Conformational stability of a peroxidase from *Artocarpus lakoocha*: Effect of pH, chaotrophs and temperature.

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Abstract

A novel heme-peroxidase has been isolated from the medicinally important plant Artocarpus lakoocha and preliminary studies suggest it has anti-inflammatory and wound healing properties. The protein's structure, dynamics, and stability have been examined using Far-UV Circular Dichroism, fluorescence spectroscopic studies, and activity measurements. These studies revealed the existence of seven folding states for this peroxidase, including a native state (N) at pH 7, pre-molten globule state at pH 2, acid-unfolded state (UA) at pH 1.5 or lower, acid-refolded (A-state) at pH 0.5, the molten globule state (MG) at pH 2.0 with 1 M GuHCl, alkaline denatured state (UB state) in the pH range of 8.5-12 and the complete denatured state (D) beyond 5 M GuHCl concentration. This is the first report of such multiple partially folded intermediate states for A. lakoocha peroxidase, which differ in their amount of secondary structure, stability, and compactness. The absorbance studies showed the presence of free heme in the pH range of 1-2. The protein's structural integrity is maintained over a wide range of pH (3-10), temperature (65 oC), and high concentrations of GuHCl (5 M) and urea (8 M).

Keywords: *Artocarpus lakoocha*, Circular dichroism, Molten globule, Peroxidase.

1. Introduction

Artocarpus lakoocha (*A. lakoocha*) is a medicinal plant of the Moraceae family. It is a widely used medicinal plant of India for the treatment of many diseases like intestinal fluke *Haplorchis* taichui (Wongsawad, Wongsawad, Luangphai, & Kumchoo, 2005), taeniasis (Charoenlarp, Radomyos, & Bunnag, 1989), and cosmetics (Theorell, 1941). In earlier report we have purified and characterized a novel peroxidase from *A. lakoocha.* The preliminary biophysical studies on *A. lakoocha* peroxidase revealed that the isolated novel enzyme from *A. lakoocha* is a stable heme-peroxidase with anti-inflammatory and wound healing properties (Sonkar et al., 2015). These broad applications provide a stimulus for detailed characterization of this peroxidase regarding folding behavior. Proteins undergo extensive conformational changes as part of their functionality, tracing of these changes is important for understanding the mechanism of proteins function.

Folding and conformational stability of protein is primarily based on its amino acid sequence as well as cellular external variables such as temperature, pH, and chemical denaturants (Anfinsen, 1973; Masson & Lushchekina, 2022). To predict the behavior of protein folding it is important to understand the effect of these external variables on the thermodynamics of the folding process. Several other forces such as hydrogen bonding, disulfide bonding, entropic effects, hydrophobic, van der Waals, and electrostatic interactions, stabilize the folded state of the protein.

From unfolded chain to functional three dimensional state, the protein undergoes through various non-native intermediate forms which are involved in various cell phenomena such as chaperone action (Díaz-Villanueva, Díaz-Molina, & García-González, 2015; Uversky, 2010), amyloid fibril formation in pathological cells(Almeida & Brito, 2020). Better understanding of protein folding mechanism can help in treating diseases associated with protein misfolding, such as Alzheimer's, Parkinson's and prion diseases (Rochet & Lansbury, 2000; Stefani & Dobson, 2003). The protein folding pathway is characterized through the accumulation of many intermediates between the native (N) and the fully unfolded (U) states. Understanding of these intermediate states is important to know how and when various forces come into play in directing the protein to attain three-dimensional functional structures. Studying these intermediates will help to explain the mechanism of folding of protein in to the specific biologically active confirmations (Benaim & Villalobo, 2002).Protein folding is a sequential

ordered process with the existence of stable 'molten globule' (MG) conformations between fully folded and unfolded states [3]. Molten globule serves as a common equilibrium intermediate state in the protein folding pathway, which is acquired under mild denaturing conditions. These molten globule states are believed to give information on early events in the folding process, at a stage when specific side-chain interactions are not yet formed. Molten globule was considered less compact than native state and more compact than unfolded state with prominent secondary structure but slight or no tertiary packing (Baldwin & Rose, 2013; Bhattacharyya & Varadarajan, 2013). The characteristics of the molten globule state is exposed hydrophobic surface that binds a hydrophobic dye. This dye helps to yield experimental evidence concerning the structure of intermediates throughout protein folding state(Judy & Kishore, 2019). The exact role of molten globule state is controversial among the proteins. Same protein may have varied molten globule states in the folding pathway (Bychkova, Dolgikh, Balobanov, & Finkelstein, 2022). The experimental techniques typically used to detect molten globule states are, far-UV and near-UV CD spectroscopy that detect the secondary and tertiary structures of protein. The hydrophobic dye, such as ANS (8-anilino-naphtalene-1-sulfonate) binding experiments detect the formation of a loose hydrophobic core and estimate the extent of hydrophobic area exposed to the solvent.

With this background, conformational studies with *A. lakoocha* peroxidase have been examined in wide range of pH temperature and in presence of various chemical denaturants (GuHCl). The present analysis shed light on the biophysical properties of *A. lakoocha* peroxidase, to found the correlation between its structure and function and the root and logic behind its distinct physiochemical properties. The conformational stability of the native protein as well as intermediate states have been evaluated, compared and discussed. Several intermediates with distinct spectroscopic properties are populated under various conditions.

Earliest biochemical analysis have revealed that the isolated peroxidase has molecular mass of 53 kDa and its molecular structure consists of four disulfide bridges, fourteen tyrosine and seventeen tryptophan residues with a extinction coefficient ($\varepsilon_{280}^{1\%}$) of the enzyme at 16.3 (Sonkar et al., 2015). This Peroxidase showed remarkable stability under various denaturing conditions. In this section, conformational studies and other factors responsible for such high stability of peroxidase against various denaturing conditions, such as high concentration of urea as well as guanidine hydrochloride, and wide range of pH and temperature have been reported. Several equilibrium intermediate states with different stabilities have been obtained and

characterized to increase the understanding of folding aspects of peroxidase molecule. The structural statistics about transition-state intermediates have been examined to identify whole protein folding method of peroxidase. These studies establish a stable folded state of protein, which reflect its functional stability and are aimed to provide an insight into the folding of proteins in general which may find application in biotechnology and other fields of sciences.

2. Materials and methods

2.1 Materials

Peroxidase was purified from fresh latex of *Artocarpus lakoocha* plant using the method of Sonkar et al(Sonkar et al., 2015). ANS (8-anilino-1-naphthalene sulphonate) and GuHCl were purchased from Sigma Chemicals, USA. Other reagents used were of analytical grade. The samples were centrifuged before spectroscopic measurements and the exact concentration of the protein and pH of the buffer were determined before the measurements were taken.

2.2 Methods

2.2.1 Absorbance spectroscopy

Spectroscopic analysis was performed using a Beckman DU-640B spectrophotometer with a constant-temperature cell holder. A protein concentration of 0.1mg/ml was used for all absorbance measurements. The concentration of the enzyme was determined spectrophotometrically using an extinction coefficient (ε2801%) of 16.3. Far-UV Circular Dichroism (CD) measurements were done using a JASCO J-815 spectropolarimeter (JASCO Corporation, Tokyo, Japan) equipped with a constant temperature cell holder. The instrument was calibrated using ammonium (+)-10-camphor sulfonate, and conformational changes in the secondary structure of the peroxidase were monitored in the 200-260 nm range. After subtracting appropriate blanks, mean residue ellipticity was calculated using the equation provided by Balasubramanian et al (Balasubramanian & Kumar, 1976) [θ] MRW = θobs x MRW/10cl; where θobs is the observed ellipticity in degrees, MRW represents mean residue weight, c represents the concentration of protein (mg/ml) and l represents the path length in centimeters. The mean weight of amino acid residues (MRW) was taken as 112. Sensitivities of 1 and 2 millidegrees/cm were used for far-UV CD measurements.

2.2.2 Fluorescence spectroscopy

Fluorescence measurements were carried out using an Eclipse Cary Varian UV-Vis spectrofluorometer (Varian Inc., Palo Alto, California, USA) attached to a Peltier temperature controller. For intrinsic tryptophan fluorescence, the protein was excited at 292 nm, and the fluorescence emission was monitored in the 300-400 nm range. For ANS fluorescence, the excitation was done at 380 nm, and the emission was monitored between 400-600 nm. For all experiments, a protein concentration of 1 mM and excitation and emission slit widths of 10 and 5 nm were used respectively. All the fluorescence spectra were recorded at 25oC unless stated otherwise. The activity of A. lakoocha peroxidase under various conditions of pH and chemical denaturant was monitored using hydrogen peroxide as a hydrogen acceptor, and other compounds such as guaiacol as hydrogen donor. Twenty micrograms of peroxidase were added to 0.2 mM of hydrogen peroxide (substrate I) with guaiacol in a 50 mM acetate buffer (substrate II). The rate of change of absorbance of guaiacol was determined at 470 nm.

