Thermal Inactivation of Mushroom Polyphenoloxidase Employing 2450 MHz Microwave Radiation

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Browning reactions in fruits and vegetables are a serious problem for the food industry. In mushrooms, the principal enzyme responsible for the browning reaction is polyphenoloxidase (PPO). A microwave applicator has been designed and used for studying mushroom PPO inactivation. The effects of microwaves and conventional heating on the kinetics of the monophenolase and diphenolase activities of PPO were studied. Conventional and microwave treatments produce different enzyme intermediates with different stability and kinetic properties. We describe how considerable time can be saved during microwave inactivation of the enzyme compared with the time needed when conventional hot-water treatment is used, resulting in greater profitability and enhanced quality. The short exposure time required for samples irradiated with microwaves is very important for maintaining the quality of mushrooms. The fast microwave treatment used resulted in an increase in antioxidant content and a considerable decrease in browning.

Keywords: *Mushroom; polyphenoloxidase; tyrosinase; diphenols; monophenols; thermal inactivation; blanching; microwave heating*

INTRODUCTION

Browning reactions in fruits and vegetables are recognized as a serious problem in the food industry. In mushroom, the principal enzyme responsible for the browning reactions is polyphenoloxidase (Zhang and Flurkey, 1997). Polyphenoloxidase (PPO, monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a widely distributed copper-containing protein that catalyses two different reactions, both of which use molecular oxygen: hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) (Mason, 1955; Makino and Mason, 1973). The quinones thus formed lead by polymerization to the formation of brown pigments, which generally decrease the quality of processed food (Prota, 1988).

Fresh mushrooms are extremely perishable and can be preserved only if properly processed. They contain water, mineral salts, vitamins, typical phenol compounds, and various enzymes, including PPO. Current conventional techniques to avoid browning include autoclave and blanching methods, whereby the food products are immersed in a liquid at 80−90 °C for 10−12 min or passed through a forced steam flow. These conventional processes are inherently linked to important weight and nutritional quality losses in the product (Konanayakam and Sastry, 1988), pointing to the need for alternative industrial blanching techniques. One of the alternatives that has been proposed is microwave energy. Few published works exist regarding food blanching using microwave heating techniques, and most of them are solely dedicated to final product quality analyses for diverse power regimes and process times in domestic microwave ovens (Ponne et al., 1994). The most restrictive factor for the application of microwave heating techniques to industrial blanching processes is the temperature gradients generated within the samples during microwave heating (Decareau, 1985). The main effect of these temperature gradients is the overheating of some areas, while in colder areas the enzyme may not be completely inactivated. To overcome this restriction, combined conventional−microwave heating techniques are envisaged since surface enzymes may not be completely inactivated by microwave treatment alone unless internal thermal stress is caused. The application of a combined microwave hot-water treatment for industrial mushroom blanching has shown some improvement on final quality, weight loss, and processing times (Devece et al., 1997). Nevertheless, the rigorous control of microwave heating effects on enzymes requires a homogeneous heating of the sample and a strict temperature control. A microwave−hot air combination has also been used for drying mushrooms (Riva et al., 1991). The consequent reduction in processing time led to improved water diffusivity of the dried product and better rehydration and flavor retention.

The aim of this study was to elucidate the heating inactivation kinetics of mushroom PPO using 2450 MHz microwave radiation. The different phenomena that occur while employing microwave heating during mushroom blanching are presented, and promising results

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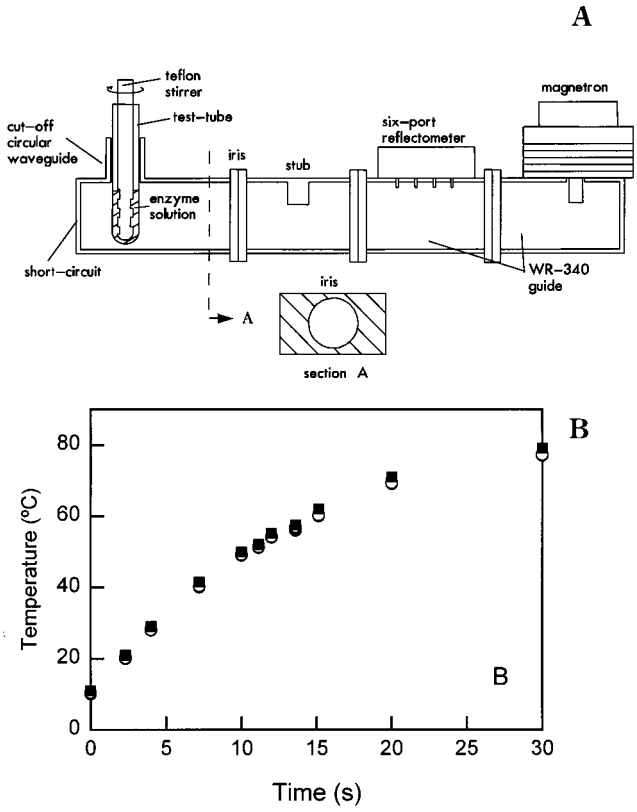


Figure 1. (A) Schematic representation of a monomode microwave applicator designed for liquid food blanching evaluation. (B) Temperature variation within enzyme solution for the applicator depicted in part A: ●, ○, and ■ represent the temperature value measured with three temperature probes placed at different points within the enzyme solution. The standard deviation for each point was ±0.3.

are obtained in terms of the residual and antioxidant activity of treated mushroom solutions.

MATERIALS AND METHODS

Reagents. Edible mushrooms (*Agaricus bisporus*) were kindly supplied by COVEMUR, S.A. (Archena, Murcia, Spain) and lyophilized on reception. Six grams of lyophilized mushroom was ground with 150 mL of extraction buffer comprising 30 mM phosphate buffer (pH 7.0) with a blender operated at maximum speed. The homogenate was centrifuged at 100000*g* for 15 min. The supernatant was filtered, collected, and used as source of PPO. The purification steps were kept to minimum in order to maintain PPO with its natural substrates and to facilitate the study of browning after different thermal treatments.

Mushroom PPO was purchased from Sigma Chemical Co. (Madrid, Spain) and purified by the procedure of Duckworth and Coleman (1970). PPO substrates and MBTH were purchased from Sigma Chemical Co. Reducing substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. All other chemicals were of analytical grade and supplied by Merck (Germany).

