

## Reactivation of Broccoli Peroxidases: Structural Changes of Partially Denatured Isoenzymes

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Structural changes involved in the reactivation of peroxidases (PODs) from broccoli and horseradish (HRP) following heat denaturation were investigated by using circular dichroism and absorption spectroscopy. Cooling heat-treated enzymes resulted in rapid refolding of the secondary structure into an inactive structural species, similar in conformation to the native enzyme. Reassociation of heme to the refolded peroxidase, as well as molecular rearrangement of the structure around the heme, occurs during incubation at ~25 °C and results in the return of biological activity. The secondary structure of neutral broccoli POD (N) is relatively heat labile, resulting in a rapid loss of activity, but the level of reactivation is high because the structure at the heme pocket is relatively stable. Acidic broccoli POD and HRP are more heat stable than N, but have a low degree of reactivation. Loss of activity is due primarily to alteration of the structure at the heme pocket. Effects of bovine serum albumin and pH on reactivation of PODs are also discussed.

**KEYWORDS:** Peroxidase; reactivation; horseradish; broccoli; circular dichroism; absorption spectroscopy

### INTRODUCTION

Heat-treated peroxidases (PODs) from several plant sources have shown an ability to recover their activity while being stored at ambient temperature after heat treatment (1, 2). Many studies have revealed that residual or reactivated POD can cause significant deterioration in the quality of various high-temperature–short-time (HTST) processed foods (1–4). The ability of POD to reactivate after it is denatured by heat varies with treatment conditions and the species of vegetable and may differ between isoenzymes of the same species. In HRP, reactivation has been reported to take place after partial inactivation at 70, 90, or 110 °C (5). Rodrigo et al. (6) showed that HRP (RZ = 3.1) treated at high temperatures (115 and 130 °C) for short times (5–85 s) can recover up to 22% of its activity during incubation at 25 °C. An earlier study by Joffe and Ball (7) on reactivation of POD was performed with highly purified HRP (RZ = 3.02), which gave a significant amount of reactivation. The temperatures used in this study were high, for example, 120–150 °C. In a more recent study, a 15–30% restoration of original POD activity was noted for the cationic isoenzyme (pI ~ 9) of green peas after several hours of incubation at 25 °C following a 50–70 °C heat treatment for 1–10 min (8). However, several other studies have found no appreciable

recovery of activity in purified PODs derived from spring cabbage and Brussel sprouts (9) or green peas (8).

Previous studies have shown that heat inactivation characteristics differed for acidic (A), neutral (N), and basic (B) broccoli PODs (10). At 65 °C, A was the most heat stable followed by N and B. Reactivation of N occurred within 10 min after the heated enzyme was cooled and incubated at room temperature. Other broccoli isoenzymes as well as HRP regained their activity after heat treatment, but the extent of reactivation varied from 0 to 50% depending on the isoenzyme and heating conditions (temperature and time). The denaturation temperature allowing for the maximum reactivation varied depending on the isoenzyme. In all cases, heat treatment at low temperatures for long times prevented reactivation of the heated enzymes (10). This result is in agreement with other studies by Schwimmer (1), Lu and Whitaker (2), Adams (3), and Rodrigo (4), which showed that reactivation occurs more readily if the time taken to reach the desired treatment temperature during heat treatment is short.

It has been suggested that reactivation of the peroxidase enzyme is a complex process that is influenced by several factors. To explain how the types of isoenzyme and treatment conditions affect recovery of activity following heat treatment, it is necessary to understand the changes that occur in the enzyme structure during heat inactivation and reactivation. In this study, we investigate the structural changes involved in the heat inactivation and reactivation of this enzyme by using circular dichroism (CD) and absorption spectroscopy. CD has

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proven to be a useful tool to study protein structure, because the far-UV CD (200–250 nm) of a protein reflects its secondary structure. Thermal unfolding and heat stability of the enzyme can be studied by observing a decrease in the CD intensity, particularly at 222 nm at various temperatures (11, 12). Due to the presence of heme, PODs have absorption bands in the visible and near-ultraviolet region. The Soret band has the highest extinction coefficient with its maximum at ~403 nm. Change in absorbance in the Soret band allows us to determine the effect of temperature on the tertiary structure of the overall protein and the tertiary structure around the heme active site. In this study, we use CD and absorption spectroscopy to investigate structural changes of the reactivated broccoli isoenzymes, A and N, as well as HRP. Due to the limited amount of B available, it was not used in this study.

## MATERIALS AND METHODS

**Enzyme Purification.** POD isoenzymes were prepared from soluble extracts of broccoli stems and purified according to the procedure described by Thongsook and Barrett (13). The isoenzymes obtained from broccoli stems are distinguished by their *pI* and are referred to as acidic (anionic, A), neutral (N), and basic (cationic, B). HRP with *RZ* = 2.9 was purchased from Sigma (St. Louis, MO). A, N, and HRP have molecular masses of ~48, 43, and 44 kDa and their isoelectric points are <3, 5, and >10, respectively.

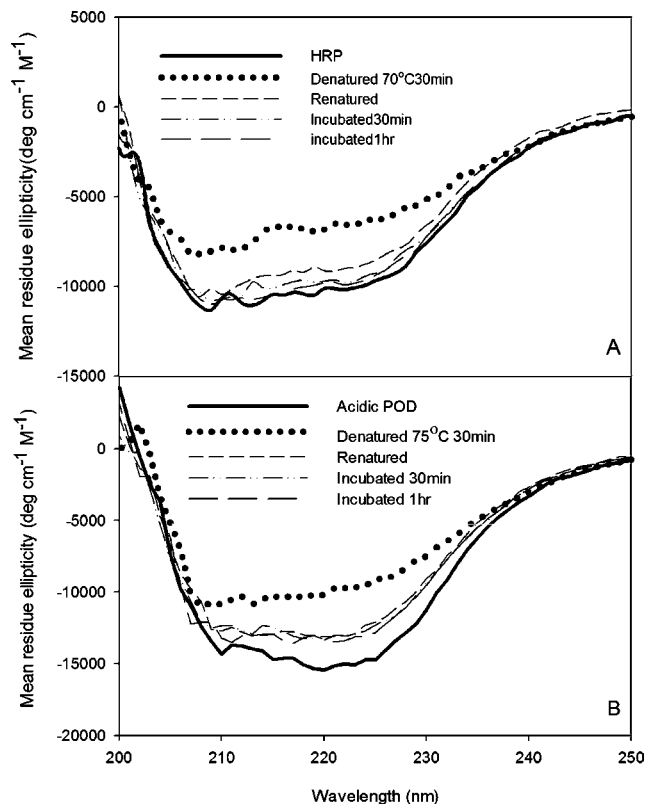
**Circular Dichroism.** CD experiments were carried out using a Jasco J720 spectropolarimeter. CD in the far-UV region (250–190 nm) was monitored in a water-jacketed cylindrical cell of 10 mm path length. All samples were prepared in 10 mM sodium phosphate buffer, pH 7.0. Structural changes in POD were observed during heating, cooling, and incubation. To study the rate of reactivation of the enzyme following heat treatment, the temperature was increased using a preheated water bath set at the desired temperature and held for a specific time. Spectra reported were taken during heating and after cooling of the heated samples in ice water. Spectra were also taken at various times during incubation at room temperature. Spectra were averages of four scans taken at a scan rate of 50 nm/min. Spectra were analyzed using SELCON3 software. The CD data were expressed in terms of mean residue ellipticity per residue,  $[\theta]$ , in  $M^{-1} \text{ cm}^{-1}$  units, according to the equation

$$[\theta] = \theta_{\text{obs}} / 10 \times C \times l \times n$$

where  $\theta_{\text{obs}}$  is the ellipticity measured (degree) at wavelength  $\lambda$ ,  $C$  is protein concentration (M),  $l$  is the optical path length of the cell (cm), and  $n$  is the number of amino acid residues of the protein. Every treatment was repeated at least twice. For thermal unfolding of POD isoenzymes, the temperature was raised from 25 to 90 °C in increments of 5 °C with an equilibration time of 2 min at each temperature before the spectrum was taken. The rate of temperature increase was 1.8 °C/min.

