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Purification of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213

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Abstract

The purification of the multienzyme system producing the lipodecapeptide fengycin in *Bacillus subtilis* b213 was investigated. By gel filtration of a cell free extract of this organism three enzyme fractions were obtained from which five multifunctional components of fengycin synthetase were separated by high resolution anion-exchange FPLC procedures. These proteins were characterized by their thioester formation activities with ^{14}C-labeled substrate amino acids and by N-terminal sequencing. Correlation of these data with the DNA sequences of the *pps (fen)* operons in three *B. subtilis* strains provided detailed knowledge on the structural and functional organization of fengycin synthetase.

Keywords: Purification; *Bacillus subtilis* b213; Enzymes; Fengycin synthetase

1. Introduction

Bacillus subtilis strains produce a broad spectrum of bioactive peptides with a high potential for biotechnological and pharmaceutical applications. A prominent class of such compounds are the lipopeptides surfactin [1–3], fengycin [4] and the members of the iturin family (iturin, mycosubtilin, bacillomycin) [5–8] (see Table 1). They represent amphiphilic membrane active biosurfactants and peptide antibiotics with potent antimicrobial activities. Fengycin is a lipodecapeptide containing a β-hydroxy fatty acid in its side chain. It specifically inhibits filamentous fungi [4,9]. It appears as a mixture of isoforms with variations in both length and branching of the β-hydroxy fatty acid moiety and as well in the amino-acid composition of its peptide ring [4]. Fig. 1 shows a MALDI-mass spectrum of the fengycin cluster. The mass numbers m/z of the isoforms of the protonated species and their alkali metal adducts were found between 1435 and 1529 [10].

The biosynthesis of such compounds is catalyzed by large multienzyme systems which produce bioactive peptides according to the 'Multiple Carrier Thiotemplate Mechanism' [11,12]. These processes involve a two step mechanism for the activation of the amino-acid substrates. In the first step amino-acyl adenylates are formed followed by thioester formation with the sulfhydryl group of a 4′-phosphopantetheine (4′-ppan) cofactor which is posttranslationally attached to a reactive serine at each reaction center. These 4′-ppan-carrier arms function as the thiotemplate sites and manage the assembly of the growing peptide chain in a series of transpeptidation reactions. Such multienzymes which represent cellular factories show a typical modular structure [12]. They consist of amino acid activating modules

