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Biosynthesis of bacillomycin D by *Bacillus subtilis*

Evidence for amino acid-activating enzymes by the use of affinity chromatography

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Bacillomycin D is an antifungal lipopeptide produced by *B. subtilis*. The formation of the peptidyl bonds of bacillomycin D occurs non-ribosomally, as demonstrated by the use of chloramphenicol, an inhibitor of protein biosynthesis. Amino acid-activating enzymes were found in *B. subtilis* cell lysates purified by affinity chromatography on a gel containing L-Pro, an amino acid of bacillomycin D. Presence of ATP during this purification increases the binding of enzymatic proteins and their activity. An enzyme, with an apparent molecular weight of 230 kDa, catalyzed ATP–PPi exchange reactions, which were mediated by specific amino acids, corresponding to a partial sequence of bacillomycin D.

Bacillomycin D; Antibiotic lipopeptide biosynthesis; Amino acid activation; Affinity chromatography; *Bacillus subtilis*

1. INTRODUCTION

Bacillus species are well-known producers of antibiotics, and a multi-enzymatic system has been shown to be responsible for the biosynthesis of some peptide or lipopeptide antibiotics, such as gramicidins A and S, tyrocidine, edeine from *Bacillus brevis*, mycobacillin from *Bacillus subtilis*, bacitracin from *Bacillus licheniformis* and polymyxin from *Bacillus polymyxa* [1,2]. In the case of bacillomycin D, a lipopeptide antibiotic from *B. subtilis* which contains β-amino fatty acids [3,4] (Fig. 1), such an enzymatic system has not yet been described. During our study on the biosynthesis of bacillomycin D, we characterized enzymes which give ATP–pyrophosphate (PPi) exchange reactions mediated by some of the amino acid components of bacillomycin D.

2. MATERIALS AND METHODS

2.1. *Chemicals and radioactive chemicals*

The U-^{14}C-labeled amino acids were obtained from CEA (Saclay, France), $Na_2H^{32}PO_4$ and $Na_4{}^{32}P_2O_7$ were purchased from NEN Products (Boston, USA). Standard bacillomycin D was prepared as in [5] and β-amino fatty acids were prepared after total hydrolysis of bacillomycin D according to [6].

Abbreviations: BSA, bovine serum albumin; CP preparation, crude protein preparation; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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2.2. *Strain and culture conditions*

The strain of *B. subtilis* producing bacillomycin D was grown in a brain-heart medium (Bio-Mérieux, France) as previously described [7].

2.3. *In vivo incorporation of radioactive amino acid precursor into bacillomycin D and proteins*

After 24 h of growth, 1 ml samples of the culture were withdrawn and [^{14}C]Pro (48 MBq/ml) were added, with or without chloramphenicol. After 1 h incubation at 35°C, the assays were tested for [^{14}C]Pro incorporation either into proteins [8] or into bacillomycin D, as previously described for iturin [9].

2.4. *Preparation of a cell-free extract from* B. subtilis

The *B. subtilis* cells (corresponding to 1 l of culture) were harvested in the late exponential phase of growth, washed once with 0.9% NaCl and then with 5 mM $MgCl_2$. Cells were suspended in 50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol (buffer I) to which lysozyme (from Sigma) was added at 1 mg/ml. After 20 min at 30°C, the cell-free system, obtained by 20,000 × *g* centrifugation, was treated with 10% streptomycin sulfate to make a final concentration of 1%; the supernatant was precipitated by $(NH_4)_2SO_4$ at 60% saturation. The precipitate was dialyzed according to [10]; this corresponds to the crude protein (CP) preparation. The in vitro bacillomycin D synthesis was tested by incubating the CP fraction in a 10 mM Tris-HCl buffer, pH 7.8, containing 0.4 mM ATP, 10 mM $MgCl_2$, 10 μM β-amino fatty acids and [^{14}C]amino acid constituents of bacillomycin D (10 μM, 37 kBq/ml, each).

2.5. *Determination of protein concentration*

Protein concentration was determined by the method of Bradford [11] with BSA as the standard.

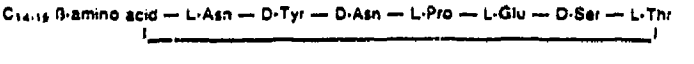

Fig. 1. Structure of bacillomycin D.

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2.6. *Assay for the ATP-phosphate and ATP-pyrophosphate exchanges*
The ATP-Pi and ATP-PPi exchanges were determined as in [10,12]; the total amount of radioactivity in each incubation mixture was 4 kBq of [^{32}P]Pi or [^{32}P]PPi.

