J Fluoresc (2009) 19:239–248
DOI 10.1007/s10895-008-0409-z

ORIGINAL PAPER

# Comparative Studies of Two Araceous Lectins by Steady State and Time-Resolved Fluorescence and CD Spectroscopy

**Poorva N. Dharker · Sushama M. Gaikwad · C. G. Suresh · Vikram Dhuna · M. Islam Khan · Jatinder Singh · Sukhdev Singh Kamboj**

Received: 17 June 2008 / Accepted: 11 August 2008 / Published online: 26 August 2008


**Abstract** Transitions in the tryptophan microenvironment and secondary structure of two monocot lectins from *Sauromatum guttatum* and *Arisaema tortuosum* under different denaturing conditions were studied by steady state and time resolved fluorescence and CD spectroscopy. The lectins exist as tetramers with a single tryptophan residue estimated per monomer, present in a polar environment. Quenching with ionic quenchers showed predominantly electropositive environment for tryptophan residues. Acrylamide had maximum quenching effect. A decrease in KI quenching due to lectin denaturation indicated redistribution of charges as a result of possible conformational change. The two values for lifetimes of tryptophanyl population (1.2–1.4 and 6.3–6.4 ns) reduced substantially on quenching or denaturation. Similarly, both the lectins showed a drastic loss of secondary structure in 5 M Gdn-HCl or 6 M Urea or at pH 2.0 and below. For the first time araceous lectins, like legume lectins are shown to bind adenine. The presence of a compact structure at alkaline pH 10.0–12.0 was observed in CD spectra.

**Keywords** Araceae lectins · Fluorescence · Lifetime spectroscopy · Denaturation · Secondary structure

P. N. Dharker · S. M. Gaikwad (✉) · C. G. Suresh · M. I. Khan
Department of Biochemistry, National Chemical Laboratory, Pune 411008 Maharashtra, India
e-mail: sm.gaikwad@ncl.res.in

V. Dhuna · J. Singh · S. S. Kamboj
Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar 143005 Punjab, India

## Introduction

Tryptophan residues in proteins are good molecular probes to study structural dynamics of proteins in solution. The physical and dynamic properties of tryptophan microenvironment and certain structural features and behavior of protein as a whole can be studied by monitoring tryptophan fluorescence [1–3]. Small chemical moieties that quench or decrease the fluorescence intensity of tryptophan residues in a protein molecule are employed to characterize the microenvironment of tryptophans.

Lectins from araceae have been grouped in the superfamily of single monocot mannose binding lectins on the basis of their sequence homology, structural features and sugar specificity. Some members of this superfamily from Liliaceae [4], Amarylidaceae, Alliaceae [5–8], Araceae [9, 10] Gramineae and Orchidaceae [6] have been characterized. In the past few years, Araceae has been identified as a lectin-rich family with lectins constituting 70–80% of storage proteins in their tubers [7]. Among the Araceae lectins those from *Arisaema tortuosum* schott [11], *Arisaema consanguineum* Schott (ACA), *Arisaema curvature* Kunth (ACmA), *Gonatanthus pumilus* (GPA), *Sauromatum guttatum* Schott (SGA) [10] and *Alocasia cucullata* [12] have been purified and characterized.

Although, extensive studies on three-dimensional structures of lectins and lectin-carbohydrate interactions have been carried out, till date there is no report of folding-unfolding as well as characterization of tryptophan environment of monocot lectins belonging to araceae. The two Araceae lectins compared here are *S. guttatum* lectin (Voodoo lily; SGA) and *A. tortuosum* lectin (Himalayan cobra lily; ATL). Both are non-mannose binding monocot lectins isolated from the tubers of the respective araceous plants. SGA and ATL consist of a mixture of isolectins

differing in charge, similar to *Alocasia indica* lectin [13], WGA [14] and those from Amaryllidaceae and Alliaceae [6]. Recently, SGA [15] as well as some more lectins from Arisaema sp. [11, 16–19] have been found to show antiproliferative as well as mitogenic activity

In this paper, we report the structural studies of the *S. guttatum* and *A. tortuosum* lectins by using steady-state and time resolved fluorescence and CD spectroscopy. The two lectins have been characterized with respect to their tryptophan environment and secondary structure under chemical, pH and thermal denaturing conditions. The study presents a comparison of the structural stability and dynamics of the two proteins.

## Experimental

### Purification of the lectins from *S. guttatum* (SGA) and *A. tortuosum* (ATL)

The lectins were extracted from the tubers of plants *S. guttatum* Schott (SGA) and *A. tortuosum* and purified by affinity chromatography as described by Shangary et al. [10] on a column of asialofetuin-linked amino activated silica column (0.8×3 cm). The unbound protein was removed by washing with 0.01 M PBS (pH 7.2), followed by elution with 0.1 M glycine-HC1 buffer (pH 2.5) with a flow rate of 40 ml $h^{-1}$. Hemaglutination assays carried out with rabbit erythrocytes gave consistent results as previously reported [10, 11].

### N-bromosuccinimide Modification of Trp residues

Both the lectins (300 μg) in 100 mM sodium acetate buffer pH 4.5 were titrated with 0.02 mM N-bromosuccinimide (NBS) prepared in the same buffer. The reagent was added in five installments (5 μl each) and the reaction was monitored spectrophotometrically by monitoring the decrease in absorbance at 280 nm. The number of tryptophan residues modified was determined by assuming a molar absorption coefficient of 5500 $M^{-1}cm^{-1}$ [20].

### Steady-state fluorescence spectroscopy

The intrinsic fluorescence of the protein was analyzed at 30 °C in PerkinElmer LS-50B spectrophotometer equipped with a thermostatically controlled sample holder. The effect of chemical denaturants was studied by incubating the protein samples (1.8 μM) in 0–6 M Gdn-HCl at 30 °C and pH 7.0 for 4, 8, and 24 h. Protein samples were also incubated in 0.05 M each of glycine-HCl buffer pH 1.0, potassium phosphate buffer pH 7.0, and glycine-NaOH buffer pH 10.0 at 25 °C for 16 h. The fluorescence emission of the protein was monitored by recording the scans after every stipulated period in 1 cm quartz cell in 300–400 nm range after excitation at 280 nm. Excitation and emission band pass of 7 nm was used. Reaction mixtures with protein absent were used to correct base line.

### Steady state fluorescence quenching

Fluorescence titrations were carried out by adding 3–5 μl of acrylamide (5 M), potassium iodide (5 M), cesium chloride (5 M) and 2.5 M of succinimide to the protein sample (2 μM) prepared in 20 mM phosphate buffer pH 7.2. Fluorescence intensity was recorded after each addition. The iodide solution contained sodium thiosulfate (200 μM) to suppress triiodate formation. The excitation wavelength was set at 295 nm; the emission spectra were recorded in the range 300 to 400 nm with both the slit widths as 7 nm at a scan speed of 200 nm/min. To eliminate contribution from background emission, the signal produced by buffer solution was subtracted.

