A fluorescence spectroscopic study of a coagulating protein extracted from *Moringa oleifera* seeds

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Abstract

The fluorescence studies of coagulating protein extracted from *Moringa oleifera* seeds have been studied using steady-state intrinsic fluorescence. The fluorescence spectra are dominated by tryptophan emission and the emission peak maximum (λ_{max} = 343 ± 2 nm) indicated that the tryptophan residue is not located in the hydrophobic core of the protein. Changes in solution pH affected the protein conformation as indicated by changes in the tryptophan fluorescence above pH 9 whereas the ionic strength had minimal effect. The exposure and environments of the tryptophan residue were determined using collisional quenchers.

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1. Introduction

Moringa oleifera is a multipurpose tropical tree with most of its parts being useful for medicinal and commercial applications in addition to its nutritional value [1–7]. In the case of water treatment, coagulants/flocculants have to be applied to transform water constituents into forms, through the aggregation/flocculation of particles which are present in water, which can be separated physically [4,8]. The seed kernels contain significant quantities of a series of low molecular-weight, water-soluble proteins. The crushed seed powder, when mixed with water, yields water-soluble proteins that possess a net positive charge. The solution acts as a natural cationic polyelectrolyte during treatment [9]. The water-soluble proteins have been proposed to bind to predominantly negatively charged particles (silt, clay, bacteria, etc. suspended in a colloidal form), that make raw waters turbid [8].

Numerous laboratory studies have so far shown that *M. oleifera* seeds possess effective coagulation properties [2,10–14]. However, different nature and the properties of the coagulant component seem to be reported, therefore leading to a belief that a number of coagulant components are present in *Moringa* seeds. Several reports have described the main water-extractable component as proteinaceous. It is described as dimeric cationic proteins with molecular mass of 12–14 kilodaltons (kDa) and isoelectric point between 10 and 11 [11] as a cationic protein with isoelectric point greater than 9.6 and molecular mass less than 6.5 kDa [14]. Others reported a molecular mass of 6.5 kDa and isoelectric point greater than 10 [10]. In addition, a non-proteic active component was purified from *Moringa* seeds. Okuda et al. [13] reported that the active component from an aqueous salt extraction was not a protein, polysaccharide or lipid, but an organic polyelectrolyte with molecular weight of about 3.0 kDa.

Ndabigengesere et al. [11] purified the coagulant proteins from *M. oleifera* seeds. They observed that under non-reducing conditions, the active agents of coagulation are dimeric cationic proteins of molecular weight of approximately 13 kDa having an isoelectric point between 10 and 11, but under reducing conditions, the molecular weight was found to be 6.5 kDa. They suggested that the active protein is actually composed of two 6.5 kDa subunits, connected with an S–S bond that is cleaved when protein extraction occurs in reducing conditions, and that the mechanism of coagulation with *M. oleifera* consist of adsorption and charge neutralization of the colloidal charges. Ndabigengesere and Narasiah [2] examined various parameters of the quality of the water treated by coagulation using *M. oleifera* seeds. They suggested that *M. oleifera* seeds be used

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as a coagulant in water and wastewater treatment only after an adequate purification of the active protein.

Fluorescence of proteins originates almost entirely from tyrosyl and tryptophanyl residues. Fluorescence spectroscopy of amino acid residues is a very sensitive probe in detecting protein molecular conformational changes. In our previous work, fluorescence were used to monitor (i) the structure changes of the coagulant protein when interacting with anionic sodium dodecyl sulphate (SDS) surfactant and (ii) the changes in the accessibility of the hydrophobic regions of the molecules through the binding of the fluorescence hydrophobic probe anilinonaphthalene sulphonic acid (ANS) [15]. In this work, we report the effect of ionic strength, pH and type of quencher on the intrinsic fluorescence of the coagulant protein. The processing protocol of dry *M. oleifera* seeds by Ndabigengesere and Narasiah was used for extraction and purification of the coagulant protein [2].

