BIOSYNTHESIS OF ALAMETHICIN

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1. Introduction

Alamethicin is a cyclic octadecapeptide containing 2-methylalanine (aminoisobutyric acid, Aib), an unusual component. All other constituent amino acids are in the L-form. It has the structure [1]:

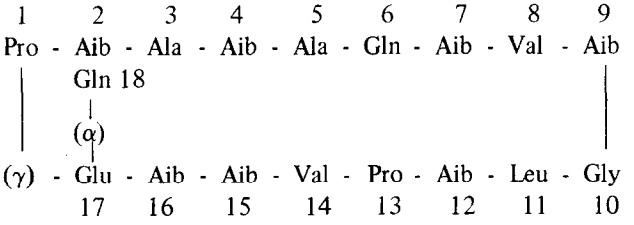

Fig.1. Structure of alamethicin

Recent spectrometric studies suggest a linear structure for this molecule: The amino terminus at Pro-1 is protected by an acetyl group and the α-carboxyl at Glu-17 by a phenylalaninol molecule [2,3].

Alamethicin is synthesized at the end of the exponential growth phase of *Trichoderma viride*-cultures and was first detected in the nutrient broth of submerged cultures in 1967 [4]. Reusser showed that cycloheximid failed to inhibit the in vivo formation of alamethicin, and took this as a proof of non-ribosomal biosynthesis [5]. We have recently shown that the in vitro synthesis of alamethicin is neither influenced by RNase nor puromycin and that the constituent amino acids are activated in the form of thioesters on the synthesizing enzyme [6].

Fractionation of alamethicin synthetase from extracts of *Trichoderma viride* by gel filtration on Sephadex G200 was also described [6]. This present paper describes the further purification and separation of the synthetase into two fractions by chromatography on hydroxyapatite. The amino acid dependent ATP–$^{32}PP_i$ exchange reaction and the actual synthesis of alamethicin were used as a measure of enzyme activity.

2. Materials and methods

2.1. *Enzyme preparation and purification*

Cultivation of the organism, preparation of cell-free extracts and partial purification of the alamethicin synthesizing enzyme system by gel filtration on Sephadex G200 were carried out as described [6]. The active fractions of the gel filtration were pooled and applied to a hydroxyapatite column (1.5 × 15 cm, Hypatite C, Clarkson Chemical Comp., Williamsport). The column was washed with 100 ml of buffer A, and then eluted

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with 400 ml of a linear gradient from 10 mM to 200 mM of phosphate buffer, pH 7.0, 5 ml fractions being collected. Amino acid dependent ATP–$^{32}PP_i$ exchanges were tested as described by Gevers et al. [7]. Radioactivity was counted in toluene scintillation liquid containing 2.8 g/l PPO and 0.9 g/l POPOP in a Nuclear Chicago Mark II scintillation counter. The buffer (A) used was 10 mM phosphate, pH 7.0, containing 10 mM Mg^{++}, 0.5 mM EDTA, 5 mM 2-mercaptoethanol and 10% sucrose.

2.2. *Aminoacyl-tRNA-synthetase identification*

The tests were carried out according to Vold [8]. The soluble tRNA from *E. coli* (Boehringer, Mannheim) was used. The incubation mixture contained 100 μl of the enzyme solution and 100 μl of a solution containing 300 μg tRNA plus 50 μg bovine serum albumin, 0.2 μmol ATP, 1 μmol Mg^{++}, and 0.2 μCi of a ^{14}C-labeled amino acid in buffer A. In the control experiments the tRNA was omitted. Incubation was carried out for 30 min at 37°C. The reaction was stopped by the addition of 2 ml of cold 5% trichloroacetic acid. After 30 min in the cold, the precipitate was collected on a glass fiber filter and washed with 150 ml of cold 5% TCA, and then 30 ml of ethanol-ether (1:1). After drying at 50°C, radioactivity was counted under toluene scintillation liquid in the Nuclear Chicago liquid scintillation counter.

2.3. *In vitro synthesis of alamethicin*

The 250 μl of incubation mixture contained 200 μl of enzyme solution and 50 μl of a solution of buffer A containing 2.5 μmol ATP, 2.5 μmol Mg^{++}. 0.2 μmol of each of seven of the alamethicin constituent amino acids and 0.2 μCi of the eighth ^{14}C-labelled amino acid. Incubation was carried out for 30 min at 37°C. The mixture was lyophilized and the residue extracted with 2 ml *n*-butanol–chloroform (4:1). The solvent was removed by evaporation and the residue dissolved in 200 μl of ethanol, then applied to silica gel thin-layer plates (Kieselgel F 1500, Schleicher & Schüll, Dassel), and chromatographed.

