ENZYMATIC SYNTHESIS OF D(−)-α-AMINOBENZYLPENICILLIN BY *KLUYVERA CITROPHILA*

Sir:

D(−)-α-Aminobenzylpenicillin (ampicillin) is a chemotherapeutic drug of importance because of its acid stability and broad-spectrum activity. It is now industrially manufactured by chemical synthesis from 6-aminopenicillanic acid (6-APA), as first reported by DOYLE *et al.*[1]

Two approaches to prepare ampicillin by microbial processes can be considered: (1) direct fermentative production by adding appropriate precursors to *Penicillium chrysogenum* fermentations and (2) enzymatic synthesis from 6-APA. As pointed out by MORTIMER and JOHNSON[2], numerous experiments to prepare biosynthetic penicillins by the supplement of α-substituted phenylacetic acids to *P. chrysogenum* fermentations have been unsuccessful presumably because of steric hindrance to these precursors. In fact all our efforts to synthesize ampicillin by adding various phenylglycine derivatives to *P. chrysogenum* cultures have also been in vain.

Subsequently the authors tried to synthesize ampicillin enzymatically from 6-APA. Our attempts for this purpose are based upon the assumption that a microbial enzyme, penicillin acylase (E. C. 3.5.1.11), capable of cleaving penicillin G into 6-APA most efficiently, may also be able to most readily synthesize ampicillin, in its reverse reaction, from 6-APA and phenylglycine derivatives. Indeed KAUFMANN *et al.* first achieved enzymatic acylation of 6-APA by use of a penicillin acylase from *Escherichia coli*[3~5] and COLE has very recently reported enzymic formation of a few penicillins by *E. coli* acylase.[6~9]

First of all, though a number of works on penicillin acylases have been published by ROLINSON *et al.*[10], CLARIDGE *et al.*[11], HUANG[12], HUANG *et al.*[13], MURAO *et al.*[14] and HAMILTON-MILLER[15], the authors searched for a penicillin acylase adequate for ampicillin synthesis among a variety of microorganisms. As a result, *Kluyvera citrophila* KY 3641 was selected as one of the most potent acylase producers.

The Gram-negative bacterium, known to accumulate α-keto-glutaric acid[16], was found to be very strong in penicillin acylase: that is, about 50 mg/ml of 6-APA were formed in 80~90 % of theoretical yield from penicillin G by use of its intact cells. It was further found that the *K. citrophila* enzyme was able to efficiently carry out the acylation of 6-APA with phenylglycine ester leading to ampicillin. The enzyme was cell-bound and not leaked out into the culture liquid. Its optimal pH for ampicillin synthesis was 6.5 in contrast with pH 4.0~6.0 optimal for penicillin G synthesis by *E. coli* enzyme[4].

The procedures for preparing ampicillin by *K. citrophila* are as follows: a loopful of *K. citrophila* stock slant was inoculated into 30 ml of a liquid growth medium in a 250 ml Erlenmeyer flask, which was then shaken at 30°C by a rotary shaker. The medium consisted of yeast extract 1 %, peptone 1 %, meat extract 0.5 %, NaCl 0.25 % and Na-L-glutamic acid 0.5 %. Its pH was 7.2 before autoclaving. Grown cultures were harvested 2 days after inoculation, washed once with deionized water, suspended in an

Table 1. Ampicillin yields from various phenylglycine derivatives

Phenylglycine derivatives	Concentration mg/ml	Incubation time, hours	Ampicillin yields, mg/ml
D,L-Phenylglycine·methylester·HCl	25.0	20	6.1
D-Phenylglycine·methylester·HCl	25.0	20	10.7
L-Phenylglycine·methylester·HCl	25.0	20	2.0
D,L-Phenylglycine·amide·HCl	17.4	20	2.5
D,L-Phenylglycine·butylester·HCl	22.5	4	5.0
D-Phenylglycine·butylester·HCl	22.5	4	8.9
D,L-Phenylglycine·thioglycolic acid ester·HCl	25.0	4	7.2

A reaction mixture used was: 10 mg/ml of 6-APA, phenylglycine ester indicated in the table, and about 30 mg/ml of *K. citrophila* cells. Total volume 3 ml in 1/30 M phosphate buffer (pH 6.5). Incubated at 35°C for 4~20 hrs.

