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Enhanced Enzymatic Production of Cephalexin at High Substrate Concentration with *in situ* Product Removal by Complexation

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Summary

Cephalexin (CEX) was synthesized with 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) and D(–)-phenylglycine methyl ester (PGME) using immobilized penicillin G acylase from *Escherichia coli*. It was found that substrate concentration and *in situ* product could remarkably influence the ratio of synthesis to hydrolysis (S/H) and the efficiency of CEX synthesis. The optimal ratio of enzyme to substrate was 65 IU/mM 7-ADCA. High substrate concentration improved the 7-ADCA conversion from 61 to 81 % in the process without *in situ* product removal (ISPR), while in the synthetic process with ISPR, high substrate concentration increased the 7-ADCA conversion from 88 to 98 %. CEX was easily separated from CEX/β-naphthol complex and its purity and overall yield were 99 and 70 %, respectively.

Key words: cephalexin, complexation, enzymatic synthesis, *in situ* product removal, penicillin G acylase

Introduction

Semisynthetic β-lactam antibiotics are important pharmaceuticals, corresponding to 65 % of the rising worldwide production of antibiotics (*1*). Traditional methods of industrial production of these drugs by chemical route have several disadvantages, for example, low temperature equipment (–30 °C or even lower), complicated modification procedures (*e.g.* protection and deprotection of side groups), hazardous chemicals and solvents (such as silylation reagents and methylene chloride), and a lot of non-recyclable waste generated (*2*). In recent years, biocatalytic strategy has been considered as an alternative to replace the chemical method for the production of semisynthetic β-lactam antibiotics due to its high specificity and low environmental pollution (*3*). Penicillin G acylase (PGA, EC 3.5.1.11) is the most commonly used catalyst in pharmaceutical industry to produce 6-aminopenicillanic acid (6-APA) or 7-amino-3-deacetoxycephalosporanic acid (7-ADCA), which are the key intermediates for production of various important semisynthetic antibiotics.

Enzymatic synthesis of β-lactam antibiotics can be conducted under thermodynamic or kinetic control. The thermodynamic strategy is the direct condensation of the nucleophile and the acyl donor. Non-ionic forms of the substrates are required for the condensation reaction, and the yield is determined by the thermodynamic equilibrium of the reaction. Cosolvents may be helpful in this strategy by altering the p*K* value of the carboxylic acids and by depressing the water activity. However, the main drawbacks of this strategy are low activity and stability of the enzyme caused by organic solvent (*4*). In contrast to the thermodynamic control, the kinetic

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control is an interesting strategy used in the synthetic process. To achieve a non-equilibrium concentration of the product, the substrate (acyl donor) must be activated as an amide, ester or anhydride. By this means the amino group can be partly uncharged at the optimal pH value of the enzyme (*5*).

Cephalexin (CEX) is one of the semisynthetic cephalosporins that are widely used for therapeutic purposes. In the kinetic control synthesis of CEX, the hydrolysis of the activated acyl donor and the product take place simultaneously (Fig. 1). The efficiency of the synthetic reaction can be characterized using two parameters, the yield of 7-ADCA (the more expensive substrate) and the ratio of synthesis to hydrolysis (S/H) ([CEX]/[PG]) (*6*). By mathematical modeling of batch and semi-batch reactors for enzymatic synthesis of amoxicillin, Gonçalves *et al.* (*7*) found that the semi-continuous operation of the integrated reactor (reaction+crystallization) provided better results than the batch mode. *In situ* product removal (ISPR) techniques have been increasingly applied in biocatalytic fields by removing the product as soon as it is formed (*8*). In earlier literature, ISPR techniques were performed by complexation (*6,9*), adsorption using resins, (*e.g.* Amberlite XAD) (*10*) and aqueous two-phase systems (*11*). In contrast to the adsorption and aqueous two--phase systems, the complexation technique was an efficient removal technique because only the CEX was bound in the whole reaction process. It was reported that cephalosporins could complex efficiently with aryl compounds to form insoluble clathrate-type compounds, *e.g.* β-naphthol for CEX (*6*) and α-naphthol for cefaclor (*9*). Recently, Youshko *et al.* (*12*) have indicated that the penicillin acylase-catalyzed ampicillin synthesis in aqueous solution was highly dependent on the initial substrate concentrations. There were several reports about catalytic reactions with PGA at high substrate concentration or substrate supersaturation system (*13,14*), whereas the results for combining high substrate concentration with ISPR have almost no reports. In this work, kinetic control synthesis of CEX with PGA was studied at high substrate concentration system by ISPR using β-naphthol as a complexing agent (Fig. 2), and the separation of CEX from CEX/β-naphthol complex was also studied.