2.2.3 pH denaturation of A. lakoocha peroxidase

Acid denaturation of peroxidase was observed at a broad pH range using various buffers, such as KCl–HCl buffer (pH 0.5–1.5), Gly–HCl buffer (pH 2.0–3.5), sodium acetate buffer (pH 4.0–5.5), sodium phosphate buffer (pH 6.0–8.0), Tris–HCl buffer (pH 8.5–10.5), and Gly–NaOH buffer (pH 11.0–12.5). The concentration of all the buffers used in this study was 50 mM. A stock solution of the protein was added to the appropriate buffer, and the mixture was incubated for 24 hr at 25 °C. The pH of the final suspension and concentration of the protein in each sample were confirmed by measurement at the final steps.

2.2.4 8-anilino-1-naphthalene sulfonate (ANS) binding assay

ANS is highly soluble in methanol (Hawe, Sutter, & Jiskoot, 2008) and minimal amount of methanol does not affect the structure of the protein; as *A. lakoocha* peroxidase shows activity and stability up to 50 % methanol (Sonkar et al., 2015). Based on this, ANS solution was prepared in methanol. An extinction coefficient value of 5000M−1 cm−1 at 350 nm was used for ANS concentration measurement. A molar ratio of 1:100 for protein to ANS was used, and the suspension was incubated for 30 minutes in the dark. Subsequently, the extrinsic fluorescence of the protein-ANS complex was monitored by recording the emission spectrum between 400-600 nm using an excitation wavelength of 380 nm. Protein concentration 2 µM, and slit widths of excitation and emission 10 and 5 nm were used respectively.

2.2.5 Guanidine hydrochloride and urea induced unfolding

Concentrations of GuHCl solutions were determined from the refractive index of the solution. As the concentration of the chemical denaturant increased, denaturation of the enzyme was observed at different pH conditions. To reach equilibrium, the peroxidase sample was incubated at the corresponding denaturant concentration for 24 hr at 25 °C. To obtain transition midpoints, as well as to judge the cooperativity or non-cooperativity of the transitions, the method used by Shirley (1995) was followed for the analysis of unfolding transitions. To compare the results from different measurements (molar ellipticity, fluorescence emission maximum, and relative activity), all the results were normalized and expressed as a fraction unfolded. The formula used for fraction unfolded calculation is:

$$\textbf{Fraction unfolded} = (F_{obs} - F_n) / (F_u - F_n)$$

Where F_{obs} represent the observed signal at a given denaturant concentration, F_n represents the signal for the native protein and Fu represent the respective signal of the denatured protein. The values for Fn and Fu are obtained by extrapolating the linear dependence of the signal on the concentration of the denaturant before and after the transition, respectively. Denaturant blanks are used to ensure that the observed signal is specific to the peroxidase and not due to the presence of the denaturant. This equation is used in a technique called Spectroscopic Denaturation, which is used to study the stability and unfolding of proteins.

2.2.6 Thermal unfolding of peroxidase

Temperature-induced denaturation of the enzyme, under specified conditions was performed as a function of increasing temperature. The enzyme samples are heated to increasing temperatures and kept at each temperature for 15 minutes before measurements are taken. The temperature of the samples is monitored using a thermocouple and a digital multimeter to ensure that the desired temperature is reached and maintained. Additionally, the samples are checked for protein aggregation using light scattering measurements, which can indicate if the proteins have undergone irreversible denaturation due to heat. This method allows for the study of how temperature affects the stability and folding of the enzyme, which can provide insight into its function and potential for thermal inactivation.

2.2.7 Data analysis

The denaturation curves were plotted with ratio of fluorescence intensity at the emission wavelength maxima of the native protein and the denatured protein against the denaturant molarity or temperature. A two-state $N \rightleftharpoons U$ unfolding mechanism is assumed, meaning that at any point on the curve, only the folded and unfolded conformations are present in significant concentrations. The method used by Shirley (Shirley, 1995) is followed to obtain the transition midpoints and judge the cooperativity or non-cooperativity of the transitions. Shirley's method is a widely used way to analyze protein denaturation and it helps to identify the thermodynamic parameters of the transition and the mechanism of unfolding. For comparison of results obtained from different measurements such as molar ellipticity, fluorescence emission maximum, and relative activity, all the results are normalized and expressed as the fraction of unfolded protein. The fraction of unfolded protein, at any denaturant concentration, is calculated using the equation: fraction unfolded = (Fobs – Fn)/ (Fu – Fn), as previously mentioned.

3. Results

3.1 Absorbance spectroscopy

The structure-function relationship of *A.lakoocha* peroxidase can be studied using .various biophysical tools. These tools can be used to observe the intermediates of the enzyme at different pH levels and during denaturation, in order to understand how the structure of the enzyme affects its function.

The absorbance spectra of peroxidase showed four bands in the visible and ultraviolet (UV) regions: the α-band at ~630nm, the β-band at ~502nm, the γ-band or Soret band at 404nm and the absorbance spectra of peroxidase at 280 nm. These bands can be used as a tool to investigate structural and functional properties of *A.lakoocha* peroxidase. Changes in the wavelength or intensity of these bands upon ligand binding or unfolding can provide insight into the conformational changes of the enzyme. For example, substantial changes at 404 nm were observed upon ligand binding or unfolding, indicating changes in the tertiary and/or quaternary structure of the enzyme. However, changes at 280 nm were not observed under native and unfolded conditions, indicating that the secondary structure of the enzyme remains unchanged.

The shifts at 404 nm wavelength are evaluated to study the conformational change of the peroxidase. The absorbance spectra of *A. lakoocha* peroxidase in its native state, at pH 2.0 and in its unfolded state (6 M GuHCl) (fig.1B) are compared to observe the changes in the spectra.

Under native conditions, the absorbance spectra shows a soret peak at 404 nm, which is absent in the unfolded state. This indicates that the conformational change of the enzyme occurs upon unfolding. Additionally, Fig.1C shows the peroxidase absorbance maxima measured at 404 nm under varying pH. The bell-shaped pattern suggests that the peroxidase is stable in the range of pH 3.5 to 8.5, which corresponds to the enzyme's physiological pH range. This information is useful for understanding the stability and activity of the enzyme under different conditions.

3.2 . Fluorescence spectroscopy

The fluorescence spectra of peroxidase under native condition, at pH 2.0, 11.0 and with 6 M GuHCl have been shown in fig.2A. The intrinsic fluorescence spectrum of peroxidase in native state has an emission maximum at 333.07 nm indicating hydrophilic environment of its tryptophan residues. Under acidic and alkaline conditions, the fluorescence emission maximum remains the same but compared to the native protein, the intensity of fluorescence increases by 57.4% at pH 2.0 (178.43 to 280.88) and decreases by 16.42% at pH 11.0 (178.43 to 149.13). This indicates that changes in pH can affect the microenvironment of the tryptophan residues and influence their fluorescence properties. On complete unfolding of the protein in the presence of 6 M GuHCl, the emission maximum of peroxidase suffers a red shift of 8 nm (333.07 nm to 341.07 nm) along with approximately 175% increase in fluorescence intensity (from 178.43 to 491.04) compared to the native condition. This suggests that upon unfolding the tryptophan residues are exposed to a more hydrophobic environment, resulting in an increase in fluorescence intensity and a red shift in the emission maximum. The effect of different pH on A. lakoocha peroxidase was also observed by intrinsic fluorescence emission scale. The wavelength maximum of peroxidase as a function of pH has been shown in (Fig.2B). The transition curve did not show much change in the emission maximum under varying pH and appeared to occur through three state transitions. The first transition shows a red shift of 1 nm on decreasing pH from 4.0 to 2.0 under acid unfolded state. The second transition occurred between 6.0 and 8.5 pH along with a red shift of 2nm. On increasing the pH to the basic region leads to a third transition in the range of 9.0 to 10.5 pH, with a blue shift of 4 nm in the fluorescence emission maxima, resulting in the alkaline denatured state of peroxidase. The pH-induced transition is non-cooperative with slight change in transition maxima because tryptophan residues even in native state seem to be exposed

to the solvent. Changes in the fluorescence intensity as a function of pH are shown in fig.2C. Fluorescence intensity spectra displayed two transition phase. The first transition occurs from the native state to acid unfolded state (between pH 4.0 to pH 2.0) with a transition mid-point at pH 3.0, where the fluorescence intensity is increased marginally. The second transition towards basic pH is incomplete and takes place from pH 8.0 to 12.0. This study of intrinsic fluorescence spectra of peroxidase under different pH conditions provide insight into the conformational changes occurring in the enzyme and its stability at different pH.

