Purification and Partial Characterization of Three Turnip (*Brassica napus* L. var. esculenta D.C.) Peroxidases

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Three turnip peroxidases (fractions C1, C2, and C3) were partially purified and characterized, to permit study of their feasibility for use in clinical and enzyme immunoassays. These fractions represented 20% of the initial activity, and fractions C1 and C2 were purified to homogeneity. The optimum pH was between 5.0 and 5.5, while optimum temperature ranged from 40 to 55 °C. The ABTS K_m values for the two acidic fractions (C2 and C3) were 0.70 and 0.42 mM, respectively; about 5 times lower than that reported for the acidic commercial horseradish peroxidase (HRP). Fraction C3 had 4 times higher K_m value than commercial cationic HRP. The molecular weights determined by SDS−PAGE ranged from 39.2 to 42.5 kDa. Activation energies for inactivation were 113 (C1), 130 (C2), and 172 kJ/mol (C3) which are higher or comparable to other peroxidase isoenzymes reported. Fractions C1 and C3 represent an alternative source of peroxidase because of their higher purification yield and specific activity, when compared to fraction C2.

Keywords: *Peroxidase isoenzymes; turnip; protein purification, Brassica napus L.*

INTRODUCTION

Peroxidases are found in bacteria, fungi, plants, and animals. On the basis of sequence similarity, peroxidases containing a heme group can be categorized into two superfamilies: fungal, plant, and bacterial peroxidases and animal enzymes (Welinder, 1992).

In plants, peroxidases have been implicated in numerous physiological processes including lignification (Lagrimini, 1991), cross-linking of cell wall polysaccharides (Fry, 1986), pathogen resistance (Stintzi et al., 1993), and general stress response (Castillo and Greppin, 1986) as well as the phytohormone's indole-3-acetic acid catabolism (Smith et al., 1982).

This enzyme has attracted interest in the food industry because of its ability to bring about desirable and sometimes undesirable changes. Development of off-flavor, color, texture, and/or decreased nutritional value (Fils et al., 1985) can be contrasted with the desirable texture obtained through the formation of protein cross-linking (Matheis and Whitaker, 1984).

Peroxidase is well-suited for the preparation of enzyme-conjugated antibodies (ELISA), due to its ability to yield chromogenic products at low concentrations and to its relatively good stability characteristics (Krell, 1991).

Horseradish (*Armoracia rusticana* L.), the traditional source of peroxidases, does not grow well in Mexico. In our laboratory we are testing alternative sources more locally available and studying their properties as possible substitutes for horseradish peroxidase (HRP).

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MATERIALS AND METHODS

Fresh turnip roots were obtained from a local market. The soil was removed from the roots, and they were sorted, rinsed with distilled water, drained, frozen, and stored at −20 °C until used.

Crude Extract. Turnip roots (700 g) were removed from frozen storage and homogenized at 4 °C using 1 L of 10 mM potassium acetate buffer, pH 6. The extract was centrifuged at 12 000*g*, and the supernatant was used for further purification.

Protein Determination. Protein was quantitated based on the dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Peroxidase Activity. Activity was determined spectrophotometrically by the change in absorbance at 414 nm using ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] as substrate (Childs and Bardsley, 1975). The final reaction mixture contained 1 mM ABTS, 50 μL of enzyme, 5 mM H_2O_2, and 10 mM potassium phosphate buffer, pH 6.0, in a volume of 1.5 mL. The assay was performed at 25 °C using a Lambda 2S spectrophotometer. One unit of enzyme was defined as the amount of substrate (μg) consumed in 1 min.

Precipitation. Precipitation was carried out by using ammonium sulfate first with 10% saturation and centrifugation, and then saturation was increased to 90% followed by centrifugation. The redissolved precipitate was dialyzed against deionized water and precipitated using cold acetone (−20 °C; ratio 2:1 of acetone:sample) at an overall temperature of 4 °C. The precipitate was collected by centrifugation and was redissolved in 0.05 M Tris-HCl, pH 8.6, and dialyzed overnight versus the same buffer.

