

Purification of tyrosinase from edible mushroom

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Abstract

A simple preparative method was developed for purification of Tyrosinase from edible mushroom (*Agaricus bispora*). A homogenized extract of mushroom was first saturated by ammonium sulfate. The desired precipitate was mixed thoroughly with DEAE-Cellulose (DE-52) and washed out to produce melanin free precipitate. The obtained protein solution was dialyzed against running water for 4 hrs, then, concentrated and chromatographed on a DE-52 column. On the basis of the activities assay, the eluted fractions by 150 mM salt solution were selected for further purification. The collected fractions were pooled and chromatographed on a Sephadex G-200 column. Polyacrylamide gel electrophoresis (PAGE) of the purified tyrosinase produced a single band right beside the commercial sample obtained from Sigma Company at 128 kDa. The lyophilized form of the purified Tyrosinase had a purification degree of 104 and showed strong cresolase and catecholase activities when compared to a commercially available tyrosinase.

Keywords: Tyrosinase, Edible mushroom, Purification, Extraction

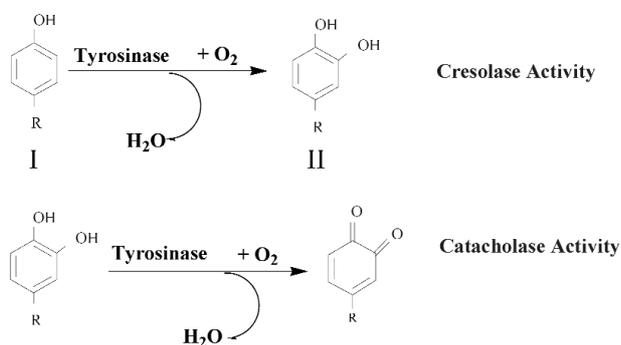
INTRODUCTION

There is an increasing demand for different enzymes in modern industries (Dordick, 1991). Besides, in spite of the outstanding progress in chemistry, there is not yet efficient reagent for synthesizing some chemical substances. *ortho*-Hydroxylation of phenolic compounds is a good example of such reactions (Smith and March, 2001). This reaction happens easily and repeatedly in almost all organisms and results in formation of important biochemical such as neurotransmitters of L-dopa family, coumestrol, polyphenolic acids and tannins

and (Seo *et al.*, 2003). However, chemistry has failed to introduce a decent reagent for this seemingly simple reaction up to now. As a result, scientists have paid attention to biotransformation. Except tyrosine hydroxylase, tyrosinase (EC.1.14. 18.1) is the only known enzyme, which carries out this reaction effortlessly and efficiently (Kazandjian and Kilbanov, 1985). Therefore, a great deal of effort has been put on applying this enzyme in biotransformation of the desired phenolic compounds to their corresponding *ortho*-dihydroxy substances both in aqueous and non-aqueous media (Casella *et al.*, 2004).

Tyrosinase (also called monophenol mono-oxygenase; polyphenol oxidase; catechol oxidase; oxygen oxidoreductase) is a cuproenzyme responsible for formation of the pigments of skin, hair, and eye (Passi and Nazzaro-Porro, 1981). It carries out two successive reactions known as cresolase and catecholase which are illustrated in figure 1. Tyrosinase is found throughout the phylogenetic tree and has been extracted from different sources such as fungi, fruits, and mammalian melanoma tumors. However, edible mushroom is considered as a clean, enriched, and cheap source of this enzyme. The active site of mushroom tyrosinase (MT) is close to the surface of the protein skeleton. Hence, it is accessible to small and large substrates (Marumo and Waite, 1986). There are considerable reports indicating the great potential of this enzyme for food, medicine and agricultural industries as well as analytical and environmental purposes (Seo *et al.*, 2003). Furthermore, MT like mammalian tyrosinase has a tetrameric structure and can be used for clinical purposes (Gelder *et al.*, 1997 and Baharav *et al.*, 1996). All these features have made MT an apt tool for today's biotechnology. For this purpose, we decided to develop simple procedure for (Fig. 2) purification of this enzyme from fresh edible mushroom (*Agaricus bispora*).

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R = -N=N-ph-CH₃

I = 4-[(4-Methylphenyl)azo]-phenol (MePAPh)

II = 4-[(4-methylbenzo)azo]-1,2-benzenediol (MeBACat)

Figure 1. Cresolase (mono-oxygenase) and catecholase (oxidase) activities of tyrosinase. “I” and “II” represent the synthetic substrates used for assaying the cresolase and catecholase activities of MT, respectively. See the Methods section for the details.

