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Biosynthesis of β-Lactam Antibiotics by Cell-free Extract from *Lysobacter lactamgenus*

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Abstract □ Using cell-free extract of *Lysobacter lactamgenus*, enzymatic conversion of δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV), the first substrate of β-lactam biosynthesis, into antibiotic compounds was attempted. In high performance liquid chromatographic (HPLC) analysis, the biosynthetic intermediates for cephalosporin antibiotics including isopenicillin N, deacetoxycephalosporin C, deacetylcephalosporin C and unknown cephem compound were detected in reaction mixtures. It implies that cephabacin compounds from *L. lactamgenus* could be produced by biosynthetic routes through penicillin ring formation and its expansion to cephalosporin ring, likely as cephalosporin C from *Cephalosporium* or cephamycin C from *Streptomyces*. Among biosynthetic enzyme in cell-free extract, the ring formation activity (isopenicillin N synthetase activity) was separated in 50-60% of ammonium sulfate fraction, and ring expansion activity (deacetoxycephalosporin C synthetase activity) was found to be in 40-50% fraction. The partially purified isopenicillin N synthetase could convert as much as 90% ACV to isopenicillin N during 6-hour reaction.

Keywords □ β-lactam biosynthesis, *Lysobacter lactamgenus*, isopenicillin N synthetase, deacetoxycephalosporin C synthetase.

During last decade, lots of efforts to elucidate the biosynthetic routes of β-lactam antibiotics including penicillins from *Penicillium*, cephalosporins from *Cephalosporium* and cephamycins from *Streptomyces* have been made by several research groups. Throughout their works, these β-lactam antibiotics has been known to be produced through the common pathway at initial biosynthetic stage; synthesis of tripeptide δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV), penam ring formation to isopenicillin N (IPN) and its epimerization to penicillin N[1-4] (Fig. 1). The organisms producing cephalosporins or cephamycins convert penicillin N to deacetoxycephalosporin C (DAOC) by ring expansion reaction and then process it to final cephalosporin compounds, whereas *Penicillium* producing penicillins exchanges the side chain of penicillin N with an appropriate one such as phenylacetyl group.

Recently an eubacterium, *Lysobacter lactamgenus*, has been reported to produce novel β-lactam antibiotics called cephabacins[5-7]. These compounds have 7-formylamino group and 3-peptidyl chain on cephalosporin backbone. In this paper, we report the fundamental data on cephabacin biosynthesis obtained by high performance liquid chromatographic analysis of reaction mixtures using cell-free extract from *L. lactamgenus*.

MATERIALS AND METHODS

Materials

The substrate compound was obtained as *bis*-δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (*bis*-ACV) from Peninsula Laboratory, Belmont, CA, USA, and isopenicillin N (IPN) was a gift from Gist Brocades, Delft, Netherland. The biosynthetic intermediates of β-lactam antibiotics, sodium deacetoxycephalosporin C (DAOC), sodium deacetylcepohalosporin C

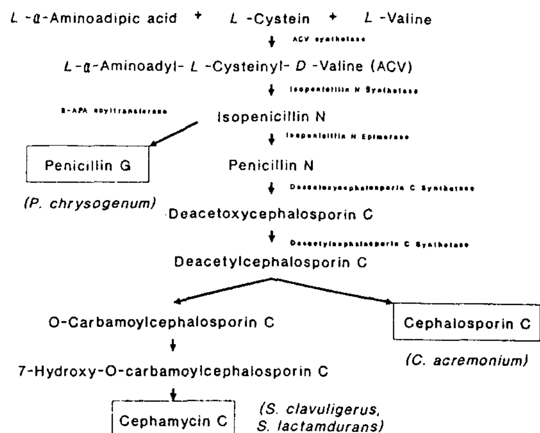

Fig. 1. The biosynthetic pathway of β-lactam antibiotics.

(DAC) and potassium cephalosporin C were kindly supplied by Ciba-Geigy AG, Basel, Switzerland.

