Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 101 (2007) 778–785
Food Chemistry
www.elsevier.com/locate/foodchem

Comparative characterization of monophenolase and diphenolase activities from a wild edible mushroom (*Macrolepiota mastoidea*)

Yakup Kolcuoğlu [a], Ahmet Colak [a,*], Ertuğrul Sesli [b], Melike Yildirim [a], Nagihan Saglam [a]

[a] *Department of Chemistry, Karadeniz Technical University, 61080 Trabzon, Turkey*
[b] *Department of Science Education, Karadeniz Technical University, 61335 Trabzon, Turkey*

Received 4 April 2005; received in revised form 16 February 2006; accepted 16 February 2006

Abstract

In this study, *Macrolepiota mastoidea*, a wild edible mushroom, was evaluated for its polyphenol oxidase potential. Native electrophoresis, stained by L-dihydroxyphenylalanine, of the crude extracts from this species showed two bands having R_f values of 0.38 (minor) and 0.50 (major), supporting a polyphenol oxidase potential. The crude extracts showed monophenolase activity against 3-(4-hydroxyphenyl)-propionic acid and diphenolase activity against 4-methylcatechol as substrates. Monophenolase and diphenolase activities of enzyme extract prepared from *M. mastoidea* showed pH optimum values at pH 6.0 and pH 4.0, respectively. The extracts retained about 100% and 60% of their original monophenolase and diphenolase activities at their optimum pH values, respectively. It was estimated from thermodynamic data that *M. mastoidea* had a thermostable monophenolase activity. Thiourea and ascorbic acid were highly potential inhibitors for monophenolase, and ascorbic acid and sodium metabisulphite for diphenolase activity. It is clear from the present results that the enzyme extracts prepared from *M. mastoidea* possess polyphenol oxidase activities with interesting properties.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Diphenolase; Monophenolase; *Macrolepiota mastoidea*; Mushroom

1. Introduction

Polyphenol oxidase (PPO) is a copper enzyme which catalyzes two different reactions in the presence of oxygen: the hydroxylation of monophenol to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red, or black pigments (Mason, 1948; Prota, 1988). PPOs are essential for melanization (Mayer & Harel, 1979). In mammals, melanins are mainly found in skin and hair, and they have a protective function against UV radiation. In lower organisms, melanins are also protective polymers that constitute a primary response against chemicals and free radicals, toxic metal ions (Jacobson, 2000). PPO activity has also been found in plants and plays an important role in plant metabolism, including the respiration system, intermediary metabolism, regulation of the oxidation–reduction potential, antibiotic effects, and wound-healing (Mayer, 1987). In addition, enzymatic browning is the main function of PPOs in fruits and vegetables, and it is often undesirable and responsible for unpleasant sensory qualities and losses in nutrient quality (Sanchez-Amat & Solano, 1997).

PPO, especially having monophenolase activity, is very important for use in the removal of phenols from wastewater (Duran & Esposito, 2000). A PPO-modified solid composite biosensor has been developed, and its application for the determination of phenol and related compounds in environmental samples was studied (Svitel & Miertus, 1998). In addition, monophenolase activity of PPO also attracts scientific interest for use in the synthesis of

* Corresponding author. Tel.: +90 462 3772489; fax: +90 462 3773661.
E-mail address: acolak@ktu.edu.tr (A. Colak).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.02.035

L-DOPA, used for the treatment of Parkinson's disease (Ahmed & Vulphson, 1994).

The fruiting bodies of *Macrolepiota mastoidea* (Fr.) Singer which is a member of *Agaricaceae* family grow, solitary to gregarious, in open hardwood forests, under brush, on soil among grasses and herbs in collecting areas. The growing season of the mushroom is summer-fall in Turkey, and it can be found in large quantities in the collection area (Maçka, Trabzon, Turkey). The pileus of fructification organs is 70–150 mm across, pale ochre to brown, ovoid when young, later campanulate, plane when old, with a papilla. The flesh of the mushroom is white, thick in the centre of the pileus, thin toward the margin, with faint odour, mild taste. The stipe of the fruiting body is 80–150 × 8–15 mm, cylindrical, solid when young, hollow when old, pale ochre and floccose to smooth. *Macrolepiota* species are used as medicine for stomach and heart diseases by the people in the collection area. *M. mastoidea* is one of the commonly-consumed wild mushrooms in the collection area for its nutritional value (33% protein, 55% total carbohydrate, 4.5% crude fat, unpublished data) when it is abundantly available. Some local people dry fresh mushrooms in order to preserve them for future use. All of these factors increase its economic importance.

In this work, the characterization of PPO from *M. mastoidea*, a wild edible mushroom, 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 activity were studied.

2. Materials and methods

2.1. Materials and chemicals

M. mastoidea (Fr.) Singer, Lilloa 22: 417(1951) [1949], *Lepista nuda* (Bull.:Fr.) Corumooke, *Handkea excipuliformis* (scop.) Pers., Nova Hedwigia 48 (3–4): 283 (1989), and *Amanita rubescens* var . *rubescens* (Pers.) Gray, Tent. disp. meth. Fung.: 71 (1797) were collected directly from the Lişer Plateau-Maçka (Trabzon, Turkey). The mushrooms were carried into the laboratory in liquid nitrogen, with a nitrogen Dewar flask. The extracts were prepared as quickly as possible after the mushrooms were brought into the laboratory 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 and used as obtained.

