JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Heat Inactivation and Reactivation of Broccoli Peroxidase

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Heat inactivation characteristics differed for acidic (A), neutral (N), and basic (B) broccoli peroxidase. At 65 °C, A was the most heat stable followed by N and B. The activation energies for denaturation were 388, 189, and 269 kJ/mol for A, N, and B, respectively. Reactivation of N occurred rapidly, within 10 min after the heated enzyme was cooled and incubated at room temperature. The extent of reactivation varied from 0 to 50% depending on the isoenzyme and heating conditions (temperature and time). The denaturation temperature allowing the maximum reactivation was 90 °C for A and horseradish peroxidase (HRP) and 70 and 80 °C for B and N, respectively. In all cases, heat treatment at low temperatures for long times prevented reactivation of the heated enzymes. Calcium (5 mM) increased the thermal stability of N and B but had no effect on reactivation. The presence of 0.05% bovine serum albumin decreased thermal stability but increased the extent of reactivation of A..

KEYWORDS: Broccoli; peroxidase; inactivation; reactivation

1. INTRODUCTION

Peroxidase (POD) is an enzyme found in many plant-based foods. The enzyme concerns food processors because of its high thermostability and its involvement in the oxidation of many organic compounds, leading to deterioration in flavor, color, and nutritional quality. These qualitative changes have been found to occur particularly in canned and frozen fruits and vegetables during storage (*1*−*4*). POD is also used as an index of the adequacy of fruit and vegetable blanching due to its presence in most plant tissues, its high thermal stability, and the simplicity of its measurement (*5*, *6*).

Heat treatment is commonly used to inactivate an active enzyme. However, it is well-known that POD can recover its activity after heat treatment (*5*, *7*). 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 (*5*, *7*−*9*). Reactivation of the enzyme is suggested to be a complex process and is influenced by several factors. In horseradish peroxidase (HRP), reactivation has been reported to take place after partial inactivation at 70, 90, or 110 °C (*10*). One factor affecting its reactivation is the time taken to reach the desired treatment temperature. If this time is short, reactivation occurs more easily (*5*, *7*−*9*). This fact poses a problem in HTST treatment of acid and low-acid vegetables that are subsequently frozen or canned (*5*).

The ability of POD to regain activity after being denatured by heat varies with the species of vegetable and may even differ between isoenzymes of the same species (*8*, *9*, *11*, *12*). As with heat inactivation, reactivation is pH dependent (*5*, *10*). Previous investigators found that HRP was inactivated very rapidly at 76 °C and pH 4.5 or below, and there was no measurable regain in activity. However, the rate of reactivation increased from none at pH 5.0 to a maximum at pH 9.0 (*5*). Studies have also indicated that Ca^{2+} is important for both the activity and maintenance of the protein structure (*13*). The presence of Ca^{2+} during reactivation affects the extent of reactivation. In corn steep water, a byproduct of the corn-steeping process, the recovery of POD following inactivation for 60 min at 75 °C and incubation for 20 h at 4 °C increases nearly 80% when Ca^{2+} is added to the heated enzyme solution compared to 42.2% for the sample without addition of Ca^{2+} (*14*).

Most of the published work on the reactivation of peroxidase has been done on horseradish, which has high POD activity but is not of consequence as a vegetable consumed in great quantities. There has been much less study of PODs in more economically important vegetable crops, such as broccoli. Although the reactivation of POD has been commonly observed in heat-treated plant-based foods, little work has been attempted to investigate the detailed mechanisms involved in the heat inactivation and reactivation of this enzyme. Knowledge of the mechanism of POD reactivation in vegetables important to the food industry may be used to prevent enzyme reactivation, which would help prolong the shelf life of processed vegetable products.

Three peroxidase isoenzymes were previously purified from the soluble extract of broccoli stems (*15*). The acidic and neutral PODs were purified to homogeneity with ion exchange and hydrophobic chromatography. The basic POD was purified by cation exchange and gel filtration chromatography. The three isoenzymes differ in their molecular weight, p*I*, and reaction with various substrates. The objective of this work was to study the room temperature reactivation of these three POD isoenzymes following thermal inactivation in Tris−acetate buffer (pH

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10.1021/jf0481610 CCC: $30.25
Published on Web 03/24/2005

7.0) over a wide range of temperatures (55−100 °C). Effects of additional calcium ion, calcium chelator, and bovine serum albumin (BSA) on the rate and extent of reactivation of POD isoenzymes from broccoli and commercial HRP were also studied.

2. MATERIALS AND METHODS

2.1. Enzyme Purification. Acidic (A), neutral (N), and basic (B) peroxidases were prepared from soluble extracts of broccoli stems and purified according to the procedure described previously (*15*).

2.2. Peroxidase Activity Determination. Activity was determined by monitoring the time course of the change in absorbance at 420 nm upon oxidation of the substrates catalyzed by the enzyme. Guaiacol (Sigma, St. Louis, MO) was used as substrate. The final reaction mixture contained 50 mM guaiacol, 50 μL of enzyme, 10 mM H_2O_2, and 50 mM Tris−acetate buffer, pH 6.0, in a volume of 1.5 mL (*5*). The assay was performed at 25 °C using a UV−vis scanning spectrophotometer (UV-2101PC Shimadzu) connected to a temperature controller. Absorbance increase at 420 nm was monitored for up to 3 min with the slope of the linear portion of the curve used to determine activity. One unit of enzyme was defined as the amount of substrate (micrograms) consumed in 1 min.

