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EFFECTS OF SODIUM PHOSPHATE BUFFER ON HORSERADISH PEROXIDASE THERMAL STABILITY

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Thermal stability of horseradish peroxidase (HRP) was studied by differential scanning calorimetry, tryptophan fluorescence, the heme absorption and enzymatic activity analysis while the concentrations of sodium phosphate buffer ranged from 2.5 to 50 mM at pH 7.0.

The results showed that the denaturation temperature (T_m) values decreased and the intrinsic tryptophan fluorescence intensity of denatured HRP increased as sodium phosphate buffer concentration increased. Furthermore, the heme absorbance at 403 nm and enzymatic activity of HRP decreased with the increasing buffer concentrations. According to data obtained in this experiment, it can be concluded that sodium phosphate accelerated the denaturation process of HRP and reduced the thermal stability of HRP.

Keywords: *DSC, enzymatic activity, fluorescence, heme absorption, horseradish peroxidase, sodium phosphate buffer, thermal stability*

Introduction

Horseradish peroxidase (HRP EC 1. 11. 1. 7) is one of the most intensively studied members of the family of heme-containing enzymes that catalyzes the oxidation of a variety of substrates by hydrogen peroxide [1]. It has been found of great diagnostic, analytical, biochemical and biotechnological applications [2–5] due to its unusually high stability in aqueous solution. HRP (44 kDa) is a monomeric heme-protein with a single tryptophan residue at position 117. Since HRP belongs to class peroxidases [1], its location of trytophan in the three-dimensional structure was found in the extended structure connecting helices D and D' [6–8]. The previous spectroscopic and chemical modification studies indicate that it is not a part of the catalytic site of the enzyme [9]. However, the distance between the tryptophan and the nearest heme edge is consistent with the mechanism of quenching [8, 9] and the heme is regarded as a quencher in the native HRP. When the enzyme is denatured, the distance increases and the heme quenching effect of tryptophan emission is weakened, which lead to the increase in the fluorescence intensity. Therefore, thermal stability in the tertiary structure of HRP can be studied by measuring the changes in the intrinsic tryptophan fluorescence emission.

Class III peroxidases have Fe(III) protoporphyrin IX as the prosthetic group, which play an important role in the catalytic mechanism. The structural features of HRP include four disulfide bonds, two Ca^{2+} binding sites located distal and proximal to heme and eight glucans [10–13]. The heme cavity is the catalysis group of the enzyme. The heme of native HRP has absorption in the Soret region and its maximum absorption at 403 nm [14]. Several papers have reported the absorbance changes in the Soret region corresponds to changes in the tertiary structure around the heme in proxidases [8, 9, 15–20].

In recent years, differential scanning calorimetry (DSC) has established itself as the primary technique for studying the thermal stability of proteins, especially since the availability of ultrasensitive microcalorimeter and convenient deconvolution algorithms have developed. When DSC studies the thermal denaturation of a globular protein, the thermodynamic parameters (heat capacity, enthalpy, entropy and free energy) of protein folding/unfolding transition can be obtained [21–27]. This technique when used in conjugation with information obtained by spectroscopic techniques provides a more detailed analysis of the process [28–30]. In several literatures [17–20] DSC combining with steady-state fluorescence and circular dichroism (CD) has been used for studying the thermal denaturation of HRP.

Several studies have analyzed the thermal stability of HRP affected by glycosylation [31], calcium ion [19], temperature [18] and pH [8, 17, 20, 32]. However, no paper has reported effects of sodium phosphate buffer on thermal stability of HRP, although a majority of studies are performed in this buffer solution. In this paper, thermal stability of HRP was studied at various concentrations of sodium phosphate buffer, pH 7.0, and using calorimetric and spectroscopic techniques.