3.3. ANS binding assay

ANS (8-anilino-1-naphthalene sulphonate) is also known as a polarity-sensitive extrinsic fluorescent probe. The effect of pH on ANS fluorescence of peroxidase at different pH is shown in fig.3A and B. ANS emission spectra were recorded with excitation at 380 nm. At pH 2.0, the protein shows partial ANS binding. On further decreasing the pH below 2.0, an increase in fluorescence intensity was observed. At pH 1.0, enzymes showed maximum ANS binding, compared to native state and shows up to 5-fold increases in fluorescence intensity with a blue shift in fluorescence maxima from 508.05 nm (pH 7.0) to 468.03 nm (pH 1.0). ANS fluorescence decreases on either side of 1.0 pH. At alkaline denatured state (UB state) ANS binding is almost absent and stable up to pH 12.0.

3.4. Circular dichroism

The effect of varying pH on the secondary structure of peroxidase was also studied. The CD spectra of *A. lakoocha* peroxidase at acidic, neutral and under denatured conditions (6 M GuHCl) are presented in fig.4A. At pH 7.0 in the far-UV region, the CD spectrum of peroxidase comprises of strong negative ellipticity at 208 and 222 nm, suggesting that the molecule may consists of α-helix and β-sheet rich regions and belongs to the α + β class of proteins (Kumar, Tripathi, de Moraes, Caruso, & Jagannadham, 2014; Manavalan, 1983). Fig.4A display that the mean residue ellipticity at 222 nm is -5300.2 deg.cm^2 dmol^{-1} in the native condition (Fig.4A) while under acidic state at 1.0 pH ellipticity at 222 nm shifts to -3779.9 deg.cm^2 dmol^{-1}.

However, under similar conditions, all the spectral features of peroxidase are lost in the presence of 6 M GuHCl, this indicate that the enzyme drops the secondary structural

organization under denaturing condition.

The structural changes of peroxidase according to pH are indicated by shifts in ellipticity at 208 and 222 nm as shown in fig.4B. These structural changes display that the secondary structural contents of protein remain same in the range of 4.0-9.5 pH and decrease on either side of this range, without changing the shape of the spectra. The changes are same in magnitude at lower pH and in the basic range.

3.5. Guanidine hydrochloride induced unfolding of peroxidase

Guanidine hydrochloride (GuHCl) induced unfolding of peroxidase under neutral conditions, when examined by alterations in secondary structure, absorbance and fluorescence, showed structural stability till 3.0 M GuHCl (Fig.5A). Under natural condition, GuHCl-induced unfolding results in cooperative sigmoidal curves (Fig.5A) with a transition midpoint at 4.5 ± 0.1 M GuHCl. However, at pH 2.0 enzymes displayed structural stability till 1.5 M GuHCl (Fig.5B). The transitions as measured by different spectroscopic techniques are cooperative and non-coincidental (Fig.5B). This observation illustrated that fluorescence intensity is lost first, followed by loss in secondary structure. Loss in secondary structure took place between 2.0 and 5.0 M of GuHCl, whereas, the changes in intrinsic fluorescence occurred between 1.5 and 5.0 M of GuHCl at pH 2 .0. The transition mid-points (Cm) of denaturation followed by far UV CD are 3.5 ± 0.1 M and by absorbance and fluorescence intensity is approximately 3.25 ± 0.1 M. The effect of GuHCl on the alkaline denatured state of a protein at pH 11.0 is similarly studied. The transitions as measured by different spectroscopic techniques are cooperative and coincidental (Fig.5C). Loss of secondary structure and intrinsic fluorescence took place between 2.5 and 5.0 M of GuHCl and enzyme showed structural stability till 2.0 M of GuHCl.

When ANS fluorescence was used to monitor the denaturation process under native and alkaline denatured state at pH 11.0, peroxidase does not specify any ANS binding, even after a prolonged exposure. That may be due to absence of contact with hydrophobic surface. These characteristic indicate that *A. lakoocha* peroxidase is more susceptible to denaturation in acidic conditions in comparison to alkaline conditions. So, it is meaningful to observe the structural integrity of the molecule at acidic pH. Fig.6A shows ANS binding pattern of peroxidase at pH 2.0 with increasing concentration of GuHCl. Under this acid denatured state, a decrease in ANS fluorescence intensity by increasing molarity of GuHCl coupled with red shift in its wavelength

maximum was found with GuHCl at pH 2.0.

ANS binding to peroxidase at pH 2.0 in the presence of 2.5M GuHCl and above suggest the absence of surface hydrophobic patches, since no ANS binding was observed. While, on decreasing the concentration of GuHCl, one intermediate was found at 1.0 M GuHCl. Here the protein shows maximum ANS binding.

3.6. Urea induced unfolding of peroxidase

Under neutral conditions, urea does not cause any structural agitation in the protein, as enzyme retains its maximum structural and functional parameters even in 5.5 M urea concentration (Fig.7A). The urea induced unfolding studies are therefore carried out at low and high pH, where enzyme is more susceptible to denaturants. However, urea induced unfolding at lower pH yielded some interesting facts. The denaturation is followed by absorbance, far UV CD and fluorescence. At pH 2.0, the urea induced unfolding is cooperative, but non-coincidental (Fig.7B). The transition mid-points for intrinsic fluorescence and absorbance are 4.25 ± 0.1 M and for secondary structure are 4.5 ± 0.3 M. The loss in secondary structure occurred between 2.0 and 7.0 M urea. But, at pH 11.0, where enzyme is in alkaline denatured state, the unfolding transitions by different probes are cooperative with a transition mid-point at 4.75 ± 0.1M (Fig.7C).

The cooperative but non-coincidental transitions at pH 2.0, along with an increase in ANS binding at low concentration of urea (Fig.8) indicate that a discrete intermediate state exists in the urea induced unfolding of peroxidase at pH 2.0. Increase in ANS fluorescence Intensity coupled with red shift in its wavelength maximum of peroxidase at pH 2.0 is shown in fig.8A and B.

3.7. Thermal denaturation of peroxidase

Temperature, the oldest mode of protein denaturation can induce structural changes and hence provide ample information about a protein molecule. The temperature induced unfolding of peroxidase at pH 7.0 was measured (Fig.9). The transition midpoints of temperature induced unfolding of peroxidase are very high and comes in the range 62.5 ± 0.5°C. Further, the structure of the protein melts at much higher temperature at 75°C indicating marked structural agitations already induced by increased concentration of protons.

4. Discussion

In the era of proteomics, it is becoming very important to understand the structure-function relationship of proteins using various biophysical tools (Neet & Lee, 2002). Despite the importance of peroxidase, relatively few biophysical studies have been carried out on this class of proteins. Because of the dependency of catalytic activity on pH; stability of an enzyme decides its functionality and may limits its wide application. Therefore, pH denaturation of peroxidase was monitored by various spectroscopic techniques to probe the different aspects of conformational changes in its structure and identification of populated intermediates at extremes of pH, during denaturation.

4.1. Effect of pH on absorbance spectra of A. lakoocha peroxidase

The absorbance technique is used to measure the absorption of radiation by a sample, as a function of wavelength or frequency. The absorbance spectra of the protein at different pH levels are shown in Fig.1A, which shows spectra of the protein under neutral conditions that are similar to characteristic heme peroxidase spectra. The absorption bands in the visual spectrum region (α- and β-peaks) and at the boundary of the visual and UV parts (Soret γ) are due to the intensively colored prosthetic groups of peroxidase. Other absorption bands of peroxidase solution are located in the UV region: φ-band with the maximum of 280 nm, indicating a significant contribution from tryptophan and tyrosine residues also due to the light absorption by aromatic amino acids in protein. As revealed from Fig.1A, peroxidase spectra do not show much change in the range of pH 2.5 to 8.5, which indicate good stability of peroxidase in this state. But at lower pH, heme peroxidase spectra express a left shift with a declined absorbance at 404 nm due to denaturation, which is a clear indication of free heme in the range of pH 1.0 to 2.0. This result specifies that at lower pH peroxidase releases its prosthetic group (heme). Whereas beyond pH 8, the heme group of peroxidase shows OH- or H2O binding, with an increase in pH and results in right shifting of soret band. At higher pH, Q-band of peroxidase gets split and the soret peak shifted to the right. Though the shift in absorbance intensity at 404 nm (Soret peak) gives information regarding the stability and activity of peroxidase, this shift at 404 nm wavelength is investigated further to follow the conformational change of the protein in the present investigation.

The spectra in Fig.1B show a decrease in the soret band of peroxidase at pH 2.0 and 6 M

GuHCl, indicating the loss of the heme group and denaturation of the protein. Fig.1C shows that the enzyme retains proteolytic activity in the pH range of 3.5-8.5 and drops on either side of it, suggesting that the native structure and functional stability of the protein is maintained in this pH range. The decline in proteolytic activity outside of this range is likely due to the repulsion of columbic forces from the positive charges of the polypeptide chains.

