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Activity and stability of cross-linked tyrosinase aggregates in aqueous and nonaqueous media

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ABSTRACT

Cross-linked tyrosinase aggregates were prepared by precipitating the enzyme with ammonium sulfate and subsequent cross-linking with glutaraldehyde. Both activity and stability of these cross-linked enzyme aggregates (CLEAs) in aqueous solution, organic solvents, and ionic liquids have been investigated. Immobilization effectively improved the stability of the enzyme in aqueous solution against various deactivating conditions such as pH, temperature, denaturants, inhibitors, and organic solvents. The stability of the CLEAs in various organic solvents such as *tert*-butanol ($t_{1/2}$ = 326.7 h at 40 °C) was significantly enhanced relative to that in aqueous solution ($t_{1/2}$ = 5.5 h). The effect of thermodynamic water activity (a_w) on the CLEA activity in organic media was examined, demonstrating that the enzyme incorporated into CLEAs required an extensive hydration (with an a_w approaching 1.0) for optimizing its activity. The impact of ionic liquids on the CLEA activity in aqueous solution was also assessed.

1. Introduction

Being more advantageous than the normal carrier-bound strategies, cross-linked enzyme aggregates (CLEAs) have emerged as a novel, versatile, and effective methodology for enzyme immobilization (Sheldon, 2007). This method involves precipitation of the enzyme from aqueous buffer solution followed by cross-linking of the resulting physical aggregates with a bifunctional reagent such as glutaraldehyde, combining purification and immobilization into a single operation to provide stable, recyclable catalysts exhibiting high catalyst productivities. It is applicable to a variety of enzymes such as hydrolases, lyases, and oxidoreductases.

Although CLEAs have found broad applications (Sheldon, 2007), the fundamental research on CLEAs has basically engaged in the optimization of the CLEA preparation and the comparison of the activity and thermostability of CLEAs with soluble enzymes in aqueous solution (Gaur et al., 2006; Cabana et al., 2007; Zhao et al., 2008). Very few authors (Cao et al., 2001; Toral et al., 2007; Sangeetha and Abraham, 2008) have worked on applying CLEAs to nonaqueous environments such as organic solvents and ionic liquids (ILs), a new type of promising solvent for biocatalysis (Yang and Pan, 2005), and the factors affecting the CLEA's catalytic performance in such media have not been assessed yet.

Mushroom tyrosinase was selected as a model enzyme in this study. It is an oxidoreductase catalyzing the hydroxylation of a variety of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones (Robb, 1984), and has found broad applications in waste water treatment (Tikhonov et al., 2010), biosensor design (Nistor et al., 1999), and pharmaceutical production (Pialis and Saville, 1998). So far, only one report has been found regarding the preparation of tyrosinase CLEAs (Aytar and Bakir, 2008). These authors have worked on the characterization of the CLEAs in terms of their K_m and V_{max} measurements, pH and temperature optima, and thermal and storage stability in aqueous solution. The objectives of the study reported herein were: (1) to demonstrate the maintenance of the enzyme's activity and stability of the tyrosinase CLEAs in different media such as aqueous solution, organic solvents, and ionic liquids; (2) to examine the superior stabilizing effect of the CLEAs against various deactivating conditions including pH, temperature, denaturants, inhibitors, and organic solvents; and (3) to investigate the impact of some factors, such as water and water activity, type of organic solvents, and ionic nature of ionic liquids, on the catalytic performance of the CLEAs in nonaqueous environments.

2. Materials and methods

2.1. Materials

Crude enzyme solution was obtained from fresh mushrooms as described in Yang et al. (2009). 3,4-Dihydroxyl-phenylalanine (L-

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DOPA), 4-methylcatechol, and glutaraldehyde (50 wt.% in water) were purchased from Sigma–Aldrich China Inc. Ionic liquids (99%) were purchased from ShangHai Cheng Jie Chemical Co. Ltd. Other reagents used were of analytical grade from local manufacturers in China.

2.2. CLEA preparation

Ammonium sulfate was progressively added to the tyrosinase solution (100 ml, extracted from 50 g fresh mushrooms) to reach 90% saturation, and the mixture was magnetically stirred at 4 °C for 10 min before 2 ml of glutaraldehyde (12.5 wt.%) was added dropwise. The mixture was subjected to magnetic stirring at 4 °C for 16 h. After centrifugation, the recovered pellets were washed with Na phosphate buffer (50 mM, pH 6.0), vacuum-dried, and smashed with a mortar and pestle before being stored at 4 °C. Both enzyme activity and protein content were determined before and after the preparation.

2.3. Activity assays in aqueous solution

The activity of free tyrosinase and its CLEAs in aqueous solution was determined spectrophotometrically by following the oxidation of L-DOPA to dopachrome (Yang and Robb, 1993). A typical reaction for determining the CLEA activity was carried out by addition of 5.0 mg CLEAs to 25 ml Na phosphate buffer (50 mM, pH 6.0) containing 0.8 mM DOPA in a beaker, which was magnetically stirred at 25 °C. For determining CLEA activity in IL-containing aqueous solution, Na phosphate buffer (50 mM, pH 6.0) containing 5% (w/v) of each IL was readjusted to pH 6.0 prior to use, and CLEAs (5.0 mg) were added to a capped test tube containing 9.2 ml of this buffer solution and 0.8 ml of the DOPA solution (10.0 mM) to start the reaction, which was conducted in a shaker at 30 °C with agitation of 250 rpm. All tests were repeated at least twice with standard deviations not greater than 5%.

