitogenicity for either the T-cells or B-cells, or both. However, PHA and ConA remain the most widely used mitogens. Almost all of these mitogens are inhibited by simple sugars. Lectins can stimulate a large number of polyclones and this greatly facilitates the detection and study of changes associated with proliferation. The exact mechanism of mitogenic stimulation by lectins is not known, but the phenomenon has clinical applications such as in the production of polyclonal antibodies [Kilpatrick, 1991].

Lectin-induced cytotoxicity

In the presence of mitogenic lectins, cytotoxic T-lymphocytes could lyse a wide variety of cells that are not their corresponding target cells. This phenomenon is called lectin-induced cytotoxicity. Several lectins including ConA, wheat germ agglutinin and phytohemagglutinin are toxic to mammalian cells [Lis & Sharon, 1981, 1984]. Toxic lectins are generally selective in their action on cells. Transformed cells are usually more sensitive to cytotoxic effects of lectins than normal ones [Nicolson, 1974].

Insulinomimetic activity

ConA, wheat germ agglutinin and several other lectins mimic the effects of insulin on adipocytes. The receptor for insulin is a glycoprotein and these lectins are able to compete with insulin in binding with fat cells. So it was suggested that lectins also bind to the insulin receptor [Lis & Sharon, 1986].

RIP activity

Some lectins have the capacity to inactivate eukaryotic ribosomes and are called ribosome-inactivating proteins. Recently they are considered as polynucleotide: adenosine glycosidases that can use various polynucleotides as a substrate for deadenylation [Barbieri *et al.*, 1993]. RIPs are subdivided into type1 and type 2 RIPs. Type 1 RIPs consist of a single polypeptide of about 30 kDa with polynucleotide: adenosine glycosidase-activity, whereas type 2 RIPs contain an enzymatically active A chain and a carbohydrate binding B chain with lectin activity. The A and B chains remain covalently linked by a disulfide bond.

Lectins with enzymatic properties

Galactose specific mung bean lectin is the first lectin reported to have enzymatic properties [Hankins & Shannon, 1978]. Later on a few more lectins from legume family were reported to exhibit glycosidase activity. Of these most of the lectins have sugar binding and enzyme activity closely related such that they also get defined as glycosidases with lectin like behavior. For example, mung bean lectin binds for short while to erythrocytes because upon binding to the carbohydrate on the cell surface, they proceed to hydrolyze them as well. The lectin from *Vicia faba* alone clearly distinguishes between enzymatic specificity and its carbohydrate binding specificity, indicating the presence of multiple domains with different function in the same protein [Dey *et al.*, 1982].

Lectin-induced agglutination of glycolipid vesicles/membrane fusion

Lectins cross-link glycolipid receptors on liposomes leading to their agglutination. This property has been used to study the liposome-lectin interactions [Surolia *et al.*, 1975; Surolia & Bachhawat, 1978; Grant & Peters, 1984]. Their studies have shown that receptor concentration in the membrane is very critical and sensitive determinant of agglutinability when the lectin receptors are glycolipids. Receptor

density in the membrane is also a critical factor in lectin agglutination via glycolipid as receptors. Redwood & Polefka [1976] have shown that WGA agglutinates model membranes that have lectin receptors on their surface and induces fusion between the vesicles.

Some biological functions of plant lectins

Lectins as plant storage proteins

As more and more information became available on the developmental control of plant lectins, their spatial and temporal distribution, as well as on their cellular and subcellular location, evidence has accumulated supporting the notion that many lectins function as typical storage proteins. Especially those lectins, which are abundantly present in either seeds or different kind of vegetative storage tissues exhibit such a storage protein like behavior, it was proposed that they function as reserve proteins. These lectins are synthesized during seed development together with the seed storage proteins. During germination and seedling growth, both storage proteins and lectins are broken down to provide amino acids for the growing seedling [Etzler, 1986]. Advantage of these, sugar-binding, storage proteins over the normal storage proteins is that they perform a dual function. As long as they reside in the plant, they act as storage proteins but once they are released from the plant they play a role in plant defense [reviewed in Van Damme *et al.*, 1998]. This is evidenced from the fact that PHA causes severe harm upon oral administration to animals. Another example is that several lectins such as WGA are toxic for particular insects [Chrispeels & Raikhel, 1991].

Lectins in plant defense

The toxicity of various plant lectins for animals and their growth inhibitory effect on fungi are the basis for the assumption that they function in the defense of plants

against pathogenic fungi and predatory animals. Much of the information about the toxic effects of plant lectins on animals comes from feeding experiments with PHA and accidental poisoning of humans by raw or insufficiently cooked beans [Peumans & Van Damme, 1995; Pusztai & Bardocz, 1996]. Ingested PHA binds to the border cells of the intestine, where it is rapidly endocytosed. Upon entering the cells, the lectin enhances their metabolic activity, which eventually leads to hyperplasia and hypertrophy of small intestine [Pusztai *et al.*, 1990]. Moreover, ingestion of PHA or raw beans causes acute nausea followed by vomiting and diarrhea. The bark lectins of black locust and elderberry provoke similar toxic effects. Type 2 RIPs are known to be potent cytotoxic agents. They are toxic to all eukaryotes if they reach cytoplasm. While fungi seem to be unaffected by them except when chitinase is present, bacteria, viruses, certain insects and higher animals show great sensitivity towards these proteins [reviewed in Peumans & Van Damme, 1995]. These examples illustrate the potential of lectins in protection against predators. Another argument in favor of defense role of plant lectins is their marked stability under unfavorable conditions. Most of these lectins are stable over a wide range of pH, are able to withstand heat, and are resistant to animal and insect proteases. Their role in defense mechanism has also been inferred from the fact that many of these lectins show remarkable specificity for oligosaccharides not found within plant system but abundant in other organisms (for example, chitin in the cell wall of fungi).

Lectins as cryoprotective agents

Some cryoprotective lectins in plants have been correlated with cryoprotective properties in the tissues that they accumulate in [Hincha *et al.*, 1993]. For example the leaves of mistletoe contain three Gal/GalNAc specific lectins, two of which show strong cryoprotectivity during freezing and thawing of isolated spinach

thylakoid membranes [Hincha *et al.*, 1997]. It has also been shown that accumulation of these lectins in the leaves is seasonally regulated.

Lectins as metabolic signals for gut

It has been known for a while that red kidney bean is toxic to a variety of animals and humans when eaten raw. Further research has shown that lectins such as those from snowdrop bulb and the elderberry tree bark have effects similar to that of red kidney bean. This has led to interesting research on lectin receptors and the consequences of lectin binding to epithelium in mammals. The results suggest that lectins in the gut may act via either class-I or the class-II receptors. In the former case the binding of lectin triggers off a second messenger molecule that acts as a signal to elicit response from the system. This action is weak compared to that of the latter. In the latter case, subsequent to lectin binding, the lectin itself gets endocytosed. The antinutritional effect appears to be proportional to their ability to stimulate growth of the gut at the cost of growth of the animal itself and the ones endocytosed appear to be more efficient at this stimulation [reviewed in Liener, 1986; Peumans & Van Damme, 1995; Pusztai *et al.*, 1991; Pusztai, 1993].

Lectins in Rhizobium-legume interactions

Lectins function in the establishment of symbiosis between nitrogen fixing bacteria, mainly rhizobia, and leguminous plants, which is of importance in both the nitrogen cycle of terrestrial life and in agriculture [Diaz *et al.*, 1989]. When rhizobia encounter root hairs in the soil, several profound developmental events take place in the infected roots. The invasion into root hair requires a highly specific association between the bacteria and root hair surface. It is assumed that rhizobial attachment to plant roots occur by the interaction between rhizobial surface carbohydrates and lectins present in the roots of legume plants. This is known as 'lectin recognition

hypotheses'. Molecular genetic experiments favor this hypothesis [reviewed in Lis & Sharon, 1998].

Applications

Hemagglutination of lectins by which they show ability of distinguishing between different blood groups, has been popular as a means of achieving blood typing [Kilpatrick & Green, 1992]. The ready availability of large number of lectins with different carbohydrate specificities has led to their extensive utilization as reagents for study of simple and complex carbohydrates in solution and on cell surfaces. Lectins have been used for the study of carbohydrate-binding sites in proteins [Goldstein *et al.,* 1997, Lis & Sharon, 1996]. They act as recognition determinants in diverse biological processes [Sharon & Lis, 1989,1993]. Lectin binding has been used to demonstrate that membrane receptors for hormones, growth factors, neurotransmitters and toxins are glycoconjugates. Lectin derivatives are employed as histochemical and cytochemical reagents for detection of glycoconjugates in tissue sections, on cells and subcellular organelles, and in investigation of intracellular pathways of protein glycosylation [Rhodes & Milton, 1998].

Lectins are used for the isolation, purification and structural studies of glycoconjugates. Lectin-based affinity chromatography has found wide applications in the purification and isolation of glycoproteins, glycopeptides, and oligosacharides [Carlson, 1994; Hasselbeck & Hosel, 1993; Yamamoto & Tsuji, 1993]. The use of ConA for resolving mixtures of closely related glycopeptides such as those found in proteolytic digests of glycoproteins is well established [Baenziger & Fiete, 1979; Krusius, 1976; Narasimhan *et al.*, 1979].

The presence of lectin receptors on the cells is readily demonstrated with use of suitable lectin derivatives using the techniques involved in the study of cell-

surface antigens [Lotan, 1979]. Radioactively labeled lectins may be used to measure the number of lectin receptor sites on the cell surface and the affinity of lectin-receptor interactions.

Agglutination of a microorganism from a primary isolate with a particular lectin may be used for the identification of the organism, making it possible to replace the expensive and time-consuming culturing or serological methods. *Neisseria gonorrhoeae* can be differentiated from other *Neisseria* species and related bacteria by its agglutination with wheat germ agglutinin [Schaefer *et al.*, 1979]. *Bacillus anthracis* and *Bacillus mycoides* can be separated from other strains of *Bacillus* due to their agglutination by soybean agglutinin. These two can also be separated by their difference in agglutination with *Helix pomatia* agglutinin [Cole *et al.*, 1984].

Lectins are also widely used to study the complex carbohydrate structure on cell surfaces of animals, plants and microorganisms. First application of lectin to cell separation (leukocytes from erythrocytes in human blood with the aid of PHA) was reported over 50 years ago [Li & Osgood, 1949]. PNA is widely used in the separation human thymocytes [Sharon, 1983]. Separation with PNA provides a means to examine *in vitro* their developmental and functional relationship. PNA and *Amaranthus leucocarpus* lectin discriminate between memory and naive/quiescent porcine lymphocytes [Hernandez *et al.*, 2002]. Selective agglutination of SBA permits separation of B and T mouse splenocytes. The main application of this lectin is purging human bone marrow for transplantation [Aversa *et al.*, 1994]. Horseradish peroxidase conjugated lectins have proved to be useful markers in mapping central neuronal pathways, since the conjugate is taken by neurons and transported within the axons [Mesulam, 1982].

The ability of some lectins to interact preferentially with certain transformed cells has led to use of these compounds as carriers for chemotherapeutic agents. Examples of such conjugates are the chimeric toxins, consisting of Con A and the α chain of diphtheria toxin [Gilliland *et al.*, 1978] or ricin [Yamaguchi *et al.*, 1979], in which the lectin serves to direct the cytotoxic agent to target cells. There have been attempts at the saccharide-assisted delivery of cytotoxic liposomes to human tumor cells [Vodovozova *et al.*, 1998] and it remains to be seen whether similar attempts will be successful with other lectins. Certain lectins such as peanut lectin or Jacalin have been shown to bind the T-antigenic structural determinant, Galβ 13GalNAc, with high selectivity. Site directed mutagenesis could be used to enhance this specificity as demonstrated in the case of peanut lectin [Sharma *et al.*, 1996]. There have been attempts to replace toxic chain of lectin with polypeptide chains of the drug, which can then be endocytosed into tissue [Pusztai *et al.*, 1991].

Lectins are used in the study of lysosomal storage disorders that are involved in the accumulation of carbohydrates in the cells due to defective catabolism [Alory *et al.*, 1991]. They can also be used to modify the structure and function of the absorptive surface of the gut. When specific lectins are introduced into the diet, they can compete with the harmful bacteria for specific binding sites or they may alter the surface of the receptors required for the adhesion of the harmful bacteria. Some lectins are known to possess antiviral, antifungal and antibacterial properties. Using genetic engineering it is possible to find application of these properties in crop protection (Peumans & Van Damme, 1995; Peumans *et al.*, 1997).

Certain lectins are potent mitogens. For example, PHA and Con A stimulate T lymphocytes while pokeweed mitogen stimulates both T and B cells [Borrebaeck & Carlsson, 1989; Di Sabato *et al.*, 1987]. Mitogenic stimulation by

lectins is useful for studying the immunocompetence of patients suffering from a diversity of diseases, including AIDS. It is also employed in the preparation of chromosome maps for different purposes, such as karyotyping, sex determination, and detection of chromosome defects, since the chromosomes are easily visualized in the stimulated cells [Reviewed in Lis & Sharon, 1998].

Motivation and focus of the present work

Now lectins are established to be ubiquitous, their presence being demonstrated in a variety of plant and animal species, from bacteria to human beings. New lectins are being regularly discovered and new properties of even well characterized lectins are being investigated by a large number of groups all over the world. Legume lectins have received considerable attention over the years and a great deal of information including primary structure and crystal structure for many of them is now available. These lectins therefore are now widely used in applications such as structural and functional investigation of complex carbohydrates, especially glycoproteins, and for the examination of changes that occur on cell surfaces during physiological processes, from cell differentiation to cancer [Goldstein *et al.,* 1997; Lis & Sharon, 1996]. It has also been possible to get a detailed understanding of the similarities between them in terms of primary, secondary and tertiary structure as well as at the level of three-dimensional structure resulting in the unraveling of evolutionary relationships between them. It is hoped that by isolating new lectins and undertaking their systematic physicochemical characterization will eventually lead to similar understanding and exploitation of lectins from other plant families as well.

Being an important part of the diet in tropics, cucurbits have received a great deal of attention in nutrition research. However, not many lectins, particularly from

the seeds of different species in this family, have received as much attention. Therefore in our laboratory we have undertaken a project on the purification and characterization of seed lectins from Cucurbitaceae family. The screening, for lectin activity, of seeds from a large number species has been carried out. *T. anguina* seed lectin has been purified and characterized in considerable detail with respect to physicochemical properties [Komath *et al*, 1996]. Also, purification and preliminary characterization of a lectin from the seeds of *T. cucumerina* has been achieved [Padma *et al*., 1999]. A comparison of the properties the two lectins show remarkable similarities between the two. Immunodiffusion and Western blot techniques have shown that they share antigenic similarity. Both lectins exhibit a preference for the β-anomer of galactose over the α-anomer. Sugar-binding studies by hemagglutination inhibition show that they follow a similar trend in binding.

In this regard it is interesting to pursue the research on *T. cucumerina* lectin and extend this study for the further characterization of the lectin in order to achieve a better understanding of the structure-function relationships of this protein. With this objective, physicochemical and saccharide-binding studies have been performed on the *Trichosanthes cucumerina* seed lectin. Chemical modification of the side chains of tryptophan, tyrosine, lysine, cysteine, arginine and histidine residues of TCSL are carried out with group specific reagents in order to identify the amino acids involved in the carbohydrate-binding and hemagglutination activity of TCSL. Tryptophan exposure and environment in TCSL has been probed by steady state and time-resolved fluorescence spectroscopic studies. With the objective of exploring the possibility of application of lectins in the field of medicine, and also to understand the nature of probable hydrophobic ligands for lectins in biological systems interaction of several metallo-porphyrins with *Trichosanthes cucumeirna* seed lectin has been investigated.

Chapter II

Physicochemical and Saccharide Binding Studies on the Galactose-Specific Seed Lectin from *Trichosanthes cucumerina*

SUMMARY

Physicochemical and saccharide-binding studies have been performed on the *Trichosanthes cucumerina* seed lectin. The agglutination activity of TCSL is highest in the pH range of 8.0 - 11.0, whereas below pH 7.0 it decreases quite rapidly. The lectin activity was unaffected between 0 and 60°C, but a sharp decline is seen at higher temperatures. Isoelectric focusing studies show that TCSL has three isoforms with pI values of 5.3, 6.2 and 7.1, with the isoform of pI 6.2 being the most abundant. DSC studies show that the protein unfolds at 82.0 °C in the absence of any sugar, and at 86.9 °C, in the presence of 0.1 M lactose, clearly indicating that ligand binding stabilizes the protein. Circular dichroism spectroscopic studies reveal that TCSL contains about 28.4% β-sheet, 10.6% turns, 7% polyproline type 2 structure and rest unordered structure, while the α-helix content is negligible. Binding of 4-methylumbelliferyl-β-D-galactopyranoside (MeUmbβGal) to TCSL resulted in a significant increase in the fluorescence intensity of the ligand, and this change has been used to obtain the association constant for the interaction. At 25°C, the association constant, K_a, for the TCSL-MeUmbβGal interaction was determined as 6.9×10^4 M^{-1}. Binding of non-fluorescent, inhibitory sugars was studied by monitoring their ability to reverse the fluorescence changes observed when MeUmbβGal was titrated with TCSL.

INTRODUCTION

Due to their presence in large quantities in plant seeds, the plant seed lectins, especially a large number from the legume seeds have been characterized well with respect to physico-chemical properties and carbohydrate-binding specificity and the forces that govern their interaction with various saccharides. Structural studies on legume lectins also led to a deeper understanding on the evolutionary relationships among the legume species [Sharon, 1993; Elgavish & Shannan, 1997; Sharma & Surolia, 1997; Vijayan & Chandra, 1999]. Much of this may be attributed to the fact that many legumes are commercially important and are produced in large quantities. Studies characterizing the lectins from other plant families have been considerably fewer and correspondingly the knowledge available on them is also much less. Among the other plant families, many species from Cucurbitaceae are cultivated in large quantities in many countries. Lectins have been isolated from the phloem exudates of *Cucurbita*, *Citrullus*, *Cucumis*, *Sechium*, *Luffa* and *Coccinia* species of this family. Reports show that remarkable similarities exist between the lectins from these different species (Sabnis & Hart, 1978). However, until recently only the *Momordica charantia* lectin and *Trichosanthes kirilowii* lectin, among the cucurbit seed lectins, were characterized in detail with respect to macromolecular and carbohydrate-binding properties [Mazumder *et al.*, 1981; Khan *et al.*, 1981; Das *et al.*, 1981; Falasca *et al.*, 1989]. So it is interesting to explore the presence of lectins in the cucurbit seeds and investigate similarities among the seed lectins from this family. In view of this lacuna, our laboratory took up a long-term research program to investigate lectins from Cucurbitaceae seeds and developed affinity-based high-yield purification methodologies for two cucurbit seed lectins, namely the snake gourd (*T. anguina*) seed lectin and *T. cucumerina* seed lectin and characterized them

in considerable detail [Komath *et al.*, 1996, 1998, 2000, 2001; Komath & Swamy, 1998, 1999; Padma *et al.*, 1999].

The *T. cucumerina* seed lectin is a hetero-dimeric glycoprotein of $M_r \sim 62$ kDa with ca. 3.0% covalently bound neutral sugar. The two subunits of this protein (of $M_r \sim 41$ and 22 kDa) are linked by disulfide bridge(s). The specificity of TCSL is directed towards galactose with *p*-nitrophenyl-β-D-galactopyranoside being the best inhibitor for this lectin among a variety of sugars investigated [Padma *et al.*, 1999]. This lectin exhibits immunological cross-reactivity with SGSL [Padma *et al.*, 1999]. Also a comparison of sugar-binding affinities of SGSL and TCSL with *T. kirilowii* and *M. charantia* lectins indicates a common pattern in their carbohydrate-binding affinities.

In view of the foregoing, it is important to characterize TCSL further with respect to macromolecular and carbohydrate-binding properties. Such studies will lead to a better understanding of the structure-function relationships of this protein. In the present study we have characterized this lectin further with respect to pH and temperature optima, isolectin profile and secondary structure content. Binding of a fluorescently labeled sugar, 4-methylumbelliferyl-β-D-galactopyranoside, has been investigated by fluorescence spectroscopy and binding of unlabelled saccharides has been studied by reversal titrations using MeUmbβGal as an indicator ligand. The results obtained are discussed in this chapter.

MATERIALS AND METHODS

Materials

T. cucumerina seeds were obtained from United Chemicals and Allied Products (Kolkata, India). Guar gum, galactose, lactose, MeUmbαGal, MeUmbβGal,

MeαGal, MeβGal, GalNAc and melibiose, as well as the ampholytes used in isoelectric focusing experiments were purchased from Sigma (St. Louis, MO, USA).

Purification of *T. cucumerina* seed lectin

TCSL was purified by a combination of ammonium sulphate precipitation and affinity chromatography on cross-linked guar gum [Appukuttan *et al.*, 1971], essentially as described earlier [Padma *et al.*, 1999]. The homogeneity of the affinity-purified protein was assessed by polyacrylamide gel electrophoresis under native conditions as well as in the presence of sodium dodecyl sulphate and β-mercaptoethanol [Laemmli, 1970]. The activity of the lectin was assessed by hemagglutination and hemagglutination-inhibition assays as described in [Padma *et al.*, 1999].

Agglutination assay

Cell-agglutination activity of TCSL was assayed by the hemagglutination technique as described previously [Padma *et al.*, 1999]. A 4% suspension of human A (+) erythrocytes in 20mM PBS buffer was mixed with serially diluted samples of the lectin in a 96-well ELISA plate and incubated at 4° C for 1 h. The agglutination titer was scored visually.

Concentration determination

TCSL concentration was determined as described by Lowry *et al.* [1951] with BSA as standard. Concentration of 4-methylumbelliferyl galactopyranosides (both anomers) was determined using their molar extinction coefficient, $\varepsilon_{318nm} = 1.36 \times 10^4 M^{-1}.cm^{-1}$ [Loontiens *et al.*, 1977; De Castel *et al.*, 1984].

pH dependence and thermal inactivation of TCSL

The pH-dependence of agglutination activity was assessed by dialyzing the lectin extensively against a buffer of desired pH, followed by the haemagglutination assay at 4° C. The erythrocyte suspension used for the haemagglutination assay was also prepared in the same buffer. The following buffers were used: 50 mM citrate (pH 3.0), 50 mM phosphate (pH 6.0-7.0), Tris/HCl (pH 8.0) and carbonate/bicarbonate (pH 9.0-11.0).

To investigate the effect of temperature on the agglutination activity of TCSL, samples of the lectin in PBS were incubated at different temperatures for 30 min. The samples were then centrifuged and the clear supernatants tested for activity at 25° C as described above.

Isoelectric focusing (IEF)

IEF experiments were performed using a Bio-Rad Rotofor system with a mini-rotofor chamber at 10W constant power [Komath & Swamy, 1998]. The preparative IEF apparatus consists of a horizontal focusing chamber equipped with a cold finger, an anode chamber with a cation exchange membrane and a cathode chamber with an anion exchange membrane. The anode and cathode chambers were filled with 0.1 M NaOH and 0.1 M H_3PO_4 and the membranes were allowed to soak overnight in the electrolytes. The sample was mixed with ampholytes and injected into the focusing chamber through the ports provided on it. Focusing with TCSL was done in the pH range of 3-10 over a period of ca. 4 hours whilst circulating ice-cold water through the cold finger to cool the focusing chamber. The chamber was continuously rotated about its longitudinal axis during the focusing run in order to ensure that neither gravity, nor any convection currents affect the focusing of the protein. The focusing chamber was physically partitioned into 20 different

compartments by a physical barrier and elution was done by vacuum suction from each section. Each fraction collected was checked for its pH and the protein concentration by measuring absorption at 280 nm.

Absorption spectroscopy and fluorescence spectroscopy

Absorption spectra were recorded on a Shimadzu UV-3101PC UV-Vis-NIR spectrometer using 1-cm path-length cells. Fluorescence measurements were made on a Spex Fluoromax 3 fluorescence spectrophotometer. Slit width of 3.5 nm was used on the excitation and emission monochromators. All samples were centrifuged just before use, and clear supernatants were used for studies.

Binding of 4-methylumbelliferyl galactopyranosides to TCSL

For fluorescence titrations, the concentration of the fluorescent sugars was kept below 5 μM. Samples placed in 1.0 × 1.0 × 4.5 cm quartz cuvettes were excited at 318 nm, and emission was recorded in the wavelength range of 350-450 nm. Titrations were carried out at 25°C. After the spectrum of free umbelliferyl sugar had been recorded, small aliquots from a lectin stock solution (≈ 30 $mg.mL^{-1}$) were added to 2.0 mL of the ligand solution, mixed, and the fluorescence intensity measured after incubation in the sample compartment for 2 min. The data were analysed by the method of Chipman *et al* [1967].

Binding of non-fluorescent sugars

Binding of non-fluorescent competing ligands to TCSL was investigated by monitoring their ability to dissociate MeUmbβGal from its complex with TCSL as described by Khan *et al.* [1981]. These reversal titrations were performed at 25°C by the addition of small aliquots of the competing ligand to a sample containing MeUmbβGal (< 5 μM) that had been pre-incubated with a known concentration of

TCSL, and the fluorescence intensity in the absence and presence of the lectin was recorded. The mixture was then titrated with small aliquots of the competing ligand, and the fluorescence intensity was recorded after addition of each aliquot. It was observed that addition of the competing ligand led to a decrease in fluorescence intensity as a result of release of fluorescent ligand from the lectin-combining site. The fluorescence intensity was first corrected for dilution, and then were analysed as described earlier [Khan *et al.*, 1981, Bessler *et al.*, 1974].

Differential scanning calorimetry (DSC)

DSC measurements were made on a Microcal VP-DSC differential scanning calorimeter, equipped with two fixed 0.5 ml cells, a reference cell and a sample cell. Measurements were made with a 1.55 mg/ml sample of TCSL in the absence and in the presence of 0.1 M lactose. All DSC experiments were carried out in 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl at a scan rate of $60^{o}.h^{-1}$.

