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Purification and Characterization of a Cephalexin-Synthesizing Enzyme from *Gluconobacter oxydans* CCRC10383

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A cephalexin-synthesizing enzyme which catalyzes the synthesis of cephalexin from two substrates, 7-amino-3-deacetoxy cephalosporanic acid (7-ADCA) and D-phenylglycine methyl ester (D-MEPG) was isolated, purified and characterized from *G. oxydans* CCRC 10383 by ammonium sulfate precipitation, CM-Fractogel and Sephadex G-200 chromatography. The molecular weight of the enzyme was estimated to be 270 kd by gel filtration. From the analysis of SDS-PAGE, the enzyme is a tetramer and consists of two 53 kd subunits and two 73 kd subunits. The isoelectric point of the enzyme was estimated to be 7.5. The optimal pH and temperature for the synthetic reaction of cephalexin were 5-7 and 30-50 °C, respectively, with a maxium reaction rate at pH 6 or 50 °C. Metal ions are not essential for the enzymatic activity because EDTA (ethylenediaminetetraacetic acid) exerts no influence upon the enzyme activity. The growth medium containing 0.25% DL-phenylglycine (DL-PG) or 0.25% D-phenylglycine (D-PG) as inducers could obtain 1.4 times higher enzyme activity than the growth medium without inducers. The values of K_m, K_{cat}, V_{max} and bimolecular constant K_{cat}/K_m were 19 mM, 1.2×10^4 s^{-1}, 30 unit/mg of protein and 6.2×10^5 $M^{-1}s^{-1}$, respectively. The K_m values for D-MEPG and 7-ADCA were determined as 13.9 mM and 3.08 mM, respectively. The conversion of cephalexin was found to be 60% when the synthesis was carried out in the 0.1 M phosphate buffer solution (pH 6.2) containing 40 mM of 7-ADCA and 118 mM of MEPG at 37 °C.

INTRODUCTION

There is much interest in the use of enzymes as industrial catalysts in the synthesis of β-lactam antibiotics. Cephalexin is an important chemotherapeutic drug because of its acid stability and broad antimicrobial spectrum against the pathogens most frequently encountered in clinical practice.

Cephalexin has been synthesized by chemical N-acylation of 7-amino-3-cephem compounds with corresponding organic acids. But the processes for the chemical synthesis of cephalexin required many reaction steps, including the protection of the amino group of D-phenylglycine (D-PG) and the carboxyl group of 7-ADCA for N-acylation, the deblocking of the protected cephalexin and the use of highly toxic solvents.[1,2] Several microorganisms have been observed to produce enzymes capable of synthesizing semi-synthetic cephalosporins.[2,3-6] After screening, the enzyme from *G. oxydans* CCRC10383 was found to synthesize cephalexin from 7-ADCA (amine nucleophile) and DL-MEPG (acyl donor) (Scheme I) at higher activity than those from other sources (*Acetobacter*, *Xanthomonas*, *Pseudomonas*). From the previous reports for the synthesis of cephalexin by the enzymatic reaction, the optimal molar ratio of D-MEPG to 7-ADCA was determined to be about 2.5 because the hydrolysis of D-MEPG was about three times as fast as the synthesis of cephalexin.[7,8] The cephalexin-synthesizing enzyme from *Gluconobacter oxydans* has not been fully reported yet. In this article, the purification, basic reaction kinetics and some characteristics of the cephalexin-synthesizing enzyme from *G. oxydans* CCRC10383 will be described.

