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Covalent Assembly of Penicillin Acylase in Mesoporous Silica Based on Macromolecular Crowding Theory*

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Abstract To improve the covalent immobilization of penicillin acylase (PA), macromolecular crowding theory was applied to its immobilization. Influence of mass ratio of enzyme to the silica, as well as, activation time with glutaraldehyde on the activity of assembled PA, was studied. In the mesopores, the effect of β-cyclodextrin (β-CD) on the immobilization of the enzyme was also investigated. It was remarkable that the coupled yield and relative activity reached 99.5% and 92.3%, respectively, when penicillin acylase assembled covalently in the mesopores. The results here indicate that mimicked macromolecule crowding could significantly ameliorate the performance of covalently immobilized PA.

Keywords enzyme immobilization, penicillin acylase, β-cyclodextrin, macromolecule crowding

1 INTRODUCTION

Because of its importance in the antibiotics industry, penicillin acylase (PA, EC 3.5.1.11) has been immobilized with many new methods[1]. Maria Chong *et al.*[2] has reported that the activity of PA immobilized with adsorbent linkage was observed to be 200% of that of the free enzyme. However, it is more stable in the covalently immobilized enzyme than that obtained with the adsorbed method[3—5]. Activity of the covalently immobilized enzyme is often much lower than that obtained in the adsorptive method[1]. It has been the goal of scientists to improve the covalent immobilization of the enzyme.

Molecular crowding theory and modelling efforts have predicted that a protein inside a confined space will be stabilized by some folding forces not present in proteins in bulk solutions[6]. In this article, the macromolecular crowding theory[7] is applied to the covalent immobilization of PA, to improve its enzyme activity. Penicillin acylase assembles covalently in the confined space of mesoporous silica with the help of glutaraldehyde. β-cyclodextrin also coassembles with PA using *p*-benzoquinone as the crosslinking reagent, which is expected to improve the assembly of PA, by the interaction between cyclodextrin and enzyme.

2 EXPERIMENTAL

2.1 Preparation and characterization of mesoporous silica monolith

A mesoporous silica monolith was prepared by the co-condensation of tetramethoxysilane (TMOS) with organosilane (aminopropyl)triethoxysilane (APTES) via the modified sol-gel process[8]. The volume ratio of TMOS to APTES was 5 : 6. The template of PEG was removed by using solvent extraction, with anhydrous ethanol, in a Soxhlet apparatus for 24h. Amino groups on the surface of the mesoporous silica were determined as reported[9]. The mesoporous morphology was observed through a scanning electron microscopy system (SEM, JSM-5900, JEOL). Nitrogen adsorption and desorption of the mesoporous silica samples were measured at 77K with ASAP 2010 Micromeritics apparatus.

2.2 Covalent assembly of penicillin acylase in the mesoporous silica

300mg of mesoporous silica were activated in a solution of glutaraldehyde (5%), in a phosphate buffer (PB) of pH 8.0 for 2h. Next, the activited mesoporous silica were dispersed in 15ml of penicillin acylase solution (0.025%) and mixed at 0—4℃ for a few hours. Finally, the assembled penicillin acylase was separated and washed with PB. The protein content of the supernatant was measured using the Bradford assay[10]. In the coassembly of β-CD, and PA in the mesoporous silica, *p*-benzoquinine ($0.1 mol \cdot L^{-1}$) was used as the coupling reagent.

2.3 Enzyme activity assay

The activity of the immobilized PA was determined by titrating phenylacetic acid (PAA), an acidic byproduct produced from the hydrolysis of penicillin G potassium salt[2], with $0.1 mol \cdot L^{-1}$ NaOH solution, to maintain a constant pH of 8.0.The activity yield, the coupled yield, and the relative activity were calculated as in Ref.[11].

3 RESULTS AND DISCUSSION

Figure 1(a) shows that the ethanol extracted

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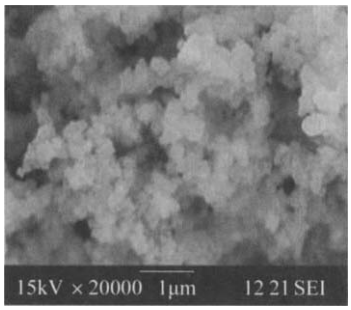
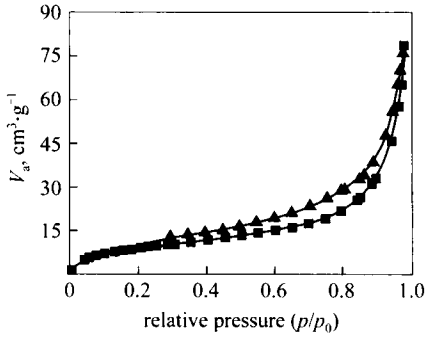


