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Cell-Free Cyclization of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine to Isopenicillin N

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Cell-free cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N by lytic enzyme extracts of *Cephalosporium acremonium* M-0198 was stimulated by ferrous ions. The optimum concentration of $FeSO_4$ was 80 μM. No additional stimulation was observed with ascorbate, adenosine 5'-triphosphate, or α-ketoglutarate, but Triton X-100 and sonication of the extracts increased activity. $ZnSO_4$ was very inhibitory to enzyme activity; $CuSO_4$ was somewhat less inhibitory, and the least effective of the three was $MnCl_2$. The dimer of the tripeptide was converted to a penicillin that has the biological spectrum of isopenicillin N, and this reaction was also stimulated by $FeSO_4$. We found that sonication can be used directly to prepare extracts with cyclization activity from mycelia, without preparing protoplast lysates. The kinetics of cyclase appearance and disappearance during fermentation were similar to those of ring-expansion activity, i.e., enzyme appeared and peaked 13 h after growth ceased and then disappeared.

Both our group (7) and Abraham's group (9) have shown, independently, that δ-(L-α(aminoadipyl)-L-cysteinyl-D-valine (LLD-tripeptide) is cyclized to isopenicillin N by extracts of *Cephalosporium acremonium* (Fig. 1). O'Sullivan et al. (9) isolated the product of the cell-free conversion and determined that the side chain of the penicillin was L-α-aminoadipic acid. Because oxidation of deacetoxycephalosporin C to deacetylcephalosporin C is stimulated by ferrous ions, ascorbate, and α-ketoglutarate (12) and because oxidative ring-expansion of penicillin N to deacetoxycephalosporin C is stimulated by ferrous ions and ascorbate (4, 10) we checked the effect of these additives on the oxidative ring-closure reaction. We also tested the effect of Triton X-100, which, we found, stimulates ring-expansion by these same cell-free extracts (11).

We also wanted to determine whether the dimer of LLD-tripeptide, i.e., bis[δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine]disulfide, could be converted to isopenicillin N. This dimer is known to be produced by certain blocked mutants of *C. acremonium* (5), but its significance as an intermediate in β-lactam biosynthesis is not known.

MATERIALS AND METHODS

Culture. The culture used was *C. acremonium* M-0198 (NRRL 11418), an antibiotic-negative mutant of *C. acremonium* CW-19 (*Acremonium strictum* ATCC 36255). The parental culture produces both cephalosporin C and penicillin N.

Media and culture conditions. In all cases, media were prepared with glass-distilled water. *Cephalosporium* cultures were maintained on agar slants containing 1/10 strength LePage-Campbell medium (8).

Seed cultures were inoculated from agar slants and shaken (Psychrotherm model G26; New Brunswick Scientific Co., New Brunswick, N.J.) at 25°C and 250 rpm in 250-ml Erlenmeyer flasks containing 40 ml of the seed medium. The seed medium contained 30 g of corn steep liquor, 10 g of glucose, 30 g of soluble starch, 5 g of calcium carbonate, and distilled water to 1 liter (pH adjusted to 7.0 before autoclaving). The contents of 4-day-old seed flasks were pooled before being used as the inoculum. Fermentations were at 25°C and 250 rpm in a chemically-defined medium modified from Demain et al. (3) and containing 36 g of sucrose, 27 g of glucose, 3 g of DL-methionine, 7.5 g of $(NH_4)_2SO_4$, 1.5 g of oleic acid, 7.5 ml of salts mixture 1, 135 ml of salts mixture 2, and water to 1 liter. This medium, minus sugars and oleic acid, was adjusted to pH 7.4. The sugar mixture was prepared separately, as was the oleic acid. Salts mixture 1 contained 2 g of ferrous ammonium sulfate hexahydrate and water to 100 ml. Salts mixture 2 contained 208 g of dibasic potassium phosphate, 204 g of monobasic potassium phosphate, 22.7 g of sodium sulfate decahydrate, 4.9 g of magnesium sulfate heptahydrate, 400 mg of zinc sulfate heptahydrate, 400 mg of manganese sulfate monohydrate, 100 mg of cupric sulfate pentahydrate, 1 g of calcium chloride dihydrate, and water to 1.8 liters. The salts were added in reverse order, and each was dissolved completely before adding the next. The basal medium, sugar mixture, and oleic acid were each autoclaved separately. The basal medium and sugar solution were combined in a large sterile flask, and the inoculum was

