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Journal of Molecular Catalysis B: Enzymatic 1 (1996) 201–211

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

# Penicillin acylase-catalyzed synthesis of β-lactam antibiotics in water–methanol mixtures: effect of cosolvent content and chemical nature of substrate on reaction rates and yields

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Received 11 December 1995; accepted 7 February 1996

**Abstract**

The synthesis of four β-lactam antibiotics (penicillin G, pivaloyloxymethyl ester of penicillin G, ampicillin and pivampicillin) catalyzed by *Escherichia coli* penicillin acylase has been investigated in water–methanol mixtures. The enzyme reactions were either thermodynamically or kinetically controlled at the same conditions using phenylacetic acid and D-α-phenylglycine methyl ester as acyl donors and 6-aminopenicillanic acid and pivaloyloxymethyl 6-aminopenicillanic acid as acyl acceptors. It has been found that the influences of the cosolvent content on the reaction rates and synthetic yields are significantly different depending on the substrates used in the experiments. On the other hand, within certain ranges of the methanol content (up to ca. 40% (v/v)) the residual activities of the enzymes in water–methanol mixtures were only slightly lower than those in aqueous media. To analyze the factors that determine the reaction rate in water–cosolvent mixtures, the effect of methanol on the apparent p$K$ values of the substrates has been investigated, and a mathematical model has been developed on the basis of the assumption that the enzyme binds non-ionized substrates. Model simulation results indicate that the solvent effect on reaction rates is mainly attributed to the kinetic effects of changes in apparent p$K$ values.

*Keywords:* Penicillin acylase; Penicillin G; Ampicillin; Pivampicillin; β-Lactam antibiotic synthesis; Water–cosolvent mixtures; Solvent effects on reaction rates and yields; Solvent effects on apparent p$K$ values

## 1. Introduction

Enzymatic synthesis of β-lactam antibiotics has long been a subject of many researchers, although industrial production of semisynthetic β-lactam antibiotics by enzymatic methods has not yet been realized [1–3]. The main drawback of the enzyme process may be the low synthetic yield of the product. Recently, the use of organic solvent systems in enzymatic organic synthesis has attracted much interest because the synthetic yield can be improved by increasing the solubility of substrates, reducing the water activity and altering the p$K$ values of reactants (thus increasing the concentration of the uncharged, reactive substrate) [4–11]. In consequence, it would be of interest to examine the synthesis of β-lactam antibiotics in the presence of organic solvents.

The enzymatic syntheses of β-lactam antibiotics in water–cosolvent mixtures have previously been investigated [12–14]. Kasche et al. briefly reported the influence of organic solvents (20% acetone, 20% DMSO) on synthetic

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*PII* S1381-1177(96)00007-0

yields in the equilibrium and kinetically controlled synthesis of penicillin G [12]. According to their report, the yield of penicillin G could be enhanced by 2–3 times for an equilibrium controlled synthesis whereas there was no or little improvement in the maximum yield for a kinetically controlled synthesis. More detailed studies on the synthesis of β-lactam antibiotics in organic solvents have been made by Guisan and his coworkers [13,14]. They investigated the equilibrium controlled synthesis of penicillin G [13] and cephalothin [14] in the presence of higher concentration of polar solvents (e.g., 50% DMF). They were able to reach more than 95% of synthetic yields for the production of penicillin G and cephalothin under optimal conditions in which excess amounts of acyl donors were used.

Recently, we have investigated the kinetically controlled synthesis of pivampicillin using *Escherichia coli* penicillin acylase (penicillin amidase, penicillin amidohydrolase; EC 3.5.1.11) [15,16]. Unlike the previously reported success in improving the yield of β-lactam antibiotics and peptides [9–14], however, the maximum yields of pivampicillin synthesis were not enhanced by the addition of water-miscible organic solvents [15]. Furthermore, negligible or no synthesis of pivampicillin was observed in water-immiscible, hydrophobic solvents such as ethyl acetate, butyl acetate, and *n*-octanol, despite higher solubilities of the substrate in these solvents. It has been found that the catalytic activity of penicillin acylase can be considerably lowered in the presence of hydrophobic solvents due to binding of organic solvents to the enzyme [16].

Viewing these contradictory effects of the solvent on penicillin acylase-catalyzed synthesis reactions, it is considered that more systematic analysis of the factors that influence the catalytic efficiency of enzymes in organic solvents is required. Most previous works have been carried out at a fixed amount of organic solvents for a single product, and thus the effects of solvent concentrations and chemical properties of the substrate on β-lactam antibiotic synthesis have not yet been fully assessed.

