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A new metal-chelated beads for reversible use in uricase adsorption

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Abstract

Poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) [poly(EGDMA-VIM)] beads (average diameter 150–200 μm) was prepared by copolymerizing ethylene glycol dimethacrylate (EGDMA) with *n*-vinyl imidazole (VIM). Average pore size of poly(EGDMA-VIM) beads was 550 nm. The copolymer beads composition was characterized by elemental analysis and found to contain five EGDMA monomer units each VIM monomer unit. Poly(EGDMA-VIM) beads had a specific surface area of 59.8 m^2/g. Poly-(EGDMA-VIM) beads were characterized by swelling studies and SEM. Cu^{2+} ions were chelated on the poly(EGDMA-VIM) beads, then these beads were used in the adsorption of uricase from Porcine Liver in batch system. The maximum uricase adsorption capacity of the poly(EGDMA-VIM)-Cu^{2+} beads was observed as 118.3 mg/g at pH 6.0. The K_m values for immobilized uricase (poly(EGDMA-VIM)-Cu^{2+}) (91.95×10^{-3} mM) was higher than that of free enzyme (7.5×10^{-3} mM). V_{max} was calculated as 0.012 μmol/min mg protein for the free enzyme. For the immobilized enzyme, V_{max} was calculated as 1.44 μmol/min mg protein. Free enzyme lose all of original activity in 35 days. On the other hand immobilized enzyme preserved 80% of original activity in same time. Storage stability was found to increase with immobilization. It was observed that enzyme could be repeatedly adsorbed and desorbed without significant loss in adsorption capacity or enzyme activity.

Keywords: Uricase; Enzyme immobilization; IMAC

1. Introduction

The advantages of the use of immobilized enzymes are many, and some of them have a special relevance in the area of food technology [1–3]. In this industrial area the control of the expenses must be very strict because of the low added value of products [4]. Different procedures have been developed for enzyme immobilization [5–11]. These include adsorption to insoluble materials, entrapment in polymeric matrix, encapsulation, crosslinking with a bifunctional reagent, or covalent linking to an insoluble carrier. Among these, adsorption to a solid support material is the most general, easiest to perform and oldest protocol of physical immobilization methods. The most important advantages of this method are the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method [12–14]. Reversible enzyme immobilization is a very powerful tool that may be considered to solve this cost problem. Reversible immobilization could provide the possibility of using such enzymes in an immobilized form and, in this way, having the advantages of the use of immobilized enzymes, saving time and cost [15,16]. The support matrix for binding of chelator ligand should consist of a molecular network that is permeable to proteins, therefore this method provide one step purification of proteins [17,18]. Among reversible methodologies, metal-chelate immobilization seems to be the simplest way to immobilized proteins [15]. However, scarce work is found referring to the reversible metal-chelate immobilization. Because of the easily polarised nature of their d-electron shells due to orbital valancies, 1st row transition metal ions such as Cu^{2+}, Zn^{2+} and Ni^{2+} function as soft or borderline Lewis acids according to the Lewis acid–Lewis base concepts of Pearson [19].

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide, and hydrogen peroxide. The equation of

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the enzymatic reaction is as follows;

$$\text{Uric acid} + O_2 + H_2O \rightarrow \text{Allantoin} + H_2O_2 + CO_2$$

Uricase is an enzyme participating in the final step of purine degradation. Uric acid represents the major catabolite of purine breakdown in humans and for this reason remains an important marker molecule for disorders associated with purine metabolism, most notably gout, hyperuric aemia and the Lesch–Nyhan syndrome [20]. Determining the urate concentration in blood and urine is very important for these reasons [21]. Uricase is useful for enzymatic determination of urate in clinical analysis by coupling with 4-aminoantipyrineperoxidase system [22]. As a copper protein, this enzyme is a tetramer composed of two types of different subunits with a final molecular weight in the range 145–150 kDa [23–26]. The subunit size, as calculated from the cDNA sequence, is 35 kDa [26,27]. Uricase can be also used as a protein drug to reduce the toxic urate accumulation [28–30].

