Some Problems Involved in Ampicillin Formation by *Kluyvera's* Penicillin Acylase†

By Seigo TAKASAWA, Ryo OKACHI, Isao KAWAMOTO,
Mitsuyoshi YAMAMOTO and Takashi NARA

*Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.
Asahimachi, Machidashi, Tokyo*

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The cell growth of *Kluyvera citrophila* KY3641, capable of producing α -aminobenzylpenicillin (APc) from 6-aminopenicillanic acid (6-APA) and phenylglycine, was stimulated by glutamic acid, serine or proline, or by pH control with tartaric acid or fumaric acid.

Penicillinase produced in an early stage of growth or pH-controlled culture was inactivated by alkaline treatment (incubation of cells at 40°C for 5 to 24 hr in pH 7.5 to 9.5) without inactivation of penicillin acylase. Surface active agents enhanced APc production. On the other hand, phenylalanine and some inorganic compounds inhibited this production.

This bacterium formed APc from penicillin G, but amounts of APc formed were only 9 μ g from 20 mg of penicillin G.

It was previously reported that the intact cells of *Kluyvera citrophila* KY3641 decomposed penicillin G to form 6-aminopenicillanic acid (6-APA) in alkaline side^{1,2)} and formed α -aminobenzylpenicillin (APc) from 6-APA and phenylglycine derivatives in acidic side.^{3,4)}

During the investigation of APc production, some problems arose: (1) the growth of *K. citrophila* was very poor, (2) this strain contained penicillinase like enzyme which decomposed penicillin G to form penicilloic acid in the early stage of growth (3) how the productivity of APc is raised and (4) whether APc can be formed directly from penicillin G. In this paper, some results pertaining to those problems are reported.

METHODS

Strain and culture condition. *Kluyvera citrophila* (KY3641) was employed. This strain was cultured with shaking at 30°C for 1 to 3 days in the following medium, polypeptone 1%, yeast extract 1% and NaCl 0.25% at pH 7.0. In some experiments, some materials were added to this medium as described in each experiment. For the 5 liter-jar fermentation, the strain was cultured under the following conditions, temperature 30°C, aeration 5 liter/min and agitation, 350 rpm.

Conditions of APc formation. Reaction mixture used was: 6-APA 10 mg, DL-phenylglycine methyl-ester 25 mg and cells 30 mg in 1.0 ml of M/30 phosphate buffer at pH 6.5. In some experiments, components of the reaction mixture and pH were changed as described in each experiment. Cells were prepared as following: cells were collected by centrifugation (3000 rpm) from culture broth and washed twice with water by centrifugation. The reaction was run at 35°C for 24 hr. The reaction was stopped by the removal of the cells from the reaction mixture with centrifugation.

Assay method. Assay methods were described in previous papers.^{1,2,4)} Cell growth was determined as DCW by the turbidity of broth at 660 m μ . APc

† Enzymatic Synthesis of D[–]- α -Aminobenzylpenicillin [Part 4] (see reference^{1,2,4)})

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Abbreviation: APc; D[–]- α -Aminobenzylpenicillin (Ampicillin), 6-APA; 6-Aminopenicillanic acid, DCW; Dry cell weight.

formed was determined both by the cylinder plate method using *E. coli* as a test organism and by silicagel TLC by use of a solvent system consisting of *n*-butyl-lactate-*n*-butanol-acetic acid-water (80:15:40:24). APc was detected both by the bioautography using *E. coli* and by the colored reaction¹⁾ with starch-0.01 M iodine. In this solvent system, the following *R_f* values were obtained, penicillin G, 0.8, penicilloic acid 0.5, APc 0.4 and 6-APA 0.2.

RESULTS AND DISCUSSION

Effects of amino acids, organic acids and sugars on the growth of K. citrophila

The growth level of *K. citrophila* was only 3~4 mg/ml as DCW in the medium described in Methods. Effects of amino acids, organic acids and sugars on the growth were therefore tested to stimulate it. In this experiment, 0.5% of each compound was added to the basal medium and *K. citrophila* was grown at 30°C for 24 hr. As shown in Table I, glutamic acid, serine and proline increased

TABLE I. EFFECTS OF ADDITIONS ON CELL GROWTH AND AMPICILLIN FORMATION

K. citrophila was cultured in the basal medium containing 0.5% of additions at 30°C for 24 hr.

Additions	Cell growth	Formation of APc
Sucrose	+	±
Glucose	—	—
Na-Fumarate	+	+
Na-Tartarate	+	+
Na-Lactate	+	±
Na-Succinate	+	±
Na-Glutamate	++	±
Na-Aspartate	+	±
Asparagine	+	+
Serine	++	+
Proline	++	±
Methionine	±	+

— <90% of control (no addition) ± 90~110% of control + 110~130% of control ++ 130~150% of control

the growth up to 130~150% and organic acids, aspartic acid and asparagine increased

to 110~130% but glucose did not support the cell growth.

