

## Purification and partial characterization of tyrosinase from straw mushroom

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### Abstract

Tyrosinase from straw mushroom was purified to homogeneity in a two step procedure. After purification, the enzyme purity was increased by 4.81 fold with a specific activity of 9.68 unit/mg protein and the recovery yield of 0.12%. The molecular mass of purified tyrosinase estimated by SDS-PAGE electrophoresis was approximately 31 kDa. The purified tyrosinase showed the highest activity at pH 8.0 and 60 °C. The activity of the enzyme was enhanced by the addition of 10 mM Cu<sup>2+</sup> or Zn<sup>2+</sup> whereas the EDTA and 1,10-phenanthroline strongly inhibited the activity. The apparent K<sub>m</sub> and V<sub>max</sub> of the purified tyrosinase for L-DOPA at pH 8.0 and 60 °C were calculated to be 7.08 mM, 1.2 μM min<sup>-1</sup> and the apparent catalytic constant (k<sub>cat</sub>) was 1651 min<sup>-1</sup>.

### บทคัดย่อ

งานวิจัยนี้ได้ศึกษาการแยกบริสุทธิ์เอนไซม์ไทโรซิเนสจากเห็ดฟาง เอนไซม์ถูกแยกบริสุทธิ์ได้ใน 2 ขั้นตอนด้วยวิธีตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟตและผ่านคอลัมน์ Sephadex G-100 หลังจากแยกบริสุทธิ์แล้วพบว่าเอนไซม์มีบริสุทธิ์เพิ่มขึ้น 4.81 เท่า มีค่ากิจกรรมเอนไซม์จำเพาะเท่ากับ 9.68 U/mg protein และมีผลได้ของการแยกบริสุทธิ์เท่ากับ 0.12% น้ำหนักโมเลกุลของเอนไซม์จากการประมาณด้วยวิธี SDS-PAGE เท่ากับ 31 kDa เอนไซม์ไทโรซิเนสที่ผ่านการแยกบริสุทธิ์แล้วมีสถานะที่เหมาะสมสำหรับการเร่งปฏิกิริยาที่พีเอช 8.0 อุณหภูมิ 60 °C กิจกรรมเอนไซม์ไทโรซิเนสถูกกระตุ้นด้วยไอออนของ Cu<sup>2+</sup> และ Zn<sup>2+</sup> ที่ความเข้มข้น 10 mM และถูกยับยั้งด้วย EDTA และ 1,10-phenanthroline การศึกษาจลนพลศาสตร์ของเอนไซม์ไทโรซิเนสที่ผ่านการแยกบริสุทธิ์พบว่าค่า K<sub>m</sub> และ V<sub>max</sub> ของเอนไซม์เท่ากับ 7.08 mM และ 1.2 μM min<sup>-1</sup> ตามลำดับ และมีค่า catalytic constant (k<sub>cat</sub>) เท่ากับ 1651 min<sup>-1</sup>

**Keywords:** tyrosinase / purification / straw mushroom / L-DOPA

### Introduction

Tyrosinase (E.C. 1.14.18.1) is a ubiquitous enzyme involved in pigmentation. It catalyzes hydroxylation of monophenols (cresolase activity) and oxidation of diphenols (catecholase activity) in the presence of molecular oxygen. The catalytic activity of tyrosinase is potentially attractive with respect to its biotechnological applications, for example, the production of L-DOPA (3,4-dihydroxyphenylalanine) and the treatment of phenolic wastes (Hearing, 1987)

Although tyrosinases are widely distributed in microorganisms, much of the current interest in the development of biotechnological applications has been focused on the use of tyrosinases from

mushroom. Several aspects of mushroom tyrosinases such as their biochemical characteristics and some of their potential biotechnological applications have been intensively reviewed (Seo et al., 2003). However, relatively little attention has been given to the purification of tyrosinases straw mushroom, *Volvariella volvacea*. In this study, the purification of tyrosinase from straw mushroom and some characterizations of the purified tyrosinase were reported. The kinetics parameters of the purified enzyme for L-DOPA were also examined.

## Objective

This study was aimed for the purification and characterization of tyrosinase from straw mushrooms (*Volvariella volvacea*).

