

**Original article:**

**CONFORMATIONAL CHANGES OF A CHEMICALLY  
MODIFIED HRP: FORMATION OF A MOLTEN GLOBULE  
LIKE STRUCTURE AT pH 5**

Kourosh Bamdad<sup>1,\*</sup>, Bijan Ranjbar<sup>2</sup>, Hossein Naderi-Manesh<sup>2</sup>, Mehdi Sadeghi<sup>2</sup>

<sup>1</sup> Department of Biology, Payame Noor University (PNU), Tehran, Iran

<sup>2</sup> Department of Biophysics, Faculty of Biological Sciences,  
Tarbiat Modares University, Tehran 14115-154, Iran

\* Corresponding author: Kourosh Bamdad; Tel/Fax: +987125230710;  
E-mail: k.bamdad@farspnu.ac.ir

**ABSTRACT**

Horseradish peroxidase is an all alpha-helical enzyme, which widely used in biochemistry applications mainly because of its ability to enhance the weak signals of target molecules. This monomeric heme-containing plant peroxidase is also used as a reagent for the organic synthesis, biotransformation, chemiluminescent assays, immunoassays, bioremediation, and treatment of wastewaters as well. Accordingly, enhancing stability and catalytic activity of this protein for biotechnological uses has been one of the important issues in the field of biological investigations in recent years. In this study, pH-induced structural alterations of native (HRP), and modified (MHRP) forms of Horseradish peroxidase have been investigated. Based on the results, dramatic loss of the tertiary structure and also the enzymatic activity for both forms of enzymes recorded at pH values lower than 6 and higher than 8. Ellipticity measurements, however, indicated very slight variations in the secondary structure for MHRP at pH 5. Spectroscopic analysis also indicated that melting of the tertiary structure of MHRP at pH 5 starts at around 45° C, which is associated to the pK<sub>a</sub> of His 42 that has a serious role in keeping of the heme prosthetic group in its native position through natural hydrogen bond network in the enzyme structure.

According to our data, a molten globule like structure of a chemically modified form of Horseradish peroxidase at pH 5 with initial steps of conformational transition in tertiary structure with almost no changes in the secondary structure has been detected. Despite of some conformational changes in the tertiary structure of MHRP at pH 5, this modified form still keeps its catalytic activity to some extent besides enhanced thermal stability. These findings also indicated that a molten globular state does not necessarily preclude efficient catalytic activity.

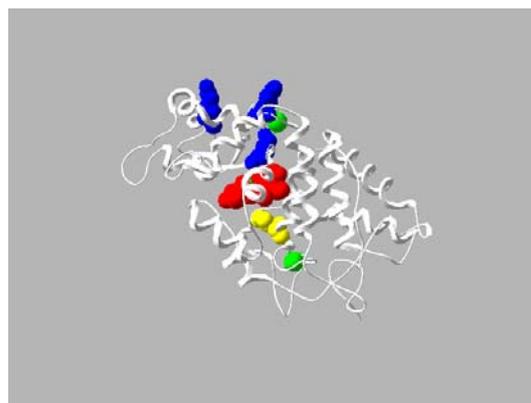
**Keywords:** Horseradish peroxidase, conformational transition, molten globule like structure

**INTRODUCTION**

Peroxidases are a class of heme-containing enzymes that are catalytically active in the ferric form, oxidizing several substrates such as cytochrome c, substituted phenols, and some of the more negative

methoxybenzenes (Sakurada et al., 1986; Kersten et al., 1990). According to the origin, peroxidases are generally divided into three classes including prokaryotes (class I), fungi (class II), and plant peroxidases (class III) (Welinder, 1992). Horseradish peroxidase isoenzyme C (HRP, EC 1.11.1.7), one

of the best-characterized peroxidases, belongs to class III, which its X-ray structure has been reported in Protein Data Bank (Gajhede et al., 1997). The structure of this enzyme, like the other peroxidases such as peanut peroxidase (Schuller et al., 1996), and the major peroxidases from barley (Henriksen et al., 1998), shows the similar overall protein fold with two  $\text{Ca}^{2+}$  ions buried in the proximal and distal portions of the heme pocket (Figure 1). This monomeric heme-containing plant peroxidase is widely used as a reagent for the organic synthesis, biotransformation, chemiluminescent assays, immunoassays, bioremediation, and treatment of wastewaters (Veitch and Smith, 2001; Krieg and Halbhuber, 2003; Veitch, 2004). Several investigations have been performed in order to increase the enzyme's structural stability and functionality as well. Based on some of these studies, the structural  $\text{Ca}^{2+}$  ions play a very important role in the activity and the thermal stability of the enzyme. NMR experimental studies have also indicated that the  $\text{Ca}^{2+}$  ions are essential in maintaining the native fold structure of the protein and furthermore, the refolding of the recombinant HRP is dependent on the presence of these ions in the buffer solution (Garguilo et al., 1993; Pappa and Cass, 1993). Several strategies have been employed to thermodynamically and kinetically increasing the stability of this enzyme, using several approaches such as site-directed mutagenesis, directed evolution (Hult and Berglund, 2003; DeSantis and Jones, 1999), and chemical modifications as well (Davis, 2003; Hassani et al., 2006). Chemical modification approaches are useful tools to determine the physicochemical properties of the individual amino acids, their participation in the native folded state (Torchilin et al., 1979), protein stabilization (Ryan et al., 1994; Miland et al., 1996a, b; Mozhaev et al., 1988, 1992), and also their transition into the molten globule structures (Hosseinkhani et al., 2004; Naseem et al., 2004; Khatunhaq et al., 2002). In the previous investigations, significant stabilization achieved using chemical modi-



**Figure 1:** Schematic representation of the tertiary structure of HRP (PDB accession code: 6ATJ). Three Lys residues 174, 232, and 241 that have been modified by citraconic anhydride are depicted in blue, two structural calcium ions in green, heme prosthetic group in red, and the His 42 in yellow.

fications (Mozhaev et al., 1988; Wong and Wong, 1992), and surface modifications have also shown to stabilize the native fold of the proteins (Hassani et al., 2006; Khajeh et al., 2001a, b). In the present study, using citraconic anhydride, modification of the  $\epsilon$ -amino groups of the Lys residues in horseradish peroxidase has been performed. The following induced structural changes have been measured by means of circular dichroism and fluorescence spectroscopy. According to the results, we can suggest that the formation of a molten globule-like structure occurs due to the chemical modification at slightly acidic pH conditions. The results of thermal studies have also shown different transition phases for the protein structure.

