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Simultaneous purification and immobilization of mushroom tyrosinase on an immunoaffinity support

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Abstract

The use of immobilized and stable enzymes has immense potential in the enzymic analysis of clinical, industrial and environmental samples. But their widespread use is limited due to the high cost of their production. In the present study, an effort has been made to immobilize tyrosinase directly from ammonium sulphate precipitated proteins of the mushroom (*Agaricus bisporus*) on a polyclonal antibody bound to Seralose 4B support. Polyclonal antibodies were raised in male albino rabbits by injecting commercially available mushroom tyrosinase in the presence of Freund's adjuvants. Antibodies were purified from antisera by ammonium sulphate fractionation followed by DEAE-cellulose chromatography. Two distinct bands of light and heavy chains of purified IgG, appeared on SDS-PAGE. The homogeneity of the purified IgG was further confirmed by Ouchterlony double immunodiffusion. One milliliter of cyanogen bromide-activated Seralose 4B bound 9.0 mg purified IgG and retained nearly 573 tyrosinase units. Immunoaffinity bound tyrosinase was more stable against heat and pH inactivation compared to the soluble enzyme. Immobilized enzyme exhibited no change in temperature optima between 30–35 °C whereas soluble tyrosinase has temperature optima of 35 °C. Immunoaffinity bound tyrosinase retained greater fraction of activity on both sides of temperature-optima compared to soluble enzyme. However, the broadening in pH-optima was observed from pH 5.5 to 6.0 for immobilized enzyme. Moreover, immobilized tyrosinase preparation exhibited remarkably high resistance against denaturation induced by urea and water-miscible organic solvents.

Keywords: Tyrosinase; Immobilization; Stabilization; Organic solvents; Polyclonal antibodies; Immunoaffinity support; Seralose 4B

1. Introduction

Enzymic biosensors are useful tools in satisfying analytical requirements, particularly those of specificity for biochemistry, pharmacology, industrial and environmental science [1,2]. Enzyme immobilization on sensing electrode surfaces is one of the most important points to be considered in biosensor design. The selected procedure of immobilizing enzyme should be able to stabilize the macromolecules and allow easier diffusion of substrates and products to ensure an efficient electron transfer. However, among immobilization methods being employed, very few can control the spatial distribution of catalyst. Stabilization of enzymes against various forms of inactivation has been accomplished using a multitude of immobilization strategies including covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation and bioaffinity etc. [3–5]. Among the immobilization procedures entrapment is most suitable for high yield immobilization of enzymes but sometimes the leaching of the enzyme out of the gel beads take place [6–8]. Another disadvantage of immobilization of polyphenol oxidase inside the polymeric network is that the enzyme catalyzes the conversion of simple aromatic compounds into complex soluble oligomers and insoluble polymers, which cannot move out of the beads easily due to their high molecular mass, accumulating the products inside the beads and inhibiting further entry of the substrate. It slows down the enzyme catalyzed analysis or detoxification/decolorization of toxic pollutants [9]. In view of the difficulty faced by the

Abbreviations: DMF, dimethyl formamide; DMSO, dimethyl sulphoxide; DEAE, diethyl aminoethyl; SDS, sodium dodecyl sulphate

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entrapment method for the immobilization of polyphenoloxidases, surface based methods of enzyme immobilization are preferred. Bioaffinity based methods have several advantages over other methods of surface based immobilization. These procedures in view of their reversibility, lack of chemical modification and the usually accompanying stability enhancement, are emerging as powerful strategies for immobilization of various enzymes [10–13]. It gives oriented immobilization to enzymes that facilitates good expression of activity and possibility of direct enzyme immobilization from partially purified preparation or even crude homogenate [12].