Table 1
Acyl donors and acyl acceptors used for the synthesis of β-lactam antibiotics [a]

| Acyl donor | Acyl acceptor | Condensed product |
|---|---|---|
| *Equilibrium controlled synthesis* | | |
| PAA | 6-APA | penicillin G |
| PAA | POM–6-APA | POM–PG |
| *Kinetically controlled synthesis* | | |
| PGM | 6-APA | ampicillin |
| PGM | POM–6-APA | pivampicillin |

[a] Abbreviations: PAA; phenylacetic acid, 6-APA; 6-aminopenicillanic acid, POM–6-APA; pivaloyloxymethyl 6-aminopenicillanic acid, POM–PG; pivaloyloxymethyl ester of penicillin G, PGM; D-α-phenylglycine methyl ester.

In this work, we have investigated the penicillin acylase-catalyzed synthesis of four different kinds of β-lactam antibiotics in water–methanol binary systems, which include penicillin G, pivaloyloxymethyl ester of penicillin G (POM–PG), ampicillin and pivampicillin. The synthesis of these β-lactam antibiotics can be carried out using each one of two types of acyl donors and acyl acceptors as shown in Table 1. Therefore, it is considered that this study can provide valuable information on the effect of the substrate properties on penicillin acylase-catalyzed reactions in water–cosolvent systems. In the present study, we also made an attempt to analyze the factors that determine the enzyme activity in organic solvents. For this purpose, a correlation equation which relates the reaction rate to changes in the fraction of non-ionized, reactive substrate, water activity of the reaction medium and inhibitory effect of the solvent has been developed, and predictions of the proposed relationship have been compared with experimental observations.

## 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, and ampicillin were purchased from Sigma (USA), D-α-phenylglycine methyl ester from Wako (Japan), and phenylacetic acid and chloromethyl pivalate from Aldrich (USA). Pivampicillin was obtained from Choong Wae Pharmaceutical Co. (Korea). All other reagents used were of analytical grade.

POM–6-APA was synthesized from 6-APA and chloromethyl pivalate according to the method developed by Daehne et al. [17] as described in a previous paper [15]. POM–PG was synthesized from penicillin G and chloromethyl pivalate by the same procedures that were applied to the synthesis of POM–6-APA. Both POM–6-APA and POM–PG prepared in this way eluted as single peaks in HPLC.

### 2.2. Enzyme reactions

#### 2.2.1. Synthesis of β-lactam antibiotics

All reactions were carried out at 20°C in 30 ml vials shaken at 150 rpm in a temperature-controlled rotary shaker (New Brunswick Scientific, USA). In the standard experiments, the reaction mixture (5 ml) was composed of 5 mM acyl donors, 10 mM acyl acceptors, and 50 mM of 2-(*N*-morpholino)ethanesulphonic acid–NaOH (MES) buffer (pH 6.2) with or without methanol. The reactions were started by adding 20 mg of enzyme suspensions (Eupergit PcA) to the reaction mixture. For HPLC analysis, 50 μl of the reaction mixture was withdrawn at predetermined time intervals.

The initial reaction rates were evaluated according to the following procedure: first, the experimental data (initial four to five data points) for the reaction course were fitted to a third-order polynomial equation, then the first derivative was obtained and the initial rate evaluated from the resulting equation. The synthetic yields of condensed products were determined based on the initial concentration of acyl donors and expressed as a percentage.

#### 2.2.2. Determination of residual activities

The residual activity of the enzyme was determined after the reaction was completed. The enzyme suspension was washed with MES buffer solution (pH 6.2) two to three times, and the residual penicillin acylase activity was determined by measuring the rate of penicillin G hydrolysis in buffer solution. 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.

### 2.3. 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. 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.

For the analysis of penicillin G and POM–PG, the eluent composed of 40% (v/v) acetonitrile and 60% (v/v) deionized water (pH 2.6) was used. In the case of pivampicillin synthesis, 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.

### 2.4. Determination of dissociation constants

The apparent dissociation constants (p$K$ values) of PAA, PGM, 6-APA, and POM–6-APA were determined at room temperature using an

automatic titrator (Mettler DL 25). The electrode (DG 111-SC) was soaked in the appropriate cosolvent mixtures for 20 min prior to use. The compounds (5–10 mM) were dissolved in water or water–methanol mixtures and titrated with 0.1 N NaOH or 0.1 N HCl. All p$K$ values were obtained from the average of four measurements. The glass electrode was calibrated in aqueous buffer solutions and no correction for solvent medium effects was made. Thus, the pH and p$K$ values in aqueous–organic mixtures refer to the apparent values.

