ENZYMATIC SYNTHESIS OF PHENOXYMETHYLPENICILLIN USING *ERWINIA AROIDEAE* ENZYME

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Enzymatic synthesis of phenoxymethylpenicillin from 6-aminopenicillanic acid and phenoxyacetic acid methyl ester was attempted by using partially purified α-acylamino-β-lactam acylhydrolase I (ALAHase I) enzyme from *Erwinia aroideae* NRRL B-138. The reaction rates were carefully followed by determination of 6-aminopenicillanic acid (6-APA), phenoxymethylpenicillin (PNV), phenoxyacetic acid (POA), phenoxyacetic acid methyl ester (POM), and phenoxyacetylglycine (POG) using high performance liquid chromatography. Among the acyl donors tested, POM gave the highest yield (12.2% based on 6-APA). The overall conversion increased almost linearly with an increase in molar ratio of POM to 6-APA up to 4:1. The effects of organic solvents on the overall yield were also evaluated. Some improvement of PNV yield was observed when ethanol, 2-propanol, and acetone were used. ALAHase I was found to carry out three reactions simultaneously: transfer of acyl group to acyl acceptor to form semisynthetic β-lactam antibiotic; hydrolysis of acyl donor in amide or ester bond, and hydrolysis of semisynthetic β-lactam antibiotic which was produced by the enzyme. It was also observed that the hydrolysis reactions of POM and PNV were irreversible in this reaction system. The optimal pH for the three reactions was different. They were: pH 9.0 for POM hydrolysis, 6.8 for the transfer of phenoxyacetyl group to 6-APA, and 6.0 for the PNV hydrolysis. The apparent K_m values for POM, 6-APA and PNV were estimated as 33, 25 and 31 mM, respectively.

During the last decade, significant laboratory advances have been made on the enzymatic synthesis of semisynthetic β-lactam antibiotics. They include: ampicillin[1,2], amoxicillin[3], cephalexin[4,5,6], cephaloglycin[7], cefadroxil[8] and cephacetrile[9]. Enzymatic preparation of semisynthetic antibiotics has some advantages over chemical synthesis, *i.e.*, high conversion yield, transformation of only specific compounds, one-step reactions, and mild reaction conditions.

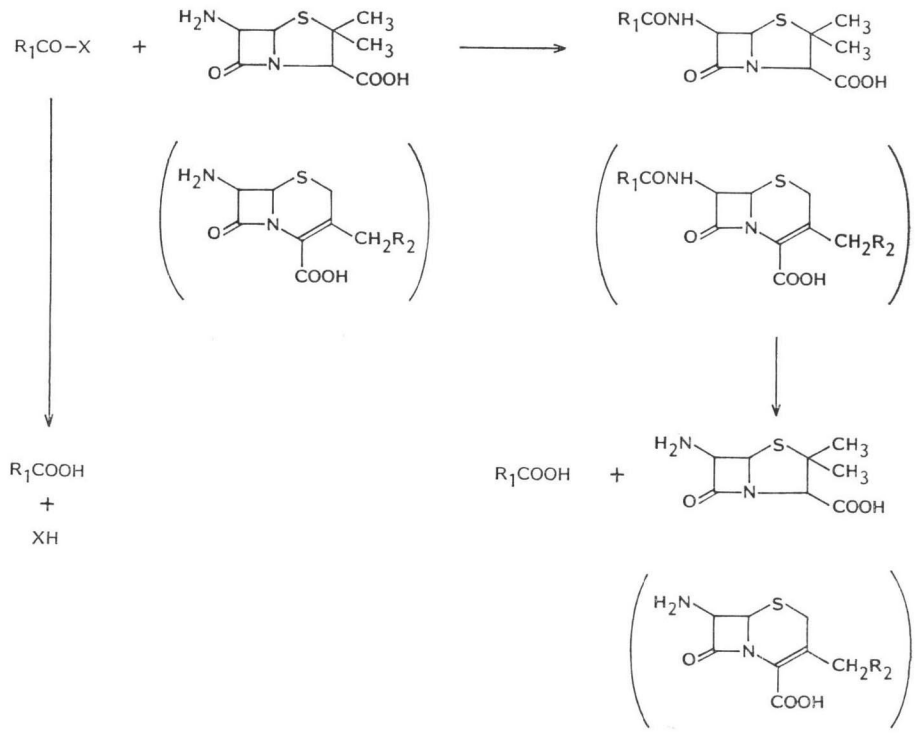
It has been revealed that β-lactam antibiotics can be hydrolyzed to acid forms of their side chain groups and their nuclei, although it can be synthesized from their nuclei and ester or amide forms of their side chain compounds (Fig. 1), especially in case of benzylpenicillin[10], cephalexin[4], cephaloglycin[7] and cephacetrile[9]. As a part of our endeavor to ascertain this reaction kinetics, enzymatic synthesis of phenoxymethylpenicillin (PNV) from 6-aminopenicillanic acid (6-APA) and phenoxyacetic acid methyl ester (POM) was carried out using α-acylamino-β-lactam acylhydrolase I (ALAHase I), which is more commonly known as phenoxymethylpenicillin acylase.