Solvent 1: *n*-butanol–acetic acid–water (4:1:1); solvent 2: ethylacetate–pyridine–acetic acid–water (60:20:6:11). The plate was first developed with solvent 1 and then, after drying, with solvent 2, into the same direction. Radioactivity was located with a Berthold scanner. Radioactive bands in the range of the R_f value of authentic alamethicin were scraped off and extracted with ethanol. After removal of the solvent, the residue was hydrolyzed in 250 μl of 6 N HCl (110°C, 16 h). The HCl was evaporated, and the residue was dissolved in 100 μl of aqueous ethanol, chromatographed and autoradiographed as described above.

3. Results and discussion

Fig.2 shows the separation of alamethicin synthetase by gel filtration on Sephadex G200. The ATP–$^{32}PP_i$ exchange stimulated by Aib was used for the assay. The effect of each of the eight constituent amino acids of alamethicin as well as of some other amino acids on the ATP–$^{32}PP_i$ exchange is shown in table 1. For comparison, the aminoacyl-tRNA activities with the various amino acids are shown in table 2. It is clear that some,

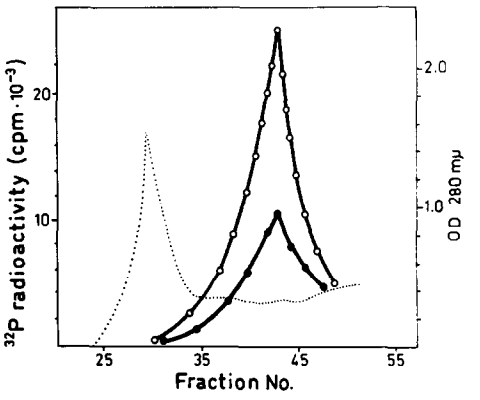

Fig.2. Separation of the alamethicin synthesizing system by gel filtration on Sephadex G 200 and identification by ATP–$^{32}PP_i$ exchange. The 55% $(NH)_2SO_4$ precipitate obtained from the extract of 20 g wet mycelium was taken up in 5 ml buffer A and put on a column (2.6 × 90 cm) equilibrated with the same buffer. Elution was carried out with buffer A. Four ml-fractions were collected. (. . .) Absorbance at 280 nm; (o——o) represents the ATP–$^{32}PP_i$ exchange activity dependent on L-Aib, (•———•) the amino acid independent exchange activity. The assay was carried out according to Gevers et al. [7].

Table 1
Amino acid dependent ATP–$^{32}PP_i$ exchange (cpm X 10^{-3})

Amino acid	Sephadex G 200 peak fraction	Hydroxyapatite fractionation peak 1	peak 2
Aib	12.0	4.1	0.18
L-Ala	8.6	1.0	0.16
L-Gln	1.6	1.3	0.13
L-Glu	5.6	0	0.10
L-Gly	2.0	1.5	0.10
L-Leu	53.5	3.5	2.90
L-Pro	9.5	3.5	0.12
L-Val	111.0	1.2	9.40
L-Asn	4.8	0	0
L-Asp	2.6	0	0
L-Ile	46.8	0	0
L-Phe	77.6	0	0
L-Ser	0.8	0	0
L-Trp	0.9	0	0
L-Thr	15.2	0	0

The incubation mixture contained 100 μl of enzyme solution and 100 μl of a solution of buffer A containing 0.2 μmol ATP, 0.2 μmol $^{32}PP_i$ (approx. 0.1 μCi) and 0.2 μmol of one of the amino acids. The assay is described in [6].

Table 2
Aminoacyl tRNA activity with various amino acids

	pmoles attached to tRNA		
^{14}C-labelled amino acid	Sephadex G 200 peak fraction	Hydroxyapatite fractionation peak 1	peak 2
Aib	0	0	0
L-Ala	28.8	0	0
L-Gln	0.2	0	0
L-Glu	0	0	0
L-Gly	33.9	0	0
L-Leu	47.5	8.1	19.0
L-Pro	2.7	0.5	0
L-Val	81.0	19.0	0
L-Ile	0	–	–
L-Orn	0	–	–
L-Phe	252.0	–	–
L-Ser	0	–	–
L-Thr	28.0	–	–

The assay is described under Materials and methods. 0.25 μCi of the labelled amino acids were added to the incubation mixture which contained 300 μg of *E. coli*-tRNA. Incubation was carried out for 30 min at 37°C.

but not all of the ATP–$^{32}PP_i$ exchange activity can be attributed to aminoacyl tRNA ligase activity. Thus, the ligases activating Leu and Val are the most active. This may explain the high ATP–$^{32}PP_i$ exchange rates for these two amino acids compared with the others. The extremely high exchanges for Phe, Thr and Ile are remarkable. For Phe and Thr, they are obviously caused by tRNA-ligases (compare tables 1 and 2). The Ile dependent ATP–$^{32}PP_i$ exchange can not be explained by the action of an Ile-tRNA-ligase. It may be attributed to a certain unspecificity of the Val activating enzymes, the alamethicin synthetase and/or the Val-tRNA-ligase. The opposite case is well known; for example the Ile-tRNA-ligase from E. coli is capable of activating Val [9]. We could not detect any influence of phenylalaninol on the in vitro synthesis of alamethicin.

