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Isolation and Thermal Characterization of an Acidic Isoperoxidase from Turnip Roots

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An acidic peroxidase (p*I* ~2.5) was purified from turnip roots (TAP), and its thermal properties were evaluated. TAP is a monomeric protein having a molecular weight (MW) of 49 kDa and a carbohydrate content accounting for 18% of the MW. The yield of pure TAP was relatively high (~2 mg/kg of fresh roots), with a specific activity of 1810 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) units/mg at pH 6. The activity increased 4-fold at the optimum pH (4.0) to 7250 ABTS units/mg, higher than that of most peroxidases. TAP was heat stable; heat treatment of 25 min at 60 °C resulted in 90% initial activity retention, whereas an activity of 20% was retained after 25 min of heating at 80 °C. TAP regained 85% of its original activity within 90 min of incubation at 25 °C, following heat treatment at 70 °C for 25 min. Thermal inactivation caused noticeable changes in the heme environment as evaluated by circular dichroism and visible spectrophotometry. TAP was rapidly denatured by heating in the presence of 1.0 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, but the Soret band and activity were fully recovered by adding an excess of Ca^{2+}. This is further evidence that Ca^{2+} plays an important role in the stability of TAP. The high specific activity of TAP, together with its relatively high thermal stability, has high potential for applications in which a thermally stable enzyme is required.

KEYWORDS: Peroxidase isoenzymes; protein purification; thermal characterization

INTRODUCTION

Peroxidases (peroxidase, EC 1.11.1.7) are hemoproteins that catalyze the oxidation of a wide variety of substrates, using H_2O_2. They are widely distributed in nature. Peroxidase activity has been identified in plants, microorganisms, and animals, in which they play important roles. Deteriorative effects in some harvested fruits and vegetables have been atributed to this enzyme, such as changes in flavor and texture and the development of off-flavors (*1*, *2*).

Plant foods are generally exposed to a blanching treatment to inactivate deteriorative enzymes that may produce quality losses during storage. The deteriorative enzyme requiring the most severe heat treatment may be used, in thermal food processing, as an indicator for the adequacy of heat treatment, leading to an improved product stability. Due to its relatively high thermal stability and wide distribution, peroxidase has been widely used as an indicator enzyme for many plant foods (*3*−*5*). However, partial regeneration of peroxidase activity following heat treatment and cooling has been reported (*5*, *6*). This partially reversible inactivation results in flavor losses and off-flavor development in vegetables during storage (*7*, *8*). The kinetics of inactivation and reactivation of peroxidase involves both the heme dissociation and reversible denaturation of the protein (*9*, *10*).

We have previously reported on neutral isoperoxidase isolated from turnip roots (*11*). The objective of this work was to isolate an acidic isoperoxidase, test its thermal stability, and evaluate the fate of the heme group after heating of this isozyme. A peroxidase with as high a thermal stability as the one purified and reported here may offer some advantages in processes in which heat stability is crucial, such as in the removal of phenols and aromatic amines from polluted waste waters (*12*) and in phenolic resin synthesis (*13*).

MATERIALS AND METHODS

Plant Materials. Fresh turnip roots were purchased from the local market. Soil was removed physically from the roots, and they were

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sorted, rinsed with distilled water, drained, frozen, and stored at −20 °C until used.

Crude Extract. Turnip roots (1 kg) were homogenized at 4 °C in a blender using 10 mM potassium acetate buffer, pH 6. The extract was passed through a cheesecloth and centrifuged at 12000*g* for 15 min, and the supernatant was used for further purification. Protein was quantitated according to the dye-binding method of Bradford (*14*) using bovine serum albumin (BSA) as standard.

Peroxidase Activity. Peroxidase activity was determined by the change in absorbance at 414 nm due to 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) oxidation in the presence of H_2O_2 (*15*), at 25 °C. The reaction mixture consisted of 1 mM ABTS, 50 μL of enzyme (added last), 5 mM H_2O_2, and 10 mM potassium phosphate buffer, pH 6.0, in a total volume of 1.5 mL. One unit of enzyme was defined as the amount of substrate ABTS (micrograms) consumed in 1 min.

