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INHIBITION OF POLYPHENOL OXIDASE BY COPPER-METALLOTHIONEIN FROM *ASPERGILLUS NIGER*

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Abstract—Metallothionein from *Aspergillus niger* was found to be an inhibitor, in a model system, for the enzymic activity of a commercially purified mushroom tyrosinase. The inhibitory effect of metallothionein was higher on catechin oxidation compared with that on chlorogenic acid. The degree of inhibition of enzymic colour formation by metallothionein, using catechin as substrate, was lower than that of oxygen uptake; however, a reverse pattern was observed with chlorogenic acid as substrate. The demetallization of metallothionein did not increase its inhibitory effect on polyphenol oxidase whereas pre-incubation of the enzyme and metallothionein increased this inhibitory effect.

INTRODUCTION

Metallothioneins are ubiquitous cytosolic proteins, usually characterized by their low M_rs, selective binding of large amount of heavy metal ions such as Zn^{2+}, Cu^{2+}, Cd^{2+} or Hg^{2+} and high content of cysteine [1–3]. *Neurospora crassa* copper-metallothionein was reported as a metal donor for apotyrosinase [1].

Enzymic browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic compounds catalysed by polyphenol oxidase (PPO). The PPOs (EC 1.14.18.1) are copper containing enzymes that catalyse, in the presence of oxygen, the oxidation of diphenols of *o*-quinones, which upon further reactions lead to brownish pigments [4]. The most widespread methodology used in food industries for the control of enzymic browning is the addition of sulphiting agents. However, the adverse health effects of sulphites as well as increased regulatory scrutiny [5] have created the need for alternatives to chemical agents.

Chelating agents such as EDTA are reported to be used as inhibitors for PPO activity. The chelating agents are believed either to bind to the active site copper of PPO or reduce the level of copper available for incorporation into the holoenzyme [6]. On the other hand, several thiol-containing compounds have been investigated as inhibitors for the enzymic browning [7, 8). The literature has reported on two major mechanisms for the inhibition of PPO by cysteine, a direct inhibition of PPO [9] and the formation of colourless *cis*-quinone adducts [8, 10, 11].

A low M_r copper-chelating peptide from *Dactylium dendroides* was reported to inhibit mushroom tyrosinase [12]. Under conditions of high exposure, induced metallothioneins could provide highly effective demetallizing agents because of their strong avidity for the metal ion [13]. In addition, metallothioneins exhibit strong reduction activities [14]. Since PPO contains copper in the active site, metallothionein could be used as an inhibitor of enzyme activity. Procedures for the induction and purification of metallothionein from *Aspergillus niger* were previously developed in our laboratory [15, 16]. The aim of this study was to investigate the degree of inhibition of PPO by fungal metallothionein preparations in a model system, using a commercially purified enzyme and catechin and chlorogenic acid as substrates with comparative spectrophotometric and polarographic methods.

RESULTS

Inhibitory effect of partially purified metallothionein on tyrosinase activity

The inhibitory effect of the partially purified metallothionein (M1) was assessed by both polarographic and spectrophotometric methods, using chlorogenic acid and catechin as substrates at a concentration of 5 mM. Under these conditions, the substrate concentration was much higher than K_m values, 0.91 and 0.73 mM for catechin and 0.20 and 0.31 mM for chlorogenic acid, using spectrophotometric and polarographic methods, respectively. These results suggest

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that, at the beginning of the assay, the enzymic reaction was of zero order.

Using catechin as substrate, the spectrophotometric results (Fig. 1A) show that the highest rate of formation of enzymic coloured end-products by PPO was achieved at the beginning of the reaction before its decrease after 4 min, with a maximum of absorbance reached at 20 min. The addition of metallothionein M1 to the reaction media (Fig. 1A) resulted in the appearance of a lag period in the formation of coloured products, followed by a low rate of this formation. The polarographic results (Fig. 1B) demonstrate that the addition of M1 to the PPO enzymic assay resulted in the absence of a lag period and a decrease in the rate of oxygen uptake.

Using chlorogenic acid as substrate (Fig. 2A and B), a similar pattern as for catechin was observed without metallothionein. The addition of M1 to the reaction media (Fig. 2A) extended the lag time and resulted in a low rate of enzymic browning with a concomitant immediate decrease in the rate of oxygen consumption (Fig. 2B).

