ISOLATION AND PARTIAL CHARACTERISATION OF ACV SYNTHETASE FROM *CEPHALOSPORIUM ACREMONIUM* AND *STREPTOMYCES CLAVULIGERUS*

Sir:

δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine (ACV) synthetase catalyses the formation of the tripeptide ACV from its constituent amino acids. This tripeptide is the common progenitor in the biosynthesis of the penicillins and cephalosporins[1]. Several enzymes in the β-lactam antibiotic pathway have been purified and extensively studied. Relatively little information is however, available regarding the tripeptide formation, the key initial step in the pathway. Exogenous feeding experiments to *Cephalosporium acremonium* of [4-$^{2}H_{6}$,$^{18}O_{2}$]-valine have demonstrated that an intracellular exchange of one and both valine oxygens occurs in the *in vivo* formation of ACV[2]. Cell-free synthesis of ACV has been detected in extracts from *C. acremonium*[3,4] and *Streptomyces clavuligerus*[5] and recently a single enzyme has been purified from *Aspergillus nidulans* which is capable of catalysing ACV formation[6].

Here, we report the isolation and initial characterisation of ATP dependent proteins from *C. acremonium* (ATCC 60777) and *S. clavuligerus* SC. 2 which incorporate L-[^{14}C]valine into the tripeptide [^{14}C]-ACV.

ACV synthetase was purified from both *S. clavuligerus* and *C. acremonium* by similar procedures. Extraction of mycelia into a high glycerol buffer was followed by ammonium sulfate fractionation, gel filtration chromatography (AcA 34) and anion-exchange chromatography (Mono Q). Incorporation of [^{14}C]-valine into [^{14}C]-ACV was measured at each stage[6] (Table 1) and the final preparation was apparently homogenous when analysed by SDS-polyacrylamide gel electrophoresis. Using purified ACV synthetase, tripeptide formation was also coupled with purified isopenicillin N synthase (IPNS)[7] and β-lactam production was monitored, by a β-lactamase induction assay[8].

This fractionation procedure resulted in a *ca.* 300-fold increase in specific activity for both enzymes, although total activity measured was higher in extracts from *C. acremonium*. The MW of both enzymes, (under reducing and dissociating conditions) was estimated to be 300 kD by SDS-polyacrylamide gel electrophoresis. This is larger than the reported MW of the enzyme from *A. nidulans*. It has been noted that ACV synthetase activity isolated in this study elutes from AcA 34 gel filtration in the void volume (exclusion limit: 750 kD) indicating protein aggregation.

Table 1. Purification of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*.

Fraction	Protein (mg)	Specific activity	Recovery (%)	Fold purification
C. acremonium[a]				
Crude[c]	721.5	—	—	—
Ammonium sulfate	94.6	5	100	1
AcA 34	12.5	428	20	86
Mono Q	0.26	1,527	13	305
S. clavuligerus[b]				
Crude[c]	334	—	—	—
Ammonium sulfate	133	2.21	100	1
AcA 34	3.1	20.8	22	9.4
Mono Q	0.05	610	10.4	277

Specific activities are expressed as nmol ACV formed per mg protein per hour.

[a] Extract obtained from 3 g of freeze dried mycelia.

[b] Extract obtained from 12 g of damp mycelia.

[c] No reliable activity measurements could be obtained using crude extracts.

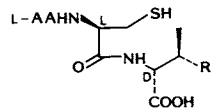
Table 2. Incubations to determine the substrate specificity of ACV synthetase from both *Cephalosporium acremonium* and *Streptomyces clavuligerus*.

Amino acids	β-Lactam formation	
	C. acremonium	*S. clavuligerus*
L-α-A + L-Cys + L-Val	*****	****
L-α-A + L-Cys + D-Val	—	—
L,L-AC + L-Val	***	—
L-α-A + L-Cys + L-α-AB	**	***

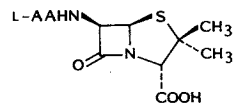
Tripeptide formation was monitored by coupling the incubation with IPNS and detection of β-lactam production. The number of asterisks crudely indicate the relative amount of β-lactam produced in each incubation. L-α-A: L-α-aminoadipate, L,L-AC: δ-(L-α-aminoadipoyl)-L-cysteine, L-α-AB: L-α-aminobutyrate.

Incubation of purified ACV synthetase with various amino acids and the dipeptide δ-(L-α-aminoadipoyl)-L-cysteine, indicated that, under our assay conditions, some differences exist in the substrate specificity of the enzymes isolated from the two sources (Table 2). In both cases, D-valine was not incorporated into the tripeptide ACV. The dipeptide δ-(L-α-aminoadipoyl)-L-cysteine was not

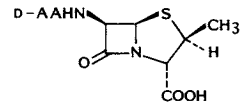
Fig. 1. Structures of ACV, ACAB, isopenicillin N, penicillin (1) and cepham (2).



ACV R=CH_3
ACAB R=H



Isopenicillin N



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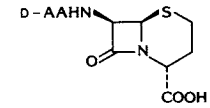

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L-AAHN: L-α-Aminoadipoyl, D-AAHN: D-α-aminoadipoyl.

incorporated into ACV when incubated with purified ACV synthetase from *S. clavuligerus*, whereas some ACV formation was detected in similar incubations with ACV synthetase from *C. acremonium*. Breakdown of the dipeptide AC to individual amino acids was not detected by ^{1}H NMR (500 MHz) studies and thin layer chromatography analysis during the time course of these incubations. Furthermore, the coupling of ACV production to the IPNS catalysed cyclisation (to give isopenicillin N) demonstrates that the stereochemistry of the *in vitro* produced ACV was L,L,D, since L,L,L-ACV is not a substrate for IPNS[1]. When L-α-aminobutyrate was substituted for L-valine, tripeptide formation could be detected in incubations with ACV synthetase from *S. clavuligerus* and to a lesser extent in incubations with the enzyme from *C. acremonium*. It is of interest, that the β-methyl penicillin (1) and an associated cepham (2), presumably derived from the reaction of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-aminobutyrate (ACAB) with IPNS, followed by the epimerisation of the α-aminoadipoyl side chain, have recently been isolated from *Streptomyces* ACC 13285[9] (Fig. 1). These results indicate that the substrate specificity of purified ACV synthetase from *C. acremonium* differs from that of the corresponding enzyme from *S. clavuligerus* but is similar to that reported for crude extracts displaying ACV synthetase activity from *C. acremonium* C10[3,4] and *S. clavuligerus*[5]. Previous reports have indicated that phosphopantotheine is associated with peptide antibiotic synthesizing enzymes *e.g.* gramicidin S synthetase[10] and bacitracin synthetase[11]. Therefore, it was of interest to determine if pantothenic acid could be detected in preparations of purified ACV synthetase from both organisms. After treatment of protein fractions with alkali and alkaline phosphatase, release of pantothenic acid was measured using a pantothenate requiring organism *Lactobacillus plantarum*[10]. The results indicated that approximately 0.8 and 0.95 mol of pantothenic acid could be liberated per mol of ACV synthetase from *S. clavuligerus* and *C. acremonium*, respectively. These results suggest that tripeptide formation may involve a phosphopantotheine arm which may transport each activated amino acid to the site of the next activated amino acid to allow di- and tri-peptide formation[12].

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