2.2. Enzyme extraction

Crude enzyme fractions were prepared as reported previously (Dincer, Colak, Aydın, Kadioglu, & Güner, 2002; Colak, Özen, Dincer, Güner, & Ayaz, 2005). Mushrooms (10 g) were placed in a Dewar flask under liquid nitrogen for 15 min in order to decompose cell membranes. The cold mushrooms were separately homogenized by using a blender in 10 ml of acetate buffer (pH 4.0 and pH 5.0), phosphate buffer (pH 6.0 and pH 7.0), or Tris–HCl buffer (pH 8.0) containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 6% (w/v) Triton X-114, for 10 min at 4 °C. All of the buffers were cold and at 50 mM concentration. The homogenate was filtered and kept at 4 °C for 60 min before being centrifuged at 17.000*g* for 20 min at 4 °C. The supernatant was stored at −30 °C and used as crude enzyme.

2.3. Protein determination

Protein quantity in the enzyme extracts was determined according to the Lowry method with bovine serum albumin as standard (Lowry, Rosebrough, Farr, & Randall, 1951). The values were obtained by graphic interpolation on a calibration curve at 650 nm and it was found that the crude extract from *M. mastoidea* had an average of 2.9 ± 0.1 mg/ml of protein.

2.4. Enzyme assay

Polyphenol oxidase (PPO) activity was assayed by measuring the rate of increase in absorbance at a given wavelength, using an ATI Unicam UV2-100 double beam UV–Vis spectrophotometer (ATI Unicam, Cambridge, UK), as described previously (Colak et al., 2005; Dincer et al., 2002; Yildirim et al., 2005). The activity was determined by using different mono- or diphenolic 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 contained substrates (stock 100 mM), an equal volume of MBTH (stock 10 mM), and 20 μl of dimethylformamide (DMF), and the solution was diluted to 950 μl with buffer. After dilution, 50 μl of enzyme extract were added. The reference cuvette included all the reactants except the crude enzyme. One unit of PPO activity was defined as 1 μM of product formed per min in 1 ml of reaction mixture. Specific activity was defined as units of enzyme activity per mg of protein (Kong, Hong, Choi, Kim, & Cho, 2000; Yildirim et al., 2005).

2.5. Screening of mushrooms for their PPO potentials

The mushrooms were screened for their PPO potentials by the method reported previously (Colak et al., 2005; Dincer et al., 2002; Espin et al., 1995; Özen, Colak, Dincer, & Güner, 2004). In the crude enzyme extracts prepared within different buffers, as described above, the PPO activity was determined by using 50 mM phosphate buffer, pH 7.0 as buffer and 4-methylcatechol as substrate.

2.6. Characteristics of the crude enzyme

2.6.1. Substrate specificity and enzyme kinetics

PPO activity was determined by using L-tyrosine and 3-(4-hydroxyphenyl)-propionic acid (PHPPA) as monophenolic substrates and 4-methylcatechol, catechol and L-3, 4-dihydroxyphenylalanine (L-DOPA) as diphenolic substrates with MBTH (Espin et al., 1995) in 50 mM buffer at optimum pH of each substrate.

The kinetic data were plotted as reciprocals of activities vs. substrate concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined as the reciprocal absolute values of the intercepts on the x- and y-axes, respectively, of the linear regression curve. Substrate specificity (V_{max}/K_m) was calculated by using the data obtained on a Lineweaver–Burk plot (Lineweaver & Burk, 1934).

2.6.2. Effect of pH on PPO activity and pH stability

The effects of pH on the monophenolase and diphenolase activities were determined by using PHPPA and 4-methylcatechol, respectively, with the following buffers (50 mM) at the indicated pH; glycine–HCl buffer (pH 3.0), acetate buffer (pH 4.0 and pH 5.0), phosphate buffer (pH 6.0 and pH 7.0) and Tris–HCl buffer (pH 8.0 and pH 9.0). The optimum pH obtained for both substrates was used for determining thermal properties and other parameters (Colak et al., 2005; Özen et al., 2004).

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 by using 50 mM phosphate buffer, pH 6.0, for PHPPA and 50 mM acetate buffer, and pH 4.0 for 4-methylcatechol (Colak et al., 2005; Özen et al., 2004).

2.6.3. Effect of temperature on PPO activity and thermal stability

PPO activity was assayed at various temperatures over the range 10–70 °C, using a circulation water bath. The reaction mixtures at the optimum pH of each substrate, containing all the reagents except crude enzyme, were incubated for 5 min at various temperatures as indicated above. The enzyme extract was added to the mixtures and the activity of PPO was determined spectrophotometrically at 500 nm for PHPPA and 494 nm for 4-methylcatechol as rapidly as possible.