Peroxidase Purification. The first stages of turnip acid peroxidase (TAP) purification including anion-exchange chromatography (AEC) were conducted as detailed by Duarte-Vázquez et al. (*11*). After AEC, the fractions corresponding to a retained peroxidase peak having about half of the injected activity were pooled, dialyzed against deionized water, and freeze-dried (Labconco, Freezone 18 equipment). The dry sample was redissolved in a 0.05 M potassium phosphate buffer containing 0.3 M NaCl and applied directly to a column packed with Sephadex G-100 (Pharmacia, medium pore size) previously equilibrated with the same buffer using a flow rate of 15 mL/h. Fractions (4 mL) of the active peroxidase peak were pooled, dialyzed against distilled water, and freeze-dried. The material was redissolved in 2 mL of 0.02 M diethanolamine buffer, pH 11, and 0.5 mL was injected onto a Resource-Q column (Pharmacia) fitted to an FPLC system (Pharmacia). The column was previously equilibrated with the same buffer, using a 60 mL/h flow rate. The retained protein was eluted at the same flow rate using a linear gradient of 0.0−1 M NaCl in the above buffer. Fractions (1 mL) eluted from the column were monitored for absorbance at two wavelengths, 280 and 403 nm, and tested for peroxidase activity. All chromatographic steps were performed at room temperature.

Enzyme Characterization. *SDS-PAGE.* Purity, molecular weight, and glycosylation of peroxidase fractions were analyzed by SDS-PAGE as described previously by Duarte-Vázquez et al. (*11*).

Isoelectric Focusing. The isoelectric point (p*I*) was determined using a cooled horizontal electrophoresis cell (Biophoresis, Bio-Rad). A gel was prepared using 7.5 T (percent of the mixture acrylamide plus *N*,*N*′-methylenebisacrylamide, w/v), and 3 C (percent of *N*,*N*′-methylenebisacrylamide in the monomers mixture, w/w). Pharmalyte (Pharmacia) carrier ampholytes with a p*I* range of 3−6 were used. After prefocusing of the ampholytes, the protein sample was added close to the cathode. Initial voltage was set at 504 V and current intensity at 10 mA, whereas the final conditions after 2 h were 1639 V and 4 mA, respectively.

Thermal Stability. Heat inactivation studies were conducted at temperatures of 60, 70, and 80 °C using Pyrex glass tubes (i.d. = 9 mm, wall thickness = 1 mm). Tubes containing 3 mL of 20 mM Tris-HCl buffer, pH 7.5, were preheated to the required temperature in a water bath (Shell Lab). Once the buffer solution reached the specific temperature, a 0.3 mL aliquot of purified enzyme (0.2 mg/mL) was added to each tube, vortexed, and immersed again into the water bath. After heating for a designated time at the fixed temperature, the tubes were cooled in an ice−water bath and immediately assayed for peroxidase activity at 25 °C.

Reactivation. Samples of TAP in 0.5 mL of 0.02 M Tris-HCl buffer, pH 7.5, were heated for 25 min at 70 or 80 °C. Heated samples were cooled immediately, and one set was assayed for remaining peroxidase activity as zero-time samples. The remaining samples were incubated in a water bath at 25 or 4 °C and assayed for peroxidase activity at time intervals.

Thermal Unfolding of TAP. Changes of the heme group were evaluated by recording the peroxidase spectrum from 320 to 700 nm on a Lambda 40 spectrophotometer fitted with a peltier temperature control. TAP (0.2 mg/mL) samples were heat treated as described previously and immediately cooled in ice−water, followed by recording of their UV−visible spectra at time intervals during reactivation at 25 °C.

The structural calcium ions present in plant and fungal peroxidases are released during unfolding and can be efficiently bound by ethylenediaminetetraacetate (EDTA), leading to irreversible conditions. This permitted the measurement of the unfolding rate constant (k_u) independently of the folding rate constant (k_f) during irreversible temperature-induced unfolding of TAP (*16*).

