JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Kinetics and Thermodynamics of the Thermal Inactivation of Polyphenol Oxidase in an Aqueous Extract from *Agaricus bisporus*

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ABSTRACT: The kinetics and thermodynamics of the thermal inactivation of polyphenol oxidase (PPO) in an aqueous extract from mushroom *Agaricus bisporus* (J.E. Lange) Imbach was studied, using pyrocatechol as a substrate. Optimal conditions for enzymatic studies were determined to be pH 7.0 and 35−40 °C. The kinetics of PPO-catalyzed oxidation of pyrocatechol followed the Haldane model with an optimum substrate concentration of 20 mM. Thermal inactivation of PPO was examined in more detail between 50 and 73 °C and in relation to exposure time. Obtained monophasic kinetics were adequately described by a first-order model, with significant inactivation occurring with increasing temperature (less than 10% preserved activity after 6 min at 65 °C). Arrhenius plot determination and calculated thermodynamic parameters suggest that the PPO in aqueous extract from *Agaricus bisporus* mushroom is a structurally robust yet temperature-sensitive biocatalyst whose inactivation process is mainly entropy-driven.

KEYWORDS: *Agaricus bisporus, polyphenol oxidase, thermal inactivation, kinetics, thermodynamics*

INTRODUCTION

Agaricus bisporus (J. E. Lange) Imbach, also called white button mushroom, has registered tremendous increase in interest over the recent years, due to its delicacy, flavor, nutritional value, and possible source of bioactive species.[1−3] In particular, several studies have been devoted to the effect of drying procedures, irradiation process, and storage conditions on the quality of the product.[4−6] In fact, the shelf life of the commercial *Agaricus bisporus* is limited to a few days because of enzymatic browning during postharvest storage or processing.[7] These browning reactions have been linked to mechanical damage during handling and processing, abrasions, washing, senescence, and bacterial infestations. In white button mushroom (*Agaricus bisporus*), polyphenol oxidase (PPO, monophenol, dihydroxy-L-phenylalanine/oxygen oxidoreductase, EC 1.14.18.1) is considered as the primary enzyme responsible for browning.[8] PPO is a widely distributed copper-containing protein that catalyzes two different reactions, both of which involve molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity).[9] The *o*-quinones undergo polymerization reactions leading to the formation of black, brown, or red pigments. Apart from this color change, enzymatic browning results in the development of off-flavors and a reduction of nutritional and market value. Hence, the possibility to inhibit enzymatic browning is a great challenge in the food industry.[10]

It is currently accepted that inactivation of PPO by heat treatment is the most effective method to control enzymatic browning but is limited by alterations in sensory characteristics and the loss of nutrients so that optimization of time and temperature must be achieved.[11] In fact, PPO enzyme does not belong to an "extremely heat-stable enzyme" group, and short exposures of product to temperatures between 70 and 90 °C are sufficient to inactivate the enzyme.[8] Although there are many studies published on heat inactivation of PPOs,[12−17] this information is scarce for *Agaricus bisporus*.[18] In addition to its interest for food preservation, these data are of primary importance for other applications in biocatalysis and biosensing where enzymes from white mushroom have attracted increased attention due to their easy access, low price, and good efficiency.[19]

The objective of this work was therefore to achieve a full characterization of the thermal behavior of PPO extracted from mushroom *Agaricus bisporus*, not only in terms of kinetics but also for the first time to our knowledge via the determination of the thermodynamic parameters (free energy (ΔH), enthalpy (ΔG), and entropy (ΔS)) of heat inactivation. These data allow not only the comparison of this specific PPO with similar enzymes from other sources but also contribute to the clarification of the mechanism behind the thermodenaturation process, with significant implications for browning inhibition and enzyme-based biotechnological devices.

MATERIALS AND METHODS

Material. PPO was obtained from the common commercially cultivated mushroom (*Agaricus bisporus*) bought fresh on the free market. Mushrooms were chosen to be white, fresh, and as young as possible. The carpophores were cleaned and stored at 4 °C until they were used as the source for enzyme extraction.

Reagents. Pyrocatechol was obtained from Fluka. Stock solution of pyrocatechol was prepared in 0.5 mM phosphoric acid to prevent autoxidation. All other chemicals and reagents were of analytical grade. Distilled water was used in all experiments.