In order to determine the thermal stability of the enzyme, the enzyme extracts in Eppendorf tubes were incubated at various temperatures of 20–80 °C with 10 °C increments for 60 min, rapidly cooled in an ice bath for 5 min, and then brought to 25 °C. After the mixture reached room temperature, the enzyme activity was assayed under the assay conditions. The percentage residual PPO activity was calculated by comparison with unincubated enzyme (Colak et al., 2005; Dincer et al., 2002; Yildirim et al., 2005). The data obtained from the thermal stability profile were used to analyze some thermodynamic parameters.

2.6.4. Effect of protein concentration on PPO activity

PPO activity, as a function of protein concentration, was determined in a protein concentration range of 0.01–0.5 mg/ml for PHPPA and 0.005–0.25 mg/ml for 4-methylcatechol. The activity was assayed under standard conditions using various volumes of the enzyme extract (Yildirim et al., 2005).

2.6.5. Effect of various inhibitors on enzyme activity

Sodium azide, benzoic acid, sodium metabisulfite, ascorbic acid, thiourea, and cysteine were evaluated for their effectiveness as inhibitors of PPO activity. An aliquot of each inhibitor at various final concentrations was added to the standard reaction mixture immediately before the addition of enzyme extract. Relative enzymatic activity was calculated as a percentage of the activity in the absence of inhibitor. The concentration of inhibitor giving 50% inhibition (IC_{50}) was determined from plot of residual activity against inhibitor concentration (Colak et al., 2005; Yildirim et al., 2005).

2.6.6. Effect of metal ions on PPO activity

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

2.6.7. Native polyacrylamide gel electrophoresis

Before electrophoresis, the enzyme extract was precipitated using an equal volume of cold acetone (−30 °C). 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 6.0). After being stirred for 5 min, the suspension was centrifuged at 5000 rpm for 5 min. The supernatant was used as the enzyme sample for electrophoresis (Duangmal & Owusu Apenten, 1999).

Native polyacrylamide gel electrophoresis was performed on a Hoeffer SE 600 Series Electrophoresis dual slab cell unit (California, USA), using preparative 8% polyacrylamide gels (Laemmli, 1970) under native conditions. After electrophoresis, the gel was stained for PPO activity in 24 mM L-DOPA.

3. Results and discussion

In the present study, polyphenol oxidase (PPO) potentials of four mushroom species were evaluated. The screening of PPO activities in the presence of 4-methylcatechol showed that *M. mastoidea* possessed the greatest PPO activity (Table 1). In all of the studies, the *M. mastoidea* enzyme extract prepared with 50 mM acetate buffer, pH

Table 1
Polyphenol oxidase potentials of the mushroom species in the presence of 4-methylcatechol as a substrate

Extraction pH	Specific activity (μM min^{-1} mg protein^{-1})				
	4.0	5.0	6.0	7.0	8.0
Macrolepiota mastoidea	164 ± 2.2	145 ± 2.0	78.9 ± 0.8	17.3 ± 0.5	10.2 ± 0.5
Lepista nuda	99.5 ± 1.5	99.4 ± 1.6	82.1 ± 0.8	77.4 ± 1.2	75.2 ± 1.4
Handkea excipuliformis	17.2 ± 0.5	15.0 ± 0.2	4.3 ± 0.2	3.7 ± 0.2	No activity
Amanita rubescens	97.2 ± 1.7	83.7 ± 1.3	No activity	No activity	No activity

4.0 was used because the PPO activity was highest in this crude enzyme extract.

Native polyacrylamide gel electrophoresis resulted in two isoforms of *M. mastoidea* PPO, having R_f values of 0.38 (minor) and 0.50 (major), respectively, indicating the presence of at least two PPO isoenzymes (Fig. 1). Two to four PPO isoenzymes have been reported for different fruits. Park and Luh (1985) reported four forms of PPO as isoenzymes in kiwis. Rivas and Whitaker (1973) reported the existence of two types of PPO in Bartlett pears and Flurkey and Jen (1980) reported three types of PPO in peach.

3.1. Substrate specificity and enzyme kinetics

L-Tyrosine and 3-(4-hydroxyphenyl)-propionic acid (PHPPA) as monophenolic substrates, and 4-methylcatechol, catechol and L-3,4-dihydroxyphenylalanine (L-DOPA), as diphenolic substrates were tested for substrate specificity of the *M. mastoidea* PPO. While 4-methylcatechol, catechol, L-DOPA, and PHPPA were oxidized by crude *M. mastoidea* PPO, there was no significant oxidation of L-tyrosine (Table 2). The crude enzyme showed the greatest activity toward PHPPA as monophenolic substrate and 4-methylcatechol as diphenolic substrate. The relative activities of the enzyme, based on the wavelength maximum of the product, were compared with the activities in the presence of PHPPA as 100% for monophenolase activity and in the presence of 4-methylcatechol as 100% for diphenolase activity. These results are consistent with the previous reports on the mushroom PPOs containing both monophenolase and diphenolase activities (Fenol et al., 2000; Ratcliffe, Flurkey, Kuglin, & Dawley, 1994; Zhang & Flurkey, 1997; Seo, Sharma, & Sharma, 2003). In addition, extracted PPO from different sources has been shown to have varying substrate specificity (Wong, Luh, & Whitaker, 1971; Jen & Kahler, 1974).