**Absorption Spectroscopy.** Changes in Soret band absorbance at 403 nm were monitored using a UV–vis scanning spectrophotometer (UV-2101PC, Shimadzu). The enzyme solutions were prepared in 50 mM Tris–acetate buffer, pH 7.0. Heat treatments were performed in capillary tubes (~5 mm diameter) in a circulating water bath (model 20B, Julabo, Allentown, PA) for the temperatures and times specified. The come-up time for the capillaries has been determined to be <10 s (14). Heat-treated enzyme solutions were cooled in ice water and transferred to a cuvette. Absorbance (200–800 nm) of the heated enzyme solution was measured before and after heat treatments. Spectra were also taken at various times during incubation at room temperature. Every treatment was repeated at least twice. Concentrations of the enzymes were chosen arbitrarily to yield comparable absorptions at the Soret band.

**Peroxidase Activity.** Activity was determined by monitoring the time course of the change in absorbance at 420 nm upon enzyme-catalyzed oxidation of the substrate. Guaiacol (Sigma) and hydrogen peroxide were used as substrates. The final reaction mixture contained



**Figure 1.** Secondary structure of native, thermally denatured, and reactivated peroxidase for HRP (0.03 mg/mL HRP in 10 mM sodium phosphate buffer, pH 7.0) (A) and for acidic broccoli POD (0.026 mg/mL in 10 mM sodium phosphate buffer, pH 7.0) (B) as determined by CD spectroscopy. Denaturation was carried out at 70 °C for 30 min for HRP and at 75 °C for 30 min for acidic POD. Renatured spectra were obtained on the enzyme solution after cooling in ice water. Incubated spectra were obtained for the enzyme solution during holding at 25 °C.

50 mM guaiacol, 50  $\mu\text{L}$  of enzyme, 10 mM  $\text{H}_2\text{O}_2$ , and 50 mM Tris–acetate buffer, pH 6.0, in a volume of 1.5 mL. The assay was performed at 25 °C using a UV–visible scanning spectrophotometer (UV-2101PC Shimadzu). One unit of enzyme was defined as the amount of substrate (micrograms of guaiacol) consumed in 1 min using an extinction coefficient of 6650  $M^{-1} \text{ cm}^{-1}$ , for tetraguaiacol, the product.

**Effect of Bovine Serum Albumin (BSA) and pH on Reactivation.** HRP and A were prepared in 50 mM Tris–acetate buffer, pH 7.0, in the presence and absence of 0.05% BSA. Heat treatments were carried out at 68 °C for 1 h for HRP and at 75 °C for 5 min for A. Absorption spectroscopy and CD experiments were performed as described above. CD and absorbance spectra were obtained at pH 4.0 for HRP samples prepared in phosphate buffer, pH 4.0.

**Statistical Analysis.** Data were analyzed using one-way analysis of variance (ANOVA) by SPSS software. Means were compared using Duncan's multiple range *t* test.

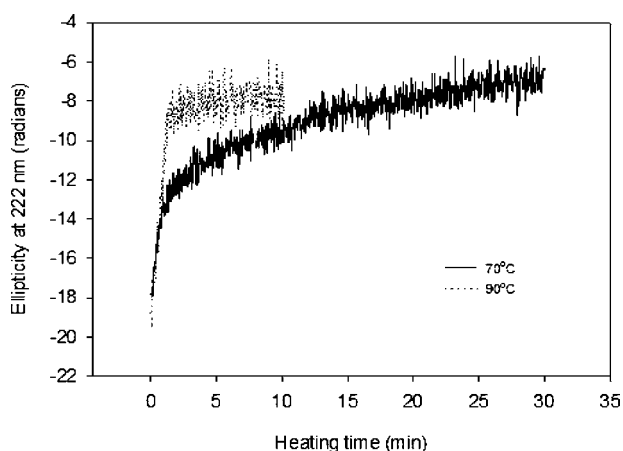
## RESULTS AND DISCUSSION

**Secondary Structure and Reactivation of Thermally Denatured Peroxidases.** Far-UV CD spectra of native, thermally denatured, and reactivated HRP at pH 7.0 are given in Figure 1A. The far-UV CD spectra of HRP showed two minima at approximately 210 and 222 nm, confirming the helical structure of the enzyme. Computer simulation of the CD spectra using the program SELCON3 allowed determination of the fractions of  $\alpha$ -helix,  $\beta$ -strands, turns, and unordered secondary structure of the native, thermally denatured, and reactivated HRP at neutral pH (Table 1). The structure of heme PODs from plants is formed by 10–11  $\alpha$ -helices (ca. 30–40% of the total

**Table 1.** Secondary Structure of Native, Thermally Denatured, and Renatured HRP (0.03 mg/mL HRP in 10 mM Sodium Phosphate Buffer, pH 7.0) As Determined by CD Spectroscopy<sup>a</sup>

protein	helix (%)	$\beta$ -strand (%)	turn (%)	unordered (%)	activity (%)
<b>A,<sup>b</sup> native</b>	30.75 $\pm$ 2.47	16.05 $\pm$ 1.91	20.35 $\pm$ 0.35	33.65 $\pm$ 1.06	100
denatured at 70 °C for 30 min	22.95 $\pm$ 2.19	26.45 $\pm$ 7.85	15.75 $\pm$ 1.10	32.35 $\pm$ 4.06	nd <sup>c</sup>
renatured	27.75 $\pm$ 2.33	17.55 $\pm$ 1.20	19.45 $\pm$ 0.04	34.45 $\pm$ 0.95	48.70 $\pm$ 0.42
incubated for 1 h	29.65 $\pm$ 2.90	16.60 $\pm$ 1.27	19.95 $\pm$ 0.32	34.10 $\pm$ 0.42	73.65 $\pm$ 4.74
<b>B,<sup>b</sup> native</b>	33.57 $\pm$ 1.79	13.48 $\pm$ 1.11	20.40 $\pm$ 0.14	31.80 $\pm$ 0.28	100
denatured at 90 °C for 3 min	17.60 $\pm$ 5.09	24.90 $\pm$ 6.93	17.55 $\pm$ 1.48	40.85 $\pm$ 1.20	nd
renatured	32.30 $\pm$ 0.42	13.95 $\pm$ 1.34	20.20 $\pm$ 0.56	34.95 $\pm$ 1.48	36.20 $\pm$ 5.94
incubated for 1 h	33.50 $\pm$ 0.71	13.90 $\pm$ 1.27	20.36 $\pm$ 0.33	34.70 $\pm$ 1.84	63.05 $\pm$ 1.48
<b>C,<sup>d</sup> native</b>	35.00 $\pm$ 4.94	14.33 $\pm$ 1.76	20.97 $\pm$ 0.47	31.57 $\pm$ 2.84	100
denatured at 95 °C for 5 min	23.23 $\pm$ 2.97	15.00 $\pm$ 3.61	17.60 $\pm$ 3.50	36.30 $\pm$ 5.31	nd
renatured	31.00 $\pm$ 5.56	19.33 $\pm$ 1.27	19.10 $\pm$ 0.82	32.20 $\pm$ 0.87	23.37 $\pm$ 3.59
incubated for 1 h	32.37 $\pm$ 4.96	15.10 $\pm$ 3.81	19.40 $\pm$ 0.20	33.83 $\pm$ 0.15	61.57 $\pm$ 3.61

<sup>a</sup> Denaturation was carried out at 70 °C for 30 min, at 90 °C for 3 min, and at 95 °C for 5 min. <sup>b</sup> Mean  $\pm$  SD ( $n = 2$ ). <sup>c</sup> Not determined. <sup>d</sup> Mean  $\pm$  SD ( $n = 3$ ).