2.3. Thermal Inactivation. Thermal inactivation of the purified enzymes was performed in capillary tubes (1 mm i.d., 200 μL total volume). Samples were heated in a circulating water bath (model 20B, Julabo, Allentown, PA) to the indicated temperatures (between 50 and 100 °C depending on the enzyme) for the times specified. The come-up time for the capillary tubes was determined to be <10 s (*6*). Heated samples were cooled immediately in ice−water, and the residual enzymatic activity was evaluated as described previously. Rate constants were calculated for POD inactivation in samples treated at specified temperatures and times. The rate constant for inactivation (k) was obtained from the slope of a plot of the natural log of residual activity versus time of heat exposure. The values of activation energy for inactivation of the enzymes were calculated from the slope of the straight lines obtained using Arrhenius plots of the logarithm of the first-order rate constant for inactivation (log k) versus the reciprocal of absolute temperature ($1/T$).

2.4. Reactivation of Peroxidase. Reactivation of acidic, neutral, and basic PODs from broccoli and HRP (Sigma) was studied. Aliquots of purified enzymes in 0.05 M Tris−acetate buffer, pH 7.0, were filled in capillary tubes (1 mm i.d., 200 μL total volume) and heated in a circulating water bath. Concentration of purified enzymes used was at the level that produced a constant reaction rate (e.g., straight line) for 2 min with the POD assay using guaiacol as a substrate. Heat treatment for the times specified was performed in a circulating water bath for the temperature range from 50 to 80 °C and in an oil bath for the temperature range from 80 to 100 °C. After heating, the tubes were rapidly cooled in an ice−water bath. Residual activity was determined immediately. The enzyme solution was then transferred to an Eppendorf tube and incubated at room temperature or in water at the desired temperature. Residual activity was determined after different periods of time.

2.5. Effect of Calcium Ion on Reactivation. Enzyme solutions were prepared as described above and treated at the temperature and time specified. The heat-treated enzyme solution was cooled immediately in ice−water and then divided into two portions. The first part was mixed with $CaCl_2$ in 0.05 M Tris−acetate buffer, pH 7.0, to give a final concentration of 0.005 M $CaCl_2$. The second part was mixed with buffer solution without $CaCl_2$. This step was performed at low temperature (4 °C). The mixtures were incubated for 30 min at room temperature before enzyme activities were determined.

2.6. Effect of Ethylene Glycol Tetraacetic Acid (EGTA) on Reactivation. Acidic, neutral, and basic PODs from broccoli were prepared in 0.005 M EGTA in 0.05 M Tris−acetate buffer, pH 7.0. Heat treatment and reactivation of the enzyme were performed as previously described.

2.7. Effect of Bovine Serum Albumin on Reactivation. HRP (Sigma, RZ = 3.1) was prepared in 0.05 M Tris−acetate buffer, pH 7.0, in the presence of various concentrations of BSA, ranging from 0.0005 to 0.75%. Acidic POD from broccoli was prepared in 0.05% BSA in 0.05 M Tris−acetate buffer, pH 7.0. Heat treatment and reactivation of the enzymes were again performed as previously described.

Table 1. Some Properties of Acidic, Neutral, and Basic Broccoli PODs (*15*)

	acidic	neutral	basic
molecular weight (kDa)	48	43	43
p*I*	<4	5	8
K_m (guaiacol) (mM)	0.305	0.711	8.789

3. RESULTS AND DISCUSSION

The isoenzymes obtained from broccoli stems are easily distinguished by their p*I* and are referred to as acidic (anionic), neutral, and basic (cationic) (**Table 1**).

3.1. Selection of Conditions for Enzyme Assay. Reaction mixtures with various guaiacol and H_2O_2 concentrations were prepared, and the initial reaction rates for each condition were determined. For neutral POD, it was observed that the initial reaction rates were constant when guaiacol concentrations were between 20 and 40 mM at H_2O_2 concentrations between 1 and 10 mM. At H_2O_2 concentrations of 0.5 and 15 mM, the initial rates were significantly lower than those with H_2O_2 concentrations between 1 and 10 mM. For acidic POD, the initial reaction rates increased as the guaiacol concentration increased, and the rates were constant as guaiacol concentrations reached 20 mM. H_2O_2 concentrations from 1 to 15 mM had no effect on the reaction rate, whereas low H_2O_2 concentrations (0.5 mM) lowered the reaction rate. Because neutral POD is sensitive to extreme levels of H_2O_2, the H_2O_2 concentration used for the measurement of enzyme activity in the next experiments was limited to 10 mM.

3.2. Thermal Inactivation. Semilog plots of residual activity versus heating time were linear over the first 80−85% loss in activity, indicating that inactivation is first order over this range (**Figure 1**). After this, a trace of activity remained during prolonged heat treatment. The heat treatment was performed to <85% loss of activity, but the data are not shown. Deviation from first-order kinetics has been reported for HRP and purified POD from other sources (*5*). This nonlinear heat inactivation of POD may occur because there are two steps involved, that is, dissociation of heme from the active enzyme and a conformational change in the apoenzyme (*16*). Plant extracts contain a mixture of POD isoenzymes with various heat stabilities and often give a nonlinear heat inactivation plot. A nonlinear heat inactivation plot has also been reported for the thermostable isoenzyme A purified from cauliflower (*17*).

The rate constant was calculated from the slope of the linear section of the inactivation line and plotted in an Arrhenius plot (**Figure 2**). The Arrhenius plots of all three isoenzymes gave straight lines. The activation energies, E_a, were calculated by least-squares analysis to be 388, 189, and 269 kJ/mol for the acidic, neutral, and basic forms, respectively.

Published data on POD heat inactivation have shown a wide variation in heat inactivation characteristics, depending on the plant source of the enzyme, and among the isozymes present. In the present study, it was determined that at 65 °C, acidic POD was the most heat stable with a $t_{1/2}$ of 17.3 min. Neutral POD was the second most heat stable with a $t_{1/2}$ at 65 °C of 8.1 min, and basic POD was relatively heat labile with a $t_{1/2}$ at 65 °C of 0.5 min.