Preparation of Crude PPO Extract. The extraction process was adapted from a previously reported procedure.[20] The mushroom

Received: October 10, 2011
Revised: December 5, 2011
Accepted: December 10, 2011
Published: December 10, 2011

Agaricus bisporus (J. E. Lange) Imbach, of commercial maturity, was washed abundantly with distilled water, freshly harvested, and kept for 2 days in the deep-freezer (−20 °C) before extracting PPO. To prepare the crude extract, 260 g of mushroom were cut quickly into thin slices and homogenized in a Waring blender containing 430 mL of prechilled acetone (−15 °C) for 2 min. The pulp was filtered through cheesecloth and was manually pressed until obtaining a dry residue (acetone powder). The acetone powder, which weighed 19.65 g, was frozen in contact with dry ice for at least 3 h. The frozen acetone powder was then broken up, suspended in 280 mL of distilled water, and allowed to stand overnight in the refrigerator at about 5 °C. The suspension was filtered through five layers cheesecloth to remove solid particles. The separated filtrate (148 mL) was subsequently centrifuged at 4000*g* for 15 min. The resulting supernatant constituted the crude PPO extract for this study and was kept frozen at −15 °C until use. Because of the remaining presence of phenolic substances originating from the acetone precipitation process, the recovered powder was slightly brown, but its color remains stable over several weeks. The darkening process was faster (within a few days) in the suspension, so care was taken to prepare fresh solutions at regular intervals of time.

PPO Activity Assay. PPO activity was determined by measuring the initial rate of *o*-benzoquinone formation, as indicated by an increase in absorbance at 410 nm using pyrocatechol as substrate. A UV−visible spectrophotometer (Thermo Scientific Heλlios γ, England) was employed throughout the investigation. The sample cuvette contained 2 mL of 10 mM pyrocatechol solution in 0.05 M sodium phosphate buffer at pH 7.0 and 10 μL of undiluted crude PPO extract. The blank sample contained only 2 mL of substrate solution. PPO activity was followed by recording the absorbance at 10 s intervals for a period of 1 min.

The substrate and enzyme fractions were mixed after a preincubation period of 5 min at 30 °C. Unless otherwise indicated, PPO was the last component added to the reaction mixture. The linear portion of the absorbance vs time curve was used to determine the initial rates (v).[20] It was checked that autoxidation of pyrocatechol did not interfere with the activity measurement. All activity analyses performed in this work were carried out in triplicate, and the averages of data were considered. The error bars represent standard deviation (SD).

Effect of pH on Enzyme Activity. The PPO activity profile was analyzed over a range of pH 3.75−8.15 using 0.1 M citric acid−0.2 M disodium phosphate buffer. Pyrocatechol was used as substrate at 10 mM. PPO activity was measured according to the method described above and expressed as a percentage of the maximum activity. The pH value corresponding to the highest enzyme activity was taken as the optimal pH and used in all other studies. Because the rapid nonenzymatic browning of substrates may occur at higher pH values by rapid autoxidation, the upper limit of pH range was set up at pH 8.15.

Enzymatic Kinetics. In order to determine the Michaelis−Menten constant (K_m), substrate inhibition constant (K_s), and maximum velocity (V_{max}), PPO activities were measured using pyrocatechol as substrate at concentrations ranging from 0.2 to 50 mM. The kinetic parameter, K_m, K_s, and V_{max}, values of the enzyme were estimated both by linear and nonlinear regression analysis using the Haldane (eq 1) and Lineweaver−Burk (eq 2) equations. The kinetics expressions were as follows:

$$v = \frac{V_{\max}\cdot S}{K_m + S + \frac{S^2}{K_S}} \tag{1}$$

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{1}{S}\cdot\left(\frac{K_m}{V_{\max}} + \frac{S^2}{K_S\cdot V_{\max}}\right) \tag{2}$$

Thermal Stability. The thermal stability was determined by heating 2 mL of enzyme solution in a test tube in a thermostatted water-bath at various temperatures ranging from 30 to 70 °C (in 5 °C increments, except for the upper value due to equipment limitation) for 30 min periods. The enzyme solution was rapidly cooled in ice, and the remaining activity was essayed in the above conditions. Residual PPO activity was expressed as relative to the maximal activity. The optimal temperature obtained was used in all subsequent experiments.

