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Immobilization of penicillin G acylase on macro-mesoporous silica spheres

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ABSTRACT

In this study, macro-mesoporous silica spheres were prepared with a micro-device and used as the support for the immobilization of penicillin G acylase (PGA). To measure the enzymatic activity, the silica spheres with immobilized PGA were placed into a packed-bed reactor, in which the hydrolysis of penicillin G was carried out. The influences of the residence time, the initial concentration of the substrate, the accumulation of the target product 6-aminopenicillanic acid, and the enzyme loading amount on the performance of the immobilized PGA were investigated. The introduction of macropores increased the enzyme loading amount and decreased the internal mass transfer resistance, and the results showed that the enzyme loading amount reached 895 mg/g (dry support), and the apparent enzymatic activity achieved up to 1033 U/g (dry support). In addition, the immobilized PGA was found to have great stability.

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1. Introduction

Penicillin G acylase (PGA; EC 3.5.1.11), an N-terminal nucleophile hydrolase with molecular dimensions of $7.0 \times 5.0 \times 5.5\ nm^3$, is one of the key enzymes used in the pharmaceutical industry for the production of β-lactum antibiotics (Wei and Yang, 2003; Sio and Quax, 2004; Zhang et al., 2006; Cheng et al., 2007; Chandel et al., 2008). In recent years, many efforts have been devoted to using mesoporous silica materials as the supports for enzyme immobilization because of their large surface area and pore volume, tunable pore size and structures, openness to a wide variety of chemical modifications, convenience of reutilization, and environmental friendliness (Huang et al., 2003; Xu et al., 2003; Bootsma et al., 2008; Yang et al., 2008; Chouyyok et al., 2009; Salis et al., 2009; Bautista et al., 2010; Kumar et al., 2010; Wang et al., 2010). PGA enzymes have been immobilized on MCM-41 (He et al., 2000; Xue et al., 2004), MCM-48 (Xue et al., 2004), cubic *Ia*3d mesoporous silica (Lü et al., 2007, 2008), SBA-15 (Chong and Zhao, 2004a,b; Lü et al., 2008; Shah et al., 2008; Sun et al., 2009), and mesostructured cellular foams (Xue et al., 2008; Zhao et al., 2010) with different pore structures and surface properties. The experimental results showed that the mesoporous silica was a suitable support for the immobilization of PGA. However, morphologies of the supports used in these works were powdered, and it is well known that the submicrometer-sized powders are difficult to separate and recover in industrial processes. Thus, mesoporous silica spheres may be more promising supports.

However, compared with mesoporous silica powders, mesoporous silica spheres used as the supports for the immobilization of PGA may bring about larger internal mass transfer resistance for the hydrolysis of Pen G, which is a fast reaction with the diffusions of the substrate and products as the rate-determining steps. To solve this problem, macropores (>50 nm) were introduced into the mesoporous silica spheres, thus in this work, macro-mesoporous silica spheres were prepared with a micro-device based on our previous researches (Zhai et al., 2008, 2009). There, the performance of this kind of silica spheres as the adsorbents for two kinds of proteins – bovine serum albumin (BSA, $4 \times 4 \times 14\ nm^3$) and lysozyme (LYS, $3 \times 3 \times 4.5\ nm^3$) was investigated, and the results showed that the introduction of macropores increased the adsorbed amounts of BSA and LYS and significantly decreased the adsorption equilibrium time, implying that the macro-mesoporous silica spheres might be effective supports for the immobilization of PGA ($7.0 \times 5.0 \times 5.5\ nm^3$). This is because the macropores could make the PGA molecules penetrate into the internal mesopores of the silica spheres more easily, producing a high enzyme loading amount; moreover, the macropores could provide a highway for the mass transfer of the substrate and products toward and away from the active sites of the immobilized PGA molecules, obtaining a lower internal mass transfer resistance and a higher apparent activity.

