CYCLIZATION OF δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE TO PENICILLINS BY CELL-FREE EXTRACTS OF *STREPTOMYCES CLAVULIGERUS*

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Cell-free extracts prepared by sonication of *Streptomyces clavuligerus* cyclized δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) into a penicillin-type antibiotic. The antibacterial spectrum of this antibiotic suggested it was a mixture of isopenicillin N and penicillin N indicating that both cyclization and racemase activities were present. Cyclization activity was optimal in extracts prepared from 48 hours cultures. Extracts incubated at 20°C produced antibiotic for 2 hours before activity ceased. Cyclization activity showed an absolute requirement for dithiothreitol (DTT) and O_2 and was stimulated by ascorbic acid and $FeSO_4$. No requirement for ATP was observed.

The cyclization of ACV in cell-free enzyme systems derived from the beta-lactam antibiotic-producing eucaryotic fungi has been reported[1~8]. The product of this cyclization reaction has been identified as isopenicillin N, both in *Cephalosporium acremonium* and in *Penicillium chrysogenum*[5~7]. This initial ring-forming reaction is therefore a common step in the biosynthetic pathway to penicillin and cephalosporin-type antibiotics.

Streptomyces clavuligerus is a procaryotic organism which produces penicillin N, deacetoxycephalosporin C and cephamycin C in addition to the non-classical beta-lactam compound clavulanic acid[9]. Cell-free extracts from this organism have been used to study the carbamoylation, hydroxylation and methoxylation reactions which give rise to cephamycin C[10~12]. No investigation of cyclization activity in these cell-free systems was reported.

The present study describes the preparation of a cell-free extract from *S. clavuligerus* which cyclizes ACV into a penicillin-type antibiotic. Factors affecting this activity are investigated and compared to the similar systems from *C. acremonium*.

Materials and Methods

Materials

Bis-ACV was chemically synthesized as previously described[13]. Penicillin G (potassium salt), cephalosporin C (sodium salt) and penicillin N were gifts from D. HOOK, Bristol Laboratories, Syracuse, N Y, U. S. A. Clavulanic acid was a gift from A. BROWN, Beecham Pharmaceuticals, Betchworth Surrey, U. K. ATP (disodium salt), ascorbic acid (sodium salt), isoascorbic acid and dithiothreitol (DTT) were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. and penicillinase from Difco Laboratories, Detroit, MI, U.S.A. All other chemicals were of the highest grade commercially available.

Culture Conditions

S. clavuligerus NRRL 3585 was maintained on a sporulation medium described by HIGGINS *et al.*[14]. Inoculated plates were incubated 7~10 days at 28°C. Spores were scraped off into sterile distilled water

(5 ml/plate) and used to inoculate, 2% v/v, 25 ml of seed medium in a 125-ml flask. Inoculated seed medium, (O'SULLIVAN *et al.*[15]), was incubated 3 days and used to inoculate, 2% v/v, 100 ml amounts of production medium in 500-ml flasks. Production medium was developed based on the findings of AHARONOWITZ *et al.*[16~18] and consisted of: soluble starch 10 g, L-asparagine 2 g, 3-*N*-morpholinopropanesulfonic acid 21 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, K_2HPO_4 4.4 g, $FeSO_4 \cdot 7H_2O$ 1 mg, $MnCl_2 \cdot 4H_2O$ 1 mg, $ZnSO_4 \cdot 7H_2O$ 1 mg, and $CaCl_2 \cdot 2H_2O$ 1.3 mg in 1 liter of water, pH 6.8. Inoculated production medium was incubated 48 hours and the cells were then collected by filtration and used to prepare cell-free extracts. All incubations were at 27°C on a gyrotory shaker (250 rpm, eccentricity 19 mm).

