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Truffle thio-flavours reversibly inhibit truffle tyrosinase

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Abstract

Tyrosinase is an enzyme having two copper atoms at the reactive site occurring in prokaryotic and eukaryotic organisms. In animals tyrosinase is responsible for pigmentation, in plants for protection of injured tissues or, as in fungi, to harden cell walls. Some of us have previously shown that tyrosinase is involved in truffle development and differentiation. Here we present the purification, the molecular properties and the reversible inhibition of *Tuber melanosporum* tyrosinase by dimethyl-sulfide and bis[methylthio]methane, the main flavour compounds of black and whitish truffles. The MW_r is 39 000. L-3,4-dihydroxyphenylalanine and L-tyrosine stain corresponding bands as expected for a true tyrosinase. Phenylthiourea, diethyldithiocarbamate and mimosine inhibit L-tyrosine and L-3,4-dihydroxyphenylalanine oxidation.

Keywords: Development; Melanin; Mycorrhiza; Truffles; *Tuber*; Tyrosinase

1. Introduction

Monophenol, *o*-diphenol oxygen oxidoreductase (EC 1.14.18.1) referred to with the trivial name tyrosinase is an enzyme occurring throughout the phylogenetic scale, from bacteria to higher vertebrates including man [1–3]. Tyrosinase is a metalloenzyme with a dinuclear copper active site as suggested and demonstrated by several investigations [4–6] and related to the active site of the oxygen carrier protein hemocyanin that shows in some forms also diphenol oxidase activity [7–8]. This enzyme is involved in many biological processes such as defence, mimetism, protection from UV light, hardening of cell walls in fungi or of exoskeleton in Arthropods and in general in the production of melanins [1–2,9].

The reactions catalyzed by tyrosinase are indicated:

Monophenol+O_2 → *o*-quinone+H_2O (A).

2-*o*-diphenol+O_2 → 2-*o*-quinone+2H_2O (B).

The reaction (A) is referred to as cresolase activity while the reaction (B) as the catecholase activity.

The tyrosinases from the different organisms are all able to oxidize L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) but show variable specificity for the different monophenols and *o*-diphenols [10–11]; for example the substrates for the tyrosinases of several fungi are 1,8-dihydroxynaphthalene and/or γ-butylhydroxybenzene [12–13]. It is known that tyrosinase is inhibited by thiols [14–15] and a possible role by cysteine and glutathione on tyrosinase regulation has been suggested [16–17].

Truffles of the genus *Tuber* are ectomycorrhizal Ascomycotina fungi [18], whose tyrosinase expression is related to the reproductive differentiation like *Neurospora crassa* [19–23]. Black and white truffle flavours, such as dimethylsulfide (DMS) and bis[methylthio]methane (BMTM) [24–25] have been shown to inhibit reversibly *Agaricus bisporus* tyrosinase [23]. This point has been supported recently by kinetic studies concerning DMS inhibition of *A. bisporus* tyrosinase [26], a model tyrosinase from organisms that do not evidently produce DMS or BMTM. The aim of this work is to make clear if the thio-flavours (sulfides) of truffles inhibit truffle tyrosinase, thus making biological sense to the inhibition of an enzyme critical to the fruit body development. As shown in other fungi melanin and differentiation are related [27].

In this page, we describe the purification, molecular

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characterization and some of the kinetic properties and the reversible inhibition by thionic truffle flavours of *Tuber melanosporum* Vittad. tyrosinase. The interest concerning truffle biology arises from their organoleptic properties and economic value and as a model in vivo of adaptation to a microaerobic environment [23].

2. Materials and methods

2.1. Truffles

Immature ascocarps (fruit bodies) of *T. melanosporum* were collected near L'Aquila, Abruzzi, Italy. The truffles were used soon after collection or after a few days stored at −25°C.

2.2. Tyrosinase purification from truffle ascocarps

Step 1. Extraction. (a) 50 g of truffles was homogenized, after exhaustive washing with distilled water, suspended 1/10 (w/v) with 0.1 M sodium phosphate buffer at pH 6.8 containing 1% Brij in Ultraturrax Waring blendor for 45 min at 4°C; (b) the homogenate was added with 4% polyvinylpyrrolidone (PVP) and homogenized in Potter Elvehijem homogenizer for five pestle strokes at velocity 3 and three pestle strokes at velocity 6 motor driven by a Black and Decker power drill; (c) the homogenate was centrifuged 10 min at $370\times g$; (d) the pellet was resuspended with 200 ml of the same buffer used for homogenization and centrifuged 10 min at $370\times g$; the two supernatants (502 ml) were pooled; (e) the supernatant was recovered and centrifuged 20 min at $20\,750\times g$; the supernatant (500 ml) was recovered.

