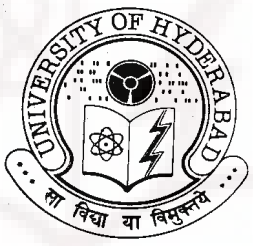
Purification and Characterization of Chitooligosaccharide-Specific Lectins from the Phloem Exudate of *Cucurbita maxima* and *Trichosanthes anguina*

A thesis
Submitted for the degree of
DOCTOR OF PHILOSOPHY

By

Akkaladevi Narahari



School of Chemistry
University of Hyderabad
Hyderabad – 500 046
INDIA

February 2009

CONTENTS

Statement		i
Certificate		ii
Acknowledgments		iii
Abbreviations		v
Chapter 1:	Introduction	1
Chapter 2:	Rapid affinity-purification and physico-chemical characterization of pumpkin (*Cucurbita maxima*) phloem exudate lectin.	27
Chapter 3:	Energetics of chitooligosaccharide binding to pumpkin (*Cucurbita maxima*) phloem exudate lectin.	49
Chapter 4:	Steady-state and time-resolved fluorescence studies on the chitooligosaccharide-specific lectin from the phloem exudate of pumpkin (*Cucurbita maxima*).	65
Chapter 5:	Purification and physico-chemical characterization of a new chitooligosaccharide-specific lectin from snake gourd (*Trichosnathes anguina*) phloem exudate.	89
Chapter 6:	Thermodynamics of the interaction of chitooligosaccharides with snake gourd (*Trichosanthes anguina*) phloem exudate lectin. Isothermal titration calorimetric and fluorescence spectroscopic studies.	111
Chapter 7:	General discussion and conclusions	129
References		139
List of Publication		159

School of Chemistry
University of Hyderabad
Hyderabad – 500 046

STATEMENT

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

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever the work described is based on the finding of other investigators. Any omission which might have occurred by oversight or error is regretted.

Hyderabad

February 2009 **A. Narahari**

School of Chemistry
University of Hyderabad
Hyderabad – 500 046

CERTIFICATE

Certified that the work embodied in this thesis entitled **"Purification and Characterization of Chitooligosaccharide-Specifc Lectins from the Phloem Exudate of *Cucurbita maxima* and *Trichosanthes anguina*"** has been carried out by **Mr. A. Narahari** under my supervision and the same has not been submitted elsewhere for any degree.

Hyderabad

February 2009

Prof. Musti J. Swamy
(Thesis Supervisor)

Dean
School of Chemistry

ACKNOWLEDGEMENT

I especially want to thank my supervisor Prof. Musti J. Swamy for his guidance, constant encouragement, support, freedom and valuable suggestions given me throughout the Ph.D program. It is a good opportunity for me to work with him during the course of my work and it was a remarkably educative experience for me. I thank him again to accept me in his group as a Ph.D student in 2004, when I was in a critical position as Prof. Bhaskar G. Maiya, with whom I was working was passed away.

My sincere thanks to Prof. D. Basavaiah, Dean, School of Chemistry as well as past deans of the School for providing the facilities to carry out research work. I thank all the faculty members of the School for their help at various times during the Ph.D. program as well as during my M.Sc course. I also thank all of my teachers, who have guided me to come to this stage.

I thank all the non-teaching staff of the School of Chemistry and I am also grateful to the Central Instrumental Laboratory, University of Hyderabad for providing an excellent work environment and my special thanks to Mr. Murthy, Mr. Suresh and other staff for their cheerful assistance.

I am grateful to Prof. Samanta and his student Ravi, for allowing me to use their time-resolved fluorescence spectrophotometer. I also thank Dr. M. V. Jagannadham (Centre for Cellular and Molecular Biology, Hyderabad, India) and Prof. Dipankar Chatterji (Molecular Biophysics Unit, I. I. Sc. Bangalore) for the MALDI-TOF mass spectral data.

I would like to express my thanks to all of my colleagues: Kavitha, Pradip, Rajeshwar, Kishore, Thirupathi Reddy and Pavan Kumar for making the lab such a peaceful environment. I would like to thank my seniors Dr. Roopa, Dr. Nabil, Dr.Anbu, Dr. Ravikanth and Dr. Rajani for their help and critical suggestions. My special thanks to Dr. Kavitha who helped me when I was switched from porphyrin chemistry to protein chemistry as well as in various other things.

I thank late Prof. Bhaskar G. Maiya, where I started my early stage research and had experience in the synthesis of porphyrins. My special thanks to Dr. Premaladha, who was supportive and helped me in various things. I also thank Dr. Prashanth, Dr. Sastry, Dr. R. Reddy, Dr. Ashok, Dr. Mariappan, Dr. Tamal and Prathap.

I would like to thank my M.Sc friends Bhuvan, Kavitha, Kiran, Ravi, Veeru, Nagi reddy, Murthy, Yadaiah, Anwar, D.K., K.K., Jayaprakash, L.K., Prasad, Narasimha, Mallu, Girish etc. I thank Chandrashekar, Devender, Ramesh Reddy and rest of COSIST friends. I also thank to Rajesh, Anil and Shasi for their help and useful discussion.

It gives me immense pleasure to thank my childhood friends Sudhakar, A.V., Kistaiah, Venkanna, Indhira, Premanand, Chandram, Ravi, Ramesh, Suresh and Naresh for their cooperation and encouragement. My special thanks to Sudhakar for his incredible friendship, affection and caring.

I thank my parents Narsaiah and Samrajyamma for giving me life in the first place, their immeasurable support, affection and caring. I thank my brothers Srihari and Nagendra for their love and support. I thank my uncles, peddhamma and my cousins for their help and encouragement in various things.

I would like to acknowledge my beloved wife Srilatha who stepped into my life during my Ph.D. She is very encouraging, supportive and affectionate friend. She is devotee of Lord Saibaba and I was impressed as lot with her prayers to Baba for me. I was blessed with my daughter Honey who filled life with cheerfulness with her cute smile. I want to thank my in-laws Shankaraiah, Suryakala and their family members for caring. My special thanks to Shailaja, who took care of my daughter when I was busy with my thesis.

I thank CSIR for the fellowship. Finally, I would like to thank everybody who contributed to the successful realization of this thesis. My apologies if missed acknowledging anyone.

ABBREVATIONS

AcAMP	Antimicrobial peptides from *Amaranthus caudatus*
AIDS	Acquired immune deficiency syndrome
CBD	Carbohydrate binding domain
CD	Circular dichroism
CIA	*Coccinia indica* agglutinin
CMA	*Chelidonium majus* agglutinin
Con A	Concanavalin A
DPM	Double Prediction Method
DSC	Differential scanning calorimetry
ELISA	Enzyme linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
FACS	Fluorescence-activated cell sorter
Gdn.HCl	Guanidine hydrochloride
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* (snowdrop) agglutinin
ITC	Isothermal titration calorimetry
LAA	*Luffa accutangula* agglutinin
MALDI-TOF	Matrix assisted laser desorption /ionization- time of flight
MCL	*Momordica charantia* lectin
MLL	*Moluccella laevis* lectin
MPRs	Mannose-6-phosphate receptors
OSA	*Oryza sativa* agglutinin
PAGE	Polyacrylamide gel electrophoresis
PA-IIL	*Pseudomonas aeruginosa* lectin-II
PBS	Phosphate buffer saline
PHA	Phytohemagglutinin

PNA	Pea nut agglutinin
PP1	Phloem protein 1
PP2	Phloem protein 2
PPL	Pumpkin phloem lectin
PWM	Pokeweed mitogen
RCA	*Ricinus communis* agglutinin
REES	Red-edge excitation shift
RIP	Ribosome inactivating protein
SA	Sinapinic acid
SBA	Soybean agglutinin
SDS	Sodium dodecyl sulfate
SGPL	Snake gourd phloem lectin
SGSL	Snake gourd seed lectin
SOPMA	Self-Optimized Prediction Method from Alignments
STA	*Solanum tuberosum* agglutinin
TCSL	*Trichosanthes cordata* seed lectin
TDSL	*Trichosnathes dioica* seed lectin
TFA	Trifluoroacetic acid
T_m	Transition temperature
Trp	Tryptophan
Tyr	Tyrosine
UDA	*Urtica dioica* agglutinin
UEA-II	*Ulex europaeus* agglutinin II
ViscalbCBP	*Viscum album* chitin-binding protein
WGA	Wheat germ agglutinin
βME	β-mercaptoethanol

Chapter 1

Introduction

Lectins

Definition

Lectins were first designated as phytohemagglutinins, as these agglutinating proteins were almost exclusively found in plants. Based on the ability of some of these plant agglutinins to determine the blood group of a given erythrocyte sample, Boyd and Shapleigh in 1954 proposed the name "lectin" from the Latin word '*legere*' which means to pick out or choose [Boyd & Shapleigh, 1954]. Goldstein et al. [1980] defined lectins as 'sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates'. This definition acknowledged polyvalency of lectins. Kocourek and Horejsi modified this definition proposing that 'lectins are carbohydrate binding proteins that do not modify the carbohydrates which they bind' [Kocourek & Horejsi, 1981]. The definition of a lectin was further modified by Barondes, who proposed that in addition to a carbohydrate binding site, a lectin may contain one or more non-carbohydrate binding sites [Barondes, 1988].

Historical Background

Lectins were first identified towards the end of 19th century by William Hermann Stillmark in his doctoral dissertation presented to the University of Dorpat in 1888. Couple of years later, H. Hellin reported on a second agglutinating protein called abrin isolated from jequirity beans (*Abrus precatorius*). These two lectins were used by Ehrlich as model antigens to demonstrate specific immunity against these proteins, and demonstrated that the antibody raised against them was able to quantitatively inhibit hemagglutination [as mentioned in Sharon & Lis, 2003]. Later James B. Sumner crystallized the lectin from jack bean (*Canavalia ensiformis*) and named it concanavalin A in 1919. He found that this lectin can

recognize and bind carbohydrates [Sumner & Howell, 1936] and identified the requirement of divalent metal ions in the specificity of concanavalin A. Two decades later Peter C. Nowell reported on the mitogenic property of lectins wherein the lectins stimulate lymphocytes to undergo mitosis, defying the notion prevalent at that time that lymphocytes are dead end cells that could neither divide nor differentiate further [Nowell, 1960]. The same decade also saw the introduction of affinity chromatography for the purification of concanavalin A from the crude extracts of jack bean meal by adsorption on a Sephadex column and elution with glucose [Agrawal & Goldstein, 1965; Goldstein, 1990]. This development turned out to be highly useful in lectin research as affinity chromatography became the method of choice for the purification of lectins from a variety of sources.

Classification

Plant lectins can be defined as plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide [Peumans & Van Damme, 1995]. This heterogeneous group of carbohydrate-binding proteins comprises at least seven distinct families of structurally and evolutionarily related proteins [Van Damme et al., 1998a, b]. Four of these families, namely, the legume lectins, the type-II ribosome-inactivating proteins (RIPs), the chitin-binding lectins containing hevein domains and the monocot mannose-binding lectins are considered to be ‘large’ families. The amaranthins, the Cucurbitaceae phloem lectins and the jacalin-related lectins comprise at present only a small number of individual lectins and accordingly are considered as ‘small’ families. These seven families can be grouped under four types depending on the number and activity of subunits as merolectins, which contain a single carbohydrate binding domain (CBD), hololectins that have at least two identical carbohydrate binding domains with the same specificity, and chimerolectins, which are fusion proteins, which

consist of a lectin domain linked to some other domain independent in action from the CBD of the protein (e.g., Type-II RIPs) and super lectins, which possess more than one CBD but with different specificities, e.g., the tulip bulb lectin [Van Damme et al., 1998a].

Carbohydrate binding

The ability of lectins to bind carbohydrates specifically and reversibly has made them important tools in biochemical and immunochemical studies. It is important to characterize the carbohydrate binding specificity of a lectin and evaluate the thermodynamic parameters that are associated with carbohydrate binding in order to understand its biological functions. Hemagglutination-inhibition and precipitation-inhibition techniques have been used to determine the relative affinity and specificity of lectins for carbohydrates, which gives approximate affinities of the tested sugars to the lectin [Goldstein et al., 1986; Sharon & Lis, 2003]. However, in order to accurately determine the binding affinity and the thermodynamic parameters associated with lectin-sugar interaction it is necessary to use biophysical approaches employing spectroscopic and other physical methods. The most widely used techniques are spectrophotometry, spectrofluorimetry, microcalorimetry [Dam & Brewer, 2002], nuclear magnetic resonance [Poveda & Jimenez-Barbero, 1998], surface plasmon resonance [Haseley et al., 1999] and frontal affinity chromatography [Hirabayashi et al., 2002].

Lectins can be divided into two classes, namely that those bind monosaccharides and oligosaccharides. Lectins which are specific to monosaccharides can be divided into five groups; these are, those that recognize mannose, galactose/*N*-acetylgalactosamine, *N*-acetylglucosamine, fucose and sialic acid. Binding of monosaccharides to lectins are typically characterized by association constants in the range of 10^3 to 10^4 M^{-1}; in exceptional cases, however

stronger association constants have been found. For example, the association constant for PA-IIL-fucose interaction is ~10^7 M^{-1} and that of MLL to *N*-acetylgalactosamine is ~10^5 M^{-1}. Oligosaccharides in most cases have higher affinity for lectins than monosaccharides [Goldstein & Poretz, 1986].

Certain lectins from the same specificity group possess anomeric specificity, i.e. they can distinguish between α or β- anomers of a sugar, with selective affinity for one of the anomers. For example, the lectins from *Griffinia simplicifolia* (B4) [Hayes & Goldstein, 1974] and lima bean lectin [Roberts & Goldstein, 1984] specifically bind to the α-anomer of galactose with a significantly higher avidity than the corresponding β-anomer. Galactose-specific lectins from the seeds of Cucurbitaceae species preferentially bind to the β-anomer of galactose than the α-anomer [Komath et al., 1998; Sultan & Swamy, 2005a]. An important family of galactose specific lectins is the galectins; all of them bind to β-galactosidies such as lactose and *N*-acetyllactosamine, but do not react with *N*-acetylgalactosamine [Leffler & Barondes, 1986; Sparrow et al., 1987]. WGA binds *N*-acetylglucosamine and its β1→4 linked oligomers with affinities that increase with increase in the size of the saccharide [Bains et al., 1992]. Lectins from Solanaceae family (Potato, tomoto and D*atura stramonium*) and Cucurbitaceae phloem exudate lectins specifically bind to chitooligosaccharides [Allen & Neuberger, 1973; Kilpatrick & Yeoman, 1978; Nachbar et al., 1980; Read & Northcote, 1983; Anantharam et al., 1986; Sanadi & Surolia, 1994]. A novel lectin isolated from dendritic cells, called as dectin-1 binds to glucose oligosaccharides [Ariizumi et al., 2000; Willment et al., 2001]. Some lectins are unusual in that they interact with monosaccharides belonging to different specificity groups at the same combining site. For example, WGA is specific for *N*-acetylglucosamine and *N*-acetylneuraminic acid and UEA-II recognizes both galactose and *N*-

acetylglucosamine [Loris et al., 2000]. Although the lectins from Dutch iris (*Iris hollandica*) are Gal/GalNAc specific, they are able to recognize mannose also [Hao et al., 2001]. Similarly, jacalin which is specific to galactose also binds to mannose [Barre et al., 2000].

The overall process of binding of a ligand to lectin involves interaction between solvated combining site and solvated polyhydroxylated carbohydrates. The difference in hydrogen bond energies between protein-water, carbohydrate-water and protein-carbohydrate complexes plays a main role for binding energy. Additional contributions may also originate from changes in protein conformation, van der Waalls' interactions, hydrophobic effects and entropic effects [Toone, 1994; Lemieux, 1996; Burkhalter et al., 2000; Dam et al., 2000]. Water molecules at the combining site of lectins are in a special perturbed state of higher energy than bulk water and release of this water to bulk provides driving forces for lectin-sugar complexation [Lemieux, 1996]. The importance of water in lectin-sugar interactions has been demonstrated by isothermal titration calorimetry in the presence of increasing osmotic stress [Swaminathan et al., 1998].

Isothermal titration calorimetric data reveal that protein-carbohydrate interactions are enthalpy driven, with the enthalpy of binding being more negative or equal to free energy change. Enhancement of the affinity of lectins for multivalent ligands may however be due to relatively positive $T\Delta S$ contribution with respect to monovalent sugar binding [Dam et al., 2000]. The binding data also show linear enthalpy-entropy compensation for several carbohydrate lectin interactions [Sharma et al., 1998; Rani et al., 1999; Sultan & Swamy, 2005b; see also Chapters 3, 6]. The loss of entropy resulting from changes in rotational degrees of freedom such as torsion angles around the glycosidic bonds, which are

frozen upon binding, is compensated by change in enthalpy due to removal of ligated water.

Non-carbohydrate ligand binding

Besides the carbohydrate binding site(s) some of the lectins possess one or more binding sites for non-carbohydrate ligands, which are independent from the carbohydrate ligand binding sites. A number of plant lectins were found to strongly bind, *in vitro*, some hydrophobic biomolecules like adenine and adenine-based plant hormones [Roberts and Goldstein, 1982, 1983a, b; Maliarik and Goldstein, 1988; Gegg et al., 1992; Srinivas et al., 2000]. Equilibrium studies in solution showed that legume lectins bind adenine and related molecules in a stoichiometry of one (or two) per lectin molecule. Interestingly, the crystal structure of *Dolichos biflorus* seed lectin complex with adenine shows two identical hydrophobic cavities within the lectin matrix, with each cavity accommodating two adenine molecules [Hamelryck et al., 1999]. MCL was also found to bind adenine with moderate affinity, suggesting that it might be playing a role in adenine-based plant hormones transport within its natural source [Kavitha et al., 2009]. Besides adenine and adenine-related phytohormones, binding of other biomolecules such as kinetin, zeatin, isopentyl adenine as well as abscisic and gibberellic acids to WGA has been reported [Bogoeva et al., 2004].

Porphyrins are a class of predominantly hydrophobic molecules, which have been shown to bind to several plant lectins such as Con A, pea lectin, jacalin, SGSL, TCSL and MCL with affinities comparable to that observed for the specific monosaccharide ligand [reviewed in Komath et al., 2006]. This binding takes place in the presence of saturating concentrations of a specific sugar as well as in the absence of sugar, indicating that the interaction with porphyrins occurs at binding sites that are distinct from carbohydrate binding sites [Bhanu et al., 1997; Komath

et al., 2000a, b; Kenoth et al., 2001; Sultan et al., 2004b]. However, in the crystal of concanavalin A and jacalin porphyrins bind at the carbohydrate binding site, whereas peanut agglutinin was found to bind the porphyrins at sites well removed from the carbohydrate binding site [Goel et al., 2001, 2004, 2005; reviewed in Komath et al., 2006].

Functions of lectins

Plant lectins

The functions of lectins identified so far are based on their specific and reversible binding of carbohydrate structures of glycoconjugates that are present on cell surfaces. Although plant lectins have been known for a long time, *in vivo* functions of the lectins in the plants remain largely unclear. They are thought to play a role in plant defense, as storage proteins and for the establishment of symbiosis between nitrogen-fixing bacteria and rhizobia in leguminous plants. In *Pisum sativum* (pea), apart from seeds, lectin is also present in small amounts in the tip of developing root hairs. The presence of lectin is responsible for the specific association of bacteria and the root hair surface. This theory is known as the "lectin recognition hypothesis" [Kijne, 1996; Hirsch, 1999].

Plant lectins are toxic for insects and higher animals, and they act as inhibitors for fungal growth. The basic assumption is that these proteins may function in plant defence against predatory animals and different kinds of pathogens [Peumans & Van Damme, 1995; Pusztai & Bardocz, 1996]. Ricin and ricin-related toxins were reported to protect plants against herbivorous animal and phytophagous invertebrates [Peumans et al., 2001]. Similarly WGA and GNA are toxic to the brown planthopper, which is a pest that affects rice plant, but other lectins such as those from potato and pea had no effect on them [Gatehouse et al., 1995].

Animal lectins

Recently a variety of functions have been demonstrated for animal lectins. Galectins are animal lectins, which are specific for β-galactosides such as lactose and *N*-acetyllactosamine. Galectins are thought to be important for the normal differentiation and growth of all multicellular animals. They have the ability to induce cell proliferation and apoptosis, and have been implicated in tumor cell metastasis, leukocyte trafficking, organ morphogenesis, immune response and inflammation, as well as recognition of extracellular matrix [Perillo et al., 1998; Rabinovich, 1999; Leffler, 2001].

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

Microbial lectins

The major function of the microbial lectins is to mediate the adhesion of the organism to host cells, an initial stage of infection. These lectins also function in mycoparacitism by mediating the attachment of parasitic fungi to plant pathogenic fungi and nematodes. In viruses and bacteria, cell surface lectins help in adhesion

of the organisms to host cells. This is a prerequisite for infection to occur [Sharon & Lis, 2003]. This infection by bacteria can be prevented by pre-incubation with specific sugars. Adhesion of lectin carrying bacteria to human polymorphonuclear cells is often followed by their ingestion and killing of bacteria, a process known as lectinophagocytosis [Ofek et al., 1995]. Lectins on bacterial surface are useful in the attachment of bacteria to primitive organisms and inanimate matter. For example, a galactose specific lectin present on the surface of the pathogen *Vibrio shiloi*, mediates the attachment of bacteria to the coral surface [Banin et al., 2001].

Applications

Lectins are useful in the isolation and characterization of glycoproteins and polysaccharides in solution and on cell surfaces. Separation of glycoproteins can be done by employing Ouchterlony double diffusion technique, in which lectins are used in place of antibodies [Carter & Sharon, 1977]. The blotting technique is highly useful for the detection and identification of glycoproteins on cells, which serve as receptors for bacterial cell surface lectins [Sharon & Ofek, 1995]. Glycoproteins containing mannose can be identified by overlaying the blots with concanvalin A and those containing galactose with PNA or RCA [Gershoni & Palade, 1982]. Lectins are useful for the isolation and affinity chromatographic purification of glycoconjugates like glycoproteins, glycopeptides, and oligosaccharides. Con A has been used for the separation of closely related glycopeptides which are found in proteolytic digests of glycoproteins [Baenziger & Fiete, 1979; Narasimhan et al., 1979]. Lectins are extremely useful for the isolation and purification of membrane glycoproteins, because lectins can show binding activity in the presence of mild detergents [Lotan & Nicolson, 1979], which are required for the solubilization of membrane glycoproteins. Lectins are useful in mapping of neuronal pathways and radioactive iodine labeled WGA and PHA were

used for this application. These two lectins were used as tracers in neuroanatomical studies and these are most popular reagents for this purpose [Wouterlood & Groenewegen, 1985; Sawchenko & Gerfen, 1985].

The ability of lectins to preferentially agglutinate certain cells, based on their surface sugars, is used to separate cells of interest from a mixture of cells. The agglutinated cells are then obtained intact for further studies by adding a specific sugar recognized by the lectin. PNA and SBA have been found to be valuable tools for cell separation. PNA was used for separation of human or mouse thymocytes into immature and mature populations using fluorescence-activated cell sorter (FACS) [Sharon, 1983]. By selective agglutination using SBA, mouse splenocytes were separated into B and T cells [Reisner et al., 1976].

Certain lectins are able to induce quiescent lymphocytes to grow and divide, a process termed mitogenic stimulation or mitogenicity [Kilpatrick, 1998]. Most mitogenic lectins are from plant sources and only a limited number of lectins from animals and microorganisms have been found to exhibit mitogenicity. PHA and concanavalin A stimulate T lymphocytes only, while artocarpin and pokeweed mitogen (PWM) stimulate both T and B cells [Sharon & Lis, 2003]. Mitogenic lectins such as concanavalin A, PHA and PWM were used in clinical laboratory to assess immunocompetence of patients suffering from a diversity of diseases and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations [Di Sabato, 1987; Kilpatrick, 1998; Fletcher et al., 1987]. SBA was used for removal of T-cells responsible for the lethal graft-versus-host diseases, from bone marrow of immonologically non-identical donors, so that it can be safely transplanted into children born with severe combined immune deficiency. A ricin-linked anti-CD45 antibody was able to kill CD_4^+ T cells, latently infected with

human immunodeficiency virus (HIV) in the blood of HIV-positive persons [Saavedra-Lozano et al., 2002].

Cucurbitaceae phloem lectins

Occurrence

Lectins have been isolated from different vegetative tissues of cucurbits. Among them a few lectins isolated from the phloem exudate have been studied in some detail. High hemagglutination activity has been reported about three decades ago in the phloem exudate from three cucurbit species, namely *Cucurbita maxima, Cucumis sativus* and *Cucumis melo* [Sabnis & Hart, 1978]. Among these, a lectin specific for chitooligosaccharides has been purified from the phloem exudate of *Cucurbita maxima* (pumpkin) by conventional chromatographic methods [Read & Northcote, 1983]. In 1979 Allen reported a lectin from the fruit exudate of the vegetable marrow (*Cucurbita pepo*) that is specific for β-1,4-linked N-acetylglucosamine oligosaccharides [Allen, 1979]. Surolia and coworkers purified lectins that are specific to chitooligosaccharides from the phloem exudate of ridge gourd (*Luffa accutangula*) and *Coccinia indica* [Anatharam et al., 1986; Sanadi & Surolia, 1994]. Another lectin was identified in the phloem exudate of *Sechium edule* fruit (Chayote) [Magdolna et al., 1992]. The laboratory of M. J. Swamy (where the present work was carried out) has developed a rapid method for purification of pumpkin phloem lectin (PPL) using affinity chromatography on chitin and characterized the lectin in considerable detail (see Chapters 2-4 of this thesis). Additionally, a lectin present in the phloem exudate of snake gourd (*Trichosanthes anguina*) has been identified and purified by affinity chromatography and it's physicochemical and carbohydrate binding properties were characterized by this group (Chapters 4, 5).

Purification of Cucurbitaceae phloem lectins

Different experimental approaches were used by different groups for the purification of Cucurbitaceae phloem lectins. Pumpkin (*Cucurbita maxima*) phloem exudate lectin was purified by conventional chromatographic techniques such as gel filtration on Sephedex G-100 followed by cation exchange chromatography on CM Sepharose CL 6B [Sabnis & Hart, 1978; Read & Northcote, 1983]. Another phloem lectin from vegetable marrow was purified by affinity chromatography on chitooligosaccharides covalently attached to Sepharose [Allen, 1979]. Two other Cucurbitaceae phloem lectins, namely *Luffa accutangula* agglutinin (LAA) and *Coccinia indica* agglutinin (CIA) were purified by affinity chromatography on soybean agglutinin coupled to Sepharose 6B column. A lectin from the exudate of *Sechium edule* fruit (Chayote) was purified by ammonium sulphate precipitation followed by gel filtration on Sephadex G-100 and affinity chromatography on fetuin-agarose [Magdolna et al., 1992].

Macromolecular properties

Pumpkin phloem exudate lectin is a chitooligosaccharide-specific, dimeric protein with a subunit mass of 24 kDa [Read & Narthcote, 1983]. Sequence analysis of PPL cDNA shows that it is a polypeptide of 218 aminoacids with eight tryptophan and six tyrosine residues [Bostwick et al., 1992, 1994] and suggests that the protein may be hydrophobic in nature. Vegetable marrow lectin consists of a single poly peptide chain of about 20 kDa molecular weight. It is not a glycoprotein and it was reported to an anti-parasitic function [Allen, 1979]. Ridge gourd phloem lectin is a homodimer with a molecular weight of 48 kDa and subunit mass of 24 kDa. It does not contain covalently bound sugar. The subunits in this dimeric lectin are held together solely by noncovalent interactions. Secondary structural analysis by circular dichroism measurements showed that in consists of 31% α-helix

[Anantharam et al., 1986]. *Coccinia indica* agglutinin is a homodimeric protein with a molecular weight of 32 kDa and chemical modification studies shows that tryptophan residues are involved in the carbohydrate binding site [Sanadi & Surolia, 1994]. *Sechium edule* fruit exudate lectin consists of two identical non-covalently bound subunits and has a molecular weight of 44 kDa. The subunit mass was estimated as 22 kDa by SDS-PAGE and also by gel filtration [Magdolna et al., 1992].

Thermodynamic analysis

Among the Cucurbitaceae phloem lectins discussed above, thermodynamic parameters for the interaction of chitooligosaccharides to ridge gourd phloem lectin and *Coccinia indica* agglutinin were reported. Thermodynamic parameters associated with the binding of chitooligosaccharides to ridge gourd phloem exudate lectin were studied by fluorescence spectroscopy and circular dichroism spectroscopy [Anantharam et al., 1986]. Binding of chitooligosaccharides to LAA resulted in a significant increase in the fluorescence intensity as well as the molar ellipticity in the near UV region of the lectin. The binding constants obtained for chitooligosaccharides are 1.4×10^3, 12.6×10^3, 97.0×10^3 and 650×10^3 M^{-1} for chitobiose, chitotriose, chitotetraose and chitopentaose, respectively. The binding reaction for $[(GlcNAc)_{2\text{-}5}]$ with LAA were essentially enthalpy driven with the values of -41.0, -47.9, -55.9 and -56.0 $kJ.mol^{-1}$ at 25 °C, whereas the entropic contribution to the binding reaction is negative. These thermodynamic data are consistent with an extended binding site in this lectin, which accommodates a tetrasaccharide [Anantharam et al., 1986]. Binding of 4-methylumbelliferyl chitooligosaccharides to *Coccinia indica* agglutinin was studied by fluorescence spectroscopy. Thermodynamic analysis shows that the binding of labeled tetrasaccharide is very strongly entropically driven, with the terminal, nonreducing

sugar residue protruding from the binding pocket [Sanadi & Surolia, 1994]. Analysis of the ligand size dependence of the fluorescence spectra of CIA together with thermodynamic parameters for the lectin revealed that its combining site spans the chitotetraose. They identified that the fourth sugar residue of tetrasaccharide is proximal to a highly fluorescent tryptophan. The schematic model for the binding site of CIA with bound chitotetraose is shown in Fig. 1.1. [Sanadi et al., 1998].

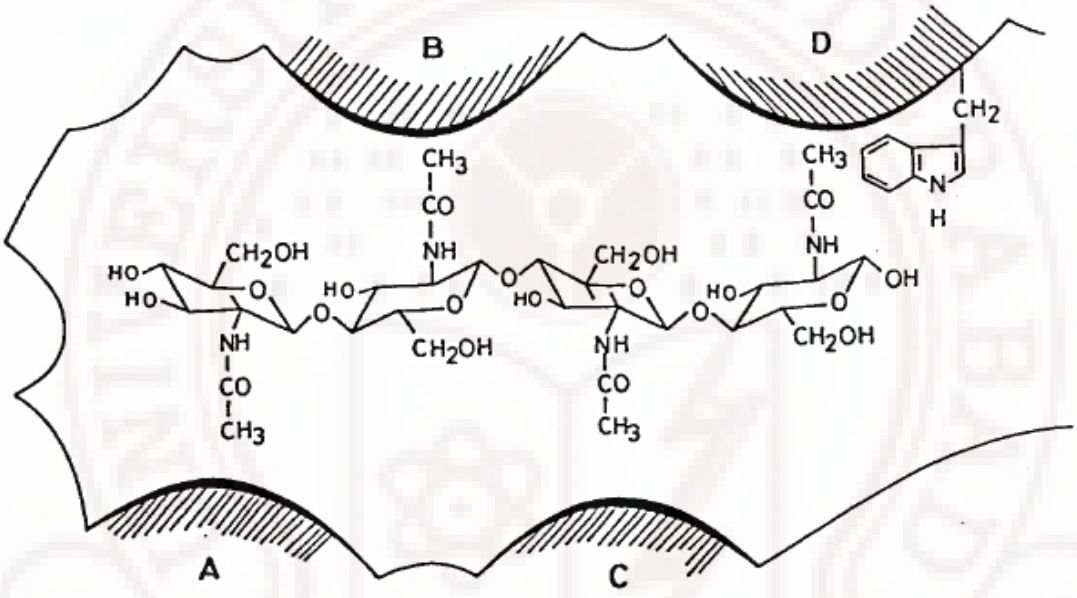


Fig. 1.1. Model of the binding site of CIA with bound chitotetraose. A, B, C, and D refer to the different subsites. Reproduced from Sanadi et al., 1998 (Copyright (1998) International Union of Pure and Applied Chemistry).

Other chitin binding lectins

In addition to their occurrence in the phloem exudate of Cucurbitaceae species, chitooligosaccharide-specific lectins have been isolated from various other sources such as seeds, embryo, latex, fruit, root, rhizome and tuber of different plant species [Van Damme et al., 1998a]. The hemagglutination activity of potato tuber was first investigated in 1926 by Marcusson-Begun and its partial purification was reported

later [Marinkovich, 1964]. Wheat germ agglutinin was the first chitin-binding lectin to be purified to homogeneity and characterized in detail including the determination of the three-dimensional structure [Nagata & Burger, 1972; LeVine et al., 1972; Wright, 1977]. WGA was also the first protein to be cloned among the chitin binding lectins [Raikhel & Wilkins, 1987].

The family of chitin binding lectins includes merolectins, hololectins and chimerolectins [Van damme et al., 1998a]. Schematic representations of the molecular structures of these proteins are shown in Fig. 1.2. Merolectins consist of a single hevein domain and they are divided into two types based on antimicrobial activity. The first types are hevein and hevein-like proteins. Hevein, which is an abundant latex protein of the rubber tree, contains a single polypeptide chain of 43 amino acids [Waljuno et al., 1975]. The second type have a single hevein domain and exhibit antimicrobial activity. This type of chitin binding domains were identified from *Amaranthus caudatus* (AcAMP). AcAMP1 and AcAMP2 are single chain polypeptides of 29 and 30 amino acid residues, respectively [Broekaert et al., 1992].

The chitin binding hololectins are composed of polypeptides containing two, three, four, or seven hevein domains. These include *Urtica dioica* (stinging nettle) agglutinin (UDA) which consists of two hevein repeats separated by a four aminoacid hinge region [Peumans et al., 1984; Beintema & Peumans, 1992]. *Viscum album* chitin-binding protein (ViscalbCBP) is a homodimer and contains two hevein repeats [Peumans et al., 1996]. Another chitin binding lectin called *Chelidonium majus* agglutinin (CMA) is a dimeric lectin and each dimer contains two hevein repeats [Peumans et al., 1985]. Roots of *Phytolacca americana* consist of a complex mixture of chitin binding lectins. These are Pa-5/PL-D, Pa-4/PL-c and PL-B, which contain two, three and seven hevein domains, respectively

[Yamaguchi et al., 1995, 1996, 1997]. WGA and other Gramineae lectins characterized so far consist of four repetitive hevein domains for each monomer [Raikhel et al., 1993].

