A study of the biosynthesis of the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine in a β -lactam-negative mutant of *Cephalosporium acremonium*

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A cell-free extract of *Cephalosporium acremonium* (Takeda N-2) was obtained that synthesized the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine and also the dipeptide δ -(L- α -aminoadipyl)-L-cysteine from the corresponding L-amino acids.

It has been established that the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (L-Aad-L-Cys-D-Val) is the precursor of isopenicillin N in cell-free extracts of *Cephalosporium acremonium* (Konomi *et al.*, 1979). The biosynthesis of this tripeptide is, however, less well understood. Three amino acids, i.e. L- α -aminoadipic acid, L-cysteine and L-valine, have been shown to be incorporated into the tripeptide by intact cells (Aberhardt, 1977), and the reaction between δ -(L- α -aminoadipyl)-L-cysteine (L-Aad-L-Cys) and L-valine in a broken-cell system has been studied (Fawcett & Abraham, 1975).

The present work shows that a soluble activity capable of the synthesis of both L-Aad-L-Cys-D-Val and L-Aad-L-Cys from the above amino acids is present in the supernatant fraction from broken cells of the β -lactam-negative mutant N-2 derived from *C. acremonium* (A.T.C.C. 14553) (Shirafuji *et al.*, 1979).

Experimental

Peptides and amino acids

The synthetic peptides L-Aad-L-Cys-D-Val, L-Aad-L-Cys and L-Cys-D-Val were prepared by reported methods (Baldwin *et al.*, 1981). DL-Amino-[6- 14 C]adipic acid was obtained from Fluorochem, Glossop, Derbyshire, U.K. L-[U- 14 C]Valine and L-[3,3'- 3 H]cystine hydrochloride were purchased from Amersham International, Amersham, Bucks., U.K.

Cephalosporium acremonium Takeda Mutant N-2

Growth conditions. The organism was cultured on slopes of modified Le Page–Campbell agar [1.0 g of Oxoid yeast extract, 1.0 g of D-glucose, 0.5 g of

NaCl, 10.0 g of CaCl₂, 20.0 g of Oxoid no. 1 agar and distilled water to 1 litre, pH 6.8 (before sterilization)], for 10 days before transfer to refrigeration at 4°C.

A mycelial suspension in sterile distilled water was prepared from well-grown slopes, and 5 ml of this was used to inoculate each flask of seed medium (80 ml in 500 ml Erlenmeyer flasks).

Seed medium. The seed medium was made up of 30 g of sucrose, 15.0 g of Oxoid Lab Lemco, 5.0 g of corn-steep liquor, 1.5 g of CaCO₃ and distilled water to 1 litre, pH 6.8 (before sterilization).

Seed cultures were incubated at 25°C for 5 days at 200 rev./min on a Gyrotory shaker with an eccentric throw of 5 cm. Then 10 ml of this material was taken to inoculate each flask of growth medium.

Growth medium. Solution 1 was made up of 27.0 g of D-glucose and distilled water to 100 ml. Solution 2 was made up of 135 ml of salt solution A, 7.5 ml of salt solution B, 5.0 g of DL-methionine, 36.0 g of sucrose and distilled water to 900 ml, pH adjusted to 7.4 before the addition of 7.5 g of L-asparagine. [Salt solution A was made up of 115.0 g of KH₂PO₄, 156.0 g of K₂HPO₄, 5.6 g of Na₂SO₄, 1.3 g of MgSO₄·7H₂O, 220 mg of ZnSO₄·7H₂O, 220 mg of MnSO₄·4H₂O, 55 mg of CuSO₄·5H₂O, 425 mg of CaCl₂ and distilled water to 1 litre. Salt solution B was made up of 20.0 g of Fe(NH₄)₂(SO₄)₂ and distilled water to 1 litre.]

L-Aad-L-Cys-D-Val

Abbreviation used: Aad-, δ -(α -aminoadipyl)-.

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Solution 1 (10ml) and solution 2 (90ml) were sterilized separately and combined immediately before inoculation, and 0.1 ml of sterile oleic acid was added to each flask at this time.

After inoculation, the flasks were incubated at 25°C at 200 rev./min on a Gyrotory shaker with an eccentric throw of 5 cm.

The growth of the culture was monitored by measuring the absorption of the culture broth at 550 nm with a Pye/Unicam model SP.6-550 spectrophotometer.

After 52 h growth the flasks were harvested, and the mycelium was separated by filtration and washed with distilled water (three times).