## MATERIALS AND METHODS

### Chemicals

Lyophilized powder of horseradish peroxidase isoenzyme C was purchased from Sigma chemical company (St. Louis, USA) and used without further purifications. The purity of the peroxidase preparations was determined by assessing the ratio of the heme absorbance at 403 nm to the protein absorbance at 280 nm, which is denoted as the  $R_Z$  value (Hassani et al., 2006). The  $R_Z$  of the protein solution used for the experiments was above 3.0. The concentration of HRP

was determined spectrophotometrically using the extinction coefficient of  $102 \text{ M}^{-1} \cdot \text{cm}^{-1}$  at 403 nm (Hassani et al., 2006; Goto et al., 1990a, b). All of the reagents were of analytical grade and supplied by Merck (Darmstadt, Germany) or Sigma.

### **Spectroscopic studies**

The pH-induced conformational changes of HRP were measured by fluorescence and CD spectroscopy. Intrinsic fluorescence intensity measurements were carried out using a PerkinElmer (LS-50 B) fluorimeter with a 1 cm light-path cell. Tryptophan fluorescence was induced by the excitation of the sample at 295 nm and the emission was recorded between 320 and 400 nm. Extrinsic fluorescence studies were carried out using 1-anilino-8-naphthalenesulfonic acid as a fluorescent probe (Hosseinkhani et al., 2004). All of the experiments were carried out at 25° C with ANS and protein concentrations of 50  $\mu\text{M}$  and 1  $\mu\text{M}$  in 0.02 M phosphate buffer. An excitation wavelength of 380 nm was used and the emission recording was scanned from 400 to 600 nm. CD measurements were carried out using a Jascospectropolarimeter, model J-715. The ellipticity values were obtained in millidegrees directly from the instrument and converted to the molecular ellipticity,  $[\theta]_{\text{MRW}}$ , expressed in  $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$  (Goto et al., 1990a; b; Strickland, 1968), based on a mean amino acid residue weight (MRW), assuming the average weight for HRP to be 110. The molar ellipticity was determined using the equation:

$$[\theta]_{\text{MRW}} = \frac{\theta \times 100 \text{ MRW}}{c \times l}$$

where  $c$  is the protein concentration in mg/ml,  $l$  is the light path length in centimeters, and  $\theta$  is the measured ellipticity in degrees at wavelength  $\lambda$ . The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming  $[\theta]_{291} = 7820 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$  (Hewlett et al., 1991), and with Jascostandard nonhydroscopic ammonium (+)-10-camphorsulfonate assuming  $[\theta]_{290.5} = 7910 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$  (Merrill et al., 1990). Noise

in the data was smoothed using the Jasco (J-715) software including the fast Fourier-transform noise reduction routine, which allows refinement of the recorded spectra without distorting the peak shapes (Merrill et al., 1993). The far-UV CD spectra were measured using a rectangular quartz cell of 1 mm path length with a sample concentration of 0.15 mg/ml. Each spectrum was an average of at least three scans between 250 and 200 nm. The resultant ellipticities of the HRP solutions were calculated by subtracting the ellipticity of the buffer solution. The visible CD spectra were measured using a rectangular quartz cell of 1 cm path length and a sample concentration of 2 mg/ml. Each spectrum was an average of at least three scans between 450 and 350 nm. The wavelengths of 222 and 407 nm were used to monitor the thermal denaturation in the far-UV and the visible CD range, respectively. In the thermal studies, the temperature was raised stepwise from 30° C to 90° C with an equilibration time of 1 min for each 2° C. pH values were measured before and after of each run and its variations were not greater than  $\pm 0.1$  pH unit.

### **Activity assays**

All assays of the enzymatic activity were carried out in 96-well flat-bottomed microtiter plates (Ryan et al., 1994). 20  $\mu\text{l}$  of HRP ( $5 \times 10^{-4}$  mg/ml) solution in 0.02 M phosphate buffer was dispensed into each well and followed by 180  $\mu\text{l}$  of buffered substrate solution (0.2 M phosphate buffer, containing 0.0017 M hydrogen peroxide and 0.0025 M 4-aminoantipyrine with 0.17 M phenol) (Parker et al., 1994). Reactions took place at 25° C for 4 min.  $A_{495}$  values were then read in an Anthos 2020 ELISA reader instrument. All of the kinetic parameters for the enzyme were determined from the average of at least three substrate measurements at each substrate concentration and pH. Values for  $K_m$  and  $k_{\text{cat}}$  were obtained from the Lineweaver–Burk equation. The dependence of the initial velocity upon substrate concentration was hyperbolic at each pH value under investiga-

tion and all of the Lineweaver–Burk plots were linear.

### **Modification of Lysine residues**

The modification process was carried out using citraconic anhydride as a specific blocking agent for the Lysine residues following the standard procedure (Steer and Merrill, 1994). The protein was used at 5 mg/ml in 5 ml of 0.1 M borate buffer at pH 8 and the procedure was carried out at room temperature with stepwise addition of 3  $\mu$ l aliquots of the modifier up to a total volume of 20  $\mu$ l. Finally, the sample was dialyzed against 0.02 M phosphate buffer at pH8 for 48 h. To determine the number of the modified Lysine residues, the number of the free amino groups was measured following the standard methods (Steer and Merrill, 1995). Three Lysine residues out of the total of the six Lysines were found to be modified (Hassani et al., 2006; Chattopadhyay and Mazumdar, 2000).