### 2.5. Estimation of thermodynamic activity

The activity of species $i$, denoted by $a_i$, is defined as:

$$a_i = x_i \gamma_i \tag{1}$$

where $x_i$ and $\gamma_i$ are the mole fraction and activity coefficient of component $i$, respectively. In this study, the NRTL equation proposed by Renon and Prausnitz [18] has been used for the estimation of thermodynamic activity of water and methanol. For a binary mixture system the NRTL equation can be expressed as:

$$\ln \gamma_i = x_j^2 \left\{ \tau_{ji} \left[ G_{ji} / \left( x_i + x_j G_{ji} \right) \right]^2 + \tau_{ij} G_{ij} / \left( x_j + x_i G_{ij} \right)^2 \right\} \tag{2}$$

where $G_{ij} = \exp(-\alpha_{12}\tau_{ij})$ and $\tau_{ij} = A_{ij}/RT$ ($R$ = 1.987 cal/mol K). Three NRTL equation parameters required for a water–methanol binary system ($A_{12} = -287.950$, $A_{21} = 694.875$, and $\alpha_{12} = 0.2442$) were obtained from Gmehling et al. [19]. Densities of water (0.998 g/cm$^3$) and methanol (0.791 g/cm$^3$) at 20°C were taken from Riddick et al. [20].

## 3. Results and discussion

### 3.1. Synthesis of β-lactam antibiotics in water–methanol mixtures

To compare the reaction profiles of four β-lactam antibiotics (penicillin G, POM–PG, ampicillin and pivampicillin) on the same basis, all enzyme reactions were carried out under identical conditions in a medium composed of water (50 mM MES buffer) and methanol.

It is known that the apparent pH of the buffer solutions can be changed by the presence of organic cosolvents, e.g., [21]. Phosphate [12] or acetate buffer [13,14] has previously been used as an aqueous medium for the synthesis of β-lactam antibiotics in water–cosolvent mixtures. However, the apparent pH of these buffer solutions increased markedly with increasing methanol content (Fig. 1). On the other hand, the apparent pH of MES buffer was almost invariant with the methanol content (Fig. 1). In this work, therefore, MES buffer was used as an aqueous medium to eliminate a possible change in the reaction rate caused by a pH shift. (At higher content of methanol, however, the apparent pH of the medium was slightly changed (max. 0.3 unit) by the addition of substrates, and in such cases the pH was readjusted to 6.2 after dissolving the substrates.)

In Fig. 2, the time courses of the synthesis of penicillin G and ampicillin are shown as an example of the equilibrium controlled and kinetically controlled reactions, respectively. In the case of penicillin G and POM–PG, the reactions apparently approached an equilibrium and the synthetic yields of the condensation products were enhanced by the presence of cosolvent

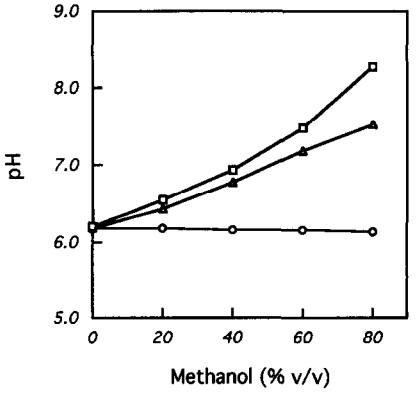


Fig. 1. The apparent pH of buffer solutions in the presence of methanol. □: phosphate buffer (0.1 M); △: acetate buffer (0.05 M); ○: MES buffer (0.05 M).

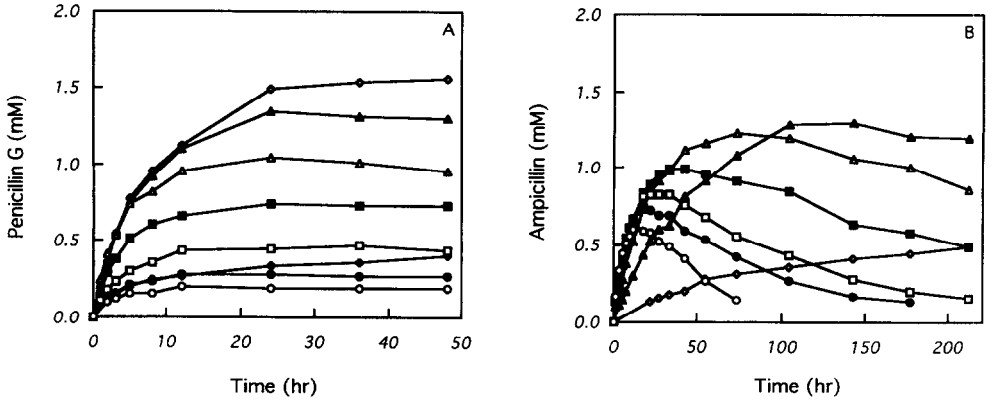


Fig. 2. Time courses of penicillin acylase-catalyzed synthesis of penicillin G and ampicillin in water–methanol mixtures. (a) Penicillin G synthesis. Volume percent of methanol: ○: 0% (MES buffer); ●: 10%; □: 20%; ■: 30%; △: 40%; ▲: 50%; ◇: 60%; ◆: 70%. (b) Ampicillin synthesis. Volume percent of methanol: ○: 0% (MES buffer); ●: 5%; □: 10%; ■: 20%; △: 30%; ▲: 40%; ◇: 50%.