Step 2. Ammonium sulfate precipitation. (a) The supernatant recovered was added with $(NH_4)_2SO_4$ to 40% at room temperature and stirred for 2 h., then centrifuged 30 min at $18\,000\times g$; (b) the supernatant was added with $(NH_4)_2SO_4$ to 80% saturation and the suspension stirred overnight at room temperature and subsequently centrifuged at $20\,600\times g$ for 30 min; (c) the pellet was suspended with distilled water (15 ml); (d) the suspension was desalted up to 20% $(NH_4)_2SO_4$ with Amicon Centriplus 3000.

Step 3. G-25 column. The suspension was put onto a G-25 ϕ 2.5×45-cm column to remove $(NH_4)_2SO_4$, that strongly inhibits enzyme activity, and eluted with 10 mM sodium citrate and 1 mM sodium benzoate at pH 5.0. The 106 ml of the activity peak was concentrated with Amicon Centriplus 3000 to 10 ml.

Step 4. CM-Sepharose chromatography. (a) The eluate was put onto a CM-Sepharose ϕ 1.6×40-cm column equilibrated with 10 mM sodium citrate and 1 mM sodium benzoate at pH 5.0 (by pumping 1.3 ml min^{-1}) and eluted with a linear gradient of 100 mM sodium citrate, 1 mM sodium benzoate at pH 5.0 and 0.5 M NaCl (by pumping 1.3 ml min^{-1}; the fractions were of 5.3 ml); (b) the fraction showing tyrosinase activity (38 ml) was concentrated with Amicon Centriplus 3000 to 6.5 ml.

Step 5. G-25 column. The concentrate was put onto a G-25 column ϕ 2.5×45 cm and eluted with 20 mM sodium phosphate at pH 7.2.

Step 6. DEAE-Sephadex Chromatography (a) The eluate was put onto a DEAE-Sephadex column ϕ 1.6×15 cm, washed with 10 ml of 20 mM sodium phosphate, pH 7.2, then eluted with a linear gradient of 20 mM sodium phosphate and 1 M NaCl at pH 7.2, at a velocity of 0.4 ml min^{-1} (fractions of 3.2 ml per 8 min); (b) the peak showing enzyme activity (19 ml) was recovered and concentrated to 2.8 ml with Amicon Centriplus 3000; (c) the eluate was desalted by passing it through a G-25 1.6× 40-cm column and eluted with 0.1 M sodium phosphate at pH 6.8 for the enzymatic assays and electrophoresis. Each step was carried out at 4°C.

2.3. Tyrosinase activity assay

Tyrosinase activity was measured spectrophotometrically by recording the change in absorbance at A_{475} due to dopachrome formation from L-tyrosine or L-DOPA. The reaction mixture for cresolase activity consisted of 1 mM L-tyrosine containing 10^{-6} M L-DOPA as a cofactor [28–30] in sodium phosphate buffer at pH 6.8 and 25°C. The reaction mixture for catecholase activity contained 5 mM L-DOPA in 100 mM sodium phosphate buffer at pH 6.8 and 25°C [10,23,30]. The A_{475} of dopachrome is 3.6×10^3 M^{-1} cm^{-1} at pH 6.8 [10]. One unit (U) of cresolase or catecholase activity is the amount of enzyme that produces 1 μmol of dopachrome min^{-1} at 25°C starting from L-tyrosine or L-DOPA. DMS and BMTM were taken by a syringe from the corked bottle and added to the parafilm-stopped reaction cuvette up to the selected concentration. As the extracts enzyme activity increased with time we suspected a release of an endogenous inhibitor. In order to preliminarily investigate the presence of an endogenous inhibitor the truffle homogenate supernatant at $20\,750\times g$, containing or not 4% PVP was also passed through a G-25 column in order to remove the suspected endogenous inhibitor s^{-1}; the elution buffer was 0.1 M sodium phosphate at pH 6.8 and each peak at A_{280} was recovered. 1.5 ml of each peak, not coincident with the tyrosinase activity peak, was assayed for inhibitory activity after lyophilization and resuspension in distilled H_2O (0.8 ml). These peaks from the G-25 column (MW range 1000–5000 Da) were treated at 100°C for 10 min but were thermostable. The same peaks gave precipitates when treated with 100 mM $HgCl_2$ or $ZnCl_2$ and showed an A_{280} peak.

