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Fluorescence Spectroscopic Studies on Ovis Lactoperoxidase

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Abstract. Ovis lactoperoxidase (sLP), on excitation at 280 nm shows fluorescence emission of a single broad maximum at 332 nm. The conformational stability was measured by unfolding studies in urea and guanidine hydrochloride. The fluorescence intensity gradually decreased with increase in urea concentrations. The decline might have been caused by partial unfolding, affecting some of the tryptophan residues. In 5M GuHCl concentrations, a red shift in emission maximum to 356 nm was observed. It indicates that tryptophan is buried in the interior of the hydrophobic environment in native folded state and inaccessible to solvent water but on unfolding all get exposed to aqueous environment. Acrylamide is an efficient quencher and the quenching process is essentially homogenous with all tryptophan being accessible. A little quenching is observed for KI is interpreted as sLP has tryptophan residues that are buried inside the core of the protein.

Keywords: Fluorescence, Unfolding, Quenching, Ovis Lactoperoxidase

Introduction

Lactoperoxidase (LP) (EC 1.11.1.7) is a constituent of mammalian exocrine gland secretions *viz*. milk, tears, saliva etc..[1] LP, a glycoprotein consists of a single polypeptide chain of 612 amino acid residues, [2, 3] a molecular mass of approximately 78000 Da,[4] of which about 10 % is due to carbohydrate,[5] a covalently bound heme and metal ions.[6]

Industrial application of LP in dairy industry for the preservation of raw milk during transportation to the processing plants[7, 8] is of some significance. Biological application of LP can be found in cosmetics, ophthalmic solutions and dental and wound treatment for antiseptic purposes.[9, 10] It also functions as antitumour and antiviral agent.[11] In view of important applications of this enzyme, it is pertinent to carry out detailed structural studies. In the present study, intrinsic fluorescence of ovis lactoperoxidase (sLP) has been examined. Fluorescence changes occurring during denaturation, in the presence of urea and guanidine hydrochloride (GuHCl) were studied to provide information concerning the stability of sLP. Fluorescence quenching studies of sLP were also performed.

Materials and Methods

LP was isolated and purified from sheep colostrum by simple procedures including ion exchange chromatography on CM Sephadex C-50 (Sigma) and Gel filtration chromatography on Sephadex G-100 (Sigma). The presence of sLP containing fractions were detected by ABTS (Azinobis (3 ethyl benzthiazoline 6-sulphonic acid) assay method.[12] The specific activity of sLP was measured to be 92.2 IU mg^{-1} at 30° C. The fractions with sLP were pooled, lyophilized and was used for spectroscopic studies. Fluorescence spectra of sLP were recorded on SPEX-FLUOROLOG-spectrofluorimeter equipped with software for data acquiring and processing with excitation at 280 nm and emission was scanned in wave length region between 300 -400 nm at 20°C. The scan speed was 60 nm/min. Urea and GuHCl induced unfolding studies were done. All measurements were made on 2μM sample and excitation and emission bandwidth of 3 nm was used. Aliquots of urea and GuHCl with sLP in 0.05 M Tris HCl buffer, pH 8 were incubated for one hour at each concentration before recording the spectra.

Fluorescence quenching studies by acrylamide and potassium iodide were performed for native protein.[13] Acrylamide was recrystallised from ethyl acetate. Aliquots from 4 M acrylamide were added to the sLP sample in 0.05 M Tris HCl buffer, pH 8 in the cuvette at 20°C. Emission intensities at 332 nm were monitored and slit width was 3 nm. Quenching of fluorescence was also performed with 2 M potassium iodide in the presence and absence of denaturants. Quenching studies as above were repeated after adding 1 M GuHCl, with various concentrations of potassium iodide.

Results

The unfolding of enzyme was studied by monitoring the changes in the intrinsic tryptophan fluorescence at varying concentrations of urea (**Figure 1**).

