Kinetic Study of the Irreversible Thermal Deactivation of Palmito (*Acanthophoenix rubra*) Polyphenol Oxidase and Effect of pH

Christine M. Robert,[†,‡] Frédéric R. Cadet,*[,†] Claude C. Rouch,[†] Michel Pabion,[†] and Florence Richard-Forget[§]

Laboratoire de Biochimie, Faculté des Sciences, Université de la Réunion, 15 Avenue René Cassin, B.P. 7151, 97715 Saint-Denis Messag Cedex 9, Réunion, France, Laboratoire Research and Development, Prisma International, Réunion, France, and Station de Technologie des Produits Végétaux, Domaine Saint-Paul, Site Agroparc, 84914 Avignon Cadex 9, France

The optimal temperature of palmito (*Acanthophoenix rubra*) polyphenol oxidase (PPO) is 30 °C. The Arrhenius activation energy was calculated to be 5.41 kJ mol^{-1}. $\Delta H°$ of the reaction is −60.99 kJ mol^{-1}. At 25 °C, $\Delta G°$ and $\Delta S°$ were, respectively, 16.75 kJ mol^{-1} and −260.87 J mol^{-1} K^{-1}. The enzyme heated at temperatures above 30 °C loses its activity. Fifty percent inhibition is reached in 18 min at 70 °C, in 8 min at 75 °C, and in 2.5 min at 80 °C. The kinetics of the thermal irreversible denaturation of this enzyme is characterized by two steps: N → X(T_d) → D, where N represents the native form, X represents an intermediate form, the structure of which depends on the deactivation temperature T_d, and D is the completely denatured form of the enzyme. Our experimental results rule out a two-isoenzyme (with varying heat sensitivity) model. The thermodynamic parameters of the irreversible denaturation of the intermediate form were 102.70 and 97.10 kJ mol^{-1} for $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$, respectively, and 16.85 J mol^{-1} K^{-1} for $\Delta S^{\ddagger}$ at 60 °C. Furthermore, this paper describes the effect of pH on the activity of the PPO. Studies were carried out with 4-methylcatechol and pyrogallol as substrates. The pH profile was not a function of the nature of the substrate assayed. The pH optimum was 5.2. The plot of log$V_{max\ app}$ vs pH indicates that the oxidation of the substrates depended of the ionization of two groups in the enzyme−substrate complex with apparent pK values of 3.06 and 7.29 and 3.44 and 7.12, respectively, for 4-methylcatechol and pyrogallol. The very slight differences between the values suggest the existence of only one site on the molecule for both substrates.

Keywords: *Thermal deactivation; polyphenol oxidase; palmito*

INTRODUCTION

The brown discoloration arising in vegetables and fruits as a result of a mechanical injury during storage or processing is caused primarily by the oxidation of phenolic substrates by polyphenol oxidases (PPO; EC 1.14.18.1) present in the tissue, and their subsequent polymerization by both nonenzymatic and enzymatic reactions. The heart of palmito (*Acanthophoenix rubra*) is very susceptible to enzymatic browning. There have been many reviews dealing with polyphenol oxidases from plants, but few studies have been published on polyphenol oxidase from palmito (Lourenço et al., 1990; Demeau and Bidan, 1967; Bernhardt et al., 1978), even though this vegetable is industrially processed in many countries. To inhibit these reactions, industrial treatment consists frequently in a thermal destruction of the enzyme.

The behavior of polyphenol oxidases from fruits and vegetables when submitted to heat treatment has been studied extensively for industrial purposes (Dimick et al., 1951; Montogomery and Petropakis, 1980). It is well-known that browning reactions, initialized by the enzyme, occur mainly postharvest when the tissues are exposed either to stress conditions or to deterioration (Lourenço et al., 1990). Efforts to avoid darkening during processing have centered on establishing suitable processing conditions for polyphenol oxidase inactivations without appreciable texture losses.

To understand the kinetic behavior of polyphenol oxidase when exposed to high temperatures, a detailed analysis of the effect of temperature on the activity and stability of polyphenol oxidase was undertaken. The effect of pH with 4-methylcatechol and pyrogallol as substrates was also studied to gain some information on the active site of the enzyme. No data are available for this species (*A. rubra*); a few publications refer to *Euterpe edulis* species (Lourenço et al., 1990; Bernhardt et al., 1978).

MATERIALS AND METHODS

Extraction and Purification of Polyphenol Oxidase. The palmito was harvested 1 day before extraction. The enzyme was extracted from chilled stem palmito (heart of palmito), cut into pieces, and homogenized in a Waring Blendor containing 0.1 M sodium phosphate buffer, pH 7.5, and 1 mM cysteine (it has been determined that the residual cysteine concentration in the enzymatic assay has no effect). The solution turn slightly brown during the extraction of the enzyme. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 17000g for 20 min at 4 °C. The pellet was dissolved in the same buffer containing 1.5% Tween 20 and then centrifuged at 17000g for 20 min at 4 °C.

$(NH_4)_2SO_4$ was added to the supernatant to 20% saturation. After centrifugation (40000g, 15 min, 4 °C), $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation. The protein

* Author to whom correspondence should be addressed [fax (262) 29 00 90].
† Université de la Réunion.
‡ Prisma International.
§ Station de Technologie des Produits Végétaux.