## Materials and methods

### 1. Preparation of crude tyrosinase

1000 g of the straw mushrooms purchased from local market in Nakhon Pathom, Thailand, were sliced to thin pieces and subsequently homogenized in 500 ml of cold 10 mM phosphate buffer pH 7.0. The suspension was stirred for 30 min at room temperature and filtered through a cotton mesh. The filtrate was centrifuged at 5000 rpm for 30 min at 4°C. The resulting supernatant was subjected to ammonium sulfate precipitation.

### 2. Purification of tyrosinase

#### 2.1 Ammonium sulfate precipitation

A fine powder of ammonium sulfate was added to the collected supernatant from the former step until the final concentration of 80% saturated solution was obtained. The supernatant was stirred in an ice bucket for 30 min and left at 4°C for overnight. The protein precipitant was separated by centrifugation at 15000 rpm for 20 min at 4°C and re-dissolved in 10 mM phosphate buffer at pH 7.0.

#### 2.2 Sephadex G-100 gel filtration chromatography

5 ml of crude tyrosinase in 80% saturated ammonium sulfate solution was directly applied to a Sephadex G-100 column (1.7x70 cm) pre-equilibrated with a 25 mM phosphate buffer of pH 7.0. The protein was eluted with the same buffer at a flow rate of 0.5 mL/min. The fractions containing tyrosinase activity were pooled and subsequently lyophilized.

### 3. Assay of tyrosinase activity

The tyrosinase activity was determined spectrophotometrically by following the formation of dopachrome as described by Yang and Robb (1993) with some modifications. After addition of 10 µl enzyme solution to a 96-well plate containing 1 mL of 5 mM L-DOPA dissolved in 10 mM potassium phosphate buffer pH 7.0, the reaction was incubated at 30°C for 30 min. The formation of dopachrome was measured at 475 nm. One unit of tyrosinase was defined as the amount of the enzyme catalyzing the formation of 1 µmol dopachrome per minute under the assay conditions. The extinction coefficient of dopachrome at 475 nm was 3400 M<sup>-1</sup>cm<sup>-1</sup> (Duckworth and Coleman, 1970).

#### 4. Assay of protein content

The protein content was determined by the Bradford method (Bradford, 1976) using the Bio-Rad assay reagent (Bio-Rad, USA) and bovine serum albumin as the standard. The protein concentration in the fractions was monitored by the absorbance of 280 nm.

#### 5. Determination of molecular mass

The molecular mass of purified tyrosinase was analyzed by using 12% polyacrylamide of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under the method of Laemmli (1970). The electrophoresis was performed on constant current at 25 mA for 45 min. The proteins on SDS-PAGE were visible by staining with Coomassie Brilliant Blue R-250. The relative molecular mass of the protein was calculated by using protein standard markers (Bio-Rad, USA).

#### 6. Characterization of purified tyrosinase

##### 6.1 Effect of pH and temperature on tyrosinase activity

The effect of pH on tyrosinase activity was measured at 30°C at the pH range of 6-11 using 5 mM of L-DOPA as the substrate. The buffer systems used were 50 mM Phosphate buffer (pH 6-7.5), 50 mM Tris-HCl buffer (pH 7-9), and 50 mM glycine-NaOH buffer (pH 9-11).

The effect of temperature on tyrosinase activity was determined by using 5 mM of L-DOPA dissolved in 50 mM Tris HCl buffer at pH 8.0 as the substrate. The reaction mixture was incubated at the various temperatures (40-90°C) for 30 min and the concentration of dopachrome was determined by spectrophotometer.

##### 6.2 Effect of metal ions and inhibitors on tyrosinase activity

The purified tyrosinase was pre-incubated at room temperature with 10 mM and 2 mM of different metal ions and inhibitors for 30 min. The remaining tyrosinase activity was measured by the method as described above.

