General discussion and conclusions

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

Studies reported in the second part of the thesis (Chapters 5 and 6) deal with the purification, physico-chemical characterization, and carbohydrate specificity of a new lectin from the seeds of another Cucurbitaceae species, namely *Trichosanthes dioica*. In addition, the amino acid residues that are important for the sugar-binding activity of
this lectin were identified by chemical modification studies employing group-specific reagents. Finally, fluorescence quenching and time-resolved fluorescence studies were carried out in order to characterize the environment of the indole side chains of the tryptophan residues in this protein. The results of these studies are briefly summarized below.

The interaction of several free-base and metalloporphyrins with MCL was investigated by absorption spectroscopy. The association constants of the interaction were found comparable to that of lectin-sugar interactions and were not affected significantly by the presence of lactose in the interaction medium. Additionally, presence of porphyrins in the medium did not affect the hemagglutination activity of the lectin, indicating that both sugars and porphyrins have distinct binding sites on MCL, i.e., the lectin is capable of binding porphyrins and cells simultaneously. Similar results were obtained for several other plant lectins in this laboratory. Unlike the *Trichosanthes cucumerina* seed lectin (TCSL), the interaction of MCL with porphyrins is enthalpically driven with negative net contribution from entropy. The interaction is also characterized by enthalpy-entropy compensation. These results suggest that the mode of MCL-porphyrin interaction is most likely similar to that observed in the crystal structure of Con A-H$_2$TPPS complex in which the interaction is mediated by an extensive network of H-bonds, some of which are water-mediated whereas no hydrophobic interaction is observed.

Isothermal titration calorimetry (ITC) is a powerful technique to obtain accurate thermodynamic data on protein-ligand interactions. Therefore, this technique was chosen for investigating the binding of carbohydrates to MCL. These studies showed that each lectin tetramer (120 kDa) has two saccharide binding sites. Other seed lectins
from Cucurbitaceae such as SGSL, TCSL and TDSL, which are dimeric can be represented as ab pair of subunits with at least two binding sites per dimer. On the other hand, MCL can be regarded as a dimer of this pair, i.e., $a_2b_2$ or $(ab)_2$, however it has only two active binding sites instead of four. In this regard MCL is similar to APA and RCA which are believed to be dimers of abrin and ricin, respectively, but have only two active binding sites per tetramer. This is another aspect of the similarity between Cucurbitaceae galactose-binding lectins (CGLs) and type 2 RIPs.

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

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

Binding of 4-methylumbelliferyl $\alpha$- and $\beta$-D-galactopyranosides to MCL was investigated by fluorescence spectroscopy. Binding of these fluorescent sugars to this lectin results in a quenching of their fluorescence intensity. In comparison, SGSL and TDSL enhance the $\beta$-anomer fluorescence but only slightly quench the fluorescence of the $\alpha$-anomer. The interaction of MCL with these sugars was studied by monitoring the quenching of their fluorescence induced by binding to the lectin at different temperatures to obtain the binding constants and thermodynamic parameters ($K_b$, $\Delta H_b$, $\Delta S_b$).
ΔS_b) associated with the binding process. Analysis of these parameters indicated that the higher affinity of the lectin for the β-anomer is due to larger enthalpy of binding, which overrides a larger negative entropy of binding associated with the binding of the corresponding β-anomer.

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

All these findings, and several other observations discussed in Chapters 1 and 5, suggest that Cucurbitaceae galactose specific lectins are closely related proteins and show significant similarity to type 2 RIPs. These similarities appear to be strong enough to suggest a close structural similarity between CGLs and type 2 RIPs. However, further investigations on these proteins with regard to amino acid sequence, 3-dimensional structure and RIP activity assays need to be carried out on these lectins to confirm this hypothesis.
Hapten inhibition assays of the hemagglutination activity of TDSL by sugars showed that it is a galactose-specific lectin with preferential affinity to \( \beta \)-galactosides over the \( \alpha \)-isomers, a property shown also by the other well-characterized CGLs, namely MCL, SGSL and TCSL. However, in contrast to these CGLs, TDSL binds GalNAc stronger than galactose itself. Another noteworthy point is that, like MCL and SGSL (and unlike TCSL), TDSL binds galactosides with bulky aromatic aglycon stronger than galactose or aliphatic galactosides. Therefore, although there is a gross similarity in sugar binding properties among these lectins, there are subtle differences in the fine sugar specificity of the CGLs. In the light of this observation, it would be interesting to investigate the binding of complex oligosaccharides bearing galactose and GalNAc as terminal sugars with the CGLs and compare the binding profiles for the various lectins.

Chemical modification experiments showed that tyrosine is a key residue in the sugar binding site of TDSL. About 7 tyrosine residues could be acetylated by \( N \)-acetylimidazole under native condition (see Chapter 5), resulting in total loss of agglutination and sugar-binding activities of the lectin. The degree of activity loss was proportional to the number of tyrosine residues modified. The presence of lactose partially protected tyrosine residues from acetylation conforming the involvement of tyrosine in the sugar-binding activity. No changes in the secondary structure of the modified lectin could be observed by CD measurements indicating that the activity loss is due to the acetylation of tyrosine residues alone. No free sulfhydryl could be detected and attempt to reduce the disulfide bond(s) under native condition failed as was the case with SGSL and TCSL. No loss of activity could be detected upon chemical modification of lysine, histidine and tryptophan residues. Similar studies on SGSL and
TCSL showed histidine residues and not tyrosine residues to be essential for their sugar binding activity while in MCL tyrosine and also tryptophan residues were found essential. Therefore one can conclude from these observations that Cucurbitaceae galactose specific lectins show close saccharide specificity inspite of presence of some differences at least in the amino acid building the binding site. Legume lectins on the other hand have highly conserved binding sites both in fold and amino acid composition, but show diverse specificities. Lectins therefore appear to adopt different mechanisms for similar recognition processes.

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

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