0021-8561/95/1443-1143$09.00/0

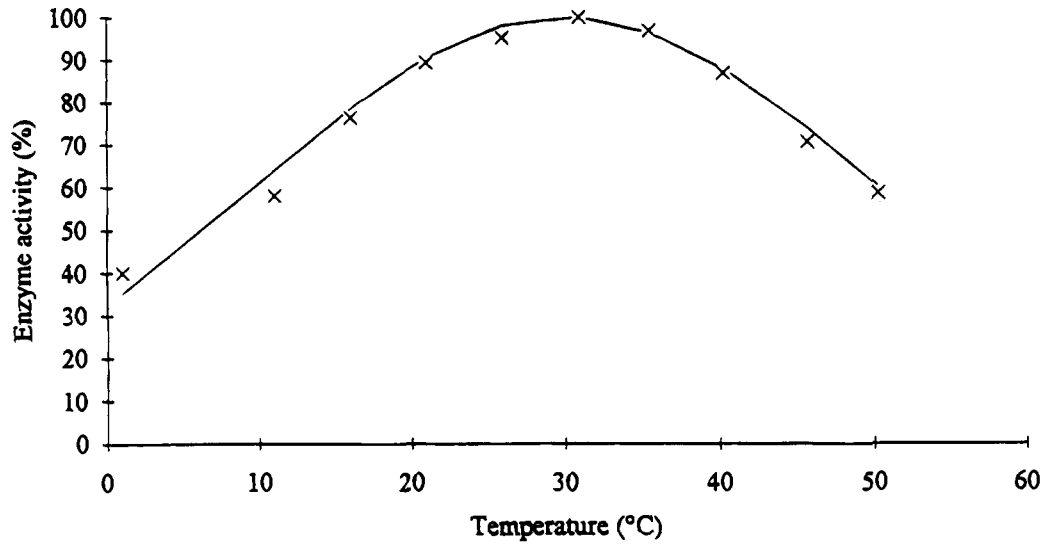

Figure 1. Optimal temperature of polyphenol oxidase (*A. rubra*). The hydrolytic activity has been studied with the 4-methylcatechol test, 0.1 M citrate–phosphate buffer, at pH 4.8 with respect to the temperature. The protein concentration in the cuvette was about 4.42 nM.

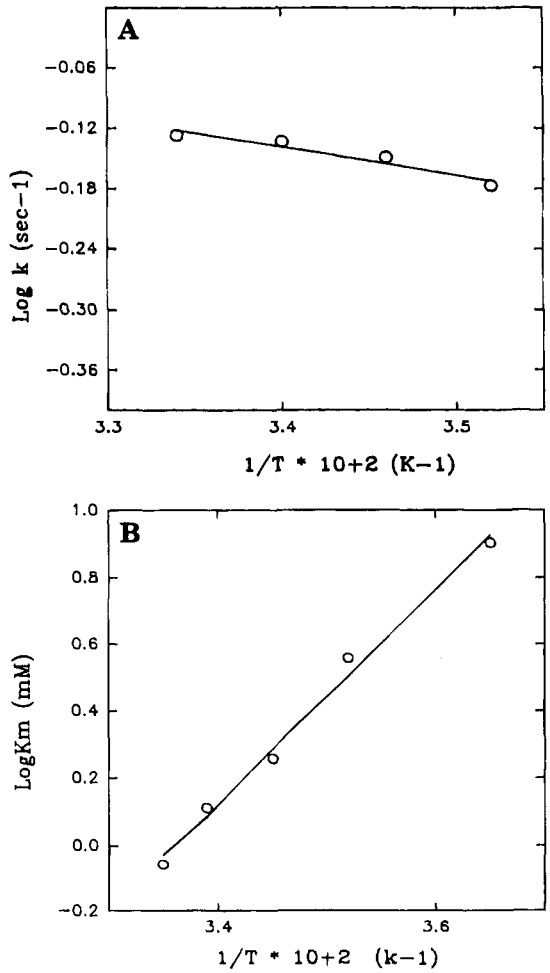

Figure 2. Thermodynamic parameters of the polyphenol oxidase. (A) Arrhenius plot. The Arrhenius pattern is drawn from date of Figure 1. (B) Van't Hoff plot. K_m has been determined using the 4-methylcatechol test which concentration varied over 0.08–20 mM.

fraction (95% of the initial activity) precipitated between 20 and 60% saturation (40000*g*) was dissolved in 0.1 M phosphate buffer, pH 7.5, and dialyzed overnight against a 5 mM phosphate buffer, pH 7.5. The dialyzed enzyme was loaded on a DEAE-cellulose column previously equilibrated with 0.1 M sodium phosphate buffer, pH 7.5, and eluted with a linear gradient from 0 to 0.5 M KCl dissolved in the equilibration buffer. The enzyme eluted at a concentration of 0.21 M KCl was highly purified and used as enzyme source for this study (Robert et al., 1995a).

Protein Assay. Protein concentration was determined with the Bio-Rad reagent according to the Bradford method (Bradford, 1976).

Kinetic Measurements. The polyphenol oxidase activity was measured by the increase of absorbance at 395 nm for 4-methylcatechol or at 422 nm for pyrogallol with a Phillips spectrophotometer PU 8730. The reaction mixture contained 17.25 mM 4-methylcatechol or 30 mM pyrogallol (for pH study, substrate concentrations were variable but always saturating) in 0.1 M citrate–phosphate buffer, pH 4.8, and an aliquot of the enzyme extract (4.42 nmol).The rate of the reaction was calculated from the initial slope of the progress curve. One unit of enzymatic activity represents the appearance of 1 μmol of product min^{-1}, under conditions giving the maximum rate, when the substrate is 4-methylcatechol. Activity is expressed in percent of maximum activity when pyrogallol is used as substrate.

Determination of the p*K* and pH Optimum Values. Polyphenol oxidase activity as a function of pH was determined using 4-methylcatechol or pyrogallol as substrate. The pH range used to determine the pH optimum was 2.5–8. The substrates were dissolved in 0.1 M citrate–phosphate buffer for pH from 2.5 to 6 and in 0.1 M sodium phosphate buffer for pH from 6.5 to 8. The protein concentration in the cuvette was about 4.42 nM. A check was made to ensure that the results were identical with the two buffers.

The pH also affects the stability of enzymes. Indeed, the decline could result from the formation of an improper ionic form of the enzyme or from the inactivation (irreversible enzyme denaturation) of the enzyme. The effect of a preincubation of the enzyme at extreme pH was monitored to verify that the decline in activity between acidic pH and optimum pH, on the one hand, and optimum pH and alkaline pH, on the other, results from the formation of an improper ionic form of the enzyme. The enzyme stability as a function of the ionic strength has been verified.

For the determination of the p*K* values, pH profiles and treatment of the data were performed according to the method of Segel (1975) by assuming that the enzyme exists in three different protonated forms, E_{n+1}, E_n, and E_{n-1}, and that only E_nS gives product. The bell-shaped curves justify this assumption.

