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Purification and characterisation of a polyphenol oxidase from *Boletus erythropus* and investigation of its catalytic efficiency in selected organic solvents

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ABSTRACT

Polyphenol oxidase (PPO) was purified from *Boletus erythropus* using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column. Optimum pH and temperature were found to be 8.0 and 20 °C, respectively, using 4-methylcatechol as a substrate. The enzyme was extremely stable between pH 3.0 and 9.0 after 24 h incubation at 4 °C. *B. erythropus* PPO was also quite stable between 10 and 30 °C after 4 h incubation. The K_m and V_{max} values were calculated as 2.8 mM and 1430 U/mg protein by Lineweaver–Burk curve, respectively. The enzyme activity was inhibited by sodium metabisulfite, ascorbic acid, sodium azide and benzoic acid. It was seen that the mushroom PPO was an effective biocatalyst in selected organic solvents, such as dichloromethane, dichloroethane and toluene, when catechin was used as a substrate. All data support that *B. erythropus* has a highly active PPO, possessing similar biochemical and kinetic characteristics to other plant PPOs.

1. Introduction

Polyphenol oxidases (PPOs) are a group of copper proteins distributed throughout microorganisms, plants and animals (Mayer, 2006). PPOs catalyse two different reactions in the presence of molecular oxygen: hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). The quinones then polymerise to melanins, which are brown, red or black pigments (Zawistowski, Biliaderis, & Eskin, 1991).

The nutritional and medicinal values of many wild edible mushrooms have long been known. While they are low in calories and fats, they contain abundantly essential fatty acids, vegetable proteins, vitamins and minerals (Agrahar-Murugkar & Subbulakshmi, 2005). Some edible mushroom species are also sources of physiological agents for medicinal applications, possessing antitumour, cardiovascular, antiviral, antibacterial and other activities (Halpern & Miller, 2002).

Fungal tyrosinases, a type of PPO, were firstly characterised from the edible mushroom *Agaricus bisporus* (Wichers, Gerritsen, & Chapelon, 1996) because of enzymatic browning during development and post-harvest storage, which particularly decreases the commercial and nutritional value of the product. Some other mushroom PPOs have also been subjected to further characterisation studies in terms of their biochemical characteristics and their potential in biotechnological applications (Colak, Sahin, Yildirim, & Sesli, 2007b; Kolcuoğlu, Colak, Sesli, Yildirim, & Saglam, 2007).

Mayer (2006) reviewed the potential use and the role of PPOs in organic synthesis reactions or biosynthetic processes. Enzymatic bioconversion in appropriate organic solvents as a reaction medium can constitute an alternative to chemical synthesis when natural compounds are required. Bioconversion of monophenols into diphenols in the presence of tyrosinase as a biocatalyst encouraged researchers to study the production of some molecules with beneficial properties as food additives or pharmaceutical drugs such as L-DOPA in this way (Halaouli, Asther, Sigoillot, Hamdi, & Lomascolo, 2006).

Studies concerning the purification and characterisation of PPOs from new sources are very important in explaining their biochemical properties and behaviour. Thus, a particular enzyme may find applications in the food or drug industries; enzyme activity causing undesired browning can be inhibited and nutritional value and shelf-life of food can be increased. Purification and characterisation of PPOs will also enlighten researchers to perform further studies such as immobilisation and bioengineering.

In this work, PPO was purified from *Boletus erythropus*, a wild and edible mushroom, by affinity chromatography and studied in terms of thermal activation and stability, pH optimum and stability, and degrees of inhibition by general PPO inhibitors, in order to help to predict the behaviour of this mushroom enzyme. In addition, catalytic efficiencies of *B. erythropus* PPO in some organic solvents were also investigated.

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2. Materials and methods

2.1. Materials and chemicals

B. erythropus Pers. was harvested directly from the Akcaabat district of Trabzon in Turkey by Prof. Dr. Ertuğrul Sesli, carried into the laboratory in liquid nitrogen and stored in deep-frozen at −34 °C.

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

Affinity gel used in this study was synthesised according to the method reported previously (Arslan, Erzengin, Sinan, & Ozensoy, 2004).