Preparation of Cell-free Extracts

Cell-free extracts, unless otherwise indicated, were prepared by washing 48-hour cells of *S. clavuligerus* in 0.05 M tris-HCl buffer, pH 7.0+0.1 mM DTT (100 ml/100 ml culture). Washed cells were resuspended to 1/10 of the original culture volume in the same buffer and disrupted by sonication in an ice water bath for 2×15 seconds at maximum intensity (300 watts, Biosonik III, Bronwill Scientific, Rochester, NY, U.S.A.). Broken cell suspensions were centrifuged 1 hour at 100,000×*g*. All cell-free extracts were stored frozen at −20°C.

C. acremonium CW-19 was cultivated and used to prepare cell-free extracts exactly as described by HOOK *et al.*[19].

Alternative Methods of Cell Breakage

1. Lysozyme-NaCl: Washed cells of *S. clavuligerus* were resuspended to 2/3 of the original culture volume in 0.05 M tris-HCl buffer, pH 7.0 containing 0.8 M NaCl+2% sucrose and lysozyme, 0.75 mg/ml. After 3 hours shaking at 27°C, 125 rpm, the "spheroplasts" were collected by centrifugation at 5,000×*g* for 10 minutes and resuspended into 0.05 M tris-HCl buffer pH 7.0+0.1 mM DTT to 1/10 of the original culture volume.

2. French pressure cell: Washed cells of *S. clavuligerus* were resuspended to 1/10 of the original culture volume in 0.05 M tris-HCl buffer, pH 7.0+0.1 mM DTT and were passed twice through a French pressure cell at 1,400 kg/cm^2.

3. Omnimixer-plastic beads: Washed cells of *S. clavuligerus* were resuspended to 1/10 of the original culture volume in 0.05 M tris-HCl buffer pH 7.0+0.1 mM DTT and were mixed with an equal weight of plastic beads (Bio-Beads S-X8, BioRad Laboratories, Richmond, CA, U.S.A.) at maximum speed in an Omnimixer (Ivan Sorvall Inc., Norwalk, CT, U.S.A.) for 1 minute. The broken cells-beads slurry was centrifuged at 3,000×*g* for 5 minutes. Broken cell suspensions from all three methods were centrifuged at 100,000×*g* for 1 hour.

Protein Estimation

Protein content of cell-free extracts was measured by the method of BRADFORD[20]. Bovine gamma globulin was used as standard.

Cyclization Assay System

Cyclization activity of cell-free extracts was measured in reaction mixtures containing: bis-ACV 0.9 mM, DTT 4 mM, Na ascorbate 2.8 mM, $FeSO_4$ 45 μM, tris-HCl buffer 0.05 M, pH 7.0, cell-free extract 0.3 ml, final volume 0.4 ml. Reaction mixtures were incubated at 20°C for 1 hour and stopped by the addition of 0.4 ml methanol.

Bioassays of Beta-lactam Compounds

Antibiotic in reaction mixtures was estimated by the agar diffusion method. One unit (Pen N) of antibiotic gives a zone of inhibition equivalent to 1 μg of penicillin N with *Micrococcus luteus* ATCC 9341 as the indicator organism. *M. luteus* is insensitive to cephalosporin-type antibiotics, so when cephamycin C-containing culture filtrates were assayed or when cephalosporins were sought in reaction mixtures, *Escherichia coli* Ess was used as the indicator organism. One unit (Ceph C) of antibiotic gives a zone of inhibition equivalent to 1 μg of cephalosporin C with *E. coli* Ess as the indicator organism. *E. coli* Ess was generously provided by A. L. DEMAIN, Massachusetts Institute of Technology, Cambridge, MA, U.S.A..

Clavulanic acid in culture filtrates was determined by an indirect bioassay for beta-lactamase inhi-

bitors. *Staphylococcus aureus* N-2 is resistant to penicillin due to its ability to produce penicillinase. Melted agar supplemented with filter-sterilized penicillin G at 1 μg/ml was inoculated with this indicator organism and poured into plates. Culture filtrate or authentic clavulanic acid standard was placed on sterile 6.5 mm filter paper discs (Schleicher and Schuell, Keene, NH, U.S.A.) on the inoculated agar plates. Zones of inhibition were measured after 24 hours at 37°C.