On the other hand, penicillin acylase activity was not enhanced by these materials, whereas tartaric acid, fumaric acid, asparagine, serine and methionine promoted the enzyme activity slightly. Other sugars (glycerol, mannitol, sorbitol, fructose, lactose and maltose), amino acids (lysine, alanine, glycine, phenylalanine, glutamine, threonine, tyrosine, tryptophan, histidine, arginine, cystine and homoserine) and organic acids (Na-citrate, Na-maleite and Na-gluconate) did not show any significant effect on the growth and enzyme activity, whereas fructose, lactose, maltose, Na-maleite, Na-gluconate, alanine, glycine, tyrosine, tryptophan, cystine and homoserine inhibited the cell growth.

Effect of pH control on the growth and penicillin acylase

Previous experiment showed that organic acids promoted the cell growth. Effects of pH control with organic acids were hence examined. pH of cultures was controlled at 7.5 with 1 M organic acids. As shown in Fig. 1, the growth was 6 mg/ml on the culture

FIG. 1. Effect of pH Control on Cell Growth and Ampicillin Formation.

●—● not controlled cell growth ▲—▲ controlled cell growth
 ●---● not controlled APc formation ▲---▲ controlled APc formation

The basal medium was used. pH was controlled to 7.5 with 1 M of tartaric acid.

medium whose pH was controlled with tartaric acid during the growth, but the growth on the uncontrolled medium was 4 mg/ml and its final pH was 9.0. The growth was stimulated by pH control with tartaric acid and, to a lesser extent, by fumaric acid and lactic acid. On the other hand, the cells from the uncontrolled culture formed 4.5 mg/ml of APc, while the cells from pH controlled culture formed only 1 mg/ml. Products in the reaction mixture of penicillin G and the cells of each stage of growth at pH 7.5 were tested on silicagel TLC. The cells of early stage of growth or pH-controlled culture were very weak in activities of APc formation. Such cells decomposed penicillin G to form penicilloic acid without 6-APA formation, while cells in a late stage of growth produced mainly 6-APA.

From these results, it follows that since the early stage of cells and pH-controlled cells had the strong penicillinase activity, they could not form APc.

When 0.5% of sodium glutamate was added to the basal medium and its pH was controlled with 1 M of tartaric acid containing 10% of sucrose, the growth was increased up to 7 mg/ml.

Inactivation of penicillinase

Even the cells possessing high penicillinase activity, if the enzyme was inactivated, may be used for APc production. Inactivation of penicillinase was therefore attempted. To inactivate the penicillinase from pH-controlled cells or early stage of cells, two methods were considered: (1) incubation at 40°C in alkaline side and (2) the addition of some solvents to the reaction mixture for APc formation.

The first case is shown in Table II. When the broth containing the cells with potent penicillinase was incubated at pH 8.5 for 10 hr or pH 9.0 for 5 hr at 40°C, those cells could give APc yields of 5 mg/ml and 5.3 mg/ml, respectively, while untreated cells formed only

TABLE II. INACTIVATION OF PENICILLINASE BY ALKALINE TREATMENT

		Ampicillin formed (μ g/ml)			
		0 ^{a)}	5 ^{a)}	10 ^{a)}	24 ^{a)}
not treated		1760			
pH	7.5		2230	3100	4430
	8.5		4080	5000	1260
	9.0		5270	3870	2650
	9.5		4140	2130	2950

^{a)} Incubation period (hour) at 40°C. pH was controlled with 28% of ammonia water.

Cells	
not treated with alkaline
treated with alkaline for 10 hrs at pH 8.5
treated with alkaline for 24 hrs at pH 8.5
pH uncontrolled culture
6-APA
Penicilloic acid
Penicillin G

FIG. 2. Detection of Penicillinase Activity with Silica Gel TLC.

Reaction mixture: 10 mg of penicillin G and 4 mg of cells in 1 ml of M/30 phosphate buffer (pH 7.5).

After the reaction for 17 hr at 35°C, the supernatant of reaction mixture was spotted on silica gel TLC, and developed with the following solvent system; *n*-butylacetate-*n*-butanol-acetic acid-water (80: 15: 40: 24).

