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Some properties of active and latent catechol oxidase of mushroom

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Abstract

Latent form of mushroom catechol oxidase was activated by 0.1% sodium dodecyl sulfate (SDS). Catalytic power of the latent form, calculated from the kinetic parameters was 1.8 times higher than that of active one. Salicyl hydroxamic acid (SHAM) appeared as a powerful inhibitor for both active and latent forms of catechol oxidase. However, in the range of 150-250 μM SHAM the inhibitory effect for active catechol oxidase was significantly higher than that for the latent one. Non-competitive and irreversible characteristics of inhibition of latent and active catechol oxidase was calculated from kinetic data. Electrophoretic analysis followed by scanning of the gels was used. The spots' absorbance was determined from a computer image of the isoenzyme band patterns. It allowed us to estimate gels quantitatively. Presence of one additional clearly defined slow moving isoform of SDS-activated catechol oxidase, differed in the respect of 3 bands for the active and 4 bands for the total.

Key words: *Agaricus bisporus*; mushroom; latent and active catechol oxidase; isoenzyme pattern.

INTRODUCTION

Enzymatic discoloration of fruits, vegetables and mushrooms is catalyzed primarily by the enzyme polyphenol oxidase (PPO - EC. 1.14.18.1). PPO has been the subject of several reviews (Mayer, 1987; Nicolas et al., 1994; Sanchez-Ferrer et al., 1995). PPO exhibits two types of activity: hydroxylation of monophenols to o-diphenols (monophenolase, cresolase) and oxidation of o-diphenols to o-quinones (catecholase, catechol oxidase) with subsequent polymerization, leading to the formation of complex brown pigments (Prota, 1988). The prevention of this reaction has always been a challenge to food scientists (Matheis, 1987). Numerous compounds have been investigated as inhibitors for enzymatic browning. Some of them act by inhibiting PPO (Kahn, Andravis, 1986; Sayavedra-Soto, Montgomery, 1986; Allan,

Walker, 1988) by reducing o-quinones to o-diphenols (Chen et al.,1991) or Cu^{+2} to Cu^{+} (Hsu et al., 1988) by interacting with the formation of o-quinone products (Ferrer et al., 1989) and by decreasing the uptake of O_2 for the reaction (Kahn et al., 1997). One of the powerful and selective inhibitor both catecholase and monophenolase activities of mushroom is salicylhydroxamic acid (SHAM) (Allan, Walker, 1988). SHAM is usually known as a selective inhibitor of cyanide resistant respiration in plants (Seidow, Berthold, 1986).

An inactive or latent diphenolase was found in the soluble fraction of the fruiting bodies of cultivated mushrooms (*Agaricus bisporus*) and constituted almost 95% of the total PPO (Yamaguchi et al., 1970). Amount of latent form of catechol oxidase increased during maturation of the basidiocarp (Yamaguchi et al., 1970; Ingebrigsten et al., 1989). The most effective preparation of mushrooms for latent diphenolase extraction is freeze drying or acetone powder preparation. Sanchez-Ferrer et al. (1989) developed novel procedure for extraction of latent grape PPO using temperature induced phase separation in Triton X-114. In some plants (broad beans, spinach) PPO exist in latent form (Kenten, 1957; Golbeck, Cammarata, 1981). The latent enzyme can be activated by different treatments including trypsin (Tolbert, 1973), fatty acids (Golbeck, Cammarata 1981), aging and low temperature (Lieberei, Biehl, 1978). The process of activation by trypsin is quick and it can be used to activate small samples of latent enzyme (Sanchez-Ferrer et al.,1989). The effect of detergents on the activity of latent PPO was tested using cationic, anionic, and nonionic ones (Sanchez-Ferrer et al., 1989). The cationic detergent (cetyltrimethylammonium bromide) was most effective followed by anionic (sodium dodecyl sulfate). The nonionic detergents (Brij 96) have little effect on activity. Latent form of grape polyphenol oxidase has also been activated by the divalent cations such as Ca^{+2}, Mn^{+2}, Mg^{+2} (Jimenez, Garcia-Carmona, 1993a) and by polyamines (Jimenez-Atienzar et al., 1991). This last activation caused a higher sensitivity of PPO to pH and temperature. Also polyglucan type elicitors (zymosan and chitosan) activated latent PPO of partially purified grapevine cell suspension culture (Jimenez, Garcia-Carmona, 1993b). Since information concerning latent mushroom catecholase is still limited this study was undertaken to compare activity, kinetic characterization, effect of inhibitor (SHAM) and electrophoretic pattern of isoenzymes of both latent and active catechol oxidase.

