Journal of Membrane Science 380 (2011) 34–40

Contents lists available at ScienceDirect

Journal of Membrane Science

journal homepage: www.elsevier.com/locate/memsci

Direct penicillin G acylase immobilization by using the self-prepared immobilized metal affinity membrane

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ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 11 June 2011
Accepted 19 June 2011
Available online 24 June 2011

Keywords:
Penicillin G acylase
Immobilized metal affinity membrane
Enzyme immbolization
Kinetic analysis
Enzyme stability test

ABSTRACT

The use of penicillin G acylase (PGA) requires efficient methods for immobilizing the enzyme to yield a biocatalyst with high activity and stability for industrial application. This study constructs the immobilized metal affinity membrane (IMAM) via a series of chemical reactions to sequentially couple epichlorohydrin (EPI), iminodiacetic acid (IDA), and copper ions on the regenerated cellulose membrane. The feasibility of direct PGA immobilization on IMAM was examined. Various conditions were tested for constructing IMAM for PGA immobilization. Under the optimal condition, a copper ion of 492.8 μmol/g with a PGA activity of 11.76 U/g was obtained for IMAM as compared to those for the commercial immobilized metal affinity resin (299.8 μmol/g and 2.99 U/g). The immobilized PGA membrane was stable in the pH range from 7 to 9 and for temperatures from 40 to 60 °C. In addition, 99% of the residual activity could be retained via a 16-times repeated use. Only little activity loss was observed after a 40 days of storage. This study also determines the kinetic parameters V_{max}, K_m, and the energy of activation (E_a) for the immobilized PGA membrane.

1. Introduction

Penicillin G acylase (PGA) is a biocatalyst capable of hydrolyzing penicillin G for the formation of 6-aminopenicillanic acid (6-APA), an important intermediate to semisynthetic β-lactam antibiotics production. Because of its unique structural characteristics, PGA has a high specificity toward the amide bond of penicillins [1]. To use PGA more economically and efficiently, it is common to immobilize this enzyme to enhance its operational stability for industrial application. In the literature, numerous methods for immobilizing PGA were reported [2–4]. Much work is still being done to search for new support materials and novel synthesis techniques. The type of support and the method of immobilization remarkably influence the activity and operational performance of immobilized PGA. An appropriate selection during the immobilization process can significantly reduce the costs of industrial processes for 6-APA production.

The immobilized metal ion affinity chromatography (IMAC) technique is one of the common methods generally applied for protein purification [5–7]. To act as a successful affinity adsorbent, the solid supports chosen should be suitable for a coupling ligand so that the target protein is bound without being seriously disturbed. Accordingly, the regenerated cellulose (RC) membrane, with its abundance of hydroxyl groups, was chosen as the appropriate support for constructing the immobilized metal affinity membrane (IMAM). While synthesizing the IMAM for PGA purification in our lab, it was found that the membrane exhibited a remarkably strong affinity to PGA, and therefore was considered to be a possible way to directly immobilize PGA [8,9]. A commercial IMAC-resin was also employed to immobilize PGA and an unsatisfactory result was obtained as can be seen in the text. In addition, no literature reported the use of IMAC to successfully immobilize enzymes due to the concern that the coordination bonds between IMAC resin and proteins are not strong enough to bind the enzymes firmly for the long term application. To overcome this issue, we attempted to prepare an IMAM membrane with high metal ions amounts to provide enough affinity for PGA immobilization. In this study, the feasibility of direct PGA immobilization on IMAM was explored. The activity and stability of the constructed immobilized PGA membrane (IPM) was investigated. The optimal hydrolytic conditions and the kinetic analysis for immobilized PGA and free PGA were also studied and discussed.

2. Materials and methods

2.1. Materials and equipments

The RC membranes (diameter of 47 mm, pore size of 0.45 μm, thickness of 160 μm, dry weight of 153 mg/disc) were purchased from Sartorius (Germany); penicillin G (PG) from MDBio (Taiwan);

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doi:10.1016/j.memsci.2011.06.027

6-aminopenicillanic acid (6-APA), p-dimethylaminobenzaldehyde (DAB), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and epichlorohydrin (EPI) were from Tedia (Fairfield, OH, USA); iminodiacetic acid (IDA) from Acros Organics (Geel, Belgium). Other reagents were of analytical grade and from local suppliers.

2.2. Preparation of crude PGA and purified PGA

Fermentation broth from *Escherichia coli* cultivation was obtained. The cells were harvested by centrifuging 100 ml of crude broth at 14,000 × *g* for 20 min (Centrifuge Z323K, Hermle, Germany). The pellet obtained was washed twice with DI water, then resuspended in lysis buffer (0.1 M KH_2PO_4, pH 8.0) and sonicated for 12 min. The solution was centrifugated at 18,000 × *g* for 20 min and the supernatant was collected as the crude PGA (diluted to a specific activity of 0.58 U/mg before use) [10]. In addition, the purification approach with IMAM proposed by Ke et al. was used to prepare the purified PGA (with a specific activity of 7.38 U/mg; purification fold: 12.7, as the free PGA) for the use on enzyme kinetic study [9].

