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SHORT COMMUNICATION

Steady State and Time-Resolved Fluorescence Studies of a Hemagglutinin from *Moringa oleifera*

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Abstract The saccharide binding and conformational characterization of a hemagglutinin, a low molecular weight protein from the seeds of *Moringa oleifera* was studied using steady state and time resolved fluorescence. The lectin binds sugars LacNAc (K_a=1380 M^{-1}) and fructose (K_a=975 M^{-1}), as determined by the fluorescence spectroscopy. It has a single tryptophan per monomer which is exposed on the surface and is in a strong electropositive environment as revealed by quenching with iodide. Quenching of the fluorescence by acrylamide involved both static (K_s=0.216 M^{-1}) and collisional (K_{sv}=8.19 M^{-1}) components. The native protein showed two different lifetimes, τ_1 (1.6 ns) and τ_2 (4.36 ns) which decrease and get converted into a single one, (2.21 ns) after quenching with 0.15 M acrylamide. The bimolecular quenching constant, k_q was 7.55×10^{11} M^{-1} s^{-1}. ANS binding studies showed that the native protein has exposed hydrophobic patches which get further exposed at extreme acidic or alkaline pH. However, they get buried in the interior of the protein in presence of 1 M GdnHCl or urea.

Keywords *Moringa oleifera* · Hemagglutinin · Saccharide binding · ANS binding · Fluorescence · Solute quenching · Lifetime

Introduction

Lectins are non-catalytic carbohydrate-binding proteins of non-immune origin that are widely distributed in micro-organisms, plants and animals. They bind glycoconjugates or cell-surface carbohydrates and cause agglutination of erythrocytes and other cells. Lectins participate in many biological processes, including cell-cell recognition, host-pathogen interaction, serum glycoprotein turnover and innate immune responses [1]. Most of the lectins are specific for simple sugars and their hemagglutination activity is inhibited by monosaccharides or oligosaccharides. In addition, plant lectins with complex specificity that are inhibited only by complex glycoproteins and not by simple sugars have also been reported; for example *Scilla campanulata* bulb lectin [2], *Acacia constricta* seed lectins [3], *Arisaema flavum* tuber lectin [4].

Moringa oleifera (drumstick or horse radish tree) is a tropical plant belonging to the family *Moringaceae*, which is widely cultivated in India to use its leaves, fruits, and roots as food and for medicinal purposes. The *Moringa oleifera* seed flour is traditionally used as a coagulant in water treatment [5]. Use of these seeds for softening the hard water has also been reported [6]. It was observed that the active agents of coagulation are dimeric cationic proteins of molecular weight of approximately 13 kDa having an isoelectric point between 10 and 11 [7]. Interfacial properties and the interaction of this coagulant protein with sodium dodecyl sulfate have been reported [8].

Studies of indole fluorescence quenching by added solutes have provided valuable information regarding the structure and dynamics of proteins in solution [9–11]. The dynamics of the steady state fluorescence of the protein can be resolved to correlate the photophysical parameters of the protein to the structural properties. The low molecular weight protein from the seeds of *Moringa oleifera*, which has been previously characterized as a coagulating protein [5, 7] has been characterized by us as a hemagglutinin with complex sugar specificity (unpublished data). The present paper describes the sugar binding and the steady state and time resolved fluorescence studies of this protein.

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Experimental

Purification

The hemagglutinin (*Moringa oleifera* lectin; MoL) was purified by ammonium sulfate precipitation at 90% saturation followed by two successive ion exchange chromatography steps using DEAE cellulose and CM sephadex.

Modification of tryptophan residues with NBS

To estimate the tryptophan content of the protein, 1 ml (400 µg) of MoL at pH 5.0 was titrated against freshly prepared *N*-bromosuccinimide (5 mM) [12]. By measuring the reduction in OD at 280 nm, the number of modified tryptophan residues was determined, assuming the molar absorption coefficient of 5,500 M^{-1} cm^{-1} for the modified tryptophan at 280 nm. To determine the total number of tryptophans modified in the unfolded state, the reaction was carried out for the protein incubated in the presence of 6 M Urea for 16 h.