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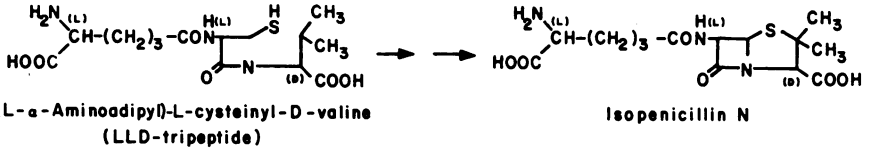

FIG. 1. *Cyclization reaction.*

added (2 ml of pooled inoculum per 40 ml of medium). After thorough mixing, individual 42-ml portions were aseptically distributed into sterile 250-ml flasks. Sterile oleic acid was added to each flask. Flasks were incubated on the shaker at 25°C and 250 rpm.

Growth estimation. After fermentation, we routinely measured absorbance in a Klett-Summerson colorimeter with the red filter. First, we diluted the whole broth with 0.01 N HCl to less than 100 Klett units to dissolve $CaCO_3$ carried over from the seed medium. The pH value of the fermentation broth was determined after filtration through paper (no. 595; Schleicher & Schuell Co., Keene, N.H.). In certain fermentations, we determined mycelial wet weight after growth. Flask contents were filtered through paper in a Buchner funnel. Mycelia were washed twice with 20 ml of distilled water and weighed. We found a direct relationship between absorbance and mycelial wet weight, namely, 1 g of wet mycelium per liter was equal to 42 Klett units.

Preparation of lytic enzyme extract. Mycelia were harvested 13 h after the start of the stationary growth phase, as measured in a Klett-Summerson colorimeter. The contents of six flasks were pooled. Mycelia were washed twice with 60 ml of sterile distilled water and suspended in a 250-ml Erlenmeyer flask containing 40 ml of 0.05 M McIlvaine buffer (pH 7.2) and 0.01 M dithiothreitol (Sigma Chemical Co., St. Louis, Mo.). The suspension was incubated for 1 h at 28°C, with shaking at 150 rpm. The mycelia were filtered, washed twice with 50 ml of chilled, sterile distilled water, and suspended in a 250-ml Erlenmeyer flask containing 40 ml of 0.05 M McIlvaine buffer (pH 7.2) plus 1.0 M NaCl, 0.02 M $MgSO_4$, 160 mg of *Cytophaga* lytic enzyme L_1 (Gallard-Schlesinger Chemicals, Carle Place, N.Y.), and 160 mg of *Arthrobacter* zymolase (Kirin Brewery, Tokyo, Japan). The suspension was incubated at 28°C for 3 h, with shaking at 120 rpm. The resulting protoplast suspension was centrifuged at 800 × *g* for 10 min. The pellet was washed three times with 60 ml of a solution containing 0.05 tris(hydroxymethyl)aminomethane buffer (pH 7.2), 0.01 M $MgSO_4$, and 1 M sucrose. After the third wash, the pellet was suspended in 6 ml of pH 8.0 tris(hydroxymethyl)aminomethane-salts [0.05 M tris(hydroxymethyl)aminomethane buffer–0.01 M KCl–0.01 M $MgSO_4$], and the suspension was placed in a freezer (−20°C) for 60 min. The suspension was homogenized with a steel spatula and centrifuged at 3,000 × *g* for 10 min. The supernatant fluid (lytic enzyme extract) was frozen (−65°C) until use. It usually contained 4 to 10 mg of protein per ml. For the ring-expansion reaction, it was diluted to less than 1 mg of protein per ml in pH 7.2 tris(hydroxymethyl)aminomethane-salts. For the cyclization reaction, the extract was not diluted.