The results (Table 1) show that the range of inhibitor concentrations that yield 50% of inhibition of PPO activity (I_{50}), varied from 42 to 104 μg of protein ml^{-1}. Using catechin as substrate, the I_{50} values indicate that the inhibitory effect of M1 was 1.3 times higher for the polarographic determination than for the spectrophotometric one. However, using chlorogenic acid as substrate, the results (Table 1) show a 2.1 times greater inhibitory effect with the spectrophometric assay compared with the polarographic one.

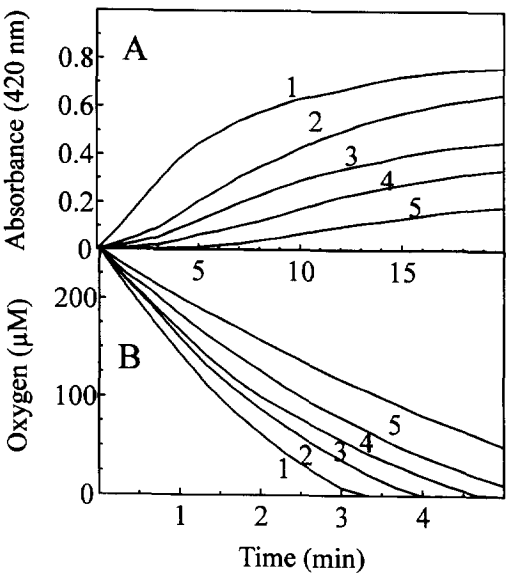

Fig. 1. Oxidation of catechin by PPO as determined by (A) spectrophotometric measurement of coloured-product formation at 420 nm, and (B) polarographic method by oxygen uptake. Metallothionein (M1) concentrations are in mg protein ml^{-1}: (1) 0; (2b) 18; (3) 28; (4) 84; (5) 156.

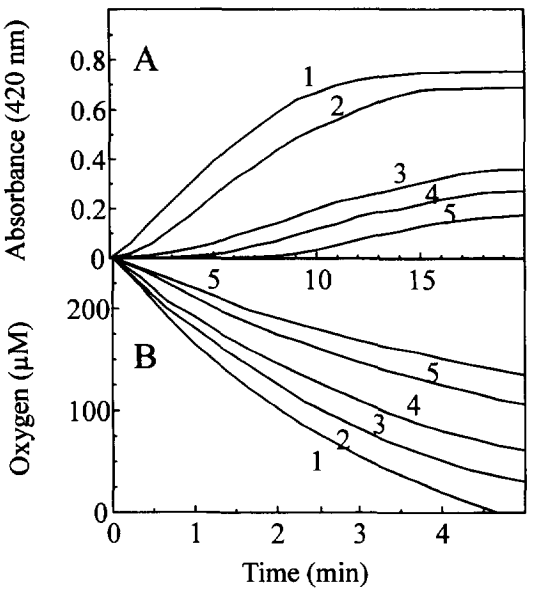

Fig. 2. Oxidation of chlorogenic acid by PPO as determined by (A) spectrophotometric measurement of coloured-product formation at 420 nm, and (B) polarographic method by oxygen uptake. Metallothionein (M1) concentrations are in mg protein ml^{-1}: (1) 0; (2) 11; (3) 28; (4) 76; (5) 270.

Kinetic studies were carried out and the type of inhibition was deduced from Lineweaver–Burk plots. Inhibitory constants (K_i) were calculated from secondary plots according to the method described by Segel [17]. The results (Table 1) indicate that, using the polarographic method, M1 exhibited a non-competitive inhibitory effect on the PPO oxidation of catechin. However, using the spectrophotometric method, a mixed type inhibition was observed for the oxidation of catechin (Table 1); the K_i value for catechin was higher for the polarographic assay than for the spectrophotometric one.

Using chlorogenic acid as substrate, the results (Table 1) indicate that M1 has an inhibitory effect of the mixed type on the PPO activity, using both spectrophotometric and polarographic methods; however, the K_i value was four times higher with the polarographic determination than with the polarographic one.

Assuming that the M_rs of metallothionein [18] and PPO subunit [19] are 10 and 34 kD, respectively, the effectiveness of binding of the inhibitor ranges from 127 to 270 mol metallothionein per mol PPO subunit (Table 1).

