การศึกษาลักษณะของไทโรซิเนสจากเห็ดฟางในสภาวะที่ถูกตรึงด้วยวิธีเชื่อมไขว้

Characterization of Cross-Linked Enzyme Aggregates (CLEAs) of Tyrosinase from *Volvariella volvacea*

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บทคัดย่อ

เห็ดเป็นแหล่งผลิตไทโรซิเนสที่มีราคาถูกซึ่งเอนไซม์ดังกล่าวสามารถนำมาใช้เป็นตัวเร่งปฏิกิริยาสำหรับการสังเคราะห์ L-3,4-dihydroxyphenylalanine (L-DOPA) ได้ เมื่อนำไทโรซิเนสจากเห็ดฟางมาทำให้เข้มข้นโดยตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต และนำไปตรึงโดยใช้ glutaraldehyde เป็นตัวเชื่อมไขว้ที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 16 ชั่วโมง พบว่าความเข้มข้นของตัวเชื่อมไขว้ที่เหมาะสมเท่ากับร้อยละ 1.5 โดยปริมาตร ไทโรซิเนสตรึงรูปที่เตรียมได้มีสภาวะที่เหมาะสมสำหรับการเร่งปฏิกิริยาที่พีเอช 7.0 อุณหภูมิ 40 องศาเซลเซียส ในขณะที่ไทโรซิเนสในสภาวะที่ไม่ถูกตรึงมีพีเอชและอุณหภูมิที่เหมาะสมเท่ากับ 6.0 และ 30 องศาเซลเซียส ตามลำดับ ค่าคงที่จลนพลศาสตร์ของไทโรซิเนสตรึงรูปเมื่อใช้ไทโรซีนเป็นสารตั้งต้นมีค่าสัมพรรคภาพและอัตราเร็วสูงสุดเท่ากับ 0.261±0.057 มิลลิโมลาร์ และ 0.053±0.0081 มิลลิโมลาร์ต่อนาที ตามลำดับ เมื่อทดสอบการนำไทโรซิเนสตรึงรูปกลับมาใช้ซ้ำพบว่าไทโรซิเนสตรึงรูปยังคงมีกิจกรรมเอนไซม์เหลืออยู่ครึ่งหนึ่งหลังผ่านการใช้งานไป 3 รอบ โดยเวลาที่ใช้ในแต่ละรอบเท่ากับ 4 ชั่วโมง ผลการสังเคราะห์ L-DOPA โดยใช้ไทโรซิเนสตรึงรูปที่มีค่ากิจกรรมจำเพาะเท่ากับ 12.3 หน่วยต่อกรัม ในสภาวะที่เหมาะสมพบว่าสามารถผลิต L-DOPA ได้ 0.0025 มิลลิโมลาร์ ในเวลา 2 ชั่วโมง

คำสำคัญ : เอนไซม์ตรึงรูป, วิธีตรึงด้วยการเชื่อมไขว้, ไทโรซิเนส, เห็ดฟาง

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Abstract

Mushroom is considered as a cheap source of tyrosinase for L-3,4-dihydroxyphenylalanine (L-DOPA) production. Tyrosinase extracted from fresh straw mushroom (*Volvariella volvacea*) was concentrated by ammonium sulfate precipitation and immobilized as cross-linked enzyme aggregates (CLEAs) with the optimal glutaraldehyde concentration of 1.5% (v/v) at 4 ^{o}C for 16 h. The optimum pH and temperature on CLEAs-tyrosinase activity was found at 7.0 and 40 ^{o}C, respectively while the free tyrosinase exhibited pH and temperature optima at 6.0 and 30 ^{o}C, respectively. The apparent K_m and V_{max} values of CLEAs-tyrosinase for L-tyrosine were 0.261±0.057 mM and 0.053±0.0081 mM min^{-1}, respectively. The CLEAs tyrosinase retained 50% activity after three reuses with 4 h of each reaction cycle. The CLEAs tyrosinase with the specific activity of 12.3 units/g could produce 0.0025 mM L-DOPA in 2 h under the optimized conditions.