Preparation of sonic extracts. Mycelia were harvested 13 h after growth ceased. The contents of six flasks were pooled. The mycelia were washed twice, each time with 60 ml of chilled, sterile distilled water. Filter paper was used to harvest cells. A 2-g (wet weight) amount of washed mycelia was suspended in 6 ml of tris(hydroxymethyl)aminomethane-salts (pH 8.0). The cells were sonicated in an ice water bath at setting no. 4 of a Branson sonifier (Cell Disruptor 200; SmithKline Corp., Danbury, Conn.) for 2 min (20-s exposures separated by 20-s intervals). The disrupted cells were centrifuged at 3,000 × *g* for 10 min at 4°C. The sonic extract was distributed among several tubes and kept frozen until use.

Protein determination. Protein in cell-free extracts was measured by the method of Bradford (2). Bovine serum albumin (Sigma) was used as the standard.

Cell-free cyclization reaction. Cell-free extract (0.82 ml), LLD-tripeptide (0.09 ml), and additive (0.09 ml) were mixed in a 10-ml Erlenmeyer flask kept on ice. The final concentration of LLD-tripeptide in the reaction mixture was 200 μg/ml; those of the additives are described in the text, tables, and figure legends. The flask was incubated at 25°C on a shaker at 250 rpm. At 0, 30, and 60 min, 25-μl samples were removed and pipetted onto duplicate paper disks (no. 740-E, 6.5-mm diameter; Schleicher & Schuell). The disks were kept at 4°C until the experiment was over, when all were placed on agar plates as described below.

Assay of isopenicillin N. The product was determined by an agar diffusion assay with *Pseudomonas aeruginosa* Pss. This is a β-lactam-supersusceptible mutant that responds equally well to cephalosporin C, deacetoxycephalosporin C, deacetylcephalosporin C, and penicillin N; isopenicillin N produces considerably less activity. Because *P. aeruginosa* Pss frequently reverts to normal susceptibility, special precautions were taken. The reverted population was recloned on agar plates. After a particular colony was determined still to be supersusceptible, it was spread on two agar plates (antibiotic medium no. 5; Difco Laboratories, Detroit, Mich.) and grown, and the cells were harvested, washed twice with saline, and then suspended in 20 ml of 50% (vol/vol) glycerol. The suspension was divided into a large number of sterile vials at 0.5 ml per vial and frozen at −65°C until use. For each assay, a tube was thawed, and its contents were suspended in molten agar at a concentration of 0.1% (vol/vol). The plates contained 8 ml of agar medium. Cephalosporin C (0.3 to 30 μg/ml) was used as the standard. Plates were incubated overnight at 37°C, and zones of inhibition were measured.

Due to the limited availability, lack of purity, and lability of isopenicillin N, we used cephalosporin C as the standard for the plate assay. We quantitated product formation by units (not micrograms) of isopenicillin N; 1 U corresponds to the amount of isopenicillin

N that produces the same size inhibitory zone on *P. aeruginosa* Pss as 1 μg of cephalosporin C per ml. As defined here, 1 U of isopenicillin N weighs considerably more than 1 μg.

Zero-time problem. Ever since we discovered the ring-expansion reaction (6), we have been troubled by the apparent presence of product at zero time. This was not as much of a problem in the ring-expansion reaction as in the cyclization reaction, and we merely subtracted the zero-time microbial assay from the later values. However, in the cyclization reaction, the zero-time value was entirely too large for such an adjustment. Zero-time activity in both reactions is thought to be due to the assay method, i.e., a microbiological assay of long duration (ca. 16 h). Apparently, even though the sample placed on the disk is taken at zero time, reactions can continue while the disk is incubating on the agar plate. The lability of penicillin N probably limits the extent of the ring-expansion reaction occurring on the plates. However, the stability of LLD-tripeptide probably allows the cyclization reaction to proceed for many hours on the agar plates, resulting in very high zero-time values. For this reason, much of the early data is presented in this paper as extent of cyclization, i.e., the maximum figure observed at 0, 30, or 60 min. During this study, a method using methanol treatment was developed that markedly reduced zero-time activity. This method is described in Results.