Anion-Exchange Chromatography. A 1.5 × 20 cm Bio-Rad column packed to 15 cm bed height with DEAE-cellulose (Sigma, exchange capacity 0.89 mequiv/g), fitted to a Gradifrac (Pharmacia) system, was equilibrated with 0.05 M Tris-HCl buffer (pH 8.6) using a 20 mL/h flow rate. Two milliliters of the enzyme solution was injected to the column. The retained protein was eluted at the same flow rate using a linear gradient of 0.0 to 1.0 M NaCl in the above buffer. Fractions of

10.1021/jf990454j CCC: $19.00
Published on Web 04/08/2000

4 mL were collected, absorbance was read at 280 nm, and peroxidase activity was measured.

HPLC−Ion-Exchange Chromatography. Fractions from the DEAE-cellulose column showing peroxidase activity were combined and dialyzed against deionized water and freeze-dried using a Virtis 5L equipment. A Resource Q column (Pharmacia) coupled to a HPLC (Waters) was equilibrated with 0.10 M phosphate buffer, pH 11.0, using a flow rate of 20 mL/h. The dried sample was redissolved in this buffer, and 500 μL was injected onto a Resource Q column. The retained enzyme was eluted at the same flow rate using a linear gradient of 0.0 to 1.0 M NaCl in the above buffer. Each fraction was monitored for protein (absorbance at 280 nm) and also for peroxidase activity. The fractions having peroxidase activity were collected and dialyzed against deionized water and freeze-dried. The dry samples were kept frozen for further study.

Enzyme Characterization. *Optimum pH.* The following buffers were used: 0.01 M citrate buffer pH 3−6; 0.01 M phosphate buffer pH 7; 0.01 M Tris-HCl buffer, pH 8, and 0.01 M borate buffer, pH 9. H_2O_2 (2 mM), 2.5 mM ABTS, and 50 μL purified fractions of enzyme were used (ionic strength kept constant at 0.43 by adjusting with KCl).

Optimum Temperature. Peroxidase activity was measured between 20 and 65 °C with 5 °C increments, using the optimum pH. Temperature was controlled using a constant temperature water circulator (Laubda Model B).

SDS−PAGE. Purity and molecular weight of the different enzyme fractions were analyzed using SDS−PAGE under reducing and nonreducing conditions. This was conducted using a Mighty Small vertical chamber (SE 250, Hoeffer Instruments). Polyacrylamide gels were prepared according to the discontinuous buffer system of Laemmli (1970). The stacking gel had 4 T (% of the mixture acrylamide plus N,N′-methylene-bis-acrylamide in the gelling solution, w/v) and 2.6 C (% N,N′-methylene-bis-acrylamide in the mixture acrylamide plus N,N′-methylene-bis-acrylamide, w/w), while the separating gel had 10 T and 2.6 C. Runs were performed at constant current (10 mA/plate in the stacking phase and changing to 15 mA/plate in the separating phase). Molecular weight markers in the range 6.5−66 kDa (Sigma) were included. The rapid silver staining technique was used (Ausubel et al., 1995). A digital camera was used to analyze the gels, and the molecular weights were determined using the 1D software for Windows version 2.0.3 (Rochester, NY).

Isoelectric Focusing. The isoelectric point (pI) was determined using a refrigerated horizontal electrophoresis cell (Biophoresis, Bio-Rad). A gel was prepared using 7.5 T, 3 C, and wide pH range ampholytes (3−10, Bio-Lyte). The gel was prepared according to the manufacturer directions. Initial voltage was set at 418 V and current intensity of 10 mA, while the final conditions after 2 h were 1639 V and 4 mA. The bands were developed by immersion in a solution prepared just before use, with the following composition: 0.2 M phosphate buffer (pH 6.0), 0.05% (w/v) ABTS, and 0.03% (v/v) H_2O_2. Activity bands generally developed within 3−5 min at which time the gel was washed with deionized water and photographed immediately. A calibration curve was obtained by measuring the pH along the length of the gel by slicing, and the peroxidase isoelectric point was estimated by interpolation from this curve.

K_m Determination. K_m values for H_2O_2 were obtained by varying H_2O_2 concentration from 0.11 to 5.0 mM using an enzyme-saturating ABTS concentration (2.5 mM). Concentrations of ABTS ranging from 0.05 to 3.25 mM were used at enzyme-saturating H_2O_2 concentration (2 mM). pH, ionic strength, and temperature were kept constant in these series of experiments. K_m of peroxidase for each substrate was calculated from a plot of [S] vs [S]/v according to Augustinsson (Cornish-Bowden, 1995).