EXPERIMENTAL SECTION

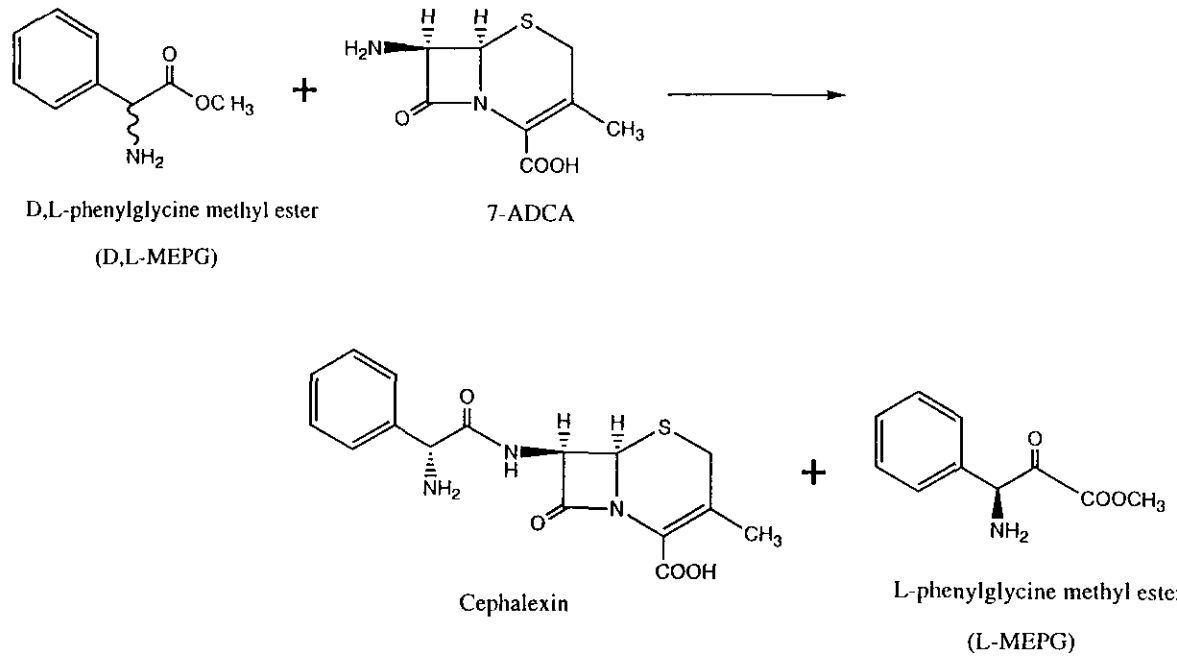
Bacterial Growth Condition and Chemicals

G. oxydans CCRC10383 was a kind gift from Profes-

Dedicated to Professor Kung-Tsung Wang on the occasion of his 70th birthday.

Abbreviation: 7-ADCA, 7-amino-3-deacetoxy cephalosporanic acid; 6-APA, 6-aminopenicillanic acid; 7-ACA, 7-amino-cephalosporanic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, High-performance Liquid Chromatograpy; D-MEPG, D-phenylglycine methyl ester; DL-MEPG, D,L-phenylglycine methyl ester; D-PG, D-phenylglycine; DL-PG, D,L-phenylglycine; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Scheme I Enzymatic Synthesis of Cephalexin



sor Liu, Y. T. (Department of Microbiology and Immunology, National Defense Medical College, Taiwan) and was used as a source of the enzyme. The culture was incubated with 150 rpm agitation and 48 h cultivation time at 28 °C on medium.[3] Cephalexin, ampicillin, D-(-)-phenylglycine, L-(+)-phenylglycine, DL-phenylglycine, D-*p*-hydroxyphenylglycine, L-*p*-hydroxy-phenylglycine, 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA) and 7-ADCA were all purchased from Sigma, USA and amino acid methyl ester were prepared by $SOCl_2$/methanol treatment. Protein molecular weight markers (α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphorylase, egg albumin and bovine albumin) were purchased from Sigma, USA. Sephadex G-200 was from Pharmacia and CM-Fractogel was from Merck, Germany. Protein molecular weight markers for gel filtration (blue dextran, thyroglobulin, ferritin, catalase) were from Pharmacia, Sweden. All solvents were HPLC grade and obtained from Alps Chemical Co., Taiwan.

