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Characterisation of tyrosinase immobilised onto spacer-arm attached glycidyl methacrylate-based reactive microbeads

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Abstract

Immobilisation of tyrosinase onto modified poly(methyl methacrylate–glycidyl methacrylate–divinyl benzene), poly(MMA–GMA–DVB), microbeads was studied. The epoxy group containing poly(MMA–MMA–DVB) microbeads were prepared by suspension polymerisation. The epoxy groups of the poly(MMA–GMA–DVB) microbeads was converted into amino groups with either ammonia or 1,6-diaminohexane (i.e., spacer-arm). Tyrosinase was then covalently immobilised on aminated and the spacer-arm-attached poly(MMA–GMA–DVB) microbeads using glutaric dialdehyde as a coupling agent. Incorporation of the spacer-arm resulted an increase in the apparent activity of the immobilised tyrosinase with respect to the enzyme immobilised on the aminated microbeads. The activity yield of the immobilised tyrosinase on the spacer-arm-attached poly(MMA–GMA–DVB) microbeads was 68%, and this was 51% for the enzyme, which was immobilised on the aminated microbeads. Both immobilised tyrosinase preparation has resistance to temperature inactivation as compared to that of the free form. The temperature profiles were broader for both immobilised preparations than that of the free enzyme. Kinetic parameters were determined for immobilised tyrosinase preparations as well as for the free enzyme. The values of the Michaels constants (K_m) for all the immobilised tyrosinase preparations were significantly larger, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} values were smaller for the both immobilised tyrosinase preparations. In a 40 h continuous operation with spacer-arm-attached poly(MMA–GMA–DVB) microbeads at 30 °C, only 3% of immobilised tyrosinase activity was lost. The operational inactivation rate constant (k_{opi}) of the immobilised tyrosinase was 1.25×10^{-5} min^{-1}.
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1. Introduction

The enzyme tyrosinase (E.C.1.14.18.1; monophenol monoxygenase) is a copper-dependent enzyme and widely distributed throughout the phylogenetic scale from bacteria to mammals [1,2]. Tyrosinase catalyses two different oxygen-dependent reaction via separate copper-dependent active sites: the *o*-hydroxylation of monophenols to yields *o*diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity) [3–5]. The enzyme has been proposed for synthesis of 3,4-dihydroxyphenylalanine (L-DOPA) [6], dephenolization of industrial wastewater [7–10], as a part of an enzyme electrode for the determination of phenol and its derivatives [11,12], bioremediation of contaminated soils [13,14], and fruit juice clarification [15].

Mushroom tyrosinase was covalently immobilised on zeolite [6], entrapped in alginate, polyacrylamide and gelatine [16], and utilised for the production of L-DOPA, which is a commonly prescribed drug for the treatment of Parkinson's disease. This disease is caused by the deficiency of a neurotransmitter dopamine and L-DOPA is a precursor of dopamine. L-DOPA has been currently produced by chemical methods [17]. Recent researches has focused on microbial production from *Erwinia herbicola* and *Escherichia coli* and enzymic production from tyrosinase hydroxylase and tyrosinase. The cresolase activity of tyrosinase catalyses the synthesis of L-DOPA from L-tyrosine in the presence of molecular oxygen. The subsequent catecholase activity can be suppressed by L-ascorbate [6,17]. Another major

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application area of the immobilised tyrosinase is removal of phenol and its derivatives from wastewaters. Atlow et al. proposed the application of mushroom tyrosinase to the treatment of phenolic wastewaters [18]. In the successive reports, tyrosinase was immobilised on polymeric supports and used in enzyme reactor to transform several phenols and its derivatives in the treated wastewaters to *o*-quinones and low molecular weight polymers [19–22].

The availability of large number of support materials and methods of enzyme immobilisation leave virtually no bioactive species without a feasible route of immobilisation. It is, thus, important that the choice of support material and immobilisation method over the free bioactive agent should be well justified [23–26]. Acrylic copolymers are especially versatile as a family of carrier materials for enzyme immobilisation that can be prepared with a wide variety of properties. Among these epoxy group carrying acrylic copolymer exhibited some significant advantages as a potential carrier matrix, i.e., easy and stable covalent linkages with different groups such as amino, thiol, and phenolic ones under mild experimental conditions [27–30].