RESULTS

Effect of ferrous ions. Table 1 shows the effect of additives on the cyclization of LLD-tripeptide. $FeSO_4$ doubled the production of isopenicillin N, but ascorbate, dithiothreitol, and α-ketoglutarate had no further effect. The optimum concentration of $FeSO_4$ was found to be 80 μM (Table 2); even at this concentration, ascorbate had no effect. Also inactive was adenosine 5′-triphosphate (alone or in the presence of an energy-generating system composed of phosphoenolpyruvate and pyruvate kinase).

Effect of other ions. Table 3 shows that other divalent cations inhibited the reaction conducted in the presence of $FeSO_4$. The inhibitions observed with Mn^{2+}, Cu^{2+}, and Zn^{2+} were 30, 70, and 80%, respectively.

Effect of Triton X-100. Triton X-100 (0.1% vol/vol) stimulated activity markedly in both the absence and presence of Fe^{2+} (Table 4).

Reduction of zero-time value. As described in Materials and Methods, a very high isopenicillin N assay was observed even at zero time, although neither LLD-tripeptide nor the cell-free extract had any antibiotic activity of its own. For testing whether this was due to the cyclization reaction occurring on the agar plates during the agar diffusion assay procedure, 25-μl samples taken at 0, 30, 60, and 120 min were first mixed with equal volumes of pure methanol (class 1B; Fisher Scientific Co., Fair Lawn, N.J.) in an ice bath to inactivate the enzyme. A 25-μl amount each of the treated samples was applied to disks and assayed in the usual way, except that assay values were doubled to compensate for dilution by methanol. Figure 2 shows that most zero-

TABLE 1. *Effect of additives on the cyclization of LLD-tripeptide*[a]

Additive	Isopenicillin N produced (U/ml)
None	2.5
40 μM $FeSO_4$	5.3
0.67 mM ascorbic acid	3.0
40 μM $FeSO_4$ + 0.67 mM ascorbic acid	5.3
40 μM $FeSO_4$ + 0.67 mM ascorbic acid + 80 μM dithiothreitol	5.5
40 μM $FeSO_4$ + 0.67 mM ascorbic acid + 160 μM α-ketoglutaric acid	4.7

[a] The cell-free extract was prepared by lytic enzyme treatment and contained 9.1 mg of protein per ml.

TABLE 2. *Effect of concentration of ferrous sulfate on the cyclization of LLD-tripeptide*[a]

$FeSO_4$ conc (μM)	Isopenicillin N produced (U/ml)
0	4.0
40	5.1
80	6.2
80[b]	6.0
160	4.7

[a] The cell-free extract was prepared by lytic enzyme treatment and contained 9.0 mg of protein per ml.
[b] In the presence of 0.67 mM ascorbate.

TABLE 3. *Inhibition of the cyclization of LLD-tripeptide by divalent cations*[a]

Additive	Isopenicillin N produced (U/ml)
None	4.6
80 μM $FeSO_4$	10.5
80 μM $FeSO_4$ + 80 μM $MnCl_2$	7.3
80 μM $FeSO_4$ + 80 μM $CuSO_4$	3.1
80 μM $FeSO_4$ + 80 μM $ZnSO_4$	1.3

[a] The cell-free extract was prepared by lytic enzyme treatment and contained 9.6 mg of protein per ml.

TABLE 4. *Stimulation of the cyclization of LLD-tripeptide by Triton X-100*[a]

Additive	Isopenicillin N produced (U/ml)
None	4.0
80 μM $FeSO_4$	6.2
0.1% Triton X-100	14.0
80 μM $FeSO_4$ + 0.1% Triton X-100	26.0

[a] The cell-free extract was prepared by lytic enzyme treatment and contained 9.0 mg of protein per ml.

