Chapter 4

Structural studies on hemagglutinin from *Moringa oleifera* using fluorimetric methods and circular dichroism (CD)
4.1. Summary

The intrinsic fluorescence of the protein was quenched upon titrating with sugars LacNAc ($K_a = 1380 \text{M}^{-1}$) and fructose ($K_a = 878 \text{M}^{-1}$), although these sugars did not inhibit the hemagglutination activity of MoL. To study the environment of the single tryptophan residue in MoL, solute quenching studies were carried out at pH 1.0, 7.2 and 10.0 and in the presence of 6 M urea using quenchers such as acrylamide, potassium iodide and cesium chloride. The results indicated that tryptophan is highly exposed to the solvent and present in a strongly electropositive environment. The study of the binding of hydrophobic dye 8-anilino-1-naphthalene sulfonic acid (ANS) using fluorescence spectroscopy showed exposed hydrophobic patches in protein that get further exposed at extreme acidic or alkaline pH but get buried in the interior in the presence of 1M GdnHCl or urea. MoL also binds adenine ($K_a = 7.76 \times 10^3 \text{M}^{-1}$). Time resolved fluorescence of the native protein showed two lifetimes indicating two different conformers of tryptophan exist which get merged into a single one after quenching with 0.15M acrylamide.

Analysis of the far-UV CD spectrum of MoL by CDPro showed secondary structure composition of $\alpha_R$ (regular $\alpha$-helix) 16%, $\alpha_D$ (distorted $\alpha$-helix) 12%, $\beta_R$ (regular $\beta$-sheet) 14%, $\beta_D$ (distorted $\beta$-sheet) 9%, turn 20% and unordered 28%. The secondary structure was not affected by extreme acidic or alkaline conditions; however, it was drastically affected by the presence of a reducing agent like dithiothreitol (DTT) (1mM) at and above pH 7.0. The far UV CD spectra of the protein incubated in the presence of different concentrations of GdnHCl showed no change in the ellipticity in the region 215 to 225 nm, although it was affected at 210 nm and below indicating an increase in the unordered structural element. The secondary structure is stable up to 3M urea concentration above which it starts changing.
4.2. Introduction

Three-dimensional structure of a protein, which is responsible for its function, is held together by several factors like hydrogen bonds, ionic, hydrophobic and van der Waals interactions as well as covalent interactions like disulfide linkages. The stability of the protein is dependant on these interactions. The conditions which interfere with these interactions lead to loss of the three-dimensional structure as well as activity of the protein. Increase in temperature and changes in pH, denaturation by chemical agents like GdnHCl and urea, reduction of disulfide linkages by DTT are some of such conditions. Changes in the native conformation of the protein in such conditions can be followed by using fluorescence and circular dichroism spectroscopic techniques. Fluorescence spectroscopy can also be used to quantitate the binding of ligands to a protein.

*Moringa oleifera* lectin (MoL) is a low molecular weight, dimeric protein with a highly basic pl of 10.0 (section 3.5.1). It is stable at comparatively high temperatures as well as in the entire pH range (section 3.5.2), and is inhibited only by glycoproteins (section 3.5.3). The studies on the conformational stability of this protein in various conditions by making use of fluorescence spectroscopy and circular dichroism have been described in this chapter. Saccharide binding as well as the accessibility of tryptophan residue of the lectin has also been studied.

4.3. Materials

Acrylamide, potassium iodide, cesium chloride, 8-anilino-1-naphthalene sulfonic acid (ANS), adenine, guanidium hydrochloride, urea, glucose, galactose, mannose, methyl α-glucose, methyl α-galactose, methyl α-mannose, fucose, LacNAc, ManNAc, Gal-β-1,3-GalNAc were purchased from Sigma, USA. All other chemicals used were of analytical grade.
Quencher solutions (acrylamide, potassium iodide, and cesium chloride) were prepared as 5 M stocks in deionised water. Potassium iodide solution was made in 200 μM sodium thiosulfate solution to prevent the formation of triiodide (I³⁻) ions. Sugar solutions were prepared as 100 mM stocks. Buffers were prepared as 1 M stocks and pH was adjusted at 25 °C. All these solutions were filtered through 0.45 μ filter from Sartorius. Protein solutions were centrifuged at 8000 g for 5 min before use.

The software Microcal Origin 6.1 was used to plot the graphs and do the related calculations.

4.4. Methods

4.4.1. Purification of MoL

MoL was purified as described in section 3.4.1. The concentration of the protein was determined according to the method of Lowry et al. (1951) using BSA as standard.

4.4.2. Fluorimetric measurements

The aromatic amino acid residues in the protein, namely tryptophan, tyrosine and phenylalanine, get excited on absorption of light energy (photons). Since the residue is unstable in the excited state, it returns to its ground state which is accompanied by the dissipation of excess energy through fluorescence. Tryptophan has much stronger fluorescence than tyrosine and phenylalanine. The intensity and the wavelength of maximum fluorescence emission of tryptophan is highly dependant on the polarity of the environment surrounding it. Hence by studying the tryptophan fluorescence, the conformational changes in the protein can be monitored.