### Hydrophobic dye binding studies

8-anilino-1-napthalene sulfonic acid (ANS) is a charged hydrophobic dye that binds to hydrophobic clusters in proteins and hence is largely employed for characterizing and detecting partially unfolded states in proteins [21, 22]. ANS emission spectra were recorded in the range 400–550 nm with excitation at 375 nm using slit widths of 5 nm for emission and excitation monochromators. The change in ANS fluorescence on binding to lectin was recorded at constant concentration of protein (2 μM) and ANS (50 μM). The spectrum of ANS in buffer was subtracted from the combined protein-ANS spectrum to yield the final spectrum.

### Adenine binding studies

Adenine is known to bind the hydrophobic amino acid residues of proteins and quench the intensity of the fluorophore. Protein (2 μM) was titrated against a 10 mM solution of adenine dissolved in 0.1 M HCl and recorded the scans. Hydrophobic ligand (5–10 μl) was added till saturation. The binding of adenine to lectins was monitored by recording changes in fluorescence after excitation at 280 nm using slit width of 7 nm at 30 °C [23]. Change in fluorescence ($\Delta F$) was noted with increasing concentrations of adenine in independent readings. The fluorescence intensity was correlated for inner filter effect due to excitation light absorbance by adenine according to the following equation:

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

In the above equation $F_0$ and $F_C$ are the fluorescence intensities of the free protein and of the protein at an

adenine concentration [$C$]. From the ordinate intercept of the double reciprocal plot of $F_0/(F_0-F_C)$ versus 1/[$C$], $F_\infty$, the fluorescence intensity upon saturation of all the adenine binding sites is obtained. 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 these interactions, the reciprocal of which gave the $K_a$ (the association constant). This analysis of the binding data was carried out according to Chipman [24].

### Circular dichroism measurements

CD measurements were performed in a Jasco 810 spectropolarimeter using a cuvette of 0.1 cm path length at 30 °C, unless otherwise stated. The solution was scanned in the wavelength range 200–250 nm (50 nm $min^{-1}$, response time 2 s) three times and the data were averaged. The protein samples (80 μg/ml) were in 20 mM phosphate buffer pH 7.2 containing 150 mM NaCl. The effect of temperature on the proteins was studied by two different methods. In one set of experiments protein samples were equilibrated at temperatures ranging from 25 to 90 °C for 10 min and the scan was recorded separately for each sample. For thermally denatured samples, after cooling the sample to room temperature a new scan was recorded under the same conditions, for testing the reversibility of thermal transitions. For determination of $T_m$, the spectropolarimeter was connected to a PTC343 Peltier circulating water bath (Jasco,Tokyo,Japan). The temperature of the protein samples was increased at the rate of 1 °C/min for the temperature ranging from 25 to 90 °C and the ellipticity was recorded at 225 nm. To study the effect of denaturants the protein samples were incubated in 0–6 M Gdn-HCl and 0–8 M urea for 4 and 16 h, respectively, and after that the far ultraviolet (UV) CD spectra were recorded. Effect of pH on the secondary structure was studied by incubating the protein samples in 50 mM buffers in pH range 1–11, as given above, for 16 h at 30 °C. All spectra were corrected by subtracting the respective buffer baseline.

### Fluorescent lifetime spectroscopy

Protein samples at a concentration of 0.5 mg/ml were employed for this experiment. Lifetime measurements were carried out on Edinburg lifetime spectrofluorimeter. For samples of native and denatured (8 M urea) lectins tryptophan residues were selectively excited at wavelength 295 nm and emission recorded at 340 nm. Protein samples containing final concentrations of acrylamide were subjected to lifetime measurements. The decay curves thus obtained were analyzed by a multiexponential iterative fitting program provided with the instrument.

## Results and discussion

The fluorescence emission maxima of SGA and ATL at pH 7.0 were at 350 nm (Fig. 1A), indicative of exposed tryptophan residues existing in polar environment. The NBS modification of tryptophan in both the native and urea denatured states of the two lectins indicated presence of single tryptophan per monomer and the lectins were tetramers in the native state. These results further confirmed the surface exposed state of tryptophans in these araceous lectins.

The far-UV CD spectroscopy for secondary structure prediction showed maxima of positive ellipticity at 201 nm

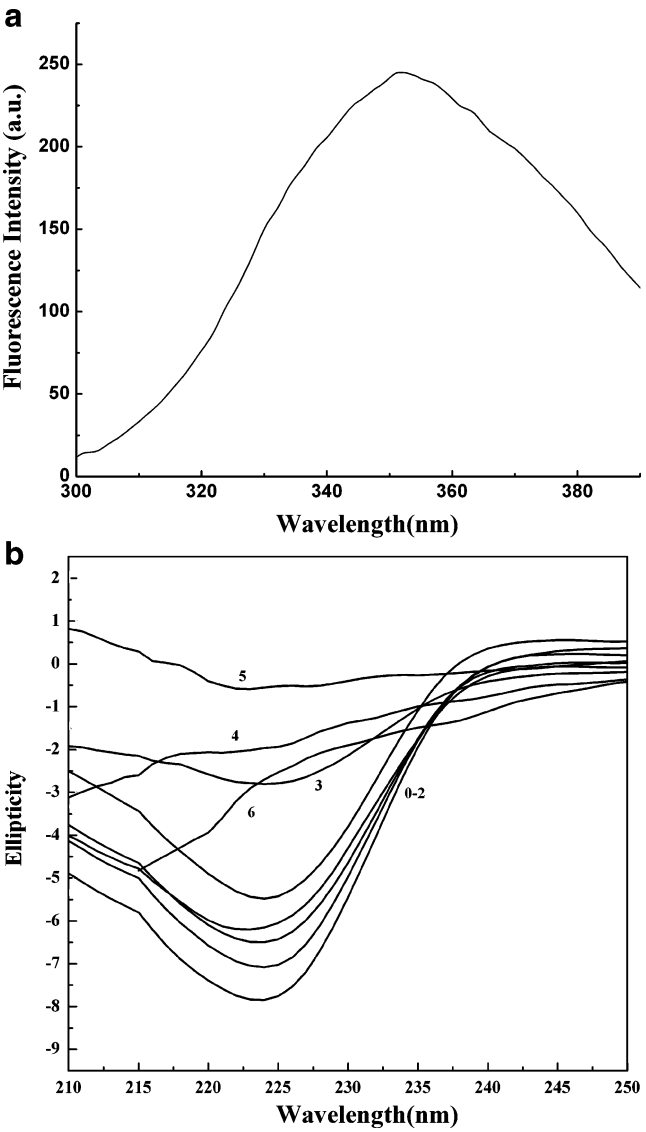


**Fig. 1** **A** Fluorescence spectra of Native SGA (1.8 μM) at pH 7.2; **B** Far-UV CD scans of SGA, incubated in 0–6 M GdN-HCl, pH 7.2. Scans were taken after incubation of the protein in the denaturant for 16 h at 30 °C. The scans were averaged after three accumulations

and minima at 225–226 for both the lectins (Fig. 1B). The percentage of secondary structural elements in SGA estimated from the far-UV CD spectra showed α-helix: 2.8%, β-sheet: 49% turns: 22.9% and random coil: 25.2%. Similar results were obtained for ATL. The far-UV CD spectra of SGA and ATL resemble the spectra of a synthetic tetrapeptide *tuftsin*, which is shown to be an important immunomodulator and stimulates phagocytosis and is shown to elicit *in vivo* as well as *in vitro* antitumor effects. A negative trough at 225 nm and maxima at 200 nm are found to be associated with type III β turns as in the case of *tuftsin* [25].