(a) SEM photograph



(b) Nitrogen adsorption-desorption isotherm

Figure 1 SEM photograph (A) and nitrogen adsorption-desorption isotherm (B) of mesoporous silica

samples exhibit co-continuous morphology of silica gel skeletons and pores. Fig.1(b) shows the nitrogen adsorption-desorption results of the prepared mesoporous silica. Calculated from the nitrogen adsorption isotherm, specific surface area and average pore size are 34.3m^2·g^{-1} and 14nm, respectiviely. Moreover, the experiment result shows that mesoporous silica has a very high superficial density of amino groups (around 1.98mmol·g^{-1}).

Influence of assembling time on the activity of assembled PA is shown in Table 1. It is remarkable that the coupled yield and relative activity of assembled PA has reached 99.5% and 92.3%, respectively. PA is possibly coupled with silica, with multi-point attachments, because of the richness of the amino. On the other hand, the molecular crowding theory and modelling efforts have predicted that a protein inside a confined space will be stabilized by some folding forces not present in bulk solutions[6]. Therefore, when the enzyme assembles in mesoporous silica,

Table 1 Influence of assembling time on the activity of assembled PA

Assembling time for PA, h	Coupled yield, %	Relative activity, %	Activity yield, %
20	88.2	94.5	83.3
24	92.7	95.3	88.4
28	99.5	92.3	91.8
32	100	90.4	90.4

Note: Assembly conditions are 300mg support, 15ml PA solution (3.8mg PA, 0.1mol·L^{-1} phosphate buffer at pH7.0), and temperature 0—4℃.

nanopore-protein interactions, confinement, and crowding dramatically alter the stability and yield of the native state in restricted spaces[12]. The authors speculate that peptide chain unfolding will also be less and is likely to occur inside the confined space because of macromolecule crowding after penicillin acylase is confined inside the mesopore.

Figure 2 shows the influence of the amount of enzyme on the activity of the assembled PA. When the mass ratio of enzyme to silica is 38mg·g^{-1}, the specific activity of the assembled PA is 860U·g^{-1}, maximum in the experiments. The result is better than that of PA coupled to polymer carriers in the epoxy group[5]. Fig.3 shows that higher operational stability is obtained by recycling the immobilized penicillin acylase. The results confirm that the stability of PA immobilized and confined in the functional mesoporous silica is good. Macromolecular crowding mimicked by the confinement of nanopores[13] restricts the movement of the backbone and side chains of the protein molecule, thereby preventing intermolecular interaction and unfolding of the polypeptide chain. These advantages possiblly improve the stability of the enzyme and retain its activity after many recycles.

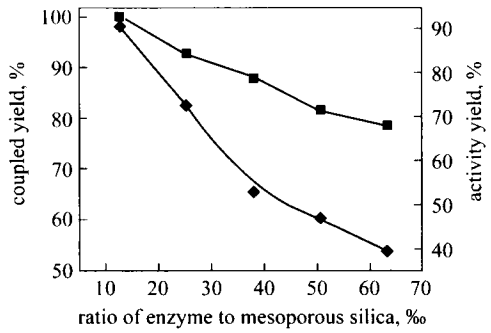


Figure 2 Effect of mass ratio of enzyme to mesoporous silica on the coupled yield and activity yield of assembled PA
■ coupled yield; ◆ activity yield

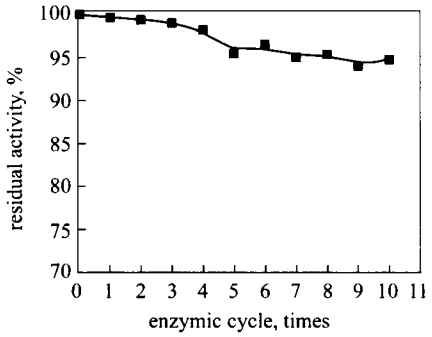


Figure 3 Operational stability of the covalent immobilized PA in the mesopores

In the coassembly of PA and β-cyclodextrin, 89.1% of PA was coupled with mesoporous silica after stirring for 24h, when the mass ratio of β-cyclodextrin to PA was 4%. The coupled yield was increased to 1.71 folds of that without cyclodextrin. It is possible that PA can be induced to approach and have a chance

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