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Theory

The Lumry–Eyring model

Generally, the thermal denaturation of proteins is often discussed in terms of the Lumry–Eyring model [33], in which a reversible unfolding step is followed by an irreversible denaturation step:

$$N \xrightarrow{k_1,k_{-1}} U \xrightarrow{k_2} D \quad (1)$$

where N is the native state, U is the reversible unfolded state and D is a final irreversible denatured state [34, 35]. k_1, k_{-1} and k_2 are the rate constants for reversible denaturation, renaturation and irreversible denaturation stage, respectively. Assuming that all the kinetic processes are first order and the reversible unfolding equilibrium constant between N and U is given by $K= k_1/k_{-1}=[U]/[N]$. Thus, in this model the rate-limiting step is determined by the relative values of the rate constants k_{-1} and k_2.

If $k_2>>k_{-1}$, this model can be converted into a single step model ($N\rightarrow D$). In this case no equilibrium between N and U is established during denaturation. Therefore, the amount of U is very low and most of U molecules are converted into D instead of refolding to form N. In this case, the rate-limiting step is the unfolding one ($N\rightarrow U$) and the formation of D is determined by a first-order rate constant, k_1:

$$N \xrightarrow{k_1} D \quad (2)$$

If $k_{-1}>>k_2$ and in addition $K<<1$, the amount of U is very low and the rate of formation of D can be determined by an apparent first-order rate constant equal to Kk_2. Thus, theoretical analysis [34] shows that this two-state irreversible model may be considered as a limiting case of the Lumry–Eyring model.

The Lumry–Eyring model has analyzed the denaturation processes of many proteins. In this paper, the effect of sodium phosphate buffer on HRP thermal stability was interpreted in terms of this classic model.

Experimental

Materials

Horseradish peroxidase with a purity index of $R_z\approx3.0$ (R_z is the ratio between the absorbance at the Soret band (403 nm) and the absorbance at 280 nm) at pH=7.0 was purchased from Chinese Academy of Science, Shanghai institution of biochemistry and used without further purification. Hydrogen peroxide (30%, *v*/*v* solution) and *o*-phenylenediamine (OPD) were of analytic grade. Stock solutions of H_2O_2 (0.98 M) and OPD (0.1 M) for enzyme assay were prepared by dissolving H_2O_2 and OPD in 50 mM sodium phosphate buffer, pH 7.0. The stock solutions were stored at 4°C.

Analytical grade disodium phosphate and sodium dihydrogen phosphate were used for preparing the different concentrations of sodium phosphate buffer solutions. The pH value of the solutions was adjusted to 7.0 by means of a pH meter (ORION Model 828). Doubly distilled water was used throughout.

Instrumental methods

Differential scanning calorimetry

DSC experiments were performed on a DSC822^e Mettler Toledo calorimeter (Schwerzenbach, Switzerland) with cell volumes of 0.025 mL. All measurements were performed at a scan rate of 5°C min^{-1} in the temperature ranges of 35–95°C. The enzyme concentration used in the experiments was 8 mg mL^{-1}. Before calorimetric measurement, HRP was dissolved in various concentrations of sodium phosphate buffer and incubated at room temperature for 1 h. The temperature of thermal denaturation (T_m) was determined at the temperature of the maximum of the endothermic peak. Data processing was performed with the Mettler Star^e program.

Fluorescence spectrophotometry

Fluorescence measurements were performed on a Hitachi F-2500 fluorescence spectrophotometer using a cuvette with a path length of 10 mm. The tryptophan fluorescence emission spectra of HRP were measured by exciting at 296 nm. The excitation wavelength was chosen at 296 nm in order to avoid the contribution of the tyrosine residues present in HRP [20]. Excitation and emission slit widths were set to 10 nm. The sample after the DSC experiment was diluted into 50 mM sodium phosphate buffer to obtain 0.075 mg mL^{-1} HRP solution and then measured the tryptophan fluorescence emission spectra at 25°C.

Spectrophotometry

Absorption spectral measurements were recorded on a TU-1901 (Beijing general instrument Co. Ltd.) double-beam spectrophotometer using rectangular quartz cell path length of 1 mm with light path length of 10 mm. Before measuring the heme absorbance at 403 nm, HRP (0.5 mg mL^{-1}) solutions were incubated in 70°C for different incubation times ranging from 10 to 60 min and then maintained 24 h at room temperature.

