(3,3'-Leu)-GRAMICIDIN S FORMATION BY GRAMICIDIN S SYNTHETASE

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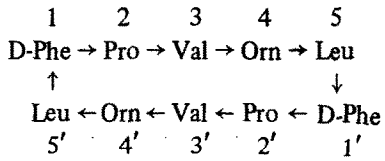
1. Introduction

The biosynthesis of gramicidin S is catalyzed by the enzyme system gramicidin S synthetase, which consists of the phenylalanine racemase (EC 5.1.1.11, 100 000 daltons) and a multienzyme (synthetase, 280 000 daltons) [1]. The enzymes activate amino acids by formation of aminoacyl-adenylates, which are accepted by specific thiol groups in an aminoacylation step. Polymerization proceeds from these active esters by the 'protein-thiotemplate mechanism' [2]. The specifity of this nucleic acid-free polymerization appears to be lower than that of the ribosomal system, since evidence for various substitutions by amino acid analogues has been reported [1,3]. In this report, the formation of a peptide in the absence of L-valine and the replacement by L-leucine [4] is confirmed by enzymatic synthesis and product analysis.

2. Materials and methods

2.1. *Cell growth*

Cells were grown in 7% bactopeptone, 7% yeast at 37°C, harvested at the end of the logarithmic phase, frozen and stored at –20°C.



gramicidin S

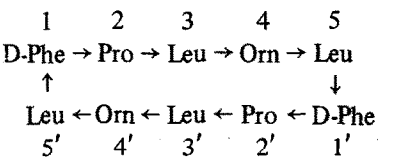
2.2. *Enzyme purification*

2.2.1. Preparation of the crude extract

Cells, 50 g wet wt, were suspended in 250 ml 50 mM TES-buffer (pH 7.0) containing 2 mg/ml lysozyme, 12 mM DTE and 1 mM PMSF. The suspension was incubated at 30°C for 20 min and centrifuged for 10 min at 25 000 × *g*. All the following steps were performed at 4°C. KCl was added to the supernatant to final conc. 0.3 M and the mixture treated with a 10% polyethyleneimine solution (neutralised and dialysed 24 h against water) to final conc. 0.4%. After 10 min stirring, the suspension was centrifuged 10 min at 25 000 × *g*. The supernatant was adjusted to 45% saturation of ammonium sulfate by the addition of a saturated solution (pH 7.2, 0.1 mM EDTA). After 20 min the precipitate was collected by centrifugation for 10 min at 45 000 × *g*, dissolved in 5 ml 20 mM buffer P (20 mM sodium phosphate, pH 7.2, 2 mM DTE, 0.25 mM EDTA).

2.2.2. Sepharose 6B-C1 filtration

The predialysed crude extract was applied to a Sepharose 6B-C1 column (5 × 80 cm) equilibrated and eluted with buffer P. The enzymes were localized by the amino acid-dependent ATP–PP_i exchange [5].



(3,3'-Leu)-gramicidin S

The overall rate of biosynthesis was estimated by the millipore assay [6] to be 3.5 nM gramicidin S/min × mg protein.

2.3. *Peptide biosynthesis*

The enzyme fractions (about 150 ml) were collected in a batch reactor. The necessary biosynthesis components had been dissolved and neutralised previously in 50 ml 20 mM sodium phosphate buffer and added to the enzyme fractions, resulting in end vol. 200 ml with the following final concentrations: ATP 20 mM; EDTA 0.25 mM; $MgCl_2$ 20 mM; DTE 10 mM; Phe, Pro and Leu 10 mM; [^{3}H]Orn (0.1 mCi/mM) 1 mM as the limiting concentration. The reaction mixture was stirred gently at 30°C under careful N_2 aeration to avoid enzyme oxidation. During the reaction aliquots were taken to calculate the amount of synthesized product by counting the incorporated radioactivity on millipore filter.

2.4. *Product isolation*

The protein denaturated during the incubation was centrifuged at 15 000 × *g* for 10 min. Supernatant and pellet were extracted twice with *n*-butanol.

Most of the synthesized product was found adherent to the pellet. The collected extracts were evaporated and dissolved in 1 ml methanol. Besides the product the extract contained impurities as Phe, Leu and phenylalanylprolyl-diketopiperazine.

The extract was applied to a Sephadex G-25 column (2.5 × 40 cm) equilibrated and eluted with 50% acetic acid [7]. The product was located by liquid scintillation counting. Purity was checked by thin-layer chromatography on silica gel (fig.3).

2.5. *Enzymatic hydrolysis*

Product, 200 μg, was dissolved in 100 μl 100 mM Tris buffer (pH 7.2) and 400 μg subtilisin Carlsberg in 400 μl Tris buffer were added. The mixture was incubated for 40 h at 37°C. The completeness of the reaction was checked by thin-layer chromatography.