2.4. SDS–PAGE electrophoresis

The M_r of *T. melanosporum* tyrosinase has been calcu-

lated with reference to M_r markers purchased from Pharmacia Biotech. Both M_r (LMW calibration kit). And truffle tyrosinase from the last step of purification (Step 6C) were treated as previously described [31]. The electrophoretic cell was a Mini-Protean 3 cell from Bio-Rad; run conditions were 45 mA constant current for 40 min: 1 μg of marker proteins or 1 μg of purified tyrosinase protein was charged onto the gel.

2.5. Tyrosinase isoelectrofocusing

The isoelectrofocusing run was performed on the tyrosinase-purified fraction and *p*I markers (Pharmacia Biotech) to detect truffle tyrosinase p*I*. The conditions for isoelectrofocusing were: (a) LKB Ampholine PAG plates with a pH range of 3.5–9.5; (b) anode electrode solution 1 M H_3PO_4; (c) cathode electrode solution: 1 M NaOH; (d) temperature 10°C; (e) $I = 50$ mA; (f) $E = 1500$ V; (g) $P = 30$ W; (h) 30 μg of p*I* markers; (i) 1 μg of purified tyrosinase; (j) 90 min run. A LKB isoelectrofocusing apparatus Ultrophor 2217 was used for the runs.

Enzyme bands were stained by incubating the gels, after the runs, with 1 mM L-tyrosine, containing 10^{-6} M L-DOPA, or 5 mM L-DOPA, dissolved in 100 mM sodium phosphate buffer at pH 6.8, for cresolase (*o*-hydroxylating activity) and catecholase (oxidase activity) activities respectively. The protein bands, of the purified tyrosinase fraction and of p*I* markers, were stained with 0.1% Coomassie blue dissolved in 45% methanol and 8% acetic acid. Tyrosinase p*I* was calculated from the distribution curve of p*I* markers.

2.6. Protein content

The protein content of the various purification steps was measured by the biuret reaction [32].

2.7. Reagents

The various reagents were purchased from Sigma, Bio-Rad and Merck. *A. bisporus* tyrosinase was a commercial preparation purchased from Sigma. DMS was purchased from Sigma while BMTM was obtained from Merck.

3. Results

3.1. Truffle tyrosinase purification and recovery

Fig. 1A,B shows the A_{280} and related tyrosinase activity profiles of the effluents from CM-Sepharose (A) and DEAE-Sephadex (B) columns. As the activity and protein profiles illustrate, enzyme activity measured as catecholase is recovered in one single peak through the passages in the columns. Table 1 reports the specific activity in the various steps of purification, its increase and the percentage of recovery. The removal of ammonium sulfate, that inhibits enzyme activity, through an G-25 column filtration produced an eluate with very much increased catecholase and cresolase activities. The DEAE-Sephadex fraction showed an increase of specific activity if compared to the crude preparation in 80% saturated ammonium sulfate suspension of about 6×10^3-fold for both L-DOPA or L-tyrosine oxidations.

3.2. Homogeneity, MW_r, isoelectric point, cresolase and catecholase activities of truffle tyrosinase

The SDS–PAGE electrophoresis of the tyrosinase fraction from the DEAE-Sephadex column and of marker proteins (Fig. 2A) showed that the tyrosinase fraction was homogeneous and its electrophoretic mobility compared to those of the marker proteins resulted in an estimated MW_r of 39 000 Da. The isoelectric point of *T. melanosporum* tyrosinase is 5.5 as deduced from the isoelectrofocusing run of tyrosinase compared to p*I* marker proteins (Fig. 2B). The comparison of Coomassie blue-stained native tyrosinase preparation (Fig. 2C, lane 2) compared to Coomassie blue-stained marker proteins (Fig. 2C, lane 1) showed that the tyrosinase band corresponded to those stained with L-tyrosine or L-DOPA (Fig. 2C, lanes 3 and 4).