Circular dichroism spectroscopy

CD spectra were recorded on a Jasco J-715 spectropolarimeter at a scan speed of 20 nm/min using a 0.2 cm path-length rectangular quartz cuvette. All spectra were recorded at 25 ^{o}C. For spectral scans in the range of 200 to 250 nm the lectin concentration was 0.77 mg/ml whereas for scans in the wavelength range of 250-300 nm, the concentration of TCSL was 5.88 mg/ml. Data points were collected with a response time of 4 s per point and a bandwidth of 5 nm. Each spectrum was an average of two consecutive scans. Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis. Spectra were recorded for native protein as well as in the presence of 50 mM lactose.

Analysis of CD spectra in the wavelength range 200-260 nm was done using the CDPro software package in order to determine the fractions of different

secondary structural elements [Sreerama & Woody, 2000]. This package, which is available at the website: http://lamar.colostate.edu/~sreeram/CDPro, contains three popular programs, namely SELCON3, CDSSTR and CONTIN, which are used for analyzing CD spectra of proteins. A basis set containing 37 reference proteins has been used for the analysis.

RESULTS

As indicated in the Introduction to this thesis, the major objectives of the present work are to characterize the macromolecular and carbohydrate-binding properties of the *T. cucumerina* seed lectin, to identify the amino acid side chains involved in its sugar-binding activity and to investigate its interaction porphyrins, which are primarily hydrophobic in nature. As part of this study, in this chapter results obtained from physico-chemical and carbohydrate binding studies on TCSL are reported.

The *T. cucumerina* seed lectin, purified by affinity chromatography was found to yield a single band in polyacrylamide gel electrophoresis and two bands corresponding to 41 kDa and 22 kDa in SDS-PAGE (Fig. 2.1), consistent with the results of Padma et al. [1999]. Results obtained from different physico-chemical and saccharide-binding studies are reported below.

pH optimum, thermal stability and isolectin profile of TCSL

The samples dialyzed against appropriate buffers in the pH range 3-11 were tested for agglutination activity at 4° C (Fig. 2.2). Haemagglutination by TCSL is rather weak at low pH, but increases steeply with increase in pH. Only 20% relative

Fig. 2.1. Electrophoresis of the *T. cucumerina* seed lectin (SDS-PAGE).

activity is observed at pH 5.0, which increases to 90% at pH 7.0 and to 100% at pH 8.0. It remains unaltered with further increase in pH up to pH 11.0 (Fig. 2.2).

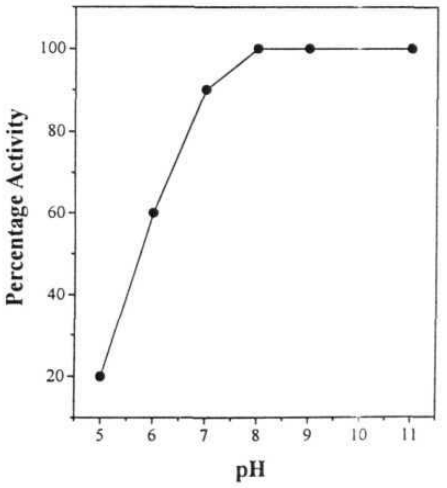


Figure 2.2. Effect of pH on the agglutination activity of *Trichosanthes cucumerina* seed lectin.

Thermal inactivation of TCSL was investigated by incubating the lectin at different temperatures for 30 min and then assaying for agglutination activity. The results obtained indicate that lectin activity is nearly unaffected between 4 and 60° C, while at higher temperatures it decreases sharply (Fig. 2.3). Incubation at 70° C

led to a 50% loss in activity whereas incubation at 80°C led to a complete loss of the hemagglutinating activity.

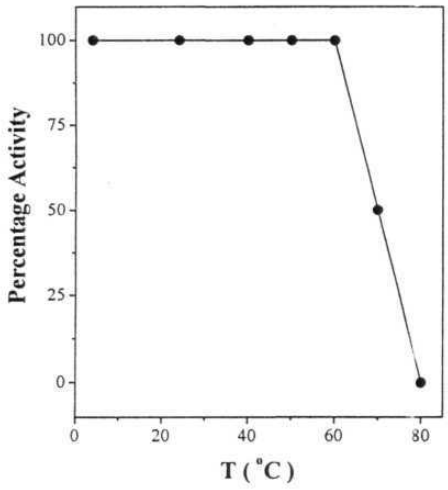


Figure 2.3. Thermal inactivation of *T. cucumerina* seed lectin.

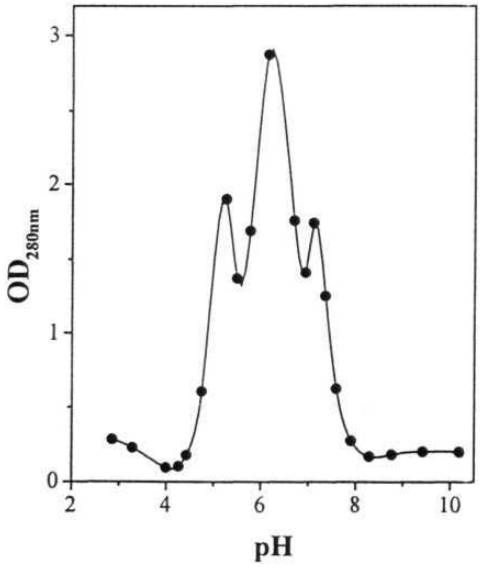


Figure 2.4. Isoelectric focusing of *Trichosanthes cucumerina* seed lectin. See 'Materials and Methods' for details.

IEF experiments performed in the broad pH range of 3-10 gave three well-separated peaks at pH 5.3, 6.2 and 7.1, suggesting that the *T. cucumerina* lectin is made up of three isolectins (Fig. 2.4). The peak at pH 6.2 is the most intense among the three peaks whereas the peaks at pH 5.3 and 7.1 are of comparable intensity, clearly indicating that the most abundant isoform has a pI of 6.2.

CD spectroscopy and determination of secondary structure

The CD spectra of TCSL in the far and near UV region are given in Fig. 2.5A and 2.5B, respectively. Spectra are shown for the native lectin as well as in the presence of 50 mM lactose. The spectrum of the native TCSL in the far UV (200-250 nm) region is characterized by a single minimum at ca. 218 nm, which is characteristic of a significant content of β-sheet. The spectrum in the near UV region exhibits a minimum at 283 nm, but is otherwise featureless.

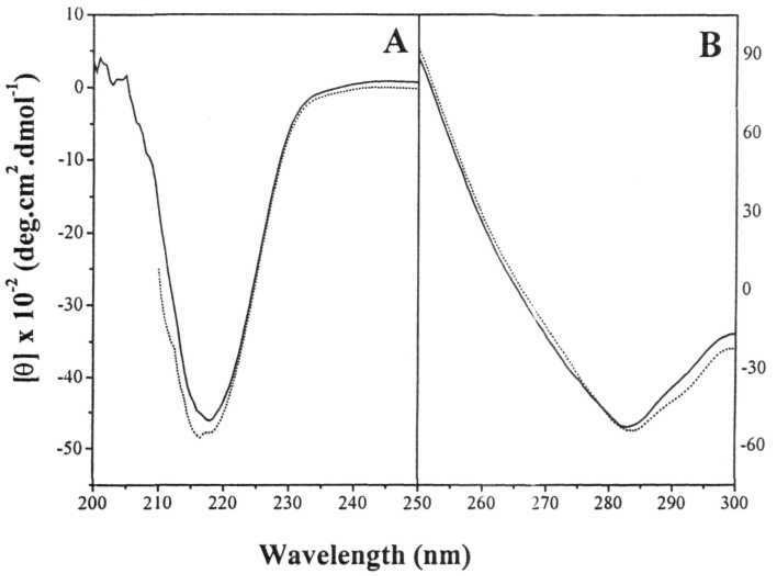


Figure 2.5. Circular dichroism spectra of *T. cucumerina* seed lectin. The CD spectra were recorded at 25°C. A. Far UV region. B. Near UV region. Concentration of the lectin was 0.77 mg/ml in A and 5.88 mg/ml in B. Solid lines correspond to TCSL alone and dotted lines correspond to TCSL in the presence of 0.1 M lactose.

Ligand binding does not lead to any significant changes in the CD spectrum of TCSL, both in the near UV region and in the far UV region (Fig. 2.5). It is therefore clear that the secondary structure of the protein is not significantly affected by saccharide binding. Also, the aromatic side chains of the protein do not seem to be perturbed by sugar binding.

Analysis of the CD spectrum of native TCSL in the wavelength range 200-260 nm by the CDPro software package [Sreerama & Woody, 2000] gave the fractions of different secondary structural elements. The results obtained from three programs, namely SELCON3, CDSSTR and CONTIN have been averaged and the average values obtained for the different secondary structural elements are as follows. α-Helix: 0.9 (± 0.6) %, β-sheet: 28.4 (± 2.4) %, β-turn: 10.6 (± 0.8) %, polyproline type II structure: 6.8 (± 0.3) % and unordered structures: 53.2 (± 3.2) %. With the exception of α-helix, which is present in negligible amounts, the standard deviations observed for the secondary structures are well below 10% of the estimated values, clearly indicating that the CD spectral data gives comparable values for the secondary structures when analysed by the three different programs.

Differential scanning calorimetry

DSC thermograms of TCSL alone and of TCSL in the presence of 0.1 M lactose are given in Fig. 2.6. Native TCSL yields a complex thermogram, which appears to consist of three overlapping transitions, with the most intense transition occurring at around 81.5 °C, preceded by two transitions of lower intensity. These three transitions in the thermogram could possibly arise due to the subunit dissociation and the unfolding of the two subunits. Another possibility is that the three isoforms of TCSL unfold at different temperatures. Binding of lactose simplifies the thermogram and it seems to contain only two transitions. In addition, the major

transition is shifted to a higher temperature, with the peak maximum being seen around 86.8 °C, clearly indicating that at least one of the subunits is stabilized by ligand binding. The transitions are irreversible because subsequent heating scans on the same samples yielded only featureless thermograms.

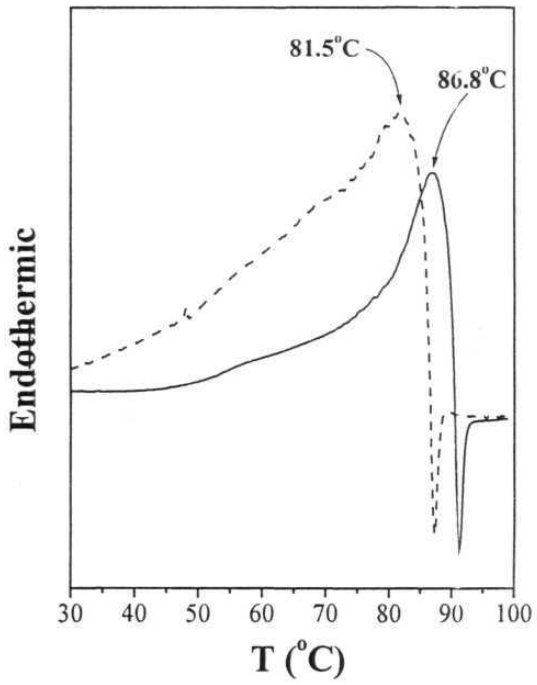


Figure 2.6. Differential scanning calorimetry of the thermal unfolding of *T. cucumerina* seed lectin. Thermograms of native TCSL (-------) and TCSL in the presence of 0.1 M lactose (———) are shown.

The DSC thermograms of TCSL in the absence as well as in the presence of lactose exhibit a sharp exothermic peak that overlaps with the major endothermic transition corresponding to the unfolding process. The origin of this exothermic transition is not clear. However, because of the overlapping of the endotherm and

the exotherm, it has not been possible to determine the enthalpy associated with the unfolding transition.

Binding of 4-methylumbelliferyl galactopyranosides to TCSL

Emission spectra of MeUmbβGal without any additives and in the presence of different concentrations of TCSL are shown in Fig. 2.7. The spectra are characterized by an emission maximum at 372nm and the fluorescence intensity at this wavelength increases on titration with TCSL. However, no shift is observed in emission maximum when the ligand is titrated with the protein.

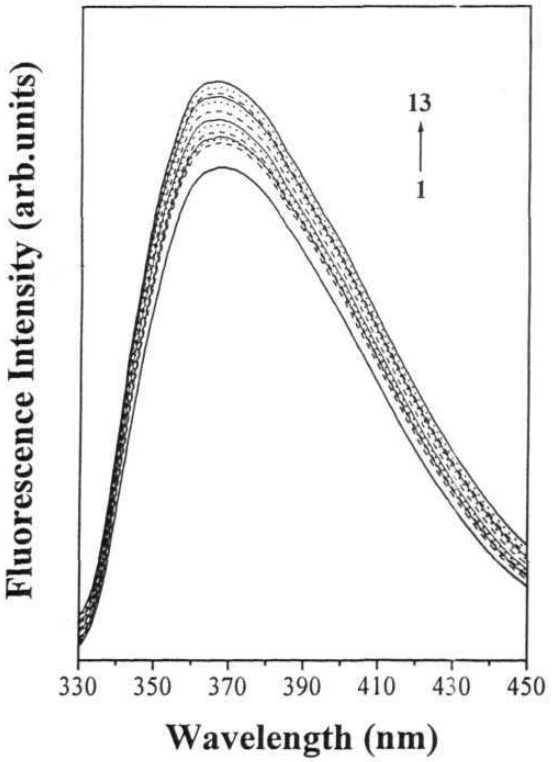


Figure 2.7. Fluorescence titration of MeUmbβGal with *T. cucumerina* seed lectin. Fluorescence spectra of MeUmbβGal in the absence (spectrum 1) and in the presence of increasing concentrations of TCSL (spectra 2-13) are shown. Temperature = 25°C.

The fluorescence titration data has been analysed in the following way. If F_o is the initial fluorescence intensity of sugar alone and F_c is the fluorescence intensity after addition of the lectin, corrected for the resulting volume change, then a plot of $F_o/\Delta F$ *versus* $[P^{-1}]$, where $\Delta F = |F_o - F_c|$ can be used to obtain the value of F_c at $1/[P] = 0$. This gives the fluorescence intensity expected in the presence of infinite concentration of protein and hence can be represented by F_∞ and the difference ($|F_o - F_\infty|$) may be designated as ΔF_∞ and indicates the maximum change in fluorescence intensity observed in the titration. From such analysis, the maximal increase in fluorescence intensity of MeUmbβGal at infinite concentration of TCSL was found to be 25.5 %. If $[P]_t$ represents total protein concentration in solution and $[P]_f$ represents the concentration of free protein (without bound ligand), then

$$[P]_f = [P]_t - \log\{[P]_t - [U]_t \, \Delta F / \Delta F_\infty\}$$

where $[U]_t$ is the total concentration of the umbelliferyl sugar. The binding constant, K_a for MeUmbβGal can be obtained from the abscissa of the plot of $\log\{\Delta F/(|F_c - F_\infty|)\}$ versus $\log[P]_f$, according to the relationship [Chipman *et al.*, 1967]:

$$\log\{\Delta F/(|F_c - F_\infty|)\} = \log K_a + \log[P]_f$$

A typical double-logarithmic plot for binding of TCSL to MeUmbβGal is shown in Fig. 2.8. The K_a value estimated from this plot is 3.1×10^4 M^{-1}. Two independent titrations yielded an average value of $4.9 (\pm 1.87) \times 10^4$ M^{-1} for the association constant. This value is presented in Table 2.1.

The fluorescence intensity of MeUmbαGal decreased slightly on titration with TCSL, but the decrease was too small to obtain any reliable data. Therefore, further experiments were not carried out with it.

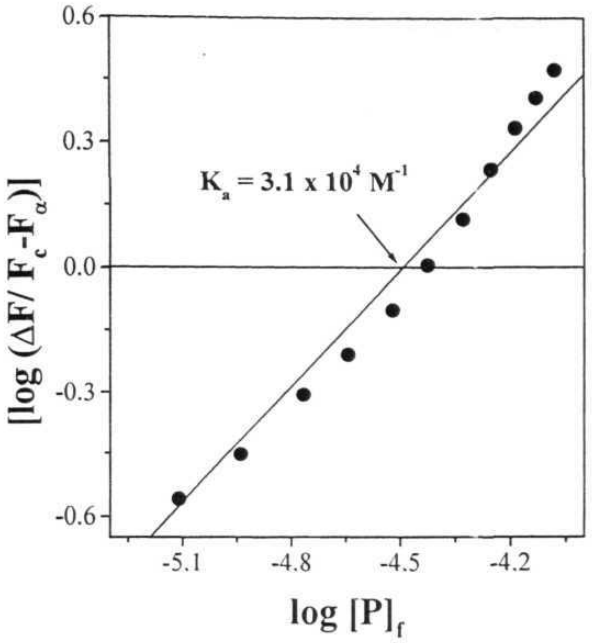


Figure 2.8. Double-logarithmic plot for the binding of MeUmbβGal to *T. cucumerina* seed lectin. The double logarithmic plot for the binding of MeUmbβGal to TCSL was obtained using the Chipman analysis. Two independent titrations gave an average K_a value of 4.9 (± 1.87) × 10^4 M^{-1}. Temperature = 25°C.

Table 2.1. Association constants, K_a, obtained from fluorescence titrations for the interaction of different sugars with *T. cucumerina* seed lectin.

Sugar	$K_a \times 10^{-3}$ (M^{-1})	Relative inhibitory potency (Galactose = 1.00)
MeUmbβGal	49.0	5.21
Galactose	9.4	1.00
MeβGal	24.8	2.64
MeαGal	24.2	2.57
GalNAc	12.0	1.28

Binding of non-fluorescent ligands

Binding of MeUmbβGal to TCSL could be completely reversed by titrating a mixture of the fluorescent ligand and lectin with competing non-fluorescent ligands such as galactose or its derivatives. This is borne out by the ability of these ligands to reverse the change in fluorescence intensity resulting from the association of MeUmbβGal with TCSL. This clearly indicates that binding of the fluorescent MeUmbβGal to TCSL is not only reversible but also saccharide specific. The reversal of changes in the fluorescence intensity resulting from the titration of the TCSL-MeUmbβGal mixture with the non-fluorescent, competing sugars can be analysed to obtain the association constants, K_a, for the binding of the competing ligands to TCSL. In this study, we have performed such reversal titrations with a few monosaccharides such as galactose, MeαGal, MeβGal and *N*-acetylgalactosamine. The titration data obtained were analysed according to the following scheme in order to obtain the association constants for the different equilibria [Khan *et al.*, 1981, Bessler *et al.*, 1974]:

$$\begin{array}{ccc} P + S + U & \rightleftarrows & PS + U \\ \uparrow\downarrow & & \uparrow\downarrow \\ PU + S & \rightleftarrows & PSU \end{array}$$

where P, S and U refer to the protein, non-fluorescent competing ligand and fluorescent sugar (MeUmbβGal in this case). PS and PU represent the non-fluorescent sugar-protein and umbelliferyl sugar-protein complexes, respectively, and PSU refers to the complex of both the fluorescent and non-fluorescent sugars bound to the same site on the lectin. K_U, K_S, K_{US} and K_{SU} are the equilibrium constants of the different equilibria possible according to the above scheme.

As binding of the umbelliferyl sugar is completely and specifically reversed by competing ligands such as lactose, [PSU] = 0 and $K_{US} = K_{SU} = 0$. Then the equilibria described above can be quantified using the expression:

$$(K_S/K_U)\,[S]_f + K_U^{-1} = [U]_f\,\{([P]_t / [PU])-1\}$$

Now, if F_o is the fluorescence intensity of free U, which on addition of the protein changes to F_P, addition of S decreases the value to F_S. If $\Delta F = (|F_S - F_o|)$ and $\Delta F' = (|F_P - F_S|)$, then $[S]_f$ can be obtained from the expression:

$$[S]_f = [S]_t - (\Delta F'/ \Delta F_\infty)$$

and $[P]_T$ and $[U]_F$ can obtained from the expression:

$$[U]_f = [U]_t - (\Delta F/ \Delta F_\infty)[U]_t$$

A plot of $[S]_f$ *vs.* $[U]_f\{([P]_t/[PU])-1\}$ has an ordinate that is equal to K_U^{-1} and a slope that gives K_S/K_U. Thus K_U can be obtained both by direct titrations of the umbelliferyl sugar with the lectin as well as from the reversal of umbelliferyl sugar binding due to competitive binding of another ligand, and the values of K_U obtained from both these approaches must be consistent.

A representative plot for the estimation of the association constant characterizing the binding of galactose to TCSL is shown in Fig. 2.9. From the slope of this plot and the association constant of 4.9×10^4 M^{-1} for MeUmbβGal obtained from the direct fluorescence titration with TCSL, the K_a value for the binding of galactose to TCSL has been determined as 9.4×10^3 M^{-1}. From the Y-intercept of the plot, association constant for MeUmbβGal was estimated as $K_U = 6.29 \times 10^4$ M^{-1}. This is in reasonable agreement with the association constant obtained from the direct titration. Similar reversal titrations were carried out with a few other monosaccharides, viz., MeαGal, MeβGal and GalNAc and the K_a values obtained from these titration data are also listed in Table 2.1. From these reversal

titrations an average value of 4.6×10^4 M^{-1} was obtained for the MeUmbβGal-TCSL interaction. This value agrees very well with the K_a value of 4.9×10^4 M^{-1}, obtained from direct titrations.

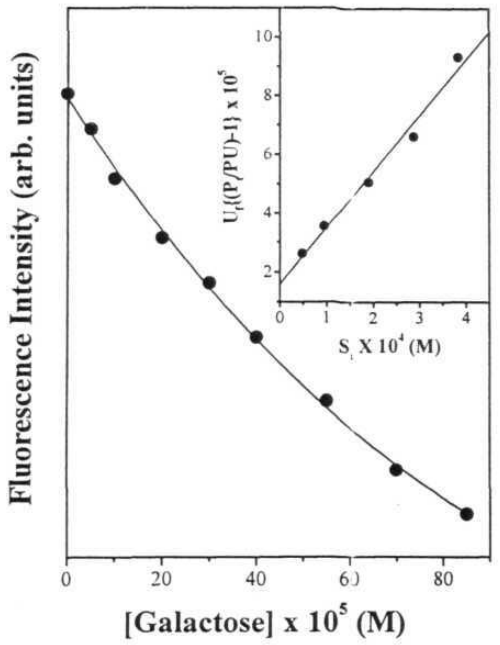


Figure 8. Reversal of the binding of MeUmbβGal to *T. cucumerina* seed lectin by competitive titration with galactose. Temperature = 25°C.

DISCUSSION

Thermal stability, pH-dependence and isolectin profile of TCSL

The agglutination activity of TCSL is virtually unchanged in the pH range 8-11. At lower pH the activity decreases sharply. This is somewhat similar to the results obtained with the snake gourd (*T. anguina*) seed lectin, which exhibited a high level of hemagglutination activity in the pH range 6-10, which decreased sharply at lower

pH values [Komath *et al.*, 2001]. Data presented in Fig. 2.2 clearly shows that the hemagglutimation activity of TCSL was unaffected by increase in the temperature up to 60°C. Incubation at higher temperatures led to inactivation of the lectin. TCSL appears to be more stable as compared to SGSL, as the latter was found to lose activity when incubated at temperatures higher than 40 °C [Komath *et al.*, 2001].

IEF experiments with TCSL gave three well-separated peaks at pH 5.3, 6.2 and 7.1 when the experiment was done in the pH range of 3-10. This indicates that TCSL has at least three isolectins, which differ considerably in their pI. SGSL, another cucurbitaceae seed lectin with which TCSL shares immunological cross-reactivity has a single isolectin [Komath & Swamy, 1998]. The present IEF results indicate that these two lectins differ in the isolectin profiles.

CD spectroscopy and secondary structure

Analysis of the CD spectra in the far UV region indicates that TCSL is primarily a β-sheet protein, with about 28.4% sheet content and 10.6% β-turns. The helix content estimated from the CD analysis is very small and is in the range of uncertainty of the CD measurements. More than 50% of the polypeptide backbone of this protein is estimated to be of unordered structure. TCSL is similar to the legume lectins in the absence of helical structure. However, the β-sheet content of this protein is somewhat lower than that found in the legume lectins, which have nearly 50% of the polypeptide chain in the β-sheet conformation [Herrmann *et al.*, 1978].

Saccharide binding

Addition of MeUmbβGal resulted in a substantial increase in fluorescence intensity but only a very small decrease in fluorescence intensity was observed when

MeUmbαGal was titrated with TCSL. Enhancement of fluorescence intensity of MeUmbβGal might be the result of the umbelliferyl moiety experiencing a more polar environment compared to the α- anomer when bound to TCSL. This suggests that the anomeric configuration of the galactoside residue bound to TCSL has a significant effect on the environment of fluorophore. These results are similar to our previous results obtained with SGSL, another galactose-specific lectin from same family [Komath *et al.*, 2001]. This suggests that the two lectins have considerable similarity in the saccharide-combining region and is consistent with the earlier results, which showed that these two lectins exhibit immunological cross-reactivity [Padma *et al.*, 1999].

Chipman analysis of the fluorescence data for the binding of MeUmbβGal gave linear plots with a slope of unity, indicating a 1:1 lectin subunit to ligand ratio. The dimeric lectin therefore has two sugar-binding sites. The association constant of 4.9×10^4 M^{-1} obtained at 25 °C for TCSL-MeUmbβGal interaction is comparable to the value of 5.8×10^4 M^{-1} obtained at 20 °C for the SGSL-MeUmbβGal system [Komath *et al.*, 2001].

The association constants, K_a for different sugars given in Table 2.1, clearly indicates that among the non-chromophoric monosaccharides studied, MeβGal exhibits the highest K_a value, closely followed by MeαGal. GalNAc and galactose bind to TCSL with affinities that are weaker by a factor of 2 and 2.6, respectively, as compared to MeβGal. These results are mostly in accord with the results of hemagglutination-inhibition studies, with the exception of galactose, which yielded a lower K_a value than MeαGal whereas the hemagglutination-inhibition data indicates that these two should be comparable in their inhibitory ability [Padma *et al.*, 1999]. However, since the hemagglutination technique is only semi-quantitative

as compared to the present fluorescence studies, which yield quantitative results for the binding data, the present results may be taken to be more definitive.

