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Substrate specifity and inhibitors of polyphenol oxidase in aspect of darkening of fresh and frozen mushrooms (*Agaricus bisporus* (Lange) Sing.)

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Abstract

Activity of mushroom polyphenol oxidase (PPO) toward 6 substrates and inhibitory effect of cysteine, 2-mercaptoethanol, benzoic acid and sodium metabisulphite were determined. The o-diphenols which appeared to be the best substrates were: catechin, DOPA (L-3,4-dihydroxyphenylalanine) and chlorogenic acid. Affinity of PPO crude preparation substrates to enzyme, expressed as inverse value of Michaelis constant was lower then affinity of catechol. Inhibitory effect depended on specifity of inhibitors and their concentration. Electrophoretic patterns of PPO of mushrooms reveals slow and fast moving 4 isoforms when DOPA was used as a substrate, 2 bands for catechin and chlorogenic acid while only one band showed activity toward tyrosine and p-cresol.

INTRODUCTION

Enzymatic discoloration of fruits, vegetables and mushrooms is catalyzed primarily by the enzyme polyphenol oxidase (PPO) and is mainly the result of the oxidation and polymerization of polyphenols. In mushrooms, polyphenol oxidase exhibits 2 types of activity: hydroxylation of monophenols to o-di-phenols (EC. 1.14.18.1) and oxidation o-diphenols to o-quinones (EC. 1.10.3.1, o-diphenol oxidase) with subsequent polymerization, leading to formation complex brown pigments. (Dawson, 1963; Swan, 1963; Walker, 1975; Lerch, 1981; Robb, 1984). Many compounds have been reported the inhibition of melanosis through the following mechanisms: by inhibiting PPO (Kahn, Andrawis, 1986; Sayavedra--Soto, Montgomery, 1986; Allan, Walker, 1988; Dawley, Flurkey,

1993; K e r m a s h a et al., 1993); by reducing o-quinones to diphenols (G o l a n - - G o l d h r i s h et al., 1984; C h e n et al., 1991) or Cu^{+2} to Cu^{+} (H s u et al., 1988); by interacting with the formation of o-quinone products (E m b s, M a r k a k i s, 1965; F e r r e r et al., 1989) and by decreasing the uptake of O_2 for the reaction (E m b s, M a r k a k i s, 1965).

Sulphur dioxide (SO_2) and its derivatives are most powerful and extremely versatile PPO inhibitors; inhibit enzymatic browning as well as nonenzymatic browning of mushrooms *Agaricus bisporus*, during storage and processing. However, they have been classified as a health hazard compounds for asthmatic (K o e i n g et al., 1983) and the need for alternatives to these additives thus becomes more imperative.

The present work reports the substrate and inhibitor specifity of mushroom PPO enzyme.

MATERIAL AND METHODS

Fresh mushrooms *Agaricus bisporus* (Lange) Sing. strains No. 22, No. 53 and H-25 were obtained from the local growers. Caffeic acid was recrystallized from water before use. Other chemicals were the best commercial grade available and were used without further purification.