compared to those obtained in aqueous buffer solutions. In the case of ampicillin and pivampicillin, the reaction mechanism follows a kinetically controlled process; the concentration of the condensation product reached a maximum and then gradually decreased due to hydrolysis of the product. The maximum yields of POM–PG and pivampicillin were much lower than that of penicillin G and ampicillin (see below). This may be ascribed to the rapid hydrolysis of POM–PG and pivampicillin during the reaction since penicillin acylase hydrolyzes hydrophobic compounds more efficiently [15].

### *3.2. Effect of methanol on reaction rates and yields*

The dependence of initial reaction rates and synthetic yields on the methanol content is summarized in Table 2. The initial reaction rates were determined from the polynomial regression of time-course profiles. Since the yields varied with reaction time (even for equilibrium controlled reactions), the yields of penicillin G and POM–PG were determined at a fixed reaction time (36 and 34 h for penicillin G and POM–PG, respectively) and of ampicillin and pivampicillin from the maximum values during the reaction.

It can be readily seen from Table 2 that the dependence of reaction rates on the methanol content is clearly different, depending on the acyl donors. In the cases of the penicillin G and POM–PG synthesis reactions where PAA was used as an acyl donor, the initial rates were gradually increased up to 40% (v/v) methanol and then decreased at higher methanol concentrations. In contrast, when PGM was used as an acyl donor (ampicillin and pivampicillin), the rates were decreased in a monotonic fashion as the methanol content was increased.

Table 2 shows that the synthetic yields are enhanced by the addition of methanol in all cases. The yields of penicillin G, POM–PG, and ampicillin under optimal methanol content were increased by 8.1-, 4.1-, and 2.1-fold respectively, as compared to the yields in aqueous media. In the case of pivampicillin, on the other hand, the addition of methanol to the reaction medium shows a marginal influence on the maximum yields; the highest yield (at 20 v/v % methanol) was only 14% greater than that obtained in an aqueous buffer solution.

It appears that the yields in water–methanol mixtures are also influenced by the acyl acceptors: the yields were much higher when 6-APA was used as an acyl acceptor (penicillin G and ampicillin) compared with the corresponding reactions with POM–6-APA (POM–PG and pivampicillin). It is also noteworthy that there is

Table 2
Initial reaction rates and yields for penicillin acylase-catalyzed synthesis of β-lactam antibiotics in water–methanol mixtures

| A. Equilibrium controlled synthesis | | | | |
|---|---|---|---|---|
| Methanol | Penicillin G | | POM–PG | |
| (v/v %) | Initial rate (mM/h) | Yield (%) | Initial rate (mM/h) | Yield (%) |
| 0 | 0.061 | 3.8 | 0.020 | 2.7 |
| 10 | 0.065 | 5.4 | 0.028 | 3.4 |
| 20 | 0.109 | 9.3 | 0.046 | 4.3 |
| 30 | 0.170 | 14.6 | 0.078 | 6.6 |
| 40 | 0.236 | 20.2 | 0.091 | 9.5 |
| 50 | 0.217 | 26.3 | 0.083 | 11.2 |
| 60 | 0.188 | 30.8 | 0.051 | 7.2 |
| 70 | 0.077 | 7.3 | 0.018 | 1.3 |
| **B. Kinetically controlled synthesis** | | | | |
| Methanol | Ampicillin | | Pivampicillin | |
| (v/v %) | Initial rate (mM/h) | Yield (%) | Initial rate (mM/h) | Yield (%) |
| 0 | 0.135 | 12.1 | 0.266 | 6.4 |
| 5 | 0.108 | 15.2 | 0.198 | 6.0 |
| 10 | 0.107 | 16.6 | 0.114 | 7.1 |
| 20 | 0.085 | 20.0 | 0.108 | 7.3 |
| 30 | 0.064 | 24.6 | 0.053 | 6.2 |
| 40 | 0.040 | 26.0 | 0.040 | 5.4 |
| 50 | 0.008 | 9.7 | 0.032 | 3.1 |

an optimal methanol content that maximizes the yield. If the (equilibrium) yield is solely governed by the water activity, a decrease of water activity should result in an increase of the yield. Although equilibrium probably was not attained at higher methanol concentrations, it is likely that, at least for pivampicillin, factors other than water activity of the medium influence the yield of the reaction.