Purification of Cephalexin-synthesizing Enzyme

Cells were collected by centrifugation, washed with distilled water and resuspended in 0.2 M phosphate buffer at pH 6.0.[9] The harvested cells were then disrupted with French Press (15000 psi, 30 sec × 2). Crude cell-free enzyme prepared by 40% ammonium sulfate precipitation was dialyzed against 0.01 M phosphate buffer (pH 6.0) at 4 °C for 24 h. The dialyzed solution was chromatographed on CM-Fractogel column (35 × 190 mm) (Merck) which was eluted first with 0.1 M phosphate buffer at pH 6.6 and then with a gradient of 0 to 1 M NaCl. After ultrafiltration (MW 10,000 cut-off), the concentrate was further purified by gel filtration on Sephadex G-200 column (18 × 580 mm) eluted with 0.1 M phosphate buffer at pH 6.6. Protein concentration was determined with a solution of Coomassie Blue (Bradford method)[10] and the isoelectric point of the enzyme was measured by solubility in different pH 3-9. The apparent molecular weight of denatured enzyme was determined by 10% SDS-PAGE.[11] The effects of temperature 15-55 °C and pH 3-9 were also detected.[12]

Estimation of Enzyme Activity

The standard reaction mixture for enzymatic activity consisted of 0.1 M sodium phosphate buffer (pH 6.2), 10 mg/mL of 7-amino-3-deacetoxy cephalosporanic acid (7-ADCA), 25 mg/mL of D-MEPG and a controlled amount of enzyme solution.[8] The reaction mixture was incubated at 37 °C for 30 min and then filtered and analyzed by HPLC. The HPLC system with a C_{18} column (7 μm particle size, 250 × 4.6 mm I.D.) was eluted with a gradient of 18-27% CH_3CN in double distilled water at 1.0 mL/min of flow rate and monitored by UV 254 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of cephalexin per min at pH 6.2. Specific activity

was defined as the amount of activity (U) per mg of protein. The enzymatic synthesis were carried out in phosphate buffer solution and were followed by HPLC analysis.[9]

Effect of Inducer

Pre-enriched bacteria were added to the medium which contained 0.25% of DL-PG or 0.25% of D-PG as inducer. The cell growth and enzyme activity at different intervals of incubation time were recorded in comparison with those of normal medium without the addition of inducers.[13] The enzyme activity was measured as described above at 37 °C for 3 h.[8]

Determination of Kinetic Parameters

A Lineweaver-Burk plot was constructed from the initial reaction rates observed with various concentrations of the two substrates, 7-ADCA or D-MEPG. All enzymatic reactions were carried out in 0.1 M phosphate buffer (pH 6.2) at 37 °C for 30 min and initial rates were determined by measuring the concentration of cephalexin in the solution of enzymatic reaction with the HPLC system described above.[9,14-17]

RESULTS

Purification and Characterization of Cephalexin-synthesizing Enzyme

The purification of the cephalexin-synthesizing enzyme is summarized in Table 1. First, the harvested cells were disrupted as described in Materials and Methods and the supernatant was collected by centrifugation. Ammonium sulphate fractionation was then performed in serial concentration ranges of saturation with the supernatant as shown in Table 2. In our cephalexin-synthesizing enzyme, 60% of the total activity could be recovered within the 50% salt fraction, but highly specific activity was shown within the 40% salt fraction. After desalting by dialysis, the enzyme was further purified first by CM-Fractogel and then by Sephadex G-200 column chromatography, as shown respectively in Figs. 1 and 2. The purified enzyme was analyzed by 10% SDS-PAGE and two bands with molecular weight of 53 kd and 73 kd were detected (Fig. 3). The apparent molecular weight of the enzyme was 270 kd as estimated from gel filtration. This implies that the enzyme has a quarter-