Peroxidase Thermal Inactivation. Thermal stability of purified peroxidase was evaluated as follows. An aliquot of enzyme solution was added to 0.5 mL of 0.01 M citrate buffer (pH 5.0) in 12 × 75 mm test tubes. The tubes were shaken in a water bath with slight agitation, while heated for a designated time at a fixed temperature. Heated samples were cooled immediately in ice−water, and the residual enzymatic activity was evaluated as described previously. To calculate the activation energy for inactivation of peroxidase (E_a), rate constants were calculated for peroxidase inactivation in samples treated at specified temperatures and various times. The rate constants (r) were obtained from the slope of a plot of log A (% original activity) versus time of heat exposure. From the slopes of the straight lines obtained using the Arrhenius plots of log(r) versus 1/T the E_a values were calculated.

Table 1. Summary of the Purification Stages of Turnip Peroxidases (Mean of Four Replicates with Standard Error within 5% of the Mean)

sample	protein (mg)	activity (U)	specif act. (U/mg)	fold	yield (%)
crude extract	1200	7800	6.50	1.00	100
ammonium sulfate precipitation	425	6100	14.0	2.20	78.0
acetone precipitation	268	5900	22.0	3.40	76.0
anion-exchange	2.90	1700	580	89.0	32.0
DEAE-cellulose nonretained fractions					
HPLC-anion exchange					
fraction C1	0.600	820	1370	210	10.5
fraction C2	0.110	100	886	136	1.20
fraction C3	0.550	650	1180	182	8.33

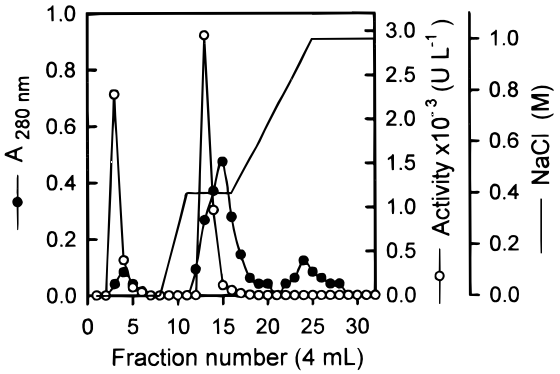

Figure 1. DEAE-cellulose chromatography of turnip roots peroxidase after acetone precipitation. The column was equilibrated with 0.05 M Tris-HCl (pH 8.6). Absorbance was measured at 280 nm, and peroxidase activity was assayed as described in the text.

RESULTS AND DISCUSSION

Peroxidase Purification. The crude extract was precipitated with different concentrations of ammonium sulfate. The precipitate formed between 35% and 80% ammonium sulfate saturation recovered most peroxidase activity. This was done because defined peaks could not be obtained. Acetone precipitation was conducted as a further step because peroxidase purification was improved, and a brownish colored-precipitate appearing at the top of the ion-exchange column was difficult to remove if this step was not used. The activity yield and purification factor using these two precipitation steps are shown in Table 1.

The next stage in the purification scheme was anion-exchange chromatography. Figure 1 indicates that peroxidase activities are mainly distributed into two peaks, apparently corresponding to basic (nonretained) and a mixture of slightly basic, neutral, and acidic (eluted with the salt gradient) isoenzymes. However, when there is charge asymmetry, localization of charge clusters on the protein surface may sometimes allow its adsorption at or beyond its isoelectric point (above its pI for cation-exchangers and vice versa for anion-exchangers), re-

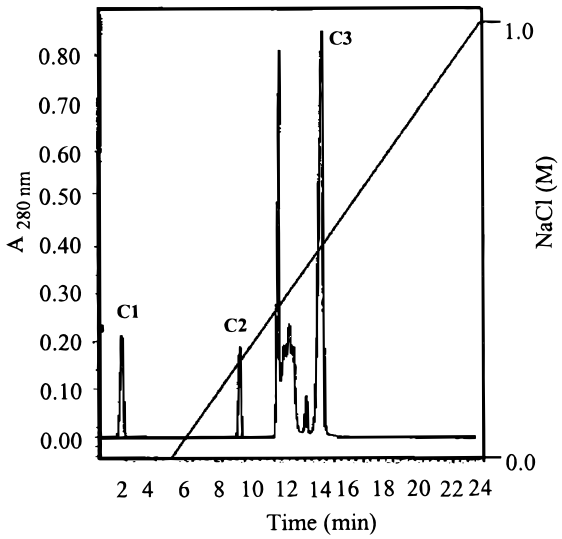

Figure 2. HPLC−anion exchange using a Resource Q (Pharmacia) column of the nonretained fractions after anion exchange using DEAE-cellulose. Peroxidase activity was assayed in each peak using ABTS as substrate. Conditions: 0.010 M phosphate buffer, pH 11.0.