Enzyme preparation and extraction was made by the method described before for apples (C z a p s k i et al., 1988), using 100 g tissue portion of mushroom caps for acetone dry powder preparation and 0.1 M phosphate buffer pH 6,1 for enzyme extraction from powder. Enzyme activity was measured according to H o p f i n g e r et al. (1984) with small modifications. Reaction mixture 2 ml contained 10 mM of substrate in 0.1 M phosphate buffer pH 6.1 and 0.05-0.2 ml of enzyme solution. In the experiments with inhibitors, 10 mM of the substrate solution and appropriate volume of inhibitor solution were mixed immediately before the addition of 0.2 ml of enzyme solution. Enzyme solution were carried out at 21°C. A Spectromom 195D spectrophotometr was used for the enzyme assay; wavelength was 400 nm. Readings were taken at intervals of 15 sec. or 30 sec. for 3 or 5 minutes. Enzyme activity was calculated as a linear change in optical density per minute per mg protein (Δ OD $\cdot$ $min.^{-1} \cdot mg^{-1}$). The enzyme was prepared in triplicate and each preparation was assayed in duplicate. The multiple enzymes forms of polyphenol oxidase were separated in 7.5 % polyacrylamide gel, using disc technique of Orstein and Davies (S a r g e n t, 1969). Samples for electrophoresis were prepared by the extraction of enzyme from powders with 0.1 M phosphate buffer pH 6.1. 50 mg of acetone powder was homogenized with 1 ml cold buffer. The electrode buffer was 0.05 M Tris-0.38 M glicyne pH 8.3. The preelectrophoresis was conducted using 2 mA current per gel tube for 0.5 hr. then 4 mA. After the run the gels were removed from the tubes placed in 10 ml substrate solutions containing $1.5 \cdot 10^{-3}$ M L-3, 4- dihydroxyphenylalanine (DOPA), catechin, tyrosine, chlorogenic acid and p-cresol in 80 % ethyl alcohol. The gels were stained for PPO isozyme patterns for about 1 hour and were finally stored

in 30 % ethyl alcohol (Constatinies, Bedford, 1967). Crude preparation of PPO substrates from mushrooms were obtained according to McCord and Kilara (1983).

Total phenolic compounds was determined using Folin-Ciocalteau reagent (Johnson, Schaal, 1957) and total soluble protein was estimated according to Czapski (1989) using amido black reagent.

RESULTS

Activities of the PPO toward 6 substrates are shown in Table 1. Caution should be used in interpreting these data since the extinction coefficients of the products of oxidation are probably different at 400 nm. However some general statement about the substrate specifity of mushroom PPO may be made. The o-diphenols which appeared to be the best substrates were: catechin, DOPA and chlorogenic acid. L-tyrosine was oxidized at a very slow rate by mushroom PPO extract. Tyrosine was used as a preferential substrate for tyrosinase activity (Mayer, Harel, 1991), however slow oxidation of DOPA but not of p-cresol by laccase was possible (Dawley, Flurkey, 1993). There were some minor differences in PPO activity between two strains No. 22 and No. 53 (Tab. 1).

Table 1

Substrate specifity of polyphenol oxidase (PPO) of two strains of mushrooms

Substrate	PPO activity (units*)	
	Strain No. 22	Strain No. 53
Catechin	16.80	19.04
L-3,4-dihydroxyphenylalanine (DOPA)	5.79	7.37
Chlorogenic acid	3.99	3.33
Caffeic acid	2.28	2.44
L-Tyrosine	0.77	0.79
p-Cresol	1.01	0.89

*one unit of PPO activity is defined as an increase in absorbance of 0.01 min^{-1} per 1 mg protein

The enzyme preparation was assayed over a range of concentration of crude preparation of PPO substrates. Total phenolic compounds of PPO substrates was determined using catechol as a standard and calculated and expressed in catechol concentrations. The results were plotted by the method of Linewaver-Burk double reciprocal plot (Fig. 1). Michealis constant K_m and maximum velocity (V) were determined. For comparison, catechol was also used as a substrate and the results plotted (Fig. 1). Calculated from figure 1 the K_m value for crude preparation of PPO substrates is higher than for catechol being, 2 mM and 1.25 mM respectively. It means that affinity PPO crude preparation substrates to enzyme, expressed as inverse value

of K_m is lower then affinity of catechol, being 0.5 and 0.8 $(mM)^{-1}$ respectively. Crude preparation of PPO substrates contained probably not only various substrates but also inhibitors. Calculated from figure 1 maximum enzyme velocity value for crude preparation of PPO substrates is equiponderant 5 μmoles of catechol per minute per 1 mg protein and it is about 66 % lower then for catechol used as a substrate.

