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# Biosynthesis of cefprozil in an aqueous two-phase system composed of pH-responsive copolymers and its crystallization analysis

Hongxia Pan, Ting Yang, Jialing Chen, Xuejun Cao*

*State Key Laboratory of Bioreactor Engineering, Department of Bioengineering, East China University of Science and Technology, Shanghai 200237, China*

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ABSTRACT

Cefprozil, a semi-synthetic antibiotic, has been used in pediatric therapy. The purpose of this study was to improve the yield of cefprozil and obtain purer product in an aqueous two-phase system composed of $P_{ADB}$ and $P_{MDB}$. $P_{ADB}$ and $P_{MDB}$ were synthesized using acrylic acid, methacrylic acid, 2-dimethylaminoethyl methacrylate, and butyl methacrylate. Cefprozil was synthesized with 7-amino-3-(1-propenyl)-4-cephalosporanic acid (7-APRA) as the main ring, (*R*)-amino-(4-hydroxyphenyl) acetic acid methyl ester hydrochloride (D-HPGME-HCl) as the acyl donor and the immobilized penicillin acylase as the catalyst. 7-APRA and D-HPGME-HCl were concentrated in the $P_{MDB}$-rich phase, while cefprozil was in the $P_{ADB}$-rich phase. The optimal partitiona coefficient of cefprozil was 1.84 with 40 mM KSCN, while the enzyme reaction yield of cefprozil reached 99.39% compared with 77.61% in the aqueous-phase system. The recovery of the two copolymers was up to 96.01% at pH 3.54. After crystallization, the purity of the product reached 88.02%, and no copolymer remained in the product. The combination of cefprozil·DMF was detected by gas chromatography-mass spectrometry, FT-IR spectroscopy, thermogravimetric analysis, and electrospray ionization mass spectrometry. The results showed that no chemical bond existed between cefprozil and DMF.

## 1. Introduction

Cefprozil is an oral cephalosporin antibiotic that inhibits the synthesis of the cell walls of gram-positive and gram-negative aerobic bacteria. Cefprozil is primarily used in the treatment of respiratory tract infections such as recurrent acute otitis media [1,2]. The safety and clinical efficacy make cefprozil a competitive antibiotic in therapy [3].

The traditional synthesis of cefprozil involves a chemical technology, which is expensive. Enzyme catalysis is an environmentally friendly approach for biotransformation [4,5], and efficient catalysts could increase the reaction rate and improve the yield. However, efficient substrate conversion remains a problem [6–8]. The accumulation of products would restrain the enzyme activity and accelerate the side reaction (hydrolysis). Many strategies were reported to improve the synthesis process of cefprozil [9,10]. In an aqueous two-phase system (ATPS), mass transform could be easily attained by the two incompatible phases. It is an extractive bioconversion option that reduces the inhibition of biocatalyst by in situ removal of product from substrate while sustaining the reaction.

We developed a method to separate cefprozil efficiently from the enzyme and substrate. An ATPS with a low interfacial tension composed of two incompatible copolymers or one copolymer and one salt was applied for efficient separation [11]. The ATPS has been used to extract biomolecules such as proteins, antibiotics, monoclonal antibodies, nucleic acid, and enzymes [10,12–16] under mild biotransformation conditions with high extraction recovery. The ATPS could be an alternative to the single-phase system. The pH-responsive ATPS is considered an effective and economical way to separate substrate and product. The laboratory-scale synthesis of $P_{MDB}$ [17] and $P_{ADB}$ [18] had already been studied in Cao's group. The $P_{ADBA}/P_{MDB}$ system was applied for cephalexin synthesis, and $P_{ADB}/P_{ADBA}$ was used to distribute demeclocycline.

$P_{ADB}$ and $P_{MDB}$ could be recycled, making the pH-responsive ATPS a competitive method for biosynthesis. In this article, $P_{ADB}$ and $P_{MDB}$ were scaled-up, and a pH-responsive ATPS was obtained. This system was used as a medium to synthesize cefprozil optimally with a high yield and crystallized purity. Moreover, the products were characterized to support the application of ATPS in the bioconversation of cefprozil. The reaction process is shown in Fig. 1.