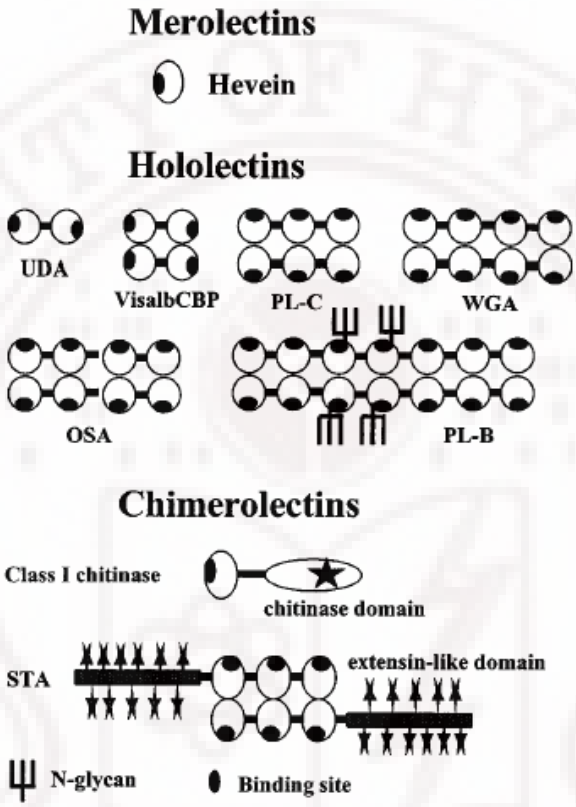


Fig. 1.2. Shematic representation of the different molecular structures of the native chitin binding lectins. Examples shown are: hevein, UDA, ViscalCBP, PL-C and PL-B, WGA, OSA, class I chitinase and STA. Reproduced from Van Damme et al. [1998a] (Copyright (1998) CRC Press LLC).

The chitin binding lectins under the group of chimerolectins are of two different types. The first type contains a single hevein domain without agglutination activity and the second type contains multiple hevein domains with agglutination activity. Class I chitinases contain a single *N*-terminal hevein domain linked through small

variable glycine/proline-rich hinge domain to the catalytically active chitinase domain [Collinge et al., 1993; Beintema, 1994]. These are monomeric proteins of 25-30 kDa and possess only one carbohydrate binding site, which is the reason for their inability to agglutinate cells. *Solanaceae* lectins are considered as the second type. Potato lectin (*Solanum tuberosum* agglutinin or STA) is a dimer of two identical or slightly different subunits of 65 kDa and consists of an *N*-terminal chitin-binding domain of three hevein repeats linked to an *O*-glycosylated serine-hydroxyproline-rich domain [Kieliszewski et al., 1994; Allen et al., 1996].

Three-dimensional structures of chitin binding lectins

The three-dimensional structure has been determined for only a few of the chitin binding lectins. Among these the three-dimensional structure of wheat germ agglutinin was solved in 1977 at 2.2 Å resolution [Wright, 1977]. Crystal structure of WGA is shown in Fig. 1.3. It shows that wheat germ agglutinin consists of eight hevein domains. Each monomer consists of four hevein-like domains and the monomers are noncovalently associated in a head-to-tail fashion to form four binding sites located at the interface of two domains. Each hevein-like domain contains a small α-helix.

Urtica dioica agglutinin is a small chitin binding lectin and its crystal structure with chitotriose was determined by X-ray analysis [Harata & Muraki, 2000]. UDA consists of two hevein like domains. The three-dimensional structure of a complex of UDA with chitotriose is shown in Fig 1.4. The arrangement of the two domains gives the molecule a dumbbell shape and the sugar binding sites are located at both ends of this structure. In both the domains, three aromatic amino acid residues and one serine residue participate in the binding of the ligand.

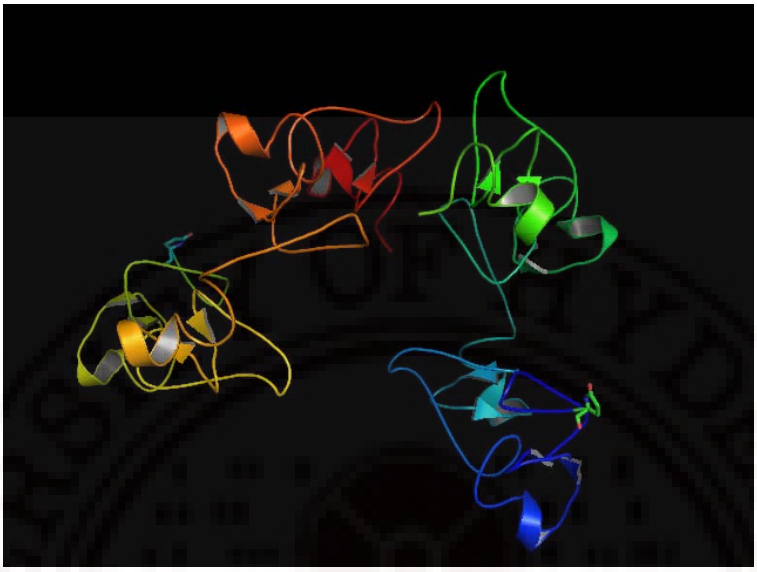


Fig. 1.3. Three-dimensional structure of the wheat germ agglutinin dimer. There are four hevein like domains for each monomer located at the interface of the two domains. Reproduced from Wright, 1992 (Copyright (1992) The American Society for Biochemistry and Molecular Biology, Inc.)

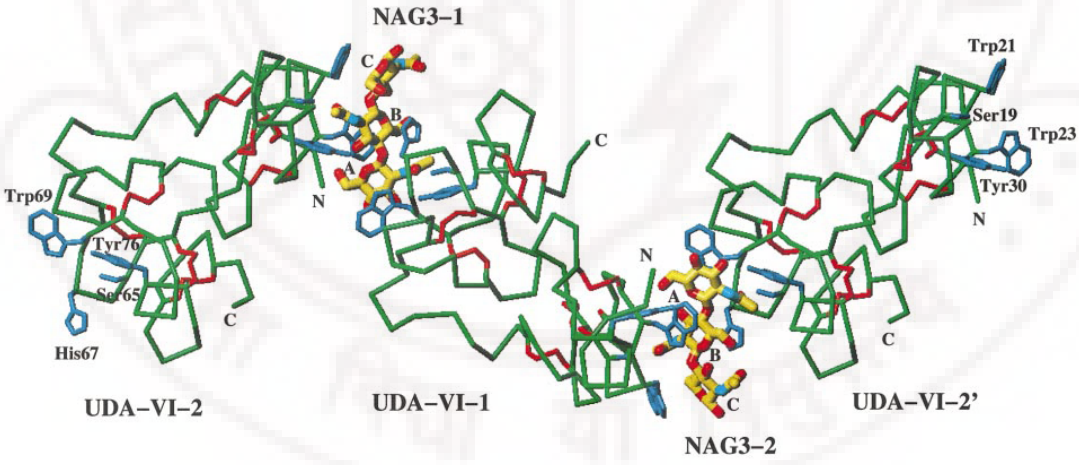


Fig. 1.4. Structure of the 2:2 complex of UDA-VI with NAG3. The proteins' backbone structure (green) and disulfide de bridges (red). Aromatic side-chain groups at the sugar-binding sites (blue). The N and C terminals of protein molecules are denoted by N and C, respectively. UDA-VI-2' denotes the UDA-VI-2 molecule related by the translation along the c axis. Reproduced from Harata & Muraki, 2000 (Copyright (2000) Academic press)

Hevein is a low molecular weight protein, isolated from the latex of *Hevea brasiliensis.* Its three-dimensional structure was determined by single-crystal X-ray diffraction at 2.8 Å resolution [Rodriguez-Romero et al., 1991]. Nearly a decade later the structure of hevein-chitin complex was solved by NMR spectroscopy and the NMR structure is shown in Fig. 1.5. [Asensio et al., 2000].

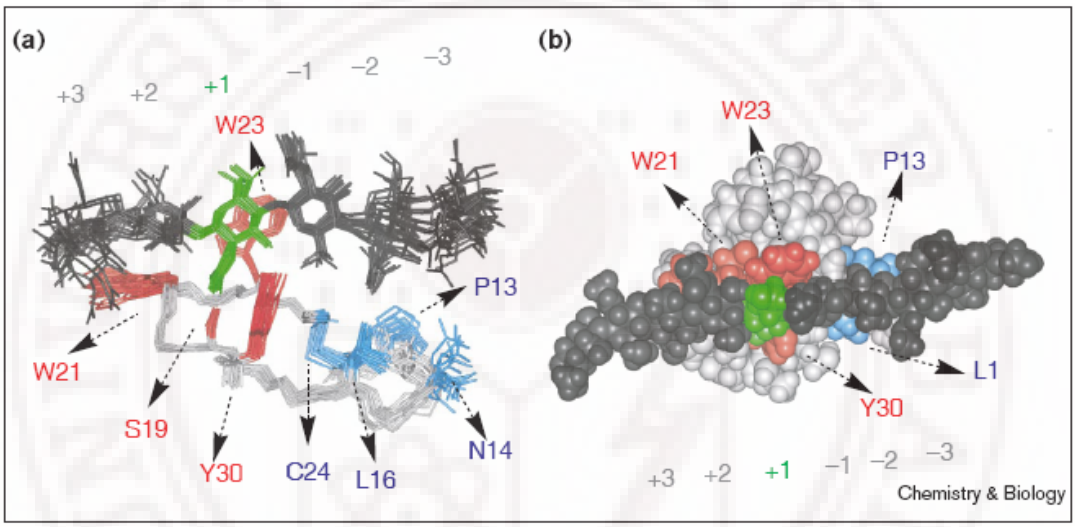


Fig. 1.5. (a) Ensemble of 16 structures obtained from the restrained molecular dynamics simulations on hevein-chitin complex. The GlcNAc unit located at subsite +1 is highlighted in green; and protein residues involved in the recognition of this GlcNAc unit are highlighted in red. (b) CPK representation of the NMR-derived model of the hevein-chitin complex. The GlcNAc unit located at subsite +1 is highlighted in green. Reproduced from Asensio et al., 2000 (Copyright (2000) Elsevier Science Ltd.)

Isothermal titration calorimetric studies on chitin binding lectins

Isothernal titration calorimetric studies were reported on wheat germ agglutinin (WGA), *Urtica dioica* agglutinin (UDA) and on hevein with chitooligosaccharides. Among these lectins only WGA binds to *N*-acetylglucosamine, whereas the other lectins require at least a disaccharide structure to recognize with detectable affinity.

Thermodynamic parameters for the binding of chitooligosaccharides to the above three lectins are listed in Table 1. While the association constants increase with increase in size of the ligand upto pentasaccharide, the enthalpy of binding increases only up to the trisaccharide [Bains et al., 1992]. These results support the "three subsite" structural binding model proposed earlier [Allen et al., 1973].

Table 1. Thermodynamic parameters for the binding of chitooligosaccharides to WGA, UDA and Hevein.

Ligand	K_a $M^{-1} \times 10^{-3}$	ΔG kcal/mol	ΔH kcal/mol	$T\Delta S$ kcal/mol/K
WGA				
GlcNAc	0.41	-3.7	-6.10	-2.40
$(GlcNAc)_2$	0.53	-5.1	-15.6	-10.5
$(GlcNAc)_3$	11.1	-5.5	-19.4	-13.9
$(GlcNAc)_4$	12.3	-5.6	-19.2	-13.6
$(GlcNAc)_5$	19.1	-5.8	-18.2	-12.4
UDA				
$(GlcNAc)_2$	0.8	-3.1	-4.7	-0.8
$(GlcNAc)_3$	6.2	-5.1	-6.3	-1.2
$(GlcNAc)_4$	14.4	-5.6	-5.1	+0.5
$(GlcNAc)_5$	26.5	-5.9	-5.1	+0.8
Hevein				
$(GlcNAc)_2$	0.616	-3.8	-6.3	-2.5
$(GlcNAc)_3$	8.520	-5.4	-8.3	-2.9
$(GlcNAc)_4$	10.80	-5.5	-9.5	-4.0
$(GlcNAc)_5$	474.0	-7.8	-9.6	-1.8

The binding site of UDA is composed of three subsites, wherein each subsite accommodates one GlcNAc residue. A Typical ITC profile for chitotriose binding to UDA is shown in Fig. 1.6. Thermodynamic parameters obtained show that while chitobiose has two independent non-interacting sites whereas, chitotriose, chitotetarose and chitopentaose have two interacting sites on each monomer of UDA [Katiyar et al., 1999; Lee et al., 1998]. Chitooligosaccharide interaction for UDA was enthalpically driven and the enthalpy values estimated are lesser than those of wheat germ agglutinin. Entropy values for binding of chitooligosaccharides to UDA were more favorable as compared to WGA, suggesting that carbohydrate ligands recognized by UDA have a higher hydrophobic component than that of WGA.

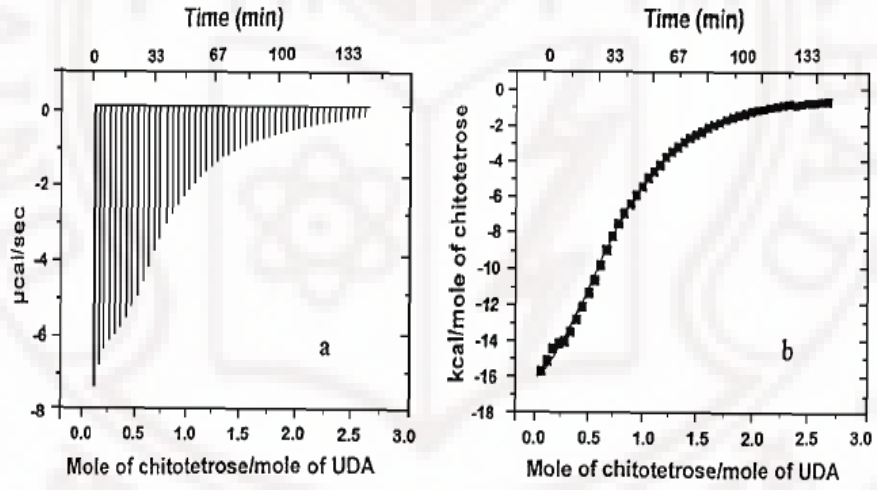


Fig. 1.6. Calorimetric titration of chitotetraose solution with UDA solution. (a) Raw data obtained from 48 automatic injections of 6.0 µl aliquots of 3.0 mM chitotriose solution into 0.28 mM UDA solution in 0.02 M PBS buffer, pH 7.2 containing 0.15 M NaCl. (b) The heat exchanged per mole of titrant versus the ratio of the total concentration of ligand to the total concentration of protein and the best least square fit of the data for two non-interacting identical sites. Reproduced from Katiyar et al., 1999 (Copyright (1999) Plenum Publishing Corporation).

Isothermal titration calorimetric studies were also carried out on chitooligosaccharide binding to hevein [Asensio et al., 2000; Garcia-Hernandez et al., 1998]. Thermodynamic parameters suggest that chitooligosaccharides bind in a multivalent fashion to extended binding sites. The association constants for chitooligosaccharides to hevein 0.6, 8.5, 10.8 and 474 mM^{-1} for chitobiose, chitotriose, chitotetraose and chitopentaose respectively. The data demonstrate that hevein binds $(GlcNAc)_{2-4}$ in 1:1 stochiometry with millimolar affinity. In contrast, for $(GlcNAc)_5$, a significant enhancement in binding affinity was observed. Analytical ultracentrifugation studies showed that the interaction of hevein-$(GlcNAc)_{5,8}$ complexes shows a major stochiometry of 2:1 in solution [Asensio, et al., 2000].

Motivation and focus of the present work

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

It is important to isolate new lectins and undertake physicochemical characterization of these lectins, which is expected to eventually lead to understanding and exploitation of lectins from other plant families as well. In previous work from our laboratory lectins from the seeds of several Cucurbitaceae species, namely T*richosanthes anguina, Trichosanthes cucumerina, Trichosanthes dioica* and *Trichsanthes cordata* have been isolated and characterized. More

recently our group has started working on lectins from the phloem exudate of cucurbit species and the present study forms a part of this programme.

In this study we have chosen to investigate on Cucurbitaceae phloem lectins. In this regard the objectives of the present study are to develop an affinity method for the purification of the pumpkin (*Cucurbita maxima*) phloem exudate lectin (PPL) and to characterize the affinity-purified protein with respect to its secondary structure, carbohydrate specificity and thermal stability. In addition, we propose to carry out fluorescence quenching and time-resolved fluorescence studies in order to probe the exposure of tryptophan residues and their microenvironment in PPL. Further the energetics of chitooligosaccharide binding to PPL will be studied by isothermal titration calorimetry.

Preliminary experiments carried out in this laboratory have identified a lectin activity that could be inhibited by chitooligosaccharides in the phloem exudate of snake gourd (*Trichosanthes anguina*). We propose to develop an affinity and chromatographic method for its purification and to investigate the purified protein with respect to macromolecular properties, carbohydrate specificity and secondary structure. The interaction between SGPL and chitooligosaccharides [$(GlcNAc)_{3-6}$] will be studied by isothermal titration calorimetry (ITC) and fluorescence spectroscopy.

Chapter 2

Rapid affinity-purification and physico-chemical characterization of pumpkin (*Cucurbita maxima*) phloem exudate lectin

Summary

The chitooligosaccharide-specific lectin from pumpkin (*Cucurbita maxima*) phloem exudate (PPL) has been purified to homogeneity by affinity chromatography on chitin. In SDS-PAGE in the presence of β-mercaptoethanol the pumpkin phloem lectin (PPL) yielded a single band corresponding to a molecular weight of 23.7 kDa, whereas MALDI-TOF mass spectrometry gave the molecular weights of the subunit and dimeric lectin as 24,812.3 and 49,721.5 Daltons, respectively. Analysis of the CD spectrum of the protein indicated that the secondary structure of PPL consists of 9.7% α-helix, 35.8% β-sheet, 22.5% β-turns and 32.3% unordered structures. Saccharide binding did not significantly affect the secondary and tertiary structures of the protein. The hemagglutinating activity of PPL was mostly unaffected in the temperature range 4-70 °C, but a sharp decrease was seen between 75 and 85 °C. Differential scanning calorimetric and CD spectroscopic studies indicate that PPL undergoes cooperative thermal unfolding process centered at ca. 81.5 °C.

Introduction

Although many species from Cucurbitaceae are cultivated in large quantities across the world because fruits of many cucurbit species are edible, only a few lectins from this family have been characterized in detail. These include *Momordica charantia* lectin (MCL) and *Trichosanthes kirilowii* lectin from the seeds, and *Luffa acutangula* agglutinin (LAA) and *Coccinia indica* agglutinin (CIA) from the phloem exudate of cucurbits [Khan et al., 1981; Mazumder et al., 1981; Falasca et al., 1989; Anantharam et al., 1986; Sanadi & Surolia, 1994]. In order to characterize the cucurbit seed lectins in greater detail a long-term program was intiated by M. J. Swamy's group in the mid 1990s and they reported on the purification, physico-chemical characterization and carbohydrate binding specificity of four new lectins from the Cucurbitaceae species, namely *Trichosanthes anguina* (snake gourd) seed lectin (SGSL), *Trichosanthes cucumerina* seed lectin (TCSL), *Trichosanthes dioica* seed lectin (TDSL) and *Trichosanthes cordata* seed lectin (TCA) characterized in greater detail, in addition carbohydrate and hydrophobic ligand binding properties for *Momordica charantia* (Bitter Gourd) seed lectin were characterized [Komath & Swamy, 1998, 1999; Kenoth & Swamy, 2003; Komath et al., 1996, 1998, 2001; Manoj et al., 2001; Padma et al., 1999; Kenoth et al., 2000, 2003; Sultan & Swamy, 2003, 2005a,b; Sultan et al., 2004a, 2009; Kavitha et al., 2009]. Additionally, the interaction of several water-soluble porphyrins with SGSL, TCSL, and MCL were investigated [Komath et al., 2000b; Kenoth et al., 2001; Sultan et al., 2004b].

In this thesis, the focus of our study is the lectins present in the phloem exudate of cucurbit species. Besides LAA and CIA which are mentioned above, strong hemagglutinating activity has been reported about three decades ago in the phloem exudate from three cucurbit species, namely *Cucurbita maxima, Cucumis sativus*

and *Cucumis melo* [Sabnis & hart, 1978]. Among these, a lectin specific for chitooligosaccharides has been purified from the phloem exudate of *Cucurbita maxima* (pumpkin) by conventional chromatographic methods [Read & Northcote, 1983]. This protein was termed PP2 as it corresponded to the second prominent protein band in polyacrylamide gel electrophoresis. However, as this protein is a lectin, it would be more appropriate to call it a lectin and therefore hereafter we shall refer to it as pumpkin phloem lectin (PPL). Analysis of the cDNA sequence yielded the primary structure of PPL and indicated that it is a polypeptide of 218 amino acids [Read & Northcote, 1983; Bostwick et al., 1992, 1994]. To the best of our knowledge, no further work has been done on this protein. Therefore, we took up a detailed characterization of the macromolecular and carbohydrate binding properties of PPL. In this chapter a rapid and efficient purification method is reported for this protein with affinity chromatography as the key step. Physico-chemical studies show that PPL is stable up to about 75 °C, whereas CD spectroscopy indicates that its secondary structure is characterized by a large amount of β-sheet and β-turns and a very small α-helical content. DSC and CD studies indicate that the protein undergoes a cooperative thermal unfolding process around 81.5 °C.

Materials and Methods

Materials

Pumpkin fruits were obtained from local vendors. Chitin (practical grade, from crab shells), 2-mecrcaptoethanol, sodium dodecyl sulfate, acrylamide, methylene bis-acrylamide, molecular weight markers and chitooligosaccharides [$(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$], were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, sodium azide, di-sodium hydrogen phosphate, sodium dihydrogen phosphate,

trichloroacetic acid, sodium deoxycholate, sodium hydroxide, ammonia solution, and acetic acid were obtained from local suppliers and were of the highest purity available.

Preparation of affinity matrix

The chitin column was prepared essentially as described earlier [Sampietro et al., 2001]. Briefly, practical grade chitin from crab shells was suspended in 0.25 M NH_4OH and incubated for 60 minutes. The supernatant was decanted and the material was washed successively with 0.2 M NaCl, double distilled water and 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 10mM β-mercaptoethanol (PBS-βME). The material was finally suspended in PBS-βME was packed in to a glass column (16 × 4 cm) and equilibrated again with the same buffer.

Extraction and purification of pumpkin phloem exudate lectin

Pumpkin fruits were first washed with water and dried. Phloem exudate was then collected into ice cold PBS-βME by longitudinal, 2-5 mm deep cuts [Anantharam et al., 1986; Allen, 1979]. The buffer containing exudate was centrifuged at 9000 rpm in an Eppendorf 5810 R refrigerated centrifuge. The resulting supernatant containing soluble protein was subjected to ammonium sulfate precipitation to give 80% saturation. This solution was allowed to stand at 4 °C overnight and centrifuged at 9000 rpm and the supernatant was discarded. The precipitate obtained was dissolved in a minimal volume of water and dialysed against PBS-βME extensively at 4 °C. The solution from the dialysis bag was then centrifuged and the precipitate was discarded. The supernatant was subjected to affinity chromatography on a column of chitin (16 × 4 cm) that was pre-equilibrated with PBS-βME. The breakthrough obtained was reloaded to ensure complete binding of

the protein. The column was then washed with PBS-βME to remove unbound proteins, monitoring absorbance of the eluant at 280 nm. When the absorbance fell below 0.01 the washing was stopped and the bound protein was eluted with 0.1 M acetic acid at room temperature. Fractions of ca. ~5 ml were collected and their absorbance at 280 nm was checked for detecting the protein. Fractions showing high concentration of protein were pooled and the eluting acid was removed by extensive dialysis against PBS-βME. Purity of the dialysed protein was assessed by SDS-PAGE according to the method of Laemmli [Laemmli, 1970] and its concentration was estimated by a modified Lowry method [Peterson, 1977].

Hemagglutination and hemagglutination-inhibition assays

Hemagglutination assays were carried out using rabbit erythrocytes in 96-well ELISA microtiter plates. All solutions were made in PBS-βME and all dilutions were carried out using the same buffer. To each well containing 100 µl of serially diluted lectin 100 µl of 4% erythrocyte suspension was added. The plate was incubated at 4 °C for 1 hour and then the agglutination titer was visually scored. Hemagglutination-inhibition assays were carried out with purified protein. For inhibition assay 1 mM saccharide solutions were used except for chitobiose (52 mM). In the first well of microtiter plate, 50 µl of saccharide solution was placed and serially 2-fold diluted. Then 50 µl of protein solution (4 µg/ml) was added to each well. After incubating the mixture at 4 °C for 15 min, 100 µl of a 4 % erythrocyte suspension was added, the plate was incubated for one hour and the titer was scored visually.

Mass spectrometry

The mass spectrum of PPL was recorded using a model 4800 MALDI-TOF-TOF mass spectrometer from Applied Biosystems (Foster City, CA, USA) in the linear

mode using sinapinic acid (SA) as the matrix. The matrix was prepared by dissolving 5 mg of SA in 1 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). About 5 picomoles of the protein was spotted on the MALDI plate and the spectrum was acquired.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www.jascoint.co.jp) essentially as described earlier [Sultan et al., 2006]. Concentration of PPL was 1.8 μM for measurements in the far UV region (250-190 nm) and 20 μM for measurements in the near UV region (250-300 nm). Samples were placed in a 2-mm pathlength rectangular quartz cell and spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 2 nm. In order to investigate the effect of carbohydrate binding on the secondary and tertiary structure of PPL, spectra were recorded for the native protein as well as in the presence of 1 mM chitotriose. To investigate any structural changes that take place during thermal unfolding of the protein, CD spectra were recorded in the near UV and far UV regions at different temperatures by means of a Peltier device. Measurements were made in PBS-βME and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

Secondary Structure Prediction

Secondary structure prediction of PPL was made using Self-Optimized Prediction Method from Alignments (SOPMA) software available at (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) [Geourjon & Deléage, 1995] and Double Prediction Method (DPM) available in the Antheprot software (http://antheprot-pbil.ibcp.fr/) [Geourjon, et al., 1991] For prediction using SOPMA the following parameters were used: window width = 9, similarity

threshold = 10 and number of states = 4. For prediction using DPM the structural class was chosen as 'all beta'. The amino acid sequence of PPL [Bostwick, et al., 1994] was imported from EMBL Nucleotide sequence database (accession number L31550).

Differential scanning calorimetry

Differential scanning calorimetric (DSC) studies were performed on a MicroCal VP differential scanning calorimeter (MicroCal LLC, Northampton, MA) with a scan speed of 60°/hr (Celsius scale). Thermograms were recorded with lectin samples of 12 μM concentration in PBS-βME. For experiments carried out in the presence of sugar, lectin samples contained 10 mM chitotriose. PPL was dialysed extensively against the buffer and degassed prior to loading into the cell. Buffer scans were subtracted from the thermograms corresponding to the lectin samples for analysis. Data were analysed by Origin^{TM} software supplied by the manufacturer.

Results and discussion

Affinity chromatographic purification of PPL

Purification of two major proteins from the phloem exudate of *Cucurbita maxima* (pumpkin), named PP1 and PP2 by a combination of ammonium sulfate precipitation, ion exchange chromatography and gel filtration was reported by Read and Northcote [Read & Northcote, 1983]. Of these two proteins, PP2 (which is more appropriately referred to here as pumpkin phloem lectin or PPL) has been shown to exhibit hemagglutination activity which could be inhibited by oligomers of *N*-acetylglucosamine. It has been proposed that the lectin plays a role in plant's defence mechanism by interacting with bacteria and fungi through its carbohydrate binding pocket [Read & Northcote, 1983]. In view of this it is important to investigate the macromolecular and carbohydrate binding properties of this protein

in detail. Such studies will be facilitated by the availability of an efficient method of purification of the lectin. Although it was reported that PPL bound to ovomucoid coupled to Sepharose CL-4B in a carbohydrate-specific manner and could be eluted with chitotetraose [Read & Northcote, 1983], since ovomucoid is rather expensive, it is desirable to develop an alternative, inexpensive affinity method for its purification. In view of this, in this study we have developed a rapid purification method for PPL based on affinity chromatography on chitin, which is an inexpensive, commercially available polysaccharide. The affinity purified protein has been characterized with respect to molecular weight, secondary structure and thermal unfolding.

Since PPL was reported to be inhibited by oligosaccharides of chitin [Read & Northcote, 1983], we thought that chitin could be used as an affinity matrix for the purification of this lectin as affinity chromatography on chitin has been used as the key step in the purification of a number of lectins that specifically recognize chitooligosaccharides including wheat germ agglutinin, *Entamoeba histolytica* lectin and chitin binding lectins from *Artocarpus integrifolia*, *Artocarpus incisa* and *Cynchomandra betecea* [Bloch & Burger, 1974; Kobiler & Mirelman, 1980; Trindade et al., 2006; Xu et al., 1992].

PPL bound to the chitin affinity matrix eluted as a single peak when 0.1 M acetic acid was passed through the column (Fig. 2.1). In a typical purification experiment, about 80 mg of the protein was obtained from 8 ml of phloem exudate, i.e., a yield of 10 mg/ml of phloem exudate, which is comparable to the yield of 10.5 mg/ml reported earlier [Read & Northcote, 1983]. The overall yield of lectin activity recovered was about 40 % (Table 2.1). Different affinity matrices such as soybean agglutinin glycopeptide coupled to Sepharose-6B and chitotriose coupled to Sepharose-4B have also been used to purify other chitooligosaccharide-specific

lectins such as potato tuber lectin and the phloem exudate lectins from ridge gourd (*Luffa acutangula*) and *Coccinia indica* [Anantharam et al., 1986; Sanadi & Surolia, 1994; Allen & Neuberger, 1973]. Sepharose-fetuin and insolubilized poly-L-leucine hog A + H blood group substance were used to purify the lectins from *Datura stramonium* and common tomato (*Lycopersicon esculentum*) [Kilpatric & Yeoman, 1978; Nachbar et al., 1980].

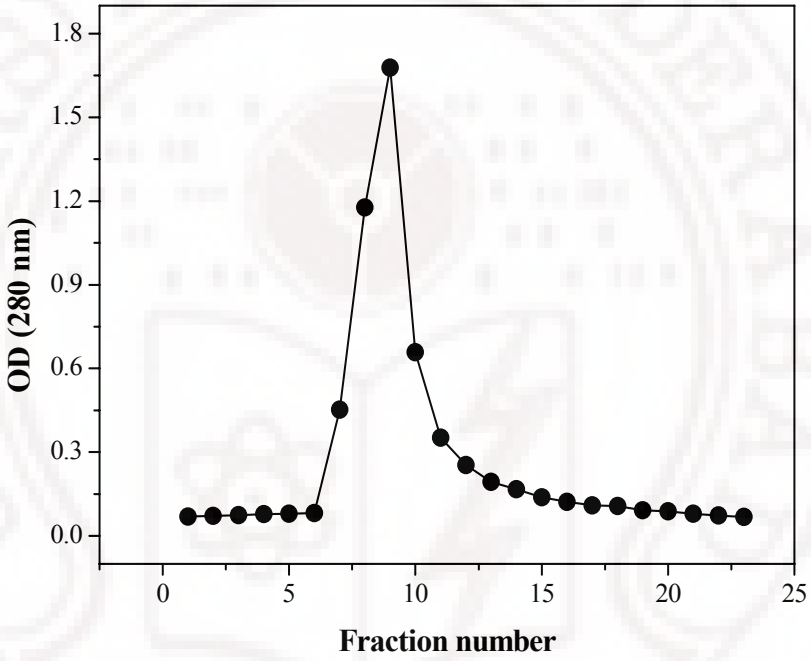


Fig. 2.1. Affinity chromatographic purification of pumpkin phloem exudate lectin (PPL) on chitin. After washing the column extensively with PBS-βME, the bound lectin was eluted with 0.1 M acetic acid. Fractions (5 ml) were collected and absorbance of the column effluent at 280 nm was plotted as a function of the fraction number.

The chitin affinity matrix used here is inexpensive and the method can be scaled up to process larger quantities of the phloem exudate and hence should be the method of choice for the purification of chitin-binding proteins. PPL yielded a single band in SDS-PAGE at pH 8.8 in the presence of β-mercaptoethanol (Fig. 2.2A, lane 2).

Table 2.1 Purification of pumpkin phloem exudate lectin (from 8 ml of phloem fluid).

Purification step	Total activity ($\times 10^4$)	Protein content (mg)	Specific activity (units/mg)	Percent recovery	Purification fold
Aqueous extract	52	707	735	100	1.0
Ammonium sulfate precipitation	31	213	1455	60	1.97
Affinity chromatography	21	88	3840	40	5.2

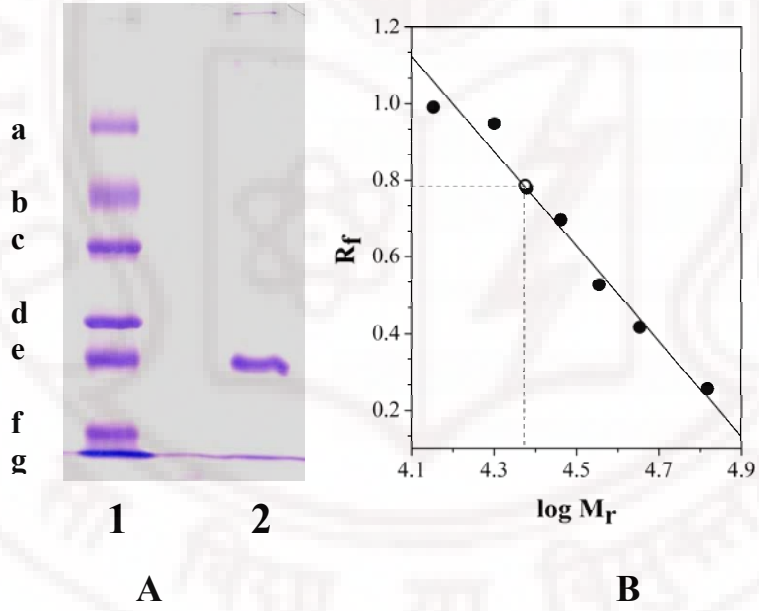


Fig. 2.2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PPL. Lane 1, molecular weight markers: (a) bovine serum albumin (66,000); (b) ovalbumin (45,000); (c) Glyceraldyhyde-3-phosphate dehydrogenase (36,000); (d) carbonic anhydrase (29,000); (e) trypsinogen (24,000); (f) trypsin inhibitor (20,000); (g) α-lactalbumin (14,200). Lane 2, PPL. (B) Plot of relative mobility (R_f) versus log M_r for the estimation of molecular weight of PPL subunit, (•) marker proteins; (o) PPL. From the plot the mass of PPL subunit was estimated as 23.7 kDa.