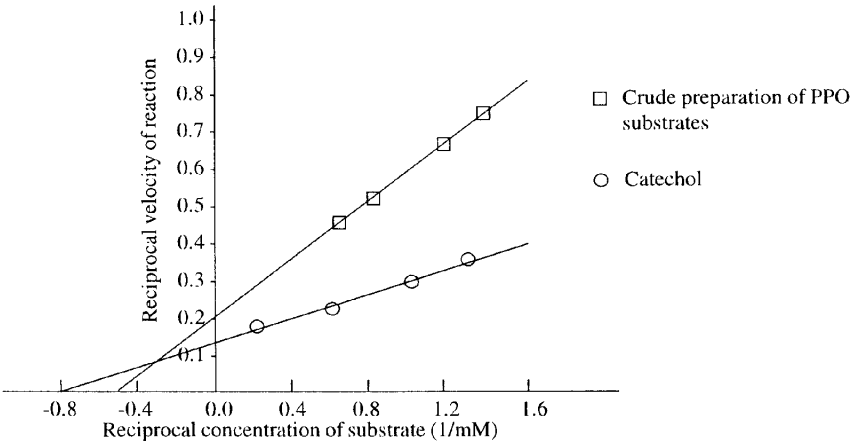

Fig. 1. Lineweaver – Burk double reciprocal plots for crude preparation of mushroom polyphenol oxidase substrates and catechol as a standard

A number of different substrates have been observed as PPO inhibitors and data about the variation of their inhibitory effect toward various substrates are presented in Table 1 and Figure 2. Completely inhibition of PPO activity by cysteine and 2-mercaptoethanol was observed at 1 mM concentration for these tested substrates. Partial inhibition of catechol, DOPA, chlorogenic acid and caffeic acid oxidation was observed at cysteine concentration 0.1 mM, while much less inhibition was apparent at 0.01 mM concentration. Substrate specifity and inhibitor concentration had various effect when 2-mercaptoethanol was tested. At 0.1 mM concentration, 2-mercaptoethanol strongly inhibits of PPO activity towards the catechin (88.1 %) and DOPA (100 %) while for chlorogenic and caffeic acids slight stimulation of activity was observed (Tab. 2). Also slight stimulation of activity was observed for 0.01 mM 2-mercaptoethanol for all except catechin substrates.

Benzoic acid was shown to inhibit mushroom PPO activity (Tab. 2). The percentage of inhibition gradually decreased with decreasing concentration of benzoic acid. More than 60 % inhibition was observed, when 1 mM benzoic acid was used and only 8-30 % activities were inhibited for 0.01 mM. Benzoic acid was also shown to have more profound inhibitory effect on the oxidation of DOPA by PPO than for catechin, chlorogenic acid and caffeic acid. PPO enzyme derived from strains No. 22 and No. 53 did not differ so much in response toward tested inhibitors. As shown in Figure 2 sodium metabisulphite strongly inhibited the PPO activity and the level of inactivation depends on the concentration of metabisulphite present in the reaction mixture. Concentration above 2 mM of sodium metabisulphite was necessary to completely inactivate the enzyme.