##### 6.3 Kinetic Analysis of the purified tyrosinase

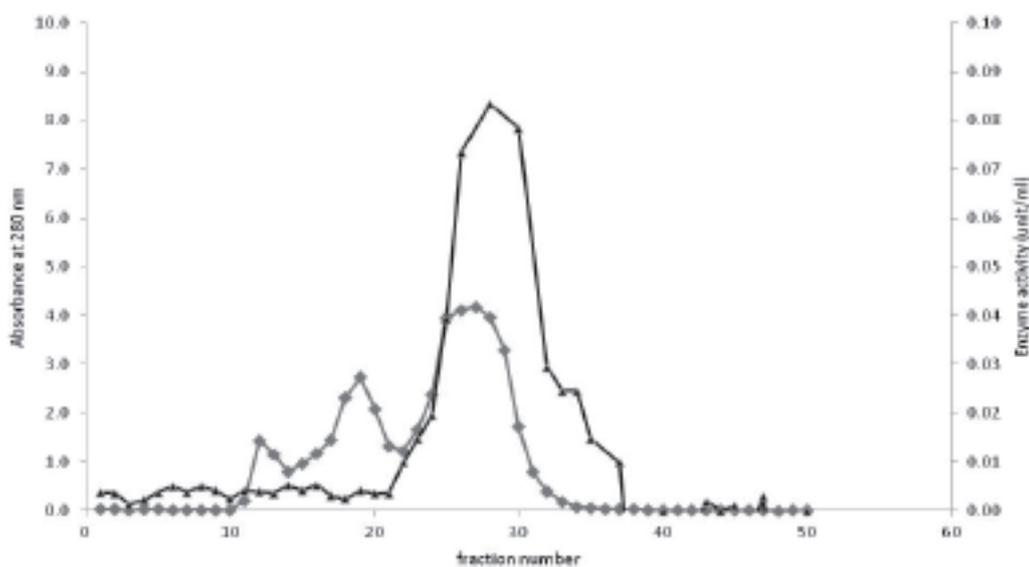
The kinetics parameters of purified tyrosinase were estimated under a range of L-DOPA concentrations (1.5 mM - 6.0 mM) at 60 °C. The apparent  $K_m$  and  $V_{max}$  value of purified tyrosinase was calculated from the Lineweaver-Burk plots relating  $1/V$  to  $1/[S]$ . The catalytic constant,  $k_{catalytic}$ , was estimated from the equation  $V_{max} = K_{cat} [E]_t$  where  $[E]_t$  is an enzyme concentration

## Results

### 1. Purification of tyrosinase

The purification of mushroom tyrosinase is moderately more difficult as the mushroom normally contains a considerable amount of various phenolic compounds which are readily oxidized and polymerized to macromolecules of melanins during the homogenizing process. However, the purification of tyrosinase from straw mushroom could be easily accomplished by two purification steps. At the first step, the crude enzyme was concentrated by precipitation with 80% ammonium sulfate resulting the recovery yield of 88.63%. For the second step of purification, the concentrated tyrosinase was directly

applied to Sephadex G-100 gel filtration column chromatography without salt removing. The tyrosinase active were found in fraction 21-37 (Fig 1) and the purification fold of 4.81 was obtained with the specific activity of 9.68 U/mg and 0.12% recovery yield (Table 1).

