HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CYCLIZATION ACTIVITY IN CELL-FREE SYSTEMS FROM *STREPTOMYCES CLAVULIGERUS*

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A thirteen-fold excess of dithiothreitol maintains δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) in its monomeric form under the conditions normally encountered in an ACV cyclization assay system, using *Streptomyces clavuligerus*. A reversed phase high performance liquid chromatographic (HPLC) system which separates ACV monomer from isopenicillin N, penicillin N and from other cyclization assay components has been developed as follows; mobile phase: 5% methanol - 95% KH_2PO_4 (0.05 M adjusted to pH 4.0 with concentrated H_3PO_4; stationary phase: μBondapak-C_{18}; flow rate: 2 ml/minute for 5 minutes, 3 ml/minute for the remainder; detection: 220 nm). Under these conditions, authentic samples of isopenicillin N and penicillin N elute with a retention time of 5.25 minutes, which coincides with a peak of newly-formed material observed in cyclization reaction mixtures. The combined concentration of isopenicillin N and penicillin N[(iso)penicillin N] in cyclization reaction mixtures corresponds closely to the concomitant decrease in the ACV monomer. Cyclization reaction mixtures, in which crude cell-free extract from *S. clavuligerus* NRRL 3585 is the enzyme source, contain (iso)penicillin N at a concentration of 43.3 μg/ml after a 1-hour incubation period. Cyclization reaction mixtures, in which salt-precipitated cell-free extract from *S. clavuligerus* is the enzyme source, contain 39.0 μg/ml (iso)penicillin N.

The cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) into penicillin-type compounds has been achieved in cell-free systems from several organisms[1~8]. Quantitation of this process can be difficult, because of the biological assay procedures usually employed to detect the antibiotic products. With *Cephalosporium acremonium*, the antibiotic product is exclusively isopenicillin N[5,7], so that cyclization activity in these systems can be quantitated by comparison of the bioactivity of reaction mixtures with that of authentic isopenicillin N. On the other hand, a cell-free system from *Streptomyces clavuligerus* was described recently which cyclizes ACV to a product whose antibacterial spectrum suggested the presence of both isopenicillin N and penicillin N[4]. Subsequent studies have confirmed that this cell-free system contains an epimerase which converts isopenicillin N to penicillin N under normal assay conditions[9]. The quantitation of mixtures of isopenicillin N and penicillin N by bioassay has proven to be difficult, because each penicillin has a different antibacterial spectrum, and no indicator organism has been found which responds equally to both. Chemical assay procedures have also been found to be unsatisfactory, either because of lack of sensitivity[10], or interference by other components of the reaction mixture[11]. For these reasons, a high performance liquid chromatographic (HPLC) procedure for the analysis of reaction mixtures and the quantitation of cyclization activity seemed desirable.

There has been much recent interest in the analysis of β-lactam antibiotics by HPLC[12~17]. With few exceptions, the compounds examined have aromatic side chains, which greatly affect their UV

absorption and chromatographic characteristics. In contrast, the products derived from ACV contain α-aminoadipic acid side chains and, therefore, require a different chromatographic strategy.

This paper describes a reversed phase HPLC system which separates isopenicillin N and penicillin N from other reaction mixture components (although not from each other) and permits the quantitation of the cyclization activity.

Materials and Methods

Materials

ACV dimer[18] was supplied as the diformate salt, and penicillin N[9] and isopenicillin N[9] were supplied as the monosodium salts. The purities of the two penicillins were determined by the arsenomolybdate assay of HOLM[11] using penicillin V as standard. The isopenicillin N was 75.7% pure, and the penicillin N was 87.9% pure. Dithiothreitol (DTT) and sodium ascorbate were from Sigma Chemical Co., St. Louis, MO., U.S.A. All other chemicals were of reagent grade.