Table 2. Enzymatic synthesis of various penicillins by *K. citrophila* cells

Acyl side chain precursors used	Corresponding penicillins	Results*
Phenylacetic acid	Benzylpenicillin	+++
p-Aminophenylacetic acid	*p*-Aminobenzylpenicillin	+++
Phenylmalonic acid monoethyl ester	α-Carboxybenzylpenicillin	+
Diethylphenylmalonate	α-Carboxybenzylpenicillin	−
Ethyl-3-phenyl-5-methylisoxazole-4-carboxylate	Methylphenylisoxazolylpenicillin	−
Methyl-3-O-chlorophenyl-5-methylisoxazole-4-carboxylate	Methylchlorophenylisoxazolylpenicillin	−
Methyl-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxylate	Methyldichlorophenylisoxazolylpenicillin	−
α-Aminosulfonylphenylacetic acid methylester	α-Sulfoaminobenzylpenicillin[17]	−
Aminocyclohexane carbonic acid methylester	Aminocyclohexylpenicillin[18]	+

* Results show a degree of qualitative formation of corresponding penicillins, which was estimated by thin-layer chromtography in various solvent systems, using corresponding reference penicillins. Experimental conditions were the same as those for ampicillin shown in Table 1.

aliquot of deionized water and then used for enzyme reaction. A reaction mixture used for ampicillin synthesis consisted of 10 mg/ml of 6-APA, 20~30 mg/ml of phenylglycine ester, 3.18 mg/ml of KH_2PO_4, 1.42 mg/ml of Na_2-HPO_4 and 20~30 mg/ml of intact cells. Its pH was adjusted to 6.5 with NaOH solution. The mixture was incubated at 35°C for 4~20 hours. Quantities of ampicillin formed were determined by cup assay by use of *E. coli* KY 8201 and Na-salt of ampicillin (manufactured by Meiji Seika Company) as a standard. Ampicillin formed was also examined by bioautography of the reaction mixtures.

The ampicillin yields from various phenylglycine esters are given in Table 1. Maximal yields of ampicillin, 10.7 mg/ml, were obtained from D-phenylglycine methylester and 6-APA. The conversion yield from 6-APA was 62.6%. A racemic mixture of the methylester and D-phenylglycine butylester also gave good yields, 6.1 and 8.9 mg/ml, respectively.

Ampicillin formed from D, L-phenylglycine·methylester and 6-APA was isolated in crystalline form after a procedure consisting of Dowex 50W×4 column chromatography, Sephadex G-10 gel filtration, activated carbon treatment, and freeze drying. The crystalline product was definitely identified as D(−)-α-aminobenzylpenicillin·$3H_2O$ from its bioautogram, paperchromatogram, thin-layer chromatogram, bioassay, chemical assay, IR spectrum, specific rotation and rotatory dispersion.

It was thus shown that the penicillin acylase of *K. citrophila* KY 3641 gave good yields of ampicillin (more than 60% of conversion ratio) from 6-APA and D-phenylglycine methylester.

The KY 3641 enzyme could form 6-APA from penicillin G, ampicillin, *p*-aminobenzylpenicillin and, to a much lesser extent, penicillin V and carboxybenzylpenicillin. The enzyme was able to liberate 7-aminocephalosporanic acid from semisynthetic cephalosporins such as cephalothin, but not from natural cephalosporin C.

Incidentally, attempts to prepare other semisynthetic penicillins were made by the use of the *Kluyvera* acylase. The experimental conditions were the same as those for ampicillin, except the supplment of various corresponding side chains precursors instead of phenylglycine·ester. As seen in Table 2, syntheses of benzylpenicillin and *p*-aminobenzylpenicillin proceeded as readily as ampicillin. Carboxybenzylpenicillin and aminocyclohexylpenicillin appeared to be synthesized to a much less degree than ampicillin. These were proven by thin-layer chromatography in various solvents, by the use of corresponding reference penicillins.

Further detailed data on the *Kluyvera* enzyme will be reported separately later.

The discovery of a good ampicillin producer, *K. citrophila* KY 3641, resulted from our attempts to search for organisms capable of splitting penicillin G into 6-APA most efficiently. Another approach to look for ampicillin producers is to screen directly organisms able to synthesize ampicillin from 6-APA and phenylglycine·methylester.

Studies along such lines will be reported shortly.

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