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Preparation of cross-linked tyrosinase aggregates

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Abstract

Tyrosinase from mushroom was immobilized as a cross-linked enzyme aggregate (CLEA) via precipitation with ammonium sulfate and cross-linking with glutaraldehyde. The effects of precipitation and cross-linking on CLEA activity were investigated and the immobilized tyrosinase was characterized. Sixty percent ammonium sulfate saturation and 2% glutaraldehyde were used; a 3-h cross-linking reaction at room temperature, at pH 7.0 was performed; particle sizes of the aggregates were reduced; consequently, 100% activity recovery was achieved in CLEAs with enhanced thermal and storage stabilities. Slight changes in optimum pH and temperature values of the enzyme were recorded after immobilization. Although immobilization did not affect V_{max}, substrate affinity of the enzyme increased. Highly stable CLEAs were also prepared from crude mushroom tyrosinase with 100% activity recovery.

Keywords: Immobilization; Mushroom tyrosinase; Cross-linked enzyme aggregates; Glutaraldehyde; Ammonium sulfate

1. Introduction

Tyrosinase (EC 1.14.18.1) is a monooxygenase which catalyzes *o*-hydroxylation of monophenols (monophenolase activity) and subsequent oxidation of the formed *o*-diphenols into *o*-quinones (diphenolase activity) in the presence of molecular oxygen [1,2]. These *o*-quinones undergo nonenzymatic reactions to form melanin [3]. Tyrosinases have a wide application area including the detection of phenols by biosensor preparation [4], detoxification of phenolic compounds [5], and pharmaceutical drug production such as L-DOPA, which is used in the treatment of Parkinson's disease [6].

Separation from the reaction medium and reuse of tyrosinases can be facilitated by immobilization. This enzyme has been so far immobilized on various types of carriers by different methods, such as by adsorption [7], covalent cross-linking [8], and entrapment [6]. However, association of enzymes with carrying matrices results in diluted enzyme activities. Enzymes can also be immobilized without using an extra carrier. This, for example, applies to the cases of cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). These preparations have concentrated enzyme activity and high stability. Preparation of CLEAs depends on the precipitating of enzymes and subsequently cross-linking of formed aggregates. These immobilized enzymes can be easily recovered from the reaction mixture by centrifugation or filtration [9]. This method is simple and robust and it does not require highly purified enzymes [10]. Because of its latter feature, several enzyme activities can be collected in a single CLEA called combi-CLEA [11]. So far, CLEAs of various enzymes such as penicillin acylases, lipases, laccases, etc. have been prepared [12–19]. However, no study has been reported on CLEAs of mushroom tyrosinase in the literature yet. Thus, in this study, use of crude mushroom tyrosinase for CLEA preparation was studied considering the industrial importance of the enzyme. For this purpose, immobilization conditions were optimized using pure tyrosinase, and the immobilized enzyme was characterized in terms of structure, optimum temperature and pH, kinetic parameters, thermal and storage stabilities. Then, CLEAs were prepared from crude mushroom tyrosinase. Finally, CLEAs, the storage stability of which was examined, were used to determine gallic acid concentration in a synthetic aqueous solution.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase (EC 1.14.18.1), glutaraldehyde (25% solution), catechol, L-lysine, bovine serum albumin, brilliant blue G, gallic acid were

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purchased from SIGMA (St. Louis, MO, USA), and ammonium sulfate was obtained from MERCK (Darmstadt, Germany). Other reagents were of analytical grade and obtained either from SIGMA or MERCK. Crude tyrosinase extract was prepared from mushroom bought from the local supermarket.

2.2. CLEA preparation

Saturated ammonium sulfate solution was added up to the final concentration of 60% saturation in a 0.2 ml enzyme solution containing 1 mg/ml pure tyrosinase, and 50 mg/ml bovine serum albumin (BSA) in 0.1 M pH 7 sodium phosphate buffer. After 5 min of mixing, glutaraldehyde was added slowly to the final concentration of 2% (v/v). At the end of a 3-h cross-linking reaction at room temperature, the suspension was centrifuged at 13,000 × *g* for 15 min at 4 °C. The recovered pellet was stored overnight in 4 ml 0.1 M L-lysine solution (in 0.1 M, pH 7.0 sodium phosphate buffer) to allow reaction with excess glutaraldehyde present [20]. On the next day, CLEAs were recovered and washed via filtration. Finally, the formed CLEAs were finely dispersed in 0.1 M, pH 7.0 sodium phosphate buffer and stored at 4 °C.

To prepare CLEAs from crude enzyme, tyrosinase was extracted from fresh mushroom. For this aim, 100 g of cap tissues of mushroom were homogenized in 125 ml 0.1 M, pH 7.0 sodium phosphate buffer; then the obtained homogenate was centrifuged at 11,000 × *g* for 10 min at 4 °C. Supernatant (2.5 ml) without BSA was used as the crude enzyme extract for the CLEA production. The rest of the procedure was as described above.