2.2. Enzyme extraction and purification

Crude enzyme extracts were prepared as reported previously (Colak, Kolcuoglu, Faiz, Özen, & Dincer, 2007a; Colak et al., 2007b). Mushrooms (*ca.* 50 g) were placed in a Dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The cold mushrooms were homogenised by using a porcelain mortar in 50 ml of 50 mM cold acetate buffer (pH 5.0) containing 2 mM EDTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 6% (w/v) Triton X-114. After the homogenate was filtered through four layers of muslin, the filtrate was centrifuged at 20.000 rpm for 30 min at 4 °C. An equal volume of cold acetone was added to the supernatant and the mixture was incubated overnight at 4 °C for precipitation of proteins. After centrifugation at 20,000 rpm for 30 min at 4 °C, the precipitate was redissolved in an appropriate volume of 50 mM acetate buffer (pH 5.0).

The enzyme solution was applied to an affinity column (1 × 15 cm), pre-equilibrated with 50 mM acetate buffer (pH 5.0). The affinity gel was washed with the same buffer, and PPO was eluted with a solution of 50 mM phosphate buffer containing 1 M NaCl (pH 8.0).

2.3. Protein determination

Protein concentration was determined according to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin (BSA) as a standard. Different concentrations of BSA and enzyme solutions were prepared by using stock BSA (1 mg/ml) and enzyme solutions. After the addition of alkaline copper sulphate reagent, all tubes were mixed well and incubated for 10 min at room temperature. Then Folin–Ciocalteau reagent was added into each tube; tubes were incubated for 30 min at room temperature. The protein values were obtained by graphic interpolation on a calibration curve at 650 nm.

2.4. Assay of polyphenol oxidase activity

PPO activity was assayed by measuring the rate of increase in absorbance at a given wavelength using a double-beam model ATI Unicam UV2-100 spectrophotometer (ATI Unicam, Cambridge, UK) as described previously (Yildirim et al., 2005). The activity was determined by 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 with buffer. Subsequently, an appropriate volume of pure enzyme eluate was added. The reference cuvette included all the reactants except the enzyme. One unit of PPO activity was defined as the amount of enzyme causing 0.001 increase of absorbance per minute in 1 ml of reaction mixture.

2.5. Properties of Boletus erythropus PPO

2.5.1. pH optimum and stability

PPO activity, as a function of pH, was determined using various buffers (all are 50 mM): citrate–phosphate (pH 3.0), acetate (pH 4.0–5.0), phosphate (pH 6.0–7.0), and Tris–HCl (pH 8.0–9.0). 4-methylcatechol was used as a substrate. The optimum pH was used in other studies.

The pH stability was determined by incubating enzyme solution in the buffer solutions named above for 24 h at 4 °C. At the end of the storage period, the activity was assayed using a standard reaction mixture at optimum values. The percentage residual PPO activity was calculated by comparison with unincubated enzyme (Colak et al., 2007b; Yildirim et al., 2005).

2.5.2. Thermal activity and stability

To determine the optimum temperature of the enzyme, PPO activity was measured at different temperatures in the range from 0 to 80 °C with 10 °C increments by using 4-methylcatechol as a substrate (Colak et al., 2007b).

In order to determine the thermal stability of the *B. erythropus* PPO, the enzyme solution in Eppendorf tubes was incubated at the temperatures shown above, up to 4 h, 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 standard assay conditions. The percentage residual PPO activity was calculated by comparison with unincubated enzyme (Yildirim et al., 2005).

2.5.3. Enzyme kinetics

Enzyme kinetics for the *B. erythropus* PPO was studied by using 4-methylcatechol as a substrate and the rate of the PPO reaction was measured at various substrate concentrations in the standard reaction mixture.

The kinetic data were plotted as reciprocals of activities versus 4-methylcatechol concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined as the reciprocal absolute values of the intercepts on the *x*- and *y*-axes, respectively, of the linear regression curve (Lineweaver & Burk, 1934).

2.5.4. Effect of inhibitors and metal ions

Sodium metabisulfite (0.02–0.125 mM), ascorbic acid (0.03–0.25 mM), sodium azide (1–50 mM) and benzoic acid (2–16 mM), were used as PPO inhibitors and the effects of inhibitors on *B. erythropus* PPO activity were determined using 4-methylcatechol as a substrate. I_{50} values were calculated from the plots of inhibitor concentration versus percentage inhibition of 4-methylcatechol oxidation at the optimum values.

