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Effect of organic solvents on penicillin acylase-catalyzed reactions: interaction of organic solvents with enzymes

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Abstract

The effects of various organic solvents on penicillin acylase-catalyzed synthesis of β-lactam antibiotics (pivampicillin and ampicillin) have been investigated in water–solvent mixtures. The rates of penicillin acylase-catalyzed reactions were found to be significantly reduced by the presence of a small amount of organic solvent. In particular, the rate of enzyme catalysis was extremely low in the presence of ring-structured solvents and acids while enzyme activities were fully restored after removing the solvents. This indicates that interactions between the solvents and the enzyme are specific and reversible. To correlate the inhibitory effects of organic solvents with solvent properties the influence of solvent hydrophobicities and solvent activity on the rate of pivampicillin synthesis was examined. The reaction rate was found to decrease with increasing solvent hydrophobicities, and a better correlation was observed between the reaction rate and solvent activity. The effects of ionic strength on the synthesis of pivampicillin and ampicillin were also examined. The ionic strength dependence indicates that electrostatic interactions are involved in the binding of ionic compounds to the enzyme. On the basis of the active site structure of penicillin acylase, a possible mechanism for molecular interactions between the enzyme and organic solvents is suggested.

Keywords: Penicillin acylase; Pivampicillin; Ampicillin; Organic solvents; Solvent hydrophobicity; Thermodynamic activity: Ionic strength

1. Introduction

The use of organic media in enzymatic organic syntheses offers several advantages over the enzyme reaction in an aqueous solution [1–3]. For instance, solubility of sparingly water-soluble substrates can be greatly enhanced compared to that in water, thermodynamic equilibrium of an enzyme reaction can be shifted toward the desired direction by reducing the water activity and/or altering the pK values of reactants, and substrate specificity and enantioselectivity of enzymes can be controlled by the solvent.

Previously, we investigated the synthesis of pivampicillin (PVM) from D-α-phenylglycine methyl ester (PGM) and pivaloyloxymethyl 6-aminopenicillanic acid (POM-6-APA) (see Scheme 1) in water–solvent mixtures using *Escherichia coli* penicillin acylase (penicillin amidohydrolase; EC 3.5.1.11) [4,5]. In an aqueous buffer solution, the synthesis yield of PVM was low due to rapid hydrolysis of the product.

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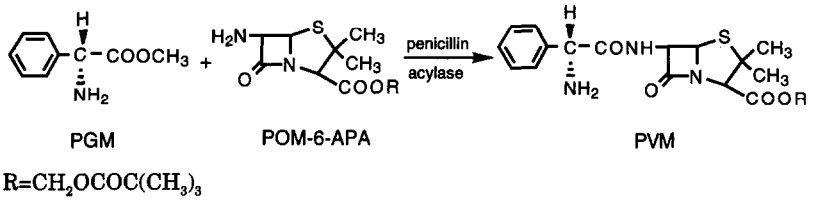

Scheme 1. Reaction mechanism for penicillin acylase-catalyzed synthesis of pivampicillin.

Since organic cosolvents can reduce the rate of hydrolysis reaction by lowering the water content (or water activity) of the reaction medium, we expected that the addition of organic cosolvents could enhance the PVM synthesis yield. However, contrary to our expectations, both initial reaction rates and maximum yields of penicillin acylase-catalyzed synthesis of PVM were reduced by the presence of a small amount of water-miscible organic cosolvents. Furthermore, almost no PVM was produced when water-immiscible hydrophobic solvents such as ethyl acetate, butyl acetate and n-octanol were used as reaction media [4,5].

It has been suggested that the effect of organic solvents on an enzyme is primarily due to interaction with the enzyme-bound essential layer of water [6,7] and that the most adequate organic media are water-immiscible hydrophobic solvents that do not strip the essential water from enzymes [6,8,9]. Recently, we have also shown that the enzyme hydration is mainly determined by the thermodynamic activity of water and that the water activity at a given water content is higher for a solvent which contains a more hydrophobic group [10–12]. Our experimental data on PVM synthesis, however, indicate that these indirect effects caused by the changes in water activity cannot be regarded as major factors that determine the rate of penicillin acylase-catalyzed reactions in water–solvent mixtures. Instead, it appears that specific interactions between the enzyme and organic solvents greatly influence the rate of enzyme catalysis in organic media.