### 3.3. Residual activities of enzymes

The effect of methanol on enzyme deactivation was investigated for two cases (penicillin G and ampicillin) by measuring the residual activities of enzymes after completing the reactions (the reaction time of penicillin G and ampicillin synthesis was 55 and 212 h, respectively). In both cases, enzymes were deactivated during the reaction as expected (Fig. 3). The residual activities of the enzymes used for ampicillin synthesis reactions were much lower than those used for penicillin G reactions due to a longer incubation time.

Fig. 3 shows that there is a threshold solvent level at which enzymes are deteriorated considerably: the residual enzyme activities were ap-

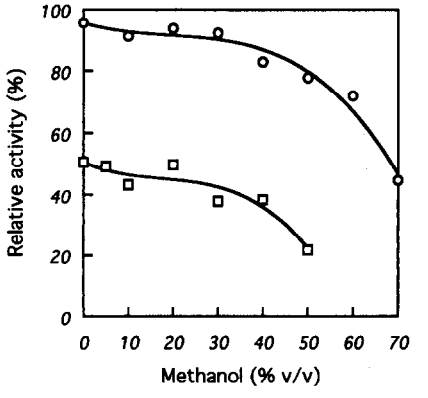


Fig. 3. Effect of methanol on the residual activities of enzymes: ○: penicillin G; □: ampicillin.

preciably reduced at the methanol content of 70 and 50% (v/v) for penicillin G and ampicillin synthesis reactions, respectively. However, it is worth noting that within certain ranges of the methanol content (up to ca. 40% (v/v)) the residual activities in water–methanol mixtures are only slightly lower than those obtained in aqueous media. Even at higher methanol content (70% (v/v) in penicillin G and 50% (v/v) in ampicillin synthesis), about half the aqueous activities were retained. One may therefore conclude that the effect of methanol on enzyme deactivation is less significant than that on reaction rates and yields (cf. Table 2).

### *3.4. Analysis of factors affecting reaction rates in water–cosolvent mixtures*

#### *3.4.1. Effect of methanol on the apparent dissociation constants*

It has been reported that the main cause of increase of synthetic yields for protease-catalyzed synthesis of peptide in water-cosolvent mixtures is an increase of the p$K$ of the carboxyl group of the reactants [5]. Also, it has been suggested that penicillin acylase, due to extreme hydrophobicity of the active site, binds only the non-ionized form of the substrate ([22]; see also [15]), the concentration of which is influenced by the p$K$ of the substrate. To investigate whether changes in the reaction rates were due quantitatively to the p$K$ perturbation, the apparent p$K$ values of the substrates were determined in water–methanol mixtures.

Fig. 4 (A) shows the changes in the apparent p$K$ values of PAA, PGM, and POM–6-APA with the volume content of methanol. It can be seen that the apparent p$K$ values of carboxyl group (PAA) are markedly affected by the presence of methanol while the p$K$ changes in amine groups (PGM and POM–6-APA) are relatively small, which are in good accord with earlier observations on the p$K$ changes of carboxyl and amine compounds in water–cosolvent mixtures [5,13,23]. Also shown in Fig. 4 is the fraction of non-ionized compound in a mixed solvent ($\phi_S$) normalized to that in aqueous media ($\phi_S^O$). The fraction of non-ionized form can be estimated by the following expressions: for carboxylic acids,

$$\phi_S = [\mathrm{RCOOH}]/([\mathrm{RCOOH}] + [\mathrm{RCOO^-}]) = 1/(1 + 10^{\mathrm{pH}-\mathrm{p}K_a}) \quad (3)$$

and for amines,

$$\phi_S = [\mathrm{RNH_2}]/([\mathrm{RNH_2}] + [\mathrm{RNH_3^+}]) = 1/(1 + 10^{\mathrm{p}K_b-\mathrm{pH}}) \quad (4)$$

where $K_a = [\mathrm{RCOO^-}][\mathrm{H^+}]/[\mathrm{RCOOH}]$ and $K_b = [\mathrm{RNH_2}][\mathrm{H^+}]/[\mathrm{RNH_3^+}]$. Fig. 4 (B) shows that the $\phi_S/\phi_S^O$ values for PAA markedly increase with the methanol content whereas those for PGM and POM–6-APA are almost invariant.

