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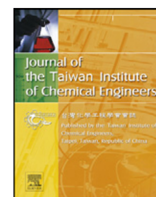

Exploring the complex effects of metal ions on D-hydantoinase purification with an immobilized metal affinity membrane

Yi-Miao Ko [a], Chih-I Chen [b], Hou-Chien Chang [a], Hsiu-Mei Chen [c], Chwen-Jen Shieh [d], Yu-Jhe Syu [a], Yung-Chuan Liu [a,*]

[a] *Department of Chemical Engineering, National Chung Hsing University, Taichung 40227, Taiwan*
[b] *Department of Chemical Engineering, Hsiuping Institute of Technology, Taichung 41280, Taiwan*
[c] *Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 10607, Taiwan*
[d] *Biotechnology Center, National Chung Hsing University, Taichung 40227, Taiwan*

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ABSTRACT

In this study, D-hydantoinase (DHTase) was purified using an immobilized metal ion affinity membrane (IMAM), on which the interactions between amino acid residues placed on the protein surface and the chelated metal ions located on the IMAM surface facilitate DHTase purification. Batch DHTase adsorption experiments showed that the adsorption capacity varied remarkably for IMAM with different metal ions. The maximum adsorption of DHTase (1.513 ± 0.12 mg) was reached when using Cu^{2+} as the chelated ion, whereas the Co^{2+} showed the highest activity with only small amounts of protein adsorption. The Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Fe^{3+} additions showed a positive effect on DHTase activity. The addition of Cu^{2+} showed a varied effect from the inhibition on original DHTase to the promotion on Ni-purified DHTase. The purification folds using IMAM chelated with Co^{2+}, Ni^{2+}, and Zn^{2+} were in the range of six to seven. SDS–PAGE analysis showed that all of the IMAM-purified DHTase exhibited the same molecular weight, meaning DHTase adsorbed on IMAM was highly specific. The DHTase purified by different metal ions showed various levels of increased activity when adding the corresponding metal ions. The addition of Mn^{2+} or Co^{2+} displayed a dramatic increase (9- to 10-fold) in activity of DHTase purified by IMAM chelated with the same ion.
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1. Introduction

D-Hydantoinase (DHTase) is an enzyme capable of selectively catalyzing the hydrolytic ring opening of D-5-substituted hydantoins into corresponding N-carbamoyl-D-amino acids. Due to the high specific selectivity of the reaction, DHTase is widely employed in the industrial production of optically resolved amino acids, and is of great interest to the pharmaceutical and chemical industries. For example, some D-amino acids are key compounds used as intermediates in the preparation of semisynthetic antibiotics, such as ampicillin and amoxicillin, and other products such as pesticides or hormones (Ogawa and Shimizu, 1999; Shigeru *et al.*, 1991; Takahashi *et al.*, 1979; Yamada *et al.*, 1978). A number of DHTase enzymes have been reported to exhibit metal ion dependence (Huang and Yang, 2002). The metal ions were also reported to show different levels of influence in DHTases obtained from varied strains (Glusker, 1991; Runser *et al.*, 1990).

Adsorption through immobilized metal affinity chromatography (IMAC) has been shown to be a relatively convenient protein purification method (Girelli and Mattei, 2005; Krajewska, 2004; Scouten *et al.*, 1995). Using metal ions as ligands, the target protein can be selectively separated from the harvested cultivation broth. In the literature, the recombinant proteins carrying a poly-His tagged residue can bind specifically to the chelated metal ions on IMAC for purification (Armisen *et al.*, 1999; Crowe *et al.*, 1995; Lin *et al.*, 2008; Liou *et al.*, 2008; Wang *et al.*, 2009). Many materials are suitable for use as support matrices for constructing IMAC, including: activated carbon, silica gel, celite, montmorillonite, resins and membranes (Yip and Hutchens, 1994). The procedure for IMAM preparation from regenerated cellulose (RC) membrane as a support has been developed in our lab (Chen *et al.*, 2007; Liu *et al.*, 2005). Compared to the conventional packed-column systems (Fitton and Santarelli, 2001; Hu *et al.*, 2006; Liu *et al.*, 2003), the IMAM process offers advantages, such as low pressure drop, no bed compaction and easier scale up.

