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pH-Controlled Synthesis of Cephalexin by a Purified *Acetobacter turbidans* Ampicillin Acylase

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Abstract It has been known that, in enzymatic synthesis of cephalexin, the conversion yield was reduced by high loading of ampicillin acylase. In order to elucidate this phenomena, pH-controlled synthesis of cephalexin was examined using a purified *Acetobacter turbidans* acylase. When the pH of the reaction mixture was maintained at 6.20±0.04, the reduction of the maximal conversion rate was not observed even with high enzyme loading. The kinetic parameters also suggest that pH drop during the enzymatic synthesis of cephalexin was mainly attributed to the rapid hydrolysis of D-α-phenylglycine methyl ester to D-α-phenylglycine, rather than the disappearance of 7-amino-3-deacetoxycephalosporanic acid for cephalexin synthesis. At higher molar ratio of two substrates, [D-α-phenylglycine methyl ester]/[7-amino-3-deacetoxycephalosporanic acid], the conversion rate was also elevated under pH-controlled enzymatic synthesis, which implies that the main reason for the pH drop is due to the production of D-α-phenylglycine. In order to reduce the hydrolysis of D-α-phenylglycine methyl ester, the effect of a water-methanol cosolvent system on the conversion profile was also examined. Even though the conversion rate was increased in 10% methanol solution, a higher than 16% methanol in the reaction mixture caused an inactivation of enzyme.

Key words: Cephalexin, ampicillin acylase, *Acetobacter turbidans*, pH-controlled synthesis, D-α-phenylglycine methyl ester, 7-amino-3-deacetoxycephalosporanic acid

Since Takahashi and his colleagues reported that microorganisms are able to catalyze N-acylation of 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) with D-α-amino acid esters to the corresponding cephalosporins [23], the development of enzymatic processes for the production of semisynthetic cephalosporins has been developed during the last two decades [1-2, 17]. Among the semisynthetic cephalosporins, enzymatic synthesis of cephalexin (CEX) from 7-ADCA and D-α-phenylglycine methyl ester (PGM) was most intensively investigated [3, 5, 12, 16].

So-called ampicillin acylase in this category produced by some Gram-negative bacilli, including *Pseudomonas melanogenum*, *Xanthomonas citri*, *Kluyvera citrophila* and *Acetobacter turbidans* [6, 20, 23], are known to have the capability of producing semisynthetic penicillins from 6-aminopenicillanic acid (6-APA) as well as semisynthetic cephalosporins from 7-aminocephalosporanic acid (7-ACA) or 7-ADCA. In fact, this category of enzymes can catalyze three reactions simultaneously; these are N-acylation of 7-ADCA, 7-ACA, or 6-APA with D-α-amino acid ester to semisynthetic β-lactam antibiotics, hydrolysis of D-α-amino acid ester to D-α-amino acid, and hydrolysis of β-lactam antibiotics to 7-ADCA, 7-ACA, or 6-APA. Nam *et al.* [13] proposed an acyl enzyme intermediate model in order to elucidate this reaction characteristic. The proposed kinetic model for the synthesis of CEX consists of rapid binding steps for substrate D-α-amino acid ester (PGM), hydrolysate D-α-amino acid (D-α-phenylglycine; PG), and product CEX to the enzyme, acylation steps of enzyme with bound PGM or CEX to form an acyl enzyme intermediate, hydrolysis of acyl enzyme with water, and nucleophilic attack of acyl enzyme with 7-ADCA (Fig. 1).

In order to develop an enzymatic process for the production of CEX, two problems have to be considered to achieve higher maximal yield of CEX from 7-ADCA and PGM. As suggested by Kasche [8], the maximal conversion is influenced by enzyme properties; the ratio of transferase activity to hydrolase activity. Thus, the kinetic control of the reaction to maximize the transferase activity by optimizing reaction pH, temperature, type of activated substrate, and

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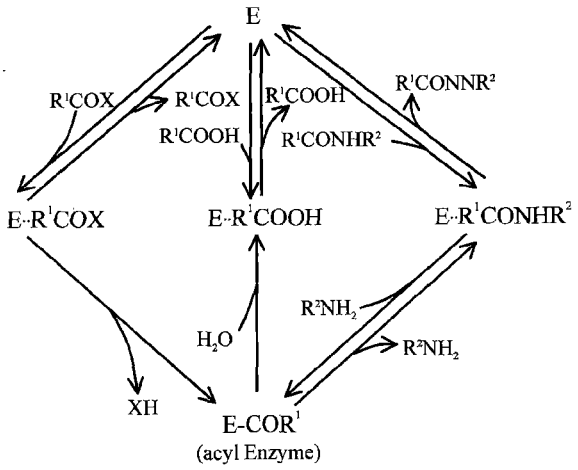