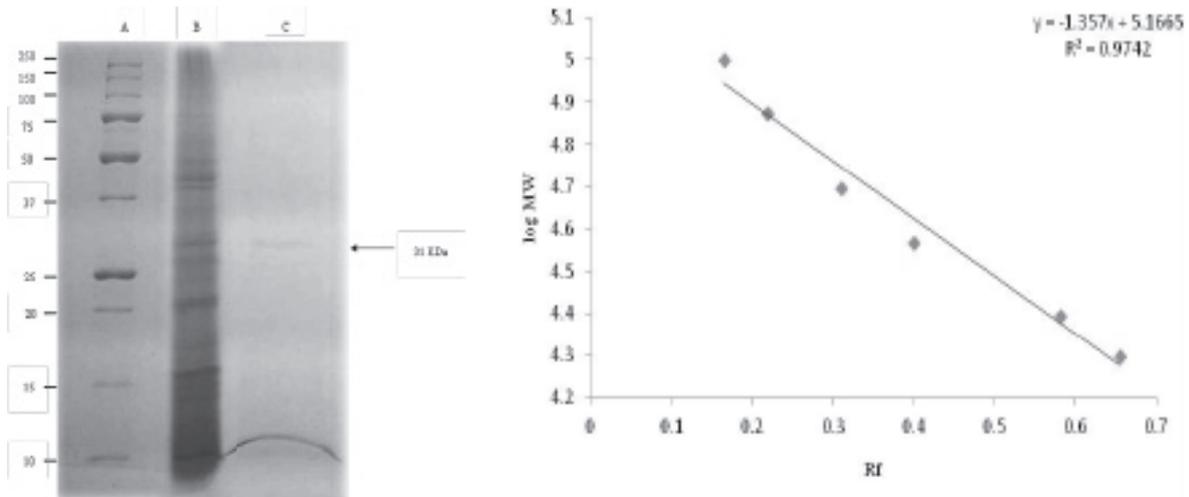


**Figure 1** The resulting chromatogram of the purified tyrosinase from Sephadex G 100 column. The flow rate was set at 0.5 ml/min (—◆— A280, —▲— tyrosinase activity)

**Table 1** Summary of tyrosinase purification from straw mushroom

fraction	Total volume (ml)	Activity (unit/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude Extract	1400	2.25	3150	1568	2.01	1.00	100
80% Ammonium Sulphate Precipitate	100	27.92	2792	1246	2.24	1.11	88.63
Sephadex G100 Gel filtration	3	1.29	3.87	0.4	9.68	4.81	0.12

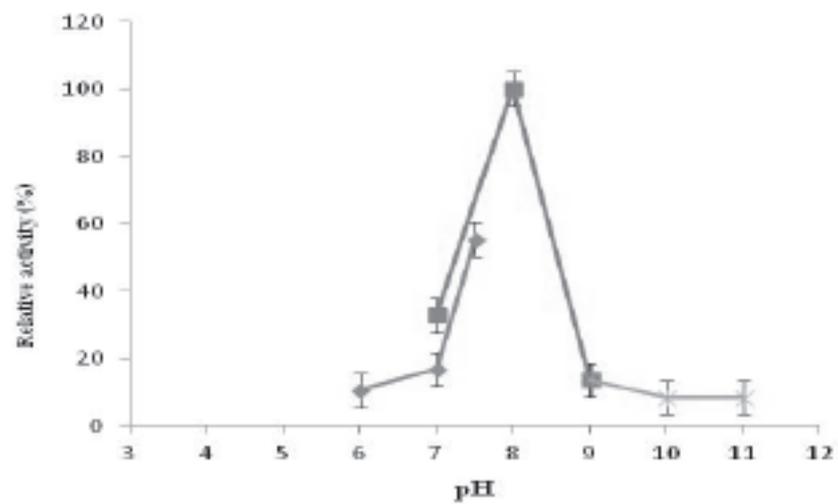
SDS-PAGE revealed only a single distinctive protein band for the pure preparation of tyrosinase with an apparent molecular weight of 31 kDa (Lane C in Fig 3). The molecular weight of tyrosinase from straw mushroom was relatively low as compared to the purified tyrosinase from gill tissue of portabella mushroom and button mushroom which had a molecular weight of 70 kDa (Yan Fan et al., 2004) and 95 KDa (Kamal et al., 2014), respectively.



**Figure 3** SDS-PAGE (12%) of tyrosinase from straw mushrooms, lane A, standard protein of molecular weight; Lane B, crude tyrosinase preparation concentrated by 80% ammonium sulfate precipitation. Lane C, purified tyrosinase from Sephadex G-100 column.