MATERIALS AND METHODS

Extracts from mushrooms prepared for study monophenolase (Czapski, 1998) were used as a source of total (T) and active (A) catechol oxidase.

Enzymic activity was measured by determining the amount of o-benzoquinone formed from catechol. Catechol was the best o-diphenolic substrate assayed because of the absence of a ring substituent (Espin et al., 1998). Instead of recommended (Espin et al., 1997) chromogenic nucleophile, 3-methyl-2-benzothiazolinone hydrazone (MBTH), which MBTH-benzoquinone adduct is not very soluble in the assay medium (Espin et al., 1998), we used L-proline as nucleophilic agent for the coupling of o-benzoquinone to produce a red compound previously described (Jackson, Kendal,

1949; Jolley, Mason, 1965; Yamaguchi et al., 1970). The color thus formed is stable at pH 6-7 within the range for maximum enzymic activity. Sodium dodecyl sulfate (SDS) in the concentration used for extraction of total catechol oxidase from acetone powder did not affect color formation. By use of this reaction, the enzymic activity was determined spectrophotometrically at 525 nm and at 25°C. The temperature was controlled with a precision of 0.1°C. The standard reaction mixture contained 2.5 mM catechol and 2.5 mM L-proline in 0.1 M phosphate buffer pH 6.5. Unless otherwise indicated the cuvette contained 2.8 - 2.9 ml standard reaction mixture and 0.2 - 0.1 ml enzyme extract. The blank sample contained 3 ml substrate solution. Change in absorbance was recorded and the enzyme activity was calculated from the linear portion of the curve. Enzyme activity was expressed as micromol catechol oxidized per minute per 1 g tissue fresh weight [$\mu mol \cdot (min\ (g)^{-1}$ fr.wt].

Latent (L) activity of catechol oxidase was calculated from total (T) minus active (A) activity: L = T - A. Value of molar absorptivity $\varepsilon = 2.23 \cdot 10^3$ $(M \cdot cm)^{-1}$ of o-benzoquinone-L-proline pigment was determined at 525 nm by fast oxidation of the low concentration of catechol (0.33 mM) by high activity (0.2 *A*/min) of commercial mushroom catechol oxidase with the presence 10 mM L-proline. This molar absorptivity was similar to value $1.79 \cdot 10^3$ (Goodenough, 1978) compared to $5 \cdot 10^3$ quoted by Yamaguchi et al., (1970).

Michaelis constant (K_m) and maximum velocity (V_{max}) was determined using substrates in various concentrations. Data was plotted according to the Lineweaver and Burk (1934) and using linear regression fitting of GraphPad Prism program for Windows.

To determine effects of salicylhydoxamic acid (SHAM) inhibitor, various concentrations of substrate and 50μM SHAM or 2.5 mM substrate and various concentrations of SHAM were used. The K_m and V_{max} were calculated as described above.

The multiple enzyme forms of active and total catechol oxidase were separated in 7.5% polyacrylamide gel, using disc technique of Orstein and Davies (Sargent, 1969). Sample was introduced together with upper gel before polymerization. Enzyme activities introduced with upper gel were: A = 179 nmol (min^{-1}; T = 675 nmol (min^{-1}. The electrode buffer was 0.05 M Tris – 0.38 M glycine, pH 8.3. Bromophenol blue (0.001%) was added as the tracking dye. The electrophoresis was running at 2°C and 2 mA per tube was applied until the bromophenol blue had reached the „running" gel, then current was increased to 4 mA. Immediately after stopping the electrophoretic run the gels were removed from the tubes and incubated in 0.1M phosphate buffer pH 6.5 for 30 min and then stained with substrate solution containing 2.5 mM catechol and 2.5 mM L-proline in 0.1 M phosphate buffer pH 6.5. Then the gels were rinsed with water and placed in deionized water and scanned as soon as possible. The ScanMan hand held scanner and Logitech ScanMan Software was used as an image source for the FotoTouch Image Editing Software. The computer image of stained gels was then printed using Hewlett-Packard LaserJet 5P printer.