Keywords: enzyme immobilization, cross linked enzyme aggregates, tyrosinase, *Volvariella volvacea*

Introduction

Tyrosinase (E.C. 1.14.18.1), also known as polyphenol oxidase, is a copper-containing oxidoreductase catalyzing hydroxylation of monophenols (cresolase activity) and oxidation of diphenols (catecholase activity) in the presence of molecular oxygen as an oxidizing agent (Robb, 1984). It is widely distributed in plants, animals and microorganisms, including mushrooms which nowadays have become a popular source of tyrosinase due to its high activity, inexpensive cultivation and commercial availability throughout the year (Seo *et al*., 2003). L-3,4-dihydroxy phenylalanine (L-DOPA) is a precursor of dopamine, an important neural message transmitter, which has been used as a preferred drug for the treatment of Parkinson's disease since 1967 (Nagatsu and Sawada, 2009). The production of L-DOPA by chemical processes involving complicated procedures under harsh conditions offers a low conversion rate and a low enatiomeric excess. Nowadays, the alternative bioconversion of tyrosine to L-DOPA using tyrosinase as a catalyst has attracted a lot of attention because of the attractive enzymatic activity and catalytic stability. Moreover, the productivity and enantioselectivity have been improved. The immobilized enzyme is considered as a powerful tool for reducing the production cost and improving the stability of catalytic process. The immobilization could be performed by many methods, including physical and chemical adsorption, entrapment and cross linking with bifunctional or multifunctional agents (Sheldon and VanPelt, 2013) The enzyme immobilization technique of cross-linked enzyme aggregates (CLEA) prepared by protein precipitation and subsequently protein cross-linking by a bifunctional agent, such as by glutaraldehyde is a rapid, gentle and cost effective method for preparing carrier-free immobilized enzymes (Migneault *et al.,* 2004). The enzyme immobilized by this technique is stable over wide ranges of pH and temperature and tolerance to organic solvents (Sheldon, 2007, 2011). Recently, this immobilized technique has been employed for combining β-galactosidase and glucose isomerase in CLEAs

for fructose syrup production (Araya *et al.,* 2019) In this study, immobilized tyrosinase from *Volvariella volvacea* was prepared in the form of cross-linked enzyme aggregates (CLEAs), aiming to apply for L-DOPA production. The characterizations of CLEAs-tyrosinase and the enzyme kinetic parameters were also investigated.

Methods

Preparation of crude tyrosinase

Fresh mushroom (*Volvariella volvacea*) was purchased from a local market in Nakhon Pathom, Thailand. L-tyrosine, L-ascorbic acid, ammonium sulfate, sodium hydroxide, sodium molybdate, and sodium nitrite were supplied by Sigma. Other chemicals used were of analytical grade. Fresh mushroom (500 g) was washed with tap water and then with ice-cold distilled water. The mushroom was homogenized by blender in 500 ml of cold 50 mM potassium phosphate buffer pH 6.0 and subsequently centrifuged at 10,000xg for 10 min at 4°C. The protein dissolved in supernatant was precipitated by adding ammonium sulfate to reach 90% saturation. After centrifugation, the protein pellets were dissolved in 50 mM potassium phosphate buffer pH 6.0 and used as crude tyrosinase.

CLEAs preparation

4 ml of crude tyrosinase from previous step was magnetically stirred at 4 °C for 10 min before 2 ml of glutaraldehyde at different concentrations was added dropwise. The mixture was subjected to magnetic stirring at 4 °C overnight. After centrifugation, the recovered pellets were washed with 50 mM potassium phosphate buffer pH 6.0, vacuum-dried, and smashed with a mortar and pestle before being stored at 4 °C.