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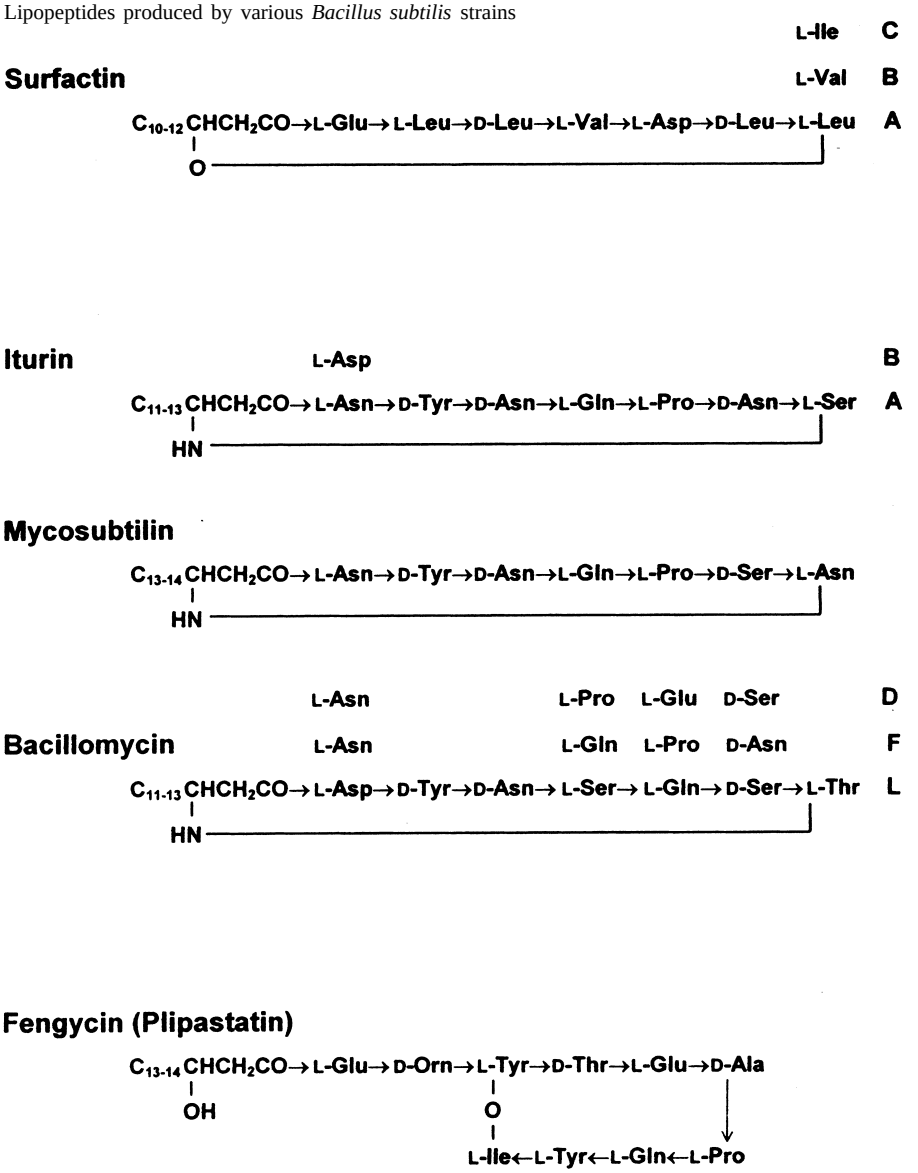
Table 1
Lipopeptides produced by various *Bacillus subtilis* strains



comprising 1000–1500 amino acid residues which can be divided into three main sections responsible (a) for substrate recognition and binding as well as for the primary aminoacyl adenylate formation, (b) for thiolation of the amino acid substrates and (c) for peptide elongation and in some cases also for amino acid epimerization.

Fengycin biosynthesis is encoded by the *pps (fen)* operon, which has been sequenced in the framework of the European *B. subtilis* genome sequencing project investigating the DNA of *B. subtilis* 168 [13,14]. Extensive sequence information is also available for the fen-operons detected in the genomes of *B. subtilis* F-29-3 and A 1/3 [15–17]. All these gene structures show five open reading frames (ORF) indicating that the peptide part of fengycin is assembled by five multifunctional enzymes Fen 1-5 [17]. The purification of such large modular peptide synthetases with relative molecular masses M_r between 100 000 and more than one million is often a

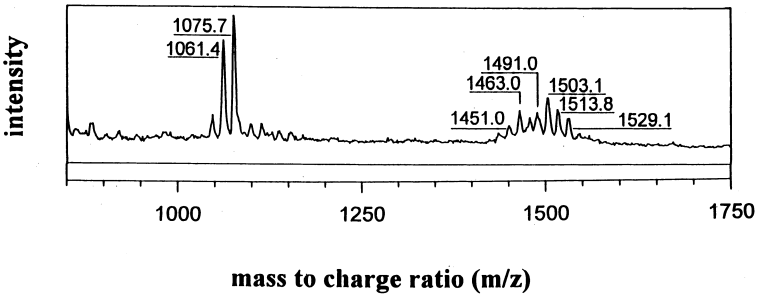


Fig. 1. MALDI-mass spectrum of lipopeptides produced by *B. subtilis* b213.

difficult problem. In particular, enzymes showing the same module multiplicity frequently differ only slightly in size and charge. Therefore, they cannot be separated by size exclusion chromatography and sometimes even by electrophoretic techniques of high resolution power. From the arsenal of chromatographic techniques high resolution–ion-exchange fast protein liquid chromatography (FPLC) is qualified to solve this delicate task efficiently. This is demonstrated in this paper for the fengycin synthetase multienzyme system from *B. subtilis* b213 which comprises a three-module enzyme, three proteins each containing two modules and an one-module enzyme [17].