Although the enzymatic synthesis of β-lactam antibiotics in water–cosolvent systems has previously been attempted [4,13,14], little is known about the inhibition effects due to the binding of organic solvents to penicillin acylase. In this study, therefore, we have investigated the effect of organic solvents on penicillin acylase-catalyzed reactions more comprehensively. In addition an attempt has been made to correlate the inhibitory effects of organic solvents with solvent properties such as solvent hydrophobicities and solvent activity.

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-aminopenicillanic acid (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 high performance liquid chromatography (HPLC) eluent were from Malinckrodt (USA). All other reagents used were of analytical grade and obtained from Aldrich (USA). POM–6-APA was synthesized from 6-APA following the procedure of Daehne et al. [15].

2.2. Penicillin acylase-catalyzed reactions

All the experiments were carried out at 30°C in 30 ml vials agitated at 150 rpm in a tempera-

ture-controlled incubator shaker (New Brunswick Scientific, USA). The reactions were started by adding immobilized penicillin acylase (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.

2.2.1. Synthesis of PVM and ampicillin

For PVM synthesis reactions, penicillin acylase (4 mg/ml Eupergit PcA) was added to the reaction mixture (5 ml) composed of 5 mM POM–6-APA, 10 mM PGM, and 50 mM MES buffer (pH 6.2) with or without organic solvents (2% v/v). The ionic strength effect on the rate was examined by adjusting the ionic strength with NaCl. When the pH of the reaction mixture was changed due to the presence of organic solvents (acids and amines), the pH was readjusted to 6.2 with 4 N HCl or 4 N NaOH. The synthesis of ampicillin from 6-APA (5 mM) and PGM (10 mM) was carried out under the same conditions that were used for PVM synthesis.

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 (pH 6.2), and the residual penicillin acylase activity was determined by measuring the initial rate of penicillin G hydrolysis reaction. The used enzymes from each reaction were separately added to a solution containing 5 mM penicillin G (in MES buffer, pH 6.2) and the reaction was carried out at 30°C for 1 h.

2.3. Analysis

Substrates and product involved in enzyme reactions were separated in a reversed-phase μ-Bondapack C_{18} column (3.9 mm × 300 mm, Waters) of a HPLC (Pharmacia) system and analyzed by a UV detector (225 nm). For the analysis of PVM 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 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. Estimation of log P

Currently the log P parameter (logarithm of the n-octanol–water partition coefficients) is most widely used as a measure of the hydrophobicity of solutes. In this work, the log P values were calculated using the hydrophobic fragment method developed by Rekker and de Kort [16].

2.5. Estimation of thermodynamic activity

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

$$a_i = f_i/f_i^0 = x_i\gamma_i \quad (1)$$

where f_i and f_i^0 are the fugacities in the mixture and in its standard state and x_i and γ_i are the mole fraction and activity coefficient of component i, respectively. If vapor–liquid equilibrium (VLE) data are available from the literature, the activity coefficient can be calculated using models such as the van Laar, Wilson, NRTL, and UNIQUAC equations. Otherwise the activity coefficient may be estimated using predictive methods, such as the regular solution theory, ASOG, or UNIFAC equations.

In this study, the activity coefficients have been estimated using the NRTL equation proposed by Renon and Prausnitz [17]. For a binary

mixture system the NRTL equation can be expressed as:

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

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 binary system (A_{12}, A_{21}, and α_{12}) were obtained from Gmehling et al. [18]. The effect of temperature on the activity coefficient has been calculated using a regular solution approximation:

$$\ln\gamma_i \propto T^{-1} \quad (3)$$

when there is a temperature difference between the VLE measurement and enzyme reaction conditions.