Heat Inactivation Kinetics of Mushroom PPO. The thermal inactivation of mushroom crude PPO was studied at constant temperature between 45 and 73 °C and at atmospheric pressure. For the study, 2 mL of crude enzyme extract was poured into a preheated test tube immersed in a water bath at the required temperature for fixed time intervals (up to 30 min). At predetermined time intervals, 100 μL aliquots were pipetted into Eppendorf tubes that were immediately immersed in an ice bath to stop thermal inactivation instantaneously.

After the sample had been cooled in the ice bath and brought to room temperature, 10 μL of the heated enzyme solution was mixed with 2 mL of 10 mM pyrocatechol and the residual activity (A_t) was determined spectrophotometrically. A nonheated enzyme sample was used as blank (A_o). The percentage residual activity was calculated using the following equation (eq 3):

$$\text{residual activity (\%)} = \left(\frac{A_t}{A_0}\right)\cdot 100 \tag{3}$$

where A_o is the initial enzyme activity and A_t is the residual enzyme activity at time t.

Kinetic data analysis of thermal inactivation of enzymes can often be described by the first-order reaction (eq 4):[21]

$$\frac{dA_t}{dt} = -k\cdot A_t \tag{4}$$

where A_t is the enzymic activity at treatment time t, and k is the reaction rate constant (min^{-1}) at the temperature studied.

For constant extrinsic/intrinsic factors, in the case of a first-order reaction, the kinetics can be described by the following equation (eq 5):

$$\ln\left(\frac{A_t}{A_0}\right) = -k\cdot t \tag{5}$$

where A_0 is the initial activity, and A_t is the remaining activity at time t, and k is the inactivation rate constant at the temperature studied. The inactivation rate constant k can be estimated by linear regression analysis of the natural logarithm of residual activity versus treatment time.

The half-life ($t_{1/2}$) value of inactivation is given by the expression (eq 6):

$$t_{1/2} = \frac{\ln(2)}{k} \tag{6}$$

The D-value is the time (min) needed to reduce the initial activity 90%. It was related to k-values by eq 7 and mathematically expressed by[22]

$$D = \frac{\ln(10)}{k} \tag{7}$$

The Z_T-value is the temperature needed to reduce the D-value one log-unit (temperature sensitivity parameter), and it is obtained by plotting the D-values on a log scale against the corresponding temperatures.[23]

Arrhenius' law is usually utilized to describe the temperature dependence of k-values, and it is algebraically given by (eq 8):

$$\ln(k) = \ln(k_0) - \frac{E_a}{R\cdot T} \tag{8}$$

where k_0 is the Arrhenius constant, E_a the activation energy (kJ/mol), R the universal gas constant (8.314 J/mol·K), and T the absolute temperature (K). The activation energy can be estimated by the slope of linear regression analysis of the natural logarithm of rate constant versus the reciprocal of the absolute temperature.

The values of the activation energy (E_a) and Arrhenius constant (k_0) allowed for the determination of different thermodynamic

parameters such as variations in enthalpy, entropy, and Gibbs free energy, ΔH, ΔS, and ΔG, respectively, according to the following expressions:[24]

$$\Delta G = -R \cdot T \cdot \ln\left(\frac{k \cdot h_p}{K_B \cdot T}\right) \quad (9)$$

$$\Delta H = E_a - R \cdot T \quad (10)$$

$$\Delta S = \left(\frac{\Delta H - \Delta G}{T}\right) \quad (11)$$

where K_B is Boltzmann's constant (1.3806×10^{-23} J/K), h_P is Planck's constant (6.6262×10^{-34} J.s), and k is the inactivation rate constant of each temperature (s^{-1}).

Data Analysis. All data analyses were performed using linear and nonlinear regression fitting by application of the programs Table Curve 2D v2.03 (Copyright 1989−1994. AISN Software) and SigmaPlot, version 12.0 (Copyright 2011 Systat Software, Inc.) for Windows.

