J Fluoresc (2010) 20:305–313
DOI 10.1007/s10895-009-0556-x

ORIGINAL PAPER

Steady State and Time Resolved Fluorescence Quenching and Chemical Modification Studies of a Lectin from Endophytic Fungus *Fusarium solani*

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Received: 28 May 2009 / Accepted: 30 September 2009 / Published online: 13 October 2009

Abstract The solute quenching studies of a lectin from endophytic fungus *Fusarium solani* were carried out using different quenchers such as acrylamide, succinimide, potassium iodide and cesium chloride. The lectin showed emission maximum at 348 nm indicating relative exposure of tryptophan. The quenchable fraction of the fluorophore was 100% with acrylamide, whereas it was only 50% with succinimide. The ionic quenchers iodide and cesium showed opposite effects at different pH. In the case of cesium, raising the pH resulted in increased quenching and accessibility of typtophan residue, while the iodide showed just opposite effect. These studies showed that the single tryptophan residue of the lectin (per monomer) is relatively exposed, and might be in the vicinity of positively charged amino acid residues. Various amino acids of the *F. solani* lectin were modified using different reagents to obtain information about the hemagglutinating site. The chemical modification studies suggested tyrosine residues can be modified using N-acetylimidazole, which results in complete loss of hemagglutination activity of the lectin. Kinetics of chemical modification suggested involvement of only 2 tyrosine residues. Modification of arginine, cysteine, histidine, lysine, aspartate, glutamate and tryptophan did not result in loss of hemagglutinating activity of the lectin.

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Keywords *Fusarium* · Lectin · Quenching · Chemical modification · Time-resolved · Fluorescence

Abbreviations

DTNB	5, 5′-Dithiobis (2-nitrobenzoic acid)
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
NAI	N-acetylimidazole
NBS	*N*-Bromosuccinimide
NTEE	Nitrotyrosine ethylester
PMSF	Phenylmethylsulphonil fluoride

Introduction

Lectins are mono- or multivalent proteins or glycoproteins of non-immunogenic origin, which recognize diverse sugar structures with a high degree of stereospecificity in a non-catalytic manner [1, 2]. Mitogenicity, blood group specificity and preferential agglutination of tumor cells are some of the important biological characteristics of lectins [3]. Lectins have been reported from all types of organisms such as animals, plants, bacteria, viruses and fungi. Extensive studies have been carried out on plant and animal lectins [4, 5], whereas very little information is available on lectins from fungal sources [6, 7]. However, over the past decade mushroom lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities [8–10]. More recently, there have been several reports of lectins from lower and pathogenic fungi but their physiological role still remains uncertain [11–15].

Carbohydrate binding property of the lectins renders their use as biomarker in cancer research [16]; and harvesting their intrinsic fluorescence property or tagging with

external fluorophore may serve the purpose. However, in either case a detailed characterization of their fluorescence property would be highly desirable. The intrinsic fluorescence of the protein is conferred by aromatic residues such as tryptophan, tyrosine and phenylalanine [17]. Fluorescence of tryptophan is influenced by its microenvironment, hence changes which affect the tryptophan environment can cause changes in fluorescence properties of the proteins [18] and such changes in the intrinsic fluorescence of proteins have also been used to obtain information regarding structure, specificity and conformation of the proteins [19–21]. The accessibility of tryptophan in a protein molecule can be measured by use of quenchers perturbing fluorescence. Solute quenching of tryptophan fluorescence provides information on the microenvironment of these residues.

Fusarium is a well known plant pathogen and has been found to be involved in loss of several economic crops. A 26 kDa dimeric lectin from endophytic fungus *Fusarium solani* was purified and characterized earlier, which showed specificity for N-linked (asialo-triantennary and asialo-biantennary) as well as O-linked glycotopes [22, 23]. The denaturation studies of the lectin revealed that it shows flexibility in tertiary structure with retention of hemagglutinating activity at acidic pH [24]. However, the effect of pH on microenvironment of tryptophan remained unexplored. Hence, we carried out fluorescent quenching studies of the lectin at various pH to elicit effect of pH on the accessibility of various quenchers inside the protein molecule. Besides this, chemical modifications of various amino acid residues and its effect on hemagglutinating activity of the lectin are also presented here.