Apparatus. The microwave applicator consisted of a short-circuited WR-340 waveguide as shown in Figure 1A. A 16 mm diameter Teflon test tube was introduced into the applicator through a cutoff circular waveguide placed on the wide side of the WR-340 waveguide and at $\lambda_g/4$ from the short circuit, where the electric field is maximum. A 10 mm diameter cylindrical Teflon stirrer was used to evenly distribute the temperature during heating. The applicator with 5 mL of enzyme solution was matched by means of a circular iris and a stub. The reflection coefficient was lower than −10 dB (VSWR = 1.93) at 2.45 GHz. The magnetron used was a NL 10250-2 from National Electronics. The power supply was adjusted to generate 113 W. A six-port reflectometer was placed between the applicator and the magnetron in order to measure the incident and reflected power. The temperature during heating was measured by means of a Luxtron 790 fluoroptic thermometer with a precision of ±0.1 °C. Three temperature probes placed at different points within the enzyme solution monitored temperature homogeneity during heating (Figure 1B).

Thermal Treatments. Microwave exposure was carried out in the system described above. A 5 mL sample of mushroom extract was placed in a Teflon tube and exposed to 2.45 GHz microwave radiation for different times. The power absorbed by the sample during heating was 113 W (22.6 W/cm^3). Once treated, the samples were quickly frozen in liquid nitrogen to avoid time-dependent inactivation of the enzyme. Conventional thermal treatment was carried out using a Tectron 3473100 circulating bath. A 10 mL sample of extract was introduced in a bath at 80 °C and maintained under continuous stirring to homogenize the extract temperature. Aliquots at different times/temperatures were collected and frozen immediately in liquid nitrogen. The temperature was checked using a Cole-Parmer digital thermometer with a precision of ±0.1 °C. Three temperature probes placed at different points within the enzyme solution monitored temperature homogeneity during conventional heating. In conventionally treated samples the difference in temperature never exceeded more than 0.5 °C.

Enzymatic Assays. PPO activities toward L-tyrosine and L-dopa were determined spectrophotometrically at 507 nm [$\epsilon_{507\ nm}$ = 38000 M^{-1} cm^{-1} (Espín et al., 1997)] by using MBTH, which is a potent nucleophile through its amino group, which attacks enzyme-generated *o*-quinones (Rodríguez-López et al., 1994; Espín et al., 1997). The standard reaction mixture included the substrate, 5 mM MBTH, and 2% DMF in 50 mM sodium phosphate buffer (pH 7.0). Diphenolase activity of mushroom PPO on TBC was determined by measuring the direct formation of its enzyme-generated *o*-quinone. TBCQ formation was followed at 400 nm [$\epsilon_{400\ nm}$ = 1150 M^{-1} cm^{-1} (Rzepecki and Waite, 1989)]. The standard reaction mixture contained the substrate in 50 mM sodium phosphate buffer (pH 7.0). One unit of enzyme is defined as the amount of enzyme that produces 1 μmol of TBCQ per min at pH 7.0 and 25 °C using 10 mM TBC.

Kinetic assays were carried out with a Perkin-Elmer Lambda-2 UV−vis spectrophotometer interfaced on-line with a compatible PC for further data analysis. Activity determination was carried out at 25 °C, and temperature was controlled with a Haake D1G circulating water bath equipped with a heater/cooler.

Kinetic Data Analysis. The K_m^D, K_m^M, V_{max}^D, and V_{max}^M values on TBC, L-dopa, and L-tyrosine were calculated from triplicate measurements of the steady-state rate, v_{ss}, for each initial substrate concentration, $[S]_0$. The kinetic parameters were obtained from Lineweaver−Burk plots, a linear transformation of the Michaelis equation.

Total Antioxidant Activity Determinations. TAA was spectrophotometrically determined using the method proposed by Cano et al. (1996). The reaction mixture contained 0.1 mM ABTS, 0.01 mM H_2O_2, and 0.25 μM HRPC in 50 mM glycine−HCl buffer (pH 4.5) in a total volume of 1 mL. The reaction was monitored at 414 nm until stable absorbance, corresponding to the ABTS radical, was obtained. Different amounts of mushroom extracts were then added, and the decrease in absorbance was determined. The relative TAA remaining in the mushroom extracts after use of the different blanching methods was determined as a percentage of the TAA calculated in an untreated sample. For conventional heating, mushroom extracts were immersed in an 80 °C hot-water bath for 10 min, while the microwave heat treatment consisted of irradiation for 20 s. Mushroom extracts were immediately cooled to 25 °C at the end of the heat treatment (zero time) by inserting the test tube in an ice bath. The extracts were kept at room temperature, and the remaining TAA was determined at different times.

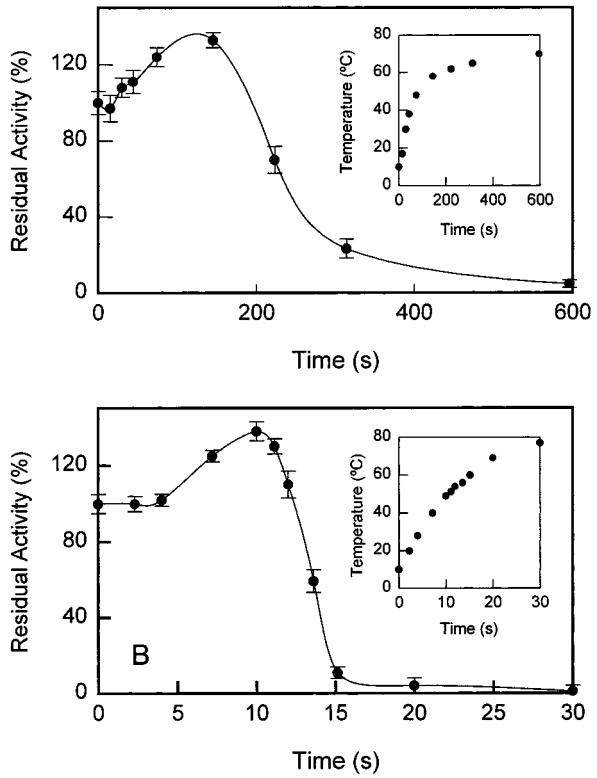


Figure 2. Time-dependent inactivation kinetics of mushroom PPO in 30 mM phosphate buffer, pH 7.0, using an 80 °C hot-water bath (A) or 22.6 W/cm^3 microwave power irradiation (B). Residual activity was revealed following the diphenolase activity of PPO on 1.6 mM TBC. Each point represents the mean of five separate experiments. Insets, temperature−time dependence for each of the heat treatments. Each point represents the mean of 10 separate determinations. The standard deviation for each point was ±0.5 and ±0.3 for conventional and microwave heating, respectively.