**Figure 2.** Changes in ellipticity (radians) of HRP (0.03 mg/mL HRP in 10 mM sodium phosphate buffer, pH 7.0) at 222 nm during heating at 70 and 90 °C.

secondary structure) linked by loops and turns, whereas the  $\beta$ -strand structures are minor components. The results show the helical structure content of the native enzyme is approximately 30%, which agrees with other studies (11, 15).

Heat treatment resulted in loss of the secondary structure. The helical structure decreased, whereas the  $\beta$ -strand and unordered structure increased significantly ( $p < 0.05$ ) (Table 1). However, heat treatment did not completely unfold the protein. For example, heat treatment of HRP at 95 °C for 5 min substantially reduced the enzyme activity to 23% of the original, yet the helical structure content remained at 65%, with only a slight increase in the unordered structure content. Previous studies have shown that heat treatment at 93 and 92 °C did not completely unfold HRPc and soybean POD, respectively, unless >30 mM dithiothreitol (DTT) was used in combination with high temperature (11, 12). Removal of interhelical disulfide bonding by DTT drastically decreased the stability of the enzyme.

Reduction of mean ellipticity at 222 nm occurred as heating time increased in the 70 °C treatment, but this relationship was not found for heat treatments at 75 and 95 °C. This may be because the decrease in the mean ellipticity at 222 nm ( $[\theta]_{222}$ ) has approached a maximum at those given heating times. Figure 2 shows a rapid loss of secondary structure when the enzyme was treated at 90 °C. The  $[\theta]_{222}$  was fairly constant after having increased rapidly within 3 min of initiation of the heat treatment. For treatment at 70 °C, the  $[\theta]_{222}$  went up more gradually as heating time increased. After heat treatment at 90 °C for 10

min and at 70 °C for 30 min,  $[\theta]_{222}$  reached approximately the same level ( $\sim 8$ ) (Figure 2).

Following heat treatments of 10, 30, and 60 min at 70 °C, the amount of helical structure decreased from 75 to 87%, whereas the enzyme lost between 34 and 78% of its activity (data not shown). After treatment at 75 °C for either 5 or 10 min, helical structure of the protein decreased between 30 and 67%, respectively, whereas the enzyme activity declined by 59–73%. Treatment at 90 °C for 3 min decreased helical structure of the protein to 35% and activity to 32% (data not shown). No clear relationship linking heating time with the extent of reduction in helical structure content or the increase in  $\beta$ -strand content was observed.

Upon cooling, the heat-treated enzyme refolded to a state with decreased ellipticity, as compared to the native state, with some variation depending on the severity of the treatment (Figure 1A). The CD spectrum remained unchanged during prolonged incubation at room temperature. Following incubation for 1 h, the helical structure increased, but the final value remained lower than the original. The  $\beta$ -strand structure increased slightly (Table 1).

Activity of the enzyme decreased due to heat treatment. The extent of decrease in enzyme activity increased as heating time increased. A recovery in enzyme activity did not occur until after cooling and holding of the heat-treated enzyme at room temperature, whereas the protein structure refolded rapidly upon cooling and remained unchanged during holding at room temperature.

The CD spectrum of acidic broccoli POD (A) was similar to that of HRP. The helical structure of the native enzyme was approximately 40%, which is slightly higher than that of HRP. However, the two enzymes showed similar heat inactivation and reactivation characteristics. A significant decrease ( $p < 0.05$ ) in the helical structure of A took place during heat treatment. The CD spectra and secondary structure content of the native, denatured, and renatured enzymes are shown in Figure 1B and Table 2. Restoration of the secondary structure occurred rapidly when the heated enzyme was cooled. The helical structure returned to the original after heat treatment at 70 °C for 30 min, whereas the helical structure returned to approximately 90% of the original in sample held at 75 °C for 30 min and 1 h. Similar to the observation for HRP, the secondary structure remained mostly constant after the cooling step, but the enzyme activity was regained more slowly, upon incubation at room temperature.

HRP and A refolded to their native structures after heat treatment was removed. Like other single-chain proteins, the information governing folding to the minimum energy, biologi-



**Table 2.** Secondary Structure of Native, Thermally Denatured, and Renatured Acidic Broccoli POD (A) (0.026 mg/mL in 10 mM Sodium Phosphate Buffer, pH 7.0) As Determined by CD Spectroscopy<sup>a</sup>

protein	helix (%)	$\beta$ -strand (%)	turn (%)	unordered (%)	activity (%)
<b>A,<sup>b</sup> native</b>	39.95 $\pm$ 2.05	14.77 $\pm$ 2.74	26.10 $\pm$ 5.66	23.44 $\pm$ 4.01	100
denatured at 70 °C for 30 min	31.10 $\pm$ 0.57	17.48 $\pm$ 0.04	20.30 $\pm$ 0.28	30.70 $\pm$ 0.14	nd <sup>c</sup>
renatured	44.85 $\pm$ 2.76	11.58 $\pm$ 0.95	17.73 $\pm$ 0.18	28.40 $\pm$ 0.71	33.00 $\pm$ 1.41
incubated for 1 h	41.30 $\pm$ 3.96	13.20 $\pm$ 5.66	15.10 $\pm$ 3.39	29.80 $\pm$ 1.27	79.70 $\pm$ 3.82
<b>B,<sup>b</sup> native</b>	43.10 $\pm$ 2.40	9.77 $\pm$ 4.33	20.10 $\pm$ 2.83	27.89 $\pm$ 2.28	100
denatured at 75 °C for 30 min	31.90 $\pm$ 0.57	17.43 $\pm$ 0.04	19.90 $\pm$ 0.28	30.90 $\pm$ 0.14	nd
renatured	40.95 $\pm$ 2.76	12.93 $\pm$ 0.95	17.98 $\pm$ 0.18	29.40 $\pm$ 0.71	31.50 $\pm$ 0.71
incubated for 1 h	40.00 $\pm$ 1.27	14.00 $\pm$ 0.42	17.30 $\pm$ 0.42	29.75 $\pm$ 0.49	65.10 $\pm$ 8.34
<b>C,<sup>b</sup> native</b>	42.65 $\pm$ 2.47	13.45 $\pm$ 2.33	18.60 $\pm$ 0.71	27.80 $\pm$ 1.27	100
denatured at 75 °C for 1 h	26.65 $\pm$ 2.90	24.50 $\pm$ 2.62	18.60 $\pm$ 5.37	28.45 $\pm$ 1.91	nd
renatured	37.05 $\pm$ 1.06	13.25 $\pm$ 2.62	19.20 $\pm$ 1.41	33.60 $\pm$ 3.25	33.72 $\pm$ 8.99
incubated for 1 h	39.30 $\pm$ 0.85	11.70 $\pm$ 1.41	16.95 $\pm$ 1.20	33.45 $\pm$ 3.32	67.22 $\pm$ 4.78

<sup>a</sup> Denaturations were carried out at 70 °C for 30 min, at 75 °C for 30 min, and at 75 °C for 1 h. <sup>b</sup> Mean  $\pm$  SD ( $n = 2$ ). <sup>c</sup> Not determined.

cally active protein is contained in the primary structure. Refolding of the secondary structure upon removal of denaturants has been reported in several studies (16, 17). HRP, denatured by guanidinium chloride, showed recovery of its secondary structure upon removal of the denaturant by dialysis (18).