4.2. Fluorescence spectroscopy

The fluorescence emission maximum of chromophores, such as tyrosine and tryptophan residues, can change when exposed to different solvent environments. The quantum yield of fluorescence can also decrease when the chromophores interact with quenching agents in the solvent or within the protein. In the case of *A. lakoocha* peroxidase, the fluorescence spectrums under different pH and denaturing conditions are shown in fig.2A. The tryptophan excitation spectra reflect the average environment of the tryptophan, and show a red shift in the emission maximum when the chromophores are exposed to solvent. The results of the experiment, as observed in fig.2A, indicate that the tryptophan residues become exposed to solvents due to unfolding and disclosure of chromophores to the solvent, particularly under denaturing conditions with 6 M GuHCl. Additionally, the intensity of fluorescence increases at pH 2.0, but decreases at pH 11.0, likely due to the presence of a heme group in the peroxidase that acts as a main quencher and quenches the energy in the native state. However, upon denaturation with 6 M GuHCl, the fluorescence intensity is amplified compared to the acidic condition, indicating the complete exposure of tryptophan residues to an aqueous environment.

the observations suggest that the fluorescence intensity of the peroxidase protein increases as the protein loses iron upon denaturation. The pH-induced transition of peroxidase in the alkaline range, as monitored by fluorescence intensity, seems to be cooperative, with a transition mid-point at pH 3.0. The fluorescence wavelength maxima remained largely unaffected throughout different pH values (Fig.2B), but the fluorescence intensity revealed changes at different pH values (Fig.2C). Lower pH values resulted in higher fluorescence intensity, while higher pH values resulted in a decrease in fluorescence intensity, likely due to quenching of tryptophan fluorescence by specific quenching groups such as protonated carboxyl and imidazole groups.

4.3. Acid-induced changes in peroxidase

ANS binding is a tool used to measure exposure of hydrophobic surfaces in enzymes. The magnitude of ANS binding can indicate the structural integrity of a protein. In the case of *A. lakoocha* peroxidase, ANS binding was absent at higher pH values (> pH 3.0) fig.3A but present at lower pH values. At pH 2.0, the protein enters a pre-molten globule state, characterized by partial ANS binding and a significant amount of secondary structure. As the pH is decreased to acidic values, the protein becomes acid-unfolded, characterized by strong ANS binding due to protonation of amino acid residues leading to charge-charge repulsions. On decreasing the pH even further, the protein refolds and forms an acid-refolded state caused by shielding of Columbic repulsions by counter ions. The results of the experiment as observed in fig.3A show that the peroxidase retains its proteolytic activity and native structure within a pH range of 3.0-10.0, indicated by the absence of ANS binding in this range, suggesting that the protein has a relatively stable and rigid native conformation in a wide range of pH values, but becomes more flexible and susceptible to changes in acidity.

4.4. Circular dichroism

The use of Circular Dichroism (CD) spectroscopy and hydrophobic dye ANS allowed for the study of the structure and stability of *A. lakoocha* peroxidase. The Far-UV CD spectra of the enzyme (Fig.4A) showed it to be consistent with the α + β class of proteins and possibly contains distinct α and β-rich domains. The peroxidase was found to be quite stable in terms of secondary structure, with only a 50% decrease in ellipticity at lower pH. However, under denaturing conditions, the peroxidase lost all prominent peaks of aromatic regions, indicating the loss of secondary structural features and the enzyme being in an unfolded state.

The shifts in ellipticity at varying pH were found to not follow a simple two-state transition, but rather a three-step transition (Fig.4B). The first transition from the native state to acid unfolded state occurred between pH 4.0 – 1.0, with a half bell shape in the range of pH 1.0 – 4.0, revealing the loss of some prominent peaks. This loss of secondary structure, as well as reduced activity, represents the enzyme acid unfolded state. The second transition occurred between pH 4.0 to 8.0 under native conditions, and the third transition occurred between pH 8.0 - 12.0 with a mid-point at 10.5pH. The use of hydrophobic dye ANS also revealed that despite the

melting of its secondary structure, large amount of hydrophobic clusters were left over in the acid-unfolded state(Fig.3A),.

4.5. GuHCl induced unfolding of peroxidase

The analysis of denaturation curves induced by GuHCl can provide information about the conformational stability of the enzyme. The denaturation of peroxidase by GuHCl at neutral pH is a cooperative and two-state process with a high transition mid-point of about 4.5 ± 0.1M fig.5A. However, at pH 2.0 and 11.0, the transition mid-points are less as compared to the same parameters at pH 7.0. When ANS fluorescence was used to monitor the denaturation process, enzyme only reveals ANS binding with acidic condition (pH 2.0). At pH 2.0 partial ANS binding as well as other spectroscopic properties exist due to increase of distance from the heme group, which serves as a quencher in heme containing peroxidase fig.6B. There is absence of ANS binding to peroxidase at 3 M GuHCl and above, which suggests the absence of surface hydrophobic patches and refolding and stabilization due to specific stabilizing interactions between the intermediate state and GuHCl. In the native and alkaline denatured state protein, there is no ANS binding and the alkaline denatured state has significantly reduced secondary structure compared to acid unfolded state, suggesting it to be a distinct intermediate.

4.6. Urea induced unfolding of peroxidase

The stability of the enzyme peroxidase against urea was evaluated under different pH conditions. Results revealed that at neutral pH, the enzyme retained its native structure and function even when exposed to 5.5M urea (Fig.7A), indicating stability against urea. However, at acidic pH (2.0), urea caused the enzyme to unfold cooperatively, with transition midpoints for secondary and tertiary structures at 4.5 ± 0.3M urea and 4.25 ± 0.1M urea (Fig.7B), respectively. The unfolding process was observed using far-UV CD and fluorescence, which revealed cooperative and non-overlapping transitions (Fig.7B) along with an increase in ANS binding at low concentrations of urea (Fig.8A & B). Additionally, ANS fluorescence intensity, which is highest at pH 2.0 in the absence of denaturant, decreased parallel with an increase in urea concentration. The sigmoidal change in fluorescence intensity with urea at pH 2.0 is attributed to

configurational changes in the amino acids. The unfolding process at pH 2.0 showed the same changes in far-UV CD and wavelength maxima as at pH 7.0. However, complete loss of activity above 5M urea, sigmoidal increase in fluorescence intensity, ANS binding, and conversion to beta-sheet-like spectra seen by far-UV CD above 3.5M urea, were due to perturbation of the enzyme's functional structure (Fig.7B). These results suggest that a discrete intermediate state exists in the urea-induced unfolding of peroxidase.

Overall, the study found that the enzyme peroxidase unfolds differently when exposed to urea and guanidine hydrochloride (GuHCl) under similar conditions. The results showed that urea is less effective than GuHCl in reducing the mid-points of unfolding at a given concentration. This difference in behavior is likely due to the ionic character of the two denaturants. Urea and GuHCl interact with peptide bonds but have different electrostatic and hydrophobic properties. GuHCl, which is positively charged, may stabilize the protein through its anionic effect, while urea does not have this effect. Additionally, the counter-anion of GuHCl, chloride, may shield the electrostatic repulsion and allow forces that favor the folding and stability of the protein. However, the stabilizing effect of GuHCl may not always be predominantly ionic in nature(Zarrine-Afsar, Mittermaier, Kay, & Davidson, 2006). The study also discussed the effects of temperature on the structure and activity of peroxidase from *A. lakoocha*. It was found that the enzyme retained approximately 45% structure under neutral conditions and had a high stability, with a transition midpoint (Tm) of 62.5±0.5°C (Fig.9). The study summarized the complete unfolding Parameters of *A. lakoocha* peroxidase in Table 1 and presented a model for the folding-unfolding of the enzyme based on data's and observations which can be presented as shown in fig.10. It is found that peroxidase may possess two domains which are behaving differently in various denaturing conditions leading to the formation of a number of intermediate states.

5. Conclusion

In this study, seven different folding states of *A. Lakoocha* peroxidase were observed using various spectroscopic techniques under various temperature, pH, and denaturing conditions. At pH 7.0, the enzyme is in the native state with proteolytic activity, intact secondary structure, and no hydrophobic patches. At lower pH values, the enzyme transitions through intermediate states such as the pre-molten globule state and the acid-unfolded state. Under

denaturing conditions, the enzyme can reach a complete denatured state. This study is the first to report the existence of multiple partially folded intermediate states of *A. Lakoocha* peroxidase and suggests that it could be a useful model system for further studies on folding and unfolding factors, as well as applications in biotechnology and pharmaceuticals due to its stability under a wide range of conditions.

Conflict of interest

The authors declare no competing financial interest.

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Figure captions:

Fig.1. pH-induced conformational changes of peroxidase: (A) UV absorbance spectra at different pH (B) Spectra at pH 7.0 and at 6 M GuHCl have been compared with spectra at pH 2.0 (free of heme) (C) The absorbance spectrum at 404 nm under different pH.

Fig.2. pH-induced conformational changes of peroxidase: (A) fluorescence spectra at different pH and in 6.0M GuHCl (B) fluorescence wavelength maxima (C) fluorescence intensity at different pH.

Fig.3. Effect of pH on ANS fluorescence of A. lakoocha peroxidase: (A) ANS binding to peroxidase were studied at pH 1.0, pH 1.5, pH 2.0, pH 2.5, pH 7.0 and in the presence of 6M GuHCl. (B) For intrinsic fluorescence measurement samples were incubated for 24 h at 25oC. ANS emission spectra were recorded with excitation at 380nm.