## **RESULTS AND DISCUSSION**

### ***pH-dependent enzymatic activity***

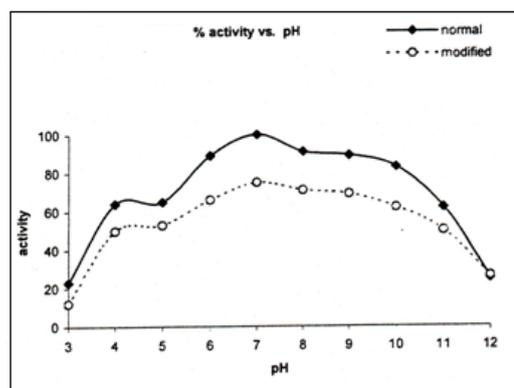
Enzymatic activity versus different pH values was recorded with a maximum value for this parameter between pH 6 and pH 8 (Figure 2), which is decreased on either side of this pH range for both Horseradish peroxidase (HRP) and the modified form (MHRP) of this enzyme. As it may be supposed, the general trend is a bell shaped graph, however, the path following by MHRP is under the one recorded by HRP, which implies that modification process induced some irreversible structural changes to the native form of the enzyme affecting the catalytic activity of MHRP. The impact of pHs 4 to 10 on kinetic parameters for both forms are also listed in Table 1. These parameters implied that some changes must be occurred in the protein structure due to the chemical modification. Analysis of the kinetic constants for MHRP at pH 5 showed that the modified form of the enzyme at this pH possesses the maximum value for the  $K_m$ , and the minimum value for  $k_{cat}/K_m$ . As shown in Table 1,

the values of the different kinetic constants for MHRP at pH 5 generally differ from those recorded in other experiments. It could be suggested that the chemical modification significantly affect the catalytic constant ( $k_{cat}$ ), and the substrate affinity ( $K_m$ ) of MHRP at this pH. Based on the results the catalytic efficiency ( $k_{cat}/K_m$ ) of MHRP at pH 5 is significantly lower than that of the native and modified form at the other pH values. It can be related to the pH-induced conformational changes in the secondary/tertiary, or both structures. The probability of the molten globule-like structure formation could not be also excluded, which usually arise at slightly acidic conditions and mildly ionic strengths (Pina et al., 2001; Carvalho et al., 2003).

### ***pH-dependent structural changes***

Circular dichroism spectroscopy has been used to provide more information on the structural changes of the protein molecule (Shanon et al., 1966). We have also used these data to detect the occurred changes in the HRP structure using the following protocol:

- (1) Far-UV CD (190–250 nm): changes in the secondary structure of the apoprotein.
- (2) Near-UV CD (250–320 nm): changes in the tertiary structure of the apoprotein.



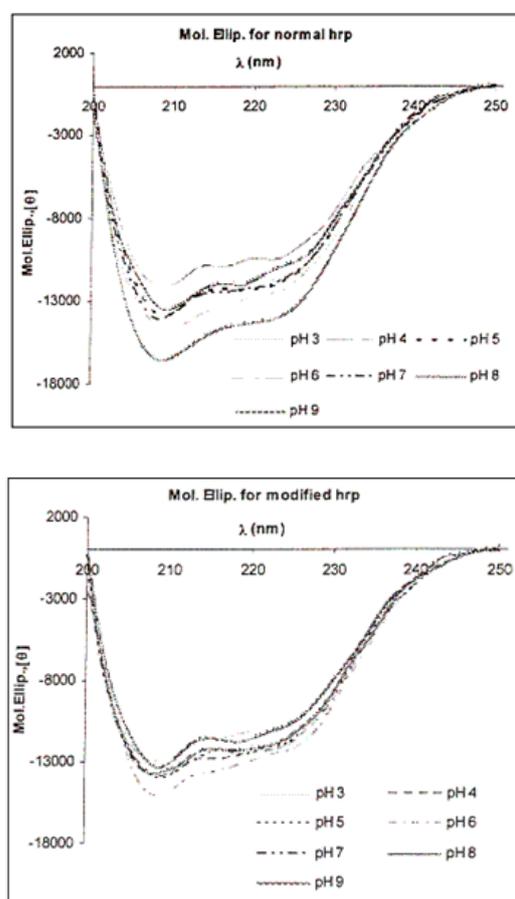
**Figure 2:** Enzymatic activity of HRP and MHRP versus different pH values. The general trend is a bell shaped graph with the maximum activity in pH values between 6 – 8 for both forms, however, modification induced some structural changes to the MHRP that caused its catalytic activity to be suppressed.

