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Improvement of the catalytic performance of immobilized penicillin acylase through assembly of macromolecular reagents in nanopore to create a crowding environment

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Abstract−Macromolecular reagents were co-assembled with penicillin acylase (PA) and immobilized in mesocellular siliceous foams (MCFs) to resemble living cells. Types and concentrations of macromolecules were studied. The catalytic characteristic and stability of PA preparations were also investigated. PA assembled with dextran 10 k in MCFs showed maximum specific activity, 1.32-fold of that of the solely immobilized PA. The optimum pH of dextran and BSA derivatives shifted to neutrality, and the optimum temperature increased by 10 °C. Also, Km of BSA derivative of PA declined 56.7% compared to solely immobilized PA, while the Kcat/Km of PA assembled with BSA was enhanced to 147%. After incubation at 50 °C for 6 h, residual activity of PA assembled with BSA exhibited 53.0%. The ficoll derivative showed 82.8% of its initial activity at 4 °C after 8-week storage. The results indicated that macromolecular reagents assembled with PA in MCFs could dramatically improve the catalytic performance and stability of im- mobilized enzyme.

Key words: Penicillin Acylase, Immobilization, Covalent, Mesocellular Siliceous Foams, Macromolecular Crowding

INTRODUCTION

Penicillin acylase (PA) is one of the major industrial biocatalysts in the enzymatic production of semisynthetic β-lactam antibiotics [1]. PA catalyzes the hydrolysis of penicillin G to produce 6-aminopenicillin acid (6-APA), which is a major pharmaceutical intermediate. Efficient immobilization of PA is one of the keys to successful adoption of the enzymatic cleavage of penicillin G [2,3]. The development of biocatalysts is necessary to make the production of 6-APA economically feasible. Nowadays, technologies have been focused on modification of carriers [4] and immobilization techniques [5,6].

Stabilization of enzyme is closely related to its structure and microenvironment [7,8]. Minton [9] pointed out that biochemical reactions within cells differed from those in test tubes. Cells contain numerous macromolecules, especially proteins, nucleic acids and complex sugars. These macromolecules, which create crowding environments, have been induced into enzymatic catalysis. The addition of natural or synthetic macromolecules to buffers enables crowding to be mimicked in vitro, and should be encouraged as a routine alternative to study [10,11]. Kazan et al. [12] investigated that dextran polymers could improve the thermal stabilization of PA, but they did not alter the optimum catalytic pH and temperature. Mislovicova et al. [13] reported that PA derivatives modified with various polysaccharides were stabile at pH 5-8, and the activities increased from 4% to 44%; however, the activity of modified PA was poor (5.78 U/mg protein).

In this study, we co-assembled macromolecules with PA on the wall of mesocellular siliceous foams (MCFs), to mimic the microenvironment resembling living cells (Fig. 1). Types and concentrations of macromolecules were optimized, the effects of temperature and pH on the catalytic activity of immobilized enzyme were studied, and their thermal and storage stability were also carefully investigated.

MATERIALS AND METHODS

1. Chemicals

Penicillin acylase (EC 3.5.1.11) from *Bacillus megaterium* was supplied by Shunfeng haider (Zhejiang, P.R. China), with a specific activity of 796 U/ml, and the protein concentraion of 10 mg/ml. Penicillin G sodium was bought from Xinhua Medicines Factory (Hebei, P.R. China). Bovine serum albumins (BSA, 67,000), dextran 10,000 (Dex 10k), dextran 40,000 (Dex 40k), and ficoll 70,000 (ficoll) were purchased from Sigma-Aldrich (Steinheim, Germany). Other chemicals were provided by Sinopharm Chemical Reagent (Shanghai, P.R. China) and all were analytical grade. Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