The intrinsic fluorescence of the protein was measured using a PerkinElmer Life Sciences LS50 fluorescence spectrophotometer. The samples were kept in a quartz cuvette, at a constant temperature (± 0.1 °C) using a Julabo F20 circulating water bath.
To eliminate the background emission, the signal produced by either buffer solution, or buffer containing the appropriate quantity of denaturants was subtracted. The protein solution (~0.025 mg ml$^{-1}$) was excited at 295 nm and the emission was recorded in the range of wavelengths 300-400 nm. Each spectrum was an average of 5 accumulations. Both the excitation and emission spectra were obtained setting the slit-width at 5 nm, and speed 100 nm min$^{-1}$.

### 4.4.3. Sugar binding studies

The binding of sugars to MoL was studied by titrating the protein against sugar solutions and monitoring the fluorescence. The temperature was maintained at 25 $^\circ$C throughout the experiment using the waterbath. To 2 ml of lectin sample (concentration 0.04 mg ml$^{-1}$) in 50 mM phosphate buffer, pH 7.2, 3-10 μl aliquots of the sugar solutions (of concentration 10-100 mM) were added. The samples were excited at 295 nm and the fluorescence emission intensity at 351 nm ($\lambda_{\text{max}}$ of the protein) was monitored. Corrections were also made to compensate the dilution effect upon addition of sugar to lectin. At the highest concentration of the saccharide, the volume change was less than 5% of the solution in the cuvette. Each data point was an average of three independent sets of experiments with standard deviation (SD) less than 5%.

The following equation was used to determine the association constant ($K_a$) (Chipman et al., 1967).

$$\log [C]_t = \log [K_a] + \log [(F_0-F_C)/(F_C-F_\infty)] \quad (\text{Eq. 4.1})$$

From the ordinate intercept of the double reciprocal plot of $F_0/(F_0-F_C)$ versus $1/[C]$, where $F_0$ and $F_C$ are the fluorescence intensities of the free protein and of the protein at a sugar concentration $[C]$, $F_\infty$, the fluorescence intensity upon saturation of all the sugar binding sites is obtained. In the plot of $\log[(F_0-F_C)/(F_C-F_\infty)]$ versus $\log[C]$, the
abscissa intercept yielded the $K_d$ value (the dissociation constant) for the protein-sugar interactions, which is the reciprocal of $K_a$ (the association constant).

The free energy changes of the association ($\Delta G$) were calculated by using the equation:

$$-\Delta G = RT \ln(K_a) \quad \text{(Eq. 4.2)}$$

### 4.4.4. Solute quenching studies

Interaction between a fluorophore and a molecule induces perturbation or modification in the fluorescence parameters like intensity, quantum yield and lifetime. Two types of fluorescence quenching can be observed when the interaction takes place between the fluorophore and the quencher molecule. Collisional quenching occurs when the fluorophore and another molecule diffuse in the solution and collide with each other. In this case, the two molecules do not form a complex. In static quenching, on the other hand, two molecules bind one to the other forming a complex (Albani, 2004).

The most commonly used quenching molecules are acrylamide, succinimide (neutral), iodide (cationic) and cesium (anionic). Acrylamide, being a small uncharged molecule, can diffuse within a protein and can quench the fluorescence of buried tryptophans as well. Iodide and cesium ions, on the other hand, quench the fluorescence of tryptophans present at or near the surface of the protein. Being charged ions, their quenching efficiency also depends on the charge surrounding the tryptophan (Albani, 2004).

Fluorescence quenching experiments on MoL were carried out at 27 °C. Protein samples (0.05 mg ml$^{-1}$) incubated for 16 h in 50 mM buffers (pH 1.0, 7.2 and 10.0) as well as in 6M urea were titrated with small aliquots (5-10 μl) of 5M quencher solution (acrylamide, potassium iodide or cesium chloride). The samples were excited at 295 nm
and the emission spectra were recorded in the range of 300-400 nm. Each spectrum was an average of 5 accumulations. Quenching of the fluorescence was monitored at 351 nm (emission maximum of MoL) and corrections were made to compensate the dilution effect upon addition of quencher solutions.

Quenching data for all these quenchers were analyzed by the Stern-Volmer (4.3) equation (Lehrer, 1971):

$$\frac{F_0}{F_C} = 1 + K_{sv} [Q]$$

(Eq. 4.3)

Where $F_0$ and $F_C$ are the relative fluorescence intensities, corrected for dilution, in the absence and presence of quencher respectively, $[Q]$ is the resultant concentration of the quencher and $K_{sv}$ is the Stern-Volmer constant for the given quencher. Slopes of Stern-Volmer plots yield $K_{sv}$ values.

