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Penicillin acylase-catalyzed synthesis of pivampicillin: Effect of reaction variables and organic cosolvents

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Abstract

Enzymatic synthesis of pivampicillin (PVM) from D-α-phenylglycine methyl ester (PGM) and pivaloyloxymethyl 6-aminopenicillanic acid (POM–6-APA) was investigated using an immobilized *Escherichia coli* penicillin acylase. The effects of reaction variables such as enzyme concentration, pH, temperature, and molar ratio of the substrates on PVM synthesis were investigated. The time-course profiles of the PVM synthesis reaction followed a typical pattern of the kinetically-controlled synthesis of β-lactam antibiotics: the concentration of PVM reached a maximum and then decreased gradually. By lowering the reaction temperature, the maximum yield was enhanced significantly. This was mainly attributed to the suppressed hydrolysis of PVM and PGM at low temperatures. A higher yield of PVM was also attained with increasing the molar ratio of PGM to POM–6-APA. When the molar ratio of PGM to POM–6-APA was 10, the maximum yield of 61.8% was obtained at 4°C. The addition of organic cosolvents, on the other hand, showed no improvement in the PVM synthesis yields due to inhibitory effect of the solvent; both synthetic and hydrolytic activities of penicillin acylase were reduced by organic solvents. The degree of inhibition was found to be more profound in the presence of less polar solvents.

Keywords: Penicillin acylase; Pivampicillin; Kinetically controlled synthesis; Organic solvents

1. Introduction

The enzymatic synthesis of β-lactam antibiotics has been a subject of many researchers since Kaufman and Bauer [1] demonstrated the formation of penicillin G from phenylacetic acid and 6-aminopenicillanic acid (6-APA) by *Escherichia coli* penicillin acylase. Penicillin acylase (penicillin amidohydrolase; EC 3.5.1.11) from *E. coli*, *Bacillus megaterium* and *Kluyvera citrophila*, or α-amino acid ester hydrolase from *Xanthomonas citri* and *Acetobacter turbidance*, have been used for the synthesis of β-lactam antibiotics [2–14] which include penicillin G [2,10], ampicillin [3,4,7], amoxicillin [6], cephalexin [5,8,9], cephalothin [11], cefamandole [12], and cefazolin [13].

The advantage of enzymatic process in β-lactam antibiotics synthesis may be that one can use one-step reaction under mild conditions, while the chemical process often requires many reaction steps including protection/deprotection of functional groups and uses of toxic chemicals. In addition to this, a continuous or semi-

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continuous process can be employed for a large-scale production using an immobilized enzyme reactor.

In this work, we have investigated the enzymatic synthesis of pivampicillin (PVM), a prodrug of ampicillin, from pivaloyloxymethyl 6-aminopenicillanic acid (POM–6-APA) and D-α-phenylglycine methyl ester (PGM) using an immobilized *E. coli* penicillin acylase. Currently, PVM is produced by a chemical method [15], and the enzymatic synthesis of PVM has not yet been reported. To optimize the reaction conditions, we have examined the effect of reaction variables such as enzyme concentration, pH, temperature, and molar ratio of the substrates on PVM synthesis. Since the acyl-acceptor substrate (POM–6-APA) for the PVM synthesis is more hydrophobic than 6-APA, this study could provide information on the effect of the acyl-acceptor structure in penicillin acylase-catalyzed synthesis of β-lactam antibiotics.

Another objective of the present study was the investigation of the effects of organic solvents on penicillin acylase-catalyzed synthesis of PVM. The use of organic solvent systems in enzymatic organic synthesis has attracted many interests because the synthetic yield can be improved by reducing the water activity and/or altering the pK values of reactants (thus increasing the concentration of the uncharged, reactive substrate) [16–18]. Unlike the previously reported success in improving the yield of β-lactam antibiotics and peptides [10,11,19–21], this study shows that the reaction rate and maximum yield of PVM synthesis are decreased by adding organic solvents.

2. Materials and methods

2.1. Materials

Eupergit PcA, an *E. coli* penicillin acylase immobilized on Eupergit C, was purchased from Rohm Pharma (Germany) and used as an enzyme. The activity of Eupergit PcA was ca. 100 IU/g (wet). Penicillin G, 6-APA, D-α-phenylglycine, and ampicillin were purchased from Sigma (USA), D-α-phenylglycine methyl ester hydrochloride from Wako (Japan), and chloromethyl pivalate from Aldrich (USA). Pivampicillin was obtained from Choong Wae Pharmaceutical Co. (Korea). Acetonitrile and methanol used for HPLC eluent were from Malinckrodt (USA). All other reagents used were of analytical grade and obtained from Aldrich (USA).