Upon cooling of heat-treated HRP and A, the enzyme refolded but the secondary structure of the renatured enzymes did not completely return to the original, indicating that modification of the enzyme structure occurred. Holzbaur et al. (19) used Fourier transform infrared (FTIR) spectroscopy to examine the thermal denaturation of the Fe(III) form of HRP in phosphate buffer at pH 7.0. Their results indicated that the denatured form of Fe(III) HRP does not undergo aggregation or gel formation after treatment, but the secondary structures of native and renatured HRPs are not identical. This result is consistent with our observation that changes in the secondary structure content were found in the heat-treated samples of both HRP and A, especially the significant increase ( $p < 0.05$ ) in the unordered structure in all treatments.

In general, restoration of the secondary structure of a protein may or may not be complete depending on the severity of heating conditions, as indicated by the recovered activity after heat treatment of HRP and A. In all cases, the secondary structure returned almost to that of the original (Figure 1), whereas the activity regained varied depending on treatment conditions. During incubation at room temperature, heated enzymes regained their activity without observable changes in the secondary structure. Refolding of the unfolded enzyme occurred immediately after the heated enzyme was cooled, whereas recovery of the enzyme activity occurred at warmer temperatures such as 25–35 °C during incubation. Although it seems that refolding of secondary structure is not directly correlated to reactivation of the heat-treated enzymes and that other processes occur during reactivation, it appears that reformation of the secondary structure is a requirement but not sufficient for recovery of the enzyme activity. Disulfide bonds were probably maintained during heat treatments in our study and therefore prevented complete unfolding of the secondary structure and most likely assisted in refolding of the structure upon cooling.

**Absorption Spectroscopy and Reactivation of Thermally Denatured Peroxidases.** Due to the presence of the heme group, PODs have absorption bands in the visible and near-ultraviolet region due to either  $\pi \rightarrow \pi^*$  or charge-transfer transitions as a result of interaction between the porphyrin and iron (20). The Soret band has the highest extinction with a maximum at  $\sim 403$

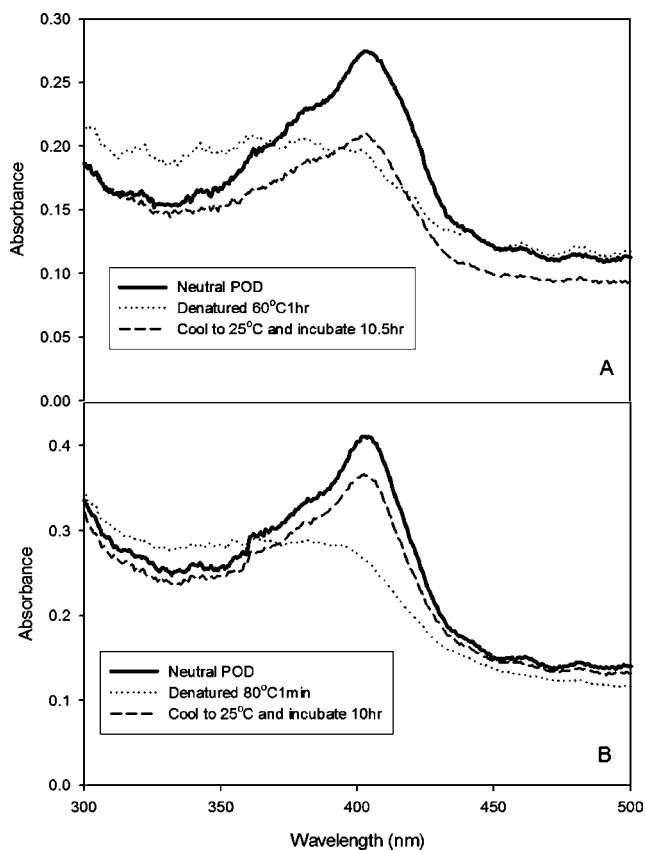
nm. In the absence of heme, apoproteins have no absorption in the visible region. Although the polypeptide chain has no absorbance in the visible range, its conformation affects the spectroscopic properties of the heme group (21). Therefore, changes in absorbance within the Soret band correlate with the conformational and functional stability of the enzyme. Heat treatments result in a reduction in the Soret band absorption and are often interpreted in terms of heme dissociation from the protein (5, 15, 22). In this part of the study, Soret band absorption was investigated to observe changes in heme structure during reactivation of POD following heat treatments.

*Neutral Broccoli POD Heated at Low and High Temperatures.* Previous study (10) on the reactivation of N treated at low temperatures for long times and at high temperatures for short times showed that the latter resulted in a higher extent of reactivation. In the present study, we looked at the effect of these different time/temperature treatments on changes in the structure of the enzyme at the heme pocket. Heat treatment resulted in a reduction in Soret band absorbance, which correlated with a reduction in enzyme activity.

Following heat treatment at 60 °C for 1 h and at 80 °C for 1 min, absorbance at 403 nm was decreased to 81 and 71% of the original native enzyme absorbance, whereas enzyme activity decreased to 32 and 21% of initial activity, respectively. Therefore, there is a smaller percentage change in absorbance at 403 nm than in enzyme activity as a result of heating. The Soret band absorption of N during incubation at 25 °C following heat treatment is shown in Figure 3. No shift in the maximum absorption was observed for the reactivated enzyme. During short (30 min) incubation times following heat treatment at 80 °C, the shape of the spectrum tended to broaden, indicating structural modification of the enzyme, but the spectra returned almost to the original shape, with a diminished intensity, with longer incubation times (5–10 h).

During incubation at room temperature (25 °C) following 1 min of heating, the total increase in the Soret band absorbance during incubation was 9 and 25%, whereas enzyme activity increased 26 and 73% for heat treatments of 60 and 80 °C, respectively (Figure 4). After the 80 °C treatment, both the enzyme activity and the absorbance at 403 nm increased significantly during the first 30 min of incubation at 25 °C, as compared to the 60 °C ( $p < 0.05$ ), which remained fairly constant during prolonged incubation.

Changes in the Soret band absorbance correlated well with changes in POD activity. In a study on unfolding of *Coprinus cinereus* POD at high temperatures, Tams and Welinder (23) found that in urea and at high pH the change in POD activity



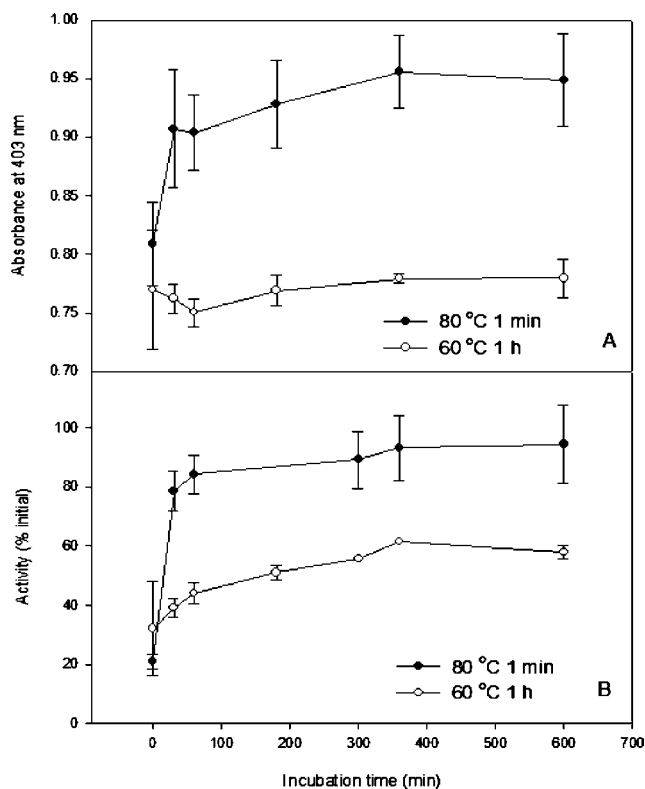
**Figure 3.** Absorption spectra of neutral broccoli POD (N) (~0.19 mg/mL) during heating at 60 °C for 1 h (A) and at 80 °C for 1 min (B), after cooling, and during incubation at 25 °C for 10 h.

paralleled that in Soret band absorbance. Tamura and Morita (5) also found that changes in absorbance at 404 nm coincided well with the activity curve, where absorbance decreased during heat inactivation of Japanese radish POD.

