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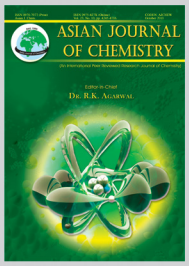

Characterization of a Polyphenol Oxidase Having Monophenolase and Diphenolase Activities from a Wild Edible Mushroom, *Russula delica*

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A polyphenol oxidase (PPO) extracted from a wild edible mushroom, *Russula delica*, was characterized spectrophotometrically. Native polyacrylamide gel, stained with L-dihydroxyphenylalanine (L-DOPA), showed two bands supporting a polyphenol oxidase potential. pH and temperature optima were determined as 5.0 and 30 ºC, respectively, for both of 3-(4-hydroxyphenyl)-propionic acid (PHPPA) and 4-methylcatechol (4-MC). After incubating at this pH at 4 ºC for 24 h, the crude extract retained about 90 % of its original monophenolase and diphenolase activities. The crude extract conserved about 90 % of its activities after 1 h incubation at 30 ºC. V_{max} and K_m values were calculated as 769.2 U/mg protein and 0.92 mM, respectively, for monophenolase and 71.4 U/mg protein and 0.27 mM, respectively, for diphenolase activity. It was found that benzoic acid was a potent inhibitor for both activities and some metal ions affected the activities. It is clear that *R. delica* possess polyphenol oxidase activities having interesting properties.

Key Words: Diphenolase, Monophenolase, Polyphenol oxidase, *Russula delica*.

INTRODUCTION

Polyphenol oxidases (PPOs) are a group of copper proteins distributed throughout microorganisms, plants and animals[1]. Polyphenol oxidases have at least two distinct activities linked with each other as monophenol monooxygenase or tyrosinase (monophenolase) (EC 1.14.18.1) and catechol oxidase or *o*-diphenol:oxygen oxidoreductase (diphenolase) (EC 1.10.3.1)[2]. The quinones are formed after the consecution reactions catalayzed by monophenolase and diphenolase and then polymerize to melanins which are brown, red or black pigments[3] which lead to organoleptic and nutritional modifications and diminish food product quality[4]. These reactions highly influence consumer acceptance, storage life and value of plant products[5]. So researchers have paid much attention to polyphenol oxidase. Polyphenol oxidase enzyme activity causing undesired browning can be inhibited and nutritional value and shelf-life of food can be increased. Fungal tyrosinases were firstly characterized from the edible mushroom *Agaricus bisporus*[6] because of enzymatic browning during development and post-harvest storage, which particularly decreases the commercial and nutritional value of the product.

Although *Russula delica* is an important edible mushroom, there are no conclusive reports on the polyphenol oxidase activity of this mushroom. The main aim of this study was to investigate the polyphenol oxidase potential of *Russula delica*. Polyphenol oxidase activitity was biochemically characterized from the extract preparing from the fruiting body of *Russula delica* by determining pH and temperature optima, pH and thermal stability, kinetic parameters and effects of some metal ions and chemical compounds on the enzyme activity.

EXPERIMENTAL

Russula delica Fr. was harvested directly from Macka district of Trabzon in Turkey, carried into the laboratory in liquid nitrogen and stored in deep freeze at -34 ºC. Substrates were purchased from Sigma Chemical Co. (St. Louis). All other reagents were of analytical grade and used as obtained.

Enzyme extraction: Crude enzyme extracts were prepared as reported previously with slight modifications[7,8]. Mushrooms (10 g) were placed in a Dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The cold mushrooms were homogenized by using a porcelain mortar in 10 mL of 50 mM cold phosphate buffer (pH 7.0) containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF). After the homogenate was filtered through four layers of muslin, the filtrate was centrifuged at 15.000 rpm for 20 min at 4 ºC. The equal volume of cold acetone (- 20 ºC) was added to the supernatant and the mixture was incubated overnight at 4 ºC for the precipitation of proteins. After centrifugation at

8.000 rpm for 10 min at 4 ºC, the precipitate was resuspended in appropriate volume of 50 mM phosphate buffer (pH 7.0). Then the solution was centrifuged again at 8.000 rpm for 10 min at 4 ºC and the supernatant was used as crude enzyme extract.