Experimental

Material

Acrylamide, CsCl, KI and succinimide, N-Acetylimidazole, 2,4,6-trinitrobenzenesulphonic acid, diethylpyrocarbonate, phenylmethyl-sulphonilfluoride, N-bromosuccinimide, phenylglyoxal, 5,5′-dithiobis-2-nitrobenzoic acid and sodium borohydride from Sigma Chemical Co. St. Louis, USA were used. Triantennary-N-glycans were prepared from fetuin [25, 26]. All other reagents were of analytical grade.

Hemagglutination assays

Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 μl aliquot of the erythrocytes suspension was mixed with 50 μl of serially diluted lectin and agglutination was examined visually after incubation for one hour. A unit of hemagglutination activity (U) is expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units/mg of the protein.

Protein concentration determination

Protein concentrations were determined using bovine serum albumin (BSA) as standard [27].

Purification of F. solani lectin

Cultivation of *F. solani* and purification of the lectin was carried out, and the purity of the preparation was confirmed as described earlier [22].

Fluorescence measurements and solute quenching

Fluorescence measurements were carried out using a Perkin Elmer LS-50B spectrofluorimeter, with slit width of 7 nm for both the monochromators and scan speed 100 nm/min. Titration of *F. solani* lectin (2 μM) with acrylamide, CsCl, KI and succinimide were performed in the absence and presence of 100 μM asialo-triantennary N-glycan. The iodide solution contained sodium thiosulfate (200 μM) to suppress tri-iodate formation. Defined amounts of the quencher (5–10 μl) were added from a stock of 5 M to 2 ml (2 μM) lectin solution. The titrations were carried out at pH 4 (20 mM acetate buffer), pH 6 (20 mM phosphate buffer) and pH 10 (20 mM glycine-NaOH buffer), 27 °C. The fluorescence spectra were recorded after 3 min and each spectrum was an average of 3 accumulations. The excitation wavelength was 295 nm, slit width was 7 nm, the emission spectra were recorded in the range of wavelength 300 to 400 nm with slit width of 7 nm and scan speed 100 nm/min. Quenching data for all the quenchers used in this study were analyzed by the Stern-Volmer Eq. 1 as well as by the modified Stern-Volmer Eq. 2 [28].

$$F_0/F_c = 1 + K_{sv}[Q] \tag{1}$$

$$F_0/\Delta F = f_a^{-1} + \left(K_q f_a\right)^{-1}[Q]^{-1} \tag{2}$$

Where F_0 and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and presence of quencher, [Q] is the quencher concentration, K_{sv} is the Stern-Volmer quenching constant of the lectin for a given quencher, f_a refers to the fraction of the total fluorescence that is accessible to the quencher and K_q is the corresponding quenching constant. Slopes of Stern-Volmer

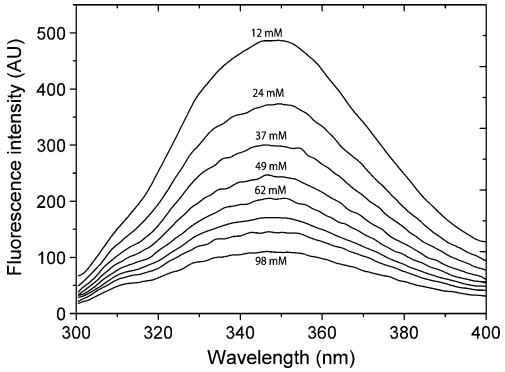

Fig. 1 Fluorescence emission spectra of *F. solani* lectin with increasing concentrations of acrylamide as indicated on the top of the spectrum

plots yield K_{sv} values, whereas the slopes of modified Stern-Volmer plots give $(K_q f_a)^{-1}$ and their ordinate give values of f_a.