2. Experimental

Measurements of steady-state fluorescence for protein solutions were performed using Shimadzu RF1501 spectrofluorophotometer with fast scan speed and low sensitivity. The intrinsic fluorescence of the proteins arises from the aromatic amino acid residues such as tryptophan, tyrosine and phenylalanine. Emission spectra were recorded from 300 to 450 nm. The monochromator slit width was kept at 10 nm in excitation and emission measurements.

The emission wavelength of the coagulant protein extracted from *M. oleifera* seeds was not known. The peak excitation wavelength was determined between 260 and 297 nm. The protein concentration in water was 0.1 mg/mL. Wavelength of 280 nm was the excitation wavelength giving maximum fluorescence emission signal. The emission spectrum of the protein was recorded from 300 to 450 nm with excitation wavelength of 280 nm. The emission maximum was observed at 343 ± 2 nm. Excitation wavelength of 295 nm and emission wavelength of 343 nm were used for subsequent analysis of the coagulant protein. The fluorescence excitation wavelength of 295 nm was used to eliminate contribution from amino acids other than tryptophan [16–20]. The fluorescence measurements of the protein samples were carried out with an optical density of less than 0.1 at 295 nm to avoid the inner filter effects.

Linearity of fluorescence intensity was tested for possible inner filter effects. Fluorescence intensity is typically directly proportional to sample concentration. To test for linearity, a fluorescence intensity reading for a high concentration of the protein sample in water (1 mg/mL) was taken. The solution was then diluted by a factor of half. The readings were obtained for the dilute sample. Sequential twofold dilutions were done to cover the concentration range studied. Fluorescence intensity was monitored at the wavelength of emission maximum.

Ionic strength, pH and presence of surfactants can affect protein conformational stability [16,19]. The influence of ionic strength on protein fluorescence in water was investigated by addition of varying amounts of 1.0 M NaCl to the protein solution. In order to study the effect of pH on the protein fluorescence, fluorescence intensity of the samples were measured. The fluorescence spectra of samples were also recorded. Solution pH was varied between 4 and 12 by addition of 0.1 M NaOH or 0.1 M HCl. The pH was measured using a Hanna Instruments model 209 pH meter.

The solvent accessibility of the tryptophan residues of the protein was studied by tryptophan fluorescence quenching experiments carried out for protein samples in water. All the protein concentration for all solutions used was kept at 0.08 mg/mL. Quenching stock solutions used were 1 M acrylamide, 0.45 M KI, 0.45 M CsCl and 0.45 M $NaNO_3$. The ionic strengths of solutions quenched by KI, CsCl and $NaNO_3$ were diluted to 0.045 M by addition of 0.2 M NaCl. The KI solution contained 0.1 mM sodium thiosulphate to prevent formation of triiodide (I_3^-). This was necessary because I_3^- also absorbs in the region of tryptophan fluorescence [17,19,20]. The quenching process of the tryptophan fluorescence was described by Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in absence and presence of quencher Q, respectively, $[Q]$ the concentration of the quencher and K_{SV} is the Stern–Volmer quenching constant. This equation describes the well-known concept that fluorophores with larger lifetimes are quenched more than those with shorter lifetimes [21]. If the relative intensity F_0/F increases linearly with $[Q]$, the Stern–Volmer constant K_{SV} can be obtained from the (initial) slope of the linear Stern–Volmer plot [21]. Non-linear Stern–Volmer plot can also be observed in a multi-tryptophan protein with different tryptophan residues (buried and exposed). The fraction of total fluorophore accessible to the quencher can be calculated from the modified Stern–Volmer plot:

$$\frac{F_0}{\Delta F} = \frac{1}{K_Q f_a[Q]} + \frac{1}{f_a} \quad (2)$$

where ΔF is the change in the fluorescence intensity due to quenching, F_0 and $[Q]$ have the same meaning as in Eq. (1), K_Q the Stern–Volmer quenching constant of the exposed tryptophan residues and f_a is the fraction of the initial fluorescence, which is accessible to the quencher.