## Denaturation studies by steady state and lifetime fluorescence and CD spectroscopy

### *Gdn-HCl induced denaturation*

Characterization of the structural and conformational changes occurring in both these araceaous lectins in the presence of Gdn-HCl was carried out to probe the changes occurring in the tryptophan environment. A red shift in $\lambda_{max}$ of fluorescence of the protein from 350 to 356 nm with increasing concentration of Gdn-HCl was observed in the spectrum indicating increased polarity of tryptophan environment. A 50% loss of hemaglutination activity of ATL has been reported in presence of 3 M Gdn-HCl concentration [11]. A simultaneous drop in the fluorescence intensity observed could be due to structural changes in protein with increasing concentration of Gdn-HCl (1–6 M) leading to solvent shielding of Trp residues (data not shown).

The change in negative ellipticity caused by Gdn-HCl in case of SGA is depicted in Fig. 1B. At 6 M concentration of the denaturant major distortion in the secondary structure along with a corresponding drop in the ellipticity at 225 nm was observed. ATL shows similar Gdn-HCl mediated denaturation profiles. Three molar Gdn-HCl caused only partial unfolding of both the lectins with concomitant loss of hemagglutination activity indicating disruption of structural and functional integrity.

### *Effect of pH*

A 2–3 nm blue shift in $\lambda_{max}$ to 347 nm at pH 3, and a 2–3 nm red shift in $\lambda_{max}$ to 353 nm at alkaline pH (10–12) were observed for both the proteins. The far-UV CD scans of SGA at various pH are shown in Fig. 2A. The spectra of both the lectins suggest a compact structure existing at alkaline pH (10–12). The secondary structures of ATL as well as SGA were drastically affected at acidic pH as evident from the change in ellipticity at 210 nm in far-UV CD spectra. Both these lectins retain hemagglutination activity at both acidic (pH 2–4) and alkaline pH (10–12) [10, 11]. Thus, it may be reasonable to assume that the apparent structural loss of the proteins at acidic pH observed in the CD spectra can be attributed to some disruption away from the sugar binding site and not affecting its integrity.

### *Hydrophobic dye binding*

Enhancement of the intrinsic fluorescence of ANS upon binding to proteins at acidic and alkaline pH was studied. Although the secondary structure at pH 2.0 gets distorted as

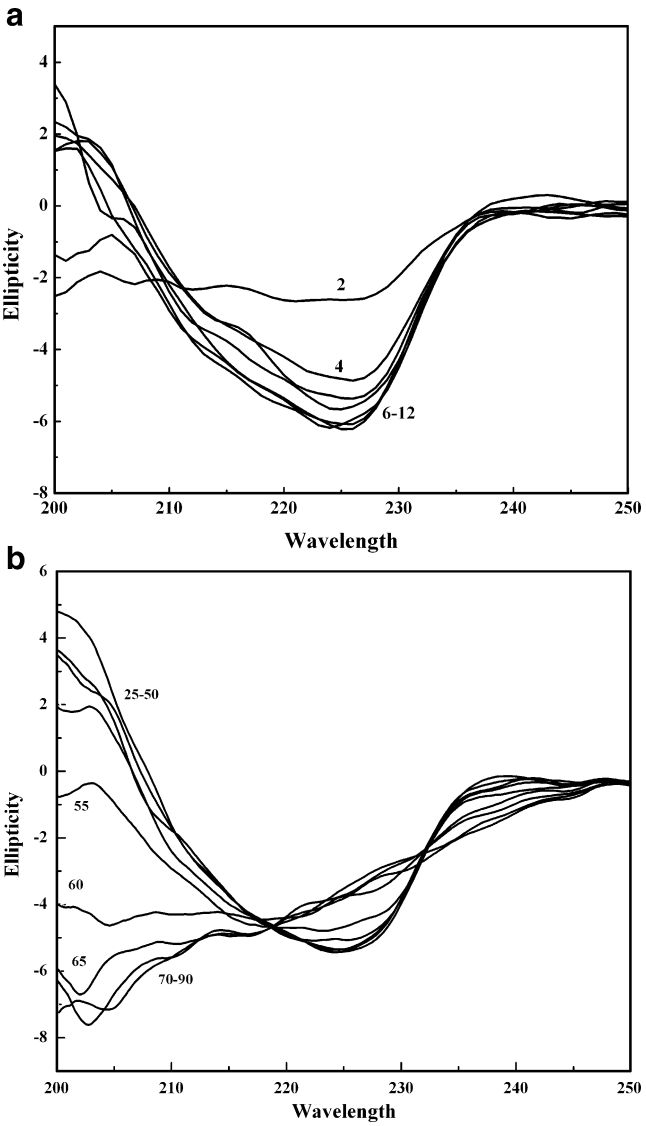


**Fig. 2** **A** Far-UV CD spectra of SGA (1.8 μM) incubated in buffers of pH from 2–12 for 16 h at 30 °C. **B** Effect of temperature on the secondary structure of SGA (1.8 μM): Far-UV CD scans of SGA at temperatures ranging from 25–90 °C. Each sample was incubated at the respective temperature for 10 min after which scans were recorded

**Table 1** The percentage quenching obtained with different quenchers in the native and 8 M urea denatured SGA and ATL

| Quencher (final concentration) | SGA quenching % (native) | SGA quenching% (denatured) | ATL quenching % (native) | ATL quenching % (denatured) |
|---|---|---|---|---|
| Acrylamide (0.25 M) | 80 (±1.5) | 80.21 (±1.3) | 83.03 (±1.9) | 80.39 (±1.8) |
| Succinimide (0.3 M) | 65.2 (±1.2) | 61.64 (±1.6) | 72.05 (±2.0) | NA |
| KI (0.4 M) | 72.49 (±2.0) | 65.18 (±1.7) | 74.74 (±1.2) | 59.66 (±1.5) |
| CsCl (0.33 M) | 29.5 (±1.1) | 34.34 (±1.1) | 15.38 (±0.9) | 15.7 (±1.0) |

Calculated from raw data

evident from the far-UV CD spectrum, this unfolded structure does not result in the exposure of any ANS accessible hydrophobic patches. Even the compact structure at alkaline pH did not exhibit fluorescence enhancement in bound hydrophobic dye. Hence this compact structure at extreme alkaline pH cannot be categorized as a molten globule like intermediate.