This is consistent with the results of Read and Northcote [Read & Northcote, 1983], who reported that the lectin gave a single band in SDS-PAGE under reducing conditions at pH 8.8, but could be resolved into two closely-spaced bands at pH 9.2. By comparing the mobility of PPL with that of standard proteins (Fig. 2.2A, lane 1), the molecular weight of the subunit was estimated as ~23.7 kDa (Fig. 2.2B). In order to get the exact mass of the protein, PPL was subjected to mass spectrometric analysis. The MALDI-TOF mass spectrum of PPL is shown in Fig. 2.3. The base peak in the spectrum, seen at 24,812.3 Daltons corresponds to the subunit of PPL whereas the other prominent peak, corresponding to a mass of 49,721.5 Daltons is consistent with the dimeric protein.

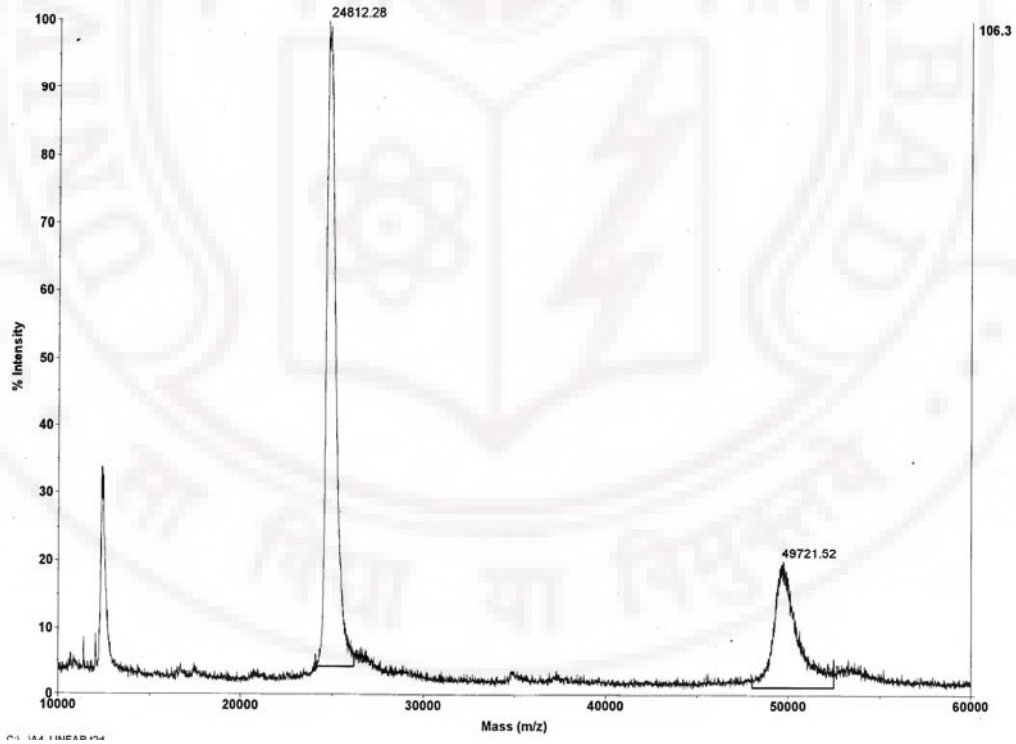


Fig. 2.3. MALDI-TOF mass spectrum of PPL. The molecular ion of the subunit is seen at 24,812.3 Daltons. The peak at 49,721.5 Daltons corresponds to the dimer.

Carbohydrate binding specificity

The hemagglutination activity of PPL was reported to be inhibited efficiently by chitooligosaccharides whereas a large number of other sugars investigated were ineffective [Sabnis & Hart, 1978; Read & Northcote, 1983]. To investigate the agglutination activity of the affinity purified PPL and its relative affinity towards various chitooligosaccharides, hemagglutination-inhibition assays were performed.

Table 2.2. Inhibition of the agglutination activity of PPL by chitooligosaccharides.

Sugar	Minimum concentration for 50% inhibition (μM)	Relative inhibitory potency
chitobiose	700	1
chiototriose	100	7
chitotetraose	50	14
chitopentaose	12	60
chitohexaose	10	70

The results obtained are presented in Table 2.2. The lectin activity could not be inhibited by *N*-acetylglucosamine, but strong inhibition is seen when chitooligosaccharides are used as ligands, with the inhibitory potency of the ligands increasing with increase in the oligosaccharide size up to chitohexaose. While the concentration of chitobiose for 50% inhibition of the hemagglutination activity of PPL is about 700 μM, chitohexaose is able to achieve the same level of inhibition at only 10 μM concentration. Thus chitotriose, chitotetraose, chitopentaose and chitohexaose are 7, 14, 60 and 70 times more potent in their ability to inhibit the hemagglutination activity of PPL and suggest the presence of an extended combining site on each subunit of the lectin. These observations are qualitatively

consistent with the results of Read & Northcote [Read & Northcote, 1983], who investigated the ability of chitobiose, chitotriose and chitotetraose to inhibit the activity of the pumpkin phloem lectin. Lectins from the phloem exudate of *Luffa acutangula* and *Coccinia indica* also exhibited stronger affinity for the higher oligomers of chitin [Anantharam et al., 1986; Sanadi & Surolia, 1994]. The amino acid sequence of a chitooligosaccharide-specific, phytohormone-inducible lectin (Nictaba), isolated from the leaves of tobacco plant (*Nicotiana tabacum*), has been reported to be homologous to the primary structure of PPL [Chen et al., 2002]. The presence of a fraction of Nictaba in the nucleus suggested that it may be involved in a specific regulatory process in the nucleus. Glycan array screening has shown that this lectin exhibits a strong affinity for high-mannose and complex *N*-glycans and is most complementary to the core glycan i.e., the Manβ1-4GlcNAcβ1-4GlcNAcβ-*N*-Asn moiety [Lannoo et al., 2006]. Similarly, another Cucurbitaceae phloem exudate lectin, LAA was also shown to interact with the *N*-linked glycans of fetuin, ovalbumin and soybean agglutinin, which contain an internal chitobiosyl moiety in core glycan structure [Anantharam et al., 1986]. The binding of LAA to the above *N*-linked glycans has been interpreted as arising, most likely, due to the interaction of the lectin with the core chitobiosyl moiety, with some additional interactions of the protein with some of the other sugar residues of the oligosaccharide [Anantharam et al., 1986]. It is, therefore, likely that PPL also binds to *N*-linked glycans by interacting with the chitobiosyl moiety of the core glycan structure. Such binding may be relevant to the endogenous functions of these cucurbit phloem exudate lectins. In the light of this, it would be interesting to investigate if PPL and other Cucurbitaceae phloem exudate lectins can also interact with *N*-linked glycans and other sugar structures by the glycan array approach, which may shed further light on the roles played by them in their endogenous functions.

Effect of temperature on the activity of PPL

In order to investigate the effect of temperature on the activity of PPL, samples of the lectin were incubated at various temperatures for 15 minutes, followed by cooling to room temperature and then subjected to hemagglutination assay. The results obtained are presented in Fig. 2.4. It is seen from this figure that incubation at different temperatures up to 70 °C did not affect the activity of the lectin at all.

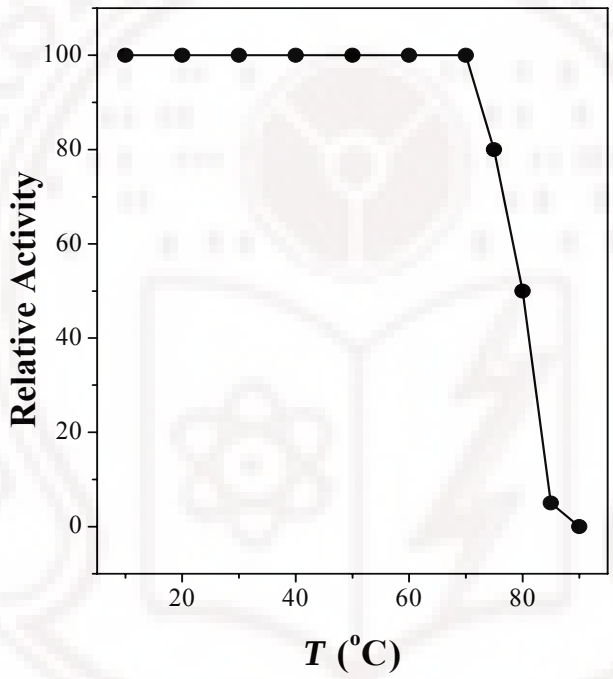


Fig. 2.4. Thermal inactivation of the agglutination activity of PPL. To investigate the effect of temperature on activity, the lectin samples were incubated at the desired temperature for 15 min. After cooling to room temperature, the sample was centrifuged, and the clear supernatant was tested for hemagglutination activity.

The activity decreased quite sharply between 75 and 85 °C; PPL incubated at 75 °C exhibits about 80% activity as compared to the native protein whereas the sample incubated at 85 °C has less than 10% activity. Incubation at 90 °C led to a complete loss of activity of the protein.

CD spectroscopy and secondary structure of PPL

Circular dichroic spectra of PPL in native state (solid line) and in the presence of chitotriose (dotted line) are shown in Fig. 2.5. The far UV spectrum of the native protein (Fig. 2.5A) is characterized by a minimum around 216 nm and a maximum around 196 nm, suggesting a predominantly β-sheet conformation for the lectin. The near UV CD spectrum of PPL (Fig. 2.5B) is characterized by three maxima at 275, 282 and 289.5 nm, which most likely arise due to the aromatic amino acids Tyr and Trp present in the protein.

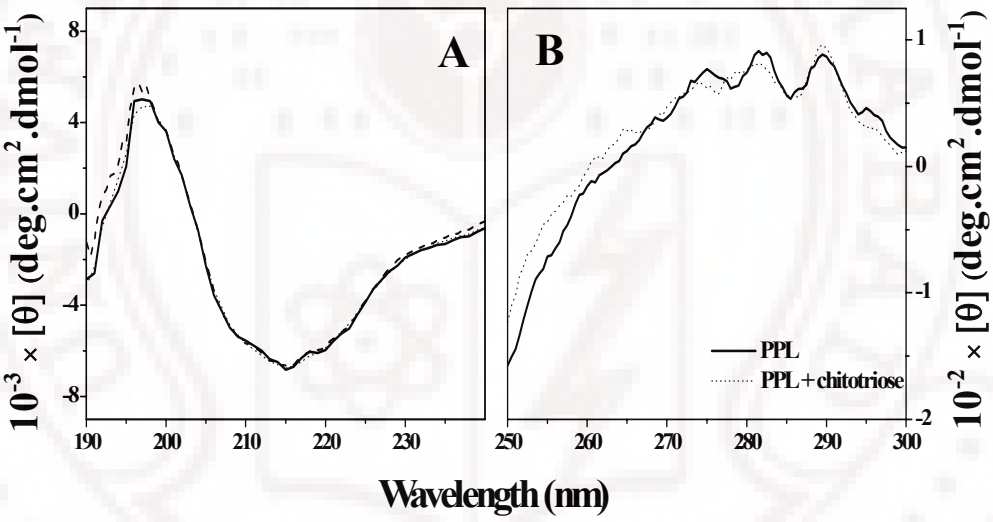


Fig. 2.5. CD spectra of PPL. (A) Far UV region, (B) Near UV region. (——) lectin alone, (…….) lectin with chitotriose, (------) calculated fit obtained by using CDSSTR program.

In order to derive information on the content of different structural elements in this protein, the CD spectrum was analyzed by three different methods, namely CDSSTR [Compton & Johnson., 1986; Sreerama & Woody, 2000] CONTINLL [Provencher & Glockner, 1981; Van Stokkum et al., 1990] and SELCON3 [Sreerama & Woody, 1993; Sreerama et al., 1999] employing the software routines

available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Lobley & Wallace, 2001; Lobley et al., 2002]. A basis set containing 43 proteins was used as a reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 2.3. Among the three methods mentioned above, the best fit was obtained with CDSSTR and the resulting fitted spectrum is shown in Fig. 2.5A (dashed line) and it is seen that the fit is in good agreement with the experimental spectrum.

Table 2.3. Secondary structure of PPL determined from CD spectral analysis and theoretical prediction.

Method	α (%)	β (%)	Turns	Unordered
From CD spectral analysis				
CDSSTR	8.0	42.0	22.0	31.0
CONTINLL	9.6	34.4	23.0	33.4
SELCON3	11.6	34.0	22.6	32.6
Average	9.7	36.8	22.5	32.3
From theoretical prediction				
SOPMA	12.8	30.7	17.4	39.0
DPM	14.0	37.0	15.0	34.0
Average	13.4	33.9	16.2	36.5

The content of various secondary structures obtained from the CDSSTR analysis are: 2 % regular α-helix, 6 % distorted α-helix (giving a total α helical content of 8 %), 26 % regular β-sheet and 13 % distorted β-sheet (adding to a total of 39 % of pleated sheet structure), 22 % β-turns and 31 % unordered structures. These values along with the values obtained from the other two methods, namely CONTINLL and SELCON3 are presented in Table 2.3; the average values obtained from the

above three methods are also given in this Table. From the above data it is clear that although CDSSTR gave the best fit to the experimental spectrum, values of secondary structures obtained from the other two methods are also in good agreement. The relatively small standard deviations obtained with the average values are quite consistent with this. Overall the results obtained from analyzing the CD spectral data indicate that PPL is a predominantly β-sheet protein with a relatively small α-helical content. LAA, another phloem exudate lectin from Cucurbitaceae was reported to contain 31% α-helix [Anantharam et al., 1986]. The far UV CD spectrum of PPL recorded in the presence of 1 mM chitotriose, is nearly identical to the spectrum of native PPL, suggesting that the secondary structure of the protein is unaltered upon ligand binding. Very minor changes are seen in the near UV CD spectrum of the protein upon binding of chitotriose, indicating that the environment of the side chains of Tyr and Trp residues is also largely unaffected by ligand binding. In contrast, significant changes were observed in the near UV CD spectrum of LAA upon binding of chitooligosaccharides [Anantharam et al., 1986]. This observation, taken together with the observation that LAA contains significant α-helical content, suggests that despite very similar sugar specificity, the two lectins differ significantly in the secondary and tertiary structures.

Secondary structure prediction

The results of secondary structure prediction based on the primary structure of PPL using two different methods, namely SOPMA and DPM for PPL are listed in Table 2.3. The prediction results from SOPMA indicate that 28 amino acid residues adopt α-helical structure among the 218 residues, corresponding to 12.8%. Likewise 67, 38 and 85 residues are predicted to be involved in β-sheet, β-turn and random coil, respectively. These values correspond to about 30.7% β-sheet, 17.4% β-turn and 39% random coil. Prediction by DPM gave 14, 37, 15 and 34

percentages for α-helix, β-sheet, β-turn and random coil. Secondary structure prediction values form these two methods are comparable to experimental results form circular dichroism spectroscopy.

Thermal unfolding: DSC and CD studies

Thermal unfolding of PPL was investigated using DSC and CD spectroscopy. Thermograms of PPL in the absence and in the presence of 10 mM chitotriose are shown in Fig. 2.6.

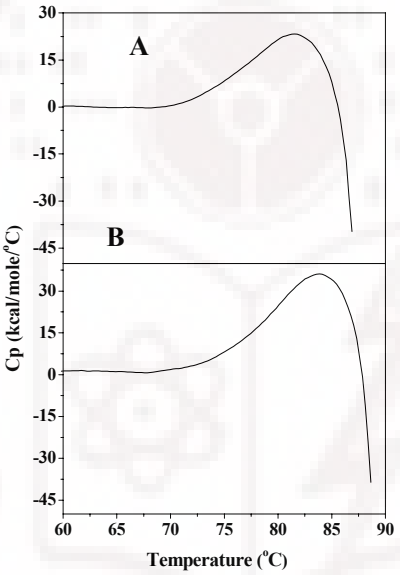


Fig. 2.6. DSC thermograms of PPL. A) PPL alone, B) PPL + 10 mM chitotriose.

DSC thermogram of PPL alone exhibits an unfolding transition centered at about 81.5 °C, which shifts to 83.8 °C in the presence of 10 mM chitotriose. It was not possible to determine the enthalpy of the transition unambiguously because the baseline at the end of the transition could not be established clearly; this could be because of aggregation of the unfolded protein as a white precipitate was observed when the sample was examined at the end of the calorimetric scan. Addition of carbohydrate ligand, chitotriose or additives such as putrescene,

hexafluoroisopropanol, spermine, and spermidine did not have any detectable effect on the aggregation of PPL during thermal denaturation (data not shown).

Far UV CD spectra of PPL recorded at various temperatures are shown in Fig. 2.7. Only minor differences are seen in the spectra recorded between 30 and 80 °C, indicating that the secondary structure of PPL is essentially unaltered in this temperature region.

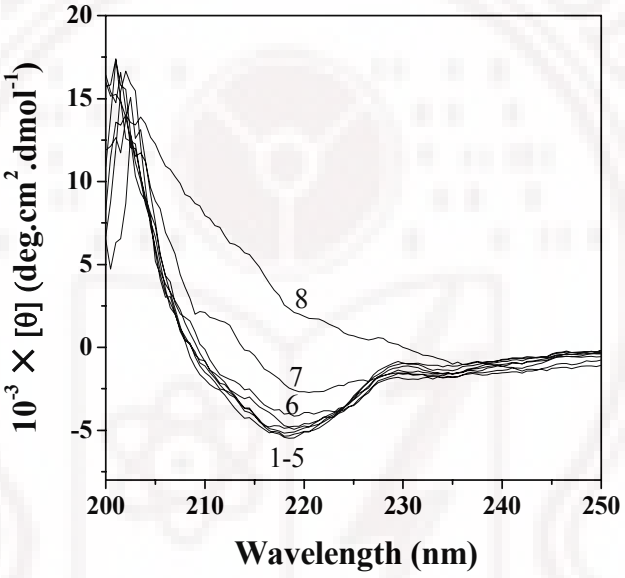


Fig. 2.7. Far UV CD spectra of PPL recorded at different temperatures. Spectra 1-8 corresponding to 30, 45, 60, 70, 75, 80, 85 and 90 °C respectively.

On the other hand, the spectrum recorded at 85 ^{o}C has a significantly (negative) lower intensity and differs substantially from that recorded at 80 °C, clearly indicating that the protein secondary structure undergoes significant changes above 80 °C. The spectrum obtained at 90 °C exhibits further decrease in the intensity indicating a near total loss of secondary structure. These results are fully consistent with the DSC data presented in Fig. 2.6. (see above).

In summary, the present study reports a rapid affinity purification method for the pumpkin phloem lectin. The lectin activity was inhibited by chitooligosaccharides, with chitohexaose being the best ligand. CD spectroscopic studies indicate that the secondary structure of PPL contains mostly β-sheet and β-turns and very little α-helix, which has been supported by theoretical prediction of the secondary structure from the amino acid sequence of the protein. DSC and CD spectroscopic studies indicate that PPL undergoes thermal unfolding at ca. 81.5 °C. Carbohydrate binding has a slight stabilizing effect on the structure of PPL.

Chapter 3

Energetics of chitooligosaccharide binding to pumpkin (*Cucurbita maxima*) phloem exudate Lectin

Summary

Binding of chitooligosaccharides [$(GlcNAc)_{2-6}$] to pumpkin phloem exudate lectin (PPL) have been studied by isothermal titration calorimetry (ITC). The titration data show that the dimeric PPL binds to two molecules of chitotriose, chitotetraose and chitopentaose with comparable binding constants of 1.8×10^5, 1.4×10^5 and 0.9×10^5 M^{-1} at 25 °C whereas chitobiose exhibits a ~100 fold lower affinity with the binding constant being 2.9×10^3 M^{-1}. However, chitohexaose shows 12 to 15 times higher affinity with an association constant of 4.16×10^6 M^{-1}. The binding reaction for [$(GlcNAc)_{2-6}$] investigated were essentially enthalpy driven with the binding enthalpy (ΔH_b) at 25 °C for the different chitooligosaccharides ranging between -27.9 and -10.25 kcal.mol^{-1}, whereas the entropic contribution to the binding reaction is negative, with the values of binding entropy (ΔS_b) being in the range of -63.3 to -18.5 cal.mol^{-1}.K^{-1} at 25 °C. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with PPL are hydrogen bonding and van der Waals' interactions. The stoichiometry of binding decreases from ca. 2.2 for chitobiose to about 1.3 for chitohexaose, suggesting the formation of higher order complexes with the higher oligomers of GlcNAc, i.e., the larger oligosaccharides probably interact simultaneously with two molecules of the protein.

Introduction

Cucurbitaceae phloem lectins are a small family of chitooligosaccharide specific lectins found in the phloem exudate of Cucurbitaceae species. They are not related to other Cucurbitaceae lectins and do not have hevein domain [Van Damme et al., 1998]. The occurrence of high heamagglutinating activity in the phloem exudate from three cucurbit species, *Cucurbita maxima, Cucumis sativus* and *Cucumis melo* has been reported [Sabnis et al., 1978]. Phloem lectins from other cucurbitaceae species, which specifically recognize chitooligosaccharides, have been well characterized [Anantharam et al., 1986, Sanadi et al., 1994]. Previously thermodynamic studies were reported on the interaction of chitooligosaccharides with *Urtica dioica* agglutinin and wheat germ agglutinin using isothermal titration calorimetry [Lee et al., 1998, Katiyar et al., 1999; Bains et al., 1992]. Calorimetric studies were also reported on the binding of the AVR4 elicitor of *Cladosporium fulvum* to chitotriose and association of havein to oligomers of *N*-acetylglucosamine [van den Burg et al., 2004; Asensio et al., 2000; Garcia et al., 1997].

Pumpkin phloem exudate lectin (PPL) is a chitooligosaccharide-specific, dimeric protein with a subunit mass of 24 kDa. Analysis of PPL cDNA sequence shows that it is a polypeptide of 218 aminoacids [Bostwick et al., 1992; 1994]. In Chapter 2 of this thesis a rapid purification method was presented for this protein using affinity chromatography on chitin as the key step. Circular dichroism spectroscopic studies indicate that its secondary structure is made up of 9.7% α-helix, 35.8% β-sheet, 22.5% β-turns and 32.3% unordered structures. Whereas DSC studies have shown that the protein undergoes a cooperative thermal unfolding at ca. 81.5 °C. In the studies reported in this Chapter, the interaction of

chitooligosaccharides with this lectin has been investigated by isothermal titration calorimetry. In order to investigate the effect of temperature on the thermodynamic parameters associated with the binding, ITC measurements were carried out at three different temperatures for different chitooligosaccharides.

Materials and methods

Materials

Pumpkin fruits were obtained from local vendors. 2-mecrcaptoethanol, chitin (from crab shells) and chitooligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, calcium chloride, di-sodium hydrogen phosphate, sodium dihydrogen phosphate and acetic acid were obtained from local suppliers and were of the highest purity available.

Pumpkin phloem exudate lectin (PPL)

The lectin from the pumpkin phloem exudate has been purified by affinity chromatography on chitin as described in Chapter 2. The affinity eluted lectin was dialyzed thoroughly against 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 10 mM β-mercaptoethanol (PBS-βME). The lectin thus obtained gave a single band in SDS-PAGE in the presence of β-mercaptoethanol. Lectin concentration was estimated according to Petersen [1977].

Isothermal titration calorimetry

The calorimetric titrations were performed at three different temperatures with a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA, USA). Briefly, 7 to 10 μl aliquots of a 2.0 to 5.5 mM chitooligosaccharide solution were added via a rotating stirrer syringe to 60-100 μM lectin solution contained in a

1.445 ml sample cell. Samples were dialyzed against 20 mM phosphate buffer at pH 7.4 containing 150 mM sodium chloride, 2% sodium azide and 10 mM β-mercaptoethenol (PBS-βME). Samples were degassed prior to loading into the cell. The additions were 4 min apart to allow the exothermic heat peak accompanying each addition to return to the baseline prior to next addition as described previously [Sultan & Swamy, 2005]. Usually the first injection was found to be inaccurate; therefore, a 1 or 2 μl injection was added first and the resultant point was deleted before the remaining data were analyzed as described below.

For a system of one set of identical binding sites, the total heat evolved (or absorbed) during the binding process at the end of the *i*th injection [Wiseman et al., 1989], Q(*i*), is given by Equation (3.1)

$$Q(i) = nP_t\Delta H_b V\{1+X_t/nP_t+1/nK_bP_t-[(1+X_t/nP_t+1/nK_bP_t)2-4X_t/nP_t]½\}/2 \qquad (3.1)$$

Where n is the number of binding sites, P_t is the total protein concentration, X_t is the total ligand concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. The heat corresponding to the *i*th injection, ΔQ (*i*), is equal to the difference between Q(*i*) and Q(*i-1*) and is given by the Equation (3.2), which involves the necessary correction factor for the displaced volume (the injection volume dV_i):

$$\Delta Q(i) = Q(i) + dV_i/2V[Q(i) + Q(i\text{-}1)] - Q(i\text{-}1) \qquad (3.2)$$

The ITC unit measures ΔQ(*i*) value for every injection. These values are then fitted to equations (1) and (2) by a nonlinear least squares method using the data analysis program Origin® [MicroCal™]. The fit process involves initial guess of n, K_b and ΔH_b which allows calculation of ΔQ(*i*) values as mentioned above for all injections and comparing them with the corresponding experimentally determined values. Based on this comparison the initial guess of n, K_b and ΔH_b is improved

and the process is repeated till no further significant improvement in the fit can be obtained. The thermodynamic parameters, ΔG^{o}_{b} and ΔS_b are calculated according to the basic thermodynamic Equations (3.3) and (3.4):

$$\Delta G^{o}_{b} = -RT\ln K_b \qquad (3.3)$$

$$\Delta G^{o}_{b} = \Delta H_b - T\Delta S_b \qquad (3.4)$$

Results

Thermodynamics of carbohydrate binding

Thermodynamic parameters associated with the binding of chitooligosaccharides to the pumpkin phloem exudate lectin (PPL) have been characterized by isothermal titration calorimetry at different temperautures in the range 15-25 °C. Results of representative calorimetric titrations obtained for the binding of chitotriose and chitopentaose to PPL are shown in Fig. 1A and 1B, respectively. In these experiments 7 μl aliquots of a 2 mM solution of the appropriate chitooliogosaccharide (chitotriose to chitohexaose) were added to a 1.445 ml sample of 60 μM PPL in the calorimeter cell at intervals of 240 seconds. In the case of chitobiose, however, 10 μl aliquots of a 5 mM concentration ligand were added to 100 μM protein in view of ~100 fold lower affinity of the protein for this ligand as compared to that for chitotriose. In these two figures the upper panels show the exothermic heat released upon binding at each injection, which decreases monotonically with successive injections until saturation is achieved. A plot of the incremental heat released as a function of chitotriose and chitopentaose to SGPL ratio is shown in the lower panels of Fig. 3.1A and 3.1B, together with a non-linear least square fit of the data to Equation (3.1). The experimental data could be fitted satisfactorily to the *'one set of sites'* model available in the Origin software

provided by the instrument manufacturer. The fits obtained for the data are shown as solid lines in the lower panels of Fig. 3.1A and 3.1B.

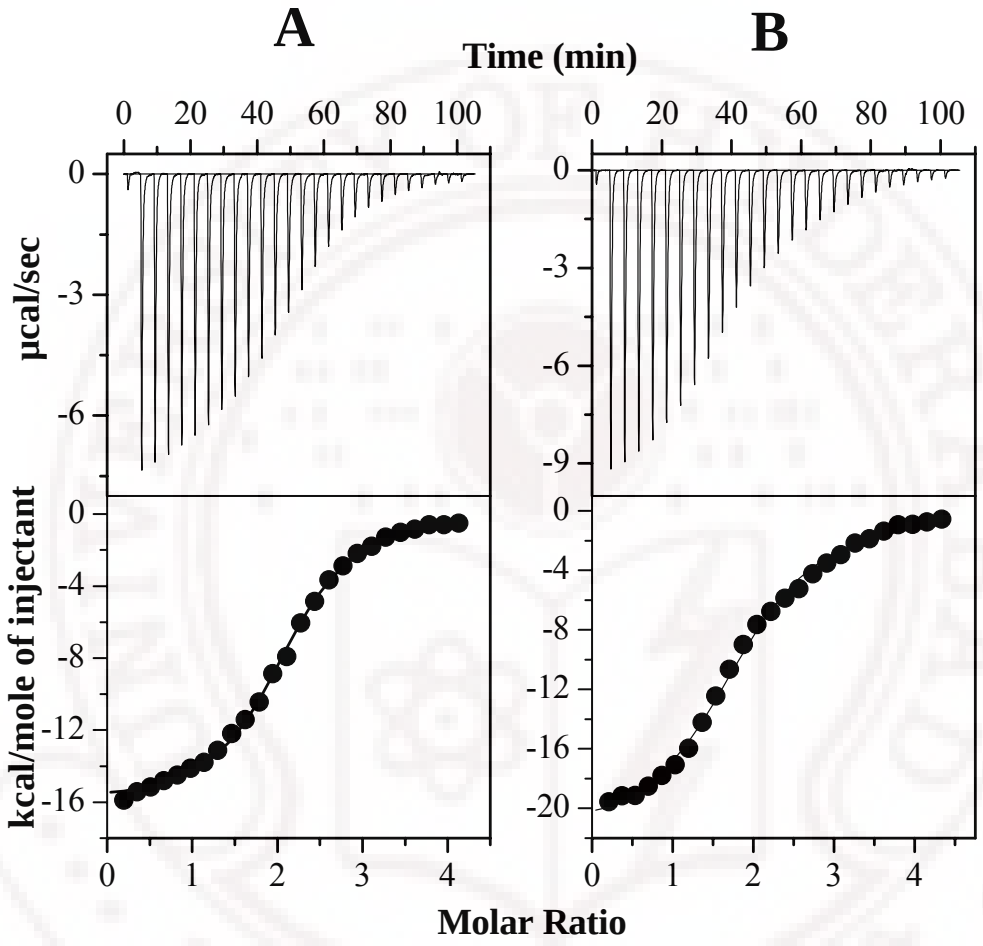


Fig. 3. 1. (A) Calorimetric titration of PPL with chitotriose and (B) with chitopentaose at 298.15 K. Upper panels show the ITC raw data obtained from 30 automatic injections of 7 µl aliquots of 2 mM chitotriose and chitopentaose into 60 µM of PPL. Lower panels show the integrated data obtained from raw data shown in the upper panels.

The fit in Fig. 3.1A yielded the values of various parameters such as number of binding sites, n = 2.06 (± 0.012); binding constant, K_b = 1.81 (± 0.1) × 10^5 M^{-1}; enthalpy of binding, ΔH_b = -16.95 (± 0.129) kcal.mol^{-1}; entropy of binding, ΔS_b = -

33.30 cal.mlo^{-1}.K^{-1} for the association of chitotriose to PPL. From the fit shown in Fig. 3.1B the corresponding parameters for the binding of chitopentaose to PPL were obtained as n = 1.5 (± 0.034); binding constant, K_b = 1.23 (± 0.18) × 10^5 M^{-1}; enthalpy of binding, ΔH_b = -24.5 (± 0.075) kcal.mol^{-1}; entropy of binding, ΔS_b = -58.9 cal.mlo^{-1}.K^{-1}. These values as well as the corresponding values obtained for the calorimetric titrations performed with the other chitooligosaccharides, namely chitobiose, chitotetraose and chitohexaose at various temperatures are listed in Table 3.1. In addition, values of Gibbs free energy $\Delta G^o_{b,}$ are also listed in Table. 3.1.

Table. 3.1. Binding constants (K_b) and thermodynamic parameters for the binding of chitooligosaccharides to pumpkin phloem lectin PPL.

Sugar	T (K)	n	K_b ×10^{-5} (M^{-1})	-ΔG° (kcal.mol^{-1})	-ΔH° (kcal.mol^{-1})	-ΔS° (cal.mol^{-1}.K^{-1})
$(GlcNAc)_2$	298	2.26	0.029	4.73	10.25	18.5
$(GlcNAc)_3$	288	2.22	3.24	7.27	15.68	29.2
	293	2.17	2.37	7.20	16.20	30.7
	298	2.06	1.81	7.03	16.95	33.3
$(GlcNAc)_4$	288	1.84	1.26	6.72	20.69	48.5
	293	1.85	1.10	6.77	21.10	48.9
	298	1.82	1.03	6.83	21.80	50.2
$(GlcNAc)_5$	288	1.59	1.65	6.88	21.63	51.2
	293	1.53	1.40	6.91	23.06	55.1
	298	1.50	1.23	6.94	24.50	58.9
$(GlcNAc)_6$	298	1.27	41.60	9.03	27.90	63.3

From the results presented in Table 3.1, it is interesting to note that the binding stoichiometry obtained for different oligosaccharides decreases with increasing ligand size (Table 3.1). When the observed stoichiometry was plotted as a function of the number of GlcNAc units in the oligosaccharide, a linear dependence was observed (Fig. 3.2).

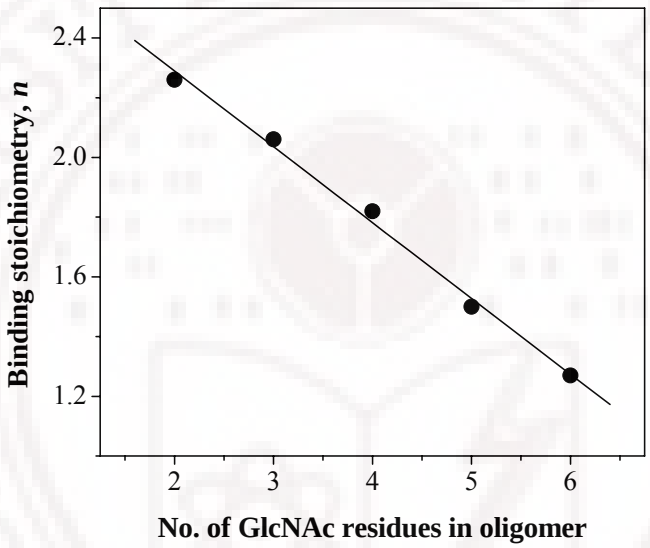


Fig. 3.2. Dependence of binding stoichiometry on the size of oligosaccharide for the binding of chitooligosaccharides to PPL. Stoichiometry (*n*) corresponds to the number of ligand molecules that bind to each molecule of PPL (dimer).