3. Results and discussion

3.1. Effect of organic solvents on reaction rate

As mentioned earlier, we previously observed that the rate of PVM synthesis reaction in water–solvent mixtures could be significantly reduced at higher concentration of solvents [5]. In this work, therefore, we examined the effect of organic solvents on penicillin-acylase catalyzed reactions at 2% (v/v) solvent concentrations. The time courses of PVM synthesis in water and water–solvent mixtures (methanol, 2-propanol, tert-pentanol) are shown in Fig. 1. The PVM synthesis reaction follows the typical pattern of a kinetically-controlled reaction in that the concentration of PVM reaches a maximum and then decreases gradually, as described elsewhere in detail [5]. Fig. 1 shows that both the reaction rate and the maximum yield can be significantly affected by the presence of only 2% (v/v) of organic solvents.

To examine the effect of organic solvents more comprehensively, experiments were carried out for the penicillin acylase-catalyzed synthesis in 33 other solvents (the aqueous solubilities of the solvents used here are higher than 2% (v/v)). The relative reaction rates of PVM synthesis (v/v^0) determined from the regression of time-course profiles are summarized in Table 1. The v/v^0 values presented in Table 1 exhibit a strong dependence of the reaction rate on the chemical nature of the solvent. In particular, the rate of PVM synthesis is extremely low in the presence of ring-structured compounds, which include cycloaliphatics (cyclohexanol, cyclohexanone), aliphatics (dioxane, tetrahydrofuran), and aromatics (pyridine, phenol). Unexpectedly, acidic solvents (isobutyric acid, methacrylic acid), which have anionic charges under the reaction condition (pH 6.2), also completely inhibit the enzyme reaction. It appears that strong inhibitory effects of anionic compounds are not restricted to acidic solvents. For instance, we have recently found that Aerosol OT, an anionic surfactant frequently used for the formation of reversed micelle, is also a potent inhibitor for penicillin acylase-catalyzed reactions (data not shown).

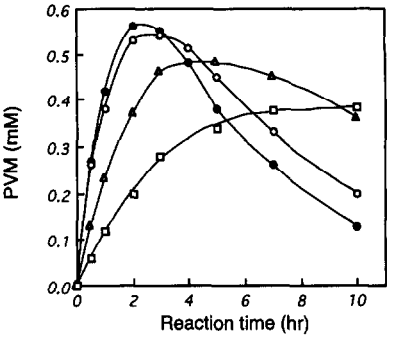

Fig. 1. Effect of organic solvents on penicillin acylase-catalyzed synthesis of PVM. (●) control (MES buffer), (○) 2% (v/v) methanol,(△) 2% (v/v) tert-pentanol, (□) 2% (v/v) 2-propanol.

To establish the generality of the inhibitory effects observed for the PVM synthesis, we investigated further the synthesis of another β-lactam antibiotic, ampicillin, in some of the solvents used in Table 1 (two additional acids, acetic acid and propionic acid, were also tested). In this experiment, 6-APA instead of POM–6-

Table 1
Relative reaction rates of PVM synthesis and log P values of the solvent

Solvent (2% v/v)	v/v^0 (%) [a]	log P (−)	Solvent (2% v/v)	v/v^0 (%) [a]	log P (−)
Glycerol	102	−3.04	Methyl acetate	49	0.15
1,2-Ethanediol	97	−1.90	Ethyl acetate	31	0.67
Methanol	90	−0.77	Ethyl ether	85	0.85
Ethanol	77	−0.25	EGDE [c]	74	−0.75
1-Propanol	11	0.27	2-Methoxyethanol	65	−1.33
2-Propanol	24	0.27	2-Ethoxyethanol	76	−0.81
1-Butanol	6.2	0.79	Methacrylic acid	< 0.1	0.44
2-Butanol	12	0.79	Isobutyric acid	< 0.1	0.80
tert-Butanol	27	0.79	Butylamine	41	0.32
1-Pentanol	5.5	1.31	Triethylamine	66	1.58
2-Pentanol	29	1.31	2-Cyanoethanol	81	−1.47
tert-Pentanol	48	1.31	Acetonitrile	69	−0.34
Cyclohexanol	1.6	1.46	Dimethyl sulfoxide	91	−1.35
Allyl alcohol	11	−0.10	Dimethylformamide	63	−1.00
HMP [b]	95	−0.34	Dioxane	0.5	−1.11
Acetone	25	−0.24	Tetrahydrofuran	< 0.1	0.48
2-Butanone	38	0.28	Pyridine	< 0.1	0.76
Cyclohexanone	2.3	0.98	Phenol	< 0.1	1.53

[a] The reaction rate was determined in the presence of 2% (v/v) solvents (v) and normalized to that obtained in buffer solution (v^0).
[b] 4-Hydroxy-4-methyl-2-pentanone.
[c] Ethylene glycol dimethyl ether.