In this study, acrylic copolymer supports were synthesised in the bead form from the monomers methyl methacrylate (MMA), glycidyl methacrylate (GMA) and divinyl benzene (DVB). The epoxy groups of poly(MMA–GMA–DVB) microbeads were modified into amino group using ammonia or/and 1,6-diaminohexane (i.e., spacer-arm). Tyrosinase was then immobilised onto aminated and spacer-arm attached poly(MMA–GMA–DVB) microbead using glutaric dialdehyde as coupling agent. The Michaelis–Menten kinetics constants (K_m and V_{max}), optimum pH and temperature for the free and immobilised enzymes were investigated. Thermal deactivation of the free and immobilised enzymes at various temperatures was studied and the half-lives of the immobilised tyrosinase under these operation conditions were calculated. Finally, the immobilised enzyme system was applied to a packed-bed reactor to study the behaviour of the immobilised enzyme in a continuous system.

2. Experimental

2.1. Materials

Tyrosinase [EC 1.14.18.1; polyphenol oxidase; monophenol monooxygenase, from mushroom, 2000 U mg^{-1} solid], L-tyrosine, bovine serum albumin (BSA), 1,6-diaminohexane, glutaric dialdehyde, DVB and maleic anhydride were all obtained from the Sigma Chemical Company (St. Louis, USA). MMA, glycidyl methacrylate (methacrylic acid 2,3-epoxypropyl isopropyl ether; GMA), and α,α'-azoisobisbutyronitrile (AIBN) were obtained from Fluka Chemie, AG (Buchs, Switzerland) and the monomers distilled under reduced pressure before use. All the other analytical grade chemicals were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of poly(methyl methacrylate-co-glycidylmethacrylate) microbeads

Poly(methyl methacrylate-co-glycidyl methacrylate) microbeads were prepared via suspension polymerisation. The aqueous continuous phase with suspension stabiliser was obtained by the following method. Styrene–maleic anhydride (0.74 g) alternating copolymer, NaOH (0.30 g), Na_2SO_4 (4.0 g) and distilled water (300 ml) were transferred in to a three-necked reactor (1.0 l) and stirred at 70 °C for 1 h until a clear solution was obtained. The reaction product (i.e., sodium salt of styrene–maleic anhydride copolymer) was cooled to room temperature and used as suspension stabiliser. The reactor was then equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The organic phase contained MMA (26.5 ml; 0.25 mol), glycidyl methacrylate (26.4 ml; 0.2 mol) and DVB (7.1 ml; 0.05 mol; cross-linker) were mixed together with 1.0 g of AIBN in 60 ml of toluene. The reactor was placed in a water bath, heated to 65 °C and stirred at 375 ± 25 rpm under a nitrogen atmosphere. The polymerisation mixture was placed into a dropping funnel and was introduced drop wise into the reactor in about 30 min. The polymerisation reaction was maintained at 65 °C for 6 h. After the reaction, the beads were separated by decanting simply and washed with distilled water and methanol. The product was dried under vacuum for 24 h at room temperature. The total polymerisation yield was 58.6. The beads were sieved and 105–210 μm size of fraction (28.7 g) was used in further reactions.

2.3. Immobilisation of tyrosinase onto poly(MMA–GMA–DVB) microbeads

The epoxy groups carrying poly(MMA–GMA–DVB) microbeads were aminated with 0.5 M ammonia or 1,6-diaminohexane solution (i.e., spacer-arm) at 65 °C in a reactor containing 25 g microbeads and stirred magnetically for 5 h. After the reaction, the aminated and/or spacer-arm attached poly(MMA–GMA–DVB) microbeads were washed with distilled water.

The aminated and/or spacer-arm attached poly(MMA–GMA–DVB) microbead (5 g) were equilibrated in phosphate buffer (10 ml, 50 mM, pH 7.0) for 18 h, and transferred to the same fresh medium containing glutaric dialdehyde (20 ml, 0.5%, v/v). The activation reaction was carried out at 25 °C for 12 h, while continuously stirring the medium. After the reaction period, the excess glutaric dialdehyde was removed by washing sequentially the microbeads with distilled water, acetic acid solution (100 mM, 100 ml) and phosphate buffer (100 mM, pH 7.0). The resulting modified poly(MMA–GMA–DVB) microbeads were dried in a vacuum oven at 40 °C.

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