Regardless of the heating conditions, heme absorbance and activity did not return to the original values following cooling and incubation. The cause is suggested to be incomplete recapturing of the heme. Incomplete heme binding was found for HRP previously unfolded in urea and in guanidine hydrochloride at pH 7.0 after the concentration of the denaturants was reduced (24). Addition of exogenous heme has been shown to quench fluorescence to the level obtained before denaturation and result in 100% heme loading of HRP (24). A similar study by Pappa and Cass (18) on the folding mechanism of HRP showed that not all of the reactivated molecules contained heme, because <100% of the heme was incorporated into the protein. In most cases, incomplete heme incorporation is suggested to be caused by some degree of heme-heme association (18, 24).

The extent of enzyme activity recovered depended on the thermal inactivation and reactivation conditions. The yield of active enzyme when the refolding procedure was done by the heme addition after the recombinant HRP apoenzyme reactivation was about twice as high as that for the direct refolding of the recombinant HRP holoenzyme (25). This study also suggests the possibility of heme aggregation during refolding of the protein.

In aqueous solutions, hemins as well as the porphyrin IX species tend to aggregate among themselves (26, 27). Studies on interactions of porphyrins with hemoglobin as well as with myoglobin also suggest that various aggregation states of the porphyrin (dimer or other higher order species) may be bound

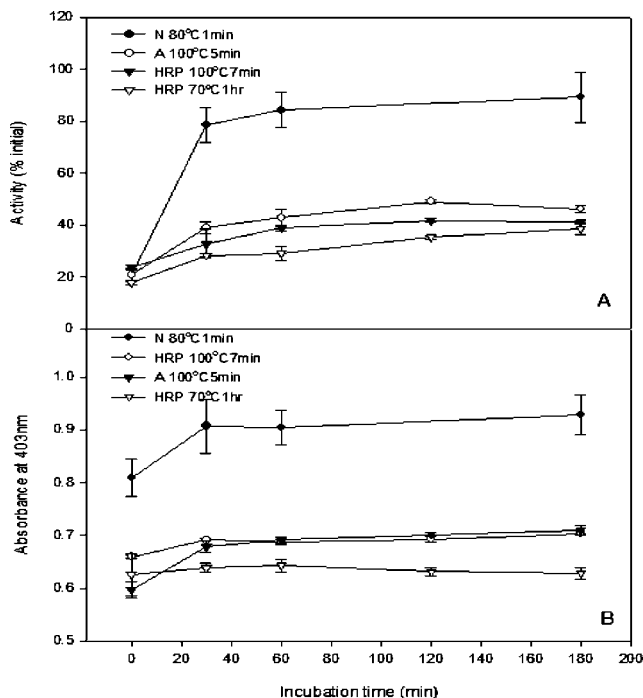


**Figure 4.** Changes in absorbance at 403 nm (A) and enzyme activity (B) of neutral broccoli POD during incubation at 25 °C for various times following heat treatment at 60 °C for 1 h and at 80 °C for 1 min. The points at 0 min indicate absorbance and activity of the enzyme at the initiation of cooling following heat treatment.

to different binding sites on the protein. The binding parameters of protoporphyrin (PP) and hematoporphyrin (HP) with hemoglobin or myoglobin change with the state of aggregation of the porphyrins. The binding affinity decreases, but the number of binding sites increases with increasing concentrations of porphyrins (27).

Heme dissociates from the protein moiety during heat treatment. In some cases, non-covalent bonding of heme to the protein moiety in regions other than the proper ligation position can occur. If heme aggregation occurs during heat treatment, it is likely that a high-temperature-short-time treatment causes less aggregation of the heme. Long heating times would tend to promote heme aggregation. The aggregated heme may also bind to the wrong site on the protein, therefore reducing the availability of the heme to return to the active site and, in turn, decreasing reactivation of the enzyme.

*Reactivation of HRP and Broccoli Peroxidase Isoenzymes.* Because each POD studied has a different heat stability, HRP, A, and N were heat treated under different conditions as illustrated in Figure 5. Heat treatments of the enzymes were performed until their activities were reduced to the same extent, ~20% of the original activity. The Soret band absorbance decreased to a different extent as a result of each heat treatment, depending on the enzyme, indicating the loss of heme, or at least loss of the native coordination structure. The least decrease was found following heat treatment of N at 80 °C for 1 min, which resulted in retention of 80% of the original absorbance at 403 nm. The absorbance at this wavelength decreased to 60, 55, and 65% of the original after heat treatment of A at 100 °C for 5 min and of HRP for 1 h at 70 °C and for 7 min at 100 °C, respectively.



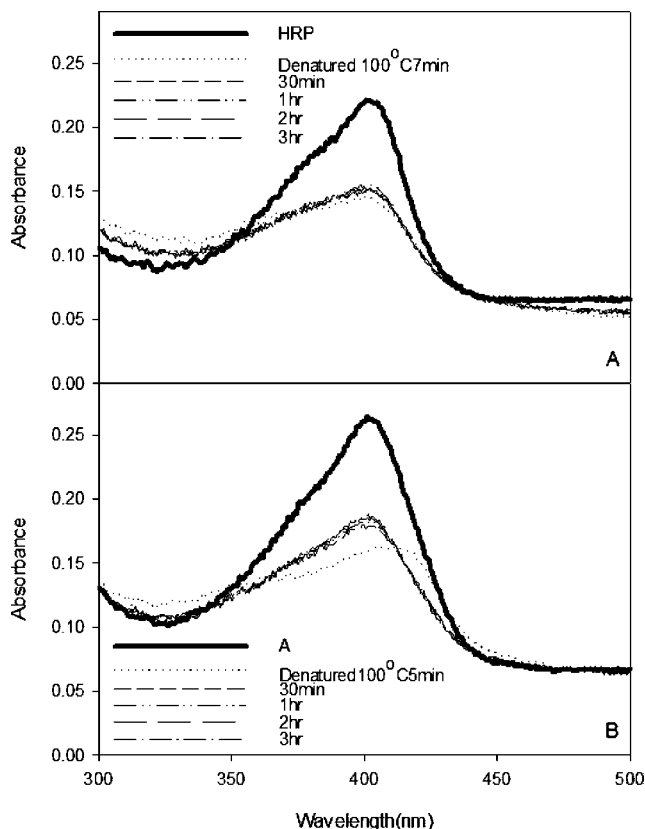
**Figure 5.** Changes in activity (A) and absorbance at 403 nm (B) during incubation at 25 °C for various times following heat treatment at 70 °C for 1 h and at 100 °C for 7 min for HRP, at 100 °C for 5 min for acidic broccoli POD, and at 80 °C for 1 min for neutral broccoli POD.