APA was used as the acyl-acceptor (cf. Scheme 1).

As can be seen from Table 2, the rates of ampicillin synthesis are also significantly reduced by ring-structured solvents and acids, similar to the PVM synthesis reaction. In the presence of methacrylic acid, tetrahydrofuran, pyridine, and phenol, ampicillin production could hardly be detected in the reaction mixtures for the duration of the 30 h reaction time. When comparing the data for alkanoic acids, one can see the dependence of inhibition on the hydrophobicity of acids (acetic acid < propionic acid < isobutyric acid) as measured by their log P values.

Since enzymes could be deactivated by organic solvents, we determined the activity retention of enzymes after completing the reaction. Thus the used enzymes were washed with buffer solutions prior to measuring the residual activity. As shown in Table 2, enzyme activities were fully restored after removing the solvent in all cases. This indicates that the reduction of enzyme activities in the presence of organic solvents is not attributed to the inactivation of the enzyme. It is also likely that the solvent denaturation of enzymes, if any, may be insignificant at 2% (v/v) of solvents.

Table 2
Relative reaction rate of ampicillin synthesis in the presence of organic solvents (v/v^0), activity retention of used enzymes (%) and log P values of the solvent

Solvent (2% v/v)	v/v^0 (%) [a]	Activity retention (%)	log P (−)
Methanol	95	97	−0.77
Cyclohexanol	2.7	98	1.46
Cyclohexanone	21	106	0.95
Acetic acid	36	99	−0.24
Propionic acid	6.3	97	0.28
Isobutyric acid	2.1	106	0.80
Methacrylic acid	< 0.1	94	0.44
Dimethyl sulfoxide	83	98	−1.35
Dimethylformamide	40	105	−1.00
Dioxane	4.0	95	−1.11
Tetrahydrofuran	< 0.1	92	0.48
Pyridine	< 0.1	102	0.76
Phenol	< 0.1	96	1.53

[a] See footnote in Table 1.

3.2. Relationship between reaction rate and solvent properties

If the reduction of penicillin acylase activity with the solvent is caused by the (reversible) binding of the solvent to the enzyme, then the inhibitory effects of the solvent should be related to the physicochemical properties of the solvent. To examine the dependence of the rate of PVM synthesis on solvent hydrophobicity, the log P values of the solvent were calculated using the hydrophobic fragment method [16]. When the relative activity versus log P of the solvent is plotted, the v/v^0 values roughly correlate with solvent hydrophobicity, i.e. the reaction rates are lower in the presence of more hydrophobic solvents (Fig. 2).

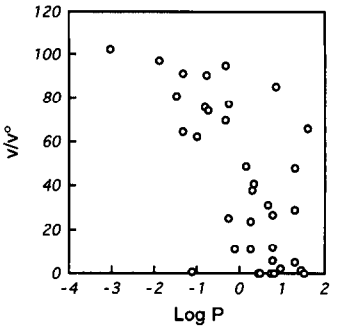

Fig. 2. Dependence of relative reaction rates (v/v^0) of penicillin acylase-catalyzed synthesis of PVM on solvent hydrophobicity (log P).

It has been suggested that the use of thermodynamic activity may be more desirable than the log P parameter in describing enzyme reactions in organic solvents [11,12]. Thus, the thermodynamic activities of water (a_W) and the solvent (a_{SOLV}) used were estimated using the NRTL equation [17] and the dependence of reaction rates on thermodynamic activities was examined. Since the NRTL parameters of aliphatic alcohols are well-established and more reliable than other solvents, the correlation of reaction rates with thermodynamic activities was first examined for aliphatic alcohols as a class example. The calculated thermodynamic activities and the reference of the NRTL parameters used in this work are listed in Table 3. (2-Pentanol is not included because of the uncertainties in the calculated values due to a large temperature difference between the VLE measurement and reaction condition.)