Protein determination: Protein concentration was determined according to the Lowry method with bovine serum albumin as a standard[9]. The values were obtained by graphic interpolation on a calibration curve at 650 nm.

Enzyme assay: Different mono- and diphenolic substrates were tested for the activity determination by using an ATI Unicam UV2-100 double beam UV-VIS spectrophotometer. In this assay method, the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) traps *o*-quinone products originating from the oxidation of phenolic compounds by polyphenol oxidase. The stable MBTH-quinones are kept in solution by addition of 2 % dimethyl formamide and the increase in absorbance is measured for MBTH-quinone complex[10].

Polyphenol oxidase activity of *Russula delica* was assayed by measuring the rate of increase in absorbance at 494 nm for 4-methylcatechol, 507 nm for L-tyrosine and L-DOPA and 500 nm for all other substrates[8]. The assay mixture containing 100 µL of substrate (stock 100 mM), an equal volume of MBTH (stock 10 mM) and 20 µL dimethyl formamide was diluted with 730 µL of desired buffer. After then, 50 µL of crude enzyme extract was added to start the reaction. The reference cuvette included all the reactants except the enzyme. One unit of polyphenol oxidase activity was defined as 1 µM of product produced per min. Specific activity was defined as units of enzyme activity per mg of protein.

Native polyacrylamide gel electrophoresis: Non-denaturing polyacrylamide gel electrophoresis was performed at 4 ºC by using a 10 % separating gel. Approximately 30 µg protein sample was loaded into each well. The 25 mM current was applied to the gel approximately 1 h. After electrophoresis, the gel was stained for polyphenol oxidase activity in 24 mM L-DOPA for 0.5 h[11].

Characteristics of the crude enzyme

Substrate specificity: Polyphenol oxidase activity was assayed by using L-tyrosine and 3-(4-hydroxyphenyl)-propionic acid (PHPPA) as monophenolic substrates and catechol, 4-methylcatechol (4-MC), L-DOPA and 3-(3,4-dihydroxyphenyl)propionic acid (DHPPA) as diphenolic substrates[10]. Stock solutions of all substrates were separately prepared at the concentration of 100 mM. The activity was determined as described above.

pH optimum and stability: The effect of pH on monophenolase and diphenolase activity of *Russula delica* polyphenol oxidase was determined by using 3-(4-hydroxyphenyl)propionic acid as a monophenolic substrate and 4-methylcatechol as a diphenolic substrate. The reactions were performed with the following buffers (50 mM) at the indicated pH; glycine-HCl (pH 3.0), acetate (pH 4.0 and pH 5.0), phosphate (pH 6.0, pH 7.0 and pH 8.0) and Tris-HCl buffer (pH 9.0). The determined optimum pH was used in futher studies[8].

To determine the pH stability of *Russula delica* monophenolase and diphenolase activities, the enzyme extracts were mixed separately with buffers mentioned above in the ratio of 1:1 and the mixtures were incubated at 4 ºC for 24 h. At the end of the storage period, the activity was assayed by using standard reaction mixture at optimum values. The percentage residual polyphenol oxidase activity was calculated by comparison with the activity of unincubated enzyme[11,12].

Thermal activity and stability: Polyphenol oxidase activity, as a function of temperature, was determined by performing enzymatic reactions at various temperatures over the range of 10-80 ºC with 10 ºC increments. Reaction cuvette containing all the reagents except crude enzyme extract was incubated for 5 min at desired temperature. Then crude extract was added into the incubated mixture and activity was assayed as quickly as possible[11].

In order to determine the thermal stability of the *Russula delica* polyphenol oxidase, the enzyme solution in Eppendorf tubes was incubated at 10-70 ºC with 10 ºC increments for 1 h and rapidly cooled in an ice bath for 5 min and then brought to 25 ºC. After the mixture reached room temperature, the enzyme activity was assayed under the standard assay conditions. The percentage residual polyphenol oxidase activity was calculated by comparison with the activity of unincubated enzyme[11].

Effect of protein concentration on polyphenol oxidase activity: To determine the effect of protein concentration on the polyphenol oxidase activity of *Russula delica*, reactions were performed at various protein concentrations and the activities were assayed under standard reaction conditions.