Fig.4. pH induced conformational changes of peroxidase: (A) far-UV CD spectra at different pH and in 6.0 M GuHCl (B) Ellipticity at 208 nm and 222 nm as a function of pH.

Fig.5. GuHCl induced unfolding of peroxidase at different pH: (A) pH 7.0, (B) pH 2.0 and (C) pH 11.0, by following far-UV CD, Fluorescence and UV absorbance.

Fig.6. Effect of pH on ANS fluorescence of *A. lakoocha* peroxidase: (A) ANS binding spectra, as a function of increasing concentration of GuHCl at pH 2.0 (B) ANS fluorescence intensity and wavelength maxima at pH 2.0 as a function of increasing concentration of GuHCl

Fig.7. Urea induced unfolding of peroxidase at different pH: (A) pH 7.0, (B) pH 2.0 and (C) pH 11.0, by following far-UV CD, Fluorescence and UV absorbance

Fig.8. Effect of pH on ANS fluorescence of *A. lakoocha* peroxidase: (A) ANS binding spectra, as a function of increasing concentration of Urea at pH 2.0 (B) ANS fluorescence intensity and wavelength maxima at pH 2.0 as a function of increasing concentration of Urea

Fig.9. Temperature induced unfolding of peroxidase at pH 7.0: The structural changes are followed by UV absorbance, far- UV CD, and fluorescence.

Fig.10. Scheme: unfolding factor found under several experimental circumstances like pH, chemical denaturant and organic solvents.

Fig.1

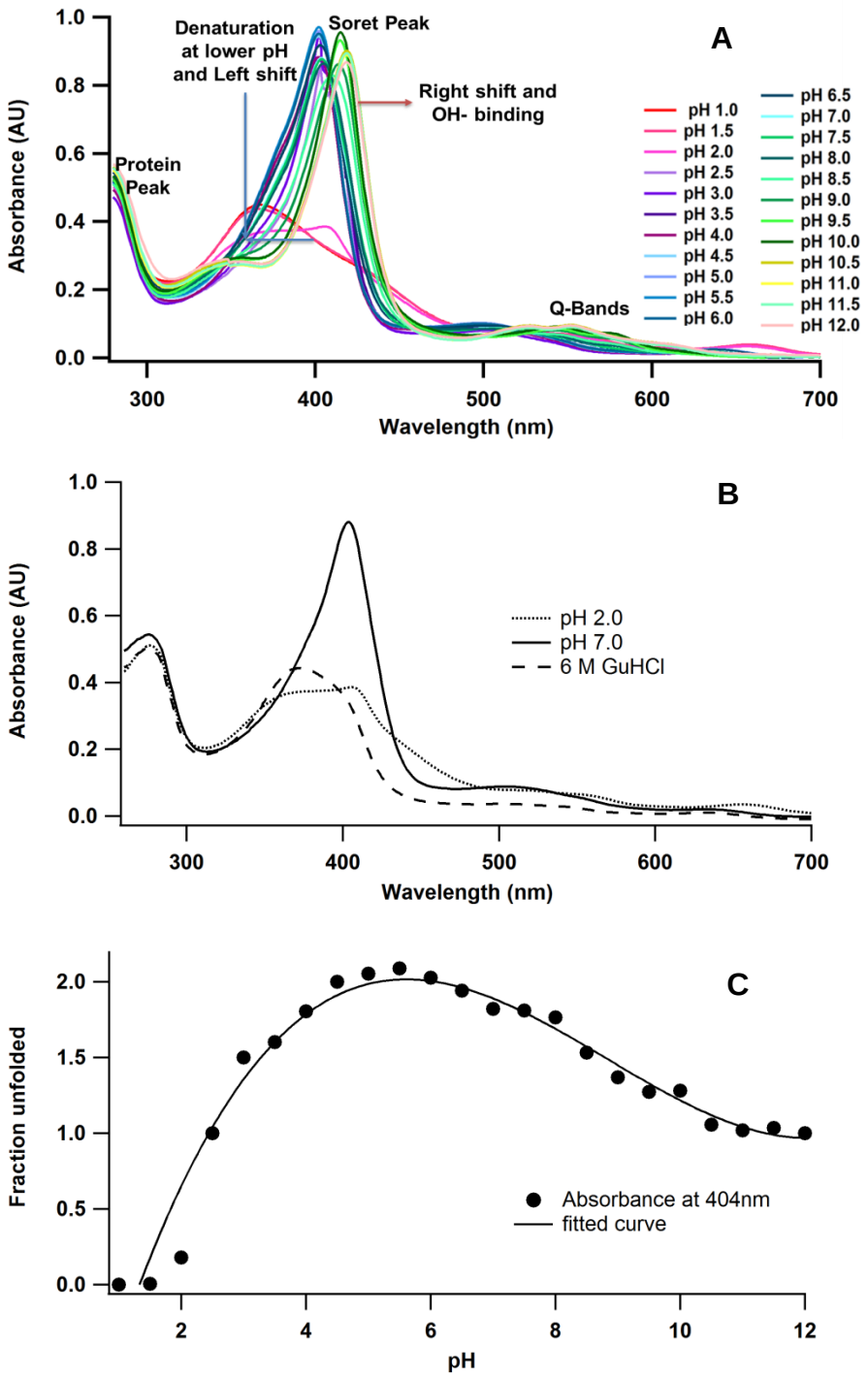

A
Denaturation at lower pH and Left shift
Soret Peak
Right shift and OH- binding
Protein Peak
Q-Bands
pH 1.0
pH 1.5
pH 2.0
pH 2.5
pH 3.0
pH 3.5
pH 4.0
pH 4.5
pH 5.0
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
pH 9.0
pH 9.5
pH 10.0
pH 10.5
pH 11.0
pH 11.5
pH 12.0
Absorbance (AU)
Wavelength (nm)
B
pH 2.0
pH 7.0
6 M GuHCl
Absorbance (AU)
Wavelength (nm)
C
Absorbance at 404nm
fitted curve
Fraction unfolded
pH