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

2.5.5. Native and SDS polyacrylamide gel electrophoresis

Electrophoresis was performed using a P8DS electrophoresis unit (Owl Scientific Inc., Woburn, MA). Polyacrylamide gel (12%) was prepared according to Laemmli (1970) under native and denaturating conditions, separately. After running, gel was incubated in 24 mM *L*-DOPA for 1 h and then in 1 mM ascorbic acid

solution until appearance of a protein band for native electrophoresis. Gel was dyed with Comassie Brillant Blue R-250 for SDS–PAGE.

2.6. Effect of selected organic solvents of PPO activity

The effects of dichloromethane, dichloroethane and toluene (all organic solvents were dried in anhydrous sodium sulphate) on the PPO activity were studied by using catechin (400 mM stock solution in methanol) as a substrate. Pure enzyme and substrate solution at appropriate concentrations was incubated for 5 min in each organic solvent, separately. After this time, the reaction was stopped by adding acetone. The reference cuvette included all the reactants except the enzyme. The absorbances of end products of each reaction were measured over a wavelength range of 330–700 nm (Kermasha, Bao, & Bisakowski, 2001).

3. Results and discussion

The present study was aimed at screening PPO potential of *B. erythropus* and catalytic efficiency of the enzyme in selected organic solvents.

3.1. Enzyme purification

B. erythropus PPO was purified by using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column. Protein content determined spectrophotometrically at 280 nm and activity measurement described above were performed for each fraction (Fig. 1). The fraction having the greatest protein amount and PPO activity was used for performing all other studies such as protein determination, electrophoresis and enzyme characterisation.

After determination of protein amounts (3.80 and 0.40 mg/ml, respectively) and PPO activities for crude enzyme extract and pure enzyme eluate, specific activities were calculated and it was seen that the enzyme was purified 28.5 times (data not presented). PPO was purified 74-fold from mulberry fruit by using the same affinity column (Arslan et al., 2004). PPOs have also been purified from different vegetables and fruits by using other chromatographic techniques, such as ion exchange and hydrophobic interaction (Paul & Gowda, 2000; Zhou & Feng, 1991).

3.2. Native and SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis stained with Comassie Brillant Blue R-250 showed that *B. erythropus* crude enzyme extract and pure enzyme solution had protein bands of approximately 40 kDa molecular weight and PPO was purified successfully by using the affinity column (Fig. 2A). Native polyacrylamide gel electrophoresis stained with *L*-DOPA showed only one band having the same R_f value for crude enzyme extract and pure enzyme eluate (Fig. 2B). This result showed that both crude enzyme extract and pure enzyme eluate had an active PPO.

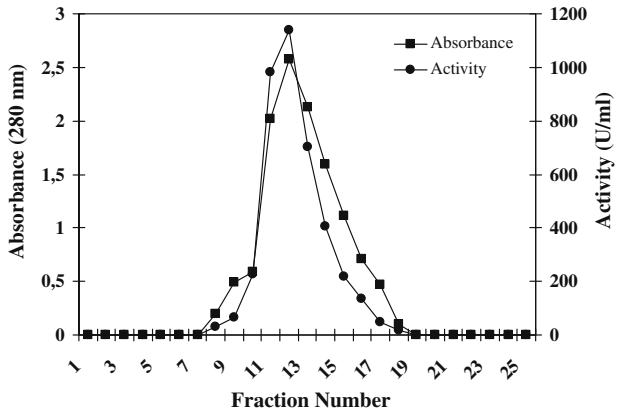

Fig. 1. Purification of *B. erythropus* PPO by Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column.

3.3. Substrate specificity and enzyme kinetics

L-Tyrosine as a monophenolic substrate and 4-methylcatechol, catechol, *L*-3,4-dihydroxyphenylalanine (L-DOPA) and 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA) as di-phenolic substrates were tested for substrate specificity of the *B. erythropus* PPO. The highest activity was observed in the presence of 4-methylcatechol. These results are consistent with previous reports on mushroom PPOs (Colak et al., 2007b; Kolcuoğlu et al., 2007).

Substrate saturation curves for 4-methylcatechol indicated that the *B. erythropus* PPO followed simple Michaelis–Menten kinetics. The Lineweaver–Burk plot analysis of the pure enzyme showed 2.8 mM K_m value and 1430 U/mg protein V_{max} value for 4-methylcatechol. In the presence of 4-methylcatechol as a substrate, enzyme activity was characterised for purified PPOs from quince and *Portabella* mushroom and the K_m values were found to be 4.54 and 2.1 mM, respectively (Concellon, Anon, & Chaves, 2004; Yagar & Sagiroglu, 2002).