Following cooling and 30 min of incubation of enzymes that had been heated to 25 °C, the POD activity and the Soret band absorbance increased to a point and then remained fairly constant during prolonged incubation. Incubation of heat-treated enzymes at 25 °C resulted in an increase in the enzyme activity to 80% of the original activity for N, and the total reactivation was 60%. A and HRP reactivated to 30–40% of the original. Although the activity increased 60% for N and 20% for A and HRP, in all cases, the increases in the Soret band absorbance were the same, accounting for ~10% increase from the points after heating (Figure 5). These results show that reactivation of partially inactivated POD is related to the increase in the Soret band absorbance and is the result of reassociation of the heme to the protein moiety, although these relationships were not directly proportional.

Figure 6 shows the absorbance spectra of the native, denatured, and reactivated enzymes. In all cases, following heat treatment, besides the reduction in the Soret band absorption, the shapes of the spectra were altered due to changes in the enzyme structure. As a result, recovered activity was low. The activity increased as the heated enzymes were incubated at 25 °C. The spectra in most cases returned to the original shape but with a reduced maximum because not all of the heme reassociated with the protein.

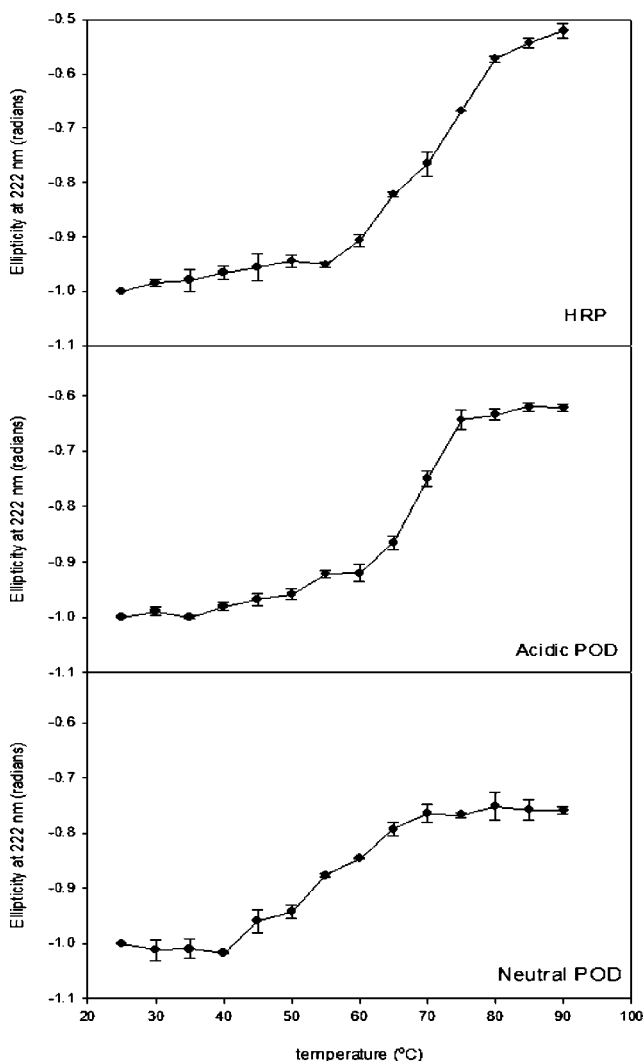
In our previous work, N was found to be the least heat stable isozyme (10). The loss of enzyme activity occurred at a much greater rate than did the heme loss; therefore, we saw loss in the activity while most of the heme appeared to be attached to the protein. Loss of activity was reversible, and because most of the heme remained attached to the protein, a higher level of activity recovery was found (Figure 5). The amount of activity recovered correlated with the amount of the refolded protein, which contained heme.

To further investigate the effect of different temperatures on the structure of POD isoenzymes, thermal unfolding of the



**Figure 6.** Absorption spectra of native, denatured, and reactivated POD after cooling and during incubation at 25 °C for 3 h: (A) HRP (0.2 mg/mL) treated at 100 °C for 7 min; (B) acidic broccoli POD (0.12 mg/mL) treated at 100 °C for 5 min.

secondary structures of HRP, A, and N was studied. The change in ellipticity of the backbone absorption at 222 nm versus temperature is shown in Figure 7. The unfolding processes of the backbone of HRP and A were similar. They exhibited more than one phase of thermal unfolding. The loss of the secondary structure began slowly as temperature increased. At 55 °C, at which the second phase began, a transition with a rapid loss in the secondary structure was observed. Unlike HRP and A, N showed one phase of thermal transition. N lost its secondary structure gradually as temperature increased, starting at 40 °C with the midpoint of the transition [an apparent melting temperature ( $T_m$ )] of approximately 55 °C. HRP, A, and N lost 50, 40, and 15%, respectively, of their original folded protein backbone following the 90 °C heat treatment. These results suggest that the unfolding of N differed from that of HRP and A. N lost its secondary structure more readily (i.e., at a lower temperature) than did HRP and A; however, its secondary structure was maintained to a greater extent at high temperature compared to the other two enzymes as judged by circular dichroism (Figure 8). Previous experiments (10) showed that HRP was the most heat stable, followed by A, whereas N was heat labile. In contrast to its low heat stability, N reactivated to a greater degree as compared to A and HRP. In this case, the rapid loss of the folded backbone structure of N is likely to contribute to the loss of its enzyme activity at lower temperatures. Despite its ready loss of secondary structure, the structure remained to a greater degree following heat treatment at high temperatures, even at 90 °C, unlike that of HRP and N, for which loss of their secondary structure was more profound. Therefore, the higher extent of reactivation for N may be

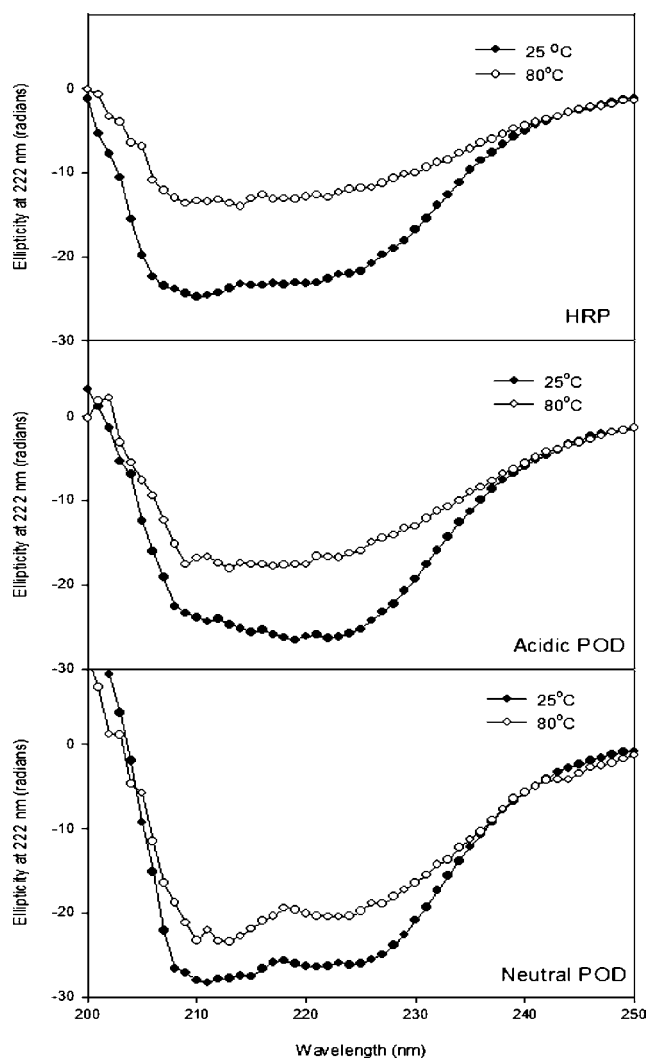


**Figure 7.** Change in the ellipticity of HRP and acidic (A) and neutral (N) broccoli PODs with temperature. The path length is 10 mm. Concentration of HRP was 0.03 mg/mL,  $\sim 0.04$  mg/mL for A and  $\sim 0.026$  mg/mL for N. All samples were prepared in 10 mM phosphate buffer, pH 7.0. Temperature was raised from 25 to 90 °C (1.8 °C/min) in steps of 5 °C with an equilibration time of 2 min at each temperature before the spectrum was taken.

explained by its more stable secondary structure, which may prevent heme from detaching from the protein.