Enzyme kinetics: Enzyme kinetic parameters of the *Russula delica* polyphenol oxidase were obtained by measuring the rate of 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol oxidation at various substrate concentrations in 50 mM acetate buffer (pH 5.0). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined from the Lineweaver-Burk plot using the Microsoft Excel software.

Effect of some metal ions on polyphenol oxidase activity: The activities were measured in the presence of K^+, Mn^{2+}, Co^{2+}, Cd^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+} and Al^{3+} at 1 mM final concentration under the standard reaction conditions. Stock solutions of all metal ions (5 mM) were prepared from chloride salts of them. The percentage remaining activities were expressed by comparison with standard assay mixture with no metal ion added[11].

Effect of some chemicals on polyphenol oxidase activity: Sodium metabisulfite, ascorbic acid, benzoic acid and cysteine were tested as inhibitors of monophenolase and diphenolase activities of *Russula delica* polyphenol oxidase. Reactions were performed at concentration range should be mentioned of inhibitors. The percentage remaining activities were determined by comparison with the activities assayed with no inhibitor added. I_{50} values for each inhibitor were determined from the plot of inhibitor concentration against residual activity[11].

RESULTS AND DISCUSSION

In this study, *Russula delica*, a wild edible mushroom, was evaluated for its polyphenol oxidase potential. The crude enzyme extracted from *Russula delica* was able to catalyze

both of the hydroxylation of monophenols and the oxidation of *o*-diphenols.

Native polyacrylamide gel electrophoresis: Native polyacrylamide gel stained with L-DOPA showed two bands (Fig. 1) having R_f values of 0.34 (major) and 0.64 (minor) indicating the presence of at least two polyphenol oxidase isoenzymes. Two or more isoenzymes have been reported for polyphenol oxidases extracted from different sources. The presence of two isoenzymes of polyphenol oxidase was reported in *Macrolepiota mastoidea* and in the first and third maturity stage of *Mespilus germanica*[8,13]. In addition, the presence of three and two isoenzymes in *Armillaria mellea* and *Hypholoma fasciculare*, respectively, was reported previously[14].

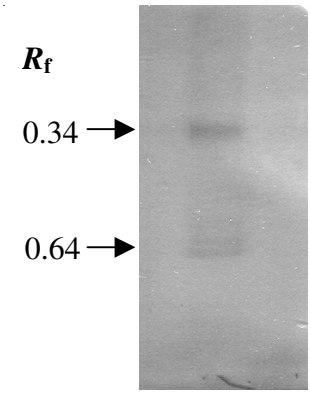

Fig. 1. Native PAGE profile of crude extract of *Russula delica* stained with 24 mM L-DOPA

Substrate specificity: All of the mono- or diphenolic substrates tested were oxidized by the polyphenol oxidase extracted from *Russula delica* (Table-1). Specific activitity values before acetone precipitation for both 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol were also found 0.07 and 3.9 U/mg protein, respectively.

TABLE-1
SUBSTRATE SPECIFICATIONS OF POLYPHENOL OXIDASES EXTRACTED FROM *Russula delica*

Substrate	Specific activity (U/mg protein)
Monophenols	
3-(4-Hydroxyphenyl)-propionic acid (PHPPA)	0.2
L-Tyrosine	0.1
Diphenols	
4-Methylcathecol (4-MC)	11.3
Catechol	8.5
L-3,4-Dihyrdoxyphenylalanine (L-DOPA)	2.4
3-(3,4-Dihydroxyphenyl)-propionic acid (DHPPA)	8.6

The enzyme showed the highest activity in the presence of 3-(4-hydroxyphenyl)-propionic acid as a monophenolic substrate and 4-methylcatechol as a diphenolic substrate. Tyrosine is also oxidized by the enzyme (Table-1). Although polyphenol oxidases extracted from animal sources are more specific to L-tyrosine and L-DOPA than the other phenolic compounds, polyphenol oxidases extracted from mushrooms or plants show activity against various mono- or diphenolics[15]. It was reported that polyphenol oxidases from *A. mellea*, *Lepista nuda*, *H. fasciculare* and *Boletus erythropus* showed the highest activity in the presence of 4-methylcatechol as a diphenolic substrate[14,16]. *M. mastoidea* polyphenol oxidase has both monophenolase and diphenolase activities with 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol as substrates, respectively[8]. It can be concluded from these results that although mushroom polyphenol oxidases generally have diphenolase activity, some of them may have both activities.

pH optimum and stability: pH optimum of *Russula delica* polyphenol oxidase was determined as 5 for both 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol (Fig. 2). The pH stability of the enzyme was tested by incubating the enzyme at different pH values at 4 ºC for 24 h (Fig. 3).