RESULTS AND DISCUSSION

Determination of Optimum Experimental Conditions. Setting up adequate conditions to investigate the thermal behavior of enzymes from foodstuff faces many challenges. Noticeable variations can be observed depending on the extraction/purification procedure, the selection of the substrate as well as the buffer conditions.[18,25] High degree of purity must be achieved for structural studies[26] but may be less relevant due to the absence of other molecules that may impact its behavior within the vegetal tissue. Moreover, if the enzymatic activity is the key parameter, for instance in biosensing,[19] then purification may decrease availability and increase cost. Preferred substrates should indeed be selected among natural ones.[13] However, alternative molecules may have been preferred for analytical reasons (absorption band in the visible region, better stability toward oxidation under air, and commercial availability) and constitute reference substrates for comparison with the earlier literature data. For these reasons, a crude extract of PPO from *Agaricus bisporus* was selected in the present work and pyrocatechol chosen as a substrate, as a widely used member of the catechol family (such as chlorogenic acid, L-epicatechin, or dopamine) that represent the major natural substrates of PPO in fruits.[9] Noticeably, it was previously shown that the aqueous extract studied here exhibited both creolase and catecholase enzymatic activities, demonstrating that it behaves as a true tyrosinase, but higher oxidation rates were found using pyrocatechol as a substrate, whereas lower activities were measured for L-tyrosine, phenol, resorcinol, phloroglucinol, and pyrogallol.[20]

In a first step, it was important to determine the optimum conditions for studying PPO extract activity, in terms of pH, temperature, and substrate concentration. The activity of PPO was measured at different pH levels by using pyrocatechol (20 mM) as a substrate. It was found that PPO activity increased drastically from pH 5.8, reaching maximum activity at pH 7.0, and then again decreased at pH 7.7 (Figure 1). At pH 8.15, the activity dropped down by only 10%, and below pH 5.8, the enzyme showed less than 25% activity. In general, PPO from plants, vegetables, and fruits show maximum activity at or near neutral pH values,[8] although more acidic (3.4 for vanilla)[12] or alkaline (8.0 for rosemary)[27] optimal pHs were found using pyrocatechol as a substrate.

The highest stability of PPO from edible mushroom *Agaricus bisporus* was found in the 30−35 °C range, and no remaining activity could be found after incubation of the enzyme at 65 °C for 30 min (Figure 2). At temperatures above 45 °C, the decline in PPO activity was very drastic. At 50 °C, 50% of PPO activity in mushroom was lost after 30 min of heating, whereas at 55 °C more than 80% of the PPO activity was lost over the same delay.

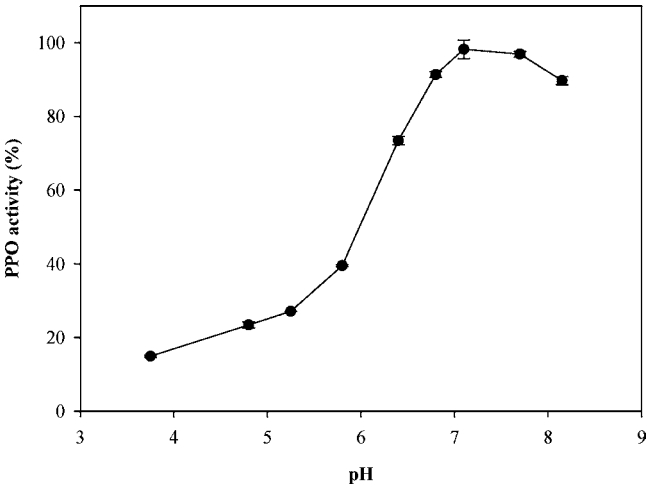

Figure 1. Activity of mushroom PPO crude extract as a function of pH. Assay conditions: pyrocatechol 20 mM in 0.1 M citric acid−0.2 M disodium phosphate buffer (pH 3.75−8.15), 30 °C, 5 μL of enzyme extract. Activity was expressed as relative activity (%) compared with activity determined at pH 7.0. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