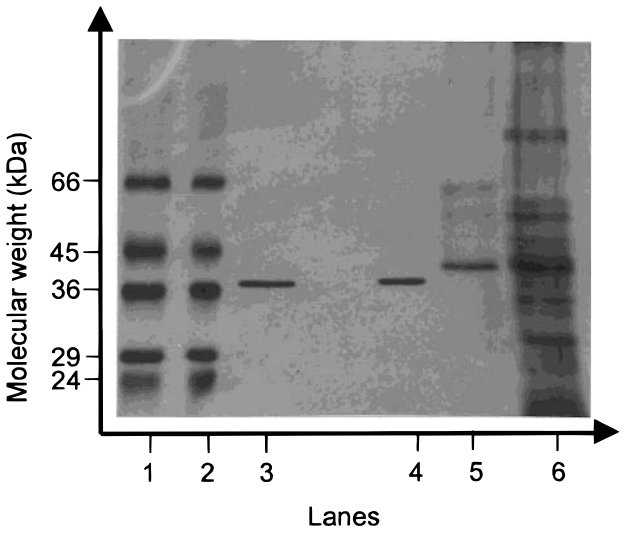

Figure 3. SDS−PAGE analysis of turnip peroxidase isoenzymes stained with the rapid silver technique. Lanes 1 and 2, molecular weight markers; lane 3, fraction C1; lane 4, fraction C2; lane 5, fraction C3; lane 6, crude extract.

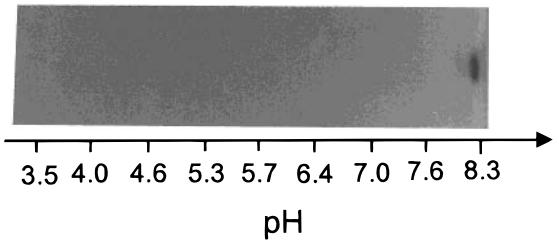

Figure 4. Isoelectric focusing of the fraction C1 in polyacrylamide gel. The pH measured along the gel is shown. The protein pI corresponds to the pH where the band appears.

gardless of the mobile-phase pH (Kopaciewicz et al., 1983; Algar and Scopes, 1979). Hydrophobic interactions may partly contribute to this effect (Scopes, 1994).

The specific activity and the R_z values (A_{403}/A_{280}) of the nonretained pooled fractions were greater than those of the retained pooled fractions, and thus they were chosen for further purification. The R_z value (0.62) of the nonretained turnip roots peroxidases obtained so far indicated a fair degree of purification. Wang and Luh (1983) reported R_z values for soluble and ionically bound asparagus peroxidases of 0.25 and 0.59, respectively; after ammonium sulfate precipitation and chromatographic steps with Sephacryl S-200 and Con-A Sepharose 4B. Anion exchange chromatography gave a great improvement in specific activity and purification factor (Table 1).

The nonretained pooled fractions were injected into a Resource Q column on a HPLC. The elution profile (Figure 2) showed mainly three peaks having peroxidase activity and were labeled as C1, C2, and C3. Peak C1 was a nonretained fraction, while fractions C2 and C3 eluted with the salt gradient. These fractions had high specific activities and purification factors (Table 1).

Peroxidases Characterization. *Molecular Weight.* The purified fractions were assayed for purity and molecular weight using SDS−PAGE (Figure 3). Fractions C1 and C2 were homogeneous, because the silver-stained gel showed only one band for each fraction (Figure 3, lanes 3 and 4). The molecular weight of the denatured peroxidases were 39.2 kDa for isoenzyme C1, 39.4 kDa for isoenzyme C2, and 42.5 for the more defined band of fraction C3 (Figure 3, lane 5). The molecular weight determined by SDS−PAGE in the presence of 2-mercaptoethanol was found to be very similar to that obtained under nonreducing conditions, indicating that the isoenzymes C1 and C2 are a single polypeptide proteins. According to Vamos-Vigiazo (1981), the molecular weight of peroxidases from a variety of fruits and vegetables range from 30 to 54 kDa, which agree with the results obtained here.