Fluorimetric measurements

The intrinsic fluorescence of the protein was measured using a PerkinElmer Life Sciences LS50 fluorescence spectrophotometer connected to a Julabo F20 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) was excited at 295 nm and the emission was recorded in the range of wavelengths 300–400 nm. Both the excitation and emission spectra were obtained setting the slit-width at 5 nm, and speed 100 nm min^{-1}.

Sugar binding studies

The binding of sugars to MoL was studied by intrinsic fluorescence titrations. To 2 ml of lectin sample (concentration 0.04 mg/ml) in 50 mM phosphate buffer, pH 7.2, 5–100 µl aliquots of the sugar solutions were added and the fluorescence intensity was monitored. The following equation was used to determine the association constant (K_a) [13].

$$\log{[C]_f} = -\log{[K_a]} + \log{[(F_0 - F_C)/(F_C - F_\infty)]}$$

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_∞, the fluorescence intensity upon saturation of all the sugar binding sites was 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 energies for binding were calculated by using the equation $-\Delta G = RT \ln(K_a)$.

Solute quenching studies by steady state fluorescence

Protein samples (0.05 mg/ml) incubated for 16 h in 50 mM buffers (pH 1.0, 7.2 and 10.0) as well as in 6 M urea were titrated with small aliquots (3–5 ul) of 5 M quencher solution (acrylamide, potassium iodide or caesium chloride) and fluorescence spectra were recorded after each addition. Relative fluorescence intensities were measured at the wavelength corresponding to the emission maximum (351 nm) of the protein [14] and they were corrected for dilution. All measurements were carried out at 27 °C. Iodide stock solution contained 0.2 M sodium thiosulfate to prevent formation of tri-iodide (I^{-3}).

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) 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.

Binding of ANS to MoL

The binding of the hydrophobic dye ANS to MoL was analyzed by measuring the fluorescence of MoL incubated at pH 1.0, 7.2 and 10.0 on the Fluorescence Spectrophotometer. 15 µl of 20 mM ANS was mixed with 2 ml of protein (0.05 mg/ml) which was then excited at 375 nm and the emission recorded between 450–550 nm. Reference spectrum with ANS in each of the condition was subtracted from the spectrum of the sample.

Results and discussion

Purification

MoL was purified by two successive ion exchange chromatography steps. The highly basic lectin eluted as an

unbound protein from the anion exchanger DEAE-cellulose column, got bound very tightly to the cation exchanger CM Sephadex column and got eluted with 1 M NaCl. The final yield of the protein was 800 mg per 100 g of dried seeds with a specific activity of 160 HU mg^{-1}.

Fluorimetric analysis of sugar binding

Sugar binding studies were carried out using fluorescence spectroscopy. Specific binding of the sugar might change the microenvironment of tryptophan either by enhancing [15] or by quenching [16] of the fluorescence. This can be used to calculate the binding affinity of the sugar towards protein. Addition of glucose, galactose, mannose, methyl α-glucose, methyl α-galactose, methyl α-mannose to MoL did not significantly change the fluorescence intensity of the protein. However, quenching upon addition of *N*-acetyllactosamine (LacNAc) and fructose was observed 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% quenching of the fluorescence, while titration with fructose resulted in 8% quenching in the fluorescence.

The plots of $F_0/\Delta F$ vs $1/C$ and the double logarithmic plots for LacNAc have been shown in Fig. 1 (a and b, respectively). The binding constants for binding of fructose and LacNAc to MoL are K_a=975±29 and 1,380±41 M^{-1}, respectively, which are low, as the binding constants of lectin-sugar interactions with high affinity range between 10^4 to 10^5 [15]. The ΔG values for binding of fructose and LacNAc to MoL are −17.16 and −18.03 kJ mol^{-1}, respectively, which indicates spontaneous nature of binding.