In the application of IMAM to the purification of various recombinant proteins, the purified proteins would display a stable performance after the purification. However, it is also interesting to find that for some proteins, especially the metal-enzymes, the metal ions used in IMAM not only influence the purification efficiency but also influence the properties of the purified enzyme. Although the IMAM is used in protein purification, rare of the

* Corresponding author. Tel.: +886 4 22853769; fax: +886 4 22854734.
E-mail address: ycliu@dragon.nchu.edu.tw (Y.-C. Liu).

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studies mentioned about the role of metal ions on the performance of purified proteins. In a preliminary test, it is found that the recombinant DHTase purified via the IMAM process displayed a significant variation in activity compared to the original enzyme. Therefore, DHTase is chosen as the target protein in this study. Metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+} were employed. The stimulatory effects of metal ions on DHTase activity before and after IMAM purification were tested. Analysis on residual metal ions contents in DHTase purified with various metal ions was conducted. The roles of metal ions on DHTase activity expression were compared and discussed.

2. Materials and methods

2.1. Materials and chemicals

RC membrane (47 mm in diameter, pore size of 0.45 μm, and a thickness of 160 μm) was purchased from Sartorius (Germany). Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and epichlorohydrin (EPI) were obtained from Tedia (Fairfield, OH, USA); iminodiacetic acid (IDA) from Acros Organics (Geel, Belgium); 5-(4-hydroxyphenyl) hydantoin (HDT) from TCI (Tokyo kasei, Japan). N-carbamoyl-D-p-hydroxyphenylglycine (D-CpHPG) and the *E. coli* strain harboring DHTase gene were gifts from Prof. Wen-Hwei Hsu (Institute of Molecular Biology College of Life Science, National Chung Hsing University, ROC). Other reagents were of analytical grade and obtained from local suppliers.

2.2. Preparation of DHTase enzyme

Recombinant *E. coli* strain was activated in a Luria-Bertani (LB) agar plate. A loop of strain was inoculated into a shake flask containing LB medium, and cultivation was conducted at 37 °C and 100 rpm for 12 h for use as the seed culture. To perform the fermentation, 1% of the seed culture was inoculated into the flask containing 100 ml of fresh LB medium. The cultivation was carried out at 37 °C and 100 rpm for 3 h. When the biomass reached optical density (600 nm) of 1.0, the culture was induced with the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG) for overexpression of recombinant DHTase. After further induction at 15 °C for 1 day, cells were harvested by centrifugation (7000 × *g*, 10 min). The pellet was washed once and resuspended with 100 mM Tris–HCl buffer (pH 8). The resuspended cells were disrupted using a cell disruption system (NN11 4SD, Constant Systems Limited, UK). The solution was then centrifuged at 7000 × *g* for 10 min and the supernatant was collected as a crude enzyme for use. In the metal ions addition test, the crude enzyme was further purified via the salting-out (60% ammonia sulfate) and dialysis (against 0.1 M Tris–HCl buffer) process. Afterwards, an ultrafiltration (50–100 kDa, Amicon ultra, Millipore Co., USA) process was used to remove the impurity protein. The targeted DHTase enzyme was then collected as the original enzyme for the metal ions tests.