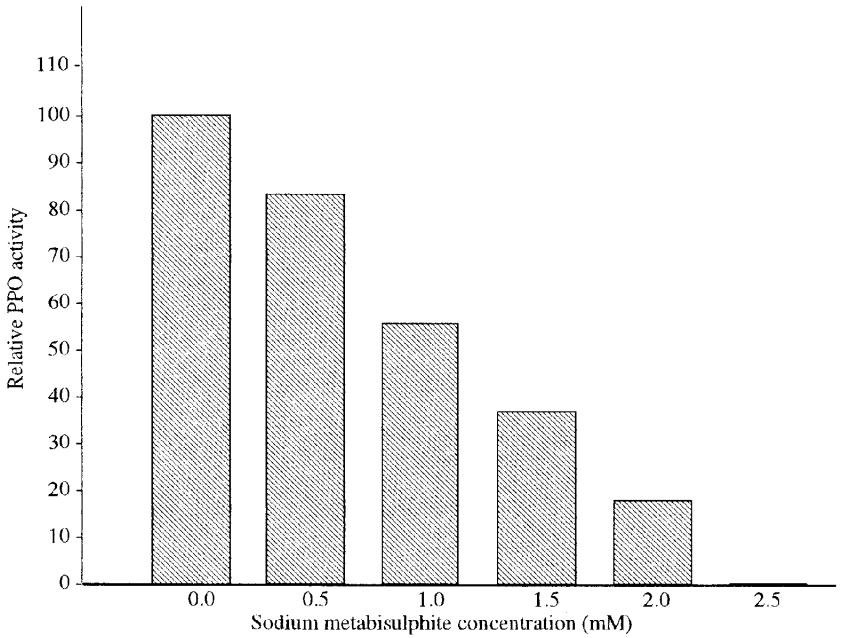

Fig. 2. Effect of sodium metabisulphite on mushroom polyphenol oxidase activity toward catechol as a substrate

Table 2

Effect of inhibitors on mashroom polyphenol oxidase (PPO) relative activity (%)* toward various substrates

Inhibitor concentrations (mM)	Catechin		DOPA		Chlorogenic acid		Caffeic acid	
	No. 22	No. 53	No. 22	No. 53	No. 22	No. 53	No. 22	No. 53
Cysteine HCl								
1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	43.4	51.6	49.1	47.9	38.4	45.6	50.4	28.2
0.01	100.0	90.4	92.3	89.0	79.9	78.6	88.8	76.4
2-Mercapto-ethanol								
1.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0
0.1	11.9	–	0.0	0.0	103.8	102.6	128.1	122.0
0.01	76.2	–	110.2	115.0	117.4	138.4	132.5	100.0
Benzoic acid								
1.0	33.3	32.9	22.6	24.7	32.5	44.1	39.5	33.9
0.1	60.2	68.4	60.0	56.1	57.7	75.8	58.1	51.5
0.01	79.3	86.1	72.0	68.5	78.4	92.4	79.4	86.4

*Relative PPO activity without inhibitors was assumed as 100 %

The PPO from mushrooms has been shown to contain numerous isoenzymes with various substrates (Fig. 3). Characteristic slow- and fast-moving 4 isoforms was present when DOPA was used as a substrate. Electrophoretic patterns of PPO of mushrooms reveals 2 bands when catechin and chlorogenic acid were used as substrates, while only one band showed tyrosine and p-cresol activity as shown on Figure 3. One most active towards DOPA isoenzyme (relative mobility Rm of 0.5) oxidized all the substrates, appears to be capable of oxidizing both monophenols (p-cresol) and diphenols.

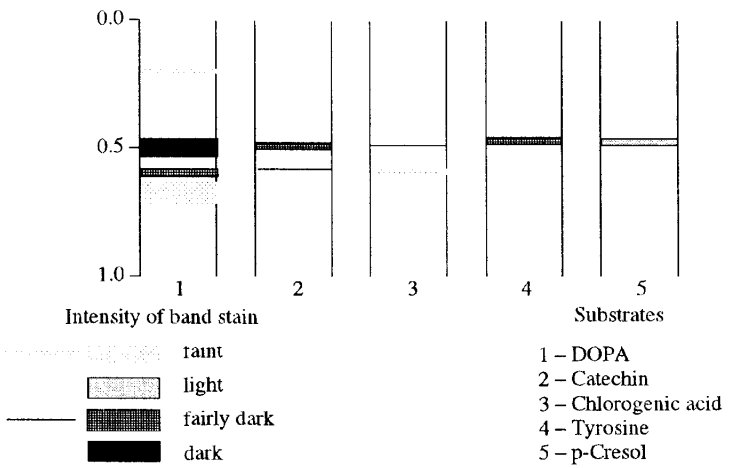

Fig. 3. Electrophoretic patterns of mushroom polyphenol oxidase toward variuos substrates