2.2. Preparation of POM–6-APA

POM–6-APA was synthesized from 6-APA according to the method developed by Daehne et al. [13]. Triethylamine (49 ml, 0.35 mol) was added to a suspension of 6-APA (54 g, 0.25 mol) in DMF (250 ml) and, after stirring for 30 min, chloromethyl pivalate (74 ml, 0.35 mol) was added. After stirring at room temperature for 4 h, the mixture was diluted with ethyl acetate (750 ml). The precipitated triethylamine hydrochloride was filtered off and the filtrate was washed four times with water (250 ml) to remove DMF and unreacted 6-APA. The organic layer was concentrated to about half the volume by evaporation at 30–35°C. The crystalline hydrochloride of POM–6-APA was obtained by adding 1 N HCl in isopropanol to the concentrated POM–6-APA solution.

2.3. Enzyme reactions

All the experiments were carried out in 30 ml vials agitated at 150 rpm in a temperature-controlled incubator shaker (New Brunswick Scientific, USA). The reactions were started by adding enzyme suspensions (Eupergit PcA), which were preincubated in 50 mM MES buffer solution, to the reaction mixture. For HPLC analysis, 50 μl of the reaction mixture was withdrawn at predetermined time intervals. The initial reaction rate was determined from the polynomial regression of time-course profiles.

2.3.1. Synthesis of PVM and ampicillin

In the standard experiments, the reaction mixture (5 ml) composed of 50 mM MES buffer (with or without organic cosolvents), 5 mM POM–6-APA, and 10 mM PGM was used for PVM synthesis. The synthetic yield of PVM was determined based on the initial concentration of POM–6-APA and expressed as a percentage. The synthesis of ampicillin was carried out under the same conditions used for PVM synthesis except that 6-APA (5 mM) was used as an acyl acceptor.

2.3.2. Hydrolysis of PVM and PGM

The hydrolysis reactions of PVM and PGM were carried out using 2 mM PVM and 10 mM PGM (in MES buffer, pH 6.2), respectively. The reactions were initiated by adding 20 mg of Eupergit PcA to the reaction mixture (5 ml).

2.3.3. Determination of residual activities

In cases that PVM synthesis reactions were carried out in the presence of organic solvents, the residual activity of the enzyme was determined after the reaction was completed. The enzyme suspension was washed with MES buffer (pH 6.2), and the residual penicillin acylase activity was determined by measuring the initial rate of penicillin G hydrolysis reaction. The used enzymes were added to a solution containing 5 mM penicillin G (in MES buffer, pH 6.2) and the reaction was carried out at 30°C and 150 rpm for 1 h.

2.4. Analysis

2.4.1. HPLC analysis

Substrates and products were identified and analyzed by HPLC (Dionex Bio LC) and a UV detector (225 nm) with a μ-Bondapak C_{18} column (3.9 mm × 300 mm, Waters). To increase the resolution of elution peaks, three different eluents were used in this work. For the analysis of PVM synthesis, PVM hydrolysis, and PGM hydrolysis reactions, the eluent composed of 35% (v/v) acetonitrile, 20% (v/v) methanol and 45% (v/v) deionized water (pH 3.0) was used. Octanesulfonic acid (1 g/l), which forms an ion pair with cations, was added to the eluent to increase the resolution. The eluent used for ampicillin synthesis reaction was 20% (v/v) acetonitrile, 25% (v/v) methanol and 55% (v/v) deionized water (pH 2.4) with 1 g/l octanesulfonic acid. In the case of penicillin G hydrolysis reaction, the eluent composed of 40% (v/v) acetonitrile and 60% (v/v) deionized water (pH 2.6) was used. The pH of the eluent solution was adjusted with phosphoric acid and the flow rate of the eluent was 1.0 ml/min in all cases.

2.4.2. Determination of dissociation constants

Dissociation constants (pK values) of PGM, POM–6-APA, and PVM were determined at room temperature using an automatic titrator (Mettler DL 25). Each compound (5–10 mM) was dissolved in water or water–methanol mixtures and titrated with 0.1 N NaOH.