MATERIALS AND METHODS

MT and bovine serum albumin were purchased from Sigma Chemical Co. (USA). Sephadex G-50 and G-200 were purchased from Pharmacia and Diethylaminoethyl cellulose (DE-52) from Whatman™ (Maidstone, UK). Phenyl methyl sulphonyl fluoride (PMSF) was obtained from Merck™. 4-[(4-Methylphenyl)azo]-phenol (MePAPh) and 4-[(4-methylbenzo)azo]-1,2-benzenediol (MeBACat), assigned as “I” and “II” in figure 1, were prepared as described earlier (Haghbeen and Tan, 1998). Fresh edible mushroom was bought from the market and frozen at -20°C. All the other chemicals and reagents, needed for electrophoresis and buffer preparation, were taken from the authentic samples. Double-distilled water was used for preparing the desired solutions.

Assay of MT activities: In this study, all the enzymatic reactions were run in phosphate buffer solution, PBS, (10 mM) at pH 6.8 and constant temperature, 20±0.1°C. The final volume of all the reaction mixtures was 3 ml filling up three quarters of the conventional UV-Vis cuvette, 1-cm width. Freshly prepared MT was used for both the cresolase and catecholase activities. Using a Beckman (DU Series 70) spectrophotometer, the rate of the enzymatic reaction of MT was monitored as described previously (Haghbeen and Tan, 2003). Therefore, the cresolase and catecholase activities were assayed through the depletion of MePAPh ($\lambda_{\max} = 352$ nm) and MeBACat ($\lambda_{\max} = 364$ nm), respectively. The results presented in this article

are the averages of, at least, triplicate measurements.

Protein extraction: 250 g of the frozen mushroom was homogenized in 300 ml of Tris-HCl buffer (50 mM, pH 5.8) containing 1 mM of PMSF. The suspension was stirred for 30 min at room temperature and filtered through a cotton mesh. The filtrate was centrifuged at 13500×g for 30 min at 4°C using a Beckman Centrifuge, J-21 model. The resulting supernatant was separated and subjected to ammonium sulfate precipitation.

Ammonium sulfate precipitation: Ammonium sulfate powder was added to the collected supernatant from the former step to make a 35% saturated solution. The resulting solution was stirred in an ice bucket for 30 min, then, centrifuged for 30 min at 1500×g and 4°C. Ammonium sulfate was added to the obtained supernatant to make a 70% saturated solution. The solution was left stirring on ice for 2h, then, centrifuged for 30 min at 1500×g and 4°C. The supernatant was discarded and the resulting precipitate was dissolved in Tris-HCl (50 mM, pH 5.8).

Desalting and chromatography: In order to desalt the obtained protein solution from the previous stage, it was chromatographed on a G-50 column (2.6×45cm, Pharmacia) using Tris-HCl buffer (50 mM, pH 5.8) as the mobile phase. The output of the column was monitored spectrophotometrically at $\lambda_{\max} = 280$ nm. Then, the collected fractions were checked for the cresolase and catecholase activities in the presence of MePAPh and MeBACat, respectively. The fractions with high activities were pooled. Desalting was also achieved by dialysis. Dialysis was carried out against Tris-HCl buffer (50 mM, pH 6.8) at 4°C over night.

Ion exchange chromatography: The dialyzed protein solution from the previous step was loaded onto ion exchange (DE-52) column and passed through by Tris-HCl buffer (50 mM, pH 6.8). Then, the proteins were eluted from the column using NaCl solution. The collected protein fractions at 150 mM salt eluent were pooled and concentrated by lyophilization.

Gel filtration: To purify further the lyophilized sample obtained from the previous step, it was dissolved in PBS and loaded on a Sephadex G-200 column (10×100 mm Pharmacia). The column washed with PBS. The ensuing fractions were collected and checked for the presence of the protein. The quality of the purified tyrosinase was evaluated by PAGE and activities assays results.