Determination of the Optimal Temperature. The enzyme was incubated at the desired temperature ranging

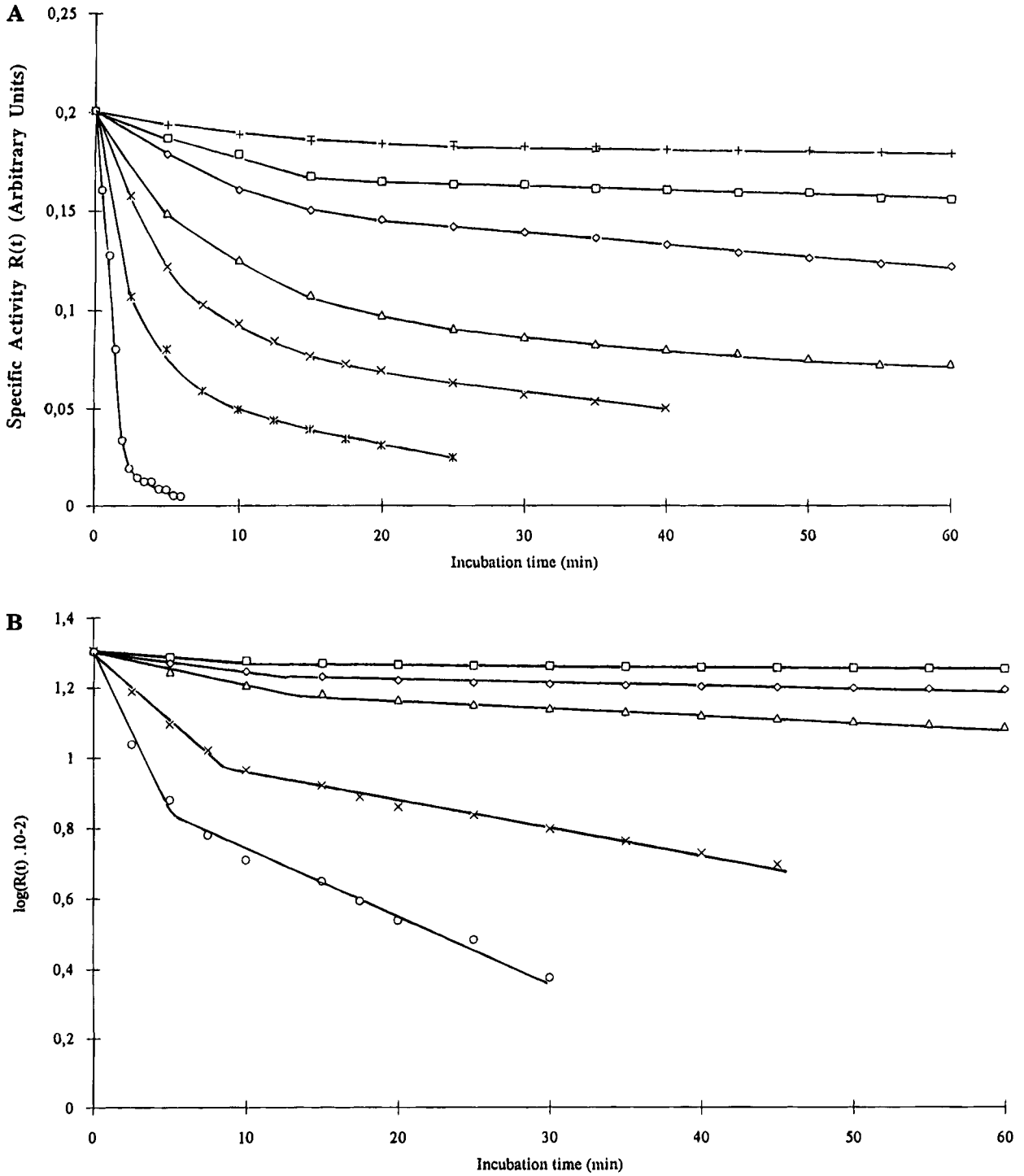

Figure 3. Thermostability of *A. rubra* polyphenol oxidase. (A) Enzyme activity is shown as a function of the deactivation time. All of the kinetic tests were performed at 30 °C in 0.1 M citrate–phosphate buffer, pH 4.8, with 17.25 mM 4-methylcatechol. The deactivation temperatures varied over 40–90 °C: (+) 40 °C; (□) 50 °C; (◇) 60 °C; (×) 75 °C; (*) 80 °C; (○) 90 °C. (B) Log(v) vs the deactivation time: (+) 40 °C; (□) 50 °C; (◇) 60 °C; (×) 75 °C; (*) 80 °C. The enzyme concentration in the cuvette was about 4.42 μM.

from 1 to 50 °C for 10 min. Then an aliquot was taken out and the activity was measured at the same temperature as the incubation temperature. The buffer was 0.1 M citrate–phosphate, pH 4.8.

Evaluation of the Kinetic and Thermodynamic Activation and Standard Parameters. The kinetic (V_m and K_m) and activation parameters were calculated from the least-squares-fitted Lineweaver–Burk and Arrhenius plots, respectively (Segel, 1975). All Arrhenius plots were linear over the range of temperature used.

E_a was obtained from the slope of the Arrhenius plots.

Thermodynamic functions were calculated according to the following relationships:

$$\Delta H^{\ddagger} = E_a - RT$$

$$\Delta G^{\ddagger} = -RT \ln(k)h/K_B T$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T$$

h is the Planck constant, K_B is the Boltzmann constant, and k is a rate constant. $\Delta H°$ is obtained from the slope of the van't Hoff plot. $\Delta G° = RT \ln K_m$, and $\Delta S° = (\Delta H° - \Delta G°)/T$.

Heat Inactivation Study. The temperature ranged from 40 to 90 °C. There are three ways to calculate the thermo-

Table 1. Calculated Values of the Kinetics Parameters for Both Deactivation Models for *A. rubra* Polyphenol Oxidase

	specific activity (μmol s^{-1} μmol^{-1}) × 100				rate constant (s^{-1}) × 100	
	two-isoenzyme model		two-step model			
temp (°C)	R_1	R_2	R_1	R_2	k_1	k_2
40	6.55 ± 0.36	71.51 ± 0.35	78.06 ± 0.17	71.25 ± 0.31	12.20 ± 1.29	0.04 ± 0.01
50	14.45 ± 1.50	63.78 ± 1.52	78.23 ± 0.59	63.29 ± 1.31	10.52 ± 1.94	0.08 ± 0.04
60	59.99 ± 1.11	18.28 ± 1.48	78.28 ± 0.53	58.18 ± 1.09	13.11 ± 1.54	0.39 ± 0.04
69	30.87 ± 2.93	47.33 ± 2.70	78.19 ± 1.46	44.55 ± 2.64	18.13 ± 3.37	1.06 ± 0.14
75	34.60 ± 1.68	43.80 ± 1.65	78.40 ± 0.64	13.21 ± 1.42	20.05 ± 1.26	1.45 ± 0.17
80	31.98 ± 2.64	45.89 ± 2.74	77.88 ± 0.93	28.60 ± 2.32	47.00 ± 4.81	4.99 ± 0.55

[a] k_1, k_2, R_1, and R_2 were obtained by nonlinear fitting of the experimental results to eqs 1 and 2.