The association constants determined at 25 °C exhibit interesting trends. The K_b value increases from 2.9×10^3 M^{-1} for chitobiose to 1.81×10^5 M^{-1} for chitotriose, i.e., a 62-fold increase. However, the K_b value then decreases for chitotetraose, but increases marginally for chitopentaose, whereas a 34-fold increase is seen for the binding of chitohexaose as compared to chitopentaose.

The data presented in Table 3.1 also indicates that the enthalpy of binding and entropy of binding increase with increasing size of the chitooligosaccharides. The increase in enthalpy with increasing number of GlcNAc residues in the ligand indicates that the lectin combining site contains several subsites which interact with the individual monosaccharide units of the oligosaccharide. In order to investigate this further the contributions of the different monosaccharide units of the chitooligosaccharides to the binding enthalpy, entropy and free energy have been calculated from the thermodynamic data presented in Table 3.1, by subtracting the values corresponding to the oligosaccharide containing '(*n*-1)' monosaccharide units from the values corresponding to the oligosaccharide containing '*n*' GlcNAc residues. The results obtained are presented in Table 3.2.

Table 3.2. Change in enthalpy, entropy and free energy per additional saccharide for the binding of chitooligosaccharides to PPL.

Sugar*	*T* °C	$-\Delta\Delta H°$ (kcal.mol^{-1})	$-\Delta\Delta S°$ (cal.mol^{-1}.K^{-1})	$-\Delta\Delta G°$ (kcal.mol^{-1})
$(GlcNAc)_2$	25	10.25	18.50	4.73
$(GlcNAc)_3$	25	6.70	14.8	2.30
$(GlcNAc)_4$	25	4.85	16.9	-0.20
$(GlcNAc)_5$	25	2.70	8.70	0.11
$(GlcNAc)_6$	25	3.40	4.40	2.09

∗Values for $(GlcNAc)_2$ are in comparison for the lectin alone. Hence the $-\Delta\Delta H°$, $\Delta\Delta S°$ and $-\Delta\Delta G°$ values are for the two GlcNAc residues of the trisaccharide.

From the data presented in Table 3.2 it can be seen that the addition of the 3rd, 4th, 5th and 6th *N*-acetylglucosamine residues to chitobiose increases the enthalpy of binding by 6.7, 4.85, 2.7 and 3.4 kcal/mol, respectively. This shows that the

contributions of the 3rd, 4th and 5th GlcNAc residues towards the overall binding enthalpy decrease gradually whereas the contribution of the 6th GlcNAc residue increases slightly. However, due to larger negative contribution from the entropy of binding from the 4th GlcNAc residue, the K_b value decreases for chitotetraose as compared to chitotriose. The incremental enthalpy due to the 5th GlcNAc residue is almost nearly compensated by the incremental entropy due to it, resulting in only a marginal increase in the K_b value for chitopentaose as compared to chitotetraose. Addition of the 6th GlcNAc residue, however, leads to a slightly larger increase in the enthalpy of binding whereas the corresponding increase in the entropy of binding is relatively smaller, which leads to an approximately 34-fold increase in the association constant for chitohexaose over chitopentaose.

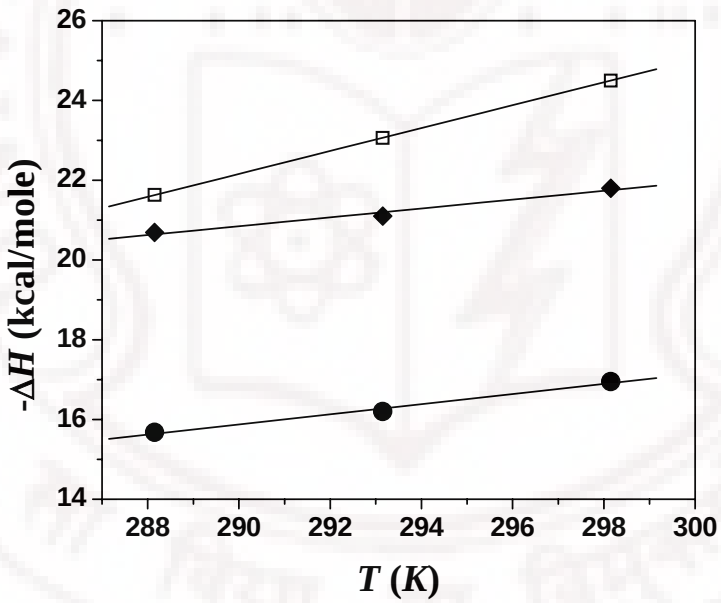


Fig. 3.3. Plot of the - ΔH_b versus T for the binding of chitotriose (•), chitotetraose (♦) and chitopentaose (□) to PPL. From the slope of linear least square fit the ΔC_p value is obtained.

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

different temperatures for chitooligosaccharides. The data presented in Table 3.1 show that the binding enthalpies for the three saccharides increase slightly with temperature. Plots of binding enthalpy versus temperature for chitotriose, chitotetraose and chitopentaose based on the ITC data obtained at three different temperatures is shown in Fig. 3.3. It is seen from this plot that ΔH_b values for the three saccharides exhibit linear dependence in the temperature range studied. Assuming ΔC_p to be temperature independent, as clearly indicated by the linearity of the ΔH_b *versus T* plots shown in Fig. 3.3, the ΔC_p values were estimated as -127, -118 and -287 $cal.mol^{-1}.K^{-1}$ for chitotriose, chitotetraose and chitopentaose, respectively, from the slope of the linear fits shown in (Fig. 3.3).

Discussion

The pumpkin phloem lectin is a homodimeric protein and its agglutinating activity is strongly inhibited by chitooligosaccharides [Read & Northcote, 1983]. Development of an affinity chromatographic purification of for this protein, characterization of its secondary structure by CD spectroscopy and investigation of its thermal unfolding by differential scanning calorimetry and CD spectroscopy are reported in Chapter 2. With the objective of investigating the binding of chitooligosaccharides to this lectin and determine the association constants for the binding as well as the associated thermodynamic parameters, isothermal titration calorimetric studies have been performed and the results obtained are discussed here.

Analysis of the thermodynamic data associated with the binding of chitooligosaccharides of different chainlengths, presented in Tables 3.1 and 3.2, show that the carbohydrate binding site of PPL is an extended one, with several subsites. Based on thermodynamic analysis of carbohydrate binding, two other phloem exudate lectins, namely *Luffa acutangula* agglutinin (LAA) and *Coccinia*

indica agglutinin (CIA), which specifically recognize chitooligosaccharides, have been shown earlier to contain extended binding sites, made up of a number of subsites. ITC studies on the interaction of *Urtica dioica* agglutinin and wheat germ agglutinin have shown that these two lectins also contain extended binding sites that are complementary to a trisaccharide and a tetrasaccharide, respectively [Bains et al., 1992; Lee et al., 1998; Katiyar et al., 1999]. Titration calorimetric studies on the binding of chitooligosaccharides to hevein also demonstrated the presence of an extended binding site in the protein [Asensio et al., 2000]. The association constants determined for PPL-chitooligosaccharide interaction (Table 3.1) are about an order of magnitude higher than the corresponding values obtained with *Urtica dioica* lectin and WGA, but are in the same range as the values obtained with LAA and CIA.

The results of calorimetric titrations presented in Table 3.1 clearly show that the dimeric PPL has two identical binding sites for chitobiose and chitotriose. However, the binding stoichiometry decreases substantially with increase in the size of the chitooligosaccharide, i.e., the number of GlcNAc residues in the oligosaccharide. This is quite clear from Fig. 3.2, which shows that the stoichiometry of binding decreases almost linearly when the number of GlcNAc residues in the chitooligosaccharide is increased, reaching a value of 1.27 ligand molecules per protein dimer. This decrease in stoichiometry suggests the formation of higher order structures, wherein some of the ligand molecules simultaneously interact with two binding sites, most likely located on two protein molecules. Thus while chitotriose interacts with only one lectin combining site, a majority of the chitohexaose molecules most likely interact with two lectin combining sites. Similar observations have been made regarding the binding of AVR4 elicitor of *Cladosporium fulvum* to chitotriose [van den Burg et al., 2004].

The binding reaction for [$(GlcNAc)_{2-6}$] investigated were essentially enthalpy driven with the binding enthalpy (ΔH_b) at 298.15 K for the different chitooligosaccharides ranging between –10.25 to –27.9 kcal.mol^{-1}, whereas the entropic contribution to the binding reaction is negative, with the value of binding entropy (ΔS_b) being -18.5 to -63.3 cal.mol^{-1}.K^{-1} (Table 3.1). The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with PPL are hydrogen bonding and van der Waals' interactions.

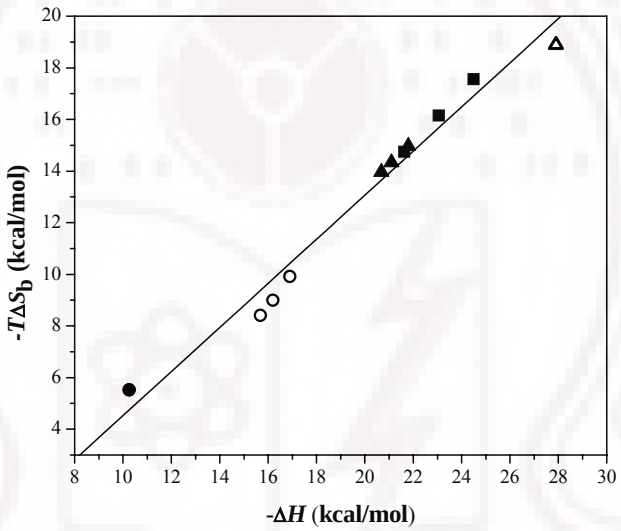


Fig. 3.4. Enthalpy–Entropy compensation plot for PPL-chitooligosaccharide interaction. The ligands investigated are: chitobiose (•), chitotriose (o), chitotetrose (▲), chitopentose (■) chitohexaose (Δ). The straight line corresponds to linear least squares fit of the data. Slope of the line is 0.86 and correlation coefficient (R) is 0.987.

Close examination of the values of ΔH_b and ΔS_b determined for different oligomers of chitin at different temperatures given in Table 3.1 indicate that the changes in enthalpy and entropy are compensatory in nature. This is clearly seen in a plot of $-\Delta H_b$ versus $-T\Delta S_b$ values corresponding to the different chitooligosaccharides at different temperatures (Fig. 3.4). The data plotted here

could be fitted to a straight line with a slope of 0.86 with a correlation coefficient (R) of 0.987. This indicates that in the temperature range (288.15 - 298.15 K) in which the measurements have been made, binding of chitooligosaccharide to PPL follows enthalpy-entropy compensation. Enthalpy-entropy compensation has been observed earlier for the interaction of a number of lectins with carbohydrates [Schwarz et al., 1993; Surolia et al., 1996; Gupta et al., 1996; Swaminathan et al., 1997; Komath et al., 2001; Sultan & Swamy, 2005b]. A widely accepted model for enthalpy-entropy compensation in protein-ligand interaction is related to the reorganization of water structure around the binding site of the protein and the carbohydrate ligand [Spohr et al., 1992; Lemieux, 1996]. Since a number of studies indicate that water molecules play a crucial role in carbohydrate binding by lectins and since ITC studies provided direct evidence for the involvement of water molecules in the interaction of mannooligosaccharides by Con A [Swaminathan et al., 1998], the enthalpy-entropy compensation observed here can be explained in terms of changes in water structure associated with carbohydrate binding.

In summary, the ITC studies reported here indicate that the pumpkin phloem exudate lectin has an extended combining site that can accommodate at least a trisaccharide (chitotriose) moiety. Most likely higher order structures are formed between PPL and larger chitooligosaccharides such as chitohexaose. Binding of chitooligosaccharides to PPL is governed by enthalpic forces with negative contribution from binding entropy. Enthalpy-entropy compensation has been observed in PPL-chitooligosaccharide interaction, underscoring the role of water structure in the binding process.

Chapter 4

Steady-state and time-resolved fluorescence studies on the chitooligosaccharide-specific lectin from the phloem exudate of pumpkin (*Cucurbita maxima*)

Summary

Fluorescence quenching and time-resolved fluorescence studies were carried out on the chitooligosaccharide-specific lectin from pumpkin (*Cucurbita maxima*) phloem exudate. The emission λ_{max} of native pumpkin phloem lectin (PPL) seen at 338 nm was red-shifted to 348 nm upon denaturation by 6 M Gdn.HCl in the presence of 10 mM β-mercaptoethanol, whereas a blue-shift to 335 nm was observed in the presence of saturating concentrations of chitotriose. The extent of quenching was maximum with the neutral molecule, acrylamide whereas the ionic species, iodide and Cs^+ led to significantly lower quenching. Binding of chitotriose did not affect the quenching profile significantly. The Stern-Volmer plot for acrylamide was linear for native PPL and upon ligand binding, but became upward curving upon denaturation, indicating that the quenching occurs via a combination of static and dynamic mechanisms. A red-edge excitation shift (REES) of 4 nm was observed for the native protein, which decreased to 3 nm upon ligand binding and further to 2 nm when denatured with 6M Gdn.HCl. In time-resolved fluorescence experiments, the decay curves could be best fit to biexponential patterns, with life times of 1.20 and 4.35 ns for native PPL. In all cases both lifetimes systematically decreased with increasing acrylamide concentrations, indicating that quenching occurs predominantly via a dynamic process.

Introduction

In studies reported in Chapter 2 of this thesis a rapid affinity method has been reported for the purification of the pumpkin (*Cucurbita maxima*) phloem exudate lectin (PPL) using chitin as the affinity matrix. Circular dichroism spectroscopic studies indicated that PPL contains predominantly β-sheets, β-turns and unordered structures and very little α-helix. DSC and CD studies have shown that the thermal unfolding of PPL is a cooperative process centered at ca. 82 °C, which shifts to slightly higher temperatures upon ligand binding. Thermodynamic studies on the interaction of chitooligosaccharides with PPL investigated by isothermal titration calorimetry have been reported in Chapter 3. These studies have shown that PPL has an extended binding site and that binding of chitooligosaccharides to this protein is enthalpically governed with negative contribution from entropic factors.

The fluorescence spectral characteristics of indole side chains of tryptophan residues in a protein are highly sensitive to their environment and can be monitored to obtain information regarding the protein structure and conformation [Lakowicz, 1999; Eftnik & Ghiron, 1981; Grinvald & Steinberg, 1976]. In a protein molecule that has both tryptophan and tyrosine residues, the Trp residues can be excited specifically by irradiating at 295 nm, which allows the study of fluorescence from the Trp residues alone [Teale, 1960]. In previous work from our laboratory the fluorescence characteristics of snake gourd seed lectin (*Trichosanthes anguina*), *Trichosanthes cucumerina* seed lectin, *Trichosanthes dioica* seed lectin, *Trichosanthes cordata* seed lectin as well as a galactose-specific lectin from *Dolichos lablab* seeds were investigated by steady-state and time-resolved fluorescence measurements [Komath & Swamy, 1999; Kenoth & Swamy, 2003; Sultan & Swamy, 2005a; Sultan et al., 2006].

In the present study we have investigated the tryptophan exposure and environment of PPL in the absence and presence of a specific carbohydrate ligand, chitotriose, and upon denaturation with 6 M Gdn.HCl, using steady state and time-resolved fluorescence quenching measurements as well as red-edge excitation shift (REES) studies. The results obtained indicate that the Trp residues are partially buried in native PPL and are not directly involved in carbohydrate binding. The cationic quencher Cs^+ is unable to quench some of the Trp residues even upon denaturation due to repulsion by positive charges on adjacent residues.

Materials and methods

Materials

Pumpkin fruits were obtained from local vegetable vendors. Chitin (from crab shells), 2-mecrcaptoethanol, acrylamide, potassium iodide, cesium chloride, and chitooligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, di-sodium hydrogen phosphate, sodium dihydrogen phosphate and acetic acid were obtained from local suppliers and were of the highest purity available.

Pumpkin phloem exudate lectin (PPL)

The lectin from the pumpkin phloem exudate has been purified by affinity chromatography on chitin as described in Chapter 2. The affinity-eluted lectin was dialyzed thoroughly against 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 10 mM β-mercaptoethanol (PBS-βME). The lectin thus obtained gave a single band in SDS-PAGE in the presence of β-mercaptoethanol. Lectin concentration was estimated according to Petersen [1977].

Steady-state fluorescence spectroscopy

All emission spectra were recorded on a Spex Fluoromax-3 fluorescence spectrometer. Slit widths of 3 and 5 nm were used on the excitation and emission monochromators, respectively. Lectin samples (~ 0.1 OD) in PBS-βME were irradiated at 295 nm to selectively excite tryptophan residues of the protein and emission spectra were collected above 305 nm. In fluorescence quenching experiments, small aliquots of the quencher from a 5 M stock solution were added to the protein sample and fluorescence spectra were recorded after each addition. The stock solution of iodide ion contained 0.2 mM sodium thiosulphate to prevent the formation of triodide (I_3^-). Fluorescence spectra were corrected for volume changes before further analysis of quenching data. All measurements were performed at room temperature and carried out in duplicate, which showed high reproducibility and the average results have been reported. Red-edge excitation shift (REES) experiments were performed by varying the excitation wavelength between 280 nm and 307 nm and the emission spectra were recorded between 320 and 450 nm [Anbazhagan et al., 2008]. For REES studies the excitation and emission slits were set to 2 and 3 nm, respectively.

In order to correct the fluorescence intensity values for inner filter effect, the absorbance at emission and excitation wavelengths of the experimental solutions were measured. Correction was made using Equation (4.1) [Lakowicz, 1999]:

$$F_{corr} = F_{obs} \times \text{antilog}\,[\{OD_{ex} + OD_{em}\}/2] \qquad (4.1)$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} is the observed fluorescence intensity and OD_{ex} and OD_{em} are the absorbance intensities of the solution at the excitation and emission wavelengths, respectively.

Time-resolved fluorescence studies

Fluorescence lifetime measurements were performed on an IBH-5000 single photon counting spectrofluorimeter equipped with a NanoLED excitation source and a cooled microchannel plate photomultiplier tube from Hamamatsu (Model R0839U-50). The time resolution of the spectrometer was ~50 ps. Protein samples in PBS-βME (OD_{280nm} < 0.1) were exited at 281 nm and emission was monitored at 338 nm for native PPL and at 348 nm for PPL in the presence of 6M Gdn.HCl. All experiments were performed using excitation and emission slits with a nominal bandpass of 12 nm or less. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. The signal/noise ratio was optimized by collecting at least 5000 photon counts in the peak channel. The fluorescence decay curves thus obtained were analyzed by a multiexponential iterative fitting program supplied by IBH.

Results

Quenching of the intrinsic fluorescence emission of PPL

Quenching of the intrinsic fluorescence of PPL was investigated using a neutral quencher (acrylamide), a cationic quencher (cesium ion) and an anionic quencher (iodide ion). Quenching studies were carried out with PPL under native conditions, upon binding of chitooligosaccharides and upon denaturation with 6 M Gdn.HCl. The emission spectra of the native protein recorded in the absence and presence of increasing concentrations of acrylamide, iodide ion and cesium ion are shown in Fig. 4. 1A, B and C, respectively. In these figures spectrum 1 corresponds to the lectin alone, while the spectra numbered 2-20 correspond to those recorded in the presence of increasing concentrations of the quencher. The spectra in Fig. 4.1 also show that the emission λ_{max} of native PPL is seen at 338 nm. Binding of chitotriose

results in a blue shift in the emission maximum to 335 nm whereas a red shift to 348 nm is seen upon denaturation with 6 M Gdn.HCl (spectra not shown). An observation of the spectra in Fig. 4.1 shows that the extent of quenching by acrylamide is higher than that due to iodide ion which in turn is significantly higher than the extent of quenching by cesium ion.

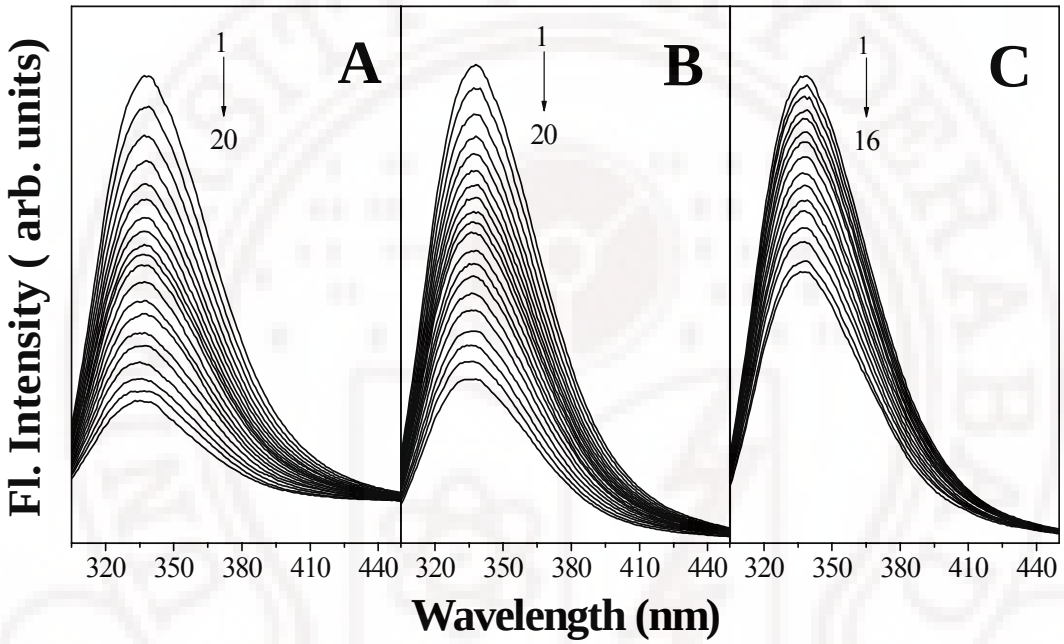


Fig. 4.1. Fluorescence spectra of PPL in the absence and presence of various quenchers. (A) acrylamide, (B) iodide ion, and (C) cesium ion. In each panel spectrum 1 corresponds to PPL alone and spectra 2-20 in A and B, and 2-16 in C correspond to increasing concentrations of the respective quencher.

The extent of quenching observed in each case, at a resultant quencher concentration of 0.5 M, is shown in Table 4.1. The data show that quenching by acrylamide (75.4%) is significantly higher than that by I^- (62.5%) and Cs^+ (34.6%), clearly indicating that some of the Trp residues are not accessible to the ionic quenchers, which cannot penetrate into the protein interior.

Table 4.1. Extent of intrinsic fluorescence quenching of PPL by different quenchers and quenching parameters. The final quencher concentration in each case was 0.5 M.

Quencher	%	K_{SV1}	$K_{q1} \times 10^{-9}$	f_a (%)	K_a
Acrylamide					
Native	75.4	6.28	1.79	97.6	6.58
Denatured	84.5	---	---	99.2	7.82
With 1mM chitotriose	78.3	6.90	1.65	98.2	6.81
With 1 mM chitotetraose	76.9	6.31	---	97.7	6.20
With 1 mM chitopentaose	75.8	6.02	---	95.4	6.36
Iodide					
Native	62.5	3.24	0.92	80.0	6.02
With 1 mM chitotriose	64.5	3.58	0.86	85.0	5.78
Denatured	63.2	3.38	1.07	92.8	3.89
Cesium ion					
Native	34.6	0.99	0.28	55.4	3.02
With 1 mM chitotriose	38.1	1.22	0.29	54.2	3.06
Denatured	32.8	1.05	0.33	59.0	1.98

Further, between the two ionic quenchers the extent of quenching is more with iodide as compared to Cs^+, which may be attributed to the lower quenching efficiency of Cs^+ as compared to I^- or due to the presence of positively charged residues near one or more tryptophan residues or a combination of both. The extent of quenching observed with Cs^+ did not change significantly under denaturing conditions. The percentage of quenching observed with acrylamide for PPL under native conditions and in the presence of different chitooligosaccharides (Table 4.1)

is comparable, suggesting that tryptophan residues are probably not involved in the carbohydrate binding site.

Analysis of the steady-state fluorescence quenching

The steady-state fluorescence quenching data were analyzed using Stern-Volmer Equation (4.2) as well as modified Stern-Volmer Equation (4.3) [Lehrer, 1971].

$$F_o/F = 1 + K_{SV}[Q] \qquad (4.2)$$

$$F_o/\Delta F = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \qquad (4.3)$$

where F_o and F are the relative fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$ is the quencher concentration, K_{SV} is Stern-Volmer quenching constant of the lectin for a given quencher, ΔF (= F_o-F) is the change in fluorescence intensity at any point in the titration, f_a is the fraction of total fluorescence intensity that is accessible to the quencher and K_a is the corresponding Stern-Volmer quenching constant for the accessible fraction of the fluorophores. Equation (4.2) indicates that the slope of a plot of F_o/F versus $[Q]$ is equal to the Stern-Volmer constant (K_{SV}), whereas Equation (4.3) shows that the slope of a plot of $F_o/\Delta F$ versus $[Q]^{-1}$ (modified Stern-Volmer plots) gives the value of $(K_a f_a)^{-1}$ and its Y-intercept gives the value of f_a^{-1}.

Stern-Volmer plots for the quenching of PPL with all the three quenchers used in this study under different conditions are shown in Fig 4.2. The Stern-Volmer plots for acrylamide quenching of the protein under native conditions and in the presence of chitotriose are linear indicating that the quenching mechanism is dynamic in nature and that all the tryptophan residues that are quenched by this neutral quencher have comparable accessibility. From the slopes of these plots the corresponding Stern-Volmer constants, K_{SV}, were obtained and listed in Table 4.1.

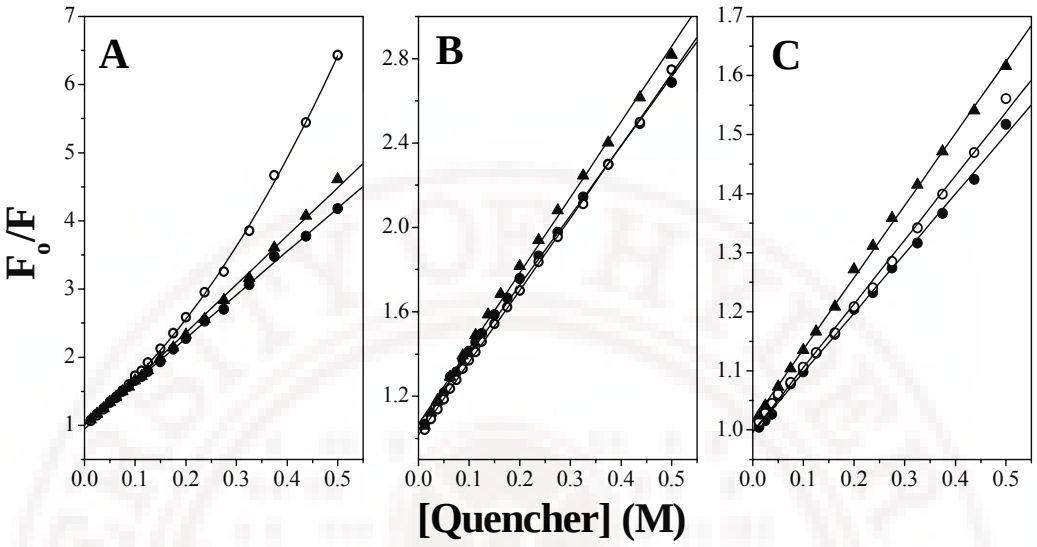


Fig. 4.2. Stern-Volmer plots for fluorescence quenching of PPL with (A) acrylamide, (B) iodide ion, (C) cesium ion. (●) native protein, (○) upon denaturation with 6 M Gdn.HCl, (▲) in the presence of chitotriose.

Interestingly, for the denatured lectin the Stern-Volmer plot exhibits upward curvature (Fig. 4.2A), suggesting that the quenching process involves both dynamic and static components. The static mechanism involves complex formation, while dynamic mechanism involves collisions with acrylamide during the lifetime of tryptophan in excited state. In such cases the quenching data can be analysed by Equation (4.4), which allows resolution of the static and dynamic components [Lakowicz, 1999]:

$$F_o/F = (1+K_{SV}[Q])(1+K_S[Q]) \qquad (4.4)$$

where K_{SV} is the Stern-Volmer (dynamic) quenching constant, K_S is the static quenching constant and $[Q]$ is the quencher concentration. The dynamic component of the quenching can be determined by fluorescence lifetime measurements, according to the following equation [Lakowicz & Weber, 1973]:

$$\tau_o/\tau = 1 + K_{SV}[Q] \qquad (4.5)$$

where τ_o is the average lifetime in the absence of the quencher and τ is the average lifetime in the presence of the quencher at a concentration, [*Q*]. The average lifetimes were determined from the time-resolved fluorescence measurements obtained at different concentrations of acrylamide by using Equation (4.7) (see below). These values were then used to obtain a plot of τ_o/τ versus [*Q*] (Fig. 4.3A) and the slope of this plot yielded the K_{SV} value for acrylamide quenching as 3.86 M^{-1}. The static quenching constant, K_S was then obtained by incorporating this value in Equation (4.4) as 2.46 M^{-1}. The fit, given by the solid curved line in Fig. 4.2A, shows that the data are in excellent agreement with Equation (4.4). The static and dynamic components of quenching can also be resolved by analyzing the equilibrium quenching data in the following way [Lakowicz, 1999].

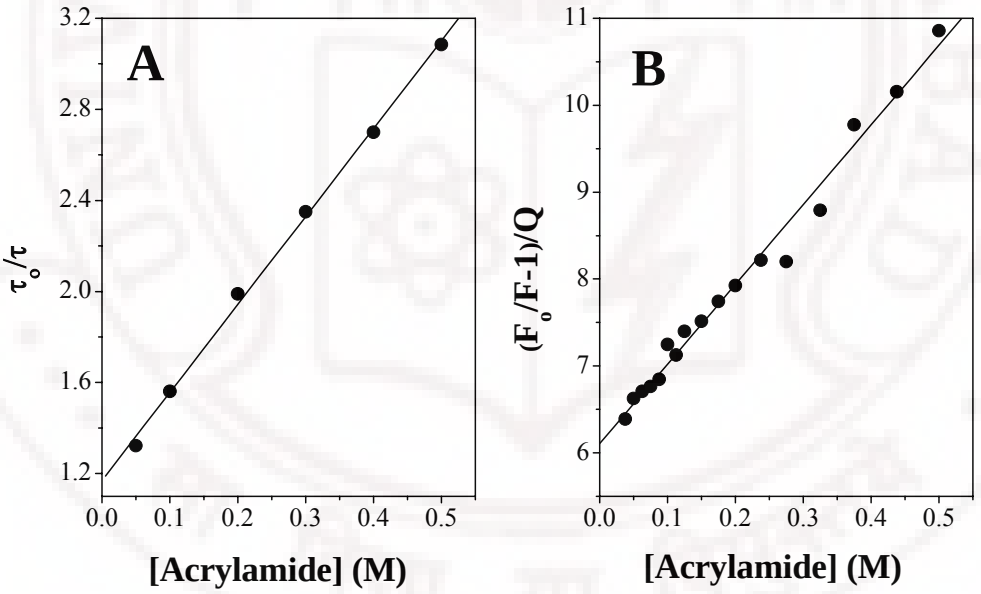


Fig. 4.3. Resolution of static and dynamic components of acrylamide quenching of PPL under denaturing conditions. (A) Plot for τ_o/τ versus [*Q*]. (B) A plot of $(F_o/F\text{-}1)/[Q]$ versus [*Q*], where [*Q*] is acrylamide concentration.

A plot of $[F_o/F - 1]/[Q]$ versus $[Q]$ yields a straight line with an intercept (I) of (K_{SV}+K_S) and a slope (S) of $K_{SV}K_S$ [Lakowicz, 1999]. Such a plot for acrylamide quenching of PPL is shown in Fig. 4.3B. The slope and intercept values obtained from this plot are 9.17 M^{-1} and 6.11 M^{-1}, respectively.

Value of the static quenching constant K_S can then be obtained by incorporating the values of S and I in the quadratic Equation (4.6):

$$K_S^2 - K_S I + S = 0 \qquad (4.6)$$

From this analysis, the static quenching constant, K_S and dynamic quenching constant, K_{SV} were resolved as 2.67 M^{-1} and 3.43 M^{-1}, respectively. These values are in good agreement with the values of 2.46 M^{-1} and 3.86 M^{-1} for K_S and K_{SV}, respectively, obtained using the time-resolved measurements and Equation (4.4), presented above.

Stern-Volmer plots obtained for quenching by I^- and Cs^+ were linear under all conditions, indicating that these ionic quenchers also see a homogeneous population of fluorescent tryptophan residues of the lectin. From the slopes of these plots the corresponding Stern-Volmer constants were obtained and listed in Table 4.1. Additionally, the corresponding bimolecular quenching constants, k_q (=K_{SV}/τ_o, where τ_o is the average lifetime of fluorescence decay) were also calculated and listed in Table 4.1.

Modified Stern-Volmer plots obtained with all the three quenchers are shown in Fig. 4.4. From the Y-intercepts of these plots, f_a, the fraction of accessible Trp residues in each case was determined and using these values and the values of the slopes of the linear modified Stern-Volmer plots the corresponding quenching constants, K_a, were calculated using Equation (4.3). These values are also given in Table 4.1. It can be clearly seen from the data that the Trp residues are almost completely accessible to this neutral quencher under all conditions. Iodide ion is

able to access about 80% of the Trp fluorescence under native conditions which increases to about 85% upon binding of chitotriose whereas denaturation further increases the accessible fraction of fluorescence to 92.8%. For cesium ion the accessibility is about 55% with the native protein as well as in the presence of chitotriose, which increases only marginally to ca. 59% upon denaturation with 6 M Gdn.HCl.