2.7. *Assay for covalent binding of substrate amino acids by amino acid-activating enzyme*
The reaction mixture contained, in a final volume of 250 μl, 50 mM Tris-HCl, pH 7.8, 10% glycerol, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 4 kBq of 20 μM ^{14}C-labeled amino acids and 200 μl of enzyme. Samples were incubated for 10 min at 37°C. The reaction was stopped by addition of 5 ml of cold 5% TCA. After 30 min at 4°C, the precipitate was collected on 0.45 μm Millipore filters. Filters were treated and radioactivity was determined as described in [8].

2.8. *Affinity chromatography*
Two affinity gels were used: Commercial L-Pro agarose (from Sigma), where the amino acid was bound through its amine moiety, and L-Pro affigel, where the amino acid was bound by its carboxyl moiety. In this case, the esterification of L-Pro by the affigel 102 (Bio-Rad) was carried out according to the procedure described by the manufacturers. The affinity column (about 5 ml) was equilibrated with buffer I and 1 ml fractions were collected at a flow rate of 6 ml/h.

2.9. *Molecular weight determination*
The molecular weight of the enzyme was determined by gel permeation HPLC with a LKB HPLC apparatus on an Ultropac TSK G3000 column (7.5 × 600 mm) which affords separation of 10-300 kDa proteins. Elution was performed with buffer I without glycerol at a flow rate of 0.5 ml/min and 1 ml fractions were collected. A gel permeation chromatography was also carried out with an AcA22 column (40 × 2.5 cm) which affords separation of 100–1200 kDa proteins. Elution was performed at 4°C with buffer I at a flow rate of 6 ml/h and 2 ml fractions were collected. This column was calibrated with a protein calibration kit from Pharmacia containing thyroglobulin (667 kDa), ferritin (440 kDa), catalase (232 kDa) and BSA (67 kDa).

3. RESULTS AND DISCUSSION

It had been shown previously that bacillomycin D production began at the end of the exponential phase of growth and reached a maximum level during the stationary phase [7]. The synthesis of bacillomycin D is independent of the ribosomal process as the addition of chloramphenicol, a protein synthesis inhibitor, to the culture medium did not modify the incorporation of radioactive proline into bacillomycin D. Several assays for the characterization of a cell-free system giving in vitro synthesis of bacillomycin D were unsuccessful. Similar results have been previously reported for other lipopeptides [13,14] and the detection of the enzymes involved in their synthesis was made by the activation of the amino acids present in the lipopeptide.

3.1. *Purification of the CP preparation*

The enzyme system responsible for bacillomycin D synthesis was purified by affinity chromatography on an affigel containing, as ligand, one of the amino acids of bacillomycin D, L-Pro, bound through its carboxyl group. When the CP preparation was passed through L-Pro affigel in the absence of ATP, a protein fraction was retained on the column and was eluted by the addition of 0.1 M NaCl to the buffer (Fig. 2, fraction II). No further elution was obtained with 0.1–1 M NaCl gradient. The UV spectrum of fraction II showed the protein absorbance band at 280 nm and the absence of nucleic acids at 260 nm. Quantification of the proteins of fraction II gave about 16% of the proteins loaded on the column. The influence of the unbound function of L-Pro during the affinity chromatography was tested by using L-Pro agarose, where the amino acid was bound through its amine group. When the CP preparation was passed through the L-Pro agarose column, the addition of NaCl 0.1 M or a 0.1–1 M NaCl gradient to the buffer did not elute any protein. Thus, the purification of the amino acid-activating enzyme can be realized by affinity chromatography only when the amine function was free.

3.2. *Activation of the amino acids*

The eluates of fraction II were screened for activation of the amino acid components of bacillomycin D by measuring the ATP-Pi and the ATP-PPi exchange reactions, the two most common amino acid mechanisms in non-ribosomal peptide synthesis [2,15]. No ATP-Pi exchange depending on the presence of amino acid was detected with the bacillomycin D components. In contrast, several enzyme activities were observed with the ATP-PPi exchange technique (Fig. 2). Four amino acid components of bacillomycin D, L-Glu, L-Ser, L-Thr and L-Asn, were activated by fraction II. Three of these amino acids correspond to a partial sequence, L-Glu → D-Ser → L-Thr, of bacillomycin D (Fig. 1). An unexpected result was the absence of activation of L-Pro, a component of bacillomycin D, by fraction II even though this fraction bound [^{14}C]L-Pro (Fig. 2).