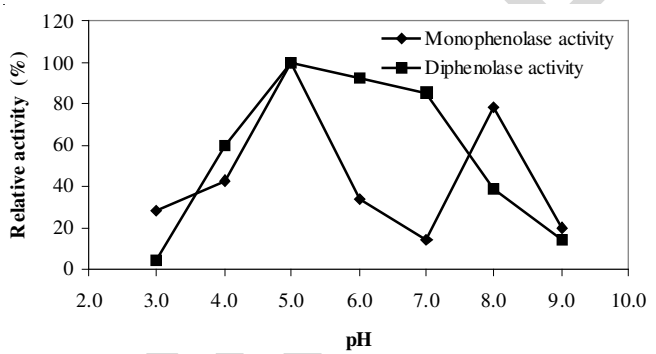

Fig. 2. Effect of pH on the monophenolase and diphenolase activities of *Russula delica* polyphenol oxidase. Assays were performed in 50 mM of different buffer systems at indicated pH values; glycine-HCl (pH 3.0), acetate (pH 4.0 and pH 5.0), phosphate (pH 6.0, pH 7.0 and pH 8.0), tris-HCl (pH 9.0). 3-(4-Hydroxyphenyl)-propionic acid (PHPPA) and 4-methylcatechol (4-MC) were used as substrate for monophenolase and diphenolase activities, respectively

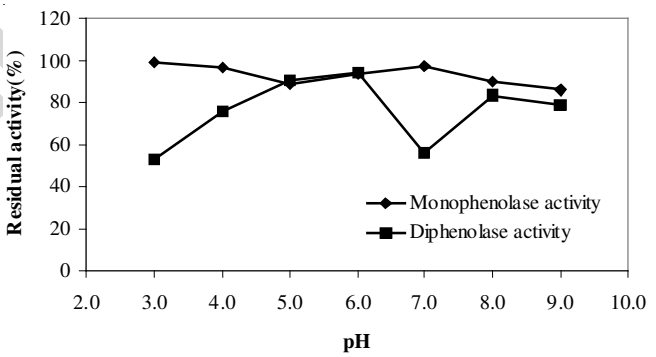

Fig. 3. pH stability profile of monophenolase and diphenolase activities of *Russula delica* polyphenol oxidase. Residual activity was determined under standard conditions after incubation for 24 h at indicated pH at 4 ºC

pH optimum of *Russula delica* polyphenol oxidase was determined as 5.0 for both 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol. The second peak around pH 8.0 in the presence of 3-(4-hydroxyphenyl)-propionic acid may be caused by the isoforms of polyphenol oxidase existing in the enzyme extract. Similar results were also reported for *M. mastoidea*[8], almond[17] and eggplant[18]. In addition, the optimum pHs of polyphenol oxidases from *A. mellea*, *L. nuda* and *H. fasciculare* were found to be 7.0[14]. It was reported that polyphenol oxidase activity varied with pH depending on the origin of the material, extraction method, the purity of enzyme, the type of buffer used and substrate[19].

As shown in Fig. 3, the residual percentage activity of the enzyme with 3-(4-hydroxyphenyl)-propionic acid as substrate was almost retained at all of the tested pH values. Diphenolase activity of the enzyme retained 90 and 95 % of its original activity when kept at pH 5 and 6, respectively. At pH 3 and pH 7, the enzyme lost half of its original diphenolase activity. Incubation of the enzyme at pH 8 and 9 caused 20 % loss of its original diphenolase activity. It was reported that *B. erythropus* polyphenol oxidase was extremely stable in the range of pH 3-9 after 24 h of incubation at 4 ºC[16]. It was also reported that *A. mellea* polyphenol oxidase conserved approximately 84 % of its original activity at pH 3.014, *M. mastoidea* diphenolase retained more than 95 % of its original activity at physiological pH values[8] and banana peel polyphenol oxidase was stable over 90 % at pH 5.0-11.0 after 48 h incubation at 4 ºC[20]. Results earlier reported show that mushroom polyphenol oxidases could retain their activities over 50 % at pH values between 3 and 9[8,14,16].

