DEACETYLCEPHALOSPORIN C SYNTHESIS BY CELL-FREE EXTRACTS OF *CEPHALOSPORIUM ACREMONIUM*

Sir:

In our previous paper[1], it was demonstrated that deacetylcephalosporin C (DCPC) negative mutant No. 40–20 of *Cephalosporium acremonium* mainly accumulated deacetoxycephalosporin C (DOCPC) in the culture broth. This observation suggested that DOCPC might be the precursor of DCPC in the cephalosporin C (CPC) biosynthesis. Recently, LIERSCH *et al.*[2] observed the formation of labeled CPC from DOCPC in the presence of acetyl -1-^{14}C-CoA, NADH and cell-free extract of *C. acremonium*. We have now succeeded in establishing the formation of DCPC from DOCPC with cell-free extracts of *C. acremonium* using *Pseudomonas aeruginosa* PsC^{ss} as assay organism. This mutant obtained by KITANO *et al.*[3] is specifically hypersensitive to various kinds of β-lactam antibiotics. Our results show directly that cell-free extracts of the fungus catalyze the synthesis of DCPC from DOCPC, and suggest the existence of DOCPC hydroxylase in the extracts.

A potent CPC-producing mutant of *C. acremonium*, No. 52–54, was grown in a soluble medium[4] at 28°C for 4 days on a rotary shaker (200 rpm). Cells were harvested by centrifugation at 9,000 × *g* for 20 minutes, washed twice with 0.01 M Tris-HCl buffer (pH 7.0) containing 1mM 2-mercaptoethanol, and suspended in the same buffer. The cells were disrupted with a French press at 2,000 kg/cm^2 below 4°C. The cell debris was removed by centrifugation at 20,200 × *g* for 30 minutes. The supernatant fluid was used as the cell-free extract.

The hydroxylating enzyme activity was assayed by incubating a mixture containing 5 μl of 1 M Tris-HCl buffer (pH 7.5), 20 μl of 20 mM DOCPC, 20 μl of 20 mM NADH and the cell-free extract in a total volume of 100 μl at 37°C for 10 hours. After incubation, the whole mixture was streaked on a thin-layer cellulose plate. The plate was developed with *n*-butanol - acetic acid - water (3 : 1 : 1, v/v) and air-dried. The DCPC corresponding zone was scraped off from the plate and suspended in 1.5 ml of 0.01 M potassium phosphate buffer (pH 7.0). After centrifugation at 2,000 × *g* for 15 minutes, the amount of DCPC in the supernatant was determined by the paper disc method using *P. aeruginosa* PsC^{ss} as test organism. Under the assay conditions described here, the rate of the DCPC-forming reaction was proportional to enzyme concentration (Fig. 1). The optimum pH was 7.0 to 7.5 (Fig. 2) and the reaction required NADH or NADPH as essential co-factor. DCPC formation was also dependent on the addition of DOCPC and the cell-free extract to the reaction system (Table 1).

To identify the product of the enzyme reaction, 45 ml of the reaction mixture containing 2.5 ml of 1 M Tris-HCl buffer (pH 7.5), 10 ml of 20 mM DOCPC, 10 ml of 20 mM NADH and 20 ml of the cell-free extract was incubated for 20 hours at 37°C. The mixture was filtered through a

Fig. 1. Effect of incubation time (A) and enzyme concentration (B) on the enzyme activity

The reaction was carried out under the standard assay conditions except for the enzyme concentration or incubation time. In Fig. 1A, 1 mg of the enzyme was used.

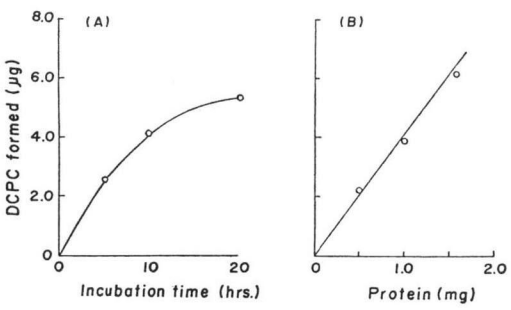

Fig. 2. Effect of pH on the enzyme activity

The enzyme activity was measured under the standard assay conditions using the following buffers (1M): potassium phosphate buffer (○) or Tris-HCl buffer (●) at various pH values.

