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Polyphenol oxidase potentials of three wild mushroom species harvested from Lişer High Plateau, Trabzon

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Abstract

Crude enzyme extracts were prepared from *Armillaria mellea* (*A. mellea*), *Lepista nuda* (*L. nuda*) and *Hypholoma fasciculare* (*H. fasciculare*), which were harvested from the Lişer High Plateau-Maçka (Trabzon, Turkey). The crude polyphenol oxidase (PPO) extracts from each mushroom were highly active against 4-methylcatechol. Native polyacrylamide gel electrophoresis, stained by *L*-3,4-dihydroxyphenylalanine, showed the polyphenol oxidase potentials. The optimum pH value, for each enzyme, was 7.0. When enzyme extracts were incubated at pH 7.0 for 24 h at 4 °C, it was observed that *L. nuda* and *H. fasciculare* enzyme activities decreased by about 26% and 18%, respectively, but, *A. mellea* enzyme activity increased by about 11%. The temperature optima of *A. mellea*, *L. nuda* and *H. fasciculare* were, respectively, 30, 30 and 20 °C. Cr^{3+} and Cu^{2+} ions inhibited each activity. Also, sodium metabisulphite and ascorbic acid were strong inhibitors of the enzyme activities.

Keywords: *Armillaria mellea*; *Lepista nuda*; *Hypholoma fasciculare*; Polyphenol oxidase; Mushroom

1. Introduction

Polyphenol oxidase, or tyrosinase (EC 1.14.18.1), is a copper enzyme, widely distributed throughout microorganism, plants and animals. It is of central importance in such processes as vertebrate pigmentation and browning of fruits and vegetables (Mason, 1948; Prota, 1988). The enzyme uses the copper ion as a prosthetic group which participates in several redox reactions. Oxygen is required for the catalytic processes that lead to hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to highly reactive *o*-quinones (diphenolase activity) (Mayer & Harel, 1979). The *o*-quinones polymerize to form melanin through a series of subsequent enzymatic and nonenzymatic reactions. Although the physiological function of polyphenol oxidase (PPO) in fungi is not yet understood, melanin synthesis is correlated with differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury (Gadd, 1980; Bell & Wheeler, 1986; Howard, Ferrari, Roach, & Money, 1991; Miranda et al., 1997). In addition, PPO is responsible for the undesired enzymatic browning of mushrooms that takes place during senescence or damage during post-harvest handling (Pérez-Gilabert, Morte, Honrubia, & García-Carmona, 2001).

Mushrooms are valuable health foods. While they are low in calories and fats, they contain abundant essential fatty acids, vegetable proteins, vitamins and minerals (Agrahar-Murugkar & Subbulakshmi, 2005; Bobek, Ginter, Jurcovicova, & Kuniak, 1991; Manzi, Aguzzi, & Pizzoferrato, 2001; Breene, 1990). Mushrooms also have a long history of use in traditional Chinese medicine. Their legendary effects in promoting good health and vitality and increasing the body's adaptive response have been supported by recent studies.

Armillaria mellea (*A. mellea*) is well known as a fungus. It has been reportedly used in the treatment of geriatric

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patients with palsy, dizziness, headache, neurasthenia, insomnia, numbness in limbs, and infantile convulsion, and also reportedly exerts neuroprotective effects (Yang et al., 1989). *A. mellea* is edible and considered good by many people. The acrid or bitter taste of fresh specimens disappears with cooking.

Lepista nuda (*L. nuda*), which is an edible mushroom, is used in traditional medicine. The sporophore of *L. nuda* contains Vitamin B_1. Infusion of this macrofungus is used for preventing beriberi (Ying, Xiaolan, Yichen, & Huaan, 1987). In addition, the decoction is used for the treatment of abscesses and wounds (Hanssen & Schadler, 1982).

Hypholoma fasciculare (*H. fasciculare*), also known as sulphur tuft, is poisonous. *H. fasciculare* is often abundant in Bay Area woodlands, forming bright yellow clusters on both hardwood and conifer wood.