**4.4.5. Lifetime measurement of fluorescence decay**

Lifetime measurements were carried out on an FLS920 spectrometer supplied by Edinburgh Instruments. A xenon flash lamp of pulse width 1 ns was used for excitation and a single photon counting photomultiplier was used for detection of fluorescence. The diluted Ludox solution was used for measuring Instrument Response Function (IRF). The samples (1 mg ml$^{-1}$) were excited at 295 nm and emission was recorded at 343 nm. Slit widths of 15 nm each were used on the excitation and emission monochromators. The resultant decay curves were analyzed by a multiexponential iterative fitting program provided by Edinburgh Instruments.

**4.4.6. Binding of ANS and adenine to MoL**

8-anilino-1-naphthalene sulfonic acid (ANS) is a fluorescent molecule which can bind to the exposed hydrophobic patches on the protein surface. ANS alone shows a weak fluorescence at 515 nm, when excited at 375 nm. When this dye binds to the
exposed hydrophobic patches of the protein, its fluorescence intensity increases considerably with a blue shift in the fluorescence maximum to 495 nm. This property can be used to estimate the exposed hydrophobic patches on the protein under various conditions (Daniel and Weber, 1966; Cardamon and Puri, 1992). Since the hydrophobic patches are usually buried in the interior of the protein and do not get exposed until the protein is unfolded by thermal/pH denaturation, ANS binding studies provide valuable information about the unfolding behavior of the protein, as well as about the intermediates formed during unfolding and refolding of the protein.

The binding of ANS to MoL was analyzed by measuring the fluorescence of MoL incubated in various conditions of temperature, pH and denaturants on the fluorescence spectrophotometer. 15 µl of 20 mM ANS was mixed with 2 ml of protein (0.05 mg ml⁻¹) which was then excited at 375 nm and the emission recorded between 450-550 nm. Reference spectrum of the buffer alone with same amount of ANS added in each of the condition was subtracted from the spectrum of the sample.

The binding of adenine to MoL was studied by intrinsic fluorescence titrations, in a way similar to that used to study the binding of sugars (section 4.4.3).

4.4.7. Circular Dichroism analysis

Circular dichroism spectroscopy measures the differences in the absorption of left- and right-handed polarized light by molecules which arise due to structural asymmetry in them. This property is exhibited by all optically active molecules like sugars and amino acids. Secondary structure elements in proteins and nucleic acids (α helix, β sheet, double helix etc.) also give rise to characteristic CD spectra. Using CD spectra of proteins in the far-ultraviolet region (180-250 nm), the secondary structure elements present in the protein can be estimated. At these wavelengths, peptide bonds in the protein act as chromophores and the signal produced by them is characteristic of
the secondary structure. Far UV CD spectra can also be used to follow the conformational changes in the protein.

In the near UV region (250-300 nm), the aromatic amino acids and the disulfide bonds act as chromophores. Phenylalanine gives signals in the region from 250-270 nm, tyrosine gives signals in the region from 270-290 nm and signals from 280-300 nm are given by tryptophan. Disulfide bonds give weak but broad signals throughout the near-UV spectrum. The CD signals in near UV region are sensitive to the overall tertiary structure of the protein.

CD spectra of the lectin samples were recorded on a JASCO-715 spectropolarimeter, at 25 °C, in the range of wavelengths 190–260 nm at a scan speed of 100 nm min⁻¹ with a response time of 1 s and slit width 1 nm. The sensitivity was 20 mdeg. A rectangular quartz cell of 1 mm path length was used. All measurements were recorded at a lectin concentration of 0.08 mg ml⁻¹. 6 successive scans were collected for each spectrum, and their average was used for further analysis. Measurements were made in 25 mM buffers of pH 2.0, 4.0, 6.0, 7.2, 8.0, 10.0 and 12.0 and buffer scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

Mean residue ellipticity (MRE) was calculated as:

\[
MRE = \frac{100 \times \theta \times MW}{l \times c \times N} \text{ deg cm}^2 \text{ dmol}^{-1} \\
\text{(Eq. 4.5)}
\]

Where \( \theta \) is ellipticity in mdeg, \( MW \) is the molecular weight of the protein in daltons, \( l \) is the length of the light path in cm, \( c \) is the concentration of the protein in mg ml⁻¹ and \( N \) is the number of amino acids in the protein.

To monitor the tertiary structure of the protein (0.8 mg ml⁻¹ at pH 1.0, 7.2 and 10.0), CD spectra were recorded in the range of 250-300nm using a cuvette of path length 1 cm.
Effect of temperature on the secondary structure of MoL was studied by heating the protein at pH 7.2 from 30 to 90 °C (1 °C per min) and monitoring the ellipticity at 222 nm. Similarly, renaturation of MoL was studied by cooling the protein from 90 to 30 °C (1 °C per minute) and monitoring the ellipticity at 222 nm.