Using CD, Soret-CD, and tryptophan fluorescence of HRP, Chattopadhyay and Mazumdar (11) showed that thermal unfolding of the enzyme consists of two phases. The first phase (35–55 °C) involves a change in the local conformation of the heme active site with a little or no change in the secondary structure. The second phase involves a significant change in secondary structure, which is a result of the complete removal of the heme active site (50–93 °C). Similar to their results, in the present study HRP and A exhibited more than one phase of thermal unfolding. The secondary structure seems to be more resilient than the structure at the heme pocket, because in most cases it returns to the original after heating, whereas heme is usually lost and does not reassociate.

N showed a distinctly different thermal unfolding from the other two enzymes (HRP and A). It is likely that unfolding of POD enzymes may proceed via different pathways depending on the particular enzyme. Rapid loss in the structure of N was most likely involved in its ready loss of enzyme activity.

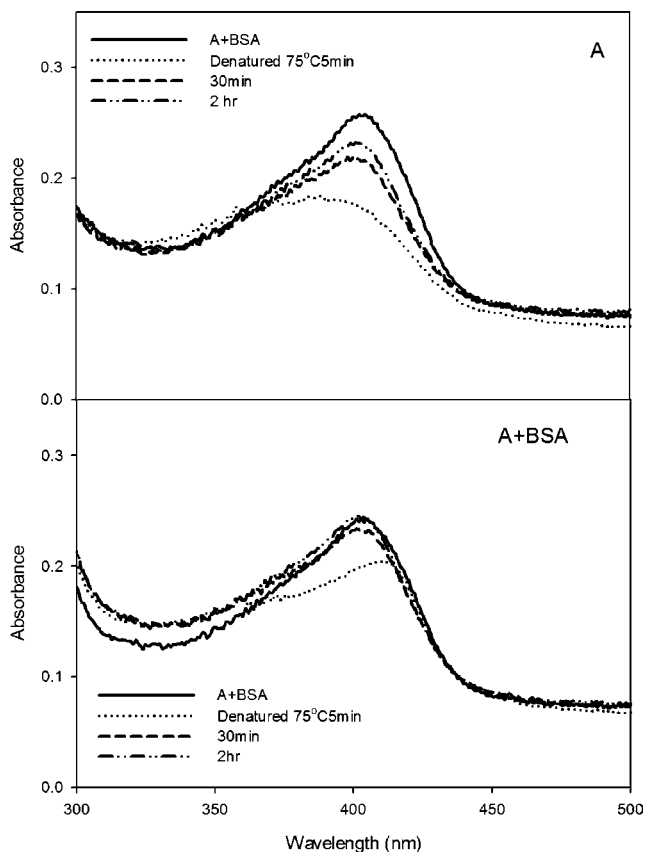


**Figure 8.** UV CD spectra of HRP and acidic and neutral broccoli PODs at 25 and 80 °C. The path length was 10 mm. The concentration of HRP was 0.03 mg/mL,  $\sim 0.04$  mg/mL for A, and  $\sim 0.026$  mg/mL for N. All samples were prepared in 10 mM phosphate buffer, pH 7.0.

Because heme was shown to bind to the protein structure under the same conditions that the enzyme lost its activity, the loss of activity may involve another part of the protein other than the heme structure. Sutherland and Aust (28) showed that inactivation of manganese POD (MnP) involved two steps. The first step involved the loss of  $\text{Ca}^{2+}$ , which caused conformational changes resulting in additional loss of MnP activity. The second step was believed to involve further structural loss that results in the loss of  $\text{Ca}^{2+}$ .

Studies have shown that  $\text{Ca}^{2+}$  is crucial for the stability of plant POD structure. Depletion of  $\text{Ca}^{2+}$  ions resulted in marked decrease in the stability of the secondary structure of HRP (11). However, the situation may be different for each POD. Our previous study showed that the presence of EGTA in N reduced the activity of the enzyme by 5 times, even before heat treatment was performed. In addition, different PODs require different levels of treatment to remove  $\text{Ca}^{2+}$ . HRP and peanut PODs contain much more tightly bound  $\text{Ca}^{2+}$  than does MnP, but  $\text{Ca}^{2+}$  is not required for the activity of HRP and peanut PODs, whereas for MnP the loss of  $\text{Ca}^{2+}$  appears to be responsible for the loss of activity (22, 28, 29). This observation suggests differences in binding affinity of the ion to the enzyme. It is likely that  $\text{Ca}^{2+}$  in neutral POD may not be tightly bound or



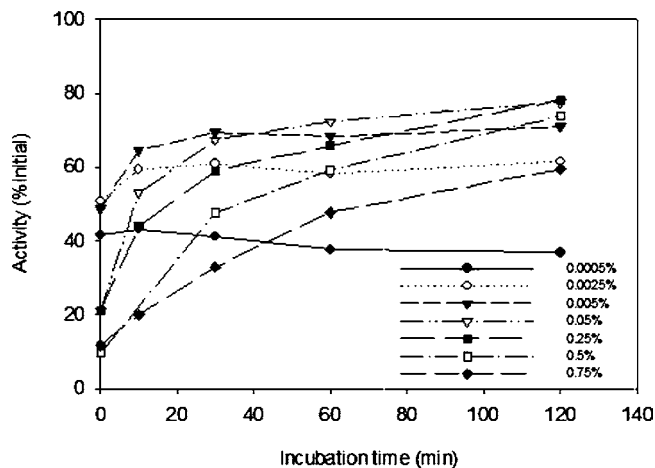


**Figure 9.** Absorption spectra of 0.12 mg/mL acidic broccoli POD in Tris-acetate buffer, pH 7.0, in the absence (A) and presence (B) of 0.05% BSA. Samples were heated at 75 °C for 5 min, and the spectrum was taken after heat treatment, cooling, and during incubation at room temperature.

may be located in the exterior part of the enzyme and thereby be more readily removed during heating, as compared to A. As a result, N lost its structure more readily. However, this secondary structure loss is reversible. The structure may reform during cooling, and because the heme is still mostly bound, N showed a higher extent of reactivation. This is similar to the case of MnP, for which reactivation studies revealed that the amount of activity that could be recovered upon the addition of  $\text{Ca}^{2+}$  was correlated with the amount of MnP, which still contained heme.

**Effect of BSA on Reactivation.** The presence of 0.05% BSA has no effect on the absorbance spectrum at the heme pocket (400 nm) or on the activity of the enzyme. However, the presence of BSA decreased the heat stability of the enzyme. Heat treatment at 75 °C for 5 min of A decreased the POD activity to 20% of the original activity for the samples containing BSA but only to 30% of the original in the absence of BSA. In contrast to the activity, the Soret band absorbance decreased to only 83% of the original in the presence of BSA, as compared to 70% in the absence of BSA.

During refolding, the Soret band absorbance increased to almost that of the original in the samples containing BSA and to 90% of the original for the samples without BSA early in the incubation at 25 °C and remained fairly stable during prolonged incubation (Figure 9). POD activity increased corresponding to the increase in the Soret band absorption. In the presence of BSA, POD activity returned to the original. The effect of BSA on HRP was similar to what was found for A but the data are not shown.