2. Preparation of PA Immobilized in MCFs with Macromolecular Reagents

Mesocellular Siliceous Foams (MCFs) was prepared by Schmidt-Winkel et al. [14] and our previous work [15,16]. The surface of the pristine support was functionalized by Kim et al. [17]. A portion of 20 mg of functionalized MCFs was activated by means of p-benzoquinone (2 mL, 0.1 mol/L) in 20% (v/v) alcohol solutions for 1 h. The activated supports were immersed in 2 mL phosphate buffers (PBS, pH 8.0, 0.1 mol/L) containing 150 μL PA solutions and suitable amount of macromolecular reagents. BSA, Dex10k, Dex40k and ficoll were chosen as the macromolecular reagents. Immobilization was carried out at 25 °C for 20 h in a gently shaking incuba-

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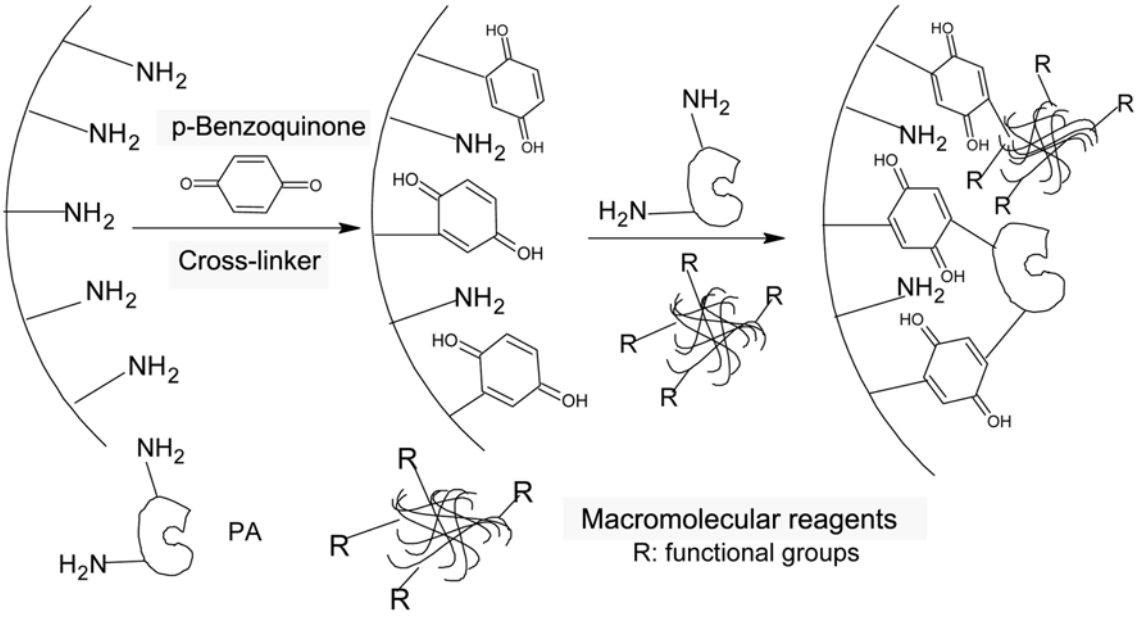

Fig. 1. Immobilization of penicillin acylase with macromolecular reagents in MCFs.

tor. Then the suspensions were separated by centrifugation. Washing with 0.01 mol/L PBS of pH 7.0 leached the extra protein in the composite out, until no protein could be detected in the supernatant. The remaining protein in the washing supernatant was checked by Bradford assay [18].

3. Enzymatic Activity Assay for PA Preparations

The hydrolytic activity of PA was measured by using PDAB (p-dimethylaminobenzaldehyde) method [19]. The reaction mixture was composed of 2% (w/v) penicillin G sodium, 5 mL of PBS (pH 8.0, 0.1 mol/L) and appropriate amount of soluble PA (20 μL) or immobilized PA preparations (5-10 mg), and then incubated at 37 °C for 3 min. One unit of enzymatic activity (U) is defined as the amount of enzyme required to produce 1 μmol of 6-APA. The supernatant protein was measured to determine the percentage of the assembled PA in MCFs. The coupled yield, relative activity and activity yield were calculated as the following equations.

$$\text{Coupled yield } (\%) = \frac{(A-B)}{A} \times 100 \quad (1)$$

$$\text{Relative activity } (\%) = \frac{C}{(A-B)} \times 100 \quad (2)$$

$$\text{Activity yield } (\%) = \frac{C}{A} \times 100 \quad (3)$$

Here, A represents the total activity of enzyme added before immobilization; B is the activity of the same amount of enzyme that remains in the supernatant after immobilization; C expresses the activity of the immobilized enzyme. All the experiments were performed in triplicate, and the standard error was never over 4%.