3.3. *Purification of CP preparation in the presence of ATP*

ATP had been found to have a positive effect on the purification of the gramicidin S synthases by affinity chromatography [16]. Thus, the influence of ATP on the binding of amino acid-activating enzymes on the L-Pro affigel column was tested. In the presence of 3 mM ATP, a protein fraction was retained on the column and was eluted by the addition of 0.1 M NaCl to the buffer (Fig. 3, fraction II'). Quantification of the proteins of fraction II' showed that they represented half the proteins loaded on the column. Thus, the presence of ATP during purification on L-Pro affigel increased the binding of proteins by a factor of 3. The eluates of fraction II' were screened for ATP-PPi exchange reactions mediated by L-Glu or L-Pro (Fig. 3). A significant activation of L-Glu and a very weak activation of L-Pro were observed. When other amino acid components of bacillomycin D were tested (Table I), only L-Thr gave a significant ATP-PPi exchange. The apparent molecular weight of the amino acid activating enzyme was moni-

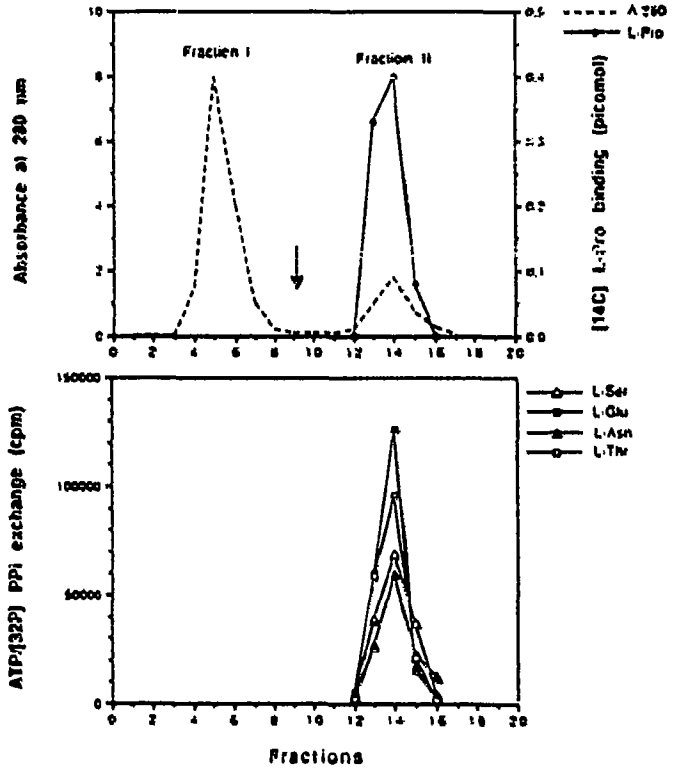

Fig. 2. L-Pro affinity chromatography of the CP preparation carried out in the absence of ATP. The [^{32}P]PPi–ATP exchange activities were measured on 100 μl of the fraction incubated in the presence of amino acids; the values were corrected with blanks, obtained by incubating the same volume of each fraction without amino acids. The binding of [^{14}C]L-Pro was determined as described in section 2; the values were corrected with blanks obtained with boiled enzyme. The arrow indicates the addition of NaCl to the elution buffer.

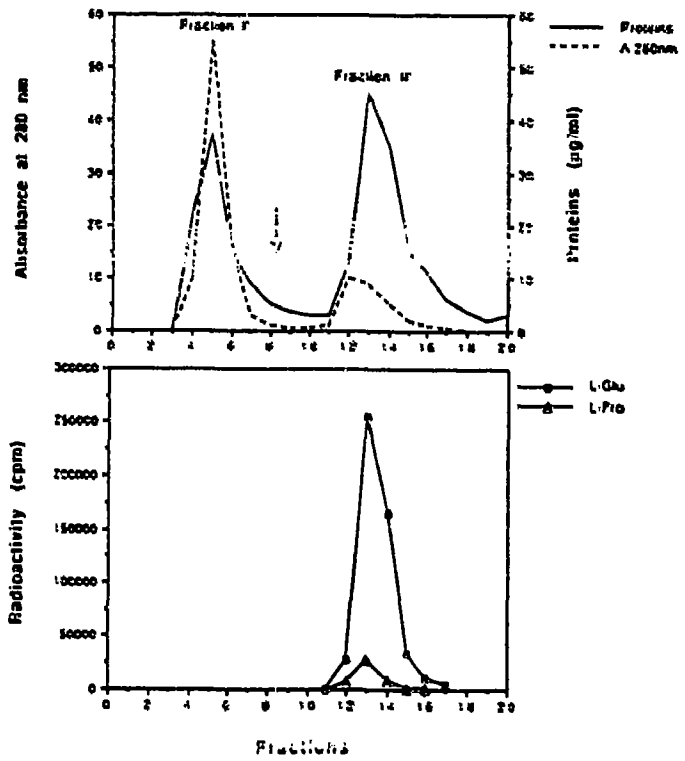

Fig. 3. L-Pro affinity chromatography of the CP preparation carried out in the presence of ATP. The elution profile was determined by measuring the absorbance at 280 nm, which detected both ATP and proteins, and by protein titration. The [^{32}P]PPi–ATP exchange activities were measured on 20 μl of the fraction incubated in the presence of amino acids; the values were corrected with blanks, obtained by incubating the same volume of each fraction without amino acids. The arrow indicates the addition of NaCl to the elution buffer.