The peak fraction of the gel filtration catalyzes the formation of alamethicin. Fig. 3 shows the incorporation of [^{14}C]Aib into the peptide. The product synthesized in vitro possessed the same R_f values as the marker, alamethicin, when analyzed

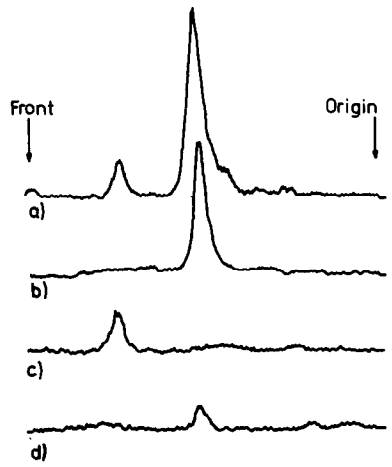

Fig.3. In vitro synthesis of alamethicin and separation by thin layer chromatography. Identification by autoradiography with a Berthold scanner. The assay is described under Materials and methods. (a) Incorporation of [^{14}C]Aib; (b) marker [^{14}C]Aib; (c) marker [^{14}C]alamethicin; (d) hydrolysate of the labelled alamethicin of (a). The t.l.c. plates were first developed with *n*-butanol – acetic acid – water (4:1:1), then dried and developed in the same direction with pyridine – ethylacetate – acetic acid – water (20:60:6:11).

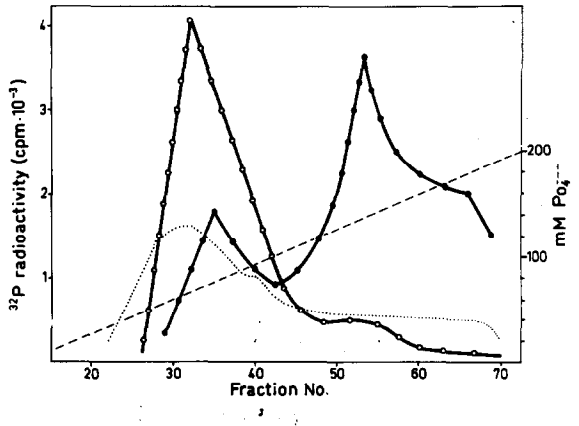

Fig.4. Fractionation of the alamethicin synthesizing system by chromatography on hydroxyapatite. The Sephadex G 200 peak fractions were pooled and applied to a hydroxyapatite column (1.5 X 15 cm). After washing with 100 ml of buffer A the column was eluted with 400 ml of a linear phosphate gradient, pH 7.0, containing 10 mM Mg^{2+}, 0.5 mM EDTA, 5 mM mercaptoethanol and 10% sucrose. (o——o) and (•——•) represent the ATP–$^{32}PP_i$ exchange activities of Aib and L-Val, respectively, (. . .) represents the optical density and (- - -) the phosphate gradient.

by thin layer chromatography with the two solvents specified in 'Materials and methods' section. After hydrolysis of the labelled substance [^{14}C]Aib was regained. In an earlier work we demonstrated the incorporation of other alamethicin constituent amino acids [6].

The peak fraction of the gel filtration was fractionated into two peaks by chromatography on hydroxyapatite (fig.4). Peak 1 catalyzed the ATP–$^{32}PP_i$ exchange for 7 of the 8 amino acids (all but Glu), and peak 2 activated Val and Leu (table 1). Peak 1 still contained some tRNA-ligase activity for Leu, Pro and Val. Peak 2 contained ligase activity for Leu, but not Val (table 2). Both peaks are required for alamethicin synthesis (table 3), i.e. a Val-activating subunit of the alamethicin synthetase has been split off under the conditions of hydroxyapatite chromatography. It is expected that the enzyme complex may be further fractionated by suitable methods. One has to consider that alamethicin is an octadecapeptide; the molecule is considerably larger than for example gramicidin S or tyrocidin, whose synthetases consist of two or three subunits, respectively [10,11].

Table 3
Dependence of alamethicin synthesis on two fractions

Peak number	Alamethicin synthesized (cpm)
I	120
II	50
I + III	1450

The incubation mixture contained either 400 μl of peak I or II or 200 μl of each peak. The incubation conditions are described in Materials and methods. The in vitro synthesized alamethicin was separated by thin layer chromatography as described in the legend of fig.3, then extracted with ethanol. After evaporation of the alcohol radioactivity was counted under toluene scintillation liquid.

The results published here and previously [6] indicate that there is an analogy between the first steps of the biosynthesis of alamethicin and the formation of gramidicin S and tyrocidine [12,13]; the synthetase consists of several complementary subunits; the different amino acids are activated in the forms of aminoacyl adenylates and then they are bound to the synthesizing enzyme as thioesters. The polymerisation of the enzyme linked aminoacyl thioesters to form alamethicin requires further clarification.

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