3.4. pH optimum and stability

The pH-relative activity (%) profile of PPO activity was determined by using 4-methylcatechol as a substrate and optimum pH was found to be 8.0 (Fig. 3). PPO activity varies with pH depending upon the origin of the material, extraction method, the purity of enzyme, the type of buffer used and substrate (Aylward & Haisman, 1969). An optimum pH of 7.0 for Anamur banana PPO (Ünal, 2007), and pH 8.0 for raspberry PPO (Gonzales, Ancos, & Cano, 1999) has been reported.

The pH stability was examined by incubating the pure enzyme eluate at different pH values at 4 °C for 24 h (Fig. 3, inset). The *B. erythropus* PPO was extremely stable, retaining at least 85% of its original activity in the range of pH 3.0–9.0 after 24 h of incubation at 4 °C. It was reported that *Armillaria mellea* PPO retained approximately 84% of its original activity at pH 3.0 (Colak et al., 2007a). It was also reported that banana peel PPO was over 90% stable at pH 5.0–11.0 after 48 h incubation at 4 °C (Yang et al., 2001).

3.5. Optimum temperature and thermal stability

The dependence of PPO activity on temperature was studied between 0 and 80 °C and the optimum temperature of *B. erythropus* PPO was found to be 20 °C (Fig. 4A). The enzyme activity declined as the temperature increased but the enzyme was not completely inactivated even at 80 °C. Optimal temperatures for PPO activities were reported by other authors to be between 20 and 40 °C (Colak et al., 2007a; Kolcuoğlu et al., 2007; Siddiq, Sinha, & Cash, 1992; Ünal, 2007).

The thermal stability profile for purified *B. erythropus* PPO, presented in the form of the residual percentage activity, is shown in Fig. 4B. *B. erythropus* PPO was most stable at 10 °C and the enzyme lost 60% of its original activity above 40 °C after 1 h incubation (data not shown). The enzyme conserved approximately 80% of its original activity between 0 and 30 °C and it was completely inactivated at 80 °C after 4 h incubation (Fig. 4B). It has been reported that *Allium* sp. PPO was stable at 40 °C for 30 min (Arslan, Temur, & Tozlu, 1997). Stanley plum PPO (Siddiq et al., 1992) was stable for 30 min at 70 °C. It has been noted that heat stability

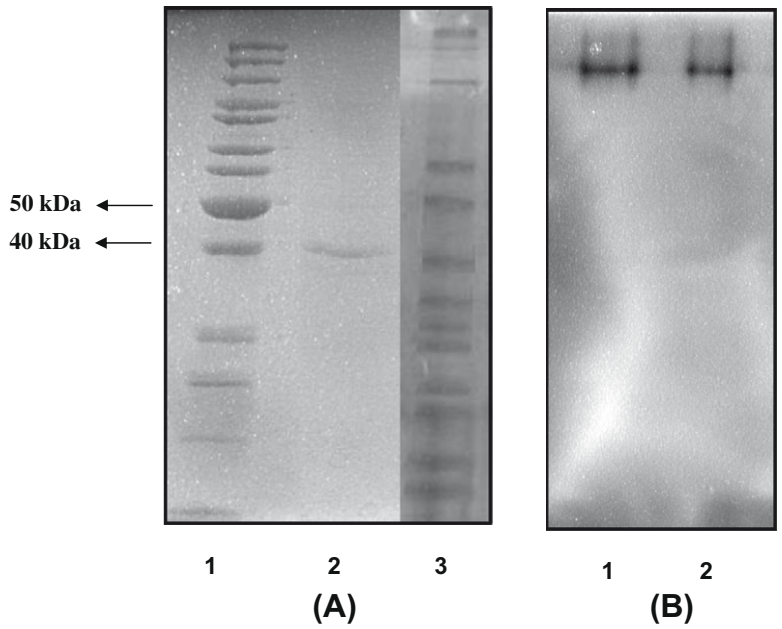

Fig. 2. (A) SDS–PAGE of *B. erythropus* PPO purified by affinity gel. 1: Molecular weight marker, 2: Purified PPO, 3: *B. erythropus* crude extract. (B) Native electrophoresis of *B. erythropus* crude extract (1) and purified PPO (2) stained with 24 mM L-DOPA.