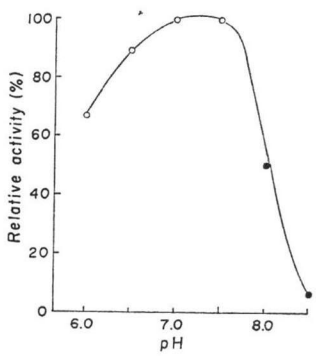

Table 1. Effect of reaction components on DCPC formation

Condition	DCPC formed (μg)
Complete system	4.1
Minus DOCPC	0.4
Minus NADH	0.9
Minus NADH, Plus NADPH	2.5
Minus enzyme	0.3
Blank (zero time)	0.3

The standard assay method was used except for the omission of the indicated substances.

Table 2. Thin-layer chromatography of the reaction product

Solvent system	Rf value	
	Reaction product	Authentic DCPC
(A)	0.16	0.16
(B)	0.22	0.22
(C)	0.03	0.03
(D)	0.25	0.25
(E)	0.13	0.13
(F)	0.19	0.19

The following solvent systems were used: (A) *n*-butanol - acetic acid - water (3: 1: 1, v/v); (B) *n*-propanol - water (7: 3, v/v); (C) acetonitrile - water (8: 2, v/v); (D) isopropanol - water - pyridine (65: 30: 5, v/v); (E) methanol - *n*-propanol - water (6: 2: 1, v/v); (F) ethyl acetate - acetone - water (2: 4: 2, v/v).

Millipore filter and the filtrate was concentrated to 3 ml *in vacuo*. The concentrate was passed through a Sephadex G-15 column (3.0 × 137 cm) and the above fraction was lyophilized. The lyophilized powder was dissolved in a small volume of distilled water. The solution was streaked on thin-layer cellulose plates (20 × 20 cm, layer thickness 0.1 mm, E. Merck) and the plates were developed with the solvent system described above. The product zone was scraped off, eluted with distilled water and lyophilized. For further purification, the product was subjected to paper electrophoresis (95 V/cm, 60 minutes) in 10% acetic acid (pH 2.2) on a sheet of Whatman No. 1 filter paper (12 × 42 cm). The product zone was cut off from the paper, and the antibiotic was eluted with distilled water. The eluate was concentrated to 2 ml *in vacuo*. The concentrate was applied to the Sephadex G-15 column, and the product fraction was lyophilized to give a colorless powder. The purified product was examined by thin-layer chromatography. Rf values of the product were identical with those of authentic DCPC (Table 2). The UV spectra of the product and DCPC in 0.01 M potassium phosphate buffer (pH 7.0) were identical and the absorption band with λ_{max} at 261 nm disappeared rapidly upon addition of cephalosporinase, which was partially purified from *Enterobacter cloacae* IFO 12937, mutant No. 3.[5] The IR spectrum of the product was in good agreement with that of DCPC. From these data, the product was identified as DCPC.

Table 3. Deacetoxycephalosporin C hydroxylase activity in the cell-free extracts of some strains of *C. acremonium*

Strain	DCPC formed (μg)
CPC-producing strain No. 52–54	4.0
DCPC-producing mutant No. 40	3.2
DOCPC-producing mutant No. 40–20	0.4

The cell-free extracts were prepared as described in the text. Enzyme activity was determined under the standard assay conditions.

Cell-free extracts were prepared from two other mutants of *C. acremonium* and assayed for the enzyme activity. As shown in Table 3, the hydroxylase activity was observed in the CPC- or DCPC-producing strains[5], but not in the DOCPC-producing mutant. These findings suggest that the accumulation of DOCPC by the DCPC negative mutant No. 40–20 is due to the lack of DOCPC hydroxylase. Therefore, the terminal biosynthesis of CPC in *C. acremonium* is considered to proceed as follows: DOCPC→DCPC→CPC.

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