The rate of change from native to unfolded protein was evaluated by the decrease in absorbance of the Soret band at 403 nm for 25 min at 80 °C, in the presence of 20 mM EDTA (irreversible conditions). The decrease in the Soret band absorbance was used as a convenient probe for conformational stability of *Coprinus cinereus* peroxidase (CIP; *16*, *17*) using high pH, urea, and heat as denaturants. This assumption was also employed by Lige et al. (*18*) to determine the rate of irreversible unfolding of wild-type and mutant peanut cationic peroxidases using guanidinium chloride. The remaining fraction of native peroxidase (RP), that is, the concentration of native peroxidase at time *t* divided by the initial concentration at time 0, is described by eq 1

$$\mathrm{RP} = [\mathrm{native}]_t/[\mathrm{native}]_0 = (A_t - A_\infty)/(A_0 - A_\infty) \qquad (1)$$

where A_t is the absorbance at time *t* (during the unfolding reaction), A_0 is the initial absorbance at time 0, and A_∞ is the absorbance at the end of the reaction.

The guanidium chloride induced unfolding of peanut peroxidase followed first-order kinetics, and we assumed that TAP denaturation by heat could be modeled using a similar approach (*17*, *18*). Therefore, a plot of ln(RP) versus time gave a straight line, and from the negative slope the rate constant of the unfolding reaction (k_u), from native to unfolded TAP, was calculated.

The transition state free energy (ΔG^*) between native and unfolded forms of TAP was calculated according to the theory of absolute reaction rates (*19*)

$$\Delta G^* = -\mathrm{RT} \ln [k_u h/(k_b T)] \qquad (1)$$

where *h* is the Planck constant (6.626×10^{-34} J/K) and k_b is the Boltzmann constant (1.381×10^{-23} J/K). Using this approach ΔG^* could be calculated for a single temperature.

Thermal denaturation of TAP was also monitored by using circular dichroism (CD) employing a Jasco J715 spectropolarimeter fitted with a peltier temperature control. Far-UV (250−200 nm, CD-far-UV) determinations were conducted using 1 mm path quartz cells and a protein concentration of 0.1 mg/mL. A sample concentration of 0.2 mg/mL was used for visible spectra (450−350 nm, Soret-CD) with 5 mm cells. The CD spectra were corrected by subtracting the appropriate blanks, and data were expressed in terms of ellipticity (θ). The temperature was raised from 20 to 90 °C in steps of 5 °C, with 2 min of equilibration at each temperature.

The melting temperature (midpoint of the transition, T_m) of the secondary structure and conformational changes around the heme group of TAP were evaluated by heating the sample at a constant rate of 1 °C/min while monitoring the ellipticity at 222 nm (secondary structure) and 403 nm (tertiary structure around the heme group). The sample was stirred in a 3 mL quartz cuvette throughout the experiment. The onset temperature of denaturation was defined as that where 5% of the ellipticity signal is lost.

RESULTS AND DISCUSSION

Peroxidase Purification. Two peaks with peroxidase activity were obtained from DEAE-cellulose AEC; one was eluted in the void volume, whereas the other eluted at 0.5 M NaCl gradient (results not shown). The nonretained fractions contained a neutral turnip peroxidase, which has been already purified and characterized (*11*). Thus, the retained pooled fractions (RPF) containing mainly acidic peroxidase isoforms were chosen for further purification.

The RPF were injected onto a Sephadex G-100 gel filtration column. The elution profile showed three protein peaks (A_{280}; results not shown), but only the second one showed peroxidase

Table 1. Summary of the Purification Steps of TAP (Mean of Five Replicates with Standard Error within 5% of the Mean)

sample	protein (mg)	activity (units)	specific activity (units/mg)	fold[a]	yield (%)
1. crude extract	6060	34800	5.74	1.00	100
2. ultrafiltration	4370	33400	7.64	1.33	96.0
3. acetone precipitation	3020	31100	10.3	1.79	89.2
4. AEC[b]	36.0	18100	502	87.5	52.0
5. gel filtration	8.80	10400	1180	205	29.8
6. FPLC, AEC[c]	4.00	7080	1810	315	20.3

[a] Ratio of actual to initial specific activity. [b] Anion-exchange chromatography (DEAE-cellulose), retained fraction. [c] Resource Q column fraction 15 (TAP).