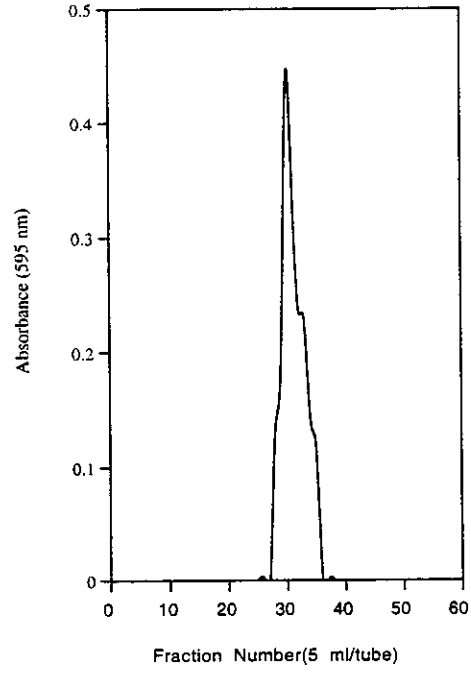


Fig. 1. Elution profile of CM-Fractogel column chromatography. The elution conditions were a gradient of 0-1 N NaCl in 0.1 M phosphate buffer, pH 6.6. Five mL of each fraction was collected and the protein concentration was determined by Bradford dye-binding procedure. The enzymatic activity was consistent with the profile of protein concentration.

Table 1. Purification of the Cephalexin-synthesizing Enzyme from *G. oxydans* CCRC10383

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell debrix	305	1896	6.2	
Cell-free extract	231	1416	6.1	100
40% Ammonium sulfate	76	992	13	70
CM-Fractogel column	21	741	35	52
Sephadex G200	7	392	56	27

1 Unit: 1 μmole cephalexin/min, 37 °C, pH 6.2

Table 2. Ammonium Sulfate Precipitation of the Cephalexin-synthesizing Enzyme from *G. oxydans* CCRC10383

% Ammonium sulfate	Total activity (U)	Specific activity[a] (U/mg)
0-20	16.4	17.3
30-40	16.6	15.5
40-50	12.8	2.8
50-60	9.1	0.8
60-80	11.8	1.5
80-100	9.1	1.8

[a] Specific activity = total unit (U)/total proteins (mg)

nary structure and consists of two subunits of 53 kd and two subunits of 73 kd. The isoelectric point of enzyme was estimated to be 7.5 by solubility experiment. The enzyme could produce cephalexin under pH 5-7 and 30-50 °C, and the optimum pH and temperature for its synthesis were found to be pH 6.0 and 50 °C. The purified enzyme maintained its high activity within 30 min in the reaction solution. The specific activity of the purified enzyme was 56 U/mg protein compared to 6.1 U/mg protein in the initial cell debris. This indicated that high enzyme activity in crude extract was recovered with about 9 fold purification finally. N-Terminal analysis of the enzyme failed to be determined by an automatic instrument (477A protein sequencer, Applied Biosystem) probably because of blockage of N-terminal.

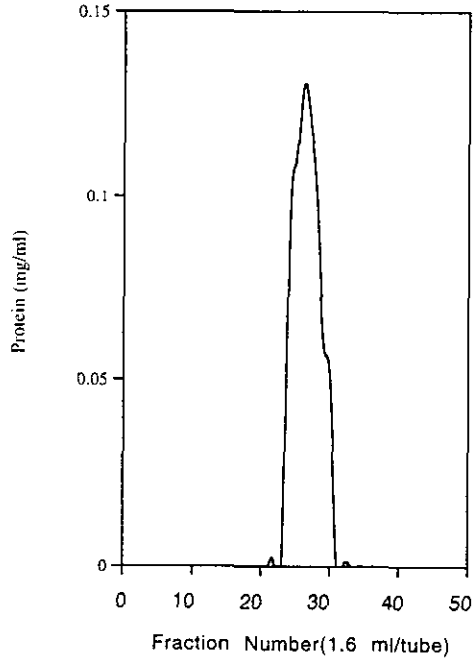


Fig. 2. Elution profile of Sephadex G-200 column chromatography. The eluent was 0.1 M phosphate buffer, pH 6.6, 1.6 mL of each fraction was collected and the protein concentration was determined by Bradford dye-binding procedure. The enzymatic activity was consistent with the profile of protein concentration.