In summary, in the present study the physicochemical properties of TCSL were investigated by a variety of approaches. The lectin has been found to be optimally active in the pH range of 8.0-11.0 and is stable up to 60 °C. Its secondary structure is characterized by a significant content of β-sheet whereas the α-helix content is negligible. Binding of 4-methylumbelliferyl-β-D-galactopyranoside to TCSL leads to an increase in the fluorescence intensity of the fluorophore, indicating an increase in the polarity of the microenvironment of the umbelliferyl moiety and the association constant for the binding of this sugar could be determined from fluorescence titrations. Reversal of the fluorescence increase by the addition of competing ligands provides a convenient method for the determination of the association constants for the non-fluorescent ligands.

Chapter-III

Chemical Modification Studies on *Trichosanthes cucumerina* Seed Lectin

SUMMARY

Chemical modification studies with group specific reagents indicated that the imidazole side chains of histidine residues are involved in the carbohydrate-binding activity of *Trichosanthes cucumerina* seed lectin (TCSL). A total of 9.8 (± 1.8) histidine residues could be modified by reaction with diethyl pyrocarbonate when the reaction was carried out for 2 hours, which resulted in a total loss of the carbohydrate binding and hemagglutinating activities of the lectin. Reversing the modification by treating the histidine-modified protein with hydroxylamine resulted in a complete recovery of the activity. Presence of the specific sugar (0.2 M galactose) afforded a partial protection in that only 4.6 His residues could be modified in the same period of time. In the presence of 6 M guanidinium hydrochloride, 15.8 (± 1.5) His residues were modified. In immunodiffusion experiments the histidine-modified lectin cross-reacted with rabbit anti-TCSL antiserum, indicating that the conformation of the modified lectin is unaltered and that the loss of activity is not a consequence of structural changes. Modification of the side chains of lysine, tyrosine and cysteine residues did not affect the hemagglutination activity of the lectin. The tryptophan residues of native TCSL could not be modified by *N*-bromosuccinimide, suggesting that these residues are deeply buried within the protein matrix. However, modification with this reagent in the presence of 8 M urea indicated that there are 4.6 (± 0.4) Trp residues in the dimeric lectin.

INTRODUCTION

The hemagglutinating activity of lectins is attributed to their interaction with specific sugars. Specific amino acid residues are essential for maintaining the carbohydrate-binding and hemagglutinating activities of lectins. Chemical modification with group-specific reagents serves as a useful tool to identify the amino acid residues involved in the functional or active sites of proteins including lectins [Means & Feeney, 1971; Glazer *et al.*, 1976]. In lectins the loss in hemagglutination or sugar-binding activity upon modification of specific residues suggests that these residues are probably essential for the carbohydrate-binding activity. However, it does not indicate the direct involvement of specific residues in the sugar-binding site. Further insight into understanding the binding site of a lectin can be obtained if modifications are carried out in the presence of inhibitory sugar. Protection against modification as well as lower loss of lectin activity suggests the involvement of these amino acids in the binding site of lectin. The reduction in loss of lectin activity could be due to the fact that the inhibitor binds at the sugar-binding site of the lectin and prevents the specific chemical reagents from modifying the amino acid residues. The total number of residues of a specific amino acid in a protein can also be determined by performing the modifications in the presence of a denaturing agent.

However, there are a few limitations to this technique. Selective modification is possible only if the amino acid has a side chain with a reactive functional group. Therefore, modification of amino acids such as tryptophan, histidine or lysine can be achieved with considerable selectivity while others such as glycine, alanine or valine cannot be studied by this method. Even when the reagent has high selectivity for a particular amino acid, side reactions may occur. In order to achieve selective modifications, conditions such as concentration of reagents, pH and temperature, and in some cases, group protective reagents have to be carefully controlled. It is very

Table. 3.1: Some commonly used reagents for chemical modification of amino acid side chains.

Reagents used/ pH range	Amino acid modified	Other reactive groups	Reference
N-Bromosuccinimide, pH 4.0	Trp	Tyr, His, Met, Lys	Spande & Witkop [1967]
2- Hydroxy-5-nitrobenzyl bromide, pH 2.7	Trp	Cys	Barman & Koshland [1967]
2, 4, 6- Trinitrobenzyl sulphonate (TNBS), pH 9.5	Lys	N-terminal amine	Habeeb [1966]
Citraconic anhydride, pH 8.0	Lys	N-terminal amine	Dixon & Perham [1968]
5, 5'- Dithiobis (2- nitro benzoate) (DTNB), pH 8.6	Cys	Met, R-S-S-R	Glazer *et al.* [1976]
Iodoacetamide, pH 8.6	Cys	Asp, Glu, His, Lys, Met	Glazer *et al.* [1976]
N-acetylimidazole, pH 8.0	Tyr	- - -	Riordan *et al.* [1965]
Diethylpyrocarbonate (DEPC), pH 7.0	His	Asp, Glu, Lys, Cys, Met	Melchior & Fahrney [1970], Anderson & Ebner [1979]
p-Nitrophenyl glyoxal, pH 8.0	Arg	- - -	Yamasaki *et al.* [1981]
1, 2-cyclohexanedione, pH 8.0-9.0	Arg	- - -	Pathy & Smith [1975]
Glycine methyl ester; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), pH 5.0-6.0	Asp, Glu	Tyr	Hassing *et al.* [1971]
EDAC, Nitrotyrosyl ethyl ester (NTEE), pH4.75-5.0	Asp, Glu	Tyr	Pho *et al.* [1977]

important to make sure that the specific modification leading to loss of activity is clearly due to modification of the amino acid residues and not due to changes in overall conformation of protein. In cases where modification is reversible, it is also necessary to test the protein activity with adequate controls.

Though the recently developed technology of genetic engineering using site directed mutagenesis has been growing as an alternative method to chemical modification because of its ability to alter the functional groups much more specifically, this approach is limited by the need for information regarding the residues in a protein that are likely to be involved in its function. Chemical modification studies can provide this necessary information. Therefore, chemical modification studies can be used to identify the residues likely to be involved in the activity and genetic engineering can then be used to elucidate in detail their roles in biological function. Thus both these techniques are complementary.

Because plant lectins are stable over a wide range of temperature and pH, they have been particularly amenable to study by chemical modification. Additionally, in most cases lectin activity is easy to monitor via haemagglutination and does not require sophisticated techniques to monitor activity changes. Hence this method has been employed to study a large number of lectins from a variety of sources [Mazumder *et al.,* 1981; Jimbo & Matsumoto, 1982; Patanjali *et al.*, 1984; Desai *et al.*, 1988; Swamy & Surolia, 1989; Komath *et al.*, 1998]. Some of the commonly used reagents for modification of some of the amino acid side-chains are listed in Table. 3.1. In the present study chemical modification of the side chains of tryptophan, tyrosine, lysine, cysteine, arginine and histidine residues of TCSL have been carried out with group specific reagents.

MATERIALS AND METHODS

Materials

Diethyl pyrocarbonate (ethoxyformic anhydride), citraconic anhydride, 2,4,6-trinitrobenzenesulfonate, 5, 5'-dithiobis(2-nitrobenzoate), *N*-acetylimidazole, sodium dodecyl sulfate, 2-mercaptoethanol, acrylamide, bisacrylamide, TEMED, bovine serum albumin, galactose and lactose were obtained from Sigma Chemical Co., St. Louis (MO, USA). Sephadex G-50 was obtained from Pharmacia, Uppsala (Sweden). *N*-Bromosuccinimide was purchased from Sisco Research Laboratories, Mumbai (India) and recrystallised prior to use. All other chemicals were of the highest purity available.

Modification of lysine residues and estimation

The ε-amino side chains of lysine residues of TCSL were modified by citraconic anhydride according to Dixon & Perham [1968]. A 2.0 mg/ml sample of lectin in saturated sodium bicarbonate, pH 8.0 was allowed to react with 400-fold molar excess of citraconic anhydride for one hour at 4°C. The reaction mixture was then loaded on a column Sephadex G-50 (1.2 × 40 cm), pre-equilibrated with saturated sodium bicarbonate. The modified protein eluted as a single peak as monitored by OD_{280nm}. The peak fractions were pooled, dialyzed against PBS and then tested for agglutination activity and estimated for extent of modification.

Modification of the amino side chains of lysine residues was also performed by adding 5-fold molar excess of acetic anhydride to a 1.5 mg/ml solution of the protein in 6 M sodium acetate (pH 8.0). The reaction was allowed to proceed for two hours under constant stirring at room temperature. Excess reagent was removed by

passing the reaction mixture through a Sephadex G-50 column (1.2 × 40 cm). Peak fractions were tested for haemagglutination activity.

Estimation of the extent of modification was done according to the method of Habeeb [1966]. To 1.0 ml of protein solution (1.0 mg/ml), 1.0 ml of 0.05 M borate buffer (pH 9.5) was added. After 20 minutes, 0.1% TNBS was added, followed immediately by 1.0 ml of 10% SDS to ensure solubilization of any denatured protein. SDS added prior to TNBS can interfere with the assay. The sample was incubated at 40°C for two hours, cooled and 0.5 ml of 1N HCl was added to it. The mixture was vortexed and the absorbance of the mixture at 335nm was monitored. Blanks, which did not contain protein but contained all other reagents, were also run. BSA (ε_{335}=1.0 × 10^4 M^{-1}) was used as standard.

Modification and estimation of tyrosine residues

The phenoxy side chains of tyrosine residues were modified using *N*-acetylimidazole [Riordan *et al.* 1965]. A 2.5 mg/ml concentration lectin sample in 10 mM Tris/HCl buffer, pH 7.5 was incubated with a 60-fold molar excess of the reagent for 1 hour at room temperature, under constant stirring. The sample was then passed through a column of Sephadex G-50 (1.2 × 40 cm), pre-equilibrated with the same buffer in order to remove the excess reagent. The protein eluted as a single peak and the peak fraction was assessed for activity. The extent of modification was estimated according to Riordan *et al.* [1965]. Briefly, the reaction was reversed by incubating the modified sample with 0.5 M hydroxylamine for two hours and the increase in absorbance at 278nm was noted. Using $\Delta\varepsilon_{278}$ =1160 per mole, concentration of tyrosine residues that were modified was calculated.

Cysteine modification and estimation

The thiol side chains of cysteine residues were modified according to the procedure of Glazer *et al.* [1976] using Ellman's reagent (DTNB) under denaturing conditions. A 2.0 ml protein sample (~1.0 mg/ml) was taken in Tris-EDTA buffer (0.1 M Tris-HCl, 0.01 M EDTA, pH 8.4) containing 8 M Urea. A 100 µl aliquot of DTNB (1.25 mM stock) was added to both reference and sample cuvettes and the maximum increase in absorbance at 412 nm was monitored until no further increase was observed. Using ε_{412} = 14290 $M^{-1}cm^{-1}$, the concentration of free thionitrobenzoate ions in solution was calculated. Since this is directly proportional to concentration of thionitrobenzoate incorporated in the protein, it is possible to obtain the number of thiols modified by DTNB.

Modification of the native protein was done as described by Janatova *et al.* [1968]. A 1mg/ml TCSL solution in 7.4 mM phosphate buffer, pH 8.0 (2ml) was allowed to react with 500 µl of DTNB (0.01 M DTNB in 0.037 M phosphate buffer, pH 8.0) in the presence of 130 µl of 25 mM EDTA solution. Absorbance of the reaction mixture was read against a reagent blank in the spectrophotometer at 412 nm after the reaction was allowed to proceed for 45 min. The blank contained all other reagents except the protein.

Thiol group modification with iodoacetamide was done after reducing the disulfide bonds with β–mercaptoethanol. The disulfide bonds in TCSL could not be reduced under native conditions. Therefore, the lectin was first denatured with 8 M urea and then the disulfide bonds were reduced with β–mercaptoethanol. The free thiol groups were then modified with iodoacetamide. The modified sample was dialyzed extensively against PBS and the lectin activity was checked by the

haemagglutination assay. The free thiol groups in the modified lectin were estimated as described above with DTNB.

Modification of the guanidino side chains of arginine residues

Arginine modification was done using cyclohexanedione according to the method of Patthy & Smith [1975]. The lectin (3.8 mg/ml) in 0.5 M borate buffer, pH 9.0 was allowed to react with a 200-fold molar excess of a methanolic solution of the reagent in the dark under nitrogen atmosphere. After 18 hours of reaction, the excess reagent was removed by passing the sample through a column of Sephadex G-50 and the peak lectin fraction was tested for activity.

Modification and estimation of tryptophan residues

Tryptophan modification was done in citrate buffer, pH 4.0, with *N*-bromosuccinimide. The modification reaction was done in a spectrophotometer cuvette on a ~1.0 mg/ml concentration protein sample. Small aliquots were added from a freshly prepared aqueous solution of the reagent (10mM) to the sample cuvette as well as the reference cell. Modification was monitored by following the changes in the absorption at 278 nm. Addition of the reagent was stopped when further addition led to deviation from isosbestic point at 263 nm. Since the Trp residues could not be modified in the native TCSL, modification was done on the protein denatured with 8 M urea in the same buffer in order to estimate the total number of Trp residues present in the protein. The degree of modification was calculated according to Spande & Witkop [1967] using the relation:

$$n = 1.31 \times \Delta A_{278} / (5500 \times c)$$

where n is number of residues modified, ΔA_{278} is the difference in absorbance of the sample at 278 nm upon addition of NBS and c is the molar concentration of protein.

Histidine modification and estimation

The imidazole side chains of histidine residues of TCSL were modified at room temperature by diethyl pyrocarbonate [Melchior & Fahrney, 1970; Anderson & Ebner, 1979]. Ten µl of DEPC was diluted 60-fold with 95% ethanol and the diluted reagent was used for the modification reaction. The reagent was added to a 2.0 mg /ml protein sample in 25 µl aliquots such that the final concentration of the reagent did not exceed 3 mM and the reaction was allowed to proceed for 2 hours. Aliquots were withdrawn at different time intervals and mixed with an equal volume of 10 mM histidine in order to quench the reaction at those intervals. The reaction was also carried out in the presence of 0.2 M galactose in order to check whether the sugar could protect histidine residues from modification. In each case, the modified protein was freed from the excess reagent by gel filtration on a column of Sephadex G-50 and the peak fraction was tested for activity. The extent of modification was estimated by using the ε_{250} value of ethoxyformyl histidine to be 1.6×10^3 $M^{-1}.cm^{-1}$. The modification was reversed by treating the modified lectin with 50 mM hydroxylamine at room temperature for 10 minutes [Anderson & Ebner, 1979].

Immuno-diffusion experiments

Ouchterlony double immuno-diffusion experiments were performed in petri dishes (7.5 cm diameter) containing 1% agar in PBS with anti-TCSL antiserum raised in New Zealand white rabbits [Ouchterlony, 1948]. The antibody against purified TCSL was raised in rabbits in the following manner. Purified lectin (0.5 mg) in 0.5 ml of 0.9% saline was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits. At subsequent intervals of 4, 6 and 8 weeks, a booster dose of 0.5 mg of lectin in Freund's incomplete adjuvant was given. Blood was collected from the ear vein one week after the second and

subsequent injections, allowed to clot and the serum was collected and stored at -20°C.

RESULTS

Modification of lysine, tyrosine, arginine and cysteine residues

Modification of amino groups of TCSL by TNBS under native conditions indicated that there are about 20 surface-exposed amino groups in this dimeric protein (Table 3.2). All of them could also be modified by citraconic anhydride, because after citraconylation no further free amino groups were available for modification with TNBS. Since there are two polypeptide chains in the protein, it indicates that there are at least 18 surface-exposed ε-amino groups of lysine residues in the protein. Haemagglutination experiments showed that modification with either reagent did not lead to any loss of activity of the lectin.

Reaction with *N*-acetylimidazole resulted in the modification of 9.3 tyrosine residues in the native TCSL dimer, whereas in the presence of denaturant (8 M urea) 18.2 residues could be modified (table. 3.2). Reversal of modification was achieved by treating the modified lectin with 0.5 M hydroxylamine for two hours at room temperature. In either case the modification did not result in any alteration in the activity of the lectin, as assessed by the hemagglutination assay.

Modification of the guanidino group of arginine residues of TCSL with cyclohexanedione did not result in any alteration of the hemagglutination activity of the protein. Since the extent of modification could not be estimated, this result should be considered as only qualitative.

Treatment of the *T. cucumerina* seed lectin with DTNB under native conditions and in the presence of 8 M urea led to the modification of 0.3 thiol groups

Table 3.2. Summary of results obtained from the chemical modification studies on *Trichosanthes cucumerina* seed lectin.

Reagent	Residue Modified	Number of residues per molecule modified or % modification	Effect on agglutination activity
Diethylpyrocarbonate (DEPC)			
Without denaturant and sugar	Histidine	9.8 (± 1.8)	No activity
Without denaturant but with 0.2 M Galactose		4.6	65%
With 8 M urea		15.8 ((± 1.5)	No activity
2, 4, 6- trinitrobenzene sulfonic acid (in borate buffer)	Lysine	20.1 (± 1.5)	Unchanged
Citraconic anhydride*	Lysine		100%
N-acetylimidazole			
Without denaturant	Tyrosine	9.3 (± 2.0)	Unchanged
With 6M Gdn.HCl		18.2 (± 4.4)	Unchanged
5,5'-Dithiobis(2-nitrobenzoic acid)			
Without urea	Cysteine	0.30 (±0.05)	Unchanged
With 8M urea		0.33	Unchanged
Iodoacetamide With 8M urea and 10 mM β-ME	Cysteine	1.8 (± 0.2)	No activity
N-bromosuccinimide With 8M urea	Tryptophan	4.6 (± 0.4)	No activity

*The % modification value was arrived at based on the fact that subsequent to the reaction with citraconic anhydride, no more free amino groups were available for modification with TNBS.

per dimer of M_r 60,000, indicating that there are no free sulfhydryl groups in the lectin. Upon denaturation with urea and reduction of the disulfide bonds in the

protein, 1.8 (±0.2) thiol groups could be modified with DTNB, indicating that the two-polypeptide chains are covalently linked by a single disulfide bridge (table. 3.2). The modified sample did not exhibit any hemagglutination activity.

Modification of indole side chains of tryptophan residues

Treatment of native TCSL with NBS did not result in the modification of the tryptophan residues of the protein. The absorption spectra of the lectin recorded upon addition of successive aliquots of the reagent did not yield the characteristic isosbestic point seen when Trp residues alone are selectively modified. On the contrary, the sample was seen to become turbid, suggesting that the polypeptide is getting cleaved by the reaction of the reagent with other residues. Therefore, it was assumed that the tryptophan residues of this lectin are buried in the protein matrix and are not accessible to the reagent. Therefore the modification reaction was carried out after denaturing the protein with 8 M urea.

The absorption spectrum of TCSL denatured with 8 M urea and the spectra obtained upon treating the denatured lectin with increasing concentrations of NBS are shown in Fig. 3.1. While the absorption intensity at 278 nm decreased upon NBS treatment, that at 250 nm was found to increase. A distinct isosbestic point was seen around 263 nm, clearly indicating that the modification is selective for tryptophan under these conditions. Reaction was stopped when deviation from isosbestic point was noticed because it indicates that NBS is attacking other residues, especially tyrosine [Spande & Witkop 1967; Patanjali *et al.* 1984]. The experiment was done thrice and a total of 4.6 (± 0.4) Trp residues could be modified in the presence of 8 M urea (table. 3.2).

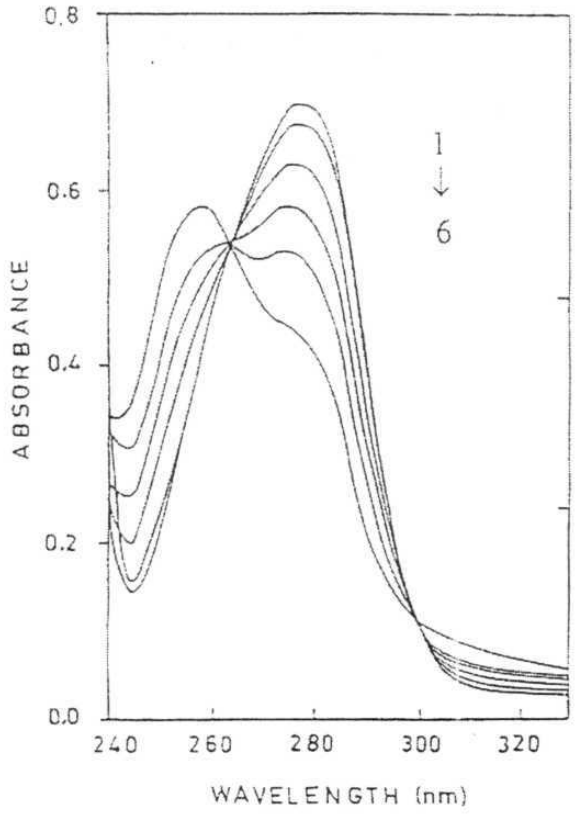


Fig. 3.1: Typical absorption spectra of native and tryptophan modified samples of *T. cucumerina* seed lectin. The modification reaction was carried out with *N*-bromosuccinimide at pH 4.0 on TCSL denatured with 8 M urea. Spectrum 1 corresponds to TCSL denatured with 8 M urea and spectrum 6 corresponds to denatured TCSL in which 4.6 Trp residues were modified. Spectra 2-5 correspond to samples in which the extent of modification was in between these two extremes.

Modification of imidazole side chains of histidine residues

When the *T. cucumerina* lectin was reacted with diethyl pyrocarbonate under native conditions a maximum of 10 histidine residues could be modified when the reaction was continued for a total of 2 hours (table 3.2). When the modification reaction was performed in the presence of 8 M urea, 15.8 His residues could be modified. Treatment of the lectin with additional amounts of the reagent or increasing the reaction time did not increase the extent of modification. This suggested that there

are about 16 His residues in TCSL, of which 10 residues are accessible to the reagent in the native protein, while denaturation rendered the remaining 6 residues accessible to the reagent.

Modification of all the accessible His residues (10 residues/molecule) in the native TCSL led to a complete loss in the hemagglutination activity of the lectin, suggesting that the imidazole side chains are involved in its carbohydrate-binding activity. When the hemagglutination activity was assayed as a function of the extent of modification, it was observed that the activity decreased with increasing modification (Fig. 3.2). Modification of about 4 residues/dimer reduced the activity to half, whereas modification of 5 residues reduced the activity to 25%. Protein

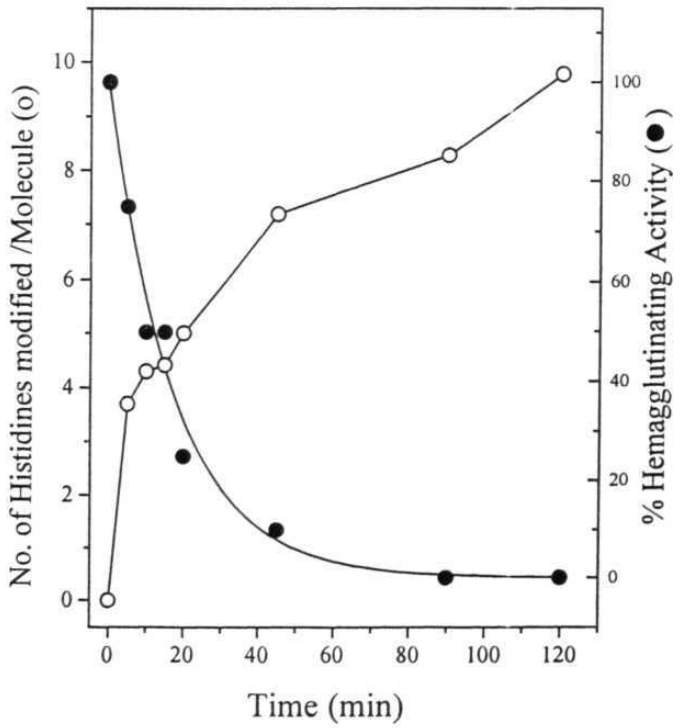


Fig. 3.2: Kinetics of the histidine modification and its effect on the hemagglutination activity of *T. cucumerina* seed lectin. (O) Number of histidine residues modified/molecule (dimer of M_r 60,000); (●) percent hemagglutination activity remaining.

sample in which 8 His residues/dimer were modified did not exhibit any hemagglutination activity. Further, the His modified protein (5.7 residues/dimer) did not bind to cross-linked guar gum, suggesting that the loss of hemagglutination activity coincides with the abolition of carbohydrate-binding activity.

In order to verify whether presence of the specific sugar has any effect on histidine modification, the modification reaction was performed in the presence of 0.2 M galactose. The results obtained are shown in Fig. 3.3. It is very clear from this figure that the reactivity of His residues of TCSL is significantly reduced in the presence of galactose. Thus, while a total of about 10 His residues could be modified in 120 minutes in the absence of galactose, only 4.6 residues could be modified in the

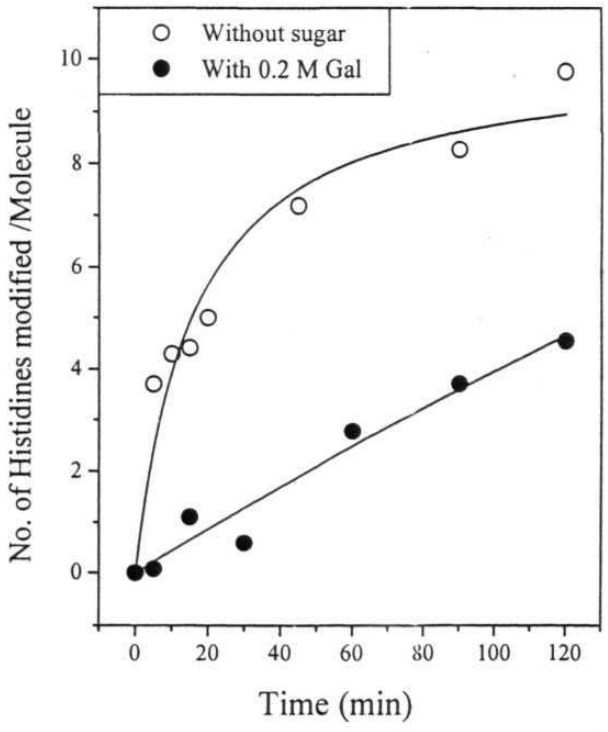


Fig. 3.3: Protection of histidine modification of *T. cucumerina* seed lectin by galactose. Data for modification of the native protein in the absence of sugar (O) and in the presence of 0.2 M galactose (●) are shown.

same time when the reaction was carried out in the presence of 0.2 M galactose, with all other conditions being identical.