Materials and Methods

Materials

Potassium phenoxymethylpenicillin (K-PNV) and 6-aminopenicillanic acid were kindly provided by the Wyeth Laboratories Inc. (West Chester, PA, USA). Phenoxyacetic acid methyl ester was sup-

Fig. 1. Reactions catalyzed by α-acylamino-β-lactam acylhydrolase in case of enzymatic synthesis of β-lactam antibiotics.



plied by the Tokyo Kasei Kogyo Ltd. Co. (Tokyo, Japan), and phenoxyacetic acid (POA) was purchased from Aldrich Chemical Co. (St. Louis, MO, USA). Phenoxyacetylglycin (POG) was prepared using phenoxyacetyl chloride (Tokyo Kasei Kogyo Ltd. Co., Tokyo, Japan) and glycine according to the procedure described by HONGO *et al.*[11] mp 119~120°C. IR (KBr) 3440, 3395, 1695, 1600, 1548 (-CONH-), 1713 (-COOH) cm^{-1}; NMR (DMSO) δ 3.80 (d, J=6.2 Hz, N-CH_2), 4.54 (s, O-CH_2), 6.87~7.40 (m, C_6H_5-), 8.15~8.33 (m, -CONH-, -COOH).

Enzyme Preparation

Erwinia aroideae NRRL B-138 strain was used for preparation of α-acylamino-β-lactam acylhydrolase I. The culture medium and fermentation conditions used were the same as those described by VANDAMME and VOETS[12,13]. After cultivation for 24 hours at 28°C, the cells were harvested by centrifugation, washed twice with distilled water, and resuspended in 0.1 M phosphate buffer (pH 7.0). The total activity was 2,447 U. The resuspended cells were disrupted by sonication for 15 minutes and the cell debris were removed by centrifugation at 9,000 rpm. The supernatant contained 2,092 U of enzyme (0.45 U/mg protein). Nucleic acids were precipitated from the supernatant by adding 2.0% streptomycin sulfate[13]. Following removal of precipitates by centrifugation, the clear solution was treated with ammonium sulfate. The precipitated fractions from 30~70% ammonium sulfate solution were collected and redissolved in 0.1 M phosphate buffer (pH 7.0), and then dialyzed at 4°C against 1 mM phosphate buffer (pH 7.0). The recovery yield was 1,399 U of enzyme (1.25 U/mg protein). The solution was passed through a column of DEAE-Sephadex A-50 at 4°C and eluted with linear gradient of sodium chloride (0~0.1 M) in 10 mM phosphate buffer (pH 6.5). The fractions having enzyme activity were collected and dialyzed against water to yield 576 mg of enzyme having a specific activity of 1.92 U/mg protein. This enzyme preparation was used throughout this work.

Estimation of Enzyme Activity

The standard reaction mixture consisted of 0.1 M phosphate buffer (pH 7.0), 10 mM PNV and 6.9

U/ml of enzyme. After reaction for 10 minutes at 36°C, the enzyme was precipitated by heat denaturation at 100°C for 1 minute, and filtered through a Millipore membrane (0.45 μm). The filtrate was assayed by a colorimetric method using *p*-dimethylaminobenzaldehyde (*p*-DAB)[14]. One U of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of 6-APA from PNV per hour.

In POM hydrolysis system, 30 mM of POM was employed as a substrate. For PNV synthesis, 10 mM of 6-APA and 30 mM of POM were used in the reaction system. The overall conversion was determined after reaction for 1 hour at 36°C.