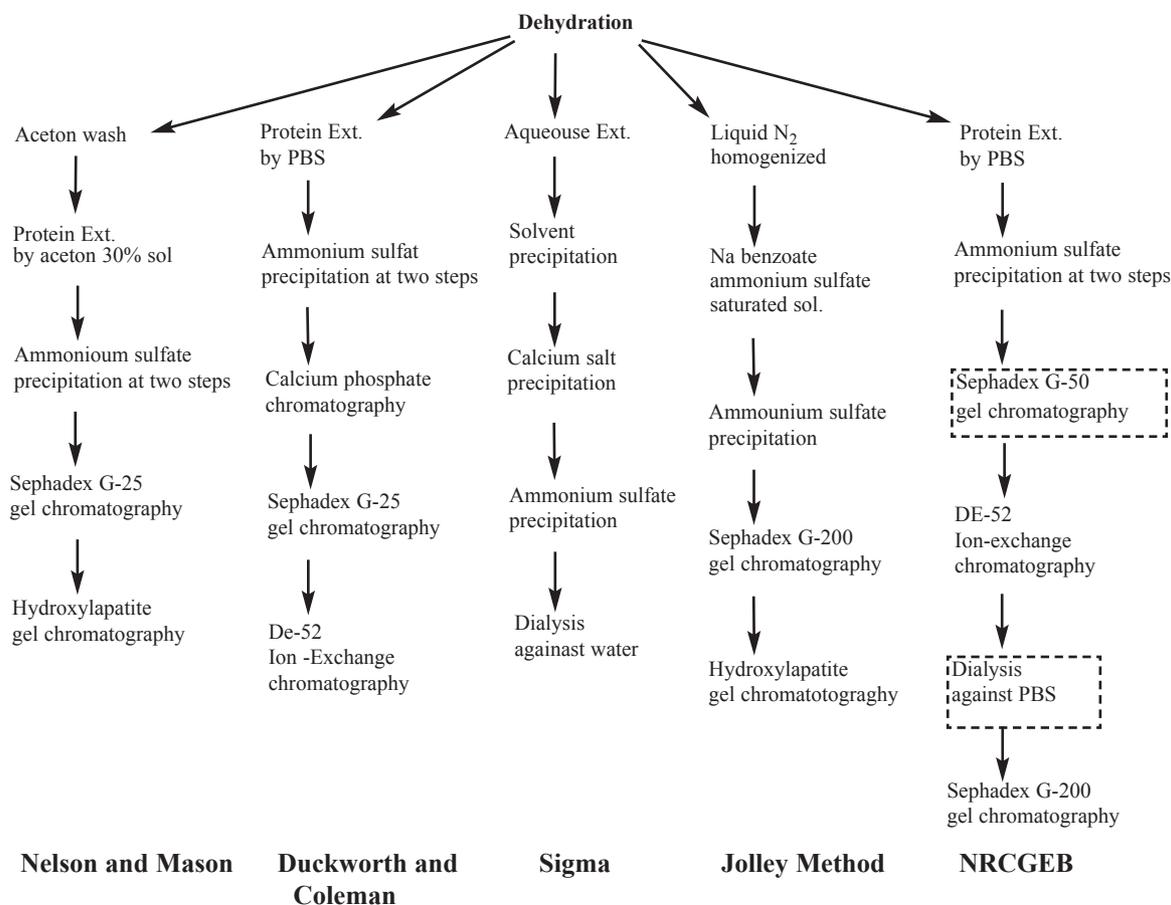


Figure 2. Schematic illustration of the discussed purification procedures. See the results and discussion section for the details.

Electrophoresis and determination of protein concentration: Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970). Accordingly, the protein solution was subjected to electrophoresis on 10% resolving and 4% stacking polyacrylamide gel at a constant electrophoretic field of 200 V. The protein bands were visualized by staining with Coomassie brilliant blue G250. The observations were double-checked by silver nitrate-stain method. The protein concentration was determined using Bradford method (Bradford, 1976).

RESULTS

Removal of melanins: The accompanying melanins which are usually built up during preparation of the homogenized extract could be extensively removed from the protein mixture using ion-exchange material. See the discussion section for the detail.

Ion-exchange chromatography: The eluted fractions obtained by salt solution (150 mM) from a DE-52 col-

umn showed high catecholase activity and tyrosinase content. These results are illustrated in figures 3 and 4, respectively.

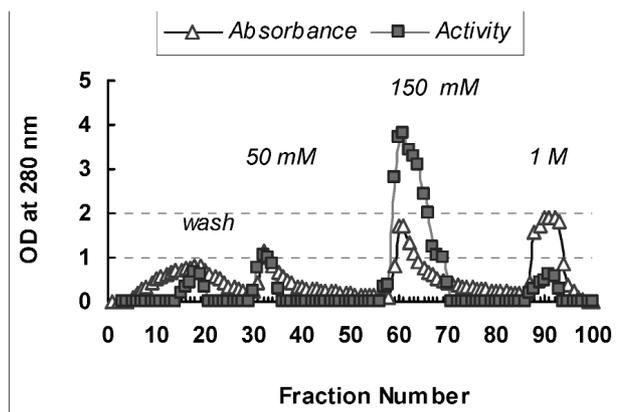


Figure 3. The chromatogram of the ion-exchange chromatography stage. Absorbance (Δ) refers to the optical density for each collected fraction at $\lambda_{\max} = 280$ nm and activity (\bullet) means the rate of the catecholase reaction for each fraction. See the methods section for the detail.