Microorganism and cultivation

Lysobacter lactamgenus IFO 14,288 (YK-90) was used as a cephabacin producer throughout this work. The seed culture was prepared by cultivating in the medium containing 0.2% yeast extract, 1.0 peptone and 0.1% magnesium sulfate (pH 7.0), and transferred to fermentation medium composed of 2.0% glucose, 1.0% casamino acid, 0.1% sodium thiosulfate and 0.01% nickel nitrate[8], which was cultured for 48 hr at 30℃ with shaking at 300 rpm.

Micrococcus luteus ATCC 9341, the test organism for antimicrobial activity assay, was grown in nutrient broth medium by overnight cultivation.

Preparation of cell-free extract

Cell-free extract of *L. lactamgenus* was obtained by sonication of washed cell suspension in 25 mM Tris buffer (pH 7.2) and further centrifugation of the broken cells at 5,000 rpm for 20 min. The sonication was done for 30 seconds five times with cooling on ice, with employing Sonicator XL2010 (Heat Systems Inc., NY, USA). The final supernatant after centrifugation, cell-free extract was stored as aliquots at −70℃ until use.

Ammonium sulfate fractionation

The cell-free extract obtained above was treated with ammonium sulfate on ice by step-wisely increasing concentration of 10%. The precipitates at each steps were separated by centrifugation and dialyzed against 25 mM Tris buffer (pH 7.2).

Enzyme reaction

First, 100 μg of substrate, *bis*-ACV, was activated to monomeric form (ACV) by treating with 80 μl of 4 mM dithiothreitol in 0.5 M Tris buffer (pH 7.2). To this, 200 μl of cell-free extract, 40 μl of 1 mM ferrous sulfate, and 40 μl of 30 mM ascorbic acid were added in the order, and made finally up to 400 μl. This mixture was incubated at 27℃ with vigorous shaking on rotary shaker (250 rpm)[9,10]. At the end of reaction, 400 μl of methanol was added to terminate enzyme action, and the precipitates were removed by centrifugation for reaction analysis.

Analysis of enzyme reaction mixture

The enzyme reaction mixture was analyzed by high performance liquid chromatography (HPLC) (System Prep 550, Pharmacia LKB Biotechnology, Sweden). 20 μl of each samples was eluted by 0.05 M phosphate buffer (pH 5.0) through μBondapak C_{18} column (125 Å, 10 μm, 3.9×150 mm; Waters, MA, USA) with the flow rate of 1.0 ml/min. The eluted antibiotics were detected at 220 nm employing Pharmacia LKB variable wavelength monitor (VWM 2141). The standard curves for ACV and IPN were made by peak areas of authenic samples. In case of IPN the initial concentration in cell-free extract was substracted from the measured amount.

The antimicrobial activity in the reaction mixture was also assayed by disk diffusion method using *Micrococcus luteus* ATCC 9341 as the test organism. The filter paper disks (Whatman No. 1, diameter= 0.68 cm) loaded 50 μl of samples were placed on the nutrient broth agar already spread with test organism, and incubated overnight at 30℃.

RESULTS AND DISCUSSION

Identification of biosynthetic intermediates for cephabacin

In order to analyze the enzyme reaction mixture by HPLC, the separation profile of substrate (ACV) and biosynthetic intermediates (IPN, DAOC and DAC) was examined with varying the composition and/or pH of eluting phosphate buffer. Even though Jensen's group[11] used 5% methanol − 95% phos-

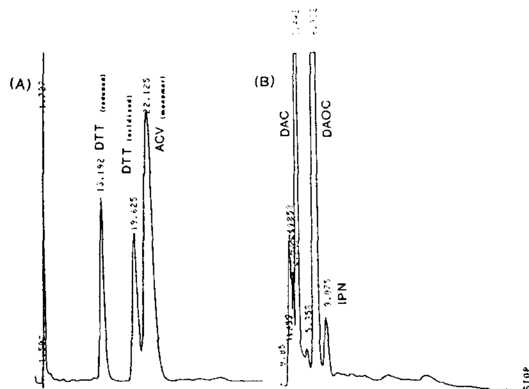

Fig. 2. Separation profiles for substrates and products of isopenicillin N synthetase, and the other biosynthetic intermediates for cephalosporin C by high performance liquid chromatography.
Running buffer; 0.05 M phosphate buffer (pH 5.0), flow rate; 1.0 ml/min, detection; 220 nm.