Because PPO has many applications in industry, and mushrooms are not only valuable foods but also good enzyme sources, it is interesting to study PPO activity from different mushroom species. In this work, the characterization of PPOs from *A. mellea* and *L. nuda* and *H. fasciculare* was studied in terms of substrate specificities, thermal activation and stability, pH optimum and stability, and degrees of inhibition by general PPO inhibitors. In addition, the effects of some metal ions on PPO activities were studied.

2. Materials and methods

2.1. Materials and chemicals

Armillaria mellea (Vahl.: Fries) Quélet, (Champ. Jura Vosg. 1:75.1872), *Lepista nuda* (Bull.:Fr.) Corumooke and *Hypholoma fasciculare* (Huds.) Quél., Führer fürPilzfreunde (Zwickau): 21, 72 (1871) were collected directly from the Lişer High Plateau-Maçka (Trabzon, Turkey). The mushrooms were carried into the laboratory in liquid nitrogen, in a nitrogen Dewar flask. After that, the extracts were prepared as quickly as possible and stored deep-frozen at −30 °C until used.

Substrates and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the other reagents were of analytical grade.

2.2. Crude enzyme preparation

Mushrooms (10 g) were placed in a Dewar flask under liquid nitrogen for 10 min and then homogenized by using a blender in 10 ml of phosphate buffer (pH 7.0, 50 mM) containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 6% (w/v) Triton X-114 for 10 min. The homogenate was filtered and kept at 4 °C for 1 h before being centrifuged at 10.000 rpm for 20 min at 4 °C. The supernatant was stored at −30 °C and used as crude enzyme (Kolcuoğlu, Colak, Sesli, Yildirim, & Saglam, 2007; Özen, Colak, Dincer, & Güner, 2004; Dincer, Colak, Aydın, Kadioglu, & Güner, 2002; Colak, Özen, Dincer, Güner, & Ayaz, 2005).

2.3. PPO activity assay

The enzyme activity was assayed by measuring the rate of increase in absorbance at a given wavelength, as described previously (Kolcuoğlu et al., 2007; Yildirim et al., 2005). An ATI Unicam UV2-100 double beam UV–vis spectrophotometer (ATI Unicam, Cambridge, UK) was employed throughout the investigation. The activity was determined, using different mono- or di- phenolic compounds, by measuring the increase in absorbance at 494 nm for 4-methylcatechol and 500 nm for all other substrates (Espin, Morales, Varon, Tudela, & Garcia-Canovas, 1995). The assay mixture, containing substrate (stock 100 mM), an equal volume of MBTH (stock 10 mM), and 20 μl dimethylformamide (DMF), was diluted to 950 μl with buffer. After that, 50 μl of crude enzyme extract were added. The reference cuvette included all the reactants except the crude enzyme. One unit of PPO activity was defined as 1 μmol of product formed per min. Specific activity was defined as the units of enzyme activity per mg of protein (Kong, Hong, Choi, Kim, & Cho, 2000).

2.4. Native polyacrylamide gel electrophoresis

First, the enzyme extract was added to an equal volume of cold acetone (−30 °C) and left for 1 h at 4 °C to allow precipitation of the proteins. The acetone precipitate was collected and left for 24 h at 4 °C, to remove acetone. After that, the precipitate was dissolved in 50 mM phosphate buffer (pH 7.0, 50 mM). After vortexing for 5 min, the suspension was centrifuged at 5.000 rpm for 5 min. The supernatant was used as the enzyme sample for electrophoresis (Duangmal & Owusu Apenten, 1999).

Nondenaturing polyacrylamide gel electrophoresis was carried out at 4 °C, using preparative 8% polyacrylamide gels (Laemmli, 1970) under native conditions in a P8DS Electrophoresis Unit (Owl Scientific Inc., Woburn, USA). After the electrophoresis run, the gel was stained in 24 mM *L*-DOPA.

2.5. Protein determination

Protein content was determined, according to the Lowry method, using bovine serum albumin as standard (Lowry, Rosebrough, Farr, & Randall, 1951). The values were obtained by graphic interpolation on a calibration curve at 650 nm.