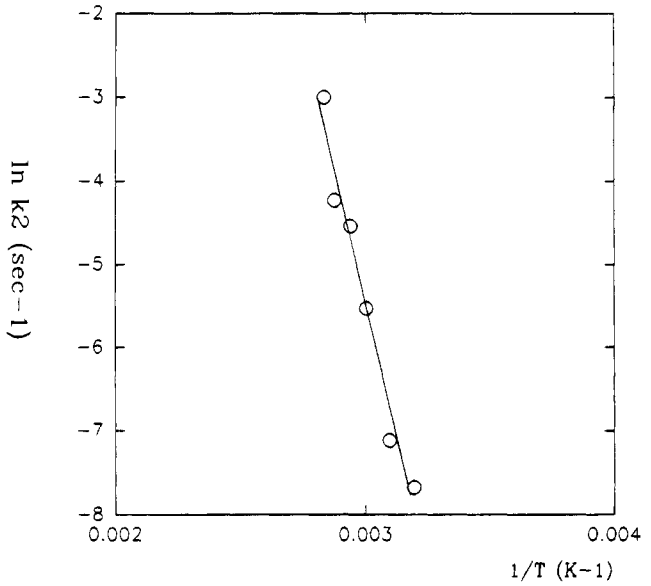

Figure 4. Arrhenius plot for the irreversible denaturation: Ln k_2 (two-step model) vs $1/T$ (data from Table 1).

dynamic parameters of the irreversible thermal denaturation of an enzyme: (i) The enzyme is incubated at different deactivation temperatures, T_d, and its activity measured at one test temperature, T_t. (ii) The enzyme is incubated at different T_d values, and its activity is measured at the same temperature (T_t equals T_d). (iii) The enzyme incubated at one temperature (T_d) is assayed at a different T_t. Only the results given by the first method are unambiguous for the choice of the model (Tanford, 1968). Therefore, to discriminate between the two models, we have used the method indicated in (i).

The enzyme was incubated at the desired inactivation temperature. At intervals, an aliquot of enzyme was taken out and quickly cooled on ice (15 min). The remaining activity was monitored at a constant temperature of 30 °C.

The remaining enzyme activity reached a constant value immediately after cooling on ice and maintained this activity for at least several hours. Caution was taken to prevent pH changes when the temperature of the sample was modified.

The reaction mixture contained 4-methylcatechol in 0.1 M citrate–phosphate buffer, pH 4.8, and an aliquot of the enzyme extract (4.42 nmol).

Mathematical treatment was performed on a NEC personal computer with Sigma Plot (Jandel Scientific Software).

RESULTS AND DISCUSSION

Temperature Dependence of Enzyme Activity. Experiments conducted to study polyphenol oxidase activity as a function of temperature showed a maximum at 30 °C using 4-methylcatechol as substrate at pH 4.8 (Figure 1). Below and above 30 °C the enzyme activity fell gradually, with 40% activity still left at 1 °C and nearly 60% at 50 °C. The optimum temperature obtained in this study is within the range values reported for polyphenol oxidases from other plants (Augustin et al., 1985; Lamikanra et al., 1992; Siddig et al., 1992) between 20 and 45 °C. The effect of temperature on enzyme activity was presented as Arrhenius and van't Hoff plots showing the temperature dependence of V and K_m (Figure 2A,B). Both relationships are linear over the temperature range from 5 to 25 °C. The Arrhenius activation energy was calculated to be 5.41 kJ mol^{-1}. The activation energy obtained here was lower than that found for *E. edulis* by Lourenço et al. (1990) (66.5 kJ mol^{-1}) with chlorogenic acid as substrate and that for potato with pyrogallol (54.5 kJ mol^{-1}) (Mihalyi et al., 1978) but was comparable to the value of activation energy obtained for banana with a catechol as substrate (18.6 kJ mol^{-1}) (Palmer, 1963). $\Delta H°$ of the reaction is −60.99 kJ mol^{-1}. At 25 °C, $\Delta G°$ and $\Delta S°$ were, respectively, 16.75 kJ mol^{-1} and −260.87 J mol^{-1} K^{-1}.

Table 2. Partial Purification of Palmito (*A. rubra*) Polyphenol Oxidase

fraction	activity (units)	protein (mg)	specific activity	recovery (%)	purification factor
crude extract	62	246	0.25	100	1
dialyzed (20–60%) $(NH_4)_2SO_4$	16	33	0.48	26.11	1.93
DEAE-cellulose (first peak)	6	2	3	8.7	11.96

The thermodynamic activation parameters over the temperature range (1–30 °C) were almost identical and equal to 2.93 kJ mol^{-1}, 73.64 kJ mol^{-1}, and −237.28 J mol^{-1} K^{-1} for $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$, respectively (at 25 °C).

The negative value for $\Delta S^{\ddagger}$ indicates that the transition state presents a more ordered structure than the ground state.

Thermostability. The effect of increasing temperatures with respect to incubation time is shown in Figure 3. The activity remained reasonably constant at 30 (data not shown) and 40 °C. A significant thermal denaturation began from 50 °C. The curves show a relatively high stability of the palmito polyphenol oxidase at high temperature. Indeed, in 1 h of incubation, the activity inhibition is only 23%; 50% inhibition is reached in 18 min at 70 °C, in 8 min at 75 °C, and in 2.5 min at 80 °C. A 3 min treatment is necessary to inhibit 90% of polyphenol oxidase activity at 90 °C. This polyphenol oxidase seems to be more stable than that of *E. edulis* (Lourenço et al., 1990) or other plants (Augustin et al., 1985; Zhou and Feng, 1991). Silva and Nogueira (1983) reported that when palmito *E. edulis* was heated at 90 °C for 2 min, polyphenol oxidase lost 97% of its activity.