Table 1
Enzyme activities of the subsequent steps of *T. melanosporum* tyrosinase purification

Step	Volume (ml)	Total activity (U)		Total proteins (mg)	Specific activity (U mg^{-1})		Yield (%)	
		L-DOPA	TYR		L-DOPA	TYR		
Pellet 80%	15	1.95	0.135	59.55	0.033	0.0023		
I G-25 eluate	106	543.8	42.4	26.50	20.52	1.6	100	100
CM-Sepharose eluate concentrated	6.5	273	19.31	13	21	1.48	50.2	45.54
II G-25 eluate	15.6	164	14.82	4.91	33.40	3.02	30.15	34.90
DEAE-Sephadex eluate concentrated	2.8	84.7	5.29	0.392	216.07	13.49	15.57	12.47

Both catecholase (L-DOPA) and cresolase (TYR) activities are presented. Activities are expressed as units and are the means of quadruplicate assays; S.E.M. never exceeded 5% of the mean.

3.3. Kinetic properties of truffle tyrosinase K_m, V_{max}

The K_m versus L-tyrosine of *T. melanosporum* purified tyrosinase is 0.29 mM and V_{max} 9 U mg^{-1} protein; the K_m versus L-DOPA is 0.34 mM while the V_{max} is 139 U mg^{-1} protein. The kinetic constants K_m and V_{max} were calculated by the Hanes–Woolf plot and by means of polynomial regression of the line.

3.4. Inhibition of truffle tyrosinase by endogenous and exogenous inhibitors

Fig. 3D shows the elution profile of truffle supernatants at 20 750 × *g* from a G-25 column. The same profiles were found with and without PVP. Peak A is that of tyrosinase activity, while the peaks B and C show inhibitory activity versus truffle tyrosinase (Fig. 3A). The MW range should be 1000–5000. The boiling of peaks B and C at 100°C for 10 min did not affect the inhibitory activity (Fig. 3A). Fig. 3B,C shows the inhibition by peaks B and C on the catecholase and cresolase activities of *A. bisporus* tyrosinase.

Fig. 4 presents cresolase (A) and catecholase (B) activities of the supernatants at 20 750 × g, with or without PVP, and of peak A, eluted from a G-25 column (Fig. 3D), versus days after preparation.

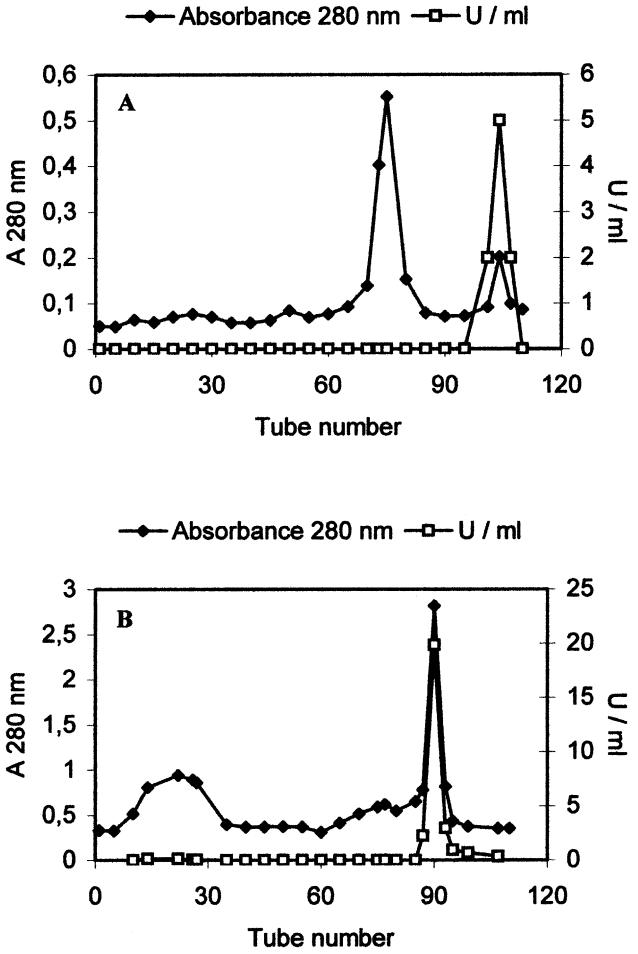

Fig. 1. Elution profiles of proteins and tyrosinase activity (A,B) of *T. melanosporum* tyrosinase preparation from CM-Sepharose (A) or DEAE-Sephadex (B) columns; each tube collected 2 ml of eluate.