Fig. 1. The kinetic model for the enzymatic synthesis of cephalexin proposed by Nam *et al.* [11].
R^1, D-α-phenylglycyl group; R^2, 3-deacetoxycephalosporanic acid; X, methyl group. Thus, E, ampicillin acylase in the synthesis of cephalexin; R^1COX, D-α-phenylglycine methyl ester (PGM); R^1COOH, D-α-phenylglycine (PG); XH, methanol; R^2NH_2, 7-amino-3-deacetoxycephalosporanic acid (7-ADCA); R^1CONHR^2, cephalexin (CEX). E··R^1COX, E··R^1COOH, and E··R^1CONHR^2 mean enzymes bound with substrates, and E-COR^1 represents the acyl enzyme intermediate.

organic solvent is important to make higher conversion to CEX [9].

Another interesting feature in the enzymatic synthesis of CEX is that the maximal conversion yield is significantly reduced by employing a high concentration of enzyme, even though kinetically controlled synthesis was attempted [14, 16-17]. This problem still remains in question. Recently, Ospina *et al.* [15] examined the effect of pH on the synthesis of ampicillin by penicillin acylase, but they could not explain the reason why there was a decrease of maximal conversion with higher enzyme loading.

Under the assumption that the pH of the reaction mixture can be lowered by disappearance of the amino group in 7-ADCA and appearance of the acid form of PG during reaction, we tried to maintain the reaction pH at 6.20±0.04 by adjusting it with sodium hydroxide. Here, the effect of pH-controlled enzymatic synthesis of CEX on the maximal conversion yield is reported.

Ampicillin acylase from *Acetobacter turbidans* ATCC 9325 was employed in the synthesis of CEX from 7-ADCA and PGM [10, 22]. The specific activity of the purified enzyme by the procedure reported previously [18] was 1,300 units per mg of protein, where one unit of enzyme activity was defined as the amount required to produce 1 μmole of CEX per minute at pH 6.2 and 30°C.

The chemicals used in this experiment were PGM hydrochloride from Wako Pure Chemical Industries Ltd. (Osaka, Japan), CEX and PG from Sigma Chemical Co. (MO, U.S.A.), and 7-ADCA from Chong Keun Dang Pharmaceutical Corporation (Cheonan, Korea).

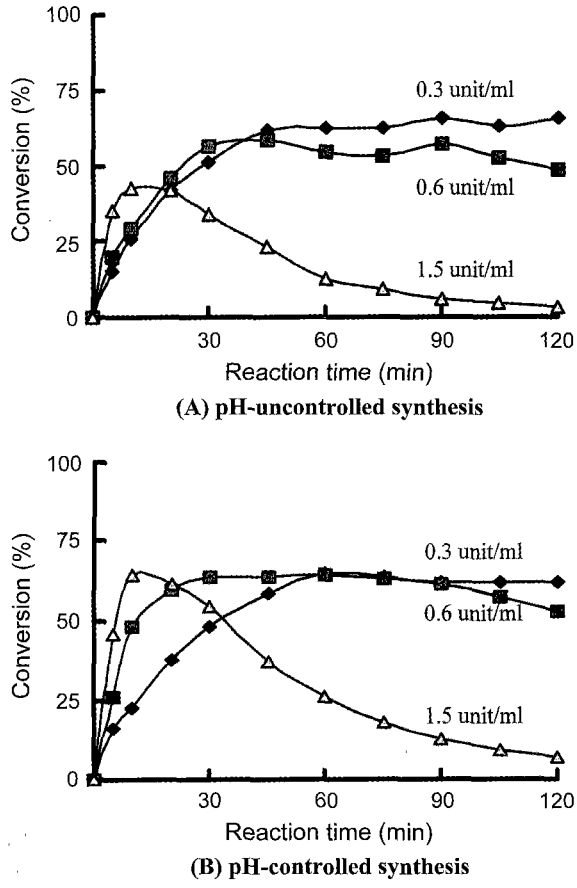

Fig. 2. Conversion profile of enzymatic synthesis of cephalexin with different enzyme loading.
The reaction was carried out by using 20 mM 7-ADCA and 40 mM PGM in 0.1 M phosphate buffer (pH 6.2) with loading of 0.3 unit/ml (◆), 0.6 unit/ml (■), and 1.5 unit/ml (▲) of *A. turbidans* acylase. (A), Conversion profile obtained without pH control; (B), conversion profile observed with pH control at 6.20±0.04.