2. Materials and methods

2.1. Materials

L-[U-^{14}C]glutamine (280 mCi mmol^{-1}), L-[U-^{14}C]glutamate (249 mCi mmol^{-1}), L-[U-^{14}C]threonine (228 mCi mmol^{-1}), L-[U-^{14}C]tyrosine (464 mCi mmol^{-1}), L-[U-^{14}C]alanine (156 mCi mmol^{-1}), L-[U-^{14}C] isoleucine (330 mCi mmol^{-1}), L-[U-^{14}C]proline (264 mCi mmol^{-1}), and L-[U-^{14}C]valine (262 mCi mmol^{-1}) were purchased from Amersham Life Sciences (Braunschweig, Germany). L-[2,3-^{3}H] ornithine (53.4 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals (St. Louis, USA). Ultrogel AcA 34 was from BioSepra (Frankfurt a.M., Germany). Anion-exchange chromatography was performed using Q-Sepharose Fast Flow and the FPLC columns MonoQ HR5/5, ResourceQ and Smart MonoQ PC 1.6/5 from Amersham Pharmacia Biotech (Freiburg, Germany). All other chemicals were reagent grade.

Bacillus subtilis b213 cells were cultivated in an optimized sucrose/yeast extract/peptone medium [18] as described previously [17].

2.2. Methods

2.2.1. Preparation of the crude cell free extract

All steps were carried out at 4°C. *B. subtilis* b213 cells were suspended in three volumes of 50 m*M* Tris–HCl buffer pH 7.5 containing 10 m*M* DTE, 3 m*M* EDTA, 5 m*M* benzamidine and 20% sucrose. Protoplasts were generated by lysozyme treatment (4000 U ml^{-1} cell suspension, 30°C, 20 min) and passed through a French press at a cell pressure of 124 MPa. Cell debris was removed by centrifugation (25 000 *g*, 20 min). Nucleic acids were precipitated by addition of streptomycin sulfate to a final concentration of 1% (w/v) and stirring for 25 min. They were pelleted by centrifugation at 25 000 *g* for 20 min. The proteins in the supernatant were salted out with ammonium sulfate at 70% saturation, dissolved in a minimum volume of buffer (50 m*M* Tris–HCl, pH 7.5, 5 m*M* DTE, 3 m*M* EDTA and 10% sucrose) and dialysed against the same buffer. Forty ml of the crude enzyme extract was loaded on an Ultrogel AcA 34 column (75×5 cm) and eluted with the same buffer (flow-rate 100 ml h^{-1}, fraction size 13 ml).

2.2.2. Methods for enzyme analysis

Thioester binding of the amino acid components of fengycin synthetase were measured using the

procedure described for the characterization of surfactin synthetase [19].

Protein concentration was measured using the procedure of Bradford [20]. SDS-PAGE was performed according to Laemmli [21]. Relative molecular masses M_r of denaturated proteins were determined by SDS-PAGE in a mini-gel apparatus (Hoefer, San Francisco, USA). Marker proteins were gramicidin S synthetase 2 (GS2, 512 000), surfactin synthetase 3 (SrfB, 405 000) and a high molecular weight standard mixture (M_r=30–200 000, Sigma, Deisenhofen, Germany).

After preparative SDS-PAGE as described in [19] the gels were electroblotted using a polyvinylidene difluoride membrane in a semi-dry Novablot apparatus (LKB, Bromma, Sweden) according to the protocols of the manufacturers. The blotted proteins were stained with a 0.2% (w/v) Ponceau S solution. Bands of the fengycin synthetase components were excised and submitted to Edman degradation analysis using a Procise Sequencer (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany) for N-terminal sequence determination.

For autoradiography of a SDS-polyacrylamide gel containing radiolabeled fengycin components, about 100 μl of a protein fraction was incubated with 0.05 μCi of [^{14}C] amino acid, 8.3 m*M* $MgCl_2$ and 12.3 m*M* ATP in a total volume of 121 μl for 15 min at 37°C. The protein was then precipitated with trichloroacetic acid (TCA) overnight. The pellet was washed with acetone and resuspended in 20 μl Laemmli-SDS-PAGE buffer [21]. The mixture was loaded on a polyacrylamide-SDS slab gel which was stained with Coomassie Blue and dried in a gel dryer. Finally it was subjected to autoradiography by exposure to a X-ray film (Konica, Japan) for 4 weeks.