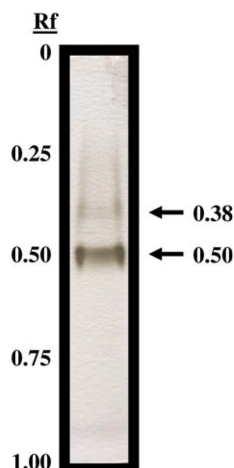

Fig. 1. Activity staining for PPO from *M. mastoidea* mushroom with 24 mM L-DOPA.

Table 2
Substrate specificities of *M. mastoidea* crude polyphenol oxidase

Substrate	Wavelength (nm)[a]	Relative Activity (%)
Monophenols		
L-Tyrosine	500	1.8 ± 0.3
PHPPA	500	100 ± 2.4
Diphenols		
4-Methylcatechol	494	100 ± 2.5
Catechol	500	66.8 ± 1.6
L-DOPA	500	13.5 ± 0.5

[a] Espin et al., 1995.

Substrate saturation curves for PHPPA and 4-methylcatechol indicated that the *M. mastoidea* PPO follows simple Michaelis–Menten kinetics. Michaelis–Menten constants (K_m) and maximum reaction velocities (V_{max}) were determined using PHPPA and 4-methylcatechol at various concentrations. The Lineweaver–Burk plot analysis of this enzyme preparation showed 2.7 and 5.3 mM K_m values, and 41.0 and 384.6 μM min^{-1} mg protein^{-1} V_{max} values for PHPPA and 4-methylcatechol, respectively. Abukharma and Woolhouse (1966) reported that 4-methylcatechol was the preferred substrate for PPO from the King Edward variety of potato. Walker (1995) also noted that 4-methylcatechol was usually the best substrate for plant diphenolases. However, there have been few studies on monophenolase activity (Rodriguez-Lopez, Tudela, Varon, Garcia-Carmona, & Garcia-Canovas,1992; Rodriguez-Lopez et al., 1992).

3.2. Effect of pH on PPO activity and stability

Different pH optima were determined for each substrate. The pH optima were 6.0 in the presence of PHPPA as a monophenolic substrate and 4.0 in the presence of 4-methylcatechol as a diphenolic substrate (Fig. 2). The pH optimum for 4-methylcatechol was found to be very broad between 3.0 and 5.0, with more than 90% of the maximum

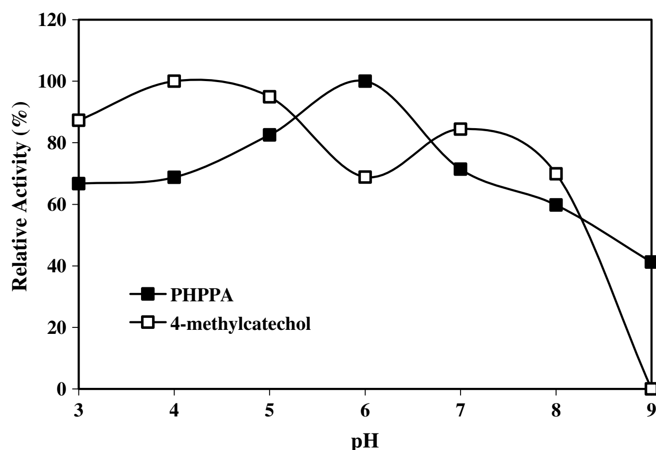

Fig. 2. pH-activity profile for *M. mastoidea* PPO in 50 mM Glycine–HCl buffer (pH 3.0), in 50 mM acetate buffer (pH 4.0 and pH 5.0), in 50 mM phosphate buffer (pH 6.0 and pH 7.0), and in 50 mM Tris–HCl buffer (pH 8.0 and pH 9.0).

activity occurring between these limits. The second peak of diphenolase activity at pH 7.0 might indicate different enzymatically-active diphenolase isoforms present in the crude enzyme preparations (Van Gelder, Flurkey, & Wichers, 1997). Differences in PPO pH optima with various substrates were reported (Aylward & Haisman, 1969), varying from 4.0 to 7.0, depending on the origin of the material, extraction method and substrate used for assay.

The pH stability of the enzyme was examined by incubating the crude enzyme extract at different pH values at 4 °C for 24 h (Fig. 3). *M. mastoidea* monophenolase activity was stimulated by approximately 71% and 12%, by keeping at pH 4.0 and pH 7.0, respectively, and retained all of its original activity at pH 6.0 and 8.0 but was not stable at pH 3.0. It was reported that pear PPO was unstable at pH values below 3.5 (Rivas & Whitaker, 1973) and plum PPO was unstable below 4.0 (Siddig, Sinha, & Cash, 1992). *M. mastoidea* diphenolase activity retained more than 95% of its original activity at physiological pH values. Incubation of the enzyme for 24 h at pH 4.0, 5.0 and 6.0 caused 41%, 51% and 27% loss of activity, respectively.