To study the effect of denaturants on the secondary structure of MoL, the protein (0.08 mg ml⁻¹) was incubated in the presence of 0.25-6 M GdnHCl or 0.25-8 M urea at pH 7.2 for 4 h at 25 °C and the scans were recorded in the range of 210-250 nm. The data at wavelengths lower than 210 nm could not be recorded due to increase in the noise and high tension. The effect of reducing agent dithiothreitol (DTT) on the secondary structure of MoL was studied by incubating the protein (0.08 mg ml⁻¹) at pH 2, 4, 6, 7.2, 8, 10 and 12 in the presence of 1 mM DTT for 4 h and recording the scans in the wavelength range 200-250 nm.

4.5. Results and discussion

MoL has a single tryptophan per monomer, as estimated by titration of the protein with NBS. When excited at 295 nm, the fluorescence emission spectrum of MoL showed maximum intensity at 351 nm, indicating that the tryptophans in the protein are in polar environment and are exposed to the solvent. Tryptophans fully exposed to the solvents show an emission maximum at 356 nm.

The far-UV CD spectrum of the native lectin was analyzed to derive quantitative information regarding secondary structural elements of the lectin by using three different methods, viz. CDSSTR, CONTINLL, and SELCON3 (Sreerama and Woody, 2000) available at http://lamar.colostate.edu/~sreeram/cdpro/main.html. A basis set containing spectra of 43 proteins was used as data for fitting the experimental spectrum. CDSSTR for the best fit values estimated that the lectin contained αR (regular α-helix) 16%, αD
(distorted α-helix) 12%, βR (regular β-sheet) 14%, βD (distorted β-sheet) 9%, turn 20% and unordered structure 28% (NRMSD= 0.069). Thus MoL is an alpha-beta protein.

4.5.1. Thermal stability of MoL

Effects of temperature on the conformation of MoL at pH 7.2 were studied by monitoring the changes in the intrinsic fluorescence, ANS binding as well as changes in the ellipticity at 222 nm. Increase in the temperature reduced the fluorescence intensity of the protein linearly (Fig. 4.1 A), without any change in the λmax (Fig. 4.1 B). The native protein shows binding to the hydrophobic dye ANS at 30 °C, which reduces considerably with increasing temperatures (Fig. 4.1 C). The negative ellipticity at 222 nm does not change much with temperature, only a marginal decrease of 1 mdeg (from -12.5 to -11.5) is observed which gets reversed by cooling (Fig. 4.1 D).

The decrease in the fluorescence intensity at higher temperatures could be due to the thermal deactivation of the fluorophore. Reduced ANS binding to MoL with increase in temperature shows that the hydrophobic patches are less accessible at higher temperature. The secondary structure of MoL remains stable at higher temperatures.
Fig. 4.1. Temperature stability of MoL

(A) Fluorescence intensity at 350 nm of MoL (0.04 mg ml$^{-1}$) as a function of temperature.

(B) Fluorescence emission spectra of MoL (0.04 mg ml$^{-1}$) incubated at various temperatures for 15 minutes. The temperatures are indicated on the spectra.

(C) Binding of ANS to MoL (0.05 mg ml$^{-1}$) at various temperatures. The temperatures are indicated on the spectra.

(D) Ellipticity of MoL (0.08 mg ml$^{-1}$) at 222 nm as a function of temperature. Solid line represents the denaturation, whereas the dashed line represents the renaturation of the protein by cooling.
4.5.2. Effect of pH on stability of MoL

Conformational stability of MoL at various pH was studied by fluorimetry as well as circular dichroism. The fluorescence $\lambda_{\text{max}}$ of MoL does not change with pH. However, at acidic pH and at pH 8, MoL shows maximum fluorescence intensity while at extreme alkaline pH it reduces considerably (Fig. 4.2 A). When ANS binds to the native protein it shows a blue shift in the $\lambda_{\text{max}}$ from 520 to 480 nm and an increase in the fluorescence intensity (Fig. 4.2 B). Compared to the binding at pH 7.2, there is a four times enhancement in the binding at pH 1.0 and 2.5 times enhancement at pH 10.0. The secondary structure of the protein is not affected by extreme acidic and alkaline conditions as seen in the far UV CD spectra (Fig. 4.2 C). The near UV CD spectrum (Fig. 4.2 D) of native protein shows that MoL possesses ordered tertiary structure. At alkaline and extreme acidic pH it gets affected only with respect to tryptophan and tyrosine environment (280-290 nm).

All these studies indicate that variation in pH in a wide range has little effect on the structural features of MoL. It was already shown that MoL retained its hemagglutination activity even at extreme pH values (section 3.5.2). The working pH for hemagglutination assay is 7.2; hence it could be possible that although MoL underwent structural changes at various pH, it regained its native structure when the pH was readjusted to 7.2 in the assay condition. However, conformational studies using fluorimetry and CD confirm that there are no major structural changes occurring in MoL even under extreme pH conditions.

Very few plant lectins are known to be stable in such a wide range of pH, some of them are: lectin from Pinto beans (pH range: 3-12) (Wong et al., 2006); Alocasia cucullata lectin (pH range: 2-12) (Kaur et al., 2005a) and Arisaema tortuosum lectin (pH range: 2-10) (Dhuna et al., 2005).
Fig. 4.2. Conformational stability of MoL at various pH. In each diagram, numbers on the spectra indicate the pH.