3. Results and discussion

Isolation, purification and characterization of multicomponent peptide synthetases is a difficult task, in particular, because of their sensitivity against proteolytic attack and because enzyme components of the same module multiplicity show very similar masses and charges. These obstacles can be overcome by the application of fast protein liquid chromatography (FPLC) techniques with high resolution power. Following this concept we isolated and

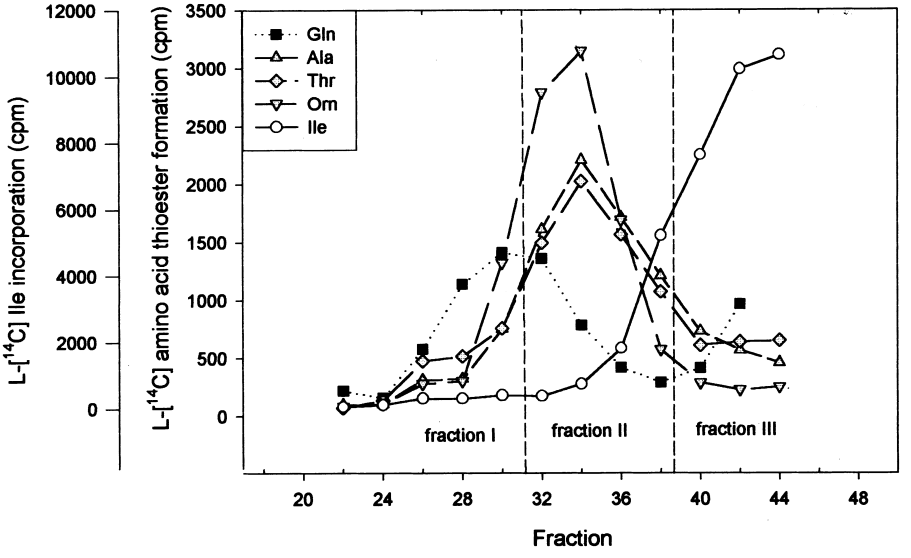


Fig. 2. Gel filtration of a cell free crude extract of *B. subtilis* b213 on Ultrogel AcA34. Three enzyme fractions (I–III) of the fengycin synthetase were distinguished by thioester-binding studies. Proteins were eluted with 50 m*M* Tris–HCl buffer, pH 7.5, 5 m*M* DTE, 3 m*M* EDTA, 10% sucrose (flow-rate: 100 ml h^{-1}; fraction size: 13 ml).

purified the fengycin synthetases multienzyme system from *B. subtilis* b213. Cells were harvested at the end of the logarithmic phase of their growth. A crude extract was prepared as described under Materials and methods. Proteins were salted out with 70% ammonium sulfate, dissolved in 50 m*M* Tris–HCl buffer, pH 7.5 containing 5 m*M* DTE, 3 m*M* EDTA and 10% sucrose and were fractionated by size exclusion chromatography on Ultrogel AcA 34 in the same buffer. Fengycin synthetase enzymes were detected by screening the obtained fractions for thioester formation reactions with the fengycin substrate amino acid components and by in gel-detection of these proteins covalently labeled with their [^{14}C] substrate amino acids as thioester. Three high molecular fractions were obtained. In fraction I which appeared near the exclusion limit of the column at molecular masses higher than 300 000 thioester formation reactions with L-Pro, L-Tyr and L-Gln were detected. In fraction II (mass range: 250–300 000) thioester incorporation of tyrosine, threonine, glutamic acid, alanine, valine and ornithine was observed. Fraction III contained a L-isoleucine activating enzyme that eluted at a volume corresponding to a molecular mass in the range of 120–160 000. Fig. 2 shows the substrate amino acid incorporation profile of these fractions after the gel filtration. Fractions I–III were purified separately to obtain an as high as possible purification grade of the fengycin synthetase components.