Thermal activity and stability: The effect of temperature on polyphenol oxidase activity is presented in Fig. 4. The optimum temperature was determined as 30 ºC for both of activities. It is clear that the diphenolase activity is more sensitive to temperature above 50 ºC. Temperature is an important factor that significantly influences the catalytic activity of the polyphenol oxidase[21]. The optimum temperature of polyphenol oxidase varies for different plant sources. Optimal temperatures for polyphenol oxidase activity were reported as 30, 30, 20 and 35 ºC for *M. mastoidea*, *H. fasciculare*, *B. erythropus* and medlar fruits, respectively[22,8,16,14]. In addition, optimum temperatures for polyphenol oxidase activity were reported as 5, 20, 60 and 70 ºC for *Asimina triloba*, *Morus alba*, *Cucumis melo* and *Eriobotrya japonica*, respectively[23-26].

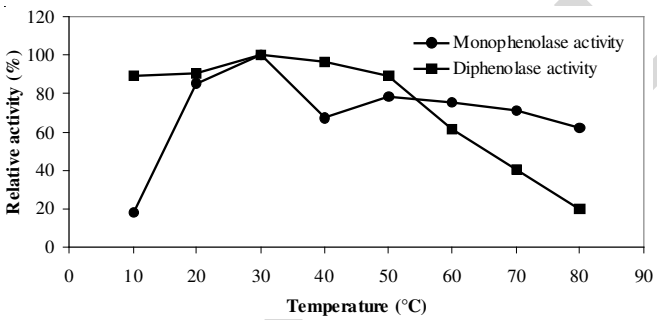

Fig. 4. Effect of temperature on monophenolase and diphenolase activities of *Russula delica* polyphenol oxidase. Reactions were carried out at different temperatures by using 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol as substrate for monophenolase and diphenolase activities, respectively, in 50 mM acetate buffer (pH 5.0)

The pH stability of the enzyme tested by incubating the enzyme solutions at 10-70 ºC is shown in Figs. 5 and 6.

Both activities of the *Russula delica* enzyme were quite stable near optimum temperature and retained nearly 90 % of its original activity when incubated at 10, 20 and 30 ºC for 1 h (Fig. 5). It is clear that diphenolase activity of the enzyme was more stable above optimum temperature than monophenolase activity. After incubation at 40 ºC for 1h, the enzyme lost 75 % of its monophenolase activity. Incubation above 40 ºC almost completely inactivated the monophenolase activity of the enzyme.

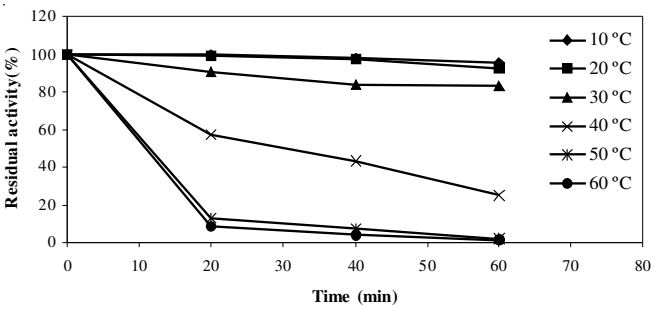

Fig. 5. Thermal stability profiles of monophenolase activity. Crude enzyme extracts were incubated for 20/40 or 1 h at various temperatures in the range of 10-60 ºC for 3-(4-hydroxyphenyl)-propionic acid. The percentage residual activities were calculated by comparing with unincubated enzyme