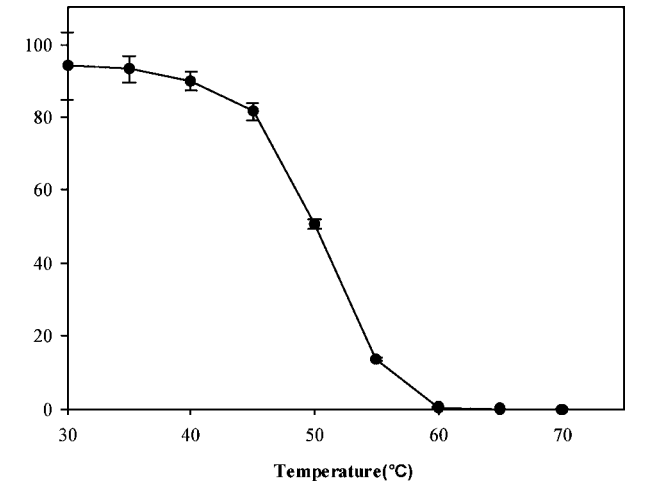

Figure 2. Thermal stability of mushroom PPO crude extract. The enzyme was held at various temperatures (30−70 °C) for 30 min prior to cooling and assay at 30 °C. Assay conditions: pyrocatechol 20 mM in phosphate buffer (0.1 M, pH 7.0) at 30 °C. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

The Michaelis constant (K_m), substrate inhibition constant (K_s), and maximum reaction velocity (V_{max}) were determined using pyrocatechol as a substrate at various concentrations and under optimum conditions (pH 7.0 and 30 °C). The plot of the rate of oxidation reaction of pyrocatechol versus substrate concentration was hyperbolic in the first part of the curve, suggesting Michaelis−Menten kinetics (Figure 3). This point was confirmed by the linearity of the Lineweaver−Burk plot. In a second part, activity decreased for a pyrocatechol

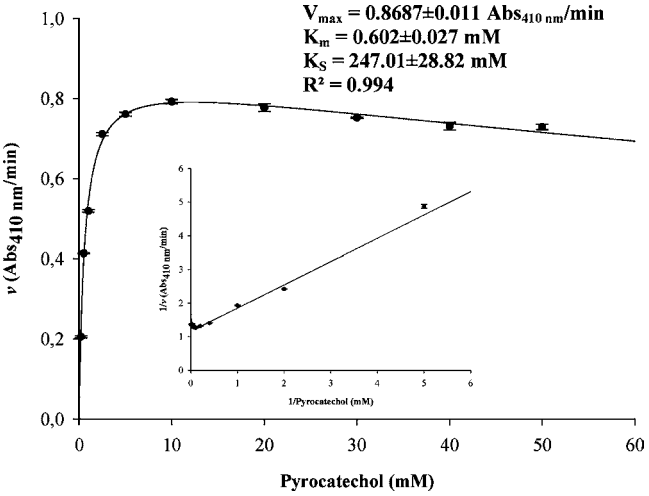

Figure 3. Determination of mushroom PPO V_{max}, K_m, and K_s by Haldane equation. The reaction mixture included phosphate buffer (0.1 M, pH 7.0) with various concentrations of pyrocatechol (0.2−50 mM). The inset curve is a Lineweaver−Burk plot. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

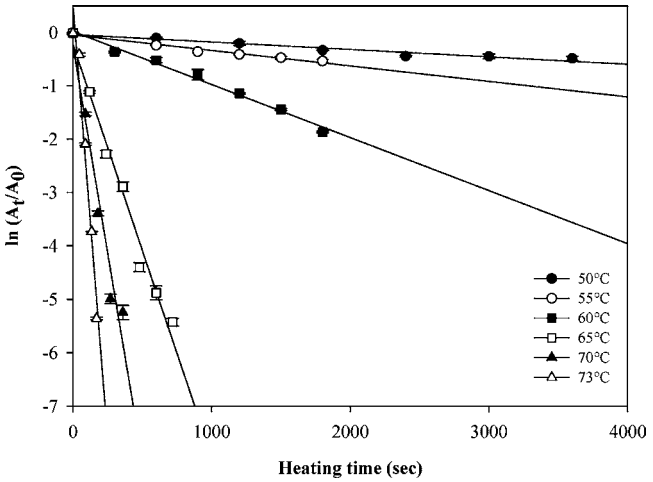

Figure 4. Thermal inactivation curves of mushroom PPO crude extract in the temperature range 50−73 °C. Enzyme aliquots (2 mL) were incubated at the indicated temperatures. The remaining activity was determined with pyrocatechol as substrate at pH 7.0 (phosphate buffer 0.1 M) and 30 °C. A_0 and A_t are initial and residual activities at the time measured, respectively. The rate constants (k) for inactivation were determined from the slopes of the logarithmic plot of activity against time. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

concentration higher than 20 mM, as indicated by the upturn observed in the Lineweaver−Burk plot (Figure 3, insert). Hence, mushroom PPO did not follow pure Michaelis−Menten kinetics. The forms of theses graphs were typical of enzymes exhibiting substrate inhibition. A K_m of 0.60 mM, K_s of 247 mM, and V_{max} of 0.86 $Abs_{410\ nm}$/min for mushroom PPO were obtained. Noticeably, this K_m-value is lower than most other reported values for various fruits,[28,29] indicating that the mushroom PPO has a relatively high affinity for pyrocatechol.

