SYNTHESIS OF BENZYLPENICILLIN BY CELL-FREE EXTRACTS FROM *STREPTOMYCES CLAVULIGERUS*

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Cell-free enzyme concentrates from *Streptomyces clavuligerus* were found to convert phenylacetyl-L-cysteinyl-D-valine (PCV) directly into benzylpenicillin when incubated under reaction conditions which support the activity of isopenicillin N synthetase. The formation of benzylpenicillin was detected both by biological assay and by high performance liquid chromatography. Supplementation of PCV-containing reaction mixtures with cofactors required for ring expansion activity did not result in the production of cephalosporins. Incubation of phenoxyacetyl-L-cysteinyl-D-valine (PoCV) under the same reaction conditions did not result in the formation of penicillin V or any cephalosporin product.

Cell-free production of penicillins and cephalosporins has been demonstrated using cell extracts from a variety of β-lactam producing organisms including *Streptomyces clavuligerus* and *Cephalosporium acremonium*[1~4]. When *S. clavuligerus* is employed as the source of the cell-free extract, penicillins and cephalosporins are formed from tripeptide precursors by a multi-step process involving four different enzymes of the penicillin - cephalosporin biosynthetic pathway, *viz.*, isopenicillin N synthetase (IPS), epimerase, deacetoxycephalosporin C synthetase (DAOCS) and deacetoxycephalosporin C hydroxylase[5]. On the other hand, cell extracts from *C. acremonium*, unless freshly prepared, do not contain significant epimerase activity so that penicillins, but not cephalosporins are formed from tripeptide precursors[6].

Cell-free extracts from either of these organisms can synthesize both natural and unnatural antibiotics depending on the nature of the tripeptide substrate presented to the system. Tripeptide precursors other than the natural substrate δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) have been used to investigate the substrate specificity requirements of IPS. Substrate analogs in which the α-aminoadipyl side chain is shortened (replaced by glutamate, aspartate or adipate) or lengthened (by acetylation or glycylation of the amino group) are either inactive or exhibit greatly reduced activity as substrates[7,8]. Replacement of the α-aminoadipyl group of ACV by *S*-carboxymethyl-L-cysteine affords a substrate which displays high levels of activity, about 50% of that of ACV[9,10]. The substrate specificity characteristics are similar for cell-free extracts derived from both *S. clavuligerus* and *C. acremonium*. Based on these observations, we believed IPS to have a rigid requirement for a 6-carbon side chain or an isostere thereof.

Recently, however, it was reported that cell-free extracts from *Penicillium chrysogenum* and *Acremonium chrysogenum* (*C. acremonium*) convert phenylacetyl-L-cysteinyl-D-valine, an ACV analog containing phenylacetate in place of α-aminoadipate, directly into benzylpenicillin, a process that presumably involves the action of IPS[11]. Since this observation is in marked contrast to the substrate

specificities encountered previously and the result has many implications, we have investigated the same reaction using cell-free extracts from *S. clavuligerus*, and also have examined the behavior of phenoxyacetyl-L-cysteinyl-D-valine, the analog containing phenoxyacetate in place of α-aminoadipate.

Materials and Methods

Materials

Benzylpenicillin and penicillin V were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and penicillinase was from Difco Laboratories, Detroit, MI., U.S.A. Bis-ACV was chemically synthesized as previously described[12]. Bis-phenylacetyl-L-cysteinyl-D-valine (PCV) and bis-phenoxyacetyl-L-cysteinyl-D-valine (PoCV) were synthesized by coupling either phenylacetic acid or phenoxyacetic acid respectively, to benzhydryl-*S*-trityl-L-cysteinyl-D-valine[8], followed by deprotection in the usual manner[8,12].

Organism and Cultural Conditions

S. clavuligerus NRRL 3585 was maintained and cultivated for the preparation of enzyme extracts as described previously[3].

Enzyme Preparations

Cell-free extract was prepared from *S. clavuligerus* and partially purified and concentrated by streptomycin sulfate and ammonium sulfate precipitation as previously described[8].

Enzyme Assays

Penicillin formation due to IPS activity, was measured in a 0.2-ml assay system prepared as follows: 100 μg of ACV or 250 μg of PCV or PoCV was exposed to 12.5 μl of 66.7 mM dithiothreitol for 15 minutes at 21°C prior to the addition of ascorbic acid 2.8 mM, $FeSO_4$ 45 μM, Tris-HCl buffer (pH 7.0) 50.0 mM, and enzyme 0.15 ml. The mixtures were incubated for 2 hours at 20°C and the reactions were then terminated by the addition of 0.2 ml of MeOH. Under these conditions, the amount of enzyme used represents a ten-fold excess of the amount required to consume 100 μg of ACV completely.