Time-resolved fluorescence measurement

Fluorescence lifetime measurements were performed using FLS920 single photon counting spectrofluorimeter from Edinburgh Instruments, Livingston, United Kingdom. A nanosecond flash lamp (nF900) was used for excitation. Samples were excited at 295 nm and emission intensities were recorded at λ_{max} (348 nm) of the lectin. Slit widths of 15 nm were used on both the excitation and emission monochromators. The resultant decay curves were analyzed by a multiexponential iterative fitting program supplied by Edinburgh Instruments.

The dynamic portion of the quenching was determined by lifetime measurements, according to the equation [17],

$$\tau_0/\tau = 1 + K_{sv2}[Q] \quad (3)$$

where K_{sv2} is dynamic quenching constant, τ_0 is average lifetime in the absence of a quencher.

The average lifetimes τ and τ_0 were calculated by following expression [29, 30]:

$$\tau = \sum_i \alpha_i \tau_i \Big/ \sum_i \alpha_i \quad (4)$$

where τ_i are the values of different lifetimes and α_i are the corresponding weighting factors.

Chemical modification studies

The modification of serine was carried out using phenylmethylsulphonil fluoride (PMSF) in 20 mM Tris-HCl buffer, pH 8.0 [31]. The cystein residues were modified by using 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 20 mM phosphate buffer pH 8.0 [32]. Arginine residues were modified with phenylglyoxal [33]. Tryptophan residues were modified using *N*-bromosuccinimide (NBS) in 100 mM sodium acetate buffer (pH 5.5) [34]. Carboxylate residues were modified by Woodward's Reagent K (WRK in 50 mM phosphate buffer, pH 7.0) [35] as well as by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ Nitrotyrosine ethylester (NTEE) [36] in MES/HEPES buffer (pH 6.0). The amino groups were modified by acetylation, succinilation and reductive methylation [37]. Tyrosine modification was performed with N-acetylimidazole (NAI) [38]. The tyrosine residues modified were determined spectrophotometrically, using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm. Histidine residues were modified by diethylpyrocarbonate (DEP) [39]. For all the reactions a lectin sample incubated at the same pH and temperature in the absence of modifying reagent served as control, and each experiment was repeated thrice. The hemagglutinating activity of the control sample was considered to be 100%. The excess reagent was removed by either dialysis or gel filtration on Sephadex G-25 column from the reaction mixture, and appropriate corrections were made for dilution. The reactivation of NAI modified lectin was done by hydroxylamine [40]. Ligand protection was carried out by pre-incubating the *F. solani* lectin with triantennary N-glycan (400 fold molar excess) followed by treatment with NAI. Both modified and unmodified protein samples were

Table 1 Summary of parameters obtained from the intrinsic fluorescence quenching of *F. solani* lectin with different quenchers

Quencher and condition	K_{sv} M^{-1}	K_q M^{-1}	f_a
Acrylamide			
pH 4.0	25.5	17.90	1
pH 6.0	24.8	19.43	1
pH 10.0	24.3	18.13	1
With ligand	35.9	20.06	1
Succinimide			
pH 4.0	4.29	14.77	0.49
pH 6.0	4.75	12.06	0.57
pH 10.0	3.50	11.66	0.43
With ligand	4.34	11.75	0.41
KI			
pH 4.0	3.66	6.07	0.67
pH 6.0	2.97	5.73	0.61
pH 10.0	2.08	4.28	0.56
With ligand	2.32	4.74	0.56
CsCl			
pH 4.0	0.60	4.13	0.19
pH 6.0	0.61	4.92	0.19
pH 10.0	0.67	5.63	0.20
With ligand	0.56	4.75	0.19

dialyzed in 20 mM phosphate buffer at pH 7.0, then the residual activity was determined.

Circular Dichroism (CD) measurements

Circular Dichroism (CD) spectra of the native and chemically modified lectin samples were recorded on a JASCO-715 spectropolarimeter, at 25 °C, in the range of 190–260 nm at a scan speed of 200 nm/min with a response time of 1 s and slit width of 1 nm. A cylindrical quartz cell of 1 mm path length was used. All measurements were made at a lectin concentration of 0.4 mg/ml. For each spectrum, fifteen successive scans were collected and the averaged spectra were used for further analysis.