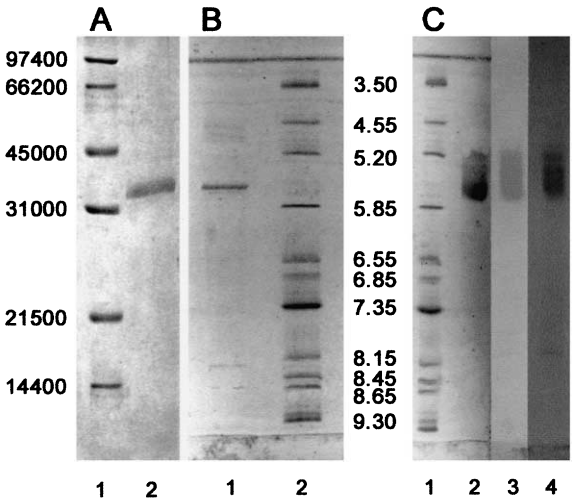

Fig. 2. Molecular characteristics of *T. melanosporum* tyrosinase. A: SDS–PAGE MW_r detection of *T. melanosporum* tyrosinase; (lane 1) MW standards, (lane 2) *T. melanosporum* tyrosinase purified to homogeneity according the method reported in Section 2. B: Isoelectric point measure of *T. melanosporum* tyrosinase; (lane 1) *T. melanosporum* tyrosinase, (lane 2) isoelectric point standards. C: Isoelectrofocusing of standard proteins (lane 1) and of *T. melanosporum* tyrosinase (lane 2) stained with Coomassie blue; lanes 3 and 4 show the L-tyrosine and L-DOPA stainings of *T. melanosporum* tyrosinase after isoelectrofocusing.

Both cresolase (Fig. 4A) and catecholase (Fig. 4B) activities increased with time; cresolase activity was about 10–15 times lower than catecholase activity.

The *T. melanosporum* tyrosinase activity inhibition in the presence of L-tyrosine or L-DOPA as substrates by some other substances has been also studied here. 0.1 mM phenylthiourea (PTU) or diethyldithiocarbamate inhibited almost completely enzyme activity (98–100% inhibition); 0.1 mM L-mimosine is a less strong inhibitor of both cresolase or catecholase activities as compared to PTU and diethyldithiocarbamate (79–87% inhibition). 5 mM BMTM or DMS, the truffle thio-flavours, inhibited L-DOPA and L-tyrosine oxidations by 76 and 79% and 79 and 86%, respectively. In Fig. 5 the inhibition of cresolase activity of truffle tyrosinase is presented versus inhibitor concentration. DMS at 1 mM was a stronger inhibitor of enzyme activity than BMTM (Fig. 5B).

After addition of 2 mM DMS or BMTM to the reaction mix for tyrosinase to inhibit the activity the enzyme inhibition by the two volatile substances decreased and tyrosinase was reactivated over time (Fig. 5C). The enzyme reactivation was higher for the enzyme inhibited by DMS than for that inhibited by BMTM.

The K_m values for L-tyrosine in the presence of DMS or BMTM were 0.29 mM and 0.25 mM respectively. The V_{max} values decreased to 4.4 U mg^{-1} protein and 2.66 U mg^{-1} protein as expected for non-competitive inhibition in the presence of 2.5 mM DMS or 5.0 mM BMTM respec-