3. Results

3.1. Time-course profiles of PVM synthesis

Enzymatic synthesis of PVM from POM–6-APA and PGM was carried out at 30°C and pH 6.2 using *E. coli* penicillin acylase. As the reaction time had passed, D-α-phenylglycine (PG) as a hydrolytic product and PVM as a condensation product were detected in the HPLC chromatograms, similarly to other kinetically-controlled synthesis of β-lactam antibiotics [22,23]. The elution times of the new (product) peaks were 3.15 and 8.38 min and were identical to those of authentic samples of PG and PVM, respectively.

The time-courses of the PVM synthesis reaction also followed a typical pattern of the kinetically-controlled synthesis of condensation product. The concentration of PVM reached a maximum and then decreased gradually due to hydrolysis of the product (Fig. 1).

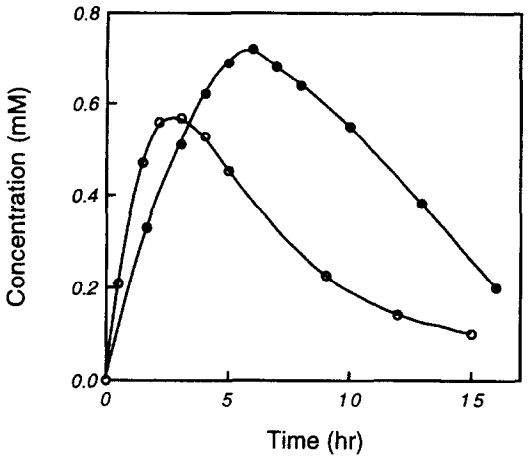


Fig. 1. Time courses of penicillin acylase-catalyzed synthesis of PVM and ampicillin. (○) PVM, (●) ampicillin. Reaction conditions: 5 mM POM-6-APA or 6-APA, 10 mM PGM, 4 mg/ml Eupergit PcA, 30°C, pH 6.2.

To examine the effect of acyl-acceptor structure on the reaction rate, the synthesis of ampicillin from 6-APA and PGM was carried out under the same conditions employed for the PVM synthesis except that 6-APA instead of POM-6-APA was used as an acyl acceptor. As compared in Fig. 1, the initial reaction rate of PVM synthesis was higher than that of ampicillin synthesis while the maximum yield of PVM was lower than that of ampicillin. This may indicate that hydrolysis of the synthesized PVM is very rapid as compared to the ampicillin hydrolysis reaction. From the separate experiment, we have found that the hydrolysis rate of PVM (at pH 6.2, 30°C) is about 1.6-fold higher than that of ampicillin when comparing at the substrate concentration of 1 mM (data not shown).

3.2. Effect of enzyme concentration

The effect of enzyme concentration (i.e., the amount of Eupergit PcA) on the rate and the maximum yield of PVM synthesis was studied at pH 6.2 and 30°C. As shown in Fig. 2, the PVM synthesis rates were increased by increasing the enzyme concentration: the initial reaction rates were 0.17, 0.48 and 0.66 mM/h at 1, 4 and 10 mg of Eupergit PcA/ml, respectively. However, the maximum yields were not significantly improved by increasing the amount of immobilized enzymes. This may be attributed to the enhanced hydrolysis of PVM with increasing enzyme concentrations. As can be seen from Fig. 2, PVM is hydrolyzed more rapidly at higher enzyme concentrations. Since the maximum yields obtained at enzyme concentrations of 4 and 10 mg/ml were not significantly different, we used an enzyme concentration of 4 mg/ml in subsequent experiments.

3.3. Effect of pH and temperature

The pH of reaction media influences not only the catalytic activity of an enzyme but also the ionization states of substrates, which affects their affinities for enzymes and solubilities in reaction media. The PVM synthesis reactions were carried out at three different pHs. As shown in Fig. 3, not only the initial syntheses of PVM but the secondary hydrolyses of PVM were faster at higher pHs. Initial reaction rates and maximum yields were 0.15, 0.25, and 0.46 mM/h and 8.2, 11.0, and 11.5% at pH 5.5, 5.8, and 6.2, respectively. Further increase in pH was undesirable

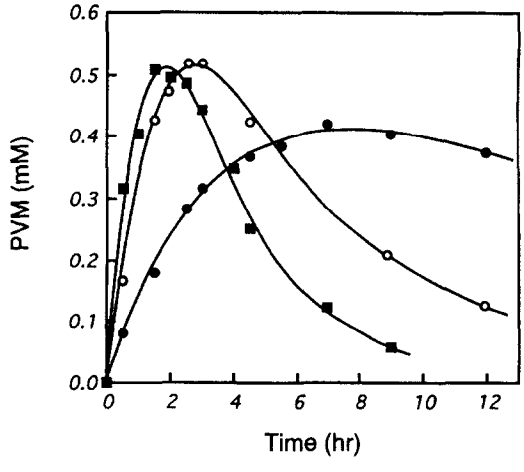


Fig. 2. Dependence of PVM synthesis on enzyme concentration. (●) 1 mg/ml, (○) 4 mg/ml, (■) 10 mg/ml of Eupergit PcA. Reaction conditions: 5 mM POM-6-APA, 10 mM PGM, 30°C, pH 6.2.