Tyrosinase activity and protein assay

The tyrosinase activity and protein content were determined before and after the preparation.The reaction mixture (1.0 ml) containing 2 mM L-tyrosine as substrate (0.45 ml) and 4 mM L-ascorbic acid in 50 mM potassium phosphate buffer pH 6.0 (0.45 ml) and enzyme solution (0.1 ml) was shaken at 150 rpm. After incubation at 30 °C for 30 min, the L-DOPA concentration was determined at the wavelength of 425 nm by the method of Arnow (Arnow, 1937). One unit of tyrosinase activity was defined as the amount of enzyme which catalyzed the formation of 1 umole of L-DOPA per min. The activity of CLEAs-tyrosinase was determined by adding 10 mg CLEAs to a 2 ml test tube containing 2 mM L-tyrosine (0.45 ml) and 4 mM L-ascorbic acid dissolved in 50 mM sodium phosphate buffer pH 6.0. The reaction was conducted in a shaker (thermomixer, eppendorf) at 30 °C with agitation of 150 rpm. The L-DOPA was assayed as described above and the tyrosinase activity was expressed as units/g CLEAs Protein was determined by the method of Lowry (Lowry *et al*., 1951) with bovine serum albumin as a standard.

Effect of pH and temperature on free and immobilized tyrosinase activity

The activity of free and CLEAs-tyrosinase was evaluated at different pH values in the range between pH 4.0 and 9.0 under assay conditions and the amount of L-DOPA was determined. Buffers used were 50 mM of citrate phosphate buffer (pH 3.0–5.0), potassium phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0), and glycine-NaOH buffer (pH 9.0-10.0). Optimum temperature for free and immobilized tyrosinase activity was determined by incubating the reaction mixture at the temperatures ranging from 20 to 90°C.

Kinetics of CLEAs tyrosinase

The kinetic properties of CLEAs tyrosinase were determined using various concentrations of L-tyrosine as substrates. The reaction was performed at 40 °C by adding CLEAs-tyrosinase with the specific activity of 10 units/mg CLEAs to L-tyrosine dissolved in 50 mM potassium phosphate buffer pH 7.0. For tyrosinase in free form, the reaction was carried out at pH 6.0 and 30 °C with the specific activity of 10 units/mg protein. The concentration of L-tyrosine was varied in the range of 0.06-2.0 mM. The initial reaction rate of L-DOPA synthesis was measured by UV-VIS spectroscopy at 425 nm for 10 min. All experiments were conducted in triplicate. The kinetic constants, Km, Vmax were assessed using Lineweaver-Burk plots.

Reusability tests

About 20 mg of CLEAs were reacted with 20 ml of 2.0 mM L-tyrosine solution containing 4.0 mM of L-ascorbic acid in 50 mM phosphate buffer pH 7.0 at 40 °C for 4 h before a 1 ml of sample was taken for residual tyrosinase activity assay. The reaction mixture was then centrifuged at 6,000xg, the supernatant was decanted, and 20 ml of a fresh L-tyrosine and L-ascorbic acid solution was added for the next-round reaction (4 h each cycle).

Production of L-DOPA by CLEAs tyrosinase

The production of L-DOPA was carried out at 40 °C in 50 mM potassium phosphate buffer pH 7.0. The reaction was started by adding 10 mg of CLEAs tyrosinase (12.3 units/g) in shake flask containing 2.0 mM mM L-tyrosine and 4.0 mM ascorbic acid. At specific time intervals, aliquots were withdrawn and subsequently analyzed L-DOPA concentration by the method described above.

Results

Tyrosinase from fresh mushroom (*Volvariella volvacea*) was precipitated by 90% ammonium sulfate saturation and then cross-linked with different glutaraldehyde concentrations. As shown in Figure 1, the concentration of glutaraldehyde had a significant effect on activity of CLEAs. When the glutaraldehyde concentration increased, higher amount of CLEAs tyrosinase activity was obtained. The optimal concentration of glutaraldehyde was found at 1.5% (v/v) with the tyrosinase activity of 7.2 units/g.

