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Enhanced D-hydantoinase activity performance via immobilized cobalt ion affinity membrane and its kinetic study

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ABSTRACT

Various immobilized metal ions affinity membranes (IMAMs) were prepared from the regenerated cellulose membrane (RC membrane) and chelated with various metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}. The D-hydantoin-hydrolyzing enzyme (DHTase) harboring a poly-His tagged residue was used as a model protein to be immobilized on the prepared IMAMs through the direct metal–protein interaction forces. The adsorption isotherm and the kinetic parameters V_{max}, $K_{m,app}$ of DHTase on IMAMs were studied. The cobalt ions chelated IMAM (Co-IMAM) was found to yield the highest specific activity of DHTase. Under the immobilization condition, the cobalt ion chelated amount was 161.4 ± 4.7 μmol/disk with a DHTase activity of 4.1 ± 0.1 U/disk. As compared to the free DHTase, the immobilized DHTase membrane could achieve a broader pH tolerance and higher thermal stability. In addition, 98% of the residual activity could be retained for 7-times repeated use. Only little activity loss was observed within 36-day storage at 4 °C. This is the first report concerning about using cobalt ion as the effective chelated metal ion for simultaneous purification and immobilization operation.

1. Introduction

Enantiomerically pure D-amino acids are considered to be important chiral building blocks for a variety of biologically active compounds such as pesticides, semi-synthetic β-lactam antibiotics, enzyme inhibitors, and peptide hormones [1–3]. The enzyme D-hydantoinase (DHTase) is an important enzyme to enantiospecifically catalyze the ring opening of hydantoins (HDTs) to the corresponding N-carbamoyl-D-amino acids. However, this enzyme is known to be unstable in its free form which limits its application in industrial processes. In order to use DHTase more economically and efficiently, it is general to immobilize DHTase to enhance its operational stability in the industrial application. Various reports concerned about DHTase immobilization in different supports, such as DEAE-cellulose [4], polyacrylamide [5], activated charcoal [6], calcium alginate beads [7,8], polystyrene anion exchange resins [9], and amino propyl functionalized glass bead [10]. These methods could be categorized into three types: encapsulation, covalent attachment, and noncovalent adsorption. Encapsulation is the physical confinement of the guest enzyme into a host support matrix. The size of enzyme, the loading amount and leaking issues might limit its application. The covalent attachment of enzymes to its support is quite popularly applied for its extensive surface area and strong enzyme binding ability [11,12]. However, the use of linking reagents might seriously result in loss of enzyme activity and sometimes poor reusability [13]. The noncovalent adsorption approach via the coordination bonds formed between the immobilized metal ions and amino acid residues of DHTase avoids the above-mentioned drawbacks and is considered to be a useful and efficient tools for proteins and peptides immobilization.

The immobilized metal ion affinity chromatography (IMAC) technique is one popular separation method generally applied for recombinant protein purification [14,15]. IMAC exhibits the high specific affinity via the immobilized metal ions as ligands in solid support to link the specific amino acid residues such as histidine, cysteine, tryptophan, aspartic acid, and glutamic acid of the protein. To extend the IMAC to membrane application, the immobilized metal affinity membrane (IMAM) was constructed via modifying the regenerated cellulose membrane with a series of reagents [16–18]. The structure of IMAM membrane displays a high flow-rate, low pressure drop, and high productivity than the traditional bead-packed column and resin in industry application.

Abbreviations: RC membrane, regenerated cellulose membrane; IMAC, immobilized metal ion affinity chromatography; IMAMs, immobilized metal ions affinity membranes; Co-IMAM, cobalt ions chelated IMAM; DHTase, D-hydantoin-hydrolyzing enzyme; IDM, immobilized DHTase membrane; Co-IDM, cobalt ions immobilized DHTase membrane; HDT, 5-(4-hydroxyphenyl) hydantoin.

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The additional advantages of easy packing and unlikely fouling clogging make it a good alternative process for enzyme purification and immobilization. The prepared IMAMs with transition metal ions such as Zn^{2+}, Ni^{2+}, Cu^{2+} as ligands were generally adopted for binding with amino acids in enzyme purification process.

In our lab, it was found that DHTase directly immobilized on IMAMs with various metal ions displayed significantly distinctive enzymatic performance. Enzyme structure and its activity might be dramatically changed by metal–protein interaction during the process of immobilization. The effect of metal ion species as ligands for DHTase immobilization was studied. In the premilinary result, the cobalt ion chelated IMAM (Co-IMAM) was found to yield a specially high specific activity. The Co-IMAM is considered worthy to be further studied for immobilization of DHTase (carrying poly His-tagged). This is also the first report concerning about using cobalt ion as the chelated ligand for IMAM to be an immobilization matrix.