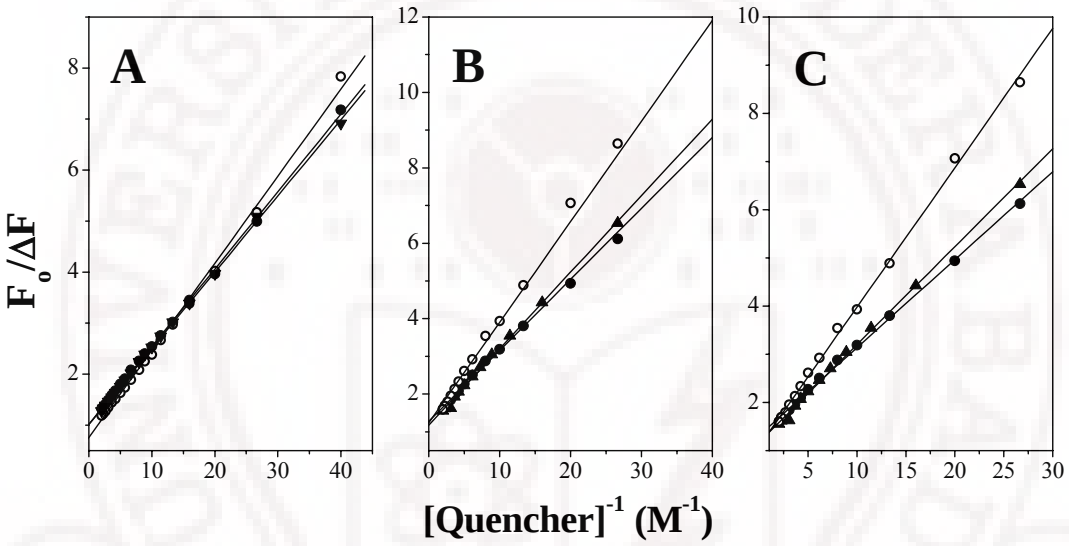


Fig. 4.4. Modified Stern-Volmer plots for the quenching of the intrinsic fluorescence of PPL with (A) acrylamide, (B) iodide ion, (C) cesium ion. (●) Under native conditions, (○) upon denaturation with 6 M Gdn.HCl, (▲) in the presence of chitotriose.

Red-edge excitation shift studies

The shifts in the emission maxima of PPL as a function of excitation wavelength are shown in Fig. 4.5. Upon excitation at 280 nm, the emission maximum of PPL under native conditions, in the ligand-bound state (in the presence of 1 mM chitotriose) and upon denaturation is seen at 338, 335 and 348 nm, respectively. As the excitation wavelength is changed from 280 nm to 307 nm, these emission

maxima are red-shifted to 342, 338 and 350 nm, respectively, which correspond to a REES of 4 nm for native PPL, 3 nm for ligand bound protein and 2 nm for the denatured lectin.

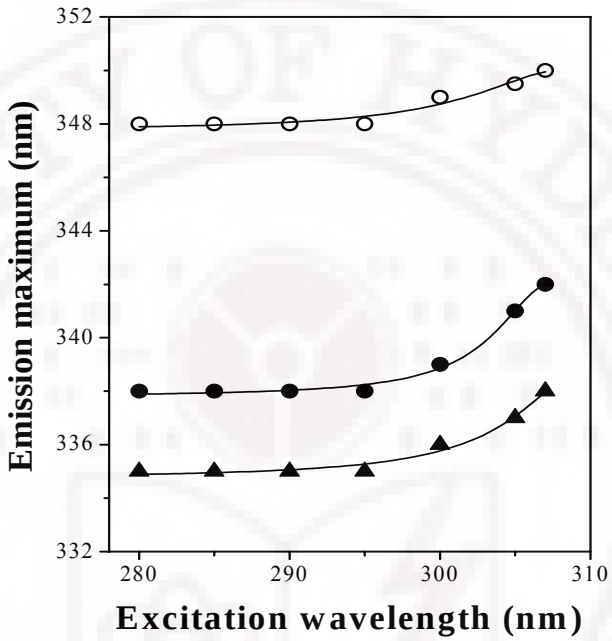


Fig. 4.5. Effect of changing excitation wavelength on the emission maximum of PPL. (●) Under native conditions, (O) upon denaturation with 6 M Gdn.HCl, (▲) in the presence of 1 mM chitotriose.

Fluorescence decay and excited state lifetimes

The fluorescence decay curves of PPL in the native state, in the presence of chitotriose, and upon denaturation, obtained from time-resolved measurements, are given in Fig. 4.6. In all cases the decay profiles could be best fitted to a biexponential function ($\chi^2 \leq 1.6$). Mono-exponential fits gave significantly larger 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 PPL, the two lifetimes (τ_1 and τ_2) were obtained as 1.2 and 4.35 ns, respectively.

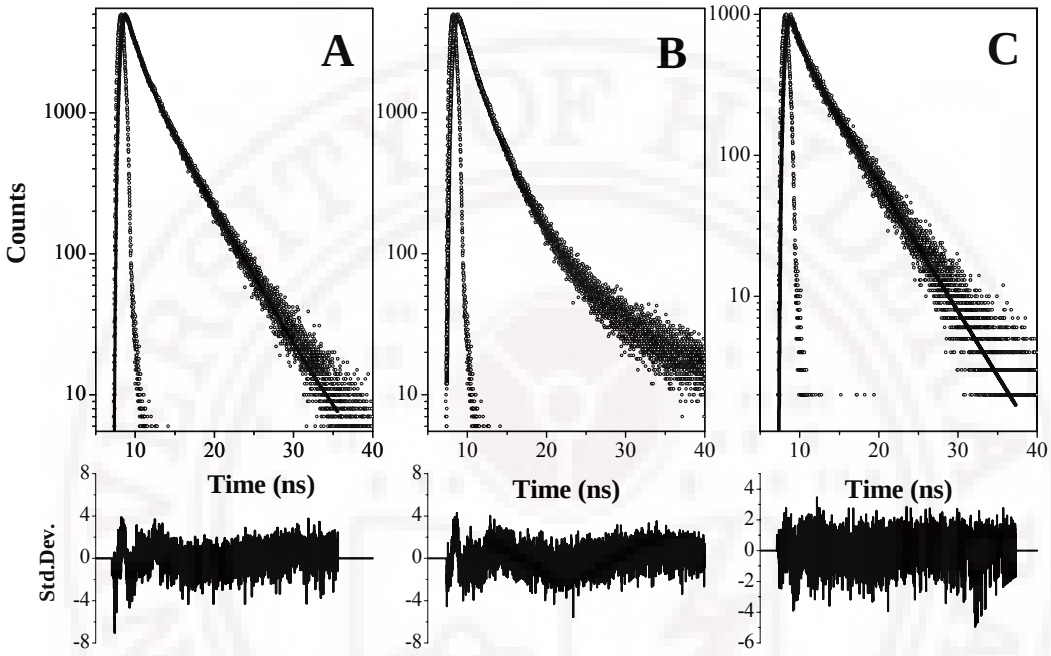


Fig. 4.6. Time-resolved fluorescence decay profiles of PPL. (A) Under native conditions, (B) upon denaturation with 6 M Gdn.HCl, (C) in the presence of 1 mM chitotriose. The solid lines correspond to the nonlinear least square fit of the experimental data to biexponential function. The lower panels in A, B and C represent the residuals.

These values together with the corresponding values obtained under different conditions (in the presence of chitotriose, upon denaturation and in the presence of different concentrations of acrylamide) are listed in Table 4.2. Both τ_1 and τ_2 showed a systematic decrease with increasing acrylamide concentration in all cases, clearly indicating that the dominant contribution by the dynamic quenching mechanism for this quencher, although presence of some static component is suggested by the upward curving Stern-Volmer plots (Fig. 4.2A).

Table 4.2. Lifetimes of fluorescence decay of PPL and the corresponding weighting factors determined under different conditions from time-resolved fluorescence measurements. The average lifetimes τ and <τ> were calculated using Equations (4.3) and (4.4).

Acrylamide Conc. (mM)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	τ	<τ>	χ^2
Native PPL with acrylamide at different concentrations (μM)							
0	0.57	1.20	0.43	4.35	2.55	3.51	1.41
50	0.61	1.06	0.39	3.52	2.02	2.73	1.40
100	0.65	1.00	0.35	3.14	1.75	2.34	1.47
200	0.75	0.94	0.25	2.80	1.40	1.87	1.50
300	0.74	0.83	0.26	2.48	1.26	1.67	1.40
400	0.76	0.80	0.24	2.32	1.17	1.53	1.44
500	0.77	0.72	0.23	2.12	1.04	1.38	1.58
Denatured PPL with acrylamide at different concentrations (μM)							
0	0.81	1.87	0.19	5.13	2.49	3.15	1.40
50	0.67	1.23	0.33	3.21	1.88	2.34	1.33
100	0.68	1.07	0.32	2.71	1.60	1.97	1.30
200	0.77	0.92	0.23	2.36	1.25	1.55	1.38
300	0.82	0.78	0.18	2.18	1.06	1.28	1.52
400	0.88	0.73	0.12	2.33	0.92	1.25	1.60
500	0.90	0.65	0.10	2.22	0.81	1.08	1.68
Native PPL + 1 mM chitotriose with acrylamide at different concentrations (μM)							
0	0.46	1.00	0.54	4.74	3.02	4.17	1.10
50	0.47	0.97	0.53	3.72	2.43	3.20	1.04
100	0.48	0.72	0.52	3.03	1.92	2.61	1.07
200	0.50	0.74	0.50	2.37	1.56	1.98	1.10
300	0.53	0.68	0.47	2.00	1.30	1.64	1.08
400	0.55	0.63	0.45	1.70	1.13	1.35	1.03
500	0.58	0.60	0.42	1.60	1.02	1.26	1.05

Analysis of lifetime decay profiles also yielded weighting factors α_1 and α_2 which represent the relative contributions of each component to the total fluorescence intensity. For native PPL the component with shorter lifetime of 1.2 ns (α_1) contributed 57% of the total fluorescence intensity while the component with longer lifetime of 4.35 ns (α_2) contributed 43% of the total intensity. These values together with the corresponding values obtained under different conditions are also listed in Table 4.2. In the presence of acrylamide, the relative contribution of α_1 was found to increase whereas the contribution of α_2 steadily decreased with increasing quencher concentration. Upon ligand binding α_1 increases moderately with increasing quencher concentration while α_2 decreases correspondingly. In the denatured state α_1 was found to be about 4 to 5 fold larger than α_2. The average lifetimes of fluorescence decay for PPL under different conditions were calculated from the data shown in Table 4.2 using Equations (4.7) and (4.8) [Lakowicz & Weber, 1973; Grinvald & Steinberg, 1974]:

$$\tau = \sum_i \alpha_i \tau_i / \sum_i \alpha_i \qquad (4.7)$$

$$\langle\tau\rangle = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i \qquad (4.8)$$

where τ and $\langle\tau\rangle$ are the average fluorescence lifetimes estimated by the two different approaches. For native PPL, τ and $\langle\tau\rangle$ were obtained as 2.55 and 3.51 ns, respectively. These values are also presented in Table 4.2 along with the corresponding values obtained under different conditions.

Discussion

In this chapter, the exposure and microenvironment of the indole side chains of tryptophan residues in PPL have been investigated by quenching the protein intrinsic fluorescence by acrylamide, iodide ion and cesium ion. Quenching by these species occurs primarily through physical interaction between the quencher and the fluorophore; hence it is directly dependent on the extent to which the

quencher can access the fluorophore in the protein. The neutral quencher, acrylamide can penetrate into the relatively hydrophobic interior of the folded protein and can quench even partially buried tryptophan residues, whereas charged quenchers such as I^- and Cs^+ can quench only the surface exposed tryptophan residues. Therefore, by using all the three quenchers mentioned above,considerable insights can be derived on the environment and exposure of tryptophan residues in proteins.

The primary structure of PPL derived from its cDNA sequence shows that there are 8 tryptophan residues in the PPL subunit containing 218 amino acid residues [Bostwick et al., 1992]. The emission maximum of native PPL, which is seen at 338 nm (Fig. 4.1), strongly suggests that a majority of these tryptophan residues are considerably exposed to the aqueous environment because Trp residues that are buried in the hydrophobic interior of folded proteins have emission maxima below 320 nm [Lakowicz, 1999]. However, the Trp residues of PPL are not fully exposed to the aqueous medium as Trp residues of completely denatured proteins, which are fully exposed to the aqueous medium exhibit fluorescence emission spectra centered around 350 nm [Lakowicz, 1999]. The large red shift in the emission maximum of the protein to 348 nm upon incubation with 6 M Gdn.HCl (Fig. 4.1) indicates greater exposure of the tryptophan residues to the aqueous environment and suggests that the protein is completely unfolded. This is also evident from the increase in the extent of quenching observed with different quenchers in the presence of 6 M Gdn.HCl (see Table 4.1). Similar results were obtained earlier with several seed lectins from Cucurbitaceae species, namely those from *Trichosanthes anguina* (snake gourd), *T. cucumerina* and *T. dioica* [Komath & Swamy, 1999; Kenoth & Swamy, 2003; Sulatan & Swamy, 2005a].

The extent of quenching is not altered significantly in the presence of chitooligosaccharides (Table 4.1). This suggests that tryptophan residues are not

directly involved in carbohydrate binding by PPL because if they were to directly interact with the bound sugar, it would be expected that ligand binding would at least partially shield the Trp residues that are interacting with the sugar, resulting in a decrease in accessibility of the indole side chains of those Trp residues, leading to a decrease in the extent of quenching. This is in agreement with the results of circular dichroism studies on PPL, where no structural changes were observed in the secondary and tertiary structures of PPL upon ligand binding [Chapter 2]. However, the blue shift in the emission λ_{max} from 338 nm to 335 nm resulting from the binding of chitotriose clearly indicates that sugar binding leads to a tightening of the protein structure which makes the environment of the tryptophan residues more hydrophobic (i.e., less accessible to the aqueous medium). For native PPL (Table 4.1), fluorescence quenching by iodide and cesium ions is comparatively small, clearly indicating that some of the Trp residues are not accessible to the ionic quenchers, which cannot penetrate into the protein interior. Interestingly, even after incubating the lectin in 6 M Gdn.HCl the fluorescence quenching parameters obtained with these ionic quenchers are similar to those obtained with the native protein. This is rather surprising because the red shift of the emission λ_{max} to 348 nm after incubation with the denaturant clearly indicates the protein is almost completely denatured. The possibility of disulfide bonds being responsible for some local order does not exist as the studies were conducted in buffer containing 10 mM β-mercaptoethanol. This suggested that the ionic quenchers are probably unable to access some of the fluorescent tryptophan residues because of the presence of charged amino acid residues in close proximity. A close examination of the primary structure of PPL [Bostwick et al., 1992] showed that tryptophan residues W-87 and W-89 are adjacent to a lysine residue (K-88) and W-199 precedes two positive residues, K-200 and R-201. Since Cs^+ will be repelled by the positively charged side chains of these neighbouring residues, it is clear that even

upon denaturation this cationic quencher is unable to access all the Trp residues in PPL; hence the accessible fraction of the total fluorescence (f_a) for cesium ion is only 59%. On the other hand, there are no acidic residues adjacent to any of the Trp residues of the lectin, although three Trp residues (W-80, W-105 and W-168) have acidic residues as next nearest neighbours. It is likely that these residues may have some influence on the ability of I^- to quench the Trp residues. Accordingly, iodide ion is able to access about 92.8% of the total fluorescence in the denatured PPL, whereas the neutral acrylamide can access nearly all the fluorescence intensity (f_a = 99.2%).

The Stern-Volmer plots for acrylamide are monophasic for PPL under native conditions as well as in the presence of chitooligosaccharides, suggesting that the quenching mechanism is dynamic in nature. But for the denatured protein this plot shows upward curvature (Fig. 4.1A), indicating that the quenching process involves both static and dynamic components. Such upward curving profiles have been reported for several other proteins [Eftink & Ghiron, 1976a, b]. From the fluorescence quenching data static and dynamic quenching constants obtained are 2.67 and 3.43 M^{-1} for acrylamide under denaturing conditions. The dynamic quenching constant value (3.43 M^{-1}) obtained from fluorescence quenching measurements for the denatured protein is in good agreement with the value of dynamic quenching constant obtained from time resolved measurements (3.86 M^{-1}).

In red edge excitation shift studies a REES value of 4 nm was obtained for the tryptophan residues of PPL in the native state. This indicates that tryptophan residues are localized in a microenvironment of restricted mobility, and that the regions surrounding the Trp residues in this protein offer considerable restriction to the reorientational motion of the water dipoles around the excited state tryptophans. Ligand bound PPL exhibits a REES of 3 nm which is slightly lower as compared to the REES value observed with the native protein, indicating that the dynamical

environment of solvent water molecules around the tryptophan residues becomes less restricted upon ligand binding. Since the emission λ_{max} of the protein decreases upon ligand binding, indicating a decrease in the polarity of the Trp environment, it is likely that some of the water molecules near the Trp residues are removed due to a possibly tighter packing of the protein when the ligand binds to it. Such removal of water molecules would be expected to result in a decrease of the REES value.

In the presence of 6 M Gdn.HCl the REES value decreases to 2 nm, suggesting that while denaturation results in a decrease in the restrictions imposed on the relaxation of the solvent water molecules in the vicinity of some of the Trp residues of PPL, those that are in contact with some other Trp residues still appear to experience significantly restricted relaxation. This is rather unexpected because even the disulfide bonds are reduced in the protein because all the fluorescence measurements were performed in the presence of 10 mM β-mercaptoethanol. In a number of denatured proteins the Trp fluorescence does not exhibit excitation wavelength dependence, because the solvent relaxation around the Trp residues in the denatured state is rather fast [Guha et al., 1996]. It is pertinent to mention that in the case of the major protein from bovine seminal plasma, PDC-109 although the REES (4 nm) did not decrease in the presence of denaturant, reduction of the disulfide bonds resulted in a decrease of the REES to 0.5 nm [Anbhazagan et al., 2008]. On the other hand, erythroid spectrin exhibited REES values of 4 nm under native conditions, which decreased to 3 nm upon denaturation [Chattopadhyay et al., 2003]. The present results indicate that at least some of the trypotophan residues in PPL are in an intermediate state.

Since time-resolved fluorescence studies yielded two different lifetimes for the Trp residues of PPL under different conditions, it appears that the Trp residues of PPL may fall into two categories, one group which has a shorter decay time and the second group which has a longer decay time. Although it is well known that

tryptophan alone in aqueous solution yields two different lifetimes, it is unlikely that all the 8 tryptophan residues of the protein will behave similarly with respect to fluorescence decay, especially since each of these Trp residues has different amino acid residues as its immediate neighbours, as seen from the primary structure of PPL [Read & Northcote, 1983]. It might seem that this is in contradiction to the observations made from the steady-state fluorescence quenching, where linear Stern-Volmer plots were obtained with acrylamide. However, it must be noted that while linear Stern-Volmer plots indicate that the Trp residues are physically accessible to acrylamide, the presence of different charged residues near different Trp residues (as discussed above) may not have any bearing on the quenching ability of this neutral quencher.

Time-resolved fluorescence studies carried out in the presence of different concentrations of the quencher show that the lifetime of fluorescence decay decreases for both the components, suggesting that acrylamide quenching of Trp fluorescence in PPL is predominantly a collisional process. Acrylamide is generally known to quench via a collisional mechanism [Eftink & Ghiron, 1975; Rayner & Szabo, 1978] although at high concentrations weak interaction (complexation) occurs with some proteins [Punyiczki et al., 1993]. Such interaction with acrylamide was also observed with the *T. cucumerina* seed lectin in the denatured state [Kenoth & Swamy, 2003].

In summary, the fluorescence studies reported in this chapter demonstrate that tryptophan residues of PPL are partially exposed to aqueous environment. Binding of chitooligosahharides, which are specifically recognized by the lectin, does not significantly alter the quenching pattern with any of the quenchers used, indicating that Trp residues are probably not directly involved in carbohydrate binding. Quenching data obtained with I^- and Cs^+ indicate that charged amino acids present

in the vicinity of some of the tryptophan residues reduce the accessibility of these charged quenchers to the fluorophores.

Chapter 5

Purification and physico-chemical characterization of a new chitooligosaccharide-specific lectin from snake gourd (*Trichosanthes anguina*) phloem exudate

Summary

A new chitooligosaccharide-specific lectin has been purified to homogeneity from the phloem exudate of snake gourd (*Trichosanthes anguina*) by affinity chromatography on chitin. The lectin, christened snake gourd phloem-exudate lectin (SGPL), agglutinated rabbit erythrocytes and the agglutination activity could be inhibited only by oligomers of *N*-acetylglucosamine among a battery of mono- di- and oligosaccharides. The inhibitory potency of chitooligosaccharides increased with increase in size from chitobiose to chitohexaose. SGPL yielded a single band on native PAGE, whereas in SDS-PAGE in the presence of β- mercaptoethanol it gave two bands of comparable intensity corresponding to masses of 48 and 53 kDa, suggesting that it is a heterodimer. MALDI-TOF mass spectrometric analysis supported the above observation and gave the molecular weights of the two subunits as 50,597 and 55,399 Daltons. Analysis of the far UV CD spectrum of SGPL showed that the secondary structure of the lectin consists of 9% α-helix, 39.6% β-sheet, 20.4% β-turns and 32.4% unordered structures. Saccharide binding did not significantly affect the secondary and tertiary structures of SGPL. The agglutination activity of the lectin was unaffected between pH 5 and pH 10, but decreased steeply below pH 5.0. The lectin activity was mostly unaffected in the temperature range 4-50 °C, but decreased steadily between 60-80 °C, which has been correlated with structural changes of the protein as seen in circular dichroism spectroscopy and unfolding temperature of the protein monitored by differential scanning calorimetry.

Introduction

In chapters 2, 3 and 4 of this thesis, the purification, physico-chemical characterization, thermodynamic analysis of carbohydrate binding to pumpkin phloem exudate lectin (PPL) have been reported. In addition the exposure and accessibility of the tryptophan residues of this protein have been investigated by fluorescence quenching, time-resolved fluorescence and red-edge excitation shift (REES) studies. These studied have fulfilled some of the objectives indicated in chapter 1.

In this chapter, the purification and characterization of a new chitooligosaccharide-specific lectin from snake gourd (*Trichosanthes anguina*) phloem exudate (SGPL) are reported. The affinity purified SGPL shows a single band on native PAGE and two bands with comparable intensity on SDS-PAGE, indicating that it is a heterodimer. The hemagglutination activity of this lectin could be inhibited by chitooligosaccharides with chitohexaose being the best ligand, suggesting that the lectin has an extended combining site. Circular dichroism spectral analysis of SGPL shows that secondary structure of this lectin consists predominantly β-sheet with relatively less α-helical content. Temperature dependent CD spectroscopic and differential scanning calorimetric studies indicate that the protein undergoes thermal unfolding around 70 °C.

Materials and methods

Materials

Snake gourd fruits were obtained from local vendors. 2-Mecrcaptoethanol, chitin (from crab shells), chitooligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulphate, calcium chloride, di-sodium

hydrogen phosphate, sodium dihydrogen phosphate, trichloroacetic acid, sodium deoxycholate and acetic acid were obtained from local suppliers and were of highest purity available.

Preparation of affinity matrix

The chitin column was prepared essentially as described earlier [Sampietro et al., 2001]. Briefly, practical grade chitin from crab shells was suspended in 0.25 M NH_4OH and incubated for 60 minutes. The supernatant was decanted and the material was washed successively with 0.2 M NaCl, double distilled water and 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 10 mM β-mercaptoethanol (PBS-βME). The material was finally suspended in PBS-βME was packed in to a glass column (16 × 4 cm) and equilibrated again with the same buffer.

Extraction

Snake gourd fruits were bled by 2-3 mm deep longitudinal cuts [Anantharam et al., 1986; Chapter 2] and the exudate obtained was collected into PBS- βME. The buffer containing exudate was centrifuged at 9000 rpm in an Eppendorf 5810 R refrigerated centrifuge. The resulting supernatant containing soluble protein was subjected to ammonium sulfate precipitation as described below.

Ammonium sulfate precipitation

The volume of the supernatant obtained in the above step was measured and to it solid ammonium sulfate was added in small quantities while the solution was being stirred to give 80% saturation. This solution was allowed to stand at 4 °C overnight and was centrifuged at 9000 rpm and the supernatant was discarded. The precipitate obtained was dissolved in minimum amount of water and was dialyzed against PBS-βME extensively at 4 °C. The solution from the dialysis bag was

centrifuged again and the precipitate was discarded. The supernatant was subjected to affinity chromatography as described below.

Affinity chromatography

Affinity chromatographic purification of SGPL was carried out on a column of chitin (16 × 4 cm) that was pre-equilibrated with PBS-βME as described above. The supernatant obtained in the previous step was passed through the column at 4 °C at a flow rate of ca. 20 ml/hour. The breakthrough obtained was reloaded to ensure complete binding of the protein. The column was then washed with PBS-βME to remove unbound proteins, monitoring absorbance of the eluant at 280 nm. When the absorbance of the column effluent fell below 0.01, the bound protein was eluted with 0.1 M acetic acid at room temperature. Fractions of ca. ~5 ml were collected and their absorbance at 280 nm was noted to check for the presence of protein. Fractions showing high concentration of protein were pooled and the eluting acid was removed by extensive dialysis against PBS-βME.

Preparation of erythrocyte suspension

Alsevier's solution was prepared by dissolving 2.05 grams of dextrose, 590 mg of sodium citrate, 420 mg of NaCl and 50 mg of citric acid in 100 ml of distilled water. Freshly drawn human blood was mixed with 5 volumes of Alsevier's solution to prevent coagulation. This solution was centrifuged on a bench centrifuge to pellet the erythrocytes. The supernatant was discarded and the pelleted erythrocytes were mixed with 10 volumes of PBS, centrifuged and the supernatant was again discarded. This washing process was repeated thrice to ensure the complete removal of plasma proteins, which would otherwise lead to coagulation. The erythrocyte pellet obtained after washing was mixed with buffer

to make a 4% solution. This erythrocyte suspension was stable for 4-5 days when stored at 4 °C.

Hemagglutination and hemagglutination-inhibition assays

Hemagglutination assays were carried out in 96 well ELISA microtitre plates. To each well containing 100 µl of serially diluted lectin or crude extract, 100 µl of a 4% erythrocyte suspension was added and mixed. The plate was then incubated at 4 °C for 1 hour and then the agglutination titre was visually scored. For inhibition assays, 1 mM saccharide stock solutions were used except for chitobiose (for which a 52 mM stock was used). In the first well of microtiter plate, 50 µl of saccharide solution was placed and serially 2-fold diluted. Then 50 µl of protein solution (30µg/ml) or an appropriate amount of crude extract was added to each well. After incubating the mixture at 4 °C for 15 min, 100 µl of a 4 % erythrocyte suspension was added, the plate was incubated for one hour and the titer was scored visually.

Effect of pH and thermal inactivation of SGPL

The dependence of agglutination activity of SGPL on the pH of the medium was investigated by dialyzing the lectin with buffer of desired pH and then assaying for hemagglutination activity. The buffers used for different pH values are: 20 mM KCl-HCl (pH 2), 20 mM citrate-phosphate (pH 3-6), 20 mM phosphate (pH 7.4), 20 mM Tris-HCl (pH 8-9) and 20 mM glycine-NaOH (pH 10). All buffers contained 0.15 M NaCl and 10 mM β-mercaptoethanol.

To investigate the effect of temperature on the activity of SGPL, protein samples were incubated at different temperatures for 15 min and cooled to room temperature. The samples were then centrifuged and the clear supernatants obtained were assayed for agglutination activity as described above.

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [1970] on slab gels with 5% and 10% acrylamide in stacking and resolving gels, respectively. PAGE under non-denaturing conditions was performed in 12% slab gels using Tris-glycine buffer at basic pH (8.8) as described in [Laemmli, 1970]. The gels were stained with Coomassie Brilliant Blue R-250. Protein concentration was estimated by the modified Lowry method of Peterson [1977].

Mass spectrometry

The mass spectrum of SGPL was recorded using a model 4800 MALDI-TOF-TOF mass spectrometer from Applied Biosystems (Foster City, CA, USA) in the linear mode using sinapinic acid (SA) as the matrix. The matrix was prepared by dissolving 5 mg of SA in 1 ml of 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). About 5 picomoles of the protein was spotted on the MALDI plate and the spectrum was acquired.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www.jascoint.co.jp), equipped with a Peltier thermostat supplied by the manufacturer. Concentration of SGPL was 0.7 μM for measurements in the far UV region (250-190 nm) and 13 μM for measurements in the near UV region (300-250 nm). Samples were placed in a 2-mm pathlength rectangular quartz cell and spectra were recorded at a scan speed of 20 nm/min with a response time of 4 s and a slit width of 2 nm. In order to investigate the effect of carbohydrate binding on the secondary and tertiary structure of SGPL, spectra were recorded for the native protein as well as in the

presence of 1 mM chitotetraose. To investigate the structural changes that take place in the protein during thermal unfolding of the protein, CD spectra were recorded at different temperatures. Measurements were made in PBS-βME and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) studies were performed on a MicroCal VP differential scanning calorimeter (MicroCal LLC, Northampton, MA) with a scan speed of 60°/hr (Celsius scale). Thermograms were recorded with lectin samples of 9 μM concentration in PBS-βME. SGPL was dialysed extensively against the buffer and degassed prior to loading into the cell. Buffer scans were subtracted from the thermograms corresponding to the lectin samples for analysis. Data were analysed by Origin™ software supplied by the manufacturer.

Results

Purification and macromolecular properties of snake gourd phloem lectin

The snake gourd phloem lectin was purified by ammonium sulpfate precipitation followed by affinity chromatography on chitin. The lectin eluted as a single peak from the affinity column when 0.1 M acetic acid was used as the eluant (Fig. 5.1). In a typical purification experiment, about 32 mg of SGPL was obtained from 10 ml of phloem exudate, i.e., a yield of ~3 mg/ml of phloem exudate. The overall yield of lectin activity recovered was about 30 % (Table 5.1). The affinity purified SGPL yielded a single band on native PAGE (5.2A), whereas in SDS-PAGE in the presence of β-mercaptoethanol it gave two bands of comparable intensity, suggesting that lectin is a heterodimer (Fig. 5.2B, lane 2).

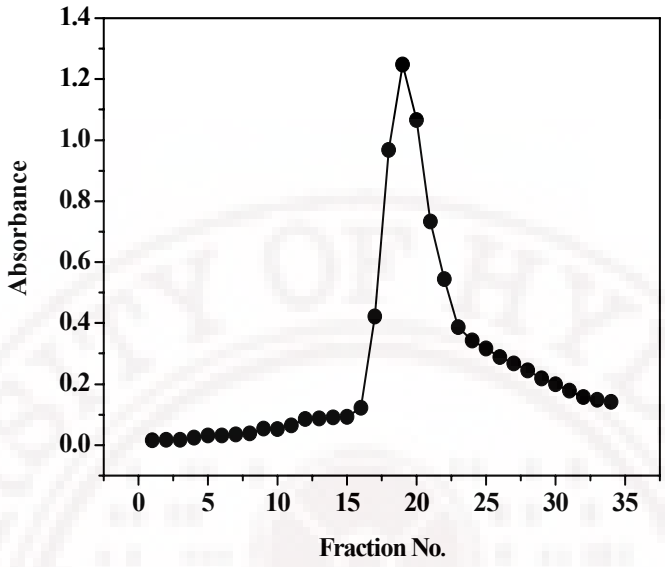


Fig. 5.1. Affinity chromatographic purification of snake gourd phloem exudate lectin (SGPL) on chitin. After washing the column extensively with PBS-βME, the bound lectin was eluted with 0.1 M acetic acid. Fractions (5 ml) were collected and absorbance of the column effluent at 280 nm was plotted as a function of the fraction number.

Table 5.1. Purification of snake gourd phloem exudate lectin (from 10 ml of phloem fluid).

Purification step	Total activity ($\times 10^4$)	Protein content (mg)	Specific activity (units/mg)	Percent recovery	Purification fold
Aqueous extract	66	1287	51	100	1.0
Ammonium sulfate precipitation	42	765	55	64	1.1
Affinity chromatography	20	32	854	30	16

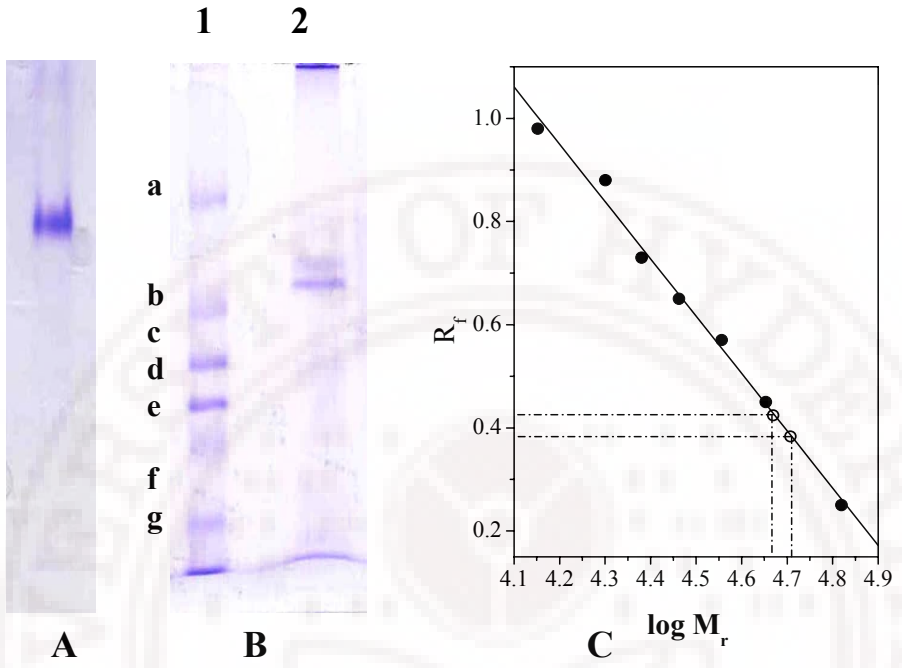


Fig. 5.2. Gel electrophoresis of SGPL. (**A**) native PAGE. (**B**) Lane (1), molecular weight markers: (a) bovine serum albumin (66,000); (b) ovalbumin (45,000); (c) glyceraldyhyde-3-phosphate dehydrogenase (36,000); (d) carbonic anhydrase (29,000); (e) trypsinogen (24,000); (f) trypsin inhibitor (20,000); (g) α-lactalbumin (14,200). Lane (2) SDS-PAGE of SGPL. (**C**) Plot of relative mobility (R_f) versus log M_r for the estimation of molecular weight of SGPL subunits, (•) marker proteins, (o) SGPL.

By comparing the mobility of SGPL with that of standard proteins (Fig. 5.2B, lane 1), the molecular weights of subunits were estimated as 48 and 53 kDa (Fig. 5.2C). In order to get the exact mass of the protein, SGPL was subjected to mass spectrometric analysis. The MALDI-TOF mass spectrum of SGPL is shown in Fig. 5.3. The peaks seen at 50,597 and 55,399 Daltons correspond to the subunits of SGPL.