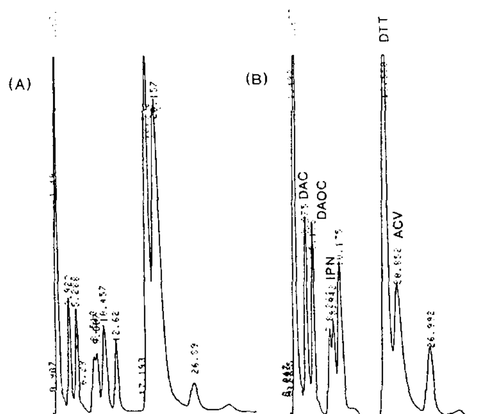

Fig. 3. High performance liquid chromatographic analysis of the reaction mixture by cell-free extract of *Lysobacter lactamgenus*.
Running buffer; 0.05 M phosphate buffer (pH 5.0), flow rate; 1.0 ml/min, detection; 220 nm. The substate, LLD-ACV tripeptide, was incubated with cell-free extract of *L. lactamgenus* at 27℃ for 2 hr with vigorous stirring (B). The sample denoted (A) was control of 0-hr reaction.

phate buffer (pH 4.0) as a mobile phase for the analysis of IPN synthetase (ring cyclase) activity, 100 % phosphate buffer (pH 5.0) gave the best separation results for IPN, DAC and DAOC as well as ACV and dithiothreitol among tested (Fig. 2).

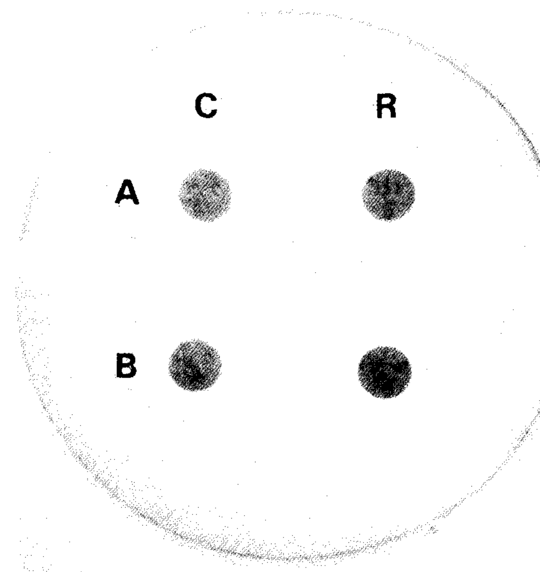

Fig. 4. Bioassay of isopenicillin N synthetase activity by disc diffusion method.
The antibacterial activity in reaction mixture was detected by growth inhibition zone against *Micrococcus luteus* ATCC 9341 on nutrient broth agar plate after incubation overnight at 30℃. The substrate, LLD-ACV tripeptide, was incubated with cell-free extract of *L. lactamgenus* (A) or partially purified enzyme by ammonium sulfate fractionation (B) at 27℃ for 3 hr with vigorous stirring (R). The samples denoted (C) were controls of 0-hr reaction in each cases.

Using this mobile eluent on μ Bondapak C_{18} column, the components in reaction mixture of cell-free extract from *L. lactamgenus* were separated. As seen in Fig. 3, the substrate ACV was observed to disappear as much as 60% after 3 hr reaction. In spite of the interference on interpretation of chromatographic results by initial contamination of small amounts of biosynthetic intermediates in cell-free extract (These peaks almost diminished after ammonium sulfate precipitation of cell-free extract), the peak areas of DAC, DAOC and IPN were significantly increased after reaction. Another new peak appeared at the retention time of 26 min, which is presumably a biosynthetic intermediate further processed.