Enzymatic activity measurements

The enzymatic activity assay of HRP was performed on LKB-2107 batch microcalorimetry system by measuring the initial exothermic rate of the oxidation of OPD

by H_2O_2 in 50 mM sodium phosphate buffer, pH 7.0 and at 25°C. The details of the performance and the structure of instrument were previously reported [36]. HRP solutions after thermal treatment as in the absorption spectral measurements were diluted into 50 mM sodium phosphate buffer. The final concentration of the enzyme was 1.5 nM in reaction solutions. In reaction solutions the concentrations of OPD and H_2O_2 were 0.1 and 0.3 mM, respectively. Reported activity values were the activities relative to native enzyme at 25°C.

Results and discussion

DSC studies

Comparison with the spectroscopic techniques, calorimetry is the only technique that directly measures the heat of protein unfolding as a function of temperature [22, 23]. Pina [18] and Carvalho *et al.* [8, 20] have studied the effects of the scan rate and the pH value on the thermal denaturation of HRP by DSC. In this paper, using DSC method, the effects of sodium phosphate buffer on the denaturation temperatures (T_m, the temperature at the maximum of the heat flow profile) for HRP were studied. Figure 1 showed the DSC curves of thermal denaturation of HRP in different buffer concentrations. The curve and further analysis showed in Fig. 2 indicated that the T_m values decreased linearly with the increasing buffer concentration. It demonstrated that sodium phosphate could accelerate the unfolding process of the protein.

In addition, the second scan was carried out after the HRP solution was cooled to 20°C. Since a lower endothermic peak was observed in the second scan, it suggested that HRP unfolding was partially reversible in these experimental conditions. The literatures [8, 20] obtained the same results for HRPA at pH 7 and 10.

Furthermore, to elucidate whether the thermal denaturation of HRP include a step of oligomerization, we checked the dependence of DSC curve on the protein concentration. Our results showed that the T_m value was not affected by the concentration of the protein in a range of 2.0–8.8 mg mL^{-1}. It was significant that oligomerization did not contribute in thermal denaturation of HRP. On the other hand, the absence of any exothermic peak in DSC profiles also indicated the lack of aggregation in the process.

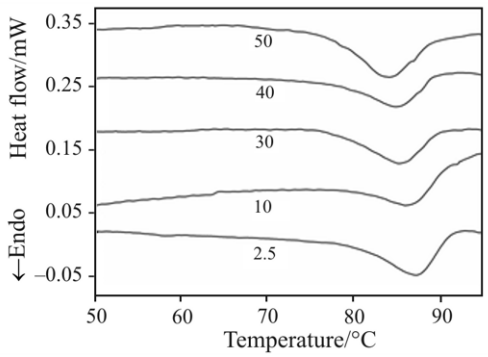

Fig. 1 DSC profiles of HRP (8 mg mL^{-1}) thermal denaturation in 2.5, 10, 30, 40 and 50 mM sodium phosphate buffer, pH 7.0. The scan rate was 5°C min^{-1}

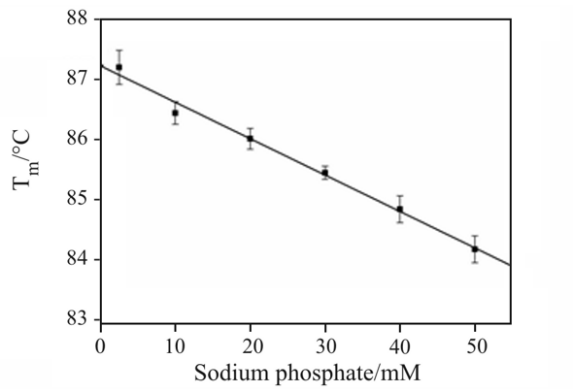

Fig. 2 Relationship of the T_m value of HRP (8 mg mL^{-1}) thermal denaturation with sodium phosphate buffer concentration at a scan rate of 5°C min^{-1}