Substrate Specificity

Metal ions are not essential for the activity of the enzyme because EDTA exerts no influence upon the enzyme activity. Cephalexin could not be synthesized directly from D-PG or their analogs (eg. phenylalanine methyl ester, *p*-hydroxyl-phenylglycine methyl ester) probably because of

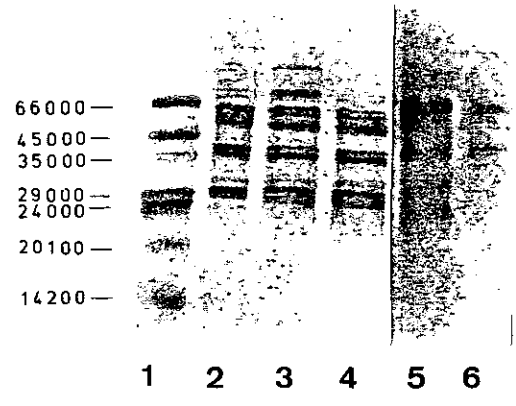


Fig. 3. Gel electrophoresis of purification of cephalexin-synthesizing enzyme in various steps under denaturing conditions (SDS-PAGE, 10%). Lane 1: protein markers (14.2 kd, α-lactalbumin; 20.1 kd, soybean trypsin inhibitor; 24 kd, trypsinogen; 29 kd, carbonic anhydrase; 35 kd, glyceraldehyde-3-phosphorylase; 45 kd, egg albumin; 66 kd, bovine serum albumin). Lane 2: cell-free supernatant; Lane 3: 50% ammonium sulphate; Lane 4: 50-100% ammonium sulphate; Lane 5: CM-Fractogel column; Lane 6: Sephadex G-200 column. The gels were stained with Coomassie blue.

high rigidity of the active site of the enzyme or low solubility of substrates.[16,18] The yield of cephalexin by the enzymatic synthesis with D-MEPG as substrate is about three times as high as with DL-MEPG as substrate. Ampicillin or cephaloglycine could be synthesized from D-MEPG and 6-APA or 7-ACA by the enzyme, but their formation rates are much lower than that of cephalexin. Either D,L-form of *p*-hydroxyphenylglycine methyl ester or L-MEPG could not be the substrates of the enzyme. In addition, neither benzylpenicillin (Penicillin G) nor cephalexin would be hydrolyzed by the enzyme. The inducer (0.25% DL-PG or 0.25% D-PG) could affect the enzyme activity in the bacterial growth stage, by inhibiting the initial cell growth and increasing the lag time. It increased up to 140% the activity of the enzyme with 1 day incubation. Compared with the proteins from non-induced incubation, there is no apparent difference between their protein patterns by SDS-PAGE analysis.

Synthesis of Cephalexin from D-MEPG and 7-ADCA

At a fixed concentration of 7-ADCA (14 mM), the rate of the enzymatic reaction increased as the concentration of D-MEPG was higher. The enzymatic rate could reach the maximal point, when the concentration of D-MEPG was 40 mM. When the concentration of D-MEPG was over 40 mM, the enzymatic rate would start to be reduced (Fig. 4). The maximum conversion of 7-ADCA to cephalexin could reach 60% based on the molar concentration of 7-ADCA, when the reaction solution were at pH 6.2 and the initial molar ratio of two substrates, D-MEPG and 7-ADCA, was 2.95:1. Further increase of the acyl donor does not result in higher conversion of the acyl acceptor. The synthesis with soluble enzyme at different levels of enzyme loading was evaluated. A higher enzyme loading increased the initial rate of reaction, but the equilibrium concentration of cephalexin was not affected by the amount of enzyme loading (data not shown). Recognizing that the cephalexin-synthesizing enzyme is involved in two-substrate reaction, the kinetic parameters were evaluated for the rate of expression for two substrates.[19] Lineweaver-Burk plots were constructed from the kinetic data obtained with various concentration of 7-ADCA and D-MEPG. The intercepts on the ordinates of Figs. 5a and 6a were reploted against the inverse of concentration of MEPG and 7-ADCA. From the intercepts and slopes of plots (Figs. 5b and 6b), the kinetic constants, $K_{7\text{-ADCA}}$, K_{MEPG} and V_{max}, were determined as 3.08 mM, 13.9 mM and 30 unit/per mg of protein, respectively.