2.6. Substrate specificity

PPO activity was determined by using catechol, 4-methylcatechol, *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and 3-

(3,4-dihydroxyphenyl) propionic acid (DHPPA) as diphenolic substrates and *L*-tyrosine and 3-(4-hydroxyphenyl)-propionic acid (PHPPA) as monophenolic substrates in 50 mM phosphate buffer (pH 7.0) (Kolcuoğlu et al., 2007).

2.7. pH optimum and stability

PPO activity, as a function of pH, was determined using various buffers (all at 50 mM): glycine–HCl (pH 3.0), acetate (pH 4.0, 5.0), phosphate (pH 6.0–8.0), and Tris–HCl (pH 9.0). 4-Methylcatechol was used as substrate.

The pH stability was determined by incubating the enzyme extract in the above buffer for 24 h at 4 °C. At the end of the storage period, the activity was assayed under standard conditions: 50 mM phosphate buffer (pH 7.0) as buffer and 4-methylcatechol as substrate (Yildirim et al., 2005).

2.8. Temperature optimum and thermal stability

PPO activity, as a function of temperature, was determined under standard assay conditions, using temperatures from 10 to 80 °C. The reaction mixtures, containing all the reagents except crude enzyme, were incubated for 5 min at various temperatures, as indicated above. After the enzyme extract was added to the incubated reaction mixture, the activity of PPO was determined spectrophotometrically at 494 nm as rapidly as possible.

To determine the thermal stability of the crude enzyme, the enzyme extracts, in Eppendorf tubes, were incubated at various temperatures from 20–70 °C with 10 °C increments for 1 h, rapidly cooled in an ice bath for 5 min, and then brought to 25 °C. After that, the enzyme activity was assayed under standard conditions. The percentage residual activity was calculated by comparison with unheated enzyme. The data obtained from the thermal stability profile were used to analyze some thermodynamic parameters (Kolcuoğlu et al., 2007).

2.9. Effect of substrate concentration on PPO activity and enzyme kinetics

To study the effect of substrate concentration, 4-methylcatechol was used at various final concentrations. The activity was assayed at pH 7.0. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values of the enzyme were determined from a plot of $1/V$ versus 1/[S] by the method of Lineweaver and Burk (Lineweaver & Burk, 1934). Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver–Burk plot.

2.10. Effect of protein concentration on PPO activity

PPO activity, as a function of protein concentration, was determined in a protein concentration range of 0.001–0.200 mg/ml for *A. mellea*, 0.002–0.300 mg/ml for *L. nuda* and 0.001–0.400 mg/ml for *H. fasciculare,* using 4-methylcatechol as substrate. The activity was assayed under standard conditions, using various volumes of the enzyme extracts (Yildirim et al., 2005).

2.11. Effect of general PPO inhibitors and some metal ions on crude enzyme activity

The following compounds were evaluated for their effectiveness as inhibitors of PPO activity, using 4-methylcatechol as substrate: benzoic acid, sodium metabisulphite, ascorbic acid, sodium azide and *L*-cysteine. An aliquot of each inhibitor at various final concentrations was added to the standard reaction solution immediately before the addition of enzyme extract. The concentration of inhibitor giving 50% inhibition (I_{50}) was determined from a plot of residual activity against inhibitor concentration (Kolcuoğlu et al., 2007).

PPO activity was measured in the presence (1 mM final concentration) of various ionic compounds under the standard assay conditions. The ionic compounds included K^+, Co^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+} and Cr^{3+}. The percentage remaining activities were expressed by comparison with standard assay mixture with no metal ion added (Yildirim et al., 2005).

2.12. Statistical analysis of data

Analysis of variance of the data was performed with SPSS 10.0 for Windows (USA). The LSD multiple range test was employed to determine the statistical analysis (Çolak et al., 2005). In all Figures and Tables, data points represent means of three determinations ($p \leqslant 0.05$).