**Table 1:** Kinetic parameters for the native and modified horseradish peroxidase in pH values between 4 - 10

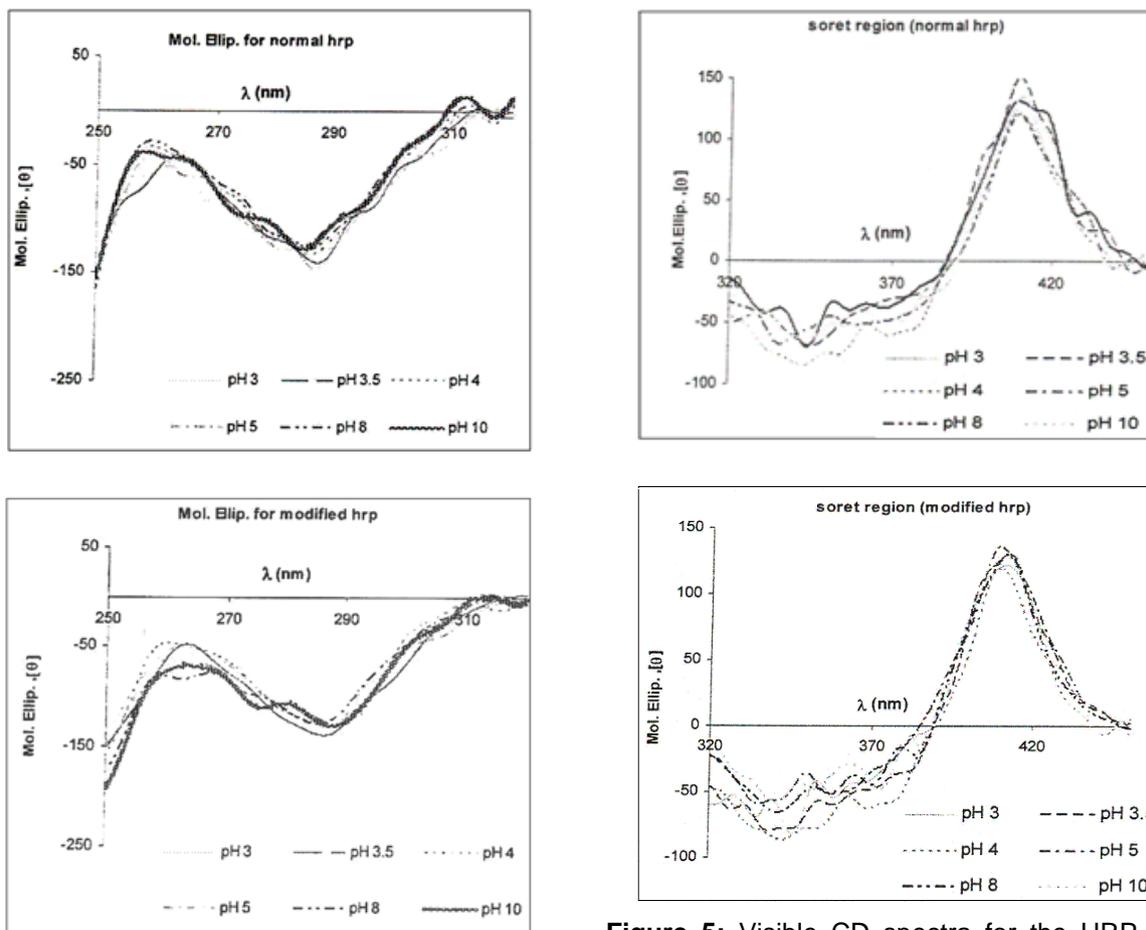
pH	HRP			MHRP		
	$K_m(\text{mM})$	$k_{\text{cat}}(\text{min}^{-1})$	$k_{\text{cat}}/K_m(\text{mM}^{-1}\text{min}^{-1})$	$K_m(\text{mM})$	$k_{\text{cat}}(\text{min}^{-1})$	$k_{\text{cat}}/K_m(\text{mM}^{-1}\text{min}^{-1})$
4	1.30	1985	1533	0.66	1570	2378
5	2.50	1477	583	4.05	1185	292
6	0.45	2399	5292	0.65	2398	3654
7	0.79	2895	3640	0.89	2688	2990
8	1.15	2770	2400	1.82	2564	1404
9	0.85	2688	3180	1.55	2316	1488
10	2.25	1446	642	0.55	1322	2411

(3) Visible CD (350–450 nm): dissociation of the prosthetic heme group from the apoprotein and structural changes in regions that surrounding the prosthetic heme.

Figure 3 shows the pH-dependent secondary structural changes for both forms of the enzyme. Comparisons indicated that the percentage of the secondary structure of MHRP is similar to that of the HRP at pH 5. Thus the modification was inert to the secondary structure at least in this pH value. However, according to the Near-UV, and Visible CD spectra it could be concluded that the percentage of the tertiary structure for MHRP in comparison to the HRP has been reduced in some extent (Figures 4 and 5). Tertiary structural changes have been also assessed by means of the fluorescence spectroscopy, using Trp residues as fluorophores to measure the introduced changes in the local microenvironment of hydrophobic patches (Welinder, 1979; Schippers and Dekkers, 1981; Takakuwa et al., 1985; Protasevich et al., 1997; Trinder, 1969). It is known that the intrinsic fluorescence of the enzyme is dependent on the fluorescence energy transferring from a Trp residue into the heme prosthetic group (Dixon and Perham, 1968; Fields, 1971). Accordingly, changes in the structure and folding of the heme cavity of the enzyme may affect the orientation or the distance between the heme group and the Trp residue, which can also alter the intensity of the intrinsic fluorescence emission of the en-



**Figure 3:** Far-UV CD spectra for the HRP (a), and MHRP (b) in different pH values. Comparisons indicate that the secondary structures of HRP and MHRP are almost similar in pH values 5, 7, 9, and 10. In pH 6 the secondary structure of HRP is reduced comparing to that of the MHRP. Although, in pH 8 the secondary structure of the native form is enhanced comparing to the modified enzyme.