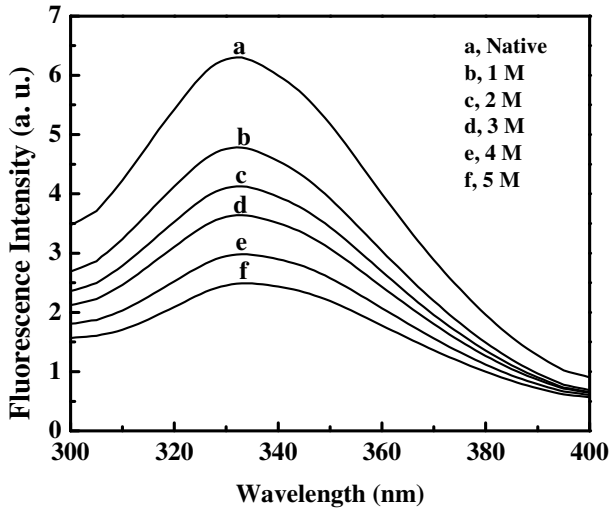

Figure 1. Fluorescence spectra of sLP at varying concentrations of urea.
(a) Native sLP (b) 1M (c) 2M (d) 3M (e) 4M (f) 5M

On excitation at 280 nm, the fluorescence emission of sLP shows a single broad maximum at 332 nm. The emission maxima vary from 332 nm to 334 nm with different concentrations of urea from 1 M to 5 M. The fluorescence intensity is gradually decreased with increase in urea concentrations. The transition in sLP as monitored by natural tryptophan fluorescence is extremely broad and suggestive of changes in the environment of tryphtophan residues over the entire range of urea concentrations.

There is a decline in emission intensity at different concentrations of urea. The decline might have been caused by partial unfolding, affecting some of the tryptophan residues.

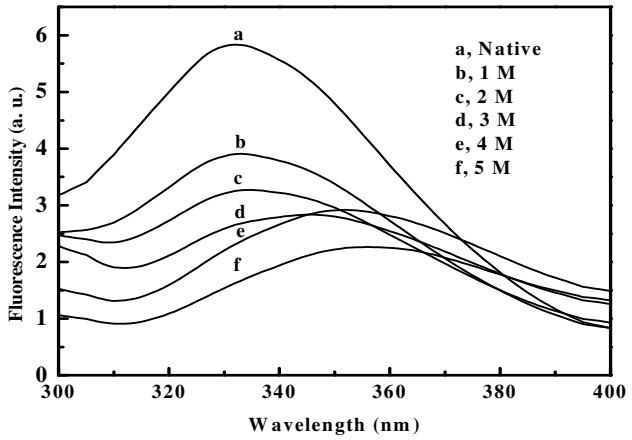

Figure 2. Fluorescence spectra of sLP at varying concentrations of GuHCl.
(a) Native sLP (b) 1M (c) 2M (d) 3M (e) 4M (f) 5M

Figure 2. shows fluorescence spectra of sLP at varying concentrations of GuHCl. Native sLP has an emission maximum at 332nm. At higher GuHCl concentrations, a decrease in fluorescence intensity takes place. A red shift in emission maximum to 356 nm was also observed. The shift of emission maximum from 332 nm to 356 nm, correlated with $\pi^* \rightarrow n$ energy transfer, results in unfolding of protein. Most proteins show a red shift upon unfolding and it is employed for studying the denaturation processes.[14] In order to probe the changes in tryptophan environment and their exposure to solvent during unfolding, fluorescence quenching studies were done.

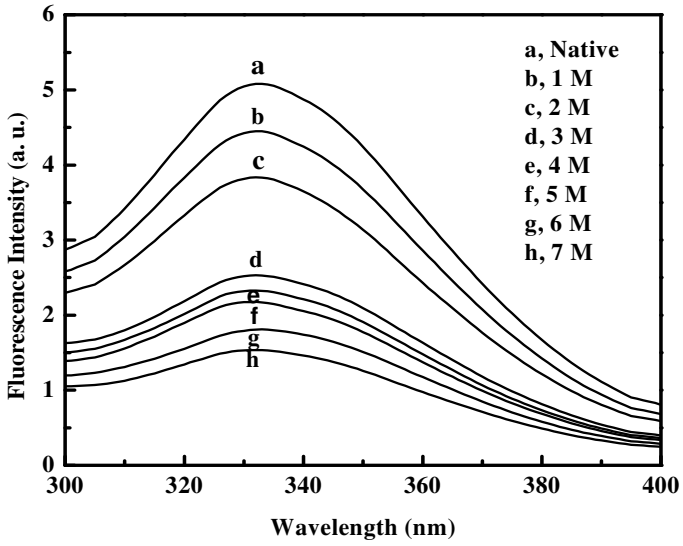

Figure 3. Quenching of tryptophan fluorescence of sLP at various Acrylamide concentrations
(a) Native sLP (b) 0.05M (c) 0.1M (d)0.15M (e)0.18M (f) 0.22M (g) 0.26 M (h) 0.3M