Measurement of Browning. Samples were prepared as for TAA determination, but soluble pigments were evaluated by the measuring absorbance at 400 nm (Weurman and Swain, 1955).

RESULTS AND DISCUSSION

Time-Course of PPO Inactivation. Figure 2 shows the time required for total PPO inactivation using both microwave and conventional heating treatments. Residual activity was determined by measuring the oxidation of TBC by mushroom PPO (diphenolase activity) and then calculating this as a percentage of a control not subjected to heating. PPO inactivation was complete after 20 s of microwave irradiation, whereas conventional methods needed more than 6 min for thermal inactivation to be complete. Since we were interested in studying the time-course of PPO inactivation during conventional blanching methods and, at the same time, we wanted to compare the results with microwave inactivation, we followed the following methodology. For conventional treatments, samples were introduced in an 80 °C water bath and aliquots were taken at different times/temperatures (see Materials and Methods section). For microwave treatments, mushroom extracts were exposed to 2.45 GHz microwave radiation for different times. The temperature−time dependence for each of the heat treatments is observed in the insets of Figure 2. Differences in the PPO inactivation time using conventional and microwave treatments are explained

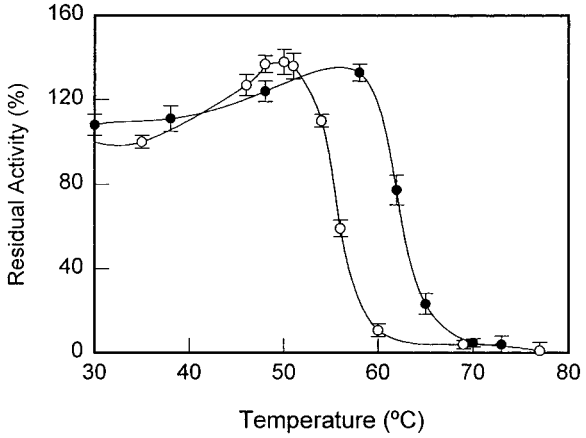
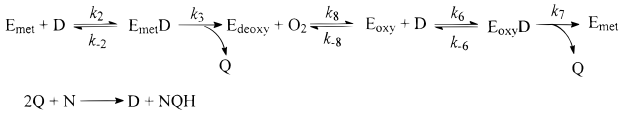


Figure 3. Temperature dependence of the inactivation of mushroom PPO in 30 mM phosphate buffer, pH 7.0, after (●) conventional hot-water or (○) microwave treatments. Residual activity was revealed following the diphenolase activity of PPO on 1.6 mM TBC. Each point represents the mean of five separate experiments.

in terms of the different principles of heating. Heat diffusion from the hot water to the mushroom extract is the main factor in a conventional heating process. However, the basic principle of microwave heating is the interaction of polar molecules with the electric component of the electromagnetic field, which generates heat due to the friction produced as the molecules attempt to orientate themselves within the oscillating field. In the next sections, we study the effect of conventional and microwave treatments on the kinetics of mushroom PPO, as well as the effect on the TAA and browning of mushroom extracts.

Temperature Stability of Mushroom PPO. From the temperature titration curve (Figure 3) a new parameter ($T_{1/2}$), representing the temperature at which residual activity is 50% of the initial activity can be determined. A $T_{1/2}$ of 63.5 °C was obtained for the mushroom crude extract treated in a conventional hot-water bath. Differences in $T_{1/2}$ with respect to previous reports (McCord and Kilara, 1983; Weemaes et al., 1997) can be explained by the different methods used. In previous studies (McCord and Kilara, 1983; Weemaes et al., 1997) aliquots of mushroom PPO were held at various temperatures for 10 min periods prior to cooling and assaying. Samples treated by microwave showed a lower $T_{1/2}$ (56.4 °C) than those heated by a hot-water bath (Figure 3). A difference in $T_{1/2}$ of 7.1 °C was observed between microwave and hot-water thermal inactivation. Such a difference was obtained after careful design of the microwave applicator in order to avoid experimental artifacts due to temperature differences in the sample. The same difference in $T_{1/2}$ was observed by using a purified sample of commercial mushroom PPO, indicating that other factors in the crude extracts such as proteases, lipids, etc., are not responsible for the different effects observed with microwave and conventional treatments. Moreover, we also observed differences in $T_{1/2}$ after treating apple PPO with microwave and conventional hot-water methods. In this case, the differences were higher, with a $T_{1/2}$ of 75 °C being obtained in conventional hot-water methods and 62 °C when treated by microwaves. The different effects on both apple and mushroom PPO indicate that activation is dependent on the nature of the protein and that

Scheme 1. Kinetic Reaction Mechanism for the Diphenolase Activity of Mushroom PPO[a]



[a] Abbreviations: D, *o*-diphenol; Q, *o*-quinone; N, nucleophile (MBTH); NQH, MBTH−quinone adduct.

microwave and conventional heating produce different enzyme intermediates of different thermal stability.

The effect of microwave energy on enzyme inactivation has generally been attributed to thermal effects, although there is some evidence that nonthermal effects also exist (Henderson et al., 1975; Porcelli et al., 1997). The latter effects are regarded as controversial since the energy associated with microwaves is many orders of magnitude below that required to break covalent bonds. However, microwave energy could break down lower energetic interactions such as the hydrogen bonding between the protein and water molecules associated with protein structure or polar interactions. For example, the faster inactivation of wheat germ lipase by microwaves with respect to conventional methods (Kermasha et al., 1993) was explained in terms of the interaction of the electrical component of the microwave field (Tajchakavit and Ramaswamy, 1995) and the numerous polar and/or charge moieties associated with the protein molecule. On the other hand, the differences observed in our experiments could also be explained by a kinetic thermal effect. Thus, the faster heating in the case of microwave treatment might stabilize different enzyme intermediates with faster inactivation rate constants. A similar effect has been described for other proteins in the contrary process (cooling), the folding of carbonic anhydrase II yielding a greater percentage of refolding when the unfolded protein was quickly cooled to 4 °C (Xie and Wetlaufer, 1996). In any case, the discrimination between thermal or nonthermal effects was not the main objective of this study, and further experiments to determine whether the different thermal inactivation profiles of PPO treated by microwave irradiation is due to one or the other effect are currently being undertaken.