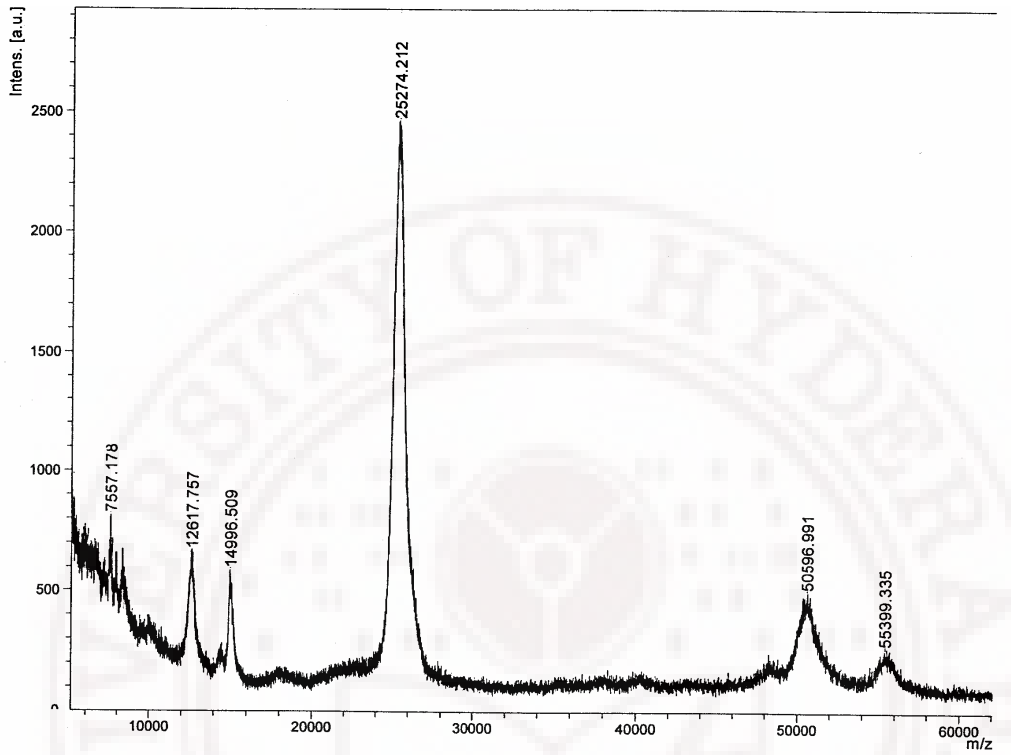


Fig. 5.3. MALDI-TOF mass spectrum of SGPL. The molecular ions of the subunits are seen at 50,597 and 55,399 Dalton.

Carbohydrate binding specificity

Hemagglutination-inhibition experiments were carried out on SGPL to probe its carbohydrate specificity and the results were summarised in Table 5.2. The hemagglutination activity of SGPL could be inhibited efficiently by chitooligosaccharides among the sugars studied here. To investigate the ability of different chitooligosaccharides to inhibit the agglutination activity of the affinity purified lectin obtained in this study, hemagglutination-inhibition assays were performed with various chitooligosaccharides, from chitobiose to chitohexaose. The results obtained are presented in Table 5.2. The data show that the lectin activity could not be inhibited by *N*-acetylglucosamine, but strong inhibition is seen

when chitooligosaccharides are used as ligands, with the inhibitory potency of the ligands increasing with increase in the oligosaccharide size up to chitohexaose.

Table. 5.2. Inhibitory power of the various sugars on the hemagglutination by SGPL.

Sugar*	**Concentration giving 50% inhibition (μM)**	**Relative inhibitory Power**
$(GlcNAc)_2$	6500.0	1
$(GlcNAc)_3$	31.3	208
$(GlcNAc)_4$	15.6	416
$(GlcNAc)_5$	7.8	832
$(GlcNAc)_6$	3.9	1662

∗The following sugars did not inhibit the hemagglunation activity of SGPL at upto 100 mM resultant concentration: N-acetylglucosamine, N-acetygalactosamine, glucose, galactose, fructose, and methyl-α-D-glucopyranoside.

Among the chitooligosaccharides investigated chitobiose has the lowest inhibitory potency and exhibits 50% inhibition at ca. 6.5 mM concentration, whereas chitohexaose is the best ligand and requires only 3.9 μM concentration for 50% inhibition of the agglutination activity of SGPL. Thus chitotriose, chitotetraose, chitopentaose and chitohexaose are 208, 416, 832 and 1662 times more potent than chitobiose in their ability to inhibit the hemagglutination activity of SGPL and suggest the presence of an extended combining site on each subunit of the lectin.

Thermal stability and pH dependence of SGPL

In order to investigate the effect pH of the medium on the activity of the lectin, samples of SGPL dialyzed against appropriate buffers in the pH range 2.0-10.0 were tested for agglutination activity at 4 °C (5.4A). The lectin has no activity at pH 2.0 – 3.0. However, the relative activity of the lectin increases steeply above

pH 3.0 and reaches 50% at pH 4.0, and 100% at pH 5.0 and remains constant at the same level up to pH 10.0. These results indicate that the agglutination activity of SGPL is maximal in the pH range 5.0-10.0.

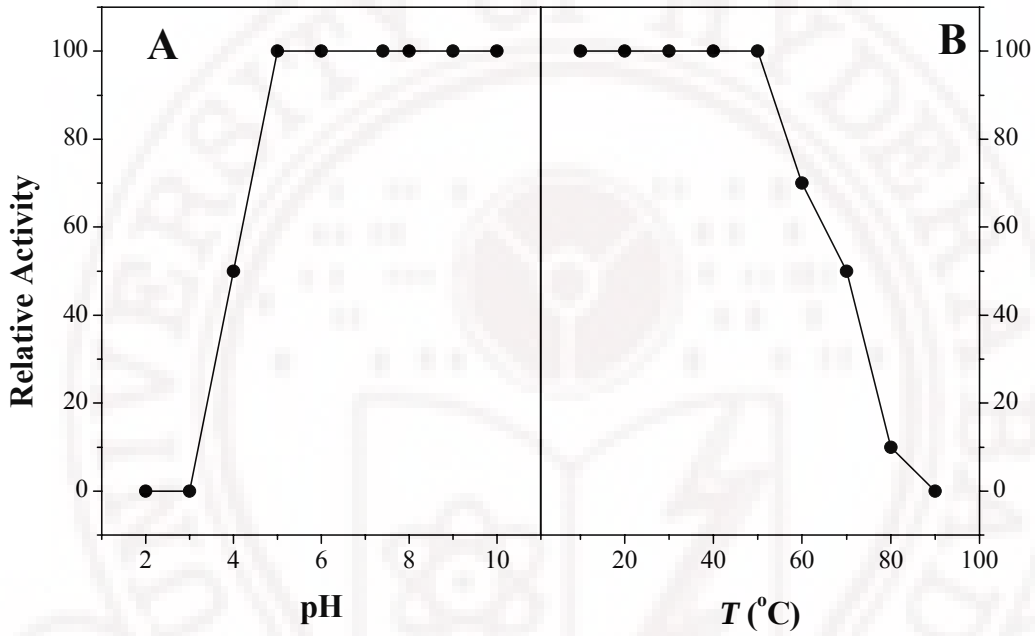


Fig. 5.4. Effect of pH (A) and temperature (B) on the agglutination activity of snake gourd phloem exudate lectin.

Thermal inactivation of SGPL was investigated by incubating lectin samples at different temperatures for 15 minutes followed by cooling to room temperature and then subjected to hemagglutination assay. The results obtained are presented in Fig. 5.4B. It is seen from this figure that incubation at different temperatures up to 50 °C did not affect the activity of the lectin. However the activity decreased quite sharply between 60 and 80 °C; SGPL incubated at 60 and 70 °C exhibits about 75 and 50% activity as compared to the native protein, whereas the sample incubated

at 80 °C has less than 10% activity. Incubation at 90 °C led to a complete loss of activity of the protein.

CD spectroscopy and secondary structure of SGPL

Circular dichroic spectra of SGPL in native state (solid line) and in the presence of chitotriose (dashed line) are shown in Fig. 5.5. The far UV spectrum of the native protein (Fig. 5.5A) is characterized by a minimum around 216 nm and a small maximum around 197 nm, suggesting a predominantly β-sheet conformation for the lectin.

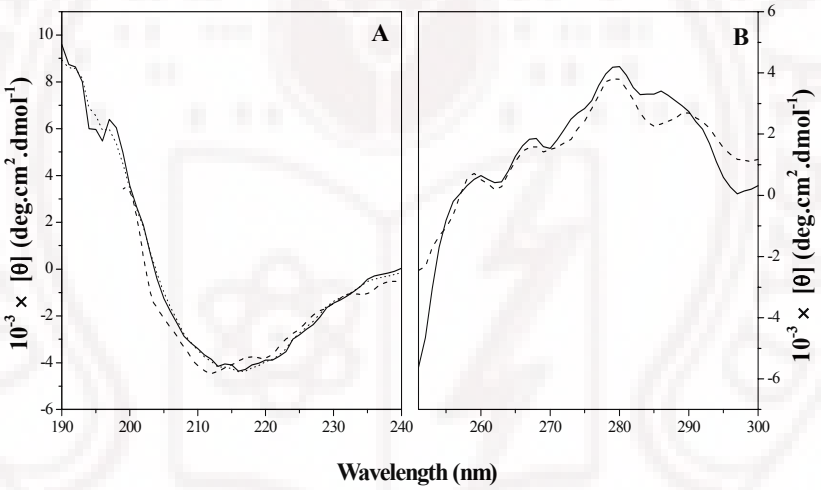


Fig. 5.5. CD spectra of SGPL. (A) Far UV region, (B) Near UV region. (——) lectin alone, (------) lectin with chitotriose, (·····) calculated fit obtained by using CDSSTR program.

The near UV CD spectrum of SGPL (Fig. 5.5B) is characterized by three maxima at 267, 280 and 286 nm, which most likely arise due to the aromatic amino acids Tyr and Trp present in the protein. In order to derive information on the content of different structural elements in this protein, the CD spectrum was analyzed by three different methods, namely CDSSTR [Compton & Johnson, 1986; Sreerama &

Woody, 2000] CONTINLL [Provencher & Glockner, 1981; Van Stokkum et al., 1990] and SELCON3 [Sreerama & Woody, 1993; Sreerama et al., 1999] employing the software routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html/) [Lobley & Wallace, 2001; Lobley et al., 2002]. A basis set containing 43 proteins was used as a reference for fitting the experimental spectrum. The results obtained from this analysis are given in Table 5.3. Among the three methods mentioned above, the best fit was obtained with CDSSTR and the resulting fitted spectrum is shown in Fig. 5.5A (dotted line) and it is seen that the fit is in good agreement with the experimental spectrum. The content of various secondary structures obtained from the CDSSTR analysis are: 2 % regular α-helix, 5 % distorted α-helix (giving a total α helical content of 7 %), 26 % regular β-sheet and 14 % distorted β-sheet (adding to a total of 40 % of pleated sheet structure), 21 % β-turns and 31 % unordered structures. These values along with the values obtained from the other two methods, namely CONTINLL and SELCON3 are presented in Table 5.3; the average values obtained from the above three methods are also given in this table.

Table 5.3. Secondary structure of SGPL determined from CD spectral analysis

Method	**α (%)**	**β (%)**	**Turns**	**Unordered**
CDSSTR	7.0	40	21	31
CONTINLL	7.6	39.9	20.3	32.1
SELCON3	11.7	38.9	19.9	34.2
Average	9 (2.3)	39.6 (0.6)	20.4 (0.5)	32.4 (1.6)

From the above data it is clear that although CDSSTR gave the best fit to the experimental spectrum, values of secondary structures obtained from the other two methods are also in good agreement. The relatively small standard deviations obtained with the average values are quite consistent with this. Overall the results obtained from analyzing the CD spectral data indicate that SGPL is a predominantly β-sheet protein with a relatively small α-helical content. The far UV CD spectrum of SGPL recorded in the presence of 1 mM chitotetraose is nearly identical to the spectrum of native SGPL, suggesting that the secondary structure of the protein is unaltered upon ligand binding. Very minor changes are seen in the near UV CD spectrum of the protein upon binding of chitotetraose, indicating that the environment of the side chains of Tyr and Trp residues are also largely unaffected by ligand binding.

Thermal unfolding: CD and DSC studies

To investigate thermal stability of SGPL, near and far-UV CD spectra of the lectin were recorded at different temperatures. Spectra recorded at 30, 40, 50, 60, 70, 80 and 90 °C are shown in Fig. 5.6. The secondary structute of the protein is not altered in the temperature range of 30-60 °C and the spectra recorded at 70 °C has significantly lower signal as compared to spectra recorded at 60 °C and indicates that the secondary structure of the protein undergoes significant changes above 60 °C. The temperature dependence of CD signal intensity at 216 nm gave a sigmoidal curve centered around 75 °C, indicating that unfolding transition temperature of the protein is 75 °C. Near-UV CD spectra indicate that changes in the tertiary structure of the protein occurs at somewhat lower temperatures as compared to changes in the secondary structure. No significant changes are seen in the tertiary structure of the protein spectra recorded between 30 and 50 °C.

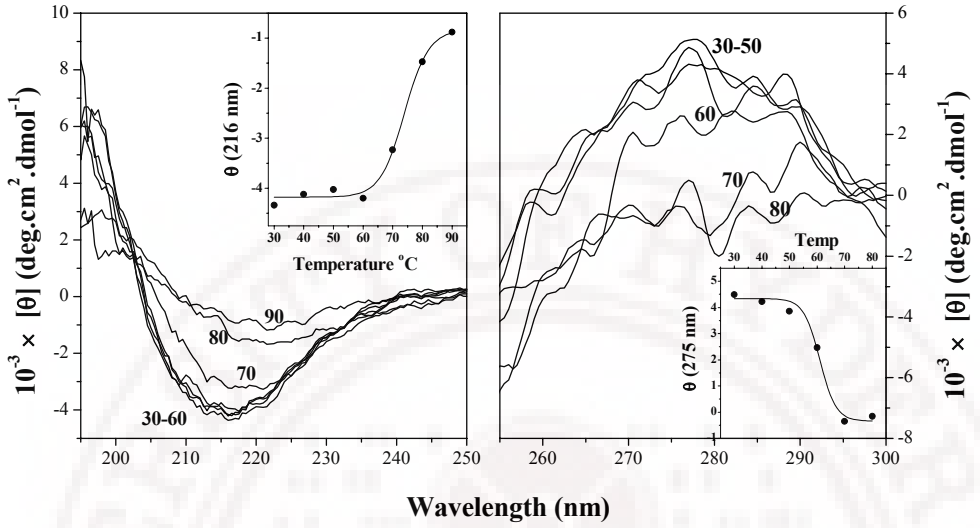


Fig. 5.6. CD spectra of SGPL recorded at different temperatures. (A) far-UV region and (B) near-UV region. Insets are plot for the temperature versus CD signal intensity at 216 nm for secondary structure and at 275 nm for tertiary structure.

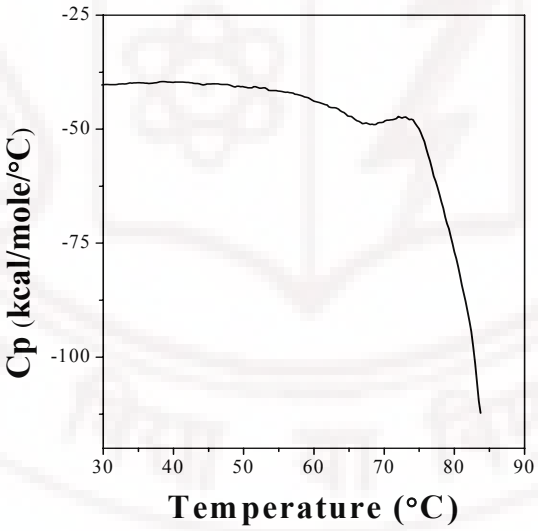


Fig. 5.7. DSC thermogram of snake gourd phloem lectin in 20 mM phosphate buffer containing 150 mM NaCl and 10 mM β-mercaptoethanol at a scan rate of 60 °C h^{-1}.

The spectrum recorded at 60 °C shows a lower signal intensity as compared to that recorded at 50 °C, indicating that the tertiary structure of the protein is significantly altered at 60 °C. Significantly larger changes are seen in the spectrum recorded at 70 °C. The spectrum obtained at 80 °C exhibits further decrease in the intensity, indicating a near total loss of tertiary structure. The plot for the temperature versus CD signal intensity at 275 nm is also sigmoidal in shape and is centered around 65-70 °C.

Similar result was obtained from differential scanning calorimetry. The DSC thermogram of SGPL exhibits an unfolding transition centered at about 73 °C (Fig 5.7). It was not possible to determine the enthalpy of the transition, because the baseline at the end of the transition could not be established clearly; this could be because of aggregation of the unfolded protein as a white precipitate was observed when the sample was examined at the end of the calorimetric scan.

Discussion

In the Cucurbitaceae family several seed lectins and few phloem exudate lectins have been previously identified and characterized in detail. However, so far there have not been any reports of the presence of both seed and phloem exudate lectins from the same species. In previous work from our laboratory a galactose-specific seed lectin has been purified from the seeds of snake gourd (*Trichsanthes anguina*), which was characterized in considerable detail [Komath et al., 1996, 1998, 2001; Komath & Swamy, 1998, 1999.]. In the present study, a lectin activity is identified in the phloem exudate of snake gourd fruits. The lectin activity could be inhibited by chitotriose, clearly indicating that the lectin specifically recognizes the β(1→4) linked oligosaccharides of *N*-acetylglucosamine. Therefore we have chosen affinity chromatography for the purification of snake gourd phloem lectin (SGPL) and

purified it to homogeneity on chitin. In the work reported in chapter 2 of this thesis, the pumpkin phloem exudate lectin (PPL) was purified in good yield by affinity chromatography on chitin. Previously, several other groups also used chitin affinity column as a key step to purify lectins such as wheat germ agglutinin, *Entamoeba histolytica* lectin and chitin binding lectins from *Artocarpus integrifolia*, *Artocarpus incisa* and *Cynchomandra betecea* [Bloch & Burger, 1974; Kobiler & Mirelman, 1980; Trindade et al., 2006; Xu et al., 1992].

But two other phloem exudate lectins from ridge gourd (*Luffa acutangula*) and *Coccinia indica* [Anantharam et al., 1986; Sanadi & Surolia, 1994] have been purified using soybean agglutinin glycopeptide coupled to Sepharose-6B column. Likewise chitotriose coupled to Sepharose-4B, Sepharose-fetuin and insolubilized poly-L-leucine hog A + H blood group substance were used as matrices to purify chitooligosaccharide specific lectins from potato tuber, *Datura stramonium* and common tomato (*Lycopersicon esculentum*) [Allen & Neuberger, 1973; Kilpatric & Yeoman, 1978; Nachbar et al. 1980].

Affinity purified SGPL showed a single band on native PAGE, whereas in SDS-PAGE it moved as two bands indicating that the protein is a heterodimer (Fig. 5.2A). This lectin thus appears to be different in subunit composition from other cucurbit phloem exudate lectins such as those from pumkin, ridge gourd and *Coccinia indica*, which are all homodimers with subunit molecular weights around 18-24 kDa [Read & Northcote, 1983; Anantharam et al., 1986; Sanadi & Surolia, 1994; Chapter 2]. However, SGPL is similar to the above lectins in having an extended carbohydrate binding site for chitooligosacchrides. The yield of SGPL is around 30 mg from 10 ml of phloem exudate, this recovery is lesser than to pumpkin phloem lectin and comparable to ridge gourd phloem lectin.

Thermal inactivation studies show that SGPL is active up to 50 °C, which decreases upon heating to 60 °C onwards. Activity decreased quite sharply between 60 and 80 °C (Fig. 5.4A). Complete loss of activity was observed when the sample was incubated at 80 °C. Previously similar results were observed for pumpkin phloem exudate lectin, which showed agglutination activity up to 70 °C [Chapter 2]. But the galactose specific seed lectin from snake gourd seeds (SGSL) was rapidly inactivated at temperatures above 40 °C [Komath et al., 2001]. This shows that SGPL is more thermostable than SGSL. Agglutination activity of SGPL is maximal in the pH range 5.0-10.0 and showed 50% activity at pH 4, where as SGSL shows maximum activity in the pH range 6-10 [Komath et al., 2001].

Secondary structural analysis shows that SGPL is predominantly β-sheet protein with a relatively small α-helical content and that there is no structural change upon ligand binding (Fig. 5.5). Chitooligosaccharide specific lectins such as PPL have 37% β-sheet with less α-helical content and potato lectin has 40% β-sheet with no α-helical content [Chapter 2; Matsumoto et al., 1983]. In contrast, the ridge gourd phloem exudate lectin has 31% α-helix, which is significantly higher than the helical content estimated in SGPL and PPL by CD spectroscopy [Anantharam et al., 1986]. This difference may be the reason for SGPL has no structural change upon ligand binding whereas ridge gourd phloem lectin undergoes significant structural changes upon binding of chitooligosaccharides.

Thermal unfolding studies using circular dichroism spectroscopy have shown the secondary structure of SGPL is practically unaltered when heated upto 60 °C, whereas significant changes were observed in both secondary and tertiary structure at higher temperatures. This is correlated with thermal inactivation as 25% activity was lost when sample was heated up to 60 °C and complete loss of secondary and tertiary structures was observed above 70 °C. The plots of temperature versus CD

signal at 216 nm for secondary structure, at 275 nm for tertiary structure show that lectin unfolds around 70 °C. Differential scanning calorimetry studies supported above observation as unfolding temperature (T_m) is 73 °C.

In summary, the results presented in this chapter report the identification, isolation and affinity purification of a novel chito-oligosaccharide specific lectin from the phloem exudate of snake gourd (Trichosanthes anguina). The lectin is a hetero dimer with subunit masses of 50 KDa and 55 KDa. The snake gourd phloem lectin (SGPL) is a predominantly β-sheet protein with very low α-helical content. DSC and CD studies indicate that SGPL undergoes a cooperative unfolding transition centered at ca. 73 ºC.

Chapter 6

Thermodynamics of the interaction of chitooligosaccharides with snake gourd (*Trichosanthes anguina*) phloem exudate lectin. Isothermal titration calorimetric and fluorescence spectroscopic studies

Summary

The interaction between snake gourd phloem exudate lectin (SGPL) and chitooligosaccharides [$(GlcNAc)_{3-6}$] was studied by isothermal titration calorimetry (ITC) and fluorescence spectroscopy. Calorimetric titrations indicate that the dimeric lectin binds to two molecules of the ligand with association constants determined at 25 °C being 1.75×10^5, 1.39×10^5, 1.45×10^5 and 3.70×10^5 M^{-1}, for chitotriose, chitotetraose, chitopentaose and chitohexaose, respectively. The binding reaction was essentially enthalpy driven with the binding enthalpy (ΔH_b) at 25 °C for the different chitooligosaccharides ranging between -17.36 and -13.82 $kcal.mol^{-1}$, whereas the entropic contribution to the binding reaction is negative, with the value of binding entropy (ΔS_b) being in the range of -32.7 and -22.3 $cal.mol^{-1}.K^{-1}$. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals' interactions. Fluorescence titrations indicate that the protein intrinsic fluorescence decreases upon binding of the chitooligosaccharides, with a 3 nm blue shift in the emission maximum. Association constants determined from an analysis of the ligand-induced changes in the fluorescence intensity are in good agreement with those obtained from ITC.

Introduction

In the work reported in Chapter 5 of this thesis, purification and characterization of some of the macromolecular properties of a new chitooligosaccharide-specific lectin from the phloem exudate of snake gourd (*Trichosanthes anguina*) has been reported. The snake gourd phloem exudate lectin (SGPL) is a heterodimer with subunit masses of 50 and 55 kDa. Secondary structural analysis has shown that it consists of 9.0% α-helix, 39.6% β-sheet, 20.4% β-turns and 32.4% unordered structures (Chapter 5). Thermal inactivation studies shows that agglutination activity of SGPL is maximal in the pH range of 5.0-10.0. Thermal unfolding studies using circular dichroism spectroscopy and differential scanning calorimetry have shown that SGPL undergoes a cooperative, thermal unfolding transition at ca. 70 °C.

In the studies reported in this chapter the interaction of snake gourd phloem lectin with chitooligosaccharides was investigated by isothermal titration calorimetry (ITC) and fluorescence spectroscopy. The results suggest that the dimeric SGPL binds to two ligand molecules of chitotriose, chitotetraose, chitopentaose and chitohexaose. The binding constants were comparable for chitotriose, chitotetraose and chitopentaose but increase by a factor of 2 for chitohexaose. Both types of titrations yielded similar results with respect to binding constant and Gibb's free energy change values. The binding reactions for chitooligosaccharides to SGPL were enthapically driven with negative entropic contribution. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals' interactions.

Materials and methods

Materials

Snake gourd fruits were obtained from local vendors. 2-Mecrcaptoethanol, chitin (from crab shells) and chitooligosaccharides $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$ and $(GlcNAc)_6$, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, ammonium sulfate, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, copper sulfate, potassium tartrate, sodium carbonate, ammonia solution and acetic acid were obtained from local suppliers and were of the highest purity available.

Sanke gourd phloem exudate lectin (SGPL)

The lectin from the snake gourd phloem exudate has been purified by affinity chromatography on chitin as described in Chapter 5. The affinity eluted lectin was dialyzed thoroughly against 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 10 mM β-mercaptoethanol (PBS-βME). The lectin thus obtained gave a single band in native PAGE where as in SDS-PAGE in the presence of β-mercaptoethanol it yielded two bands with comparable intensity. Lectin concentration was estimated according to the modified Lowry method of Peterson [1977].

Isothermal titration calorimetry

Calorimetric titrations were performed at 298 K on a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA, USA). Briefly, 7 μl aliquots of the chitooligosaccharides solution were added from a 1.5 mM stock solution via a rotating stirrer syringe to a 30 μM lectin solution contained in a 1.445 ml sample cell. Samples were dialyzed against 20 mM phosphate buffer, pH 7.4, containing 150 mM sodium chloride, 2% sodium azide and 10 mM β-mercaptoethanol (PBS-

βME). Samples were degassed prior to loading into the cell. The additions were made at 4 min intervals to allow the exothermic heat peak accompanying each addition to return to the baseline prior to next addition. Usually the first injection was found to be inaccurate; therefore, a 1 or 2 μl injection was added first and the resultant point was deleted before the remaining data were analyzed as described below.

For a system of one set of identical binding sites, the total heat evolved (or absorbed) during the binding process at the end of the *i*th injection [Wiseman et al., 1989], Q(*i*), is given by Equation (6.1)

$$Q(i)=nP_t\Delta H_b V\{1+X_t/nP_t+1/nK_bP_t-[(1+X_t/nP_t+1/nK_bP_t)2-4X_t/nP_t]½\}/2 \qquad (6.1)$$

Where n is the number of binding sites, P_t is the total protein concentration, X_t is the total ligand concentration, V is the cell volume, K_b is the binding constant and ΔH_b is the binding enthalpy. Obviously the heat corresponding to the *i*th injection only, $\Delta Q\ (i)$, is equal to the difference between Q(*i*) and Q(*i-1*) and is given by Equation (6.2), which involves the necessary correction factor for the displaced volume (the injection volume d*Vi*):

$$\Delta Q(i) = Q(i) + dV_i/2V[Q(i) + Q(i\text{-}1)] - Q(i\text{-}1) \qquad (6.2)$$

The ITC unit measures $\Delta Q(i)$ value for every injection. These values are then fitted to Equations (6.1) and (6.2) by a nonlinear least squares method using the data analysis program Origin® [MicroCal™]. The fit process involves initial guess of n, K_b and ΔH_b which allows calculation of $\Delta Q(i)$ values as mentioned above for all injections and comparing them with the corresponding experimentally determined values. Based on this comparison the initial guess of n, K_b and ΔH_b is improved and the process is repeated till no further significant improvement in the

fit can be obtained. The thermodynamic parameters, ΔG^{o}_{b} and ΔS_b are calculated according to the basic thermodynamic Equations (6.3) and (6.4):

$$\Delta G^{o}_{b} = -RT\ln K_b \qquad (6.3)$$

$$\Delta G^{o}_{b} = \Delta H_b - T\Delta S_b \qquad (6.4)$$

Fluorescence spectroscopy

Binding of chitooligosaccharides to snake gourd phloem lectin was also investigated by monitoring changes in the fluorescence intensity of the protein induced by saccharide binding. Measurements were performed on a Spex Fluoromax 4 fluorescence spectrometer using slit widths of 2 and 3 nm, on the excitation and emission monochromators, respectively. Samples were excited at 280 nm and emission spectra were recorded between 290 and 400 nm. Titrations were carried out by adding small aliquots of sugar from a 500 μM stock solution in PBS to 2.0 ml of the lectin with $A_{280} \leq 0.1$ in the same buffer. Fluorescence spectra were recorded for the protein alone and after each addition of the ligand. An equilibration period of 2 minutes was given after the addition of each aliquot. All spectra were corrected for volume changes before further analysis.

Results

Isothermal titration calorimetry

Thermodynamic parameters for the binding of chitooligosaccharides to snake gourd phloem lectin was investigated by isothermal titration calorimetry. Representative calorimetric titrations for the binding of chitotriose and chitohexaose to SGPL are given in the upper panels of Fig. 6.1A and 6.1B, respectively. From this figure it is seen that the exothermic heat of binding decreases monotonically with successive injections until saturation is achieved. A plot of the incremental heat released as a

function of the ratio of chitotriose and chitohexaose to SGPL is shown in lower panels of Fig. 6.1A and 6.1B, together with a non-linear least square fit of the data to Equation (6.1). The experimental data could be fitted satisfactorily to the '*one set of sites*' model available in the Origin software provided by the instrument manufacturer.

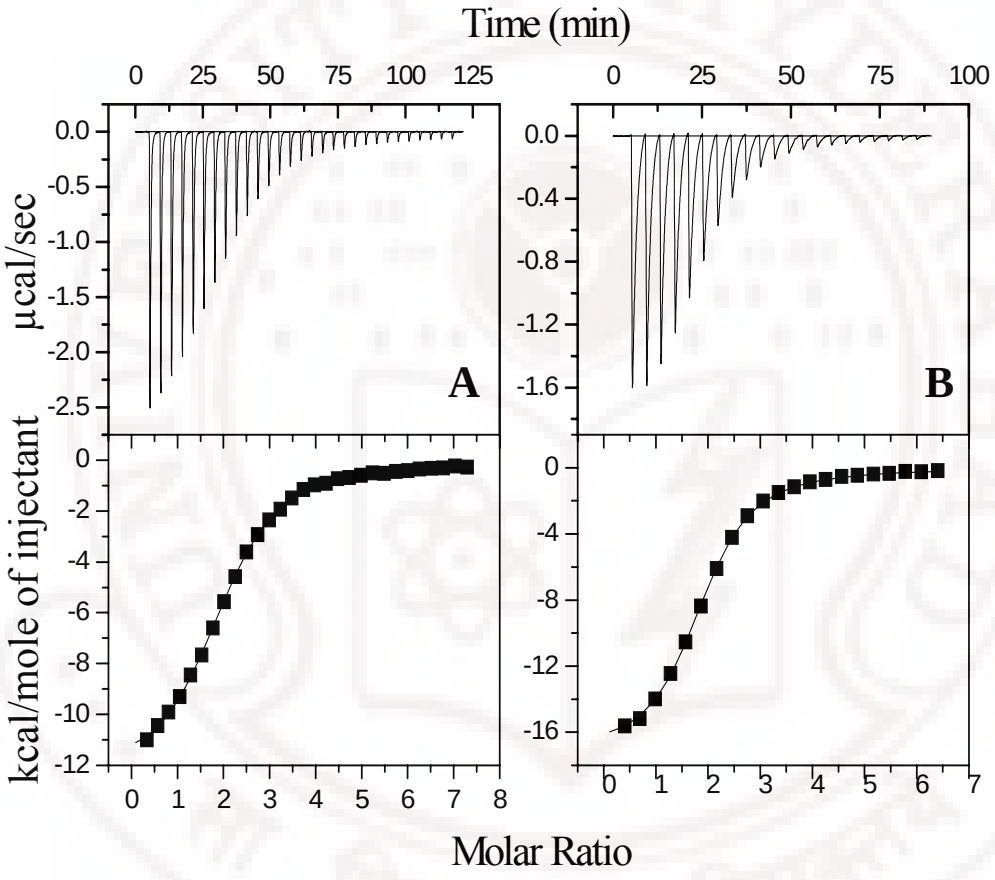


Fig. 6.1. (A) Calorimetric titration of SGPL with chitotriose and (B) with chitohexaose at 298.15 K. Upper panels show the ITC raw data obtained from 20 to 30 automatic injections of 7 µl aliquots of 1.5 mM chitotriose and chitohexaose into 30 µM of SGPL. Lower panels show the integrated data obtained from raw data shown in the upper panels.

The fits obtained for the data are shown as solid lines in the lower panels of Fig. 6.1A and 6.1B. The fits yielded the values of various parameters such as number of binding sites, n = 2.10 (± 0.012); binding constant, K_b = 1.75 (± 0.1) × 10^5 M^{-1}; enthalpy of binding, ΔH_b = -13.82 (± 0.13) kcal.mol^{-1}; entropy of binding, ΔS_b = -22.30 cal.mol^{-1}.K^{-1} for the binding of chitotriose to SGPL. Corresponding values obtained with chitohexaose are: n = 1.84 (0.007), K_b = 3.7 (0.1) × 10^5 M^{-1}, ΔH_b = -17.36 (0.09) kcal.mol^{-1}; entropy of binding, ΔS_b = -32.7 cal.mol^{-1}.K^{-1}. These values as well as the corresponding values obtained for the calorimetric titrations performed with different oligosaccharides and at different temperatures (for chitotriose) are listed in Table 6.1. In addition, values of Gibb's free energy $\Delta G_b°$, are also listed in the above table. The stoichiometry of binding was found to be in the range between 1.84 and 2.35 ligand molecules per protein dimer, indicating that the dimeric lectin binds two chitooligosaccharide molecules.

Table. 6.1. Binding constants (K_b) and thermodynamic parameters for the binding of chitooligosaccharides to snake gourd phloem lectin SGPL.