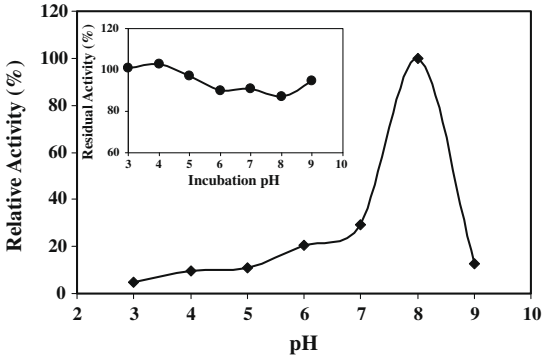

Fig. 3. Effect of pH on the activity of purified *B. erythropus* PPO and pH stability of the enzyme (inset).

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 (Zhou & Feng, 1991).

3.6. Effect of inhibitors and metal ions on B. erythropus PPO activity

The effects of sodium metabisulfite, ascorbic acid, sodium azide and benzoic acid on the purified PPO activity were investigated by using 4-methylcatechol as a substrate. I_{50} values were obtained of 0.07, 0.08, 10.00 and 10.20 mM, respectively.

Of all the inhibitors tested in this study, sodium metabisulfite was the most effective for the inhibition of *B. erythropus* PPO activity. It has been reported that sodium metabisulfite and ascorbic acid were the potent inhibitors of mulberry, *A. mellea*, *Hypholoma fasciculare* and *Lepista nuda* PPOs (Colak et al., 2007a, 2007b). *Anoxybacillus kestanbolensis* K1 and K4^T catecholases were fully inhibited by the addition of 0.01 mM sodium metabisulfite and ascorbic acid (Yildirim et al., 2005).

The effects of Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}, Cr^{+2} and Al^{3+} on *B. erythropus* PPO activity are shown in Table 1. While the enzyme activity was inhibited by Li^+, Ca^{2+}, Mn^{2+}, Cu^{2+} and Ni^{2+}, it was stimulated by Al^{3+}. Similar results were reported for *Macrolepiota mastoidea* PPO (Kolcuoğlu et al., 2007). Since metal ions may have different coordination numbers, geometry in their coordination compounds, and potentials as Lewis acids, they may behave differently towards proteins as ligands. These differences may also result in metals binding to different sites, and therefore, perturb the enzyme structure in different ways (DiTusa, Christensen, McCall, Fierke, & Toone, 2001).

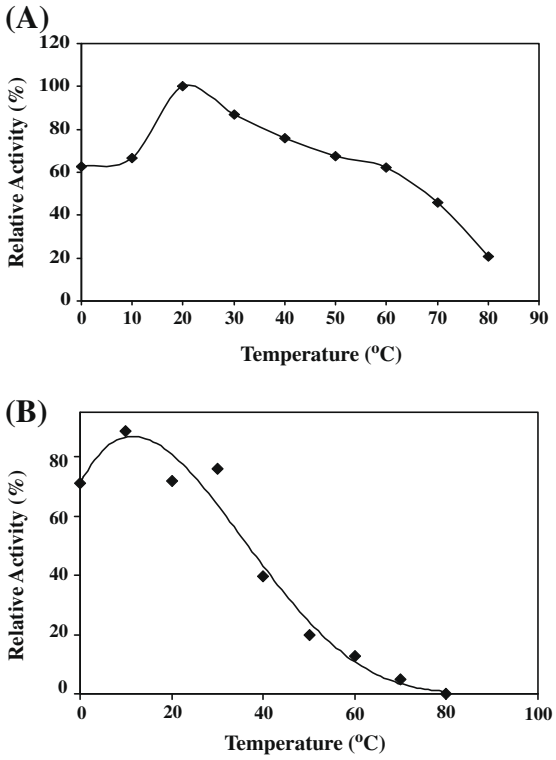

Fig. 4. Temperature optimum (A) and thermal stability (B) of purified *B. erythropus* PPO using 4-methylcatechol as a substrate.

Table 1
Effect of various metal ions on *B. erythropus* PPO activity.