In this study, uricase was immobilized onto a new support via adsorption. For this purpose, poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) [poly(EGDMA-VIM)] beads was prepared by copolymerizing ethylene glycol dimethacrylate with *n*-vinyl imidazole. Cu^{2+}-poly(EGDMA-VIM) chelate matrix was prepared by adding poly(EGDMA-VIM) beads to the aqueous solution of metal ion. Cu^{2+} ions coordinate to the vinyl imidazole chelating-ligand and the enzyme binds the polymer via the chelated metal ion. This approach for the preparation of enzyme carrier has several advantages over conventional immobilization methods. An expensive, time consuming and critical step in the preparation of immobilized metal-affinity carrier is coupling of a chelating ligand to the adsorption matrix. In this procedure, comonomer VIM acted as the metal-chelating ligand, and it is possible to load metal ions directly on the beads without further modification steps. In the present work, the protein adsorption capacity, coupling efficiency and enzymatic activity, reuse and storage stability of immobilized uricase were analyzed.

2. Experimental

2.1. Materials

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) obtained from Sigma was used in this study. Ethylene glycol dimethacrylate (EGDMA) was obtained from Merck (Darmstadt, Germany), purified by passing through active alumina and stored at 4 °C until use. *N*-vinyl imidazole (VIM, Aldrich, Steinheim, Germany) was distilled under vacuum (74–76 °C, 10 mmHg). 2,2′-Azobisisobutyronitrile (AIBN) was obtained from Fluka A.G. (Buchs, Switzerland). Poly(vinyl alcohol) (PVAL; M_w: 100,000, 98% hydrolyzed) was supplied from Aldrich Chem. Co. (USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the metal chelation experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed bed system.

2.2. Preparation of poly(EGDMA-VIM) beads

The poly(EGDMA-VIM) beads were selected as the carrier for the synthesis of metal-chelate affinity adsorbent for enzyme adsorption. The poly(EGDMA-VIM) beads were produced by suspension polymerization technique in an aqueous medium as described in previous article of our research group [31]. EGDMA and VIM were copolymerized in suspension by using AIBN and poly(vinyl alcohol) as the initiator and the stabilizer, respectively. Toluene was included in the polymerization recipe as the diluent (as a pore former). A typical preparation procedure was given below. Continuous mediumwas prepared by dissolving poly(vinyl alcohol) (200 mg) in the purified water (50 ml). For the preparation of dispersion phase, EGDMA (6 ml; 30 mmol) and toluene (4 ml) were stirred magnetically at 250 rpm for 15 min at room temperature. Then, VIM (3 ml; 30 mmol) and AIBN (100 mg) were dissolved in the homogeneous organic phase. The organic phase was dispersed in the aqueous medium by stirring the mixture magnetically (400 rpm), in a sealed pyrex polymerization reactor. The reactor content was heated to polymerization temperature (i.e. 70 °C) within 4 h and the polymerization was conducted for 2 h with a 600 rpm stirring rate at 90 °C. Final beads were extensively washed with ethanol and water to remove any unreacted monomer or diluent and then stored in distilled water at 4 °C.

2.3. Chelation of Cu^{2+} ions

Chelates of Cu^{2+} ions with poly(EGDMA-VIM) beads were prepared as follows: 1.0 g of the beads were mixed with 50 ml of aqueous solutions containing 50 ppm Cu^{2+} ions, at constant pH of 5.0 (adjusted with HCl and NaOH), which was the optimum pH for Cu^{2+} chelate formation at room temperature. A 1000 ppm atomic absorption standard solution (containing 10% HNO_3) was used as the source of Cu^{2+} ions. The flask was stirred magnetically at 100 rpm for 1 h (sufficient to reach equilibrium). The concentration of the Cu^{2+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (Analyst 800/Perkin-Elmer, USA). The amount of adsorbed Cu^{2+} ionswas calculated by using the concentrations of the Cu^{2+} ions in the initial solution and in the equilibrium.

Cu^{2+} leakage from the poly(EGDMA-VIM) beadswas investigated with media pH (3.0–7.0), and also in a medium containing 1.0 M NaCl. The bead suspensions were stirred 24 h at room temperature. Cu^{2+} ion concentration was then determined in the supernatants using an atomic absorption spectrophotometer. It should be also noted that metal-chelated beads were stored at 4 °C in the 10 mM Tris–HCl buffer (pH 7.4).

2.4. Uricase adsorption studies

Uricase adsorption of the Cu^{2+}-chelated poly(EGDMA-VIM) beads was studied at various pH values, either in acetate