Finally, we studied the reversibility of the inactivation process. It appears that thermal inactivation was irreversible in all cases, suggesting that the loss of mushroom PPO activity was due to a change in the overall conformation of the enzyme, as was described recently (Weemaes et al., 1997).

Effects of Thermal Treatments on the Diphenolase Activity of Mushroom PPO. Mushroom PPO is known to exhibit wide substrate specificity and oxidize a number of *o*-diphenolic compounds. This enzyme requires one molecule of oxygen to oxidize two molecules of *o*-diphenol to two molecules of *o*-quinone. The diphenolase mechanism of PPO is a lineal system (Scheme 1) involving several rate constants. By applying the steady-state approach to Scheme 1 and considering that mushroom PPO is saturated at 0.26 mM O_2 (Rodríguez-López et al., 1993), the analytical expression for the initial rate of product formation has been obtained (Rodríguez-López et al., 1992):

$$v_0 = (V_{max}{}^{D}[D]_0)/(K_m{}^{D} + [D]_0) \quad (1)$$

Taking into account several simplifications (Rodríguez-López et al., 1992) based on experimental data

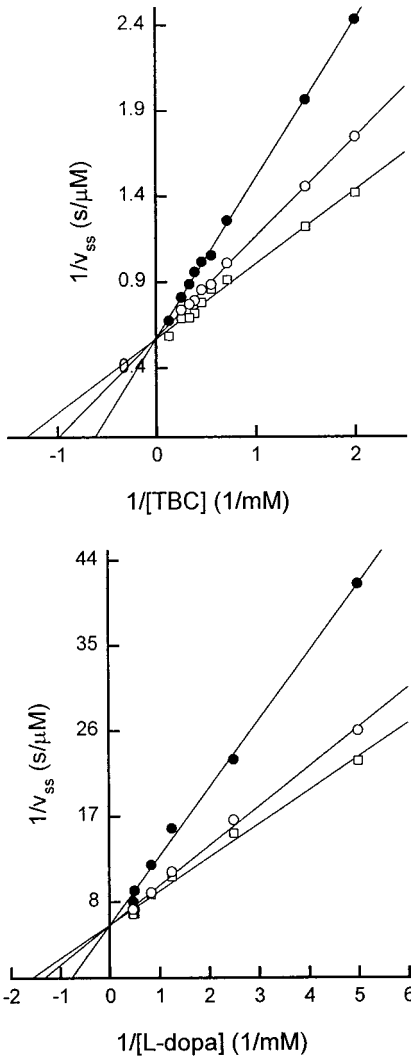


Figure 4. Lineweaver−Burk plots of the reaction of mushroom PPO (0.1 unit/mL) with TBC and L-dopa: (●) control, PPO no subject to heating; (□) PPO heated at 60 °C in a water bath; (○) PPO heated at 50 °C by microwaves.

(Makino and Mason, 1973; García-Cánovas et al., 1987) the following expressions for $V_{max}{}^{D}$ and $K_m{}^{D}$ can be derived (Rodríguez-López et al., 1992)

$$V_{max}{}^{D} = k_3[E]_0 \quad (2)$$

$$K_m{}^{D} = K_2 \quad (3)$$

where k_3 is the catalytic constant corresponding to the E_{met} reduction by D (Scheme 1), K_2 is the dissociation constant of E_{met} toward D ($K_2 = k_{-2}/k_2$; Scheme 1), and $[E]_0$ represents the initial concentration of active PPO. As can be seen, the $V_{max}{}^{D}$ expression involves only a rate constant that rules a transformation step in the reaction mechanism, whereas the $K_m{}^{D}$ expression implies a binding step in the same reaction mechanism.

When PPO was not subjected to any thermal treatment it showed a $K_m{}^{D}$ value of 1.28 mM acting on L-dopa as *o*-diphenolic substrate (Figure 4; Table 1). A typical thermal inactivation profile representing the v_{ss} versus temperature can be observed in Figure 5. In samples treated by conventional or microwave methods, the enzyme suffered an initial thermal activation prior to its irreversible inactivation, when the activity was

Table 1. Kinetic Parameters for the Oxidation of TBC, L-Dopa, and L-Tyrosine by Mushroom PPO Subject to Different Thermal Treatments[a]

substrate	treatment method	V_{max} (μM/s)	K_m (mM)
TBC	control[b]	(1.73 ± 0.05)	(1.61 ± 0.10)
	hot-water bath[c]	(1.74 ± 0.06)	(0.76 ± 0.05)
	microwave[d]	(1.73 ± 0.05)	(0.99 ± 0.06)
L-dopa	control	(0.18 ± 0.01)	(1.28 ± 0.10)
	hot-water bath	(0.18 ± 0.01)	(0.63 ± 0.04)
	microwave	(0.18 ± 0.01)	(0.75 ± 0.05)
L-tyrosine	control	(0.014 ± 0.001)	(0.25 ± 0.02)
	hot-water bath	(0.022 ± 0.001)	(0.37 ± 0.02)
	microwave	(0.030 ± 0.002)	(0.46 ± 0.02)

[a] The parameters were obtained from Figures 4 and 7. [b] Mushroom PPO was not subject to thermal treatment. [c] Mushroom PPO was heated at 60 °C in a hot-water bath. [d] Mushroom PPO was heated at 50 °C by microwaves.