4. Influence of pH and Temperature on Activity of PA Preparations

The optimum pH of PA preparations was determined by hydrolysis of penicillin G sodium in PBS from pH 6.0 to 9.0. The optimum temperature was determined by checking the enzyme activity ranging from 25 °C to 60 °C. The enzymatic assays were according to the procedure described above.

5. Determination of Kinetic Characterization of PA Preparations

Kinetic parameters of PA preparations were determined in 0.1 mol/L PBS (pH 8.0). Km and Vmax were calculated by assaying the rates of producing 6-APA at different substrate concentrations ranging from 0.5% to 3.0% (w/v) and by plotting the Lineweaver Burk plots. The turnover number Kcat, which represents the maximum number of substrate molecules that the enzyme can turn over to produce in a set time, can be calculated according to the equation, Kcat=Vmax/[E], where [E] is the enzyme concentration at zero time of reaction. The specificity constant Kcat/Km represents the relative rate of reaction at low substrate concentrations ([S]<<Km), which is derived from equations above.

6. Thermal Stability and Storage Stability of PA Preparations

Soluble and immobilized preparations of PA were determined by measuring their residual activities after being incubated at 50 °C for 6 h in PBS (pH 8.0). A sample of PA preparations was removed at one-hour intervals and immediately cooled at room temperature. Substrates were added and assayed for enzymatic activity. PA preparations were also sealed and stored at 4 °C at intervals without further protection. Aliquots were taken periodically and their residual activities were determined. The initial value of enzymatic activity in each set was assigned the value of 100% activity.

RESULTS AND DISCUSSION

1. Effects of Content of Macromolecules on Immobilized PA

Fig. 2 shows the effects of content of macromolecules on the activity of PA preparations. The optimum macromolecular concentration was 7.5% (w/w) of the content of enzyme. Macromolecular reagents exert a lesser influence on the coupled yield of the immobilized enzyme (above 95%). Maximum coupled yield (98.9%) was

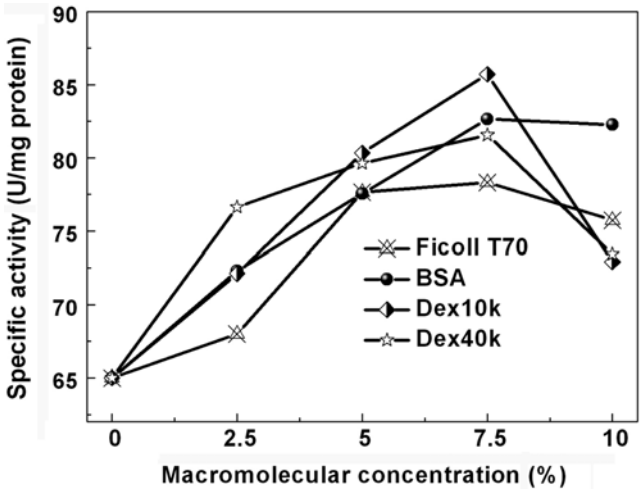

Fig. 2. Effect of content of macromolecules on the catalytic activity (U/mg protein) of PA preparations.