(A) Fluorescence emission spectra of MoL (0.04 mg ml\(^{-1}\)) incubated at various pH for 16 h.

(B) ANS Binding to MoL (0.05 mg ml\(^{-1}\)) at various pH.

(C) Far-UV CD spectra (protein concentration 0.08 mg ml\(^{-1}\)) at pH 2.0, 7.2, and 10.0.

(D) Near-UV CD spectra (protein concentration 0.8 mg ml\(^{-1}\)) at pH 1.0, 7.2 and 10.0.
4.5.3. Effect of denaturants and reducing agent on MoL

A. Guanidium hydrochloride (GdnHCl)

In the presence of GdnHCl, the fluorescence intensity of MoL increased with increase in GdnHCl concentration up to 1-2 M, along with slight red shift in $\lambda_{\text{max}}$, indicating increased exposure of the tryptophan to polar environment. However, with further increase in the concentration of GdnHCl, the intensity decreased and at 6 M concentration, a much broader spectrum was observed, indicating partial unfolding of the protein (Fig. 4.3 A). ANS binding decreases even at a low concentration (1M) of GdnHCl (Fig. 4.3 B) indicating that the minor variation in the ionic or hydrophobic interactions in the structure of the protein tend to modify the exposure of hydrophobic side chains. Thus the hydrophobic pockets in the protein seem to respond to even small changes in the environment. No wonder, MoL also loses hemagglutination activity in the presence of lower concentrations (0.25 M) of GdnHCl.

The CD spectra of the protein in the far UV region incubated in the presence of the different concentrations of GdnHCl are shown in Fig. 4.3 C. Although the ellipticity in the region between 215 to 225 nm appears unchanged, that at 210 nm or below (not shown due to noise) seems to be affected indicating the increase in the unordered structural element. Hemagglutinating activity of the protein might be getting abolished due to these structural changes.
Fig. 4.3. Conformational stability of MoL in presence of various concentrations of GdnHCl. Concentrations of GdnHCl are indicated on the respective spectra.

(A) Fluorescence emission spectra of MoL (0.04 mg ml⁻¹) incubated for 16 h with different concentrations of GdnHCl.

(B) ANS Binding to MoL (0.05 mg ml⁻¹).

(C) Far-UV CD spectra (protein concentration 0.08 mg ml⁻¹) recorded after incubating the protein sample with different concentrations of GdnHCl for 4 h.
B. Urea

Fluorescence intensity of MoL remained constant up to 2M urea concentration, without any change in the $\lambda_{\text{max}}$. However, at 4 M concentration, there was a slight red shift in the emission spectrum with an increase in the intensity. After this, the intensity decreased further with any increase in urea concentration (Fig. 4.4 A). ANS failed to bind to protein above 2 M urea concentration (Fig. 4.4 B), whereas the secondary structure was found to be stable up to 3M urea concentration after which it started getting affected (Fig. 4.4 C). Above 6 M urea concentration, the secondary structure was drastically affected.

Urea, being a milder denaturant than GdnHCl, requires a higher concentration to be effective. It also explains, in this case, the retention of hemagglutinating activity of MoL below 3 M urea concentration.
Fig. 4.3. Conformational stability of MoL in the presence of various concentrations of Urea. Concentrations of Urea have been indicated on the respective spectra.

(A) Fluorescence emission spectra of MoL (0.04 mg ml⁻¹) incubated for 16h with various concentrations of urea.

(B) ANS Binding to MoL (0.05 mg ml⁻¹).

(C) Far-UV CD spectra (protein concentration 0.08 mg ml⁻¹) recorded after incubating the protein sample with different concentrations of urea for 4 h.
C. Dithiothreitol

Chemical modification studies on MoL indicated that the native dimer contains three disulfide linkages. To study the role of these disulfide bonds on the structural stability of MoL, CD analysis was carried out in the presence of the reducing agent dithiothreitol (DTT). The secondary structure of MoL was drastically affected in the presence of 1 mM DTT at and above pH 7.2 (Fig. 4.4). The protection of the structure at lower pH values is merely due to the fact that DTT needs an alkaline environment to function as a reducing agent. This explains the loss of hemagglutination activity of MoL in the presence of DTT. The disulfide linkages present in the protein seem to hold the structure intact and offer the required conformational stability essential for the hemagglutination activity.

![Fig. 4.4. Far-UV CD spectra of MoL (protein concentration 0.08 mg ml⁻¹) in the presence of 1 mM DTT recorded at various pH. Numbers on the spectra indicate the pH.](image)
4.5.4. Fluorimetric analysis of sugar binding

Specific binding of the sugar to a lectin can change the environment of tryptophan either by enhancing (Gaikwad and Khan, 2006) or by quenching (Khan et al., 2007) the fluorescence. This property can be used to calculate the binding affinity of the sugar towards protein.