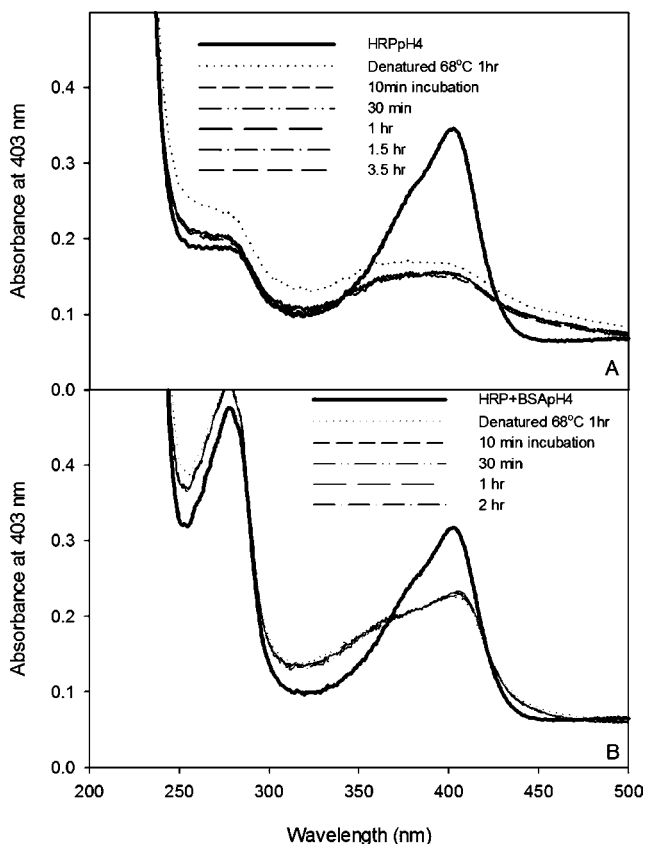
**Figure 10.** Changes in activity of HRP (RZ = 2.7) during incubation at 35 °C after heat treatment at 76 °C for 1 min in the presence of various concentrations of BSA.

Serum albumins are the major soluble protein constituents of animal circulatory systems and have many physiological functions. BSA has been one of the most extensively studied of this group of proteins because of its structural homology with human serum albumins (HSA) (30). The most important property of this group of proteins is that they serve as depot protein and as a transport protein for a variety of compounds, because they have an exceptional binding capacity for a wide range of endogenous and exogenous ligands (31). There are a number of review articles on ligand binding by albumin (32–34).

Binding sites on BSA for heterocyclic or aromatic compounds and metals have been extensively studied (35–38). Hematin and porphyrins are known to bind to serum albumin. A study on hemin–albumin interaction was carried out by Beaven et al. (39), who demonstrated the existence of one high-affinity hemin-binding site ( $K_a = 5 \times 10^7 \text{ M}^{-1}$ ) and a number of weaker binding sites. The high-affinity site was specific for hemin binding, but the weaker sites also bound fatty acid anions and other hydrophobic molecules. Adams and Berman (26) showed that the kinetics of binding of monomeric hemin to HSA indicated a two-stage single-intermediate process. The first stage of the reaction is a rapid uptake of hemin (hematin) by HSA, followed by a slower molecular arrangement to the final product. The slower phase is entropy-driven, implying hydrophobic bonding (40). A metal component in the porphyrin has little effect on binding. The  $K_a$  values for protoporphyrin without a central metal ion and hematin with Fe(II), Fe(III), or Zn(II) were similar (41). The binding of albumin to heme modified heme catalytic reactivity. Grinberg et al. (42) found that the peroxidative and catalytic activities of hemin were inhibited by 50–60% when bound to either HSA or BSA (1-to-1 ratio). This is because the binding reduced the amount of catalytically active heme available for substrates to attack.

The above information suggests the possibility of binding between BSA and heme. Because the addition of BSA to the POD solution had no effect on the activity of the enzyme, binding occurred after unfolding of POD during heating. The result was a rapid decrease in the enzyme activity, which was much more profound than when BSA was absent because the binding of BSA limited the amount of actively available POD. The effect was more pronounced when the concentration of BSA was increased. Figure 10 shows that the heat inactivation rate increased with increasing BSA concentration, suggesting that





**Figure 11.** Absorption spectra of 0.2 mg/mL acidic broccoli POD in Tris–acetate buffer, pH 4.0, in the absence (A) and presence (B) of 0.05% BSA. The samples were heated at 68 °C for 1 h, and the spectrum was taken after the heat treatment, cooling, and during incubation at room temperature (25 °C).

more heme was sequestered by BSA. However, binding of BSA may help to stabilize the heme pocket by keeping the heme in the vicinity of the unfolded enzyme as well as preventing aggregation of the heme molecule. As shown in **Figure 10**, the Soret band absorption was higher when BSA was present in the solution.

Hemopexin (Hx), a heme-binding protein, has a high affinity to heme, with a dissociation constant ( $K_d$ ) of  $10^{-13}$  M, which is 5 orders of magnitude higher than the affinity of albumin for heme ( $K_d \sim 10^{-8}$  M). This affinity is similar to that of globin to heme in hemoglobin (41, 42). In a related study, the binding of hematin to Hx was 30 times as rapid as to albumin, and hematin would readily leave albumin for Hx in plasma (43). Grinberg et al. (42) also showed that binding of Hx to heme inhibited 80–90% of the peroxidative and catalytic activity of hemin. Therefore, it is possible that when the tertiary structure of the heated POD was largely reestablished during the cooling step, binding of the heme to the refolded POD is more favorable than binding to the BSA. Heme bound to the POD, as shown in **Figure 9**, where the increase in the Soret band absorption was found during incubation of the heat-treated enzyme. When the concentration of BSA was increased, the rate of reactivation was much slower compared to that at lower BSA concentrations (**Figure 10**). In addition, at high concentrations of BSA, precipitation of the protein was clearly seen during heat treatment. It took longer for the heme to detach from the BSA and return to the refolded POD, presumably due to more association with the BSA when it is in high concentration.

Because more heme remained attached to the enzyme, in the presence of BSA, the extent of reactivation was shown to

be higher than in samples without BSA. It was noticed in previous experiments as well that following heat treatment if the structure around the heme was maintained to a greater degree (higher Soret band absorption), such as in the case of neutral POD and in the presence of BSA, reactivation occurred to a greater extent. This fact supports the importance of the structure at the heme on the reactivation of POD.

**Effect of pH on Reactivation.** Low pH has been shown to decrease the heat stability of the enzyme (2, 5). A substantial (45%) decrease in the Soret band absorbance was observed after heat treatment of HRP at 68 °C for 1 h. This is 12% lower than the absorbance obtained when the enzyme is at neutral pH. In the presence of BSA, the Soret band absorbance was maintained at a higher level, compared to that in the absence of BSA. The Soret band absorbance and POD activity remained unchanged during incubation of the heat-treated enzymes at 25 °C, regardless of the presence of BSA (**Figure 11**). At this pH, the secondary structure returned almost completely during incubation at room temperature following heat treatment (data not shown). Low pH (4.0) prevented reassociation of the heme with the protein, resulting in the prevention of reactivation of the heated enzymes.

pH has a significant influence on the stability and functionality of PODs. At very low pH values of approximately 2, breakage of the iron–histidine bond takes place, and this completely releases the heme from the protein. At slightly lower pH values, such as were used in this study, the structure at the heme pocket was weakened as well, because HRP has a very extensive hydrogen-bonding network in the proximal and distal region of the heme, which stabilizes the structure around the heme (11, 44). By lowering the pH the network is broken, leading to a decrease in stability of the heme pocket and preventing the reassociation of the heme to the enzyme.

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