Results and discussion

Steady state solute quenching

The intrinsic fluorescence emissions of aromatic amino acids in proteins are highly affected by surrounding microenvironment and are therefore very often employed to investigate structural changes in proteins [17]. Especifically the tryptophan microenvironment and its exposure have been investigated by fluorescence quenching, using small molecules such as acrylamide, succinimide, iodide, cesium and oxygen termed as quenchers [41, 42].

The *F. solani* lectin showed λ_{max} at 348 nm upon excitation at 295 nm, which indicates relative exposure of tryptophan (Fig. 1). Titration of the *F. solani* lectin was carried out with acrylamide, succinimide, KI and CsCl, which resulted in 100%, 57%, 61% and 19% quenchable fraction of fluorophore, respectively (Fig. 1 and Table 1). The *F. solani* lectin was found to have a single, relatively exposed, tryptophan (per monomer), which showed λ_{max} of emission at 348 nm. For a single tryptophan monophasic Stern-Volmer plot can be expected, and indeed it was found in the case of *F. solani* lectin, as no downward curvature was observed for any of the quenchers studied (Fig. 2). For acrylamide, there was no considerable difference in the K_q and f_a at different pH and in the presence of the ligand (Fig. 3 and Table 1). The total accessible fluorophore fraction was near unity. Succinimide, being bulkier, showed 50% quenching of the total