The plots of log(remaining activity) vs incubation time (Figure 3B) were characterized by two straight lines, and the denaturation process cannot be described in terms of a single-exponential decay. Several research-

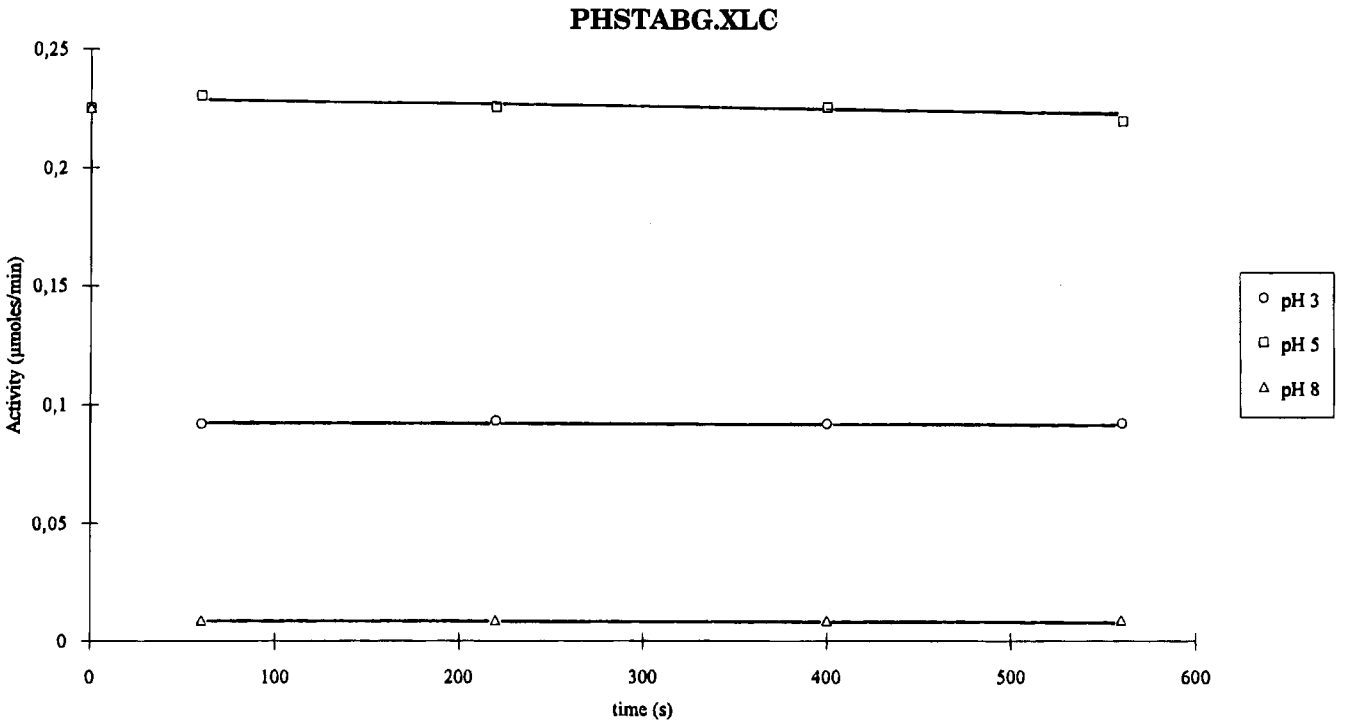

Figure 5. pH stability of *A. rubra* polyphenol oxidase. Enzyme activity is shown as a function of incubation time. Polyphenol oxidase is incubated at 30 °C at different pH values. The remaining activity was tested at 30 °C using the 4-methylcatechol test: (○) pH 3; (□) pH 5; (△) pH 8.

ers have reported the heat inactivation of polyphenol oxidases isolated from different sources to be a biphasic process (Lourenço et al., 1990; McCord and Kilara, 1983; Park and Luh, 1985), but the irreversible deactivation process has not been studied to date. Two models can explain these curves: First, there are two isoenzymes which behave differently when submitted to high temperatures; the model is

$$N_1 \xrightarrow{k_1} D_1 \quad N_2 \xrightarrow{k_2} D_1$$

and, second, the denaturation of the enzyme proceeds in two steps as

$$N \xrightarrow{k_1} X \xrightarrow{k_2} D$$

where N, X, and D are the native, intermediate, and denatured forms of the enzyme, respectively.

For model 1, it is obvious that the specific activity of the mixture, $R(t)$, varies with time t according to

$$R(t) = R_1 e^{-k_1 t} + R_2 e^{-k_2 t} \quad (1)$$

For model 2

$$R(t) = \left(R_1 - R_2 \frac{k_1}{k_1 - k_2}\right) e^{-k_1 t} + \left(R_2 \frac{k_1}{k_1 - k_2}\right) e^{-k_2 t} \quad (2)$$

where R_1 and R_2 are the specific activities of the two native isoenzymes (model 1) or of N and X (model 2) and k_1 and k_2 are the corresponding deactivation constants determined for each temperature. The calculation of the four parameters of both models was effected by fitting the data to eqs 1 and 2. An estimation of the initial parameters was obtained according to the method of Tanford (1968). For each model, if the absolute value of the difference between the norm of the residuals (square root of the sum of squares of the residuals) from one iteration to the next is less than the tolerance value (0.0001), then the iteration will stop. This usually means that a minimum has been found and corresponds to the best fit.

The choice of one of the models was made according to the values of the specific activity (R_1, R_2) and the deactivation constant k_1 and k_2. These values are presented in Table 1. The values of k_1 and k_2 given by the fitting are identical for both models. The analysis of the variation of R_1 and R_2 with respect to temperature led us to discriminate between the two-step model and the two-isoenzyme model.

The thermal denaturation followed the first model if R_1 and R_2 were independent of temperature, since they are the specific activities of two native isoenzymes (N_1 and N_2). If not, the thermal denaturation follows the two-step model.

In the case of the supposed two-isoenzyme model, values given in Table 1 show that R_1 and R_2 vary as a function of T_d. So this model must be ruled out. Only the two-step model can explain our experimental data. This is in accordance with the fact that R_1, which is the specific activity of the native enzyme (N), is constant whatever the T_d.

The second model accounts for the data if we assume that the structure of the intermediate (X) depends on the deactivation temperature. Increasing the deactivation temperature induces further deterioration in the structure of X.