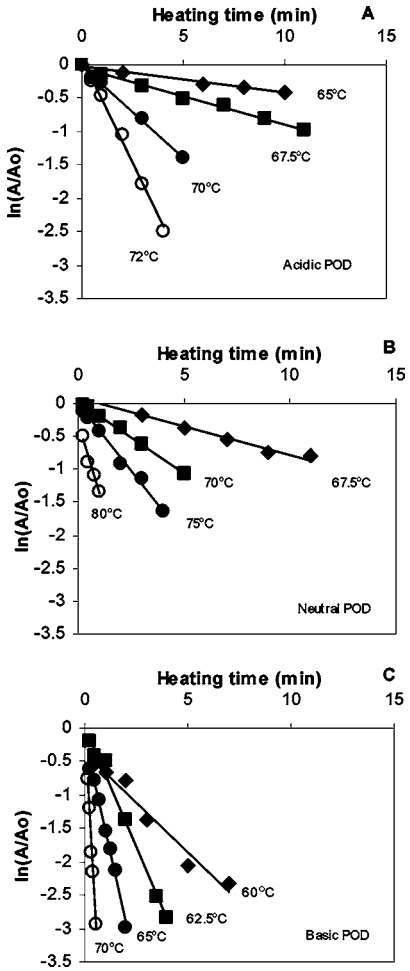

Figure 1. Rate of heat inactivation of acidic broccoli POD (**A**), neutral POD (**B**), and basic POD (**C**). The ordinate represents the natural log of relative activity, the ratio of the activity (A) to the original activity (A_0) before heat treatment.

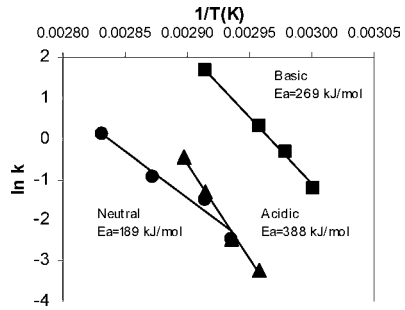

Figure 2. Arrhenius plot showing the effect of temperature on the rate constant for the thermal inactivation of acidic, neutral, and basic broccoli PODs.

For *Brassica* species, the soluble POD activity (containing mainly the acidic isoenzyme) has generally been shown to be more heat stable than that associated with the basic isoenzymes extracted from ionically bound fractions (*18*). Similarly, extracts containing mainly basic isoenzymes such as those from apples (*19*), pears (*20*), and grapes (*21*) were heat labile. The cationic (basic) POD isoenzyme, which accounted for 94% of the total POD activity in cauliflower, was found to be very heat labile, having a $t_{1/2}$ at 50 °C of 3 min compared to the anionic (acidic) isoenzyme, which had a $t_{1/2}$ at the same temperature of 30 min (*17*). Similar observations were discussed by Vamos-Vigyazo (*22*). In experiments carried out with winter spinach, heat

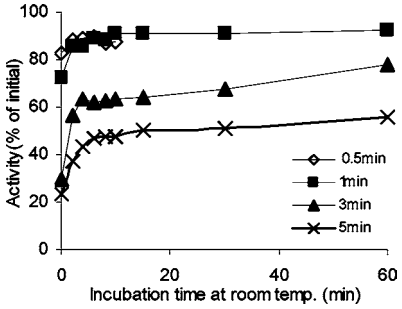

Figure 3. Changes in activity during incubation at room temperature for various times for the neutral POD after heat treatment at 75 °C for 0.5, 1, 3, or 5 min. Values are described by percent of initial activity before heat treatment.

treatment for 1 min at 70 °C inactivated all of the isoenzymes with pI values in the range of 5−6, whereas the acidic group (pI 3.5−5) proved to be much more stable, showing traces of activity even at 70 °C.

Turnip POD isoenzymes C1, C2, and C3 (all basic) were found to be heat labile ($t_{1/2}$ for C1 at 50 °C was 3 min), and E_a values were 113, 130, and 172 kJ/mol, respectively (*23*). The E_a values for PODs from various plant sources have been found to be HRP, 114 kJ/mol (*5*); asparagus, 140 kJ/mol (*24*); heat-resistant cauliflower, 193 kJ/mol (*17*); and Brussels sprouts, A1, 172 kJ/mol (*25*). In the present study, the E_a of neutral broccoli POD was 189 kJ/mol, which is in the same range as the above values, but the other forms were higher than this range, for example, 388 kJ/mol for acidic POD and 269 kJ/mol for basic POD. E_a values of crude extracts have been found to be higher than those of the purified enzymes. Crude preparations from carrot and tomatoes, for example, had E_a values of 480 and 478 kJ/mol, respectively, probably due to differences in the surrounding environment (*6*).

Variability in the heat inactivation of different isoenzymes can be attributed largely to the particular enzyme structure. Noncovalent electrostatic and hydrophobic interactions of individual isoenzymes determine enzyme folding and stability. Extra ion pairs and hydrogen bonds contribute significantly to the enzyme stability (*26*). Glycosylation of POD has also been found to play a role in the enzyme stability. Tams and Welinder (*27*) showed that fully glycosylated HRP was 2−3-fold more stable than deglycosylated HRP. Distinct E_a values, therefore, are likely to be affected by the differential stability of individual enzymes.

3.3. Reactivation. Reactivation of peroxidase in a broccoli homogenate during storage at room temperature after heating at 75 °C for 1 and 5 min was noted in preliminary studies to be about 20 and 10%, respectively. Purified PODs from broccoli also reactivate during storage after heat inactivation.