The molecular weights reported here are higher than those found for wheat germ peroxidase (35 kDa; Converso and Fernández, 1995) and green asparagus peroxidase (34 kDa; Wang and Luh, 1983), but lower than those for opuntia peroxidase (58 kDa; Padiglia et al., 1995) and pepper fruit acidic peroxidase (50 kDa; Pomar et al., 1997).

Isoelectric Focusing. The purified fractions from HPLC−anion exchange were isoelectric focused in polyacrylamide gels containing wide range ampholytes (pH 3−10) (Figure 4). Fraction C1 appeared as a single band which migrated to the cathode end of the gel; the isoelectric point value was 8.5 according to the measured pH gradient along the gel. Fractions C2 and C3 migrated to the anode, indicating these isoenzymes have a pI around 3; therefore, they did not enter the focusing part of the gel.

Peroxidases found in higher plants include isoenzymes with basic, neutral, or acidic pI and a single vegetable may have several isoenzymes with different pI values (Wang and Luh, 1983; Halpin et al., 1989; Lee and Kim, 1994). Acidic peroxidases have been identified in turnip roots (Agostini et al., 1997); pepper fruit, pI 3.8 (Pomar et al., 1997), and Brussels sprouts, pI 3.9−4.7 (Regalado et al., 1999). Basic peroxidases have been found in turnip roots, pI 11.0 (Welinder and Mazza, 1975), green peas, pI 9.0 (Halpin et al., 1989), and strawberry fruit, pI 9.5−10 (Civello et al., 1995).

Anionic and/or cationic isoenzymes in a single vegetable have been related with different physiological roles of peroxidase. Basic peroxidases are effective in indole-3-acetic acid catabolism and ethylene biosynthesis, both of which are plant hormones (Gaspar et al., 1985). On the other hand, acidic peroxidases participate

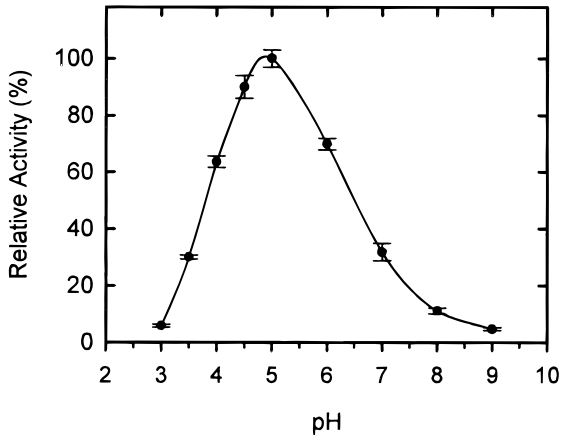

Figure 5. Effect of pH on enzymatic activity of turnip peroxidase (fraction C2) with ABTS as substrate. The ordinate represents relative activity, i.e., the ratio of the activity to the maximum activity expressed as percentage. Vertical bars indicate standard deviation.

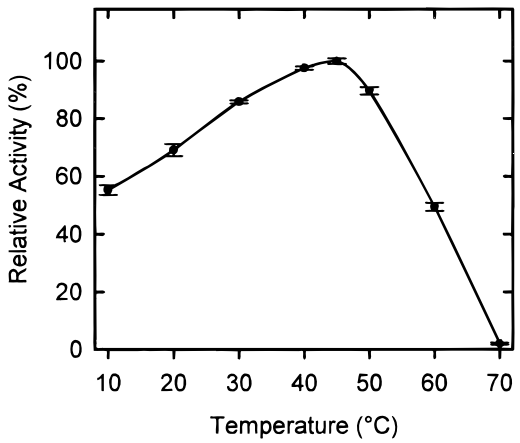

Figure 6. Effect of temperature on enzymatic activity of turnip peroxidase (fraction C2). The ordinate represents relative activity, i.e., the ratio of the activity to the maximum activity expressed as percentage. Vertical bars indicate standard deviation.

in the last stage of lignin biosynthesis (Wakamatsu and Takahama, 1993).