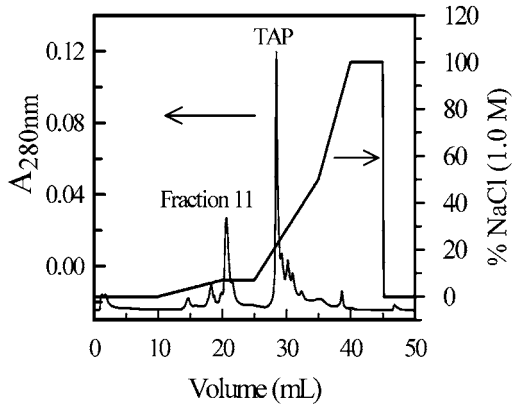

Figure 1. FPLC AEC (Resource Q) of pooled fractions from gel filtration. Buffer used was 0.02 M diethanolamine, pH 11.0. The same buffer with added 1.0 M NaCl was used for gradient elution.

activity. A large amount of the 280 nm absorbing material from the RPF was removed using this purification step, leading to a high peroxidase specific activity (**Table 1**).

The pooled fractions of the second peak showed high activity over a narrow pH range (3.5−5.0), having a maximum activity of 7250 units/mg at pH 4.0 (results not shown), ∼4 times higher than that observed at pH 6.0. However, activity measurements were carried out at pH 6.0, at which peroxidase is used in most applications.

Further purification of the pooled fractions was achieved by using the Resource-Q column, where two peaks showed peroxidase activity (fraction 11 and TAP; **Figure 1**). TAP had a higher protein content, specific activity, and purification factor than fraction 11. The RZ (A_{403}/A_{280}) value of TAP was 3.1. Because highly purified horseradish peroxidase (HRP) has an RZ = 3.0 (*20*), our extract had a high degree of purity (**Table 1**). This fraction was dialyzed, freeze-dried, and stored for further studies.

The purified peroxidase fractions were analyzed by SDS-PAGE, and after silver staining, TAP showed a single band (**Figure 2**). The molecular weight calculated from a plot of log R_f versus protein standard was 49 kDa and is very similar to that of HRP (40−46 kDa; *21*) and pepper fruit acidic peroxidase (50 kDa; *22*).

We have purified three peroxidases from turnip roots with molecular weights ranging from 36 to 39 kDa (*11*, *23*). The variability in molecular weight among plant peroxidases from the same source has been attributed to the post-translational modification of the polypeptide sequence, including the number and composition of the added glycan chains (*24*).

Isoelectric Focusing. After isoelectric focusing, TAP appeared as a single band with minor amounts of other peroxidase isozymes and migrated to the anode end of the gel. The pH corresponding to this section of the gel was 2.5, indicating an acidic peroxidase (results not shown). In turnip roots, peroxidase isozymes with a wide range of p*I* values have been identified: p*I* 4.5−5.0 (*25*), p*I* 8.3 (*23*), and p*I* 7.2 (*11*).

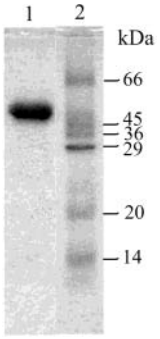

Figure 2. SDS-PAGE of purified turnip acid peroxidase: lane 1, TAP (fraction 15 from AEC using an FPLC); lane 2, molecular weight markers (Sigma).

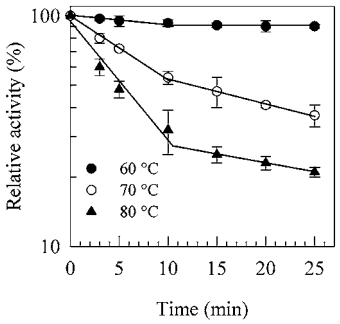

Figure 3. Heat inactivation profile of TAP (0.3 mg/mL). The ordinate represents relative activity, that is, the ratio of the activity to the initial activity before heating expressed as percentage.

Glycoprotein Nature and Carbohydrate Content. After SDS-PAGE and specific staining with a glycoprotein detection kit, TAP developed a pink color, characteristic of a glycoprotein. From the phenol−sulfuric acid reaction (*26*), TAP had 18% carbohydrate content, similar to peroxidases from soybean (15%; *27*) and peanut (17%; *28*) and lower than HRP (22%; *29*).