In this study, IMAMs chelated with various metal ions, the enzymatic kinetics and adsorption performance were investigated. The optimal hydrolytic conditions and activation energy for immobilized DHTase membrane (IDM, prepared from Co-IMAM) and free DHTase were studied and discussed. The reusability and storage tests for the IDM were also investigated.

2. Materials and methods

2.1. Chemicals

5-(4-Hydroxyphenyl) hydantoin (HDT) was obtained from TCI, Japan. Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and epichlorohydrin (EPI) were purchased from Tedia Company, USA; iminodiacetic acid (IDA) and sodium hydroxide were from Acros, Belgium. All other chemicals used were of analytical grade and obtained commercially.

2.2. Materials and microorganism cultivation

A RC membrane with a diameter of 47 mm, an average pore size of 0.45 μm, an area of 17.32 cm^2 and a thickness of 160 μm was purchased from Sartorius, Germany. The recombinant *E. coli* strain with D-hydantoinase gene was obtained from Prof. Wen-Hwei Hsu (National Chung Hsing University, ROC). The strain was used to produce DHTase and the cultural conditions were described following the report of Ko et al. [19].

2.3. Preparation of IMAM and IDM

The RC membrane was modified via series reactions to form IMAM chelated with various metal ions [17–19]. One piece of IMAM was immersed in 15 ml crude DHTase (protein content of 1.21 ± 0.1 mg/ml) followed by shaking at 18 °C and 100 rpm for 14 h to immobilize DHTase to form IDM.

2.4. DHTase purification with Co-IMAM

The purified DHTase was obtained using 250 mM imidazole as the eluent via IMAM procedure as reported by Ko et al. [19,20] and the purified DHTase has a specific activity of 1.36 U/mg.

2.5. Estimation of adsorption isotherm

To estimate the adsorption effect, the modified Langmuir equation

$$g_e = \frac{g_m K_L C_e}{1 + K_L C_e}$$

is applied.

After rearrangement, it becomes

$$\frac{C_e}{g_e} = \frac{1}{g_m K_L} + \left(\frac{1}{g_m}\right) C_e,$$

where C_e and g_e are loading protein and adsorbed protein concentrations (mg/ml) at adsorption equilibrium; K_L is the modified Langmuir constant (ml/mg), and g_m (mg/ml) is the maximal concentration of the adsorbed DHTase corresponding to the monolayer coverage (mg/ml) [21].

2.6. Estimation of reaction kinetics parameters

2.6.1. Determination of $K_{m,app}$ and V_{max}

The Michaelis–Menten equation: $V = V_m S/(K_{m,app} + S)$ (where V is the reaction rate and S is the substrate concentration) was used to determine the reaction kinetic parameters. Plotting $1/V$ vs. $1/S$ (Lineweaver–Burk plot) gives an appropriate linear fit, which is used to determine the maximum reaction rate of the enzymatic reaction (V_{max}) and the apparent Michaelis–Menten constant ($K_{m,app}$) [2].

2.6.2. Determination of energy of activation (E_a)

The values of activation energy (E_a) of free and immobilized DHTase were calculated according to the Arrhenius equation: $r = K \exp(-E_a/RT)$, where r was enzyme activity, K was the pre-exponential factor, E_a was the activation energy, T was the absolute temperature and R was the universal gas constant. By plotting $\ln(r)$ versus $(1/T)$ gives a linear relationship to calculate the E_a by the equation: $E_a = -\text{slope} \times R$ [22].

2.7. Assays

The concentration of Co^{2+} was determined at 515 nm by using a UV–vis spectrophotometer (Metertek SP-830, Metertech Inc., Taiwan) [19,23]. Protein concentration was detected with the Bradford protein assay kits (Bio-rad, Munich, German) [19]. The DHTase activities were determined by measuring the amount of N-carbamoyl-D-p-hydroxyphenylglycine (D-CpHPG) produced in the enzyme reaction with the high performance liquid chromatography (HPLC) [19,20]. The unit (U) of enzyme activity was defined as 1 μmole of D-CpHPG produced per min at 50 °C [19].

2.8. pH and thermal effects and stability

The pH and temperature effects and stability tests for Co-IDM were performed following the description reported by Ko et al. [19]. One piece of the prepared Co-IDM contains 0.08 ± 0.05 mg protein and displays DHTase activity of 4.10 ± 0.10 U per membrane. The relative activity was calculated as the ratio of the residual enzyme activity after incubation to that of the maximum activity.

2.9. Reusability and storage stability

For determination the reusability of Co-IDM, one piece of Co-IDM was used in enzyme reaction with Tris–HCl buffer (0.1 M, pH 8). The reaction was carried out at 50 °C for 40 min. When the conversion yield reached a predetermined level (82%), the Co-IDM was removed and washed three times with 0.1 M, pH 8.0 Tris–HCl buffer. The relative activity was defined as the ratio of the residual Co-IDM activity after the test to that of its initial activity. The storage stability was estimated by measuring the residual activities of Co-IDM after storage to that of its original activity [19].