In Ouchterlony double-immunodiffusion experiments (Fig. 3.4), the histidine modified *T. cucumerina* seed lectin cross-reacted with rabbit antiserum raised against native TCSL, suggesting that the modification did not result in any alterations in the overall structure of the lectin. This shows that the loss of activity resulting from modification reaction is not due to any structural changes in the protein.

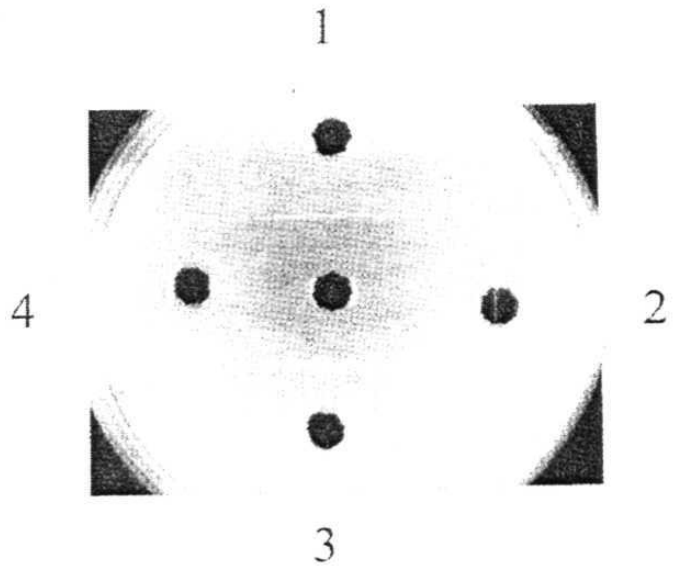


Fig. 3.4: Immunodiffusion of native and histidine-modified *T. cucumerina* seed lectin against rabbit anti-TCSL antiserum. The antiserum raised against native TCSL was placed in the central well. The samples in the other wells are: 1) native lectin; 3) histidine-modified (9.6 residues/dimer) lectin; 2 & 4, PBS.

DISCUSSION

Modification of the ε-amino groups of lysine residues, guanidino groups of arginine residues, hydroxy groups of tyrosine residues and the thiol side chains of cysteine

residues did not alter the hemagglutination activity of *T. cucumerina* seed lectin (TCSL). This clearly demonstrates that these residues are not directly involved in the sugar-binding activity of the lectin.

Under native conditions the Trp residues of TCSL could not be modified by NBS. This clearly suggests that they are deeply buried in the hydrophobic interior of the protein matrix and hence are inaccessible to the reagent. Consistent with this interpretation, 4.6 (± 0.4) Trp residues could be oxidized by NBS when the protein was denatured by 8 M urea. Thus in the case of *T. cucumerina* lectin all the Trp residues have been found to be inaccessible to NBS in the native state, but could be modified upon denaturation.

Modification of the imidazole side chains of histidine residues of TCSL resulted in a total loss of its hemagglutination activity. Further, the histidine-modified lectin failed to bind to cross-linked guar gum, which is used as the affinity matrix for the purification of this lectin. These observations suggest that the imidazole side chains of histidine residues are crucial for the carbohydrate binding and hemagglutinating activities of the *T. cucumerina* lectin. Presence of the specific sugar, namely galactose, provided a partial protection to the histidine residues from the modification reagent (Fig. 3.3). Additionally, the modification of histidine residues could be reversed by treating the modified protein with hydroxylamine or by incubating the modified protein at 37°C for 2 hours. Such reversal of modification resulted in a near complete recovery of the hemagglutination activity of the lectin, clearly indicating that the imidazole side chains of His residues are directly involved in the carbohydrate-binding activity of TCSL. Finally, in Ouchterlony double-immunodiffusion experiments, the modified lectin reacted with the antiserum raised against the native TCSL (Fig. 3.4), suggesting that the loss of agglutination activity of the His-modified lectin is not due to any conformational changes. All these

experimental data are in support of the involvement of His residues in TCSL in its carbohydrate-binding activity. Thus the *T. cucumerina* lectin resembles the *T. anguina* seed lectin, with which it shares immunological cross-reactivity, in its requirement of His residues for the sugar-binding activity [Komath *et al.* 1998].

In summary, the present chemical modifications clearly indicate that the imidazole side chains of histidine residues in the *T. cucumerina* seed lectin are necessary for its carbohydrate-binding and hemagglutinating activities. The side chains of lysine, tyrosine, arginine, cysteine and tryptophan do not seem to be directly involved in the activity of the lectin.

Chapter IV

Steady-State and Time-Resolved Fluorescence Studies on *Trichosanthes cucumerina* Seed Lectin

SUMMARY

Steady state and time-resolved fluorescence spectroscopic studies have been carried out on the *Trichosanthes cucumerina* seed lectin. The fluorescence emission maximum of TCSL in the native state as well as in the presence of 0.1 M lactose is centered around 331 nm, which shifts to 347 nm upon denaturation with 8 M urea, indicating that all the tryptophan residues of this protein in the native state are in a predominantly hydrophobic environment. The exposure and accessibility of the tryptophan residues of TCSL and the effect of ligand binding on them have been probed by quenching studies employing two neutral quenchers (acrylamide and succinimide), an anionic quencher (I^-) and a cationic quencher (Cs^+). At a quencher concentration of 0.5 M, the extent of quenching observed with acrylamide, succinimide, I^- and Cs^+ was 57.5%, 48.2%, 11.5% and 15.7%, respectively, for the native lectin. In the presence of 0.1 M lactose quenching with acrylamide and iodide increased to 62.1% and 17.1%, respectively, whereas quenching with succinimide and cesium ion was not significantly affected. When TCSL was denatured with 8 M urea, both acrylamide and succinimide yielded upward-curving Stern-Volmer plots, indicating that the quenching mechanism involves both dynamic and static components. Quenching data obtained with I^- and Cs^+ on the urea-denatured protein suggests that charged residues could be present in close proximity to some of the Trp residues. In time-resolved fluorescence experiments, the decay curves could be best fit to biexponential patterns, with lifetimes of 1.78 and 4.75 ns for the native protein and 2.15 and 5.14 ns in the presence of 0.1 M lactose.

INTRODUCTION

Intrinsic fluorescence of proteins is predominantly due to the presence of tryptophan residues present in the protein. A valuable feature of protein fluorescence is the high sensitivity of tryptophan residues to its local environment. Tryptophan residues can be selectively excited at 295 nm, avoiding excitation of tyrosine residues, such that fluorescence properties of tryptophan alone can be studied. Studies on the intrinsic fluorescence properties have been widely used to obtain information about protein structure and conformational changes induced by alteration of environment and/ or ligand binding [Lakowicz, J. R, 1999; Eftink & Ghiron, 1981; Grinvald & Steinberg, 1976]. Trp residues that are in a nonpolar medium or environment fluoresce around 320 nm, whereas those that reside in a polar environment emit at a considerably higher wavelength.

Tryptophan appears to be uniquely sensitive to collisional quenching, due to a tendency of indole to donate electrons while in the excited state. Tryptophan can be quenched by externally added quenchers or by nearby groups in the protein. Hence there are many reports on the study of tryptophan fluorescence. Tryptophan residues present in the active site of a protein or on the exterior are relatively easy to study and a large number of studies using chemical modifications and fluorescence techniques have been published, elucidating the environment and role of these residues in such proteins [e.g., see, Privat *et al.*, 1980; Peterman & Laidler, 1979, 1980; Patanjali *et al.*, 1984]. However, buried residues are generally much more difficult to study. All the tryptophan residues of TCSL appear to be buried deep within the protein matrix, as they were not accessible to *N*-bromosuccinimide in the chemical modification studies [as shown in Chapter III], and thus provide an interesting problem to study.

In the present study we have investigated the tryptophan exposure and environment of TCSL in the absence and presence of a specific carbohydrate ligand, lactose, and upon denaturation, using fluorescence quenching and time-resolved fluorescence measurements. Fluorescence quenching experiments on the *T. cucumerina* seed lectin were carried out using two neutral quenchers, namely acrylamide and succinimide, and two charged quenchers, I^- and Cs^+. Acrylamide and succinimide are chemically similar quenchers that differ in their average molecular radii and have been used to study the extent of burial of Trp residues in the protein matrix [Eftink & Ghiron, 1984], whereas charged quenchers have been used to study the degree of exposure of Trp residues in proteins [see, for example, Komath & Swamy, 1999; Lehrer, 1971; Steiner & Kirby, 1969; Vincenzetti *et al.*, 1999]. The neutral quenchers, acrylamide and succinimide can diffuse into the interior of the protein and quench the fluorescence of even partially buried tryptophans. Iodide and Cs^+, being charged species, can quench only surface exposed tryptophans and their quenching is affected by the presence of charged residues in the neighborhood of fluorophores.

MATERIALS AND METHODS

Materials

Analytical grade KI was obtained from Qualigens (Mumbai, India). Optical grade cesium chloride, acrylamide, lactose and galactose were from Sigma Chemical Company (St. Louis, MO, USA). TCSL was purified by affinity chromatography on cross-linked guar gum as described in Chapter II. The afinity-purified lectin yielded a single band in PAGE and two bands corresponding to M_r 41 and 22 kDa in SDS-PAGE, consistent with the report of Padma *et al.* [1999]. All other reagents used were of the highest quality available.

Absorption and fluorescence spectroscopy

Absorption measurements were performed on a Shimadzu UV3101PC double beam spectrophotometer. All fluorescence measurements were carried out on a Spex Fluoromax 3 spectrofluorimeter. Slit widths of 1 nm were routinely used on the excitation and emission monochromators. Selective excitation of Trp was achieved by fixing the excitation monochromator at 295 nm and the emission spectra were recorded from 310-400 nm. All samples were centrifuged immediately before use and the clear supernatant was used for the studies.

Fluorescence quenching

Protein samples ($OD_{280\ nm} \leq 0.1$) in PBS were titrated with small aliquots of the quencher solution and fluorescence spectra were recorded after each addition. When titrations were carried out in the presence of sugar, the samples contained 100mM lactose. All measurements were done at room temperature.

Time-resolved fluorescence studies

Lifetime measurements were carried out on an IBH-5000 single photon counting spectrofluorimeter [Komath & Swamy, 1999]. A H_2 flash lamp of pulse width 1.4 ns was used for excitation and a Hamamatasu photomultiplier 3235 was used to detect the fluorescence. The resultant decay curves were analysed by a multiexponential iterative fitting program supplied by IBH.

RESULTS

Steady-state fluorescence spectra of TCSL and quenching studies

Fluorescence spectra of native TCSL, TCSL denatured with 8 M urea and denatured and reduced TCSL are shown in Fig. 4.1. The native lectin exhibits an

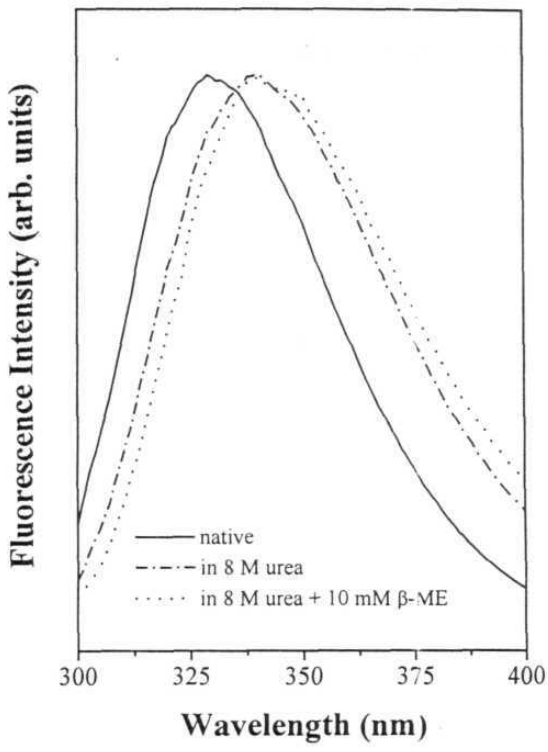


Figure. 4.1. Fluorescence spectra of TCSL under different conditions. Spectra are shown for the native protein, protein denatured with 8 M urea and for the protein denatured with 8 M urea and reduced with 10 mM β-mercaptoethanol. The spectra were normalized to the same maximum intensity to facilitate comparison.

emission maximum at 331 nm, which shifts to 346 nm upon denaturation with 8 M urea. The emission maximum shifts further by ca. 2 nm when the denatured protein is reduced with 10 mM β-mercaptoethanol (Fig. 4.1). These results indicate that denaturation renders the buried tryptophan residues of the native protein exposed to the solvent. The additional 2-nm red shift in the emission maximum upon reduction of the denatured lectin indicates that breaking the disulfide bonds leads to a further increase in the exposure of the tryptophan residues.

Fluorescence spectra of native and urea-denatured samples of TCSL in the presence of different concentrations of acrylamide are shown in Fig. 4.2A and Fig. 4.2B, respectively. These spectra indicate that there is a progressive decrease in the

emission intensity upon increasing the concentration of the quencher and the extent of quenching is increased substantially in the presence of 8 M urea as compared to the native lectin. These spectra further indicate that quenching by acrylamide is higher with the denatured protein than for the native lectin, clearly showing that exposure of the fluorescent tryptophan residues to the quencher increases when the protein is unfolded. Similar results were obtained with other quenchers also, although the maximal quenching observed was considerably smaller. Addition of charged quenchers (I^- and Cs^+) resulted in much less quenching compared to neutral quenchers (acrylamide and succinimide).

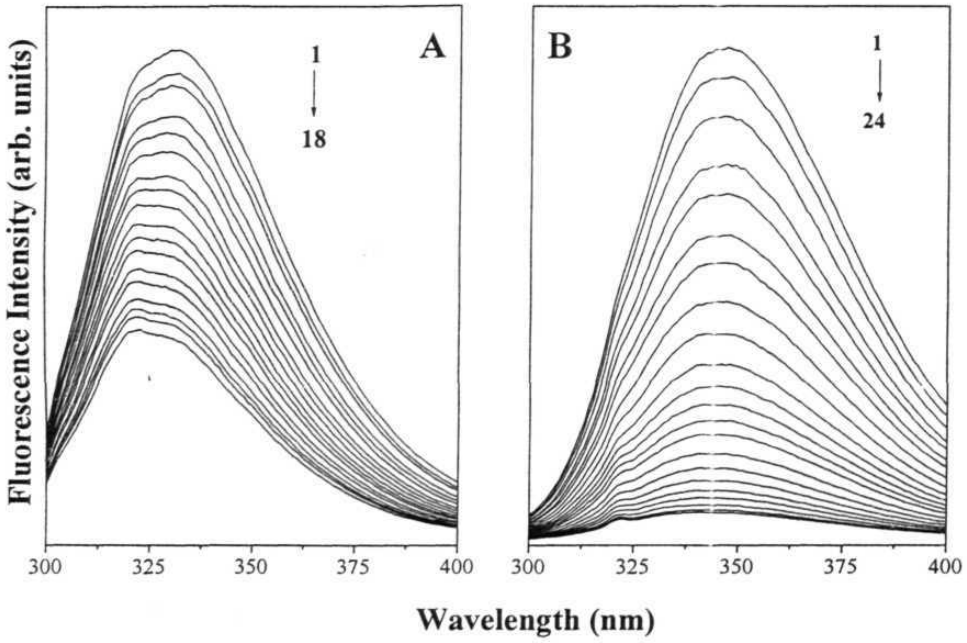


Figure 4.2. Fluorescence emission spectra of TCSL in the absence and in the presence of different concentrations of acrylamide. (A) under native conditions, (B) in the presence of 8 M urea. Spectrum 1 was recorded without acrylamide and in the remaining spectra the acrylamide concentration increases with spectrum number. The highest concentration of the quencher used was 0.5 M in each case (and corresponds to the spectrum of the lowest intensity).

The degree of quenching achieved in each case, at a resultant quencher concentration of 0.5 M, is shown in Table 4.1. Of the four quenchers used, acrylamide was the most effective, quenching 57.5% of the total intrinsic fluorescence of the lectin, whereas the bulkier succinimide quenched 48.2% of the total available fluorescence. The ionic quenchers, iodide and cesium ion, which can not penetrate into the protein matrix, were found to quench only 11.5% and 15.7%, respectively, of the total fluorescence intensity of TCSL. Upon denaturation with urea the quenching observed with the different quenchers increased to 93%, 75.3%, 57.4% and 43.3% with acrylamide, succinimide, I^- and Cs^+, respectively.

Table 4.1. Extent of quenching of tryptophan fluorescence of TCSL observed with different quenchers. The final concentration of the quencher was 0.5 M in each case.

Quencher	% quenching		
	native	with 0.1M lactose	in 8M urea
Acrylamide	57.5	62.1	93.0
Succinimide	48.2	48.2	75.3
Iodide ion (I^-)	11.5	17.1	57.4
Cesium ion (Cs^+)	15.7	9.1	43.3

Stern-Volmer analysis of quenching data

Quenching data for all the quenchers used in this study were analysed by the Stern-Volmer equation (4.1) as well as by the modified Stern-Volmer equation (4.2) [Lehrer, 1971]:

$$F_o/F_c = 1 + K_{SV}[Q] \quad (4.1)$$

$$F_o/\Delta F = f_a^{-1} + (K_a f_a)^{-1}.[Q]^{-1} \quad (4.2)$$

where F_o and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and in the presence of quencher, [Q] is the resultant quencher concentration, K_{SV} is the Stern-Volmer quenching constant of the lectin for a given quencher, f_a refers to the fraction of the total fluorescence that is accessible to the quencher and K_a is the corresponding Stern-Volmer quenching constant. Slopes of Stern-Volmer plots yield K_{SV} values, whereas the slopes of modified Stern-Volmer plots give $(K_a f_a)^{-1}$ and their ordinates give values of f_a.

The Stern-Volmer plots obtained with different quenchers are shown in Fig. 4.3. The quenching profiles obtained for the native TCSL and for TCSL in the presence of 0.1 M lactose with acrylamide, succinimide and iodide ion follow a linear dependence on the quencher concentration (Figs. 4.3A, 4.3B and 4.3C), from the slopes of which the Stern-Volmer quenching constants have been determined. The values obtained have been listed in Table 4.2. On the other hand, the profiles obtained with cesium ion exhibit a biphasic pattern (Fig. 4.3D), indicating that this cationic quencher sees heterogeneity in the environment of the surface-accessible tryptophan residues. From the slopes of the two linear components of the quenching data for cesium ion with the native protein and in the presence of 0.1 M lactose, corresponding Stern-Volmer quenching constants have been determined and these values are also listed in Table 4.2.

Quenching profiles obtained with acrylamide and succinimide on the protein denatured with 8 M urea show positive curvature, clearly indicating that the quenching has both dynamic and static components. These data therefore have been fitted to equation (4.3), which allows the resolution of the static and dynamic components and gives the corresponding quenching constants [Lakowitcz, 1999]:

$$F_o/F_c = (1 + K_{SV}[Q])(1 + K_S[Q]) \quad (4.3)$$

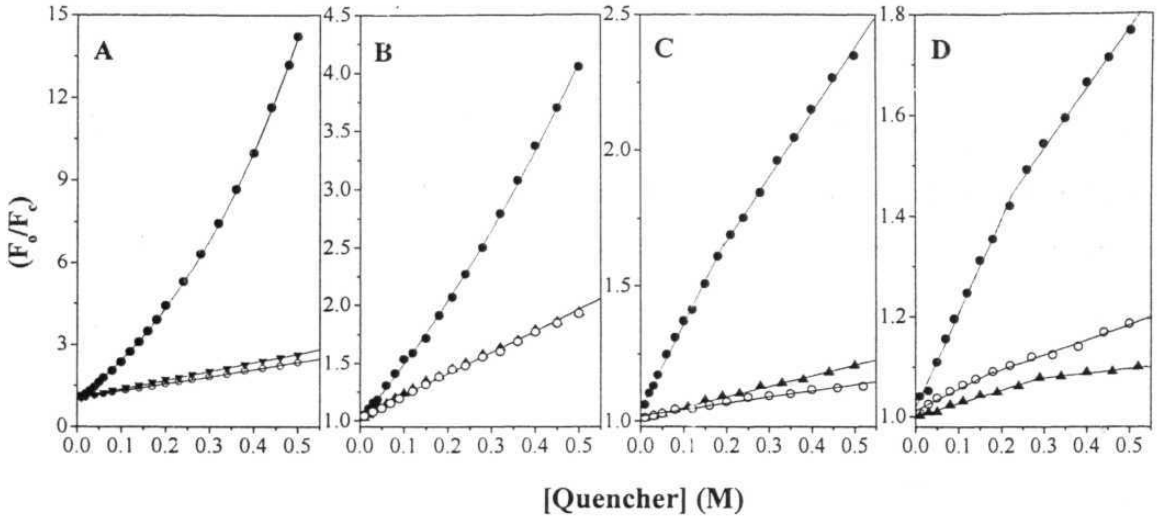


Figure 4.3. Stern-Volmer plots of the fluorescence quenching data on TCSL. Quenching profiles with (A) acrylamide, (B) succinimide, (C) iodide ion and (D) cesium ion are shown. (O) native protein, (▲) in the presence of 0.1 M lactose, (●) with 8 M urea.

where K_{SV} is the Stern-Volmer (dynamic) quenching constant, K_S is the static quenching constant and [Q] is the quencher concentration. The solid curved lines shown in Fig. 4A and Fig. 4B clearly demonstrate that the data fit very well to Eq. 4.3. From this analysis the values of K_{SV} have been obtained as 10.76 M^{-1} and 4.05 M^{-1}, for acrylamide and succinimide, respectively. Similarly, values of K_S have been obtained as 1.6 M^{-1} and 0.6 M^{-1} for acrylamide and succinimide, respectively. These values are also listed in Table 4.2.

The modified Stern-Volmer plots obtained with all the four quenchers are given in Fig. 4.4. From the Y-intercepts of these plots, f_a, the value of the fraction of accessible Trp residues in each case, has been determined and the value of the corresponding quenching constant, K_a, was calculated using Eq. 4.2. These values are also given in Table 4.2. It is clearly seen from the data presented that in the

Table 4.2. Summary of parameters obtained from intrinsic fluorescence quenching and time-resolved fluorescence measurements on the *T. cucumerina* seed lectin. K_{SV1} and K_{SV2} are Stern-Volmer quenching constants, k_{q1} and k_{q2} are bimolecular quenching constants, K_S is the static quenching constant, f_a is the fraction of accessible residues and K_a is the quenching constant obtained for the accessible fluorophores from modified Stern-Volmer analysis.

Quencher	K_{SV1} (M^{-1})	$k_{q1}\times10^{-9}$ ($M^{-1}s^{-1}$)	K_{SV2} (M^{-1})	$k_{q2}\times10^{-9}$ ($M^{-1}s^{-1}$)	K_S (M^{-1})	f_a (%)	K_a (M^{-1})
Acrylamide							
Native	2.65	0.796				76.9	4.58
with 0.1M lactose	3.25	0.922				92.1	4.22
in 8M urea	10.76	2.959			1.60	100	8.84
Succinimide							
Native	1.84	0.553				70.16	3.85
with 0.1M lactose	1.84	0.521				67.66	3.73
in 8M urea	4.05	1.114			0.60	100	5.08
Iodide ion (I^-)							
Native	0.24	0.072				12.80	5.96
with 0.1M lactose	0.40	0.113				27.89	2.25
in 8M urea	3.16	0.869	2.36	0.649		70.73	6.52
Cesium ion (Cs^+)							
Native	0.27	0.081	0.09	0.027		23.4	1.36
0.1M lactose	0.44	0.125	0.30	0.085		18.38	4.91
in 8M urea	1.88	0.517	1.21	0.333		69.84	3.37

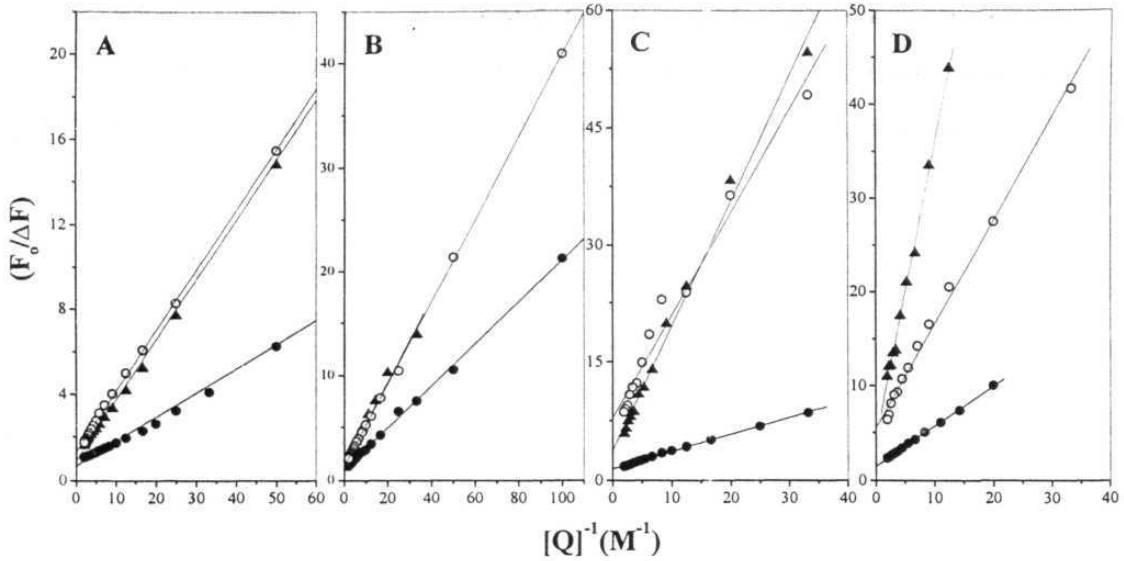


Figure 4.4. Modified Stern-Volmer plots for the quenching of the intrinsic fluorescence of TCSL. (A) acrylamide, (B) succinimide, (C) iodide and (D) cesium ion. (Ȯ) native protein, (▲) in the presence of 0.1 M lactose, (●) with 8 M urea.

native TCSL about 77% of the fluorescence intensity was accessible to acrylamide, while about 70% of the fluorescence intensity was accessible to succinimide. Iodide ion and Cs^+ could access only 12.8% and 23.4%, respectively, of the total fluorescence intensity of TCSL under native conditions. Denaturation with 8 M urea leads to 100% accessibility to acrylamide and succinimide whereas with iodide and cesium ions the fraction of accessible fluorescence intensity was about 71% and 70%, respectively. For acrylamide and iodide, the fraction of accessible fluorescence intensity increases upon binding of lactose, whereas for succinimide and cesium ion a decrease is noticed (Table 4.2).