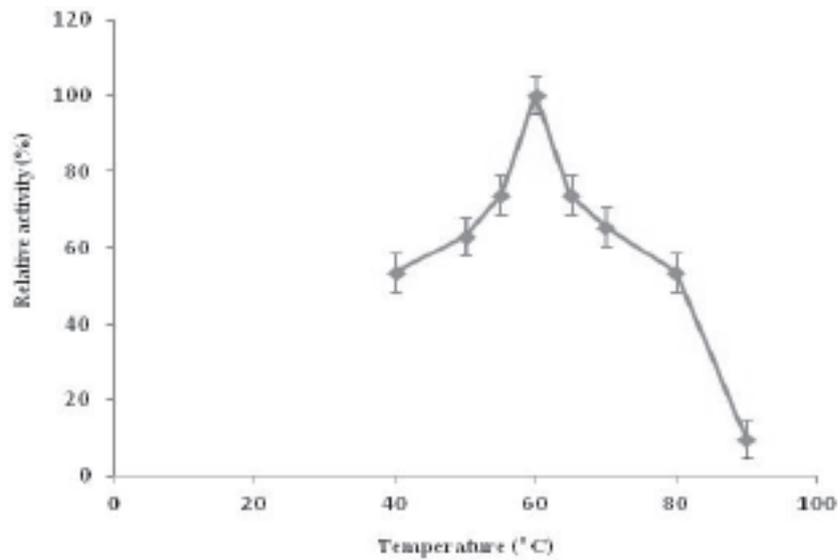
2. Characterization of purified tyrosinase

The effect of pH on tyrosinase activity was shown in Fig 2. The highest tyrosinase activity was found at pH 8.0. The optimum pH of tyrosinases from various organisms has been reported in the neutral or slightly acidic range. However, the tyrosinase from *Ipomoea batatas* and *Trichoderma reesei* has a basic pH optimum of 8 and 9, respectively (Selinheimo, 2006, Eicken, 1998).



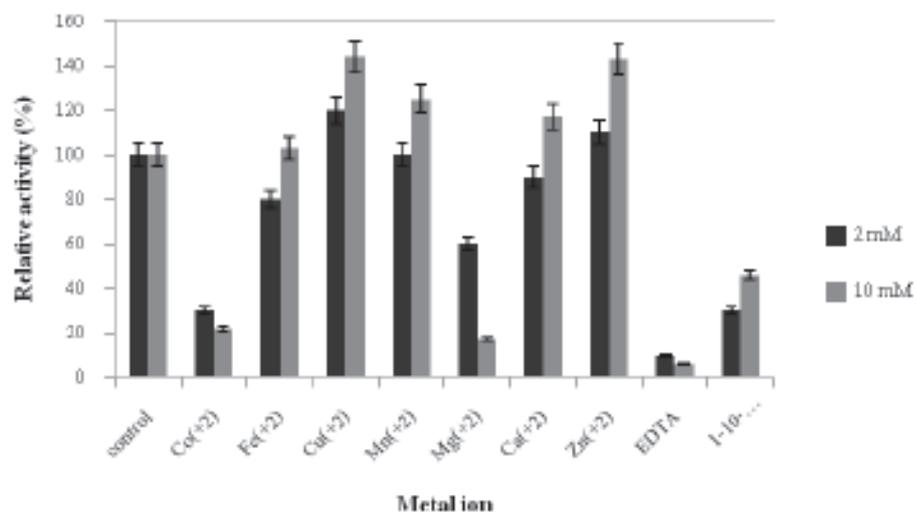
**Figure 2** Effect of pH on the tyrosinase activity of straw mushrooms. Data were obtained as mean value of the absorbance at 475 nm. Tyrosinase assays were performed at 30°C. the buffers used for the tyrosinase assay were 50 mM phosphate buffer at pH 6-7.5, 50 mM Tris-HCl buffer at pH 7-9 and 50 mM glycine-NaOH buffer at pH 9-11. The activity at pH 8.0 was taken as 100%.  
( Phosphate buffer, Tris-HCL buffer, Glycein-NaOH buffer)

The purified tyrosinase was active at a wide range of temperature from 20°C to 90°C with an optimum at 60°C (Fig 4), and about 50% of tyrosinase activity was remained at 80°C.