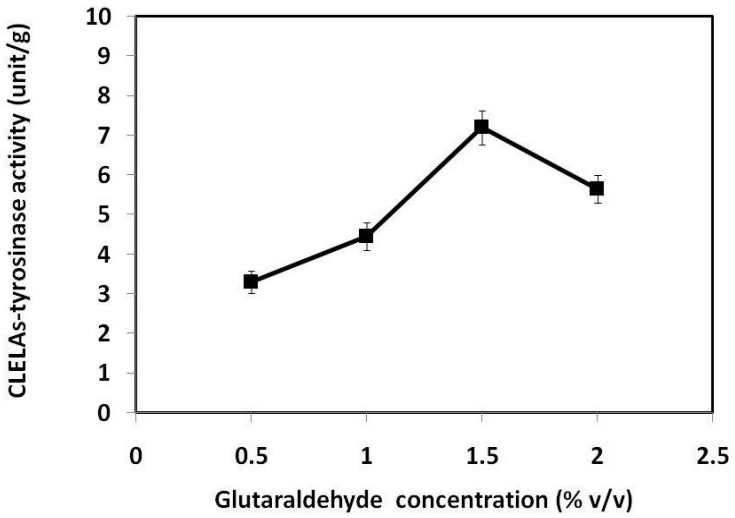

Figure 1 The effect of glutaraldehyde concentration on CLEAs tyrosinase activity. The data represented by the means of triplicate measurements under the same conditions.

The effect of pH and temperature on tyrosinase activity

The tyrosinase activity was determined in the pH range of 4-9 at 30 °C for both free and CLEAs tyrosinase. As shown in Figure 2 (A), the optimal pH values for the oxidation of tyrosine of free and immobilized enzymes were observed at pH 6.0 and 7.0, respectively.

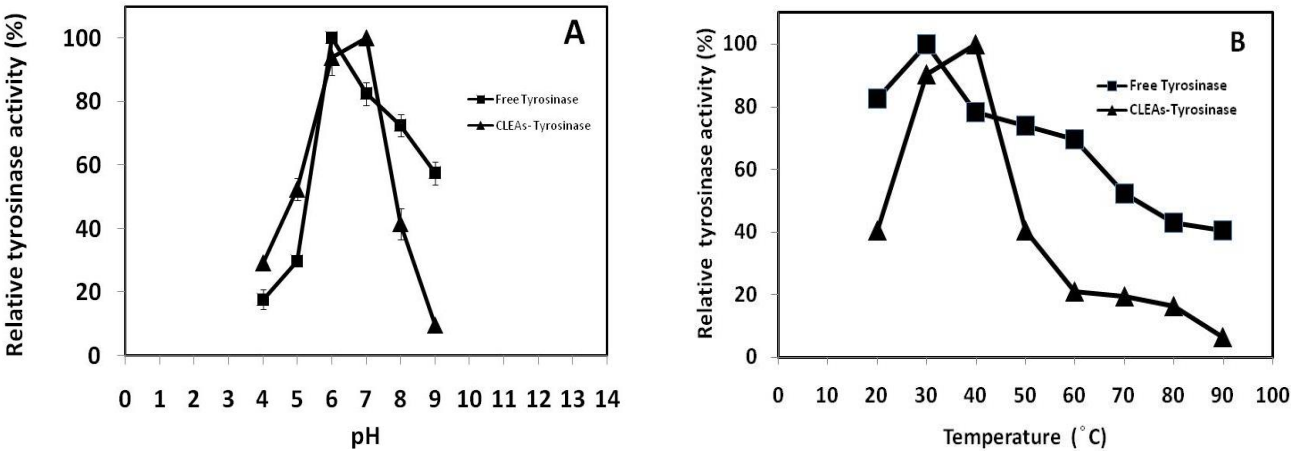

Figure 2 The effect of pH (A) and temperature (B) on free and CLEAs-tyrosinase activity. The data represented by the means of triplicate measurements under the same conditions.

The effect of temperature between 20-90 °C on the activity of free and CLEAs tyrosinase was also determined in 50 mM phosphate buffer pH 6.0 and pH 7.0 for free and immobilized tyrosinase, respectively. As demonstrated in Figure 2B, the maximum enzyme activities of free and cross-linked enzyme were at 30 and 40°C, respectively. The temperature higher than 40 °C decreased the activity of CLEAs tyrosinase rapidly as compared with the free tyrosinase. It was also found that the relative activity of CLEAs was lower than free tyrosinase at the temperature higher than 50 °C.