Inhibitory effect of purified metallothionein on tyrosinase activity

The metallothionein M1 was also subjected to further purification by affinity chromatography according to the procedure developed in our laboratory [16], which resulted in an M2 fraction with a 10-fold increase in the

Table 1. Inhibitory effects of partially purified metallothionein (M1) and purified metallothionein (M2) obtained from *A. niger* on polyphenol oxidase in a model system

Substrate	Method	Metallothionein*	I_{50}† (μg protein ml^{-1})	K_i‡ (μg protein ml)	Type of inhibition	Effectiveness of binding§ (mol M1 or M2 mol^{-1} PPO)
Catechin‖						
	Spectrophotometric¶					
		M1	55.0	31.0	Mixed	143
		M2	0.22	0.10	Mixed	0.57
	Polarographic**					
		M1	42.0	22.8	Non-competitive	109
		M2	0.15	0.04	Mixed	0.39
Chlorogenic acid‖						
	Spectrophotometric¶					
		M1	49.0	4.10	Mixed	127
		M2	20.2	0.02	Mixed	0.42
	Polarographic**					
		M1	104.0	15.8	Mixed	220
		M2	0.34	0.06	Mixed	0.88

*Partially purified metallothionein (M1) and purified metallothionein (M2) by affinity chromatography, obtained from *A. niger*.

†Inhibitor concentration that yields 50% inhibition of polyphenol oxidase activity.

‡Inhibition dissociation constant.

§Effectiveness of binding was calculated at a metallothionein (M1 or M2) concentration corresponding to I_{50}, assuming that the M_rs of M1 and PPO subunit are 10 and 34 k, respectively.

‖Two substrates of polyphenol oxidase were used at concentrations from 0.2 to 5 mM.

¶Spectrophotometric determinations were performed in triplicate with a standard error of 4%.

**Polarographic determinations were performed in duplicate with a standard error of 5%.

purification associated with a concomitant similar increase in the ratio of mole of copper per mole of protein.

Table 1 shows that the range of I_{50} values for the purified metallothionein M2 varied from 0.15 to 0.34 μg protein ml^{-1}, depending on the method of determination of PPO activity and on the substrate used. The inhibitory effect of M2 (Table 1) was higher with catechin as substrate than with chlorogenic acid. Using catechin as substrate, the inhibitory effect of M2 on PPO activity was greater with the polarographic method than with the spectrophotometric one; however, the reverse pattern was observed for chlorogenic acid.

Kinetic studies (Table 1) indicate that M2 has an inhibitory effect of the mixed type on PPO activity for both chlorogenic acid and catechin, using spectrophotometric and polarographic methods; K_i values exhibited the same trend as I_{50} values, i.e. the inhibitory effect of metallothionein decreased in the order of: catechin polarography equivalent to chlorogenic acid-spectrophotometry > catechin spectrophotometry > chlorogenic acid polarography. The effectiveness of binding of the inhibitor ranges from 0.39 to 0.88 mol metallothionein per mol PPO subunit (Table 1).

Table 2. Inhibitory effect of demetallized partially purified metallothionein (M3) obtained from *A. niger* on PPO activity in a model system

Substrate	Method	Inhibitor concentration (μg protein ml^{-1})	Residual activity* (%)
Catechin†			
	Spectrophotometric‡	90	146
		150	134
	Polarographic§	50	93
		100	89
Chlorogenic acid†			
	Spectrophotometric‡	90	85
		150	101
	Polarographic§	50	114
		100	138

*Residual activity is calculated as the ratio $[(A - A_i)/A] \times 100$ where A is the activity without inhibitor and A_i is the activity in presence of inhibitor.

†The assays were performed with a substrate concentration of 5 mM.

‡Spectrophotometric determinations were performed in triplicate with a standard error of 4%.

§Polarographic determinations were performed in duplicate with a standard error of 5%.

Inhibitory effect of demetallized metallothionein on tyrosinase activity

The results (Table 2) indicate that, using catechin as substrate and the spectrophotometric assay, the demetallized metallothionein M3 exhibited an activating effect on PPO activity; however, a limited inhibitory effect was also observed using the polarographic method. Using chlorogenic acid as substrate (Table 2), the demetallized metallothionein M3 has no inhibitory effect on PPO activity in terms of colour formation; however, an activating effect of M3 on PPO activity was observed with the polarographic assays.