Addition of glucose, galactose, lactose, sucrose, mannose, methyl α-glucose, methyl α-galactose, methyl α-mannose to MoL did not significantly change the fluorescence intensity of the protein. However, quenching was observed upon addition of N-acetyl-lactosamine (LacNAc) and fructose with no change in the emission maxima, although these sugars failed to inhibit the hemagglutination activity of MoL. Titration of MoL with LacNAc showed 9%, while titration with fructose showed 8% quenching of the fluorescence.

The plots of $F_0/\Delta F$ vs $1/C$ for LacNAc and fructose are shown in Fig. 4.5 (A and B, respectively) from which the values of $F_\infty$ have been determined and the double logarithmic plots have been shown in Fig. 4.5 (C and D, respectively). The affinities of MoL towards fructose and LacNAc are considerably low ($K_a = 975$ and $1380$ M$^{-1}$, respectively).

The corresponding $\Delta G$ values for binding of fructose and LacNAc to MoL are -17.16 and -18.03 kJ mol$^{-1}$, respectively, which indicates the spontaneous nature of this binding.
Fig. 4.5. Determination of association constant for the binding of sugars to MoL (0.04 mg/ml). Sugars used: LacNAc and Fructose, 10 mM stock each.

(A) and (B) Plots of ($F_0/\Delta F$) vs $[C]^{-1}$ for LacNAc and Fructose, respectively. Insets represent fluorescence quenching of MoL on addition of aliquots of sugars to the lectin solution.

(C) and (D) Plots of $\log(\Delta F/F_{C-F} - F_\infty)$ vs $\log(C)$ for LacNAc and Fructose, respectively.
4.5.5. Binding of adenine to MoL

The presence of exposed hydrophobic patches in the native lectin led us to check the binding of adenine to the protein. Addition of adenine resulted in quenching of the intrinsic fluorescence of tryptophan (Fig. 4.6 A inset) with \( K_a = 7.76 \times 10^3 \text{ M}^{-1} \), which is significantly less than those estimated for other lectins reported to bind adenine (lima bean lectin; \( K_a = 8.3 \times 10^4 \text{ M}^{-1} \), Phaseolus vulgaris erythroagglutinin; \( K_a = 1.2 \times 10^5 \text{ M}^{-1} \) and soybean agglutinin; \( K_a = 7.7 \times 10^4 \text{ M}^{-1} \), Dolichos biflorus seed lectins; \( K_a = 7.31 \times 10^5 \text{ M}^{-1} \) and Dolichos biflorus stem lectin; \( K_a = 1.07 \times 10^6 \text{ M}^{-1} \) (Gegg et al., 1992)). The \( \Delta G \) calculated for adenine binding to MoL is \(-22.324 \text{ kJ mol}^{-1} \). The plots of \( F_0/\Delta F \) vs \( 1/C \) and the double logarithmic plots for adenine binding are shown in Fig. 4.6 (B and C, respectively). Thus the adenine-binding site seems to be close to tryptophan. However, adenine does not inhibit the hemagglutination activity of MoL. This may be due to the known fact that the adenine binding sites can be distinctly different from the carbohydrate binding sites (Roberts and Goldstein, 1982; Hamelryck et al., 1999).

The above mentioned legume lectins also showed binding to cytokinins, which are a group of plant hormones (Roberts and Goldstein, 1983b; Gegg et al., 1992). Apart from legume lectins, wheat germ agglutinin (WGA) has also been shown to bind adenine and adenine-related phytohormones such as zeatin and kinetin as well as abscissic and gibberillic acids with affinities in the range of \( K_a = 1.6-2.3 \times 10^6 \text{ M}^{-1} \) (Bogoeva et al., 2004). MoL could also be binding in vivo cytokinins and other related plant hormones on the basis of observing adenine binding sites present in vitro.
4.5.6. Solute quenching studies

Fluorescence spectra of native MoL recorded in the presence of increasing concentrations of acrylamide and KI are shown in Fig. 4.7 (A and B, respectively). Among the ionic quenchers used, CsCl failed to quench the fluorescence of MoL, whereas KI quenched almost 85% of the fluorescence in the native condition, indicating that the environment of the tryptophan residue is highly electropositive. The extent of fluorescence quenching achieved in each case is shown in Table 4.1.

Table 4.1 Extent of quenching of intrinsic fluorescence of MoL with acrylamide and KI in various conditions.