High Performance Liquid Chromatography (HPLC)

Methanol-inactivated reaction mixtures were centrifuged at 12,000$\times g$ for 5 minutes to remove precipitated protein. Reaction mixture components were eluted from a μ Bondapak-C18 column (Rad Pak A in an RCM-100 radial compression module, Waters Scientific Co., Mississauga, Ont., Canada) using methanol - 0.05 M potassium phosphate buffer, pH 7.0 (10: 90) as mobile phase. The column was eluted at 70 kg/cm^2 at a flow rate of 2 ml/minute. UV-absorbing material was monitored at 220 nm with a Waters model 450 variable wavelength detector.

Results and Discussion

Cell-free Cyclization of ACV into Penicillin

Antibiotic product was formed in cyclization reaction mixtures containing bis-ACV, DTT, ascorbic acid, $FeSO_4$ and *S. clavuligerus* cell-free extract. This antibiotic inhibited the growth of the indicator organisms *M. luteus* and *E. coli* Ess. No antibiotic was formed when bis-ACV was omitted from reaction mixtures, or when cell-free extracts were inactivated by heating for 2 minutes at 100°C. Incorporation of penicillinase into bioassay plates at a concentration of 2×10^5 units/ml destroyed all antibiotic activity. Authentic deacetoxycephalosporin C (1 μg) was not affected by penicillinase at this concentration, and therefore the product of ACV cyclization was determined to be penicillin in nature rather than cephalosporin.

The biosynthetic pathway leading to the penicillins and cephalosporins shows the immediate product of ACV cyclization to be isopenicillin N[21]. This compound has been shown to be the predominant product of ACV cyclization in cell-free systems from *C. acremonium* and *P. chrysogenum*. Penicillin N, a second possible product, arises from isopenicillin N by the action of a racemase[5]. The penicillin product of ACV cyclization by *S. clavuligerus* was therefore examined using an array of indicator organisms known to show differential sensitivity to penicillin N *versus* isopenicillin N (Table 1). An authentic sample of penicillin N gave a pattern of sensitivity different from that of the *S. clavuligerus*

Table 1. Antibacterial activity of cyclization product against selected indicator organisms.
Penicillin N solution was made up to approximately 2.5 μg/ml. 20 μl amounts of the preparations tested were applied to paper discs placed on the surface of the seeded agar. Degree of inhibition was rated on a scale of (−)=no inhibition to (+4)=strong inhibition, zone size 26~30 mm. Inhibition scored as (±) indicated that an indistinct halo was observed.

Indicator organisms	Degree of inhibition of preparation tested		
	Penicillin N	*C. acremonium* reaction mixture (Isopenicillin N)	*S. clavuligerus* reaction mixture[a]
Micrococcus luteus ATCC 9341	+2	+3	+4
Staph. aureus ATCC 25923	±	±	±
Salm. typhimurium ATCC 13311	+1	−	+1
Comamonas terrigena ATCC 8461	+2	+1	+3
Escherichia coli Ess	+2	−	+2

[a] *S. clavuligerus* reaction mixtures did not contain clavulanic acid as determined by bioassay.

antibiotic product. Since an authentic sample of isopenicillin N was not available, a cyclization reaction mixture was prepared with *C. acremonium* cell-free extract and used as an isopenicillin N standard. Again, the pattern of sensitivity observed was quite different from that of the *S. clavuligerus* antibiotic. These results suggest that the product formed in *S. clavuligerus* reaction mixtures may be a mixture of both penicillin N and isopenicillin N, and that cell-free extracts therefore have both cyclization and racemase activities. Since *S. clavuligerus* cell-free extracts are routinely centrifuged at high speed and stored at −20°C before assay, the cyclization and racemase activities must both be "soluble" and quite stable. Racemase activity in *C. acremonium* is difficult to demonstrate in cell-free extracts and is suggested to be very labile and possibly membrane-bound[1,5,7,8,22,23]. SAWADA *et al.* have proposed that cyclization activity in *C. acremonium* is also membrane-bound based on the stimulatory effects of Triton X-100 and sonication[8]. However, we found that high speed centrifugation of cell-free extracts of *C. acremonium* caused no sedimentation of cyclization activity in agreement with ABRAHAM *et al.*[1]. We also found that neither Triton X-100 nor sonication affects cyclization in *S. clavuligerus* cell-free extracts. Perhaps sonication or Triton X-100 treatment stimulates activity only in membrane-containing cell-free extracts by activating some latent membrane-bound activity. Alternatively, Triton X-100 is reported to stabilize oxygenase enzymes[24,25].