Sugar	T (°C)	n	K_b $\times 10^{-5}$ (M^{-1})	$-\Delta G°$ (kcal.mol^{-1})	$-\Delta H°$ (kcal.mol^{-1})	$-\Delta S°$ (cal.mol^{-1}.K^{-1})
$(GlcNAc)_3$	15	2.17	2.15	7.02	12.30	18.3
	20	1.98	1.77	7.04	12.60	19.0
	25	2.10	1.75	7.26	13.82	22.3
$(GlcNAc)_4$	25	2.37	1.39	7.06	15.25	27.6
$(GlcNAc)_5$	25	2.35	1.45	7.08	16.63	32.1
$(GlcNAc)_6$	25	1.84	3.70	7.60	17.36	32.7

The association constants determined at 25 °C were nearly the same for chitotriose to chitopentaose and were found to be in the range of 1.39×10^5 to 1.77×10^5 M^{-1}, whereas the K_b value estimated for chitohexaose was twice as high. The enthalpy of binding and entropy of binding were found to increase with increasing size of the chitooligosaccharides.

The increase in enthalpy with increasing number of GlcNAc residues in the ligand indicates that the lectin combining site contains several subsites which interact with the individual monosaccharide units of the oligosaccharide. In order to investigate this further the contributions of the different monosaccharide units of the chitooligosaccharides to the binding enthalpy, entropy and free energy have been calculated from the thermodynamic data presented in Table 6.1, by subtracting the values of the oligosaccharide containing '(n-1)' monosaccharide units from the values corresponding to the oligosaccharide containing 'n' residues. The results obtained are presented in Table 6.2.

Table 6.2. Change in enthalpy, entropy and free energy per additional saccharide for the binding of chitooligosaccharides to snake gourd phloem lectin SGPL.

Sugar	T (°C)	$-\Delta\Delta H°$ (kcal.mol^{-1})	$-\Delta\Delta S°$ (cal.mol^{-1}.K^{-1})	$-\Delta\Delta G°$ (kcal.mol^{-1})
$(GlcNAc)_3$*	25	13.82	22.3	7.26
$(GlcNAc)_4$	25	1.43	5.3	-0.20
$(GlcNAc)_5$	25	1.38	4.5	0.02
$(GlcNAc)_6$	25	0.73	0.6	0.52

∗Values for chitotriose are in comparison for the lectin alone. Hence the $-\Delta\Delta H°$, $\Delta\Delta S°$ and $-\Delta\Delta G°$ values are for the three GlcNAc residues of the trisaccharide.

From the data presented in Table 6.2 it can be seen that the addition of the 4th, 5th and 6th *N*-acetylglucosamine residues to chitotriose increases the enthalpy of binding by 1.43, 1.38 and 0.73 kcal/mol. This shows that the 4th and 5th GlcNAc residues make roughly equal contribution to the binding enthalpy, whereas the contribution of the 6th GlcNAc residue is about half as much. However, in the case of the 4th and 5th GlcNAc residues, the increase in the binding enthalpy is compensated by an increase in the binding entropy, as a result of which the K_b values for chitotriose, chitotetraose and chitopentaose are comparable. On the other hand, for the binding of chitohexaose, the entropy of binding is not altered noticeably and hence the K_b value for this sugar increases by about 2.5 fold as compared to chitopentaose.

Fluorescence spectroscopy

Upon excitation at 280 nm snake gourd phloem lectin gave an emission spectrum centered at 339 nm. Titration of the protein with chitooligosaccharides resulted in a decrease in the emission intensity by 32-45% along with a 3 nm blue shift in the emission maximum. A spectrum corresponding to a representative fluorescence titration of SGPL with chitopentaose is given in Fig. 6.2. Here spectrum 1 corresponds to the protein alone and spectra 2-18 correspond to those recorded in the presence of increasing concentrations of chitopentaose. Inset of the figure gives a plot of the change in fluorescence intensity, ΔF (= $F_o - F$) as a function of the added ligand concentration, which represents the binding curve for the titration. The fluorescence intensity at saturation binding was determined from the Y-intercept of a plot of $F_o/\Delta F$ versus $1/[L]_t$, where F_o is the initial fluorescence intensity, F is the fluorescence intensity at any point during the titration, and $[L]_t$ is the total concentration of the ligand (chitopentaose). The fluorescence titration data

was then analyzed by the method of Chipman et al. [1967] according to the equation:

$$\log \{\Delta F/(F\text{-}F_\infty)\} = \log K_b + \log [L]_f \qquad (6.5)$$

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

$$[L]_f = [L]_t - \{(\Delta F/\Delta F_\infty)\,[P]_t\} \qquad (6.6)$$

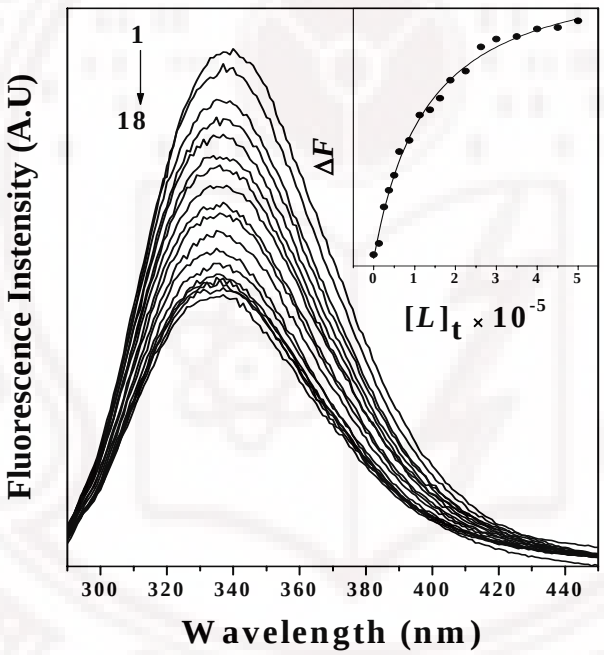


Fig. 6.2. Fluorescence spectra of SGPL in the absence and after addition of defined aliquots from 0.5 mM chitopentaose stock solution. Inset shows the binding curve.

where ΔF_∞ (= F_o- F_∞) is the change in fluorescence intensity at saturation binding and $[P]_t$ is the total protein concentration. The X-intercept of a double logarithmic plot of log $\{\Delta F/(F\text{-}F_\infty)\}$ *versus* log $[L]_f$ will yield the pK_b value for the association

reaction. Such a plot for the binding of chitopentaose to SGPL is shown in Fig. 6.3.

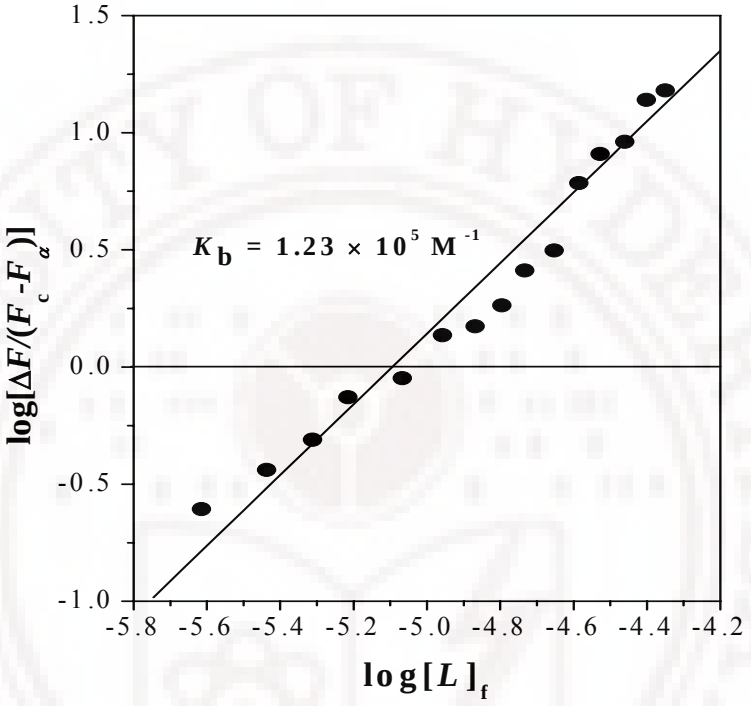


Fig. 6.3. Double-logarithmic plot for the binding of chitopentaose to snake gourd phloem lectin. The double logarithmic plot was obtained using Chipmann analysis.

From the X-intercept of this plot the association constant, K_b has been determined as 1.23×10^5 M^{-1}. This value is in reasonably good agreement with the K_b value of 1.54×10^5 M^{-1} determined at 25 °C from the ITC studies. The slope of this plot is found to be ~1.0, indicating that each lectin subunit binds one saccharide molecule. The fluorescence titration data for the interaction of the other chitooligosaccharides were also analyzed in an analogous manner and the association constants obtained are presented in Table 6.3. In addition, values of change in Gibbs' free energy, calculated according to Equation (6.3), are also listed in this Table.

Table 6.3. Binding constants, K_b, obtained for various chitooligosaccharides at room temperature with snake gourd phloem lectin and the corresponding Gibb's free energy values.

Sugar	K_b $\times 10^{-5}$ (M^{-1})	$-\Delta G°_b$ (Kcal.mol^{-1})
$(GlcNAc)_3$	1.02	6.86
$(GlcNAc)_4$	1.12	6.92
$(GlcNAc)_5$	1.23	6.97
$(GlcNAc)_6$	1.34	7.03

Discussion

The affinity chromatographic purification and general characterization of snake gourd phloem exudate lectin (SGPL), which is specific for chitooligosaccharides is described in Chapter 5 of this thesis. SGPL is a heterodimer with subunit molecular weights of ca. 50 and 55 kDa. Secondary structural analysis shows that it consists of mostly β-sheet with less α-helical content. The lectin is active in the 5-10 pH range and its thermal unfolding temperature is around 70 °C. In order to investigate the binding of chitooligosaccharides to this lectin and determine the association constants and the associated thermodynamic parameters, isothermal titration calorimetric and fluorescence ligand binding experiments were carried out. The results obtained are discussed below.

Results obtained from ITC and fluorescence studies on the interaction of chitooligosaccharides with SGPL are presented in Table 6.1 and Table 6.3,

respectively. Both these methods indicate that the dimeric lectin binds two ligand molecules with comparable affinity with chitohexaose being the best ligand. The thermodynamic data are consistent with an extended binding site in this lectin. Based on thermodynamics of chitooligosaccharide binding extended binding sites were also identified in other phloem exudate lectins, viz., *Luffa acutangula* agglutinin [Anantharam et al., 1986], *Coccinia indica* agglutinin [Sanadi & Surolia, 1994] and *Cucurbita maxima* (pumpkin) phloem exudate lectin, PPL [see Chapter 3]. However, PPL binds chitotriose with higher affinity as compared to chitotetraose and chitopentaose, whereas chitohexaose is recognized with a 12-15 fold higher affinity with a considerable decrease in the binding stochiometry suggesting the formation of higher order complex. Thus chitohexaose appears to bind to two different molecules of PPL simultaneously, whereas SGPL has a more extended binding site, which can accommodate upto the hexasaccharide. ITC studies on the interaction of chitooligosaccharides to *Urtica dioica* agglutinin and wheat germ agglutinin have shown that the binding affinity increases with increase in the size of chitooligosaccharides, suggesting that these two lectins also contain extended binding sites that can accommodate a trisaccharide and a tetrasaccharide, respectively [Bains et al., 1992; Lee et al., 1998; Katiyar et al., 1999]. Calorimetric studies on the association of hevein to oligomers of *N*-acetylglucosamine also show that hevein has an extended binding site [Asensio et al., 2000]. The binding constants obtained for the association of chitooligosaccharides with snake gourd phloem exudate lectin are approximately an order of magnitude higher than the corresponding values obtained for their interaction with the *Urtica dioica* lectin and WGA but are comparable to the values obtained with PPL and LAA. The binding reaction for [$(GlcNAc)_{3-6}$] investigated were essentially enthalpy driven with the binding enthalpy (ΔH_b) at 298.15 K for the different chitooligosaccharides ranging between –13.82 to –17.36 kcal.mol^{-1}, whereas the entropic contribution to the

binding reaction is negative, with the value of binding entropy (ΔS_b) being -22.32 to -32.7 cal.mol^{-1}.K^{-1}.

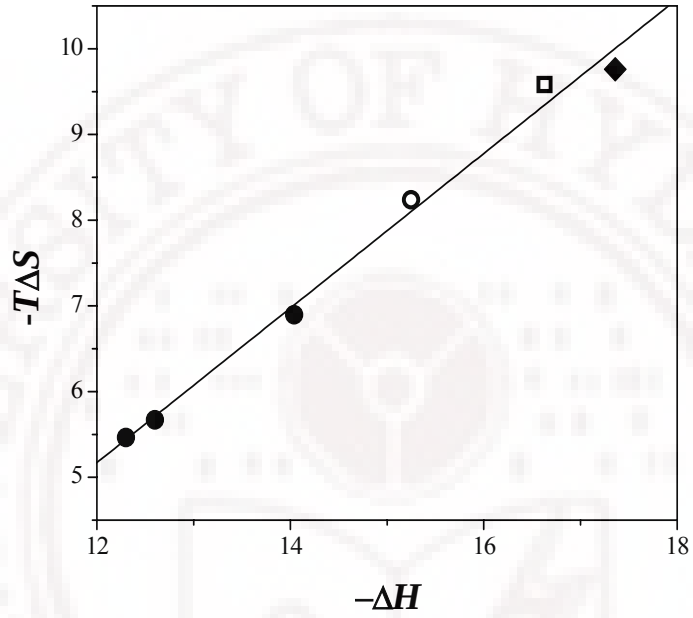


Fig. 6.4. Enthalpy–Entropy compensation plot for SGPL-chitooligosaccharide interaction. The oligosaccharides used are: chitotriose (●), chitotetraose (○), chitopentaose (□) and chitohexaose (◆). The straight line corresponds to linear least squares fit of the data. Slope of the line is 1.04 and correlation coefficient (*R*) is 0.999.

A plot for the -Δ*H* versus -*T*Δ*S* shown in Fig. 6.4 yields a straight line with slope greater than unity (slope 1.04; correlation coefficient (R) is 0.999) which indicates that the reactions for chitooligosaccharide to SGPL are enthapically driven as generally found in lectin carbohydrate interactions [Chapter 2; Sultan & Swamy, 2005b; Katiyar et al., 1999]. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals' interactions.

Results from fluorescence titrations for the binding of chitooligosaccharides to SGPL are in good agreement with the results from isothermal titration calorimetry in terms of binding constant and free energy changes. The emission maximum for SGPL seen at 339 nm exhibits a 3 nm blue shift, accompanied by a decrease in the emission intensity. In contrast, the fluorescence intensity of *L. acutangula* agglutinin was found to increase upon saccharide binding and emission maximum blue shifted to 2-4 nm [Anantharam et al., 1986]. The binding constants and Gibb's free energy values increase with increasing size of chitooligosaccharides as observed with calorimetric titrations.

In summary the titration calorimetric and fluorescence spectroscopic studies reported in this chapter clearly shows that SGPL contains an extended carbohydrate binding site that seems to accommodate upto a hexasaccharide. The binding process is governed by enthalpic forces with negative contribution from binding entropy. Enthalpy-entropy compensation was observed for the interaction between SGPL and chito-oligosaccharides attesting to the crucial role played by water structure in the overall binding process.

Chapter 7

General Discussion and Conclusions

General discussion and conclusions

In this thesis, studies on the purification and characterization of two chitooligosaccharide-specifc lectins from pumpkin (*Cucurbita maxima*) phloem exudate and snake gourd (*Trichosanthes anguina*) phloem exudate have been reported. The first of these two proteins, namely the pumpkin phloem exudate lectin (PPL) was purified previously by others and characterized in some detail. Especially, its primary structure was determined based on the cDNA sequence. It has been proposed that the lectin plays a role in the plant's defense mechanism by interacting with bacteria and fungi through its carbohydrate binding pocket. In view of this it is important to investigate the macromolecular and carbohydrate binding properties of this protein in detail. Therefore, we developed a rapid affinity method for its purification and investigated the protein with respect to macromolecular properties, secondary structure determination by CD spectroscopy, tryptophan exposure by fluorescence quenching and thermodynamic studies on ligand binding using isothermal titration calorimetry. These studies are reported in Chapters 2, 3 and 4. In previous work from this laboratory, a galactose-specific lectin was purified from the seed extracts of snake gourd (*Trichosanthes anguina*) and characterized in considerable detail. Since lectins were identified also in the phloem exudate of the fruits of a few cucurbit species, we investigated the phloem exudate of snake gourd for lectin activity and found strong hemagglutinating activity in it, which could be inhibited by chitooligosaccharides. In Chapter 5, isolation, affinity purification of the chitooligosaccharide specific lectin from the snake gourd (*Trichosanthes anguina*) are reported. The secondary structure of the lectin was characterized by CD spectroscopy and its exact mass was determined by MALDI-TOF mass spectrometry. In Chapter 6, the interaction between snake gourd phloem exudate lectin (SGPL) and chitooligosaccharides [$(GlcNAc)_{3-6}$] was

studied by isothermal titration calorimetry and fluorescence spectroscopy. The results obtained from the above studies are summarized below.

High hemagglutinating activity has been reported about three decades ago in the phloem exudate from three cucurbit species, namely *Cucurbita maxima, Cucumis sativus* and *Cucumis melo*. Among these, a lectin specific for chitooligosaccharides has been purified from the phloem exudate of *Cucurbita maxima* (pumpkin) by conventional chromatographic methods such as ion exchange chromatography and gel filtration. In the studies reported in Chapter 1, a simple and rapid affinity chromatographic method employing chitin as the affinity material has been developed for the purification of pumpkin phloem lectin. The purified lectin could be inhibited by chitooligosaccharides and chitohexaose was the best ligand. In SDS-PAGE in the presence of β-mercaptoethanol PPL yielded a single band corresponding to a molecular weight of 23.7 kDa, whereas MALDI-TOF mass spectrometry gave the molecular weights of the subunit and dimeric lectin as 24,812.3 and 49,721.5 Daltons, respectively. Analysis of the CD spectrum of the protein indicated that the secondary structure of PPL consists of 9.7% α-helix, 35.8% β-sheet, 22.5% β-turns and 32.3% unordered structures. Saccharide binding did not significantly affect the secondary and tertiary structures of the protein. Secondary structure prediction using the amino acid sequence of the protein by SOPMA and DPM methods also yielded results similar to those presented above. The hemagglutinating activity of PPL was mostly unaffected in the temperature range 4-70 °C, but a sharp decrease was seen between 75 and 85 °C. Thermal stability studies on PPL using temperature dependent CD spectroscopy showed that the secondary structure of this protein is stable in the temperature region 30-80 °C, whereas significant changes were seen in the spectra recorded above 80 °C, suggesting that the protein undergoes thermal unfolding at

higher temperatures. This is confirmed by differential scanning calorimetric studies, which showed that PPL alone exhibits an unfolding transition centered at about 81.5 °C, which shifts to 83.8 °C in the presence of 10 mM chitotriose.

In the studies reported in Chapter 3, the interaction of chitooligosaccharides with PPL was investigated by isothermal titration calorimetry. Thermodynamic parameters obtained from ITC studies show that the dimeric PPL binds to two molecules of chitotriose, chitotetraose and chitopentaose with comparable binding constants (K_b) of 1.8×10^5, 1.4×10^5 and 0.9×10^5 M^{-1} at 25 °C, respectively, whereas chitobiose exhibits ~100 fold lower affinity with a K_b value of 2.9×10^3 M^{-1}. However, chitohexaose shows about 12 to 15 times more affinity with a K_b value of 4.16×10^5 M^{-1}. The stoichiometry of binding decreases steadily from ~ 2.2 to 1.3 as the number of GlcNAc units in the chitooligosaccharide is increased from 2 to 6, suggesting that the higher oligosaccharides may interact with more than one protein molecule. The stronger binding of chitohaxaose could be partly due to the formation of such higher order complexes.

The binding of $[(GlcNAc)_{2\text{-}6}]$ to PPL is essentially enthalpy driven with the binding enthalpy (ΔH_b) at 25 °C for the different chitooligosaccharides ranging between -27.9 and -10.25 $kcal.mol^{-1}$, whereas the entropic contribution to the binding reaction is negative, with binding entropy (ΔS_b) values being in the range of -63.3 to -18.5 $cal.mol^{-1}.K^{-1}$ at 25 °C. The enthalpically driven nature of binding reactions suggests that the main factors that stabilize the interaction of saccharides with PPL are hydrogen bonding and van der Waals' interactions. In addition, enthalpy-entropy compensation was observed in the binding of various chitooligosaccharides to PPL, underscoring the role of reduced water molecules in the binding process.

Fluorescence quenching and time-resolved fluorescence studies carried out on pumpkin phloem exudate lectin with the objective of investigating the tryptophan exposure and environment in PPL form the subject of study in Chapter 4. In these studies, quenching of the intrinsic fluorescence of PPL by a neutral quencher (acrylamide) and two ionic quenchers (iodide ion and cesium ion) was investigated in the native state, in the presence of saturating concentrations of chitooligosaccharides and upon denaturation. The emission λ_{max} of native lectin seen at 338 nm was red-shifted to 348 nm upon denaturation by 6 M Gdn.HCl in the presence of 10 mM β-mercaptoethanol, whereas a blue-shift to 335 nm was observed in the presence of saturating concentrations of chitotriose. The percentage of quenching observed with different quenchers for native and denatured lectin is comparable indicating that the Trp residues are significantly exposed to the aqueous environment for native lectin. However, quenching with acrylamide was considerably higher than with I^- and Cs^+, clearly indicating that some of the Trp residues are not accessible to the ionic quenchers, which cannot penetrate into the protein interior. Further, between the two ionic quenchers the extent of quenching is more with iodide as compared to Cs^+, which is consistent with the presence of positively charged residues adjacent to three tryptophan residues, i.e., W-87, W-89 and W-199 as seen from the primary structure of the protein. The Stern-Volmer plot for acrylamide was linear for native PPL and upon ligand binding, but became upward curving upon denaturation, indicating that the quenching occurs via a combination of static and dynamic mechanisms. A red-edge excitation shift (REES) of 4 nm was observed for the native protein, which decreased to 3 nm upon ligand binding and further to 2 nm when denatured with 6M Gdn.HCl. This indicates that tryptophan residues are localized in a microenvironment of restricted mobility, and that the regions surrounding the Trp residues in this protein offer considerable restriction to the reorientational motion of the water dipoles around

the excited state tryptophans. In time-resolved fluorescence experiments, the decay curves could be best fit to biexponential patterns, with life times of 1.20 and 4.35 for native PPL. This indicates that Trp residues of PPL may fall into two categories, one group which has a shorter decay time and the second group which has a longer decay time. In all cases both lifetimes systematically decreased with increasing acrylamide concentrations, indicating that quenching occurs predominantly via a dynamic process.

In the studies reported in Chapter 5, a new lectin activity was identified in the phloem exudate of snake gourd (*Trichosanthes anguina*) fruits, which could be inhibited by chitooligosaccharides, clearly indicating that the lectin specifically recognizes β(1→4) linked oligosaccharides of *N*-acetyglucosamine. Therefore, affinity chromatography on chitin was chosen for the purification of the lectin. The affinity purified snake gourd phloem lectin (SGPL) yielded a single band on native PAGE, whereas in SDS-PAGE in the presence of β- mercaptoethanol it gave two bands of comparable intensity corresponding to masses of 48 and 53 kDa, suggesting that it is a heterodimer. MALDI-TOF mass spectrometric analysis supported the above observation and gave the molecular weights of the two subunits as 50,597 and 55,399 Daltons. Secondary structural analysis by CD spectroscopy showed that SGPL is a predominantly β-sheet protein with a relatively small α-helical content and that ligand binding does not lead to significant structural changes. The inhibitory potency of chitooligosaccharides increased with increase in size from chitobiose to chitohexaose. Agglutination activity of SGPL is maximal in the pH range 5.0-10.0 and only 50% activity was seen at pH 4. Thermal inactivation studies show that SGPL is fully active up to 50 °C and that the activity decreases quite sharply between 60 and 80 °C. Complete loss of activity observed sample incubated at 80 °C. These observations indicate

that SGPL is a relatively thermostable protein. CD spectra recorded at different temperatures show that the secondary structure of SGPL is essentially unaltered when heated up to 60 °C, whereas significant changes were observed in both secondary and tertiary structure at higher temperatures. Complete loss of secondary and tertiary structures was observed above 70 °C. DSC studies show that SGPL undergoes a cooperative unfolding transition centered at ca. 73 °C. However, due to precipitation of the protein during the unfolding process, the thermodynamic parameters associated with the unfolding process could not be delineated.

Thermodynamic studies on the interaction of chitooligosaccharides with the snake gourd (*Trichosanthes anguina*) phloem exudate lectin form the subject of study in Chapter 6. In the studies reported in this chapter, the interaction of chitooligosaccharides (n = 3-6) with SGPL were investigated using isothermal titration calorimetry and fluorescence spectroscopy. Calorimetric titrations indicate that the dimeric lectin binds to two molecules of the ligand with association constants determined at 25 °C being 1.75×10^5, 1.39×10^5, 1.45×10^5 and 3.70×10^5 M^{-1}, for chitotriose, chitotetraose, chitopentaose and chitohexaose, respectively. The binding reaction was essentially enthalpy driven with the binding enthalpy (ΔH_b) at 25 °C for the different chitooligosaccharides ranging between -17.36 and -13.82 kcal.mol^{-1}, whereas the entropic contribution to the binding reaction is negative, with the value of binding entropy (ΔS_b) being in the range of -32.7 and -22.3 cal.mol^{-1}.K^{-1}. The enthalpically driven nature of binding reaction suggests that the main factors that stabilize the interaction of saccharides with SGPL are hydrogen bonding and van der Waals' interactions. Intrinsic fluorescence studies on the lectin show that emission maximum for SGPL seen at 339 nm exhibits a 3 nm blue shift, accompanied by a decrease in the emission intensity when titrated with chitooligosaccharides. The ligand-induced fluorescence changes were

analyzed to estimate the association constants characterizing the binding of chitooligosaccharides to SGPL. The association constants obtained have been found to be in good agreement with those obtained from the isothermal titration calorimetry studies mentioned above. As demonstrated above for PPL-chitooligosaccharide interaction, enthalpy-entropy compensation was observed in the binding of chitooligosaccharides to SGPL also, which would be attributed to the involvement of water molecules in the overall binding process.

In summary, in this thesis physicochemical and carbohydrate binding studies have been carried out on two lectins isolated from the phloem exudate of the fruits of two Cucurbitaceae species. Firstly, a new affinity chromatographic method was developed for the purification of pumpkin phloem exudate lectin (PPL). Isothermal titration calorimetric studies show that carbohydrate binding site of PPL accommodates chitotriose and higher oligomers of GlcNAc with high affinity. Fluorescence quenching and time-resolved fluorescence studies carried out on PPL indicate that the tryptophan residues of this protein are significantly exposed to aqueous environment. Secondly, a new chitooligosaccharide specific lectin has been purified to homogeneity from snake gourd (*Trichosanthes anguina*) phloem exudate (SGPL). This lectin is a heterodimer with subunit mass of 50 and 55 kDa whereas other phloem lectins such as PPL, CIA, LAA and lectin from *Cucurbita pepo* were homodimers with subunit mass of 20 to 24 kDa. Even though SGPL is distinctly different from the other cucurbit phloem exudate lectins with regard to subunit composition and molecular weight, the carbohydrate specificity is similar to that of the other phloem exudate lectins, i.e., it specifically recognizes chitooligosaccharides. Among the cucurbit phloem exudate lectins, the primary structure is known only for PPL. In order to understand the evolutionary relationships among these proteins and to unravel the structural origins of their specificity towards chitooligosaccharides it would be essential to determine the

primary structure of the other lectins from this group and also to determine the three-dimensional structures of some of them.

References

Reference:

Agrawal, B. B. L. & Goldstein, I. J. (1965) Specific binding of concanavalin A to cross-linked dextran gels. *Biochem. J.* **96**: 23-25

Allen, A. K. & Neuberger, A. (1973) The purification and properties of the lectin from potato tubers, a hydroxyproline-containing glycoprotein. *Biochem. J.* **135**: 307-314.

Allen, A. K. (1979) A lectin from the exudate of the fruit of the vegetable marrow (*Cucurbita pepo*) that has a specificity for β-1,4-linked *N*-acetylglucosamine oligosaccharides. *Biochem. J.* **183:** 133-137.

Allen, A. K., Bolwell, G. P., Brown, D. S., Sidebottom, C. & Slabas, A. R. (1996) Potato lectin: a three-domain glycoprotein with novel hydroxyproline-containing sequences and sequence similarities to wheat-germ agglutinin. *Int. J. Biochem. Cell Biol.* **28**: 1285-1291.

Allen, A. K., Neuberger, A. & Sharon, N. (1973) The purification, composition and specificity of wheat germ agglutinin. *Biochem. J.* **131**: 155-162.

Anantharam, V., Patanjali, S. R., Swamy, M. J., Sanadi, A. R., Goldstein, I. J. & Surolia, A. (1986) Isolation, macromolecular properties, and combining site of a chito-oligosaccharide-specific lectin from the exudate of ridge gourd (*Luffa acutangula*). *J. Biol. Chem.* **261**: 14621-14627.

Anbazhagan, V., Damai, R. S., Paul, A. & Swamy, M. J. (2008) Interaction of the major protein from bovine seminal plasma, PDC-109 with phospholipid membranes and soluble ligands investigated by fluorescence approaches. *Biochim. Biophys. Acta* **1784**: 891-899.

Ariizumi, K., Shen, G. L., Shikano, S., Xu, S., Ritter, R., 3rd, Kumamoto, T., Edelbaum, D., Mortia, A., Bergstresser, P. R. & Takashima, A. (2000) Identification of novel, dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J. Biol. Chem.* **275**: 20157-20167.

Asensio, J. L., Cañada, F. J., Siebert, H. C., Laynez, J., Poveda, A., Nieto, P. M., Soedjanaamadja, U. M., Gabius, H. J. & Jiménez-Barbero, J. (2000) Structural basis for chitin recognition by defense proteins: GlcNAc residues are bound in a multivalent fashion by extended binding sites in hevein domains. *Chem. Biol.* **7**, 529-543.

Baenziger, J. U. & Fiete, D. (1979) Structural determination of concanavalin A specificity for oligosaccharides. *J. Biol. Chem.* **254**: 2400-2407.

Bains, G., Lee, T. R., Lee, Y. C. & Freire, E. (1992) Microcalorimetric study of wheat germ agglutinin binding to *N*-acetylglucosamine and its oligomers. *Biochemistry* **31**: 12624-12628.

Banin, E., Israely, T., Fine, M., Loya, Y. & Rosenberg, E. (2001) Role of endosymbiotic zooxanthellae and coral mucus in the adhesion of the coral-bleaching pathogen *Vibrio shiloi* to its host. *FEMS Microbiol. Lett*. **199**: 33-37.

Barondes, S. H. (1988) Biofunctional property of lectin: lectins redefined. *Trends Boichem. Sci.* **13**: 480-482.

Barre, A., Bourne, Y., Van Damme, E. J. M., Peumans, W. J. & Rouge, P. (2001) Mannose binding plant lectins: different structural scaffolds for a common sugar-recognition process. *Biochemie* **83**: 645- 651.

Beintema, J. J. & Peumans, W. J. (1992) The primary structure of stinging nettle (*Urtica dioica*) agglutinin: a two-domain member of the hevein family. *FEBS Lett*. **299**: 131-134.

Beintema, J. J. (1994) Structural features of plant chitinases and chitin-binding proteins. *FEBS Lett.* **350**: 159-163.

Bhanu, K., Komath, S. S., Maiya, B. G. & Swamy, M. J. (1997) Interaction of porphyrins with concanavalin A and pea lectin. *Curr. Sci.* **73**: 598-603.

Bloch, R. & Burger, M. M. (1974) Purification of wheat germ agglutinin using affinity chromatography on chitin. *Biochem. Biophys. Res. Commun.* **58**: 13-19.

Bogoeva, V .P., Radeva, M. A., Atanasova, L. Y., Stoitsova, S. R. & Boteva, R. N. (2004) Fluorescence analysis of hormone binding activities of wheat germ agglutinin. *Biochim. Biophys. Acta* **1698**: 213-218.

Bostwick, D. E., Dannenhoffer, J. M., Skaggs, M. I., Lister, R. M., Larkins, B. A. & Thomspon, G. A. (1992) Pumpkin phloem lectin genes are specifically expressed in companion cells. *Plant Cell* **4**: 1539-1548.

Bostwick, D. E., Skaggs, M. I. & Thompson, G. A. (1994) Organization and characterization of *Cucurbita* phloem lectin genes. *Plant Mol. Biol.* **26**: 887-897.

Boyd, W. C. & Shapleigh, E. (1954) Specific precipitating activity of plant agglutinins (lectins). *Science* **119**: 419.

Broekaert, W. F., Marien, W., Terras, F. R. G., De Bolle, M. F. C., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S. B., Vanderleyden, J. & Cammue, B. P. A. (1992) Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry* **31**: 4308-4314.

Burkhalter, N. F., Dimic, S. M. & Toone, E. J. (2000) protein carbohydrate interactions: fundamental considerations. In *Carbohydrates in Chemistry and Biology*, *Part II* (Ernst, B., Hart, G. W. & Sinaÿ, P., eds.) Vol. 2, Part 1, pp. 863-914. Wiley-VCH, Weinhein.

Carter, W. G. & Sharon, N. (1977) Properties of human erythrocyte membrane receptors for peanut and *Dolichos biflorus* lectins. *Arch. Biochem. Biophys.* **180**: 570-582.

Chattopadhyay, A., Rawat, S. S., Kelkar, D. A., Ray, S. & Chakrabarti, A. (2003) Organization and dynamics of tryptophan residues in erythroid spectrin: novel structural features of denatured spectrin revealed by wavelength-selective fluorescence approach. *Protein Sci.* **12**: 2389-2403.

Chen, Y., Peumans, W. J., Hause, B., Bras, J., Kumar, M., Proost, P., Barre, A., Rouge, P. & Van Damme, E. J. M. (2002) Jasmonic acid methyl ester induces the synthesis of a cytoplasmic/nuclear chito-oligosaccharide binding lectin in tobacco leaves. *FASEB J.* **16**: 905-907.

Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U. & Vad, K. (1993) Plant chitinases. *Plant J.* **3**: 31-40.

Compton, L. A. & Johnson, W. C., Jr. (1986) Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. *Anal. Biochem.* **155**: 155-167.

Dam, T. K. & Brewer, C. F. (2002) Thermodynamic studies of lectin-carbohydrate interactions by isothermal titration calorimetry. *Chem. Rev.* **102**: 387-429.

Dam, T. K., Roy, R., Das, S. K., Oscarson, S., & Brewer, C. F. (2000) Binding of multivalent carbohydrates to concanavalin A and *Dioclea grandiflora* lectin.

Thermodynamic analysis of the "multivalent effect". *J. Biol. Chem.* **275**: 14223-14230.