Cyclization Assay

Cyclization activity of enzyme preparations was measured in the cyclization assay system described previously[4] but with an ACV dimer concentration of 100 μg/0.4 ml (0.306 mM). Reactions were terminated by addition of 0.4 ml of methanol, and the antibiotic content was detected by agar diffusion bioassay with *Micrococcus luteus* ATCC 9341 as indicator organism or by high performance liquid chromatography (HPLC).

Cyclization Enzyme Preparations

Crude cell-free extract was prepared as previously described from 48 hours cultures of *Streptomyces clavuligerus* NRRL 3585.

Salt-precipitated cell-free extract was prepared by gradual addition of solid ammonium sulfate to crude cell-free extract with gentle stirring at 4°C until 40% saturation was reached. After 15 minutes at 4°C the suspension was centrifuged for 15 minutes at 15,000$\times g$ and the pellet was discarded. Additional ammonium sulfate was then added to the supernatant, as above, until 70% saturation was reached. Following centrifugation, the pellet was resuspended to its original volume in 0.05 M tris-HCl buffer pH 7.0 containing 0.1 mM DTT.

Both crude cell-free extract and salt-precipitated cell-free extract were stored in aliquots at −20°C.

High Performance Liquid Chromatography (HPLC)

Methanol-inactivated reaction mixtures were centrifuged at 12,000$\times g$ for 5 minutes to remove precipitated protein before analysis. The chromatographic equipment used was: M-6000A pump, UK-6 injector, M-450 variable wavelength detector, M-420 data module and μBondapak-C_{18} column (Rad Pak A in an RCM-100 radial compression module) as stationary phase. All equipment was from Waters Scientific Co., Mississauga, Ont., Canada. The mobile phase consisted of methanol - 0.05 M potassium phosphate buffer. The methanol content and pH of the mobile phase depended upon the particular separation. A short precolumn (packed with Bondapak C_{18}/Corasil) was used to guard the main column. UV Absorbing material was detected at 220 nm at a sensitivity of 0.02 aufs.

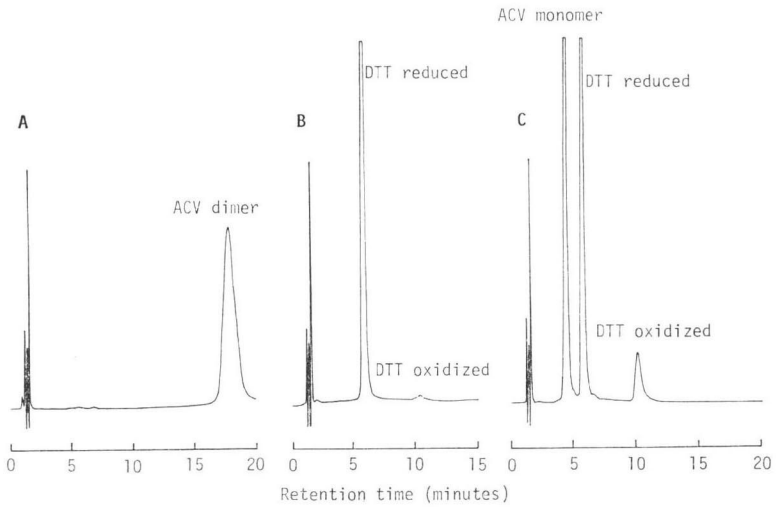
Results

The ACV substrate used in cyclization assays is synthesized[18] as a disulfide. Therefore, the first step in a cyclization assay involves the reduction of the dimer to ACV monomer with DTT. This reduction was monitored by exposure of 100 μg (0.123 μmole) of ACV dimer to 250 μg (1.62 μmole) of DTT in 25 μl of water. After 15 minutes at 21°C, the mixture was diluted with 0.375 ml of 0.05 M tris-HCl, pH 7.0, and 0.4 ml of methanol was added to bring the final concentrations of ACV and DTT to those employed in a normal cyclization reaction mixture. This mixture was analyzed by HPLC together with

Fig. 1. Reduction of ACV dimer to ACV monomer with DTT.