Computer image of isoenzyme bands on gels, printed on paper occurred in the shape of spots pattern. Spot area and absorbance was determined using Universal Densitometer Vitatron. The instrument provides the possibility of carrying out quantitative determinations of spots on TLC plates by means of transmitted light measurement. Instead of TLC plate sheet of paper with printed computer image of spots pattern was

used. Each spot was scanned in a zig-zag sine wave pattern and the photomultiplier measured the light intensity (I) continuously. The log I values were summed and the average value was passed continuously to the recorder and integrator.

RESULTS AND DISCUSSION

Concentration of 0.1% SDS in extraction buffer was most effective in activation of latent form of catechol oxidase. Lower or higher content of SDS produced lower activation (Table 1), and this agrees with the finding of Yamaguchi et al. (1970). High activation effect of SDS was also observed for latent form of grape polyphenol oxidase (Sanchez-Ferrer et al., 1989). Table 1 shows also the effect produced by the presence of different concentrations of SDS in assay medium, on activity of extract of active catechol oxidase. Final SDS concentration 0.04% increased activity 1.8 times. Concentration of SDS from 0.04% to 0.3% did not change the enzymatic activity. These results can be clarified by the findings of Yamaguchi et al. (1970), that only supernatant not particulate fraction contains latent form of mushroom catecholase. Ingebrigsten et al. (1989) reported that latent DOPA oxidase activity from mushrooms was detected by including SDS in the assay. On the other hand it was reported that enzyme assays of diphenolase from mushrooms and lettuce showed no activation by 0.05% SDS (Angleton, Flurkey, 1984). It is worth mentioning that SDS used in a concentration of 0.1% had no effect on catechol oxidase activity of commercial mushroom tyrosinase (data not shown).

Table 1

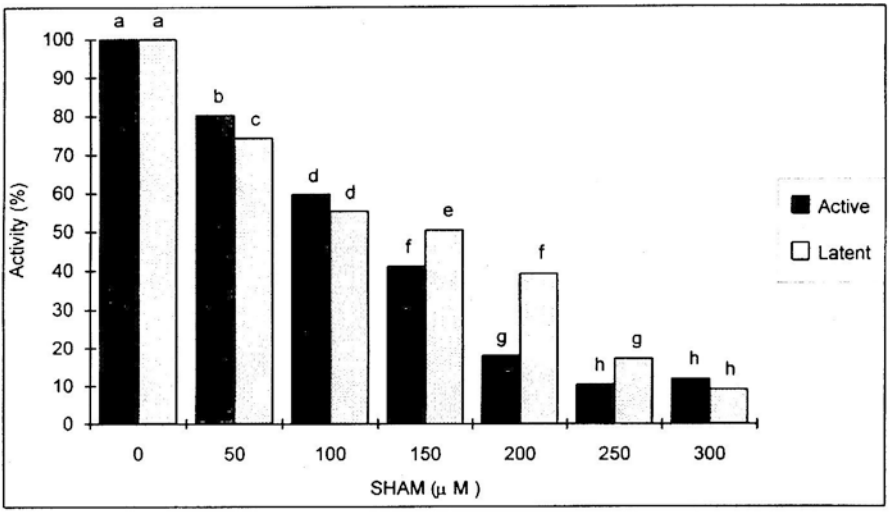
Effect of sodium dodecyl sulfate (SDS) concentration in extraction buffer and in assay medium on activation of mushroom oxidase [µmol · (min · g)$^{-1}$ fr.wt]

SDS concentration (%)	Extraction buffer		Assay medium	
	Activity	Relative activity	Activity	Relative activity
0*	9.5 d**	1.0	10.4	1.0
0.04	–	–	18.3	1.8
0.05	14.7 c	1.5	–	–
0.07	–	–	18.5	1.8
0.1	40.9 a	4.3	18.2	1.8
0.2	–	–	18.7	1.8
0.3	35.2 b	3.7	–	–

* Active catechol oxidase

** Means followed by different letters differ significantly at P = 0.95 by Newman – Keuls test

Fig. 1. Effect of SHAM concentration on active and latent relative catechol oxidase activity.
Note: Means followed by different letters differ significantly at P=0.95 by Newman – Keuls test.