Tryptophan fluorescence studies

The intrinsic fluorescence emission from tryptophan residues is quenched due to intramolecular tryptophan-heme energy transfer in the native HRPC [37, 38]. When the enzyme is denatured, the distance between the tryptophan residue and the heme group increases, so the heme quenching effect of tryptophan emission is weakened, which leads to the increase in the fluorescence intensity [16–18]. On the other hand, a change in the microenvironment surrounding the tryptophan residue can cause the shift of the wavelength of the maximum tryptophan fluorescence emission (λ_m) [39]. In most proteins, a red shift of the fluorescence spectra is resulted from the change in the tryptophan microenvironment to a more exposed and a more polar one in the unfolded form [17]. Therefore, the effects of sodium phosphate concentration on thermal stability in the environment of the tryptophan residues of the enzyme can be obtained by measuring the changes in the tryptophan fluorescence spectra.

Figure 3 showed that the tryptophan fluorescence emission spectra of HRP, which were excited at 296 nm and were measured after DSC scans in sodium phosphate buffers with different concentration. The tryptophan fluorescence intensity increased with the increase in buffer concentration. The changes in the tryptophan fluorescence intensity suggested that sodium phosphate could affect the tryptophan environment during HRP thermal denaturation. Moreover, the

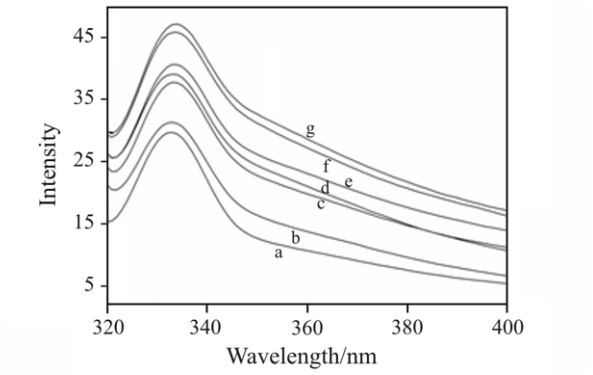

Fig. 3 Tryptophan fluorescence spectra of a – native and b–g – denatured HRP (0.075 mg mL^{-1}) at 25°C using the excitation wavelength at 296 nm. The denatured HRP solutions were thermal scanned by DSC in b – 2.5, c – 10, d – 20, e – 30, f – 40 and g – 50 mM sodium phosphate buffer up to 95°C and then cooled them to room temperature

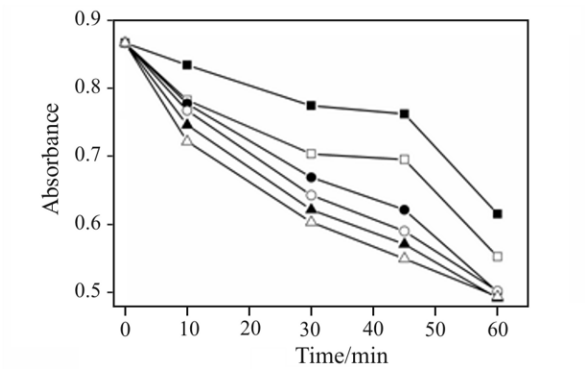

Fig. 4 Effects of sodium phosphate buffer on the absorbance at 403 nm of incubated HRP (0.5 mg mL^{-1}) at 70°C for different time and cooled to room temperature for 24 h. Symbols refer to HRP in different sodium phosphate buffer concentrations: ■ – 2.5, □ – 10, ● – 20, ○ – 30, ▲ – 40 and △– 50 mM

λ_m values were only red-shifted 1–2 nm, indicating that in the denatured state of HRP the tryptophan residue was still buried in the protein. The same phenomenon for the enzyme was found in the literatures [8, 18, 20].

UV absorption and enzymatic activity assay studies

The DSC results suggested that HRP thermal denaturation is an irreversible one with an intermediate reversible unfolded state. If a sufficient length of time, the reversible unfolded state of HRP can slowly refold to the native state and recover its activity. The HRP activity assay confirmed the results. We incubated HRP solutions at 70°C for 30 min and cooled to room temperature for different times. Enzyme activity assay showed that the HRP activity recovered with the time, and achieved maximum value after maintained 24 h at room temperature (when HRP was thermal denatured in 50 mM sodium phosphate buffer, the maximum value of HRP activity was about 53% of the native one). To determine the effect of buffer concentration on HRP thermal stability, the enzymatic activity assay and the absorbance measurement were carried out after protein solutions were incubated at 70°C for various time intervals and cooled to room temperature for 24 h.