For further purification of the product, the reaction mixture was applied to an Aminex Q-15 S column (0.9 × 30 cm, 51°C) and eluted with a gradient 0.2 N pyridine acetate (pH 3.1) to 2.0 N pyridine acetate (pH 5.0). The product was localized by radioactivity counting.

2.6. *Amino acid analysis*

Samples were hydrolyzed with 5.7 N HCl at 110°C for 22 h. Analyses were performed on a Durrum D-500 instrument.

2.7. *Sequence determinations*

Manual Edman degradations were carried out essentially as in [8], the amino acid sequence was deduced by the subtractive method. Dansyl amino acids were separated on polyamide sheets in two dimensions using the solvent systems water:formic acid = 200:3 and benzene:acetic acid = 9:1. Phenyl thiohydantoin amino acids were identified by chromatography on silica thin-layer plates in the solvent systems chloroform:*n*-propanol:iso-propanol = 98:1:1 and propionic acid:*n*-heptane:dichloroethane = 17:58:25 and visualized under ultraviolet light. Hydrazinolysis was carried out as in [9].

2.8. *Materials*

Yeast and bactopeptone for growth of *Bacillus brevis* were from Difco. *N*-Tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (TES); dithioerythritol (DTE); lysozyme and subtilisin Carlsberg were from Sigma. Phenylmethanesulfonylfluoride (PMSF) was from Merck. Polyethyleneimide-P was from BASF Ludwigshafen, Sepharose 6B-C1 was from Pharmacia. Phenylalanine, leucine, ornithine and ATP were from Merck, proline was from Sigma. [^{3}H]Ornithine was from NEN, tetrasodium $^{32}PP_i$ from Amersham. Silica gel sheets F 1500 and polyamide sheets F 1700 were obtained from Schleicher and Schüll.

3. Results

3.1. *Enzymatic peptide synthesis*

The formation of a peptide in the absence of the constituent amino acid L-valine can be observed at sufficiently high concentrations of L-leucine (fig.1). An app. K_m 1.5 mM was estimated for the complete process, which is similar to the K_m ascribed to L-leucyl-adenylate formation at the valine activation site [10]. In fig.2 the biosynthesis rate of the peptide in the absence of L-valine is compared with the biosynthesis rate of gramicidin S. In the absence of L-valine the reaction stopped after ~1 h, when 5%

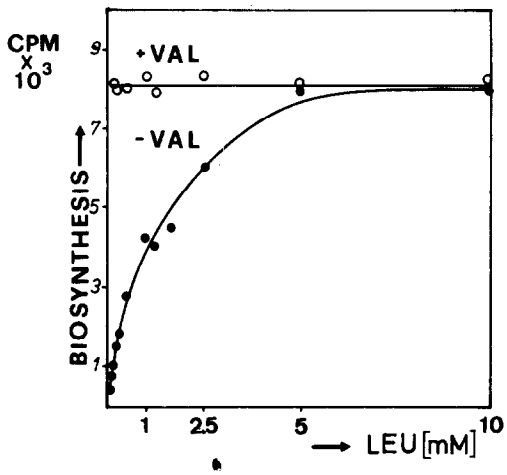


Fig.1. Rate of biosynthesis as a function of leucine concentration. Incubations were carried for 15 min at 37°C in 200 μl/test. Concentrations were: sodium phosphate (pH 7.2) 20 mM; ATP 10 mM; $MgCl_2$ 50 mM; DTE 10 mM; EDTA 1.25 mM; Phe and Pro 5 mM; [^{3}H]Orn (50 mCi/mM) 0.2 mM.

limiting amino acid ornithine had been incorporated. The reason for this limiting value is not yet understood. The product obtained showed a similar behaviour in thin-layer chromatography as gramicidin S

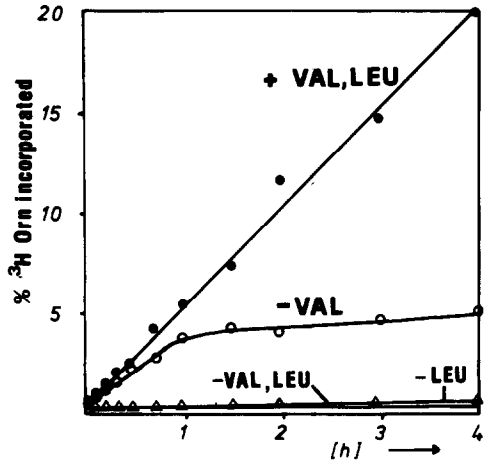


Fig.2. Rate of biosynthesis by a crude enzyme preparation under similar conditions as described in section 2. (–VAL) indicates the peptide formation in the absence of L-valine, (+VAL, LEU) indicates the gramicidin S formation in the presence of all the constituent amino acids. (–LEU) and (–VAL, LEU) are the control tests in the absence of L-leucine and both L-valine and L-leucine.