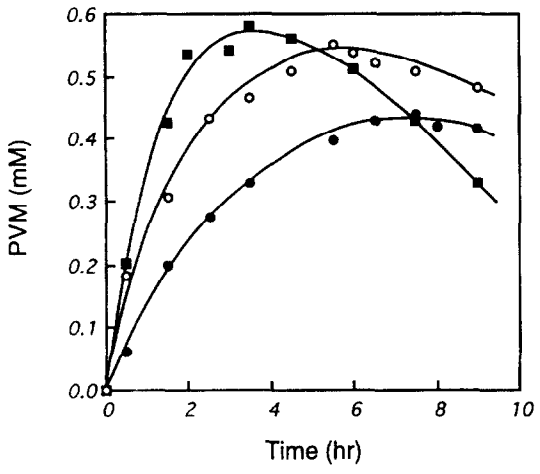


Fig. 3. Dependence of PVM synthesis on pH. (●) pH 5.5, (○) pH 5.8, (■) pH 6.2. Reaction conditions: 5 mM POM-6-APA, 10 mM PGM, 4 mg/ml Eupergit PcA, 30°C.

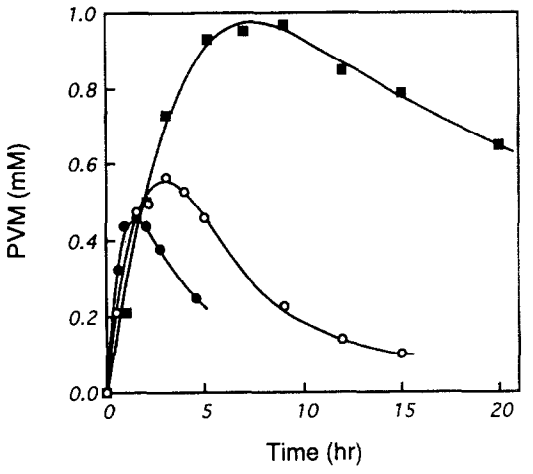


Fig. 4. Dependence of PVM synthesis on temperature. (●) 37°C, (○) 30°C, (■) 4°C. Reaction conditions: 5 mM POM-6-APA, 10 mM PGM, 4 mg/ml Eupergit PcA, pH 6.2.

due to low solubility of POM-6-APA and the nonenzymatic hydrolysis of PGM, POM-6-APA, and PVM at alkaline environments.

Ionization states of reactants are determined by pK values of each compound as well as the pH of the reaction medium, and thus dissociation constants of substrates and product were determined by titration method. The pK values of POM-6-APA, PGM and PVM were 4.45, 6.95 and 6.97, respectively. Similar pK values of PGM and PVM are expectable because both have the same amine group in the phenylglycine moiety.

The effect of temperature on PVM synthesis was examined at three different temperatures (4, 30 and 37°C). As depicted in Fig. 4, the reaction rate was lowered at low temperature, but the maximum yield was greatly increased. The maximum yield of PVM was enhanced by a factor of 1.8 by lowering the reaction temperature from 30°C to 4°C. In order to investigate the temperature effect further, the hydrolysis reaction rates of PVM and PGM were determined from separate experiments at 4°C and 30°C, respectively. The ratio of reaction rates at 4°C to 30°C (v_4/v_{30}) as well as individual reaction rates for PVM synthesis (v_s), PVM hydrolysis (v_{h1}) and PGM hydrolysis (v_{h2}) are shown in Table 1. The v_4/v_{30} values of two hydrolysis reactions (0.23–0.26) were much lower compared with that of the PVM synthesis reaction (0.61). Hence, we can infer that the increase in the maximum yield at low temperatures is due to the suppression of undesired secondary hydrolysis of PVM and PGM.