Effect of pre-incubation on PPO inhibition by metallothionein

The effect of 1 hr pre-incubation of metallothionein M1 with PPO on enzyme activity was determined under the conditions of the assay that yield 50% of inhibition for catechin and chlorogenic acid; the reaction was initiated by the addition of substrate. The results (Table 3) indicate that, using polarographic and spectrophotometric methods, the inhibitory effect of metallothionein on catechin oxidation was increased by 1.7 and 1.2, respectively; the same trend was also observed for chlorogenic acid with 1.5 and 1.6 increase in the inhibitory effect on PPO by pre-incubated M1, using polarographic and spectrophotometric assays, respectively.

DISCUSSION

The spectrophotometric and polarographic analyses of oxidation of catechin (Fig. 1) and chlorogenic acid (Fig. 2) by mushroom PPO demonstrate that the decrease in the rate of formation of enzymic coloured end-products corresponds approximately to the beginning of the anaerobic conditions [20]. For the oxidation of both catechin (Fig. 1) and chlorogenic acid (Fig. 2), M1 exhibited an inhibitory effect on enzymic colour formation, with a lag period and an immediate consumption of oxygen; however, the lag-time effect was more important for chlorogenic acid than that for catechin.

The literature [7, 21–23] indicated that, using reducing agents as inhibitors of PPO activity, spectrophotometric assays exhibited an initial lag followed by a slow increase in the reaction rate; in contrast, an immediate oxygen uptake was observed with the polarographic assays. McEvily and Iyengar [6] reported that the major role of reducing agents is their ability to reduce chemically the enzymically produced *o*-quinones; this chemical reduction is considered to be responsible for the formation of stable colourless products. Hence, the pattern observed for the inhibitory effect of metallothionein on mushroom PPO may indicate the presence of such a mechanism, involving the formation of colourless compounds.

As determined by I_{50} (Table 1), the inhibitory effect of M1 on PPO oxidation of catechin, as measured by the oxygen uptake, is higher than that obtained with the colour formation. These results suggest a direct inhibitory effect of metallothionein on the catalytic activity of the enzyme; however, a reverse pattern was observed for chlorogenic acid. Overall, these results may indicate that the inhibitory mechanism involved in the undertaken model system could have a direct effect on the catalytic PPO activity, as demonstrated by the decrease in the oxygen consumption, and by the formation of colourless compounds. Previous work [7, 23, 24] suggested that investigations with spectrophotometric and polarographic methods for the determination of PPO activity provide complementary and different interpretations.

The determination of oxygen uptake (Table 1) indicates that, using catechin as substrate, M1 is a non-competitive inhibitor of PPO activity. These results suggest that catechin and metallothionein may bind independently at different sites on the enzyme molecule where the resulting enzyme–substrate–inhibitor complex is catalytically inactive [17]. Mixed type inhibition

Table 3. Effect of pre-incubation on PPO inhibition by partially purified metallothionein (M1) obtained from *A. niger*

Substrate	Method	Rates* (units min^{-1}) No pre-incubation	Pre-incubation†
Catechin‡			
	Spectrophotometric§	0.225	0.183
	Polarographic‖	52.5	30.0
Chlorogenic acid‡			
	Spectrophotometric§	0.310	0.190
	Polarographic‖	18.7	12.5

*Rates are expressed in absorbance units min^{-1} for spectrophotometric determinations and in μM of oxygen min^{-1} for polarographic ones.

†Metallothionein and PPO were pre-incubated for 1 h at room temperature with gentle stirring.

‡Two substrates of polyphenol oxidase were used at a concentration of 5 mM.

§Spectrophotometric determinations were performed in triplicate with a standard error of 4%.

‖Polarographic determinations were performed in duplicate with a standard error of 5%.

was observed for catechin and chlorogenic acid. Mixed type inhibition is a form of non-competitive inhibition for which the presence of inhibitor affects the affinity of the enzyme for the substrate [17]; these results suggest that the binding of chlorogenic acid to the enzyme is affected by the presence of the metallothionein, resulting in a decrease of PPO affinity for the substrate. For both substrates, catechin and chlorogenic acid, the results (Table 1) indicate that variations in K_i values depended on the method used; these results may indicate that the degree of inhibition of PPO by M1 for oxygen uptake is different from that for colour formation.