DISCUSSION

Compared with the crude enzyme from *Xanthomonas citric*, the crude enzyme from *Gluconobacter* (6.1 U/mg) had much higher specific activity than that of *Xanthomonas* (2 U/mg). The enzymes from *Xanthomonas citri* IFO3825 and *Acetobacter* ATCC9325 had 66% of production, similar to our results (60%).[6,9] The synthesis of cephalexin could be increased up to 87% conversion by serial additions of D-MEPG at the appropriate time. So far, many reports have focused on cephalexin-synthesizing enzyme from *Xanthomonas* sp.,[3,7,9,14] which had specific affinity for α-amino acid esters and able to transfer the acyl group from α-amino acid esters to 7-ADCA, 7-ACA and 6-APA. The enzyme-catalyzed transfer of the acyl moiety is not from the free acid but from its derivatives (ester, amide and N-acylated phenylglycine).[6] From the viewpoint of thermodynamics, these results showed a considerable drop of the standard Gibbs energy changes and a shift of the thermodynamic pH optimum from the acidic range to a weakly acidic or neutral range for an enzymatic synthesis of antibiotics. It is also useful for

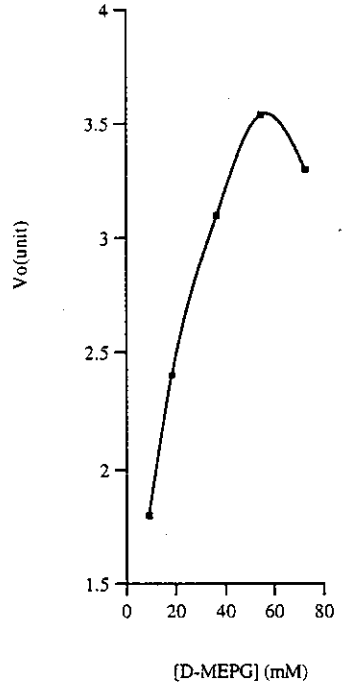


Fig. 4. Effect of molar ratio of two substrates on enzymatic rate. The enzyme assay was carried out in 0.1 M phosphate buffer (pH 6.2) at 37 °C under a fixed concentration of 7-ADCA (14 mM) and enzyme concentration of 2.38×10^{-10} mole with various concentration of D-MEPG.

the enzymatic reaction that D-MEPG has very high water solubility. D-MEPG is chosen as an acylating agent for 7-ADCA due to the kinetics of its acyl moiety transfer onto the donor catalyzed by the enzyme is preferable in comparison with other esters.[9]

The optimal pH of the synthetic reaction was nearly neutral (pH 6.0). The main problem is the absence of favorable thermodynamic conditions and very low solubility of 7-ADCA under acidic values of pH. *Pseudomonas melanogenum*[20] and *Xanthomonas citri*[9,15,16] results were similar to ours. The pI of our purified enzyme was estimated to be 7.5, almost the same as that of *Xanthomonas citri* and both of them had a quarternary structure analyzed by Sephadex G-200 column and SDS-PAGE. Biomolecular constant Kcat/Km, 6.2×10^5 $M^{-1}s^{-1}$, of the enzyme was higher than those from *Xanthomonas citri* IFO3835 (2.8×10^5 $M^{-1}s^{-1}$.