Cephalosporin formation, due to the combined activity of IPS, epimerase and DAOCS, was measured by supplementing the penicillin-forming reaction mixture described above with α-ketoglutarate 1 mM, $MgCl_2$ 7.5 mM, and KCl 7.5 mM.

Antibiotic formation was monitored by biological assay and by high performance liquid chromatographic (HPLC) analysis of reaction mixtures.

Biological Assays

Biological assays of reaction mixtures were conducted using the agar diffusion procedure with *Micrococcus luteus* ATCC 9341 and *Escherichia coli* Ess as indicator organisms as previously described[3]. *M. luteus* is sensitive to penicillins, but is relatively insensitive to cephalosporins. *E. coli* Ess is sensitive to penicillin N and cephalosporins, but is relatively insensitive to isopenicillin N. Penicillinase was included, where indicated, at a concentration of 1,000 U/ml, to distinguish penicillins from cephalosporins.

High Performance Liquid Chromatography (HPLC)

HPLC analyses were conducted on MeOH-inactivated reaction mixtures which were centrifuged for 5 minutes at 12,000$\times g$ prior to assay. The equipment used for HPLC analysis consisted of; an M 6000 A pump, a WISP Model 710B automatic injector, an M 440 fixed wave-length detector, a Model 840 system controller, and a μBondapak column in a Z module. All equipment was from Waters Scientific Ltd. The mobile phase consisted of MeOH - KH_2PO_4 (0.05 M adjusted to pH 4.0 with H_3PO_4) in proportions which varied with the particular separation. The flow rate was 2 ml/minute. Detection of UV-absorbing materials was at 214 nm at a sensitivity of 20 mvolts full scale.

Table 1. Analysis of reaction mixtures by bioassay.

Substrate	Enzyme assay*	Zone of inhibition (mm)**		
		Micrococcus luteus	*Escherichia coli* Ess	*E. coli* Ess+ penicillinase
No substrate	IPS	11.5	0	nd
ACV	IPS	30.0	32	nd
PCV	IPS	16.5	13.5	nd
PoCV	IPS	11.5	0	nd
No substrate	IPS+DAOCS	nd	0	0
ACV	IPS+DAOCS	nd	35	33.5
PCV	IPS+DAOCS	nd	14	0
PoCV	IPS+DAOCS	nd	0	0

* IPS refers to the standard reaction mixture described for assay of penicillin formation. IPS+DAOCS refers to the reaction mixture supplemented with the necessary cofactors required to support the multi-enzyme reaction leading to cephalosporins.

** Twenty microlitre amounts of methanol-inactivated reaction mixtures were bioassayed against the indicator organisms shown above.

nd: Not determined.

Results and Discussion

Cyclization reaction mixtures containing PCV as the substrate afforded an antibiotic product which was detectable by bioassay (Table 1). Reaction mixtures containing PCV together with the cofactors required for ring expansion activity gave rise to an antibiotic product which was inactivated by penicillinase, indicating that cephalosporin was not formed. This latter result, however, confirmed that the cyclization product was a penicillin. The concentration of antibiotic in the PCV reaction mixture was estimated to be 1.7 μg benzylpenicillin equivalents/ml by comparison with authentic benzylpenicillin standards. Under the same conditions antibiotic formation from PoCV could not be detected by bioassay. The 11.5 mm zone of inhibition observed in bioassays of PoCV reaction mixtures using *M. luteus* is presumably due to endogenous antibiotic production, since it is also seen in control reaction mixtures lacking substrate.

Since the bioassay data were consistent with the formation of a penicillin from PCV, reaction mixtures were analyzed by HPLC to confirm the nature of this penicillin product. Mobile phase conditions were chosen so as to permit the detection of benzylpenicillin. Analysis of authentic benzylpenicillin by HPLC, as described in Materials and Methods, and using a mobile phase consisting of MeOH - KH_2PO_4, 40: 60 (0.05 M adjusted to pH 4.0 with H_3PO_4), gave a retention time of 7.3 minutes (data not shown). When reaction mixtures containing PCV as the substrate were analyzed under the same conditions, a large peak corresponding to unreacted PCV, was seen at 8.3 minutes and a new peak was apparent at 7.3 minutes (Fig. 1). This new peak was not observed in control reaction mixtures lacking substrate. With the assumption that this peak was due to benzylpenicillin, the concentration was estimated to be $1 \sim 2$ μg/ml by comparison of peak areas with those of authentic standards. Reaction mixtures containing ACV as the substrate did not contain any peaks in the area of benzylpenicillin. Under these HPLC conditions, isopenicillin N and ACV are not retained and run with the injection peak (retention time, 1.94 minutes).

To ensure that the low level of bioactive material formed in PCV containing reaction mixtures was not the result of endogenous production of isopenicillin N and penicillin N [(iso)penicillin N], reaction

Fig. 1. Analysis of reaction mixtures by HPLC for detection of benzylpenicillin.

