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Triton X-114-aided purification of latent tyrosinase

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Abstract

Mushroom tyrosinase was partially purified using an aqueous two-phase system with Triton X-114. The purification achieved was 5.5-fold from a crude extract of mushroom pileus, with a high recovery of 84%. The phenols were reduced to 8% of the original content, avoiding pre- and post-purification tanning of the enzyme. The enzyme obtained was latent and was activated 3-fold by trypsin, 2.7-fold by changes in the pH and to different extents by cationic and anionic detergents, the latter being the more effective. There was also a synergistic effect between trypsin and detergent, at low detergent concentrations. When kinetically characterized, latent enzyme showed both monophenolase and diphenolase activities, the latter activity displaying an unexpected lag period before reaching the steady-state rate. This behaviour is characteristic of a hysteretic enzyme, and has not been previously described for this enzyme. In addition, inhibition studies with substrate analogues were carried out, tropolone being found to be the most effective inhibitor.

Keywords: Aqueous two-phase systems; Tyrosinase; Enzymes; Triton X-114

1. Introduction

Tyrosinase or polyphenoloxidase (PPO) (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a bifunctional, copper-containing enzyme widely distributed on the phylogenetic scale. This enzyme uses molecular oxygen to catalyse the oxidation of monophenols, e.g., tyrosine, to their corresponding *o*-diphenols (monophenolase–*o*-cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity). For a recent review on both activities see [1]. The *o*-quinones thus generated polymerise to form melanin through a series of subsequent enzymic and non-enzymic reactions. This bio-polymer is the main pigment found in the skin, hair and eyes of human beings, and is accumulated in specialised membranous organelles (named melanosomas) in the melanocytes [2,3]. The neoplastic transformation of such cells in human beings and other mammals leads to the clinical disease termed melanoma [4].

These malignancies are associated with significant rates of mortality and behave in an unpredictable manner [4,5]. Intensive research has been undertaken to elucidate the factor which influences the course of the disease and to design selective chemotherapeutic agents. One approach exploits the significant biochemical differences between melanotic melanoma and normal tissues. Since high tyrosinase activity and high melanin content are the principal characteristics of tumours, the agents regulating the enzyme are of potential importance [6]. Among such agents, diphenolic compounds can be used as depigmenting agents because of their ability to act as alternative

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substrates for tyrosinase catalysed oxidation [7]. The toxicity of these quinones depends on their stability, which enables them to penetrate the melanosomal membrane and initiate toxic damage in melanocytes [7].

Since different tyrosinases obtained from a variety of sources have similar structural (more than 50% homology) [8] and biochemical characteristics [9,10], and since the subsequent reactions of the enzymatically-generated toxic quinone are only chemical [11], mushroom tyrosinase is a biochemically well-characterised and inexpensive system suitable for carrying out studies with potential anti-melanoma phenolic agents.

However, commercial mushroom tyrosinase is usually tanned, i.e., modified by its self-generated quinones, which results in great enzyme heterogeneity. This modification of the commercial enzyme during the purification is also evidenced by the presence of the enzyme in fully active form [12] instead of in its natural latent inactive form [13].

In order to solve this problem, the present paper tries to develop a simple and mild Triton X-114-aided purification method for obtaining unmodified latent mushroom tyrosinase in the non-detergent phase of the aqueous two phase system formed by Triton X-114.

2. Experimental

2.1. Reagents

Biochemicals were purchased from Fluka (Madrid, Spain) and used without further purification. Inhibitors (cinnamic acid, L-mimosine, tropolone and kojic acid) were from Sigma (Madrid, Spain). Triton X-114 was also obtained from Fluka, and was condensed three times as described by Bordier [14] using 100 m*M* sodium phosphate buffer, pH 7.3. The detergent rich phase of the third condensation had a concentration of 25% Triton X-114 (w/v).

2.2. Enzyme purification

Fresh mushrooms (*Agaricus bisporus*) at the stretched-veil stage (commercial harvest maturity) were picked at a local mushroom house (Champiñones Yáñez, Bonete, Albacete, Spain) and taken to the laboratory for sampling and analysis. The white strain was used in all experiments.

A 20 g sample of pileus was cleaned by removing the earth and 40 ml of 100 m*M* sodium phosphate buffer pH 7.3 were added. The mixture was homogenised for 30 s in a high-speed blender and centrifuged at 100 000 *g* for 30 min a 4°C.