Kinetics of CLEAs-tyrosinase

The apparent kinetic values of tyrosinase in free and immobilized form were investigated by measuring initial reaction rates of L-DOPA synthesis at various L-tyrosine concentrations (0.06-2.0 mM). As shown in Figure 3, Km and Vmax values of free tyrosinase with L-tyrosine as a substrate calculated from Lineweaver Burk Plot were 0.211±0.0034 mM, 0.054±0.0022 mM min^{-1}, respectively while CLEAs tyrosinase was estimated at 0.261±0.057 mM and 0.053±0.0081 mM min^{-1}, respectively.

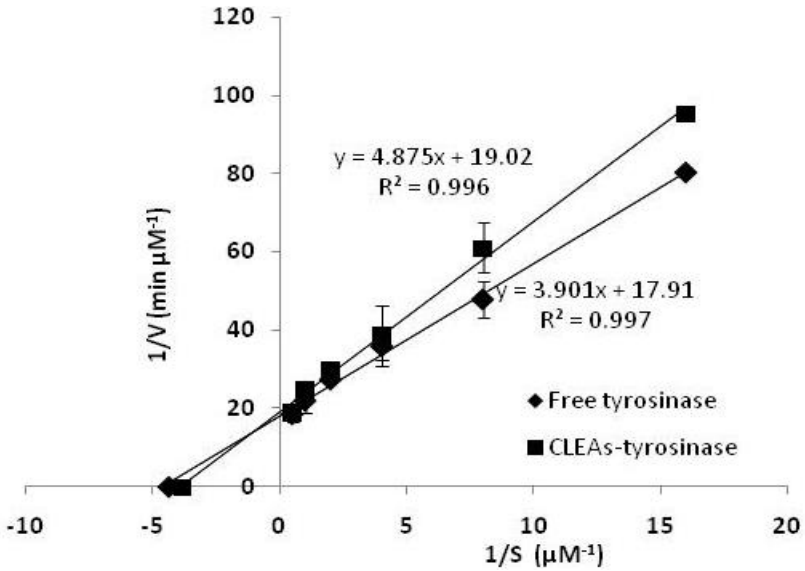

Figure 3 Lineweaver-Burk reciprocal plot of initial reaction rate of L-DOPA formation catalyzed by free and CLEAs-tyrosinase with the different concentrations of L-tyrosine. The data represented by the means of triplicate measurements under the same conditions.

Reusability of CLEAs tyrosinase

Stability of tyrosinase immobilized by cross-linked enzyme aggregation using 1.2% (v/v) glutaraldehyde was investigated in terms of reusability. The stability of CLEAs was studied up to five cycles. After each cycle, CLEAs were separated from the reaction mixture by centrifugation, washed and used again in the next cycle. In Figure 4, 50% of tyrosinase activity was remained in the third catalytic cycle and eventually decreased to 5% in the fifth cycle.

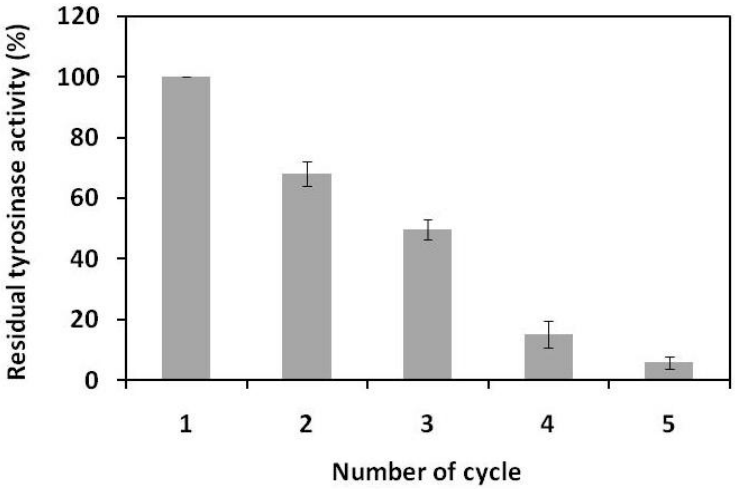

Figure 4 Reuseability of CLEAs-tyrosinase (4 h in each cycle). The experiment was performed at pH 7.0, 40 °C. The data represented by the means of triplicate measurements.