Peroxidase is known to be very stable during storage and resistant to proteolysis. Some authors (*28*, *30*) have considered that the carbohydrate moieties of peroxidase contribute to the high stability of the enzyme.

Lige et al. (*18*), using a site-directed replacement of each of three glycosylation sites of a cationic peanut peroxidase, showed that only the glycan linked to Asn185 was important for the thermal stability; its removal resulted in a rapid activity decrease at 50 °C.

Thermal Characterization. The rate of heat inactivation increased with temperature and length of heat exposure (**Figure 3**). When TAP was heated at 60 °C, ∼90% of the original activity was retained after 25 min. Even after a more drastic heat treatment, such as 25 min at 80 °C, TAP still showed peroxidase activity (∼21%, **Figure 3**). The thermal stability is very similar to that of HRP (*31*), whereas TAP appears to be more heat stable than a crude peroxidase from okra, which lost >90% of its activity within the first 15 min of heat treatment at 60 °C (*32*).

A peroxidase from Australian carrots (*Daucus carota* L.) was completely inactivated by heating at 80 °C for 4 min (*33*). A heat-resistant peroxidase from carrot (unspecified variety) homogenate lost 90% of its original activity within the first 5

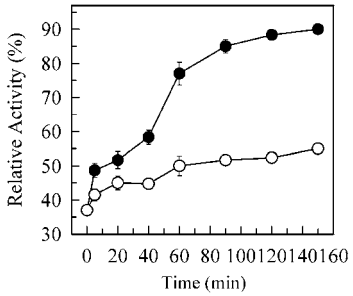

Figure 4. Activity recovery of TAP incubated at 4 °C (○) and 25 °C (●) after heat treatment for 25 min at 70 °C. The ordinate represents relative activity, that is, the ratio of the activity to the initial activity before heating expressed as percentage.

min of heat treatment at 80 °C (*34*). Thus, TAP may be considered a heat stable enzyme, comparable with a potato tuber sprout isoperoxidase that retained ~60% of the original activity after heating at 70 °C for 10 min (*35*).

It has been hypothesized that glycosidic chains linked to peroxidase increase thermal stability (*18*, *22*). From preliminary dynamic light scattering measurements, we observed that at temperatures ranging from 30 to 45 °C and at pH close to neutrality, TAP tends to form aggregates, which may result in increased thermal stability.

The thermal inactivation curves exhibited nonlinear heat inactivation rates, which have been found for peroxidase from various fruits and vegetables (*33*, *35*, *36*). This behavior has been attributed to the presence of several peroxidase isoforms with different heat stabilities (*7, 9*). Adams (*37*) attributed the nonlinearity of the inactivation curves to the regeneration of peroxidase activity, whereas Vamos-Vigyazo (*38*) proposed the formation of a new compound of higher thermostability comprising the heat-denatured peroxidase and those isoforms that remained active. However, Forsyth et al. (*39*) observed that even in purified isoperoxidases, the inactivation curves were still nonlinear, and this was attributed to different aggregation states that exhibited a range of thermal stabilities. This last hypothesis may explain our results.

Reactivation of TAP after Heating. A regain of peroxidase activity was noted after incubation of all heat-treated samples. Maximum reactivation occurred in samples previously heated at 70 °C and incubated at 25 °C, reaching 85% of the original activity within the first 90 min (**Figure 4**). Samples incubated at 4 °C reactivated to a lower extent after 120 min, because of insufficient energy for reactivation. The TAP heated at 80 °C for 25 min regenerated from 25 to 30% of the original activity by incubation at 25 °C for 120 min. It is clear that the incubation temperature had a significant effect on the extent of TAP activity regeneration, in agreement with Lopez and Burgos (*3*), who found that the most effective HRP regeneration occurred at room temperature.

Spectral Changes of TAP during Thermal Reversible Inactivation. The characteristic absorption maxima of the heme group, that is, 502 and 634 nm, disappeared after heat treatment at 80 °C for 25 min (results not shown). The effects of thermal treatment (70 °C, 25 min) on the absorption spectrum of TAP were fully reversed after incubation at 25 °C (**Figure 5B**), and the activity was almost completely recovered (85%, **Figure 4**). The reactivated enzyme had maxima at 403, 502, and 634 nm.