The neutral POD regained activity rapidly, within 10 min after the heated enzyme was cooled and incubated at room temperature. The increase in activity was very slow and remained almost unchanged during prolonged incubation (**Figure 3**). For basic POD treated at 70 °C for 30 or 40 s, activity was recovered rapidly, within 10 min of the cooling to room temperature. However, unlike the neutral form, the activity tended to decline when the incubation time was extended, indicating lower stability of the reactivated enzyme (data not shown).

Lu and Whitaker (*5*) found that, following heat treatment of HRP (pH 7.0, RZ = 0.59) at 75 °C for various times, there was regeneration to 80% of original activity within 8−10 min. This reactivation was not first or second order. The rate of recovery was similar for incubation temperatures ranging from 20 to

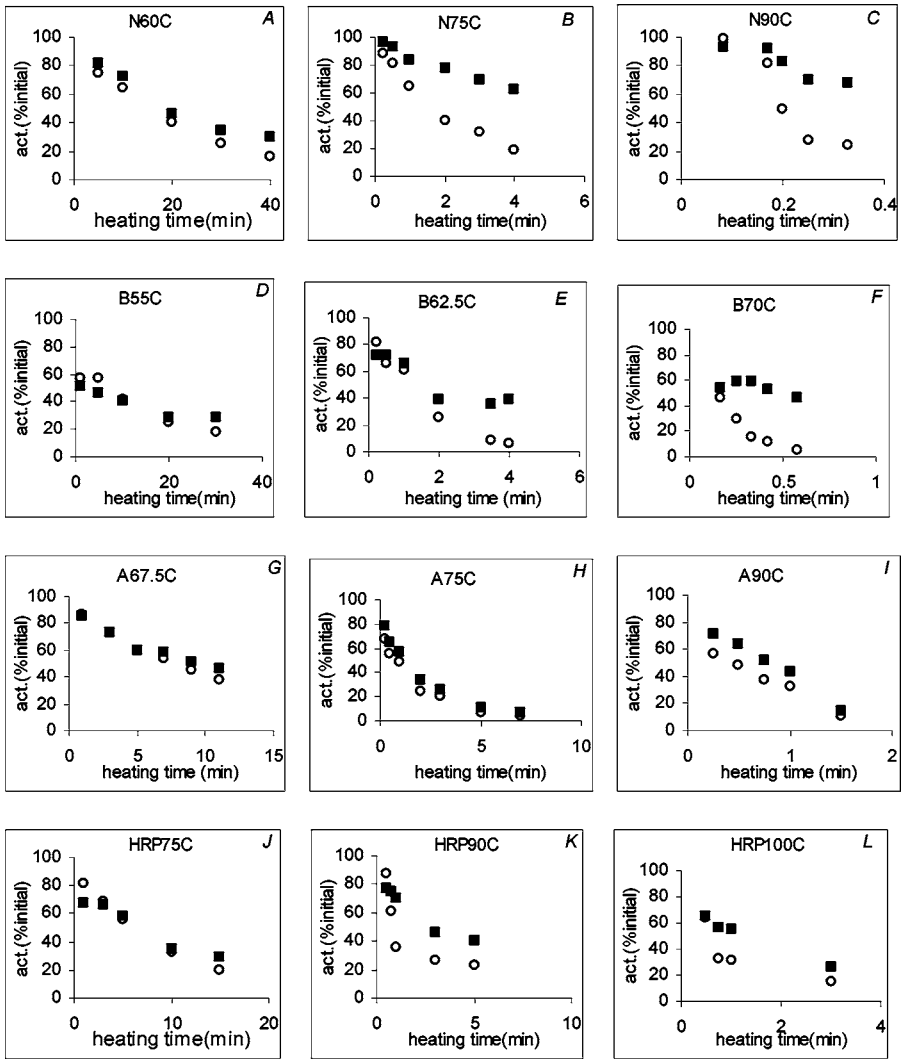

Figure 4. Difference in POD activity following heat treatment at various times and temperatures (○) and activity recovered after 30 min of incubation at room temperature (■): (**A**–**C**) neutral POD treated at 60, 75, and 90 °C; (**D**–**F**) basic POD treated at 55, 62.5, and 70 °C; (**G**–**I**) acidic POD treated at 67.5, 75, and 90 °C; (**J**–**L**) HRP treated at 75, 90, and 100 °C.

35 °C, and no regeneration was found at 0 °C. A similar finding was noted by Adams (*8*).

Because recovery of activity did not increase after 30 min of incubation, in the next part of the experiment, reactivation was studied by measuring changes in residual activity after 30 min of incubation at room temperature following a heat treatment. Results clearly show that the extent of reactivation largely depends on two major factors: the specific isoenzyme studied and treatment (time/temperature) conditions. **Figure 4** shows changes in recovered activity after 30 min of incubation at room temperature following heat treatment at various temperatures and times for the three purified broccoli POD isoenzymes and HRP.

For neutral POD from broccoli, the extent of reactivation after heat treatment from 60 to 90 °C for various times varied approximately from 10 to 50%, depending on the heating conditions. Examples are shown in **Figure 4A**−**C**. The extent of reactivation increased as heating time increased for all temperatures studied. At each heating temperature, samples with 50% loss of activity after the heat treatment reactivated to a significantly greater extent than those with >50% loss of activity. The extent of reactivation after heating from 55 to 72.5 °C for various times varied from 0 to 40%, depending on the heating conditions for basic POD (**Figure 4D**−**F**). Reactivation occurred when residual activities after heat treatment were below 50%. Similar to neutral POD, reactivation seemed to reach a certain final value, independent of the treatment temperature.

Reactivation of acidic POD after heat treatments from 65 to 100 °C and heat treatments of HRP from 70 to 100 °C for various times varied from 0 to 20%; these forms reactivated to a somewhat lower extent than the neutral and basic PODs.

