The isolation and purification of the elvapeptins

A family of peptide inhibitors of mitochondrial ATPase activity

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Elvapeptin *Efrapeptin* (Tolypocladium inflatum) *Mitochondrial ATPase inhibition* *(oxheart mitochondria)*

1. INTRODUCTION

The mould, *Tolypocladium inflatum* has been shown to produce the efrapeptins, a family of closely related hydrophobic peptide antibiotics, which are potent inhibitors of the ATPase activity of mitochondria, chloroplasts and bacteria [1,2]. It has been reported briefly that under conditions of limited aeration cultures of *T. inflatum* produce a second antibiotic activity distinct from the efrapeptins [3]. We have given the name elvapeptin to this antibiotic.

In this paper we describe the conditions required to produce the elvapeptins together with details of their extraction and purification. The elvapeptins have been shown to be a family of closely related peptides which are potent inhibitors of mitochondrial ATPase activity.

2. MATERIALS AND METHODS

The fermentation medium used routinely for antibiotic production contained glucose, 25 g/l; maize starch 10 g/l; proteose peptone 10 g/l; tryptone, 4 g/l and $MgSO_4 \cdot 7\ H_2O$, 0.5 g/l. Innocula were prepared by dislodging pieces of mycelium from an oatmeal agar slope of *T. inflatum* (IMI 202309), adding them to the fermentation medium (20 ml) and shaking in an orbital incubator at 150 rev./min for 5 days at 25°C. For large scale production a 2.5% innoculum was added to fermentation medium and shaken for a further 9–12 days at 25°C. Typically, 400 ml aliquots of medium were incubated in 21 conical flasks.

High performance liquid chromatography was carried out with a Hewlett–Packard 1084B liquid chromatograph.

Peptides were acetylated in acetic anhydride/methanol (4:1, v/v) for 3 h at 70°C. The progress of the reaction was followed by thin layer chromatographic analysis of the reaction mixture on silica gel plates developed with butan-1-ol/acetic acid/water (3:1:1, by vol.). The material was detected using ninhydrin either directly or after acid hydrolysis of the peptide on the TLC plate.

Amino acid analysis was carried out using an LKB 4400 Analyser. Gas chromatography was performed using an SP 2100 capillary column installed in a Hewlett Packard 5840A GC fitted with a flame ionisation detector. The preparation of *N*-heptafluorobutyryl amino acid butyl esters was based on the procedure described by Felker and Bandurski [4].

The soluble ATPase from ox-heart mitochondria was prepared by the method of Beechey et al. [5].

RESULTS AND DISCUSSION

3.1. *Extraction of elvapeptin*

The growth medium was separated from the mycelium by centrifugation. The mycelium was then washed twice with distilled water. The growth medium and the washings were pooled and adjusted to pH 8.5 with 1 M NaOH. The pooled