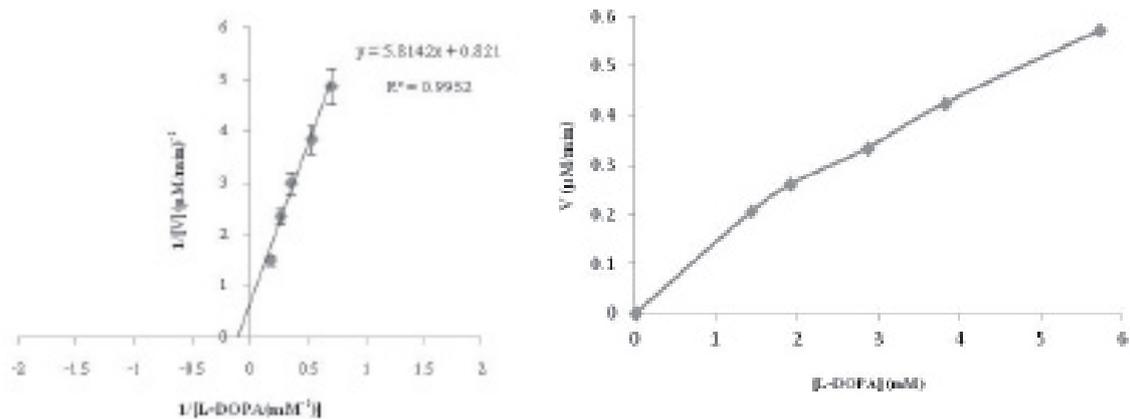
**Figure 4** Effect of temperature on the tyrosinase activity. The enzyme was assayed in 50 mM potassium phosphate buffer pH 8.0) The enzyme activity at 60°C was taken as 100%

The effect of metal ions and metal chelators at the concentration of 2 mM and 10 mM on the purified tyrosinase from straw mushroom was investigated. As shown in Fig 5, the increasing in tyrosinase activity was observed in the presence of Cu<sup>2+</sup> and Zn<sup>2+</sup>. The tyrosinase activity was retained above 80% in all the metal ions tested except for Co<sup>2+</sup> and Mg<sup>2+</sup>. The metal chelators, EDTA and 1,10-phenanthroline, had similar negative effects on tyrosinase activity, confirming that tyrosinase is a metalloenzyme.



**Figure 5** Effect of metal ions and chelating agents on tyrosinase activity from straw mushrooms

The kinetics parameters of purified tyrosinase were investigated using different concentrations of the L-DOPA as a substrate. The reaction mixture of L-DOPA at the different concentrations from 1.5 to 6.0 mM and the purified tyrosinase (0.5 mg/ml) was incubated at 60 °C for 30 min. The formation of dopachrome was measured spectrophotometrically. The Lineweaver-Burk plot shown in Fig 4 indicated the  $K_m$ ,  $V_{max}$  values of 7.08 mM, 1.2  $\mu\text{M min}^{-1}$ , respectively. The  $k_{cat}$  calculated from  $V_{max}$  and total enzyme concentration was 1651  $\text{min}^{-1}$ .



**Figure 4** Lineweaver-Burk plot of kinetics results of dopachrome formation as catalyzed by purified tyrosinase from straw mushroom. The enzyme (0.5 mg/ml) was reacted with different concentrations of L-DOPA from 1.5 to 6 mM dissolved in 50 mM Tris-HCl buffer pH 8.0 The reaction was performed at 60 °C for 30 min. Data were obtained as mean value of three independent tests

## Conclusions

Although no commercial tyrosinase-based production of L-DOPA was reported, other applications of tyrosinase are currently investigated, indicating the potential to create a large demand for the enzyme. Mushroom tyrosinases would seem to be natural candidates for the establishment of commercial processes. From the present study, straw mushroom tyrosinase was simply purified by two step procedures. The purified enzyme had a molecular weight of 31 KDa with the optimal catalytic activity at pH 8.0 and 60 °C. The kinetics parameters,  $K_m$  and  $V_{max}$ , were estimated to be 7.08 mM and 1.2  $\mu\text{M min}^{-1}$ , respectively. This indicated that the straw mushroom tyrosinase could be a prosperous source of tyrosinase for the application in bioprocess and biotechnology.

## Discussion and Recommendation

Although there are numerous methods for extracting and purifying of tyrosinases from different sources; a few methods for purification of mushroom tyrosinase have been cited repeatedly in the literature (Healey and Strothkamp, 1981; Toussaint and Lerch, 1987; Espin et al., 1997). Edible mushroom contains a considerable amount of various phenolic compounds, which are readily oxidized during the purifying process. Upon oxidation and successive polymerization of the phenolic content of the mushroom extract,

macromolecules of melanins are formed. To tackle the melanin formation problem during mushroom tyrosinase purification, solutions have been introduced in more recent works, for example, 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.

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