3.3. Effect of temperature on PPO activity and thermal stability

Thermal activity data for both enzyme activities are presented in Fig. 4. The optimum temperatures were 30 °C and 20 °C for monophenolase and diphenolase activity, respectively. It appears that the diphenolase activity is more sensitive to temperatures above 40 °C. Optimum temperature values of monophenolase and diphenolase activities were similar to those of Amasya apple (18 °C) (Oktay, Küfrevioğlu, & Şakiroğlu, 1995), artichoke (25 °C) (Aydemir, 2004), loquat (30 °C) (Ding, Chachin, Ueda, & Imahori, 1998), taro and potato (25 and 30 °C, respectively) (Duangmal & Owusu Apenten, 1999).

The thermal stability profile for crude *M. mastoidea* PPO, presented in the form of the residual percentage activity, is shown in Fig. 5. The monophenolase retained 85% of its original activity at its optimum temperature. The enzyme was quite stable at 20, 30 and 40 °C for 60 min but unstable at temperatures above 60 °C. Heating at 70 and 80 °C for 60 min almost fully inactivated monophenolase. Although *M. mastoidea* diphenolase retained all its original activity at 20 °C for a period of 60 min, the activity was stimulated by keeping at 30 and 40 °C, by approximately 33% and 46%, respectively. It has been reported that *Anoxybacillus kestanbolensis* K1 catecholase was stimulated by keeping at 80 °C (Yildirim et al., 2005).

E_a values for monophenolase and diphenolase activities were calculated to be 92.1 and 52.2 kJ mol^{-1}, respectively, from the plot of $1/T$ vs. $\ln k$ and used for the calculation of $\Delta H^{\#}$ (Table 3). The average values for $\Delta H^{\#}$ and $\Delta S^{\#}$ were 89.3 ($\pm$1.3) kJ mol^{-1} and -760 ($\pm$16) J mol^{-1} K^{-1} for monophenolase and 49.8 ($\pm$1.2) kJ mol^{-1} and -886 ($\pm$18) J mol^{-1} K^{-1} for diphenolase heat-inactivation, respectively. Results from Table 3 suggest that *M. mastoidea* has a high thermostable monophenolase activity. In

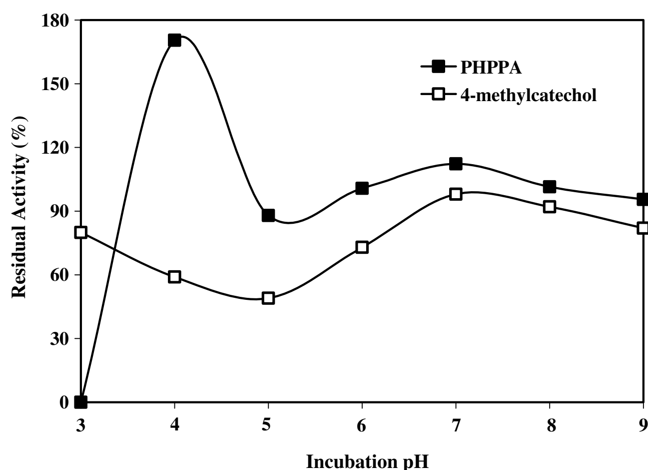

Fig. 3. pH stability of *M. mastoidea* monophenolase and diphenolase.

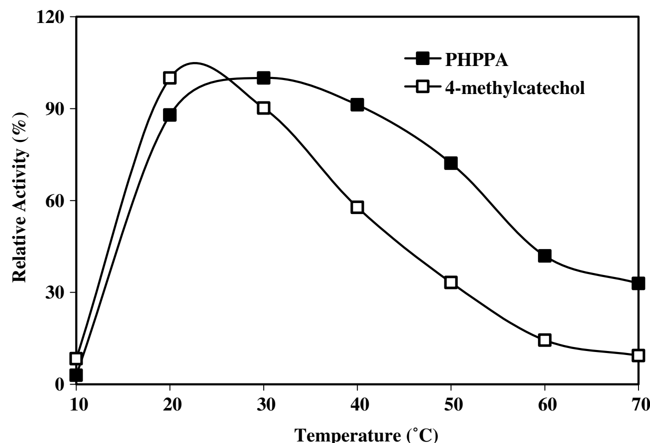

Fig. 4. Temperature optima of *M. mastoidea* monophenolase and diphenolase.

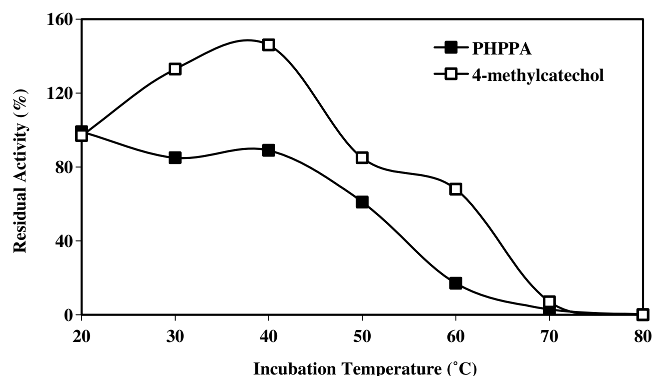

Fig. 5. Thermal stability of *M. mastoidea* monophenolase and diphenolase.

general, $\Delta H^{\#}$ is seen as a measure of the number of non-covalent bonds broken in forming a transition state for enzyme inactivation (Amiza & Apenten, 1994; Duangmal & Owusu Apenten, 1999; Mazzafera & Robinson, 2000).