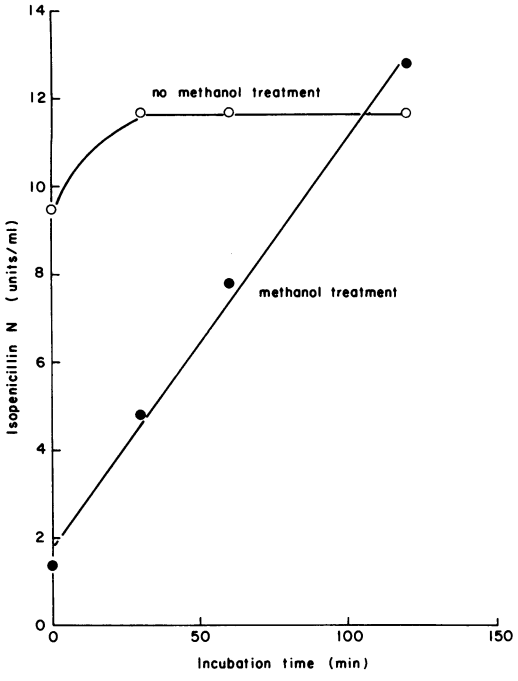

FIG. 2. *Effect of methanol treatment of samples on the time course of the* LLD*-tripeptide cyclization reaction. The cell-free extract was prepared by lytic enzyme treatment and contained 9.5 mg of protein per ml.*

time activity was eliminated by methanol treatment. For the remainder of the work, methanol treatment was done routinely, and rates, rather than extent of cyclization, were determined.

Stimulation of cyclization by sonication. Sonication for 1 min yielded an 80% increase in the activity of the lytic extract, whereas 3 min of treatment was destructive. All reaction mixtures included 80 μM $FeSO_4$. We also found that activity could be obtained from mycelia directly by 2 min of sonication, without first producing protoplasts with lytic enzymes.

Conversion of the dimer of LLD**-tripeptide.** Figure 3 shows that the dimer of LLD-tripeptide was converted to a compound with antibiotic activity by a sonic extract and that this conversion was stimulated by 80 μM $FeSO_4$. That this antibiotic was isopenicillin N rather than penicillin N was suggested by its greater activity (i.e., larger zones) against gram-positive *Staphylococcus aureus* (ATCC 25923) and *Bacillus megaterium* (MIT B-46) than against gram-negative *Salmonella typhimurium* (ATCC 13311) and *P. aeruginosa* Pss. Penicillin N shows the opposite spectrum of activity (7). Furthermore, if the product was penicillin N, it should have been converted, at least partially. to deacetoxycephalosporin C by microbial ring-

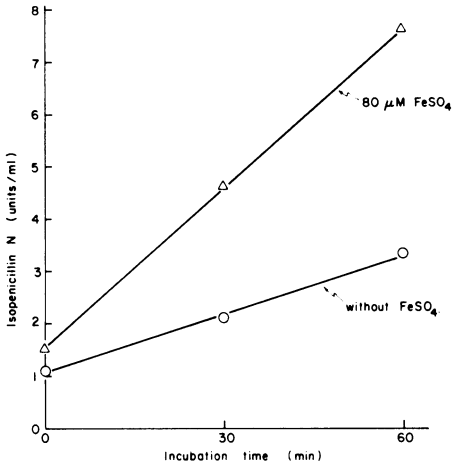

FIG. 3. *Conversion of the dimer of* LLD*-tripeptide to isopenicillin N and its stimulation by* $FeSO_4$.

expansion. However, no penicillinase-resistant product was observed in these experiments.

Formation of cyclase activity during fermentation. We determined the time course of the appearance and disappearance of cyclization activity during a fermentation. Figure 4 shows that cyclization activity was produced, reached its peak specific activity, and decreased in a manner similar to that of ring-expansion activity.

DISCUSSION

The LLD-tripeptide cyclization reaction of crude lytic enzyme extracts was found to be stimulated, like ring-expansion activity (10, 11), by Fe^{2+}, Triton X-100, and sonication. The optimum $FeSO_4$ concentration for cyclization was 80 μM as compared to 40 μM for ring-expansion (10). Other differences between the two activities include lack of stimulation of the cyclization reaction by ascorbate and adenosine 5′-triphosphate. Stimulation by Triton X-100 and sonication suggests that both reactions are catalyzed by membrane-bound enzymes; stimulation by $FeSO_4$ suggests that both are oxidative reactions.