obtained when the mass ratio of PA to MCFs was 150 mg/g. The specific activity of PA co-assembled with Dex10k in MCFs, 85.7 U/mg protein, was 1.32-fold of that of the solely immobilized PA. The activity of wet immobilized PA with BSA was 1067.1 U/g immobilized enzyme, and the activity yield of Dex10k preparation was 90.6%. PA was possibly coupled with silica by multi-point attachments because of the richness of the amino on the surface of silica. The high percentage of immobilized protein and high activity of PA preparations demonstrated the effectiveness of immobilization technique and macromolecular crowding. In free macromolecular solutions, crowding reagents did not show a similar effect on the improvement of activity of PA (shown in Table 1), which indicated the co-assembly of macromolecular reagents in MCFs. According to the crowding theory and modeling efforts, when PA was assembled in MCFs, nanopore-protein interactions, adsorption and crowding environment could dramatically alter the stability and yield of the native state of protein in confined spaces [20]. When macromolecular reagents were co-immobilized in MCFs, they enhanced refolding rates and assembly of proteins [21,22]. Therefore, PA assembled with macromolecular reagents in MCFs exhibited better catalytic activity than solely immobilized enzyme.

2. Optimum pH and Temperature of PA Preparations

Fig. 3(a) shows the optimum pH of PA preparations. The optimum pH of immobilized PA shifted from alkaline to neutrality. The optimum pH for the activity of the PA assembled with dextran and BSA was 7.0, and that of other immobilized preparations and free enzyme was 8.0 and 8.5, respectively. These declines may be attributed to the loss of charges that were present on the surface of the

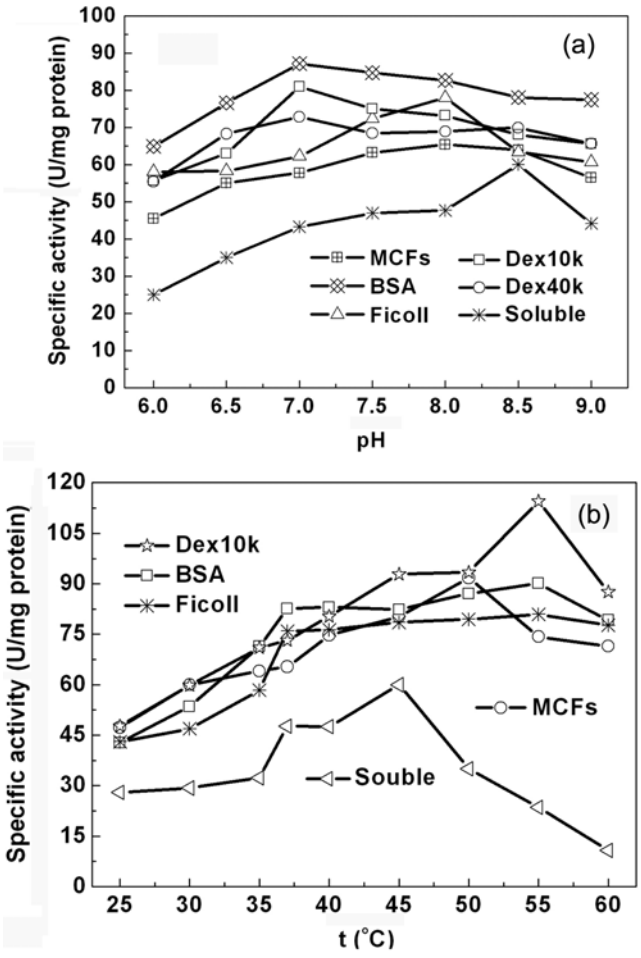

Fig. 3. Optimum pH (a) and temperature (b) of PA preparations. The immobilized preparations were prepared with 150 mg enzyme protein added to 1 g MCFs, and the amount of macromolecules was 7.5% of content of enzyme (w/w). MCFs, PA solely assembled covalently in MCFs; X, PA assembled with X covalently in MCFs (X: BSA, bovine serum albumin, 67,000; Dex 10k, dextran 10,000; Dex 40k, dextran 40,000; Ficoll, ficoll 70,000); Soluble, soluble PA. The concentration of macromolecules was 7.5% (w/w) of content of PA.