Compounds were detected by the colored reaction with starch-0.01 M iodine.

1.7 mg/ml of APc.

As seen in Fig. 2, the cells forming APc lost penicillinase but restored acylase activity, because 6-APA was formed from penicillin G with slight amounts of penicilloic acid by alkaline treated cells, whereas untreated cells formed only penicilloic acid. From these

results, it may be speculated that since acylase was more stable than penicillinase in alkaline side, it became possible to obtain the cells restoring acylase from cells of potent penicillinase by alkaline treatment.

The second method for penicillinase inactivation is seen in Table III. When xylene

TABLE III. INACTIVATION OF PENICILLINASE BY SOLVENTS

Xylene or *n*-butylacetate was added to the reaction mixture described in Methods.

Reaction was performed for 24 hr at 35°C.

Solvent		APc formed ($\mu\text{g/ml}$)
not treated		780
Xylene	10%	2700
	2%	2600
<i>n</i> -Butylacetate	10%	3600
	2%	2100

or *n*-butylacetate was added to the reaction mixture, APc formation was increased from 780 $\mu\text{g/ml}$ to about 3000 $\mu\text{g/ml}$.

In this case, it was thought that penicillinase was inhibited more strongly than acylase by these solvents. On the other hand, benzene, dioxane and phenol inactivated both penicillin acylase and penicillinase.

Conditions for APc production

In a previous paper,⁴⁾ it was reported that 10.7 mg/ml of APc was formed by the intact cells of *K. citrophila*. Some conditions for more APc production were investigated.

It was already reported⁴⁾ that the optimum pH for APc formation was 6.5, but it was found that during the reaction, pH of reaction mixture was decreased slightly. So optimum pH was studied under the pH control. In this experiment, reaction mixtures contained 1/30 M phosphate buffer of each pH, to which the pH of the reaction mixture was controlled with diluted NaOH or HCl during the reaction.

FIG. 3. Effect of pH Control on Ampicillin Formation.

●—● controlled to pH 6.5, ■—■ controlled to pH 5.5, ▲—▲ controlled to pH 4.5, ×—× not controlled

Reaction mixtures were controlled to the initial pH with N/10 HCl or N/10 NaOH.

The initial pH of uncontrolled reaction was pH 6.5.

TABLE IV. AMPICILLIN FORMATION FROM D-PHENYLGLYCINE

Reaction mixture: 10 mg of 6-APA, 25 mg of phenylglycine methylester HCl and 30 mg of cell in 1 ml of M/30 phosphate buffer (pH 6.5). Incubated at 35°C.

	APc formed ($\mu\text{g/ml}$)	
	2 hr	20 hr
D-Phenylglycine methylester HCl	7000	11000
DL-Phenylglycine methylester HCl	3400	6100
L-Phenylglycine methylester HCl	trace	2000

As seen in Fig. 3, the optimum pH was 6.5, too. It was reported by Kaufmann group and Rolinson group^{5,6)} that the optimum pH of acylase from *E. coli* was 4.5~5.5. The optimum pH of acylase from *K. citrophila* was thus found to differ from that from *E. coli*.

Effect of D-form of phenylglycine on APc production was then tested. As shown in Table IV, 11 mg/ml of APc was formed from

D-form and 6.1 mg/ml from DL-form, but from L-form, only 2 mg/ml of APc was formed. It was already found that D(–)ampicillin was produced from DL-phenylglycine. But which form of APc was produced from L-form of phenylglycine has yet remained to be decided.

Surface active agents were found to enhance APc production. As seen in Table V, cation PB-40, catiogen H, nonione E 208 and aerosol enhanced APc production, while vitamins, nucleotides, nucleosides, amino acids, sugars and inorganic compounds were not effective. As seen in Table VI, DL-phenylalanine inhibited the APc formation. The inhibitory effect of phenylalanine could be ascribed to its structurally competitive effect to phenylglycine. Some inorganic compounds, ZnCl_2 , HgCl_2 , NiCl_2 , MnSO_4 , FeSO_4 , CuSO_4 , AsO_3 , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and SnCl_2 inhibited the enzyme reaction, 100, 86, 82, 87, 82, 100, 82, 82, 92 and 67% as inhibition per cent, when 50 mM of each compound was added. However, NaCl , KCl , MgCl_2 , NH_4Cl , Na_2WO_4 and NaF did not inhibit the enzyme reaction, when 50 mM of each compound was added to the reaction mixture.

When 25 mg of D-phenylglycine methylester HCl and 0.5 μl of catiogen H were added to the reaction mixture, APc productivity was increased up to 12 mg/ml.