Determination of tryptophan residues in MoL

The titration of MoL with NBS indicated that only a single tryptophan residue per monomer in the protein gets modified both in the native and denatured conditions. In the native condition, modification of the tryptophan residue did not affect the hemagglutinating activity of the protein.

Fluorescence quenching studies on MoL

MoL is a protein containing single tryptophan residue per monomer. The protein shows fluorescence maximum at 351 nm when excited at 295 nm, indicating that the tryptophan residue is exposed to the solvent.

Fluorescence spectra of MoL in the presence of increasing concentrations of Acrylamide and KI are shown in Fig. 2 (a and b, respectively). 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 1.

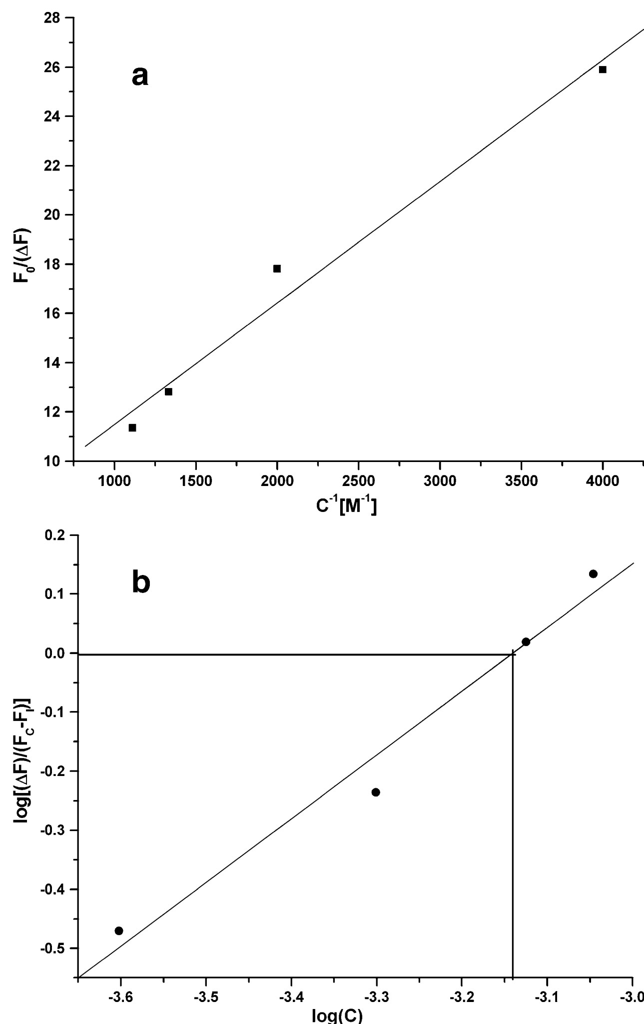

Fig. 1 Plots for the determination of association constant for the binding of LacNAc (100 mM stock) to MoL (0.04 mg/ml). **a** ($F_0/\Delta F$) vs $[c]^{-1}$. **b** $\log\left(\Delta F/F_C - F_\infty\right)$ vs $\log(c)$

Stern–Volmer analysis of the quenching data

Initially, the quenching data were analyzed by the Stern–Volmer Eq. 1, as given below [10]:

$$F_0/F_C = 1 + K_{sv}[Q] \quad (1)$$

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

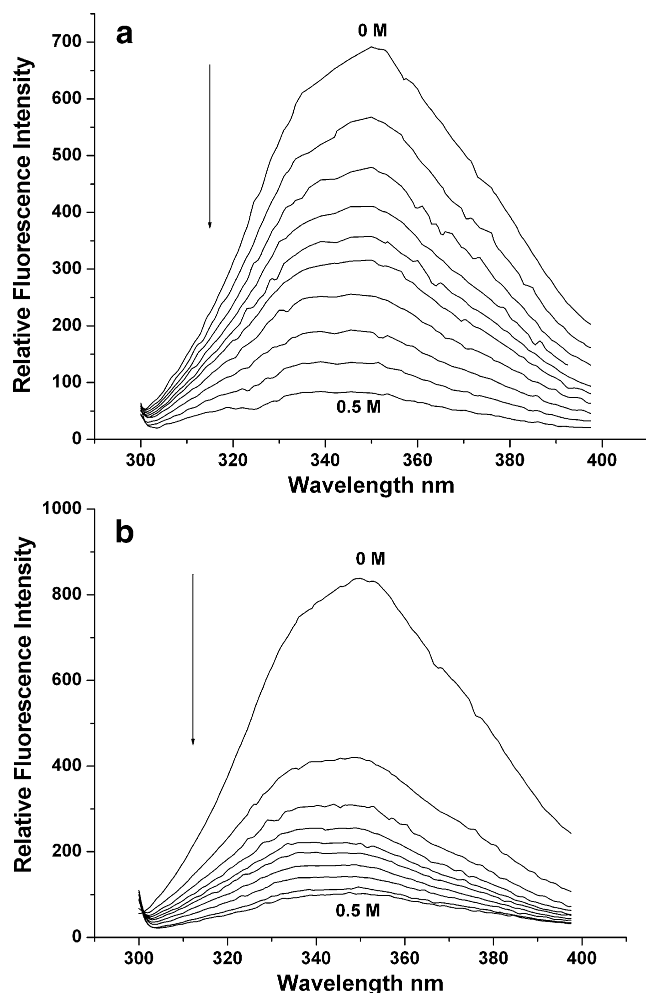

Fig. 2 Fluorescence emission spectra of MoL (0.05 mg/ml) in native condition in the absence and in the presence of quencher (**a** acrylamide; **b** potassium iodide). The quencher concentration is increased from 0 to 0.5 M, as indicated by the *arrow*

The profile obtained with acrylamide quenching shows a positive curvature (Fig. 3a), which indicates that the quenching has 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.

Table 1 Extent of quenching of intrinsic fluorescence of MoL with acrylamide and potassium iodide in various conditions

Extent of quenching %		
Quencher	Acrylamide (0.5 M)	KI (0.5 M)
Native (pH 7.2)	82.32	85.85
pH 1.0	90.42	94.81
pH 10.0	89	85.07
In 6 M urea	88.04	86.32