molecules during treatment with cross-linker and support. The positive charges might have increased the pH in the micro-environment, and resulted in the decline of the optimum pH, which was suited for PA in macro-environment. Moreover, free enzyme deactivated rapidly when pH was below 7.0. It was also proposed that the addition of crowding reagents should become as routine as controlling pH [23]. Broader adaptation to pH was observed for immobilized enzyme assembled with macromolecules, which is advantageous for

Table 1. Effects of crowding reagents on PA catalysis in free solutions

Environment for PA catalysis	PBS	BSA	Ficoll	Dex10k	Dex40k
Specific activity of free PA (U/mg protein)	48.4	47.0	48.2	51.4	48.9
Specific activity of solely immobilized PA (U/mg protein)	65.4	66.1	65.3	66.7	65.7

PBS, PBS solutions without crowding reagents; BSA, PBS solutions with BSA 67,000; Ficoll, PBS solutions with ficoll 70,000; Dex 10k, PBS solutions with dextran 10,000; Dex 40k, PBS solutions with dextran 40,000. The macromolecules were in free solutions, and their concentration was 7.5% (w/w) of the content of PA

Table 2. Kinetic parameters for PA preparations with macromolecules

Kinetic parameters	Macromolecular reagents				
	MCFs	Ficoll	Dex10k	Dex40k	BSA
Km [10^{-3}, mol/L]	6.60	4.55	9.57	4.25	3.74
Vmax [10^{-5}, mol/(L·min)]	1.05	0.82	0.97	0.84	0.81
Kcat/Km [10^{7}, mol/(L·min)]	0.85	1.23	0.60	1.24	1.48

MCFs, PA solely assembled covalently in MCFs; X, PA assembled with X covalently in MCFs (X: BSA, bovine serum albumin; Dex 10k, dextran 1,000; Dex 40k, dextran 4,000; Ficoll, ficoll 7,000). The concentration of macromolecules was 7.5% (w/w) of content of PA

the enzymatic production of 6-APA.

Fig. 3(b) shows the temperature profiles for the catalytic activity of PA preparations. The activities of immobilized preparations were greater at each temperature compared to free PA. The optimum temperature for dextran and BSA derivatives was both 55 °C, 5 °C higher than other immobilized preparations, whereas its soluble counterpart was 45 °C. Instead of a sharp peak, we observed broad peaks for the activity of immobilized enzyme with respect to temperature. The better adaptation to temperature of PA preparations may be attributed to the confinement of channels in MCFs and the crowding structures of macromolecules. Broadening of the temperature activity profile indicates that the macromolecules assembled with PA can alleviate the deactivation of enzyme to temperature.

3. Determination of Kinetic Parameters of PA Preparations

Table 2 shows kinetic parameters of immobilized PA preparations. Usually, a separate analysis for Kcat and Km was used; nevertheless, the combined term Kcat/Km would summarize the net advantage or disadvantage of the enzyme in one constant [24]. A distinct decrease in Km value of the PA co-assembled with BSA, 56.7% compared to the solely immobilized PA, showed higher affinity to substrate. The Kcat/Km of the BSA preparation was an order of magnitude larger than solely immobilized PA, indicating high reaction rate at an appropriate low substrate concentration. In existence of macromolecular association, crowding was demonstrated to increase the intrinsic catalytic efficiency of enzyme [25]. Macromolecules confined in supports would be expected to enhance the relative abundance of the substrate-enzyme complex and hence the forward reaction rate [26]. The spheral shape of BSA, analogous to the nanopore of MCFs, could account for the best catalysis performance of assembled PA with macromolecules. The combination of BSA with PA could stabilize enzyme configuration in nanopore, and improve the affinity of enzyme to substrate.