This supernatant was subjected to temperature phase partitioning by adding Triton X-114 at 4°C, so that the final detergent concentration was 6%. The mixture was kept at 4°C for 10 min and then warmed to 37°C in a thermostatic bath. After 10 min, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins and phenolic compounds. This solution was centrifuged at 10 000 *g* for 15 min at room temperature. The detergent-rich phase was discarded. The clear detergent-poor supernatant of 6% (w/v) Triton X-114 was subjected to additional phase partitioning to remove the remaining phenols. For this, fresh Triton X-114 was added to obtain a final concentration of 4% (w/v) and the mixture brought to 37°C. After centrifugation at room temperature at 10 000 *g*, the detergent-poor supernatant of 4% (w/v) Triton X-114 (Table 1), containing the soluble mushroom PPO, was brought to 25% saturation with solid $(NH_4)_2SO_4$ under continuous stirring at 4°C.

After one hour, the solution was centrifuged at 60 000 *g* for 30 min at 4°C and the pellet was discarded. $(NH_4)_2SO_4$ was added to the clear supernatant to give 75% saturation and stirred for 1 h at 4°C. The precipitate obtained between 25% and 75% was collected by centrifugation at the same rotor speed and dissolved in a minimal volume of 100 m*M* phosphate buffer, pH 7.3, containing 20% glycerol. The salt content was removed by dialysis and the enzyme stored at −20°C. An outline of the procedure is given in Scheme 1.

2.3. Enzyme activity

Catecholase and cresolase activity were determined spectrophotometrically with *tert.*-butylcatechol (TBC) and with *tert.*-butylphenol (TBP) at the quinone maximum (ϵ_{400nm}=1150 M^{-1} cm^{-1}) [15].

Table 1
Partial purification of mushroom tyrosinase

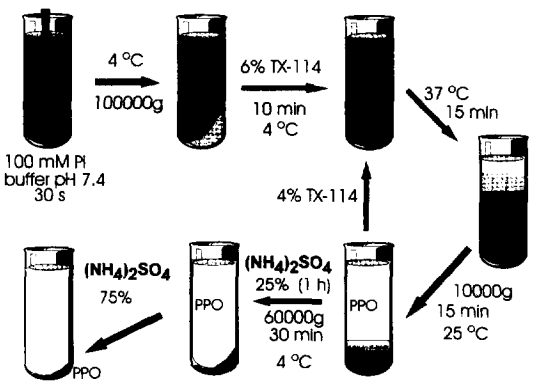
	Volume (ml)	Total protein (mg)	Total activity[a]		Specific activity[c] (U/mg)	Purification (fold)	Recovery[c] (%)	Activation (fold)	Phenolic compounds (mg/ml)
			−SDS	+SDS[b]					
Crude extract	41.0	129	153	793	6.1	1.0	100	5.2	21.4
Supernatant 6% TX-114	43.0	107	150	775	7.2	1.2	98	5.2	18.0
Supernatant 4% TX-114	44.0	99	132	716	7.2	1.2	90	5.4	14.0
25–75% $(NH_4)_2SO_4$	3.5	20	117	668	33.4	5.5	84	5.7	1.7

[a] Assayed with *tert*-butylcatechol as substrate.
[b] Assayed with 0.05% SDS.
[c] Refers to the SDS-activated form.

One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*.-butyl-*o*-quinone per min.

Unless otherwise stated, the standard reaction medium for catecholase activity at 25°C contained 6 $\mu g\ ml^{-1}$ of partially purified tyrosinase, 10 m*M* sodium phosphate, pH 6.5, and 2.5 m*M* TBC in a final volume of 1 ml. For cresolase activity, the enzyme concentration was increased by a factor of ten, and the TBP was kept at 0.1 m*M* in the presence of catalytic amounts of TBC. The activity of the latent enzyme was measured by including 0.05% SDS in the cuvette. To determine the effect of the different activating agents, the sample was preincubated with detergent (1 or 10 m*M*) or Trypsin (1000 U/ml) for 15 min, respectively.

Inhibition studies were carried out with the different substrate analogues, under the standard reaction conditions, with the appropriate concentration of inhibitor.



Scheme. 1. An outline of the enzyme purification procedure.

2.4. Other methods

The protein content was determined according to the dye-binding method of Bradford [16], using bovine serum albumin (BSA) as a standard. Phenolic compounds were measured spectrophotometrically in 80% ethanol [17]. The CMC of the detergents used in the activation studies were determined under experimental conditions using the fluorescent probe, N-phenyl-1-naphtylamine [18].

3. Results

It is difficult to purify enzymes from plant extracts because of the presence of a large variety and quantity of secondary products which can bind tightly to the enzymes, thus changing their characteristics [19,20]. To overcome this, different methods have been developed such as acetone powders, ammonium-sulfate fractionation, salts, insoluble polymers and detergents [21]. Of this last group, Triton X-114 shows the special feature of forming clear solutions in buffers at 4°C, while it separates into two phases at 20°C due to the formation of large micellar aggregates [14]. This characteristic has been used to separate integral proteins from hydrophilic proteins, since the former remain in the detergent-rich phase [22,23].