The changes in the visible absorption spectra of TAP during thermal inactivation (**Figure 5A**) were consistent with changes in the heme environment, because longer incubation times produced a lower Soret absorbance (λ = 403 nm) signal.

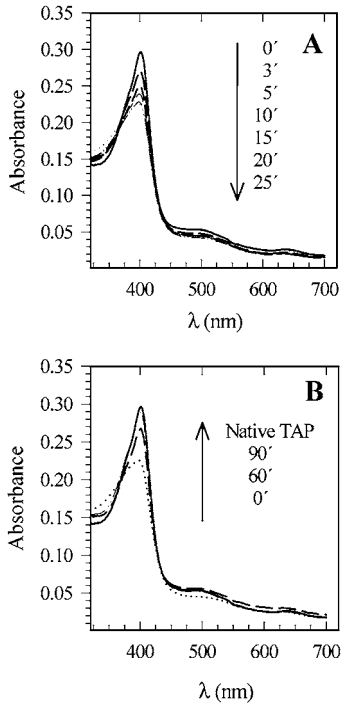

Figure 5. Absorption spectra of TAP (0.8 mg/mL) during thermal inactivation at 70 °C (A) and reactivation at 25 °C (B).

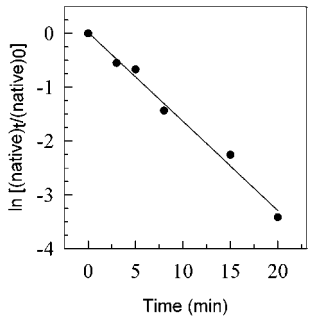

Figure 6. Unfolding of TAP (0.8 mg/mL, pH 7.5), 20 mM EDTA, at 80 °C as a function of heat treatment time. The unfolding process was evaluated as the decrease in absorption at 403 nm. The rate constant of the unfolding process ($k_u = 2.75 \times 10^{-3}$ s^{-1}) is equal to the negative slope of this line.

Samples heated at 70 °C for 25 min showed a 30% decrease in A_{403} (**Figure 5A**), whereas those heated at 80 °C for the same time had a 50% decrease. Therefore, during thermal inactivaton of TAP the steady reduction of heme absorption was consistent with a gradual calcium ion release, producing structural changes in the vicinity of the heme group and favoring a thermal equilibrium between native and unfolded states, as reported for HRP (*17*).

As suggested by Tams and Welinder (*16*, *17*) we used the Soret absorbance band as a probe for direct observation of the irreversible unfolding process. The rate constant for TAP unfolding ($k_u = 2.75 \times 10^{-3}$ s^{-1}) was obtained from the slope of the straight line resulting from a plot of eq 1 (**Figure 6**). From the unfolding rate constant, and using eq 2, the transition state free energy was 104.3 kJ/mol, at 80 °C. This indicated that TAP was more stable than CIP ($\Delta G^* = 100$ kJ/mol at 61.2 °C; *16*).

A comparison of the heat inactivation rates obtained from TAP residual activity (**Figure 3**) and those obtained from the decrease in the Soret band absorption at the same temperature (**Figure 6**) was considered to be irrelevant. The former had the

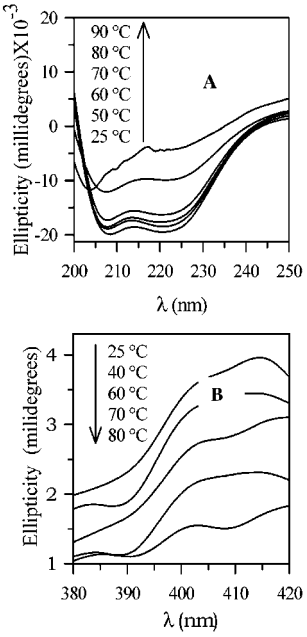

Figure 7. (A) CD-far-UV spectra of TAP (0.1 mg/mL; pH 7.5) at different temperatures. (B) Soret-CD spectra of TAP (0.2 mg/mL; pH 7.5) at different temperatures. Temperature is noted in the figure.