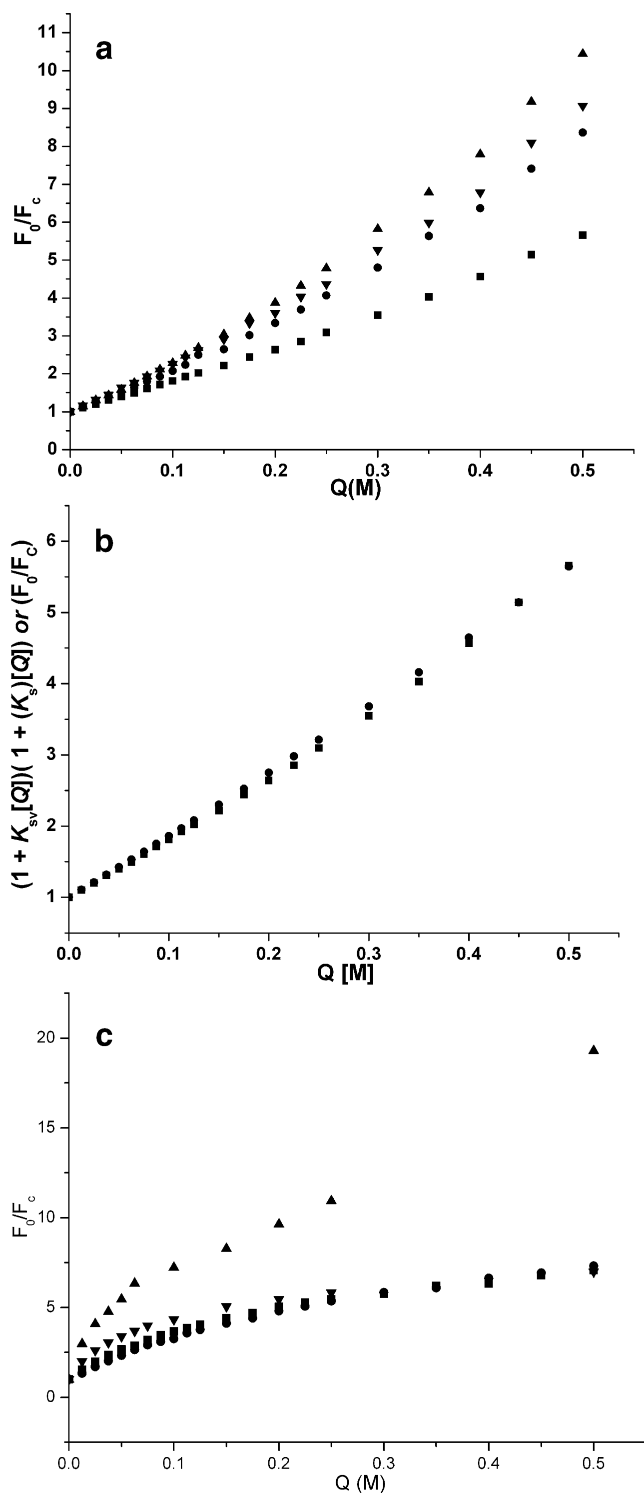

Fig. 3 **a** Stern–Volmer analysis of fluorescence quenching of MoL. Quenching profiles with acrylamide. *Square* Native; *circle* 6 M Urea; *triangle* pH 1.0; inverted *triangle* pH 10.0. **b** The plot of F_0/F_C and $(1+K_{sv}[Q])(1+K_s[Q])$ against $[Q]$ corresponding to quenching of native MoL with acrylamide. *square* F_0/F_C, *circle* $(1+K_{sv}[Q])(1+K_s[Q])$. **c** Stern–Volmer analysis of fluorescence quenching of MoL. Quenching profiles with potassium iodide. *Square* Native; *circle* 6 M Urea; *triangle* pH 1.0; inverted *triangle* pH 10.0

In such a case, the data can be analyzed by Eq. 2 by which the dynamic and the static components can be resolved [11].

$$F_0/F_C = (1 + K_{sv}[Q])(1 + K_s[Q]) \quad (2)$$

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 of the fluorophore and can be determined by the fluorescence lifetime measurements according to the equation [11]

$$\tau_0/\tau = (1 + K_{sv}[Q]) \quad (3)$$

Where τ_0 is the average lifetime in absence of the quencher and τ is the lifetime in presence of a quencher at a concentration $[Q]$. Using the average lifetimes obtained from analysis of the time resolved fluorescence data as described below in the "Lifetime measurements of fluorescence emission of MoL", the value of K_{sv} obtained for acrylamide quenching of MoL was 0.216 M^{-1}. Low value of K_{sv} suggests low collision frequency. Putting this value in Eq. 2 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} and the bimolecular quenching constant, k_q was calculated as $k_q=K_{sv}/\tau$, [9] and was found out to be 7.55×10^{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. 3b).

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. 3c). 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 around the single tryptophan in the protein leading to affinity quenching of the fluorescence rather than collisional quenching.

Lifetime measurements of fluorescence emission of MoL

The lifetime measurement of the intrinsic fluorescence of MoL from the decay curve (Fig. 4a) was done by fitting it to a biexponential function (χ^2<1.005). From this fit two