To improve the stability of the immobilized PGA, 3-aminopropyltriethoxysilane (APTS) was used as a chemical modifier to obtain aminopropyl-functionalized silica spheres, and the immobilization of PGA was performed through Schiff base reaction with glutaraldehyde. The hydrolysis of Pen G was carried out in a packed-bed reactor to determine the apparent activity of the

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immobilized PGA on the macro-mesoporous silica spheres. A packed-bed reactor was adopted here to simplify the experimental procedure, avoiding filtration or centrifugation and the mass loss of the immobilized PGA in these separation processes. Moreover, compared with stirred tank reactors, packed-bed reactors can also avoid the damages to the support caused by the mechanical stirring and the deactivation of the immobilized PGA caused by the direct contact of the immobilized enzyme and the alkaline agent that was added to neutralize the by-product phenylacetic acid (PAA). The influences of the residence time, the initial concentration of Pen G, and the accumulation of the target product 6-aminopenicillanic acid (6-APA) on the performance of the immobilized PGA were investigated. In addition, the influence of the enzyme loading amount on the catalytic efficiency of the immobilized PGA was investigated.

2. Methods

2.1. Chemicals

Tetraethyl orthosilicate (TEOS) was produced by Xilong Chemical Company (Shantou, China). Sorbitan monooleate (Span 80) was produced by China Medicine Shanghai Chemical Reagent Corporation (Shanghai, China). Trioctylamine (TOA) was purchased from Feixiang Chemical Company (Zhangjiagang, China). Poly(ethylene glycol) (PEG 20,000), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), and sodium phosphate dibasic dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) were produced by Yili Fine Chemical Company (Beijing, China). Methylcellulose (MC) and 3-aminopropyltriethoxysilane (APTS) were purchased from Acros Organics Company (New Jersey, USA). Glutaraldehyde was purchased from Bodi Chemical Company (Tianjin, China). Penicillin G acylase (PGA; EC 3.5.1.11) was purchased from Shunfeng Haideer Limited Company (Zhejiang, China). Penicillin G potassium (Pen G) was produced by North China Pharmacy Factory (Hebei, China). 6-aminopenicillanic acid (6-APA) was purchased from Alfa Aesar Chemical Limited Company (Tianjin, China). All the chemicals were used as received without further purification.

2.2. Synthesis of macro-mesoporous silica spheres

Macro-mesoporous silica spheres were synthesized with a coaxial micro-device, using a method based upon our previous researches (Zhai et al., 2008, 2009). The coaxial microdevice was fabricated on two polymethyl methacrylate (PMMA) plates (40 mm × 40 mm × 3 mm). The main channel was about 2.0 mm in diameter with a long polytetrafluoroethylene (PTFE) tube (3 m in length, 1.5 mm id × 2.0 mm od) embedded in it. A needle (0.2 mm id × 0.7 mm od) was inserted into the PTFE tube coaxially to introduce a dispersed aqueous phase. There were two side channels (1.6 mm id) with two needles (1.3 mm id × 1.6 mm od) embedded in them, and the side channels were fixed perpendicularly to the main channel to introduce the continuous phase.

The dispersed phase was prepared as follows: 0.25 g of MC and 1.0 g of PEG 20,000 were dissolved in 5.0 g of HCl aqueous solution with a concentration of 0.01 mol/L under stirring. Then 2.5 g of TEOS was added, and the resulting mixture was stirred for 3 days at room temperature. The continuous phase was a mixture containing 2 wt.% span 80, 30 wt.% TOA, and 68 wt.% octane. The dispersed and continuous phase solutions were injected in by syringe pumps at flow rates of 0.02 and 0.4 mL/min, respectively, to form monodisperse silica sol droplets. The PTFE tube was kept at 40 °C to realize the gelation of the silica sol droplets. Solidified silica spheres were obtained at the exit of the PTFE tube. Then the silica spheres together with ca. 20 mL of the continuous phase solution were transferred into an autoclave and treated at 100 °C for 24 h. After that, the silica spheres were collected by filtration, washed with water and ethanol, dried at 80 °C for 12 h, and calcined at 550 °C for 6 h. The white silica spheres obtained were designated as macro-mesoporous silica spheres (MMSS).