Kinetic Analysis of Thermal Denaturation. On the basis of these results, a detailed kinetic study of isothermal inactivation of mushroom PPO was performed in the range from 50 to 73 °C at pH 7 and under atmospheric pressure with different heating times. Figure 4 presents the isothermal inactivation curves of mushroom PPO in the temperature range from 50 to 73 °C as semilog plots. As expected, the extent of PPO denaturation increased with temperature and treatment time. Heating for 30 min at 45 °C did not cause a significant loss of enzymatic activity (data not shown). However, the heat inactivation of PPO between 65 and 73 °C was dramatically rapid. PPO activities were reduced by approximately 30% and 85% at 50 and 60 °C, respectively, after 30 min of heat treatment. In addition, residuals mushroom PPO activities were only about 5% and 0.5% at 65 and 70 °C, respectively, after only 6 min of heating, whereas a 3 min heating at 73 °C fully inactivated the enzyme. This behavior compares well with reports from the literature,[13,14] although up to 47% preserved enzymatic activity after a 30 min treatment at 70 °C was previously reported for Emir grape PPO.[15] Noticeably, the semilog plots were linear at all temperatures studied, which is consistent with inactivation by means of a simple first-order monophasic kinetics process. Moreover, all the lines extrapolate back to a common point indicating that the inactivation of the sole isoenzyme is being measured.

From the slopes of these lines, the inactivation rate constants (k) were calculated and are given in Table 1. The rate constant increased with the heating temperature, indicating that mushroom *Agaricus bisporus* PPO is less thermostable at higher temperatures (65−73 °C). The half-life ($t_{1/2}$) and the decimal

Table 1. D-, k-, and $t_{1/2}$-Values for the Thermal Inactivation of Edible Mushroom *Agaricus bisporus* Polyphenol Oxidase in the 50−73°C Temperature Range

temperature (°C)	D (min)	k (min^{-1})	$t_{1/2}$ (min)	R^2
50	267 ± 25[a]	0.009 ± 0.001	80 ± 8	0.9322
55	127 ± 17	0.018 ± 0.002	38 ± 5	0.9621
60	39.0 ± 0.7	0.059 ± 0.001	11.8 ± 0.2	0.9856
65	4.8 ± 0.2	0.48 ± 0.02	1.45 ± 0.06	0.9808
70	2.4 ± 0.2	0.95 ± 0.06	0.73 ± 0.04	0.9573
73	1.3 ± 0.1	1.8 ± 0.2	0.38 ± 0.03	0.9606

[a]Mean (n = 3) ± standard deviation.

reduction time (D value) are other important parameters commonly used in the characterization of enzyme stability. Increasing the temperature from 50 to 73 °C resulted in a decrease in $t_{1/2}$ and D values (Table 1). Comparing the D and $t_{1/2}$ values of PPO from other sources at the same temperature (70 °C), mushroom PPO ($t_{1/2}$ = 0.7 min; D = 2.4 min) was less thermostable than PPO from most other similar enzymes extracted from other sources such as Emir grape ($t_{1/2}$ = 15.9 min; D = 52.7 min),[15] vanilla bean ($t_{1/2}$ = 1.3 min; D = 4.2 min),[12] and Anamur banana ($t_{1/2}$ = 27 min; D = 92 min).[14]