The variations of R_2 with respect to T_d suggest the existence of more than one intermediate form with structures depending on the deactivation temperature according to

$$N \rightarrow X_{T_d} \rightarrow D$$

The deactivation constant k_1 does not follow the Arrhenius law. This is not surprising since in the first step N gives different intermediate forms ($X(T_d)$) issued

from various deactivation processes with its own particular constant k.

In a second step, these less active intermediate forms are also deactivated. As they may give the same product (D), it cannot be excluded that the second deactivation constant follows the Arrhenius law: noticeably, this is the case for k_2 (Figure 4)—though the correlation observed with respect to k_2 may only indicate similar reaction pathways of the proposed intermediates. The correlation coefficient is equal to 0.98. The activation energy with respect to k_2 is calculated to be 105.47 kJ mol^{-1}. The thermodynamic parameters of the irreversible denaturation of the intermediate form were 102.70 and 97.10 kJ mol^{-1} for $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ respectively, and 16.85 J mol^{-1} K^{-1} for $\Delta S^{\ddagger}$, at 60 °C.

In the case of the study described above, the degree of purity of the enzyme (i.e. the presence or absence of isoenzymes) is an important factor in obtaining reliable kinetic data. Effectively, a biphasic rate of inactivation most likely means the enzyme is not pure.

PPO is an enzyme relatively difficult to isolate and purify (Mayer and Harel, 1979; Vamos-Vigyazo, 1981). With the palmito PPO, two major peaks of activity were obtained after the DEAE-cellulose step. These isoenzymes are eluted, respectively, at 0.21 and 0.27 M KCl. The greatest specific activity is obtained for the first peak, so that this isoenzyme has been used as the enzyme source in this study. A summary of protein and activity data for the purification stages is given in Table 2. Activities were measured immediatly after each step. At the end of the chromatography on the DEAE-cellulose column, 12-fold purification was achieved. This purification factor is equivalent to the one obtained from Brazilian palmito (*E. edulis*) or other plants from which PPO has been purified (Zhou and Feng, 1991; Lourenço et al., 1990). Whereas a purification yield of 0.2% has been reported for pears (Zhou and Feng, 1991), the yield of 8.7% for PPO of *A. rubra* is comparable to that obtained for banana buds, 7.9%, or sweetpotatoes, 6.4% (Lourenço et al., 1992; Oba et al., 1992).

The fact that this isoform gives only one peak after a hydrophobic chromatography and the fact that the enzyme eluted from hydrophobic chromatography gives one peak after molecular sieving (Robert et al., 1995a) strongly suggest that only one form is present. However, it cannot be totally excluded that some isoforms are present (previous researchers reported that, under certain conditions, an association–dissociation phenomenon of PPO could take place) (Bouchilloux et al., 1963; Jolley et al., 1969); in this case we are led to suppose that these hypothetic isoforms (due to association–dissociation phenomena) have a similar behavior with regard to heat inactivation to explain the results obtained with the statistical treatment of the data.

pH Stability. It is necessary to perform a pH stability study prior to the study of pH effect on the enzyme activity. The stability is tested by preincubating the enzyme, at the indicated pH, for a period equivalent to the usual assay time.

Results exhibited in Figure 5 show that the activity is stable at pH 3 and 8—the extreme pH in our experiment. It has been checked that when the enzymatic activity is monitored at the optimum pH, the enzyme recovers its maximum activity. Thus, the bell-shaped curve observed in Figures 6A and 7A is not due to a denaturation but rather to a change in protonation of the essential groups on the active site.

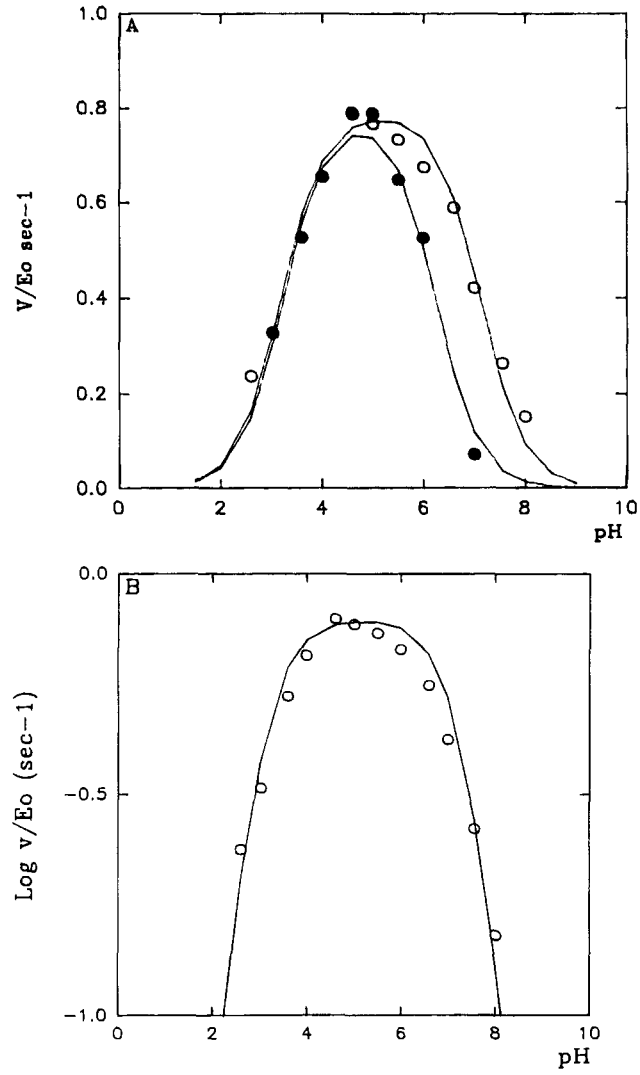

Figure 6. pH dependence with 4-methylcatechol (4-MC) as substrate. (A) (○) Substrate concentration is saturating at each pH value: pH 2.5, [4-MC] = 34.5 mM; pH 3–4.5, [4-MC] = 17.25 mM; pH 5–5.5, [4-MC] = 10.35 mM; pH 6–7, [4-MC] = 3.45 mM; pH 7.5–8, [4-MC] = 1.55 mM. (●) Substrate concentration was 17.25 mM at each pH. (B) Dixon–Webb plot with 4-methylcatechol as substrate.