*Effect of temperature*

The fluorescence intensity was found to decrease with increasing temperature without any shift in $\lambda_{max}$ for both these lectins. The decrease in the fluorescence intensity could be due to deactivation of the excited singlet state. Since, the increasing temperature has no effect on the emission maximum of the protein, the polarity of tryptophan environment can be assumed to remain same. In CD spectra the negative ellipticity of the lectins at 226 nm remains same while positive ellipticity at 200–205 nm decreases gradually with increase in the temperature till 50 °C indicating increase in the random coil element of the protein. At 55 °C and above the negative ellipticity at 226 nm also decreases indicating the subsequent unfolding of the protein (Fig. 2B). Thermal denaturation and renaturation of SGA and ATL as observed by change in the ellipticity at 225 nm showed the Tm of the proteins o be 55 and 60 °C, respectively (data not shown). Hemagglutination of SGA and ATL at 55 °C did not show change [10, 11]. The partial loss of secondary structure at these temperatures is not destroying activity fully. However, at temperatures above 55 °C far-UV CD shows distortion in secondary structure as well as loss of hemaglutination activity.

*Fluorescence quenching of SGA and ATL*

Quenching experiments on native and denatured SGA and ATL were carried out using two neutral quenchers of different sizes (acrylamide and succinimide) and two ionic quenchers (iodide and cesium ion). Acrylamide being a small molecule can penetrate into protein interior and quench the fluorescence of even buried Trps, while succinimide affects intensities of mainly partially exposed tryptophans [26]. The ionic quenchers such as iodide and cesium are also larger in size and cannot penetrate protein matrix and thus can quench fluorescence of only surface exposed tryptophan residues located in the vicinity of positively and negatively charged residues, respectively.

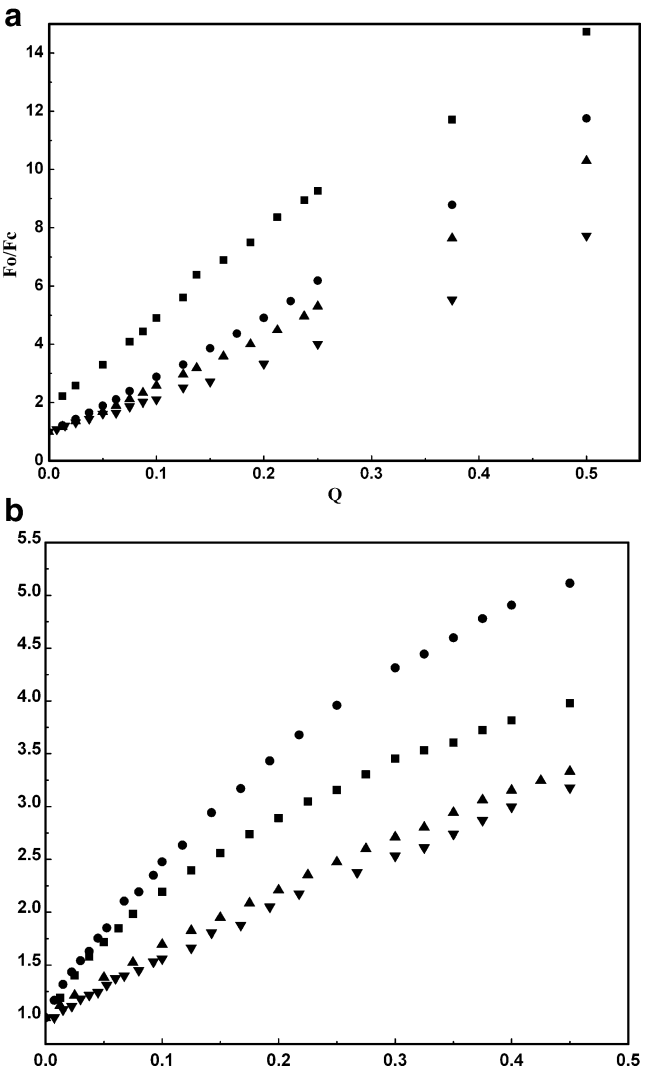


**Fig. 3** Stern volmer plot for the quenching of native and 8 M urea denatured SGA and ATL [Native SGL (*filled square*), Native ATL (*filled circle*), 8 M urea denatured SGL (*filled triangle*) ATL (*filled inverted triangle*)] **A** Acrylamide. After fitting the data the *R* value in each case was 0.99. **B** KI. The downward curves split into two linear components and the remaining data after fitting gave $R=0.99$

Table 1 shows the percentage quenching of the intrinsic fluorescence of both the proteins under native and denatured conditions with all the four quenchers. The intrinsic fluorescence of SGL and ATL lectins were quenched by all quenchers, without any change in the $\lambda_{max}$ of 350 nm. The percentage quenching in case of native SGA was 80%, 65.2%, 72.5% and 29.5% for acrylamide (0.25 M), succinimide (0.3 M), iodide (0.4 M), and cesium (0.33 M), respectively. For ATL the percentage quenching values were approximately the same except in case of succinimide and cesium ion where the values were 72.0% and 15.4%. A higher percentage of quenching obtained with succinimide is indicative of more easily accessible and non-polar tryptophans. In general, a higher percentage of fluorescence quenching was observed with the iodide ion in case of both these araceae lectins (72.5% for SGA, 74.7% for ATL) as in the case of *Moringa oleifera* lectin, 85%, [27], compared to the available values for other lectins such as *Trichosanthes anguina* lectin, 10.0%, [28], *Trichosanthes cucumerina* lectin, 11.5%, [29], *Trichosanthes dioica* lectin, 4.4%, [30], and *Doilichos biflorus* lectin, 10.6%, [31]. Higher quenching by iodide as compared to $Cs^+$ ions is an indication of predominantly electropositive environment of Trp residues.

The response to denatured lectins was negligible with most of the quenchers, presumably due to already exposed Trp residues. However, fluorescence quenching by iodide showed a decrease (from 72% to 65%) at 6 M Gdn-HCl whereas cesium quenching showed an increase (from 29% to 34%) at 8 M urea accompanied with a spectral shift of 4–5 nm. The CD analysis has already shown that the lectins get denatured at these concentrations of the denaturant. A possible explanation for the above results can be given as redistribution of charges in the vicinity of Trp residues due to the change in conformation.