3. Results and discussion

3.1. Determining emission wavelength

The fluorescence of proteins originates from tryptophan, tyrosine and phenylalanine residues [16,17]. In aqueous media, the emission peaks of phenylalanine, tyrosine and tryptophan occur at 280, 305 and 348 nm, respectively. Phenylalanine is not a significant contributor to the fluorescence spectra of most proteins, and in undenatured proteins, tyrosine emission is not usually seen. The emission of proteins is dominated by tryptophan which absorbs at the longest wavelength [17,23].

Excitation of the coagulant protein at given wavelengths resulted in emission spectra with a maximum at 343 ± 2 nm as shown in Fig. 1. This is typical of most native (folded glob-

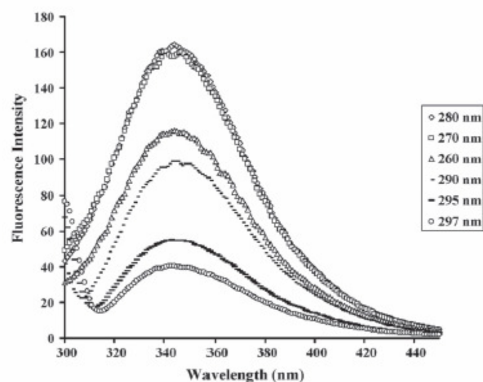

Fig. 1. Steady-state fluorescence emission spectra of coagulant protein from *Moringa* seeds in water at different excitation wavelengths. The protein concentration was 0.1 mg/mL.

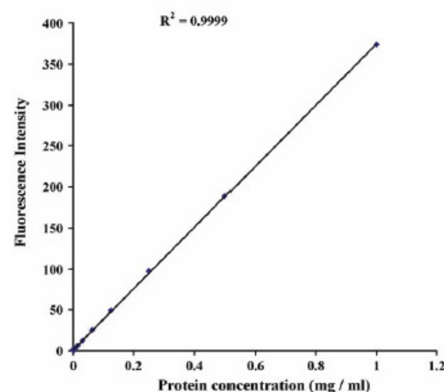

Fig. 2. Coagulant protein concentration curve. The fluorescence was determined with excitation wavelength of 295 nm and emission wavelength of 343 nm.

ular) proteins which have emission maxima in the range of 331–343 nm [24,25]. Fig. 1 also demonstrates that the position of the spectrum was independent of the excitation wavelength, suggesting the presence of one fluorescing protein type in solution. However, fluorescence intensity decreased considerably at wavelengths longer than 280 nm. Comparison of the emission spectra as a function of excitation wavelength also indicates that little tyrosine emission was observed in the range 300–315 nm at wavelengths shorter than 290 nm. However, the spectra are dominated by tryptophan emission, and the fluorescence maximum occurs at 343 ± 2 nm indicating that the tryptophan side-chain is somewhat shielded from bulk water but is not located in the hydrophobic core of the protein [16,20,25]. The maximum at 343 nm is blue shifted compared to that of free tryptophan which is about 348 nm [16,17,23] in aqueous solution. This is suggested to be a consequence of the shielding of the protein matrix [26].

The observed tryptophan emission in Fig. 1 can be supported by the composition of the lyophilizate of water extract of *Moringa* seeds reported by Kalogo et al. [27], in which trace amounts of tryptophan was detected among other amino acids. Also upon excitation at 295 nm, only tryptophan is excited [17,23]. Thus, the result in Fig. 1 indicates the presence of tryptophan residue(s) in the protein. Excitation and emission wavelength of 295 and 343 nm, respectively, were used for subsequent determinations of the coagulant protein. Excitation of 295 nm selectively excites tryptophan, and contribution of tyrosine fluorescence is minimized [16,17,19,20].