3. Results and discussion

3.1. Protein determination

Protein quantities of the *A. mellea*, *L. nuda* and *H. fasciculare* were found to be, on average, 0.86 mg/ml, 2.52 mg/ml and 0.98 mg/ml, respectively.

3.2. Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis, stained with *L*-DOPA, showed three bands with R_f values of 0.28, 0.38, and 0.42 for *A. mellea*, one single band with R_f value of 0.40 for *L. nuda*, and three bands with R_f values of 0.42, 0.46, and 0.51 for *H. fasciculare* (Fig. 1). The bands with different R_f values on stained native electrophoresis can be attributed to the presence of isoenzymes. Existing of more than two isoenzymes for PPOs has been reported for different fruits (Kolcuoğlu et al., 2007; Colak et al., 2005; Park & Luh, 1985; Rivas & Whitaker, 1973; Palmer, 1963; Pifferi & Cultrera, 1974).

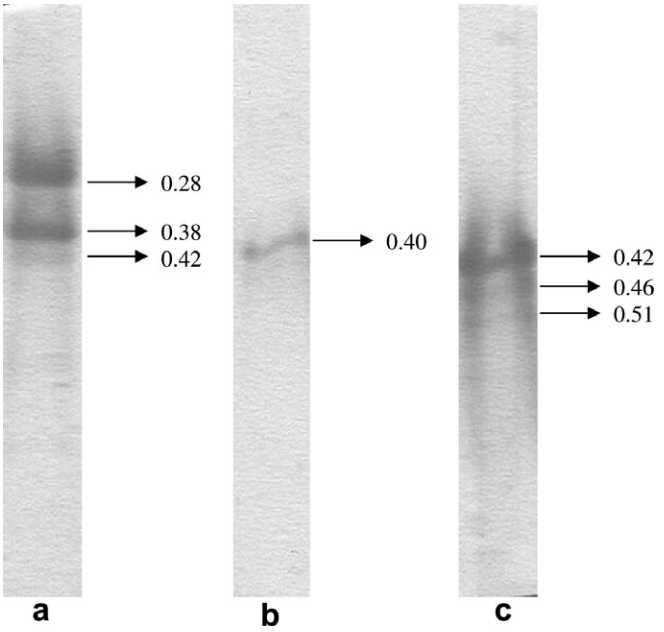

Fig. 1. Native electrophoresis of (a) *A. mellea,* (b) *L. nuda* and (c) *H. fasciculare*, stained with 24 mM *L*-DOPA.

3.3. Substrate specificity

Catechol, 4-methylcatechol, *L*-DOPA, DHPPA, *L*-tyrosine and PHPPA, at a final concentration of 10 mM, were used to monitor PPO activity. Maximum activity was achieved with 4-methylcatechol for the three crude enzymes (Table 1). There was no significant oxidation of monophenolic substrates. The inability of the enzymes to oxidize those phenolic compounds suggests that the enzymes lacked monophenolase activity and they could be diphenolases. This is similar to the findings for medlar fruit (Dincer et al., 2002), kiwi fruit (Park & Luh, 1985), and Bartlett pears (Rivas & Whitaker, 1973).

3.4. pH optimum and stability

The pH-relative activity (%) profiles of PPO activities were determined by using 4-methylcatechol as substrate. *A. mellea* PPO had two activity peaks, with higher activity at pH 7.0 than at pH 4.0. This result may be explained as an effect caused by the presence of isoenzymes. Both the *L. nuda* and the *H. fasciculare* PPOs had a maximum activity around pH 7.0 with a broad plateau between about pH 5.0 and 7.0 for *L. nuda* and 6.0 and 8.0 for *H. fasciculare* (Fig. 2).

PPO activity varies from pH 4.0–7.0, depending on the origin of the material, extraction method, the purity of enzyme, the type of buffer used and substrate (Alyward & Haisman, 1969). It is around pH 7.0 for PPO of cherry (Benjamin & Montgomery, 1973), pineapple (Das, Santhoor, & Gowda, 1997), Yali pear (Zhou & Feng, 1991), guava (Augustin, Ghazali, & Hashim, 1985), and it is around pH 4.0–5.0 for apple (Murata, Kurokami, & Homma, 1992), strawberry (Wesche-Ebeling & Montgomery, 1990) and green olive (Ben-Shamol, Kahn, Harel, & Mayer, 1977).