tored by the L-Glu-dependent ATP–PPi activity. After gel permeation HPLC of fraction II', the enzyme activating L-Glu co-migrated with catalase (230 kDa) (Fig. 4). This result was confirmed by AcA 22 chromatography.

Table 1

Influence of ATP on the purification of amino acyl activating enzymes by affinity chromatography: comparison of the ATP/[^{32}P]PPi exchange activity of Fractions II and II' obtained, respectively, in the absence and presence of ATP

Amino acid	ATP/[^{32}P]PPi exchange (cpm)*	
	Fraction II	Fraction II'
L-Asn	2,216	–
D-Asn	–	–
L-Glu	5,084	30,504
L-Pro	–	2,787
L-Ser**	2,573	557
D-Ser	–	–
L-Thr	3,588	23,715
L-Tyr**	–	–
D-Tyr	–	–

*The values correspond to the ATP–PPi exchange catalyzed by 10 μg of proteins; they are corrected with blanks obtained in the absence of amino acids.

**These L-form amino acids are components of bacillomycin D where they occur as the D form.

3.3. *Comparison of the activities of the enzymes purified in the presence and absence of ATP*

When the amino acid-activating enzymes purified in the presence of ATP (Fraction II') were compared with those purified in the absence of ATP (Fraction II), it appears that the addition of ATP increased, by a factor of 6, the activity of the L-Glu and L-Thr activating enzymes and decreased, by the factor 5, the activity of the L-Ser-activating enzyme, while L-Asn was not further activated (Table I). Indeed, L-Asn was activated by fraction I', and the major part of the L-Ser-activating activity was found in this fraction. As serine is present as D-Ser in bacillomycin D, the L-Ser substrate must undergo both racemization and activation. The decrease in activity of the L-Ser-activating enzyme, and the absence of L-Asn-activating enzyme in fraction II', even though these enzymes were present in the homologous Fraction II, demonstrates a high influence of ATP on the affinity of activating enzyme complex for L-Pro affigel. ATP could lead to the dissociation of some sub-

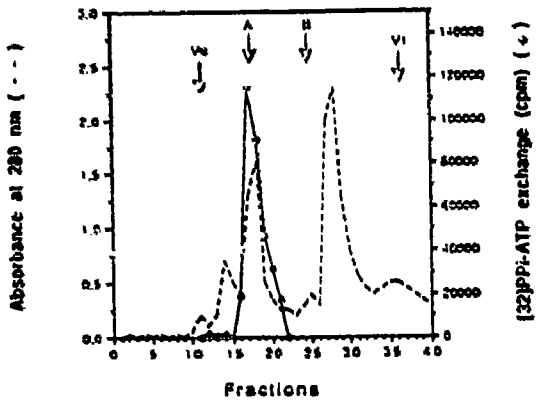

Fig. 4. Purification of the L-Glu-activating enzyme by gel permeation HPLC. The arrows indicate the calibration of the column with the exclusion volume (Ve), the total bed (Vt), the elution volume of catalase (232 kDa) (A) and BSA (67 kDa) (B). The [^{32}P]PPi–ATP exchange activities were measured on 200 μl of the fraction incubated in the presence of amino acids; the values were corrected with blanks, obtained by incubating the same volume of each fraction without amino acids.

units of this complex, especially the L-Ser racemase, the L-Ser- and L-Asn-activating enzymes. Thus, ATP is an interesting factor which could allow the separation of the enzyme subunits involved in the synthesis of the peptide moiety of bacillomycin D.

In conclusion, an enzyme fraction with an apparent molecular weight of 230 kDa was purified from the crude extract of *B. subtilis* producing bacillomycin D. This enzyme, which shows a great affinity for L-Pro, catalyzes ATP–PPi exchange mediated by amino acids present in the peptide part of bacillomycin D. These results are consistent with a peptide synthesizing system which activates amino acids as amino acyl phosphate. This amino acid activation pattern, involving an ATP–PPi exchange, is the most common for the non-ribosomal synthesis of peptidic or lipopeptidic antibiotics of *Bacillus* [2,15].

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