*Analysis of the steady state quenching data*

Quenching data thus obtained was analyzed by the Stern–Volmer Eq. 1 as well as modified Stern–Volmer Eq. 2 [26, 32] to quantify the extent of quenching.

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

$$F_0/(F_0 - F_c) = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \quad (2)$$

Where $F_0$ and $F_c$ are the respective fluorescence intensities, corrected for dilution, in the absence and presence of quencher, respectively, [Q] is the resultant quencher concentration, $K_{sv}$ is the Stern–Volmer quenching constant of the lectin for given quencher, $f_a$ refers to the fraction of the total fluorescence that is accessible to the quencher and Ka is the corresponding quenching constant. For both the lectins, Stern–Volmer plots for quenching with the various quenchers are shown in Fig. 3. Slopes of Stern–Volmer plots yield $K_{sv}$ values (Eq. 1), whereas the slopes of modified Stern–Volmer plots give $(K_a f_a)^{-1}$ and their ordinate give values of $1/f_a$ (Eq. 2). The bimolecular quenching constants $k_{q1}$ and $k_{q2}$ for both the lectins quenched by various quenchers are presented in Table 2.

**Table 2** Summary of comparative parameters for SGA and ATL obtained from Stern–Volmer and modified Stern–Volmer analysis of the intrinsic fluorescence quenching with different quenchers

| Sample description | $K_{sv1}$ ($M^{-1}$) | $k_{q1}$ ($\times 10^9$ $M^{-1}s^{-1}$) | $K_{sv2}$ ($M^{-1}$) | $k_{q2}$ ($\times 10^9$ $M^{-1}s^{-1}$) | $f_a$ | $K_a$ ($M^{-1}$) |
|---|---|---|---|---|---|---|
| *Acrylamide* | | | | | | |
| Native SGA | 17.42 (±0.12) | 5.36 (±0.08) | – | – | 0.98 | 19.08 (±0.51) |
| Native ATL | 20.32 (±0.31) | 5.51 (±0.21) | – | – | 1 | 16.55 (±0.74) |
| Denatured SGA | 15.07 (±0.14) | 4.64 (±0.10) | – | – | 1 | 13.19 (±0.17) |
| Denatured ATL | 12.94 (±0.27) | 3.51 (±0.21) | – | – | 0.98 | 11.36 (±0.29) |
| *Succinimide* | | | | | | |
| Native SGA | 6.5 (±0.15) | 2 (±0.12) | 2.25 (±0.17) | 0.7 (±0.05) | 0.77 | 12.16 (±0.44) |
| Native ATL | 9.87 (±0.07) | 2.67 (± 0.05) | – | – | 0.79 | 15.83 (±0.44) |
| Denatured SGA | 6.27 (±0.20) | 1.93 (± 0.15) | 2.41 (± 0.16) | 0.71 (± 0.05) | 0.81 | 9.7 (±0.31) |
| *KI* | | | | | | |
| Native SGA | 11.08 (±0.61) | 3.4 (±0.57) | 4.96 (±0.19) | 1.53 | 0.81 | 21.75 (±0.4) |
| Native ATL | 15.44 (±0.51) | 4.19 (±0.41) | 10.47 (±0.18) | 2.84 | 0.79 | 28.64 (±0.54) |
| Denatured SGA | 5.14 (±0.01) | 1.58 (±0.01) | – | – | 0.85 | 12.05 (±0.27) |
| Denatured ATL | 5.29 (±0.09) | 1.59 (±0.05) | – | – | 0.83 | 4.28 (±0.47) |
| *CsCl* | | | | | | |
| Native SGA | 1.44 (±0.03) | 0.44 (±0.02) | – | – | 0.83 | 2 (±0.12) |
| Native ATL | 0.99 (±0.02) | 0.27 (±0.01) | – | – | 0.72 | 1.48 (±0.16) |
| Denatured SGA | 1.59 (±0.04) | 0.9 (±0.02) | 0.77 (±0.05) | 0.24 | 0.65 | 3.07 (±0.17) |

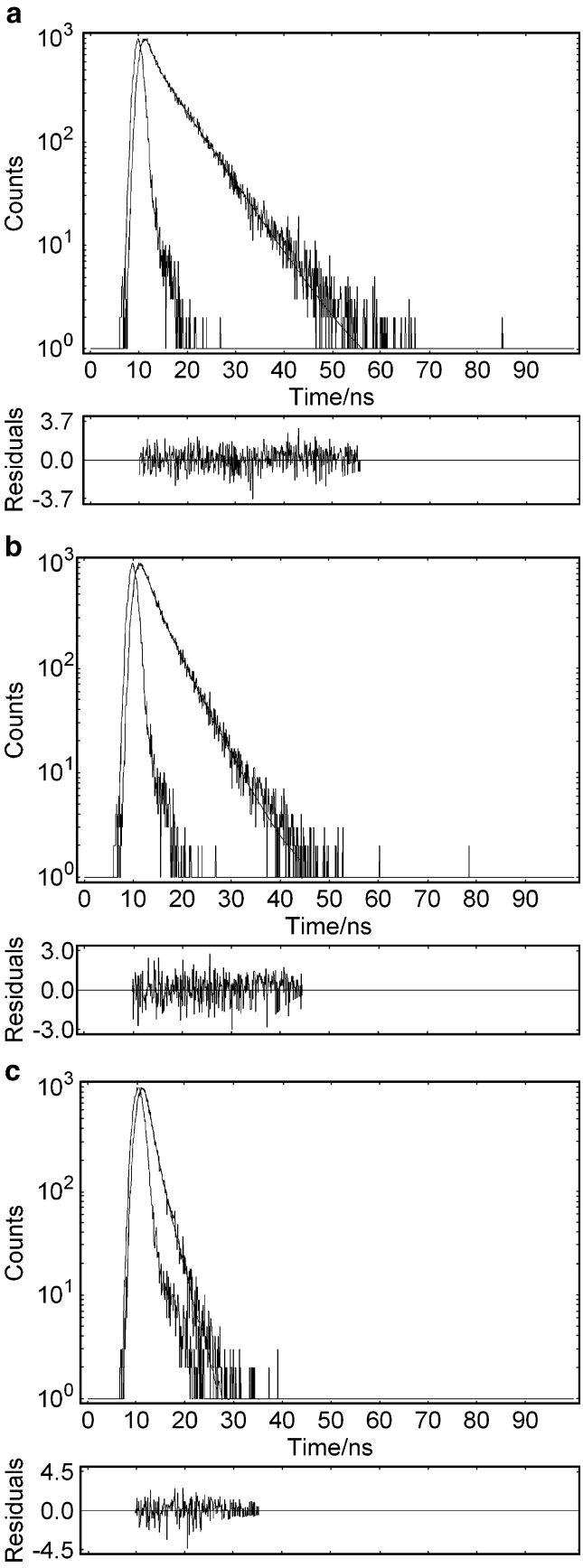


**Fig. 4** Time resolved fluorescence decay profile. **A** Native SGL ▶ **B** SGL in the presence of 6 M Gdn-HCl, **C** SGL quenched with 0.5 M Acrylamide. The *solid lines* correspond to the nonlinear least square fit of the exponential data. The *lower panel* represents the residual