Twenty microliter amounts of the following samples were analyzed by HPLC with a mobile phase of 14% methanol - 86% potassium phosphate buffer (0.05 M, pH 7.0):

A. 100 μg ACV dimer in 0.4 ml of 0.05 M tris-HCl, pH 7.0 and 0.4 ml of methanol.
B. 250 μg DTT in 0.4 ml of 0.05 M tris-HCl, pH 7.0 and 0.4 ml of methanol.
C. 100 μg ACV dimer and 250 μg DTT in 25 μl of 0.05 M tris-HCl, pH 7.0 were incubated 15 minutes at 21°C then diluted with 0.375 ml of 0.05 M tris-HCl, pH 7.0 and 0.4 ml of methanol.



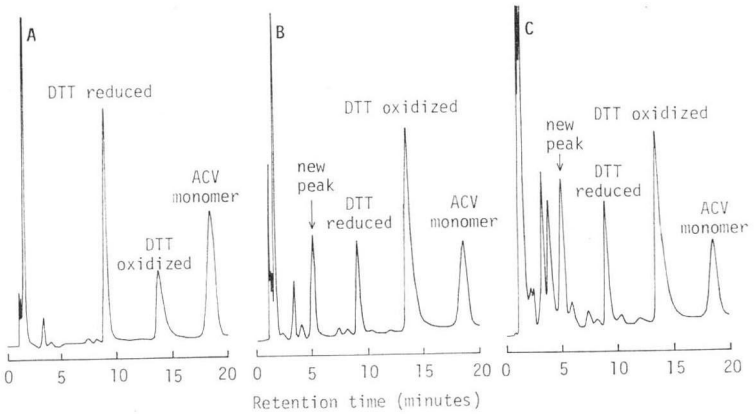
similarly prepared samples of ACV dimer alone and DTT alone (Fig. 1). The mobile phase which gave the best separation consisted of 14% methanol - 86% potassium phosphate buffer (0.05 M, pH 7.0) at a flow rate of 2 ml/minute. These chromatographic conditions afforded a good separation of ACV dimer (Fig. 1 A, 17.93 minutes) and ACV monomer (Fig. 1 C, 4.50 minutes) from each other and from DTT in its oxidized (Fig. 1 B, 10.23 minutes) and reduced (Fig. 1 B, 5.83 minutes) forms. As seen in Fig. 1 C, ACV dimer is completely converted to ACV monomer under these conditions.

Isopenicillin N and penicillin N were not retained under the chromatographic conditions described above (data not shown), and a different mobile phase was needed for the analysis of cyclization reaction mixtures. Since ACV dimer was not observed in cyclization reaction mixtures, conditions were sought for the separation of ACV monomer from isopenicillin N and penicillin N, and from other components of the reaction mixture. Optimum separation was achieved with a mobile phase of 5% methanol - 95% KH_2PO_4 (0.05 M, adjusted to pH 4.0 with concentrated H_3PO_4) and a flow rate of 2 ml/minute for 5 minutes followed by 3 ml/minute for the remaining analysis time. Fig. 2 shows the results obtained with 20 μl aliquots of three different cyclization reaction mixtures. Fig. 2 A is a zero time control prepared by addition of methanol to the reaction mixture prior to the addition of salt-precipitated cell-free extract. The ACV monomer elutes at 18.70 minutes, and DTT in its oxidized and reduced forms elutes at 13.86 minutes and 9.10 minutes, respectively. The results of Fig. 2 B refer to a reaction mixture prepared as in A, except that the mixture was incubated for 1 hour at 20°C prior to the addition of methanol. The ACV monomer peak in Fig. 2 B is reduced in area by approximately one-third compared to that in Fig. 2 A and a new peak is observed at 5.25 minutes. Similar results are obtained with crude cell-free extract as the enzyme source (Fig. 2 C), but the chromatogram is more complex because of the additional peaks associated with the crude enzyme source. Incubation of crude cell-free extract with ACV again results

Fig. 2. Analysis of cyclization reaction mixtures by HPLC.