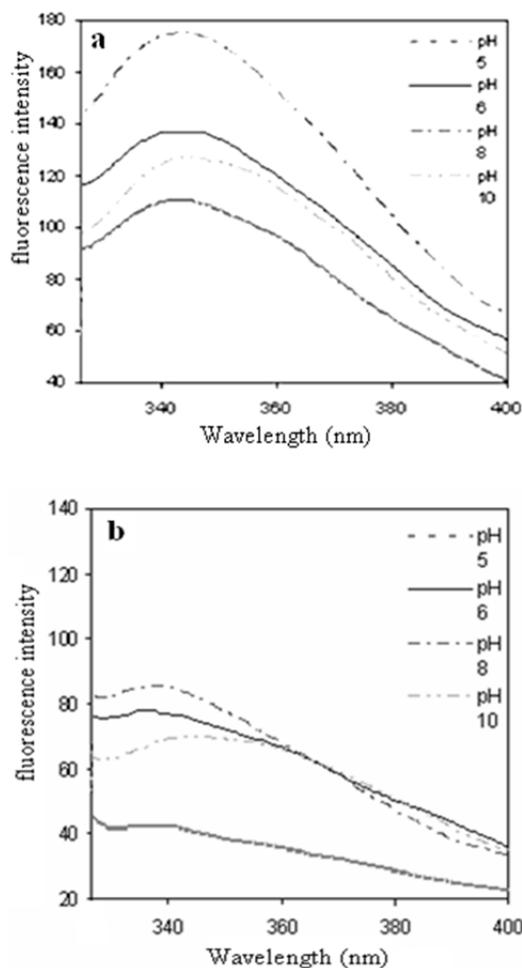


**Figure 4:** Near-UV CD spectra for the HRP (a), and MHRP (b) in different pH values. Generally, both forms of the enzymes are similar in the tertiary structure, with an exception in pH 5 that the structure of MHRP is experiencing some decrease in comparison to the native form.

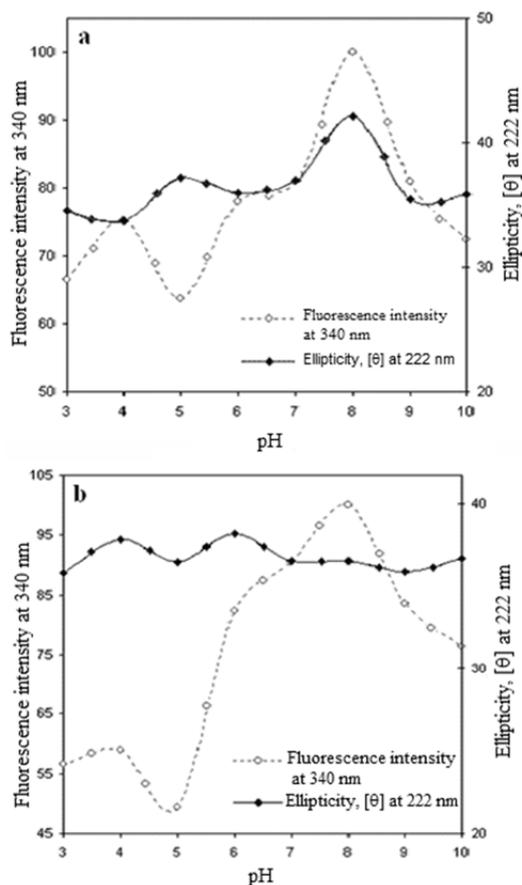
**Figure 5:** Visible CD spectra for the HRP (a), and MHRP (b) in different pH values. Comparing figures a and b indicates that a reduction in the enzyme's structure around heme region for the modified form could be detected in comparison to the native form in different pH values.

zyme. It is known that HRP contains one Trp residue (Trp 117) and five tyrosine residues (Strickland, 1968), which all of them have fluorescence emission. We chose Trp residues to probe conformational changes of the enzyme as described in other studies (Dixon and Perham, 1968). Figure 6 illustrates the fluorescence spectra at some selected pH values for the native (Figure 6a) and modified (Figure 6b) forms of the enzyme at room temperature with an excitation wavelength of 295 nm. As Figure 6b shows, the intensity of Trp fluorescence emission sharply decreases for MHRP at pH 5. It may be due to the changes in the relative orientation or distance between the heme and the Trp residue, leading to an increase in the efficiency of the en-

ergy transfer from Trp into the heme as a quenching prosthetic group (Dixon and Perham, 1968; Fraczkiewicz and Braun, 1998). Conformational changes in the secondary structure of the enzyme were also followed by assessing the alterations in the CD spectra at 222 nm. Tertiary structural changes also recorded by the fluorescence emission at 340 nm. Figure 7 shows the impact of different pH values on the conformational changes of the secondary and tertiary structure for the native (a) and modified (b) forms of the enzyme. As Figure 7 illustrates, the CD intensity at 222 nm was almost unchanged in the pH range under investigation, which means almost no variations in the secondary structure of the enzyme has been recorded.



**Figure 6:** Tryptophan fluorescence emission spectra upon excitation at 295 nm for (a) native, and (b) modified HRP in some selected pH values. Measurements were carried out at 25° C with protein concentrations of 150  $\mu$ M in 0.02 M phosphate buffer.

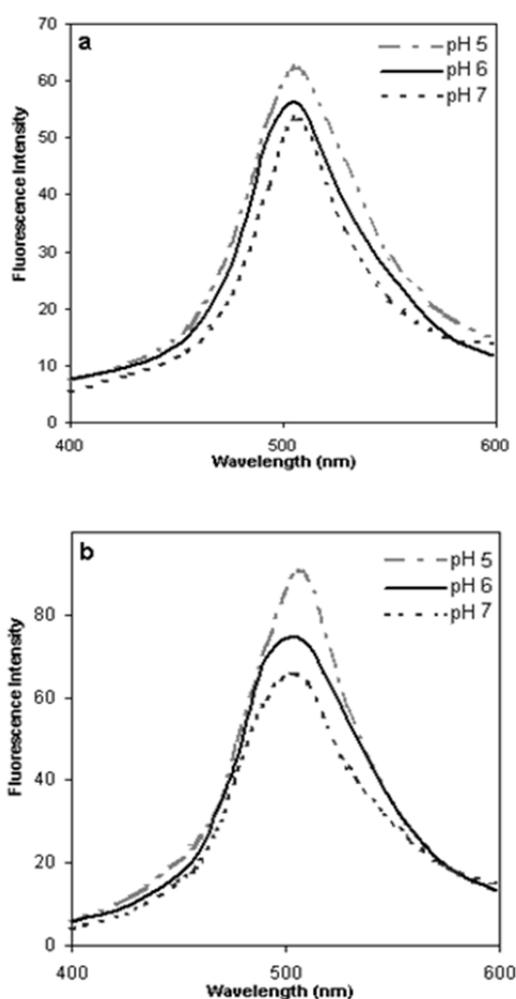


**Figure 7:** Correlation between the tertiary and the secondary structure of the (a) native and (b) modified forms of HRP followed by recording Trp emission at 340 nm. Trp fluorescence was induced by excitation of the sample at 295 nm and the CD signals at 222 nm of the enzymes were obtained in some selected pH values. Fluorescence and CD experiments were carried out at 25° C with protein concentrations of 150  $\mu$ M and 0.15 mg/ml respectively, in 0.02 M phosphate buffer