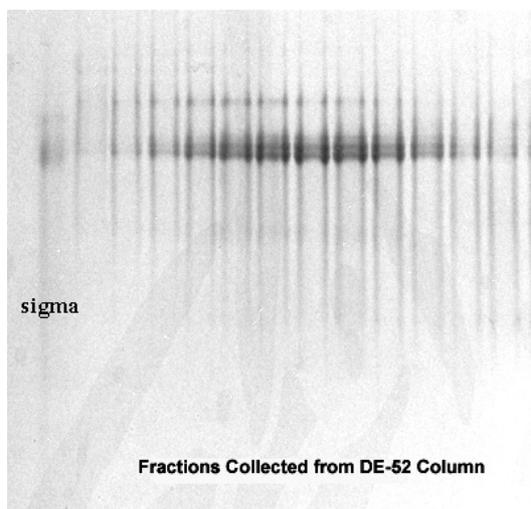


Figure 4. The PAGE result of the collected fractions eluted from the DE-52 column in comparison with the commercial (Sigma™) sample.

Gel-chromatography: The desired collected fractions from the ion-exchange chromatography step were mixed, concentrated and loaded on a Sephadex G-200 column. The purified MT at this stage produced a single band beside the commercial sample at about 128 kDa.

Activity enhancement and purification degree: The collected protein after removing of melanins and desalting had a catecholase activity of 6.258 (rate per ml) and a purification degree of 6.6. Similarly, the collected fractions from the ion-exchange chromatography stage and the purified MT collected from the gel chromatography step showed catecholase activities of 6.276 and 8.77, correspondingly, and purification degrees of 29 and 104, respectively. The detailed results are summarized in table 1.

DISCUSSION

Although there are numerous methods for extracting and purifying of tyrosinases from different sources; there are only a few methods for purification of MT which have been cited repeatedly in the literature (Healey and Strothkamp, 1981; Toussaint and Lerch, 1987; Koga *et al.*, 1992; Espin *et al.*, 1997; Fenoll *et al.*, 2004). These procedures are illustrated beside the method, which is used by Sigma™ for preparation of the commercial MT (Haghbeen, 1998), and the developed method in this lab in figure 2. Edible mushroom contains a considerable amount of various phenolic compounds, which are readily oxidized during the homogenizing process. Upon oxidation and successive polymerization of the phenolic content of the mushroom extract, macromolecules of melanins are formed. Separating the unwanted melanins from the protein content of the extract is the first, or probably the most, important task during the MT purification.

As shown in figure 2, the Nelson and Mason method (Nelson and Mason, 1970) starts with the acetone wash of the homogenized extract to collect the phenolic substances in the organic solvent at the beginning. An almost similar trick, solvent precipitating, is applied in the Sigma™ procedure. Using an organic solvent can prevent the melanin formation to a great extent, but increases the proteins denaturation risk. This is why Duckworth and Coleman had used $\text{Ca}_3(\text{PO}_4)_2$ chromatography to separate melanins (Duckworth and Coleman, 1970).

To stop all the possible chemical reactions, Jolly *et al.* had homogenized mushroom in liquid nitrogen (Jolley *et al.*, 1974). Then, they had precipitated all the protein content of the extract by a saturated sodium benzoate- ammonium sulfate solution. This method is also suffering from two clear drawbacks. First, they

Table 1. The total results of each stage of the developed method for the MT purification.

Purification stage	Total volume (ml)	Total protein (mg)	Activity (rate/ml)*	Specific activity (rate/mg)	Purification degree
Extraction	410	1640	0.87	0.21	1
Precipitation	60	1050	1.746	0.09	0.4
Desalting	90	405	6.258	1.39	6.6
Ion- exchange	156	156	6.276	6.27	29
Gel filtration	26	10.4	8.77	21.92	104

* rate refers to the change in the optical density of MeBACat at $\lambda_{\text{max}} = 364$ nm per minute during its reaction with the collected enzyme. To obtain the amount of the used substrate, the reported data has to be divided to the extinction coefficient of MeBACat at $\lambda_{\text{max}} = 364$ nm which is $15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Haghbeen and Tan, 2003).

have homogenized mushroom in liquid nitrogen at very low temperature. Under these conditions, phenolic compounds might not dissolve in liquid medium and remain absorbed on the protein and cell debris surface. Second, after carrying out the homogenizing step in liquid nitrogen, the obtained pulp has to be transferred into the aqueous medium, which inevitably triggers melanin formation.