*Quenching with acrylamide*

Quenching with acrylamide gave a linear Stern–Volmer plot in case of SGA and ATL, indicative of dynamic/collisional quenching of a homogenous population of tryptophans. After fitting the data the $R$ value in each case was 0.99. The values of the bimolecular quenching constant ($k_q$) were calculated by dividing $K_{sv}$ with $\tau_0$ which is the average fluorescence lifetime in the absence of the quencher. The values were calculated from time resolved fluorescence data described below. The value of this constant ($k_q$) for native lectin ($5.36\pm0.08\times10^9$ $M^{-1}$ $s^{-1}$) did not change much for denatured lectin ($4.64\pm0.10\times10^9$ $M^{-1}$ $s^{-1}$; Table 2). Similar values were obtained for ATL also.

*Quenching with succinimide*

The Stern–Volmer plot for succinimide quenching of SGA gave a downward curve, whereas it was straight-line for ATL. The downward curve is indicative of two populations of tryptophans, one readily accessible to the quencher and the other one slowly. The curve obtained was splitted into two linear components. The values of $K_{sv1}$ and $K_{sv2}$ calculated for SGA were $6.50\pm0.15$ $M^{-1}$ and $2.25\pm0.17$ $M^{-1}$. These values did not change for denatured SGA. The high value of $K_{sv1}$ compared to $K_{sv2}$ suggests a differentially exposed Trp population that is easily quenched. The values of bimolecular rate constants, $k_{q1}$ and $k_{q2}$ for native SGA were $2.0\pm0.12\times10^9$ $M^{-1}$ $s^{-1}$and $0.70\pm0.05\times10^9$ $M^{-1}$ $s^{-1}$, respectively, changed slightly after denaturation. For ATL the values of $K_{sv}$ and $k_q$ were $9.87\pm0.07$ $M^{-1}$ and $2.67\pm0.05\times10^9$ $M^{-1}$ $s^{-1}$, respectively. In case of native SGA and ATL the quenching constant $K_a$ calculated from modified SV plots was lesser for succinimide compared to acrylamide. The reason may be the lesser quenching efficiency and lesser penetrating capacity of succinimide compared to acrylamide. As expected, the $K_a$ values were lesser for denatured lectins.

*Quenching with Iodide*

For Iodide quenching, the Stern–Volmer plot curves downwards for both SGA and ATL, whereas it is a straight line for urea denatured SGA and ATL. The downward curves split into two linear components and the remaining data after fitting gave $R$=0.99. The values of $K_{sv1}$ and $K_{sv2}$ are $11.08\pm0.61$ and $4.96\pm0.19$ $M^{-1}$ for native SGA, and $15.44\pm0.51$ and $10.47\pm0.18$ $M^{-1}$ for native ATL. The

higher values of $K_{sv1}$ suggests an easily accessible and rapidly quenching population of tryptophans while a lower value is indicative of a late and slower quenching Trp population. The $K_{sv}$ for the straight line plot of denatured SGA and ATL is 5.14±0.01 and 5.29±0.09 $M^{-1}$, respectively. The estimated $K_a$ values were 21.75±0.4 and 2.0±0.12 $M^{-1}$ for SGA and 28.64±0.54 and 1.48±0.16 $M^{-1}$ for ATL, when quenched by KI and CsCl, respectively. For both the lectins the value of $k_{q1}$ for KI quenching is found to decrease on denaturing the protein. Since $k_q$ values can be associated with collisional frequency, a decrease in collisional frequency with denaturation in some cases is only nonspecific.

*Adenine binding*

Titrations of SGA and ATL were carried out with Adenine and the decrease in the fluorescence intensity was measured. Adenine-binding resulted in considerable quenching of the fluorescence intensity (80–90%) in both the lectins. The estimated binding constant $K_a$ was $6.61\pm0.2\times10^3$ $M^{-1}$ for native SGA and $1.0\pm0.11\times10^5$ $M^{-1}$ for ATL.

Adenine and adenine-derived plant growth regulators are hydrophobic ligands known to bind lectin in sites different from the carbohydrate binding site and this binding could be significant for the storage of plant growth regulators [33, 34]. The binding affinity is reported to be of the order of $10^5$–$10^6$ and hence may be of physiological importance in these plants. Both *D. biflorus* lectin [33] ($K_a=7.31\times10^5$ $M^{-1}$) and Lima bean lectin from *Phaseolus lunatus* [35] ($K_a=8.3\times10^4$ $M^{-1}$) have high affinity for adenine. Winged bean agglutinin also exhibits adenine-quenching without any shift in its $\lambda_{max}$ at 330 nm, $K_a=1.5\times10^4$ $M^{-1}$ [36]. Thus, SGA and ATL behave like legume lectins in binding adenine. As far as we know, this is the first report of any araceae lectin binding adenine.

*Fluorescent lifetimes*

The fluorescent decay of the tryptophan residues on a nanosecond time scale for both the lectins in native, denatured and quenched states, obtained from time resolved measurements are presented in Fig. 4A. Biexponential curve could be fitted to time resolved fluorescence profiles of both lectins ($\chi^2=1.03$) giving values of shorter ($\tau_1$) and longer ($\tau_2$) lifetimes for SGA (1.25±0.055 and 6.48±0.086 ns) which are similar for ATL (Table 3). The relative contributions of these components to the overall fluorescence were ~20% for shorter and ~80% for longer component. Thus majority of the Trp residues take longer time to decay. The values of individual tryptophan lifetimes and their averages for SGA are tabulated in Table 3. The corresponding values for urea denatured SGA were 1.26±0.15 and 4.26±0.17 ns. The values for ATL under the same conditions were similar. On denaturation the population components shifted to ~30% for shorter and ~70% for the longer lifetime. The observed change in values of relative amplitudes ($\alpha_1$ and $\alpha_2$) could be due to change in the protein conformation after subjecting to urea denaturation.

The decay times $\tau_1$ and $\tau_2$ for Gdn-HCl-denatured SGA were 1.52±0.15 and 3.50±0.065 ns with populations 66% and 34% (Fig. 4B), respectively. Denatured ATL also showed two lifetimes, which changed like SGA. For both these lectins a marginal increase in the shorter lifetime (1.25±0.055 to 1.52±0.15 ns for SGA) and a pronounced decrease in the longer lifetime (6.48±0.086 to 3.50±0.065 ns for SGA) were observed. The remarkable change in the percentage contributions on denaturation is suggestive of conformational changes affecting the local Trp environment.