Recently, Triton X-114 has found another use in plant biochemistry [21,24,25] in the removal of

phenolic compounds from fruits [26], leaves [27–29] and tubers [30,31].

The method described in this paper used a combination of the detergent Triton X-114, and ammonium-sulfate fractionation to avoid enzymatic browning in mushrooms during tyrosinase purification (Scheme 1). The main difference from other methods already published lay in the first two steps. In the first, no acetone [9,13,32,33], Ca^{2+} ions [32,34] or reducing agents [35] (i.e., metabisulfite, ascorbate or cysteine) were added. Browning was avoided by increasing the pH to 7.3 and by keeping the extract at 4°C. Long periods of manipulation can led to browning of the extract and subsequent ammonium-sulfate fractionation can tan the enzyme. To avoid this, Triton X-114 was added during the second step to achieve a concentration of 6% (w/v). This kept the supernatant clear during manipulation and removed 20% of phenols with little protein loss during temperature-induced phase separation. To take full advantage of the capacity of Triton X-114 to remove phenols and hydrophobic proteins, a second phase-partition was carried out by adding fresh Triton X-114 so that the final Triton X-114 concentration was 4% (w/v). The last two-phase step produced another 20% reduction of the phenolic content. The following step, ammonium-sulfate fractionation, rendered a 5.5-fold purification with 84% recovery (Table 1).

Up to this ammonium fractionation step, the degree of purification obtained was equal to that obtained using the most frequently reported purification procedure of Nelson and Mason [33] but with higher recovery (84% vs. 62%), perhaps due to the fact that Pb-acetate was not used to remove contaminant brown pigments, which can cause significant loss of enzymatic activity. Furthermore, the used of Triton X-114 avoided the need of several time-consuming re-extraction steps, as described in the improved method of Papa et al. [9], based on Nelson and Mason's method [33]. This improved method gives a lower recovery than Triton X-114 but a higher degree of purification (34-fold vs. 5.5-fold). The problem is that an intermediate step, involving acetone powder is needed before ammonium-sulfate fractionation [9], which results in a fully active (i.e., not latent) enzyme being obtained. In addition, the Triton X-114 method produces a latent enzyme which can be activated 5–6-fold by SDS (Table 1). This degree of activation is maintained throughout the procedure, indicating that Triton X-114 is very gentle with the latent enzyme.

The reduction of phenolic compounds to only 7–8% of the original content without the aid of special polymeric resins prevents tanning [19,20]. This remaining percentage of phenols is similar to that described in the Triton X-114 purification of potato tuber PPO, a starting material which also has a high phenolic content [30]. This low percentage may represent a fraction of the highly hydrophilic compounds which are attached to sugars. The removal of phenols by Triton X-114 was sufficient to avoid browning of the enzyme solution even after many cycles of freezing and thawing, or after months of storage at −20°C. When ammonium-sulfate was used without a previous phase-separation step, the partially purified enzyme turned black.

The partially-purified, soluble, mushroom-tyrosinase was a real polyphenoloxidase, since it had both diphenolase (Fig. 1) and monophenolase activity (Fig. 2). When diphenolase activity of latent mushroom PPO was assayed at pH 6.5, the enzyme activity increased with time, reaching a steady-state

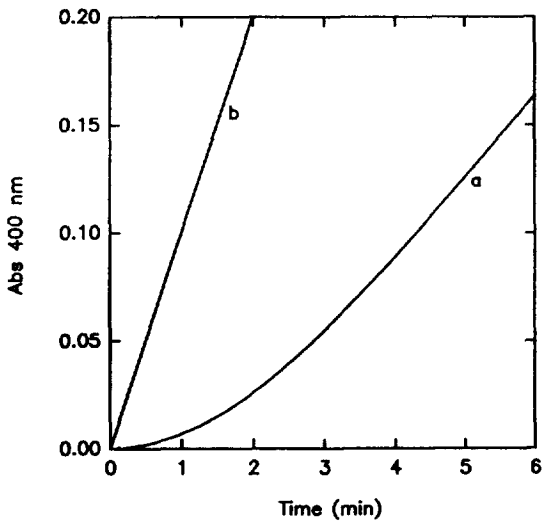


Fig. 1. Progress curves for diphenolase activity of latent mushroom tyrosinase in (a) 10 m*M* phosphate buffer pH 6.5 and (b) in 10 m*M* acetate buffer, pH 4.0, under the standard reaction-medium conditions.