The observed insensitivity of *E. coli* Ess to isopenicillin N made it unsuitable as an indicator organism for *S. clavuligerus* reaction mixtures since a portion of the antibiotic produced is isopenicillin N. For this reason, *M. luteus* was used for the remainder of the studies.

Physical Factors Affecting Cyclization Activity

The method of cell breakage used to prepare cell-free extracts affected resulting cyclization activity (Table 2). Extracts prepared by the formation and lysis of spheroplasts (lysozyme-NaCl) were low in both protein content and cyclization activity indicating that spheroplast formation was incomplete. Although related procedures are routinely used to prepare cell-free extracts from *C. acremonium* the formation and lysis of spheroplasts was not suitable for preparation of cell-free extracts from *S. clavuligerus*. The three remaining methods of cell breakage all yielded extracts with good levels of cyclization activity. Sonication was the easiest method tried, gave high levels of enzyme activity and was therefore used for all subsequent studies.

Table 2. Methods of cell breakage used to prepare cell-free extracts from *S. clavuligerus*.

Method of cell breakage	Specific cyclization activity [units(Pen N)/mg protein]
Sonication	4.01
French pressure cell	3.54
Omnimixer-plastic beads	1.58

The age of the culture at the time of cell-free extract preparation also affected cyclization activity (Fig. 1). Eight 100 ml amounts of inoculated production medium were incubated and 100 ml samples removed at intervals. Growth was estimated as absorbance at 600 nm. Cells were used to prepare cell-free extracts and culture filtrate was bioassayed for antibiotic and clavulanic acid content as described above. Cyclization activity of extracts, rises to a peak at 48 hours, then falls to undetectably low levels by 96 hours. Extracellular antibiotic reaches high levels by 56 hours. Clavulanic acid is produced earlier than antibiotics in this medium. Fairly high levels of cyclization activity are seen at 24~40 hours when extracellular antibiotic is not increasing but clavulanic acid is rising rapidly. This tends to support the hypothesis put forward by ELSON and OLIVER[9] that a single beta-lactam ring-forming enzyme may synthesize a variety of beta-lactam compounds depending on the nature of the precursors present.

Fig. 1. Cyclization activity of cell-free extracts from *S. clavuligerus* cultures of different ages.

S. clavuligerus cultures were harvested at the indicated times. Growth was measured as absorbance at 600 nm. Specific cyclization activity of cell-free extracts, units (Pen N)/mg protein was measured in the standard cyclization assay system described in Materials and Methods. Extracellular antibiotic, units (Ceph C)/ml, and clavulanic acid, μg/ml, were measured using bioassay techniques described in Materials and Methods.

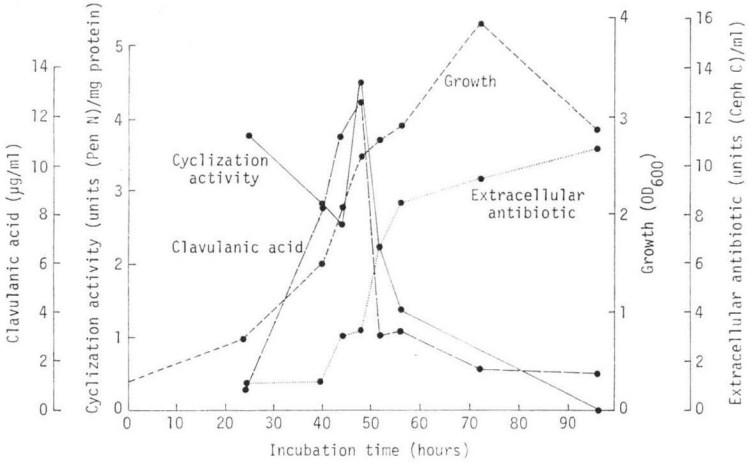


Fig. 2. Production of antibiotic by cell-free extracts of *S. clavuligerus* during a 4-hour incubation period.