Fig.2

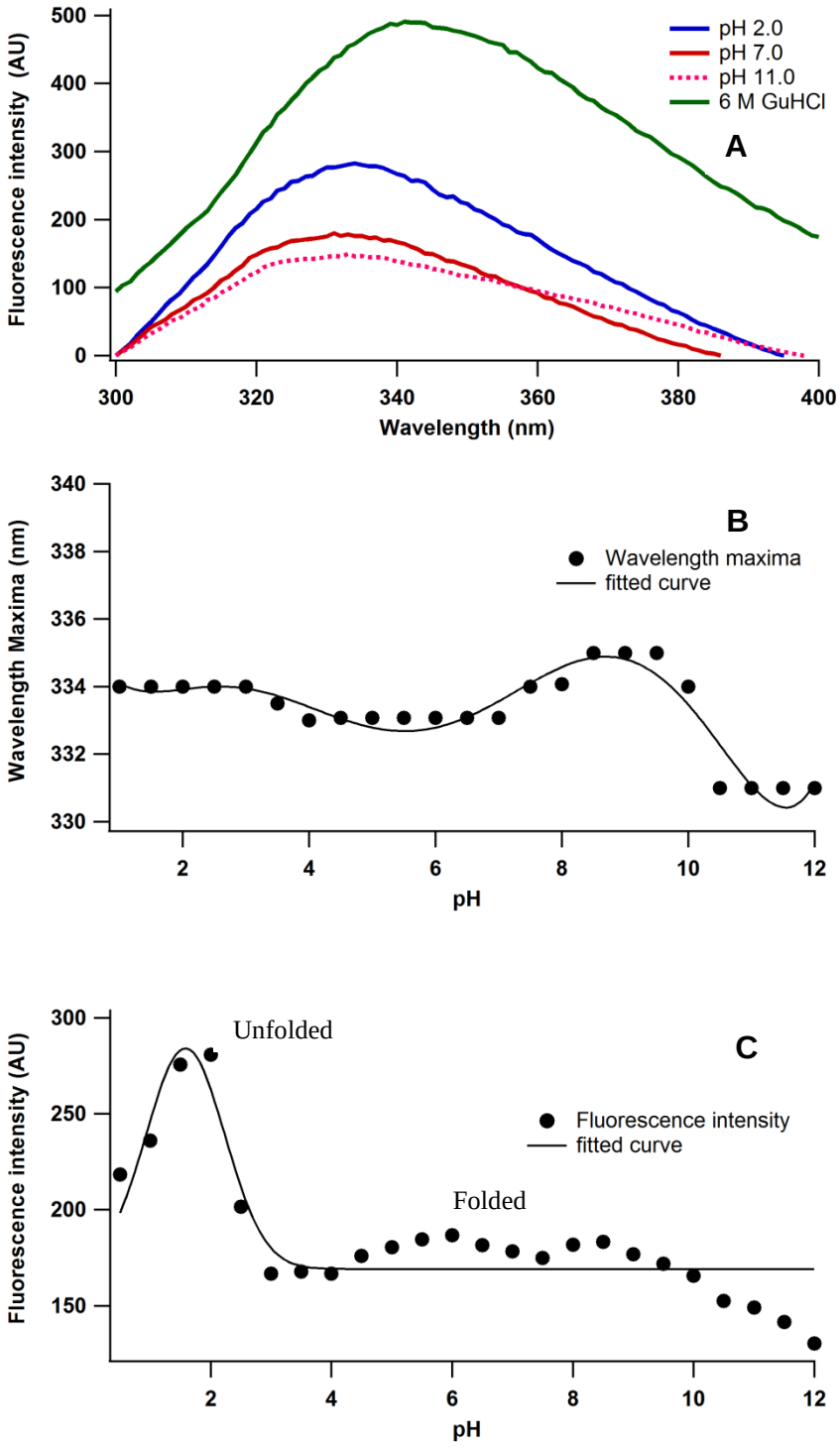

A
pH 2.0
pH 7.0
pH 11.0
6 M GuHCl
Fluorescence intensity (AU)
Wavelength (nm)
B
Wavelength maxima
fitted curve
Wavelength Maxima (nm)
pH
C
Unfolded
Folded
Fluorescence intensity
fitted curve
Fluorescence intensity (AU)
pH

Fig.3

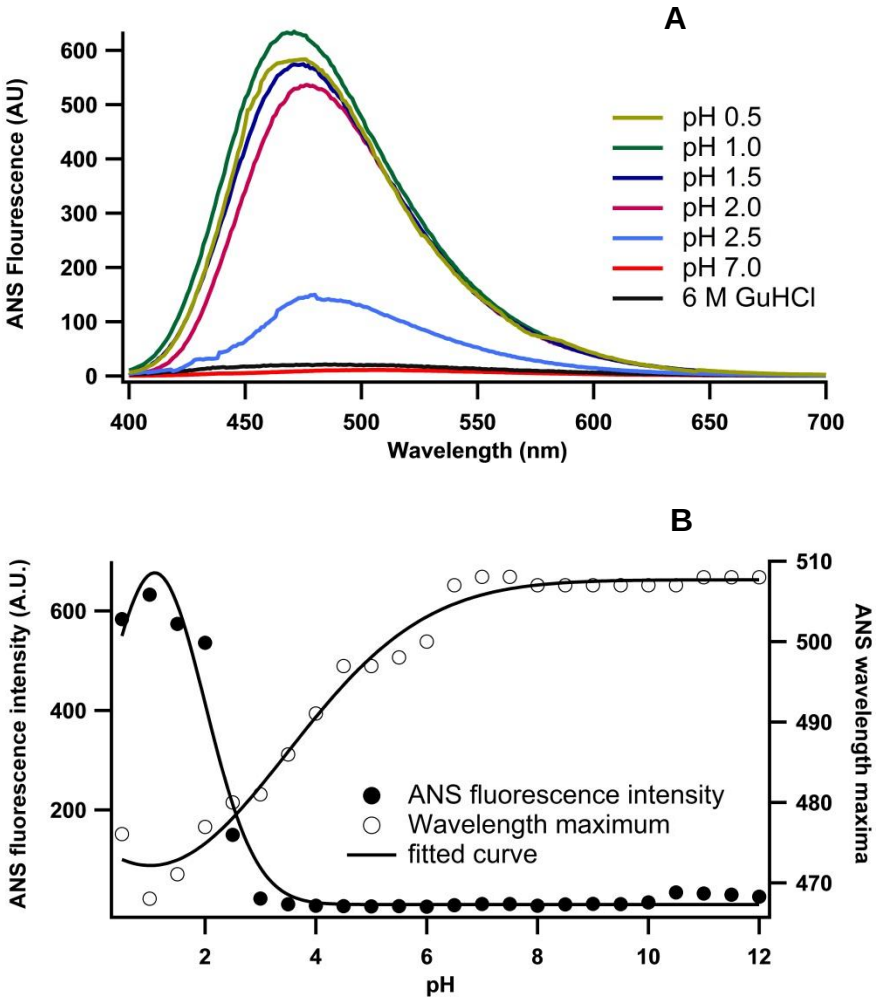

A
ANS Flourescence (AU)
Wavelength (nm)
pH 0.5
pH 1.0
pH 1.5
pH 2.0
pH 2.5
pH 7.0
6 M GuHCl
B
ANS fluorescence intensity (A.U.)
ANS wavelength maxima
pH
ANS fluorescence intensity
Wavelength maximum
fitted curve

Fig.4

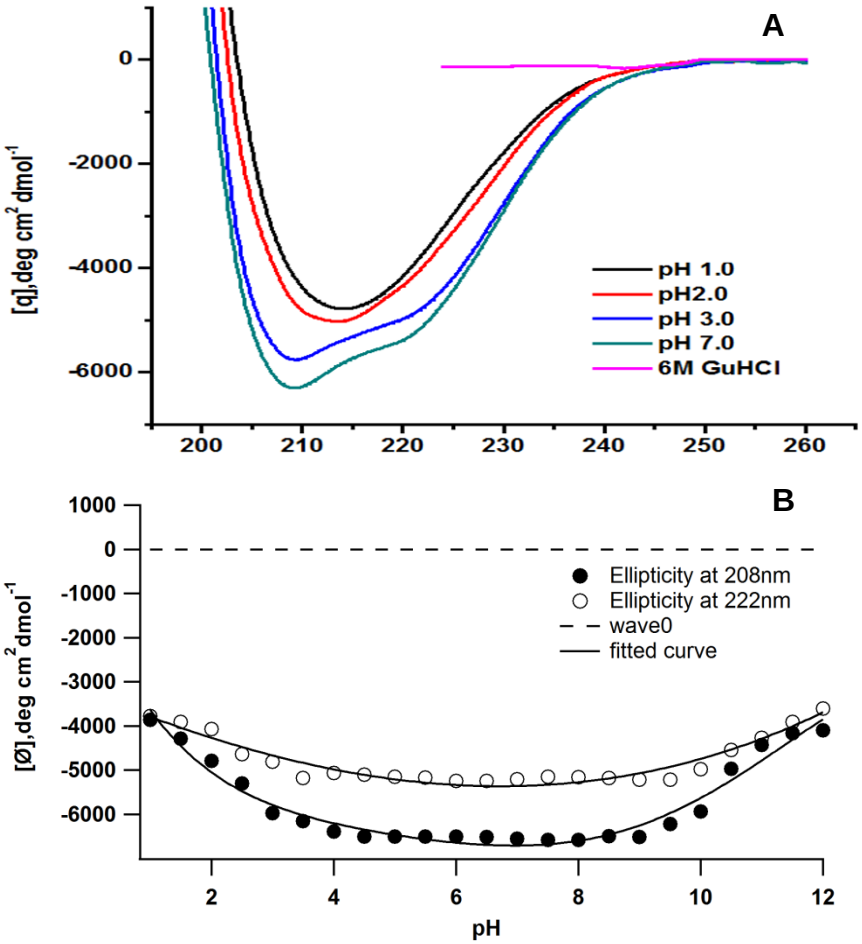
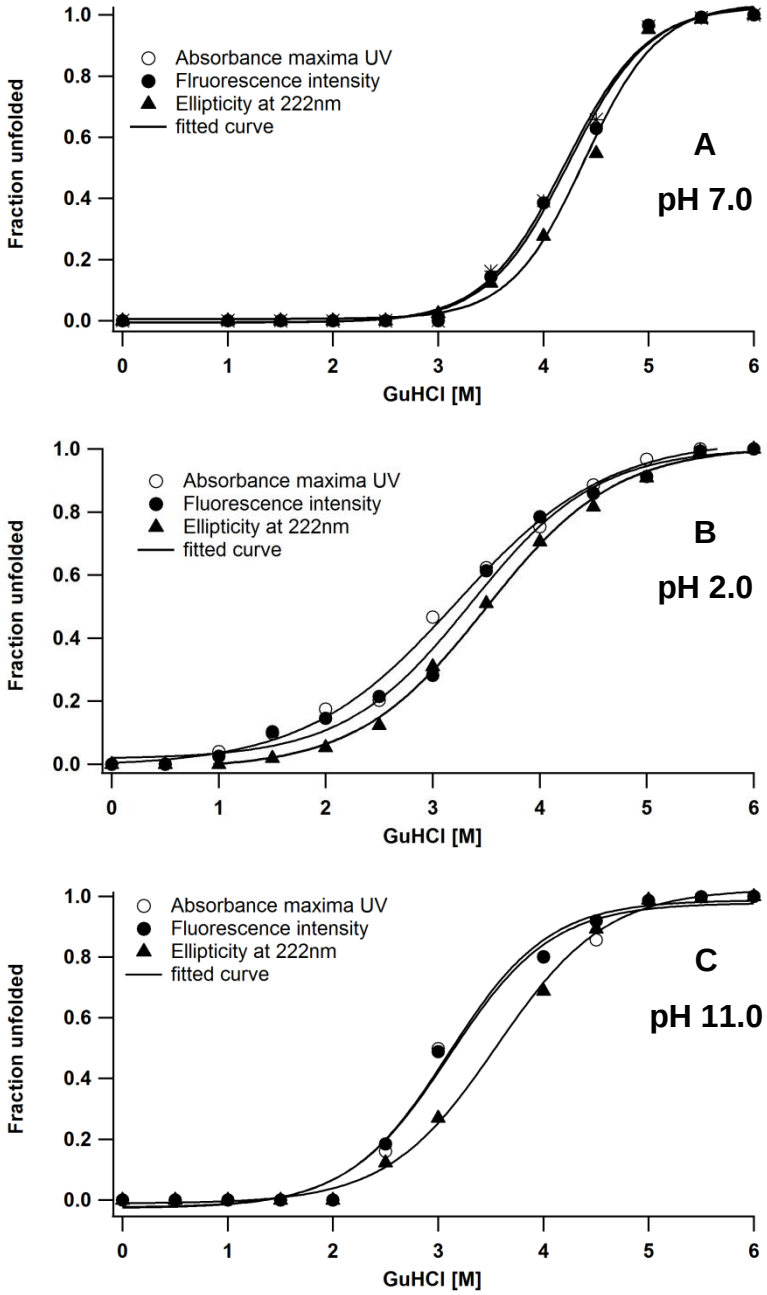