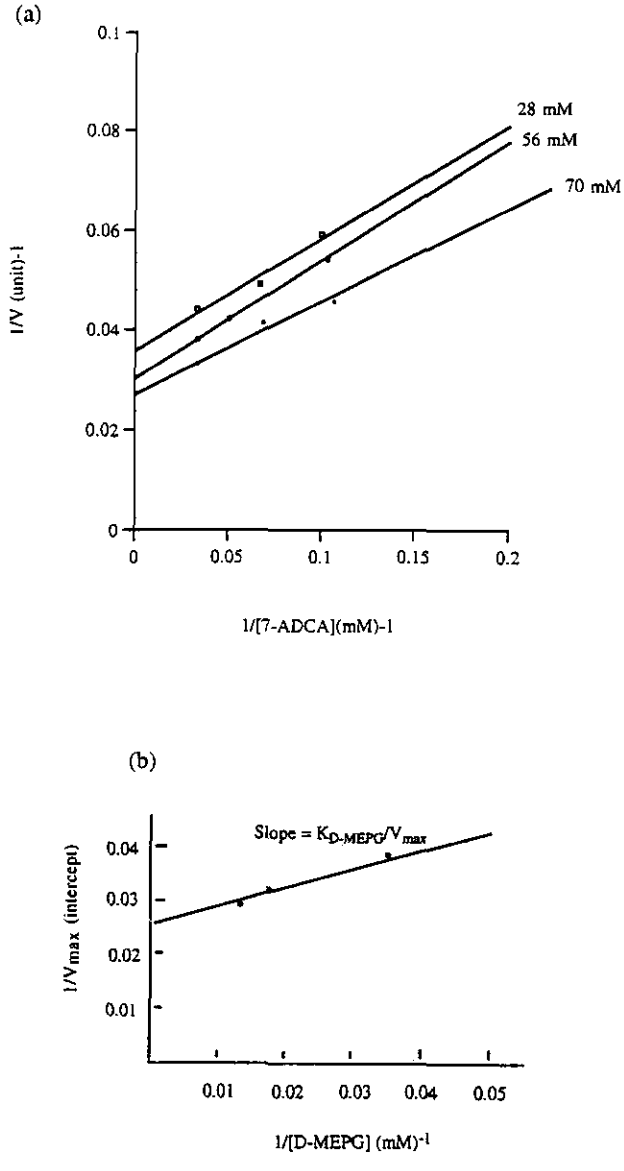


Fig. 5. Determination of Michaelis constant for D-MEPG, $K_{D\text{-}MEPG}$. The enzymatic rate was measured with various concentrations of 7-ADCA and three constant concentrations of D-MEPG (28 mM; 56 mM and 70 mM) in 0.1 M phosphate buffer (pH 6.2) at 37 °C. (a) plot of 1/v versus 1/[7-ADCA] (b) replot of 1/v-axis intercept versus 1/[D-MEPG].