<table>
<thead>
<tr>
<th>Quencher</th>
<th>Acrylamide (0.5 M)</th>
<th>KI (0.5 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (pH 7.2)</td>
<td>82 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>pH 1.0</td>
<td>90 ± 3</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>pH 10.0</td>
<td>89 ± 3</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>In 6 M urea</td>
<td>88 ± 3</td>
<td>86 ± 3</td>
</tr>
</tbody>
</table>
4.5.7. Stern-Volmer analysis of the quenching data

The Stern-Volmer plots (Eq. 4.3) obtained with acrylamide quenching showed a positive curvature (Fig. 4.8 A), which indicates that the quenching has both dynamic and static components. The static mechanism involves complex formation between tryptophan and acrylamide, while dynamic mechanism involves collisions with acrylamide during the lifetime of tryptophan in excited state. In such a case, the data can be analyzed by Eq. 4.6 by which the dynamic and the static components can be resolved (Lakowicz and Weber, 1973).

\[
\frac{F_0}{F_C} = (1 + K_{sv}[Q])(1 + K_s[Q]) \quad (\text{Eq. 4.6})
\]

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 quenching constant reflects the degree to which the quencher achieves the encounter distance from the fluorophore and can be determined by the fluorescence lifetime measurements according to the equation (Lakowicz and Weber, 1973)

\[
\frac{\tau_0}{\tau} = (1 + K_{sv}[Q]) \quad (\text{Eq. 4.7})
\]
Where $\tau_0$ is the average lifetime in absence of the quencher and $\tau$ is the lifetime in the presence of a quencher at a concentration $[Q]$. Using the average lifetimes obtained from analysis of the time resolved fluorescence data, as described in the section 4.5.8, the value of $K_{sv}$ obtained for acrylamide quenching of MoL was 0.216 M$^{-1}$. The low value of $K_{sv}$ suggests low collision frequency. By substituting this value in Eq. 4.6 and plotting a graph of $(F_0/F_C)/(1+K_{sv}[Q])$ against $[Q]$, the value of the static quenching constant ($K_s$) was obtained as 8.19 M$^{-1}$. The bimolecular quenching constant, $k_q$ was calculated as $k_q = K_{sv}/\tau$, (Lehrer, 1971) and was found to be $7.55 \times 10^{11}$ M$^{-1}$s$^{-1}$. Incorporating the values of $K_{sv}$ and $K_s$ in the expression $(1 + K_{sv}[Q])(1 + K_s[Q])$, the values obtained were plotted against $[Q]$. It was observed that the values of $F_0/F_C$ and $(1 + K_{sv}[Q])(1 + K_s[Q])$ match very well (Fig. 4.8 B).

Fig. 4.8. (A) Stern Volmer analysis of fluorescence quenching of MoL. Plots of quenching profiles with acrylamide, in conditions  ■ Native; ● 6 M Urea; ▼ pH 10.0; ▲ pH 1.0.

(B) The plot of $F_0/F_C$ and $(1 + K_{sv}[Q])(1 + K_s[Q])$ against $[Q]$ corresponding to the quenching of native MoL with acrylamide. ■ $F_0/F_C$, ● $(1 + K_{sv}[Q])(1 + K_s[Q])$. 
4.5.8. Quenching of MoL fluorescence with iodide

Iodide exhibited a strong quenching effect on the fluorescence of MoL (85%), which was quite unusual. Stern-Volmer analysis showed a sharp downward curvature of the plot, which could not be resolved into linear components (Fig. 4.9). This cannot be correlated with the single tryptophan in the protein. Thus, apart from the strong positive charge around the tryptophan, there could be some non-specific binding of iodide to the protein. Iodide having a large ionic radius and being negatively charged, probably binds to the positively charged amino acid residues present in the neighborhood of the single tryptophan in the protein leading to affinity quenching of the fluorescence rather than collisional quenching.

![Fig. 4.9 Stern Volmer analysis of fluorescence quenching of MoL. Plots of quenching profiles with potassium iodide, in conditions ■ Native; ● 6 M Urea; ▼ pH 10.0; ▲ pH 1.0.](image.png)

4.5.9. Lifetime measurements of fluorescence emission of MoL

The lifetime measurements of the fluorescence due to MoL from the decay curve (Fig. 4.10 A) was done by fitting it to a biexponential function ($\chi^2<1.005$). From this fit two decay times $\tau_1$ and $\tau_2$ with their corresponding weight factors $\alpha_1$ and $\alpha_2$ were
obtained (Table 4.2). The native lectin showed two lifetimes, $\tau_1$ (1.6 ns) and $\tau_2$ (4.36 ns) with 54% and 46% contributions, respectively, indicating presence of two conformers of the single tryptophan (Martinho et al., 2003). The shorter lifetime component contributed more to the fluorescence than the longer lifetime component. Normally the shorter lifetime component is due to fluorophors exposed on the surface while the longer one is due to fluorophors buried in the interior of the protein.

From the life time measurements of the quenching of the intrinsic fluorescence of MoL by acrylamide, the decay curve (Fig. 4.10 B) could be fitted to a bi-exponential function ($\chi^2<1.005$) for concentrations of acrylamide below 0.1 M, above which they could be fitted well with a mono-exponential function, indicating that there is only one conformer of tryptophan remaining after a certain concentration of acrylamide is reached. Both $\tau_1$ and $\tau_2$ tend to decrease with increasing acrylamide till 0.1 M concentration of the acrylamide was reached, above which only one lifetime was observed which also decreased from 2.21 to 2.07 ns at 0.45 M concentration of acrylamide. The decrease in the average lifetime from 2.86 to 2.07 ns could be due to low collisional frequency.