Materials and Methods

Enzyme and chemicals

Penicillin G acylase from *Escherichia coli*, immobilized on oxirane acrylic beads (diameter 150–300 μm) with enzyme activity of approx. 380 IU/g of wet mass, was purchased from Hunan Flag Ltd., China. D(–)-Phenylglycine was from the Shanghai Industrial Chemical Co. Ltd., China. D(–)-Phenylglycine methyl ester hydrochloride (PGME) and CEX hydrate were purchased from Sigma Chemical Co. Inc., USA. Penicillin G potassium salt and 7-aminodeacetoxymethylsporanic acid (7-ADCA) were kindly donated by North China Pharmaceutical Co. Ltd., China. β-Naphthol and all other reagents were of analytical grade and commercially available.

HPLC analysis

Samples were analyzed by high-performance liquid chromatography (HPLC) using an Agilent G1311A pump, an Agilent Zorbax XDB C18 column (4.6×250 mm, 5-μm particle size and 8-nm pore size) and an Agilent G1315B UV detector. The mobile phase consisted of 15 % (by volume) acetonitrile and 85 % (by volume) phosphate sodium buffer (20 mM, pH=6.5). The flow rate was 1 mL/min. Detection was done simultaneously at 254 nm for 7-ADCA and CEX and 214 nm for PGME and PG.

Cephalexin synthesis

CEX synthesis was done according to Yang *et al.* (*15*) with slight modification and it is demonstrated in Fig. 2. Reactions were carried out in a thermostated cell with a pH controller at 25 °C under permanent stirring. An aqueous solution containing appropriate amounts of PGME and 7-ADCA was incubated for 10 min at pH=6.5. The reaction was started by adding the immobilized en-

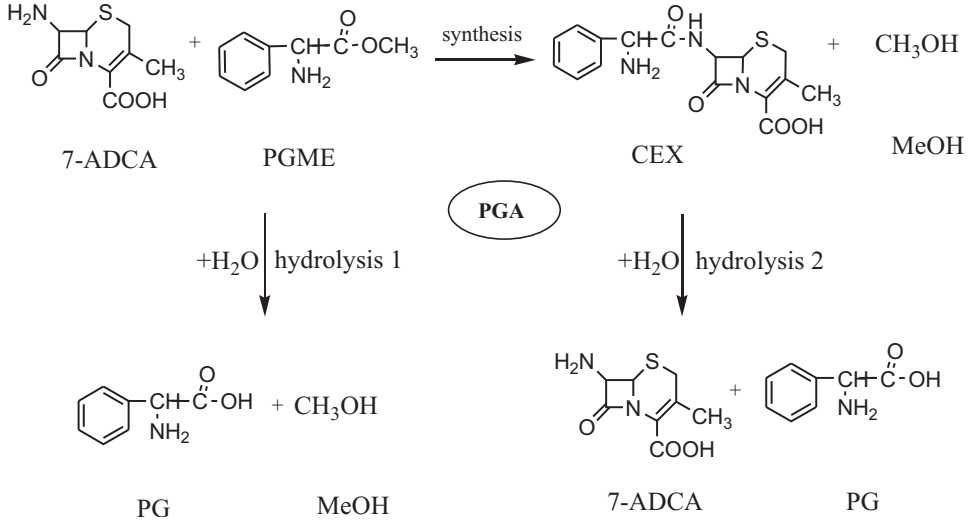

Fig. 1. Reaction mechanism of PGA-catalyzed synthesis of cephalexin. 7-ADCA=7-amino-3-deacetoxycephalosporanic acid, PGME= =D(–)-phenylglycine methyl ester, CEX=cephalexin, MeOH=methanol, PG=D(–)-phenylglycine