DISCUSSION

Two types of phenol oxidase have been isolated from plant sources. One type (tyrosinase, phenolase, polyphenol oxidase, catechol oxidase) oxidized o-diphenols to their corresponding qiunones in the presence of molecular oxygen. The second type (p-diphenol oxidase, laccase) oxidizes p-diphenols to their corresponding quinones. This classification is still rather confusing though the enzymes that catalyse these reactions have different properties, physical characteristics and substrate preferences (M a y e r, H a r e l, 1991). In general, 4-methylcatechol and DOPA have been used as selective substrates for polyphenol oxidase while for laccase most often included the use of p-quinol syringaldazine (D a w l e y, F l u r k e y, 1993). In fact, when mixture of the two types of enzyme are present, it can be very difficult to measure the enzyme activity associated with a specific enzyme or to differentiate between the two types of diphenol oxidase.

Several thiol containing compounds and aromatic carboxylic acids have been investigated as inhibitors for the enzymatic browning (K e r m a s h a et al., 1993 a). The results presented by K e r m a s h a et al. (1993 b) indicate clearly that the type and degree of inhibition of mushroom PPO activity by cysteine and aromatic acids

are dependent on the method used. Since in our investigations the spectrophotometric method was used for the determination of the absorbance of end products, the formation of these adducts may explain the high observed inhibitory effect of cysteine on PPO activity. The inhibitory effect of cysteine monitored by the polarographic method (Kermasha et al., 1993 a) suggests a direct inhibition of the enzyme activity and that cysteine is a noncompetitive inhibitor of the mixed type.

Information in the mode of action of sulphite has been relatively limited. Evidence of the mechanisms of inhibition of enzymatic browning by sulphite such as formation of quinone-sulphite complexes and the inactivation of PPO has been found (Embs, Markakis, 1965; Haisman, 1974). Other evidence presented by Sayavedra-Soto and Montgomery (1986) suggests that the major mode of direct irreversible of PPO was modification of the protein structure, with retention of its molecular unity.

REFERENCES

Allan A. C., Walker J. R. L., 1988. The selective inhibition of catechol oxidase by salicylhydroxamic acid. Phytochemistry 27: 3075-3076.

Chen J. S., Wei C., Marshall R., 1991. Inhibition mechanism of koijc acid on polyphenol oxidase. J. Agric. Food Chem. 39: 1897-1901.

Constantinides S. M., Bedford C. L., 1967. Multiple forms of phenoloxidase. J. Food Sci. 32: 446-450.

Czapski J., 1989. A simple rapid method for protein determination. Acta Agrobot. 42: 183-190.

Czapski J., Saniewski M., Puchalski J., Lange E., Nowacki J., 1988. The effect of methyl jasmonate on activity and electrophoretic pattern of polyphenol oxidase in postclimacteric apples cv. Jonathan. Fruit Sci. Reports 15: 103-110.

Dawson C. R., 1963. On the pathway the catechol-tyrosinase reaction. Ann. N. Y. Acad. Sci. 100: 937-950.

Dawley R. M., Flurkey W., 1993. Differentiation of tyrosinase and laccase using 4-hexylresorciol a tyrosinase inhibitor. Phytochemistry 33: 281-284.

Embs R. J., Markakis P., 1965. The mechanism of sulphite inhibition of browning caused by polyphenol oxidase. I. Food Sci. 30: 753-758.

Ferrer O. J., Kouburger J. A., Ottwell W. S., Gleeson R. A., Simpson B. K., Marshall M. R., 1989. Phenoloxidase from the cuticle of Florida spiny lobster (*Panulirus argus*): mode of activation and characterization. J. Food Sci. 54: 63-66.