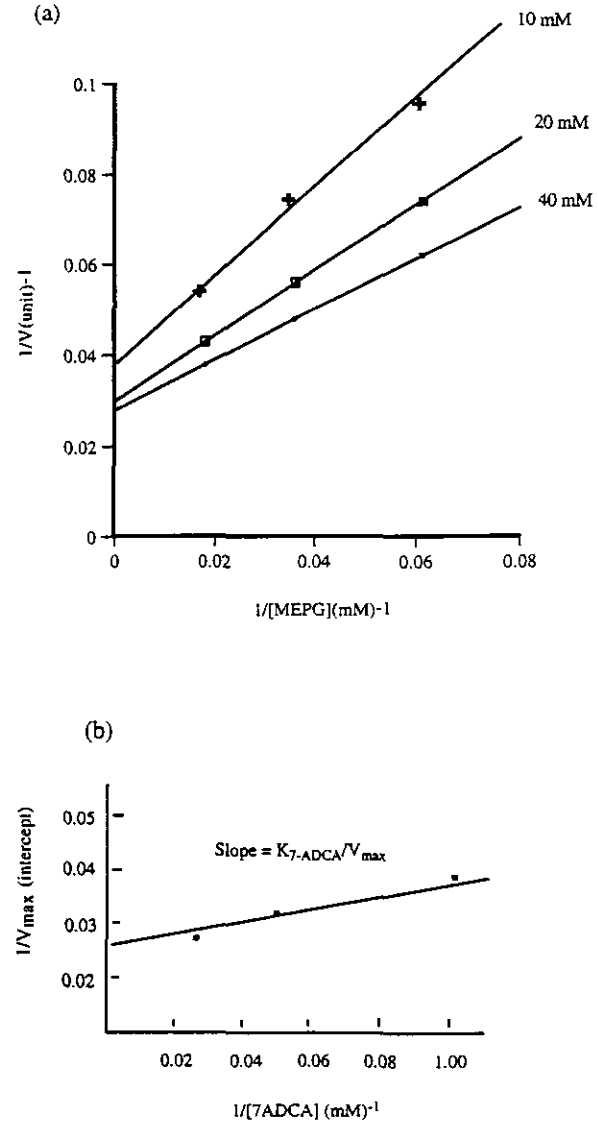


Fig. 6. Determination of Michaelis constant for 7-ADCA, $K_{7\text{-}ACDA}$. The enzymatic rate was measured with various concentrations of D-MEPG and three fixed concentrations of 7-ADCA (10 mM; 20 mM and 40 mM) in 0.1 M phosphate buffer (pH 6.2) at 37 °C. (a) plot of 1/v versus 1/[D-MEPG] (b) replot of 1/v-axis intercept versus 1/[7-ADCA].

D-MEPG could react with 7-ADCA and 7-ACA. This result indicates that the enzyme has a strong binding with dihydrothiazine or thiazo-lidine rings. The activity of the enzyme could be induced by DL-PG and D-PG, and increased about 1.4 fold, but not very significantly. It has been reported that the high inducer concentration for enzyme production inhibited initial cell grow and increased lag time, whereas the low inducer concentration stimulates growth, similar to our results.[12,13] Penicillin acylases of microorganisms particularity of *E. coli* have been reported to be induced by the presence of phenylglycine in growth medium and repressed by glucose.[13]

According to kinetic studies,[19] the intersection of the reciprocal plots (Fig. 5, 6) excluded the Ping-Pong mechanism, which results in a family of parallel lines. In the case of a random Bi-Bi mechanism, reciprocal plots are theoretically nonlinear. Hence, the enzyme-catalyzed synthesis of cephalexin from 7-ADCA and D-MEPG is an ordered reaction.[9] The Km values for D-MEPG and 7-ADCA were 13.9 mM and 3.08 mM, similar to those from *Xanthomonas citri* IFO3835 (14.5 mM, 3.7 mM).[14]

In the industrial production of cephalexin, D-MEPG is an important chiral moiety to react with 7-ADCA for the formation of cephalexin. Therefore, the preparation of D-MEPG is a key step for production of cephalexin. The preparation of D-MEPG from the resolution of racemic phenylglycine or from the asymmetric synthesis is costly and tedious. The cephalexin-synthesizing enzyme from *Gluconobacter oxydans* CCRC10383 could use DL-MEPG and 7-ADCA as substrates to produce cephalexin. It means that the enzyme has the ability not only to synthesize cephalexin but also to resolve DL-MEPG. If the enzyme is used for the production of cephalexin in industry, DL-MEPG can be directly used as substrates so that the preparation of D-MEPG will be avoided. On the basis of the preliminary results, the yield of cephalexin by the enzymatic synthesis with D-MEPG as substrate is about three time as high as with DL-MEPG as substrate. It implied that L-MEPG could inhibit the yield of cephalexin. This inhibition of the enzymatic reaction is the main difficulty for industrial use. In our future work, for the purpose of industrial use in the production of cephalexin, several steps in basic studies should be taken: (a) Although the N-terminal of the enzyme is blocked, the partial amino acid sequence should be determined and the gene of the enzyme should be cloned; (b) The enzyme is expressed in large quantity for the study of its tertiary structure; (c) The inhibition of L-MEPG in the production of cephalexin should be studied in detail and then the enzyme could be modified by site-directed mutagenesis based on its tertiary structure to eliminate the inhibition of L-MEPG.

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Key Words

Cephalexin; Cephalexin-synthesizing enzyme; Enzymatic synthesis; β-Lactam antibiotics.

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