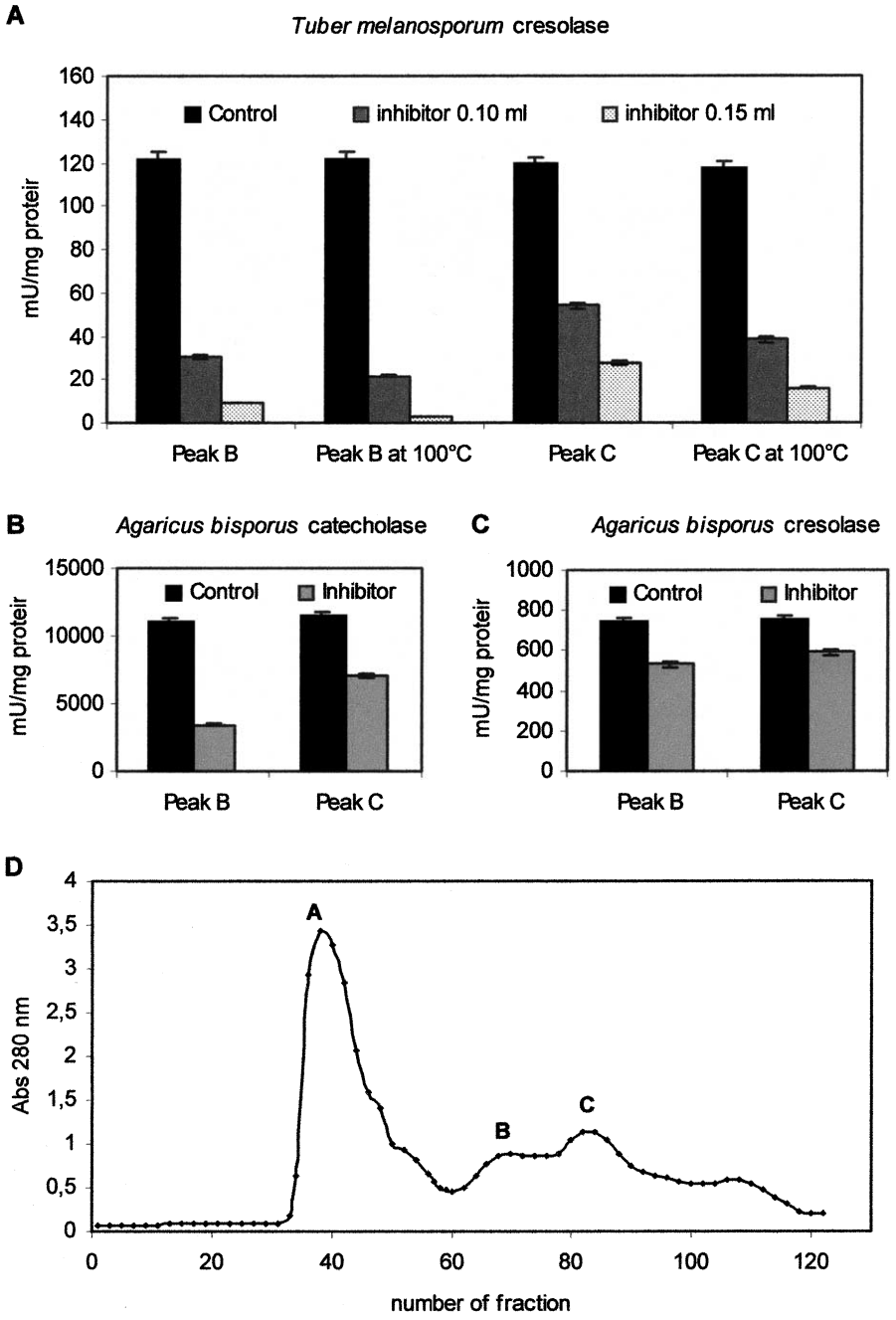

Fig. 3. Inhibition of cresolase activity of *T. melanosporum* tyrosinase (A) from peak A (D) and catecholase and cresolase activities of *A. bisporus* (B,C) by the lyophilized peaks B and C (D) (resuspended with distilled H_2O) of the eluate from a G-25 column charged with truffle homogenate supernatant at 20 750 × *g*. The values reported are means of triplicate assays; bars are S.E.M. The reaction mixtures for enzyme activity are reported in the Section 2. *A. bisporus* tyrosinase used/assay was 5 μl of a solution of 0.5 mg ml^{-1}.

tively. With L-DOPA as substrate DMS and BMTM affected the K_m values, which rose to 2.97 mM and 4.82 mM respectively, while the V_{max} values did not change significantly, as expected for competitive inhibition, and were 149 U mg^{-1} protein and 121 U mg^{-1} protein, respectively.

4. Discussion

Truffles of the genus *Tuber* and in particular *T. melanosporum* show true tyrosinases as shown by the cresolase and catecholase activities. The molecular properties are comparable with those of *N. crassa* tyrosinase, which is constituted of a single polypeptide chain like that of *T. melanosporum*. The molecular mass of *T. melanosporum* tyrosinase was about 15% lower than that of *N. crassa*. Both truffles of the genus *Tuber* and *N. crassa* are Ascomycetes.