3.4. Effect of protein concentration on PPO activity

The enzyme activities were found to be protein concentration-dependent. For both activities, the plots of final protein concentration in the assay mixture vs. activity exhibited hyperbolic curves. Monophenolase and diphenolase activities increased until the final protein concentration, under the standard assay conditions, reached 0.2 and 0.1 mg/ml, respectively, and remained constant after these values for each.

3.5. Effects of various inhibitors on enzyme activity

Sodium azide, benzoic acid, sodium metabisulfite, ascorbic acid, thiourea, and cysteine were examined to determine their potentials for inhibition of *M. mastoidea* PPO activity. Their potentials for the inhibition of *M. mastoidea* monophenolase and diphenolase activities were presented as IC_{50} values, calculated from the plots of inhibitor concentrations vs. percentage inhibition of PHPPA and 4-methylcatechol oxidation (Table 4).

Table 4
Inhibition of *M. mastoidea* monophenolase and diphenolase activities by some general PPO inhibitors

Inhibitors	IC_{50} (mM)	
	Monophenolase activity	Diphenolase activity
Sodium azide	1.40	0.27
Benzoic acid	0.84	0.64
Sodium metabisulfite	0.47	0.06
Ascorbic acid	0.04	0.04
Thiourea	0.01	0.26
Cysteine	0.37	4.90

All the compounds used in this study inhibited both activities. Inhibition assays indicated that ascorbic acid and sulphur-containing compounds, such as thiourea and cysteine, with low IC_{50} values, were potent inhibitors of the *M. mastoidea* monophenolase. The strongest inhibition of PPO occurred with thiourea, as previously reported (Zhou, Smith, & Lee, 1993). For the diphenolase activity of *M. mastoidea* PPO, the most effective inhibitor was ascorbic acid, followed by sodium metabisulfite and thiourea. These results were consistent with the earlier reports (Ding et al., 1998; Friedman & Bautista, 1995; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000).

The mechanism of inhibition by ascorbate may involve reduction of quinonoid compounds produced by the diphenolases, and chelation or reduction of copper centres at the active site of the enzyme (Martinez & Whitaker, 1995; Sapers, 1993; Zawistowski, Biliaderis, & Eskin, 1991). Ascorbic acid has also been reported to cause irreversible inhibition (Golan-Goldhirsch & Whitaker, 1984). Inhibition by the thiol compounds is attributed to either the stable colourless products formed by addition reaction with *o*-quinones (Ikediobi & Obasuyi, 1982) or binding to the active centre of PPO, e.g. metabisulfite (Valero & Garcia-Carmona, 1992).

3.6. Effect of metal ions on PPO activity

The effect of various metal ions on the both activities of *M. mastoidea* PPO are shown in Table 5. The concentrations of all metal ions tested were 1 mM and the enzyme activity was assayed under standard conditions. While Co^{2+} stimulated *M. mastoidea* monophenolase by 46%,

Table 3
Thermodynamic parameters for thermal inactivation of *M. mastoidea* monophenolase and diphenolase activities

Temperature (°C)	Monophenolase activity			Diphenolase activity		
	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})
20	297	90	−706	300	50	−854
30	314	90	−741	316	50	−878
40	324	89	−748	327	50	−886
50	338	89	−770	335	50	−884
60	352	89	−790	348	49	−896
70	365	89	−804	364	49	−917
Mean (±SD)	332 (±24.2)	89.3 (±1.3)	−760 (±16)	332 (±25.3)	49.8 (±1.2)	−886 (±18)

Note: E_a values for monophenolase and diphenolase activities were calculated to be 92.1 and 52.2 kJ mol^{-1}, respectively, from the plot of $1/T$ vs. $\ln k$ and used for the calculation of $\Delta H^{\#}$.

Table 5
Effects of various metal ions on both activities of *M. mastoidea*

Metal ion	Relative activity (%)	
	Monophenolase Activity	Diphenolase Activity
None	100	100
K^+	97.4	126
Mn^{2+}	132	134
Co^{2+}	146	122
Cd^{2+}	82	156
Ca^{2+}	93.6	130
Ni^{2+}	97.4	127
Cu^{2+}	3.4	95.5
Zn^{2+}	77.3	110
Hg^{2+}	0.9	100
Al^{3+}	134	136

Cu^{2+} and Hg^{2+} caused almost complete inhibition of the enzyme. *M. mastoidea* diphenolase was activated by all of the metal ions used except Hg^{2+} and Cu^{2+}, while Cu^{2+} inhibited diphenolase activity by approximately 4.5% and Hg^{2+} had no effect on the activity. Since metal ions may have different coordination numbers and geometries in their coordination compounds, and potentials as Lewis acids, they may behave differently toward proteins as ligands. These differences may also result in metal binding to different sites, and therefore, perturb the enzyme structure in different ways (Bock, Katz, Markham, & Glusker, 1999; DiTusa, Christensen, McCall, Fierke, & Toone, 2001).