The calculations in Table 3 show that a_{SOLV} is significantly different depending on the nature of the solvent at the same volume percent of organic cosolvents (2% v/v). When the relative reaction rate (v/v^0) was plotted as a function of solvent activity (log a_{SOLV}), a better fit was observed (Fig. 3(b)) compared to the v/v^0

Table 3
The activity of water and the solvent calculated by the NRTL equation and the source of NRTL parameters

Solvent (2% v/v)	a_W	a_{SOLV}	log a_{SOLV}	NRTL Ref. [a]
Glycerol	0.995	2.52×10^{-3}	−2.60	1b: 186
1,2-Ethanediol	0.994	5.47×10^{-3}	−2.26	1b: 120
Methanol	0.991	1.52×10^{-2}	−1.82	1b: 29
Ethanol	0.994	3.04×10^{-2}	−1.52	1b: 114
1-Propanol	0.995	1.15×10^{-1}	−0.94	1a: 240
2-Propanol	0.995	5.22×10^{-2}	−1.28	1b: 180
1-Butanol	0.996	3.95×10^{-1}	−0.40	1b: 247
2-Butanol	0.996	1.94×10^{-1}	−0.71	1b: 256
tert-Butanol	0.996	7.77×10^{-2}	−1.11	1a: 341
1-Pentanol	0.997	7.41×10^{-1}	−0.13	1a: 383
tert-Pentanol	0.997	3.92×10^{-2}	−1.41	1a: 384
Cyclohexanol	0.997	3.37×10^{-1}	−0.47	1a: 414
Allyl alcohol	0.995	9.27×10^{-2}	−1.03	1a: 204

[a] Volume and page number in Gmehling et al., [18].

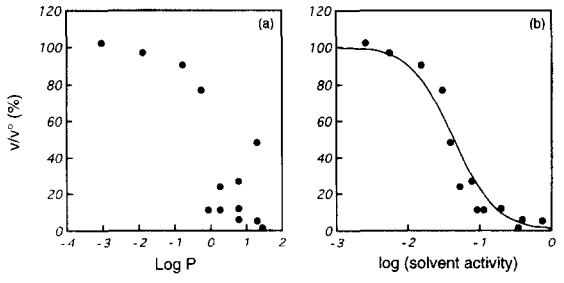

Fig. 3. Effect of aliphatic alcohols on the rate of PVM synthesis reaction. (a) Dependence of v/v^0 on solvent hydrophobicity. (b) Dependence of v/v^0 on solvent activity. The curve corresponds to the equation $\log[(v^0 - v)/v] = 1.51 \log a_{SOLV} + 2.04$ (see text).

versus $\log P$ plot (Fig. 3(a)). The curve in Fig. 3(b) represents the semi-empirical relationship between v/v^0 and a_{SOLV}, which was obtained from the regression of the $\log [(v^0 - v)/v]$ versus $\log a_{SOLV}$ plot (glycerol data were excluded from the regression analysis since $v > v^0$):

$$\log[(v^0 - v)/v] = 1.51 \log a_{SOLV} + 2.04 \quad (4)$$

for which $n = 12$ and $r = 0.939$. It is also noteworthy that there is a parallel relationship between $\log a_{SOLV}$ and $\log P$; for 13 alcohols in Table 3, we obtain

$$\log a_{SOLV} = 0.48 \log P - 1.24 \quad (5)$$

for which $n = 13$ and $r = 0.883$.

We have extended our analysis to include 15 other solvents where the NRTL parameters are available in the literature [18]. Although the NRTL parameters of one additional solvent, ethyl ether, were available, this solvent was not included in the analysis since the accuracy of v/v^0 value was uncertain due to extreme volatility (bp = 34.4°C). The calculated solvent activity and the reference of the NRTL parameters used for calculation are listed in Table 4 ($a_W \simeq 1.0$ in all cases). It can be seen from Fig. 4 that the data for other solvents than aliphatic alcohols are also reasonably fitted to the predicted relationship (Eq. (4)). Such a correlation in Fig. 4 is encouraging, when compared with the v/v^0 versus $\log P$ plot (Fig. 2), and thus the solvent activity can be used as a measure of the solvent property at a given solvent composition and temperature (see Eq. (2)).