3.2. *Linearity of intrinsic fluorescence intensity*

Fluorescence intensity is typically directly proportional to concentration. There are, however, factors that affect this linear relationship such as inner filter effect. An unknown sample needs to be tested for linearity of its fluorescence intensity. Using the determined emission wavelength, fluorescence was measured for several dilutions of the coagulant protein. As seen in Fig. 2, the fluorescent signal of the coagulant protein is directly proportional to protein concentration in the range tested. Using a least squares linear regression, the correlation coefficient (R^2) value found is 0.9999, suggesting good linear fit since it is close to unity. This indicates the absence of concentration quenching up to protein concentration of 1 mg/mL. However, very low protein concentrations were avoided for analysis because relative contribution of background fluorescence begins to be significant; giving unreliable fluorescence intensity values [17].

In conclusion, position of the emission peak maximum was observed to be independent of excitation wavelength. The spectra are dominated by tryptophan emission, and the fluorescence maximum occur at 343 ± 2 nm indicating that the tryptophan side-chain is somewhat shielded from bulk water but is not located in the hydrophobic core of the protein. There was no concentration quenching up to protein concentration of 1 mg/mL.

3.3. *Influence of solution environment*

The fluorescence intensity and spectral peak maximum of tryptophan residues in a protein are sensitive to local environment of tryptophan [16,17,19,26,28].

3.3.1. *Effect of ionic strength*

Solution ionic strength is an important factor which may affects protein conformational stability [16]. In order to study the effect of ionic strength on the fluorescence of a coagulant protein from *M. oleifera* seeds, the tryptophan fluorescence intensity of the protein was measured in water at different salt concentrations (0–0.7 M). The ionic strength was adjusted by addition of sodium chloride, a simple 1:1 salt often used for such studies in literature. Ionic strength zero is for protein solution without sodium chloride added. The fluorescence intensity of the protein (0.08 mg/mL) as a function of ionic strength is shown in Fig. 3(a). It shows that there is no significant change in the coagulant protein fluorescence intensity as the solution ionic

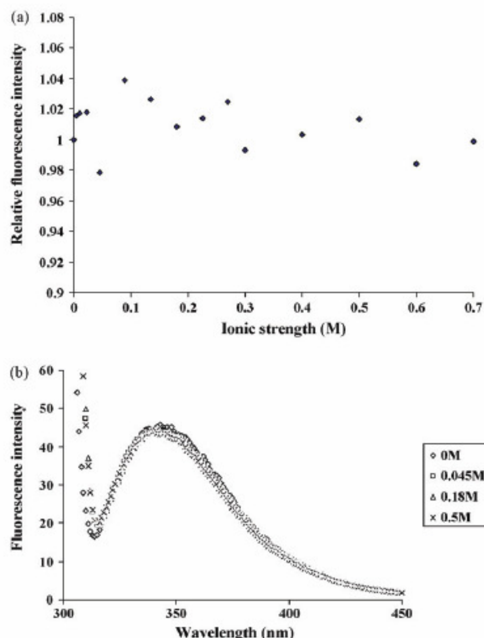

Fig. 3. Effect of ionic strength on the fluorescence intensity (a) and on the fluorescence spectra (b) of a coagulant protein from *Moringa oleifera* seeds in water on excitation at 295 nm and emission at 343 nm. The protein concentration was 0.08 mg/mL, and solution ionic strength was adjusted by addition of sodium chloride.