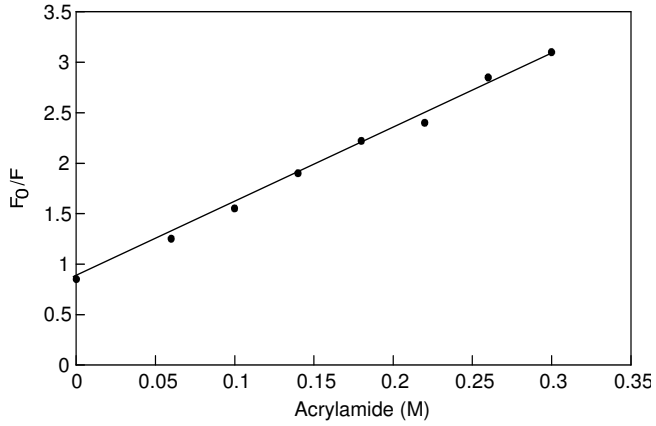

Figure 4. Stern Volmer plot for Acrylamide quenching of sLP fluorescence.
F_0 and F are the fluorescence intensities in the absence and presence of the quencher

From the graph, Stern-Volmer quenching constant (K_D) for sLP was found to be 7.5 M^{-1}. If only external residues are quenched, the smaller quenching constant for blue shifted residues would be obtained, as was observed for quenching by acrylamide.[15] A Linear Stern-Volmer plot indicates the acrylamide may permeate the matrices of protein (**Figure 4**). Acrylamide preferentially quenches the exposed tryptophan residues. The emission spectra of native protein are blue shifted upon quenching. Tryptophan residues are non-polar. Some of the tryptophan residues are expected to be in the interior of the protein and hence inaccessible to quencher in the external aqueous phase.

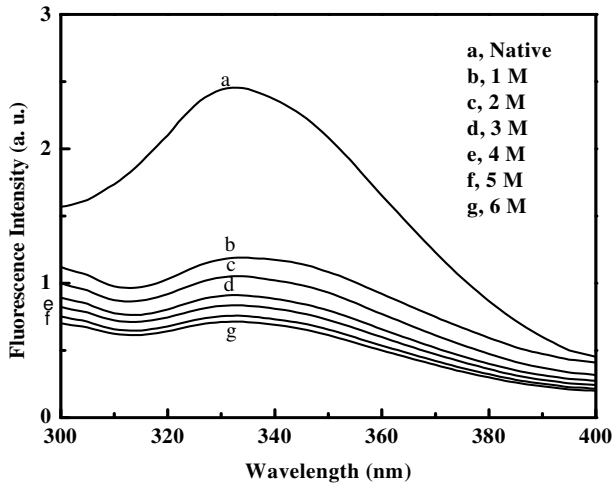

Figure 5. Quenching of tryptophan fluorescence of sLP at various concentrations of KI
(a) Native sLP (b) 0.05M (c) 0.1M (d) 0.15M (e) 0.2M (f) 0.25M (g) 0.3M

Figure 5 shows quenching of tryptophan fluorescence of sLP at pH 8 using various potassium iodide concentrations. The spectral shift observed upon quenching, is an evidence for the selective quenching of a specific population of fluorophores.[16]

Quenching of sLP in the presence of 1M GuHCl using various concentrations of potassium iodide was done and represented in the Figure 6. It is observed that unfolding of sLP with GuHCl has no impact on the quenching processes, when potassium iodide in different concentrations was used as a quencher, suggesting the quencher permeation to tryptophan residues.