A practical result of these experiments is that sonication can be used to prepare active cyclizing extracts directly, without going through protoplast formation and lysis. This had previously been found with ring-expansion activity (11).

The biosynthetic significance of the conversion of the dimer of LLD-tripeptide to a penicillin with the antibacterial spectrum of isopenicillin N is unclear at the present time because the possibility exists that the dimer is first reduced to the thiol form (i.e., LLD-tripeptide) before being converted. However, highly aerobic conditions were used for the reaction. Baldwin and

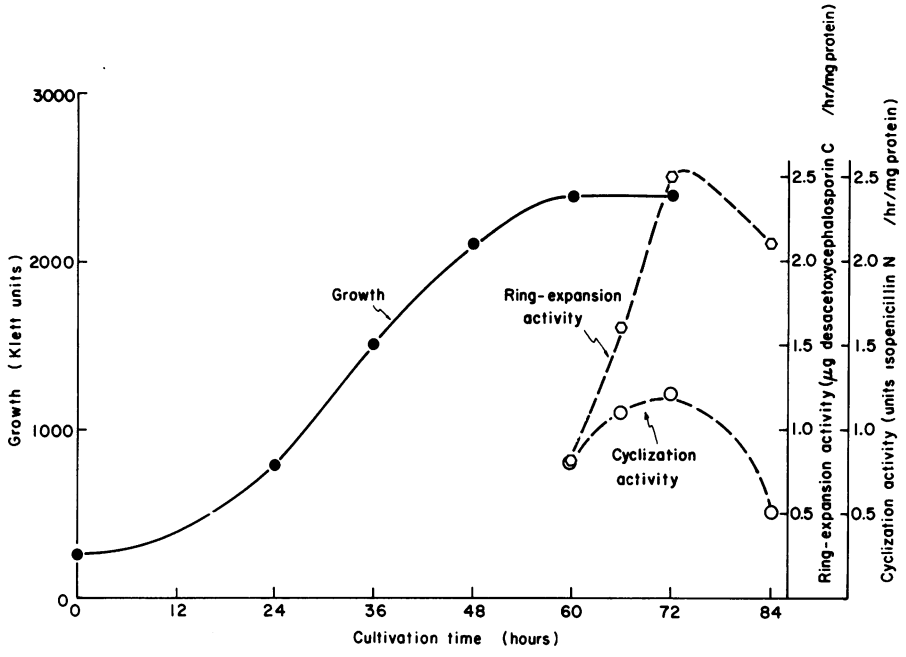

FIG. 4. *Production of LLD-tripeptide cyclization activity by C. acremonium M-0198 as compared with the growth and production of ring-expansion activity. Growth was followed by absorbance in the Klett-Summerson colorimeter with the red filter. Ring-expansion activity was assayed as described by Sawada et al. (10). Extracts were prepared by sonic treatment.*

Wan (1) have presented a model of penicillin biosynthesis that involves a disulfide intermediate.

High zero-time values were diminished by treating all samples with methanol. This supports our hypothesis that zero-time activity is due to the conversion of the tripeptide to isopenicillin N during overnight incubation of the agar plates.

It should be noted that in the experiments reported here the reaction ceased at the isopenicillin N stage. This cessation was established by checking the activity of the reaction product mixture on plates containing 500 U of penicillinase (Difco) per ml. In all cases, no zones were observed; thus, deacetoxycephalosporin C was not produced. The probable reason is the lability of the hypothetical racemase reaction converting isopenicillin N to penicillin N. From a practical viewpoint, the cessation of the reaction at the isopenicillin N stage could be useful for producing this compound, which is not available commercially. Isopenicillin N will be needed for future studies on the hypothetical racemase.

Figure 4 shows that the peak activities of ring expansion and cyclization occurred at the same time in fermentation. Although this finding might suggest that the two activities are functions of a single enzyme complex, we observed different ratios of the two activities in different extracts.

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