2.3. Preparation of IMAM

The RC membrane was modified via a series of chemical reactions to couple EPI, IDA and copper ions sequentially on the membrane surface [9,11]. All reactions were carried out in a 200 ml glass bottle. A disc of RC membrane was immersed in a mixture containing 20 ml, 1.4 M NaOH and 5 ml EPI, and shaken at 24 °C, 150 rpm for 12 h and then rinsed twice with DI water. The amount of epoxy group (about 184.72 μmol/disc) could be detected on the membrane. After that, the membrane was immersed in 1 M IDA solution (dissolved in 1 M sodium carbonate) and shaken at 24 °C for 12 h to couple the carboxyl group (176.78 μmol/disc) on the membrane. For coupling Cu^{2+}, the membrane was reacted with 25 ml of 25 mM $CuSO_4$ for 1 h and then rinsed twice with the loading buffer (10 mM phosphate buffer, pH 6.0) to remove the unbound copper ions. Cu^{2+} (about 75.5 μmol/disc) chelated on the prepared metal affinity immobilization membrane was observed. The prepared IMAM was preserved in phosphate buffer (0.1% NaN_3, 50 mM phosphate buffer, pH 8) at 4 °C before use. To couple various amounts of copper ions on IMAM, the membrane was dipped in 25 ml of 25 mM $CuSO_4$ for various time intervals at 24 °C.

2.4. Preparation of IPM and immobilized PGA resin

To adsorb PGA on the IMAM, one disc of IMAM was immersed into a 200 ml glass bottle and 25 ml of crude PGA was loaded. Adsorption was performed at 18 °C and 100 rpm for 12 h. Then, the membrane was rinsed twice with DI water and the resultant immobilized PGA membrane (IPM) was preserved in 50 mM phosphate buffer, pH 8.0 at 4 °C before use. The PGA activity of IPM was determined according to the method in Section 2.8.3. A schematic illustration of the above-mentioned procedure is shown in Fig. 1.

As for the preparation of IMAC resin, the procedure follows the instruction manual of Merck Co. (user protocol: TB054 E0405, Fractogel His-Bind resin, Merck). In brief, 1 ml resin (containing 452 mg resin in dry weight) was immersed in 25 mM $CuSO_4$ for 1 h. After washing 3 times with DI water, the IMAC was immersed into 25 ml of PGA solution (0.1 U/ml) for 12 h. Then the immobilized PGA resin was rinsed twice with 50 mM phosphate buffer (pH 8.0) and preserved at the same buffer in 4 °C before use.

2.5. The repeated-use test for immobilized PGA

To test the stability of the immobilized PGA, a disc of the prepared IPM or 1 ml immobilized PGA resin (containing 452 mg resin in dry weight) was used in the repeated-use (16 times) test. The residual PGA activity was determined according to the method in Section 2.8.3. The relative activity was calculated as the ratio of the residual PGA activity after the repeated use to that of the original PGA activity.

2.6. pH and thermal stability of free PGA and immobilized PGA

The pH stability was studied by incubating 1 ml of free PGA or 1 disc of IPM at varying pH (3–10) for 1 h and then the PGA activity was determined according to Section 2.8.3. The relative activity was calculated as the ratio of the residual activity of enzyme after incubation to that of the maximum activity. The thermal stability was tested by incubating 1 ml of free PGA or 1 disc of IPM at varying

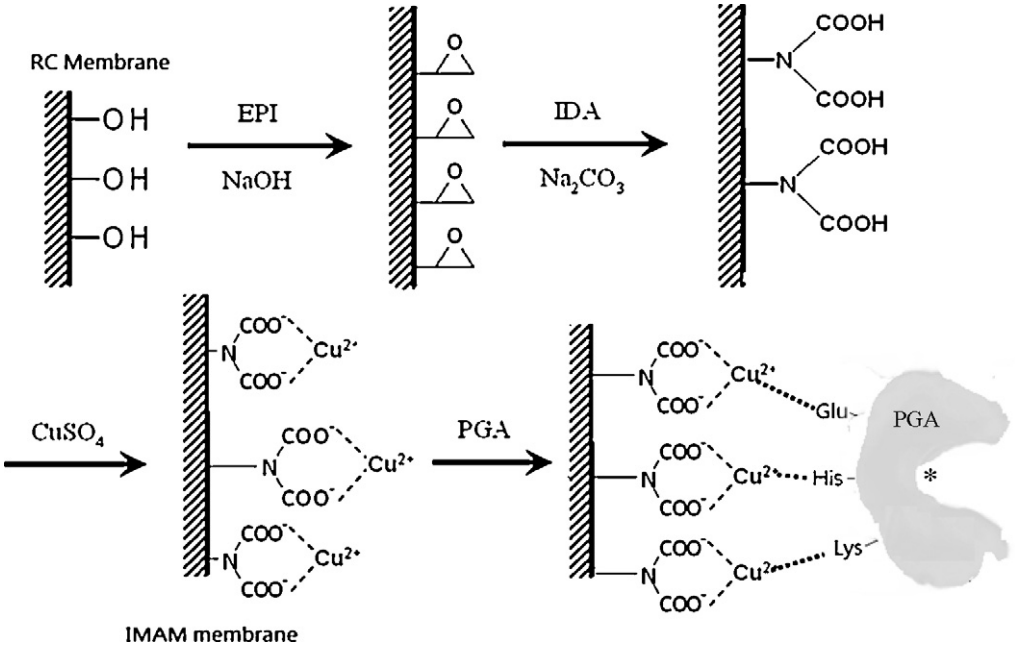

Fig. 1. Schematic illustration of IMAM preparation and PGA immobilization, where * represents the location of active site.