A
0
-2000
-4000
-6000
[q],deg cm2 dmol-1
200
210
220
230
240
250
260
pH 1.0
pH2.0
pH 3.0
pH 7.0
6M GuHCl
B
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
[Ø],deg cm2 dmol-1
2
4
6
8
10
12
pH
Ellipticity at 208nm
Ellipticity at 222nm
wave0
fitted curve

Fig.5


Absorbance maxima UV
Flruorescence intensity
Ellipticity at 222nm
fitted curve
A
pH 7.0
Fraction unfolded
GuHCl [M]
Absorbance maxima UV
Fluorescence intensity
Ellipticity at 222nm
fitted curve
B
pH 2.0
Fraction unfolded
GuHCl [M]
Absorbance maxima UV
Fluorescence intensity
Ellipticity at 222nm
fitted curve
C
pH 11.0
Fraction unfolded
GuHCl [M]

*F*ig.6

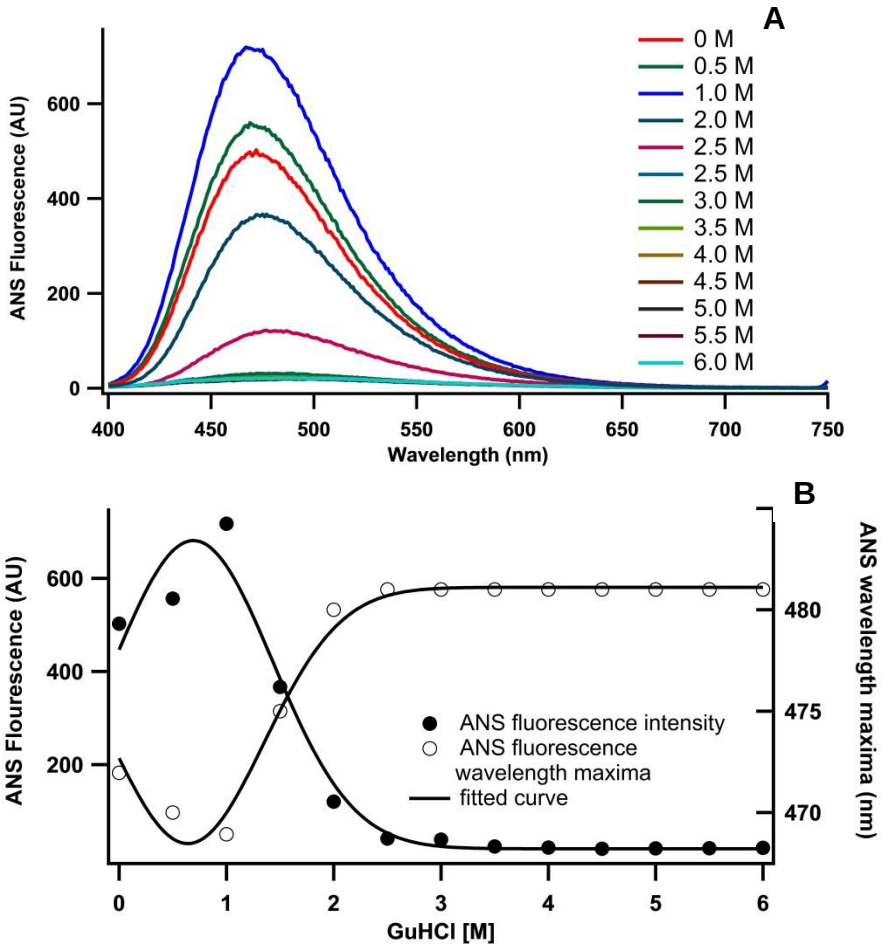

A
0 M
0.5 M
1.0 M
2.0 M
2.5 M
2.5 M
3.0 M
3.5 M
4.0 M
4.5 M
5.0 M
5.5 M
6.0 M
ANS Fluorescence (AU)
Wavelength (nm)
B
ANS Flourescence (AU)
ANS wavelength maxima (nm)
ANS fluorescence intensity
ANS fluorescence wavelength maxima
fitted curve
GuHCl [M]

Fig.7

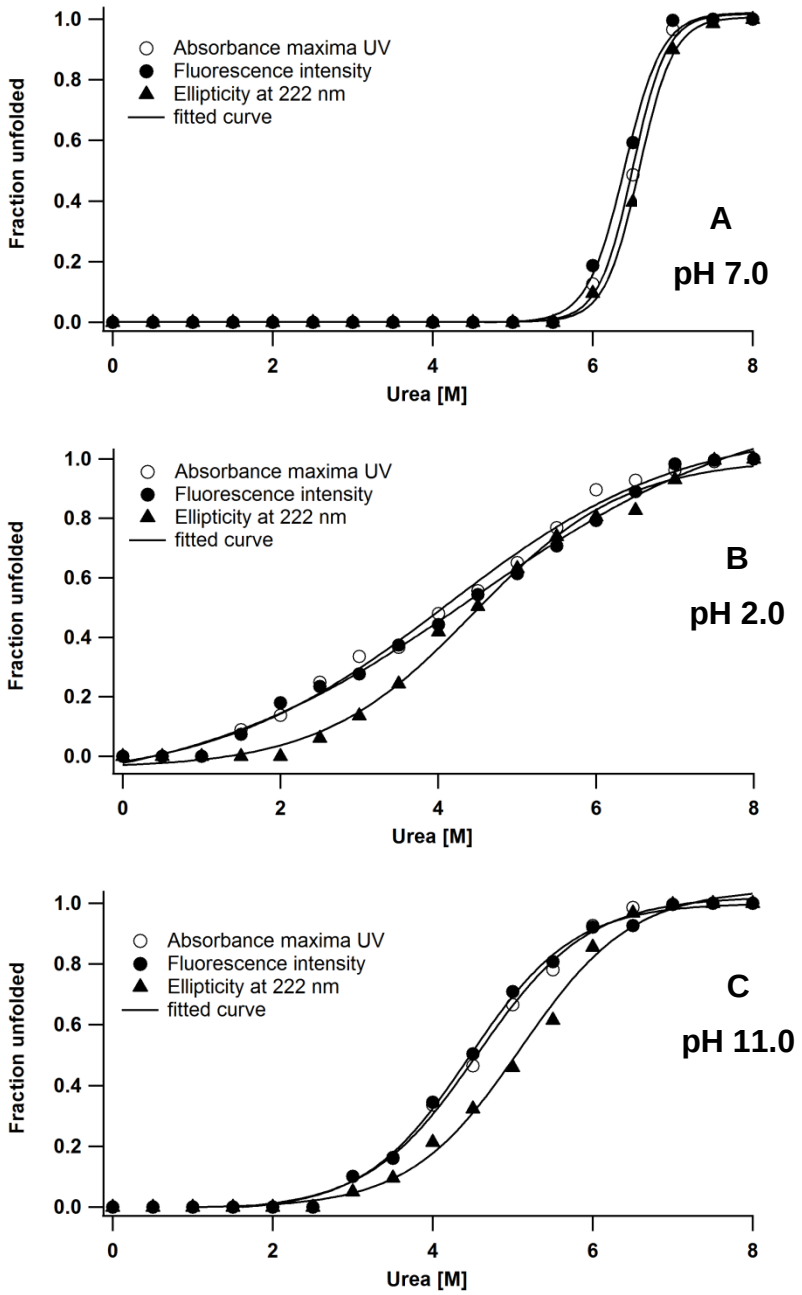

Absorbance maxima UV
Fluorescence intensity
Ellipticity at 222 nm
fitted curve
Fraction unfolded
Urea [M]
A
pH 7.0
B
pH 2.0
C
pH 11.0