Metal ion (1 mM)	Residual activity (%)	Metal ion (1 mM)	Residual activity (%)
None	100	Mn^{2+}	62.9
Li^{+}	72.5	Cu^{2+}	88.3
Na^{+}	94.6	Cd^{2+}	103.0
K^{+}	103.8	Zn^{2+}	93.2
Mg^{2+}	101.8	Cr^{2+}	95.5
Ca^{2+}	74.2	Ni^{2+}	56.6
Al^{3+}	127.3		

3.7. Effect of selected organic solvents on PPO activity

Fig. 5 shows the spectrophotometric scanning of the coloured compounds resulting from the enzymatically-oxidised end products of catechin in the selected organic solvents such as dichloromethane, dichloroethane and toluene. While in the presence of the enzyme in the reaction mixture, absorption peaks with maxima at 350, 360 and 360 nm were seen for dichloromethane (Fig. 5A), dichloroethane (Fig. 5B) and toluene (Fig. 5C), respectively; there was no peak in the absence of the enzyme. This suggested that conversion of catechin into *o*-quinones was performed with *B. erythropus* PPO. It was reported that the oxidation of catechin in chloroform, dichloromethane, dichloroethane and toluene medium yielded coloured compounds which gave peaks of maximum absorption at 372, 375, 375 and 379 nm, respectively (Goodenough, Kessell, Lea, & Loeffler, 1983; Kermasha et al., 2001).

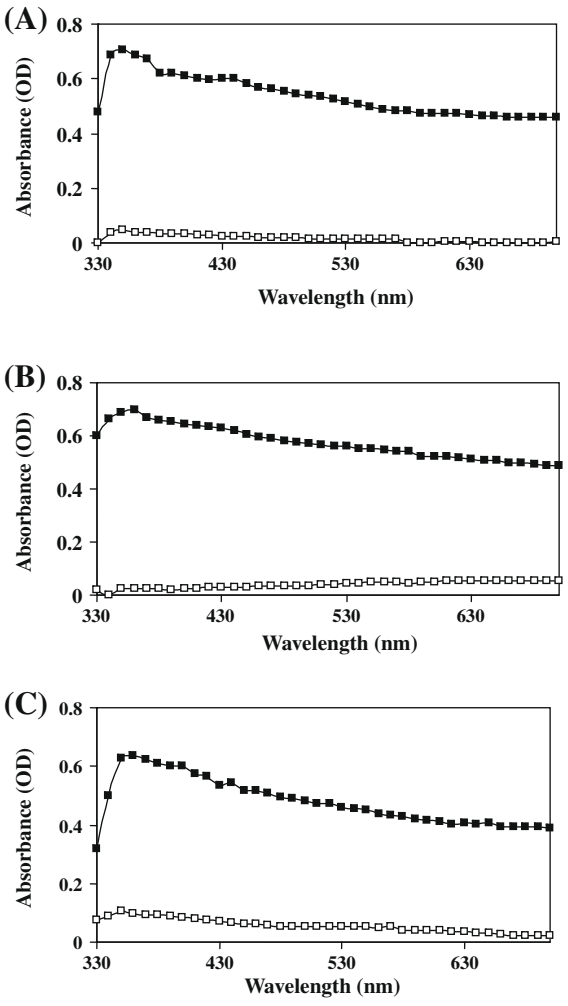

Fig. 5. Scanning profiles of catechin (□—□) and the corresponding pigments of the *B. erythropus* PPO-catalysed end products (■—■) in the selected organic solvent media of dichloromethane (A), dichloroethane (B) and toluene (C).

It can be concluded that the pure enzyme eluate prepared from *B. erythropus* possesses diphenolase activity, having greatest substrate specificity to 4-methylcatechol. The enzyme appears to share some biochemical characteristics of several plant PPOs in terms of substrate specificity, pH and temperature optima and stability. In addition, the enzyme activity was very sensitive to some general PPO inhibitors, especially sodium metabisulfite and ascorbic acid. In this study, the conversion of catechin into *o*-quinones was successfully performed with *B. erythropus* PPO in dichloromethane, dichloroethane and toluene as organic reaction media. The oxidation of organic substrates with molecular oxygen under mild conditions is of great interest for industrial and synthetic processes from an economical point of view. Hence, it can be speculated that the wild mushroom PPO purified and studied in this work may be interesting for industrial applications.

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