The amounts of POA, POM and PNV in the reaction mixture were determined by using high performance liquid chromatography (HPLC) (Model 440LC, Waters Associates Inc., Bedford, MA, USA). Waters Associates μBondapak C_{18} column was employed using running solvent (0.1 M phosphate buffer (pH 7.8) - methanol, 70: 30) at 1.0 ml/minute of flow rate and UV absorption detector (254 nm). The typical retention times of POA, POM and PNV were 4, 17.6 and 24.7 minutes, respectively.

Results

Enzymatic Synthesis of Phenoxymethylpenicillin

The preparation of PNV from POM and 6-APA using ALAHase I enzyme obtained from *E. aroideae* was confirmed by HPLC. Its retention time was the same as authentic PNV standard.

Among the substrates tested, POM was the best as a phenoxyacetyl donor to 6-APA, which yielded nearly 12% conversion of 6-APA to PNV (Table 1). When POG was employed as an acyl donor, only 0.6% of overall conversion was achieved. It was also observed that the overall conversion increased linearly with an increase in molar ratio of POM to 6-APA up to 4: 1, and no more significant increase in conversion was observed beyond this ratio. During enzymatic synthesis of PNV, the amount of PNV produced increased at a high rate initially, but decreased gradually after 90 minutes, while the amount of POA continued to increase due to the hydrolysis of POM (Fig. 2).

In order to increase the overall conversion of 6-APA to PNV, the effect of some organic solvents was investigated by adding them to the reaction (10%) (Table 2). It was expected that organic solvents would increase the solubility and reduce the hydrolysis of POM. Some improvement of PNV yield was observed in the presence of ethanol, 2-propanol and acetone. The addition of 1,4-dioxane,

Fig. 2. Conversion profile of 6-APA to PNV.
Reaction condition; pH 6.8, 30 mM of POM, 10 mM of 6-APA, 6.9 U/ml of enzyme was loaded.
●: POA yield, ○: PNV yield.

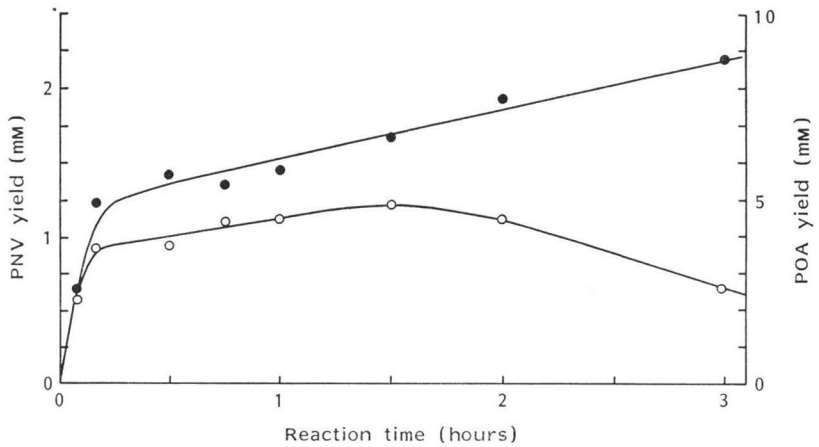


Table 1. Effect of kinds and concentrations of acyl donors on PNV production at 37°C and pH 7.0*.

Acyl donor	Concentration (mM)	Overall conversion (%)
POM	10	3.6
	20	4.8
	30	8.0
	40	11.0
	50	11.2
	60	11.8
	80	12.2
POG	30	0.6
POA	30	0

* 10 mM of 6-APA was employed as an acyl acceptor.

Table 2. Effect of different solvents on PNV production.

Solvent (10%)	Overall conversion (%)
None	10.5
MeOH	9.1
EtOH	14.5
1-PrOH	7.7
2-PrOH	14.0
1-BuOH	4.7
2-BuOH	0.5
Acetone	14.0
DMSO	7.7
DMF	10.5
1,4-Dioxane	0.0
Acetonitrile	1.1

Fig. 3. Effect of pH on enzyme activity of *E. aroideae*.

○: POM hydrolysis, ▲: PNV hydrolysis, ●: PNV hydrolysis, —: 0.1 M phosphate buffer was employed, ---: 0.1 M Tris buffer was employed.