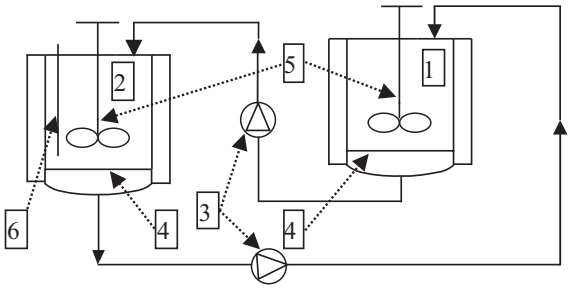

Fig. 2. Diagram of PGA-catalyzed synthesis of cephalexin with *in situ* product removal. 1. complexing reactor, 2. enzyme reactor, 3. peristaltic pump, 4. sintered glass, 5. mechanical stirrer, 6. pH controller

zyme to the reaction mixture. The pH was kept constant by automatic titration of 1 M ammonia solution. The whole reaction volume was 100 mL and the flow rate of solution by peristaltic pump was 15 mL/min. Samples of 100 μL were taken from the reaction mixture at different times, added to the eluent (900 μL) in order to dilute the sample and to stop the enzymatic reaction, and subjected to HPLC analysis. In the case of high substrate concentration system, two samples were analyzed: one was removed by 0.45-μm filter in order to separate solids from the solution, and the other aliquot of the system was taken for characterization of the whole reaction mixture.

Cephalexin separation

The reaction was stopped at the maximum conversion of 7-ADCA. CEX/β-naphthol complex in complexing reactor was filtrated, suspended in 200 mL of equal volumes of butyl acetate and sulphuric acid solution (pH=1.0). The mixed solution was stirred for 1 h at room temperature. The aqueous layer was concentrated to a yellow sticky solution under reduced pressure and then the residue was crystallized from equal volumes of isopropanol and water to form CEX hydrate.

Determination of penicillin G acylase activity

PGA activity was determined by a spectrophotometric assay with *p*-dimethylaminobenzaldehyde (PDAB) as a colorimetric substrate (*16*). One unit of PGA was defined as the amount of enzyme required to produce 1 μmol of 6-APA per minute in 4 % (mass per volume) solution of penicillin G at 37 °C and 0.1 M phosphate buffer (pH=7.8).

Determination of 7-ADCA solubility

The solubility of 7-ADCA at different PGME concentrations was measured according to Illanes *et al.* (*14*). Substances were added to the point of exceeding saturation and kept under agitation at controlled 25 °C and pH=6.5 (in 20 mM phosphate buffer). After 2 h, solids were removed by centrifugation and the supernatant was assayed by HPLC. Controls were run at the same time with dissolved 7-ADCA to determine any chemical degradation.

Determination of the ratio of synthesis to hydrolysis

The ratio of synthesis to hydrolysis (S/H) was determined according to Youshko *et al.* (*12*) by measuring the initial accumulation rates of CEX and PG in the same reaction medium.

Results and Discussion

Solubility of 7-ADCA at different PGME concentrations

Generally, solubility of 7-ADCA increased with high temperature and high pH of the reaction system. However, the high temperature and high pH could also increase the reaction rate for hydrolysis of PGME. At the same time, the stability of the enzyme decreased. According to literature (*17*), the optimal pH for the synthesis of CEX is 6.0–7.0. In addition, taking into consideration that CEX is unstable at the pH over 6.5, the pH of the reaction system was determined at 6.5. The solubility of 7-ADCA at different PGME concentrations was investigated (results shown in Fig. 3). and it was found that it was only about 52 mM in the aqueous solution, while it exceeded 120 mM at 0.8 M PGME. Therefore, it was concluded that the solubility of 7-ADCA increases in the presence of PGME. A similar effect was reported about the solubility of 6-APA in the presence of acyl donor (*14*). From the above results, it was deduced that this increase may be caused by instantaneous change of pH of the solution. Because 7-ADCA is a zwitterion, the pH of the solution could be altered instantaneously by the presence of PGME hydrochloride. In order to prove this supposition, the solubility of 7-ADCA at different concentrations of hydrochloric acid was further studied. Unfortunately, the solubility of 7-ADCA was not affected by the hydrochloric acid (data not shown), so the mechanism of the increase of 7-ADCA solubility driven by PGME has remained obscure and needs to be further investigated. However, this phenomenon may have a positive effect on the acyl transfer from PGME to the β-lactam nucleus in the synthetic process.