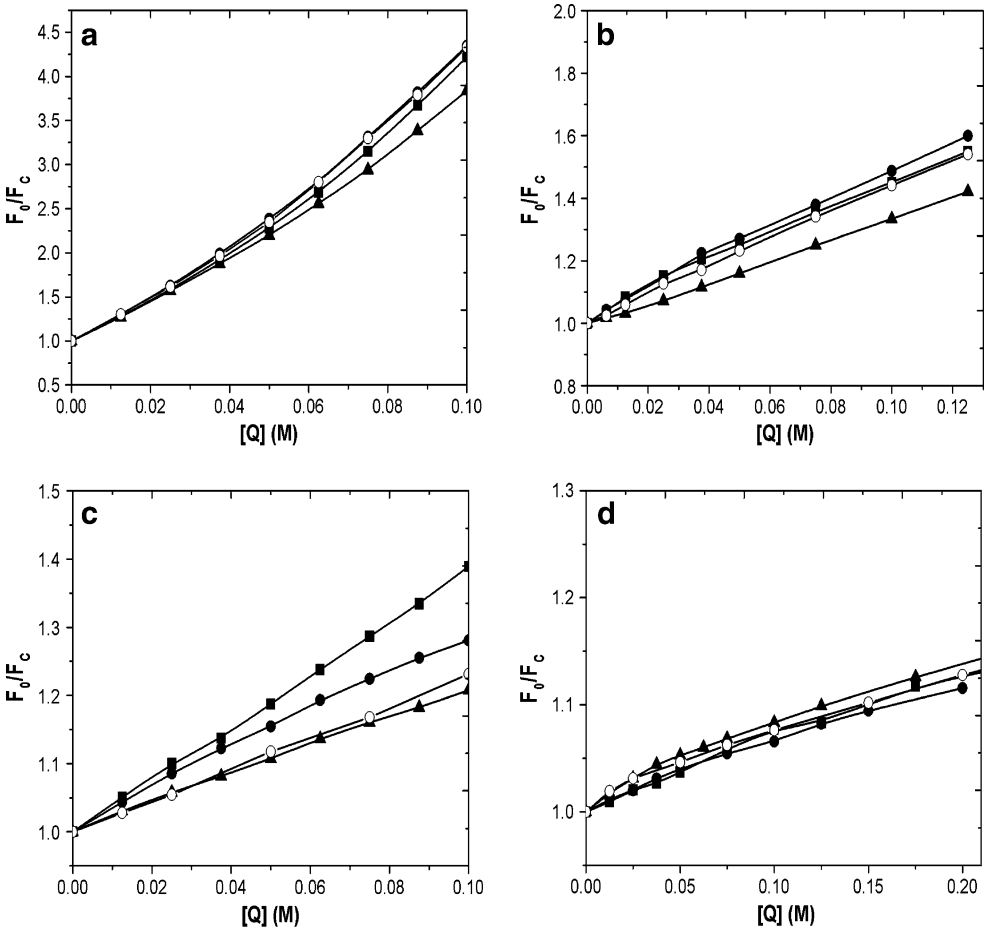

Fig. 2 Stern-Volmer plots of fluorescence quenching for *F. solani* lectin. The different quenchers used are **a** acrylamide **b** succinimide **c** iodide ion and **d** cesium ion. Lectin at (■) pH 4.0, (●) pH 6.0, (▲) pH 10.0 and (○) in the presence of ligand

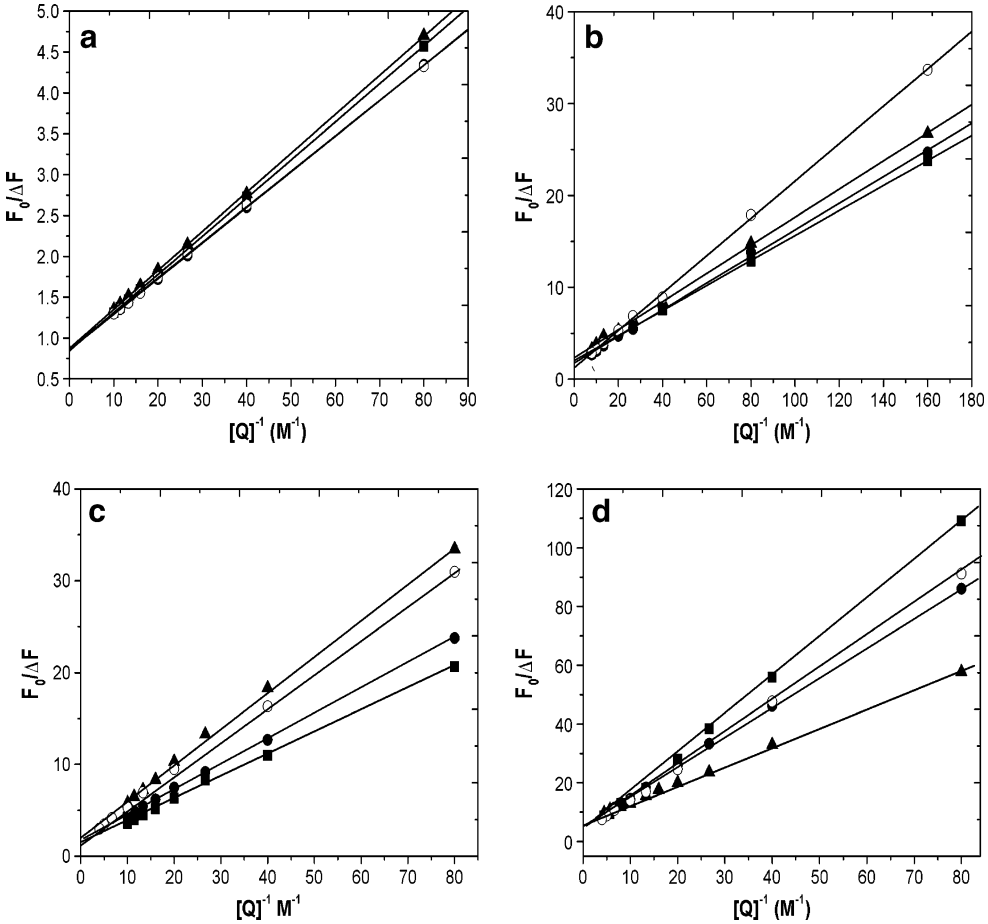

Fig. 3 Modified Stern-Volmer plots of fluorescence quenching for *F. solani* lectin. The different quenchers used are **a** acrylamide **b** succinimide **c** iodide ion and **d** cesium ion. Lectin at (■) pH 4.0, (●) pH 6.0, (▲) pH 10.0 and (○) in the presence of ligand

available fluorescence. Acrylamide and succinimide both are polar but neutral quenchers so the presence of local charges in the vicinity of tryptophan microenvironment does not affect their quenching ability. The K_{sv} for succinimide (4.75 M^{-1}) as compared to acrylamide (24.8 M^{-1}) at pH 6.0 reflected both the inefficiency of quenching by the former as well as its restricted accessibility to tryptophan (Table 1).