2.3. Activity measurements

The activity measurements of free tyrosinases and CLEAs were conducted spectrophotometrically at 35 °C by measuring the initial reaction rate of catechol oxidation with a double-beam UV–vis spectrophotometer (Thermo Electron Cooperation Evolution 100). The reaction was started by the addition of 0.1 M catechol solution (in 0.1 M pH 7.0 sodium phosphate buffer) to the enzyme solution. In the case of CLEA, samples were taken from the reaction mixture and immediately filtered (Schleicher & Schuell Grade 589/1 black ribbon filter paper) into a cuvette, whereas, the reaction of the free enzyme with substrate was conducted directly in the cuvette. Then, the change of absorbance was recorded at 420 nm. Activity assays were performed at least for two times. One unit of the enzyme activity (U) was defined as the amount of the enzyme producing 1 μmol product per minute under the given reaction conditions. The extinction coefficient for *o*-quinone formation from catechol at 420 nm was taken as $\varepsilon_{420} = 3450\ M^{-1}\ cm^{-1}$ [21]. The activity recovery in CLEAs was calculated as given in Eq. (1):

$$\text{acitivity recovery}\ (\%) = \frac{\text{total activity of CLEA (U)}}{\text{total free enzyme activity used for CLEA production (U)}} \times 100 \quad (1)$$

2.4. Effect of temperature and pH on tyrosinase activity

To determine the optimum temperature of the free and immobilized tyrosinases, enzyme activities were measured in the temperature range of 25–35 °C. The effect of pH on enzyme activity was determined in the pH range of 3–8. In the range of 3–6, 0.1 M citrate–phosphate buffer, and in the range of 6–8, 0.1 M sodium phosphate buffer were utilized. The results for optimum temperature and pH were given in relative form with the highest value being 100% activity.

Thermal stability experiments of both the free and immobilized tyrosinases were conducted by incubating the enzyme solutions at 45, 55 and 65 °C at various time intervals. Thermal stability results were given as residual activities, calculated by taking the initial activity of the enzyme as 100%.

2.5. Kinetic analysis

Kinetic parameters of free tyrosinase and CLEA were determined by using different catechol concentrations in the range of 0.05–2.5 mM in 0.1 M pH 7 sodium phosphate buffer at 35 °C. K_m, V_{max} values of free and K_m^{app}, V_{max}^{app} values of immobilized tyrosinases were calculated from nonlinear regression fitting of the initial reaction rate results corresponding to different catechol concentrations by SigmaPlot 10.0 (Systat Software Inc.).

2.6. Scanning electron microscopy (SEM) analysis

CLEA solution was spread over a microscope slide. After drying at room temperature, CLEAs were coated with gold. Scanning electron microscope images were recorded with JSM-6400 Electron Microscope (JEOL), equipped with NORAN System 6 X-ray Microanalysis System & Semafore Digitizer.

2.7. Measurement of gallic acid concentration by means of CLEAs of crude mushroom extract

To determine gallic acid concentration in an aqueous solution using tyrosinase, gallic acid was converted completely to quinone by oxidation with excess tyrosinase. The oxidation reaction was followed spectrophotometrically at 380 nm, which was reported to be the maximum absorption wavelength of quinone, oxidized from gallic acid [22]. This fast enzyme assay was reported to be well correlated with the Folin-ciocalteau assay, a common phenol detection method [22]. A calibration curve was constructed using free tyrosinase (0.37 U/ml) in a gallic acid concentration range of 0.5–5 mM. The same reaction was carried out with CLEA particles (0.37 U/ml) using 2.5 mM of gallic acid.

3. Results and discussion

3.1. Optimization of CLEA preparation

3.1.1. Effect of bovine serum albumin addition as proteic feeder

To analyze the effect of inert protein addition in CLEA production, an enzyme solution was prepared containing 1 mg/ml tyrosinase, and 50 mg/ml bovine serum albumin (BSA) [20]. CLEAs, prepared in the absence of BSA, recovered only 31 ± 0.1% of the free enzyme activity, whereas with the addition of BSA, this recovery increased to 101 ± 8%.

As reported by Shah et al. [16], BSA concentration had to be optimized to increase activity recovery. However, BSA concentration optimization was not performed considering nearly 100% activity recovery in CLEAs obtained using 50 mg/ml BSA.

Addition of BSA as a lysine rich proteic feeder to pure and dilute tyrosinase solution increases protein and consequently, free amino group concentration. Hence, when glutaraldehyde is applied to this concentrated protein solution, insoluble and active CLEA particles can easily be produced due to the presence of sufficient free amino groups, which both allow efficient cross-linking and prevent a possible damage of the enzyme structure by reducing the risk of the extensive cross-linking of the enzyme molecules [16].

3.1.2. Effect of ammonium sulfate concentration

During the CLEA preparation, dissolved tyrosinase was precipitated utilizing ammonium sulfate. Fig. 1 shows that as ammonium sulfate saturation was increased, activity recovery in the CLEAs also increased and 100% recovery was achieved at 60% ammonium sulfate saturation. Precipitation increases the stability of proteins, since it decreases the surface area that