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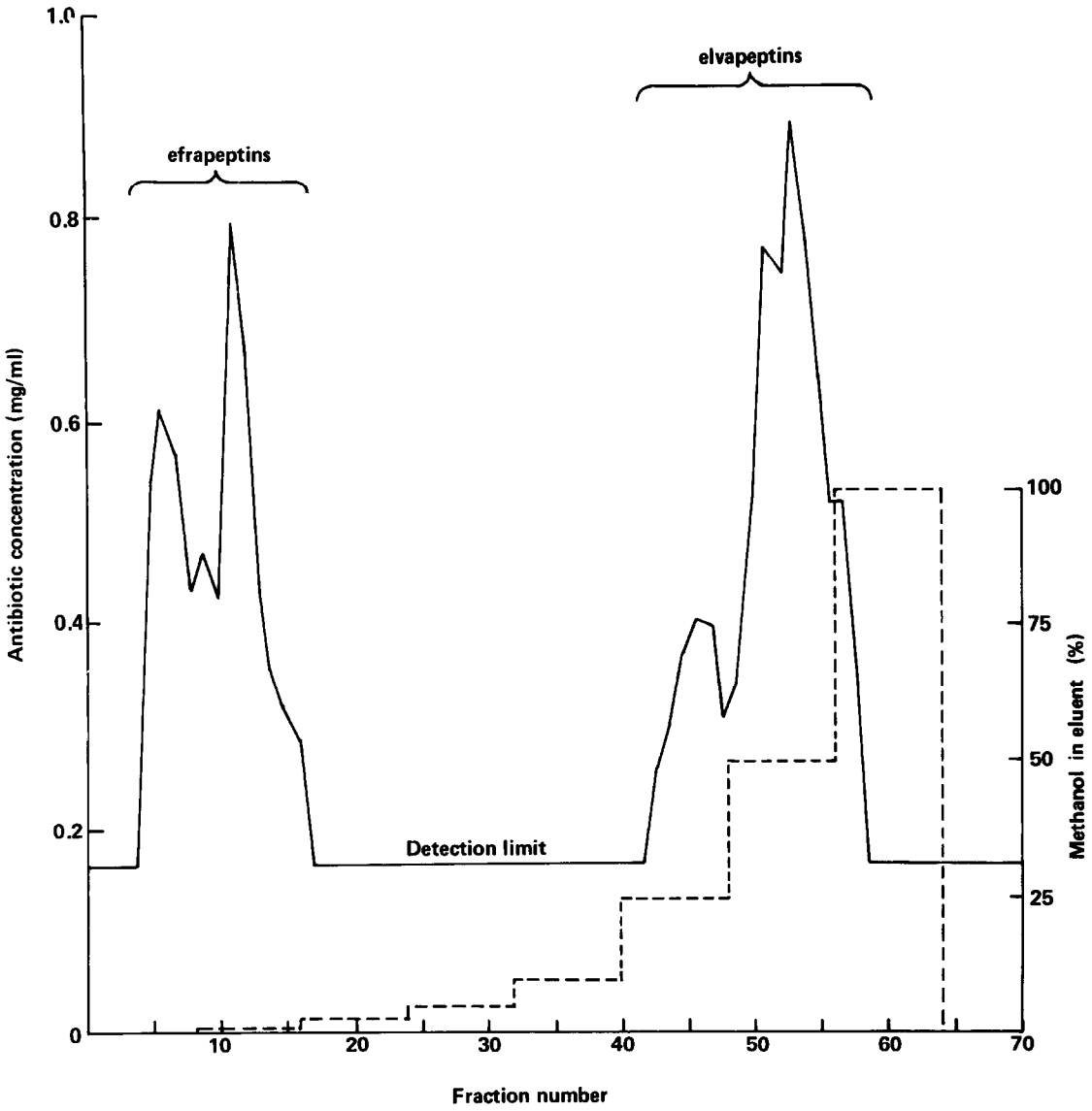

Fig.1. Isolation of the elvapeptins from an extract of *T. inflatum*. Crude antibiotic extract from five 400 ml cultures of *T. inflatum* grown in 2 litre unbaffled flasks as described in the methods section was applied to a column of basic alumina (12.5 × 1 cm) packed in chloroform. It was eluted at a flow rate of 2 ml/min with increasing concentrations of methanol in chloroform. The antibiotic activity of 50 μl samples from each 10 ml fraction was determined using the microbiological assay.

aqueous phase was extracted successively with three half-volumes of chloroform. The pooled chloroform extracts were then evaporated under reduced pressure to yield a crude antibiotic preparation. The combined yields of crude elvapeptin and efrapeptin produced by *T. inflatum* was about 100 mg/l of culture medium.

3.2. *Purification and properties of the elvapeptins*

The crude extract of the growth medium was

dissolved in chloroform and applied to a column of basic alumina. The adsorbed material was eluted with a step gradient of increasing concentrations of methanol in chloroform, see fig.1. Fractions of the eluate were applied to filter paper discs for microbiological assay of antibiotic activity, using *Bacillus subtilis* as the test organism [7].