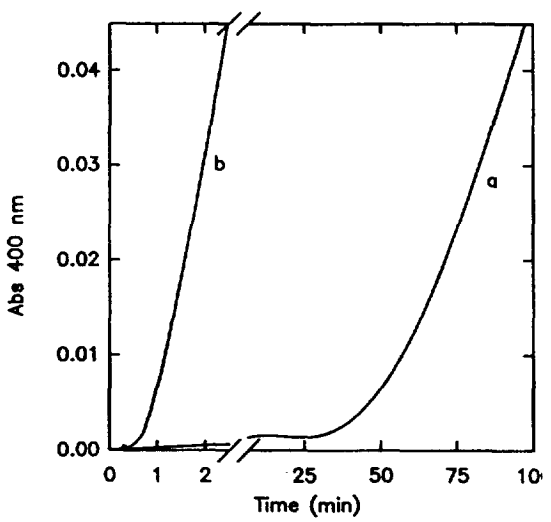


Fig. 2. Progress curves for monophenolase activity of latent mushroom tyrosinase in the absence (a), and in the presence (b), of 0.05% SDS under the standard reaction conditions.

after a discernible lag phase (Fig. 1, curve a). This lag period was abolished when the enzyme was assayed at pH 4.0, with a 2.7-fold increase in activity Fig. 1, curve b). This lag period in the expression of diphenolase activity of tyrosinase has been previously described for latent grape PPO [36], and this response of the enzyme, to pH changes in the medium, is a characteristic property of a hysteretic enzyme undergoing slow transition to another kinetically different form during catalysis [36,37].

The monophenolase activity was also present in the latent enzyme, more than 30 min being necessary to reach the steady-state (Fig. 2a). This lag is characteristic of monophenolase activity [31] and was reduced when 0.05% SDS was included in the reaction medium (Fig. 2b). A 31-fold activation was obtained for this activity compared with the 5.5-fold of diphenolase.

Inhibition studies were also carried out with specific inhibitors (Table 2). Among these, the substrate analogue, tropolone, was the most effective inhibitor at both pH values used (for latent and activated forms). This inhibitor of the 2-hydroxy-1-one family, completely inhibited the enzyme at 100 μM, which is in agreement with previously published data concerning this compound acting as inhibitor of mushroom tyrosinase [38]. Mimosine had little inhibiting effect at either pH, whereas inhibition by kojic and cinnamic acids were pH-dependent, the former being more effective at pH 6.5 and the latter at pH 4.5. A similar overall inhibition profile with these four compounds has been described for potato tuber tyrosinase [30].

The activity of latent mushroom tyrosinase can be enhanced by a variety of treatments, as has already been mentioned. These include changes in pH, detergents (SDS) and proteases (Table 3). In fact, trypsin increased latent enzyme activity 3-fold, a little less than when 0.05% SDS was used. However, the most interesting aspect of this enzyme was the striking effect which lipids and detergents had on the catalytic activity, as is shown in Table 3.

The interaction of some detergents at a final concentration of 1 mM is shown in Table 3a. Cationic detergents were the most effective activators among the detergents used, while the non-ionic and anionic detergents had little effect on the activity. After treatment by detergents, the samples were incubated with trypsin and, in all cases, a synergistic effect between the detergent and trypsin

Table 2
Percentage inhibition of partially purified mushroom tyrosinase by substrate analogues[a]

	pH 4.0				pH 6.5			
	100 μM	10 μM	1 μM	0.5 μM	100 μM	10 μM	1 μM	0.5 μM
Cinamic acid	71	37	19	15	11	9	7	5
Tropolone	100	92	68	57	100	96	87	79
L-Mimosine	14	10	9	0	14	12	4	0
Kojic acid	52	18	0	0	50	22	4	0

[a] Assayed under the standard reaction conditions with the appropiate concentration of inhibitor.

Table 3
Activation of latent mushroom PPO by anionic (SDS and 1-decanosulfonic acid), cationic (CTAB and cetylpyridinium) and non-ionic ($C_{12}E_9$ and Brij 96) detergents