3.4. Effect of substrate concentration ratio

In order to examine the effect of the ratio of acyl donor to acyl acceptor, the PVM synthesis reactions were performed at various concentrations of PGM at a given POM-6-APA concen-

Table 1
Comparison of initial reaction rates for PVM synthesis, PVM hydrolysis, and PGM hydrolysis reactions at 4°C and 30°C

Temperature	Initial reaction rate (mM/h) [a]				
(°C)	v_s	v_{h1}	v_{h2}	v_s/v_{h1}	v_s/v_{h2}
4	0.27	1.14	1.26	0.24	0.21
30	0.44	4.87	4.85	0.09	0.09
v_4/v_{30}	0.61	0.23	0.26	2.67	2.33

Reaction conditions: 4 mg/ml Eupergit PcA, pH 6.2. Substrate concentrations were 5 mM POM-6-APA and 10 mM PGM, 2 mM PVM, 10 mM PGM for v_s, v_{h1}, and v_{h2}, respectively.
[a] v_s: PVM synthesis rate, v_{h1}: PVM hydrolysis rate, v_{h2}: PGM hydrolysis rate.

Table 2
Effect of the ratio of acyl donor to acyl acceptor (R) on the synthesis of PVM [a]

R ([PGM]/[POM-6-APA])	Initial reaction rate (mM/h)		Maximum yield (%)	
	30°C	4°C	30°C	4°C
2	0.47	0.31	10.2	19.6
3	0.70	0.46	15.2	26.0
5	1.09	0.57	23.8	39.6
10	ND	0.99	ND	61.8

Reaction conditions: 5 mM POM-6-APA, 4 mg/ml Eupergit PcA, pH 6.2.
[a] ND: not determined.

tration (5 mM). The initial reaction rates and maximum yields determined at 30°C and 4°C are summarized in Table 2.

As the molar ratio of acyl donor to acyl acceptor (R = [PGM]/[POM-6-APA]) was increased, both initial reaction rates and maximum yields were increased. The enhancement of maximum yields was found to be more profound at a lower temperature. When $R = 10$, for example, we obtained a maximum yield of 61.8% at 4°C (Fig. 5). From Fig. 5, one can find that the yield reaches a maximum at nearly constant reaction time regardless of the molar ratio of acyl donor to acyl acceptor.

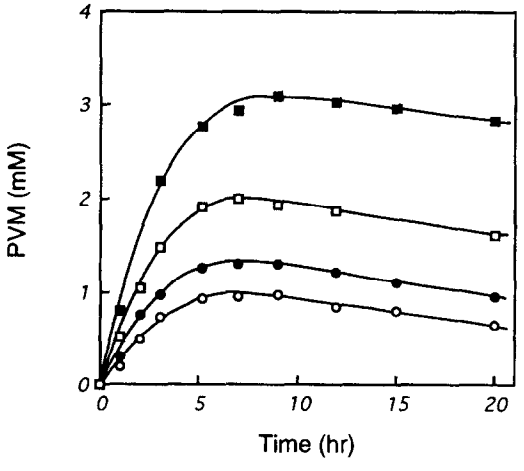


Fig. 5. Dependence of PVM synthesis on the ratio of acyl donor to acyl acceptor (R = [PGM]/[POM-6-APA]) at 4°C. (○) $R = 2$, (●) $R = 3$, (□) $R = 4$, (■) $R = 10$. Reaction conditions: 5 mM POM-6-APA, 4 mg/ml Eupergit PcA, pH 6.2.

3.5. Effect of organic cosolvents on PVM synthesis

In order to examine whether the yield of PVM synthesis could be enhanced by adding organic cosolvents, we investigated the effect of water-miscible solvents on penicillin acylase-catalyzed synthesis of PVM. Preliminary experiments in water-miscible organic solvents including methanol, acetonitrile, dimethyl sulfoxide, and t-amyl alcohol (2-methyl-2-butanol) showed that the yield of PVM synthesis as well as the reaction rates could be significantly reduced at higher concentrations of these solvents (data not shown).

Since the reaction rate of PVM synthesis was much lowered by organic solvents, we examined the time courses of PVM syntheses in the presence of 2% (v/v) of organic cosolvent. The initial reaction rates and maximum yields obtained in eight reaction media are summarized in Table 3. In all the cases examined in this work, the maximum yield of PVM synthesis was not enhanced by the presence of organic cosolvents. Initial reaction rates were affected more markedly by a small amount of organic solvents. For example, the rate in 2% (v/v) isopropanol was only one sixth of that obtained in buffer solution.