* Corresponding author.
*E-mail address:* caoxj@ecust.edu.cn (X. Cao).

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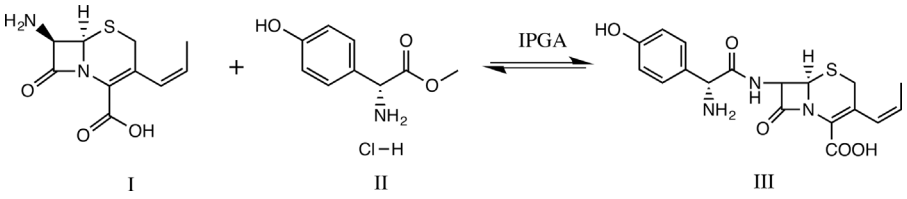


**Fig. 1.** Enzyme reaction process of cefprozil. (I) 7-APRA. (II) D-HPGME-HCl. (III) cefprozil. The abbreviations are listed in the Section 2.1.

## 2. Material and methods

### 2.1. Materials

Acrylic acid (AA), 2-dimethylaminoethyl methacrylate (DMAEMA), butyl methacrylate (BMA), methacrylic acid (MAA), ammonium persulfate (APS), and sodium disulfide ($NaHSO_3$) were obtained from Ling Feng Co., Ltd. (Shanghai, China). *N*,*N*-Dimethylformamide (DMF) was obtained from Aladdin reagents Co., Ltd. (Shanghai, China). Immobilized penicillin G acylase (IPGA) was obtained from Flag Biotechnology Co., Ltd. (Hunan, China). (6*R*,7*R*)-7-Amino-8-oxo-3-(1-propenyl)-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid (7-APRA) was obtained from Kangxin Co., Ltd. (Nantong, China). (R)-Amino-(4-hydroxyphenyl) acetic acid methyl ester hydrochloride (D-HPGME-HCl) was purchased from Vita Co., Ltd. (Shanghai, China).

### 2.2. Scale-up copolymer $P_{ADB}$ and $P_{MDB}$

Laboratory-scale synthesis of copolymer $P_{ADB}$ and $P_{MDB}$ was already reported in a 120-mL system. Here, we scaled up the synthesis to 2.4 L.

The $P_{ADB}$ scaled-up synthesis process was as follows: 2.8 g APS and 2.8 g $NaHSO_3$ were used as initiators and were added to 2.4 L water containing 100 mL AA, 90 mL DMAEMA, and 4 mL BMA. The entire synthesis process was conducted under $N_2$ protection. After polymerization at 55 °C and 200 rpm for 12 h, the precipitate was washed with ethanol three times and then dried at 55 °C. The $P_{MDB}$ scaled-up process in 2.4 L was as follows: 1.47 g APS and 1.47 g $NaHSO_3$ were used as initiators and were added to 2.4 L water containing 100 mL MAA, 10.6 mL DMAEMA, and 10 mL BMA. The entire synthesis reaction was conducted under $N_2$ protection. After polymerization at 55 °C and 200 rpm for 12 h, the polymer was dissolved in 2 M NaOH and re-precipitated in 2 M HCl by adjusting the pH and then was dried to a constant weight at 55 °C.

### 2.3. Copolymer recycling

$P_{ADB}$ and $P_{MDB}$ are pH-responsive polymers and could be recycled by regulating the pH of the copolymer solution. As the pH of the copolymer solution reached a specific value where the copolymer surface charge was zero, this value was defined as the isoelectric point (p*I*). The p*I* values of $P_{ADB}$ and $P_{MDB}$ were determined by Zetasizer Nano ZEN3600 (Malvern). The pH of $P_{ADB}$ solution was from 2.9 to 5.8 and that of the $P_{MDB}$ solution was from 2.7 to 4.6. $P_{ADB}$ and $P_{MDB}$ could be recycled by adjusting the solution pH to their respective p*I* value.