The standard enzyme reaction was carried out using 20 mM 7-ADCA and 40 mM PGM in 0.1 M phosphate buffer (pH 6.2) for 20 min at 30°C, and terminated by immersing the samples into boiling water for 30 sec. In pH-controlled synthesis of CEX, the reaction mixture was kept at pH 6.20±0.04 by adding 5 N sodium hydroxide and using a pH-stat. The amount of each reactant and product was determined by high performance liquid chromatography (Hewlett Packard HP1090, CA, U.S.A.) with isocratic flow at 0.5 ml/min of mobile phase (35% methanol in 0.1 M phosphate buffer, pH 6.2) through a Microsorb C_{18} column (4.6×220 mm; Rainin Instruments, CA, U.S.A.) at 40°C. The absorbance of the eluate was monitored at 254 nm.

As mentioned above, higher loading of *A. turbidans* acylase in the synthesis of CEX caused a decrease in the maximal conversion yield at an initial pH of 6.2, as shown in Fig. 2A. The maximal conversion of 7-ADCA to CEX was achieved to as high as 65% when 0.3 unit of enzyme/ml was loaded to the reaction mixture, whereas only 42%

Table 1. Kinetic parameters of three reactions catalyzed by a purified *A. turbidans* acylase.

Reactions	$V_m/(E)_t$ (μmol/ min · unit)	K_m (mM)	V_m/K_m (μmol/ min · mM)
PGM hydrolysis	4.0	4.0	1.0
CEX synthesis	1.6	3.0 (PGM) 7.8 (7-ADCA)	0.53 (PGM) 0.21 (7-ADCA)
CEX hydrolysis	0.45	3.0	0.15

of the maximal conversion was observed by 1.5 unit of enzyme/ml in the reaction mixture. The final pH of the reaction mixture reached to 5.8 after 2-h reaction.

Comparatively, in a case of pH-controlled enzymatic synthesis at pH 6.20±0.04, the maximal conversion was observed at almost the same value of 65%, regardless of the amount of loaded enzyme (Fig. 2B). Therefore, the change of pH during the enzymatic reaction can cause the decrease of conversion rate from 7-ADCA to CEX, probably due to the rapid hydrolysis of PGM to PG by high loading of enzyme rather than CEX synthesis from 7-ADCA.

In order to ascertain the main reason for the pH drop during the enzyme reaction, the maximal reaction velocity (V_m) was determined in three reactions which were simultaneously catalyzed by *A. turbidans* enzyme. As shown in Table 1, the kinetic parameters for PGM hydrolysis showed much greater values compared to the CEX synthesis, which is consistent with the previous report [19]. It means that the major reason for the pH drop is the rapid production of acidic form of PG from PGM rather than the disappearance of 7-ADCA by CEX synthesis. Thus, a higher loading of enzyme caused the rapid drop of pH by PG production that resulted in inhibition of CEX synthesis.

It has been generally accepted that the molar ratio of two substrates, [PGM]/[7-ADCA], can significantly affect the enzymatic synthesis of CEX. Initially, Takahashi *et al.* [21] reported that the optimal ratio for CEX synthesis by *X. citri* acylase was 2, and the ratio greater than 2 caused a drastic decrease of the conversion rate. However, other investigators reported the optimal molar ratio of two substrates for *A. turbidans* acylase as 3 [19], and that for *Bacillus megaterium* acylase as 4 [11]. Recently, Maladkar [12] suggested that enzymatic conversion of 7-ADCA to CEX was the maximum level at a molecular ratio of 1:10 when *E. coli* acylase was employed.

Under the pH-controlled system, the conversion profile was also examined at various molar ratios of [PGM]/[7-ADCA] by using *A. turbidans* enzyme (Fig. 3). Consistent with the previous report [19], higher maximal conversion was achieved by increasing the amount of PGM from a molar ratio of 1 (20 mM) to 6 (120 mM), owing to the simultaneous consumption of PGM in CEX synthesis as well as in hydrolysis to PG. It is interesting to mention that