To examine whether enzymes were deactivated during the reaction, we determined the

Table 3
Effect of organic cosolvents on initial reaction rate and maximum yield of PVM synthesis, and hydrophobicity of cosolvents (log P)

Reaction media	Initial rate (mM/h)	Maximum yield (%)	log P
buffer	0.46	10.8	−1.38
buffer + 2% (v/v) methanol	0.42	10.8	−0.77
buffer + 2% (v/v) ethanol	0.16	10.5	−0.31
buffer + 2% (v/v) isopropanol	0.08	7.4	0.05
buffer + 2% (v/v) DMSO	0.41	10.5	−1.35
buffer + 2% (v/v) DMF	0.19	8.7	−1.01
buffer + 2% (v/v) acetonitrile	0.23	10.2	−0.34
buffer + 2% (v/v) acetone	0.15	10.1	−0.24

Reaction conditions: 5 mM POM-6-APA, 10 mM PGM, 4 mg/ml Eupergit PcA, pH 6.2, 30°C.

Table 4
The relative rates of PVM synthesis, PVM hydrolysis, and PGM hydrolysis reactions in the presence of organic cosolvents (2% v/v, 30°C) [a]

Cosolvent	v_s	v_{h1}	v_{h2}	v_{h2}/v_{h1}
methanol	0.90	0.80	0.69	0.86
t-amyl alcohol	0.48	0.56	0.43	0.77
i-propanol	0.24	0.35	0.12	0.34

[a] The rate of each reaction has been normalized to that obtained without organic cosolvents (cf. Table 1).

residual activity of the enzyme, and found that at least 95% of initial activities were retained after 12 to 15 h of reaction for all the cases examined in Table 3 (data not shown). Thus, the decrease of the reaction rate in water–cosolvent mixture was not due to the denaturing effect of the solvent. As will be discussed later, it seems likely that hydrophobic interactions between organic solvents and penicillin acylase play a major role in determining the rate of penicillin acylase-catalyzed reactions in organic solvents (see 4. Discussion).

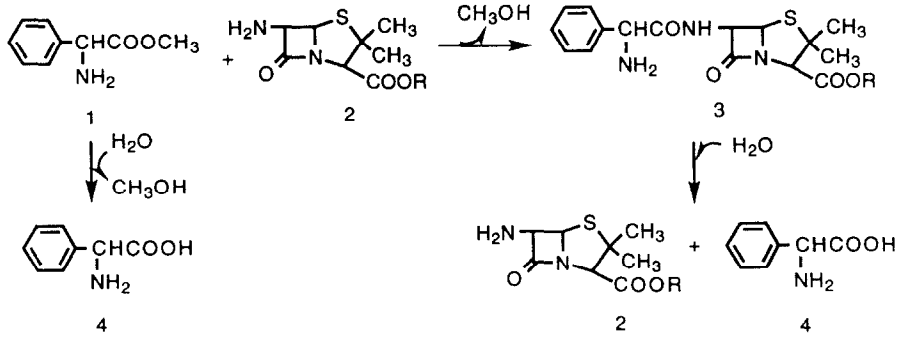
The effect of cosolvents on the hydrolysis reactions of PVM (amidase activity) and PGM (esterase activity) was also investigated in the presence of 2% (v/v) cosolvent. As presented in Table 4, both amidase activities (v_{h1}) and esterase activities (v_{h2}) were reduced by adding organic cosolvents, and the decrease of esterase activities was found to be greater than that of amidase activities.

3.6. PVM synthesis in water-immiscible solvents

We also investigated PVM synthesis in non-polar water-immiscible organic solvents saturated with a buffer solution. Contrary to our anticipation, however, no or negligible PVM synthesis was observed in water-immiscible organic solvents such as ethyl acetate, butyl acetate, and *n*-octanol, despite higher solubilities of POM–6-APA in these solvents. On the other hand, the enzyme activity was restored after removal of organic solvents, as was the case for water-miscible solvents (data not shown).

4. Discussion

The experimental results shown in the present study indicate that the mechanism of penicillin acylase-catalyzed PVM synthesis reaction is similar to that of other β-lactam antibiotics such as ampicillin and cephalexin [22,23]. PGM, an activated acyl-donor substrate, forms an acyl-enzyme intermediate with penicillin acy-



1 : D-α-phenylglycine methyl ester
2 : POM-6-APA (R=$CH_2OCOC(CH_3)_3$), 6-APA (R=H)
3 : pivampicillin (R=$CH_2OCOC(CH_3)_3$), ampicillin (R=H)
4 : D-α-phenylglycine

Scheme 1. Reaction mechanism for penicillin acylase-catalyzed synthesis of pivampicillin.

