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Effect of temperature on the enzymatic synthesis of cefaclor with in situ product removal

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Abstract

An integrated process of cefaclor synthesis from phenylglycine methyl ester (PGME) and 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7-ACCA) catalyzed by penicillin G acylase (PGA) with in situ product removal (ISPR) was established. The integrated process was more significantly influenced by temperature than the separate synthesis process as without ISPR. The difference between the overall yields with and without ISPR was minified as reaction temperatures rose. For instance, the maximum 7-ACCA conversion was 86 and 68%, respectively, in the process with and without ISPR at 5 °C. Both the maximum conversions, however, decreased to around 45% at 40 °C. The effect of substrate concentration on the overall conversion was also obviously dependent on the reaction temperature. The product cefaclor inhibited PGME hydrolysis in the enzymatic synthesis. ISPR stimulated cefaclor synthesis at lower temperatures, but was not useful at higher temperatures which accelerated PGME hydrolysis.

Keywords: Cefaclor; Enzymatic synthesis; Temperature; In situ product removal

1. Introduction

Continuous removal of products may be advantageous or even essential to improvement of the enzymatic conversion by [1,2]: (i) shifting on unfavourable reaction equilibria, (ii) preventing the hydrolysis of products (e.g. in kinetically controlled synthesis), (iii) improving the downstream processing [1,2] and (iv) preventing direct product inhibition.

Enzymatic synthesis of semisynthetic β-lactam antibiotics is receiving increased attention owing to their economical and environmental benefits compared to conventional chemical processes [3]. Cefaclor, a semisynthetic cephalosporin with a broad spectrum of antibiotic activity for oral treatment, can be enzymatically synthesized. The kinetically controlled enzymatic synthesis of cefaclor catalyzed by penicillin G acylase (PGA) is a typical example of biotransformation where continuous product removal is essential. The overall yield from this type of enzymatic processes was determined by three simultaneous reactions catalyzed by a simple enzyme: antibiotic synthesis, antibiotic hydrolysis and acyl donor hydrolysis (shown in Scheme 1), which resulted in low synthetic yield. In situ product removal (ISPR) in enzymatic synthesis process could be a convenient way to improve enzymatic conversion.

The aqueous two-phase system was often used for the enzymatic synthesis of antibiotics by extraction of product away from the media surrounding the

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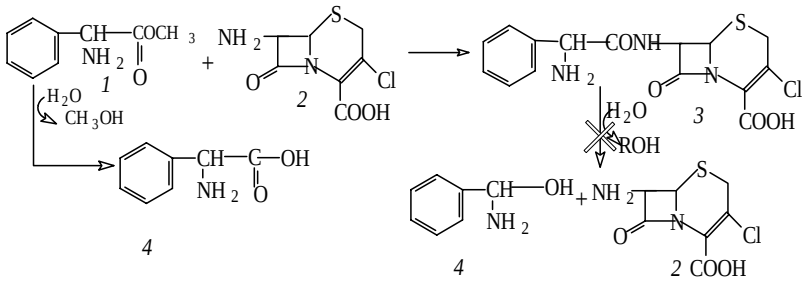

Scheme 1. Reaction scheme for penicillin acylase-catalyzed synthesis of cefaclor. (1) Phenylglycine methyl ester (PGME), (2) 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7-ACCA), (3) cefaclor, (4) phenylglycine (PG).

enzyme [4,5]. However, it was difficult to establish an adequate aqueous two-phase system to partition the product from the phase containing the enzyme. An alternative to remove the product from reaction mixture in situ is to form an insoluble complex of the antibiotic with a related complexing agent [3,6–10]. For example, cephradine or cefaclor can react with 1-naphthol, as well as cephalexin or cefadroxil with 2-naphthol, to form low solubility complexes [6,7,9,10].

Enzymatic synthesis of cefaclor, with in situ product removal (integrated process), was performed using PGME and 7-ACCA catalyzed by PGA from *Bacillus megaterium*. The ISPR in this system was achieved by formation of an insoluble complex of cefaclor with 1-naphthol. The effects of temperature on this integrated process are clarified below. Since, 7-ACCA is more expensive than PGME, the conversion of 7-ACCA was used as the primary index for estimating cefaclor synthesis.