Analysis of the decay profiles and determination of corresponding fluorescent lifetimes obtained after quenching with the neutral quenchers acrylamide (0.5 M) and succinimide (0.25 M) as well as the ionic quenchers

**Table 3** Lifetimes of fluorescent decay of SGA and ATL under different conditions and the corresponding pre-exponential factors along with the calculated average lifetimes

| Sample description | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\tau$ | $<\tau>$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| Native SGA | 0.031 | 6.48 (± 0.086) | 0.050 | 1.25 (± 0.055) | 3.25 | 5.23 | 1.003 |
| Native ATL | 0.039 | 6.36 (± 0.19) | 0.046 | 1.42 (± 0.095) | 3.69 | 5.32 | 1.003 |
| Denatured SGA (8 M Urea) | 0.039 | 1.26 (± 0.15) | 0.035 | 4.26 (± 0.17) | 2.68 | 3.65 | 1.003 |
| Denatured ATL (8 M Urea) | 0.041 | 1.85 (± 0.17) | 0.037 | 4.79 (± 0.14) | 3.24 | 3.90 | 1.001 |
| Denatured SGA (6 M GdN-HCl) | 0.045 | 1.52 (± 0.15) | 0.038 | 3.50 (± 0.065) | 2.42 | 2.82 | 1.001 |
| Denatured ATL (6 M GdN-HCl) | 0.046 | 1.27 (± 0.17) | 0.043 | 3.38 (± 0.11) | 2.23 | 2.77 | 1.004 |
| SGA +0.5 M acrylamide | 0.013 | 2.03 (± 0.057) | 0.132 | 0.71 (± 0.093) | 0.83 | 0.99 | 1.002 |
| ATL +0.5 M acrylamide | 0.134 | 0.82 (± 0.055) | 0.013 | 2.15 (± 0.25) | 0.93 | 1.09 | 1.001 |
| SGA +0.5 M succinimide | 0.133 | 0.666 (± 0.042) | 0.019 | 2.61 (± 0.144) | 0.90 | 1.36 | 1.003 |
| SGA +0.5 M KI | 0.142 | 0.757 (± 0.043) | 0.005 | 2.70(± 0.36) | 0.82 | 0.97 | 1.005 |
| SGA +0.5 M CsCl | 0.049 | 1.57 (± 0.11) | 0.027 | 4.99 (± 0.1) | 2.78 | 3.74 | 1.005 |

$I^-$ (0.5 M) and $Cs^+$(0.5 M) was also carried out (Fig. 4C). In case of acrylamide quenching for SGA it is found that the values of $\tau_1$ and $\tau_2$ were drastically reduced to 0.71±0.093 and 2.03±0.057 ns and more or less same changes have been observed for ATL also. The life of short time decay decreased from 1.25±0.055 to 0.71±0.093 ns (that is by 17.6%) whereas longer lifetime decreased from 6.48 to 2.03 ns (that is by 31.3%). Quenching with the molecule $Cs^+$ resulted in decrease in longer lifetime from 6.48±0.086 to 4.99±0.1 ns and increase in the shorter lifetime from 1.25±0.055 to 1.57±0.11 ns when compared to native SGA. Thus, the effect was not as pronounced as with neutral and anionic quenchers.

For both these lectins the average lifetimes were calculated using two different approaches ($\tau$ and $<\tau>$) from the data obtained from the fluorescent decay under various conditions and presented in Table 3, using the following Eqs. 3, 4:

$$\tau=\sum_i \alpha_i\tau_i \Big/ \sum_i \alpha_i \tag{3}$$

$$\langle\tau\rangle=\sum_i \alpha_i\tau^2 i \Big/ \sum_i \alpha_i\tau_i \tag{4}$$

Where $i$=1, 2 …

From Table 3 it can be seen that for native SGA the average lifetimes $\tau$ and $<\tau>$ are 3.25 and 5.23 ns, respectively. The average lifetimes $\tau$ and $<\tau>$ of these proteins are affected after denaturation as well as on treatment with acrylamide, succinimide and $I^-$, while they are not much affected by $Cs^+$.

In conclusion, this is the first report of structural investigations on these araceaous lectins to probe the tryptophan environment and secondary structure of the members of this monocot family. Both SGA and ATL are tetrameric lectins, containing four Trp residues that show fluorescence maxima at 350 nm corresponding to tryptophan residues exposed in the polar environment. For both these lectins the far-UV CD spectra indicate a predominant β sheet structure and type-III β-turns. Investigations into the secondary structure after various modes of denaturation reveal that these lectins possess structure that is labile at high acidic pH and stable at extreme alkaline pH. Adenine-binding, a feature of legume lectins has been observed in members of this family for the first time. This property is important for binding plant growth regulators. Complete accessibility of the tryptophans to the neutral quencher acrylamide confirmed the surface exposure of the residues. The fluorescent lifetimes were significantly altered after quenching with acrylamide. The predominantly positive environment of the tryptophans was revealed by quenching with iodide ion. Average lifetimes were not significantly affected with $Cs^+$ quenching.