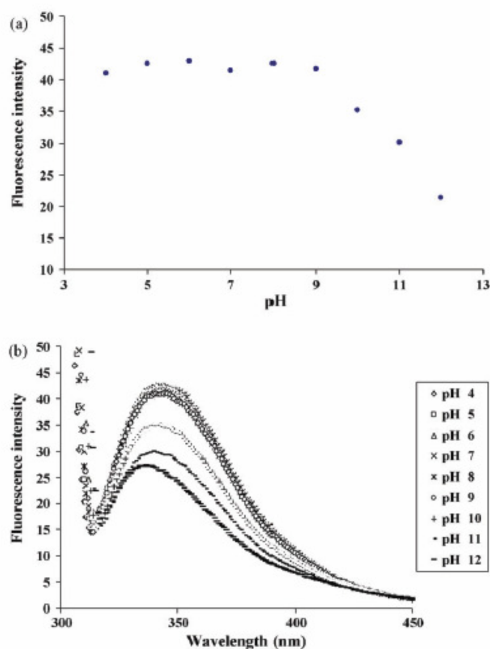

Fig. 4. Effect of pH on the fluorescence intensity (a) and on the fluorescence spectra (b) of a coagulant protein from *M. oleifera* seeds on excitation at 295 nm and emission at 343 nm. The protein concentration was 0.08 mg/mL, and solution pH was varied by addition of 0.1 M NaOH or 0.1 M HCl.

strength increases to 0.7 M. In order to study the effect of ionic strength on the protein conformation, the emission spectra of the protein were recorded from 300 to 450 nm (Fig. 3(b)). No shift in fluorescence emission maximum is observed. The absence of any shift in the fluorescence emission maximum indicates that it is not sensitive to the ionic strength of the medium under the experimental conditions used.

3.3.2. Effect of pH

Due to the presence of ionisable amino acids (such as arginine, histidine and aspartic acid), a change in pH can alter the overall charge on the protein and electrostatic interactions may change protein conformation [16]. In order to study the effect of pH on the conformation of the coagulant protein, the fluorescence of the protein was measured at different pH. Fig. 4(a) shows the fluorescence intensity of coagulant protein (0.08 mg/mL) as a function of pH (4–12). The fluorescence intensity of the coagulant protein was relatively stable to pH changes over a pH range of 4–9, although there was slight decrease at pH values 4 and 7. The reason for this decrease in fluorescence intensity is unknown, but this observation was repeatable. The observation cannot be explained on the basis of isoelectric point reported in literature to be in the range between 10 and 11 [11] for the coagulant protein. Fig. 4(a) also shows that the fluorescence intensity decreases dramatically as pH increases from pH 9 to pH 12.

In order to find out the effect of pH on the environment of tryptophan in the coagulant protein, the fluorescence spectra of the protein were also recorded at different pH and plotted as a function of pH (Fig. 4(b)). It shows that the emission peak of the coagulant protein has no significant shift as the pH decreases from pH 9 to pH 4. This result indicates that the tryptophan environment does not change significantly as the pH decreases from pH 9 to acidic pH. Fig. 4(b) also shows that the peak shifts from 343 to 337 nm as the solution pH increases from pH 9 to pH 12. It indicates that the tryptophan is in a more hydrophobic environment as the pH increases.

The dramatic decrease in fluorescence intensity and blue shift of the emission peak from pH 9 to pH 12 may indicate that the coagulant protein has lost the tertiary structure (isoelectric point of the coagulant protein is between 10 and 11). Extremes of pH from physiological operating pH of the protein may cause denaturation because sensitive areas of the protein molecule acquire more like charges, causing internal repulsion or perhaps lose charges which were previously involved in attractive forces holding the protein together [29].

Under experimental conditions used, the results of this study demonstrate that the conformation of the coagulant protein is