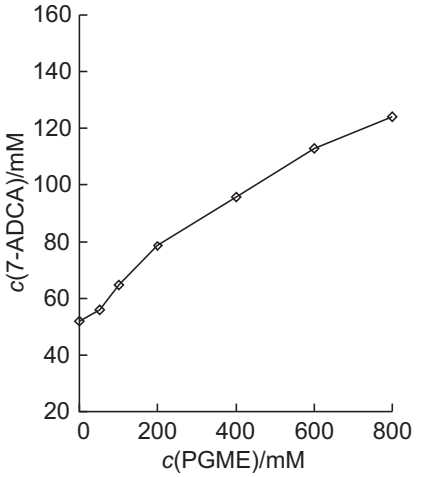

Fig. 3. Solubility of 7-ADCA at different concentrations of PGME. Reaction conditions: 25 °C and 20 mM phosphate buffer (pH=6.5)

Effects of the ratio of enzyme to substrate and substrate concentration on the S/H

High selectivity of PGA (*i.e.* S/H) is very important for kinetically controlled synthesis of β-lactam antibiotics. The results show that the S/H from initial rates increased with the increase of enzyme to substrate ratio, and the optimal ratio was found to be 65 IU/mM of 7-ADCA (Fig. 4). High substrate concentration could also improve the S/H ratio. Popular hypothesis for the synthetic scheme (Fig. 1) is that the reactions take place simultaneously, *i.e.* PGME binds to the immobilized enzyme giving an acyl-enzyme complex. This complex can either be hydrolyzed by water to give the enzyme and phenylglycine, or it can react with 7-ADCA to give CEX and the enzyme. The CEX can in turn bind to the enzyme, giving the acyl-enzyme complex that can either be hydrolyzed or it can react back to CEX (*18*). It could be concluded that water concentration was low at high substrate concentration system, which increased the probability of a nucleophilic attack on the acyl-enzyme that came from the nucleus rather than from the molecule of water, so the S/H was improved at high substrate concentration.

Cephalexin synthesis

CEX synthesis with PGA was investigated using 65 IU/mM of 7-ADCA at different substrate concentrations, and better results were obtained at higher substrate concentrations both with and without ISPR (Fig. 5). The maximum 7-ADCA conversion was only 61 % at low substrate concentration, while it increased to 81 % at high substrate concentration in the process without ISPR. When the synthetic process was performed with ISPR, the maximum 7-ADCA conversion was 88 % at low substrate concentration and then increased to 98 % at high substrate concentration. Accordingly, the 7-ADCA conversion increased 20 and 10 % in the absence and presence of the complexing agent, respectively, due to the high substrate concentration.

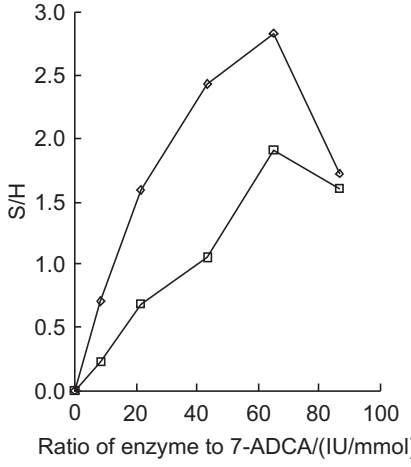

Fig. 4. Effects of the ratio of enzyme to substrate and substrate concentrations on the S/H. Reaction conditions: 25 °C, 20 mM phosphate buffer (pH=6.5) and molar ratio of PGME/7-ADCA= =2:1. (□) 50 mM 7-ADCA, (◊) 150 mM 7-ADCA

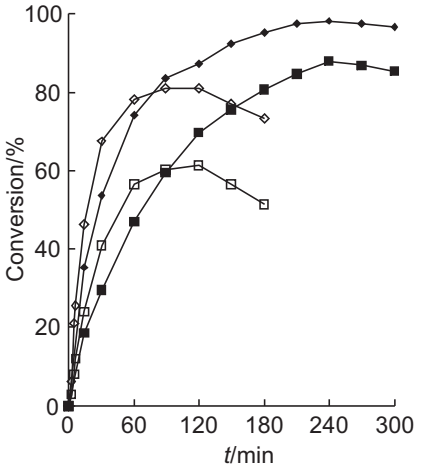

Fig. 5. 7-ADCA conversion at different substrate concentrations with or without ISPR. Reaction conditions: 25 °C, 20 mM phosphate buffer (pH=6.5) and molar ratio of PGME/7-ADCA=2:1. (□) 50 mM 7-ADCA without ISPR, (◊) 150 mM 7-ADCA without ISPR, (■) 50 mM 7-ADCA with ISPR, (♦) 150 mM 7-ADCA with ISPR