To tackle the melanin formation problem during MT purification, two other solutions have been introduced in more recent works. In the first work, Nunez-Nunez-Delicado *et al.* (1996) have trapped the hydrophobic proteins and the phenolic content of the extract into a micelle system using Triton X-114. In the second work, Rescigno *et al.* (1997) have applied a diafiltration against ascorbic acid solution. The presence of ascorbic acid avoids the oxidation of the phenolics by keeping them in the reduced form throughout the extraction phase until their complete removal. These solutions have their own disadvantages. The former method needs micelle formation and trapping phenolic compounds and hydrophobic proteins in it, which is cumbersome by its own. In the diafiltration method, a large amount of ascorbic acid or another reducing agent has to be utilized, which makes the procedure more expensive.

Experiments in this lab, however, showed that it is possible to get rid of both phenolic and melanin contaminants at the ion-exchange chromatography stage. This procedure was started by homogenizing the edible mushroom in PBS at 4°C. Therefore, no organic solvent was used at this stage. However, it was essential to carry out this step at cold temperature and as fast as possible. After removing the cell debris, the protein content of the homogenized extract was precipitated at 35% and 70% of saturation point of ammonium sulfate. The collected heavy proteins showed very weak catecholase activity and were put aside.

It is necessary to reduce the salt content of a protein mixture before loading it onto an ion-exchange column. Therefore, the collected proteins by 70% saturated ammonium sulfate were chromatographed on a Sephadex G-50 column. Doing so, not only ammonium sulfate was substituted by buffer, but also large amount of phenolic compounds were washed down. Besides, the protein components were fractionated during elution. However, this stage can be replaced by dialyzing the protein mixture against running water or buffer; if fractionation is not necessary.

Figures 3 and 4 shows the results of the ion-exchange chromatography of the collected protein mixture on a DE-52 column. As it was mentioned earlier in this paper, it is possible to omit both the pheno-

lics and melanin impurities from the protein mixture at this stage. As a matter of fact, melanins show very high affinity for DE-52 polymer. When the mixture of protein and melanin is loaded on the DE-52 column and successively washed by molar solution of NaCl, this is only the protein which is released. In other words, melanin sticks firmly to DE-52. Therefore, it is suggested to replace the Sephadex G-50 step, showed in a dashed square in figure 2, by a three-step stage; Dialysis, mixing with DE-52 and releasing the proteins and again dialysis.

The final stage in Jolly *et al.*, (1974) and Moss and Rosenblum (1972) methods involve in applying hydroxylapatite chromatography. It is known that hydroxylapatite is a strong protein absorbent which is usually used for separation of isozymes. MT is a mixture of four isozymes and all of them are capable of doing both cresolase and catecholase reactions. Therefore, there is no need to decrease the final yield to an isozyme, unless it is really needed. Considering the average molecular weight of MT, it was preferred to apply Sephadex G-200 instead of hydroxylapatite at the final stage. The PAGE result of the purified MT sample has shown in figure 5 and the results of the whole purification stages have been summarized in table 1. It is understood from the results that the amount of the total protein had been decreased to 0.64% of the initial extracted protein whereas its catecholase activity had been increased to ten-fold of the initial activity. In conclusion, it is important to mention that similar results were observed for the cresolase

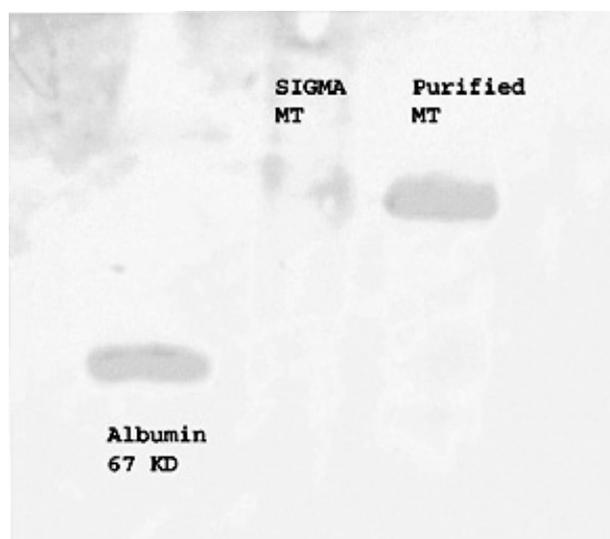


Figure 5. The PAGE result of the purified MT obtained from the G-200 column in comparison with the commercial (Sigma™) sample and BSA.

activity of the purified MT in the presence of MePAPh. However, it is easier to follow MT purification steps by monitoring its catecholase reaction. The catecholase reaction of MT is about ten times faster than its cresolase reaction.

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