Chapter 3

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

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

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

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

Momordica charantia seed lectin (MCL) is a galactose specific tetrameric glycoprotein with $\alpha_2\beta_2$ – type subunit architecture. Its macromolecular properties and carbohydrate-binding specificity towards monosaccharides and disaccharides has been studied [Sultan & Swamy, 2003; Mazumder et al., 1981; Khan et al., 1981; Das et al., 1981; Padma et al., 1998]. MCL exhibits strong type-1 and weak type-2 ribosome inactivating protein activities as well as insulinomimetic activity.
Circular dichroism studies reveal that MCL contains 36% β-sheet, 21% β-turns, 13% α-helix and rest unordered structure [Sultan et al., 2004a]. Thermodynamic studies on porphyrin binding to MCL indicate that the interaction is governed primarily by enthalpic forces [Sultan et al., 2004a]. Effect of pH and incubation at different temperatures on the hemaagglutination activity has been studied and thermodynamic parameters associated with the binding of different carbohydrates to MCL have been investigated in detail using isothermal titration calorimetry [Sultan & Swamy, 2005a]. ITC results indicate that carbohydrate binding to MCL is governed primarily by enthalpic forces, and the $\Delta C_p$ values for the interaction were found to be negative.

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

**Materials and Methods**

**Materials**

Bitter gourd seeds were obtained from local seed shops. Guar gum, lactose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside and N-acetyl-D-
galactosamine were obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade were obtained from local suppliers.

**Methods**

**Purification of *Momordica charantia* seed lectin**

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

**Differential scanning calorimetry**

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

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

Fluorescence spectroscopy

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

Results and discussion

Differential Scanning Calorimetry

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

The ratio of calorimetric enthalpy to van’t Hoff enthalpy ($\Delta H_c/\Delta H_v$) for the 1st transition is greater than 3, suggesting that four entities are involved in the first transition. For the 2nd transition $\Delta H_c/\Delta H_v$ ratio is close to 2 and suggests that two
entities are involved in the second transition. The two transitions during thermal unfolding can be analyzed by two possible models as summarized below.

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

\[
A_2B_2 \leftrightarrow A_2 + B_2 \leftrightarrow 2A_u + 2B_u \quad (3.1)
\]

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

\[
A_2B_2 \leftrightarrow 2AB \leftrightarrow 2A_u + 2B_u \quad (3.2)
\]

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

**Fig. 3.1.** DSC scan of *Momordica charantia* seed lectin in 20 mM phosphate buffer containing 150 mM NaCl at pH 7.2 at a scan rate of 40 k h\(^{-1}\). The data points are shown as open circles, and the solid lines are the best fits of the DSC data to the non-two-state transition model. Concentration of MCL tetramer is 0.0035 mM.
Among these two models, the second model describes the most likely mode of unfolding of MCL because according to the first model the dimers $A_2$ and $B_2$ will have the same unfolding temperature which is rather unlikely.

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

<table>
<thead>
<tr>
<th>Scan rate (°C/hr)</th>
<th>$T_{m1}$ (K)</th>
<th>$\Delta H_{c1}$ (kJ.mol$^{-1}$)</th>
<th>$\Delta H_{v1}$ (kJ.mol$^{-1}$)</th>
<th>$\Delta H_{c1}/\Delta H_{v1}$</th>
<th>$T_{m2}$ (K)</th>
<th>$\Delta H_{c2}$ (kJ.Mol$^{-1}$)</th>
<th>$\Delta H_{v2}$ (kJ.Mol$^{-1}$)</th>
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<td>344</td>
<td>3.39</td>
<td>337</td>
<td>977</td>
<td>1157</td>
<td>547</td>
</tr>
<tr>
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<td>1157</td>
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Unfolding parameters of MCL obtained from DSC scans performed in the presence of saturating amounts of different ligands are given in Table 3.2. In the presence of carbohydrate ligands, transition temperatures, calorimetric enthalpies and van’t Hoff enthalpies increase, suggesting that carbohydrate binding stabilizes the native conformation of MCL. Stabilization of native structure of protein by carbohydrate binding has been observed for many lectins, including abrin II, peanut lectin, winged bean acidic lectin and Erythrina corallodendron lectin [Krupakar et al., 1999; Reddy et al., 1999; Surolia et al., 1996; Srinivas et al., 1998].