The dependence of the inactivation rate constants with temperature was adequately fitted by the Arrhenius equation (R^2 = 0.9770) (Figure 5). This linearity is an indication that the inactivation in mushroom PPO occurs through a unique mechanism dependent on temperature, such as protein unfolding.[12,16] From 50 to 73 °C, the activation energy (E_a) value for thermal inactivation of the mushroom PPO was calculated to be 230 ± 6 kJ/mol. Comparable values in the 220−250 kJ/mol range were observed for PPOs in Emir grapes,[15] and were much higher than those reported for edible yam (68 kJ/mol),[16] for vanilla bean (92 kJ/mol),[12] and for Anamur banana (155 kJ/mol).[14] On this basis, PPO in

dx.doi.org/10.1021/jf204104g | J. Agric.Food Chem. 2012, 60, 500−506

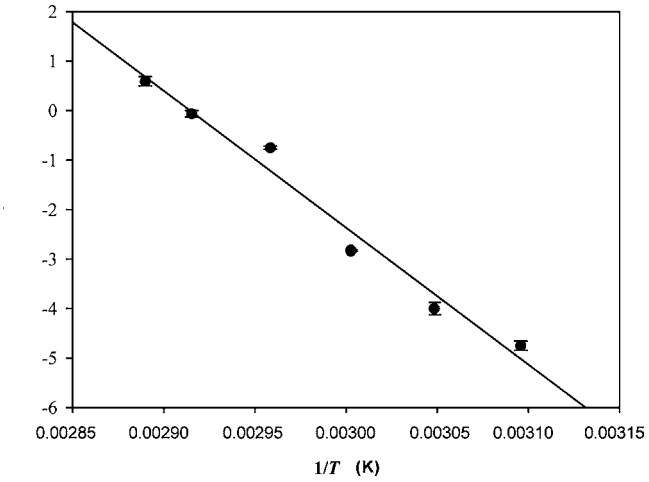

Figure 5. Arrhenius plot showing the effect of temperature on the rate constant for the thermal inactivation of mushroom PPO crude extract. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

mushroom can be classified as heat-sensitive PPO in terms of inactivation kinetics.

Figure 6 presents the relationship between decimal reduction time and temperature, where the slope of the curve represents

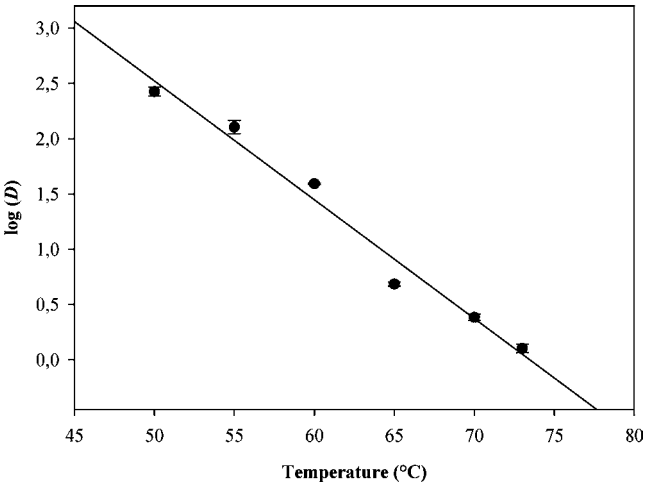

Figure 6. Variation of decimal reduction times with temperature for the mushroom PPO crude extract. Each data point is the mean of three determinations. Vertical bars represent standard deviations.

$-1/Z_T$. The estimated Z_T value of mushroom PPO was 9.3 ± 0.2 °C (R^2 = 0.9778). This result is in good agreement with previous reports indicating that Z_T values of PPO from various fruits were 8.5−10.1 °C.[8] Some investigators reported significantly higher Z_T values, up to ca. 30 °C, for the inactivation of PPO prepared from different foods.[12,14,16] In general, low Z_T values are thought to indicate greater sensitivity to heat.[12] This confirms that the PPO studied here can be considered as a heat-sensitive enzyme in this family.

Thermodynamic Analysis of Thermal Denaturation. The calculation of the thermodynamic parameters of inactivation provides information on the enzyme thermal stability for each step of the heat-induced denaturation process. This could help in detecting any secondary stabilization or destabilization effects that would go unnoticed if only the half-life times were considered.[30] These parameters include ΔG, the Gibbs free energy change considered as the energy barrier for enzyme inactivation, ΔH, the enthalpy change measuring of the number of bonds broken during inactivation, and ΔS, the entropy change that indicates the net enzyme and solvent disorder. They were calculated in the temperature range 50 to 73 °C from experimental data using eqs 9−11 (Table 2).