The average lifetimes were calculated using the following equations (Inokuti and Hirayama, 1965; Grinvald and Steinberg, 1974):

$$\tau = \sum \alpha_i \tau_i / \sum \alpha_i$$  \hspace{1cm} (Eq. 5)

$$<\tau> = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$$  \hspace{1cm} (Eq. 6)

Where $\tau$ and $<\tau>$ are the average life times obtained by two different approaches and $\alpha$ is the weighting factor.
Figure 4.10: The fluorescence lifetime decay curves for native MoL (A) and MoL quenched with 0.5 M acrylamide (B). The dotted lines correspond to the instrument response, the solid lines correspond to the experimental data and the starred lines correspond to the nonlinear biexponential fit of the experimental data to a biexponential function (in the case of native protein) or a monoexponential fit (in the case of quenched protein). The lower panels represent the corresponding residuals.

The plot of $\frac{\tau_0}{\tau}$ for the quenching data of MoL with acrylamide in native condition is shown in Fig. 4.11, from which $K_{sv}$ was calculated.

Figure 4.11: Plot of $\frac{\tau_0}{\tau}$ for the quenching data of MoL with acrylamide in native condition.
Table 4.2. The lifetimes of fluorescence decay of MoL and the corresponding pre-exponential factors along with calculated average lifetimes for acrylamide quenching.

<table>
<thead>
<tr>
<th>Q [M]</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_1$</th>
<th>$\tau_2$ (ns)</th>
<th>$\alpha_2$</th>
<th>$\bar{\tau}$ (ns)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.60</td>
<td>0.065</td>
<td>4.37</td>
<td>0.020</td>
<td>2.25</td>
<td>2.86</td>
</tr>
<tr>
<td>0.050</td>
<td>1.66</td>
<td>0.061</td>
<td>3.65</td>
<td>0.024</td>
<td>2.22</td>
<td>2.58</td>
</tr>
<tr>
<td>0.098</td>
<td>1.32</td>
<td>0.058</td>
<td>2.94</td>
<td>0.034</td>
<td>1.92</td>
<td>2.25</td>
</tr>
<tr>
<td>0.146</td>
<td>2.21</td>
<td>0.079</td>
<td>0</td>
<td>0</td>
<td>2.21</td>
<td>2.21</td>
</tr>
<tr>
<td>0.192</td>
<td>2.16</td>
<td>0.079</td>
<td>0</td>
<td>0</td>
<td>2.16</td>
<td>2.16</td>
</tr>
<tr>
<td>0.238</td>
<td>2.15</td>
<td>0.079</td>
<td>0</td>
<td>0</td>
<td>2.15</td>
<td>2.15</td>
</tr>
<tr>
<td>0.283</td>
<td>2.12</td>
<td>0.083</td>
<td>0</td>
<td>0</td>
<td>2.12</td>
<td>2.12</td>
</tr>
<tr>
<td>0.370</td>
<td>2.06</td>
<td>0.083</td>
<td>0</td>
<td>0</td>
<td>2.06</td>
<td>2.06</td>
</tr>
<tr>
<td>0.455</td>
<td>2.07</td>
<td>0.091</td>
<td>0</td>
<td>0</td>
<td>2.07</td>
<td>2.07</td>
</tr>
</tbody>
</table>
4.6. Conclusions

Conformational studies carried out on MoL using fluorescence spectroscopy and circular dichroism supported the observed stability and the hemagglutination activity of MoL at high temperatures and extreme pH. The secondary structure of MoL hardly gets affected at higher temperatures and extremes of pH. Although the intensity of the intrinsic tryptophenyl fluorescence of the protein changes with temperature and pH, the $\lambda_{\text{max}}$ of the fluorescence spectrum is not affected much, indicating no change in the overall conformation of the protein. The disulfide linkages in the protein seem to be essential for the active conformation of MoL, as both the activity and the secondary structure of MoL get severely affected by the presence of a reducing agent like DTT. Denaturants like GdnHCl and urea did affect the random coil element of the secondary structure causing some variations in the ionic or hydrophobic interactions in the structure of the protein which in turn affected the activity as well as ANS binding property of the protein.

MoL is a lectin exhibiting specificity solely for glycoproteins in hemagglutination experiments. However, in the sugar binding studies by fluorimetry, the lectin shows weak binding to sugars LacNAc and fructose. A clustering effect of multiple Gal-\(\beta\)-1,3-GalNAc or LacNAc chains might be necessary for binding, which has to be confirmed by studying the binding properties of glycopeptides of fetuin or thyroglobulin to MoL.

Binding of the hydrophobic dye ANS and adenine to the native protein indicates that MoL might have a physiological role in binding the phytohormones.

The single tryptophan per monomer in MoL is highly exposed to the solvent environment as well as surrounded by basic amino acid residues, as seen by fluorescence quenching studies. The native protein shows two fluorescence lifetimes, which get converged to a single one after quenching with acrylamide.