Earlier studies have been shown that salicylhydroxamic acid is selective inhibitor of active diphenolase (Allan, Walker, 1988; Dawley, Flurkey, 1993). As shown in Figure 1, SHAM strongly inhibited activity of active and latent catechol oxidase and the degree of this inactivation depends on the concentration of SHAM in the reaction medium. In the range of SHAM concentrations 150-250 μM the effect of inhibition of activity of active catechol oxidase is significantly higher than that for latent one, being for 200 μM SHAM 80% and 60% for active and latent respectively. A concentration of 300 μM of SHAM inhibited activity of both active and latent catechol oxidase by 90%. Measuring the initial rate of O_2 uptake using oxygen electrode, Allan and Walker (1988) found that as little as 10 μM concentration of SHAM, inhibited catechol oxidase activity by over 90% which was a lower concentration than that determined in our studies. The results of estimation of inhibition may vary depending on the method used for the determination of enzymatic activity, spectrophotometric or O_2 uptake (Kermasha et al., 1993). Similar to our results, the inhibitory effect of SHAM concentration (100 μM) was reported by Dawley and Flurkey (1993) using spectrophotometric method of determination.

Kinetic parameters (K_m and V_{max}) were obtained from the equations of Lineweaver - Burk plots and presented in Table 2. The catalytic power $V_{max} \cdot K_m^{-1}$ for the latent diphenolase activity is 1.8 times higher than that for active one, but affinity of substrate (expressed as K_m^{-1}) is 2 times lower being 0.18 and 0.36 respectively. Since K_m values were little affected by SHAM and the V_{max} were considerably reduced (Tab. 2), this suggests non-competitive inhibition of active and latent catechol oxidase by SHAM. Inhibition reversibility or irreversibility of SHAM was determined according to a mathematical method (Kermasha et al.,1993) which involved a plot (not shown here) of $v_o/(v_o-v_i)$ versus 1/[I] (where: v_o= steady state rate without inhibitor, v_i= steady state rate

in presence inhibitor, [I] = inhibitor concentration). From the equation of linear regression Y-intercept at value < 1 or ≥ 1 indicates respectively an irreversible or reversible inhibition. Calculated Y-intercept values for active and latent catechol oxidase are: 0.024 and 0.537 respectively. It suggests that SHAM has an irreversible inhibitory effect on mushroom active and latent catechol oxidase activity.

The active PPO from mushrooms has been shown to contain numerous isoenzymes with various substrates. Four characteristic slow and fast moving isoforms are present when L-3, 4-dihydroxyphenylalanine (DOPA) was used as a substrate (Choi, Sapers, 1994; Czapski, 1994) while only 2 bands were present when catechin and chlorogenic acid were used and 1 band when tyrosine was used as a substrate (Czapski, 1994). Electrophoretic analysis of total catechol oxidase isoenzymes indicated four forms present, while active one revealed the presence of three isoenzyme bands (Fig. 2).

Table 2

Kinetic parameters of active and latent mushroom catechol oxidase in the absence and presence (+) SHAM inhibitor

Catechol oxidase				+ SHAM		
	K_m	V_{max}	$V_{max} \cdot K_m^{-1}$	K_m	V_{max}	$V_{max} \cdot K_m^{-1}$
	(mM)	[μmol·(min·g)$^{-1}$]	(min)$^{-1}$	(mM)	[μmol·(min·g)$^{-1}$]	(min)$^{-1}$
Active	2.80±0.29*	22.7±3.7	8.1	3.55±0.32*	2.70±0.23	0.76
Latent	5.57±0.54	81.2±11.8	14.6	3.48±0.35	7.70±0.65	2.2