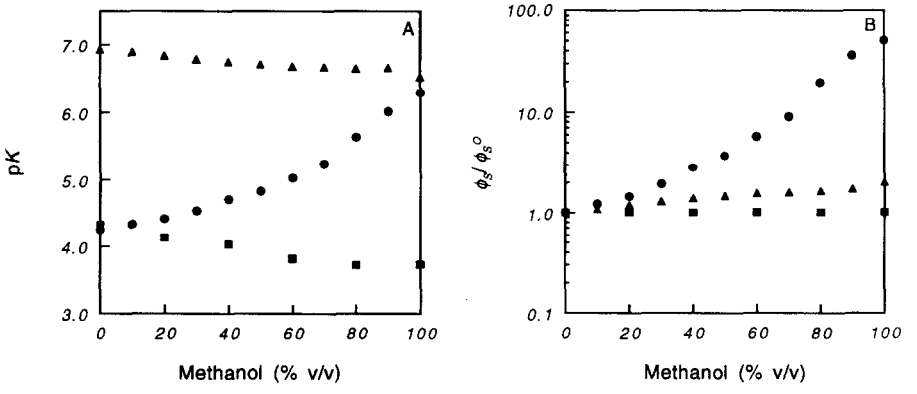


Fig. 4. (A) Effect of methanol on the apparent p$K$ values. (B) Dependence of the fraction of non-ionized form ($\phi_S/\phi_S^O$) on volume percent of methanol. ▲: PGM; ●: PAA; ■: POM–6-APA.

Table 3
Effect of methanol on the p$K$ values of 6-APA

| Methanol | 6-APA | |
|---|---|---|
| (v/v %) | p$K_a$ | p$K_b$ |
| 0 | 2.43 | 4.90 |
| 10 | 2.45 | 4.87 |
| 20 | 2.58 | 4.83 |
| 30 | 2.72 | 4.82 |
| 40 | 2.73 | 4.87 |

The p$K$ values of 6-APA are shown in Table 3 separately. Unlike other substrates used in this experiment, accurate determination of the p$K$ values of 6-APA was rather difficult when the methanol content was greater than 50% (v/v) probably owing to the zwitterionic nature of this compound. The solubility of 6-APA was lowered with increasing the methanol content and the variations of the p$K$ values between the measurements were much larger than other substrates. As can be seen from Table 3, however, the p$K$ values of carboxyl group (p$K_a$) and of amine group (p$K_b$) were changed very little. Thus we may assume that the $\phi_S$ values of 6-APA are largely invariant in water–methanol mixtures, at least up to 40% (v/v) methanol.

*3.4.2. Model simulations*

In view of the results presented in this work and those reported in the literature, the overall effect of cosolvents on enzyme activity seems to reflect the following factors: (i) changes in the concentration of the non-ionic, reactive form of the substrates [5,13,22], (ii) a decrease of enzyme activity due to a reduction of water activity in the presence of the solvent [24–27] and, (iii) deleterious effects (inhibition and/or deactivation) of organic cosolvents [28–30]. In order to analyze the solvent influence on the overall enzyme activity in water–cosolvent mixtures, we have derived a correlation equation which relates the reaction rates to these major factors (the effect of enzyme deactivation has not been considered here since the loss of enzyme activities at the initial reaction period is negligible). As described in the Appendix in detail, the ratio of the reaction rate in organic media to aqueous media ($v/v^O$) can be given by Eq. (5):

$$\frac{v}{v^O} = \frac{\kappa + 1}{\kappa\zeta + 1} \tag{5}$$

where

$$\zeta^{-1} = \left(\phi_S/\phi_S^O\right) a_W^n \{[1 + (1/K')] / [a_W^n + (1/K')]\} [1/(1 + a_I/K_I] \tag{6}$$

and

$$\kappa = K_M^O/(S) \tag{7}$$

In Eq. (6), $a_W$ and $a_I$ represent the thermodynamic activity of water and solvent, $n$ and $K'$ the apparent order of the enzyme hydration reaction and hydration constant, and $K_I$ the inhibition constant of organic cosolvent, respectively. $K_M^O$ and $(S)$ in Eq. (7) denote the Michaelis constant in aqueous media and the substrate concentration, respectively. Although all reactions investigated in this work are two-substrate reactions, it can be assumed that at fixed substrate concentrations the influence of acyl acceptors (6-APA and POM–6-APA) on the overall reaction rate is insignificant due to very little change in the $\phi_S$ values of these compounds. Therefore, it may not be unreasonable to use a single-substrate rate expression, Eq. (5), in this particular case.