Preparation of cell-free extracts

The fresh or frozen mycelium (200 g) was resuspended in 50 mM-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer, pH 7.2, containing Triton X-100 (30 g net weight/90 ml of buffer), placed in a Dynamill (Willy A. Bachofen, Basel, Switzerland) with 100 ml of 0.25–0.35 mm glass beads, and then stirred at 4500 rev./min for 100 s in a 0.15-litre glass head. The disrupted homogenate was centrifuged at 12000 g for 30 min at 0°C (Beckman J2-21 centrifuge). The crude supernatant resulting from the centrifugation was subjected to protamine sulphate treatment. Protamine sulphate (from salmon, grade II; Sigma Chemical Co.) (2%, w/v) in 50 mM-Mops buffer, pH 7.2, was added dropwise (until $\frac{1}{10}$ of total volume) to the crude supernatant with stirring. It was then centrifuged at 1200 g for 30 min, and the pellet was discarded.

The supernatant obtained after nucleotide precipitation was subjected to fractionation with $(\text{NH}_4)_2\text{SO}_4$, the salt being added with stirring over 15 min to 50% saturation (29.1 g/100 ml at 0°C). Stirring was maintained for a further period of 15 min, and the suspension was centrifuged at 12000 g for 30 min. The supernatant was subjected to a second fractionation with $(\text{NH}_4)_2\text{SO}_4$ to 85% of saturation (a further 23 g/100 ml) as described above, and centrifuged as before. The precipitate, resuspended in a minimal volume of 50 mM-imidazole/HCl buffer, pH 7.2, was applied to a column (1.5 cm $\times$ 30 cm) of Sephadex G-150, equilibrated with imidazole/HCl buffer as above. Elution was performed with this buffer (150 ml/h; 10 ml fractions) and monitored at 280 nm. Fractions showing absorption were pooled and freeze-dried.

Assay of L-Aad-L-Cys-D-Val-synthesis activity

L-Aad-L-Cys-D-Val synthesis from the three amino acids was measured by inclusion of radioactive DL- α -amino adipic acid (or L-valine or L-cystine) in mixtures containing L- α -amino adipic acid (1 mM), L-cystine (1 mM) and L-valine (1 mM). In each case

one of the three amino acids was labelled: L-[^3H]cystine (250 $\mu\text{Ci/ml}$, 3.2 Ci/mmol), L-[U- ^{14}C]valine (50 $\mu\text{Ci/ml}$, 275 mCi/mmol) or DL- α -amino-[6- ^{14}C]adipic acid (50 $\mu\text{Ci/ml}$; 46.9 mCi/mmol). All reaction mixtures were supplemented with ATP (0.8 mM), MgCl_2 (10 mM), ascorbate (1 mM), dithiothreitol (2 mM) (as final concentrations) and the cell-free extract.

In all cases, before the assay with the labelled precursor, the synthesis of L-Aad-L-Cys-D-Val from its amino acid precursors was assayed by its conversion into isopenicillin N by a purified extract of isopenicillin N synthetase from *C. acremonium* C-91 (Brownlee *et al.*, 1948) and detection via bioassay with *Bacillus subtilis* (A.T.C.C. 6633) and *Sarcina lutea* (A.T.C.C. 9341). The zones of inhibition were determined from incubations with and without the addition of precursors of L-Aad-L-Cys-D-Val to the reaction mixtures. When L-Aad-L-Cys-D-Val-synthesis activity was seen to be present, the incorporation of labelled precursors into the tripeptide was carried out.

Reaction mixtures were incubated at 27°C at 250 rev./min for different times (as indicated) and then quenched by addition of trichloroacetic acid (final concn. approx. 7.5%, w/v).

Isolation of L-Aad-L-Cys-D-Val peptide as sulphonic acid

The quenched reaction mixture was centrifuged at 3000 g for 5 min, the pellet was washed with 1 ml of 5% (w/v) trichloroacetic acid (5%, 1 ml), and it was centrifuged again. The combined supernatants were adjusted to pH 4.5 with NaOH solution, and applied to a column (1 cm $\times$ 5 cm) of Dowex 50 X8 (H^+ form) (a fresh column being used for each incubation) and washed with distilled water (10 vol.). Amino acids and peptides were eluted with aqueous 1 M-pyridine (10 vol.), freeze-dried and subjected to oxidation with performic acid [9 vol. of 98% formic acid plus 1 vol. of 30% (v/v) H_2O_2]. To each sample, 0.25 ml of 98% formic acid was added, the solution was cooled in ice and then 0.25 ml of the performic acid solution (as above) was added. Oxidation proceeded for 6 h at 0°C, after which the samples were diluted with 5 ml of distilled water and freeze-dried. They were resuspended in a minimal volume of water and applied to Dowex 50 X8 ion-exchange resin (H^+ form) again. The sulphonic acids present were not retained, and were recovered by washing with distilled water (10 vol.). The eluate was freeze-dried and resuspended in a minimal volume of water (0.3 ml) and stored at 0°C.