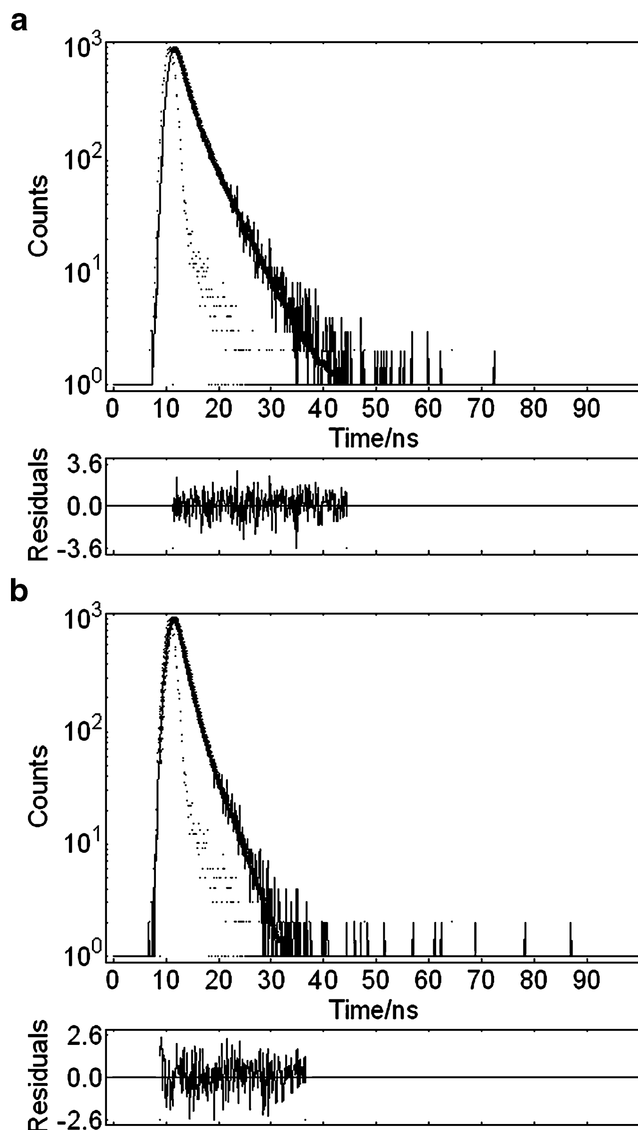

Fig. 4 The fluorescence lifetime decay curves for native MoL (**a**) and MoL quenched with 0.5 M acrylamide (**b**). The *dotted lines* correspond to the instrument response, the *solid lines* correspond to the experimental data and the starred lines correspond to the nonlinear biexponential fit of the 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 residuals

decay times τ_1 and τ_2 with their corresponding weight factors α_1 and α_2 were obtained (Table 2). The native lectin showed two lifetimes, τ_1 (1.6 ns) and τ_2 (4.36 ns) with 54 and 46% contributions, respectively, indicating presence of two conformers of the single tryptophan [17].

From the life time measurements of the quenching of the intrinsic fluorescence of MoL by acrylamide, the decay curve (Fig. 4b) could be fitted to a bi-exponential function (χ^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 τ_1 and τ_2 decreased till

Table 2 The lifetimes of fluorescence decay of MoL and the corresponding pre-exponential factors along with calculated average lifetimes for acrylamide quenching

Q [M]	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ (ns)	<τ> (ns)	χ^2
0	1.6	0.065	4.37	0.020	2.25	2.86	1.002
0.050	1.66	0.061	3.65	0.024	2.22	2.58	1.002
0.098	1.32	0.058	2.94	0.034	1.92	2.25	1.003
0.146	2.21	0.079	0	0	2.21	2.21	1.002
0.192	2.16	0.079	0	0	2.16	2.16	1.003
0.238	2.15	0.079	0	0	2.15	2.15	1.002
0.283	2.12	0.083	0	0	2.12	2.12	1.004
0.370	2.06	0.083	0	0	2.06	2.06	1.001
0.455	2.07	0.091	0	0	2.07	2.07	1.005

0.1 M concentration of the acrylamide was reached, above which only one lifetime was observed which decreased from 2.21 to 2.07 ns. 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 [18, 19]:

$$\tau = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_i \tag{4}$$

$$\langle \tau \rangle = \Sigma_i \alpha_i \tau_i^2 / \Sigma_i \alpha_i \tau_i \tag{5}$$

i=1, 2......