Table 2. Thermodynamic Parameters for the Thermal Inactivation of Crude Mushroom *Agaricus bisporus* PPO at Different Temperatures

temperature (°C)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol·K)
50	227 ± 6[a]	103.0 ± 0.3	384 ± 17
55	227 ± 6	102.7 ± 0.4	379 ± 16
60	227 ± 6	101.0 ± 0.1	378 ± 17
65	227 ± 6	96.7 ± 0.1	386 ± 16
70	227 ± 6	96.2 ± 0.2	381 ± 16
73	227 ± 6	95.2 ± 0.3	381 ± 17

[a]Mean (±SD) for triplicate experiments.

Results for ΔH show that, within the error range of our measurements, the enthalpy is independent of temperature; thus, there is no change in enzyme heat capacity (Table 2). The ΔH value found in this study (ca. 227 kJ/mol) was much higher than those reported for other PPOs, from *Lepista nuda* mushroom (13 kJ/mol)[17] to vanilla bean PPO (89 kJ/mol).[12] In general, ΔH is seen as a measure of the number of noncovalent bonds broken in forming a transition state for enzyme inactivation. Therefore, the higher the ΔH is, the larger will be the number of noncovalent bonds present in the enzyme molecule, which is going to be more stable. In fact, the stability of a protein is the result of a delicate balance between stabilizing and destabilizing forces, which may be influenced by several factors, e.g., the number of hydrogen and disulfide bridges, the folding degree and hydrophobicity of the molecule, and the amount of ionic and other interactions.[31] Our results would therefore suggest that mushroom *Agaricus bisporus* PPO is more structurally robust than the others enzymes. However, Forsyth et al. raised questions about the suitability of use of isolated ΔH values as indicators of enzyme stability.[32]

In contrast, the ΔG value is directly related to protein stability: the higher the ΔG is, the higher will be the enzyme stability. When the incubation temperature was elevated from 50 to 73 °C, there was a significant reduction of ΔG values for the mushroom PPO from ca. 103 kJ/mol to ca. 95 kJ/mol (Table 2), indicating that destabilization of this protein followed the rise in temperature. All ΔG values were in agreement with the relative constant value of 100 kJ/mol characteristic of the protein denaturation reaction as already found for other PPOs.[12,16]

Since ΔG decreases with increasing temperature whereas ΔH is overall constant, one could expect a significant contribution of entropy changes to the thermodynamics of the considered system. In fact, it was already demonstrated that activation entropy has a dominant role in thermal inactivation of proteins in aqueous solutions.[33] As indicated in Table 2, all ΔS values for thermal inactivation of the PPO from *Agaricus bisporus* are positive and similar (ca. 380 J/mol·K) in the temperature range 50 to 73 °C. This suggests an increase in disorder or randomness of the enzyme/solvent system upon denaturation. The most common cause of the heat inactivation of enzymes is the loss of the native conformation (unfolding of the active

dx.doi.org/10.1021/jf204104g | J. Agric.Food Chem. 2012, 60, 500−506

tertiary protein structure to a disordered polypeptide), a process identified as thermodenaturation, which takes place as a result of increased molecular mobility at elevated temperature.[34] Positive ΔS values also suggest that enzyme unfolding is the rate-determining step for the irreversible thermal inactivation of native PPO. It is worth noting that previous ΔS values for PPO thermodenaturation process were negative, as a result of the commonly lower reported E_a (and therefore ΔH) values.[12,16,17] The possible influence of the extraction process in terms of chemical denaturation and presence of other species associated with PPO in the system studied here cannot be fully put aside, although the activation energy obtained in this work is very similar to those obtained for PPO extracted from *Agaricus bisporus*.[18] In addition, our extraction process was mainly performed at low temperature, limiting the possibility for denaturation to occur.

The aqueous extract of PPO from *Agaricus bisporus* studied here therefore comprises a structurally robust but temperature-sensitive enzymatic system, whose thermal denaturation is mainly under entropic control. In the context of browning inhibition in *Agaricus bisporus*, it suggests that high temperature should be preferred to long heating time to achieve efficient deactivation of PPO. Moreover, it can be expected that the enzyme stability will be noticeably enhanced if thermally induced conformational changes can be restricted, for instance via specific immobilization methods,[35] which are currently under study for the design of PPO-based biocatalyst/biosensors.

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Funding

H.G. thanks the Université Amar Telidji (Algeria) for funding. C.D. is funded by the Mem-S FP7-CP-FP (no. 244967) program.

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