2.4. Activity assays in chloroform

CLEAs (20 mg) were added to a 50 ml, capped conical flask containing 20 mM 4-methylcatechol in 25 ml of chloroform (water-saturated, or dried over molecular sieves with addition of either a certain amount of water (0–0.8%, v/v) or 2.5 g of different inorganic salt hydrates), and the reaction was conducted in a shaker at 30 °C with agitation of 250 rpm. Sampling and absorbance readings were conducted following Yang and Robb (1994).

2.5. Stability tests

The stabilities of the soluble tyrosinase and its CLEAs in aqueous solution were compared by measuring the residual activity of the enzyme after incubation for a certain period in phosphate buffer (50 mM, pH 6.0) at various pHs (pH 5–8) and temperatures (40, 50, and 60 °C), or in the presence of different deactivating reagents (sodium azide (NaN_3), guanidine hydrochloride (GdHCl), sodium dodecyl sulfate (SDS), ethylene diamine tetraacetic acid (EDTA)) or hydrophilic organic solvents (methanol, dioxane, acetone) with different concentrations. The stability tests in organic solvents were done by incubating CLEAs at 40 °C in methanol, chloroform, hexane, acetone, dioxane, and *tert*-butanol. The residual activity was measured by periodic sampling for activity assay in aqueous solution as described above.

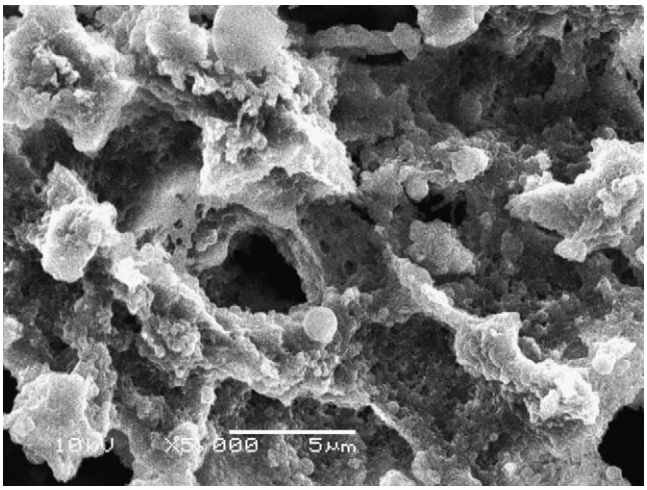

Fig. 1. Scanning electron micrograph of the tyrosinase CLEAs, obtained by using the JSM-5910LV scanning electron microscope (JEOL) in the College of Electronic Science and Technology, Shenzhen University.

3. Results and discussion

3.1. Characterization of the tyrosinase CLEAs

Fig. 1 is the scanning electron micrograph of the tyrosinase CLEAs, which shows an amorphous structure with high surface area and many cavities, thus allowing the substrate molecules to easily access the enzyme for the reaction to proceed. The progress curves of the oxidation reactions catalyzed by different amounts of the tyrosinase CLEAs are shown in Fig. 2. The slopes of the linear progress curves were taken as the initial reaction rates, which were correlated proportionally with the amount of CLEAs applied (see the inset of Fig. 2). CLEA preparation and the subsequent washing steps resulted in a marginal loss in enzyme activity (1.8%) but a considerable reduction in the total protein content (35.0%). This supports the notion that the CLEA methodology is advantageous in combining purification and immobilization into a single unit operation (Sheldon, 2007). The optimal pH for the CLEAs was the same as that for the free enzyme (pH 6.0). While the optimal temperature for the soluble enzyme was 55 °C, CLEAs presented maximal activity within the temperature range of 30–40 °C.

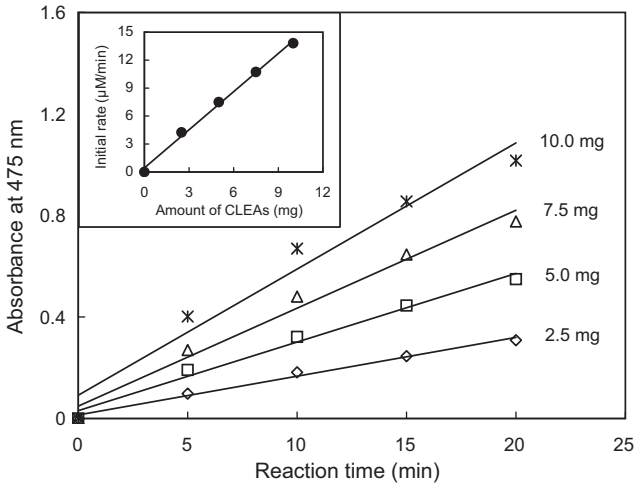

Fig. 2. Progress curves of the oxidation reactions catalyzed by different amounts of the tyrosinase CLEAs. The inset shows the linear relationship between the activity of the CLEAs and the enzyme amount.