A standard cyclization assay system was incubated at 20°C and 50 μl samples were removed at the times indicated. The reactions were stopped by addition of 50 μl of methanol and antibiotic produced was measured by bioassay.

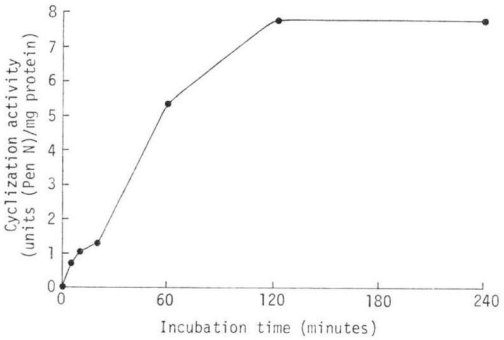
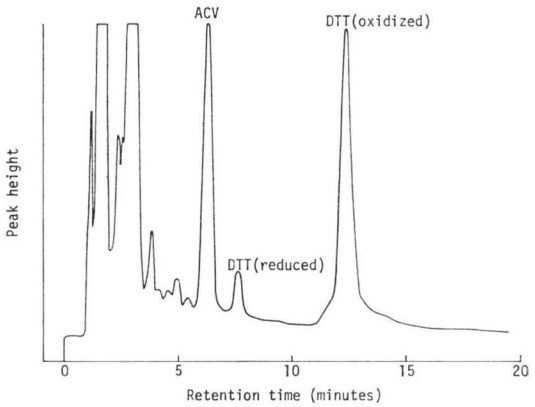


Fig. 3. HPLC profile of cyclization assay system after 4-hour incubation.

A cyclization reaction mixture was inactivated with methanol after 4-hour incubation at 20°C. A 20 μl amount of centrifuged reaction mixture was then examined by reverse-phase HPLC (see Materials and Methods for chromatographic details).



Cyclization activity was optimal in reaction mixtures incubated at 20°C. Activity dropped slowly at temperatures below 20°C and was still about half maximal at 4°C. If cyclization activity also persists at low temperatures in *C. acremonium*, this would explain the high zero time values observed in that organism when reaction mixtures are stopped by cooling on ice[8].

The effect of incubation time on antibiotic formation was also examined (Fig. 2). Antibiotic formation levels off after 2-hour incubation. While absolute quantitation by bioassay is difficult, this pro-

bably represents cyclization of about 10% of the ACV. This compares unfavorably with recently reported cyclization levels of 80~85% in *C. acremonium* extracts[1,2]. In an attempt to determine whether substrate (ACV) depletion by proteases was responsible for this low level of cyclization, reaction mixtures were examined by HPLC after 4-hour of incubation (Fig. 3). The chromatographic profile clearly shows that a large peak of ACV remains (retention time=6.15 minutes) even though activity has levelled off. Under these chromotographic conditions the penicillin product is poorly retained and elutes with other reaction mixture components at a retention time of 2~3 minutes. No reason has been established for the low level of cyclization activity but substrate depletion by non specific proteases does not appear to be a factor.

Effect of Assay System Components on Cyclization Activity

Cyclization activity was affected by the pH of the buffer used to prepare cell-free extracts. Activity was optimal over the pH range of 7.0~8.0. Comparison of a variety of organic and inorganic buffers at pH 7.0 showed that tris-HCl gave cell-free extracts with best levels of cyclization activity and with optimal stability to storage at −20°C (no loss of cyclization activity for at least 3 months).