The comparison of I_{50} values obtained with the partially purified metallothionein M1 and the purified M2 (Table 1) demonstrates that the purification procedure resulted in an increase in the inhibitory effect on PPO activity by 86–375 times, depending on the method and substrate used. The trends of the inhibitory effect of M1 on oxidation of both substrates, using spectrophotometric and polarographic methods, are similar to those obtained for M2. In addition, K_i values are, respectively, 205 to 570 times lower for M2 than those for M1. The results also indicate (Table 1) that the types of inhibition are similar for both M1 and M2. These findings suggest that the presence of metallothionein in the reaction media may affect the affinity of both substrates, catechin and chlorogenic acid, for the PPO.

The results (Table 1) indicate that the purification procedure substantially increased the effectiveness of binding of the purified metallothionein M2 when it was compared with that reported for the partially purified metallothionein M1. The increase in the purification as well as change in the ratio of moles of copper per mole of protein, reported previously [16], may account partially for the increase in the inhibitory effect of the purified metallothionein on PPO activity. Kahn and Andrawis [25] reported on the effectiveness of binding of tropolone (2,4,6-cycloheptatrien-1-one, 2-hydroxy-2,4,6-cycloheptatrienone) with mushroom tyrosinase. The demetallization procedure of metallothionein (Table 2), used throughout this study, did not increase its inhibitory effect of PPO activity. Although the demetallization procedure was carried out under nitrogen, the loss of inhibitory effect of metallothionein may be related to the oxidation of sulphhydryl groups [26].

The pre-incubation of metallothionein with PPO (Table 3) increased its inhibitory effect from 1.2 to 1.7 times, depending on the substrate and the method used. These results could be related to an interaction between the inhibitor and PPO without substrate, suggesting the formation of an enzyme–inhibitor complex rather than an enzyme–substrate one. The pre-incubation effect also indicates that enzymic catalysis is not necessary for binding the metallothionein to PPO [27]. Harel *et al.* [12] reported an appreciable inhibition of PPO activity after a pre-incubation of mushroom tyrosinase with a low M_r copper-chelating peptide from *D. dendroides*.

CONCLUSION

Overall, the present study demonstrated that metallothionein from *A. niger* could be used as an inhibitor for PPO activity. The type and degree of inhibition were dependent upon the substrate, the degree of purity of metallothionein and the method used for the determination of enzymic activity. The strong avidity of induced metallothionein to bind copper could explain the inhibitory effect of this protein, acting as a chelating agent. Another possible mechanism for such an inhibitory effect could be explained by the presence of sulphhydryl amino acids in the metallothionein molecule; the sulphhydryl group may bind with *o*-quinones to form colourless thioester compounds, acting as reducing reagents.

EXPERIMENTAL

Model system used for kinetic studies. Commercially purified mushroom tyrosinase (Sigma) was used throughout this study. One unit of enzyme activity is defined as an increase in A, at 280 nm of 0.001 min^{-1} at pH 6.5 and 25° in a reaction mix. containing L-tyrosine. The enzymic assays were performed using chlorogenic acid and catechin. To assess the enzymic reaction, the formation of coloured oxidized phenols was determined at 420 nm whereas the consumption of O_2 used by the enzyme-catalysed reaction was followed by a polarographic method. Spectrophotometric and polarographic assays were performed as described previously [23, 24], using 12 enzyme units ml^{-1} (1.31 mg protein). Unless otherwise specified, the enzymic reaction was initiated by addition of PPO and inhibitor to the temp.-equilibrated reaction mixt. The sp. act. was expressed as μmol $product^{-1}$ min^{-1} enzyme unit.

Inhibitors. A biomass of *A niger* (CBS 131.52) containing induced metallothionein was obtained according to ref. [15]. M1 was obtained by heat treatment (100°, 15 min) followed by ultrafiltration using a membrane with a 3 000 kD M_r cut-off [15]. M1 was purified by affinity chromatography, using a chelating Sepharose loaded with copper [16]. M1 was also demetallized according to a modification of the procedure described in ref. [28]. All procedures were carried out under a stream of N_2. A soln of M1 (0.3 mg ml^{-1}) was incubated in presence of a 10 mg ml^{-1} soln of diethyldithiocarbamate (DTC) for 1 hr with gentle stirring at room temp. The pptte formed with DTC and copper was removed by filtration at 0.22 μm, and the excess of unreacted DTC was removed by ultrafiltration using a membrane with a 3 000 k M_r cut-off. M1, M2 and M3 were lyophilized and used for subsequent kinetic studies.

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