It can be seen from the results presented in fig.1 that there are two regions of antibiotic activity eluted from the column. The materials eluted with 0–1% methanol in chloroform are the efrapeptins. Concentrations of methanol in chloroform greater than 25% elute a more polar antibiotic activity, the elvapeptins. They constitute about 50% of the total antibiotic activity. Other experiments have shown that if the growth medium is well aerated the proportion of the elvapeptins produced decreases significantly.

Attempts to purify the elvapeptin activity further by reversed phase HPLC using Lichrosorb

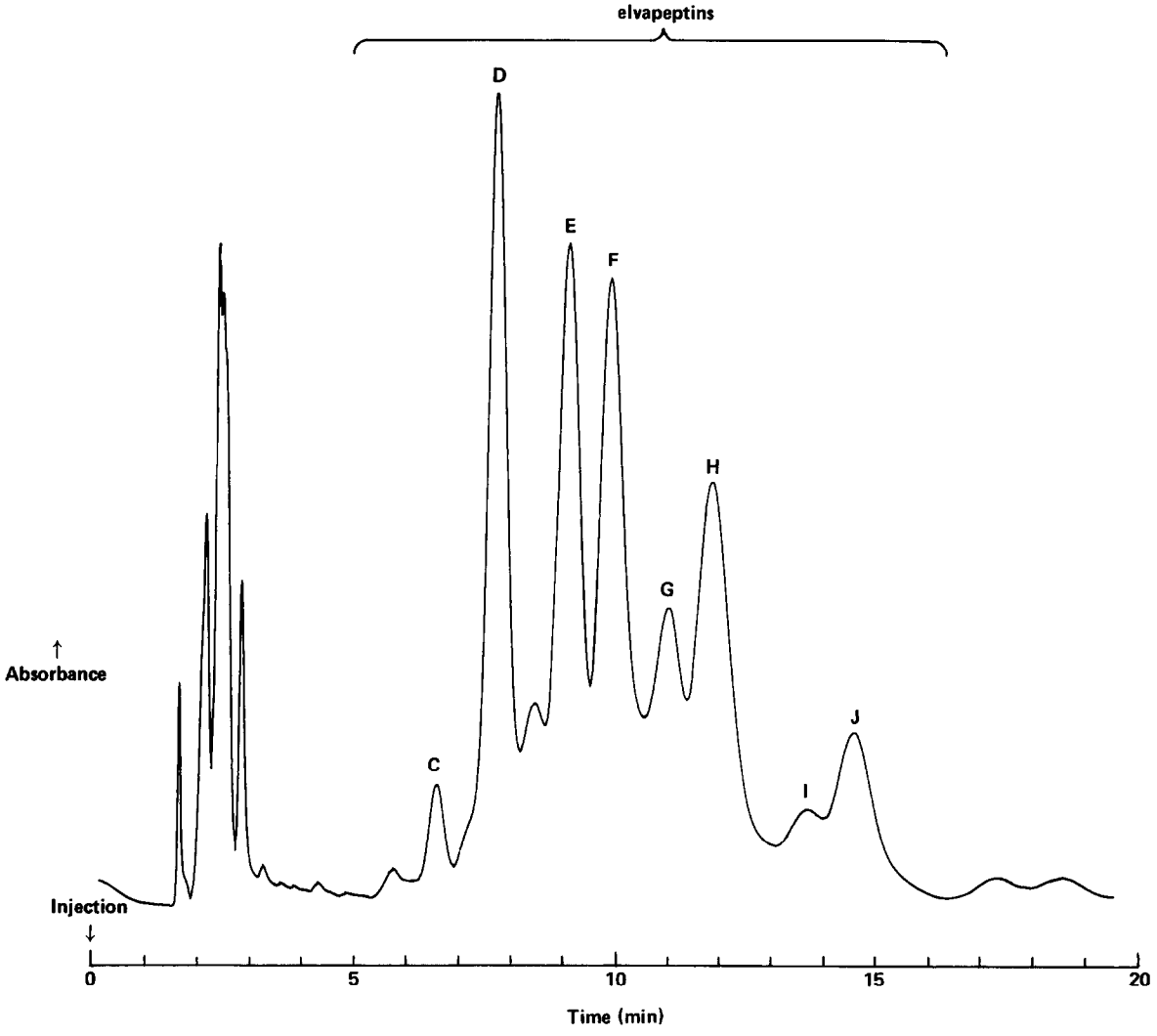

Fig.2. Resolution of acetylated elvapeptins by HPLC. A 10 μl sample of acetylated elvapeptins dissolved in methanol was injected onto a 25 × 0.45 cm column packed with Lichrosorb RP-18 (particle size 5 μm) and eluted with acetonitrile/1% aqueous phosphoric acid (11:9, v/v) at 70°C. The flow rate was 1.6 ml/min. The eluate was monitored by UV detection at 210 nm. The letters indicate the acetylated elvapeptins which have been shown to have biological activity.

RP-2, RP-8 and RP-18 columns were unsuccessful.

Analysis of the elvapeptins by high voltage electrophoresis on paper was performed at pH 2.0, 3.5 and 6.5. They could be detected with ninhydrin. The elvapeptins were positively charged over this pH range. It was found subsequently that it was possible to acetylate the material which had been eluted from the basic alumina column. The acetylated elvapeptins were resolved into several components by reversed phase HPLC on a Lichrosorb RP-18 column eluted with acetonitrile/1% aqueous phosphoric acid (11:9, v/v). A typical separation is shown in fig.2. It can be seen that the material resolves into at least eight components. Four