The K_ms for L-DOPA and L-tyrosine fell, within those reported for many tyrosinases, in the range of 10^{-4}–10^{-3}

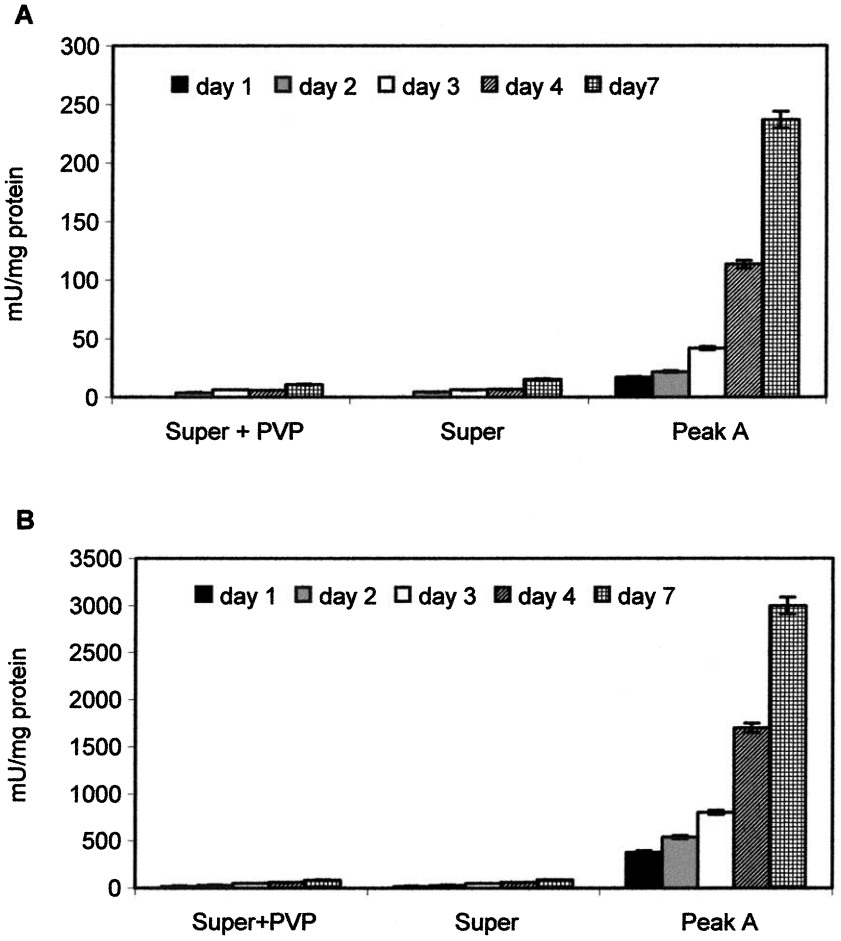

Fig. 4. Specific activity of the cresolase (A) and catecholase (B) of *T. melanosporum* tyrosinase in the homogenates and eluates at different days from the preparation. Values are means of triplicate assays ± S.E.M. Super (supernatant at 20 750 × *g*); Peak A (fractions 35–45 eluted from G-25 column charged with the supernatant at 20 750 × *g*).

M and the V_{max} for L-tyrosine was much lower than that for L-DOPA [9,29,33].