2. Materials and methods

2.1. Enzyme

The soluble PGA from *B. megaterium*, provided by the Shanghai Institute of Biochemistry of the Chinese Academy of Science, was immobilized on epoxyacrylic resin as described previously [11]. The activities of the immobilized and soluble PGA were 500 and 1060 IU ml^{-1}, respectively. A unit of PGA was defined as the amount of enzyme required to produce 1 μmol of 6-APA per minute in 4% (w/v) solution of penicillin G at pH 7.8 and temperature 37 °C. The enzyme activity was determined spectrophotometrically using *p*-dimethylaminobenzaldehyde as the substrate [12].

2.2. Chemicals

Penicillin G, D(–)-phenylglycine and phenylglycine methyl ester (PGME) were kindly donated by the North China Pharma Certical Co. Cefaclor and 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7-ACCA) were obtained from the Tiantai Pharma Co., Zhejiang, China. Epoxyacrylic resin was supplied by the Institute of Catalysis of East China University of Science and Technology. All other reagents were of analytic grade.

2.3. Methods

2.3.1. Continuous product removal during cefaclor synthesis

The experimental reactors are shown in Fig. 1, and consist mainly of two packed bed reactors, a pH

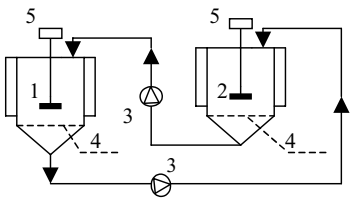

Fig. 1. Diagram of the enzymatic synthesis of cefaclor with in situ product removal. (1) Complexation reactor, (2) enzymatic reaction reactor, (3) peristaltic pump, (4) sintered-glass (G2), (5) mechanical stirrer.

controller and two peristaltic pumps. The enzymatic reaction and the complexation reaction were carried out at pH 6.3 in a fluidized bed bioreactor (200 mm height and 30 mm internal diameter) and a fixed-bed reactor (50 mm height and 30 mm internal diameter), respectively. In order to retain the solid matrices (such as the biocatalyst, 1-naphthol and complex), the bottom of both reactors was equipped with sintered glasses. Glass impellers with vertical flat blades in open clearance were designed to provide radial mixing. A pH controller was used to monitor pH values during the reaction process. The operation temperature was controlled by a circulating water bath. Prior to the reaction, immobilized enzyme and solid 1-naphthol (3:2 molar ratio to the initial 7-ACCA concentration) were added to either the fluidized bed reactor or the fixed bed reactor, respectively. The total reaction volume was 200 ml and the initial enzyme concentration was 10 IU ml^{-1}. Aqueous substrate was recirculated through fluidized bed (110 ml of working volume) and fixed bed reactors (30 ml of working volume) using peristaltic pumps (BT00-300, China) at a flow rate of 9.0 ml min^{-1}. No 1-naphthol was added to the fixed bed reactor when the enzymatic synthesis was carried out without ISPR. The stirring rate in enzyme bioreactor was 120 rpm. Sampling were carried out every 30 min and analyzed by HPLC.

2.3.2. Complexation reaction

Complex formation was performed at pH 6.3 in a stirred bioreactor fitted with a circulating water jacket. The reaction was initiated by adding 3 mmol of solid 1-naphthol to the reaction medium (50 ml) containing 30 mM cefaclor. The pH and the temperature were controlled by a pH controller and a constant temperature circulating water bath, respectively.

2.3.3. Kinetic studies

Kinetic parameters were determined by measuring the initial reaction rates at different substrate concentrations. V_{max} and K_m values were obtained from Lineweaver–Burk plots. The initial rate of PGME hydrolysis was decided from the HPLC chromatograms.