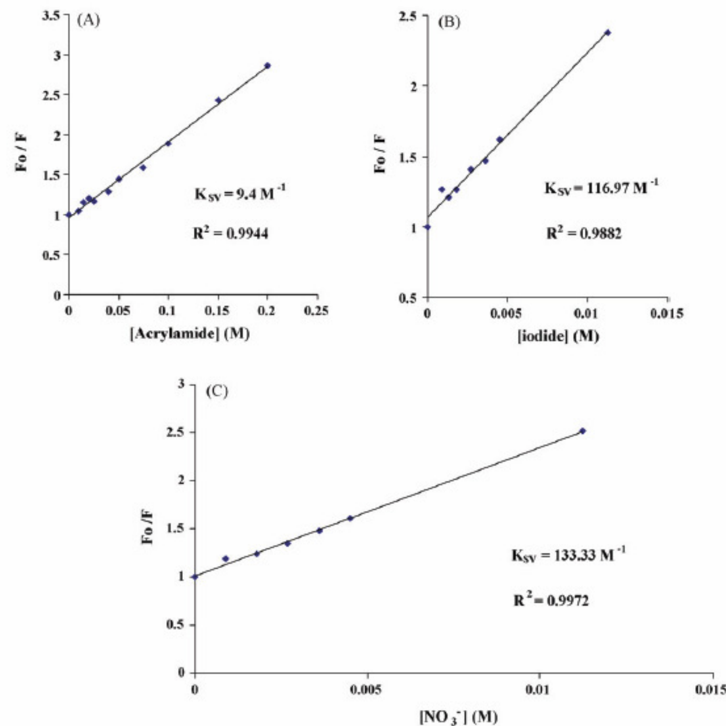

Fig. 5. Stern–Volmer plots for acrylamide quenching (A), iodide quenching (B), nitrate quenching (C) of a coagulant protein (0.08 mg/mL) from *M. oleifera* seeds in water solution on excitation at 295 nm and emission at 343 nm.

not sensitive to the ionic strength of the medium whereas pH dependence occurs above pH 9 where a significant decrease in fluorescence intensity accompanied with blue shift in the emission maximum was observed, suggesting that the protein is denatured. This presents a challenge to investigate the effect of pH on the secondary structure of the coagulant protein. Circular dichroism spectroscopy may provide an answer to the conformational changes as a function of pH [16,30–34].

3.4. Fluorescence quenching measurements

Fluorescence quenching studies were used to monitor the tryptophan environment of the coagulant protein using a polar neutral quencher (acrylamide), two anionic quenchers (iodide and nitrate), and a cationic quencher caesium ion (Cs^+). The experiments were carried out by monitoring the change in intensity at the emission maximum. The fluorescence intensity of the coagulant protein decreased depending on the concentration of a quencher added for the anionic and neutral quenchers whereas no quenching was observed with Cs^+ even up to 0.27 M under experimental conditions used. The fluorescence change was plotted according to Stern–Volmer equation (1). Only regions of low quencher concentrations were considered in the calculation of the K_{SV} constants [22,35]. Typical quenching data are exhibited in Fig. 5 and summarized in Table 1.

Protein quenching by acrylamide, a polar uncharged water-soluble molecule that can penetrate the matrix of the protein [36], gave a linear Stern–Volmer plot (Fig. 5(A)), indicating that all the tryptophan residues are accessible to acrylamide [17,20,22,37]. The value of the Stern–Volmer constant K_{SV} was found to be $9.4\,M^{-1}$. Acrylamide is known to exhibit static quenching (ground state formation of a non-fluorescent complex) at higher concentrations. The presence of such quenching is characterized by an upward curvature of Stern–Volmer plot [24]. For the concentrations used in Fig. 5(A), the Stern–Volmer plot shows linear relationship fluorescence in quenching reagent concentration. Only at very high concentration of acrylamide did the plot deviate from linearity (data not shown).

Table 1
Fluorescence quenching constants

Quencher	K_{SV} (M^{-1})	R^2
Acrylamide	9.4	0.9944
Iodide	116.9	0.9882
Nitrate	133.3	0.9972

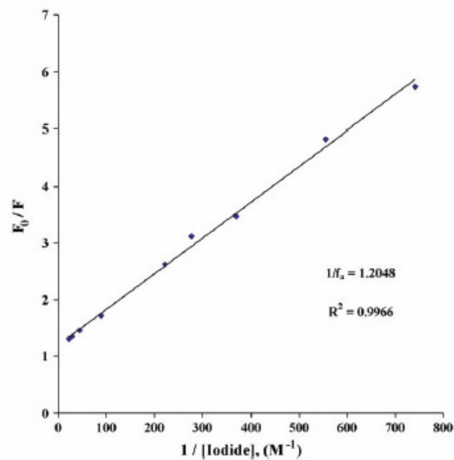

Fig. 6. Modified Stern–Volmer plot for iodide quenching of a coagulant protein (0.08 mg/mL) from *M. oleifera* seeds in water solution on excitation at 295 nm and emission at 343 nm.