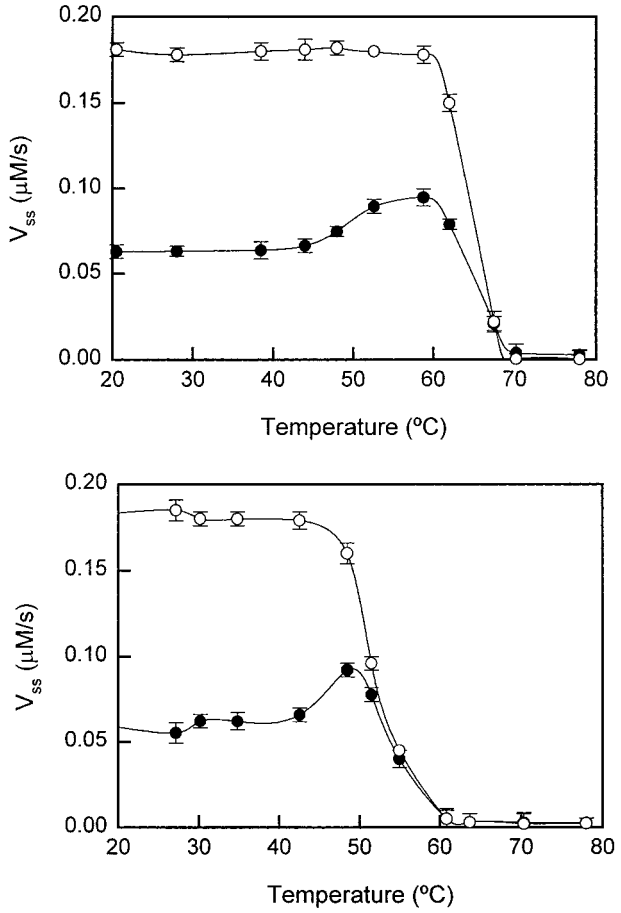


Figure 5. Temperature dependence of the inactivation of mushroom PPO in 30 mM phosphate buffer, pH 7.0, after (A) conventional hot-water or (B) microwave treatments. Activity was revealed following the diphenolase activity of PPO (0.1 units/ml) at (●) 1.28 and (○) 5 mM L-dopa. Each point represents the mean of five separate experiments.

determined using a L-dopa concentration close to the K_m^D value (Figure 5). However, this activation effect was not observed when a saturating concentration of L-dopa was used (Figure 5). Exactly the same effect was observed using TBC as substrate (data not shown). The increase in activity of PPO by conventional heating has been described for the enzyme purified from other sources (Mathew and Parpia, 1971; Lee et al., 1991; Yemenicioglu et al., 1997). It has been proposed that the increase observed in the activity of apple PPO by heating could, in part, be due to the release of latent

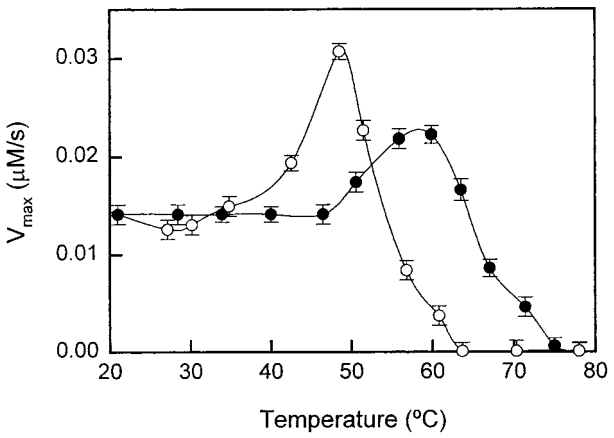


Figure 6. Temperature dependence of the V_{max}^M of mushroom PPO in 30 mM phosphate buffer, pH 7.0, after (●) conventional hot-water or (○) microwave treatments. Activity was revealed following the monophenolase activity of PPO (0.2 units/ml) at 1.2 mM L-tyrosine. Each point represents the mean of five separate experiments.

enzyme (Yemenicioglu et al., 1997), while Mathew and Parpia (1971) attributed the activation of PPO to protein association and dissociation. However, these two effects represent an increase in V_{max}^D due to an increase in the concentration of active enzyme ($[E]_0$). Samples showing higher heat activation (PPO heated at 60 and 50 °C by conventional and microwave methods, respectively) were used for kinetic determinations, and their kinetic parameters V_{max}^D and K_m^D were determined (Figure 4; Table 1). As can be seen, both thermal treatments modified the K_m^D but not the V_{max}^D values of mushroom PPO acting on TBC and L-dopa (Table 1). These data indicate that the thermal activation of the diphenolase activity of PPO after mild heat treatments is due to the structural transformation of the native enzyme into a transient form with a more *open* active site, which facilitates the access/binding of the *o*-diphenols to PPO (lower K_m^D). Conventional and microwave treatments modified the affinity of PPO for *o*-diphenolic substrates to a different extent (Table 1). These results agree with the data reported in the thermal stability section and confirm that both treatments generated different enzyme intermediates immediately prior to irreversible inactivation.

Effects of Thermal Treatments on the Monophenolase Activity of Mushroom PPO. Using L-tyrosine as monophenolic substrate and MBTH as a chromophoric agent, the monophenolase activity of mushroom PPO was determined spectrophotometrically. The presence of an induction period is a characteristic of the monophenolase activity of PPO from different sources. After this lag period, the steady-state activity can be determined. The results of all our inactivation experiments showed that the monophenolase activity of mushroom PPO increased with mild heating. This activation process was observed at both nonsaturating and saturating concentrations of L-tyrosine (Figure 6). In contrast what occurred with the diphenolase activity, the results indicated that the V_{max}^M of the reaction of PPO with L-tyrosine was affected by mild heating. The kinetic experiments carried out to determine V_{max}^M and K_m^M after different thermal treatments showed that mild heating modified both kinetic parameters (Figure 7; Table 1). These results can be explained by the mechanism that has been proposed for the monophe-

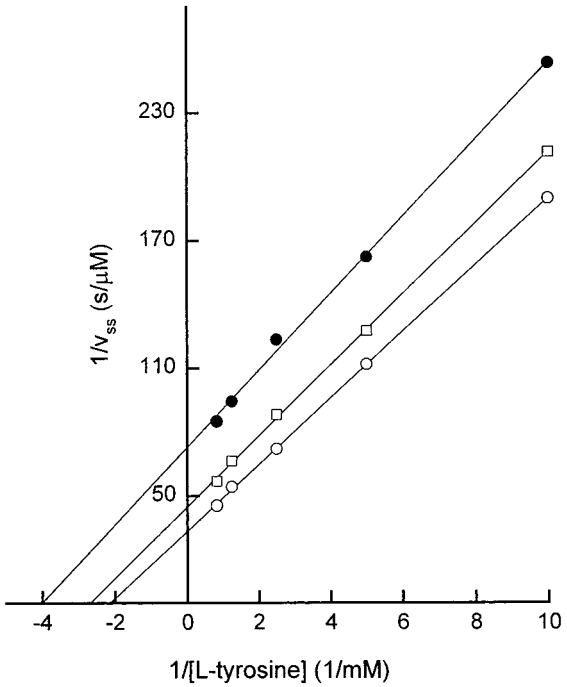
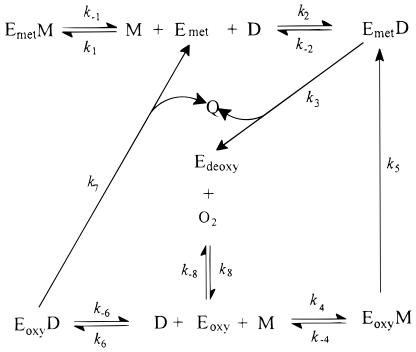


Figure 7. Lineweaver−Burk plots of the reaction of mushroom PPO (0.2 unit/mL) with L-tyrosine: (●) control, PPO no subject to heating; (□) PPO heated at 60 °C in a water-bath; (○) PPO heated at 50 °C by microwaves. Each point represents the mean of three separate experiments.