components of the acetylated crude elvapeptin (C,D,E and F) were collected separately. These have been shown to inhibit the activity of soluble mitochondrial ATPase. The major component, acetylated elvapeptin D, was analysed on Lichrosorb RP-18 to demonstrate its homogeneity, by this assay it was greater than 95% pure. This material was used for further analysis.

The amino acid composition was examined by ion exchange and gas chromatographic analysis of *N*-heptafluorobutyryl amino acid butyl esters following total acid hydrolysis (6 M HCl, 105°C for 48 h). The amino acids, α-aminoisobutyric acid, isovaline, glycine, leucine, pipecolic acid, alanine and β-alanine were shown to be present in the acid hydrolysate. These are the same amino acid residues that are found in efrapeptin D [8]. In addition, quantitative analysis of this data shows these amino acids are to be found in the same proportions as in efrapeptin D. However, elvapeptin D is clearly different from efrapeptin D. The latter peptide is uncharged at all pH values and it has an M_r of 1618 [8]. In contrast, the M_r of elvapeptin D appears to be 1639. Fast atom bombardment mass spectral analysis suggests both peptides have the same N-terminal sequences and that the major difference between these peptides lies in the nature of their C-terminal residues, the structures of which have yet to be elucidated.

Efrapeptin D is a potent inhibitor of the soluble mitochondrial ATPase activity [3]. Since acetylated elvapeptin D contains similar amino acid constituents we have investigated its ability to inhibit mitochondrial ATPase activity. It can be seen from fig.3 that acetylated elvapeptin D does inhibit the activity of soluble mitochondrial ATPase, albeit, at a potency which is somewhat less than that of efrapeptin D.