Di Sabato, G., Hall, J. M. & Thompson, L. (1987) T cell mitogens and polyclonal B cell activators. *Methods Enzymol.* **150**: 3-17.

Drickamer, K. (1994) Molecular structure of animal lectins. In *Molecular Glycobiology.* (Fukuda, M. and Hindsgaul, O. eds.), pp 53-87. Oxford University Press, Oxford.

Eftink, M. R. & Ghiron, C. A. (1975) Dynamics of a protein matrix revealed by fluorescence quenching. *Proc. Natl. Acad. Sci.* USA **9**: 3290-3294.

Eftink, M. R. & Ghiron, C. A. (1976a) Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. *Biochemistry* **15**: 672-680.

Eftink, M. R. & Ghiron, C. A. (1976b) Fluorescence quenching of indole and model micelle systems. *J. Phys. Chem.* **80**: 486-493.

Eftnik, M. R. & Ghiron, C. A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem.* **114**: 199-227.

Epstein, J., Eichbaum, Q., Sheriff, S. & Ezekowitz, R. A. (1996) The collectins in innate immunity. *Curr. Opin. Immunol*. **8**: 29-35.

Falasca, A. I., Abbondanza, A., Barbieri, A., Bolognesi, A., Rossi, C. A. & Stripe, F. (1989) Purification and partial characterization of a lectin from the seed of *Trichosanthes kirilowii* Maximowicz. *FEBS Lett.* **246**: 159-162.

Fletcher, M. A., Baron, G. C., Ashman, M. R., Fischl, M. A. & Klimas, N. G. (1987) Use of whole blood methods in assessment of immunodeficiency states. *Diag. Clin. Immunol.* **5**: 69-81.

Garcia-Hernández, E., Zubillaga R. A., Rojo-Domínguez, A., Rodriguez-Romero, A., Hernández-Arana, A. (1997) New insights into the molecular basis of lectin carbohydrate interactions: a calorimetric and structural study of the association of hevien to oligomaers of *N*-acetylglucosamine. *Proteins* **29**: 467-477.

Gatehouse, A. M. R., Powel, K. S., Peumans, W. J. & Van Damme, E. J. M. (1995) Insecticidal properties of plant lectins: their potential in plant protection. In *lectins:*

Biomedical Perspectives (Pusztai, A., Bardocz, S. ed.) pp. 35-37. Taylor & Francis, London.

Gegg, C. V., Roberts, D. D., Segel, I. H. & Etzler, M. E. (1992) Characterization of the adenine binding sites of two *Dolichos biflorus* lectins. *Biochemistry* **31**: 6938-6942.

Geourjon, C. & Deléage, G. (1995) SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Comput. Appl. Biosci.* **11**: 681-684.

Geourjon, C., Deléage, G. & Roux, B. (1991) ANTHEPROT: an interactive graphics software for analyzing protein structures from sequences. *J. Mol. Graph.* **167**: 188-190.

Gershoni, M. & Palade, G. E. (1982) Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to positively charged membrane filters *Anal. Biochem.* **124**: 396-405.

Goel, M., Anuradha, P., Kaur, K. J., Maiya, B. G., Swamy, M. J. & Salunke, D. M. (2004) Porphyrin binding to jacalin is facilitated by the inherent plasticity of the carbohydrate binding site: novel mode of lectin-ligand interaction. *Acta Cryst. D* **60**: 281-288.

Goel, M., Damai, R. S., Sethi, D. K., Kaur, K. J., Maiya, B. G., Swamy, M. J. & Salunke, D. M. (2005) Crystal structures of the PNA-porphyrin complex in the presence and absence of lactose: mapping the conformational changes on lactose binding, interacting surfaces, and supramolecular aggregations. *Biochemistry* **44**: 5588-5596.

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.

Goldstein, I. J. & Poretz, R. D. (1986) Isolation, physicochemical characterization and carbohydrate-binding specificities of lectins. In *The Lectins: Properties, Functions and Applications in Biology and Medicine* (Liener, I. E., Sharon, N., Goldstein, I. J., Eds.), pp 35-247. Academic Press, Orlando, Florida.

Goldstein, I. J. (1990). On a modest advance in an obscure area: an old timer's reminiscences. In *Lectins, Biology, Biochemistry, Clinical biochemistry* (Kocourek,

J. & Freed, D. L. J., eds.), Vol. 7, pp. 9-11. Sigma Chemical Company, st. Louis, MO.

Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. & Sharon, N. (1980) What should be called a lectin? *Nature* **285**: 66.

Grinvald, A. & Steinberg, I. Z. (1974) On the analysis of fluorescence decay kinetics by the method of least squares. *Anal. Biochem.* **59**: 583-598.

Grinvald, A. & Steinberg, I. Z. (1976) The fluorescence decay of tryptophan residues in native and denatured proteins. *Biochim. Biophys. Acta* **427**: 663-678.

Guha, S., Rawat, S. S., Chattopadhyay, A. & Bhattacharyya, B. (1996) Tubulin conformation and dynamics: A red edge excitation shift study. *Biochemistry* **35**: 13426-13433.

Gupta, D., Cho, M., Cummings, R. D. & Brewer, C. F. (1996) Thermodynamics of carbohydrate binding to galectin-1 from Chinese hamster ovary cells and two mutants. A comparison with four galactose-specific plant lectins. *Biochemistry* **35**: 15236-15243.

Hamelryck, T. W., Loris, R., Bouchaert, J., Dao-Thi, M. H., Strecker, G., Imberty, A., Fernandez, E., Wyns, L. & Etzler, M. E. (1999) Carbohydrate binding, quaternary structure and a novel hydrophobic binding site in two legume lectin oligomers from *Dolichos biflorus. J. Mol. Biol.* **286**: 1161-1177.

Hao, Q., Van Damme, E. J. M., Hause, B., Barre, A., Chen, Y., Rouge, P. & Peumans, W. J. (2001) Iris bulbs express type-1 and type-2 ribosome inactivating proteins with unusual properties. *Plant Physiol.* **125**: 866-876.

Harata, K. & Muraki, M. (2000) Crystal structure of *Urtica Dioica* agglutinin and its complex with tri-*N*-acetylchitotriose. *J. Mol. Biol.* **297**: 673-681.

Haseley, S. R., Talaga, P., Kamerling, J. P. & Vliegenthart, J. F. G. (1999) Characterization of the carbohydrate binding specificity and kinetic parameters of the lectin by using surface plasmon resonance. *Anal. Biochem.* **274**: 203-210.

Hayes, C. E. & Goldstein, I. J. (1974) An α-D-galactosyl-binding lectin from *Bandeiraca simplicifolia* seeds. Isolation by affinity chromatography and charecterization. *J. Biol. Chem*. **249**: 1904-1914.

Hirabayashi, J., Hashidate, T., Arata, Y., Nishi, N., Nakamura, T., Hirashima, M., Urashima, T., Oka, T., Futai, M., Muller, W. E., Yagi, F. & Kasai, K. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim. Biophys. Acta* **1572**: 232-254.

Hirsch, A. M. (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Curr. Opin. Plant Biol.* **2**: 320-326.

Katiyar, S., Van Damme, E. J. M., Peumans, W. J. & Surolia A. (1999) Thermodynamic analysis chitooligosaccharide binding to *Urtica dioica* agglutinin by isothermal titration calorimetry. *Biosci. Rep.* **19**: 411-419.

Kavitha, M., Sultan, N. A. M. & Swamy, M. J. (2009) Fluorescence studies on the interaction of hydrophobic ligands with *Momordica charantia* (bitter gourd) seed lectin. *J. Photochem. Photobiol B: Biol.* **94**: 59-64

Kenoth, R. & Swamy, M. J. (2003) Steady-state and time-resolved fluorescence studies on *Trichosanthes cucumerina* seed lectin. *J. Photochem. Photobiol. B: Biol.* **69**: 193-201.

Kenoth, R., Komath, S .S. & Swamy, M. J. (2003) Physicochemical and saccharide binding studies on the galactose-specific seed lectin from *Trichosanthes cucumerina*. *Arch. Biochem. Biophys.* **413**: 131-138.

Kenoth, R., Padma, P., Sarada, S. & Swamy, M. J. (2000) Role of histidine residues in the sugar-binding activity of *Trichosanthes cucumerina* seed lectin. *J. Biochem. Mol. Biol. Biophys.* **4**: 423-431.

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.

Khan, M. I., Mazumder, T., Pain, D., Gaur, N. & Surolia, A. (1981) Binding of 4-methylumbelliferyl-β-D-galactopyranoside to *Momordica charantia* agglutinin: Fluorescence quenching studies. *Eur. J. Biochem.* **113**: 471-476.

Kieliszewski, M. J., Showalter, A. M. & Leykam, J. F. (1994) Potato lectin: a modular protein sharing sequence similarities with the extensin family, the hevein lectin family, and snake venom disintegrins (platelet aggregation inhibitors). *Plant J.* **5**: 849-861.

Kijne, J. W. (1996) Functions of plant lectins. *Chemtracts-Biochem. Mol. Biol.* **6**: 180-187.

Kilpatrick, D. C. & Yeoman, M. M. (1978) Purification of the lectin from *Datura Stramonium*. *Biochem. J.* **175**: 1151-1153.

Kilpatrick, D. C. (1998) Use of lectins as mitogens for lymphocytes. In *Lectin Methods and Protocols* (Rhodes, J. M. & Milton, J. D., eds), pp. 385-392. Humana press, Totowa, NJ.

Kobiler, D. & Mirelman, D. (1980) Lectin activity in *Entamoeba histolytica* trophozoites. *Infect. Immun.* **29**: 221-225.

Kocourek, J. & Horejsi, V. (1981) Defining a lectin. *Nature* **290**: 188.

Komath, S. S. & Swamy, M. J. (1998) Further characterization of the saccharide specificity of snake gourd (*Trichosanthes anguina*) seed lectin. *Curr. Sci.* **75**: 608-611.

Komath, S. S. & Swamy, M. J. (1999) Fluorescence quenching, time-resolved fluorescence and chemical modification studies on the tryptophan residues of snake gourd (*Trichosanthes anguina*) seed lectin. *J. Photochem. Photobiol. B: Biol.* **50**: 108-118.

Komath, S. S., Bhanu, K., Maiya, B. G. & Swamy, M. J. (2000a) Binding of porphyrins by the tumor-specific lectin, Jacalin [jack fruit (*Artocarpus integrifolia*) agglutinin]. *Biosci. Rep.* **20**: 265-276.

Komath, S. S., Giribabu, L., Maiya, B. G. & Swamy, M. J. (2000b) Fluorescence and absorption spectroscopic studies on the interaction of porphyrins with snake gourd (*Trichosanthes anguina*) seed lectin. *J. Photochem. Photobiol. B: Biol.* **55**: 49-55.

Komath, S. S., Kavitha, M. & Swamy, M. J. (2006) Beyond carbohydrate binding: new directions in plant lectin research. *Org. Biomol. Chem*. **4**: 973-988.

Komath, S. S., Kenoth, R. & Swamy, M. J. (2001) Thermodynamics of saccharide binding to snake gourd (*Trichosanthes anguina*) seed lectin. Fluorescence and absorption spectroscopic studies. *Eur. J. Biochem.* **268**: 111-119.

Komath, S. S., Nadimpalli, S. K. & Swamy, M. J. (1996) Purification in high yield and characterization of the galactose-specific lectin from the seeds of snake gourd (*Trichosanthes anguina*). *Biochem. Mol. Biol. Int.* **39:** 243-252.

Komath, S. S., Nadimpalli, S. K. & Swamy, M. J. (1998) Identification of histidine residues in the sugar binding site of snake gourd (*Trichosanthes anguina*) seed lectin. *Biochem. Mol. Biol. Int.* **44**: 107-117.

Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature* **227:** 480-485.

Lakowicz, J. R. & Weber, G. (1973) Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. *Biochemistry* **12**: 4171-4179.

Lakowicz, J. R., (1999) Principles of Fluorescence Spectroscopy, 2nd Edition, Kluwer Academic Publishers, New York, 698 pp.

Lannoo, N., Peumans, W. J., Van Pamel, E., Alvarez, R., Xiong, T.-C., Hause, G., Mazars, C. & Van Damme, E. J. M. (2006) Localization and *in vitro* binding studies suggest that the cytoplasmic/nuclear tobacco lectin can interact *in situ* with high-mannose and complex *N*-glycans. *FEBS Lett.* **580**: 6329-6337.

Lee, R. T., Gabius, H. J. & Lee, Y. C. (1998) Thermodynamic parameters of the interaction of *Urtica dioica* agglutinin with *N*-acetylglucosamine and its oligomers. *Glycoconj. J.* **15**: 649-655.

Leffler, H. & Barondes, S. H. (1986) Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian β-galactosides. *J. Biol. Chem.* **261**: 10119-10126.

Leffler, H. (2001) Galectins structure and function- a synopsis. In *Mammalian Carbohydrate recognition Systems* (Crocker, P., ed.), pp. 57-83. Springer, Berlin.

Lehrer, S. S. (1971) Solute perturbation of protein fluorescence. The quenching of tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry* **10**: 3254-3263.

Lemieux, R. U. (1996) How water provides the impetus for molecular recognition in aqueous solution? *Acc. Chem. Res.* **29**: 373-380.

LeVine, D., Kaplan, M. J. & Greenaway, P. J. (1972) The purification and characterization of wheat-germ agglutinin. *Biochem. J*. **129**: 847-856.

Lobley, A. & Wallace, B. A. (2001) DICHROWEB: A website for the analysis of protein secondary structure from circular dichroism spectra. *Biophys. J.* **80**: 373a.

Lobley, A., Whitmore, L. & Wallace, B. A. (2002) DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics* **18**: 211-212.

Loris, R., De Greve, H., Dao-Thi, M. H., Messens, J., Imberty, A. & Wyns, L. (2000) Structural basis of carbohydrate recognition by lectin II from *Ulex europaeus*, a protein with promiscuous carbohydrate binding site. *J. Mol. Biol.* **301**: 987-1022.

Lotan, R. & Nicolson, G. L. (1979) Purifications of cell membrane glycoproteins by lectin affinity chromatography. *Biochim. Biophys. Acta* **559**: 329-376.

Magdolna, M. V. H., Claudia, V., Claudio, S., Simone, R., Gustavo, G. M. & Oscar, G. H. (1992) A lectin from *Sechium edule* fruit exudate. *Phytochemistry* **31**: 1477-1480.

Maliarik, M. J. & Goldstein, I. J. (1988) Photoaffinity labelling of the adenine binding sites of the lectins from lima bean, *Phaseolutus lunatus* and the kidney bean, *Phaseolus vulgaris*. *J. Biol. Chem.* **263**: 11274-11279.

Manoj, N., Jeyaprakash, A. A., Pratap, J. V., Komath, S. S., Kenoth, R., Swamy, M. J. & Vijayan, M. (2001) Crystallization and preliminary X-ray studies on snake gourd (*Trichosanthes anguina*) seed lectin. Homology with type-II ribosome inactivating proteins. *Acta Cryst. D.* **57**: 912-914.

Marinkovich, V. A. (1964) Purification and characterization of the hemagglutinin present in potatoes. *J. Immunol.* **93**: 732-741.

Matsumoto, I., Jimbo, A., Mizuno, Y. & Seno, N. (1983) Purification and characterization of potato lectin. *J. Biol. Chem.* **258**: 2886-2891.

Mazumder, T., Gaur, N. & Surolia, A. (1981) The physicochemical properties of the galactose-specific lectin from *Momordica charantia*. *Eur. J. Biochem*. **113**: 463-470.

Nachbar, M. S., Oppenheimer, J. D. & Thomas, J. O. (1980) Lectins in the U.S. Diet. Isolation and characterization of a lectin from the tomato (*Lycopersicon esculentum*). *J. Biol. Chem.* **255**: 2056-2061.

Nagata, Y. & Burger, M. M. (1972) Wheat germ agglutinin. Isolation and crystallization. *J. Biol. Chem.* **247**: 2248-2250.

Narasimhan, S., Wilson, J. R., Martin, E. & Schachter, H. (1979) A structural basis for four distinct elution profiles on concanavalin A-Sepharose affinity chromatography of glycopeptides. *Can. J. Biochem*. **57**: 83-96.

Nowell, P. C. (1960) Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res*. **20**: 462-466.

Ofek, I., Goldhar, J., Keisari, Y. & Sharon, N. (1995) Nonopsonic phagocytosis of microorganisms. *Annu. Rev. Microbiol.* **49**: 239-276.

Padma, P., Komath, S. S., Nadimpalli, S. K. & Swamy, M. J. (1999) Purification in high yield and characterisation of a new galactose-specific lectin from the seeds of *Trichosanthes cucumerina*. *Phytochemistry* **50**: 363-371.

Perillo, N. L., Marcus, M. E. & Baum, L. G. (1998) Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J. Mol. Med*. **76**: 402-412.

Peterson G. L. (1977) A simplication of the protein assay method of Lowry *et al*. which is more generally applicable. *Anal. Biochem.* **83**: 346-356.

Peumans W. J., De Ley, M., Stinissen, H. M. & Broekaert, W. F. (1985) Isolation and partial characterization of a new lectin from seeds of the greater celandine (*Chelidonium majus*). *Plant Physiol.* **78**: 379-383.

Peumans, W. J. & Van Damme, E. J. M. (1995) Lectins as plant defense proteins. *Plant Physiol*. **109**: 347-352.

Peumans, W. J., De Ley, M. & Broekaert, W. F. (1984) An unusual lectin from stinging nettle (*Urtica dioica*) rhizomes. *FEBS Lett.* **177**: 99-103.

Peumans, W. J., Hao, Q. & Van Damme, E. J. M. (2001) Ribosome-inactivating proteins from plants: more than RNA *N*-glycosidases? *FASEB. J.* **14**: 1493-1506.

Peumans, W. J., Verhaert, P., Pfüller, U. & Van Damme, E. J. M. (1996) Isolation and partial characterization of a small chitin-binding lectin from mistletoe (*Viscum album*). *FEBS Lett.* **396**: 261-265.

Poveda, A. & Jimenez-Barbero, J. (1998) NMR investigations of carbohydrate protein interactions in solutions. *Chem. Soc. Rev.* **27**: 133-143.

Provencher, S. W. & Glockner, J. (1981) Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **20**: 33-37.

Punyiczki, M., Norman, J. A. & Rosenberg, A. (1993) Interaction of acrylamide with proteins in the concentration range used for fluorescence quenching studies. *Biophys. Chem.* **47**: 9-19.

Pusztai, A. & Bardocz, S. (1996) Biological effects of plant lectins on the gastrointestinal tract: metabolic consequences and applications. *Trends Glycosci. Glycotechnol.* **8**: 149-165.

Rabinovich, G. A. (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. *Cell Death Differ*. **6**: 711-721.

Raikhel, N. V. & Wilkins, T. A. (1987) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. *Proc. Natl. Acad. Sci.* USA **84**: 6745-6749.

Raikhel, N. V., Lee, H.-I. & Broekaert, W. F. (1993) Structure and function of chitin-binding proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**: 591-615.

Rani, P. G., Bachhawat, K., Misquith, S. & Surolia, A. (1999) Thermodynamic studies of saccharide binding to artocarpin, a B-cell mitogen, reveals the extended nature of its interaction with mannotriose [3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose]. *J. Biol. Chem.* **274**: 29694-29698.

Rayner, D. M. & Szabo, A. (1978) Fluorescence decay of tryptophan conformers in aqueous solution. *Can. J. Chem.* **56**: 743-745.

Read, S. M. & Northcote, D. H. (1983) Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (pumpkin). *Eur. J. Biochem*. **134**: 561-569.

Reisner, Y., Ravid, A. & Sharon, N. (1976) Use of soybean agglutinin for the seperation of mouse B and T lymphocytes. *Biochem. Biophys. Res. Commun.* **72**: 1585-1591.

Roberts, D. D. & Goldstein, I. J. (1982) Hydrophobic binding properties of the lectin from lima beans (*Phaseolus lunatus*). *J. Biol. Chem.* **257**: 11274-11277.

Roberts, D. D. & Goldstein, I. J. (1983a) Binding of hydrophobic ligands to plant lectins: titration with arylaminonaphthalenesulfonates. *Arch. Biochem. Biophys.* **224**: 479-484.

Roberts, D. D. & Goldstein, I. J. (1983b) Adenine binding sites of the lectin from lima beans (*Phaseolus lunatus*). *J. Biol. Chem.* **258**: 13820-13824.

Roberts, D. D. & Goldstein, I. J. (1984) Effect of carbohydrate and metal ion binding on the reactivity of the essential thiol groups of lima bean lectin. *J. Biol. Chem*. **259**: 903-908.

Rodriguez-Romero, A., Ravichandran, K. G. & Soriano-Garcio, M. (1991) Crystal structure of hevein at 2.8 Å resolution. *FEBS lett.* **291**: 307-309.

Saavedra-Lozano, J., McCoig, C. Xu, J., Kao, Y., Keiser, P., Ghetie, V., Siliciano, R. F., Siliciano, J. D., Picker, L. J., Ramilo, O. & Vitetta, E. S. (2002) An anti-CD45RO immunotoxin kills latently infected human immunodeficiency virus (HIV) CD4 T cells in the blood of HIV-positive persons. *J. Infect. Dis.* **185**: 306-314.

Sabins, D. D. & Hart, J. W. (1978) The isolation and some properties of a lectin (haemagglutinin) from *cucurbita* phloem exudate. *Planta* **142**: 97-101.

Sampietro, A. R., Isla, M. I., Quiroga, E. N. & Vattuone, M. A. (2001) An *N*-acetyl glucosamine oligomer binding agglutinin (lectin) from ripe *Cyphomandra betacae* Sendt. fruits. *Plant Sci.* **160**: 659-667.

Sanadi, A. R. & Surolia, A. (1994) Studies on a chitooligosaccharide-specific lectin from *Coccinia indica*. Thermodynamics and kinetics of umbelliferyl glycoside binding. *J. Biol. Chem.* **269**: 5072-5077

Sandi, A. R., Anantharam, V. & Surolia, A. (1998) Elucidation of the combining site of *Coccina indica* agglutinin (CIA) by thermodynamic analyses of its ligand binding. *Pure & Appl. Chem.* **70**: 677-686.

Sawchenko, P. E. & Gerfen, C. R. (1985) Plant lectins and bacterial toxins as tools for tracing neuroanatomical connections. *Trends Neurosci.* **8**: 378-284.

Schwarz, F. P., Puri, K. D., Bhat, R. G. & Surolia, A. (1993) Thermodynamics of monosaccharide binding to concanavalin A, pea (*Pisum sativum*) lectin, and lentil (*Lens culinaris*) lectin. *J. Biol. Chem.* **268:** 7668-7677.

Sharma, S., Bharadwaj, S., Surolia, A. & Podder, S. K. (1998) Evaluation of the stoichiometry and energetics of carbohydrate binding to *Ricinus communis* agglutinin : a calorimetric study. *Biochem. J.* **333**: 539-542.

Sharon, N. & Lis, H. (2003) Lectins, 2nd Edition, Kluwer Academic Publishers, Dordrecht, Netherlands, 454 pp.

Sharon, N. & Ofek, I. (1995) Identification of receptors for bacterial lectins by blotting techniques. *Methods Enzymol*. **253**: 91-98.

Sharon, N. (1983) Lectin receptors as lymphocyte surface markers. *Adv. Immunol*. **34**: 213.

Sparrow, C. P., Leffler, H. & Barondes, S. H. (1987) Multiple soluble β-galactoside binding lectins from human lungs. *J. Biol. Chem.* **262**: 7383-7390.

Spohr, V., Paszkieuicz-Hnatiw, E., Morishima, N. & Lemieux, R. U. (1992) Molecular recognition XI. The synthesis of extensively deoxygenated derivatives of the H-type 2 human blood group determinant and their bonding by an anti-H-type 2 monoclonal antibody and lectin 1 of *Ulex europeus*. *Can. J. Chem.* **70**: 254-271.

Sreerama, N. & Woody, R. W. (1993) A self-consistent method for the analysis of protein secondary structure from circular dichroism. *Anal. Biochem.* **209**: 32-34

Sreerama, N. & Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal. Biochem.* **287**: 252-260.

Sreerama, N., Venyaminov, S. Y. & Woody, R. W. (1999) Estimation of the number of alpha-helical and beta-strand segments in protein using circular dichroism spectroscopy. *Protein Sci*. **8**: 370-380.

Srinivas, V. R., Acharya, S., Rawat, S., Sharma, V. & Surolia, A. (2000) The primary structure of the acidic lectin from winged bean (*Psophocarpus tetragonolobus*). *FEBS Lett.* **474**: 76-82.

Sultan, N. A. M. & Swamy, M. J. (2003) Thermodynamic analysis of binding of 4-methylumbelliferyl-α- and β-D-galactopyranosides to *Momordica charantia* lectin. *Curr. Sci*. **84:** 200-203.

Sultan, N. A. M. & Swamy, M. J. (2005a) Fluorescence quenching and time-resolved fluorescence studies on *Trichosanthes dioica* seed lectin. *J. Photochem. Photobiol. B: Biol.* **80:** 93-100.

Sultan, N. A. M. & Swamy, M. J. (2005b) Energetics of carbohydrate binding to *Momordica charantia* lectin: An isothermal titration calorimetric study. *Arch. Biochem. Biophys*. **437**: 115-125.

Sultan, N. A. M., Kavitha, M. & Swamy, M. J. (2009) Purification and physicochemical characterization of two galactose-specific isolectins from the seeds of *Trichosanthes cordata*. *IUBMB Life* (In press).

Sultan, N. A. M., Kenoth, R. & Swamy, M. J. (2004a) Purification, physicochemical characterization, saccharide specificity and chemical modification of a Gal/GalNAc specific lectin from the seeds of *Trichosanthes dioica*. *Arch. Biochem. Biophys.* **432**: 212-221.

Sultan, N. A. M., Maiya, B. G. & Swamy, M. J. (2004b) Thermodynamic analysis of porphyrin binding to *Momordica charantia* (bitter gourd) lectin. *Eur. J. Biochem.* **271**: 3274-3282.

Sultan, N. A. M., Rao, R. N., Nadimpalli, S. K. & Swamy, M. J. (2006) Tryptophan environment, secondary structure and thermal unfolding of galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies. *Biochim. Biophys. Acta* **1760**: 1001-1008.

Sumner, J. B. & Howel, S. F. (1936) Identification of hemagglutinin of jack bean with concanavalin A. *J. Bacteriol.* **32**: 227-267.

Surolia, A., Sharon, N. & Schwarz, F. P. (1996) Thermodynamics of monosaccharide and disaccharide binding to *Erythrina corallodendron* lectin. *J. Biol. Chem.* **271**: 17697-17703.

Swaminathan, C. P., Gupta, D., Sharma, V. & Surolia, A. (1997) Effect of substituents on the thermodynamics of D-galactopyranoside binding to winged bean (*Psophocarpus tetragonolobus*) basic lectin. *Biochemistry* **36**: 13428-13424.

Swaminathan, C. P., Surolia, N. & Surolia, A. (1998) Role of water in the specific binding of mannose and mannooligosaccharides to concanavalin A. *J. Am. Chem. Soc.* **21**: 5153-5159.

Teale, F. W. J. (1960) The ultraviolet fluorescence of proteins in neutral solutions. *Biochem. J.* **76**: 381-388.

Toone, E. J. (1994) Structure and energetics of protein-carbohydrate complexes. *Curr. Opin. Struct. Biol*. **4**: 719-728.

Trindade, M .B., Lopes, J. L. S., Soares-Costa, A., Monteiro-Moreira, A. C., Moreira, R. A., Oliva, M. L. V. & Beltramini, L. M. (2006) Structural characterization of novel chitin binding lectins from genus *Artocarpus* and their antifungal activity. *Biochim. Biophys. Acta* **1764**: 146-152.

Van Damme, E. J. M., Peumans, W. J., Barre, A. & Rougé, P. (1998a) Plant lectins: a composite of several distinct families of structurally and evolutionarily related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* **17**: 575-692.

Van Damme, E. J. M., Peumans, W. J., Pusztai, A. & Bardocz, S. (1998b) Handbook of Plant Lectins: Properties and Biochemical Applications. John Wiley and Sons, Chichester, UK.

Van der Burg, H. A., Spronk, C. A. E. M., Boeren, S., Kennedy, M. A., Vissers, J. P. C., Vuister, G. W., de Wit, P. J. G. M. & Vervoot, J. (2004) Binding of AVR4 elicitor of *Cladosporium fulvum* to chitotriose units is facilitated by positive allosteric protein protein interactions. *J. Biol. Chem.* **279**: 16786-16796.

Van Stokkum, I. H. M., Spoelder, H J. W., Bloemendal, M., Van Groendelle, R. & Groen, F. C. A. (1990) Estimation of protein secondary structure and error analysis from circular dichroism spetra. *Anal. Biochem.* **191:** 110-118.

Waljuno, K., Scholma, R. A., Beintema, J., Mariono, A. & Hahn, A. M. (1975) Amino acid sequence of hevein. *Proc. Int. Rubber Conf., Kualalumpur* **2**: 518-531.

Willment, J. A., Gordon, S. & Brown, G. D (2001) Characterization of the human β- glucan receptor and its alternatively spliced isoforms. *J. Biol. Chem.* **276**: 43818-43823.

Wiseman, T., Williston, S., Brandts, J. F. & Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **179**: 131-137.

Wouterlood, F. G. & Groenewegen, H. J. (1985) Neuroanatomical tracing by use of *Phaseolus vulgaris* leucoagglutinin (PHA-L): electron microscopy of PHA-L filled neuronal somata, dendrites, axons and axon terminals. *Brain Res.* **326**: 188-191.

Wright, C. S. (1977) The crystal structure of wheat germ agglutinin at 2.2 Å resolution. *J. Mol. Biol.* **111**: 439-457.

Wright, C. S. (1992) Crystal structure of wheat germ agglutinin/glycophorin-sialoglycopeptide receptor complex. *J. Bio. Chem.* **267**: 475-487.

Xu, C., Moore, C. H., Fountain, D. W. & Yu, P. L. (1992) Purification and characterization of a new lectin from tamarillo fruit (*Cynchomandra betacea*). *Plant Sci.* **81**: 183-189.

Yamaguchi, K. I., Mori, A. & Funatsu, G. (1995) The complete amino acid sequence of lectin-C from the roots of pokeweed (*Phytolacca americana*). *Biosci. Biotechnol. Biochem.* **59**: 1384-1385.

Yamaguchi, K. I., Mori, A. & Funatsu, G. (1996) Amino acid sequence and some properties of lectin-D from the roots of pokeweed (*Phytolacca americana*). *Biosci. Biotechnol. Biochem.* **60**: 1380-1382.

Yamaguchi, K. I., Yurino, N., Kino, M., Ishiguro, M. & Funatsu, G. 1997. The amino acid sequence of mitogenic lectin-B from the roots of pokeweed (*Phytolacca americana*). *Biosci. Biotechnol. Biochem*. **61**: 690-698.

List of Publications

1. Kumar, M. R., Raju, P. N., Reddy, J. T., **Narahari, A**., Reddy, M. J. R. & Vairamani, V. (2005) Evaluation of methyl 3-(4-methoxy-1-naphthyl)-(E)-2-propenoate and methyl 5-(4-methoxy-1-naphthyl)-(2E,4E)-2,4-pentadienoate as new charge-transfer matrices for matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**: 3171-3174.

2. Jagtap, D. D., **Narahari, A.,** Swamy, M. J. & Mahale, S. D. (2007) Disulphide bond reduction and *S*-carboxamidomethylation of PSP94 affects its conformation but not the ability to bind immunoglobulin. *Biochim. Biophys. Acta* **1774**: 723-731.

3. **Narahari, A.** & Swamy, M. J. (2009) Rapid affinity-purification and physico-chemical characterization of pumpkin (*Cucurbita maxima*) phloem exudate lectin. *Biosci. Rep.* (Under revision).

4. **Narahari, A.** & Swamy, M. J. (2009) Steady-state and time-resolved fluorescence studies on the chitooligosaccharide-specific lectin from the phloem exudate of pumpkin (*Cucurbita maxima*) (Communicated).

5. **Narahari, A.** & Swamy, M. J. (2009) Energetics of chitooligosaccharide binding to pumpkin (*Cucurbita maxima*) phloem exudate lectin (To be communicated).

6. **Narahari, A.** & Swamy, M. J. (2009) Purification and physico-chemical characterization of a new chitooligosaccharide-specific lectin from snake gourd (*Trichosnathes anguina*) phloem exudate (manuscript under preparation).

7. **Narahari, A.** & Swamy, M. J. (2009) Thermodynamics of the interaction of chitooligosaccharides with snake gourd (*Trichosanthes anguina*) phloem exudate lectin. Isothermal titration calorimetric and fluorescence spectroscopic studies (manuscript under preparation).

8. Srinivas, P. N. B. S., **Narahari, A.,** Swamy, M. J., Keerthi, N., Gopal, B. and Reddy, G. B. (2009) Significance of α-crystallin heteropolymer with 3:1 αA to αB ratio: Stability, oligomeric size and polydispersity (manuscript under preparation).

Participation in Symposia, Workshop etc.

1. Narahari, A. & Swamy, M. J. (2006) A new chitooligosaccharide-specific lectin from the phloem exudate of snake gourd (*T. anguina*). Purification and carbohydrate binding. *Glycans 2006 Symposium*. February 22-26, Indian institute of science (I. I. Sc.) Bangalore, India.

2. Narahari, A. & Swamy, M. J. (2006) Isothermal titration calorimetric and fluorescence studies on the phloem exudate lectin from pumpkin (*Cucurbita maxima*). *EABS & BSJ symposium***.** November 12-16, Okinawa, Japan.

3. Narahari, A. & Swamy, M. J. (2008) Isothermal titration calorimetric, fluorescence and thermal stability studies on the phloem exudate lectin from pumpkin (*Cucurbita maxima*). *8th ISCSM symposium*. January 21-25, CCMB, Hyderabad, India.

4. Narahari, A. & Swamy, M. J. (2009) A new chitooligosaccharide-specific lectin from the phloem exudate of snake gourd (*T. anguina*). purification and carbohydrate binding. *IBS-2009 symposium*. January 22-24, CCMB, Hyderabad.

5. Presented a poster in the In house symposium *Chemfest* 2007 held at School of chemistry, University of Hyderabad, India**.** Gave an oral presentation and also presented a poster on purification and thermodynamic analysis of chitooligosaccharide specific lectins from the phloem exudate of snake gourd and pumpkin.

6. **Workshop attended:** Workshop on pharmacoinformatics in drug design. April 14-26, 2005. National institute of pharmaceutical education and research (NIPER), Chandigarh, India.