Twenty microliter amounts of the following cyclization reaction mixtures were analyzed by HPLC with a mobile phase of 5% methanol - 95% KH_2PO_4 (0.05 M, adjusted to pH 4.0 with concentrated H_3PO_4):

A. Salt-precipitated cell-free extract (zero time control).
B. Salt-precipitated cell-free extract (1 hour incubation).
C. Crude cell-free extract (1 hour incubation).



in decrease of ACV monomer and the appearance of a peak at 5.25 minutes. However, the new peak is not resolved with baseline separation from other contaminating peaks. When ACV was omitted from cyclization reaction mixtures containing either crude cell-free extract or salt-precipitated cell-free extract, the resulting HPLC profiles were identical to their ACV-containing counterparts, except that both the ACV monomer peak and the new peak at 5.25 minutes were absent (data not shown).

The identity of the new peak was investigated by comparison of its retention time with those of isopenicillin N and penicillin N (Fig. 3). Under the conditions described above for Fig. 2, both isopenicillin N and penicillin N eluted with a retention time of 5.25 minutes.

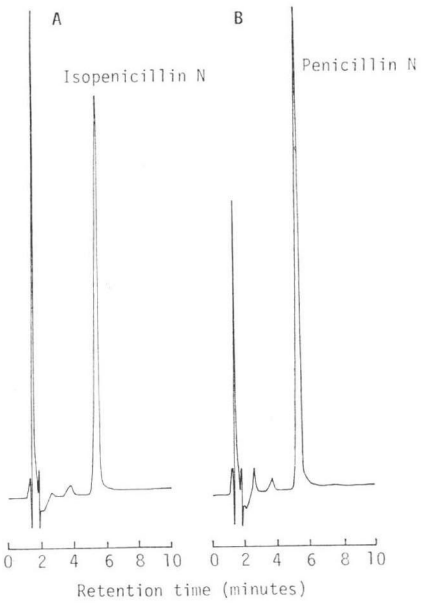


Fig. 3. Analysis of standard solutions of isopenicillin N and penicillin N by HPLC.

Weighed amount of each penicillin were dissolved in water to give a final concentration of 0.1 mg/ml. Chemical assay of these solutions indicated that the isopenicillin N and penicillin N preparations used were 75.7% and 87.9% pure respectively (actual penicillin concentrations of the solutions were 0.199 μmole/ml and 0.230 μmole/ml respectively).

Twenty microliter amounts of:

A. Isopenicillin N solution and
B. Penicillin N solution

were analyzed by HPLC with a mobile phase of 5% methanol - 95% KH_2PO_4 (0.05 M adjusted to pH 4.0 with concentrated H_3PO_4).

Table 1. HPLC analysis of isopenicillin N and penicillin N standards.

Compound analyzed (μg/ml) (μmole/ml)	Peak area[a] (μvolt second)	Actual penicillin concentration (μmole/ml)[b]	Peak area (μmole penicillin)
Isopenicillin N (100) (0.262)	1.105×10^{6}	0.199	2.78×10^{3}
Penicillin N (100) (0.262)	1.447×10^{6}	0.230	3.5×10^{3}

[a] Twenty microliters of each solution were analyzed.

[b] Based on the arseno-molybdate assay of HOLM[5], with penicillin V as standard; isopenicillin N was 75.7% pure, and penicillin N was 87.9% pure.

Table 2. Quantitation of (iso)penicillin N concentration in cyclization reaction mixtures.