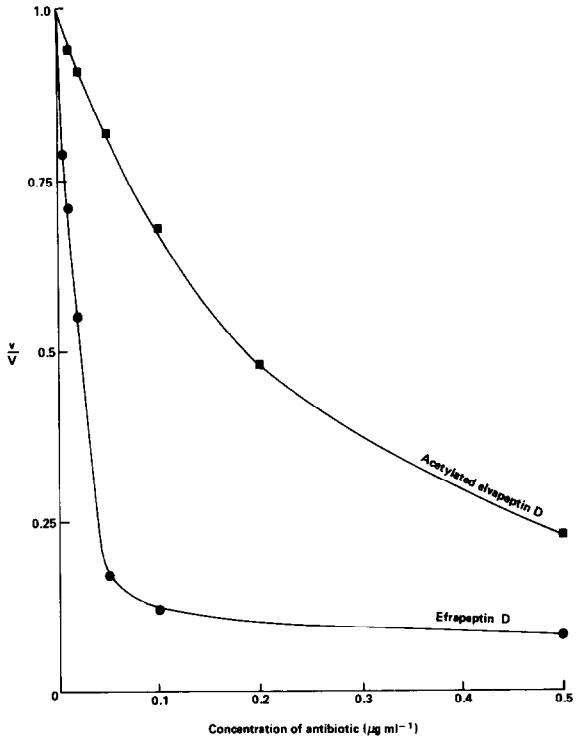

Fig.3. Inhibition of soluble mitochondrial ATPase activity by acetylated elvapeptin D. The ability of antibiotic material to inhibit the soluble ATPase from ox-heart mitochondria was measured by incubating a methanolic solution of the antibiotic with the ATPase at 30°C for 50 min, to allow equilibrium binding. The ATPase activity was measured at a constant ATP concentration using an ATP regenerating system [6].

The site of action of the acetylated elvapeptin D appears to be very similar to that of efrapeptin D.

(1) If either of the peptides and the ATPase are incubated together the ATPase activity, as measured by the coupled enzyme procedure and started by the addition of MgATP, is inhibited initially but becomes reactivated. This is followed by a return of inhibition caused by the rebinding of the peptides. Preincubation of the ATPase with either efrapeptin D or acetylated elvapeptin D together with ATP + EDTA prevents the reactivation of the inhibited enzyme on the addition of MgATP [9].

(2) An ATPase preparation that has been totally inhibited by preincubation with 4-chloro-7-nitrobenzofurazan (Nbf-Cl) and further treated with efrapeptin D or acetylated elvapeptin D can be restored to full activity by the addition of dithiothreitol. If Nbf-Cl is not present the ATPase activity remains inhibited.

The elvapeptins are a useful addition to the spectrum of inhibitors of mitochondrial ATPase activity. The presence of a functional group which can be derivatised has enabled us to prepare: (a) radiolabelled photoaffinity derivatives which we are currently using to study the site of action; (b) elvapeptins covalently bound to insoluble supports, which are useful tools for the separation of ATPase from complex mixtures.

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REFERENCES

[1] Susa, J.B. and Lardy, H.A. (1975) Mol. Pharmacol. 11, 166–173.
[2] Linnett, P.E. and Beechey, R.B. (1970) Methods Enzymol. 55, 472–518.
[3] Jackson, C.G., Linnett, P.E., Beechey, R.B. and Henderson, P.J.F. (1979) Biochem. Soc. Trans. 7, 224–226.
[4] Felker, P. and Bandurski, R.S. (1975) Anal. Biochem. 67, 245–265.
[5] Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533–537.
[6] Linnett, P.E., Mitchell, A.D., Osselton, D.P., Mulheirn, L.J. and Beechey, R.B. (1978) Biochem. J. 170, 503–510.
[7] Cooper, K.E. (1963) Anal. Microbiol. 1, 1–87.
[8] Bullough, D.A., Jackson, C.G., Henderson, P.J.F., Cottee, F.H., Beechey, R.B. and Linnett, P.E. (1982) Biochem. Int. 4, 543–549.
[9] Jackson, C.G., Beechey, R.B., Henderson, P.J.F. and Linnett, P.E. (1980) in: First European Bioenergetics Conference, Short Reports, pp. 213–214. Patron Editore, Bologna.