The stabilities of the crude enzymes were examined by incubating the extracts at different pH values at 4 °C for 24 h; as shown in Fig. 3, *A. mellea* PPO activity was slightly stimulated by keeping the enzyme extract between pH 5.0 and 9.0, and 84% of the activity was retained at pH 3.0. When kept at pH 4.0, *L. nuda* PPO activity was stimulated by about 46%. At pH 5.0 and pH 6.0, the retained activity of *L. nuda* was above 80%. *H. fasciculare* PPO was most

Table 1
Substrate specifities of *A. mellea*, *L. nuda* and *H. fasciculare*

Substrate	Specific activity (U/mg protein)		
	A. mellea	*L. nuda*	*H. fasciculare*
L-Tyrosine	0.003	0.002	No activity
3-(4-Hydroxyphenyl)-propionic acid	0.001	No activity	0.003
4-Methylcatechol	0.347	0.109	0.124
L-3,4-Dihydroxyphenylalanine	0.177	0.052	0.118
Catechol	0.187	0.067	0.111
3-(3,4-Dihydroxyphenyl) propionic acid	0.115	0.041	0.107

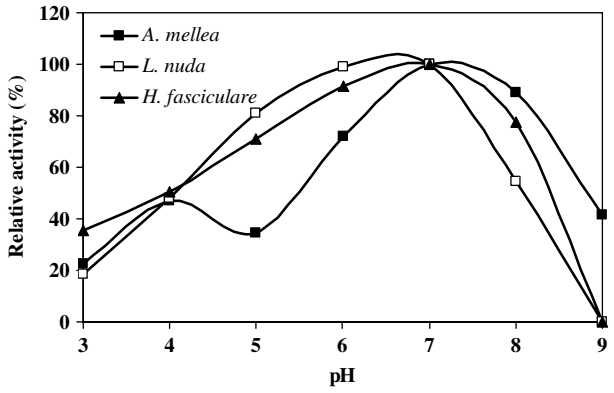

Fig. 2. Effect of pH on *A. mellea*, *L. nuda* and *H. fasciculare* PPOs.

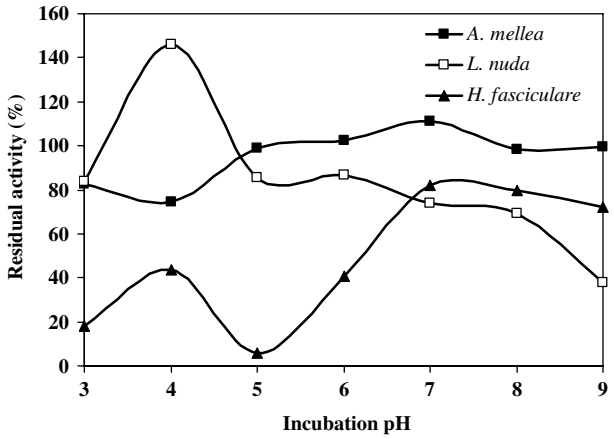

Fig. 3. pH stabilities of *A. mellea*, *L. nuda* and *H. fasciculare* PPOs.

stable at pH 7.0, and less stable at acidic pH values. It was reported that pear PPO was unstable below pH 3.5 (Rivas & Whitaker, 1973), plum PPO was unstable below pH 4.5 (Siddig, Sinha, & Cash, 1992). It was also reported that artichoke PPO was stable at pH 6.0 and 7.0 (Aydemir, 2004) and longan PPO was most stable at pH 7.0 (Jiang, 1999).