The effect of DTT on cyclization activity was examined. Since chemically-synthesized ACV is in a dimeric form, DTT was presumed necessary to reduce the dimer to the active monomeric ACV tripeptide. Recent evidence indicates that bis-ACV in the absence of DTT supports cyclization activity in *C. acremonium*, although carryover of DTT from the protoplast lysing procedure is another possible explanation[8]. We found no cyclization activity in the absence of DTT with *S. clavuligerus* cell-free extracts (Fig. 4). Activity was optimal at 4 mM which represents a 4.4 molar excess of DTT over bis-ACV. HPLC analysis of such reaction mixtures indicates that this slightly exceeds the minimum concentration needed to completely convert bis-ACV to the monomer form. In a separate examination of this DTT requirement, 5 mg of bis-ACV was combined with 5 mg DTT in 0.5 ml water pH 7.0. After 30 minutes at 22°C, DTT was removed by extraction with 5×1 ml of water-saturated ethyl acetate. Residual ethyl acetate was removed by purging the reduced ACV solution with O_2-free N_2 gas. HPLC examination of the ACV solutions before and after extraction indicated that reduction of bis-ACV to monomer was complete and that ethyl acetate extraction effectively removed all DTT while only a trace of ACV monomer was reoxidized to the dimer form. Twenty five μl amounts of these ACV solutions were then used as substrate in cyclization assays to which no additional DTT was added. Bioassays indicated that pre-reduced ACV in the absence of

Fig. 4. Effect of DTT on cyclization of ACV to antibiotic by cell-free extracts from *S. clavuligerus*.

Cyclization reaction mixtures were prepared with varying concentrations of DTT from 0~10 mM. After 1-hour incubation at 20°C, reaction mixtures were inactivated with methanol and antibiotic content determined by bioassay.

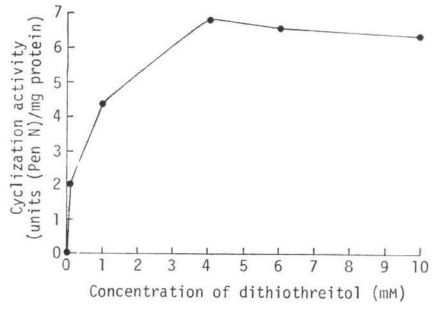


Table 3. Effect of changes in the assay system on cyclization activity.

Change in assay system	Cyclization activity remaining (%)
Normal (no change)	100
Omit ascorbic acid	53.7
Omit $FeSO_4$	78.0
Add Na_2-ATP (1 mM final concentration)	100

DTT was only 50% as effective as was ACV plus DTT. This suggests that the requirement for DTT is not solely for conversion of bis-ACV to the monomer form.

The effect of other components of the assay-system was also examined (Table 3). The normal concentration of each component is given in the cyclization assay system (see Materials and Methods) and represents the optimum concentration for this system. Activity was found to be markedly stimulated by ascorbic acid in contrast to the *C. acremonium* system[8]. Isoascorbic acid could replace ascorbic acid with no loss of activity but other ene-diol type reducing agents such as dihydroxyfumarate and rhodizonic acid were ineffective. Ferrous ion showed only a slight stimulation in this case, but the effect of ferrous ion varied markedly from one cell-free extract to another. In some cases an absolute requirement was observed. Presumably ferrous ion is required but only in trace amounts. ATP did not stimulate cyclization activity.

The stimulatory effects of ascorbic acid and ferrous ion are characteristic of oxygenase-type enzymes and prompted an investigation of the effects of aeration on cyclization activity. Reaction mixtures (0.4 ml) are normally incubated in stationary test tubes. When reaction mixtures were incubated in capped 25-ml flasks shaking at 250 rpm, the amount of antibiotic produced doubled. Conversely, when incubation was carried out in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, U.S.A.) no antibiotic product was formed. Both of these findings support a requirement for molecular oxygen in agreement with the similar requirement reported in *C. acremonium*[1].

The cyclization activity of cell-free extracts of *S. clavuligerus* shows many parallels with that same activity in extracts from *C. acremonium*, but also some interesting differences. The detection of penicillin N as a product suggests that racemase activity is also present in these extracts. In view of this possibility, the ring expansion activity of these extracts is currently under investigation.

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