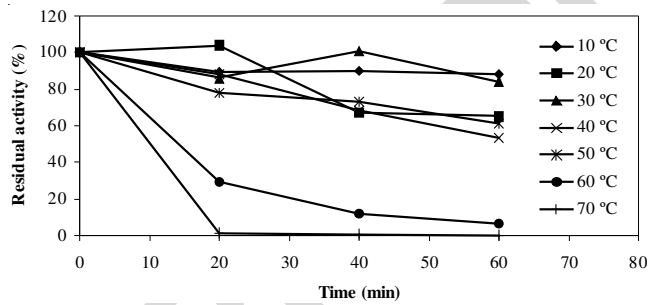

Fig. 6. Thermal stability profiles of diphenolase activity. Crude enzyme extracts were incubated for 20/40 or 1 h at various temperatures in the range of 10-70 ºC or 4-methylcatechol. The percentage residual activities were calculated by comparing with unincubated enzyme

Incubation at 40 and 50 ºC for 1h resulted in 40 % loss of diphenolase activity of the enzyme (Fig. 6). Similar results were also reported for other polyphenol oxidases. Allium sp. polyphenol oxidase was stable at 40 ºC for 0.5 h[27]. Stanley plum polyphenol oxidase[28] was stable for 0.5 h at 70 ºC. It is clear that mushroom polyphenol oxidases could retain their activities over the range of 80-90 % after 1 h incubation at 10, 20 and 30 ºC[8,16].

Effect of protein concentration on polyphenol oxidase activity: The effect of protein concentration on polyphenol oxidase activity was assayed at different protein concentrations. The protein content of the crude extract was determined as 1.13 mg/mL. Both of the activities were found to be protein concentration-dependent. Increased protein concentration increased the activity until the protein concentration reached 57 and 6 µg/mL for monophenolase and diphenolase activity, respectively.

Enzyme kinetics: Michealis-Menten constants (K_m) and maximum reaction velocities (V_{max}) were determined from the Lineweaver-Burk plots (Fig. 7a-b) by using 3-(4-hydroxyphenyl)-propionic acid and 4-methylcatechol at various concentrations. K_m and V_{max} were calculated as 0.92 mM and 769.2 U/mg protein, respectively for monophenolase activity and 0.27 mM and 71.4 U/mg protein, respectively for diphenolase activity.

Activity results obtained from the enzyme kinetics experiments for monophenolase activity did not fit well to the Lineweaver-Burk model, so the K_m and V_{max} values calculated are only the parameters estimated due to lack of linearity. Kinetic values of some mushroom polyphenol oxidase enzymes

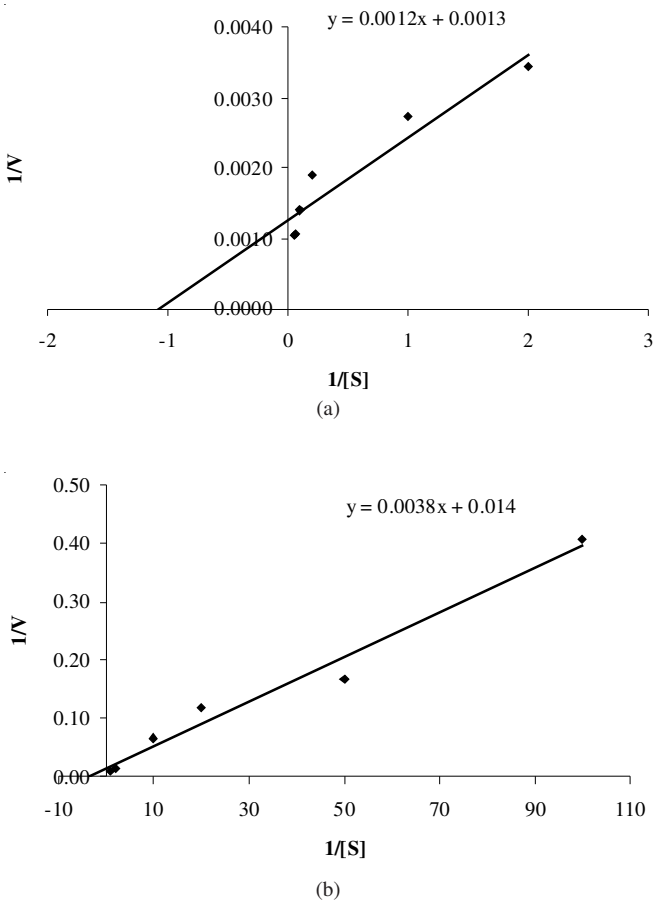

Fig. 7. Lineweaver-Burk plots. Enzyme kinetic parameters of the *Russula delica* polyphenol oxidase were determined by measuring the oxidation of 3-(4-hydroxyphenyl)-propionic acid (a) and 4-methylcatechol (b) at various substrate concentrations in 50 mM acetate buffer (pH 5.0)