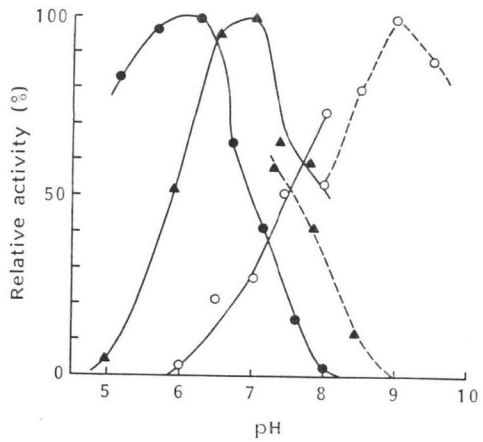
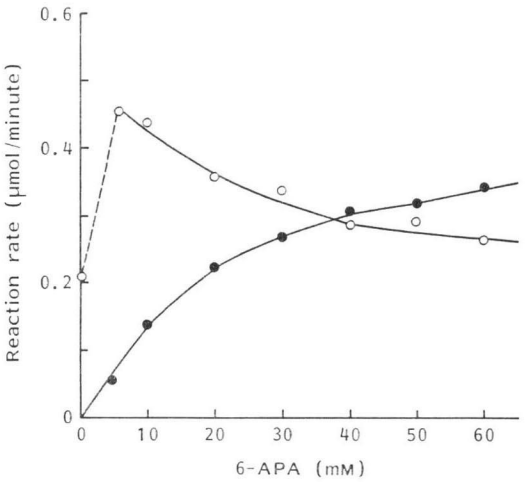


Fig. 4. Inhibitory effect of 6-APA on the rate of POM hydrolysis.

○: POM hydrolysis, ●: PNV synthesis.



acetonitile and 2-butanol resulted in a dramatic loss in yield.

Reactions Catalyzed by *E. aroideae* Enzyme

Recently, it has been reported that three reactions occur simultaneously by a single enzyme catalyzing the synthesis of semisynthetic β-lactam antibiotics; hydrolysis of acyl donor in amide or ester bond, transfer of acyl group to acyl acceptor to form semisynthetic antibiotic, and hydrolysis of produced semisynthetic antibiotic[4,7,9,10]. In the PNV synthesizing system using the *E. aroideae* enzyme, the reaction patterns were analyzed carefully by HPLC (Table 3). It was also observed that three reactions were catalyzed by the same enzyme and the hydrolysis of POM and PNV was irreversible in this system.

The optimal pH for these three reactions was shown in Fig. 3. The maximal conversions of POM hydrolysis, transfer of phenoxyacetyl group to 6-APA, and PNV hydrolysis were achieved at pH 9.0, 6.8 and 6.0, respectively.

The inhibitory effect of 6-APA on POM hydrolysis catalyzed by *E. aroideae* enzyme was also in-

Table 3. Chromatographic analysis of reaction pattern in PNV synthesizing reaction by *E. aroideae* enzyme.

Substrate	Detected compound	Expected reaction
POM	POM, POA	POM ——→ POA
POA	POA	Unreacted
PNV	PNV, 6-APA, POA	PNV ——→ 6-APA+POA
6-APA	6-APA	Unreacted
POM, 6-APA	POM, 6-APA, POA, PNV	POM+6-APA → PNV
		POM ——→ POA
POA, MeOH	POA	Unreacted
PNV, MeOH	PNV, 6-APA, POA, POM*	PNV ——→6-APA+POA
POA, 6-APA	POA, 6-APA	Unreacted

* At high concentration of MeOH (0.1 %), small quantity of POM was detected.

Table 4. Kinetic parameters for three reactions catalyzed by *E. aroideae* enzyme.

Reaction	Varying substrate	K_m (mM)	V_m (μmol/minute/mg protein)
POM hydrolysis	POM	33	0.58
PNV synthesis	6-APA	25	0.14
PNV hydrolysis	PNV	31	0.45

Table 5. Effect of chemical reagents on enzyme activity by chemical modification.