However, an interesting trend arises at pH 5 for the modified enzyme in which the secondary structure is the same as its content at pH 7, while its tertiary structure shows the minimum value in the pH range under investigation. The pH-dependent structural changes and kinetic constants of horseradish peroxidase indicate that the molten globule-like form of MHRP occurs at pH 5, revealing that these structural changes are mediated by the protonation of the ionizable groups. It may be proposed that upon slightly acidic condi-

tions, intramolecular charge repulsion is the main driving force for partial unfolding of the chemically modified protein, followed by the exposure of the hydrophobic patches out of the hydrophobic core of the protein and getting accessible to the polar water molecules of the surrounding solvent. To confirm the exposure of the hydrophobic patches of horseradish peroxidase in the modified form at pH 5, ANS was further used as a widely used hydrophobic reporter compound. This probe has been known to be a useful detector

for trapping the molten globular states, which can bind to the hydrophobic patches of the molten globule structures more strongly than the native structures, with an increasing in its fluorescence intensity (Hosseinkhani et al., 2004). The results of the ANS experiments (Figure 8) imply an enhancement of the ANS fluorescence emission for the modified form of horseradish peroxidase at pH 5 (Figure 8b), which confirms that a molten globule-like structure has been detected.

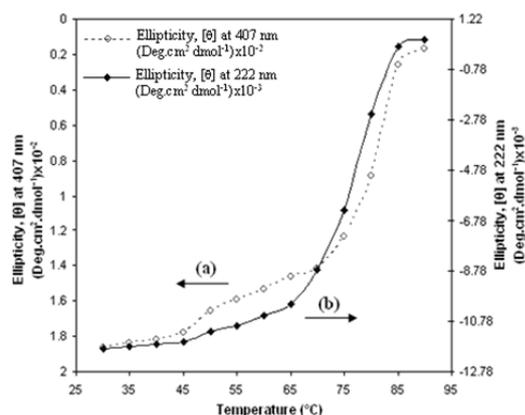


**Figure 8:** ANS fluorescence emission spectra upon excitation at 380 nm for the (a) native and (b) modified HRP in some selected pH values. The final concentration of the ANS in the enzyme solutions was 50  $\mu$ M and the molar ratio of protein to ANS was 1:50. Measurements were performed at 25° C in 0.02 M phosphate buffer. For more details please see materials and methods section.

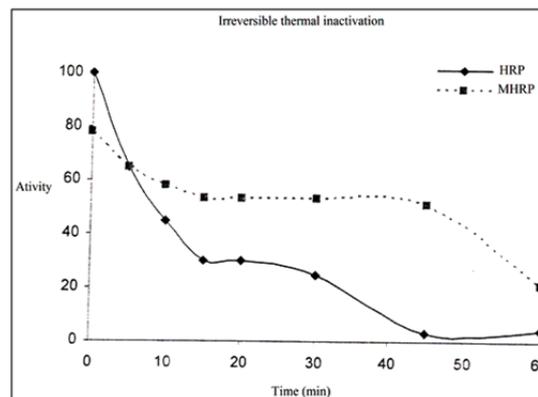
### Thermal unfolding of MHRP

Circular dichroism measurement of the heme prosthetic group is an informative strategy to study the introduced structural changes of the heme containing proteins. Accordingly, thermally induced structural phase transitions of MHRP at pH 5 were monitored by means of circular dichroism. Figure 9 shows the observed alterations in the ellipticity of MHRP at 222 nm in the slightly acidic conditions, which indicates a two-state transition pattern for the secondary structure of MHRP (curve b). CD spectra in the visible region were further monitored to determine the effects of the temperature-induced conformational changes on the tertiary structure, particularly around the heme cavity (curve a). Based on the data, two separated phases of the structural transitions for the secondary structure can be recognized. The first phase starts at around 30° C and continues to around 65° C, following by another phase of unfolding that starts at 65° C and reaches to the fully unfolded structure at around 90° C. However, according to Figure 9 curve a, three separated trends for the conformational transitions in the tertiary structure of the enzyme could be recognized. The first phase from 30 to around 45° C, the second one from 45 to at around 70° C and finally from 70 to 90° C. Comparing curves a, and b implying that a structural intermediate state of MHRP exists between temperatures 45° C and 70° C. Considering different phases of the thermally induced unfolding of MHRP demonstrates two distinct patterns in the structural transition. From Figure 9, a specific structural region between 45 to around 70° C is detectable that is associated with a significant change in the tertiary structure of the enzyme, at least around the active site, accompanied by almost little variations in the secondary structure. The second phase of the transition in the secondary structure also is related to the complete removal of the heme prosthetic group out of the enzyme's active site as detected by the complete absence of the CD signals at 407 nm. We propose that modification of the  $\epsilon$ -

amino groups of the Lysine residues ( $pK_a$  around 10) alters the global electrostatic charge of the enzyme from positive into the negative charge, which consequently leads to the presence of an intermediate molten globule-like structure at pH 5. This idea is also supported by an obvious change in the intrinsic fluorescence spectra and the disappearance of the emission intensity at 340 nm because of the exposure of Trp 117 to the polar solvent (Figure 6). These data are also in good agreement with the previous studies (Hassani et al., 2006; Hosseinkhani et al., 2004). Based on the results, the transition to a molten globule is accompanied by the loss of the tertiary interactions, while almost all of the secondary structure preserved. The results of the thermal CD also revealed that unlike the melting of the secondary structure, temperature-induced unfolding of the tertiary structure leads to the existence of an intermediate state, which promotes formation of a molten globule-like structure of Horseradish peroxidase at pH 5. Irreversible thermal inactivation experiments (Figure 10) revealed that the modified enzyme keeps its catalytic activity during the time, which means that the modification process is also capable of increasing the half-life of the enzyme.