Reaction mixture analyzed[a]	(Iso)penicillin N peak area (μvolt second)	(Iso)penicillin N produced (μmole)[b]	ACV Monomer peak area (μvolt second)	ACV consumed (μmole)
Salt-precipitated cell-free extract (zero time control)	0	0	1.753×10^{6}	0
Salt-precipitated cell-free extract (1 hour incubation)	7.022×10^{5}	0.0946	1.131×10^{6}	0.0869
Crude cell-free extract (〃)	1.236×10^{3}[c]	c	1.062×10^{6}	0.0965

[a] Twenty microliter injections.

[b] Based on the calibration of Table 1.

[c] Significant baseline contamination in this case.

Since the two pencillins are not resolved in this sytem, the total cyclization activity of reaction mixtures could be quantitated from the area of the combined isopenicillin N and penicillin N [(iso)-penicillin N] peak compared to that of authentic standards of known concentration (Table 1). Based on these standards, the peak area of (iso)penicillin N was found to be $(2.97\pm0.18)\times10^{3}$ μvolt-second/μmole. This value was then used to determine the content of (iso)penicillin N in cyclization reaction mixtures. Table 2 shows the (iso)penicillin N content of reaction mixtures, based on measurement of the peak at 5.25 minutes, and also the decrease in ACV under the same conditions.

Under identical conditions, 39.4% of the ACV is consumed using crude cell-free extract, and 35.5% is consumed using salt-precipitated material. In the latter case, this corresponds to utilization of 0.0869 μmole of ACV or the formation of 39.0 μg/ml of (iso)penicillin N if the conversion is quantitative. This decrease in the ACV is accompanied by the appearance of (iso)penicillin N having a peak area of 7.022×10^{5} for an injection of 20 μl. Since the total volume is 0.8 ml, the amount of (iso)penicillin N formed is $(7.022\times10^{5}\times40)/(2.97\times10^{3})=0.0946$ μmole, corresponding to 42.4 μg/ml. The difference of 8.7% represents experimental error and residual base-line contamination of the (iso)penicillin N peak. It can be concluded that the disappearance of ACV can be accounted for quantitatively in terms of the formation of (iso)penicillin N.

The biological assay of 20 μl amounts of the cyclization reaction mixtures described in Table 2 revealed a pattern consistent with the HPLC profiles (Table 3). The reaction mixture from the crude cell-free extract shows a slightly larger zone of inhibition than that from the salt-

Table 3. Biological assay of cyclization reaction mixtures.

Reaction mixture analyzed*	Zone of inhibition (mm)
Salt-precipitated cell-free extract (zero time control)	0
Salt-precipitated cell-free extract (1 hour incubation)	20
Crude cell-free extract (1 hour incubation)	21

* Twenty microliters of each reaction mixture were bioassayed using *Micrococcus luteus* ATCC 9341 as indicator organism.

precipitated cell-free extract. None of the controls produced zones of inhibition.

Discussion

Under the conditions encountered in a normal cyclization assay system, the ACV substrate exists entirely in the monomer form. An HPLC system which resolves ACV monomer from (iso)penicillin N and from other reaction mixture components therefore allows two independent approaches to the determination of the (iso)penicillin N concentration of reaction mixtures. Quantitation of the disappearance of ACV monomer is the more reliable approach when crude cell-free extract is used as the enzyme source because the ACV monomer is well resolved from other interfering peaks. This method requires the reasonable assumption that ACV monomer is converted exclusively to (iso)penicillin N. Evidence consistent with this assumption has been obtained.

In reaction mixtures containing crude cell-free extract, the (iso)penicillin N concentration is 43.3 μg/ml. This represents cyclization of 39.5% of the 100 μg of ACV dimer originally supplied. In reaction mixtures containing salt-precipitated cell-free extract the (iso)penicillin N concentration is 39.0 μg/ml, corresponding to cyclization of 35.5% of the ACV. These quantitative data represent a considerable improvement over the estimates of cyclization obtained previously by biological assay techniques, and will greatly facilitate enzyme purification studies currently in progress.

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