Heat-treated PODs from several plant sources have shown an ability to recover their activity while being stored at ambient temperature after heat treatment. 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. A reactivation to 16% of the original activity was observed in green asparagus extract (*9*) and Brussels sprouts and cabbage (*11*) after 2.5 h of incubation at 30 °C following heating at 75 °C. Reactivation has also been observed in purified POD. A 15−30% restoration of original POD activity was noted for the cationic isoenzyme (p*I* ∼9) of green peas after several hours of incubation at 25 °C following a 50−60 °C heat treatment, but there was no regain after a 70 °C heat treatment (*12*).

Rodrigo et al. (*28*) showed that HRP (RZ = 3.1) treated at high temperature (115 and 130 °C) for short times (5−85 s) can recover up to 22% of its activity during incubation at

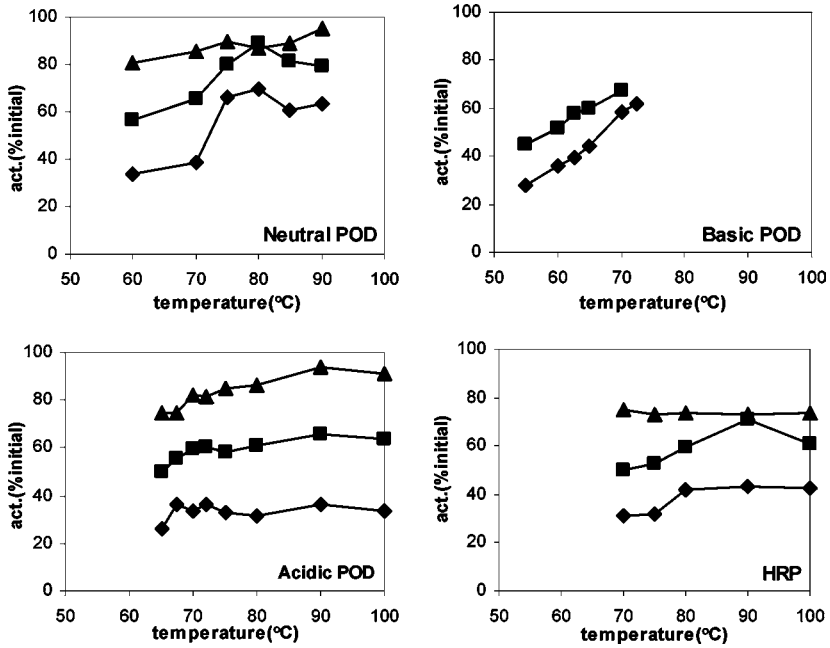

Figure 5. Recovered activity of heat-treated POD after 30 min of incubation at room temperature. Enzymes were treated at temperatures indicated on the *x*-axis for the times that resulted in 75% (▲), 50% (◆), and 25% loss of original activity (■).

25 °C. An early study on reactivation of POD was performed with highly purified HRP (RZ = 3.02), and the authors observed a significant amount of reactivation. The temperatures used in this study were high, for example, 120−150 °C (*29*). However, in the present study we found that HRP (RZ = 3.1) did not reactivate after heat treatment at 76 °C for 30 s−5 min (data not shown). Several other studies have found no appreciable recovery of activity in purified PODs derived from spring cabbage and Brussels sprouts (*30*) or green peas (*12*). The variation in the outcomes can be explained by our results (**Figure 5**), which show that treatment conditions, the source of the enzyme, and isoenzyme type all play a major role in determining the degree of reversible inactivation.

In neutral POD, the extent of reactivation was found to increase as heating temperature was increased from 60 to 80 °C. Reactivation decreased slightly as heating temperature increased to 90 °C, with the exception of samples with 25% loss of activity, where the reactivation continued to increase. After a heat treatment at 60 °C, there was only 5% recovery in activity, which is fairly low compared to 40−45% recovery after heating at 80 °C. Due to the rapid decrease in activity of the basic POD during heat treatment, it was only possible to collect data for samples with 50 and 75% loss of original activity (**Figure 5**). In general, the extent of reactivation of the basic isoenzyme continued to increase as heating temperature increased, in the range of 55−72.5 °C. It was not feasible to carry out experiments at temperatures >72.5 °C due to rapid decreases in the enzyme activity.

This increase in the extent of reactivation was also observed in the acidic POD, yet to a lower percentage as compared to the neutral and basic POD. The highest extent of reactivation was found when the enzyme was treated at 90 °C. The extent of reactivation also increased with increases in heating temperature and reached a maximum level when the enzyme was treated at 90 °C, particularly in the samples with 50% loss of activity. The sample with 25% loss of activity did not reactivate at any heating temperature, and reactivation occurred in the 50 and 75% loss samples only at heating temperatures >70 °C.

The broccoli POD isoenzymes showed variations in their reactivation behavior. However, a common characteristic of all the peroxidases studied was that reactivation following heat treatment did not occur or did so only to a very low degree when treatments were at low temperatures for long times. The extent of reactivation increased as enzymes were treated at high temperatures for short times. This is in agreement with previous observations that reactivated POD has been found to cause significant deterioration in the quality of various HTST-processed foods.

The inactivation temperature giving the maximum reactivation differed among the PODs. Highest reactivation was at 90 °C for acidic POD and HRP, whereas it was approximately 70 and 80 °C for basic and neutral PODs, respectively. This is in the same trend as observed for the stability toward heat inactivation of the enzyme, with acidic POD being the most heat stable followed by neutral and basic PODs.