The enzyme is stable in the range of the ionic strength used in the experiment (data not shown).

pH Dependence. pH dependence experiments were carried out with 4-methylcatechol or pyrogallol as substrate. The influence of pH on the activity of polyphenol oxidase with 4-methylcatechol and pyrogallol is shown in Figure 6A or 7A, respectively. At each pH, the concentration of substrate was saturating and corresponded to the maximum rate (observed before inhibition). The results show that the pH profile was independent of the nature of the substrates tested. We can note a slight pH shifting, when substrate is not saturating, toward acidic pH. The pH optimum was 5.2. A rapid loss of activity is observed below and above this optimum. For polyphenol oxidase extracted from *E. edulis*, Lourenço et al. (1990) reported an optimum pH of 5.5 when the enzyme was assayed using 4-methylcatechol as substrate. The difference can be explained by the use of only one constant substrate concentration (3 mM), by these authors, whatever the pH. Indeed, we have shown (Robert et al., 1995b) that the concentration of saturating substrate depends on pH and ranges from 1 to 34 mM from pH 8 to 2. An identical

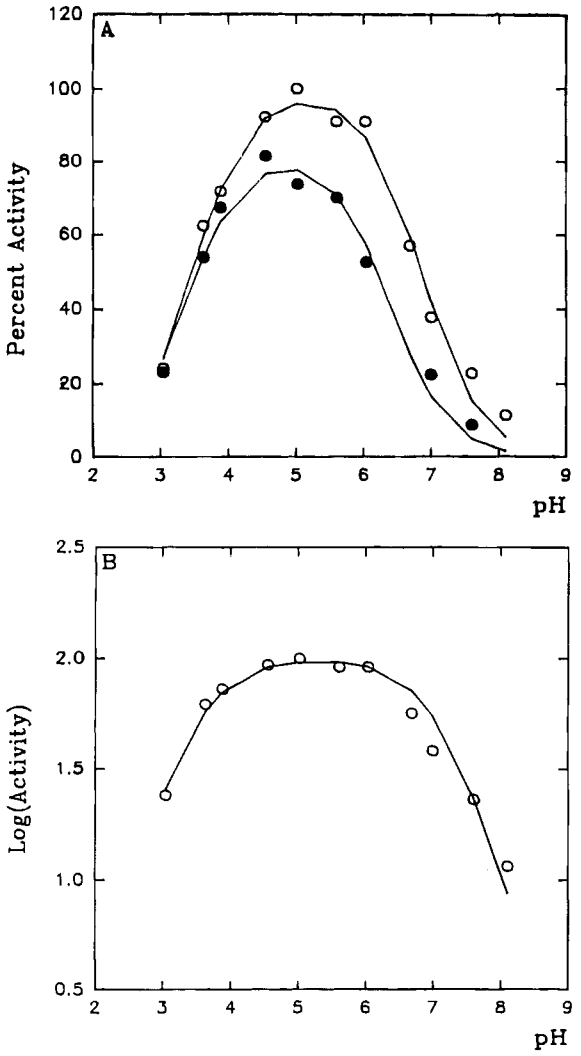

Figure 7. pH dependence with pyrogallol as substrate. (A) (○) Substrate concentration is saturating at each pH values: pH 3–6, [pyrogallol] = 30 mM; pH 6.7, [pyrogallol] = 20 mM; pH 7, [pyrogallol] = 10 mM; pH 7.5, [pyrogallol] = 5 mM. (●) Substrate concentration was 20 mM at each pH. (B) Dixon–Webb plot with pyrogallol as substrate.

behavior is probable for polyphenol oxidase extract from *E. edulis*. The constant concentration used by Lourenço et al. (1990) (3 mM) is a saturating one only at alkaline pH. The optimum pH reported here for 4-methylcatechol and pyrogallol is within the values reported for polyphenol oxidases from other sources, pH 4.5–7 (Augustin et al., 1985; Siddig et al., 1992; Palmer, 1963; McCord and Kilara, 1983). The pH optimum varies with the source of enzyme and also depends on the phenolic substrate chosen for the assay (Mayer and Harel, 1979), but the pH optimum can also be affected by the type of buffer and the purity of the enzyme (Augustin et al., 1985).

The results shown in Table 3 indicate that differences between the observed pK values (defined as pH corresponding to apparent $V/2$) were at least 3.5 in any case. Therefore, we have calculated the real pK values according to the method of Segel (1975), using the Dixon–Webb plot of $\log(V_{\text{max app}})$ vs pH (Figures 6B and 7B). The values obtained by fitting the experimental data to the equation

$$\log V_{\text{max app}} = \log V_{\text{m}} - \log\left(1 + \frac{[\text{H}^+]}{K_{\text{es1}}} + \frac{K_{\text{es2}}}{[\text{H}^+]}\right) \quad (3)$$

Table 3. pK and pH Optimum Values of *A. rubra* Polyphenol Oxidase with Both Substrates[a]

parameter	enzymes with 4-methylcatechol	enzymes with pyrogallol
pK_{1obs}	3.18	3.41
pK_{2obs}	7.12	6.91
pH_{opt}	5.15	5.16
pK_{es1}	3.06	3.44
pK_{es2}	7.29	7.12
$V_{\text{max theor}}$	0.79	100
$V_{\text{max obs}}$	0.79	100

[a] The observed pK (pK_{obs}), the observed pH (pH_{opt}), and the optimum apparent rate values ($V_{\text{max app}}$) were determined from curves of $V_{\text{max app}}$ (Figures 6A and 7A). pK_{es1} and pK_{es2} refer to the true pK of the enzyme–substrate complex, and $V_{\text{max theor}}$ refers to the rate of the reaction if all of the enzyme is in the active form (En). The values of pK and $V_{\text{max theor}}$ were calculated according to the method of Segel (1975).

are listed in the same table. Data shown indicate that in the free enzyme two ionizing groups, with pK values of 3.18 and 7.12 and 3.41 and 6.91 for, respectively, 4-methylcatechol and pyrogallol, are involved in enzyme catalysis. The plot of $\log(V_{\text{max app}})$ vs pH indicates that the oxidation of the substrates depended on the ionization of two groups in the enzyme–substrate complex with apparent pK values of 3.06 and 7.29 and 3.44 and 7.12, respectively, for 4-methylcatechol and pyrogallol.