Optimum pH. Peroxidase is specific for H_2O_2 as substrate but operates with a number of H-donor compounds. Using ABTS as H-donor, turnip peroxidases showed high activity in a narrow pH range with a maximum at pH 5.0 for fraction C2 (Figure 5); the activity versus pH profile was similar for fraction C1 (maximum at pH 5.5, results not shown). Fraction C3 had maximum activity at pH 5.0 (results not shown); the relative activities at pH's above this maximum remained high, producing a skewed bell-shaped curve, probably because of contaminating peroxidase isoenzymes (Figure 3, lane 5). The optimum pH values obtained here are similar to those previously found for two turnip anionic peroxidases (pH 4.7, Agostini et al., 1997). It is known that the optimum pH for any peroxidase depends on the hydrogen donor and buffer solution used in the activity assay (Halpin et al., 1989).

Optimum Temperature. Under the conditions used, the activity reached a maximum at 45 °C for fraction C2 (Figure 6). Fractions C1 and C3 had maximum activity at 40 and 55 °C, respectively (results not shown). Temperatures higher than 55 °C produced a sharp decrease in relative peroxidase activity, reaching

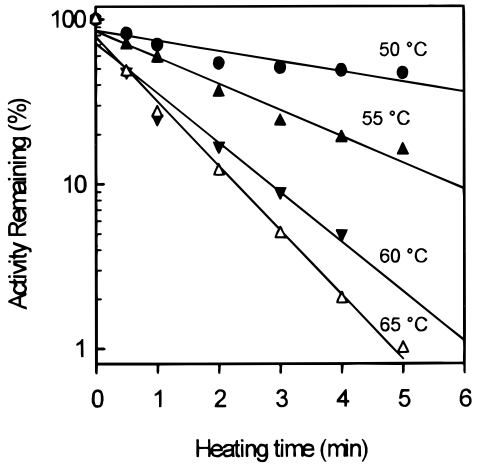

Figure 7. Rate of heat inactivation of turnip peroxidase (fraction C2).

Table 2. K_m Values of Turnip Peroxidase Isoenzymes (from Augustinsson Plots; Mean of Three Replicates with Standard Error within 5% of the Mean)

isoenzyme	ABTS (mM)	H_2O_2 (μM)
fraction C1	1.30	40.0
fraction C2	0.710	245
fraction C3	0.470	850

a value lower than 20% at 65 °C for fraction C2 and <5% for fractions C1 and C3 after 3 min heating (results not shown). The purified turnip peroxidases exhibited considerable activity at low temperatures, being about 55% for fraction C2 at 10 °C (Figure 6), and 40 and 50% for fractions C1 and C3, respectively, at 20 °C (results not shown). This behavior agrees with that reported for strawberry peroxidase (Civello et al., 1995); soluble and bound peroxidase from green asparagus (Wang and Luh, 1983) and cauliflower peroxidase (Lee et al., 1984), although they all had a different optimal temperature.

Apparently these results suggest that E_a for denaturation of the three purified peroxidases is relatively low. We are addressing this point in the thermal inactivation section, where more comments will be given on this matter.

Kinetic Constants for ABTS and H_2O_2. The purified turnip peroxidase isoenzymes followed Michaelis–Menten kinetics when hydrogen peroxide concentrations were between 0.01 and 5.0 mM. The saturation concentration of H_2O_2 was about 2.5 mM. Above this concentration, inhibition by H_2O_2 was observed, probably because of substrate inhibition combined with iron oxidation, producing inactive chromophoric compounds and eventually an inactive verdohemoprotein. This substrate inhibition has also been reported for commercial horseradish peroxidase types VI and IX (Hiner et al., 1996) and asparagus peroxidase (Rodrigo et al., 1996; Arnao et al., 1990). The extent of inhibition of peroxidase activity caused by hydrogen peroxide varies greatly with different substrates and experimental conditions.

Varying the concentration of ABTS (H-donor) and H_2O_2 as substrates, we calculated the apparent K_m from Augustinsson plots (Table 2). The ABTS K_m values for the three purified isoenzymes were four to five times lower than those reported for acidic HRP (4 mM; Hiner et al., 1996). These values were higher than for two Brussels sprouts peroxidase isoenzymes (0.2 mM; Regalado et al., 1999) and higher than commercial HRP