2.3. IMAM preparation

The RC membrane was modified via a series of chemical reactions (Liu *et al.*, 2003) to couple EPI, IDA, and metal ions sequentially on the membrane surface. All reactions were carried out in an 80 ml glass bottle. A piece of RC membrane was immersed in 20 ml, 1.4 M NaOH and 5 ml EPI, and shaken at 24 °C, 150 rpm for 14 h. Afterwards, the membrane was rinsed twice with DI water and then immersed in 1 M IDA solution (dissolved in 1 M sodium carbonate) and shaken at 24 °C for 12 h. For chelating metal ions, the membrane was reacted with 25 ml of various metal ion solutions (25 mM) for 1 h and then rinsed twice with 0.1 M Tris–HCl buffer (pH 8) to remove the unbound metal ions. The prepared IMAM membrane was preserved in 0.1 M Tris–HCl (pH 8) at 4 °C before use.

2.4. Batch DHTase purification with IMAM

For batch adsorption experiments, one piece of IMAM was put into a glass bottle (80 ml) containing 15 ml of crude enzyme (protein content of 1.21 ± 0.1 mg/ml) and then the solution was shaken at 18 °C and 100 rpm for 14 h. The membrane was then rinsed twice with Tris–HCl buffer. The bound enzyme was eluted with an eluent (200 mM imidazole) and the elution was dialyzed three times against 0.1 M Tris–HCl buffer. The enzyme activity and protein concentration of the purified DHTase were analyzed.

2.5. Metal ions addition test

100 μl of various metal ions (20 mM) were added to 900 μl of the DHTase enzyme solutions (80.0 μg/ml) and the mixtures were incubated at 4 °C for 1 h. Thereafter, the DHTase activity was measured.

2.6. Protein assay

For all adsorption experiments, the concentrations of protein before and after each step were analyzed using the Bradford method (Bradford, 1976). The protein contents were analyzed by SDS–PAGE with 12% polyacrylamide gel stained with Commassie blue and quantified using densitometry.

2.7. DHTase activity assay

Samples of free or immobilized enzymes were incubated in a reaction mixture containing 10 mM HDT as a substrate in 0.1 M Tris–HCl buffer pH 8 at 50 °C and reacted for 20 min. D-CpHPG concentration was analyzed using HPLC (RI-930, Jusco, Japan). A reversed phase BDS HYPERSIL column of 250 mm × 4.6 mm was used. The mobile phase consisted of $H_2O/CH_3CN/H_3PO_4$ in the ratio of 95/5/0.01 (v/v/v). The flow rate of the mobile phase was 1.0 ml/min and the UV detector was set at 230 nm. The unit (U) of enzyme activity was defined as μmol of D-CpHPG generated per min.

3. Results and discussion

3.1. Ions effect on DHTase

DHTase was found to be an enzyme showing great dependence on metal ions. Lee *et al.* (1995) reported that DHTase activity from *Bacillus stearothermophilus* was enhanced by Mn^{2+} and Ni^{2+}. Yamada *et al.* (1978) pointed out that DHTase from *Pseudomonas putida* was activated by Fe^{2+}. In this study, the recombinant DHTase with poly-His tag was obtained by cultivating recombinant *E. coli* harboring DHTase genes from *Brebrevibacillus argi* NCHU1002 (Kao and Hsu, 2003). The effect of metal ions on the DHTase activity was examined and the results are shown in Fig. 1(a). It was obvious that the addition of Mn^{2+}, Co^{2+}, and Ni^{2+} stimulated DHTase activity, while Cu^{2+} and Zn^{2+} inhibited its activity. Mn^{2+} exhibited the best promotion (2-fold) of enzyme activity in comparison with that without addition. Fe^{2+} and Fe^{3+} displayed no enhancement of DHTase activity. In order to examine the metal ions effect on DHTase after IMAM purification, the conventional Ni-purification procedure (in Section 2.4) was conducted. Ni-purified DHTase was obtained and used as the test protein. From Fig. 1(b), it can be seen that the addition of Mn^{2+}, Co^{2+}, and Cu^{2+} promoted DHTase activity, but that Zn^{2+} still inhibited DHTase activity. The activity of the purified DHTase was increased about 2- to 3-fold by adding either Mn^{2+} or Co^{2+}. Fe^{2+} and

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