3.5. Temperature optimum and thermal stability

The dependence of PPO activity on temperature was studied between 10 and 80 °C. It was found that *A. mellea* and *H. fasciculare* PPOs had optimum temperatures of 30 and 20 °C, respectively. *L. nuda* had a shoulder between 20 and 40°C (Fig. 4). All of the three enzyme activities declined as the temperature increased but the enzymes were not completely inactivated, even at 80 °C. Optimal temperatures for PPO activity were reported by other authors to be between 20 and 40 °C (Siddig et al., 1992; Lee, Smith, & Pennesi, 1983; Yokotsuka, Makino, & Singleton, 1988; Cash, Sistrunk, & Stutte, 1976; Nakamura, Amano, & Kagami, 1983; Jen & Kahler, 1974; Ünal, 2007).

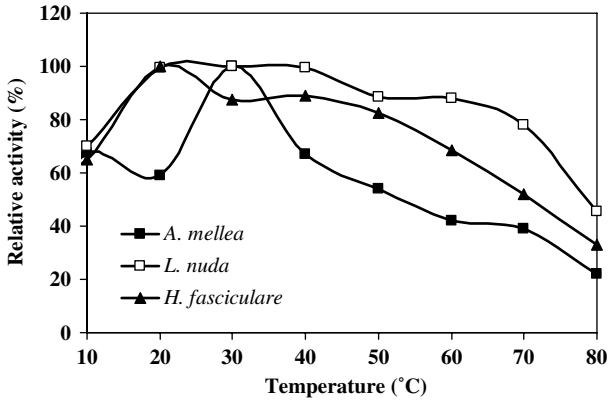

Fig. 4. Temperature optima of *A. mellea*, *L. nuda* and *H. fasciculare* PPOs for 4-methylcatechol as substrate.

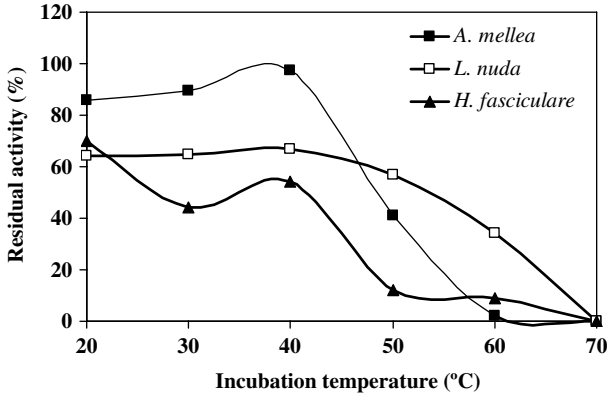

Fig. 5. Thermal stabilities of *A. mellea*, *L. nuda* and *H. fasciculare* PPOs.

The thermal stability profiles for crude *A. mellea*, *L. nuda* and *H. fasciculare* PPOs, presented in the form of the residual percentage activity, are shown in Fig. 5. *A. mellea* PPO was most stable at 40 °C after 1 h incubation. The enzyme lost of its activity rapidly above 40 °C. Rapid inactivation occurred from 50 to 60 °C and it was completely inactivated at 60 °C. *L. nuda* PPO was stable between 20 and 40 °C with nearly 65% of its original activity. About 70% of the *H. fasciculare* PPO activity remained after a heat treatment at 20 °C for 1 h. It can be concluded that it is not stable above 50 °C and completely lost its activity at 70 °C. It has been noted that heat stability of the PPO may be related to ripeness of the plant, and in some cases it is also dependent on pH. In addition, different molecular forms from the same source may also have different thermostabilities (Park & Luh, 1985; Zhou & Feng, 1991).