Reactivation has also been observed in other thermally resistant enzymes. Alkaline phosphatases, naturally occurring enzymes in milk, have been reported to reactivate under certain conditions following ultrahigh-temperature−short-time processes. The effect of processing temperature was studied, and the optimum temperature for maximum reactivation was in the range of 104.4−110 °C for 20% cream. With whole milk, the curve relating activity to pasteurization temperature was broader and flatter than the curve for cream, and the optimum temperature appeared to be 104.4−121.1 °C (*31*, *32*). The conditions studied were milk heated to 87.8−115.6 °C for 1.6 ± 0.07 s. Machado and Saraiva (*33*) studied reactivation kinetics of HRP (RZ = 0.98) and found that the rate of reactivation reached a maximum value at a certain treatment time for heating temperatures studied from 70 to 85 °C. From the previous discussion, we expect that under certain conditions any peroxidase will reactivate after heat treatment. The degree of reactivation will depend on treatment condition and the enzyme itself.

Teipel and Koshland (*34*) followed the renaturation of denatured proteins by measuring both optical rotation and fluorescence changes. Spectral analysis enabled them to observe multiple enzyme forms, such as several intermediates as well as the inactivated and native forms. Entropy changes, ΔS^*, during reactivation of milk alkaline phosphatase were calculated to be positive, which was suggested to indicate conformational

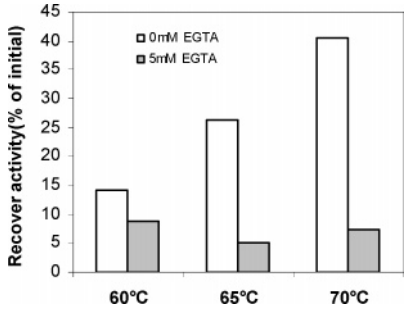

Figure 6. Changes in residual activity of heated enzyme after 30 min of incubation at room temperature. Basic POD was treated at 60, 65, and 70 °C in the presence and absence of 5 mM EGTA. All treatments resulted in residual activity in the range of 1–10% after heat treatment.

change in the enzyme molecule during the reaction (*32*). Machado and Saraiva (*33*) used a series-type model to describe the heat inactivation of HRP (RZ = 0.98). The model considers a homogeneous native enzyme population (E_N), which becomes inactivated as it forms an intermediate form, partially inactivated population (E_I), that can have different thermal resistance and specific activity compared to the native form, to yield an irreversibly inactivated form (E_D). In this work there was evidence that the rate of reactivation may be dependent on the amount of intermediate formed in the first reaction or remaining after inactivation. The heating time corresponding to the maximum rate of reactivation was when formation of the intermediate has been completed and only inactivation occurs. Studies have suggested the presence of intermediates during the reactivation process, and the formations of these intermediates were influenced by the heating process. Still, more study is needed to elucidate mechanisms involved in the reactivation of peroxidase.

3.4. Effect of Ca^{2+} on Inactivation and Reactivation. Peroxidases originating from plant sources, such as horseradish and peanuts, have been shown to bind two calcium ions (*35*, *36*). Calcium is important for both activity and maintenance of the protein structure around the heme pocket (*13*); therefore, the involvement of Ca^{2+} in heat inactivation and reactivation was investigated.

The addition of calcium had no significant effect on the activity of native POD but did have a significant effect on the thermal stability of the enzyme. The presence of 5 mM Ca^{2+} stabilized both the neutral and basic broccoli PODs to heat inactivation. The rate of heat inactivation of the neutral POD at 75 and 80 °C decreased 2.3 and 3.7 times, respectively, compared to when Ca^{2+} was absent. The thermal stability effect was much more pronounced with the basic POD. The rate of heat inactivation at 70 °C decreased 200 times when Ca^{2+} was present. Previous investigations have found that calcium-depleted cationic peanut and horseradish C PODs had substantial reductions in activity relative to untreated peroxidase and also had reduced heat sensitivities (*35*, *37*). Studies have shown that the loss of calcium corresponds to the loss of heme and that the loss of heme accounts for the loss of activity. This is the likely explanation for the protection of neutral and basic PODs. The protection against thermal denaturation has been observed for other PODs such as soybean (*38*), corn steep water derived POD (*14*), manganese peroxidase from *Phanerochaete chrysosporium* (*39*), and lignin POD (*40*). Unlike the neutral and basic forms, the presence of 5 mM Ca^{2+} did not stabilize the acidic POD to heat inactivation. The rate of heat inactivation at 75 °C was not significantly different in either case.

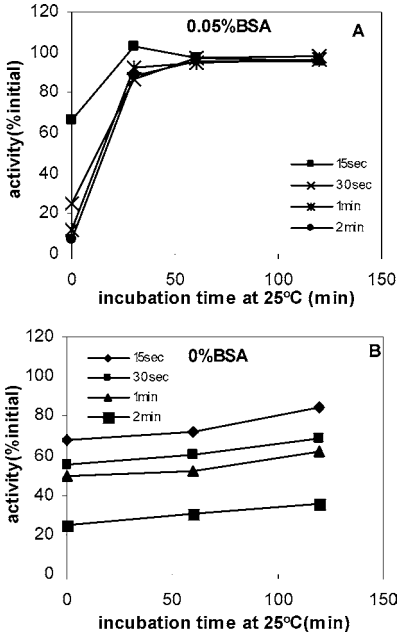

Figure 7. Changes in recovered activity during incubation at 25 °C after heat treatment of acidic POD at 75 °C for various times in the presence (**A**) and absence (**B**) of 0.05% BSA.

To study the effect of Ca^{2+} on the reactivation of the heat-inactivated enzyme, $CaCl_2$ in Tris−acetate buffer, pH 7.0, was added to the enzyme solution after heat treatment at specific temperatures and times. The heating temperatures and times that gave the lowest and highest extents of reactivation from the previous study were chosen for this study. The neutral POD was treated at 60 °C for 20 min and at 80 °C for 1 min, the basic POD was treated at 55 °C for 10 min and at 70 °C for 15 s, and the acidic POD was treated at 70 °C and 20 min and at 90 °C for 1 min. In all samples there was no significant difference between those with additional Ca^{2+} and those without Ca^{2+}. These results indicate that additional Ca^{2+} may not assist in increasing the extent of reactivation. Endogenous Ca^{2+} that is present in the enzyme was not removed during heat treatment; therefore, additional Ca^{2+} may have no effect on re-forming the enzyme structure and the return of enzyme activity.