Table 4
The activity of the solvent calculated by the NRTL equation and the source of NRTL parameters

Solvent (2% v/v)	$\log a_{SOLV}$	NRTL Ref. [a]
Acetone	−1.36	1a: 191
2-Butanone	−0.96	1b: 207
Cyclohexanone	−0.55	1b: 329
Methyl acetate	−1.01	1: 258
Ethyl acetate	−1.32	1: 395
2-Ethoxyethanol	−1.52	1b: 273
Methacrylic acid	−0.60	1b:197
Triethylamine	−1.15	1b: 347
Acetonitrile	−1.00	1a: 75
Dimethyl sulfoxide	−3.08	1b: 119
Dimethylformamide	−2.15	1a: 234
Tetrahydrofuran	−1.10	1b: 220
Dioxane	−1.46	1b: 222
Phenol	−0.58	1a: 399
Pyridine	−0.96	1b: 284

[a] Volume and page number in Gmehling et al. [18].

As mentioned earlier, organic solvents can influence the rates of enzyme catalyzed reactions indirectly by altering the water activity of the reaction medium and/or the pK values of reactants. Table 3 shows that a_W of the reaction medium is close to 1.0 independent of the solvent, and thus the effect of a_W can be excluded. Also, we have found that the changes in pK values of POM–6-APA and PGM in the presence of 2% (v/v) organic cosolvents are in-

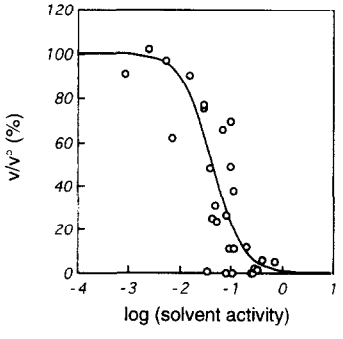

Fig. 4. Dependence of relative reaction rates (v/v^0) of penicillin acylase-catalyzed synthesis of PVM on solvent activity. The curve represents the correlation equation obtained from the aliphatic alcohol data (see Fig. 3).

significant [19]. Since a_W and pK values are not significantly changed in the presence of 2% (v/v) organic cosolvents, it is considered that the reduction of the reaction rate is mainly attributed to the direct effects of the solvent on the enzymes.

3.3. Hydrophobic and electrostatic interactions between active sites and solvents

It has been suggested that the active site of penicillin acylase is extremely hydrophobic ([20,21] and references therein). Recently, Duggleby et al. [22] have reported the active site structure of penicillin acylase. According to their analysis, hydrophobic amino acid residues such as Met (A142; at position 142 on the A chain), Phe (A146), Trp (B57), Trp (B154), and Ile (B177) lined in the binding pocket of penicillin acylase. In view of this, it is likely that hydrophobic interactions between the side chains of amino acids in the active site and organic solvents play a major role in determining the inhibitory effect of the solvent.

Strong inhibitory effects of the ring-structured solvents may also be due to interactions between these solvents and (ring-structured) aromatic amino acid residues such as Trp and Phe located in the active site. The dispersion interaction is often represented by the molar refractivity of molecules which can be estimated via the Lorentz–Lorenz equation, and the molar refractivity is in the order of alkanes < cycloalkanes < aromatics [22–25]. Hence, the dispersion interaction appears to be a dominant factor that determines the binding of ring-structured molecules to penicillin acylase.

