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Use of cross-linked tyrosinase aggregates as catalyst for synthesis of L-DOPA

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ABSTRACT

Mushroom tyrosinase immobilized as cross-linked enzyme aggregates (CLEAs) was used as the catalyst for production of L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine. The synthetic reaction catalyzed by this immobilized enzyme was investigated in different processes. In the batch process, a conversion of 53.0% was obtained during 2 h with a productivity of 209.0 mg l^{-1} h^{-1}, much superior to other batch processes catalyzed by the same enzyme immobilized with traditional carrier-bound immobilization methods. The effects of pH, temperature, and L-ascorbic acid (as the reducing agent) on the L-DOPA production were examined. Reactions can be tracked by determining the L-DOPA concentration with the spectrophotometric and HPLC methods, both giving consistent results as long as the reducing agent is in sufficient supply. In the continuous synthetic processes carried out in a continuous stirred-tank reactor and a packed bed reactor, a productivity of 103.0 and 48.9 mg l^{-1} h^{-1} was obtained, respectively. The operational stability of the tyrosinase CLEAs can be dramatically improved by entrapment into calcium alginate gels. The CLEA/alginate beads in the continuous stirred-tank reactor achieved a long life time of >104 h, producing L-DOPA with a productivity of 57.4 mg l^{-1} h^{-1}.

1. Introduction

L-3,4-Dihydroxyphenylalanine (L-DOPA) is a drug of choice for the treatment of Parkinson's disease, a degenerative brain disorder caused by deficiency of the neurotransmitter dopamine. As a precursor of dopamine, L-DOPA can pass across the blood–brain barrier (while dopamine itself cannot) to increase the dopamine level in the brain [1].

The world market for L-DOPA is a few hundred tons per year [2], but most of this is produced by chemical processes [3]. These chemical approaches involve complicated procedures, require costly chemicals and metal catalysts working under harsh conditions, and offer a low conversion rate and a low enantiomeric excess [4,5]. In order to economize the production cost and improve the productivity and enantioselectivity, recent research has centered on exploiting alternative biotechnological production methods for this drug. Processes which have shown promising results include production from L-tyrosine by intact microorganisms such as fungi [6], yeasts [7], and bacteria [8], and by isolated enzymes such as tyrosinase [9–16]. While the microbiological production of L-DOPA is time-consuming (generally over 10 days including cell culture period) and expensive as well (due to the purification processes required to remove proteins and hormones which are also produced by the cells) [17], the enzymatic biotransformation of L-tyrosine to L-DOPA takes a shorter time (within hours) and yields an exit stream containing only the desired product (which is enantiomerically pure, with a relatively higher conversion) and some unreacted L-tyrosine and reducing agent.

Tyrosinase (EC 1.14.18.1) is a copper-containing oxidoreductase catalyzing two sequential reactions: the hydroxylation of monophenols to *o*-diphenols (cresolase activity), and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity), both requiring molecular oxygen as the oxidizing agent [18]. *o*-Quinones are highly reactive compounds that will further polymerize non-enzymatically to form high-molecular weight brown pigments called melanin. In the presence of a reducing agent such as ascorbic acid, the quinone product can be recycled back to the catechol form, leaving *o*-diphenol as the sole product [19]. This well known fact has stimulated researchers to employ the enzyme as a catalyst for the production of L-DOPA from L-tyrosine (Scheme 1).

A successful production is contingent upon the enzyme's activity retention and operational stability, which can be assured by immobilization. Trials of immobilizing tyrosinase with traditional methods for the purpose of synthesizing L-DOPA have been reported, including entrapment in nylon membrane [10] and in alginate, polyacrylamide and gelatin gels [12,14], and covalent attachment to cellulose [9], chitosan [13], zeolite [11], and magnetic beads [15].

The major objective of this study was to assess the feasibility of utilizing tyrosinase immobilized in the form of cross-linked enzyme aggregates (CLEAs) as the catalyst for synthesis of L-DOPA. As a new type of immobilization method more advantageous than

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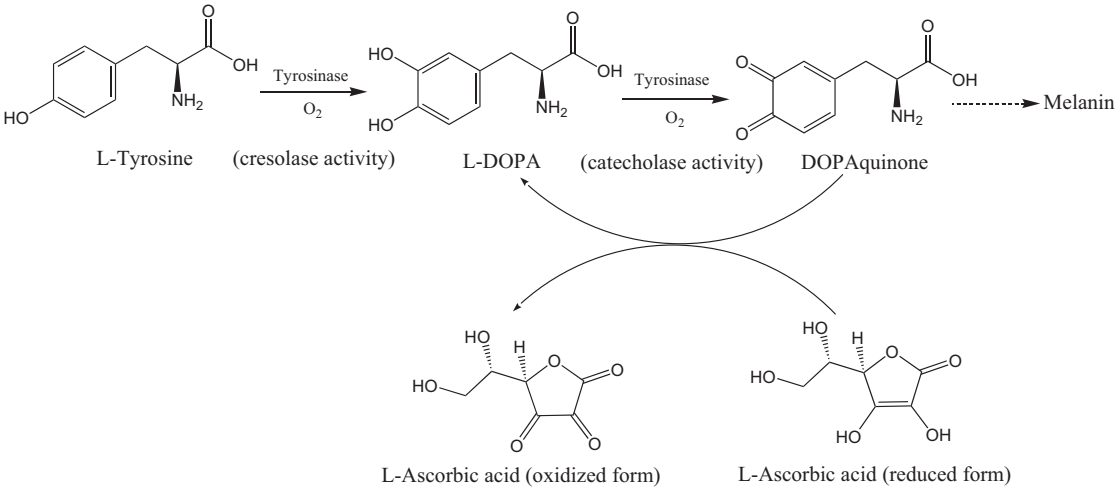