3.5. Effect of EGTA on Reactivation. The effect of EGTA, an efficient chelator of Ca^{2+}, on reactivation was also investigated. For the basic POD, addition of EGTA (5 mM) significantly decreased the stability of the enzyme toward heat inactivation and reduced the extent of reactivation (**Figure 6**). EGTA was reported to increase the rate of heat inactivation observed in manganese peroxidase (*39*) and lignin POD (*40*). This result supports the importance of Ca^{2+} in the recovery of enzyme activity following heat inactivation.

However, the presence of EGTA in neutral broccoli POD reduced the activity of the enzyme by 5 times even before a heat treatment was performed. Different PODs require different levels of treatment to remove Ca^{2+}. Other inverstigations have found that more rigorous treatment (6 M guanidine HCl and/or 50 mM EGTA) was required to remove Ca^{2+} from HRP and peanut POD compared to manganese POD (*35*, *37*, *39*). This observation suggests differences in binding affinity of the ion to the enzyme. It is likely that Ca^{2+} in neutral POD may not be tightly bound or may be located in the exterior part of the enzyme; hence, it is more readily removed compared to the basic POD.

3.6. Effect of Bovine Serum Albumin on Reactivation. The presence of 0.05% BSA significantly increased the extent of

reactivation but decreased the heat stability of acidic broccoli POD treated at 75 °C for various times (**Figure 7**). There was a rapid increase in activity within 30 min when the enzyme was incubated at room temperature regardless the heating conditions. Activity remained constant as the incubation was prolonged. In a study with HRP (unpublished data), 0.05% BSA was found to be an optimum level of BSA, showing a significant increase in the extent of reactivation. The inhibitory effect of BSA was also reported for acid deoxyribonuclease from the small intestinal mucosa of rats (*41*). The effect of BSA on 80% recovery of the original activity of denatured *Bacillus acidopullulyticus* has been reported (*42*). The mechanism of BSA-assisted refolding is unclear, and further investigation into the effect of BSA on reactivation will help to verify its mechanism.

ACKNOWLEDGMENT

We thank Dr. Gary Smith for his generous access to experimental equipment and kind suggestions throughout the experiments, as well as proof-reading the manuscript.

LITERATURE CITED

(1) Nebesky, E. A.; Esselen, W. B., Jr.; Kaplan A. M.; Fellers, C. R. Thermal destruction and stability of peroxidase in acid foods. *Food Res*. **1950**, *15*, 114.

(2) Bruemmer, J. H.; Roe, B.; Bowen, E. R. Peroxidase reactions and orange juice quality. *J. Food Sci.* **1976**, *41*, 186.

(3) Kampis, A.; Bartuczkovacs, O.; Hoschke, A.; Aosvigyazo, V. Changes in peroxidase-activity of broccoli during processing and frozen storage. *Lebensm. Wiss. Technol.* **1984**, *17*, 293−295.

(4) Robinson, D. S. Scarvenging enzyme and catalases. In *Biochemistry and Nutritional Value*; Robinson, D. S., Ed.; Longman Scientific and Technical: Harlow, U.K., 1987; pp 459−465.

(5) Lu, A. T.; Whitaker, J. R. Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. *J. Food Sci*. **1974**, *39*, 1173−1178.

(6) Anthon, G. E.; Barrett, D. M. Kinetic parameters for the thermal inactivation of quality-related enzymes in carrots and potatoes. *J. Agric. Food Chem.* **2002**, *50*, 4119−4125.

(7) Schwimmer, S. Regeneration of heat inactivated peroxidase. *J. Biol. Chem*. **1944**, *154*, 487.

(8) Adams, J. B. The inactivation and regeneration of peroxidase in relation to the high temperature-short time processing of vegetables. *J. Food Technol*. **1978**, *13*, 281−297.

(9) Rodrigo, C.; Rodrigo, M.; Alvarruiz, A.; Frigola, A. Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. *J. Food Prot*. **1996**, *59*, 1065−1071.

(10) Tamura, Y.; Morita, Y. Thermal denaturation and regeneration of Japanese-radish peroxidase. *J. Biochem. (Tokyo)* **1975**, *78*, 561−571.

(11) McLellan, K. M.; Robinson, D. S. Purification and heat-stability of Brussels-sprout peroxidase isoenzymes. *Food Chem*. **1987**, *23*, 305−319.

(12) Halpin, B.; Pressey, R.; Jen, J.; Mondy, N. Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). *J. Food Sci.* **1989**, *54*, 644−649.

(13) Barber, K. R.; Maranon, M. J. R.; Shaw, G. S.; Vanhuystee, R. B. Structural influence of calcium on the heme cavity of cationic peanut peroxidase as determined by H-1-NMR spectroscopy. *Eur. J. Biochem*. **1995**, *232*, 825−833.

(14) Gray, J. S. S.; Montgomery, R. Purification and characterization of a peroxidase from corn steep water. *J. Agric. Food Chem*. **2003**, *51*, 1592−1601.

(15) Thongsook, T.; Barrett, D. M. Purification and partial characterization of broccoli (*Brassica oleracea* var. *Italica*) peroxidases. *J. Agric. Food Chem.* **2005**, *53*, 3206−3214.

(16) Chattopadhyay, K.; Mazumdar, S. Structural and conformational stability of horseradish peroxidase: Effect of temperature and pH. *Biochemistry* **2000**, *39*, 263−270.