Enzyme activity of the cells used repeatedly

After the enzyme reaction to form APc for 4 hr, cells were separated from reaction mixture and washed with water; then, the cells were suspended again in new reaction mixture. Such a procedure was repeated five times and then APc productivity of each reaction was examined. As a result, APc productivity of the cells was not decreased in each reaction, however, the total volume of reaction mixture in the next experiment was more decreased than before (Table VII), because a part of the

TABLE V. EFFECTS OF SURFACE ACTIVE AGENTS ON AMPICILLIN FORMATION

Surface active agents were added to the reaction mixture described in Methods.
Incubated for 20 hr at 35°C.

S. A. Agent	APc formed ($\mu\text{g/ml}$)
not added	4000
Cation P.B. 40 5 $\mu\text{l/ml}^{a)}$	6400
Catiogen H 0.5 $\mu\text{l/ml}^{a)}$	7000
Nonion E 208 1 mg/ml ^{a)}	6700
Aerosol OT 1 mg/ml ^{a)}	6800

^{a)} Concentration of surface active agents in the reaction mixture.

TABLE VI. INHIBITION OF AMPICILLIN FORMATION BY PHENYLALANINE

DL-Phenylalanine was added to the reaction mixture described in Methods.
Incubated for 20 hr at 35°C.

DL-Phenylalanine added mg/ml	Inhibition (%)
0	0
0.25	16
1	38
5	51
10	60

TABLE VII. ENZYME ACTIVITY OF THE CELLS USED REPEATEDLY

Reaction mixture: 6-APA, 10 mg/ml; DL-phenylglycine methylester·HCl, 25 mg/ml; Cells, 25 mg/ml; in 1/30 M-phosphate buffer.
Incubated for 4 hr at 35°C.

Run	Volume of reaction mixture (ml)	APc ($\mu\text{g/ml}$)
1	200	3880
2	160	2850
3	120	3630
4	88	3120
5	75	3440

cells were lysed in each reaction.

Preservation of the enzymatic activity

Intact cells of *K. citrophila* were preserved

at 4°C or -17°C. It was found that the cells could maintain the acylase activity over one month in both conditions.

Acetone-dried cells were also prepared and stored for one month at room temperature. As shown in Table VIII, the acetone-dried cells were found to accumulate APc more than the intact cell.

TABLE VIII. ENZYME ACTIVITY OF ACETONE-DRIED CELLS

Reaction mixture: 6-APA, 10 mg/ml; DL-phenylglycine methylester·HCl, 25 mg/ml; in 1/30 M-phosphate buffer.

Incubated for 4 hr at 35°C.

Cells	DCW (mg/ml)	APc (μg/ml)
Acetone-dried cell	18	2600
	36	4000
Intact cell	18	2900
	36	3300

TABLE IX. FORMATION OF AMPICILLIN FROM PENICILLIN G

Reaction mixture: 10 mg of Penicillin G, 10 mg of phenylglycine amide-HCl and 20 mg of cell in 1.0 ml of 1/30 M phosphate buffer. Incubated at 35°C.

pH	Cell addition	APc formed 4 hr	(μg/ml) ^{a)} 20 hr
3.5	+	3.2	5.8
	—		0.5
4.5	+	3.2	6.8
	—		0.6
5.5	+	3.8	6.9
	—		0.8
6.5	+	2.6	4.8
	—		0.1
7.5	+	4.6	9.0
	—		1.7

^{a)} Bioassay with *Staph. aureus* 209P

Formation of ampicillin from penicillin G

In this experiment, penicillin G was added

to the reaction mixture in place of 6-APA. After the reaction, penicillin G was removed with the extraction of ethylacetate and the reaction mixture was assayed by the clinder method using *St. aureus* 209p. As seen in Table IX, as highest yield, 9.0 μg/ml of active material as APc was obtained from 20 mg/ml of Penicillin G at pH 7.5 and this material was identified as APc on silicagel TLC by use of the following three solvent systems: (1) *n*-butylacetate-*n*-butanol-acetic acid-water (80: 15: 40: 24), (2) *n*-butanol-ethylether-acetone-water (28: 9: 9: 5) and (3) isoamylacetate-methanol-formic acid-water (65: 20: 5: 10). The *R_f* values were 0.40, 0.42 and 0.20 in (1), (2) and (3) solvents, respectively. These *R_f* values were in good accord with those of APc, so that it is clear that APc was formed from penicillin G, though its more definite identification was not performed.

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