Scheme 1. Reaction scheme for tyrosinase-catalyzed production of L-DOPA from L-tyrosine.

the normal carrier-bound strategies, CLEA preparation consists of protein precipitation followed by cross-linking with glutaraldehyde, combining purification and immobilization into a single operation to provide highly stable and recyclable catalysts with remarkable catalytic efficiency. Due to these attractive features, this cheap and carrier-free immobilization method has now been successfully applied to a variety of enzymes with widespread applications [20]. Our recent study has revealed that immobilization of tyrosinase via CLEA formation can effectively improve the stability of the enzyme in aqueous solution against various deactivating conditions such as pH, temperature, denaturants, inhibitors, and organic solvents [21]. This current study is the first one to report the use of tyrosinase CLEAs as an efficient catalyst for L-DOPA synthesis, and the reactions catalyzed by this new type of immobilized enzyme have been investigated in different reactors, such as a batch reactor, a continuous stirred-tank reactor, and a packed bed reactor.

2. Materials and methods

2.1. Materials

Fresh mushrooms were obtained from a local supermarket in Shenzhen, China. L-DOPA and glutaraldehyde (50 wt.% in water) were purchased from Sigma–Aldrich China Inc. TLC plates (coated with silica gel) was obtained from Qingdao Haiyang Chemical Co., Ltd. All other reagents used were of analytical grade from local manufacturers in China.

2.2. Preparation of tyrosinase CLEAs

The enzyme solution was obtained by extraction of fresh mushrooms (50 g) into 100 ml sodium phosphate buffer (50 mM, pH 6.0), and the tyrosinase CLEAs were prepared by precipitating the enzyme with ammonium sulfate and subsequent cross-linking with glutaraldehyde, following the procedures described in [21]. Precipitation with ammonium sulfate can result in a partial purification to the enzyme without perturbation of its native structure. The subsequent cross-linking allows the formation of permanently insoluble enzyme aggregates while maintaining their protein structure, and hence, their catalytic activity [20].

2.3. Preparation of CLEA/alginate beads

Pulverized CLEA powders (600 mg) were added to 24 ml of 2.5% (w/v) sodium alginate solution. After vortex mixing, the mixture was dropped into 50 ml of 2.0% (w/v) $CaCl_2$ solution. The resulting spherical beads were allowed to stand in a refreshed $CaCl_2$ solution for 2 h for hardening, washed twice with distilled water, and then stored in a capped bottle with water at 4 °C. 1 g of the beads contained approximately 43 mg CLEA powders.

2.4. Study on reactions of tyrosinase CLEA-catalyzed L-DOPA synthesis

Unless otherwise stated, a typical reaction was started by addition of 10 mg of CLEAs into 10 ml of phosphate buffer (50 mM, pH 7.0) containing 2.0 mM L-tyrosine and 4.0 mM L-ascorbic acid in a 50 ml conical flask, which was placed in a shaker with an agitation of 250 rpm at 30 °C. Periodically, a 1 ml sample was taken and centrifuged, and the supernatant was subjected to spectrophotometric or HPLC assays as described below.

2.5. Spectrophotometric assay for L-DOPA analysis

The L-DOPA produced in the reaction mixture was determined by following a modified spectrophotometric method originally developed by Arnow [22]. To 1.0 ml sample removed from the reaction mixture, an equal volume of 1.0 M HCl was added to stop the reaction, followed by addition of 1.0 ml of 2.0 M NaOH to neutralize the solution. Then 1.0 ml of a solution containing 15% (w/v) each of $NaNO_2$ and Na_2MoO_4 was added: the nitrite reacted with L-DOPA to yield a yellow solution for spectrophotometric detection; while the molybdate was to prevent decomposition of the sample [11]. After vortex mixing for 1 min and subsequent standing for 10 min, the absorbance at 425 nm was recorded. The L-DOPA was quantified from the linear standard curve obtained (regression coefficient 0.9998).

2.6. HPLC analysis

The sample removed from the reaction system was subjected to HPLC analysis with an Agilent 1100 HPLC equipped with Agilent's C18 reverse column (Zorbax ODS, 4.6 mm ID × 250 mm, 5 μm). A solvent mixture of $H_2O/CH_3OH/H_3PO_4$ (979.5:19.5:1 by volume, pH 2.0) was employed as the mobile phase with a flow rate of 1.0 ml/min, and the absorbance at 282 nm was followed within 25 min. Linear standard curves for L-DOPA and L-tyrosine were obtained, with a regression coefficient of 0.9984 and 0.9998, respectively.