Golan-Goldhirsh A., Whitaker J. R., Kahn W., 1984. Relation between structure of polyphenol oxidase and prevention of browning. Adv. Exp. Med. Biol. 177: 437-456.

Haisman D. R., 1974. The effect of sulphur dioxide and oxidizing enzyme systems in plant tissues. J. Sci. Food Agic. 25: 808-814.

Hopfinger J. A., Poovaiah B. W., Patterson M. E., 1984. Calcium and magnesium interactions in browning of Golden Delicious apples with bitter pit. Scientia Hortic. 23: 345-351.

Hsu A. F., Shieh J. J., White K., 1988. Inhibition of mushroom polyphenol oxidase by ascorbic acid derivatives. J. Food Sci. 30: 753-758.

Johnson G., Schaal L. A., 1957. Relation of chlorogenic acid to scab resistance in potatoes. Science 115: 627-629.

Kahn V., Andrawis A., 1986. Multiple effect of hydoxylamine on mushroom tyrosinase. Phytochemistry 25: 333-337.

Kermasha S., Goetgheber M., Monfette M., Rovel B., 1993 a. Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. Phytochemistry, 34: 349-353.

Kermasha S., Goetgheber M., Monfette M., Rovel B., 1993 b. Studies on inhibition of mushrooms polyphenol oxidase using chlorogenic acid as substrate. J. Agric. Food Chem., 41: 526-531.

Koeing J. K., Pierson W. E., Horike M., Frank R., 1983. A comparison of the effects of 0.5 ppm, 1.0 ppm sulphur dioxide plus sodium chloride droplets in asthmatic adolescents. Toxicol. Environ. Health, 11: 129-136.
Lerch K., 1981. Copper monooxygenases: tyrosinase and dopamine b-monooxygenase. [In:] Metal ions in biological systems. H. Siegiel (ed.), Marcel Dekker, New York, v. 13, p. 143.
Mayer A. M., Harel E., 1991. [In:] Food enzymology. P. E. Fox (ed.) Elsevier, pp. 373-378.
McCord J. D., Kilara A., 1983. Control of enzymatic browning in processed mushrooms (*Agaricus bisporus*). J. Food Sci. 48: 1479-1483.
Robb D. A., 1984. Tyrosinase. [In:] Copper proteins and copper enzymes. R. Lontie (ed.), CRS Press, v. 2, p. 207.
Sargent J. R., 1969. Methods in zone electrophoresis. BDH Chemical Ltd., pp. 85-107.
Sayavedra-Solo L. A., Montgomery M. N., 1986. Inhibition of polyphenol oxidase by sulphite. J. Food Sci. 51: 1531-1536.
Swan G. A., 1963. Chemical structure of melanins. Ann. N. Y. Acad. Sci. 100: 1005-1019.
Walker J. R. L., 1975. Enzymatic browning in food. A review. Enzyme Technol. Dig. 4: 89-107.

Specyficzność substratowa i inhibitory oksydazy polifenolowej w aspekcie ciemnienia świeżych i mrożonych pieczarek (*Agraricus bisporus* (Lange) Sing.)

Streszczenie

Badano aktywność oksydazy polifenolowej pieczarek wobec 6 substratów oraz hamowanie aktywności enzymu przez cysteinę, 2-merkaptoetanol, kwas benzoesowy i polisiarczyn sodu.

Najbardziej efektywnymi substratami były: katechina, DOPA (L-3,4-dihydroksyfenyloalanina) i kwas chlorogenowy. Powinowactwo enzymu do endogennych substratów wyrażone odwrotnością stałej Michaelisa (K_m) było niższe niż powinowactwo do katecholu. Efekt hamowania aktywności zależał od specyfiki i stężenia inhibitora w roztworze. Elektroforetyczne rozdzielenie izozymów oksydazy polifenolowej wykazało obecność 4 izozymów wobec DOPA, dwóch wobec katechiny i kwasu chlorogenowego oraz jednego wobec L-tyrozyny i p-krezolu.