Under native conditions, the K_a value for succinimide is 3.85 M^{-1} as compared to 4.57 M^{-1} for acrylamide (Table 4.2), reflecting probably both the lower quenching efficiency of the former as well as its restricted accessibility to the tryptophan residues. The value of K_a for iodide was 5.96 M^{-1} and for Cs^+ it was 1.36 M^{-1}, clearly indicating I^- to be a much more efficient quencher than Cs^+.

From the Stern-Volmer quenching constants reported in Table 4.2 and the average lifetimes obtained from time-resolved fluorescence decay measurements (see below), the bimolecular quenching constants, k_q, were calculated. These values are also given in Table 4.2.

Lifetime measurements of fluorescence emission

The fluorescence decay curves of native TCSL and in the presence of 0.1 M lactose, obtained from the time-resolved measurements are given in Figure 4.5. The decay curves were analysed by a multi-exponential iterative program supplied by IBH [Komath & Swamy, 1999]. In both cases the decay profiles could be best fitted to a biexponential function ($\chi^2 \leq 1.1$). Mono-exponential fits gave significantly large errors ($\chi^2 \geq 2.0$), whereas fits with three exponentials did not give significantly reduced errors as compared to the biexponential fits. For native TCSL, the lifetime values were obtained as 1.78 and 4.75 ns (($\chi^2 = 1.07$), whereas in the presence of 0.1 M lactose the lifetimes obtained were 2.15 and 5.14 ns. For the protein denatured with urea the biexponential fits yielded lifetime values of 1.92 ns and 5.19 ns ($\chi^2 = 1.26$), whereas for the protein in the presence of 8 M urea and 10 mM β-ME lifetime values of 1.13 ns and 4.24 ns ($\chi^2 = 1.4$) were obtained.

Analysis of the lifetime decay profiles also yielded the relative contributions of each component to the total fluorescence intensity. For native TCSL, the component with shorter lifetime of 1.78 ns contributed 47.9% of the total fluorescence intensity whereas the component with longer lifetime of 4.75 ns contributed 52.3% of the total intensity. In the presence of 0.1 M galactose the contribution of the shorter lifetime increases to about 48.9% while in the presence of 0.1 M lactose it increases further to 54% of the total intensity. Denaturation with

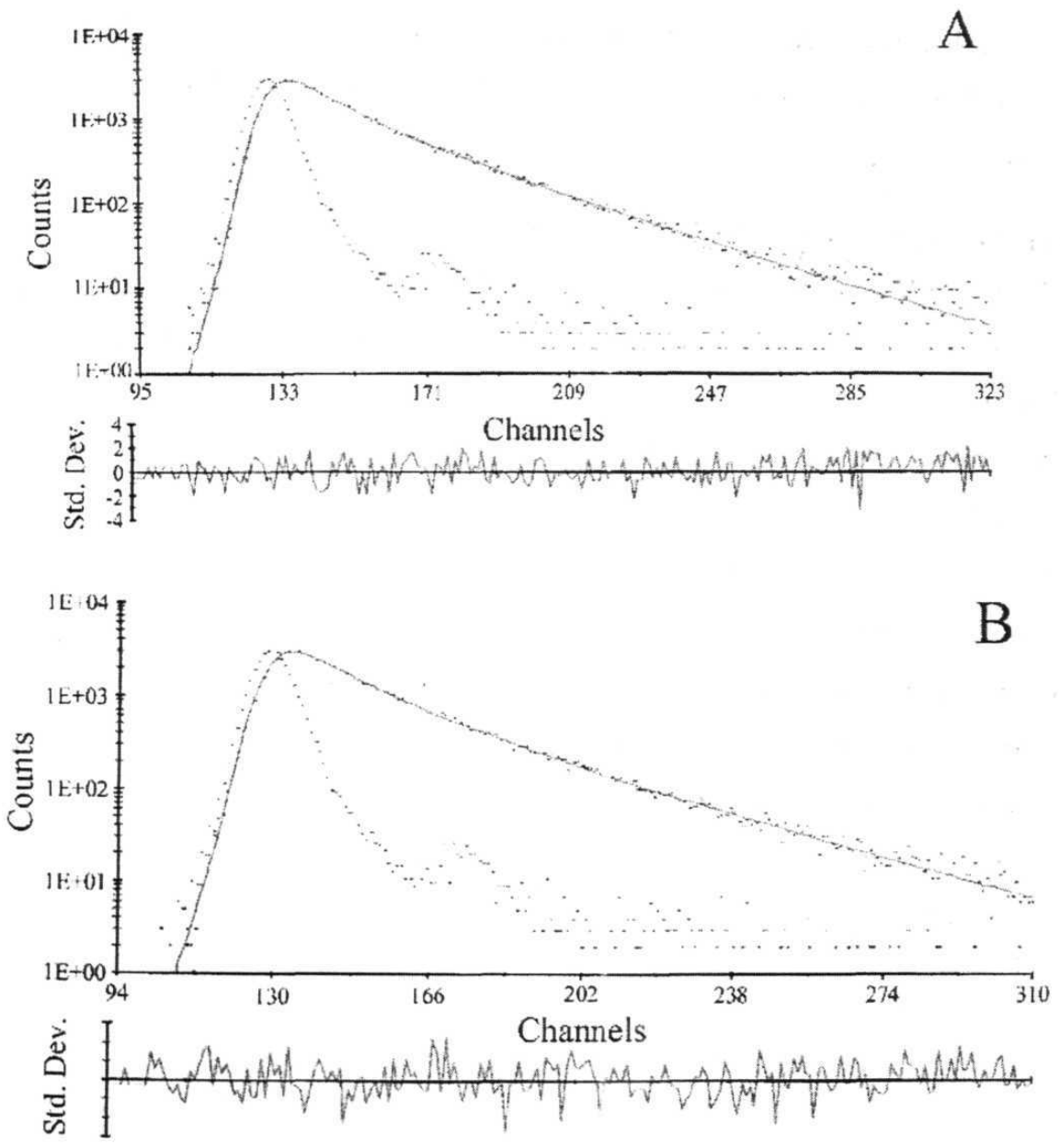


Figure 4.5. Time-resolved fluorescence decay profiles of native TCSL (A) and TCSL in the presence of 0.1 M lactose (B). The solid lines correspond to the nonlinear least square fit of the experimental data to a biexponential function. The lower panels in A and B represent the residuals.

8 M urea increases the lifetimes only marginally with practically no change in the relative contributions of the two components to the overall fluorescence intensity. However, denaturation in the presence of β-mercaptoethanol (β-ME) decreases the lifetimes of both the components to 1.13 ns and 4.24 ns, with contributions of 38.3% and 61.7%, respectively. The values of lifetimes, τ_i and the corresponding weighting factors, α_i obtained with TCSL under different conditions are listed in Table 4.3.

Average lifetimes of fluorescence decay for TCSL under different conditions were calculated from the above data using the following expressions [Grinvald & Steinberg, 1974; Inokuti & Hirayama, 1965]:

$$\tau = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i \qquad (4.4)$$

$$\langle\tau\rangle = \Sigma_i \alpha_i \tau_i^2 / \Sigma_i \alpha_i \tau_i \qquad (4.5)$$

where τ and $\langle\tau\rangle$ are the average fluorescence lifetimes estimated by the two different approaches. These values are also presented in Table 4.3. From this Table it is seen that for the native TCSL τ and $\langle\tau\rangle$ are 2.64 ns and 3.33 ns, respectively. These values change only marginally to 3.01 ns and 3.65 ns, respectively, in the presence of 0.1 M galactose and to 2.93 ns and 3.53 ns, in the presence of 0.1 M lactose. In the presence of 8 M urea also there is not much change in the average lifetimes, but denaturation with 8 M urea and reduction with 10 mM β-ME decreases the average lifetimes estimated by Equations 5.4 and 5.5 to 1.34 ns and 1.61 ns, respectively (Table 4.3).

In the presence of 0.5 M acrylamide the shorter lifetime decreases from 1.78 ns to 0.93 ns, whereas the longer lifetime decreases from 4.75 ns to 2.44 ns. On the other hand, in the presence of 0.5 M concentration of the charged quenchers, I^- and Cs^+, the shorter lifetime is largely unaffected, but the longer lifetime decreases to 3.82 ns and 3.96 ns, respectively.

Table 4.3. The lifetimes of fluorescence decay of TCSL and the corresponding pre-exponential factors along with calculated average lifetimes of acrylamide quenching.

Sample description	α_1	τ_1	$\alpha_2 \times 10^2$	τ_2	τ	$<\tau>$
Native TCSL	0.118	1.78	4.81	4.75	2.64	3.33
TCSL+ 0.1 M Galactose	0.118	2.11	5.06	5.14	3.01	3.65
TCSL + 0.1 M lactose	0.116	2.15	4.13	5.14	2.93	3.53
TCSL in 8M urea	0.114	1.92	4.68	5.19	2.87	3.64
TCSL in 8M urea with 10mM β-ME	0.143	1.13	0.006	4.24	1.34	1.61
TCSL + 0.5M acrylamide	0.083	0.93	0.12	2.44	1.47	2.37
TCSL + 0.5M KI	0.104	1.72	6.91	3.82	2.56	2.97
TCSL + 0.5M CsCl	0.118	1.92	5.14	3.96	2.54	2.89

DISCUSSION

Chemical modification studies described in Chapter III have suggested that there are 4.6 (± 0.4) Trp residues in the TCSL dimer. The emission maximum of the native protein at 331 nm clearly indicates that these residues are predominantly buried in the hydrophobic interior of the protein (Fig. 4.1). Sugar binding does not lead to any significant shift in the emission λ_{max}. The fluorescence decay profile obtained in the presence of lactose remains unaltered, as compared to that of native TCSL and the fluorescence lifetimes increase only marginally compared to the native protein when recorded in the presence of sugar. These results, which are consistent with steady-state fluorescence spectral data as well as the quenching experiments, indicate that any conformational changes resulting from lactose binding in solution do not lead to major changes in the environment of tryptophan residues. The large red shift in fluorescence emission maximum upon denaturing with 8 M urea (Fig. 4.1) indicates greater exposure of Trp residues of the lectin to the aqueous environment. This is also clear from the increase in the extent of quenching observed for all quenchers in the presence of 8 M urea. Since reduction of the disulfide bonds in the protein with mercaptoethanol increases the wavelength of the emission maximum further by 2 nm (Fig. 4.1), it appears that some residual structure is still present in the unreduced protein, which becomes unordered when the disulfide bonds are broken. Similar observations were made on the snake gourd seed lectin [Komath & Swamy, 1999], which exhibits immunological cross-reactivity with TCSL [Padma *et al.*, 1999].

As the emission λ_{max} of TCSL suggests that most of the Trp residues of this lectin are buried, the extent of quenching by different quenchers and degree of accessibility to them vary depending on their size and charge. Though acrylamide

and succinimide are both neutral quenchers, because of its larger size and structural rigidity, approach to the buried fluorophores could be considerably more hindered in case of succinimide. Hence both the extent of quenching and the accessible fraction of fluorophores decrease for succinimide as compared to acrylamide (Tables 4.1 & 4.2). The ratio ($\gamma_{S/A}$) of the initial slopes of the Stern-Volmer plots for the two quenchers (or ratio of their bimolecular quenching constants) has been used to describe the degree of burial of Trp residues in a protein matrix [Eftink & Ghiron, 1984]. For TCSL this ratio is 0.88 indicating that Trp residues in this lectin are partially buried within protein matrix. The significantly lower quenching and percentage accessibility observed with the charged quenchers Cs^+ and I^- are consistent with their inability to penetrate into the hydrophobic core of the protein and lends further support to the above interpretation, made from an analysis of the emission maximum, that most of the Trp residues of TCSL are buried in the protein interior, which is highly hydrophobic.

Binding of lactose alters the accessibility of tryptophan residues to the quenchers, indicating that ligand-induced conformational changes modify the penetration of the quencher into protein matrix. Accessibility increases in the case of acrylamide and iodide whereas it decreases in case of cesium ion on addition of sugar. For succinimide the accessibility is unaltered upon binding of lactose. The Stern-Volmer plots are monophasic with acrylamide, succinimide and iodide and the plot is biphasic with cesium when recorded in the presence or absence of sugar. The changes in accessibility induced by ligand binding are rather small and it is likely that the conformational changes which are responsible for the altered accessibility are also small and do not involve most of the tryptophan residues present in the protein. Also, these results suggest that Trp residues are most probably not directly involved in the sugar-binding activity of TCSL. This is

consistent with the chemical modification studies reported in Chapter III, where it was found that the Trp residues are not important for the carbohydrate-binding and cell-agglutinating activities of TCSL.

Greater accessibility of Trp residues is observed for all the quenchers in the presence of 8 M urea. Interestingly, the Stern-Volmer plots with acrylamide and succinimide are upward curving, and may be rationalized as arising due to a quenching mechanism that involves both dynamic and static components. In such cases, only a certain fraction of excited states is actually quenched by collisional mechanism (dynamic quenching). Some of the excited states are deactivated almost instantaneously after being formed because a quencher molecule happens to be randomly positioned in their proximity at the time they are excited. Similar upward-curving quenching profiles with acrylamide have been reported for small molecules as well as for proteins [e.g., see Eftink & Ghiron, 1976a,b; Casali *et al.*, 1990; Wasylewski *et al.*, 1995].

The Stern-Volmer plots with I^- and Cs^+ are biphasic in the presence of 8 M urea and complete accessibility could not be achieved for these ionic quenchers (Fig. 4.3). Since complete accessibility was observed with the neutral quenchers acrylamide and succinimide, this suggests that charged residues could be present in the local environment of one or more tryptophan residues. Since both the anionic iodide ion and the cationic Cs^+ could not completely access all the Trp residues, it appears likely that at least one Trp residue has a positively charged amino acid in its immediate vicinity and at least one other Trp residue has a negatively charged amino acid in close proximity.

The two fluorescence decay lifetimes observed with native TCSL suggest that the Trp residues of this protein exist, most likely in at least two different environments, although a biexponential fit is by no means a conclusive proof for

the existence of two different types of tryptophans in the protein because even single tryptophan proteins can give biexponential decay profiles [cf. Ross *et al.*, 1981]. If this is true then both these types of Trp residues are quenched by acrylamide via a collisional mechanism because both these lifetimes are reduced in the presence of this neutral quencher. On the other hand, only the Trp residues contributing to the longer lifetime seem to be quenched by iodide ion and cesium ion because presence of these two ionic quenchers reduces the longer lifetime, while the shorter lifetime is nearly unaffected by their presence (Table 4.3).

In summary, the fluorescence studies reported in this study demonstrate that the tryptophan residues of TCSL are in a predominantly hydrophobic environment. Presence of specific saccharide ligands, galactose and lactose results in only marginal differences in the quenching profiles or in the fluorescence decay lifetimes, which is consistent with the non-involvement of Trp residues in the sugar binding activity of the lectin, as previously shown by chemical modification studies [Chapter III]. Quenching data obtained with I^- and Cs^+ indicate that charged amino acids might be present in the immediate neighborhood of some of the tryptophan residues.

Chapter V

Thermodynamic and Kinetic Analysis of Porphyrin Binding to *Trichosanthes cucumerina* Seed Lectin

SUMMARY

The interaction of several metallo-porphyrins with *Trichosanthes cucumeirna* seed lectin has been investigated. Difference absorption spectroscopy revealed that significant changes occur in the *Soret* band region of the porphyrins upon binding to TCSL and these changes have been monitored to obtain association constants (K_a) and stoichiometry of binding (n). The dimeric lectin binds two porphyrin molecules and presence of the specific saccharide, lactose did not affect the porphyrin binding significantly, indicating that the sugar and the porphyrin bind at different sites. The K_a values obtained for the binding of different porphyrins with TCSL at 25 °C were in the range of 2×10^3 M^{-1} to 5×10^5 M^{-1}. Association constants for CuTPPS, a porphyrin bearing four negative charges and CuTMPyP, a porphyrin with four positive charges, were determined at several temperatures and from the temperature dependence of the association constants, the thermodynamic parameters, change in enthalpy (ΔH^o) and change in entropy (ΔS^o), associated with the binding process were estimated. The thermodynamic data indicate that porphyrin binding to the *T. cucumerina* seed lectin is largely driven by favorable entropic contribution and the enthalpic contribution is very small, suggesting that the binding process is governed primarily by hydrophobic forces. Stopped-flow spectroscopic measurements show that binding of CuTMPyP to TCSL takes place by a single-step process and at 20 °C, the association and dissociation rate constants were obtained as 1.89×10^4 $M^{-1}.s^{-1}$ and 0.29 s^{-1}, respectively.

INTRODUCTION

While the carbohydrate-recognition by lectins is a functionally significant property, considerable evidence suggests that these proteins may exhibit other, physiologically relevant interactions. Especially, the fact that for most lectins endogenous carbohydrate ligands have not been found to date lends credence to the hypothesis that these proteins may have other, non-carbohydrate endogenous ligands that are physiologically relevant. This is generally true for most lectins of plant origin, for which endogenous carbohydrate ligands are yet to be identified. Hydrophobic contacts are known to be extremely important in stabilizing protein-protein and protein-membrane interactions. But only recently such interactions have been attributed to lectins as well. The cryoprotective galactose-specific lectins from mistletoe bind to head groups of digalactolipids in thylakoid membranes and efficiency of this binding depends on hydrophobicity [Hincha *et al* 1997]. Similarly, a number of animal lectins have multifunctional domains and some of these are endogenously involved in hydrophobic interactions with receptors / ligands. There have been investigations on the lectin-peptide [Kaur *et al.*, 1997], lectin-protein [Vattuone *et al.*, 1991] and lectin-membrane [Grant & Peters, 1984] interactions to study the biological significance of such interactions as well as to explore the possibility of using lectins as tools in the study of carbohydrates and other biological systems.

Porphyrins are a group of biologically important molecules that have considerable hydrophobic character. In nature there are several examples where porphyrins are bound to polypeptide chains, e.g., hemoglobin, myoglobin and cytochrome *c*. Studies of lectin-porphyrin interactions can be important from the point of view of the effect of the lectins on porphyrin containing molecules as well as from the point of view of the effect of porphyrins on the biological systems of plants and

animals. Binding of synthetic porphyrins to proteins such as human serum albumin, bovine serum albumin and low-density lipoproteins has been investigated by several groups [Davila & Harriman, 1990; Beaven *et al.*, 1974; Reyftmann *et al.*, 1984] in order to follow the effect of these molecules on the living system as well as to explore the possibility of using proteins as specific ligand-delivery systems to target cells. We envisage that similar studies involving lectins could prove to be quite useful as well.

Recently porphyrins are being used as photosensitizers in photodynamic therapy (PDT), a new approach developed for the treatment of cancer [Klyashchitsky *et al.*, 1994; Levy, 1995]. In PDT, when excited by light of appropriate wavelength, the porphyrin photosensitizers interact with molecular oxygen and convert it to the triplet form, which then reacts with the surrounding tissue and leads to cell death. Porphyrins appear to be suitable for such an application, as they have been found to exhibit a preferential localization in tumor tissues, in addition to being biocompatible. However, in many cases the ratio of the photoactive drug in tumor tissues to that in the surrounding normal tissue is as low as 2:1 [Klyashchitsky *et al.*, 1994], thus making the use of porphyrins as such in tumor treatment less effective. Conjugating the porphyrins to another agent that can steer them towards the tumors might be a possible approach to circumvent this limitation. In this regard it is of interest to investigate whether lectin-bound porphyrins can be employed in PDT as several lectins have been shown to exhibit preferential binding to tumor cells. With the objective of exploring such a possibility, and also to understand the nature of probable hydrophobic ligands for lectins in biological systems and their clinical applications our laboratory has recently characterized the interaction of several free-base and metalloporphyrins with different plant lectins, viz., ConA, pea lectin, Jacalin and the snake gourd (*Trichosanthes anguina*) seed lectin [Bhanu *et al.*, 1997; Komath *et al.*, 2000a , b].

In this chapter, we discuss absorption spectroscopic studies on the interaction of different Cu- and Zn-porphyrins with TCSL. In order to delineate the forces that

govern the interaction of porphyrins with this lectin, the binding experiments were performed at different temperatures with some of the porphyrins and the changes in enthalpy and entropy associated with the binding process were determined. Further, the kinetics of the interaction of one of the porphyrins was investigated by stopped-flow spectroscopy to elucidate the mechanism of binding of the porphyrin to the lectin. The results indicate that porphyrin binding to TCSL is governed primarily by hydrophobic forces. The binding kinetics indicates a simple, single-step association process.

MATERIALS AND METHODS

Materials

Seeds of *T. cucumerina* were obtained from United Chemicals and Allied Products (Kolkata, India). Guar gum, sodium dodecyl sulfate, bovine serum albumin, lactose and the reagents for polyacrylamide gel electrophoresis were purchased from Sigma (St. Louis, MO, USA). Epichlorohydrin was from SD's Chemicals (Mumbai, India). *Meso* -tetra(4-sulphonatophenyl) porphyrinato zinc(II) (ZnTPPS), *meso*-tetra(4-sulphonatophenyl)porphyrinato copper(II) (CuTPPS), *meso*-tetra(4-carboxyphenyl)porphyrinato zinc(II) (ZnTCPP), *meso*-tetra(4-carboxyphenyl)porphyrinato copper(II) (CuTCPP), *meso*-tetra(4-methylpyridinium) porphyrinato zinc(II) (ZnTMPyP) and *meso*-tetra(4-methylpyridinium)porphyrinato copper (II) (CuTMPyP) were prepared and characterized as described in [Fleischer *et al.*, 1971; Kadish *et al.*, 1989; Longo *et al* 1969; Pasternack *et al.*, 1972, 1973].

Absorption spectroscopy

Absorption measurements were made on a Shimadzu model UV-3101PC UV-Vis-NIR double beam spectrophotometer using 1.0 cm path-length cells. Temperature was

maintained constant (± 0.5 °C) by means of a Peltier device supplied by the manufacturer.

Concentration determination

Concentration of TCSL was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Concentrations of the porphyrins were determined spectrophotometrically using their molar absorptivity at the λ_{max} of the *Soret* band, as described in [Komath *et al.*, 2000b].

Binding of porphyrins to *T. cucumerina* seed lectin

Porphyrin binding to TCSL was investigated by absorption titrations essentially as described earlier for studies with SGSL [Komath *et al.*, 2000b]. All binding experiments were performed in 20 mM phosphate buffer, containing 0.15 M NaCl, pH 7.4 (PBS). Porphyrin samples (2 ml) of ca. 2.0 - 4.0 μM concentration were titrated by adding small aliquots of the lectin from a concentrated stock solution. The UV-vis spectra were recorded after an equilibration period of 2 minutes following each addition. All titrations were repeated at least thrice to arrive at the average values of association constants, K_a, and number of binding sites on the lectin molecule, n.

Stopped-flow spectroscopy

Kinetics of the interaction of CuTMPyP with TCSL was investigated by the stopped-flow method. Experiments were performed on an Applied Photophysics SX-18MV stopped-flow apparatus (Leatherhead, KT227PB, U. K). The dead time of the instrument was estimated by the method described by Hiromi and coworkers (1978) and found to be 1.2 ms [Thomas *et al.*, 1998]. Concentration of CuTMPyP was fixed at 2.55 μM and the protein concentration was varied from 50 μM to 175 μM (both concentrations after mixing) and the change in absorbance at 424 nm was monitored.

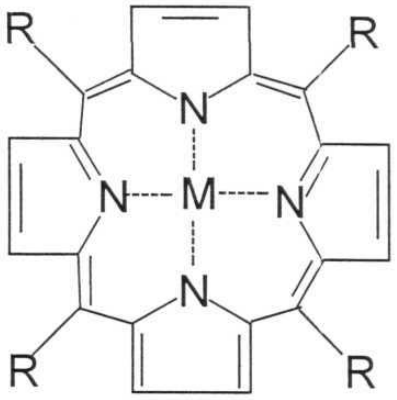
The sample syringes and the cell were maintained at 20 °C by circulating water through jackets surrounding the cell and the syringes, by means of a constant-temperature bath. Measurements were done in 20 mM phosphate buffer containing 0.15 M NaCl, pH 7.4 (PBS). All traces are averages of 5-10 independent kinetic profiles. The stopped-flow traces were analyzed by curve fitting using the Marquardt algorithm based on the routine curves. The curve-fitting and further analyses were done using an ARCON 5000, RISC workstation supplied by the manufacturer.