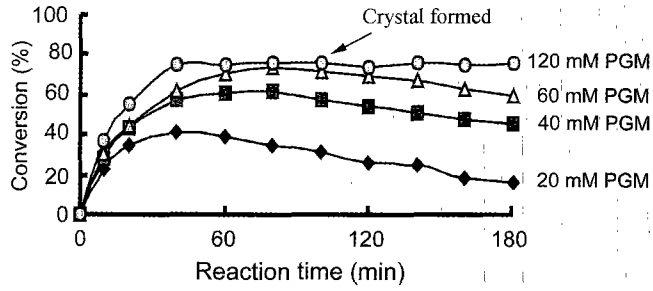

Fig. 3. Conversion profile of enzymatic synthesis of cephalexin with different molar ratios of [PGM]/[7-ADCA].
The reaction was carried out by using 20 mM 7-ADCA and 20 mM (◆), 40 mM (■), 60 mM (▲), or 120 mM (●) PGM by 0.3 unit/ml of *A. turbidans* acylase in 0.1M phosphate buffer (pH 6.2) with pH control at 6.20±0.04.

PG crystal was observed in the reaction mixture when the conversion rate reached as much as 77% by supplementing 120 mM of PGM, and that any higher conversion was not achieved at that point. The results showed that the maximal conversion rate was elevated according to the increase of the molar ratio to 6 under the pH-controlled system, different from the previous report that the optimal molar ratio for *A. turbidans* acylase was 3 [19]. It is most likely that the pH drop is mainly attributed to the amount of PG produced at a higher PGM concentration level.

In order to increase the enzymatic conversion yield to CEX by minimizing the PGM hydrolysis reaction, another effort has been made to decrease water activity by adding organic solvents in the reaction media [4, 7]. Among the solvents employed, methanol and ethanol as soft cosolvent and *N,N'*-dimethylformamide (DMF) and dimethysulfoxide (DMSO) as hard cosolvent were tested to alter the kinetic characteristics of ampicillin acylases.

Using *A. turbidans* acylase, the effect of methanol concentration in the reaction mixture on enzymatic conversion

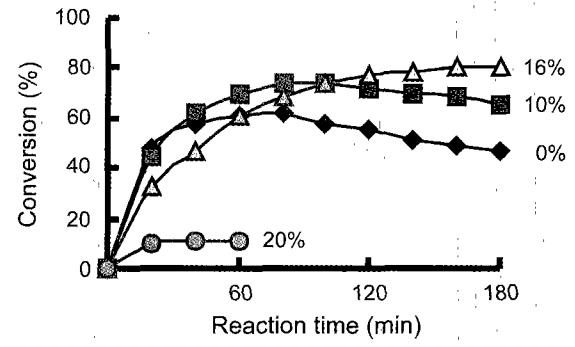

Fig. 4. Effect of methanol concentration in the reaction mixture on the conversion profile of enzymatic cephalexin synthesis.
The reaction was carried out by using 20 mM 7-ADCA and 40 mM PGM by 0.3 unit/ml of *A. turbidans* acylase in 0.1 M phosphate buffer (pH 6.2) containing 0% (◆), 10% (■), 16% (▲), or 20% (●) methanol with pH control at 6.20±0.04.

of 7-ADCA to CEX was also examined under control reaction pH (Fig. 4). By adding 10% methanol in the reaction media, an increment of maximal conversion was obtained from 62% to 74%. However, upon adding 16% methanol, the maximal conversion was somewhat improved, but the initial reaction rate was reduced, probably due to inactivation of the enzyme by methanol. The result was much clearer when supplementing with 20% methanol. In this case, the initial reaction velocity as well as the maximal conversion yield was significantly reduced.

In conclusion, it was found that the reaction pH in the enzymatic synthesis of CEX dropped by the formation of the acidic form of PG, even in the buffer solution. Since this pH drop caused the decrease of the enzymatic conversion rate to CEX, a higher conversion yield could be achieved by maintaining the optimal reaction pH, or by reducing the water activity with organic cosolvent.