The parallel experiments on the absorbance changes in the Soret region and enzymatic activity assay were carried out to determine the effects of sodium phosphate buffer on HRP thermal stability because the heme group is essential for peroxidase activity [40]. Figures 4 and 5 showed that the absorbance at 403 nm and the relative activity of denatured HRP decreased while the incubation time extended in various buffers. The results indicated that HRP thermal denaturation was an irreversible denaturation process. The effect of

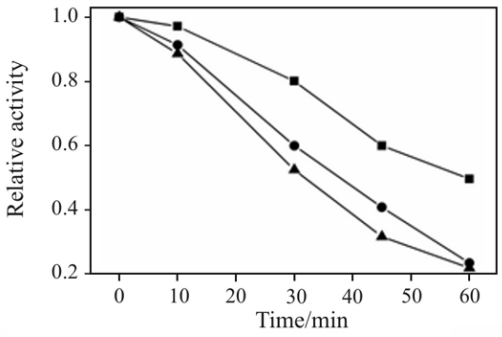

Fig. 5 Relative activity of denatured HRP in ■ – 2.5, ● – 30 and ▲ – 50 mM sodium phosphate buffer incubated at 70°C for different time. The activity was measured at 25°C

sodium phosphate buffer on the relative activity and the absorbance of denatured HRP were shown in Fig. 6. The result indicated that sodium phosphate reduced the thermal stability of HRP. In these experiments, the HRP activity assay showed the percentage of remained native HRP, which was related to the HRP irreversible denaturation rate, so Fig. 6 also indicated that the overall denaturation rates was accelerated by

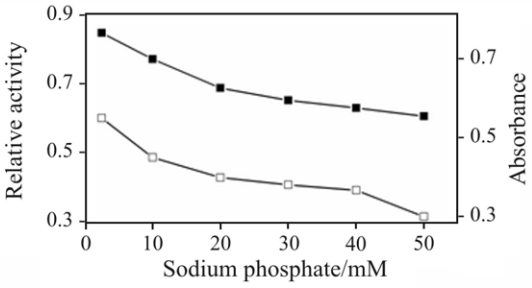

Fig. 6 Effects of sodium phosphate buffer on □ – relative activity and the absorbance at ■ – 403 nm of HRP incubated at 70°C for 45 min

sodium phosphate buffer. With the results of DSC, the acceleration of the overall denaturation rate can be determined by the unfolding step of the native HRP. Thus the HRP denaturation process can be expressed by Eq. (2), as a first-order process.

Conclusions

Different techniques such as DSC, tryptophan fluorescence, the heme absorption and enzymatic activity assay were used for analyzing the effects of sodium phosphate buffer on HRP thermal stability. In the experiments, an irreversible denaturation with an intermediate reversible unfolded state was observed. Therefore the classical denaturation model, $N \leftrightarrow U \rightarrow D$ was proposed to explain the experimental data. Since DSC is the technique for obtaining data on the thermodynamics of unfolding of the overall structure of global proteins, the T_m relates to the equilibrium between N and U. Taking into account that the T_m values depended on the concentration of sodium phosphate buffer, it suggested that sodium phosphate accelerated the transition from N to U in the denaturation process of HRP. As a result, the rate of the overall process of $N \rightarrow D$ was promoted by sodium phosphate. Therefore, sodium phosphate reduced the thermal stability of HRP. This result was proved by the data from the heme absorbance measurement and enzymatic activity assay. The whole process of HRP thermal denaturation was simplified to a two-state kinetic model ($N \rightarrow D$) and rate-limiting step of the thermal denaturation was determined by the unfolding step. The limiting case of the Lumry–Eyring model suggested that the activity-recovering rate was much slower than the denaturation rate. The contrasts of the rates can be observed in our experiments.

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