The eluate from the final column was in each case subjected to paper electrophoresis (Whatman no. 1) at pH 1.8 for 2½ h at 4 kV. After being dried, the paper was subjected to chromatography in the second dimension with butanol/acetic acid/water

(4:1:4, by vol.; upper phase) for 40 h. After staining with ninhydrin, the radioactivities of the peptide spots were counted in a scintillation counter (LKB Produkter). The positions of the spots were checked with authentic L-Aad-L-Cys-D-Val sulphonic acid derivative.

Hydrolysis of the peptides

Parts of the samples were eluted after electrophoresis, freeze-dried and hydrolysed with 6 M-HCl in stoppered test tubes under N₂ for 20 h at 110°C. The samples were then diluted and freeze-dried. Amino acid analysis was performed by electrophoresis at pH 3.5 for 1.5 h at 4 kV. The paper was again stained with ninhydrin and the radioactivity was measured. Counting of radioactivity was performed with an LKB 1215 Rackbeta liquid-scintillation counter with Lumagel scintillation fluid. The efficiency of the counter was calculated by using a ³H standard (n-[1,2-³H]hexadecane) or a ¹⁴C standard (n-[1-¹⁴C]hexadecane). The efficiencies were calculated to be 10% for the counting of ³H and 12% for the counting of ¹⁴C radioactivity.

Results

Incubations performed are outlined in Table 1.

The incubations were maintained at 27°C at 250 rev./min for the times indicated, after which they were quenched by addition of 30% trichloroacetic acid to a final concentration of 7.5% and centrifuged, and the pellets were washed with 1 ml of 5% trichloroacetic acid and centrifuged again. The combined supernatants were adjusted to pH 4.5 with NaOH solution. The samples were purified by ion-exchange chromatography (see the Experimental section), oxidized and purified to give the results shown in Table 2.

Notably radioactivity was found, not only in the L-Aad-L-Cys-D-Val tripeptide, but also (where labelled aminoadipic acid or cystine was fed) in a zone that had identical properties with the L-Aad-L-Cys dipeptide. The finding of labelled L-Aad-L-Cys in these incubations is hardly surprising, since it has been suggested (Fawcett & Abraham, 1975) that this dipeptide is an intermediate between the separate amino acids and the tripeptide. In no case was radioactive L-Cys-D-Val found when either labelled cystine or labelled valine was one of the amino acids fed to the incubation mixture. The nature of the dipeptide and tripeptide formed was corroborated by hydrolysis to their constituent amino acids, and comparison with authentic samples as outlined above.

Table 1. *Conditions for the incubations of radioactive amino acids*

Incubation mixtures A, C and E were quenched after 30 min, and incubation mixtures B, D and F after 60 min. The final concentrations of the compounds used are ATP (0.8 mM), Fe²⁺ (0.1 mM), ascorbate (1 mM), dithiothreitol (2 mM) and amino acids (each 1 mM).

Incubation	Composition of incubation mixture					
	A	B	C	D	E	F
Takeda N-2 extract	1000 μ l	1000 μ l	1000 μ l	1000 μ l	1000 μ l	1000 μ l
Cysteine	200 μ l	200 μ l	50 μ l*	50 μ l*	200 μ l	200 μ l
Aminoadipic acid	50 μ l*	50 μ l*	200 μ l	200 μ l	200 μ l	200 μ l
Valine	200 μ l	200 μ l	200 μ l	200 μ l	25 μ l*	25 μ l*
ATP/Mg ²⁺	100 μ l each	100 μ l each	100 μ l each	100 μ l each	100 μ l each	100 μ l each
Fe ²⁺ /ascorbate	100 μ l each	100 μ l each	100 μ l each	100 μ l each	100 μ l each	100 μ l each

* Radioactive precursor: L-[U-¹⁴C]valine (50 μ Ci/ml, 275 mCi/mmol), L-[3,3'-³H]cystine (250 μ Ci/ml, 3.2 Ci/mmol) or DL- α -amino[6-¹⁴C]adipic acid (50 μ Ci/ml, 46.9 mCi/mmol).

Table 2. *Incorporation of radioactivity into products*

For experimental details see the text. In all incorporations quoted, radioactivities were at least 10 times the background value.

Expt.	Labelled amino acid	Radioactivity found (as % of radioactivity added)	
		In L-Aad-L-Cys-D-Val	In L-Aad-L-Cys
A	DL-Amino[6- ¹⁴ C]adipic acid (2.5 μ Ci)	0.40	0.129
B	DL-Amino[6- ¹⁴ C]adipic acid (2.5 μ Ci)	0.355	0.145
C	L-[³ H]Cystine (12.5 μ Ci)	0.102	0.034
D	L-[³ H]Cystine (12.5 μ Ci)	0.141	0.040
E	L-[U- ¹⁴ C]Valine (1.25 μ Ci)	0.158	—
F	L-[U- ¹⁴ C]Valine (1.25 μ Ci)	0.158	—

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