* Mean ± se

Table 3

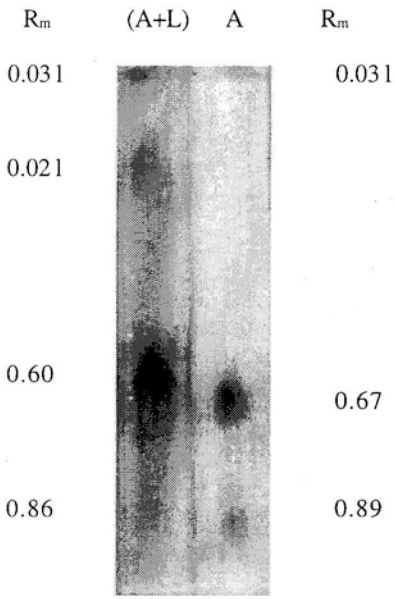
Relative intensity of individual as percentage of sum all spots intensity after electrophoretic analysis (A) and total (A + L) mushroom catechol oxidase

No of spot	Active (A)		Total (A + L)	
	R_m*	Relative intensity (%)**	R_m*	Relative intensity (%)**
1	0.031	15.4 ± 0.5	0.031	9.9 ± 0.5
2	–	–	0.21	25.3 ± 2.1
3	0.67	46.5 ± 2.0	0.60	43.1 ± 1.7
4	0.89	37.6 ± 1.9	0.86	21.7 ± 0.1

* Relative mobility (see Fig 2);

** Mean ± se

Fig. 2. Electrophoretic isoenzyme pattern of active (A) and total (A+L) mushroom catechol oxidase. All comparisons between gels were normalized to R_m among 0 and 1.



All three bands of active catecholase, two fast and one slow moving have analogues in total catechol oxidase electrophoretic pattern. Presence of one clearly defined slow moving isoform at $R_m = 0.21$ for SDS - activated catechol oxidase differs in this respect for active and total phenolase. The relative intensity of individual spot as percentage of sum all spots intensity was calculated and presented in Table 3. The relative intensity of spots for active (R_m= 0.67) and total (R_m= 0.60) forms are almost equal. Other active catecholase spots were always relatively more intense than analogous total ones. Relative intensity 25.3% of slow moving (R_m = 0.21) isoenzyme is considerably high. We can not rule out the possibility that it might be a latent form of mushroom catechol oxidase. Our results are somewhat contradictory to those of Angleton and Flurkey (1994) who found no change of any new forms generated in the presence of SDS. They agreed, however, that there was no observable change in the mobility of the individual isoenzyme forms in the presence and absence SDS. These discrepancies arose probably as result of different methods of electrophoresis and SDS treatments used. It is worth mentioning that 0.1% SDS treatment had no effect on diphenolase isoenzyme pattern of commercial tyrosinase. Only one broad spot at R_m= 0.78 - 0.87 was found (data not shown here). Yamaguchi et al. (1970) ruled out the possibility that activation of diphenolase by SDS is due to solublization of more enzyme. It might be speculated that SDS removes some tightly bound inhibitor that affects the latency of the enzyme or induces specific conformational changes in the protein complexes. One can not rule out also the possibility that SDS could dissociate proteins into subunit(s) producing electrophoretically active fraction at R_m= 0.21. Any of these possible mechanisms could explain the difference in isoenzyme pattern,

however further studies are necessary to clarify and to understand the latency and activation process of mushroom catechol oxidase.

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Niektóre właściwości aktywnej i utajonej oksydazy katecholowej grzybów

Streszczenie

Aktywowano formę utajoną oksydazy katecholowej pieczarek (*Agaricus bisporus*) 0,1 % roztworem siarczanu dodecylo - sodowego (SDS). Siła katalityczna formy utajonej była 1,8 razy większa niż formy aktywnej. Stwierdzono, że kwas salicylohydroksyamowy (SHAM) jest silnym inhibitorem aktywności obydwu form oksydazy katecholowej. Działanie SHAM w zakresie stężeń 150 - 250 µM było znacznie efektywniejsze dla formy aktywnej niż utajonej. Stwierdzono niekompetencyjny i nieodwracalny charakter inhibicji aktywności przez SHAM obydwu form enzymatycznych. Rozdział elektroforetyczny i następnie „skaningowanie" żeli pozwoliło na uzyskanie obrazu komputerowego wzoru izoenzymatycznego oraz na jego ilościową ocenę. Obecność dodatkowego izoenzymu we wzorze izozymowym obydwu form: aktywnej i utajonej łącznie (4 frakcje), odróżniało go od wzoru izoenzymatycznego tylko formy aktywnej (3 frakcje).