In the present study, an attempt has been made to immobilize tyrosinase directly from the crude acetone powder extract of mushroom on IgG-Seralose 4B. Immunoaffinity bound preparation exhibited high yield of immobilization. IgG-Seralose 4B bound tyrosinase showed enhancement in stability against temperature, pH, urea, and water-miscible organic solvents like DMF, DMSO and propanol.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase (approximately, 3000 EU/mg), protein markers and cyanogen bromide were purchased from Sigma (St. Louis, MO, USA). DEAE-cellulose, Seralose 4B, chemicals and reagents for electrophoresis and immunodiffusion were obtained from SRL Chemicals, Bombay, India. All other chemicals and reagents were of analytical grade. Mushroom was obtained from the local market.

2.2. Immunization

Commercially available highly purified mushroom tyrosinase was injected into healthy male albino rabbits weighing 2–3 kg for the production of polyclonal antityrosinase antibodies. The animals received subcutaneously 300 μg of tyrosinase dissolved in 0.5 ml of 20 mM sodium phosphate buffer, pH 7.2, mixed and emulsified with equal volume of Freund's complete adjuvant as first dose [14]. Boosters doses of 150 μg of tyrosinase mixed and emulsified with Freund's incomplete adjuvant were administered weekly after resting the animal for 15 days. After each booster dose blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 h. Serum was collected by centrifugation at 1600 × *g* for 20 min at 4 °C. It was then decomplimented by incubating at 56 °C for 30 min. After adding sodium azide (0.2%) serum was stored at −20 °C.

2.3. Purification and characterization of polyclonal antibodies

The antiserum was fractionated with 20−40% ammonium sulphate. The sample was kept overnight constant stirring at 4 °C to precipitate out proteins. The precipitated proteins were collected by centrifugation at 1600 × *g* for 20 min at 4 °C. The pellet obtained was redissolved in minimum volume of 20 mM sodium phosphate buffer, pH 7.2 and was subjected to extensive dialysis against the same buffer to remove traces of ammonium sulphate.

Antibodies against mushroom tyrosinase were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate precipitated antiserum was passed through DEAE-cellulose column (1.20 cm × 10.0 cm) and the fractions containing purified antityrosinase antibodies were pooled for further use [15].

Sodium dodecyl sulphate-polyacrylamide gel (12.5%) was run to separate proteins present in antiserum, ammonium sulphate fractionated dialyzed sample and DEAE-cellulose purified antityrosinase antibodies according to the procedure described by Laemmli [16]. The staining and de-staining was also performed by the same procedure. Low molecular weight marker proteins (ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa; and lysozyme, 14.3 kDa) were also run in one lane of the lanes to compare the molecular weight of the purified IgG.

2.4. Immunodiffusion

Ouchterlony double immunodiffusion was used to confirm the presence of antibodies against mushroom tyrosinase. Immunodiffusion was performed in 1.0% agarose prepared in normal saline [17]. The cross-reactivity of antibodies was also checked against the potato tyrosinase. The purified tyrosinase antibodies were employed for preparing immunoaffinity support.

2.5. Preparation of immunoaffinity support for the immobilization of mushroom tyrosinase

Seralose 4B was activated by the procedure described by Porath et al. [18].Five grams of Seralose 4B was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 ml of 1.0 M Na_2CO_3 and stirred slowly by placing on a magnetic stirrer at 4 °C for 30 min. One gram of CNBr dissolved in 1.0 ml of acetonitrile was added to the beaker containing Seralose 4B and was again stirred for 10 min in cold. The whole mass was then transferred immediately to a sintered funnel and washed thoroughly with sufficient volume of 0.1 M bicarbonate buffer pH 8.5, distilled water, and again with same buffer. After washing the activated Seralose 4B was dried and resuspended in 5.0 ml of 0.1 M bicarbonate buffer, pH 8.5. One hundred milligrams of purified antibodies were mixed with 5.0 g of CNBr activated Seralose 4B and stirred overnight in cold. The Seralose matrix with bound antibodies was then centrifuged to remove the unbound antibodies. Antibody bound matrix was extensively washed with 0.1 M bicarbonate buffer, pH 8.5 containing 1.0 M NaCl. This washed suspension was treated with 7.0 ml of