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References

1. Asano, Y. 2000. New enzymes acting on peptides containing D-amino acids: Their properties and application. *J. Microbiol. Biotechnol.* **10:** 573-579.
2. Bruggink, A., E. C. Roos, and E. de Vroom. 1998. Penicillin acylase in the production of β-lactam antibiotics. *Org. Proc. Res. Develop.* **2:** 128-133.
3. Choi, W. G., S. B. Lee, and D. D. Y. Ryu. 1981. Cephalexin synthesis by partially purified and immobilized enzymes. *Biotechnol. Bioeng.* **23:** 361-371.
4. Fernandez-Lafuente, R., C. M. Rosell, and J. M. Guisan. 1991. Enzyme reaction engineering: Synthesis of antibiotics catalyzed by stabilized penicillin G acylase in the presence of organic cosolvents. *Enzyme Microb. Technol.* **13:** 898-905.
5. Fujii, T., K. Matsumoto, and T. Watanabe. 1976. Enzymatic synthesis of cephalexin. *Process Biochem.* **(Oct.):** 21-24.
6. Hur, N.-Y., D.-H. Oh, J.-H. Yu, D.-H. Nam, and J.-H. Kim. 1987. Purification and reaction characteristics of ampicillin acylase from *Pseudomonas melanogenum*. *Kor. J. Appl. Microb. Biotechnol.* **15:** 9-14.
7. Hyun, C. K., J. H. Kim, and D. D. Y. Ryu. 1993. Enhancement effect of water activity on enzymatic synthesis of cephalexin. *Biotechnol. Bioeng.* **42:** 800-806.
8. Kasche, V. 1986. Mechanism and yields in enzyme catalyzed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, peptides and other condensation products. *Enzyme Microb. Technol.* **8:** 4-16.
9. Kasche, V., U. Haufler, and L. Riechmann. 1987. Equilibrium and kinetically controlled synthesis with enzymes: Semisynthesis of penicillins and peptides. *Methods Enzymol.* **136:** 280-292.
10. Kim. M. J., S. W. Kim, J. H. Bang, and D. H. Nam. 1997. Immunochemical reactivity of polyclonal antibody against ampicillin acylase of *Xanthomonas citri*. *J. Microbiol. Biotechnol.* **7:** 194-196.
11. Konecny, J., M. Sieber, and A. Schneider. 1981. Kinetics and mechanism of acyl transfer by penicillin acylase. *Biotechnol. Lett.* **3:** 507-512.
12. Maladkar, N. K. 1994. Enzymatic production of cephalexin. *Enzyme Microb. Technol.* **16:** 715-718.
13. Nam, D. H., C. Kim, and D. D. Y. Ryu. 1985. Reaction kinetics of cephalexin synthesizing enzyme from *Xanthomonas citri*. *Biotechnol. Bioeng.* **27:** 953-956.
14. Okachi, R., F. Kato, Y. Miyamura, and T. Nara. 1973. Selection of *Pseudomonas melanogenum* KY 3987 as a new ampicillin-producing bacteria. *Agric. Biol. Chem.* **37:** 1953-1957.
15. Ospina, S., E. Barzana, O. T. Ramirez, and A. Lopez-Munguia. 1996. Effect of pH in the synthesis of ampicillin by penicillin acylase. *Enzyme Microb. Technol.* **19:** 462-469.
16. Rhee, D. K., S. B. Lee, J. S. Rhee, D. D. Y. Ryu, and J. Hospodka. 1980. Enzymatic biosynthesis of cephalexin. *Biotechnol. Bioeng.* **22:** 1237-1247
17. Ryu, D. D. Y. and D. H. Nam. 1983. Enzymatic synthesis of semisynthetic β-lactam antibiotics. *Enzyme Eng. News* **9:** 30-62.
18. Ryu, Y. W. and D. D. Y. Ryu. 1987. Semisynthetic β-lactam antibiotics synthesizing enzyme from *Acetobacter turbidans*: Purification and properties. *Enzyme Microb. Technol.* **9:** 339-344.
19. Ryu, Y. W. and D. D. Y. Ryu. 1988. Semisynthetic β-lactam antibiotics synthesizing enzyme from *Acetobacter turbidans*: Catalytic properties. *Enzyme Microb. Technol.* **10:** 239-245.
20. Shimizu, M., T. Masuike, H. Fujita, K. Kimura, R. Okachi, and T. Nara. 1975. Search for microorganisms producing cephalosporin acylase and enzymatic synthesis of cephalosporins. *Agric. Biol. Chem.* **39:** 1225-1232.
21. Takahashi, T., K. Kato, Y. Yamazaki, and M. Isono. 1977. Synthesis of cephalosporins and penicillins by enzymatic acylation. *Japan. J. Antibiot.* **30:** S230-S237.
22. Takahashi, T., Y. Yamazaki, and K. Kato. 1974. Substrate specificity of an α-amino acid ester hydrolase produced by *Acetobacter turbidans* A.T.C.C. 9325. *Biochem. J.* **137:** 497-503.
23. Takahashi, T., Y. Yamazaki, K. Kato, and M. Isono. 1972. Enzymatic synthesis of cephalosporins. *J. Amer. Chem. Soc.* **94:** 4035-4037.