On the other hand, the inhibitory effect of acidic (or anionic) compounds may be caused by electrostatic interactions between the positively charged residues at or near the catalytic site and anionic molecules. In this regard, it is worth noting that Arg (B263) is located near the Ser residue (B1) which is involved in catalysis [26,27]. If electrostatic interactions are involved in the catalysis of the enzyme, the rates of PVM and ampicillin synthesis reactions are expected to be influenced differently by the ionic strength of the solution since POM–6-APA is weakly but positively charged (pK = 4.32 [28]) and 6-APA is negatively charged (pK_a = 2.43, pK_b = 4.90 [28]) at pH 6.2. For reactions between ions having identical charges the rates are expected to increase with increasing ionic strength, whereas if the charges are opposite, a decrease in the reaction rate will occur. As shown in Table 5, both the rates and yields of PVM synthesis were increased by increasing ionic strength whereas those of ampicillin synthesis were decreased. Kasche et al., [29,30] also reported that increasing ionic strength markedly decreased the rates of synthesis of ampicillin and penicillin G. The ionic strength dependence shown in Table 5 indicates that there is a specific electrostatic interaction between the active site of the enzyme and ionic compounds and that the catalytic site of penicillin acylase is positively charged.

Table 5
Ionic strength effect on the rates and maximum yields of pivampicillin and ampicillin synthesis reactions

NaCl	Initial rate (mM/h) [a]		Maximum yield (%) [b]	
(M)	Pivampicillin	Ampicillin	Pivampicillin	Ampicillin
0	0.457	0.440	7.8	8.6
0.10	0.491	0.374	8.0	7.5
0.25	0.505	0.341	8.2	7.0
0.50	0.517	0.286	9.1	6.6
1.00	0.537	0.208	10.0	6.1

[a] Correlation equations between the reaction rate (v) and ionic strength (I): $\log v = 0.068 I^{1/2} - 0.335$ ($r = 0.986$) for pivampicillin, $\log v = -0.321 I^{1/2} - 0.333$ ($r = 0.981$) for ampicillin.
[b] Correlation equations between the maximum yield (Y) and ionic strength (I): $\log Y = 0.114\ I^{1/2} + 0.876$ ($r = 0.949$) for pivampicillin, $\log Y = -0.148 I^{1/2} + 0.927$ ($r = 0.987$) for ampicillin.

4. Conclusions

Recently, there has been considerable interest in the use of organic solvents as a reaction medium for the synthesis of valuable products using the reverse reaction of hydrolytic en-

zymes. Among the reactions that are studied are esterification, peptide synthesis and carbohydrate synthesis catalyzed by lipases, proteinases and carbohydratases [1–3]. However, the use of organic solvents does not always guarantee the success of all enzyme-catalyzed reactions as shown in our earlier work [4,5]. There are many other factors to be considered in the design of the reaction medium since organic solvents can influence the enzyme catalyzed reactions directly or indirectly.

In this work, the effects of organic cosolvents on the penicillin acylase-catalyzed PVM synthesis from PGM and POM–6-APA were investigated. The rates of penicillin acylase-catalyzed reactions in water–solvent mixtures were greatly influenced by the addition of a small amount of organic solvents and were found to decrease with increasing solvent hydrophobicities due to reversible binding of the solvent to the enzyme or interactions between solvents and protein residues. In addition, a successful application of solvent activity suggests that the inhibition effects of various organic solvents can also be interpreted as a result of the changes in thermodynamic activity of the solvents.

Previously, it has been proposed that the rate of enzyme reaction in organic media is higher in a more hydrophobic environment [8,9]. In the case of penicillin acylase, however, there was a significant reduction of the reaction rate in the presence of hydrophobic solvents presumably due to hydrophobic interactions between the side chains of amino acids in the active site and organic solvents. In view of this, the direct binding of the solvent on the enzyme should be considered as a contributing factor that determines the rate of enzyme catalysis in organic media.

On the basis of the recent report [22] on the active site structure of penicillin acylase, possible molecular interactions between penicillin acylase and organic solvents have been suggested. Since the rates of synthesis of both PVM and ampicillin are significantly reduced by ring-structured solvents and acids, it is very likely that, in addition to hydrophobic interactions, dispersion and electrostatic interactions are also involved in the binding of organic molecules to the active site of penicillin acylase. Further analysis of interactions between substrates (or other inhibitors) and penicillin acylase and the possible involvement of solvents need to be studied in the future so that the mechanism of the penicillin acylase catalyzed reaction can be proposed.

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