were reported previously. The Lineweaver-Burk plot analysis of the pure Boletus erythropus polyphenol oxidase showed 2.8 mM Km value and 1428.6 U mg protein^{-1} V_{max} value for 4-methylcatechol[16]. V_{max} values of V_{max} values *Armillaria mellea*, *Lepista nuda* and *Hypholoma fasciculare* polyphenol oxidases were reported as 0.73, 0.21 and 0.25 U/mg protein, respectively. K_m values of them were 1.20, 9.19 and 0.51 mM[14].

Effect of some metal ions and chemicals on polyphenol oxidase activity: The effects of various metal ions on both activities of polyphenol oxidase extracted from *Russula delica* are shown in Table-2. IC_{50} values of sodium metabisulfite, ascorbic acid, benzoic acid and cysteine are shown in Table-3.

The metal ions can affect the enzyme by increasing or decreasing the activity. The enzyme activity was tested in the pesence of some metal ions for displaying the effect of metal ions on polyphenol oxidase activity. The final concentrations of all metal ions were 1 mM in the assay mixture. While K^+ and Hg^{2+} inhibited the monophenolase activity, Ca^{2+} did not significantly affect the activity. All other ions used activated the monophenolase activity in different ratios. Similar results were previously reported for the monophenolase activity of *M. mastoidea* polyphenol oxidase in the presence of K^+, Hg^{2+}, Mn^{2+}, Co^{2+} and Al^{3+} ions[8].

Addition of K^+, Ca^{2+} and Cu^{2+} to the reaction mixture resulted in a slight stimulation on the diphenolase activity of

TABLE-2
EFFECTS OF VARIOUS METAL IONS ON MONOPHENOLASE AND DIPHENOLASE ACTIVITIES OF POLYPHENOL OXIDASE EXTRACTED FROM *Russula delica*

Metal ion	Residual activity (%)	
	Monophenolase activity	Diphenolase activity
None	100	100
K^+	92	103
Mn^{2+}	118	90
Co^{2+}	231	79
Cd^{2+}	180	85
Ca^{2+}	99	107
Ni^{2+}	150	74
Cu^{2+}	104	103
Zn^{2+}	152	88
Hg^{2+}	26	74
Al^{3+}	124	76

TABLE-3
EFFECTS OF SOME GENERAL POLYPHENOL OXIDASE INHIBITORS ON *Russula delica* MONOPHENOLASE AND DIPHENOLASE ACTIVITIES

Inhibitors	IC_{50} (mM)	
	Monophenolase activity	Diphenolase activity
Sodium metabisulfite	1.87	6.50
Ascorbic acid	1.24	0.11
Benzoic acid	1.16	0.10
Cysteine	1.78	0.50

the *Russula delica* polyphenol oxidase. All other ions used inhibited the activity. Inhibition with Mn^{2+} and Cd^{2+} was previously reported for the Lepista nuda mushroom polyphenol oxidases. It was also reported that *A. mellea* polyphenol oxidase was slightly activated in the presence of 1 mM Ca^{2+} ions[14]. 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 metal binding to different sites and therefore, change the enzyme structure in different ways and affect the enzyme activity[29].

Both activities were inhibited by the compounds tested as inhibitors. The results clearly showed that ascorbic acid was the most effective inhibitor for both activity of polyphenol oxidase extracted from *Russula delica*. Similar results were earlier reported for *L. nuda*, *M. mastoidea*, *B. erythropus* and *M. germanica*[14,8,16,13].

Conclusion

It can be concluded from these results that polyphenol oxidase extracted from *Russula delica* had both monophenolase and diphenolase activities. The enzyme appears to share some biochemical characteristics of several mushroom and plant polyphenol oxidases in terms of substrate specificity, pH and temperature optima and stability. In addition, the enzyme activity was very sensitive to some general polyphenol oxidase inhibitors especially benzoic acid and ascorbic acid.

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