Scheme 2. Kinetic Reaction Mechanism of Mushroom PPO on Monophenols and *o*-Diphenols Coupled to Nonenzymatic Reactions from *o*-Quinone[a]



[a] Abbreviations: M, monophenol; D, *o*-diphenol; Q, *o*-quinone; N, nucleophile (MBTH); NQH, MBTH−quinone adduct.

nolase activity of mushroom PPO (Cabanes et al., 1987; Rodríguez-López et al., 1992; Sánchez-Ferrer et al., 1995). The monophenolase reaction mechanism of PPO is a cyclic system (Scheme 2) involving many kinetic constants that lead to the following expression for $V_{max}{}^{M}$ and $K_m{}^{M}$ (Rodríguez-López et al., 1992)

$$V_{max}{}^{M} = {}^{1}/_{3}(k_3k_5/k_7)(K_1K_6/K_2K_4)[E]_0 \quad (4)$$

$$K_m{}^{M} = K_1 \quad (5)$$

where k_5 and k_7 are transformation constants (Scheme 2), K_1 is the dissociation constant for the $E_{met}M$ complex, and K_4 and K_6 correspond to the dissociation constant of the E_{oxy} form toward M and D, respectively. As can be seen, the $K_m{}^{M}$ expression only involves a dissociation constant, whereas the $V_{max}{}^{M}$ expression involves kinetic constants that rule both transformation and binding steps in the reaction mechanism. Therefore, a change in the affinity of the enzyme for both M and D implies a change in the $V_{max}{}^{M}$ of the monophenolase reaction. Although the dissociation constants of the E_{oxy} form for M and D (K_4 and K_6) are present in the expression for $V_{max}{}^{M}$ (eq 4), the effect of thermal treatment on this kinetic parameter could be explained simply by the effect on K_1 and K_2. This could mean that the binding of the peroxide to the copper in E_{oxy} buffers the effect of heat on this enzymatic form.

From the data presented in Table 1, it can be seen that heating modified the interaction of mushroom PPO with monophenol and *o*-diphenols in different ways. Mild heating produced a decrease in the $K_m{}^{D}$ (K_2) but an increase in the $K_m{}^{M}$ (K_1) of mushroom PPO. Both effects produced an increase in the $V_{max}{}^{M}$ value (eq 4) in samples treated by conventional or microwave modes. Although L-tyrosine and L-dopa have the same side chain, the binding of these substrates to PPO is affected in the opposite way by thermal treatments. The binding of substrates to PPO is better explained by reference to several molecular processes involving the access of the substrate to the active center followed by nucleophilic attack of the oxygen atom from the hydroxyl groups on the copper center, probably assisted by amino acid side chains constituting the active site. The monophenolase reaction is thought to be more dependent on the structure of the enzyme (Himmelwright et al., 1980; Wilcox et al., 1985). Thus, structural changes resulting from thermal treatments could facilitate the access of monophenols and *o*-diphenols to the active site but at the some time hinder their binding to the copper center. The hydroxyl group at C-4 of L-tyrosine presents lower nucleophilic power than the hydroxyl groups at C-3 and C-4 of L-dopa (Espín et al., 1998). Therefore, deprotonation of the hydroxyl group prior to binding should be more significant for L-tyrosine than for L-dopa. These data indicate that the limiting step in the binding of *o*-diphenols and monophenols to the enzyme could differ. Thus, in L-dopa the limiting step for binding could be its access through the substrate channel but not for L-tyrosine.

The increase in the K_1 value of PPO with heating predicts a decrease in the lag period, which is characteristic of monophenolase activity. The E_{met} form of the enzymes subjected to mild conventional or microwave heating might have a lower affinity toward the monophenol, and so there would be less enzyme in the dead-end complex, $E_{met}M$, and less time would be required to reach the steady state (Ros et al., 1994). This hypothesis is supported by the dependence of the lag period on temperature (Figure 8). In any case, the increase in activity of mushroom PPO as a result of heating represents an additional problem, especially in slow bleaching methods where the enzyme remains active for a longer time in the presence of its phenolic substrates.

Effects of Thermal Treatments on TAA. Processing and storage can profoundly alter the antioxidant composition of fruits and vegetables (Halliwell, 1995). The short exposure time of samples irradiated with microwaves might be important for maintaining the quality of mushrooms. For this reason, we determined the TAA in thermally treated mushroom extracts. Table 2 shows the evolution of the relative TAA with time in mushroom extracts after the application of different

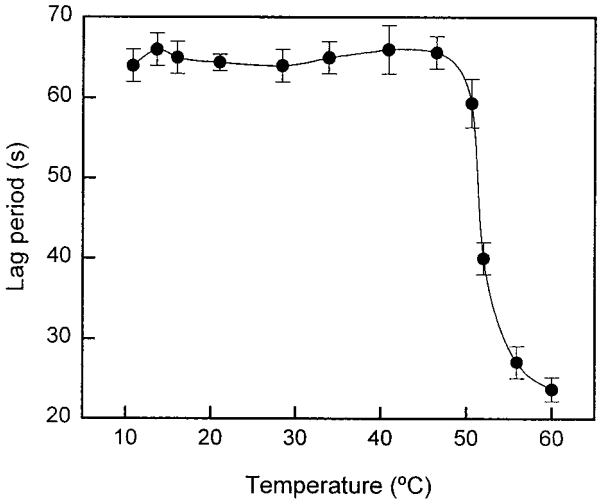


Figure 8. Temperature dependence of the lag period in L-tyrosine hydroxylation by mushroom PPO. Data correspond to mushroom extracts treated by a hot-water bath.