The isoelectric point in the acidic field (pH 5.5) may reflect the optimum of activity under quasi-anaerobic conditions when glycolysis mostly refurnishes cell energy stores and the cell interior is acidic. Truffles are known to express glycolytic enzymes [34] and to have deranged mitochondria and respiration [23]. Truffle tyrosinase is inhibited by traditional tyrosinase inhibitors such as PTU, diethyldithiocarbamate and L-mimosine [9,30,35]. In addition, a rather thermostable low-molecular-mass endogenous species inhibited enzyme activity. These endogenous inhibitors are released with time as the increasing time-dependent enzyme activity of the truffle extracts showed. Interestingly the truffle flavours DMS and BMTM, reversibly inhibited the enzyme from truffles, but also the enzyme from *A. bisporus*. As reported for *A. bisporus* tyrosinase, thio-compounds such as cysteine and glutathione affect tyrosinase activity from a 1-mM concentration, while significant inhibition by cysteine is observed at 10-mM concentrations [17]. Differently from the inhibitory thiols reported in the literature, the sulfides DMS and BMTM are inhibitors at 1 mM with DMS more inhibitory than BMTM; at a 6-mM concentration enzyme activity was almost completely inhibited by both DMS and BMTM. Thus these truffle flavours were more powerful inhibitors of tyrosinase than cysteine or glutathione. However the inhibition of enzyme activity by DMS or BMTM is reversible, and different from inhibition by PTU, diethyldithiocarbamate, dithiothreitol or 2-mercaptoethanol [2,9,14,17]. The enzyme reversibly binds the two thionic substances that may interact with the active copper center as shown by the inhibition acted versus the substrates L-tyrosine and L-DOPA. With time, the enzyme becomes almost completely reactivated after about 3 h incubation with DMS or BMTM. The non-competitive inhibition of L-tyrosine oxidation and the competitive one with L-DOPA show a different behavior of truffles tyrosinase if compared to *A. bisporus* enzyme. However one should consider that the two enzymes are structurally different; the truffle enzyme is made of a single polypeptide chain while the *A. bisporus* enzyme is a dimer of heterodimer with light regulatory and heavy catalytic polypeptide chains.

The inhibition of tyrosinase activity by DMS has been studied also with *A. bisporus* tyrosinase and the same re-

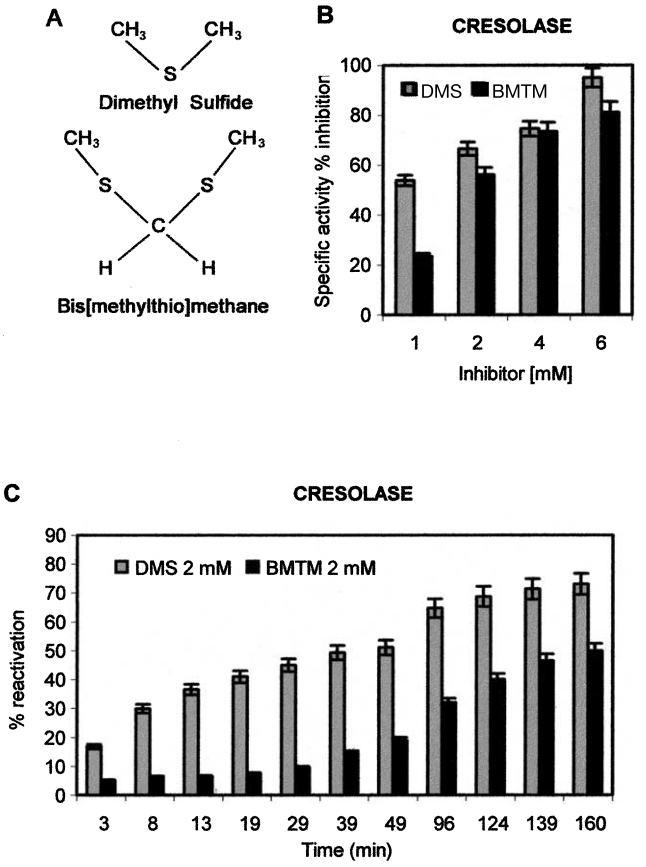

Fig. 5. *T. melanosporum* tyrosinase. Reversible inhibition by DMS and BMTM (A), the flavours of black and white truffles. B: Inhibition of *T. melanosporum* cresolase activity by DMS (*T. melanosporum* flavour) and BMTM (*Tuber magnatum* flavour). C: Reactivation of cresolase activity of *T. melanosporum* tyrosinase after inhibition by DMS or BMTM.

versible inhibition of enzyme activity with L-DOPA as substrate was found [26]. The direct copper reduction by thio derivatives with recycling of Cu(II) to Cu(I), the deoxyform of the enzyme able to bind dioxygen [6,29] has been described; previous authors found a possible reductive binding of thiols to tyrosinase copper [14–15]. The mechanism postulated for the slow binding inhibition of tyrosinase [26] may also encompass this last hypothesis if the inhibitor was removed from the reaction site as expected for volatile thio-flavours.

The reversible inhibition of truffle tyrosinase by truffle thionic flavours may correlate with fruit body maturation, as in *N. crassa* where tyrosinase activity correlates with sexual differentiation.

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