**Figure 9:** Thermal unfolding of MHRP at pH 5 followed by CD signals at 407 nm and 222 nm for probing structural phase transitions around heme cavity and secondary structure, respectively. (a): CD signals for the tertiary structure around heme prosthetic group and (b): CD signals for the secondary structure. CD spectra were measured using a sample concentration of 2 mg/ml in 0.02 M phosphate buffer at each pH and temperature.



**Figure 10:** Irreversible thermal inactivation of HRP and MHRP which is recorded for 60 minutes at pH 5. The incubation environment set at 70 °C. The modified enzyme keeps its catalytic activity during the time, which means that the modification process is capable of increasing the half-life of the enzyme.

## CONCLUSIONS

In the present study, a comprehensive investigation on kinetics and structural properties of Horseradish peroxidase has been performed. This study is important at least from two points of view. Technologically, Horseradish peroxidases have been used in industry and a wide range of biotechnological applications as well. Therefore, stabilizing experiments, like the specific chemical modification process that performed and analyzed in the present work, may clarify the impact of denaturants such as acidic/basic pH situations, and high temperatures on the functionality/efficiency of the enzyme molecule. According to the irreversible thermal inactivation, the catalytic activity of MHRP remains during the time period of the experiment, which is not detected for the native enzyme. Thus, it seems that the modification protocol is capable of increasing the half-life of the enzyme. On the other hand, there was limited data on the conformational changes and structural characteristics of this modified Horseradish peroxidase in comparison to the native form of this enzyme. According to our study, a modified structure (MHRP) of the enzyme at pH 5 with almost intact secondary structure, while reduced tertiary structure was detected that is the general feature of a

molten globule-like structure. Despite of some conformational changes in the tertiary structure of MHRP at pH 5, this modified form still keeps its catalytic activity to some extent besides enhanced thermal stability during the time period of the experiment. These findings are also in agreement to that of by Vamvaca and colleagues (2004) claiming that a molten globular state does not necessarily preclude efficient catalytic activity.

### ACKNOWLEDGEMENTS

We thank the reviewers for valuable comments. Special thanks also go to Dr. Reza Hassan-Sajedi for the comments. This work was supported by the Research Council of Tarbiat Modares University.

### REFERENCES

- Carvalho AS, Melo EP, Ferreira BS, Neves-Petersen MT, Petersen SB, Aires-Barros MR. Heme and pH-dependent stability of an anionic horseradish peroxidase. *Arch Biochem Biophys* 2003;415:257-67.
- Chattopadhyay K, Mazumdar S. Structural and conformational stability of horseradish peroxidase: effect of temperature and pH. *Biochemistry* 2000;39:263-70.
- Davis BG. Chemical modification of biocatalysts. *Curr Opin Biotechnol* 2003;14:379-86.
- DeSantis G, Jones JB. Chemical modification of enzymes for enhanced functionality. *Curr Opin Biotechnol* 1999;10:324-30.
- Dixon F, Perham RN. Reversible blocking of amino groups with citraconic anhydride. *Biochem J* 1968;109:312-3.
- Fields R. The measurement of amino groups in proteins and peptides. *Biochem J* 1971;124:581-90.
- Fraczkiewicz R, Braun W. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comp Chem* 1998;19:319-33.
- Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL. Crystal structure of horseradish peroxidase C at 2.15 Å resolution. *Nat Struct Biol* 1997;4:1032-8.
- Garguilo MG, Huynh N, Proctor A, Michael AC. Amperometric sensors for peroxide, choline, and acetylcholine based on electron transfer between horseradish peroxidase and a redox polymer. *Anal Chem* 1993;65:523-8.
- Goto Y, Calciano LJ, Fink AL. Acid-induced folding of proteins. *Proc Natl Acad Sci USA* 1990a;87:573-7.
- Goto Y, Takahashi N, Fink AL. Mechanism of acid-induced folding of proteins. *Biochemistry* 1990b;29:3480-8.
- Hassani L, Ranjbar B, Khajeh K, Naderi-Manesh H, Naderi-Manesh M, Sadeghi M. Horseradish peroxidase thermostabilization: The combinatorial effects of the surface modification and the polyols. *Enzyme Microb Technol* 2006;38:118-25.
- Henriksen A, Welinder KG, Gajhede M. Structure of barley grain peroxidase refined at 1.9-Å resolution. A plant peroxidase reversibly inactivated at neutral pH. *J Biol Chem* 1998;273:2241-8.
- Hewlett EL, Gray L, Allietta M, Ehrmann I, Gordon VM, Gray MC. Adenylatecyclase toxin from *Bordetella pertussis*. Conformational change associated with toxin activity. *J Biol Chem* 1991;266:17503-8.
- Hosseinkhani S, Ranjbar B, Naderi-Manesh H, Nemat-Gorgani M. Chemical modification of glucose oxidase: possible formation of molten globule-like intermediate structure. *FEBS Lett* 2004;561:213-6.
- Hult K, Berglund P. Engineered enzymes for improved organic synthesis. *Curr Opin Biotechnol* 2003;14:395-400.
- Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar B, Kirk TK. Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. *Biochem J* 1990;268:475-80.
- Khajeh K, Naderi-Manesh H, Ranjbar B, Moosavi-Movahedi AA, Nemat-Gorgani M. Chemical modification of lysine residues in *Bacillus alpha*-amylases: effect on activity and stability. *Enzyme Microb Technol* 2001a;28:543-9.
- Khajeh K, Ranjbar B, Naderi-Manesh H, Ebrahim-Habibi A, Nemat-Gorgani M. Chemical modification of bacterial alpha-amylases: changes in tertiary structures and the effect of additional calcium. *Biochim Biophys Acta* 2001b;2:229-37.
- Khatunhaq S, Rasheedi S, Hasan Khan R. Characterization of a partially folded intermediate of stem bromelain at low pH. *Eur J Biochem* 2002;269:47-52.