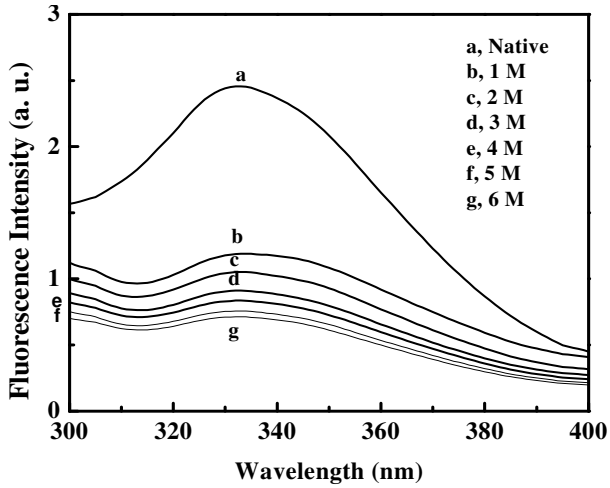

Figure 6. Quenching of tryptophan fluorescence spectra of sLP in 1M GuHCl using various concentrations of KI (a) Native sLP (b) 1M GuHCl (c) 0.05M KI (d) 0.1 KI M (e) 0.15M KI (f) 0.20M KI (g) 0.25 M KI.

Discussion and Conclusion

The conformational stability of a protein can be measured by unfolding studies in urea, guanidine hydrochloride etc. In the urea unfolding studies, sLP is not a rigid structure and it undergoes unfolding from the denaturant concentration of 1M. The unfolding phenomenon follows same trend as the concentrations range from 2M to 4M urea with emission maximum at 333 nm. In 5M urea, the emission maximum of sLP was found to be 334 nm. Similarly, in unfolding studies by GuHCl, unfolding begins at 1M concentration of GuHCl. It is observed that when GuHCl concentrations change from 1M to 5M, the emission maxima shifts from 332 to 356 respectively and it shows red shift. The emission maxima shift from 332 nm to 334 nm for same range of urea concentrations. Ovis LP may be more susceptible to GuHCl induced unfolding. This finding leads to a conclusion that GuHCl is more effective than urea in unfolding the protein. Neutral urea is reported to be less effective as denaturant than GuHCl.[17] The emission maximum of sLP in the presence of 5M GuHCl is observed at 356 nm at 20°C. Considerably red shifted in the emission maximum indicates that tryptophan is buried in the interior of hydrophobic environment in the native folded state and inaccessible to solvent water, but on unfolding, get all exposed to aqueous environment. It agrees with the previous reported results of bovine LP,[18] which states that emission maximum for bovine LP is observed at 356 nm in the presence of 9 M GuHCl. Generally proteins denature in the range of 4-6 M urea and 2-3 M GuHCl.[19-21] The present study of sLP by GuHCl and urea induced unfolding indicate the difference in their interaction with sLP.

Acrylamide is an efficient quencher and the quenching process is essentially homogenous with all the tryptophan being accessible. Acrylamide can penetrate the protein matrix. Quenching of buried tryptophan residues is not affected by the overall viscosity of the acrylamide solution. So quenching rate is limited by the diffusion of acrylamide through the protein matrix. This finding agree with results of previous studies.[22] A little quenching is observed for potassium iodide can be interpreted as the inability of the charged and hydrated iodide could not enter the non-polar interior of the protein So that sLP has tryptophan residues which are buried inside the core of the protein . Hence, comparison of quenching by charged and uncharged molecules revealed the relative permeability of the macromolecule to these substances.

The fluorescence quenching studies provide insight into the dynamic nature of protein molecule.[23] The sLP is not a rigid structure that continuously undergoes stochastic structural fluctuations that facilitate the inward diffusion of quencher. The flexibility of protein results from intermolecular nonbonded, individually weak interactions i.e., hydrogen bonds, van der Waals interactions etc. that serves to maintain the globular state of a protein. Acrylamide was found to be able to quench the fluorescence of the buried tryptophan in sLP. This neutral apolar quencher is used to sense the hydrophobic pockets in the vicinity of the flurophore. Iodide, a polar quencher, has greater access to the surface exposed tryptophan residues.

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