Table 5
Substrate specificity of penicillin acylase and hydrophobicity of substrate

Substrate	k_{cat} (s^{-1})	K_m (M)	$\log(k_{cat}/K_m)$	log P (pH 7.4)
penicillin G	48	$4.6 \cdot 10^{-6}$	7.02	−1.81
cephalothin	25	$4.2 \cdot 10^{-5}$	5.77	−2.20
cephalexin	54	$2.1 \cdot 10^{-3}$	4.41	−2.40
ampicillin	11	$5.2 \cdot 10^{-3}$	3.33	−2.60
cephaloridine	33	$1.0 \cdot 10^{-4}$	5.52	−1.62

The values of k_{cat} and K_m were taken from Margolin et al. [22] and the log P parameters were from Hansch et al. [24]

lase, and POM–6-APA and water react with an acyl-enzyme intermediate competitively to produce PVM and PG, respectively (Scheme 1).

The maximum yield of PVM synthesis was lower than that of ampicillin when compared at the same reaction conditions (Fig. 1). The low yield of PVM was ascribed to the rapid hydrolysis of PVM produced during the reaction. It is known that the active site of penicillin acylase is extremely hydrophobic [24,25], and hence the reaction rate of penicillin acylase is expected to be related to the hydrophobicity of the substrate. In Table 5, the experimental data of Margolin et al. [24] are shown along with the hydrophobicity of substrates (log P). The values of log P, the logarithm of distribution coefficient between n-octanol and water, were taken from Hansch et al. [26]. As depicted in Fig. 6, there is a close correlation between log (k_{cat}/K_m) and log P of the substrate except cephaloridine (shown as open circle). Because PVM is more hydrophobic than ampicillin due to the pivaloyloxymethyl moiety in carboxyl group, it is plausible that penicillin acylase hydrolyzes PVM more efficiently than ampicillin.

The dependence of PVM synthesis rates on pH can be interpreted by the proposition of Margolin et al. [24]. They explained the pH-dependence of k_{cat}/K_m of penicillin acylase-catalyzed hydrolysis of the two model compounds, phenylacetic and D-α-aminophenylacetic acid p-nitroanilides, by assuming that enzyme binds the nonionized, neutral form of the substrate only, due to extremely hydrophobic nature of the active site. In our case, both substrates (PGM, POM–6-APA) and product (PVM) have one ionizable amine group, and the pK values of POM–6-APA, PGM and PVM determined by the titration method were found to be 4.45, 6.95 and 6.97, respectively. At a given pH, the fraction of nonionized form (f_N) can be determined by the following expression:

$$f_N = [RNH_2]/([RNH_3^+] + [RNH_2])$$
$$= 1/(1 + 10^{pK_b - pH})$$

where $K_b = [RNH_2][H^+]/[RNH_3^+]$. The f_N values calculated at different pHs for PGM, POM–6-APA and PVM are shown in Table 6. The enhanced synthetic reaction rate and accelerated hydrolytic reaction rate of PVM at higher pHs (cf. Fig. 3) may be explained by the increased f_N values of substrates (PGM and POM–6-APA) and product (PVM), respectively.

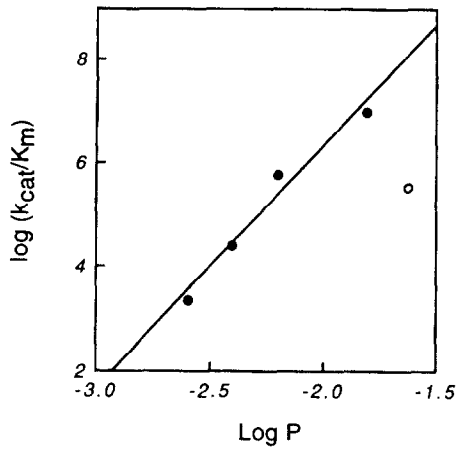


Fig. 6. Dependence of log (k_{cat}/K_m) on the hydrophobicity of substrates.

Table 6
The fraction of nonionized, reactive form (f_N) of PGM, POM–6-APA, and PVM at various pH values

pH	f_N		
	PGM	POM–6-APA	PVM
5.5	0.034	0.918	0.033
5.8	0.066	0.957	0.063
6.2	0.151	0.983	0.145

The decrease in maximum yields with temperature has generally been observed for kinetically controlled syntheses of β-lactam antibiotics and peptides [11,12,20]. As can be seen from Table 1, the enhanced maximum yield is attributed to the reduction of undesirable reactions (hydrolysis of PGM and PVM). The ratio of the initial reaction rate at 4°C to 30°C (v_4/v_{30}) was 0.23 for the hydrolysis reaction of PVM, while v_4/v_{30} for the PVM synthesis rate was 0.61. This leads to the enhancement of the ratio v_s/v_{hl} from 0.09 to 0.24, which results in an overall 2.7-fold enhancement of the synthesis reaction rate (relative to the hydrolysis reaction rate) by lowering the temperature from 30°C to 4°C.