- Krieg R, Halbhuber KJ. Recent advances in catalytic peroxidase histochemistry. *Cell Mol Biol* 2003;49:547-563.
- Merrill AR, Cohen FS, Cramer WA. On the nature of the structural change of the colicin E1 channel peptide necessary for its translocation-competent state. *Biochemistry* 1990;29:5829-5836.
- Merrill AR, Palmer LR, Szabo AG. Acrylamide quenching of the intrinsic fluorescence of tryptophan residues genetically engineered into the soluble colicin E1 channel peptide. Structural characterization of the insertion-competent state. *Biochemistry* 1993;32:6974-6981.
- Miland E, Smyth MR, Fagain CO. Increased thermal and solvent tolerance of acetylated horseradish peroxidase. *Enzyme Microb Technol* 1996a;19:63-7.
- Miland E, Smyth MR, Fagain CO. Modification of horseradish peroxidase with bifunctional N-hydroxy-succinimide esters: Effects on molecular stability. *Enzyme Microb Technol* 1996b;19:242-9.
- Mozhaev VV, Siksniš VA, Melik-Nubarov NS, Galantaitė NZ, Denis GJ, Butkus EP et al. Protein stabilization via hydrophilization. Covalent modification of trypsin and alpha-chymotrypsin. *Eur J Biochem* 1988;173:147-54.
- Mozhaev VV, Melik-Nubarov NS, Levitsky VY, Siksniš VA, Martinek K. High stability to irreversible inactivation at elevated temperatures of enzymes covalently modified by hydrophilic reagents: alpha-chymotrypsin. *Biotechnol Bioeng* 1992;40:650-62.
- Naseem F, Ahmad B, Ashraf MT, Hasan Khan R. Molten globule-like folding intermediate of asialofetuin at acidic pH. *Biochim Biophys Acta* 2004;1699:191-9.
- Pappa HS, Cass EG. A step towards understanding the folding mechanism of horseradish peroxidase. Tryptophan fluorescence and circular dichroism equilibrium studies. *Eur J Biochem* 1993;212:227-235.
- Parker MW, Buckley JT, Postma JPM, Tucker AD, Leonard K, Pattus F et al. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* 1994;367:292-5.
- Pina DG, Shnyrova AV, Gavilanes F, Rodriguez A, Leal F, Roig MG et al. Thermally induced conformational changes in horseradish peroxidase. *Eur J Biochem* 2001;268:120-6.
- Protasevich I, Ranjbar B, Lobachov V, Makarov A, Gilli R, Briand C et al. Conformation and thermal denaturation of apocalmodulin: role of electrostatic mutations. *Biochemistry* 1997;36:2017-24.
- Ryan O, Smyth MR, Fagain CO. Thermostabilized chemical derivatives of horseradish peroxidase. *Enzyme Microb Technol* 1994;16:501-5.
- Sakurada J, Takahashi S, Hosoya T. Nuclear magnetic resonance studies on the spatial relationship of aromatic donor molecules to the heme iron of horseradish peroxidase. *J Biol Chem* 1986;261:9657-62.
- Schippers PH, Dekkers HM. Direct determination of absolute circular dichroism data and calibration of commercial instruments. *Anal Chem* 1981;53:778-88.
- Schuller DJ, Ban N, vanHuystee RB, McPherson A, Poulos TL. The crystal structure of peanut peroxidase. *Structure* 1996;4:311-21.
- Shanon LM, Kay E, Lew JY. Peroxidase isoenzymes from horseradish roots: isolation and physical properties. *J Biol Chem* 1966;241:2166-72.
- Steer BA, Merrill AR. The colicin E1 insertion-competent state: detection of structural changes using fluorescence resonance energy transfer. *Biochemistry* 1994;33:1108-1015.
- Steer BA, Merrill AR. Guanidine hydrochloride-induced denaturation of the colicin E1 channel peptide: unfolding of local segments using genetically substituted tryptophan residues. *Biochemistry* 1995;34:7225-33.
- Strickland EH. Circular dichroism of horseradish peroxidase and its enzyme-substrate compounds. *Biochim Biophys Acta* 1968;151:70-5.
- Takakuwa T, Konno T, Meguro H. A new standard substance for calibration of circular dichroism: ammonium d-10-camphorsulfonate. *Anal Sci* 1985;1:215-25.
- Torchilin VP, Maksimenko AV, Smirnov VN, Berezin IV, Klivanov AM, Martinek K. The principles of enzyme stabilization: modification of 'key' functional groups in the tertiary structure of proteins. *Biochim Biophys Acta* 1979;567:1-11.
- Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 1969;6:24-7.
- Vamvaca K, Voegeli B, Kat P, Pervushin K, Hilvet D. An enzymatic molten globule: efficient coupling of folding and catalysis. *Proc Natl Acad Sci USA* 2004;101:12860-4.
- Veitch NC. Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry* 2004;65:249-59.
- Veitch NC, Smith AT. Horseradish peroxidase. *Adv Inorg Chem* 2001;51:107-62.
-

Welinder KG. Amino acid sequence studies of horseradish peroxidase. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C. *Eur J Biochem* 1979;96:483-502.

Welinder KG. Superfamily of plant, fungal and bacterial peroxidases. *Curr Opin Struct Biol* 1992;2:388-93.

Wong SS, Wong LJC. Chemical cross-linking and the stabilization of proteins and enzymes. *Enzyme Microb Technol* 1992;14:866-74.