Succinimide has a larger molecular radius than acrylamide, and is sterically much more rigid. Hence, its approach to buried tryptophan could be much more hindered and therefore, it is a less efficient quencher than acrylamide [43]. The direct Stern-Volmer plot of acrylamide quenching did not show considerable changes at different pH and in the presence of ligand, but showed a clear upward curvature, indicating a significant contribution from static quenching in the total quenching (Fig. 2).

The ionic quenchers, I^- and Cs^+ being charged, cannot penetrate into the protein interior, unlike the neutral quenchers and thus do not probe buried tryptophan, so they were found less efficient than acrylamide. Ionic quenchers are useful to explore the microenvironment of relatively exposed tryptophan. Their accessibility and quenching efficiency depend on the local charges of the tryptophan microenvironment [28, 44]. Quenching of *F. solani* lectin with Cs^+ and I^- was carried out at pH 4, 6 and 10. Considerable differences in the quenching and the accessibility was observed with Cs^+ and I^- at different pH (Fig. 2 and 3). The K_q value for I^- (5.73 M^{-1}) and Cs^+ (4.92 M^{-1}), indicate iodide to be more efficient quencher than cesium (Table 1). Cs^+ and I^- exhibited 19% and 61% quenching, respectively. In the case of Cs^+ quenching, raising the pH resulted in increased quenching and accessibility (K_q=4.13 M^{-1} at pH 4 and 5.63 M^{-1} at pH 10). Iodide a negatively charged quencher resulted in opposite effects than Cs^+ as K_q decreased with increase in pH (K_q=6.07 M^{-1} at pH 4 and to 4.28 M^{-1} at pH 10). Accessibility and quenching efficiency of Cs^+ and I^- with effect of pH suggest that the microenvironment of tryptophan is probably positively charged.

The presence of the ligand in the quenching with succinimide, Cs^+ and I^- resulted in a slight decrease in accessibility, indicating that ligand binding might result in slightly decreased penetration of the quencher into the protein matrix.