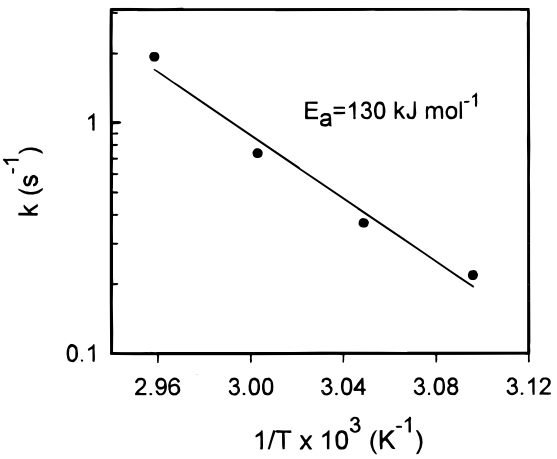

Figure 8. Arrhenius plot showing the effect of temperature on rate constant for the thermal nactivation of turnip peroxidase (fraction C2).

cationic isoenzyme (0.1 mM, Sigma type IX; Hiner et al., 1996). Apparent H_2O_2 K_m values for fractions C1, C2, and C3 (Table 2) were higher than the values of 11.4 and 6.2 μM reported for Brussels sprouts peroxidase isoenzymes A1 and A2 (Regalado et al., 1999), while fraction C1 had a K_m similar to that reported for the anionic HRP (33 μM; Pütter and Becker, 1983). These reports used hydrogen peroxide and ABTS concentrations of the same order of magnitude and therefore may be compared. These K_m values are important when peroxidase is used as a reporter enzyme for immunoassays.

Thermal Inactivation. The extent of heat inactivation of purified peroxidase fractions increased with increase in temperature and time of heat exposure. When fraction C1 was heated at 50 °C, about 50% of the activity was lost within 3 min. Thus, fraction C1 appears to be heat labile, in contrast with many previous reports showing peroxidases from various sources to be very heat stable. However, when fractions C2 (Figure 7) and C3 were heated at 50 °C, 4 and 6 min were required to destroy approximately 50% of the activity, respectively.

The heat inactivation results were used to calculate the activation energies for heat inactivation (E_a) of the purified fractions. Rate constants were calculated for the heat inactivation of peroxidase in samples treated at different specific temperatures. These constants were obtained from the slope of a semilog plot of the fraction of original activity remaining against the time of heat exposure. Arrhenius plots of ln(rate constant) against the reciprocal of absolute temperature ($1/T$) were prepared and the slope of the resulting straight lines was used to calculate the E_a (Figure 8). Fraction C1 had an E_a value (113 kJ/mol) higher than those reported by Ling and Lund (1978) for a HRP isoenzyme (88 kJ/mol) and for cauliflower peroxidase (89 kJ/mol; Lee and Pennesi, 1984). The E_a value for fraction C2 (130 kJ/mol) was higher than that reported for another HRP isoenzyme (114 kJ/mol; Lu and Whitaker, 1974) and lower than the value for asparagus peroxidase (140 kJ/mol; Ganthavorne et al., 1991). The activation energy for heat inactivation for fraction C3 (172 kJ/mol) was similar to that reported for heat resistant peroxidase isoenzyme from cauliflower (193 kJ/mol; Lee et al., 1984) and for Brussels sprouts peroxidase isoenzyme A1 (172 kJ/mol; Regalado et al., 1999). The optimum temperature results, together with those from thermal inactivation, led us to conclude that turnip peroxidase fraction C3 has a high heat stability, while fraction C2 has a medium heat stability and fraction C1 is heat labile.

Although fraction C2 had a medium ABTS K_m value, the low purification yield and specific activity are important disadvantages (Table 1). Thus, it is concluded that fractions C1 and C3 are an interesting alternative source of peroxidase. We are at present applying reverse micellar extraction of these isoenzymes to achieve a more suitable large scale purification for producing these isoenzymes

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); E_a, activation energy for inactivation (J/mol); HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; K_m, Michaelis−Menten constant; T, sum of the weights of acrylamide plus *N*,*N*′-methylene-bis-acrylamide in the gelling solution expressed as % (w/v); C, weight percent of *N*,*N*′-methylene-bis-acrylamide in the mixture of acrylamide plus *N*,*N*′-methylene-bis-acrylamide (w/w).

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Received for review May 4, 1999. Revised manuscript received January 28, 2000. Accepted February 23, 2000. This research was carried out under financial support from SIHGO, project Alim-2/96. Financial support through an MSc grant from CONACYT to M. A. Duarte-Vázquez is appreciated.

JF990454J