2.3.4. Analysis

Each reactant was identified and analyzed by HPLC using a C-18 column (250 mm length and 4.6 mm internal diameter, 5 μm particle size and 8 nm pore size). Samples were eluted at 25 °C with 85% (v/v) acetate sodium buffer (20 mM, pH 5.16) and 15% (v/v) acetonitrile at 1 ml min^{-1}, and monitored at 265 nm.

3. Results and discussion

3.1. Enzymatic synthesis of cefaclor

As shown in Fig. 2, the reaction rates of the kinetically controlled synthesis of cefaclor catalyzed by PGA were differed significantly between the reactions with and without ISPR. Cefaclor concentration was low in the integrated process but relatively higher in the separate process. Cefaclor hydrolysis was, therefore, almost negligible in the process with ISPR, but should be very rapid in the process without ISPR. A significant improvement in the maximum conversion of 7-ACCA was observed from 50 to 65% using ISPR at 20 °C.

3.2. Effect of temperature on the enzymatic conversion

The influence of the reaction temperature on the maximum 7-ACCA conversion was evaluated as shown in Fig. 3. It is evident that 7-ACCA conversion is optimal at lower temperature as is the case in the synthesis other antibiotics [13–17]. In addition, the difference between 7-ACCA conversion with and

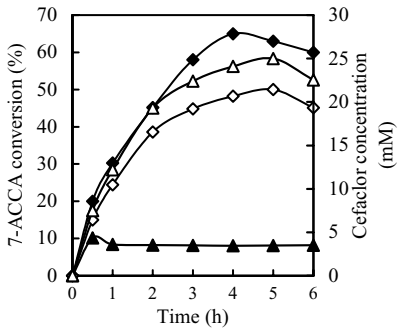

Fig. 2. Time course of the enzymatic synthesis of cefaclor with or without in situ product removal (ISPR). (◆) 7-ACCA conversion (%) with ISPR; (▲) cefaclor concentration with ISPR (mM); (◇) 7-ACCA conversion (%) without ISPR; (△) cefaclor concentration without ISPR (mM). Reaction conditions: 50 mM 7-ACCA, 100 mM PGME, 20 °C, pH 6.3.

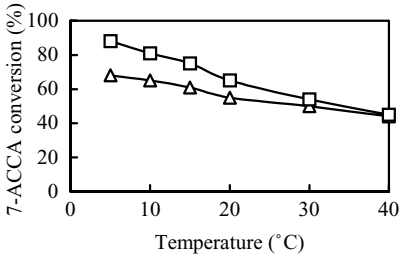

Fig. 3. Effect of temperature on 7-ACCA conversion during cefaclor synthesis with and without ISPR. (□) With ISPR; (△) without ISPR. Reaction conditions: 50 mM 7-ACCA, 100 mM PGME, pH 6.3.

without ISPR was minified as reaction temperature rose. For instance, the maximum 7-ACCA conversion was 86 and 68%, respectively, in the process with and without ISPR at 5 °C. Both the maximum conversions, however, decreased to around 45% at 40 °C.

Both the enzymatic and complexation reaction should be considered in analyzing the effect of temperature on cefaclor synthesis using ISPR, since both reactions are part of the integrated process. As shown in Table 1, both the initial rate of enzymatic synthesis and the initial rate of complexation increased slightly with the increased temperature. In the range of temperature tested, the initial complexation rates were much higher than those of the enzymatic synthesis. Therefore, the complexation reaction is not the determing step in the integrated process. However, a change of the temperature could change the cefaclor concentration existing in the system due to the effect of temperature on the reaction equilibria. As the temperature rises from 0 to 40 °C, cefaclor concentration in the integrated process

Table 1
Effect of temperature on the initial rate of enzymatic synthesis and the initial rate of product complexation in the enzymatic synthesis of cefaclor

Initial rate	Temperature (°C)				
	5	10	20	30	40
Initial synthesis rate (μmol min^{-1})	0.10	0.15	0.20	0.26	0.34
Initial complexation rate (μmol min^{-1})	1.21	1.31	1.51	1.55	1.61

Reaction conditions: 50 mM 7-ACCA, 100 mM PGME, pH 6.3, 10 IU ml^{-1} enzyme in 50 ml. The complexation reaction was performed as discussed in Section 2.3.2.