The fluorescence intensity was markedly quenched by iodide. The Stern–Volmer plot is notably curved downwards at higher concentration of the quencher, indicating that only part of the tryptophan residues is accessible to the anion [17,20,22,28]. The linear portion of the Stern–Volmer plot in Fig. 5(B) near the origin yielded a K_{SV} value of $116.9\,M^{-1}$. However, the situation for nitrate was quite different. Stern–Volmer plot for NO_3^- quenching had an upward curvature. The linear portion of the Stern–Volmer plot shown in Fig. 5(C) near the origin yielded a K_{SV} value of $133.3\,M^{-1}$. For both iodide and nitrate, protein conformational changes due to variation in the ionic strength during quenching experiments were disregarded as ionic strength was kept constant by addition of sodium chloride. High K_{SV} values suggest higher rate of quenching process, which are quite significant in the case of I^- and NO_3^-.

The quenching of intrinsic fluorescence emission of tryptophan residues in a protein requires molecular contact between the quencher and the fluorophore in either ground or excited states [17,19,20,28] and therefore can be used to determine the surface accessibility of the tryptophan using different quenchers as well as to obtain information regarding the nature of the envi-

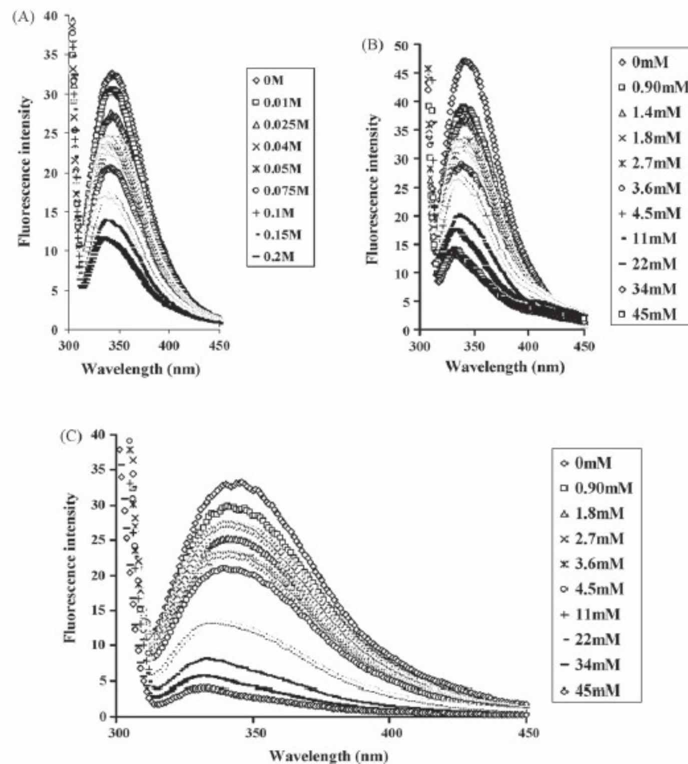

Fig. 7. Fluorescence spectra of a coagulant protein (0.08 mg/mL) in the presence of acrylamide (A), iodide (B), nitrate (C) on excitation at 295 nm and emission at 343 nm.