Shown in Fig. 5 are the model simulation results which correspond to the case for $n = 2.0$, $K' = 1.0$, and $K_I = 0.8$. In this calculation, the $\phi_S/\phi_S^O$ values were assumed to follow the data of PAA for (a)–(c) and of PGM for (d)–(f), respectively, and the thermodynamic activities of water and methanol were estimated using the NRTL equation [18] (see Eq. (2)). It can be seen that model predictions qualitatively account for the dependency of $v/v^O$ on cosolvent (cf. Table 2); the $v/v^O$ values for (a)–(c) increase and pass through a maximum, while the $v/v^O$ values for (d)–(f) gradually decrease with increasing the methanol content. Further analysis of the simulation results indicates that in cases of (a)–(c) an initial increase in the $v/v^O$ value arises

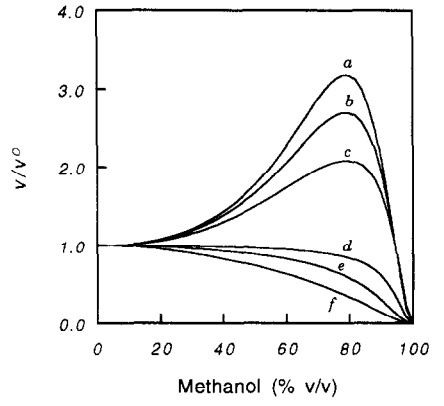


Fig. 5. Dependence of the relative reaction rate on volume percent of methanol. The acyl donors and the values of $\kappa$ used for the calculations are (a) PAA, $\kappa = 100$; (b) PAA, $\kappa = 10$; (c) PAA, $\kappa = 3$; (d) PGM, $\kappa = 0.1$; (e) PGM, $\kappa = 0.5$, (f) PGM, $\kappa = 10$.

from the increase of $\phi_S/\phi_S^O$ and a decrease at higher methanol content from the reduction of water activity. This suggests that the solvent effect on reaction rates is mainly attributed to changes in the concentration of non-ionized substrates, i.e. kinetic effects of changes in apparent p$K$ values. An attempt has been made to fit the experimental data with Eq. (5) by adjusting parameter values. However, the absolute values of $v/v^O$ and the optimal methanol contents predicted by Eq. (5) did not agree with the data.

### *3.5. Concluding remarks*

In this study, we have investigated the synthesis of four $\beta$-lactam antibiotics in water–methanol mixtures for comparative purposes, and found that dependence of the reaction rate and maximum yield on the solvent content differs depending on the nature of the substrates. For example, in the presence of methanol the initial reaction rates were enhanced when carboxylic acid (PAA) was used as an acyl donor whereas the rates were reduced when amine compound (PGM) was used as an acyl donor. It is very likely that such differences in the solvent effect on reaction rate is mainly attributed to changes in the substrate solvation in mixed solvent systems, as judged by the variation of the apparent p$K$ of substrates. Earlier, we have shown that the enzyme reaction rate in organic solvents is determined by substrate solvation as well as enzyme hydration [27].

In order to examine whether changes in the reaction rates were due quantitatively to the p$K$ variation, we also attempted to correlate reaction rates with changes in the concentration of the non-ionized substrate ($\phi_S$) calculated from the p$K$ of the substrates. Unfortunately, however, we have learned from the model simulations that it is difficult to predict the solvent effect on the enzyme reaction rate in a quantitative manner. In this regard, it should be pointed out that Eq. (5) is not thermodynamically rigorous because the $\phi_S$ (or p$K$) values are obtained from the measurements of concentrations and not thermodynamic activities (see also Ref. [5]). There is a great deal more to learn before we are ready to predict the solvent effects quantitatively due to complexities involved in the solute–solvent interactions in the presence of organic cosolvents.

## Acknowledgements

The authors thank Ms. Myung Soon Lim for her technical assistance in measuring the apparent dissociation constants. This work was supported by the Korea Science and Engineering Foundation and Bioprocess Engineering Center.

## Appendix A. Effect of organic solvents on kinetic parameters and reaction rates

### *A.1. Effect of organic solvents on kinetic parameters*

In driving a rate expression for enzyme reaction in water–cosolvent systems, we will begin with the proposition of Margolin et al. [22]. They have shown that their experimental data on the pH-dependence of $k_3/K_M$ for penicillin

acylase-catalyzed reactions can be explained only if the neutral form of the enzyme binds the non-ionized form of the substrate. On the basis of the assumption that the enzyme binds non-ionized substrates, we may write the apparent rate constants ($k_3/K_M^{app}$) in organic media in terms of the fraction of the neutral form of the enzyme ($\phi_E$) and of the substrate ($\phi_S$).

$$\left(\frac{k_3}{K_M^{app}}\right)\Big/\left(\frac{k_3}{K_M}\right) = \phi_E\phi_S \qquad \text{(A1)}$$

where $\phi_E = 1/(1 + [H^+]/K_A + K_B/[H^+])$ and $\phi_S$ is given by Eq. (3) for the acid or by Eq. (4) for the base. In order to simplify the analysis, we will assume that the influence of the solvent on the dissociation of the enzyme ionogenic groups ($K_A$ and $K_B$) is negligible.