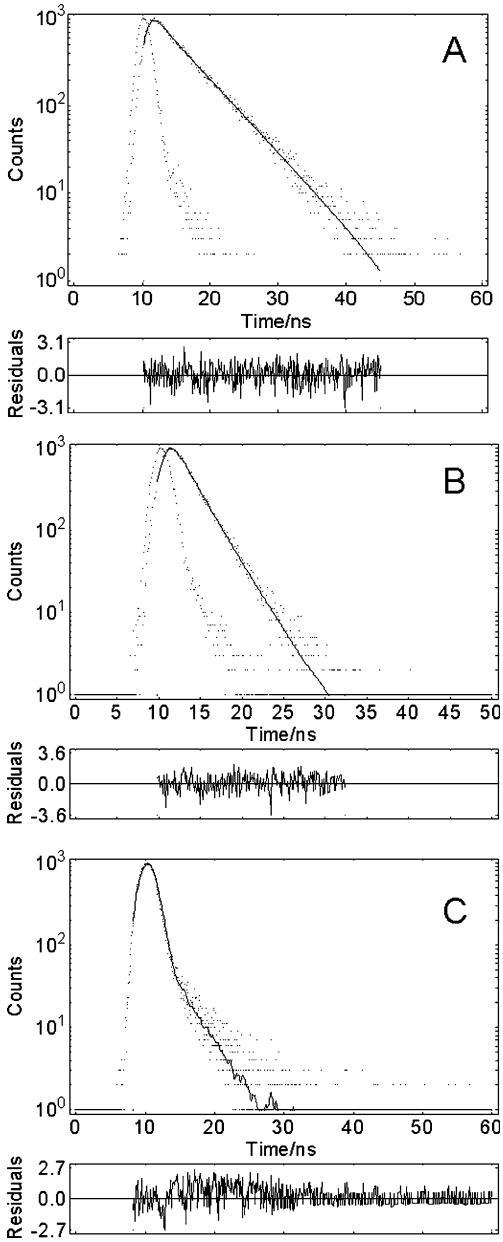

Fig. 4 Time resolved fluorescence decay profile of *F. solani* lectin. **a** Native lectin, **b** in the presence of acrylamide and (**c**) tryptophan modified lectin. The solid lines correspond to the nonlinear least square fit of the experimental data to a biexponential function. The lower panel represents the residual plot of the fit

Time resolved fluorescence quenching

The steady state quenching profile obtained with acrylamide showed positive curvature (Fig. 2), indicating that quenching has both dynamic and static components. The dynamic portion of quenching was determined by fluorescence lifetime measurements using Eq. 3. The K_{sv2} (dynamic quenching constant) value for acrylamide quenching was obtained as 8.69 M^{-1}. The fluorescence decay curves of native *F. solani* lectin and in the presence of acrylamide is given in Fig. 4. The decay curves were analyzed by a multi-exponential iterative fitting program provided with the FLS920 spectrofluorimeter. In both the cases, the decay profiles could be best fitted to a biexponential function (χ^2<1.14). Monoexponential fits gave comparatively larger errors (χ^2>1.5). Our earlier studies showed the presence of a single tryptophan (per monomer) in *F. solani* lectin [22], which should result in a monoexponential decay profile. However, single tryptophan proteins are also known to show biexponential decay profiles [45], presumably due to different rotamers of tryptophan, which are quenched to different extents by peptide backbones and amino acid side chains in the microenvironment surrounding the indole ring [46]. The native lectin exhibited two lifetimes: one longer lifetime of 5.24 ns and one shorter lifetime of 1.78 ns. An analysis of the lifetime decay profiles also provides an estimation of relative contributions of all the components in the fluorescence intensity. For native *F. solani* lectin, the component with longer lifetime has a higher contribution (89%), whereas the shorter lifetime has a relatively lower contribution (11%). In the presence of quencher acrylamide, the longer lifetime showed a significant decrease (2.61 ns), while the shorter lifetime is less affected (1.01 ns) (Table 2). The change in lifetime of both components also intended their relative contribution, as the contribution of the longer lifetime component decreased to 61% and the contribution of the shorter lifetime increased to 39%. The average lifetime (τ) was also decreased from 4.32 ns to 1.80 ns indicating significant loss of quantum yield in the presence of acrylamide.

Table 2 The lifetimes of fluorescence decay of *F. solani* lectin and the corresponding pre-exponential factors along with calculated average lifetimes for acrylamide quenching

[Q] (M^{-1})	α_1	τ_1 ns	Rel %	α_2	τ_2 ns	Rel %	Average lifetime (τ) ns	χ^2
0.00	0.047	5.24	88.97	0.017	1.78	11.03	4.32	1.144
0.03	0.052	3.99	88.24	0.016	1.69	11.76	3.44	0.980
0.07	0.054	3.32	87.94	0.022	1.11	12.06	2.68	1.10
0.10	0.055	2.91	83.30	0.021	1.05	16.7	2.39	0.983
0.13	0.039	2.80	60.79	0.038	1.04	39.27	1.93	0.989
0.17	0.041	2.61	60.86	0.042	1.01	39.14	1.80	1.020

Table 3 Effect of different modifying reagents on the hemagglutinating activity of the lectin from *F. solani*

Modification reaction	No of residues modified per molecule	Residual activity (%)
Control (Native lectin)	0	100
Tyrosine (Lectin + NAI)	8	10
Ligand protection (Lectin+ Triantennary N-glycan + NAI)	5.8	25
Reactivation (NAI modified lectin + Hydroxilamine)	0	100