Table 2
Effect of temperature on cefaclor concentration in the enzymatic synthesis of cefaclor with in situ product removal

	Temperature (°C)				
	0	10	20	30	40
Cefaclor concentration (mM)	1.2	2.5	3.5	4.3	4.8

Reaction conditions: pH 6.3, 50 mM 7-ACCA, 100 mM PGME, 10 IU ml^{-1} enzyme in 200 ml, 75 mM 1-naphthol.

increases from 1.2 to 4.8 mM (Table 2). It had been shown that cefaclor concentration below 10 mM do not influence the rate of conversion of 7-ACCA. This indicates that reduction of the difference between 7-ACCA conversion in the process with and without ISPR, as shown in Fig. 3, could result from the acceleration of PGME hydrolysis as temperature increase.

3.3. Effect of substrate concentration on cefaclor synthesis with IPSR

The effect of substrate concentration on 7-ACCA conversion was investigated in the range of 25 to 100 mM for 7-ACCA and 50 to 200 mM for PGME, respectively. As 7-ACCA concentration was fixed at 50 mM, 7-ACCA conversion increased consistently with increasing concentration of PGME at 5 °C, but decreased sharply from 100 mM of PGME at 20 °C (Fig. 4). As ratio of the concentration of 7-ACCA to that of PGME was fixed at 1:2, similar changes in reaction conversion versus 7-ACCA concentration

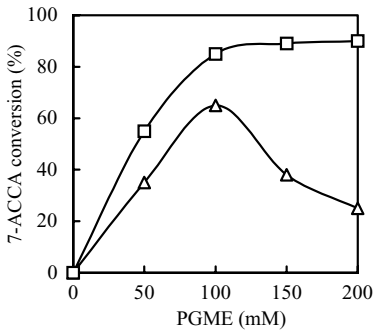

Fig. 4. Effect of PGME concentration on 7-ACCA conversion in the enzymatic synthesis of cefaclor with in situ product removal. (□) 5 °C; (△) 20 °C. Reaction conditions: 50 mM 7-ACCA, pH 6.3. Other reaction conditions were as described in Section 2.

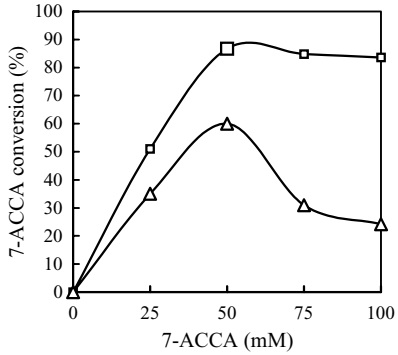

Fig. 5. Effect of 7-ACCA concentration on 7-ACCA conversion in the enzymatic synthesis of cefaclor with in situ product removal. (□) 5 °C; (△) 20 °C. Reaction conditions: 7-ACCA/PGME was 1:2. Other reaction conditions were as described in Section 2.

occurred at various temperatures (Fig. 5). This indicated that temperature is an important parameter in the integrated process.

Considering the reaction rates and the reaction equilibrium, the enzymatic reaction is the "bottleneck" in the integrated process. The main difference between the enzymatic reaction with ISPR and without ISPR was the product cefaclor concentration in the reaction mixture. Since low concentrations of cefaclor did not significantly influence the reaction equilibrium, the effects of cefaclor and 7-ACCA on PGME hydrolysis was investigated. PGME hydrolysis was shown to be retarded by cefaclor and 7-ACCA. The rate of PGME hydrolysis is described in Eq. (1), with K_m of 21.4 mM and V_{max} of 2.3 mM min^{-1}. Cefaclor behaves as a competitive inhibitor with K_{ie} of 14.2 mM (Fig. 6) and 7-ACCA as a noncompetitive inhibitor

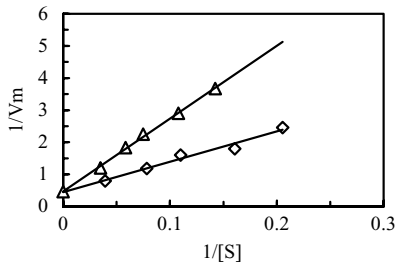

Fig. 6. Effect of cefaclor on the kinetics of PGME hydrolysis. (△) Cefaclor 20 mM; (◇) control. Reaction conditions: 100 mM PGME, 25 °C, pH 6.3.