ronment. Thus, a change in quenching properties is reflective of a change in the accessibility of the fluorophores. As the emission wavelength maximum (343 nm) of the coagulant protein suggests that the tryptophan is not located in the hydrophobic core of the protein, a larger extent of quenching accompanied by a larger degree of accessibility for the charged quenchers is expected and has been borne out by the experiments here. A striking difference between the three charged quenchers used is the lack of fluorescence quenching action by the caesium ion (Cs^+). This seems to suggest that fluorescence for the coagulant protein should be associated with the tryptophan located in close proximity to some positively charged amino acid residues [37]. Judging from the K_{SV} values, the efficiency of the quenchers to the coagulant protein was in the order $NO_3^- > I^- >$ acrylamide. The value of K_{SV} of $9.4\,M^{-1}$ is in the range observed in cases where acrylamide quenches the protein fluorescence via a collisional quenching mechanism [17]. But the high values of K_{SV} for iodide ($116.9\,M^{-1}$) and nitrate ($133.3\,M^{-1}$) might be an indication of favourable charge interactions between the quencher and positive charge on the protein in the vicinity of the tryptophan resulting in static quenching of the fluorescence [22,28].

From the modified Stern–Volmer plots, the fraction of accessible tryptophan residues can be calculated. Fig. 6 shows the modified Stern–Volmer plot for I^- quenching. The plots for both acrylamide and nitrate were linear (results not shown) with intercept ~1. The quenching efficiency of 1.0 indicates that there is equal tryptophan accessibility to both quenchers. Previous work [22] observed that NO_3^-, like acrylamide, is a contact quencher and has ability to reach any given tryptophan. However, the percentage of fluorescence quenched by iodide from the modified Stern–Volmer plot (Fig. 6) was calculated to be about 83%.

In order to study changes in the local environment of tryptophan, the fluorescence emission spectra of the coagulant protein were recorded at different concentrations of the quenchers (Fig. 7). It shows no large change in the wavelength of maximum fluorescence and shape of the spectrum for concentration ranges used in the Stern–Volmer plots. Thus, the three quenchers have no adverse conformational effects on the protein in those concentration ranges. This observation seems to indicate that the coagulant protein belongs to a family of long-wavelength-emitting proteins displaying a minimal red-edge effect. The red-edge excitation phenomenon in proteins occurs due to the dipole relaxation processes in the surrounding of the excited fluorophore. Therefore, when tryptophan residue is deeply buried in a non-polar, rigid protein matrix or is exposed to the rapidly relaxing environment (e.g. solvent) no substantial change in the emission maximum upon red-edge excitation is expected [38].

Unless additional information is provided, fluorescence quenching data obtained by intensity measurements alone cannot distinguish dynamic collisional quenching from quenching due to ground state complexation [17,22]. The measurement of fluorescence life time is the most definitive method to distinguish static and dynamic quenching [17]. On the other hand, if quenching is carried out at different temperatures, quenching caused by collision should have higher K_{SV} values at higher temperature due to higher collision frequency resulting from faster movement of molecules [16,17].

4. Conclusions

The steady-state fluorescence technique has been used to probe the spectroscopic properties of a coagulant protein extracted from *M. oleifera* seeds. The following conclusions can be drawn from this on going study:

- The intrinsic fluorescence emission is dominated by tryptophan emission, and the fluorescence maximum occur at 343 ± 2 nm indicating that the tryptophan side-chain is somewhat shielded from bulk water but is not located in the hydrophobic core of the protein.
- The result of this study demonstrated that under experimental conditions, ionic strength does not affect the conformation of the coagulant protein, but it is affected by pH above pH 9. The tryptophan environment change significantly when the solution pH is above pH 9, suggesting that the protein is denatured.
- The high accessibility of the tryptophan by ionic quencher, iodide confirmed that the tryptophan in the coagulant protein is not located in the hydrophobic core of the protein. But the anomalously high values of K_{SV} for iodide ($116.9\,M^{-1}$) and nitrate ($133.3\,M^{-1}$) might be an indication of favourable charge interactions between the quencher and positive charge on the protein in the vicinity of the tryptophan resulting in static quenching of the fluorescence.
- Quenching data from I^- and NO_3^-, and the lack of fluorescence quenching by Cs^+ on the protein suggest the presence of charged basic amino acid residues in the vicinity of the tryptophan residue.

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