The produced antibiotics were also confirmed by microbial growth inhibition test aganist *M. luteus*

Table I. Separation and purification of cephem biosynthetic enzymes of *Lysobacter lactamgenus* by ammonium sulfate fractionation

Ammonium Sulfate Fractions	Produced amount after 3-hr reaction (μg/ml) IPN	DAOC	DAC
0- 40%	0		
40- 50%	14.9	17.4	9.7
50- 60%	116	28.1	53.0
60- 70%	6.3	3.0	5.3
70-100%	0		

ATCC 9341, which is hypersensitive to penicillin compounds. As shown in Fig. 4, the clear growth inhibition zone was observed by the reaction mixture, whereas control system had no antimicrobial activity. This implies that ACV tripeptide having no antibactrial activity was converted into antibacterial β-lactam compounds including IPN by the enzyme of *L. lactamgenus*.

Because the biosynthetic steps for IPN by IPN synthetase (ring cyclase), DAOC by DAOC synthetase (ring expandase) and DAC by DAC synthetase (DAOC hydoxylase) have been known to be ferrous-mediated oxidative reactions[1-4], it could be deduced from the above results that ACV was firstly converted to penam compound IPN and further processed to cephem compounds including DAOC and DAC by one-pot reaction of cell-free extract. This results strongly suggest that cephabacin antibiotics produced by *L. lactamgenus* could be synthesized through penam ring formation followed by ring expansion to cephem, same as other cephem antibiotic producers.

Ammonium sulfate fractionation of cell-free extract

In order to examine the exact conversion pattern of ACV by *L. lactamgenus* enzymes, the cell-free extract used above was further purified by ammonium sulfate precipitation. The enzyme activities were mostly detected in the precipitates between 40% and 60% of ammonium sulfate concentration (Table I). Out of expectation, however, the enzymes involved in the biosynthesis of β-lactam antibiotics were not clearly separated. Based on the ratio of the produced amounts of IPN, DAOC and DAC, it could be identified that IPN synthetase and DAC synthetase mainly exist in 50-60% ammonium sulfate fraction whereas DAOC synthetase mostly in 40-50% fraction. These results are much consistent with other research group's who used *Penicillium*, *Cephalosporium* and *Streptomyces* as enzyme sources[12-16].

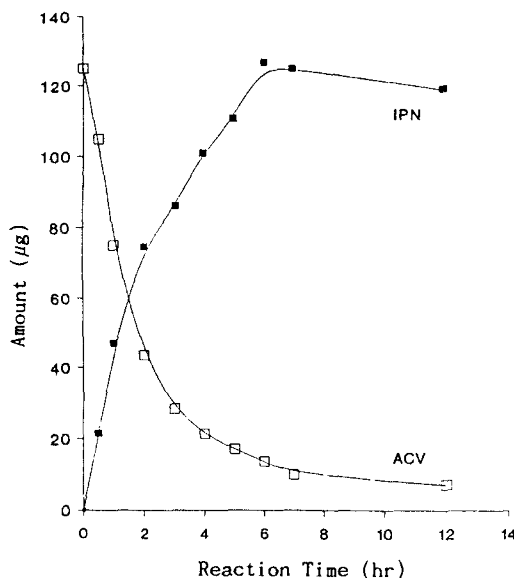

Fig. 5. The conversion profile of LLD-ACV tripeptide to isopenicillin N by partially purified isopenicillin N synthetase of *Lysobacter lactamgenus*.
The substrate, LLD-ACV tripeptide, was incubated with partially purified isopenicillin N synthetase by ammonium sulfate fractionation at 27℃ with vigorous stirring.

Simultaneous production of IPN and cephem compounds from ACV strongly implies coexistence of IPN epimerase, which can convert IPN to the substrate of DAOC synthetase, penicillin N, in cell-free extract or ammonium sulfate fractions. From other researcher's reports that this enzyme activity was found in 30-50% ammonium sulfate fractions[17,18], simultaneous precipitation of IPN epimerase by ammonium sulfate could not be excluded, either.

On examining the conversion pattern of the enzymes in 40-50% fraction, the substrate ACV was consumed almost 90% during 6 hr reaction as seen in Fig. 5. In contrast of IPN, the cephem compounds were not significantly increased throughout the reaction.

Further purification of these enzymes employing several open column chromatographic methods is necessary for the exact comprehension about the

biosynthetic route of cephabacins.

ACKNOWLEDGEMENT

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