RESULTS

Difference absorption spectra and microenvironment of porphyrin-binding site on TCSL

A schematic diagram depicting the structures of various porphyrins used in this study is shown in Fig. 5.1 along with the corresponding λ_{max} and ε_{max} values for the *Soret* band. It was observed that all the porphyrins used in this study obeyed Beer's law in the concentration range 0-5 μM, indicating that under the experimental conditions employed, the porphyrins were not aggregated [see for example, Komath *et al.*, 2000b].

Absorption spectra in the Soret band region for CuTMPyP, ZnTPPS and ZnTCPP in the absence as well as in the presence of different concentrations of TCSL are given in Figures 5.2A, 5.2B and 5.2C, respectively. In these spectra, the λ_{max} of the *Soret* band is seen at 424.9, 423.3 and 422.5 nm for CuTMPyP, ZnTPPS and ZnTCPP, respectively. Addition of TCSL results in a red shift of the λ_{max} by about 1



Porphyrin	R	M
CuTPPS	$PhSO_3^{-2}$	Cu^{+2}
ZnTPPS	$PhSO_3^{-2}$	Zn^{+2}
CuTCPP	$PhCOO^-$	Cu^{+2}
ZnTCPP	$PhCOO^-$	Zn^{+2}
CuTMPyP	Methyl-pyridinium	Cu^{+2}
ZnTMPyP	Methyl-pyridinium	Zn^{+2}

Fig. 5.1. Structures of the porphyrins used in the present study.

(± 0.2) nm in the spectra of CuTMPyP, ZnTPPS and ZnTCPP, respectively, at the highest concentration of TCSL added in the titration. Similar shifts were also observed in the *Soret* band region of the absorption spectra of CuTPPS, CuTCPP and ZnTMPyP in the presence of TCSL.

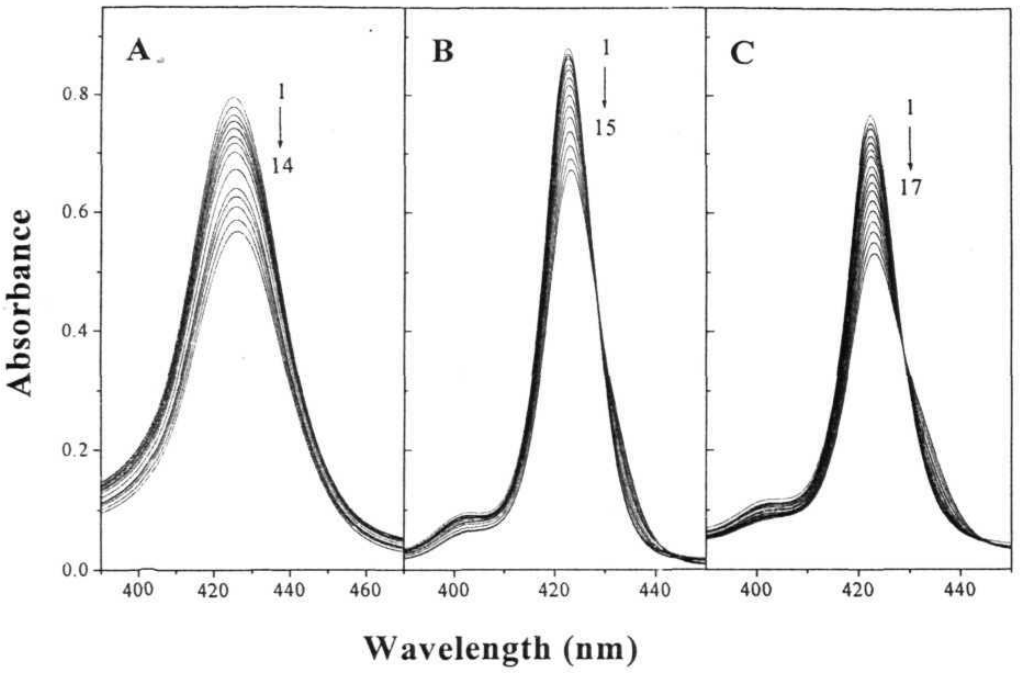


Fig. 5.2. Absorption spectral titration of porphyrin binding to *T. cucumerina* seed lectin. A) CuTMPyP, B) ZnTPPS, C) ZnTCPP. The porphyrin absorption spectra were recorded in the absence and presence of increasing concentrations of TCSL. Spectrum 1 in each panel corresponds to the porphyrin alone and the remaining spectra of decreasing intensity at the *Soret* band maximum were obtained in the presence of increasing concentrations of TCSL.

In order to further characterize the nature of the interaction of these porphyrins with TCSL, difference spectra were obtained by subtracting the spectrum of the porphyrin alone from those of the porphyrin-lectin mixtures. Difference absorption spectra thus obtained for CuTMPyP, ZnTPPS and ZnTCPP are given in Figures 5.3A, 5.3B and 5.3C, respectively. The difference spectra obtained for the titration of the zinc(II) porphyrins, namely ZnTPPS and ZnTCPP with TCSL were found to be qualitatively similar to those obtained for the titration of the corresponding copper(II)

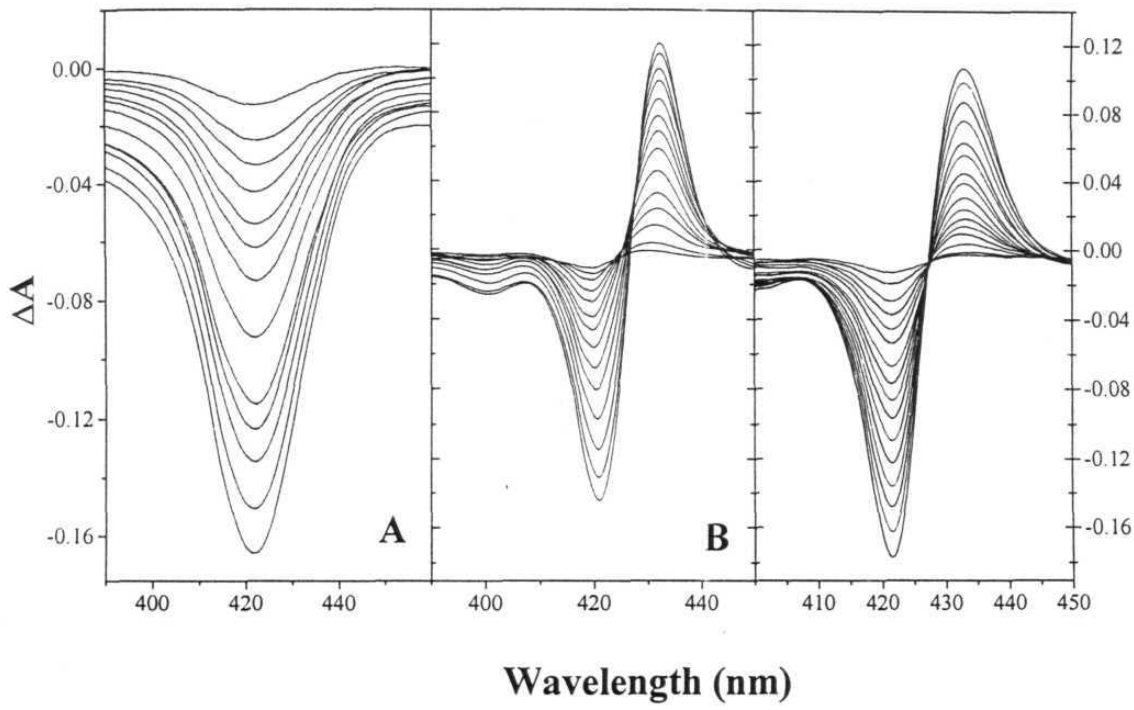


Fig. 5.3. Difference absorption spectra for porphyrin binding to TCSL. A) CuTMPyP, B) ZnTPPS, C) ZnTCPP. The difference spectra shown in panels A, B, and C were obtained by subtracting the spectrum of the porphyrin alone from the spectra of lectin-porphyrin mixtures, shown in the corresponding panel of Fig. 2. The Y-scale on the left corresponds to panel A and that on the right corresponds to panels B and C.

analogues. Similarly, the difference spectra obtained with CuTMPyP and ZnTMPyP were found to be qualitatively very similar.

Kadish and coworkers [Kadish *et al.*, 1989; Kadish & Maiya, 1991] have characterized the interaction of different porphyrins used in this study, with neutral, cationic and anionic micelles, namely Triton X-100 (TX-100), cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), respectively, and concluded that the tetra-anionic porphyrins such as H_2TPPS and its metal derivatives interact with CTAB and TX-100 via Coulombic as well as

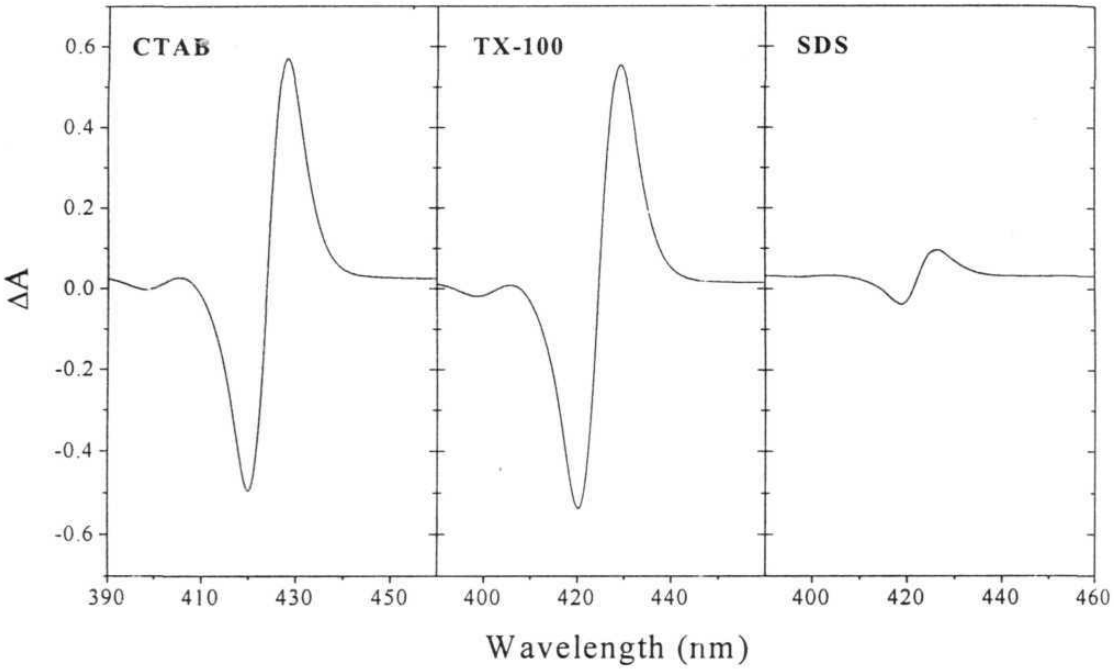


Fig. 5.4. Difference absorption spectra of ZnTPPS in the presence of different surfactants. The surfactants used are indicated in the figure.

hydrophobic interactions, whereas the interaction of tetra-cationic porphyrins such as H_2TMPyP and its metal derivatives interact with SDS only via Coulombic interactions and that hydrophobic interactions do not play a significant role in the TMPyP-micelle interaction. A significant shift in the *Soret* band region was interpreted as indicative of interaction between the porphyrin and the micelles. In order to compare the results on the interaction of porphyrins with TCSL with the results obtained on the interaction of porphyrins with micelles, difference spectra with ZnTPPS and ZnTMPyP were recorded in the presence of SDS, TX-100 and CTAB, at concentrations well above their critical micellar concentrations. Difference spectra for ZnTPPS and ZnTMPyP obtained in the presence of SDS, TX-100 and CTAB are shown in Fig. 5.4. A comparison clearly shows that the difference spectra of ZnTPPS and ZnTCPP in the

presence of the lectin are very similar to the corresponding difference spectra obtained in CTAB and TX-100 (Figs. 5.3 and 5.4). On the other hand, only minor changes were noticed in the difference spectra of ZnTMPyP in the presence of CTAB and TX-100, whereas considerably larger changes were noticed in the difference spectra obtained in the presence of SDS. These observations are consistent with the results of Kadish and coworkers [Kadish *et al.*, 1989; Kadish & Maiya, 1991], described above.

Association constants and thermodynamics of porphyrin binding to TCSL

The data obtained from the absorption titrations was analysed in the following manner to obtain the association constants (K_a) for TCSL-porphyrin interaction. A plot of (A_o/ ΔA) *vs* $[P]_t^{-1}$, where ΔA refers to the change in absorbance of the porphyrin at a given TCSL concentration during the titration and A_o corresponds to the absorbance of the sample in the absence of protein, yielded a straight line. From the ordinate of the plot, A_∞, the absorbance of the sample at infinite protein concentration was calculated. From the titration data the K_a values were obtained according to the method of Chipman *et al.* [1967] as described earlier for the binding of porphyrins to Con A and pea lectin [Bhanu *et al.*, 1997]. Briefly, a plot of log $[P]_f$ against log $\{(\Delta A)/(A_c-A_\infty)\}$ where $[P]_f$ is the free protein concentration, was obtained. A representative plot for the interaction of CuTPPS with TCSL is given in Fig. 5.5. The abscissa intercept of this plot yielded the pK_a value of the TCSL-CuTPPS interaction according to the relationship [Chipman *et al.*, 1967]:

$$\log \{(\Delta A)/(A_c-A_\infty)\} = \log K_a + \log \{[P]_t - [L]_t (\Delta A/\Delta A_\infty)\} \qquad (5.1)$$

where A_c is the absorption intensity of the sample at any point during the titration, $[L]_t$ is the total concentration of porphyrin, ΔA_∞ is the change in absorption intensity at saturation binding, and $[P]_f$, the free protein concentration, is given by:

$$[P]_f = \{[P]_t - [L]_t (\Delta A/\Delta A_\infty)\} \qquad (5.2)$$

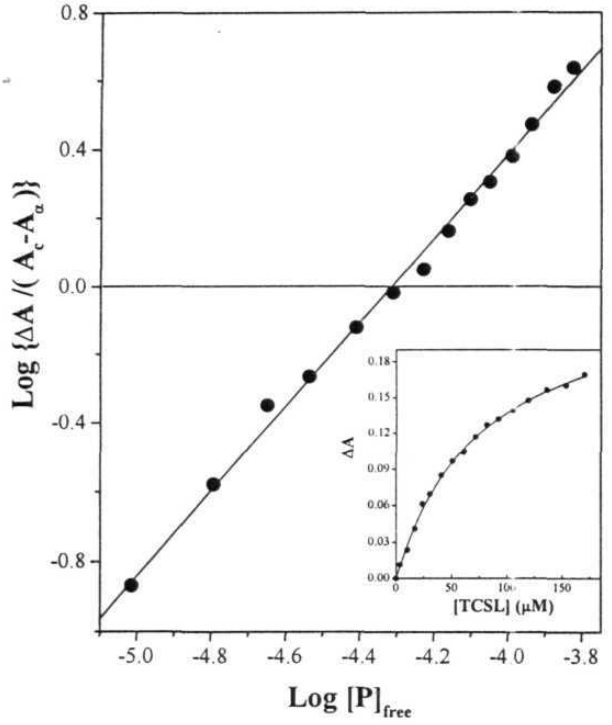


Fig. 5.5. Chipman plot for CuTPPS binding to *T. cucumerina* seed lectin. Antilog (-abscissa) gives the association constant (K_a) for the interaction. *Inset* gives a binding curve for the interaction, obtained by plotting the increase in absorption intensity at 411 nm as a function of the lectin concentration.

The slopes of such plots, obtained for the binding of different porphyrins to TCSL, were generally found to be in the range of 0.8-1.2, indicating that each lectin subunit has one porphyrin-binding site. Furthermore, the linearity of the plots indicates that the two binding sites present on the dimeric lectin interact with the porphyrin with comparable binding strength and that binding at one site does not affect binding at the other site. The gradual saturation in the decrease in the absorption intensity (corrected for dilution) upon titrating the porphyrin with the lectin is shown in the inset of Fig. 5.5. The slope, maximal change in the absorption intensity at infinite protein concentration and the corresponding association constants for binding of different porphyrins to TCSL, are listed in Table 5.1. The data obtained

with different porphyrins indicates that TCSL-porphyrin interaction is characterized by association constants in the range of $2 \times 10^3 - 5 \times 10^5$ M^{-1} and binding stoichiometry of ~1:1 per subunit, assuming an average M_r of 30,000 per subunit (Table 5.1).

Table 5.1: The maximal change in porphyrin absorbance (ΔA_∞) at infinite lectin concentration, the average slopes from double logarithmic plots and the association constants, K_a, for TCSL-porphyrin complexes at 25 °C. Experiments were performed in the absence as well as in the presence of 0.1 M lactose and the results are given in separate columns.

Porphyrin	Without sugar			With 0.1 M lactose		
	ΔA_∞ (%)	slope	$K_a \times 10^{-4}$ (M^{-1})	ΔA_∞ (%)	slope	$K_a \times 10^{-4}$ (M^{-1})
CuTPPS	25.8 ± 2.4	1.05 ±0 .02	12.11 ± 6.23	26.9 ± 3.5	1.16 ± 0.23	1.39 ± 0.15
ZnTPPS	32.2 ± 9.7	0.99 ± 0.10	1.9 ±1.086	---	---	---
CuTCPP	16.9 ± 2.9	1.03 ± 0.22	0.22 ± 1.5	---	---	---
ZnTCPP	40.0 ± 4.3	1.06 ± 0.23	0.58 ± 0.3	---	---	---
CuTMPyP	30.7 ± 7.4	0.86 ± 0.06	6.11 ± 0.5	28.5 ± 5.0	0.92±0.12	17.9 ± 0.58
ZnTMPyP*	22.0	0.7	46.8	---	---	---

*Data corresponds to a single titration

Association constants for representative examples of an anionic porphyrin (CuTPPS) and a cationic porphyrin (CuTMPyP), were also obtained in the presence of 0.1 M lactose, in order to assess the effect of the specific sugar on the porphyrin binding characteristics of TCSL (see Table 5.1). The data obtained clearly show that while considerable differences could be seen in the K_a values obtained in the absence and in the presence of the lectin, the stoichiometry of binding (as obtained from the slopes of the double logarithmic plots) remains unchanged, indicating that

sugar binding does not inhibit the porphyrin binding. In other words, the sugar and porphyrin bind to the lectin at distinctly different sites under the experimental conditions employed here.

Binding of CuTPPS and CuTMPyP was investigated at different temperatures and the K_a values obtained are listed in Table 5.2. At each temperature, at least three independent titrations were performed for each lectin-porphyrin system and the average values are given. From the temperature dependent K_a values for CuTPPS and CuTMPyP, the Gibbs free energies (ΔG^o),

Table. 5.2: Association constants, K_a, obtained at different temperatures for the interaction of CuTPPS and CuTMPyP with *T. cucumerina* seed lectin.

T (°C)	$K_a \times 10^{-4}$ (M^{-1})	
	CuTPPS	CuTMPyP
20	7.85 ± 2.70	10.48 ± 0.35
25	12.11 ± 6.23	6.11 ± 0.50
27.5	13.61 ± 4.96	nd*
30	4.85 ± 0.25	6.18 ± 0.49
32.5	7.38 ± 2.10	nd*
35	5.1 ± 1.12	8.4 ± 3.25
40	8.4 ± 4.26	7.0 ± 2.12

*nd: not determined

the enthalpy of binding (ΔH^o) and entropy of binding (ΔS^o) have been obtained by means of van't Hoff plots (fig. 5.6) using the expressions:

$$\Delta G^o = - RT \ln K_a \qquad (5.3)$$

$$\Delta G^o = \Delta H^o - T\Delta S^o \qquad (5.4)$$

For CuTPPS binding to TCSL, the values obtained at 25 °C are: ΔG^{o} = -6.93 kcal.mol^{-1}, ΔH^{o} = -3.6 (± 4.4) kcal.mol^{-1}, ΔS^{o} = 10.5 (± 14.9) cal.mol^{-1}.K^{-1}, whereas the corresponding values for CuTMPyP binding to TCSL are, -6.53 kcal.mol^{-1}, -1.8 (± 2.8) kcal.mol^{-1} and 16.2 (± 9.4) cal.mol^{-1}.K^{-1}, respectively.

Stopped-flow kinetics

As the absorption intensity of CuTMPyP in the *Soret* band region decreases upon binding to TCSL (see Fig. 5.2A), the kinetics of the corresponding interaction was monitored at 424 nm by the stopped-flow method. The apparent rate constant obtained from the absorption change of the porphyrin, k_{app}, is then related to the elementary steps involved in the binding process in a concentration dependent manner. Several possible mechanisms have been suggested for the protein-ligand interactions that do not involve covalent transformations on the protein (P) or the ligand (L) [cf. Gupta *et al.*, 1992]. Here we consider the most commonly invoked model which involves the rapid formation of an intermediate, PL_i, which isomerizes to give the final complex, PL^* (Equation 5.5). This model has been found to satisfactorily explain the stopped-flow kinetic data obtained by us.

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL^* \qquad (5.5)$$

In this case, k_{app} is related to the different rate constants and $[P]_o$ by

$$k_{app} = k_{-2} + k_2 \{[P]_o / (K_{-1} + [P]_o)\} \qquad (5.6)$$

where $K_{-1} = k_{-1}/k_1$ and $K_{-2} = k_{-2}/k_2$. Equation 5.6 predicts that k_{app} would increase linearly with $[P]_o$ but tends to saturate as $[P]_o$ increases from a value much lower than $1/K_1$ to $P >> 1/K_1$.

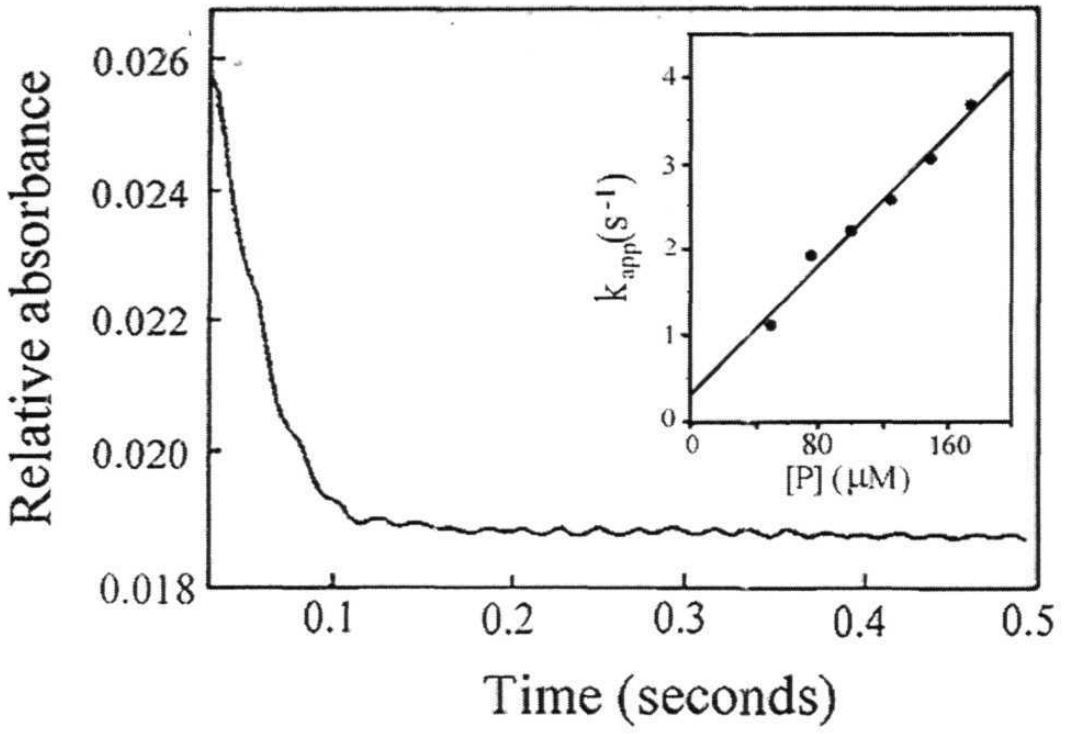


Fig. 5.6. Stopped-flow trace for the binding of CuTMPyP to *T. cucumerina* seed lectin. The change in absorption intensity at 424 nm was monitored as a functin of time. T = 20 °C. Inset gives the dependence of the apparent rate constant, k_{app}, on the lectin concentration. The slope and Y-intercept gave the k_1 and k_{-1} values as 1.89×10^4 $M^{-1}.s^{-1}$ and 0.29 s^{-1}, respectively.

Stopped-flow experiments were performed under pseudo-first order conditions, keeping the porphyrin concentration fixed (2.55 μM) and varying TCSL concentration between 50 and 175 μM (subunit). A representative stopped-flow trace recorded at 20 °C is given in Fig. 5.7. The k_{app} values obtained were found to increase linearly with increasing protein concentration. A plot of the dependence of k_{app} on the protein concentration yielded a straight line (Fig. 5.7 *Inset*). The values of k_{+1} and k_{-1} for the CuTMPyP-TCSL interaction at 20 °C were obtained as 1.89×10^4 $M^{-1}.s^{-1}$ and 0.29 s^{-1}, from the slope and intercept, respectively, of this plot.

DISCUSSION

In view of the preferential agglutination of tumor cells and the hydrophobic ligand binding exhibited by the plant lectins and also based on the low tumor tissue localization exhibited by porphyrins [Klyashchitsky *et al.*, 1994], it appeared that lectins may potentially be useful in the targeting of porphyrins to tumor tissues in photodynamic therapy. To investigate this interesting possibility initial studies from this laboratory focused on the interaction of several free-base and metallo-porphyrins with Concanavalin A, pea lectin, jacalin and snake gourd seed lectin (SGSL), in the absence and in the presence of specific saccharides that inhibit the lectin activity. In the present study, the interaction of different porphyrins with TCSL was investigated by performing the binding experiments at different temperatures to seek information on the thermodynamic forces that govern the binding. In addition, the kinetics of binding of a porphyrin was investigated by the stopped-flow technique in order to unravel the elementary steps involved in the binding process.