Fig.8

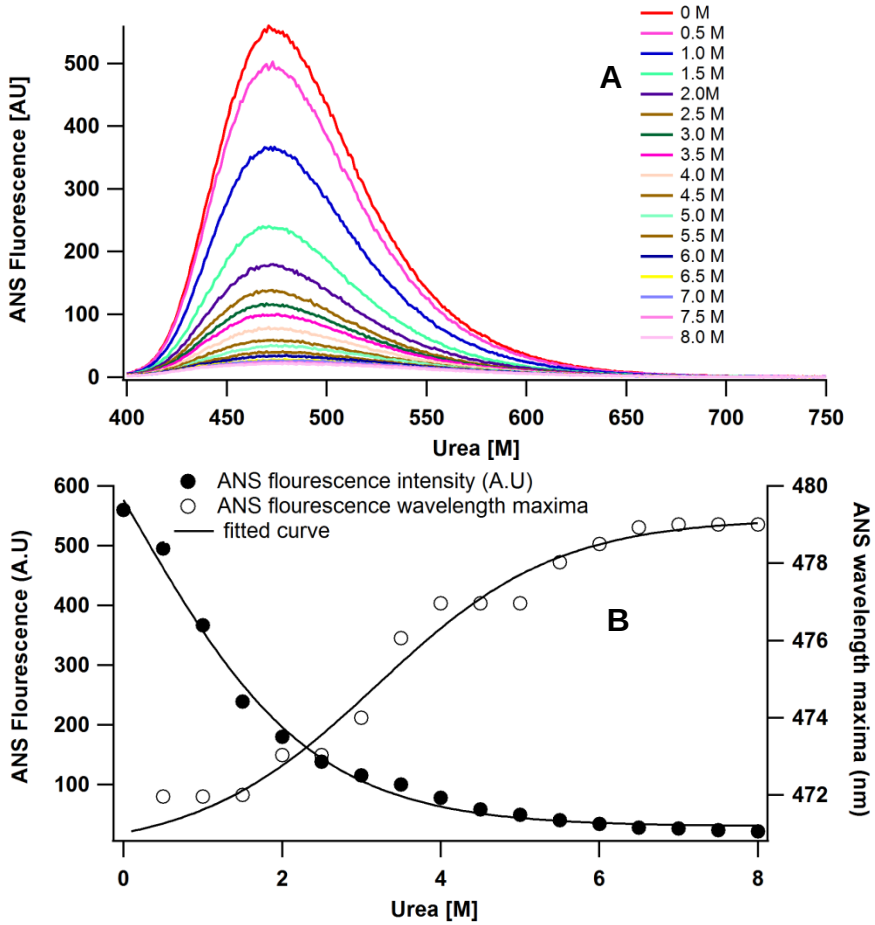

A
0 M
0.5 M
1.0 M
1.5 M
2.0M
2.5 M
3.0 M
3.5 M
4.0 M
4.5 M
5.0 M
5.5 M
6.0 M
6.5 M
7.0 M
7.5 M
8.0 M
ANS Fluorescence [AU]
Urea [M]
B
ANS flourescence intensity (A.U)
ANS flourescence wavelength maxima
fitted curve
ANS Flourescence (A.U)
ANS wavelength maxima (nm)
Urea [M]

Fig.9

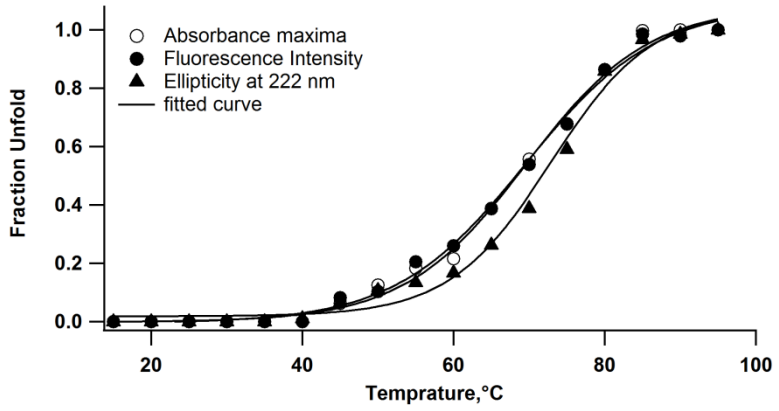

Absorbance maxima
Fluorescence Intensity
Ellipticity at 222 nm
fitted curve
Fraction Unfold
Temprature,°C

Fig.10

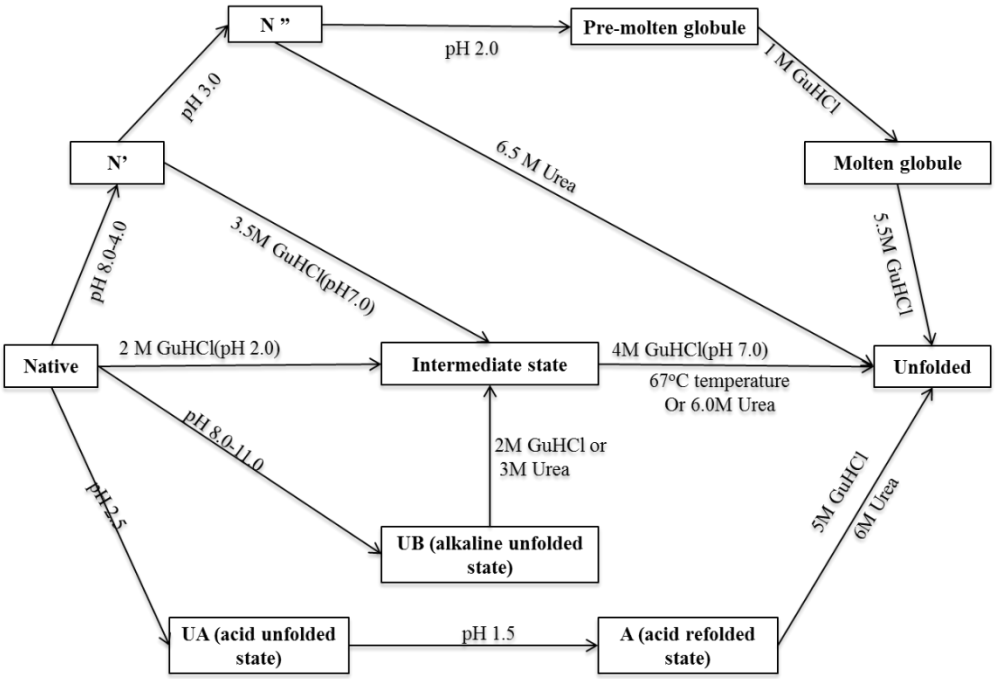

N ”
Pre-molten globule
pH 2.0
1 M GuHCl
pH 3.0
N’
6.5 M Urea
Molten globule
pH 8.0-4.0
3.5M GuHCl(pH7.0)
5.5M GuHCl
Native
2 M GuHCl(pH 2.0)
Intermediate state
4M GuHCl(pH 7.0)
67°C temperature
Or 6.0M Urea
Unfolded
pH 8.0-11.0
2M GuHCl or
3M Urea
pH 2.5
5M GuHCl
6M Urea
UB (alkaline unfolded state)
UA (acid unfolded state)
pH 1.5
A (acid refolded state)

Table.1: Unfolding parameters of *A. lakoocha* peroxidase

Condition	Denaturant	Method	Transition mid-point
pH 7.0 Native state	GuHCl	$CD[\Theta]_{222}$	4.5 ± 0.1 M
		Fluorescence	4.5 ± 0.1 M
		absorbance	4.5 ± 0.1 M
	Urea	$CD[\Theta]_{222}$	6.5 ± 0.1 M
		Fluorescence	6.5 ± 0.1 M
		absorbance	6.5 ± 0.1 M
	Temperature	CD[Θ]222	65.0 ± 0.5°C
		Fluorescence	62.5 ± 0.5°C
		absorbance	62.5 ± 0.5°C
pH 2.0 Pre-molten globule state	GuHCl	$CD[\Theta]_{222}$	3.5 ± 0.1 M
		Fluorescence	3.25 ± 0.1 M
		absorbance	3.25 ± 0.1 M
	Urea	$CD[\Theta]_{222}$	4.5 ± 0.3 M
		Fluorescence	4.25 ± 0.1 M
		absorbance	4.25 ± 0.1 M
pH 11.0 Alkaline denatured state	GuHCl	$CD[\Theta]_{222}$	3.5 ± 0.1 M
		Fluorescence	3.5 ± 0.1 M
		absorbance	3.5 ± 0.1 M
	Urea	$CD[\Theta]_{222}$	4.75 ± 0.1 M
		Fluorescence	4.75 ± 0.1 M
		absorbance	4.75 ± 0.1 M