It can be concluded that the crude extracts prepared from *M. mastoidea* mushroom possess polyphenol oxidase having greatest substrate specificity to PHPPA and 4-methylcatechol. The enzyme appears to have biochemical characteristics similar to several plant PPOs in terms of substrate specificity, pH and temperature optima, stability and kinetic parameters. Moreover, *M. mastoidea* PPO was very sensitive to some general PPO inhibitors, especially to thiourea, ascorbic acid and metabisulfite. It is, furthermore, noteworthy that *M. mastoidea* has monophenolase activity, which has been less studied than diphenolase activity.

Acknowledgement

This work was financially supported by the KTU-BAP to AC (2004.111.002.9). The authors thank Nazan Yildiz for her proof reading.

References

Abukharma, D. A., & Woolhouse, H. U. (1966). The preparation and properties of *o*-diphenol: oxygen oxidoreductase from potato tubers. *New Phytologist, 65*, 477–478.

Ahmed, G., & Vulphson, E. N. (1994). Facile synthesis of L-DOPA esters by the combined use of tyrosinase and α-chymotrypsin. *Biotechnology Letters, 16*, 367–372.

Amiza, M. A., & Apenten, R. K. O. (1994). Thermal inactivation parameters for alkaline proteinases from North Sea cod (*Gadus morhua*) and α-chymotrypsin bovine. *Journal of the Science of Food and Agriculture, 66*, 389–391.

Aydemir, T. (2004). Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads. *Food Chemistry, 87*, 59–67.

Aylward, F., & Haisman, D. R. (1969). Oxidation systems in fruits and vegetables their relation to quality of preserved products. *Advanced Food Research, 17*, 3–17.

Bock, W. C., Katz, A. K., Markham, G. D., & Glusker, J. P. (1999). Manganese as a replacement for magnesium and zinc: functional comparison of the divalent ions. *Journal of The American Chemical Society, 121*, 7360–7372.

Colak, A., Özen, A., Dincer, B., Güner, S., & Ayaz, F. A. (2005). Diphenolases from two cultivars of cherry laurel (*Laurocerasus officinalis* Roem.) fruits at early stage of maturation. *Food Chemistry, 90*, 801–807.

Dincer, B., Colak, A., Aydın, N., Kadioglu, A., & Güner, S. (2002). Characterization of polyphenol oxidase from medlar fruits (*Mespilus germanica* L. Rosaceae). *Food Chemistry, 77*, 1–7.

Ding, C. K., Chachin, K., Ueda, Y., & Imahori, Y. (1998). Purification and properties of polyphenol oxidase from loquat fruit. *Journal of Agricultural and Food Chemistry, 46*, 4144–4149.

DiTusa, C. A., Christensen, T., McCall, K. A., Fierke, C. A., & Toone, E. J. (2001). Thermodynamics of metal ion binding. 1. Metal ion binding by wild-type carbonic anhydrase. *Biochemistry, 40*, 5338–5344.

Duangmal, K., & Owusu Apenten, R. K. (1999). A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. Romano). *Food Chemistry, 64*, 351–359.

Duran, N., & Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental, 28*, 83–99.

Espin, J. C., Morales, M., Varon, R., Tudela, J., & Garcia-Canovas, F. (1995). A continuous spectrophotometric method for determining the monophenolase and diphenolase activities of apple polyphenol oxidase. *Analytical Biochemistry, 43*, 2807–2812.

Fenol, G. L., Rodriguez-Lopez, J. N., Garcia-Sevilla, F., Tudela, J., Garcia-Ruiz, A. P., Varon, R., & Garcia-Canovas, F. (2000). Oxidation by mushroom tyrosinase of monophenols generating slightly unstable *o*-quinones. *European Journal of Biochemistry, 267*, 5865–5878.

Flurkey, W. H., & Jen, J. J. (1980). Purification of peach polyphenol oxidase in the presence of added protease inhibitors. *Journal of Food Biochemistry, 4*, 29–41.

Friedman, M., & Bautista, F. F. (1995). Inhibition of polyphenol oxidase by thiols in the absence and presence of potato tissue suspensions. *Journal of Agricultural and Food Chemistry, 43*, 69–76.

Golan-Goldhirsch, A., & Whitaker, J. R. (1984). Effects of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *Journal of Agricultural and Food Chemistry, 32*(5), 1003–1009.

Ikediobi, C. O., & Obasuyi, H. N. (1982). Purification and some properties of *o*-diphenolase from white yam tubers. *Phytochemistry, 21*, 2815–2820.

Jacobson, E. S. (2000). Pathogenic roles of fungal melanins. *Clinical Microbiology Reviews, 13*, 708–717.

Jen, J. J., & Kahler, K. R. (1974). Characterization of polyphenol oxidase in peaches grown in the Southeast. *Hortscience, 9*, 590–591.