2.3. Grafting aminopropyl and glutaraldehyde groups and immobilizing PGA

The methods of grafting aminopropyl and glutaraldehyde and immobilizing PGA on MMSS were based upon our previous research (Zhao et al., 2010). The grafting of aminopropyl was accomplished as follows: 0.05 g of APTS was added to 20 g of HCl aqueous solution with a concentration of 0.1 mol/L, and the resulting mixture was stirred at room temperature for 30 min to allow the sufficient hydrolysis of APTS, after which, 0.2 g of MMSS was added. The mixture was shaken at 160 rpm and 25 °C for 8 h, transferred into an autoclave, and kept at 100 °C for 24 h. Then the resulting solid was filtrated, washed with 500 mL of deionized water and 500 mL of ethanol, and air-dried at 80 °C for 12 h. The samples obtained were designated as NH_2–MMSS.

The grafting of glutaraldehyde was carried out as follows: 0.02 g of NH_2–MMSS was packed into a glass column with a length of 1 cm and internal diameter of 0.4 cm using the dry packing method, and the column was kept at 25 °C. Glutaraldehyde aqueous solution with a concentration of 5 wt.% flowed through the column at a flow rate of 0.083 mL/min for 30 min, before and after which, deionized water flowed through the column at the same flow rate for 30 min. The samples obtained were designated as CHO–NH_2–MMSS.

To quantify the amounts of grafted aminopropyl and glutaraldehyde groups, the mass of carbon, hydrogen, and nitrogen of MMSS, NH_2–MMSS, and CHO–NH_2–MMSS was measured. Amounts of grafted aminopropyl (q_A, μmol/g dry MMSS) and glutaraldehyde (q_G, μmol/g dry MMSS) were calculated as follows:

$$q_A = \frac{N_{NH2-MMSS} - N_{MMSS}}{14} \times 10^6$$

$$q_G = \frac{C_{CHO-NH2-MMSS} - C_{NH2-MMSS}}{12 \times 5} \times 10^6$$

where, $N_{NH2-MMSS}$ (wt.%) is the mass of the nitrogen element of NH_2–MMSS; N_{MMSS} (wt.%) is the mass of the nitrogen element of MMSS; $C_{CHO-NH2-MMSS}$ (wt.%) is the mass of the carbon element of CHO–NH_2–MMSS; $C_{NH2-MMSS}$ (wt.%) is the mass of the carbon element of NH_2–MMSS. Here, an apparent grafted amount is used for glutaraldehyde because the glutaraldehyde group is considered as a single molecule to calculate the grafted amount, but it is actually in multimer form when grafted onto the support. Thus, the available amount of glutaraldehyde is much lower than the apparent grafted amount.

The covalent immobilization of PGA was accomplished as follows: 15 mL of PGA solution obtained by diluting 1 mL of enzyme liquid with 14 mL of phosphate buffer (0.2 mol/L, pH 7.9) flowed through the column packed with CHO–NH_2–MMSS at varying flow rates, before and after which, blank phosphate buffer (0.2 mol/L, pH 7.9) flowed through the column at the same flow rate for 30 min. For all these operations, the column was kept at 30 °C. The samples obtained were designated as PGA–CHO–NH_2–MMSS, and the resulting column was stored in phosphate buffer (0.2 mol/L, pH 7.9) at 4 °C when not in use. The concentration of PGA in the inlet and outlet solutions was measured by Bradford assay. The enzyme loading amount (q_E, mg/g dry MMSS) was calculated as follows:

$$q_E = \frac{m_{PGA,\ in} - m_{PGA,\ out}}{W_{support}}$$

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