## References


1. Burstein EA, Vedenkina NS, Ivkova MN (1973) Fluorescence and the location of tryptophan residues in protein molecules. Photochem Photobiol 18(4):263–279 doi:10.1111/j.1751-1097.1973.tb06422.x
2. Lakowicz EM, Weber G (1973) Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. Biochemistry 12:4171–4179 doi:10.1021/bi00745a021
3. Demchenko AP (1986) Fluorescence analysis of protein dynamics. Essays Biochem 22:120–157
4. Wright LM, Wood SD, Reynolds CD, Rizkallah PJ, Peumans WJ, Van Damme EJM et al (1996) Purification, crystallization and preliminary X-ray analysis of a mannose-binding lectin from bluebell *(Scilla campanulata)* bulbs. Acta Crystallogr D52:1021–1023
5. Van Damme EJM, Allen AK, Peumans WJ (1988) Related mannose-specific lectins from different species of the family Amaryllidaceae. Physiol Plant 73:52–57 doi:10.1111/j.1399-3054.1988.tb09192.x
6. Peumans WJ, Kellens JT, Allen AK, Van Damme EJM (1991) Isolation and characterization of a seed lectin from elderberry (*Sambucus nigra L.*) and its relationship to the bark lectins. Carbohydr Res 213:7–17 doi:10.1016/S0008-6215(00)90593-7
7. Van Damme EJM, Goossens K, Smeets K, Van Leuven F, Erhaert P, Peumans WJ (1995) The major tuber storage protein of araceae species is a lectin. Characterization and molecular cloning of the lectin from *Arum maculatum*, L. Plant Physiol 107:1147–1158 doi:10.1104/pp.107.4.1147
8. Van Damme EJM, Peumans WJ, Barre A, Rougé P (1998) Plant lectins: A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci 17:645–662 doi:10.1016/S0735-2689(98)00365-7
9. Kamboj SS, Shangary S, Singh J, Kamboj KK, Sandhu RS (1995) New lymphocyte stimulating monocot lectins from family Araceae. Immunol Invest 24(5):845–855 doi:10.3109/08820139509060711
10. Shangary S, Singh J, Kamboj SS, Kamboj KK, Sandhu RS (1995) Purification and properties of four monocot lectins from the family araceae. Phytochemistry 40:449–455 doi:10.1016/0031-9422(95)00229-Z
11. Dhuna V, Bains JS, Kamboj SS, Singh J, Shanmugavel, Saxena AJ (2005) Purification and Characterization of a Lectin from *Arisaema tortuosum* Schott Having in-vitro Anticancer Activity against Human Cancer cell lines. J Biochem Mol Biol 38(5):526–532
12. Kaur A, Kamboj SS, Singh J, Saxena AK, Dhuna V (2005) Isolation of a novel N-acetyl-D-lactosamine specific lectin from *Alocasia cucullata* (Schott.). Biotechnol Lett 27:1815–18220 doi:10.1007/s10529-005-3559-y
13. Singh J, Kamboj SS, Sandhu RS, Shangary S, Kamboj KK (1993) Purification and characterization of a tuber lectin from A*locasia indica*. Phytochemistry 33:979–983 doi:10.1016/0031-9422(93)85007-E
14. Allen AK, Neurberger A, Sharon N (1973) The purification, composition and specificity of wheat-germ agglutinin. Biochem J 131(1):155–162
15. Kamboj SS, Shangary S, Singh J, Kamboj KK, Sandhu RS (1995) New lymphocyte stimulating monocot lectins from family Araceae. Immunol. Invest. 24(5):845–855 doi:10.3109/08820139509060711
16. Shangary S, Kamboj SS, Singh J, Kamboj KK, Sandhu RS (1996) New lymphocyte stimulating monocot lectins from family Araceae. Immunol. Invest. 25(4):273–278 doi:10.3109/08820139609059310

17. Singh J, Kamboj SS (2004) A novel mitogenic and antiproliferative lectin from a wild cobra lily, *Arisaema flavum*. Biochem Biophys Res Commun 318:1057–1065 doi:10.1016/j.bbrc.2004.04.135
18. Kaur M, Rup PJ, Saxena AK, Khan RH, Ashraf MT, Kamboj SS et al (2006) A tuber lectin from *Arisaema helleborifolium* Schott with anti-insect activity against melon fruit Xy, *Bactrocera cucurbitae* (Coquillett) and anti-cancer effect on human cancer cell lines. Arch Biochem Biophys 445:156–165 doi:10.1016/j.abb.2005.10.021
19. Dhuna V, Bains JS, Kamboj SS, Singh J, Shanmugavel, Saxena AJ (2007) Purification and Characterization of a Lectin from *Arisaema tortuosum* Schott having in-vitro Anticancer Activity against Human Cancer Cell Lines. J Biochem Mol Biol 38:526–532
20. Spande TF, Witkop B (1967) Determination of the tryptophan content of proteins with *N*-bromosuccinimide. Methods Enzymol 11:498–506 doi:10.1016/S0076-6879(67)11060-4
21. Ali V, Prakash K, Kulkarni S, Ahmad A, Madhusudan KP, Bhakuni V (1999) 8-anilino-1-naphthalene sulfonic acid (ANS) induces folding of acid unfolded cytochrome c to molten globule state as a result of electrostatic interactions. Biochemistry 38 (41):13635–13642 doi:10.1021/bi9907835
22. Gasymov OK, Glasgow BJ (2007) ANS fluorescence: Potential to augment the identification of the external binding sites of proteins. Biochim Biophys Acta 1774(3):403–411
23. Watanabe K, Honjo E, Tsukamoto T, Funatsu G (1992) Fluorescence studies on the interaction of adenine with ricin A chain. FEBS Lett 304:249–251 doi:10.1016/0014-5793(92)80630-Y
24. Chipman DM, Grisaro V, Sharon N (1967) The binding of oligosaccharides containing *N*-acetylglucosamine and *N*-acetylmuramic acid to lysozyme. J Biol Chem 242:4388–4394
25. Siddiqui MZ, Sharma AK, Kumar S (1996) Solution conformation of tuftsin. Int J Biol Macromol 19(2):99–102 doi:10.1016/0141-8130(96)01108-7
26. Eftink MR, Ghiron CA (1984) Indole fluorescence quenching studies on proteins and model systems: use of the inefficient quencher succinimide. Biochemistry 23:3891–3889 doi:10.1021/bi00312a016
27. Katre UV, Suresh CG, Khan MI, Gaikwad SM (2007) Steady state and time-resolved fluorescence studies of a hemagglutinin from *Moringa oleifera*. J Fluoresc 18(2):479–485 doi:10.1007/s10895-007-0289-7
28. Komath SS, Swamy MJ (1999) Fluorescence quenching, time resolved fluorescence and chemical modification studies on the tryptophan residues of snake gourd (*Trichosanthes anguina*) seed lectin. J Photochem Photobiol B Biol 50:108–118 doi:10.1016/S1011-1344(99)00079-2
29. Kenoth R, Swamy MJ (2003) Steady-state and time-resolved fluorescence studies on *Trichosanthes cucumerina* seed lectin. J Photochem Photobiol B Biol 69:193–201
30. Sultan NAM, Swamy MJ (2005) Fluorescence quenching and time-resolved fluorescence studies on *Trichosanthes dioica* seed lectin. J Photochem Photobiol B Biol 80:93–100 doi:10.1016/j.jphotobiol.2005.03.003
31. Sultan NAM, Rao RN, Nadimpalli SK, Swamy MJ (2006) Tryptophan environment, secondary structure and thermal unfolding of the galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies. Biochim Biophys Acta 1760:1001–1008
32. Lehrer SS (1971) Solute perturbation of protein fluorescence. The quenching of tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. Biochemistry 10:3254–3263 doi:10.1021/bi00793a015
33. Etzler ME, Gupta S, Borrebaeck C (1981) Carbohydrate binding properties of the *Dolichos biflorus* lectin and its subunits. J Biol Chem 256:2367–2370
34. Gegg CV, Roberts DD, Segel IH, Etzler ME (1992) Characterization of the adenine binding sites of two *Dolichos biflorus* Lectins. Biochemistry 31:6938–6942 doi:10.1021/bi00145a011
35. Roberts DD, Goldstein IJ (1983) Adenine binding sites of the lectin from lima beans (*Phaseolus lunatus*). J Biol Chem 258:13820–13824
36. Puri KD, Surolia A (1994) Amino acid sequence of the Winged Bean (*Psophocarpus tetragonolobus*) basic lectin. Adenine binding and identification of the active-site tryptophan residue. J Biol Chem 269:30917–30926