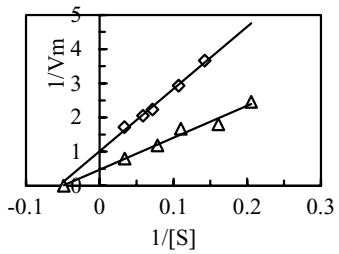

Fig. 7. Effect of 7-ACCA on the kinetics of PGME hydrolysis. (△) Control; (◇) 7-ACCA 10 mM. Reaction conditions: 100 mM PGME, 25 °C, pH 6.3.

with K_{ia} of 9.35 mM (Fig. 7),

$$-\frac{\mathrm{d[PGME]}}{\mathrm{d}t} = \frac{V_{max}[\mathrm{PGME}]}{K_m(1+([\mathrm{Cef}]/K_{ie}))(1+([\text{7-ACCA}]/K_{ia})) + [\mathrm{PGME}](1+([\text{7-ACCA}]/K_{ia}))} \quad (1)$$

where V_{max} is the maximum initial hydrolysis rate of PGME, K_m is the Michaelis–Menten constant, K_{ie} is the cefaclor inhibition constant, and K_{ia} is the 7-ACCA inhibition constant.

In Eq. (1), it is obvious that the hydrolysis of PGME could be accelerated by higher concentrations of PGME. On the other hand, the hydrolysis rate of PGME in the cefaclor synthesis in ISPR should be faster than that without ISPR due to the differences in cefaclor concentration in the ISPR versus non-ISPR reaction mixtures.

Table 3
Effect of temperature on the initial cefaclor synthesis rate and PGME hydrolysis rate

Initial rate	Temperature (°C)			
	5	10	20	30
Initial cefaclor synthesis rate (μmol min^{-1})	2.2	11.7	15.6	19.8
Initial PGME hydrolysis rate (μmol min^{-1})	14.1	24.2	42.0	72.6

Cefaclor synthesis was carried out at pH 6.3, 10 IU ml^{-1} enzyme, 50 mM 7-ACCA and 100 mM PGME in 50 ml. PGME hydrolysis was carried out at pH 6.3, 10 IU ml^{-1} enzyme and 100 mM PGME in 50 ml.

Cefaclor synthesis reaction, PGME hydrolysis and cefaclor hydrolysis were simultaneously affected by temperature. As the cefaclor hydrolysis rate was very low, due to its low concentration, in the reactions in ISPR, the effect of temperature on enzymatic synthesis and PGME hydrolysis were the focus of our study (Table 3). PGME was readily hydrolyzed by the enzyme, especially at higher temperatures. Cefaclor synthesis, however, was less influenced by temperature. Therefore, cefaclor synthesis was best performed at low temperature. A similar result with cephalexin synthesis was reported by Schroen et al.[15].

4. Conclusions

Temperature was shown to play an important role in cefaclor synthesis process. The effects of temperature was analyzed in detail by investigating the complexation and enzymatic synthesis reactions, especially PGME hydrolysis and cefaclor synthesis. Since cefaclor was a competitive inhibitor of PGME hydrolysis, the rate of PGME hydrolysis became very important when the cefaclor concentration in the reaction mixture was low. The enzymatic synthesis using ISPR promoted PGME hydrolysis because it reduced cefaclor concentration. This resulted in a low overall conversion at higher temperature. However, this was drastically improved at low temperature, where PGME hydrolysis was made slow. This observation may provide the basis for a new approach to control such enzyme processes combined with in situ product removal.

Acknowledgements

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