Production of L-DOPA by CLEAs tyrosinase

CLEAs tyrosinase was employed as a biocatalyst for L-DOPA production using L-tyrosine as a substrate. In Figure 5, L-tyrosine was converted to L-DOPA by 10 mg of CLEAs at pH 7.0, 40 °C. The maximum L-DOPA concentration of 0.0025 mM was obtained after 2 h reaction time.

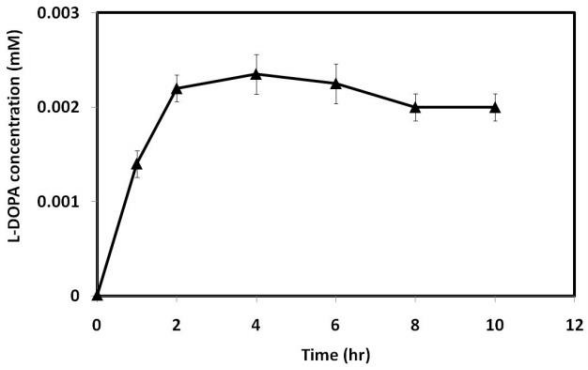

Figure 5 Time course of L-DOPA production catalyzed by CLEAs-tyrosinase at pH 7.0, 40 °C. The data represented by the means of triplicate measurements.

Discussions

Tyrosinase extracted from fresh straw mushroom was immobilized by cross-linked enzyme aggregation method. To prepare CLEAs tyrosinase, enzyme was precipitated by 90% saturation ammonium sulfate and subsequent cross-linked with glutaraldehyde. At glutaraldehyde concentration of 1.5% (v/v), the maximum tyrosinase activity was obtained. As glutaraldehyde concentration increased, the fine-grained and more structured particles with the increasing amount of cavities were obtained, which permitted better substrate transfer (Aytar and Bakir, 2008). At low glutaraldehyde concentration, the proteins could remain unstable and leach in solution. However, the excess of glutaraldehyde caused the formation of protein aggregation with a strong diffusion resistance, leading to lower CLEAs activity. (Dong *et al*., 2010; Yu *et al*., 2013) The optimum pH and temperature of CLEAs-tyrosinase were higher when compared to the free form, consistent with the previous report by Aytar and Bakir (2008). The pH and temperature shift could be resulted from the change in ionization of acid and basic amino acid side chain in the microenvironment around the active site, which was caused by the newly formed interaction between basic residues of the enzyme and glutaraldehyde during cross linking preparation (Cerdobble *et al*., 2010). This shift can be explained by covalent bond formation between proteins caused by glutaraldhyde during CLEAs formation leading to the decrease in the conformational flexibility of the enzyme and its ability to bind to its substrate (Arica *et al*., 2000).

Kinetic parameters of apparent CLEAs tyrosinase were examined by measuring initial reaction rate at various L-tyrosine concentrations. In this work, Km value of CLEAs tyrosinase extracted from *Volvariella volvacea* was 0.261±0.057 mM, which was in the same range of CLEAs tyrosinase (0.280±0.03 mM) prepared by mushroom tyrosinase extracted from *Agaricus bisporus* commercially supplied by Sigma (Aytar and Bakir, 2008). The apparent Km value of CLEAs tyrosinase was higher than free tyrosinase (0.211±0.0034 mM). This could be due to the changes in protein structure during cross-linking process, especially at the binding site, leading to the limitation of substrate accessibility to the catalytic site. (Dong *et al*., 2010; Mahmod *et al*., 2016) In terms of reusability, tyrosinase activity in CLEAs was lost about 50% of its initial activity after the third reaction cycle. This dramatic loss of catalytic activity might occur because the fine particles of CLEAs were easily lost during washing step; moreover, CLEAs were also easy to clump or did not dispersed well in solution (Xu *et al*., 2012).

Conclusions

This work was demonstrated that the concentration of glutaraldehyde had an effect on CLEAs activity. Moreover, after immobilization, optimum pH and temperature were increased. The Km and Vmax values of CLEAs-tyrosinase prepared in this work was not significantly altered as compared with the free tyrosinase. The CLEAs could be successively reused up to three reaction cycles and could catalyze the synthesis of L-DOPA as high as 0.0025 mM in two hours.

Acknowledgements

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