From the time courses of CEX and 7-ADCA, it was shown that the CEX solubility was poor in the aqueous solution. The concentration of synthesized CEX was under its saturation at low substrate concentration, so it was in dissolved form and its increase in proportion to the decrease of 7-ADCA (Fig. 6a). When the concentration of synthesized CEX was over its saturation at high substrate concentration, it took two forms, *i.e.* dissolved and precipitated CEX (Fig. 6b). In the process without ISPR, the synthesized CEX calculated by the 7-ADCA conversion matched reasonably with CEX measured by summing up dissolvable CEX and precipitated CEX (Fig. 6b, triangle). Since in the synthetic process using complexing agent most of the synthesized CEX was precipitated in CEX/β-naphthol form, CEX in the solution was only about 5 mM in the reaction system, which prevented efficiently the undesired CEX hydrolysis and therefore higher 7-ADCA conversion was obtained.

Cephalexin separation from cephalexin/β-naphthol complex

β-Naphthol was an efficient agent for *in situ* removal of CEX. Furthermore, it is also important whether CEX can be separated easily and efficiently from CEX/β-naphthol complex. That is why the CEX was further separated and crystallized by the experimental method mentioned above. The purity and the overall yield of CEX hydrate were 99 and 70 % respectively, and the results supported the application of enzymatic synthesis of β-lactam antibiotics as an alternative to traditional chemical ones.

Conclusions

In this work, it was observed that the solubility of 7-ADCA was substantially increased in the presence of PGME. The optimal ratio of enzyme to substrate was 65 IU/mM of 7-ADCA. High substrate concentration im-

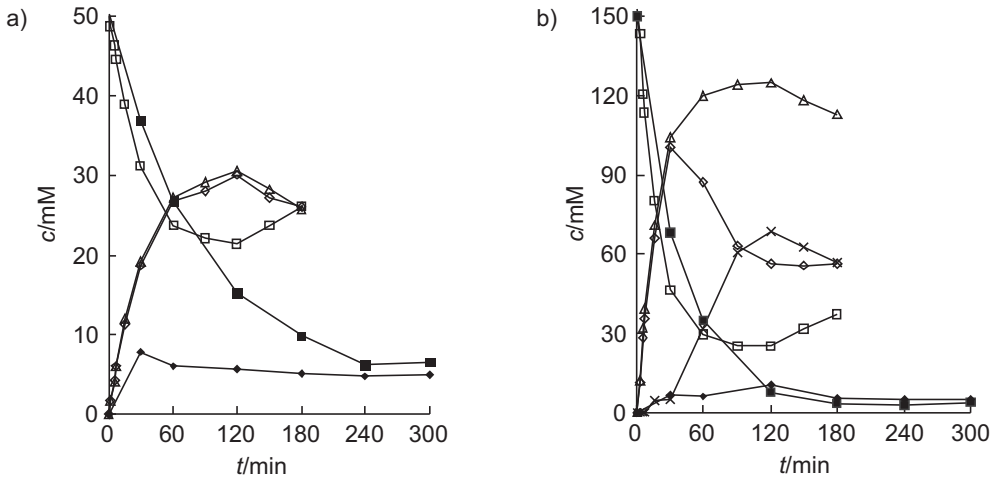

Fig. 6. Time courses of CEX and 7-ADCA at different substrate concentrations with or without ISPR. Reaction conditions: 25 °C, 20 mM phosphate buffer (pH=6.5) and molar ratio of PGME/7-ADCA=2:1. a) 50 mM 7-ADCA, b) 150 mM 7-ADCA. (□) 7-ADCA without ISPR, (■) 7-ADCA with ISPR, (◇) CEX without ISPR, (♦) CEX with ISPR; (△) CEX calculated by the conversion of 7-ADCA; (×) precipitated CEX without ISPR

proved the S/H. These results gave positive effects on the acyl group transferring to β-lactam nucleus. The synthetic process of CEX can be improved efficiently by β-naphthol complexation, especially at high substrate concentration. CEX was also easily separated from CEX/β-naphthol complex. Therefore, the strategy of using initially high substrate concentration with ISPR was easily performed and showed favourable prospects in industrial application due to its higher 7-ADCA conversion and fewer individual procedures than the other strategies.

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