$E_a$values for the three PPO activities were calculated to be 66 kJ mol^{-1} K^{-1} for *A. mellea*, 16 kJ mol^{-1} K^{-1} for *L. nuda* and 39 kJ mol^{-1} K^{-1} for *H. fasciculare* from the plot of $1/T$ vs. In K and used for the calculation of $\Delta H^{\#}$ (Table 2). At temperatures of 20–60 °C, the average values of $\Delta H^{\#}$ were 64 ± 2 kJ mol^{-1} for *A. mellea*, 13 ± 1 kJ mol^{-1} for *L. nuda* and 36 ± 2 kJ mol^{-1} for *H. fasciculare*. The value of $\Delta S^{\#}$ was -837 ± 17 J mol^{-1} K^{-1} for *A. mellea* inactivation, -1005 ± 25 J mol^{-1} K^{-1} for *L. nuda* and -936 ± 26 J mol^{-1} K^{-1} for *H. fasciculare*. These results suggest that *A. mellea* PPO is more heat-resistant than are *L. nuda* and *H. fasciculare*, apparently as a result of the larger $\Delta H^{\#}$ value for inactivation. In general, $\Delta H^{\#}$ is seen as a measure of the number of non-covalent bonds broken in forming a transition state for enzyme inactivation (Duangmal & Owusu Apenten, 1999; Amiza & Apenten, 1994; Mazzafera & Robinson, 2000).

Table 2
Thermodynamic parameters for thermal inactivation of the three mushroom PPO activities

Temperature (°C)	$\Delta G^{\#}$ (kJ mol^{-1})			$\Delta H^{\#}$ (kJ mol^{-1})			$\Delta S^{\#}$ (J mol^{-1} K^{-1})		
	A. mellea	*L. nuda*	*H. fasciculare*	*A. mellea*	*L. nuda*	*H. fasciculare*	*A. mellea*	*L. nuda*	*H. fasciculare*
20	304	306	306	64	14	36	−818	−999	−919
30	313	317	318	64	14	36	−823	−1001	−931
40	320	327	328	64	13	36	−818	−1002	−933
50	340	338	342	64	13	36	−854	−1007	−947
60	354	351	353	64	13	36	−873	−1014	−952
Mean (±SD)	326 ± 24	328 ± 23	329 ± 24	64 ± 2	13 ± 1	36 ± 2	−837 ± 17	−1005 ± 25	−936 ± 26

3.6. Effect of substrate concentration on PPO activity and enzyme kinetics

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values of the enzymes are shown in Table 3. In the literature, different K_m values were reported for 4-methylcatechol: 10 mM for strawberry (Wesche-Ebeling & Montgomery, 1990), 3.24 mM for eggplant (Concellón, Añón, & Chaves, 2004), 9.8 mM for *Thymus* (Dogan & Dogan, 2004), and 94.3 mM for field bean (Gowda & Paul, 2002). Catalytic efficiencies, using the V_{max}/K_m ratio (Table 3), indicated that both PPOs, from *A. mellea* and *H. fasciculare*, were superior to *L. nuda* PPO against 4-methylcatechol as a diphenolic substrate.

3.7. Effect of protein concentration on PPO activity

For the three PPO activities, the plots of final protein concentration in the assay mixture vs. relative activity exhibited hyperbolic curves. The specific activity increased until the final concentration reached 0.02 mg/ml for *A. mellea* and *H. fasciculare*, and 0.01 mg/ml for *L. nuda*. The activities remained constant after these values.

3.8. Effect of some compounds on crude enzyme activity

The effects of benzoic acid, sodium metabisulphite, ascorbic acid, sodium azide, and *L*-cysteine on the crude PPO activities are shown in Table 4. I_{50} values were obtained with these PPO inhibitors, using 4-methylcatechol as substrate.

Of all the inhibitors tested in this study, sodium metabisulphite was the most effective for inhibition of *A. mellea* and *H. fasciculare* PPO activities. The action of sulphite in the prevention of enzymatic browning can usually be explained by several processes. One is the action of *o*-quinones. The formation of quinonesulphite complexes prevents the quinone polymerization (Embs & Markakis, 1965). A further action of metabisulphite on PPO is directly on the enzyme structure, leading to the inactivation of PPO (Golan-Goldhirsch & Whitaker, 1984). It has been suggested that sulphite reacts with disulphite bonds within PPO. This leads to change in the tertiary structure of enzyme and inactivation (Duangmal & Owusu Apenten, 1999).