The very slight differences between the pK values for the free enzyme or the enzyme–substrate complex, relative to 4-methylcatechol and pyrogallol, suggest the existence of only one site on the polyphenol oxidase molecule for both substrates. Further proof is given by the inspection of the pH patterns. An inhibition by an excess of substrate is found with either substrate. The observed V_{max} is equal to the theorical V_{max} with either substrate. If there were two sites, there would be no reason for symmetrical behaviors. Contrary to other enzymes (Cadet et al., 1987; Cadet and Meunier, 1988), the apparent V_{max} is equivalent to the theorical V_{max}.

It is well-known that it is very difficult to assign an experimental pK value to the reactive group of an amino acid. For example, the value of the pK of the imidazole group of histidine ranges from 5.5 to 7. One of the pK values refers probably to this group, which has been described elsewhere as being at the active center of polyphenol oxidase of *Neurospora* and *Agaricus* (Gutteridge et al., 1977). The acidic pK might refer to an acidic amino acid (aspartic or glutamic acid). These hypotheses have to be confirmed by specific chemical modifications of the active center.

LITERATURE CITED

Augustin, M. A.; Ghazali, H. M.; Hashim, H. Polyphenol oxidase from guava (*Psidium guajava* L.). *J. Sci. Food Agric.* **1985**, *36*, 1259–1265.

Bernhardt, L. W.; de Lima, D. C.; Yang, J. F.; Soares, G. J. D. Volume and composition of gases occluded in heart of palm. *Colet. Instit. Tecnol. Aliment.* **1978**, *9*, 43–62.

Bouchilloux, S.; McMahill, P.; Masson, H. S. The multiple forms of mushroom tyrosinase. Purification and molecular properties of the enzymes. *J. Biol. Chem.* **1963**, *238*, 1699.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Cadet, F.; Meunier, J. C. pH and kinetic studies of chloroplast sedoheptulose-1,7-bisphosphatase from spinach (*Spinacia oleracea*). *Biochem. J.* **1988**, *253*, 249–254.

Cadet, F.; Meunier, J. C.; Ferté, N. Effect of pH and fructose-2,6-biphosphate on oxidised and reduced spinach chloro-

plastic fructose-1,6-biphosphatase. *Eur. J. Biochem.* **1987**, *162*, 393–398.
Demeau, M.; Bidan, P. Study of the inactivation of grape juice polyphenol oxidase by heat. *Ann. Technol. Agric.* **1967**, *16* (2), 75–79.
Dimick, K. P.; Ponting, J. D.; Makower. Heat inactivation of polyphenol oxidase in fruit puree. *Food Technol.* **1951**, *5*, 237–241.
Gutteridge, S.; Dickson, G.; Robb, D. Photochemical oxidation of tyrosinase. *Phytochemistry* **1977**, *16*, 517–519.
Jolley, R. L., Jr.; Robb, D. A.; Masson, H. S. The multiple forms of mushroom tyrosinase: association-dissociation phenomena. *J. Biol. Chem.* **1969**, *244*, 1593.
Lamikanra, O.; Kirby, S. D.; Musingo, M. N. Muscadine grape polyphenol oxidase: partial purification by high pressure liquid chromatography and some properties. *J. Food Sci.* **1992**, *3* (57), 686–695.
Lourenço, E. J.; de Souza Leao, J.; Neves, V. A. Heat inactivation and kinetics of polyphenoloxidase from palmito (*Euterpe edulis*). *J. Sci. Food Agric.* **1990**, *52*, 249–259.
Lourenço, J.; Neves, V. A.; Da Silva, M. Polyphenol oxidase from sweet potato and properties. *J. Agric. Food Chem.* **1992**, *40*, 2369–2373.
Mayer, A. M.; Harel, E. Review Polyphenol oxidase in plants. *Phytochemistry* **1979**, *18*, 193–215.
McCord, J. D.; Kilara, A. Control of enzymatic browning in processed mushrooms (*Agaricus bisporus*). *J. Food Sci.* **1983**, *48*, 1479–1483.
Mihalyi, K.; Vamos-Vigyazo, L.; Kiss-Kutz, N.; Babos-Sz ebenyi, E. The activities of polyphenol oxidase and peroxidase in fruits and vegetables as related to pH and temperature. *Acta Aliment.* **1978**, *7* (1), 57–67.
Montogomery, M. W.; Petropakis, H. J. Inactivation of Bartlett pear polyphenol oxidase with heat in the presence of ascorbic acid. *J. Food. Sci.* **1980**, *45*, 1090–1091.
Oba, K.; Iwatsuki, N.; Uitani, I. M.; Alvarez, A. V.; Garcia V. Partial purification and characterisation of polyphenol oxidase isoenzymes in banana bud. *Biosci., Biotechnol. Biochem.* **1992**, *56* (7), 1027–1030.
Palmer, J. K. Banana polyphenol oxidase. Preparation and properties. *Plant Physiol.* **1963**, *35*, 508–519.
Park, E. Y.; Luh, B. S. Polyphenol oxidase of kiwifruit. *J. Food Sci.* **1985**, *50*, 678–684.
Robert, C.; Richard-Forget, F.; Rouch, C.; Cadet, F.; Pabion, M. Partial purification and physicochemical characterisation of palmito (Acanthophenix rubra) polyphenol oxidases. *Plant Physiol. Biochem.* **1995a**, submitted for publication.
Robert, C.; Cadet, F.; Rouch, C.; Pabion, M. Information on the active site of polyphenol oxidase. *C. R. Acad. Sci. (Biochem.)* **1995b**, in press.
Segel, I. H. Effects of pH and temperature. In *Enzyme Kinetics*; Wiley: New York, 1975; Chapter 11.
Siddig, M.; Sinha, N. K.; Cash, J. N. Characterization of polyphenol oxidase from stanley plums. *J. Food Sci.* **1992**, *5* (57), 1177–1179.
Silva, E.; Nogueria, J. N. Effect of heat treatments on polyphenol oxidase and peroxidase activities of some fruits and vegetables. *An. ESA Luis de QUEIROZ* **1983**, *40*, 137–161.
Tanford, C. Protein denaturation. *Adv. Protein Chem.* **1968**, *33*, 121–182.
Vamos-Vigyazo, L. Polyphenol oxidase and peroxidase in fruit and vegetables. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15*, 49–127.
Zhou, H. W.; Feng, X. Polyphenol oxidase from Yali pear (*Pyrus bretschneideri*). *J. Sci. Food Agric.* **1991**, *57*, 307–313.

Received for review August 26, 1994. Revised manuscript received March 1, 1995. Accepted March 13, 1995.[⊗]

JF940490C

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1995.