The ATPS was composed of 8% (w/v) $P_{ADB}$ and 6% (w/v) $P_{MDB}$ mixed at the same volume. The recovery of $P_{ADB}/P_{MDB}$ was measured by the dry weight method depending on the p*I* of $P_{ADB}$ and $P_{MDB}$. The pH of $P_{ADB}/P_{MDB}$ solution was regulated between 2.7 and 4.3 to precipitate copolymers simultaneously.

All the precipitate was centrifuged at 6000 rpm for 30 min and was dried at 55 °C to a constant weight. All the experiments were repeated three times.

### 2.4. Partition coefficient of 7-APRA, D-HPGME-HCl, and cefprozil in $P_{ADB}/P_{MDB}$

The enzyme reaction was conducted in ATPS, which was formed by dissolving $P_{ADB}$ and $P_{MDB}$ in a 150 mM NaOH solution. Different concentrations of salts were added into the ATPS to optimize the partition coefficient of cefprozil. KSCN and NaSCN (at concentrations from 10 mM to 100 mM) were added to $P_{ADB}/P_{MDB}$. All the phase-forming processes were controlled at 37 °C and pH 5.40.

The $P_{ADB}/P_{MDB}$ solution was settled into a water bath until a distinct interphase appeared. Next, 100 μL samples were collected from the top and bottom phases, and 900 μL of water was added to the samples and the solution was filtered through 0.45 μm MCE (WondaDisc II). High-performance liquid chromatography (HPLC, SHIMADZU, Japan) was used to measure the concentration of 7-APRA, D-HPGME-HCl, and cefprozil in the top and bottom phases. The HPLC conditions were as follows: A: 0.05 M phosphate (pH 4.40)/B: methanol ($V_A$:$V_B$ = 90:10) as the mobile phase. The flow rate was 1.0 mL/min, a reversed phase C18 column (150 mm × 4.6 mm, 5 μm) was applied, and 280 nm was chosen as the determining wavelength. From the standard curve, the concentration ratio of the top phase to bottom phase was calculated as the partition coefficient [19].

### 2.5. Enzyme reaction

In this enzyme reaction, using 7-APRA as a main ring and D-HPGME-HCl as an acyl donor, cefprozil was synthesized by the catalysis of IPGA. To maximize the yield, three experiments were designed: an enzyme reaction in a single-phase system, an enzyme reaction in ATPS, and an enzyme reaction in ATPS with salt. The single-phase system consisted of a phosphate solution. The optimal enzyme reaction conditions in ATPS were as follows: 49 mmol/L 7-APRA, 98 mmol/L D-HPGME-HCl, 20 °C, pH 5.40, 4.6 U/mL IPGA. The optimal salt concentration in $P_{ADB}/P_{MDB}$ was 40 mM KSCN. During the enzyme reaction, 50 μL of reaction solution was sampled every 30 min and was diluted with 950 μL of water. These samples were detected by HPLC, and changes in the product yield with time were analyzed.

### 2.6. Crystallization

After removing the IPGA sediment, the copolymers were recycled by adjusting the solution pH. After centrifugation, the supernatant was used for crystallization. The effect of DMF amount on the cefprozil yield was studied during crystallization.

The crystallization was conducted at 4 °C for 1 h, followed by centrifugation for 30 min at 6000 rpm. The precipitate (crude product) was washed with water, and the crystal was shifted by regulating the temperature to obtain a higher purity of cefprozil. The crystal was re-centrifuged and dried at 37 °C in a vacuum oven.

### 2.7. Analysis of product

In 1 mL water, 0.5 mg dried product was dissolved. The concentration of cefprozil in this solution was measured by HPLC, and the purity of the product was calculated.

The phenomenon of phase formation is influenced by the differences in features between two copolymers such as the structure, molecular weight, viscosity, and particle size. The particle sizes of copolymers and cefprozil that crystallized in different ways were all evaluated by dynamic light scattering (DLS) at 25 °C, pH 5.40. The particle sizes of $P_{ADB}$ and $P_{MDB}$, as well as that of the residual copolymers in the product,