Table 2. Time-Course of the Relative TAA with Time in Mushroom Extracts after the Application of Different Blanching Methods[a]

time (h)	control	water bath	microwave
0	(100 ± 4)	(100 ± 5)	(100 ± 5)
1	(92 ± 3)	(70 ± 5)	(100 ± 4)
5	(85 ± 4)	(50 ± 5)	(95 ± 4)
10	(78 ± 3)	(40 ± 4)	(90 ± 4)
18	(65 ± 2)	(29 ± 4)	(81 ± 3)
24	(60 ± 2)	(25 ± 4)	(78 ± 3)

[a] Each data point represents the mean of three separate experiments.

blanching methods. After 24 h at room temperature, microwave-treated samples still showed more than 75% of their initial TAA, whereas the extracts treated by conventional methods only showed about 25% of their initial TAA. The untreated sample showed a loss of 50%. Oxidase enzymes, including PPO, are believed to be responsible for this decrease in antioxidant capacity, either directly through the oxidation of reducing substrates or indirectly through the oxidation of ascorbic acid by the quinones and the oxidized products generated in the enzymatic oxidation of their substrates (Ros et al., 1993). In the extracts treated by conventional thermal methods, PPO remained active for >6 min before being completely inactivated. After the inactivation of PPO, the oxidation product probably remains in the sample, generating melanin products and reducing the antioxidant content. The initial activation of PPO in samples heated at 80 °C in a water bath could explain why these samples lost more TAA than untreated samples. In contrast, the extracts treated with microwave techniques quickly inactivated PPO and the enzyme could not therefore generate oxidation products. The results obtained in this section confirm the direct relationship between PPO activity and loss of TAA and indicate the importance of finding rapid methods for inactivating PPO to preserve the nutritional value of mushrooms.

Effects of Thermal Treatments on Browning of Mushroom Extracts. The PPO-catalyzed enzymatic browning of phenolic compounds is of vital importance in mushroom processing due to the formation of undesirable colors and flavors and the loss of nutrients (Zawistowski et al., 1991). Figure 9 shows the relative

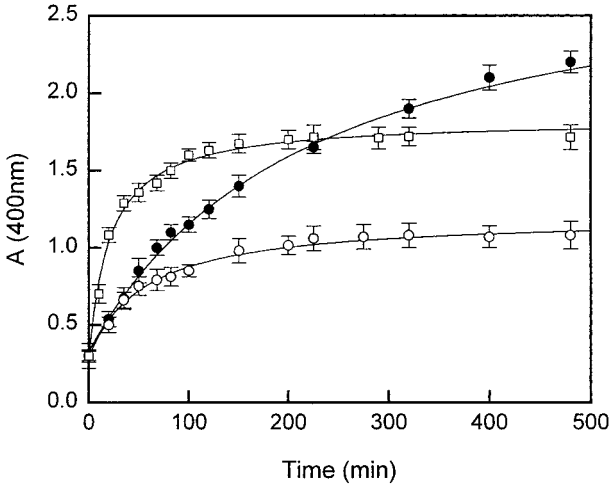


Figure 9. Rate of mushroom extract browning after the application of different blanching methods: (●) untreated extracts; (○) microwave treatment; (□) conventional hot-water treatment. Each point represents the mean of three separate experiments.

browning rates of three mushroom extracts treated differently. Although the untreated sample showed the highest overall level of browning, the mushroom extract treated with a hot-water bath had higher levels of browning than the microwave-treated sample. The extract treated with the conventional hot-water method had the faster initial rate of browning, a maximum being reached after 2 h, while the browning rates of the microwave and untreated extracts were slower. The browning in the microwave treated extract also reached at maximum after 2 h, while the reaction in the untreated control continuously increased, reaching a maximum at 10 h. The faster initial rate in both heated mushroom extracts could be related with the initial activation of PPO and the final absorbance could be related with the amount of oxidation products formed. Thus, the extracts treated by microwave energy showed a lower concentration of brown products than those heated conventionally as a consequence of the faster inactivation rate of PPO. The results described in this paper indicate that browning reactions in mushrooms are directly related to the PPO content and point to the importance of rapid PPO inactivation to reduce browning of mushroom.

CONCLUSIONS

Microwave heating techniques for the industrial blanching of mushrooms have shown promising results for improving product quality and, particularly, for shortening the processing times currently used. The microwave applicator used here is useful to study the kinetics of PPO inactivation. Mushroom PPO is inactivated differently according to whether conventional or microwave treatments are used. Nevertheless, the direct application of the microwave energy to entire mushrooms is limited by the important temperature gradients generated within the samples during heating, which can produce internal water vaporization and associated damage to the mushroom texture. Hence, to profit some of the notorious advantages that microwave heating has shown for mushroom extracts, further research involving microwave heating aided by pressure and other conventional techniques is envisaged.

ABBREVIATIONS USED

MBTH, 3-methyl-2-benzothiazolinone hydrazone; PPO, tyrosinase or polyphenoloxidase (EC 1.14.18.1); TAA, total antioxidant activity; TBC, 4-*tert*-butylcatechol, TBCQ, 4-*tert*-butyl-*o*-benzoquinone; L-dopa, L-3,4-dihydroxyphenylalanine; DMF, *N*,*N*′-dimethylformamide; ABTS, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid); HRPC, horseradish peroxidase isoenzyme C; E_{met}, met-PPO or oxidized form of PPO with $Cu^{2+}-Cu^{2+}$ in the active site; E_{deoxy}, deoxy-PPO or reduced form of PPO with $Cu^{+}-Cu^{+}$ in the active site; E_{oxy}, oxy-PPO or oxidized form of PPO with peroxide; VSWR, voltage standing wave ratio.

LITERATURE CITED

Cabanes, J.; García-Cánovas, F.; Lozano, J. A.; García-Carmona, F. A kinetic study of the melanization pathway between L-tyrosine and dopachrome. *Biochim. Biophys. Acta* **1987**, *923*, 187−195.

Cano, A.; Hernández-Ruiz, J.; García-Cánovas, F.; Arnao, M. B.; Acosta, M. A peroxidase-based method for determining the total antioxidant activity in plant material. In *Plant Peroxidases: Biochemistry and Physiology*; Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., Eds.; University of Geneva: Geneva, 1996; pp 400−403.

Decareau, R. V. *Microwaves in the Food Processing Industry*; Academic Press, Inc.: London, 1985.

Devece, C.; Catalá-Civera, J. M.; Sánchez-Hernández, D.; de los Reyes, E. Polyphenoloxidase (PPO) inactivation in industrial mushroom blanching processes employing microwave heating techniques. *14th ICECOM Int. Conf.* **1997**, *1*, 115−119.

Duckworth, H. W.; Coleman, J. E. Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.* **1970**, *245*, 1613−1625.