Usually we do not know the value of the intrinsic kinetic parameters ($k_3/K_M$), and hence it is more convenient to use the parameter value obtained in an aqueous solution as a reference. Then the ratio of the apparent rate constants in organic media to the rate constants in an aqueous solution can be expressed as:

$$\left(\frac{k_3}{K_M^{app}}\right)\Big/\left(\frac{k_3}{K_M^{O}}\right) = \left(\phi_S/\phi_S^{O}\right) \qquad \text{(A2)}$$

where $K_M^O$ and $\phi_S^O$ are the corresponding parameters obtained in aqueous media.

Let us consider the effects of water activity and inhibition by organic cosolvents. It is well known that the enzyme efficiency in organic media is greatly influenced by the water activity of the reaction medium [24–27]. We have recently developed a kinetic model for enzyme reaction in organic solvents, and have shown that the effect of water activity on reaction rate can be described by [27]

$$f(a_W) = a_W^n\{[1 + (1/K')]/[a_W^n + (1/K')]\} \qquad \text{(A3)}$$

where $a_W$ represents the thermodynamic activity of water, and $n$ and $K'$ the apparent order of the enzyme hydration reaction and hydration constant, respectively. It is relatively easy to incorporate the inhibition effect of the solvent. If organic cosolvents inhibit the enzyme competitively, the apparent $k_3/K_M$ value in the presence of cosolvents differs from $k_3/K_M^O$ by a factor of $f(a_I)$, which is

$$f(a_I) = 1/(1 + a_I/K_I) \qquad \text{(A4)}$$

In Eq. (A4), the inhibition effect due to the presence of organic solvents has been written in terms of the solvent activity ($a_I$), instead of the concentration term, and thus the inhibition constant ($K_I$) should also be given in terms of the activity.

From Eqs. (A2)–(A4), the overall rate constant can now be expressed as

$$\left(\frac{k_3}{K_M^{app}}\right)\Big/\left(\frac{k_3}{K_M^{O}}\right) = \left(\phi_S/\phi_S^{O}\right)f(a_W)f(a_I) \qquad \text{(A5)}$$

In aqueous media ($a_W = 1$, $a_I = 0$), $f(a_W)$ and $f(a_I)$ terms are dropped out, and Eq. (A5) reduces to Eq. (A2). Eq. (A5) makes specific predictions for the solvent effects on kinetic parameters ($k_3/K_M^{app}$), so separate measurements of these values can provide a further test of the proposed model.

Finally, the $\phi_S/\phi_S^O$ term in Eq. (A5) is conceptually similar to the $\gamma_S/\gamma_S^O$ term in the equations proposed previously (see Eqs. (11) and (12) in Ref. [27]), i.e. the logarithm of these terms (e.g., $RT \ln(\gamma_S/\gamma_S^O)$) represents the free energy change of substrate solvation due to the presence of organic solvents. While the $\gamma_S/\gamma_S^O$ term measures a true thermodynamic value, it is rather difficult to determine the activity coefficient of the substrate ($\gamma_S$), particularly for weak electrolytes, from the experiments. On the other hand, the $\phi_S/\phi_S^O$ term, which gives an approximate value of the changes in substrate solvation, can easily be obtained from the apparent p$K$ values. More details on the relationship between $\phi_S$ and $\gamma_S$ will be presented elsewhere [31].

### *A.2. Effect of organic solvents on reaction rates*

It is quite straightforward to derive a rate expression from Eq. (A5). Assuming that en-

zyme reaction follows Michaelis–Menten kinetics, the ratio of the reaction rate in organic media to aqueous solution ($v/v^{O}$) can be given by

$$\frac{v}{v_{O}} = \frac{k_3(E)_0/\{K_M^{app} + (S)\}}{k_3(E)_0/\{K_M^{O} + (S)\}} = \frac{K_M^{O} + (S)}{K_M^{app} + (S)} \tag{A6}$$

where $(E)_0$ and $(S)$ represent the concentration of an enzyme and the substrate, respectively. Introduction of Eq. (A5) into Eq. (A6) gives rise to

$$\frac{v}{v^{O}} = \frac{\kappa + 1}{\kappa\zeta + 1} \tag{A7}$$

where

$$\zeta = \left[(\phi_S/\phi_S^{O}) f(a_W) f(a_I)\right]^{-1} \text{ and } \kappa = K_M^{O}/(S) \tag{A8}$$

The relative reaction rate ($v/v^{O}$) is determined by two dimensionless parameters, $\zeta$ and $\kappa$. While $\zeta$ is a function of the cosolvent content, $\kappa$, which is a relative affinity of an enzyme to the substrate, is independent of the reaction medium composition.

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