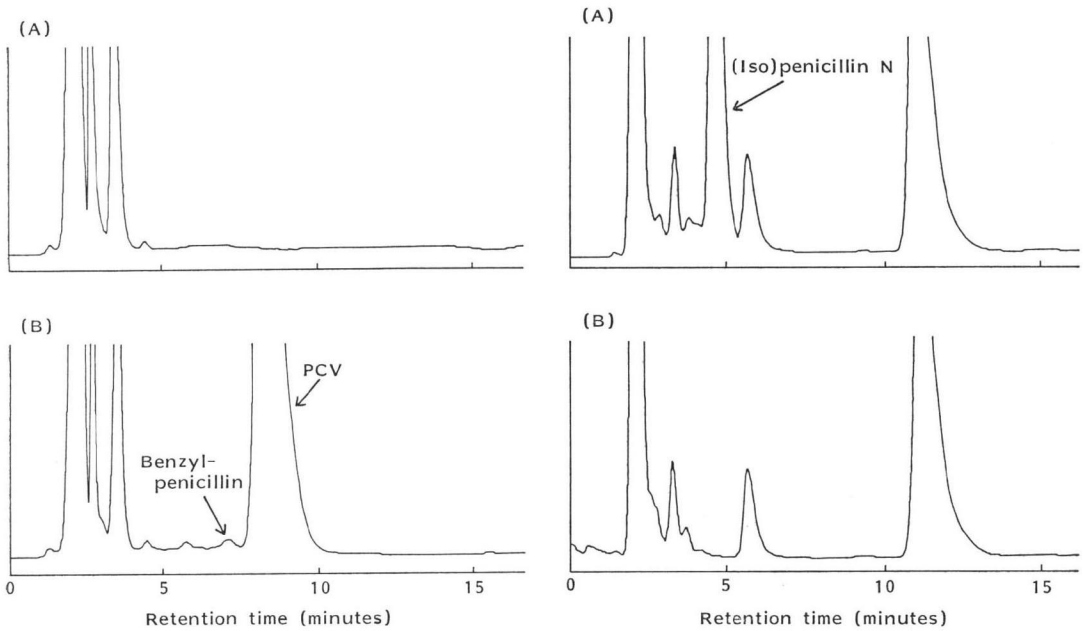
Twenty microlitre amounts of reaction mixtures were analyzed with a mobile phase of MeOH - KH_2PO_4, 40: 60 (0.05 M adjusted to pH 4.0 with H_3PO_4).

(A) Reaction mixture containing ACV as substrate. (B) Reaction mixture containing PCV as substrate.

Fig. 2. Analysis of reaction mixtures by HPLC for detection of (iso)penicillin N.

Twenty microlitre amounts of reaction mixtures were analyzed with a mobile phase of MeOH - KH_2PO_4, 5 : 95 (0.05 M adjusted to pH 4.0 with H_3PO_4).

(A) Reaction mixture containing ACV as substrate. (B) Reaction mixture containing PCV as substrate.



mixtures were reexamined under HPLC conditions that allow the detection of (iso)penicillin N. Using a mobile phase consisting of MeOH - KH_2PO_4, 5 : 95 (0.05 M adjusted to pH 4.0 with H_3PO_4), ACV-containing reaction mixtures showed a large peak due to (iso)penicillin N at a retention time of 4.7 minutes (Fig. 2). No peak due to ACV (expected retention time, 17 minutes) is seen in Fig. 2 because the amount of enzyme used is ten-fold in excess of that required to consume ACV completely. Under these analysis conditions, PCV-containing reaction mixtures gave HPLC profiles which were similar to those of control reaction mixtures lacking substrate and neither showed any evidence for the presence of (iso)penicillin N.

Based on these observations, we concur with the findings of LUENGO *et al.*[11] that benzylpenicillin can be formed by direct enzymatic cyclization of PCV, and extend their observations to include enzyme preparations from *S. clavuligerus*. Although the *Streptomyces* enzyme preparation used was prepared from a wild type strain and does not contain elevated levels of IPS, direct observation of benzylpenicillin in reaction mixtures was possible by HPLC. This was most likely a result of our use of enzyme concentrates in reaction mixtures, rather than the more dilute cell-free extracts used in the experiments of LUENGO *et al.* Under the same conditions and using the same enzyme preparations, no comparable production of penicillin V by direct cyclization of PoCV could observed. Reaction mixtures containing PCV or PoCV and supplemented with the cofactors required to support DAOCS activity also showed no evidence of cephalosporin production. This inability to detect cephalosporin formation in

PCV-containing reaction mixtures is consistent with the conclusion that the primary product is benzylpenicillin, since previous studies have shown that benzylpenicillin is not a substrate for DAOCS from *S. clavuligerus*[4].

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