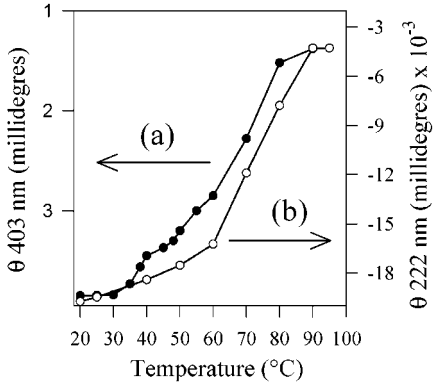

Figure 8. Change in ellipticity of TAP with temperature: (a) 0.2 mg/mL at 403 nm, path length = 5 mm; (b) 0.1 mg/mL at 222 nm, path length = 1 mm.

contributions of the intrinsic inactivation rate constant and the reversible unfolding equilibrium constant (*40*), whereas the latter measured only the rate constant of TAP irreversible unfolding.

During the thermal inactivation of TAP (**Figure 6**) the use of EDTA probably caused structural disruption of the heme environment by trapping the calcium at the most sensitive binding site (distal), producing gross activity reduction. We measured peroxidase activity as a function of the time shown on **Figure 6**. However, the activity was lost after 3 min, whereas the structural changes in the vicinity of the heme group were gradually detected by our visible absorption technique. This was attributed to the relatively low sensitivity of our visible light absorption technique or to subtle structural changes around the heme group, followed by more drastic changes in the substrate uptake mechanisms. For HRP, it has been hypothesized that the structural alterations resulting from calcium release during heat treatment promote a change in the distance between the heme and the substrate producing activity reduction (*41*). Thermal inactivation of manganese peroxidase (MnP) was attributed to the distal calcium removal, which allowed a closer interaction of His46 with the heme iron, resulting in loss of activity (*42*).

Optical activity of the α-helix in far-UV permits the use of CD studies for investigation of conformational changes in protein solutions. Ellipticity at 208 and 222 nm was used to monitor the unfolding of TAP. The far-UV spectrum did not show any significant changes at these two wavelengths after TAP had been heated at 60 °C (**Figure 7A**). However, when it was heated at 90 °C, a significant decrease in ellipticity signal at 208 and 222 nm was observed, indicating a decrease in α-helix and an increase in random coil. From the data in **Figure 8b**, TAP appeared to unfold in a single step with an apparent T_m (midpoint of the transition from helical to random structure) of 72 °C. The observed melting temperature is higher than that of CIP (T_m of 65 °C; *6*) and very close to the values obtained from temperature-dependent unfolding of HRP isozyme C (T_m of 74 °C; *43*).

The TAP prosthetic group provides a convenient means to study the conformational changes of the protein environment surrounding the heme active site. The unfolding of heme proteins generally results in disruption of the heme active site, which can be probed by changes in CD ellipticity signal in the Soret region. Thus, far-UV-CD at 222 nm gave information about changes of the protein secondary structure, whereas CD at the Soret region gave information on local changes in the heme cavity. The heme moiety of TAP exhibited a positive ellipticity, close to 403 nm (**Figure 7B**). At relatively low temperatures (40 °C) the intensity of the Soret band decreased, whereas at 70 °C it decreased to 50% of the initial value (**Figure 7B**). At 80 °C the Soret absorption almost resembled that of free hemin, because the ellipticity was very weak (**Figure 7B**). From **Figure 7B** it was observed that the heme group started to dissociate from the protein core at 40 °C and that at high temperatures (75−80 °C) the asymmetric character necessary for optical activity was almost completely lost.

Unlike the melting of the secondary structure, thermal unfolding of the tertiary structure around the heme group monitored by Soret-CD showed the existence of two distinct phases with one intermediate state (**Figure 8**). The first phase started at ∼30−40 °C and was associated with a change in the tertiary structure near the heme region, whereas the secondary structure remained fairly constant. In the second phase of the transition, maximum ellipticity lost was reached at 85 °C. Here, there were large changes in the secondary structure, resulting in much of the heme group removal from the active site as indicated by the low values of the Soret-CD at 403 nm. The intermediate formed during the first phase with partially collapsed tertiary structure and secondary structure almost intact has been related to a *pre-molten globule* intermediate. It has been observed in the thermal denaturation of HRP (*43*, *44*). These results suggest that TAP activity loss is partly associated with changes in the environment surrounding the heme group. Additionally, we added hematin to a TAP sample and observed a slower rate of thermal inactivation and an enhancement on the extent of reactivation (*45*). Therefore, we conclude that the heme group is released from the protein during heat treatment and that this occurs before the onset of changes in the secondary structure.