Chemical reagents*	POM hydrolysis	PNV synthesis	PNV hydrolysis
None	100	100	100
Phenylmethylsulfonyl fluoride	102	84	86
5-Dimethylaminonaphthylsulfonyl chloride	90	106	106
o-Phenanthroline	37	60	46
p-Mercurichlorobenzoic acid	106	82	104
N-Acetylimidazole	30	19	64

* The molar ratio of chemical reagents to enzyme was 10 : 1 from the estimation of molecular weight of enzyme as 62,000[13].

vestigated (Fig. 4). Addition of 6-APA resulted in a decrease in the rate of POM hydrolysis with an increase in the rate of phenoxyacetyl group transfer to 6-APA, and at the concentration of 6-APA above 40 mM, the rate of PNV synthesis became greater than that of POM hydrolysis.

The kinetic parameters were also evaluated using a LINEWEAVER-BURKE plot (Table 4). K_m values for POM, 6-APA and PNV were estimated as 33, 25 and 31 mM, respectively.

Inhibition of Enzyme Activity by Chemical Reagents

The partially purified enzyme of *E. aroideae* was subjected to chemical modification by several protease inhibitors (Table 5). Inhibition of enzyme activity was observed with either *o*-phenanthroline or *N*-acetylimidazole. This suggested that the enzyme requires a certain metal ion and a phenolic group such as tyrosine for its action. Other reagents which can modify either alcoholic group of serine or sulfhydryl group of cysteine did not show any significant inhibition on enzyme activity.

Discussion

Several different names for the enzyme catalyzing the hydrolysis of natural penicillins have been reported previously, including penicillin amidohydrolase, penicillin amidase, penicillin acylase, *etc.*[15]. Recent development of enzymatic process for semisynthetic cephalosporin compounds further confused the nomenclature with cephalosporin acylase[16] and α-amino acid ester hydrolase[3,4]. In a previous

report[17], α-acylamino-β-lactam acylhydrolase (ALAHase) was proposed as a new name, based on a wide substrate spectrum on a variety of β-lactam antibiotics including penicillins, cephalosporins and nocardicin, and its special kinetic behavior. This group of enzymes was also newly classified into 4 types according to their preferential substrate specificity instead of using phenoxymethylpenicillin acylase, benzylpenicillin acylase, and ampicillin acylase[18].

In order to investigate the similarity of ALAHase I (phenoxymethylpenicillin acylase) to ALAHase II (benzylpenicillin acylase) or ALAHase III (ampicillin acylase) in kinetic behavior, enzymatic synthesis of PNV from POM and 6-APA was attempted by using ALAHase I produced from *E. aroideae* NRRL B-138. The overall conversion of 6-APA to PNV achieved was only 12% on molar basis, which is much lower than those achieved by ALAHase II of *Escherichia coli* (25% for benzylpenicillin[10]) and ALAHase III of *Xanthomonas citri* (90% for cephalexin[4], 23% for amoxicillin[3] and 20% for cephaloglycin[7]). The disparity in yield may be attributed to the characteristic of POM which is only slightly soluble in water.

Similarly to ALAHase II or ALAHase III[4,7,9,10], this enzyme of *E. aroideae* catalyzed three reactions including POM hydrolysis, phenoxyacetyl group transfer to 6-APA, and PNV hydrolysis. However, *E. aroideae* enzyme showed different characteristics according to its kinetic behavior.

The inhibition study showed the participation of phenolic group of tyrosine and a certain metal. This means that this enzyme may be quite similar to exopeptidases such as aminopeptidase or carboxypeptidase[19], but different from ALAHase II enzyme produced from *E. coli*[20] or *Bacillus megaterium*[9] which has been known to be similar to serine proteases. The involvement of such metal ion as zinc had been suggested in ALAHase I of *Fusarium semitectum*[21].

The optimal pH for esterase activity and amidase activity in the reaction of ALAHase I enzyme of *E. aroideae* were different; acidic for PNV hydrolysis and alkaline for POM hydrolysis. A similar pattern has been reported in carboxypeptidase[22,23], where this difference has been explained by the different mechanistic character of enzyme such as the participation of tyrosine residue. The addition of 6-APA to POM hydrolysis system resulted in a fast increase in rate compared to its rate in the absence of 6-APA (Fig. 4), which also showed the shift of mechanistic property of *E. aroideae* enzyme. However, in the presence of 6-APA, POM hydrolysis and PNV synthesis occurred competitively.

Considerably more work is necessary for a more complete understanding of the reaction mechanism and kinetics of ALAHase enzyme and resultant yield improvement before the enzymatic synthesis of semisynthetic β-lactam antibiotics can be considered practical.

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