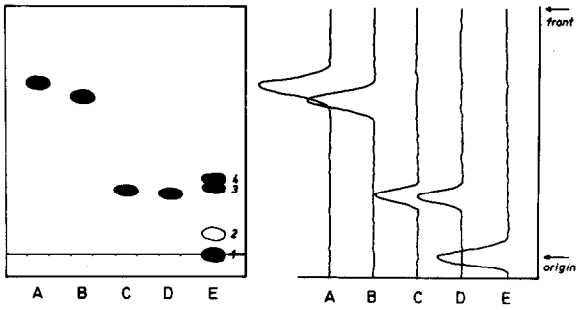


Fig.3. Thin-layer chromatography of: (A) purified product (R_F 0.69); (B) labelled gramicidin S (R_F 0.64); (C) product resulting from the enzymatic hydrolysis with subtilisin Carlsberg (R_F 0.25); (D) product resulting from the enzymatic hydrolysis of gramicidin S with subtilisin Carlsberg; (E) mixture of amino acids used for product synthesis (1 = [^{3}H]Orn, 2 = Pro, 3 = Leu, 4 = Phe). Chromatography was done on silica gel with ethylacetate:pyridine:acetic acid:water = 60:20:6:11; spots were visualized by ninhydrin reaction. The labelled compounds were detected with a Berthold thin-layer scanner.

(fig.3). On acid hydrolysis the amino acid composition Leu_2PheProOrn was obtained. The peptide was thus assumed to be (3,3′-Leu)-gramicidin S. To prove this structure, synthesis was performed on a preparative scale to obtain sufficient material for characterization. About 3–4 mg (3–4 μmol) of labelled product were isolated by one preparation.

3.2. *Enzymatic hydrolysis and sequence analysis*

Hydrolysis of gramicidin S by subtilisin Carlsberg leads to identical pentapeptides of the structure Orn–Leu–D-Phe–Pro–Val [11]. The valine-free peptide was hydrolyzed under similar conditions, and a single product was obtained (fig.3). The proposed structure Orn–Leu–D-Phe–Pro–Leu could be verified by the subtractive Edman method and, in addition, confirmed by the dansyl-Edman procedure and chromatography of the phenyl thiohydantoin amino acids (table 1). The crucial valine replacement at the carboxyl terminal end of the linear pentapeptide could be identified by hydrazinolysis unequivocally as leucine.

3.3. *Molecular weight determination and mass spectrometric analysis*

The peptide was estimated to be mol. wt 1170 ± 2 (proposed 1169.7) by field desorption mass spectro-

Table 1
Sequence of the peptapeptide

Amino acid composition	Orn →	– Leu →	– Phe →	– Pro →	– Leu ⇄
	1.0	2.0	1.0	1.0	
1. Edman	0.0	2.0	1.0	1.0	
2. Edman	–	1.2	1.0	1.0	
3. Edman	–	1.2	0.0	1.0	
4. Edman	–	1.0	–	0.3	
Hydrazinolysis	–	–	–	–	0.6

metry. The complex CI (chemical ionization) spectra showed the MH^+ peak (1171), the $(M/2)H^+$ peak (586) and fragments of mol. wt 325, 341 and 210, which were ascribed to the tripeptides Pro–Leu–Orn and Leu–Orn–Leu and the dipeptide Pro–Leu.

4. Discussion

Specifity of peptide synthesis in nucleic acid-free polyenzyme systems may be controlled at the various levels of the process, which are:

(1) Activation of amino acids as aminoacyl-adenylates.
(2) Thiol-aminoacylation.
(3) Intermediate peptide transport.
(4) Peptide bond formation.
(5) Termination reactions.

In this report the replacement of L-valine by L-leucine has been demonstrated, which apparently is controlled only at the aminoacyl-adenylate level. It has not been proven, however, that leucyl-adenylate is formed at the valine activation site. A second leucine binding site may also be involved, or two different elongation reactions may occur at the leucine activation site. According to the elongation model proposed [12] we propose that leucine is activated at the valine binding site. The leucyl-adenylate would then be accepted by the 'valine-specific' thiol group and accept the dipeptide D-Phe–Pro transported by 4′-phosphopantetheine. The following elongation reactions and the termination reactions apparently do not discriminate the amino acid substitution.

The substitution of leucine by valine on the other hand is not observed. The nature of this discrimination is not yet understood, since isoleucine and norleucine apparently may replace leucine [13], and even D-leucine is accepted in the activation reaction [14].

Acknowledgements

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