Kong, K. H., Hong, M. P., Choi, S. S., Kim, Y. T., & Cho, S. H. (2000). Purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. *Biotechnology and Applied Biochemistry, 31*, 113–118.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constant. *Journal of American Chemical Society, 56*, 658–661.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Martinez, M., & Whitaker, J. R. (1995). The biochemistry and control of enzymatic browning. *Trends in Food Science Technology, 6*, 195–200.

Mason, H. S. (1948). Chemistry of melanin. Mechanism of oxidation of 3,4-dihydroxyphenylalanine by tyrosinase. *Journal of Biological Chemistry, 72*, 83–99.

Mayer, A. M. (1987). Polyphenol oxidase in plants-recent progress. 2. *Phytochemistry, 26*, 11–20.

Mayer, A. M., & Harel, E. (1979). Polyphenoloxidase in plants. *Phytochemistry, 18*, 193–215.

Mazzafera, P., & Robinson, S. P. (2000). Characterization of polyphenol oxidase in coffee. *Phytochemistry, 55*, 285–296.

Oktay, M., Küfrevioğlu, I., & Şakiroğlu, H. (1995). Polyphenol oxidase from Amasya apple. *Journal of Food Science, 60*, 494–496.

Özen, A., Colak, A., Dincer, B., & Güner, S. (2004). A diphenolase from persimmon fruits (*Diospyros kaki* L., Ebenaceae). *Food Chemistry, 85*, 431–437.

Park, E. Y., & Luh, B. S. (1985). Polyphenol oxidase of kiwi fruit. *Journal of Food Science, 50*, 679–684.

Prota, G. (1988). Progress in the chemistry of melanins and related metabolites. *Medicinal Research Review, 8*, 525–556.

Ratcliffe, B., Flurkey, W. H., Kuglin, J., & Dawley, R. (1994). Tyrosinase, laccase, and peroxidase in mushroom. *Journal of Food Science, 59*(4), 824–827.

Rivas, N. J., & Whitaker, J. R. (1973). Purification and some properties of two polyphenol oxidase from Bartlett pears. *Plant Physiology, 52*, 501–507.

Rodriguez-Lopez, J. N., Tudela, J., Varon, R., Garcia-Carmona, F., & Garcia-Canovas, F. (1992). Analysis of a kinetic model for melanin biosynthesis pathway. *Journal of Biological Chemistry, 267*, 3801–3810.

Sanchez-Amat, A., & Solano, F. (1997). A pluripotent polyphenol oxidase from the melanogenic marine *alteromonas* shares catalytic capabilities. *Biochemical and Biophysical Research Communications, 240*, 787–792.

Sapers, G. M. (1993). Browning of foods: control by sulfites, antioxidants and other means. *Food Technology, 47*, 75–81.

Seo, S. Y., Sharma, V. K., & Sharma, N. (2003). Mushroom tyrosinase: recent prospects. *Journal of Agricultural Food Chemistry, 51*, 2837–2853.

Siddig, M., Sinha, N. K., & Cash, Y. N. (1992). Characterization of a polyphenol oxidase from Stanley plums. *Journal of Food Science, 57*, 1177–1179.

Svitel, J., & Miertus, S. (1998). Development of tyrosinase based biosensor and its application for monitoring of bioremediation of phenol and phenolic compounds. *Environmental Science and Technology, 32*, 828–835.

Valero, E., & Garcia-Carmona, F. (1992). Hysteresis and cooperative behaviour of latent plant polyphenol oxidase. *Plant Physiology, 98*, 774–776.

Van Gelder, C. W. G., Flurkey, W. H., & Wichers, H. J. (1997). Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry, 45*(7), 1309–1323.

Walker, J. R. L. (1995). Enzymatic browning in fruits: its biochemistry and control. In Y. L. Chang & J. R. Whitaker (Eds.), *Enzymatic browning and its prevention. ACS symposium series* (Vol. 600, pp. 8–22). Washington, DC: American Chemical Society.

Wong, T. C., Luh, B. S., & Whitaker, J. R. (1971). Isolation and polyphenol oxidase isoenzymes of clingstone peach. *Plant Physiology, 48*, 19–23.

Yang, C.-P., Fujita, S., Ashrafuzzaman, M. D., Nakamura, N., & Hayashi, N. (2000). Purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) pulp. *Journal of Agricultural and Food Chemistry, 48*, 2732–2735.

Yildirim, M., Col, M., Colak, A., Güner, S., Dülger, S., & Beldüz, A. O. (2005). Diphenolases from *Anoxybacillus kestanbolensis* strains K1 and $K4^T$. *World Journal of Microbiology & Biotechnology, 21*, 501–507.

Zawistowski, J., Biliaderis, C. G., & Eskin, N. A. M. (1991). Polyphenol oxidase. In D. S. Robinson & N. A. M. Eskin (Eds.), *Oxidative enzymes in foods* (pp. 217–273). London: Elsevier.

Zhang, X., & Flurkey, W. H. (1997). Phenoloxidase in Portabella mushrooms. *Journal of Science, 62*, 97–100.

Zhou, P. G., Smith, N. L., & Lee, C. Y. (1993). Partial purification and some properties of Monroe apple peel polyphenol oxidase. *Journal of Agricultural and Food Chemistry, 41*, 532–536.