Earlier studies on the interaction of porphyrins with SGSL and Jacalin indicated that both positively and negatively charged porphyrins bind to these lectins with comparable affinities, suggesting that the charge on the porphyrin does not play any significant role in the interaction [Komath *et al.*, 2000a, b]. Therefore, it appears that hydrophobic interactions largely mediate the binding interaction between these two lectins and the porphyrins. Since the tetra-anionic porphyrin, CuTPPS and the tetra-cationic porphyrin, CuTMPyP bind to TCSL with comparable affinities at different temperatures (see Table 5.2), it may be argued that porphyrin binding to TCSL also occurs via hydrophobic interactions. However, the possibility of polar interactions such as hydrogen bonding between the substituents on the porphyrin with certain functional groups on the protein cannot be ruled out in the absence of specific structural data on the porphyrin-lectin complex. For example it has been shown by X-

ray crystallographic studies that H_2TPPS binds to ConA predominantly through hydrogen bonds and water- mediated interactions [Goel *et al.*, 2001]. Comparison of the crystal structure of H_2TPPS complexed with ConA and that of ConA bound to methyl α-D-mannopyranoside provided structural evidence of molecular mimicry in the context of ligand binding.

Similarity in the difference spectra obtained when ZnTPPS and ZnTCPP were titrated with TCSL (Fig. 5.3) with those obtained for ZnTPPS in the presence of cationic and neutral surfactants, CTAB and TX-100 (Fig. 5.4), suggests that the forces involved in the interactions of these porphyrins with micelles and the protein are likely to be very similar. Since the difference spectra obtained for CuTPPS and CuTCPP in the presence of TCSL are qualitatively rather similar to the corresponding spectra of their Zn- analogues, it is likely that the interaction of the former two porphyrins with TCSL is also mediated by both hydrophobic and electrostatic interactions. Similarly, the difference spectra obtained when CuTMPyP is titrated with TCSL resembles the difference spectrum obtained with the anionic surfactant, SDS. This result suggests that forces governing the binding of CuTMPyP to TCSL are rather similar to those that come into play when this tetra-cationic porphyrin interacts with SDS.

The data presented in Table 5.1 clearly show that slopes of the double-logarithmic plots were close to unity within limits of experimental error for all the TCSL-porphyrin combinations investigated, suggesting that each lectin subunit has one binding site for the porphyrin. This is also true for experiments carried out in the presence of 0.1 M lactose, indicating that sugar binding does not interfere with the porphyrin binding. Thus, it is most likely that porphyrin binding to TCSL takes place at a site that is different from the saccharide-binding site. The K_a values obtained in the presence of lactose, however, differ somewhat from those obtained in its absence (Table 5.1). A possible reason for this is that ligand binding leads to certain changes

in the conformation of the protein, resulting in an alteration in the affinity of the protein towards the porphyrins.

The association constants obtained here for the TCSL-porphyrin complexes are in the range of 2×10^3 to 2×10^5 M^{-1} (Table 5.1) and are comparable to those observed generally with lectin-monosaccharide complexes [cf. Goldstein & Poretz, 1986] as well as those obtained for porphyrin-serum protein interactions [Davila & Harriman, 1990; Chatterjee & Srivastava, 2000; Beaven *et al.*, 1974; Andrade & Costa, 2002]. Other hydrophobic ligands and plant growth hormones, such as 2,6-toluidinylnaphthalene-sulphonic acid, adenine, auxins and cytokinins also bind to lectins with comparable affinity ($K_a \approx 1.0 \times 10^3$ M^{-1} - 1.0×10^6 M^{-1}), suggesting that some of them can also be considered as potential endogenous ligands for the lectins *in vivo* [Roberts & Goldstein, 1982, 1983; Maliarik & Goldstein, 1988; Gegg *et al.*, 1992, Puri & Surolia, 1994]. This postulate is especially attractive in view of the high concentrations of plant lectins in growing tissues, where plant growth hormones too exert their action most. Pertinently, it has been postulated that Con A could be involved in the regulation of plant cell division or germination via binding to non-polar growth factors [Hardman & Ainsworth, 1973]. Similarly association of adenine or other hydrophobic ligands with the *D. biflorus* lectin has been suggested to be biologically relevant [Gegg *et al.*, 1992]. In the light of these observations and the considerably strong interaction between TCSL and the porphyrins, it is possible that there may be some endogenous hydrophobic ligands for this lectin also.

The thermodynamic parameters, ΔH° and ΔS° associated with the binding of CuTPPS and CuTMPyP, which are obtained from the temperature dependence of the association constants, indicate that the interaction of both these porphyrins with TCSL is primarily governed by entropic forces, though there is some enthalpic contribution to the binding process. It is thus interesting to note that though the K_a values for the

binding of different porphyrins to TCSL are in the same range as those obtained for the interaction of mono- and disaccharides with different lectins, the corresponding enthalpy and entropy changes are considerably different.

Due to their preferential agglutination of tumor cells, lectins have been suggested as carriers for the targeted delivery of drugs and pharmaceuticals to tumor tissues. A number of lectin-drug conjugates have also been prepared and some were successful when tested on cell-cultures and animal models [Kitao & Hattori, 1977; Gilliland *et al.*, 1978; Yamaguchi *et al.*, 1979]. In view of the considerable affinity of TCSL for Cu(II)- and Zn(II)-porphyrins investigated in this study, it is possible to use it as a vehicle for targeting porphyrin-based drugs to tumor tissues in PDT. Studies on cell-cultures/animal models are required to further investigate this possibility.

Kinetic studies

The stopped-flow studies on the binding of CuTMPyP to TCSL indicate that this binding reaction is a relatively slow process. The k_{+1} value of 1.89×10^4 $M^{-1}.s^{-1}$ is approximately 4 orders of magnitude slower than diffusion-controlled processes, the k_{+1} values for which are in the order of 10^8-10^9 M^{-1} s^{-1} [Voet & Voet, 1995]. However, the association rate constant obtained here for the CuTMPyP-TCSL interaction is in the same range as that observed for several lectin-saccharide systems. Association rate constants between 5×10^3 and 5×10^5 $M^{-1}.s^{-1}$ have been reported for lectin-saccharide interactions [Gupta *et al.*, 1992; Grey & Glew, 1973; Brewer *et al.*, 1973; Podder *et al.*, 1978; Loontiens, 1983; Swamy *et al.*, 1986; Sastry *et al.*, 1986; Puri *et al.*, 1993]. Such slow second order rate constants are usually explained by invoking the formation of an intermediate (PL_i, see Equation 5.5) that isomerises to form the final complex (PL^*). Our failure to observe PL_i could be attributed to the low K_1 value, so that significant quantities of it do not accumulate during the course of the

reaction. Since the k_{app} *versus* $[P]_o$ plot is linear up to 175 μM of the latter, K_1 has to be lower than 5700 M^{-1}. Since the K_a value obtained from the kinetic data is in reasonable agreement with the value obtained from the equilibrium titrations, it is very unlikely that there are additional steps in the binding process that contribute significantly to the binding enthalpy.

The dissociation rate constant of 0.29 s^{-1} indicates that the dissociation reaction is a rather slow process and is comparable to the dissociation rate constants reported for the interaction of fluorescently-labeled saccharides to different lectins, viz., *N*-dansylgalactosamine binding to soybean agglutinin, 4-methylumbelliferyl-*N*-acetyl-α-galactosaminide binding to WBA-I and the interaction of 4-methylumbelliferyl β-D-galactopyranosyl (1→3)-N-acetyl β-D-galactosaminide with peanut agglutinin [Swamy *et al.*, 1986; Puri *et al.*, 1988; Loontiens, 1983].

In summary, the thermodynamic and kinetic parameters that characterize lectin-porphyrin interaction have been elucidated for the first time. The association constants obtained for the TCSL-porphyrin interaction are comparable to those observed in general for mono- and disaccharide binding to lectins and the binding appears to be favored predominantly by entropic factors. The association and dissociation rate constants for TCSL-porphyrin interaction are broadly in the same range as those observed for lectin-saccharide interaction. Further studies are required to find out if these observations could be applicable to other lectin-porphyrin systems also.

Chapter VI

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

The main objective of the present study was to characterize the *Trichosanthes cucumerina* seed lectin further with respect to macromolecular and carbohydrate binding properties employing different physico-chemical methods in order to get a better understanding of the structure-function relationships of this protein as well as its relationship to other lectins in the same family and also to explore the possibility of applying this lectin in the field of medicine. In pursuit of this goal we have carried out physico-chemical, sugar binding, chemical modification, porphyrin binding and steady-state and time-resolved fluorescence studies. The results obtained are summarized below.

The physicochemical properties of TCSL were investigated by a variety of approaches. The lectin has been found to be optimally active in the pH range of 8.0-11.0 and is stable up to 60 °C. Isoelectric focusing experiments with TCSL gave three well-separated peaks at pH 5.3, 6.2 and 7.1, indicating the presence of at least three isolectins, which differ considerably in their pI. Analysis of the circular dichroism spectra of this protein revealed that TCSL contains about 28.4% β-sheet, 10.6% turns, 7% polyproline type 2 structure and rest unordered structure, while the α-helix content is negligible. Presence of the specific sugar, lactose, which inhibits the hemagglutination activity of TCSL did not alter the CD spectrum significantly, clearly indicating that saccharide binding does not lead to any significant alterations in the lectin structure. DSC studies show that the protein unfolds at 82.0 °C in the absence of any sugar, and at 86.9 °C, in the presence of 0.1 M lactose, clearly indicating that ligand binding stabilizes the protein. The transitions were found to be irreversible.

In order to establish the carbohydrate-binding specificity of TCSL and to determine the association constants for different sugars, fluorescence spectroscopic studies were carried out. Binding of MeUmbβGal to TCSL leads to an increase in the fluorescence intensity of the fluorophore while MeUmbαGal gave a very small decrease when titrated with TCSL. Enhancement of fluorescent intensity of MeUmbβGal might be the result of the umbelliferyl moiety experiencing a more polar environment compared to the α- anomer when bound to TCSL. This suggests that the anomeric configuration of the galactoside residue bound to TCSL has a significant effect on the environment of the fluorophore. From an analysis of the titration data, it was established that the dimeric lectin has two sugar binding sites. These results are similar to the previous results obtained with SGSL in our laboratory [Komath *et al.*, 2001]. Also the association constants obtained in the present study are comparable to the values reported for SGSL. This suggests that the two lectins have considerable similarity in the saccharide-combining region, which shares immunological cross-reactivity [Padma *et al.*, 1999]. Among the non-chromophoric monosaccharides studied, MeβGal exhibits the highest K_a value, closely followed by MeαGal. GalNAc and galactose bind to TCSL with affinities that are weaker by a factor of 2 and 2.6, respectively, as compared to MeβGal. These results are mostly in accord with the results of hemagglutination-inhibition studies reported earlier [Padma *et al.*, 1999]. An exception is galactose, which yielded a lower K_a value than MeαGal whereas the hemagglutination-inhibition data indicated that these two sugars should be comparable in their inhibitory ability.

Chemical modification studies, carried out with the aim of identifying the amino acid residues involved in the activity of TCSL, clearly indicate that the imidazole side chains of histidine residues are necessary for the carbohydrate-binding and hemagglutinating activities of this lectin. While a maximum of 10

histidine residues could be modified under native conditions, 15.8 His residues could be modified in the presence of 8 M urea. Modification of 5-6 His residues in the native TCSL led to a complete loss in the hemagglutination activity of the lectin. Further, the His modified protein (5.7 residues/dimer) did not bind to cross-linked guar gum and presence of lactose provided a partial protection to the histidine residues from the modification reagent, confirming that histidine residues are directly involved in the sugar-binding activity of the lectin. Reversal of the modification resulted in a complete recovery of the lectin activity. In Ouchterlony double-immunodiffusion experiments, the histidine modified *T. cucumerina* seed lectin cross-reacted with rabbit antiserum raised against native TCSL, suggesting that the modification did not result in any alterations in the overall structure of the lectin. All these results suggest the involvement of histidine residues in the sugar-binding and hemagglutination activity of this lectin. Trp residues of native TCSL could not be modified by *N*-bromosuccinimide, indicating that the indole side chains of these residues are deeply buried in the protein matrix. However, when the modification was performed after denaturing the protein with 8 M urea a total of 4.6 (± 0.4) Trp residues could be modified per dimer. The chemical modification studies suggest that the side chains of lysine, tyrosine, arginine, cysteine and tryptophan are not directly involved in the activity of the lectin.

Because the Trp residues of TCSL are buried in the hydrophobic interior of the folded protein and could not be modified by NBS under native conditions, it is of interest to investigate tryptophan exposure and environment of TCSL using fluorescence spectroscopy. The fluorescence emission spectra of TCSL in the native state and in the presence of 0.1 M lactose show a maximum at 331 nm, which shifts to 346 nm upon denaturation. Fluorescence quenching experiments on TCSL were carried out using two neutral quenchers, namely acrylamide and

succinimide, and two charged quenchers, I^- and Cs^+ in order to study the microenvironment and exposure of the Trp residues. Neutral quenchers could completely access the Trp residues in the presence of 8 M urea. But in the case of charged quenchers complete accessibility could not be achieved even after denaturation of the lectin with 8 M urea, indicating that charged amino acids might be present in the immediate neighborhood of some of the Trp residues. Binding of lactose altered the accessibility of Trp residues to the quenchers, suggesting that ligand induced conformational changes modify the penetration of the quencher into protein matrix. The Stern-Volmer plots obtained for the native TCSL and for TCSL in the presence of 0.1 M lactose with acrylamide, succinimide and iodide ion follow a linear dependence on the quencher concentration while cesium ion exhibits a biphasic pattern. Quenching profiles obtained with acrylamide and succinimide on the protein denatured with 8 M urea showed positive curvature, indicating that the quenching has both dynamic and static components. The fluorescence decay profiles for the native lectin yielded a bi-exponential fit. Presence of specific saccharide ligands, galactose and lactose resulted in only marginal differences in the quenching profiles or in the fluorescence decay lifetimes, which is consistent with the non-involvement of Trp residues in the sugar binding activity of the lectin, discussed above.

With the objective of exploring possibilities of employing lectins in clinical investigations employing metalloporphyrins, and also to understand the nature of probable hydrophobic ligands for lectins in biological systems we have investigated the interaction of different metalloporphyrins with TCSL using absorption spectroscopy. These studies showed that both positively and negatively charged porphyrins bind to this lectin with comparable affinities, suggesting that the charge on the porphyrin does not play any significant role in the interaction. Therefore, it appears that hydrophobic interactions largely mediate the binding interaction

between TCSL and the porphyrins. These observations are consistent with earlier studies reporting the interaction of porphyrins with SGSL and Jacalin [Komath *et al.*, 2000a, b]. The difference absorption spectra obtained for the titration of the anionic zinc(II) porphyrins, ZnTPPS and ZnTCPP with TCSL were found to be qualitatively similar to those obtained for the titration of the corresponding copper(II) analogues. Similarly, the difference spectra obtained with the cationic CuTMPyP and the corresponding zinc derivative, ZnTMPyP were found to be qualitatively very similar. Similarity in the difference spectra obtained when porphyrins were titrated with TCSL with those obtained for the same porphyrins in the presence of surfactants suggests that the forces involved in the interactions of these porphyrins with micelles and the protein are likely to be very similar. Comparison of the present result with earlier studies of Kadish and coworkers [Kadish *et al.*, 1989; Kadish & Maiya, 1991] suggested that anionic porphyrins interact with TCSL via Coulombic as well as hydrophobic interactions, whereas the interaction of tetra-cationic porphyrins such as CuTMPyP with TCSL is mediated predominantly via Coulombic interactions.

The association constants obtained for the TCSL-porphyrin interaction are comparable to those observed in general for mono- and disaccharide binding to lectins. The thermodynamic parameters ΔH^{o} and ΔS^{o}, associated with the binding of CuTPPS and CuTMPyP, indicate that the interaction of both these porphyrins with TCSL is primarily governed by entropic forces. Association constants obtained in the presence 0.1 M lactose suggests that sugar and the porphyrin bind to the lectin at distinctly different sites under the experimental conditions employed here. Stopped-flow studies on the binding of CuTMPyP to TCSL indicate that this binding reaction is a relatively slow process and the corresponding association and

dissociation rate constants are in the same range as that observed for lectin-saccharide interaction.

From the above discussion it can be concluded that physical properties of TCSL are very similar to SGSL. The carbohydrate recognition site and Trp environment of TCSL were also found to be similar. It is similar to SGSL in its affinity for predominantly hydrophobic ligands such as porphyrins. Previous studies indicated a common pattern in the carbohydrate-binding affinities of TCSL, SGSL, MCL and *T. kirilowii,* although some differences exist. In view of this, it is likely that considerable structural similarities exist among the Cucurbitaceae seed lectins. Further studies, especially those aimed at determining the 3-dimensional structures of these lectins as well as seed lectins from other cucurbit species is expected to lead to a better understanding of the present scenario.

APPENDIX

Fluorescence Quenching and Time-Resolved Fluorescence Studies on Peanut Agglutinin

Summary

Peanut agglutinin (PNA) has elicited much interest due to its specific recognition of the tumor-associated disaccharide, 2-acetamido-2-deoxy-(3-O-β-D-galactopyranosyl)-D-galactopyranoside (Galβ13GalNAc; T-antigen). In this study, fluorescence quenching and time resolved fluorescence measurements have been carried out to investigate the accessibility and environment of the tryptophan residues in this protein and to study the effect of saccharide binding. Acrylamide and iodide were used as quenchers. The emission λ_{max} of native PNA is at 323 nm and is unaffected by binding of the specific ligand, lactose, but shifts to 362 nm upon denaturation with 6 M guanidinium hydrochloride (Gdn.HCl), clearly indicating that the Trp residues which are buried in the hydrophobic interior of the lectin get exposed to the aqueous environment upon unfolding. At a quencher concentration of 0.5 M, the extent of quenching observed for the native lectin with acrylamide and iodide was 25% and 5%, respectively. In presence of lactose the quenching by I^- is unaffected while that by acrylamide is decreased to 22%, indicating that diffusion of this neutral quencher into the protein matrix is decreased by ligand binding. In time-resolved fluorescence experiments, the decay curves could be fitted to biexponential patterns with lifetimes of 0.72 ns and 4.37 ns for native PNA, 0.76 ns and 4. 46 ns in presence of 0.1 M lactose, and 1.73 ns and 4.18 ns upon denaturation.

INTRODUCTION

Peanut (*Arachis hypogaea*) agglutinin (PNA), is a homotetrameric protein of M_r 110,000 with no covalently linked carbohydrate [Lotan *et al.*, 1975]. PNA recognizes the Thomsen-Freidenreich antigenic determinant (Galβ13GalNAc; T-antigen), a chemically well-defined tumor-associated antigen with a proven link to malignancy in man [Lotan *et al.*, 1975; Pereira *et al.*, 1976; Swamy *et al.*, 1991]. Since this structure is expressed in O-linked glycans by poorly differentiated cells as well as tumor cells, but is not present or modified on normal cells [Springer, 1984], probes which can specifically recognize the T-antigen are highly useful in tumor-detection. In view of this, several laboratories have been actively involved in characterizing this lectin in detail with respect to the structural as well as carbohydrate-binding properties. The primary structure of PNA is known from chemical sequencing studies [Young *et al.*, 1991] and its three-dimensional structure with and without bound ligands, including the T-antigenic disaccharide (Galβ13GalNAc) has recently been determined [Salunke *et al.*, 1985; Banerjee *et al.*, 1994, 1996; Ravishankar *et al.*, 1997]. Saccharide binding properties of this lectin have been characterized by spectroscopic techniques such as ^{13}C-NMR, ultraviolet and fluorescence spectroscopy [Swamy *et al.*, 1991; Neurohr *et al.*, 1981, 1982]. These studies reveal that among monosaccharides peanut lectin exhibits a higher preference for the α-anomer of galactose and among the disaccharides it recognizes the Thomsen-Freidenreich antigen related disaccharide, Galβ13GalNAcαOMe with an association constant that is ~20 fold higher as compared to the next best disaccharide inhibitor, lactose [Swamy *et al.*, 1991]. The genes coding for wild-type PNA and a number of specific mutants have been cloned and expressed and one of the mutants exhibits exquisite specificity for the tumor-associated T-antigen [Sharma *et al.*, 1996].

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X-ray crystallographic studies on peanut agglutinin revealed that the quaternary structure of this lectin is an open structure, unlike the other homotetrameric legume lectins such as Con A and pea lectin, which have closed structures [Banerjee *et al.*, 1994]. Differential scanning calorimetric studies have revealed that thermal unfolding of native PNA goes through two distinct phases with folded monomer being the intermediate [Reddy *et al.*, 1999], which has a molten-globule like structure and also retains carbohydrate binding ability and specificity [Reddy *et al.*, 1999b]. In view of this, it would be highly interesting to investigate the kinetics of unfolding of this protein. From the primary structure of PNA, it can be seen that there are three tryptophan residues at positions 55, 153 and 223, whose fluorescence properties can serve as a useful handle to investigate the unfolding of the native lectin since it is well known that the fluorescence intensity and emission maximum of Trp is highly sensitive to its environment. A detailed characterization of the tryptophan environment in PNA is necessary before any such studies can be taken up to investigate its unfolding process. Therefore we have carried out fluorescence quenching and time resolved fluorescence studies on PNA using acrylamide, a neutral quencher; and iodide ion (I^-), an anionic quencher, both in the absence as well as in the presence of the specific ligand, lactose and also under denaturing conditions. The results obtained are discussed in the present chapter.

Iodide ion can quench only surface exposed Trp residues and its quenching is affected by the presence of charged residues in the neighborhood of the fluorophores. On the other hand, the polar but neutral acrylamide is capable of diffusing into the protein interior and quench the fluorescence of even partially buried Trp residues.

MATERIALS AND METHODS

Materials

Peanut seeds were obtained from local vendors. Acrylamide and lactose were from Sigma (St. Louis, MO). KI was a product of Qualigens (Mumbai, India). All other chemicals were obtained from local suppliers and were of analytical grade.

Purification of peanut agglutinin

PNA was purified by a combination of ammonium sulfate precipitation and affinity chromatography on lactose-coupled to Sepharose as reported earlier [Fish *et al.*, 1978]. The affinity-purified lectin gave a single band on polyacrylamide gel electrophoresis under native conditions as well as in the presence of sodium dodecyl sulfate with a subunit molecular weight of ~28 kDa, consistent with literature reports [Lotan *et al.*, 1975]. Concentration of PNA was estimated from the $E^{1\%,1cm}$ value of 9.6 at 280 nm [Fish *et al.*, 1978].

Absorption and fluorescence spectroscopy

Absorption spectra were recorded on a Jasco 7800 double beam spectrophotometer. Fluorescence spectra were recorded at room temperature (ca. 25°C) on a Jasco FP-777 spectrofluorimeter with excitation and emission slit widths of 10 nm. For quenching studies the protein samples (OD_{295nm}<0.1) in 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) were excited at 295 nm and emission spectra recorded between 310 and 400 nm. Small aliquots of the quencher were added from 5M stock solutions in PBS. Stock solutions of KI contained 0.2 mM sodium thiosulfate to prevent the formation of triiodide. Experiments were performed in triplicate and the average results are given.

sodium thiosulfate to prevent the formation of triiodide. Experiments were performed in triplicate and the average results are given.

Time-resolved fluorescence spectroscopy

Time-resolved fluorescence measurements and analysis of the decay profiles were carried out on an IBH-5000 single photon counting spectrofluorimeter essentially as described in [Komath & Swamy, 1999]. Samples of OD_{295nm}<0.1 in 1 × 1 × 4.5 cm quartz cells were used in all experiments. The excitation wavelength was 295 nm for both native and denatured samples of PNA, whereas emission was monitored at 325 nm for the native protein and at 360 nm for the denatured form.

RESULTS

Quenching of the intrinsic fluorescence of PNA

The fluorescence spectrum of PNA in the native state exhibits an emission maximum at 323 nm, which shifts to 362 nm upon denaturation with 6M Gdn.HCl (Figure A.1 A and A.1 B, respectively). The emission λ_{max} of the native lectin is not changed in the presence of saturating amounts of lactose, a disaccharide that is specifically recognized by this lectin. Emission spectra of the lectin in the presence of different concentrations of the neutral quencher, acrylamide, are also shown in Figure A.1. These spectra indicate that while the fluorescence intensity of the lectin is quenched substantially by acrylamide in the native state, the extent of quenching increases substantially in the presence of 6M Gdn.HCl, clearly showing that the fluorescent tryptophan side chains are exposed to the quencher in the solvent when the compactly folded protein is denatured. Similar data were obtained with the charged quencher, I^- though the extent of quenching observed with it was considerably smaller for the

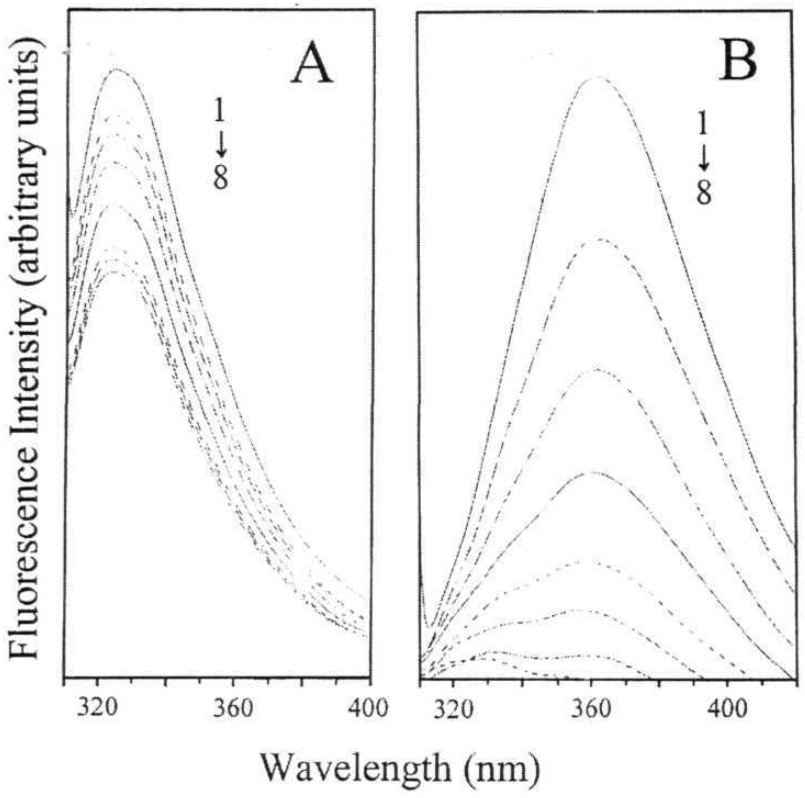

Figure A.1. Fluorescence spectra of peanut agglutinin in the absence and in the presence of increasing concentrations of acrylamide. A) native PNA; B) PNA in 6M Gdn.HCl.

native protein as compared to acrylamide. While acrylamide quenched about 25% of the fluorescence intensity at a quencher concentration of 0.5 M, the extent of quenching observed with iodide was only 5% (see Table A.I).