Chemical modification

Arginine, cysteine, histidine, lysine, aspartate, glutamate and tryptophan were not found to be involved in hemagglutinating activity of the lectin (Table 3). Purified lectin when incubated with 10 mM NAI lost 90% of its initial activity and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. Based on a molar absorption coefficient of 1160 M^{-1} cm^{-1} for tyrosine at 278 nm [38] and the molecular mass of 26 kDa for *F. solani* lectin, the total number of tyrosine residues modified were found to be 8.0. However, the plot of percentage residual activity against the number of tyrosine residues modified revealed that the loss of hemagglutination activity occurred due to the modification of 2.3 residues suggesting the probable involvement of 2 residues/mol (1 residue /monomer) in the hemagglutination activity of *F. solani* lectin (Fig. 5).

Treatment of tyrosine modified lectin with 0.15 M hydroxylamine for 6 h (which reverses the modification) led to a complete recovery of the hemagglutinating activity of the lectin, indicating that the phenolic group of the tyrosine is primarily involved in the activity of the lectin, and the loss of the hemagglutinating activity is due to the O-acetylation of Tyr residues. NAI mediated inactivation was reduced by 25% on incubation of lectin with excess of triantennary N-glycans (400 fold molar excess) prior to modification (Table 3). Moreover, the CD spectra of both native and modified lectin were almost identical showing that the loss of activity is due to the modification of tyrosine residues rather than structural changes (Fig. 6). Tyrosine has been implicated in the sugar-binding activities of a number of lectins as from *Artocarpus hirsuta* [47], *Erythrina indica* [48] and *Trichosanthes dioica* [49].

Chemical modification of tryptophan with N-bromos uccinimide opens the indole ring, which results in loss of fluorescence property of the protein. Modification of tryptophan in *F. solani* lectin resulted in tremendous decrease in the lifetime of tryptophan (0.19 ns) and total counts (Fig. 4c). This observation was also in agreement with our findings that the single tryptophan residue of the lectin can be modified and is accessible to reagents under non-denaturing conditions.

In conclusion, the fluorescence studies carried out showed that the tryptophan residue of *F. solani* lectin is relatively exposed and accessible to neutral as well as ionic quenchers and it seems to be present in the vicinity of positively charged microenvironment. Modification of

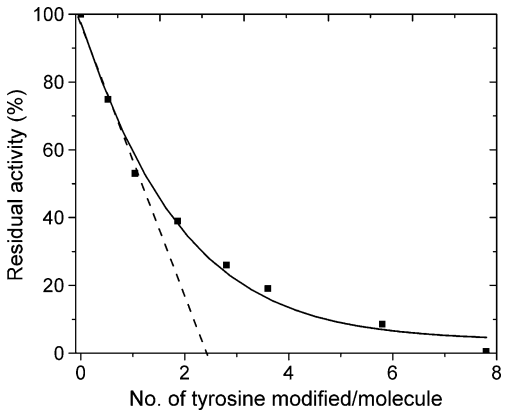

Fig. 5 Plot of percent residual activity versus number of tyrosine residues modified: The number of tyrosine residues modified were estimated as described in Methods

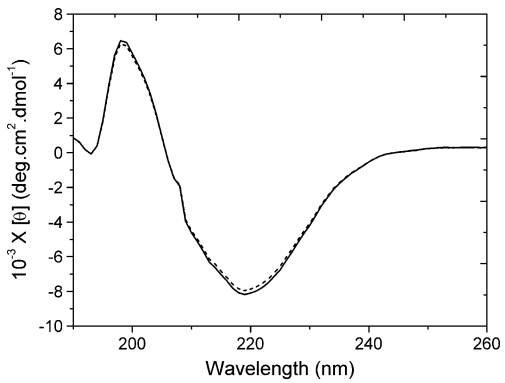

Fig. 6 The CD spectra of native and NAI modified lectin: CD measurements were performed in a 1 mm cell at a lectin concentration 19 μM. Native lectin (—), NAI treated lectin (—)

tyrosine results in the loss of hemagglutinating property of the lectin.

Acknowledgements The work was supported by a grant from Department of Biotechnology, India to MIK. F.K. was supported by research fellowship by the Council of Scientific and Industrial Research, India.

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