At saturating concentrations of lactose, the $T_m$ value increased by 8 K for both the transitions. At constant lectin concentration the denaturation transition in the presence of bound ligand can be expressed as
The thermodynamic parameters obtained from DSC measurements on MCL in presence of different concentrations of lactose. Scan rate was 30 K.h\(^{-1}\). Concentration of MCL used was 0.00497 mM in dimer. The errors were less than 0.02% for \(T_m\), and less than 10% for \(\Delta H_c\) and \(\Delta H_v\).

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

<table>
<thead>
<tr>
<th>Lactose (mM)</th>
<th>(T_{m1}) (K)</th>
<th>(\Delta H_{c1}) (kJ.Mol(^{-1}))</th>
<th>(\Delta H_{v1}) (kJ.Mol(^{-1}))</th>
<th>(\Delta H_{c1}/\Delta H_{v1})</th>
<th>(T_{m2}) (K)</th>
<th>(\Delta H_{c2}) (kJ.Mol(^{-1}))</th>
<th>(\Delta H_{v2}) (kJ.Mol(^{-1}))</th>
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A plot of \(\ln [L]\) vs \(1/T_p\) (Fig. 3.2) yielded the van’t Hoff enthalpy, where \(L\) is the concentration of ligand and \(T_p\) is the temperature at which transition peak is maximum. From this plot the van’t Hoff enthalpy was calculated as 1107 kJ. mol\(^{-1}\) for the lower-temperature transition with the m value of 2. The m value was known from ITC binding studies, which showed that MCL tetramer binds to two lactose molecules [Sultan & Swamy, 2005a]. For the higher-transition peak the calculated van’t Hoff enthalpy value is 597 kJ.mol\(^{-1}\) which is close to the van’t Hoff enthalpy values determined by non-two-state fit.
Fig. 3.2. Plots of ln[Lactose] vs 1/\(T_p\) for MCL. The lines are best linear least-square fits of ln [Lactose] against 1/Tp, for the lower temperature transition peak (●) and higher temperature transition peak (○). Scan rate was 30 °C per hour. The correlation coefficients are 0.99 for the lower-temperature and higher-temperature transition peaks.

**Effect of pH**

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

<table>
<thead>
<tr>
<th>pH</th>
<th>$T_{m1}$ (K)</th>
<th>$\Delta H_{c1}$ (kJ.mol$^{-1}$)</th>
<th>$\Delta H_{v1}$ (kJ.mol$^{-1}$)</th>
<th>$\Delta H_{c1}/\Delta H_{v1}$</th>
<th>$T_{m2}$ (K)</th>
<th>$\Delta H_{c2}$ (kJ.Mol$^{-1}$)</th>
<th>$\Delta H_{v2}$ (kJ.Mol$^{-1}$)</th>
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**pH dependent denaturation**

Fluorescence spectra of MCL at different pH are shown in Fig. 3.3 A. The emission $\lambda_{\text{max}}$ of MCL at pH 7.2 is seen at 332 nm, which is in agreement with the results reported in Chapter 2 and clearly indicates that tryptophan residues are predominantly buried in the hydrophobic interior of the protein. Fluorescence spectra of MCL samples of different pH did not show any shift in the $\lambda_{\text{max}}$, indicating that MCL is relatively stable over a wide pH range and does not unfold even at pH 1.0. The emission intensity remained almost constant in the pH range 1-8, but decreased at higher pH. The maximum decrease in fluorescence intensity was 24% at pH 2.
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Fig. 3.3. pH-dependent denaturation of MCL lectin. A) Fluorescence spectra at different pH. The pH at which each spectrum was recorded is indicated. B) Far UV CD spectra of MCL at different pH. The symbols used are: pH 2 (.....), pH 5 (----), pH 7 (-----), pH 9 (--.--.--.--), pH 11(--.--.--.--). C) Near UV CD spectra of MCL. The pH at which each spectrum was recorded is indicated.Inset of C shows pH vs CD signal intensity.

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

**Denaturation of MCL by Gdn.HCl**

Chemical denaturation of MCL by Gdn.HCl was studied by monitoring changes in the intrinsic fluorescence properties of the protein when the concentration of Gdn.HCl was varied. Fluorescence emission spectra recorded in presence of different concentrations of Gdn.HCl are shown in Fig. 3.4A. The emission $\lambda_{\text{max}}$ of MCL seen at 332 nm under native conditions (spectrum 1) exhibits a red shift as the concentration of Gdn.HCl is increased. This is accompanied by a pronounced decrease in the fluorescence intensity. The emission maximum increases with increase in the concentration of the denaturant up to 3.0 M, with a clear plateau

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

$$f_U = (F_F - F_o)/(F_F - F_U)$$  \hspace{1cm} (3.4)

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

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