It is often possible to enhance the yield of a reversible enzyme reaction by adding organic cosolvents to the aqueous reaction medium. Organic cosolvent reduces the rate of hydrolytic reactions by lowering the water activity of the reaction media and changes the pK values of the reactants favoring the synthesis reaction. Solubility of substrate(s) can also be enhanced compared to that in water by adding organic cosolvents. In the case of PVM synthesis reaction, the suppression of hydrolysis of PVM is desirable, and the solubility of POM–6-APA is low in an aqueous solution. We therefore expected that the addition of organic cosolvents could enhance the PVM synthesis yield. Contrary to our expectations, however, no increase of the maximum yield was observed by adding organic solvents to the reaction mixture. Both reaction rates and maximum yields were reduced by the presence of a small amount (2% v/v) of water-miscible organic cosolvents. Furthermore, almost no synthesis of PVM was observed in nonpolar, water-immiscible solvents.

In all cases examined in this work, most of enzyme activity was restored after removal of organic solvents. Hence, it seems likely that the organic solvent acts as a reversible inhibitor of penicillin acylase. If the reduction of reaction rate in water–solvent mixture is caused by the binding of the solvent to the enzyme, then the hydrophobicity of organic solvents may play a major role in determining their inhibitory effect on penicillin acylase due to hydrophobic nature of the active site. In Fig. 7, the dependence of the initial reaction rate of PVM synthesis on the hydrophobicity of the solvent (log P) is depicted. (The log P values of pure solvent taken from Hansch et al. [26] are listed in Table 3.) Experimental data obtained for water-miscible solvents indicate that the degree of inhibition is indeed related to the solvent hydrophobicity, showing a more profound inhibition in the presence of a less polar organic solvent (Fig. 7). Almost no synthesis of PVM in nonpolar, water-immiscible solvents may also be explained by their higher hydrophobicity. Since the solvent was saturated with buffer solution prior to use (hence, the water activity is close to 1.0 in all cases [cf. [27,28]]) and the enzyme activity was restored by removing the solvent, we can exclude the possibility that the loss of PVM synthesis activity in water-immiscible solvents comes from the effect of water activity or enzyme deactivation. More comprehensive discussion of the solvent effect on penicillin acylase-catalyzed reactions will be presented elsewhere in detail (Kim and Lee, manuscript in preparation).

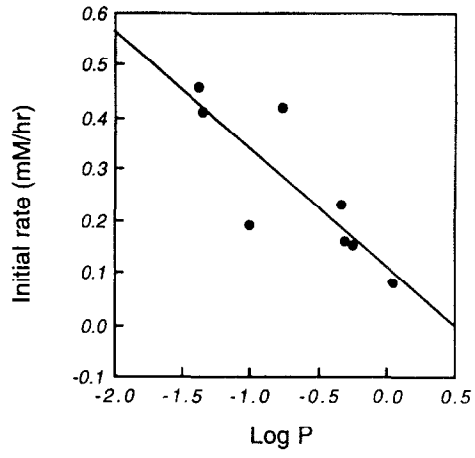


Fig. 7. Dependence of initial reaction rate on the hydrophobicity of organic solvents.

The reaction mechanism of penicillin acylase is similar to proteases in many aspects [23]. However, as can be seen from Table 4, the effect of cosolvents on penicillin acylase seems to be different from many serine and cysteine proteases. It has been reported that serine and cysteine proteases exhibit a suppressed amidase activity by organic solvents while the esterase activity remains significant [29]. Such amidase-suppressed proteases are useful for kinetically controlled peptide syntheses due to the prevention of undesired secondary hydrolysis of peptide bonds. In a kinetically controlled synthesis of PVM, however, the esterase activity (v_{h2}) as well as amidase activity (v_{h1}) of penicillin acylase was suppressed by organic solvents. Since the ratio of esterase to amidase activity (v_{h2}/v_{h1}) is smaller than 1.0 in the presence of organic solvents, we may deduce that organic solvents inhibit formation of an acyl-enzyme intermediate between PGM and enzymes more effectively than that between PVM and enzymes.

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