Where τ and <τ> are the average life times obtained by two different approaches and α is the weighting factor.

The plot of τ_0/τ for the quenching data of MoL with acrylamide in native condition is shown in Fig. 5, from which K_{sv} was calculated.

Hydrophobic dye binding

8-Anilino-1-Naphthalene Sulfonic Acid (ANS) binds to the native protein showing blue shift in the λ_{max} from 520 to 480 nm and increase in the fluorescence intensity (Fig. 6). The binding of ANS to MoL at pH 1.0, 7.2 and 10.0 has been shown in Fig. 6. Compared to pH 7.2, the binding gets four times enhanced at pH 1.0 and 2.5 times enhanced at pH 10.0. However, the protein fails to bind ANS, in the presence of low concentration of GdnHCl or urea (data not shown) indicating that the minor variation in the ionic or hydrophobic interactions in the structure of the protein does affect the exposure of the hydrophobic side chains. Thus the hydrophobic pockets in the protein seem to be flexible.

In conclusion, the low molecular weight protein from *Moringa oleifera* showing hemagglutinating activity exhibited complex sugar specificity. The tryptophan on the surface of the protein is in a strong electropositive environment, causing KI to nonspecifically quench the fluorescence. The lectin showed two lifetimes for the decay of the intrinsic fluorescence. The quenching of the fluorescence by the neutral quencher, acrylamide, showed two components, static and collisional. The protein has flexible hydrophobic side chains exposed on the surface.

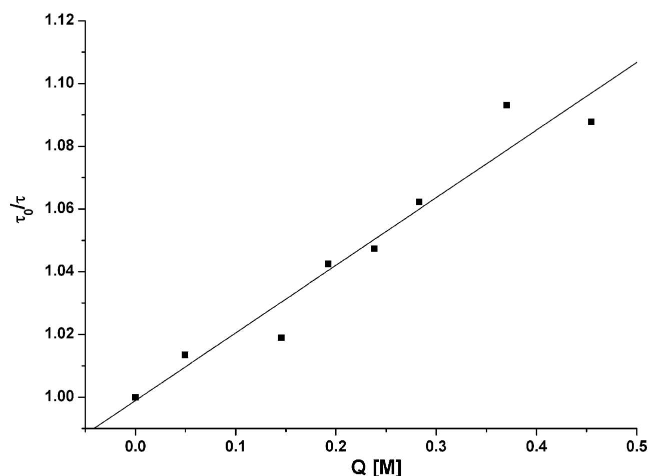

Fig. 5 Plot of τ_0/τ for the quenching data of MoL with acrylamide in native condition

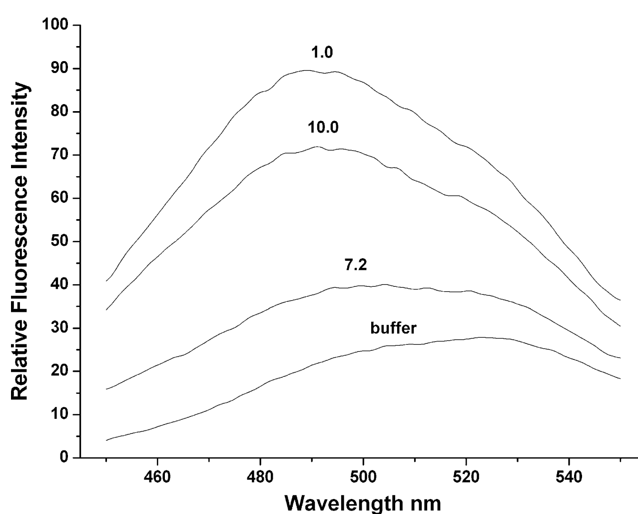

Fig. 6 The binding of ANS to MoL (0.025 mg/ml) at different pHs. Fluorescence spectra of buffer containing ANS, protein samples at pH 1.0, 7.2 and 10.0 excited at 375 nm. The numbers on the spectra indicate the respective pH of the sample

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