Previous studies have demonstrated that calcium exists in many peroxidases (*18*, *46*, *47*). Shiro et al. (*41*) and Tsaprailis et al. (*47*) showed that calcium maintains the heme microenvironment and stabilizes the enzyme. Sutherland et al. (*42*) demonstrated that the loss of enzymatic activity of manganese peroxidase (MnP) was due to the loss of calcium during heat treatment. Because thermal inactivation may be due to the release of calcium ions, the effect of calcium on TAP thermal

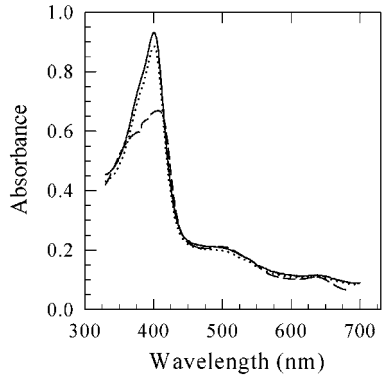

Figure 9. Absorption spectra of TAP: (—) active; (- - -) thermally inactivated at 40 °C in the presence of 1.0 mM EGTA for 5 min to 5% activity; (• • •) calcium reactivated to 90% activity following the addition of 1.5 mM $CaCl_2$ and incubation at 30 °C for 15 min. TAP concentration = 0.2 mg/mL.

inactivation and on the visible absorption spectrum was investigated.

TAP was heat treated at 40 °C during 20 min without any change in the absorption spectrum. When it was heated at 40 °C in the presence of 1.0 mM EGTA (Ca^{2+} chelator), it was denatured to 5% activity within the first 3 min of heat treatment. When $CaCl_2$ (to 1.5 mM) was added to thermally inactivated TAP, the Soret signal and activity were 90% recovered (**Figure 9**).

From these results it is clear that heat treatment caused alterations in the tertiary structure around the heme environment of TAP and that Ca^{2+} plays a very important role in TAP stability.

The crystal structure of HRP has been solved and taken as a model of plant peroxidases. The structural features include two calcium binding sites proximal and distal to the heme, four disulfide bridges (Cys11−Cys91, Cys44−Cys49, Cys97−Cys301, and Cys 177−Cys209), and N-glycosylations. This may aid in stabilization of the secondary and/or tertiary structure of the enzyme (*48*). Sutherland et al. (*42*) found that MnP was rapidly inactivated by heat in the presence of 1.0 mM EGTA to 2% activity, and it was recovered to 91% following the addition of 1.5 mM $CaCl_2$. They concluded that thermal inactivation was due to the removal of the distal calcium, which was required to maintain the structural integrity of the distal heme environment of MnP. However, cationic peanut peroxidase (PNP; *42*) and HRP (*41*) maintained 50 and 40% activity, respectively, after complete calcium removal. Thus, it appears that the role of calcium in other peroxidases may be different from that observed in MnP, although the calcium binding sites of PNP are in the same position as the distal and proximal binding sites of MnP and lignin peroxidase (LiP; *46*). The X-ray structure of PNP revealed that adjacent to the calcium ligands (Asp43 and Asp50) there was a disulfide bridge between Cys44 and Cys49 that formed a short loop encompassing the distal calcium ligand site. In MnP, this loop was found to be much longer and did not contain a disulfide bridge. The presence of this disulfide bridge may explain why the heme environment and activity of PNP were not affected as drastically as in MnP following the loss of calcium (*42*).

In a parallel work conducted at our laboratory, a partial amino acid sequence of turnip peroxidase was deduced from its cDNA (*49*) and encoded for six cysteine residues located in positions agreeing very closely with the conserved cysteines found in other plant peroxidases. This sequence lacks two of the eight well-conserved cysteine residues, and they could probably be involved in the building of the fourth disulfide bridge associated with the protection of the heme environment and activity of turnip peroxidase.

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