Presence of the specific ligand, lactose, at near saturating concentrations (0.1 M), affected the quenching by acrylamide only marginally whereas there was no detectable difference in the quenching by the charged quencher, iodide. Thus, while the maximal quenching observed with acrylamide was decreased to about 22% at a quencher concentration of 0.5 M, the extent of quenching observed with I^- at the same quencher concentration remained at 5% (Table A.I).

Table A. 1: Summary of parameters obtained by intrinsic quenching and time resolved fluorescence. K_{sv1} and K_{sv2} are the Stern-Volmer quenching constants, k_{q1} and k_{q2} are the bimolecular collisional constants, f_a is the fraction of accessible tryptophan residues and K_a is the Stern-Volmer quenching constant for the accessible fluorophores alone. The percent quenching indicated in the last column was observed at a resultant quencher concentration of 0.5 M.

Quencher/ Condition	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	$k_{q1} \times 10^{-9}$ ($M^{-1}s^{-1}$)*	$k_{q2} \times 10^{-9}$ ($M^{-1}s^{-1}$)*	f_a	K_a (M^{-1})	Percent Quenching
Acrylamide							
Native	1.21	0.77	0.3 (0.3)	0.2 (0.2)	0.28	5.95	25.0
0.1 M lactose	0.82	----	0.2 (0.2)	----	0.40	3.66	22.0
6 M Gdn.HCl	7.68	----	2.3 (2.3)	----	0.862	15.92	86.2
Iodide							
Native	0.1	----	0.02 (0.03)	----	0.062	6.15	5.0
0.1 M lactose	0.09	----	0.02 (0.02)	----	0.095	2.53	5.0
6 M Gdn.HCl	11.98	----	3.5 (3.5)	----	1.00	10.21	85.0

*The k_q values were estimated using the average lifetimes obtained by force-fitting the lifetime decay data to a single exponential. Numbers in parentheses correspond to the k_q values estimated by using the average lifetimes calculated using eq. (3).

Denaturation of PNA with 6M Gdn.HCl results in a marked increase in the extent of quenching of the tryptophan fluorescence of the lectin. The extent of quenching is increased greatly for both acrylamide and iodide. While the extent of quenching observed with 0.5 M acrylamide is 86.2%, that observed with the same concentration of iodide is 85% (see Table A.I). Thus denaturation leads to a marked

increase in the accessibility of the indole side chains of Trp residues to both the quenchers.

The quenching data for both acrylamide and iodide in the presence and absence of lactose as well as upon denaturation were analyzed by the Stern-Volmer equation (eq. A.1) and modified Stern-Volmer equation (eq. A.2) [Lehrer, 1971]:

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

$$F_o/ \Delta F = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \qquad (A.2)$$

where, F_o and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and in the presence of quencher, K_{SV} is the Stern-Volmer quenching constant of the lectin for the quencher, ΔF is the change in fluorescence intensity, [Q] is the resultant quencher concentration, f_a is the fraction of accessible fluorophores and K_a is the corresponding Stern-Volmer quenching constant (for the accessible fluorophores alone). The slopes of Stern-Volmer plots gave K_{SV}. The slopes of modified Stern-Volmer plots gave $(K_a f_a)^{-1}$ and ordinate gave f_a^{-1}.

Stern-Volmer plots for the quenching data obtained with the native lectin, in the presence of 0.1 M Lactose and with the denatured lectin are shown in Figure A.2. An expanded view corresponding to the native lectin without and with sugar is shown in Figure A.3. The modified Stern-Volmer plots are shown in Figure A.4. The Stern-Volmer quenching constants, K_{SV}, obtained from the slopes of the linear fits to the Stern-Volmer plots as well as the bimolecular quenching constants, K_q ($K_q = K_{SV}/\tau_o$ where τ_o is the average lifetime obtained from lifetime decay measurements) for acrylamide and iodide are listed in Table A.1. The fraction of accessible Trp residues and the corresponding Stern-Volmer quenching constants (K_a) were calculated from the modified Stern-Volmer plots. These values are also presented in Table A.1.

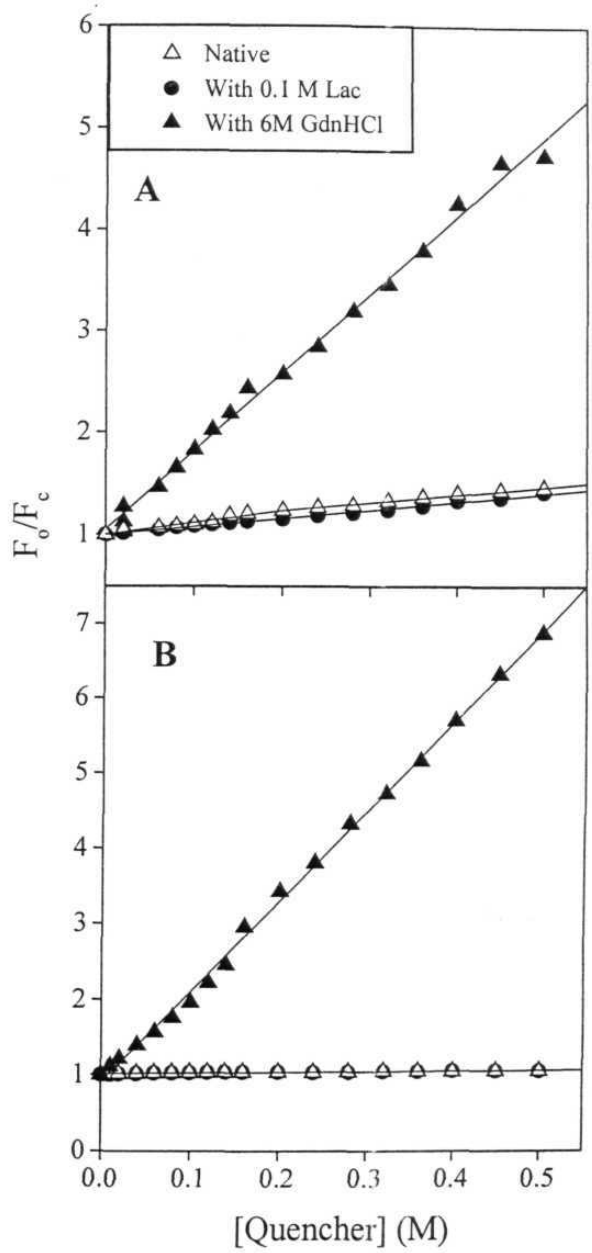

Figure A.2. Stern-Volmer analysis of the quenching of the intrinsic fluorescence of PNA. (A) with acrylamide and (B) with iodide ion (I^-). (Δ) native lectin; (●) in the presence of 0.1 M lactose; (▲) in the presence of 6.0 M Gdn.HCl.

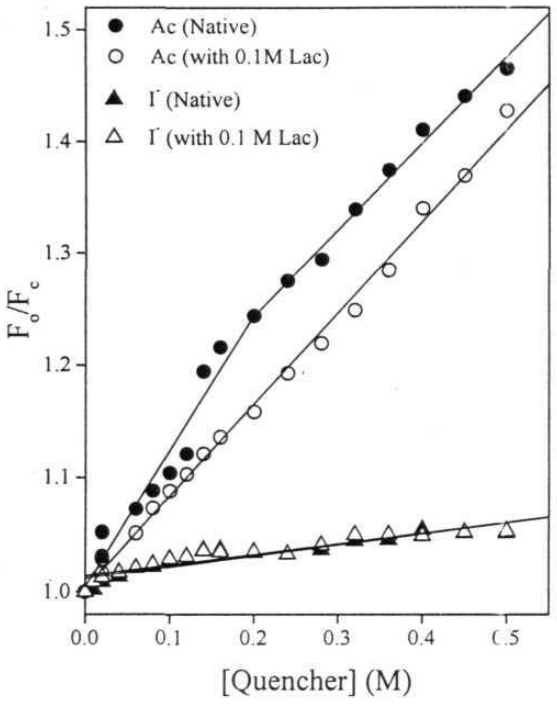

Figure A.3. Expanded view of the Stern-Volmer plots for the intrinsic fluorescence quenching of PNA with acrylamide (●, ○) and iodide ion (▲, Δ). Closed symbols are for quenching in the absence of sugar and open symbols represent quenching in the presence of 0.1 M lactose.

From Figure A.3 it can be seen that the Stern-Volmer plot with acrylamide is biphasic with native PNA, which became monophasic in the presence of 0.1 M lactose, indicating that ligand binding results in an altered exposure of the indole side chains of Trp residues. However, with iodide the plots were monophasic within the range of experimental error both in the presence and in the absence of lactose and the extent of quenching was not altered by sugar binding.

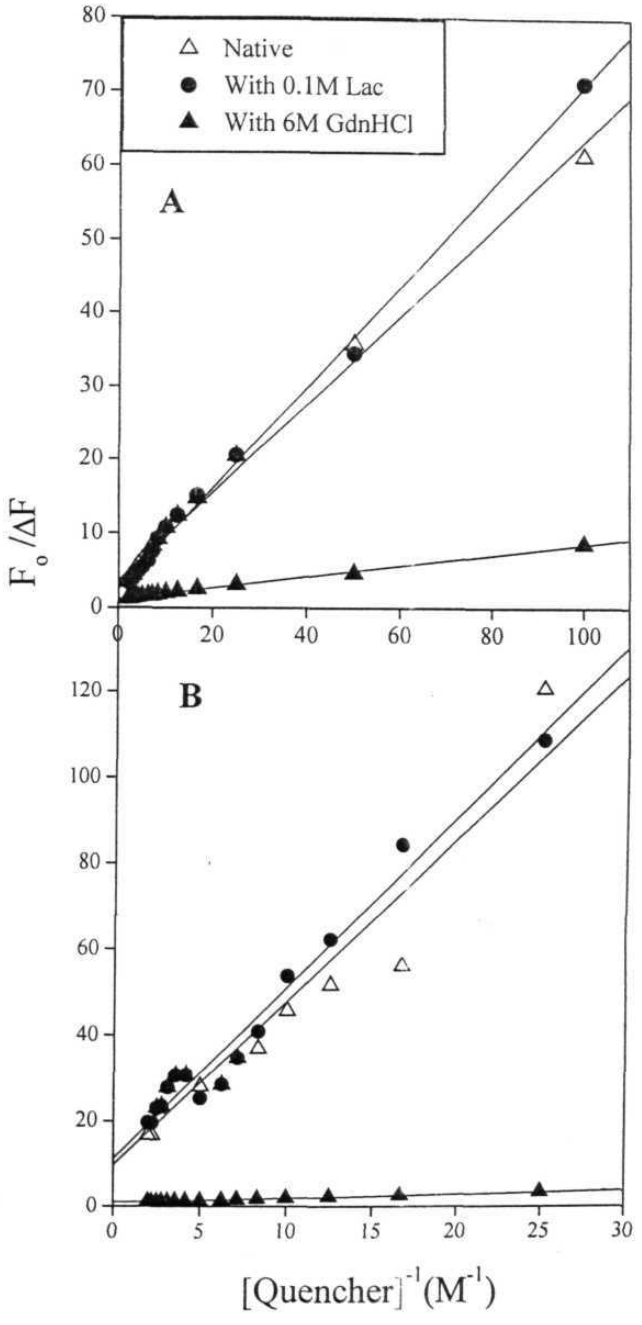

Figure A.4. Modified Stern-Volmer plots for the quenching of the intrinsic fluorescence of PNA by acrylamide (A) and iodide ion (B). (Δ) native lectin; (●) in the presence of 0.1 M lactose; (▲) in the presence of 6.0 M Gdn.HCl.

Time-resolved fluorescence measurements

Fluorescence decay curves for PNA in the absence and in the presence of 6 M Gdn.HCl are shown in Fig. A.5. The decay curves were analyzed using a multiexponential iterative program supplied by IBH [Komath & Swamy, 1999]. Under both the conditions, the decay curves could be best fitted to a bi-exponential function ($\chi^2 < 1.1$). Mono-exponential fits gave considerably larger errors ($\chi^2 > 2.0$) whereas fits with three-exponentials did not significantly alter the residuals as compared to two-exponential fits. For the native PNA, the lifetimes obtained from such analysis were 0.72 ns and 4.37 ns whereas upon denaturation with 6 M Gdn.HCl the lifetimes obtained were 1.73 ns and 4.18 ns (Table A.I). In the presence of 0.1 M lactose also, PNA gave biexponential decay profiles with lifetimes of. 0.76 ns and 4. 46 ns (not shown).

For native PNA the component with a shorter decay time of 0.72 ns contributes only 16.8% and the second component with longer lifetime of 4.37 ns contributes 83.2% towards the total fluorescence intensity. Lactose binding does not significantly alter these contributions. However, denaturation not only alters the lifetimes of the two components to 1.73 ns and 4.18 ns, but also changes the contributions of these two components to the overall fluorescence intensity to 31.2% and 68.8%. These values are also listed in Table A-1.

The average lifetimes were obtained by force-fitting the decay curves to a single exponential function as well as by calculating from the lifetimes obtained for the biexponential fits using the following equation [Inokuti & Hirayama, 1965]:

$$\tau_o = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i \text{ (where i = 1,2)} \qquad \text{(A-3)}$$

where, τ_i correspond to individual lifetimes and α_i to their respective weight factors.

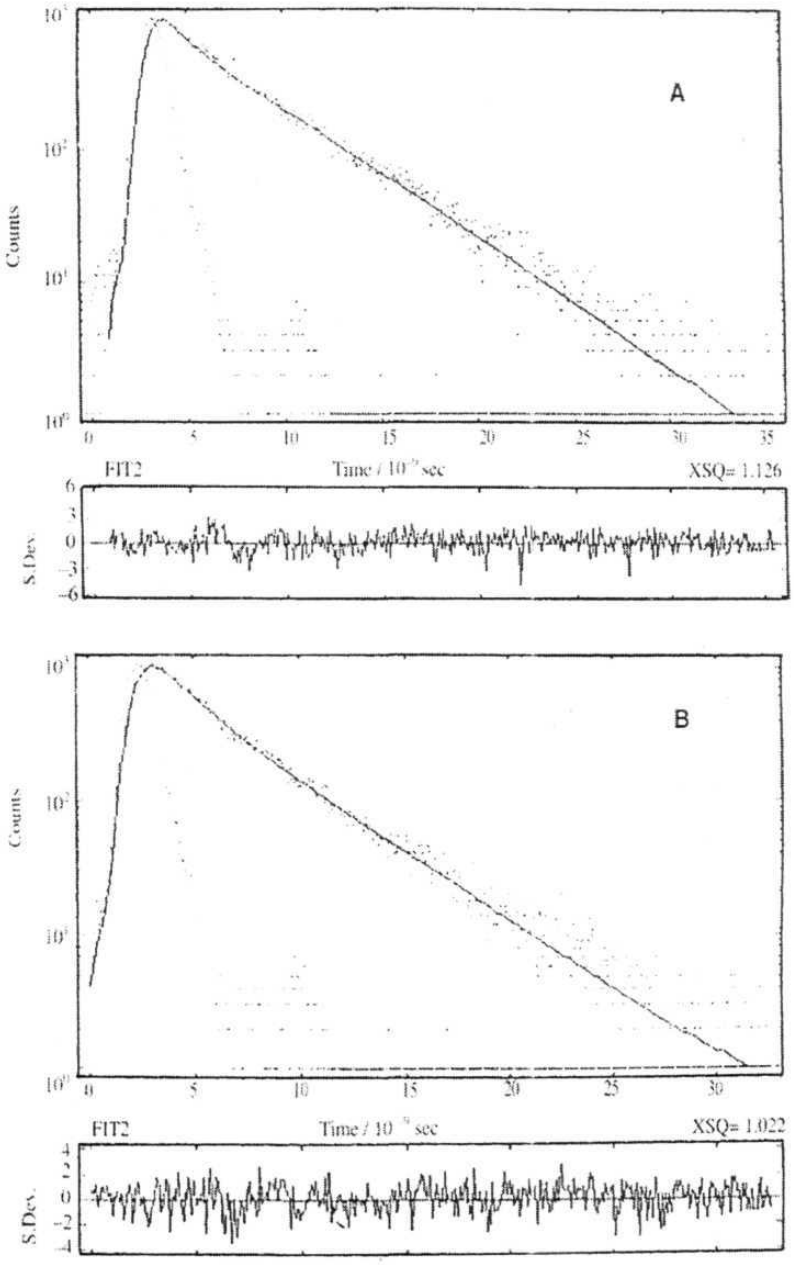

Figure A.5. Fluorescence decay profiles of native peanut agglutinin (A) and in the presence of 6 M Gdn.HCl (B). The solid lines correspond to the nonlinear least square fits of the experimental data to a biexponential function. The lower panels in A and B correspond to residual plots.

Force-fitting the decay profiles to single-exponential fits yielded the average lifetimes as 4.001 ns, 3.98 ns and 3.422 ns, respectively, for native PNA, in the presence of 0.1 M lactose, and in the presence of 6 M Gdn.HCl. Calculation according to equation (A-3) yielded the corresponding average lifetimes as 3.76 ns, 3.84 ns and 3.41 ns, respectively.

DISCUSSION

The fluorescence properties of Trp residues are highly sensitive to the environment in which they reside and therefore can serve as a tool to gain insight into the conformational changes occurring in proteins [Kronman & Holmes, 1971; Eftink & Ghiron, 1981]. In a non-polar environment the indole side chains fluoresce with the emission λ_{max} in the neighborhood of 320 nm, which exhibits a considerable red shift when the polarity of the medium is increased.

The emission λ_{max} of native PNA is at 323 nm, suggesting that all the three Trp residues (W-55, W-153 and W-223) are in a hydrophobic (nonpolar) environment. As shown in Fig. A-1, the fluorescence emission maximum of PNA shifts to 362 nm upon denaturation with 6 M Gdn.HCl, clearly indicating that unfolding of the protein results in a complete exposure of the Trp residues to the aqueous solvent. The dramatic increase in the extent of quenching with both acrylamide and iodide ion in the presence of the denaturant is also consistent with this observation.

From the crystal structure of PNA it is seen that Trp 223 is directly involved in the β-sheet that forms the backbone of the folded PNA oligomer and is thus buried in the hydrophobic core of the protein oligomer, whereas Trp-55 and Trp-183 are quite close to the surface of the protein [Banerjee *et al.*, 1994, 1996; Ravishankar *et*

al., 1997]. However, the quenching experiments described here clearly demonstrate that even the latter two residues are only partially accessible to the neutral quencher, acrylamide. On the other hand, the anionic quencher I^- is able to quench only 5% of the total fluorescence intensity of the protein, clearly indicating that the Trp residues are poorly accessed by this surface-probing quencher.

From the data shown in Figure A.3 and Table A.1 it is clear that binding of lactose alters the access of the Trp residues to acrylamide, indicating that ligand-induced conformational changes in the protein modify the penetration of this neutral quencher into the protein matrix. While in the absence of sugar the Stern-Volmer plot for this neutral quencher is biphasic with a larger K_{SV1} value at lower concentrations and a smaller K_{SV2} value at higher concentrations, in the presence of lactose the quenching pattern is uniform throughout with a single Stern-Volmer quenching constant (Table A.1). This pattern is also reflected in the values of the bimolecular quenching constants, k_q, obtained with acrylamide in the absence and presence of lactose. On the other hand, sugar binding has almost no effect on the quenching by iodide ion. The bimolecular quenching constants, k_q for this anionic quencher remain unaltered upon lactose binding (Table A.1).

Binding of lactose to PNA reduces the quenching by acrylamide by a small extent and also makes the Stern-Volmer plot monophasic. Since Trp residues are not directly present in the sugar-binding site of PNA, it appears that conformational changes resulting from ligand binding lead to a change in the accessibility of some of the Trp residues to this quencher. As quenching with I^- was unaffected by the presence of lactose, the most likely explanation for the decrease in the quenching of the lactose-bound PNA by acrylamide would be that the diffusion of this neutral quencher into the protein matrix is reduced in the presence of lactose. Since the difference in the quenching by acrylamide in the absence and presence of lactose is

rather small, it is very likely that the saccharide-induced conformational changes do not affect the accessibility of the quencher to all the three Trp residues. However, accessibility of at least one Trp residue must be reduced. Since Trp 223 is involved in the flat β-sheet at the center of the folded oligomer, it is unlikely to be affected by lactose binding to the quencher. Trp 55 is far removed from the carbohydrate-binding site and thus it is very unlikely that it would be affected by ligand binding. Trp 153 on the other hand, is directly hydrogen bonded to Asp-136, which is on the periphery of the loop comprising of residues 125-135, several of which (Tyr-125, Asn-127 and Glu-129) take part in the carbohydrate binding either directly or through water-mediated hydrogen bonds. It is therefore very likely that the accessibility of Trp-153 is altered upon sugar binding.

The nearly unaltered fluorescence decay profile obtained in the presence of lactose, as compared to that of the native PNA, indicates that the conformational changes resulting from lactose binding in solution do not result in any major alterations in the environment of the Trp residues. These observations are consistent with the steady-state fluorescence spectral data as well as the quenching data with acrylamide and iodide.

In summary, the present study shows that the Trp residues of PNA are mostly buried in the hydrophobic core of the protein. Ligand binding does not affect the quenching by I^-, which probes only the surface-exposed Trp residues, but reduces quenching by the neutral quencher, acrylamide, which can diffuse into the protein matrix, suggesting that penetration of the protein by acrylamide is slightly reduced upon ligand binding. These results provide a detailed understanding of the tryptophan environment in PNA, which should facilitate further studies aimed at delineating the kinetics and mechanism of unfolding of this protein.

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List of Publications

1. Komath, S. S., **Kenoth, R.**, Giribabu, L., Maiya B. G. & Swamy M. J. (2000) Fluorescence and Absorption Spectroscopic Studies on the Ineraction of Porphyrins with Snake Gourd (*Trichosanthes anguina*) Seed lectin. *J. Photochem. Photobiol. B: Biol.* **55**: 49-55.

2. Padma, P., **Kenoth R.**, & Swamy, M. J. (2000) Fluorescence Quenching and Time-Resolved Fluorescence Studies on the Anti-T Lectin from Peanut (*Arachis hypogaea*). *J. Biochem. Mol. Biol. Biophys.* **4**: 243-251.

3. **Kenoth, R.**, Padma, P., Sarada, S. & Swamy M. J. (2000) Requirement of Histidine Residues for the Sugar-Binding Activity of *Trichosanthes cucumerina* Seed Lectin. Evidence from Chemical Modification Studies. *J. Biochem. Mol. Biol. Biophys.* **4**: 423-431.

4. Komath, S. S., **Kenoth, R.** & Swamy, M. J. (2001) Thermodyanamic Analysis of Saccharide Binding to Snake Gourd (*Trichosanthes anguina*) seed lectin. Fluorescence and absorption spectroscopic studies. *Eur. J. Biochem.* **268**: 111-119.

5. Manoj, N., Jeyaprakash, A. A., Pratap, J. V., Komath, S. S., **Kenoth, R.**, Swamy, M. J. & M. Vijayan (2001) Crystallization and Preliminary X-Ray Studies of Snake Gourd Lectin: Homology with Type II Ribosome-Inactivating Proteins. *Acta Cryst.* **D57**: 912-914.

6. Goel, M., Jain, D., Kaur, K. J., **Kenoth, R.,** Maiya, B. G., Swamy, M. J. & Salunke, D. M. (2001) Functional Equality in the Absence of Structural

Similarity: An Added Dimension to Molecular Mimicry. *J. Biol. Chem.* **276**: 39277-39281.

7. **Kenoth, R.**, Reddy, D. R., Maiya, B. G. & Swamy, M. J. (2001) Thermodynamic and Kinetic Analysis of Porphyrin Binding to *Trichosanthes cucumerina* seed lectin. *Eur. J Biochem.* **268**: 5541-5549.

8. Ramakrishnan, M., **Kenoth, R.**, Kamlekar, R. K., Chandra, M. S., Radhakrishnan, T. P., & Swamy, M. J. (2002) *N*-Myristoylethanolamine - Cholesterol (1:1) Complex: First Evidence from Differential Scanning Calorimetry, Fast-Atom-Bombardment Mass Spectrometry and Computational Modeling. *FEBS Lett.* (In press)

9. **Kenoth, R.**, & Swamy, M. J. (2002) Fluorescence Quenching and Time-Resolved Fluorescence Studies on *Trichosanthes cucumerina* Seed Lectin (Manuscript Submitted for Publication).

10. **Kenoth**, R., Komath, S.S. & Swamy, M. J. (2002) Physicochemical and Saccharide Binding Studies on the Galactose-Specific Seed Lectin from *Trichosanthes cucumerina* (Manuscript Submitted for Publication).