Espín, J. C.; García-Ruiz, P. A.; Tudela, J.; Varón, R.; García-Cánovas, F. Monophenolase and diphenolase reaction mechanisms of apple and pear polyphenol oxidases. *J. Agric. Food Chem.* **1998**, *46*, 2968−2975.

Espín, J. C.; Morales, M.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1997**, *45*, 1084−1090.

García-Cánovas, F.; Tudela, J.; Martínez-Madrid, C.; Varón, R.; García-Carmona, F.; Lozano, J. A. Kinetic study of the suicide inactivation of tyrosinase induced by catechol. *Biochim. Biophys. Acta* **1987**, *912*, 417−423.

Halliwell, B. Antioxidants: Elixirs of life or tonics for tired sheep? *Biochemist* **1995**, Feb/Mar, 3−6.

Henderson, H. M.; Hergenroeder, K.; Stuchly, S. S. Effect of 2450 MHz microwave radiation on horseradish peroxidase. *J. Microwave Power* **1975**, *10*, 27−35.

Himmelwright, R. S.; Eickman, N. C.; LuBien, C. D.; Lerch, K.; Solomon, E. I. Chemical and spectroscopic studies of the binuclear copper active site of Neurospora Tyrosinase: Comparison to Hemocyanins. *J. Am. Chem. Soc.* **1980**, *102*, 7339−7348.

Kermasha, S.; Bisakowski, B.; Ramaswamy, H.; Van de Voort, F. Comparison of microwave, conventional and combination heat treatments on wheat germ lipase activity. *Int. J. Food Sci. Technol.* **1993**, *28*, 617−623.

Konanayakam, M.; Sastry, S. K. Kinetics of shrinkage of mushrooms during blanching. *J. Food Sci.* **1988**, *53*, 1406−1411.

Lee, P. M.; Lee, K. H.; Karim, M. I. A. Biochemical studies of cocoa bean polyphenol oxidase. *J. Sci. Food Agric.* **1991**, *55*, 251−260.

Makino, N.; Mason, H. S. Reactivity of oxytyrosinase toward substrates. *J. Biol. Chem.* **1973**, *248*, 5731−5735.

Mason, H. S. Comparative biochemistry of the phenolase complex. *Adv. Enzymol.* **1955**, *16*, 105−184.

Mathew, A. G.; Parpia, H. A. B. Food browning as a polyphenol reaction. *Adv. Food Res.* **1971**, *19*, 75−145.

McCord, J. D.; Kilara, A. Control of enzymatic browning in processed mushrooms (*Agaricus bisporus*). *J. Food Sci.* **1983**, *48*, 1479−1483.

Ponne, C. T.; Baysal, T.; Yuksel, D. Blanching leafy vegetables with electromagnetic energy. *J. Food Sci.* **1994**, *59*, 1037−1047.

Porcelli, M.; Cacciapuoti, G.; Fusco, S.; Massa, R.; d'Ambrosio, G.; Bertoldo, C.; De Rosa, M.; Zappia, V. Nonthermal effects of microwaves on proteins: thermophilic enzymes as model system. *FEBS Lett.* **1997**, *402*, 102−106.

Prota, G. Progress in the chemistry of melanins and related metabolites. *Med. Res. Rev.* **1988**, *8*, 525−556.

Riva, M.; Schiraldi, A.; Di Cesare, L. F. Drying of *Agaricus bisporus* mushrooms by microwave-hot air combination. *Lebensm. Wiss. u.-Technol.* **1991**, *24*, 479−483.

Rodríguez-López, J. N.; Escribano, J.; García-Cánovas, F. A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. *Anal. Biochem.* **1994**, *216*, 205−212.

Rodríguez-López, J. N.; Ros, J. R.; Varón, R.; García-Cánovas, F. Characterization of the Michaelis constants for oxygen in tyrosinase catalyzed oxidation of monophenols and *o*-diphenols. *Biochem. J.* **1993**, *293*, 852−866.

Rodríguez-López, J. N.; Tudela, J.; Varon, R.; García-Carmona, F.; García-Cánovas, F. Analysis of a kinetic model for melanin biosynthesis pathway. *J. Biol. Chem.* **1992**, *267*, 3801−3810.

Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. Effect of L-ascorbic acid on the monophenolase activity of tyrosinase. *Biochem. J.* **1993**, *295*, 309−312.

Ros, J. R.; Rodríguez-López, J. N.; García-Cánovas, F. Tyrosinase: kinetic analysis of the transient phase and the steady-state. *Biochim. Biophys. Acta* **1994**, *1204*, 33−42.

Rzepecki, L. M.; Waite, J. H. A chromogenic assay for catecholoxidases based on the addition of L-proline to quinones. *Anal. Biochem.* **1989**, *179*, 375−381.

Sanchez-Ferrer, A.; Rodríguez-López, J. N.; García-Cánovas, F.; García-Carmona, F. Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1−11.

Tajchakavit, S.; Ramaswamy, H. S. Continuous-flow microwave heating of orange juice: evidence of nonthermal effects. *J. Microwave Power* **1995**, *30*, 141−148.

Weemaes, C.; Rubens, P.; De Cords, S.; Ludikhuyze, L.; Van den Broeck, I.; Hendrickx, M.; Heremans, K.; Tobback, P. Temperature sensitivity and pressure resistance of mushroom polyphenoloxidase. *J. Food Sci.* **1997**, *62*, 261−266.

Weurman, C.; Swain, T. Changes in the enzymatic browning of Bramley's seedling apples during their development. *J. Sci. Food Agric.* **1955**, *6*, 186−191.

Wilcox, D. E.; Porras, A. G.; Hwang, Y. T.; Lerch, K.; Winkler, M. E.; Solomon, E. I. Substrate analogue binding to the couple binuclear copper active site of tyrosinase. *J. Am. Chem. Soc.* **1985**, *107*, 4015−4027.

Xie, Y.; Wetlaufer, D. B. Control of aggregation in protein refolding: The temperature-leap tactic. *Protein Sci.* **1996**, *5*, 517−523.

Yemenicioglu, A.; Özkan, M.; Cemeroglu, B. Heat inactivation kinetics of apple polyphenoloxidase and activation of its latent form. *J. Food Chem.* **1997**, *62*, 508−510.

Zawistowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenol Oxidase. In *Oxidative Enzymes in Foods*; Robinson, D. S., Eskin, N. A. M., Eds.; Elsevier Science: London, 1991; pp 217−273.

Zhang, X.; Flurkey, W. H. Phenoloxidases in *Portabella* Mushrooms. *J. Food Sci.* **1997**, *62*, 97−100.

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