	−Trypsin		+Trypsin	
	U/ml	Activation (fold)	*U*/ml	Activation (fold)
(a) [Detergent]=1 mM				
None	32.26	1.0	86.52	2.7
DS (CMC=14.7 m*M*)[a]	29.13	0.9	135.56	4.2
SDS (CMC=1.36 m*M*)	34.43	1.1	93.82	2.9
CTAB (CMC=0.09 m*M*)	90.61	2.8	124.09	3.8
CP (CMC>2.3 m*M*)	86.61	2.7	129.30	4.0
$C_{12}E_9$ (CMC<0.004 m*M*)	24.78	0.8	161.91	5.0
Brij 96 (CMC=0.007 m*M*)	29.74	0.9	151.39	4.7
(b) [Detergent]=10 mM				
None	32.26	1.0	86.52	2.7
DS	138.52	4.3	4.52	0.1
SDS	119.04	3.7	149.74	4.6
CTAB	70.52	2.2	50.69	1.6
CP ([CP]=2.3 m*M*)	86.96	2.7	84.52	2.6
$C_{12}E_9$	32.61	1.0	86.43	2.7
Brij 96	21.30	0.7	135.62	4.1

The latent enzyme (6 μg/ml) was incubated with detergents for 15 min and assayed for catecholase activity at pH 6.5 with and without trypsin activation using *tert.*-butylcatechol as substrate.
[a] CMC values were determined as described in the Experimental section.

was observed, with higher degrees of activation being achieved than when trypsin alone was used. The results show that the detergents at this low concentration somehow affected the conformational structure of the protein, facilitating proteolytic attack of trypsin.

The activation of latent enzyme by SDS and by the other anionic detergent, DS, was evident at 10 m*M* (Table 3b). At this higher concentration, cationic detergents produced no further increase in the activity of latent enzyme compared to the activation achieved at 1 m*M*, both activations being less than the corresponding activation using the anionics at 10 m*M*. The non-ionic detergents had a similar non-activating effect at both low and high concentrations. When the detergent-treated samples were activated with trypsin, only SDS was able to activate the enzyme completely. The above results show that the latent enzyme was activated at the maximum concentration of detergent monomers in solution. Similar results were described for broad-bean leaf, latent PPO [28], and grape berry PPO [26].

4. Discussion

Tyrosinases are known to be latent enzymes. This latency has been described in frog [39,40], insects [41] and especially in plants, where it is very common in thylakoid-bound polyphenoloxidases and has been found in spinach [27,42–44], broad bean [28,45–47], spinach beet [48], potato [30] and grape berries [26]. However, controversy has arisen regarding the latency of tyrosinase in mushroom, since sample preparation apparently has a strong influence on the extractability and stability of the latent enzyme [13]. Yamaguchi et al. [13] showed that extracts from the fresh tissue contained less latent enzyme than acetone powders and lyophilised preparations. Flurkey's group also obtained contradictory results depending on method and sample preparation [12,49]. Furthermore, they found that some commercial preparations appeared to be activated by SDS, while others were not so responsive and/or were inhibited to a slight degree, suggesting the presence of variable amounts of latent enzyme in each prepa-

ration [12]. SDS also severely inhibited the monophenolase activity of tyrosinase in all lots examined [12].

The results presented in this paper show, for the first time, that temperature-induced phase separation can be used to remove phenols from the mushroom crude extract without drastically changing the structure of the latent tyrosinase during any of the purification steps. The second phase separation at 4% Triton X-114 helps to remove the high level of phenolic compounds present in the extract, since the phenol binding capacity of Triton X-114 is saturated after the first cycle of phase separation. This removal of phenols by Triton X-114 is a prerequisite for obtaining an unmodified native enzyme, as opposed to the modified one which is usually obtained during drastic plant PPO purification methods, when up to 18 bands can exist, as has been shown in the case of potato tuber PPO [50]. Apart from its mildness, the Triton X-114 method permits both diphenolase and monophenolase activity to exist in latent form. This latency of both activities, together with the hysteretic behaviour of diphenolase activity when the pH of the medium changes, have not been previously described in mushroom. In addition, the method is also faster than the most recently improved method for purifying mushroom tyrosinase (6 h instead of 3 days).

These results confirm that Triton X-114-aided purification is an excellent method for isolating latent enzymes. However, the number of steps and the percentage of Triton X-114 necessary depend on the starting material. Initial studies on the effect of non-ionic detergents (such as Triton X-114) on the activity and properties of melanoma tyrosinase and its related proteins have recently been carried out [51]. These studies will perhaps make it possible to obtain a latent form of melanoma tyrosinase in the near future in the same way as has been described for mushroom tyrosinase in this paper.

5. Notation

PPO	polyphenoloxidase
TBP	*tert.*-butylphenol
TBC	*tert.*-butylcatechol
CTAB	cetyltrimetilamonium bromide
CP	cetylpyridinium bromide
Brij 96	poly(10)oxyethylene oleyl ether
$C_{12}E_9$	poly(9)oxyethylene lauryl ether
1-DS	1-decanesulfonic acid
SDS	sodium dodecyl sulfate
CMC	critical micelle concentration

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