4. Thermal Stability of PA Preparations

In general, thermal inactivation at process temperature is the main factor for immobilized enzyme decay in reactor operation. The effects of heating duration on the activity of PA were investigated by pre-incubating PA preparations at 50 °C (Fig. 4(a)). After incubation for 6 h, residual activity of PA co-immobilized with BSA and Dex10k remained at 53.0% and 50.5%, respectively, whereas that of the solely immobilized PA was 29.9%, compared to a sharp decrease of its soluble counterpart. The amelioration in thermal stability of the enzyme assembled with macromolecules may arise from the conformational integrity of the enzyme structures. This would make the

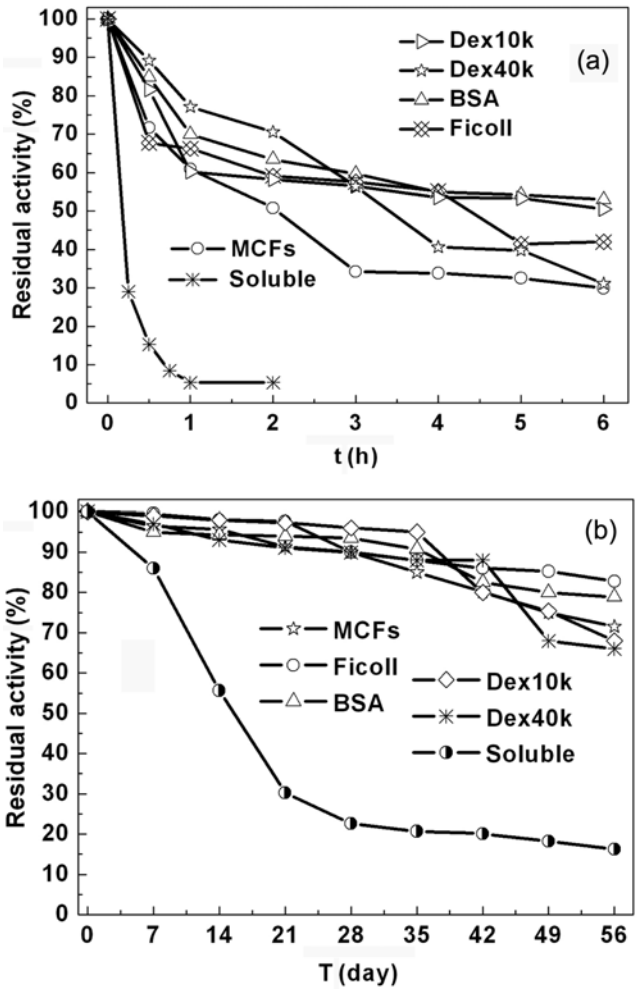

Fig. 4. Thermal stability (a) and storage stability (b) of PA preparations. Thermal treatment condition: at 50 °C in 0.1 mol/L PBS of pH 8.0; Storage condition: 4 °C in refrigerator without further management.

surface suitable for PA to modulate its structure and enhance the thermal stability of the enzyme preparations. The results showed that crowding environment may have favorable effects in stabilizing the spatial conformation of enzyme in MCFs.

5. Storage Stability of PA Preparations

The storage stability of PA preparations at 4 °C, as depicted in Fig. 4(b), was considerably higher than that of free enzyme. PA assembled with ficoll and BSA remained at 82.8% and 78.9% of initial activity after 8 weeks. In contrast, the solely assembled PA and its soluble counterpart were reduced to 71.5% and 16.2%, respectively. The improvement of protein stabilization in MCFs was consistent with that proposed by the crowding theory. Also, a suitable amount of crowding reagents, which occupied substantial volume in PA preparations, maintained the natural structure of enzyme in catalysis. The results showed that PA assembled with BSA and ficoll exhibited better storage stability than reported in the literature [27].

CONCLUSIONS

Macromolecular reagents were successfully immobilized with

PA in MCFs resembling living cells based on crowding theory. The addition of macromolecular reagents, both synthetic (dextran and ficoll) and natural (BSA), could improve the catalytic properties of immobilized PA, and protect PA against thermal damage and storage deactivation. Co-assembly of PA with appropriate macromolecular reagents could be used as a high performance biocatalyst in biotechnological processing, in particular for medical production. Therefore, the use of a suitable amount of macromolecular reagents seems to be an efficient tool to improve the control of the immobilization and the properties of final immobilized PA preparations, and more effort may be worthy to try in other biocatalysts in this regard.

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