Ascorbic acid was the most potent inhibitor of *L. nuda* PPO. In the assays, a lag period was observed before any changes in absorbance were measured. Similar findings have also been previously reported (Duangmal & Owusu Apenten, 1999; Halim & Montgomery, 1978; Ngalani, Signoret, & Crouzet, 1993). The mechanism of ascorbic acid inhibition involves the reduction of quinones generated by PPO (Sapers, 1993). PPO catalyses the oxidation of phenolic substrates to *o*-quinones, while ascorbic acid converts *o*-quinones back to phenolic compounds. After the lag period, when nearly all ascorbic acid is converted to dehydroascorbic acid, the amount of *o*-quinones formed by action of PPO increases. The *o*-quinones then polymerise and/or combine together with amino compounds to form high molecular weight brown pigments (Duangmal & Owusu Apenten, 1999).

It has been reported that sodium metabisulphite was a potent inhibitor of persimmon fruits (Özen et al., 2004), and ascorbic acid was a potent inhibitor of cherry laurel fruits (Colak et al., 2005). *Anoxybacillus kestanbolensis* K1 and K4^T catecholases were fully inhibited by addition of 0.01 mM sodium metabisulphite and ascorbic acid (Yildirim et al., 2005).

The effects of K^+, Co^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+} and Cr^{3+} on the PPO activities of *A. mellea*, *L. nuda* and *H. fasciculare* are shown in Table 5. While all three enzyme activities were inhibited by Cu^{2+} and Cr^{3+}, Co^{2+} activated all the enzymes. It was reported that *Macrolepiota mastoidea* diphenolase activity was inhibited by Cu^{2+}, and activated by Co^{2+} (Kolcuoğlu et al., 2007). Since metal ions may have different coordination numbers, different geometries in their coordination compounds, and different potentials as Lewis acids, they may behave differently toward proteins as ligands. These differences may also

Table 3
Kinetic parameters for the oxidation of 4-methylcatechol by *A. mellea*, *L. nuda* and *H. fasciculare* PPOs

	V_{max} (U mg protein^{-1})	K_m (mM)	V_{max}/K_m
A. mellea	0.73	1.20	0.61
L. nuda	0.21	9.19	0.02
H. fasciculare	0.25	0.51	0.49

Table 4
Inhibition of *A. mellea*, *L. nuda* and *H. fasciculare* PPOs by general polyphenol oxidase inhibitors

Inhibitor	I_{50} (mM)		
	A. mellea	*L. nuda*	*H. fasciculare*
Benzoic acid	6.20	10.80	6.50
Sodium metabisulphite	0.02	0.06	0.02
Ascorbic acid	0.06	0.024	0.05
Sodium azide	3.65	0.20	0.50
L-Cysteine	2.70	9.10	0.03

Table 5
Effect of various metal ions on *A. mellea*, *L. nuda* and *H. fasciculare* PPOs

Metal ion	Residual activity (%)		
	A. mellea	*L. nuda*	*H. fasciculare*
None	100	100	100
K^+	87	92	122
Co^{2+}	107	224	174
Ca^{2+}	107	81	123
Mn^{2+}	107	54	135
Cu^{2+}	23	5	15
Zn^{2+}	99	117	118
Cd^{2+}	104	71	127
Al^{3+}	98	109	113
Cr^{3+}	93	84	74

result in metal binding to different sites and, therefore, perturb the enzyme structure in different ways (DiTusa, Christensen, McCall, Fierke, & Toone, 2001).

It can be concluded that the crude extracts prepared from *A. mellea*, *L. nuda* and *H. fasciculare* possess diphenolase activities having greatest activity toward 4-methylcatechol. These enzymes appear to share some biochemical characteristics with several plant PPOs in terms of substrate specificity, pH and temperature optima, stability and kinetic parameters. In addition, the enzyme activities were very sensitive to some general PPO inhibitors, especially sodium metabisulphite and ascorbic acid. The oxidation of organic substrates with molecular oxygen under mild conditions is of great interest for industrial and synthetic prosesses, from an economic point of view (Gentshev, Möller, & Krebs, 2000). Therefore, it can be speculated that the wild mushroom PPOs studied in this work may be interesting for industrial applications.

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