(17) Lee, C. Y.; Pennesi, A. P. Isolation and further characterization of a heat-resistant peroxidase isoenzyme from cauliflower. *J. Food Sci*. **1984**, *49*, 1616−1617.

(18) Robinson, D. S.; Eskin, N. A. M. *Oxidative Enzymes in Foods*; Elsevier Applied Science: London, U.K., 1991.

(19) Moulding, P. H.; Singleton, D. E.; McLellan, K. M.; Robinson, D. S. Purification and heat-stability of Cox apple pulp peroxidase isoenzymes**.** *Int. J. Food Sci. Technol.* **1988,** *23*, 343−351.

(20) Moulding, P. H.; Goodfellow, J.; McLellan, K. M.; Robinson, D. S. The occurrence of isoperoxidases in Conference pears. *Int. J. Food Sci. Technol*. **1989**, *24*, 269−275.

(21) Robinson, D. S.; Bretherick, M. R.; Donnelly, J. K. The heat stability and isoenzyme composition of peroxidases in Ohane grapes. *Int. J. Food Sci. Technol*. **1989**, *24*, 613−618.

(22) Vamos-Vigyazo, L. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food Sci.* **1981**, 49−127.

(23) Duarte-Vazquez, M. A.; Garcia-Almendarez, B.; Regalado, C.; Whitaker, J. R. Purification and partial characterization of three turnip (*Brassica napus* L. var. *esculenta* DC) peroxidases. *J. Agric. Food Chem*. **2000**, *48*, 1574−1579.

(24) Ganthavorn, C.; Nagel, C. W.; Powers, J. R. Thermal inactivation of asparagus lipoxygenase and peroxidase. *J. Food Sci*. **1991**, *56*, 47.

(25) Regalado, C.; Arvizu, O. P.; Garcia-Almendarez, B. E.; Whitaker, J. R. Purification and properties of two acid peroxidases from brussels sprouts (*Brassica oleraceae* L.). *J. Food Biochem*. **1999**, *23*, 435−450.

(26) Adams, J. B. Review: Enzyme inactivation during heat processing of food-stuffs. *Int. J. Food Sci. Technol*. **1991**, *26*, 1−20.

(27) Tams, J. W.; Welinder, K. G. Glycosylation and thermodynamic versus kinetic stability of horseradish peroxidase. *FEBS Lett*. **1998**, *421*, 234−236.

(28) Rodrigo, C.; Rodrigo, M.; Alvarruiz, A.; Frigola, A. Inactivation and regeneration kinetics of horseradish peroxidase heated at high temperatures. *J. Food Prot*. **1997**, *60*, 961−966.

(29) Joffe, F. M.; Ball, C. O. Kinetics and energetics of thermal inactivation and the regeneration rates of a peroxidase system. *J. Food Sci*. **1962**, *27*, 587−592.

(30) McLellan, K. M.; Robinson, D. S. Purification and heat stability of Brussels sprout peroxidase isoenzymes. *Food Chem*. **1987**, *23*, 305−319.

(31) McFarren, E. F.; Thomas, R. C.; Black, L. A.; Campbell, J. E. Differentiation of reactivated from residual phosphatase in high temperature-short time pasteurization milk and cream. *J. Assoc. Off. Anal. Chem.* **1960**, *43*, 414−426.

(32) Murthy, G. K.; Cox, S.; Kaylor, L. Reactivation of alkaline phosphatase in ultra-high-temperature, short-time processed liquid milk products. *J. Dairy Sci*. **1975**, *59*, 1699−1709.

(33) Machado, M. F.; Saraiva, J. Inactivation and reactivation kinetics of horseradish peroxidase in phosphate buffer and buffer-dimethylformamide solution. *J. Mol. Catal.* **2002**, *19*−*20*, 451−457.

(34) Teipel, J. W.; Koshland, D. E., Jr. Kinetic aspects of conformational changes in protein. II. Structural changes in renaturation of denatured proteins. *Biochemistry* **1971**, *10*, 798−805.

(35) Haschke, R. H.; Friedhoff, J. M. Calcium-related properties of horseradish-peroxidase. *Biochem. Biophys. Res. Commun*. **1978**, *80*, 1039−1042.

(36) Van Huystee, R. B.; Sun, Y.; Lige, B. A. retrospective look at the cationic peanut peroxidase structure. *Crit. Rev. Biotechnol*. **2002**, *22*, 335−354.

(37) Van Huystee, R. B.; Xu, Y. J.; O'donnell, J. P. Variation in soret band absorption of peroxidase due to calcium. *Plant Physiol. Biochem*. **1992**, *30*, 293−297.

(38) McEldoon, J. P.; Dordick, J. S. Unusual thermal stability of soybean peroxidase. *Biotechnol. Prog*. **1996**, *12*, 555−558.

(39) Sutherland, G. R. J.; Aust, S. D. The effects of calcium on the thermal stability and activity of manganese peroxidase. *Arch. Biochem. Biophys*. **1996**, *332*, 128−134.

(40) Nie, G. J.; Aust, S. D. Effect of calcium on the reversible thermal inactivation of lignin peroxidase. *Arch. Biochem. Biophys*. **1997**, *337*, 225−231.
(41) Eshima, N.; Muta, A.; Anai, M. Inhibitory effect of bovine serum-albumin on acid deoxyribonuclease from rat small intestinal-mucosa. *J. Biochem*. **1983**, *94*, 345−352.
(42) Stefanova, M. E.; Schwerdtfeger, R.; Antranikian, G.; Scandurra, R. Heat-stable pullulanase from *Bacillus acidopullulyticus*: characterization and refolding after guanidinium chloride-induced unfolding. *Extremophiles* **1999**, *3*, 147−152.

Received for review November 4, 2004. Revised manuscript received February 7, 2005. Accepted February 17, 2005. T.T. has been supported by a Royal Thai Government scholarship.

JF0481610