3.1. Purification of fraction I

Fraction I was purified by fast anion-exchange liquid chromatography in two steps. First the proteins in this sample were fractionated by FPLC on Pharmacia MonoQ. Thereafter they were separated on a Smart MonoQ using the high resolution of the Pharmacia Smart System. In the active fractions thioester formation reactions with L-tyrosine, L-glutamine and L-proline were detected showing their maximum at the same position. These activities were attributed to a protein with a molecular mass of approximately 400 000 which correlated with the upper protein band in the SDS-polyacrylamide gel shown in Fig. 3. Obviously, this enzyme contains three amino acid activating modules.

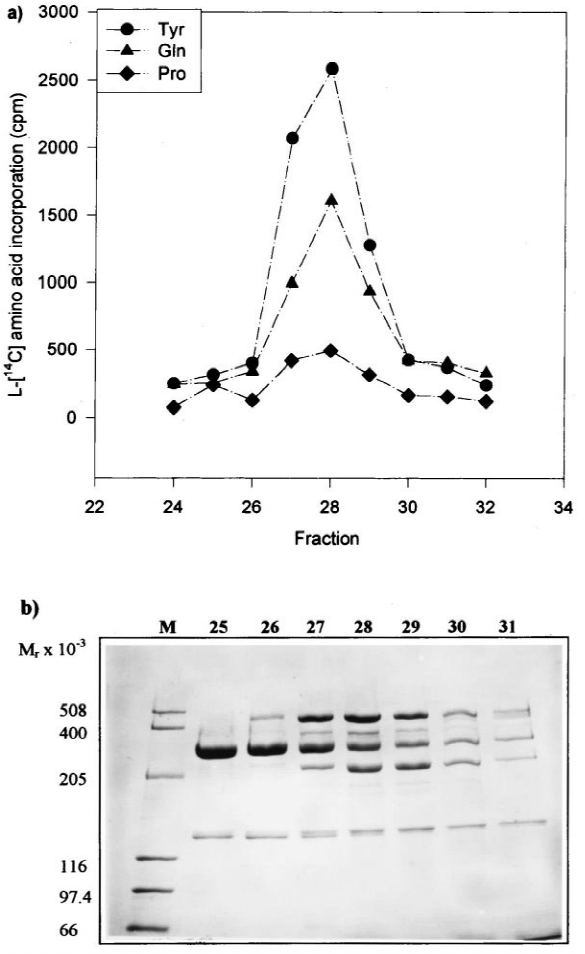


Fig. 3. High resolution anion-exchange FPLC of the fengycin synthetase component in fraction I on Pharmacia SmartQ. The protein was eluted with a linear gradient of 200–400 m*M* NaCl in 50 m*M* Tris–HCl buffer pH 7.5 containing 5 m*M* DTE. The flow-rate was 50 μl min^{-1}. (a) Thioester formation profile with L-[^{14}C]Tyr, L-[^{14}C]Gln and L-[^{14}C]Pro. (b) Coomassie-stained 5% SDS-polyacrylamide slab gel showing the protein composition of fractions tested for substrate amino acid incorporation (marker proteins: GS2, 508 000; SrfB-protein, 401 000; myosin, 205 000; β-galactosidase, 116 000; phosphorylase b, 97 400; bovine albumin, 66 000).

3.2. Purification of fraction II

After AcA 34 gel filtration fraction II was purified by anion-exchange FPLC in three steps using the Pharmacia ResourceQ- and MonoQ-columns. In this way the purity of the fengycin synthetase com-

ponents could be increased appreciably. In the final step using a MonoQ column in the Smart System with a flat salt gradient for elution three amino acid activating enzymes of very similar charge could be separated. They showed nearly the same molecular mass of about 280 000 as determined by SDS-polyacrylamide gel electrophoresis. Apparently, all these proteins represent two-module enzymes. Fig. 4 shows the chromatogram of the Smart MonoQ run and the SDS-PAGE analysis of the obtained fractions. The position of the first two enzymes in the gel was determined by autoradiographic detection of their thioester complexes with L-[^{14}C]Tyr and L-[^{14}C]Thr, respectively.

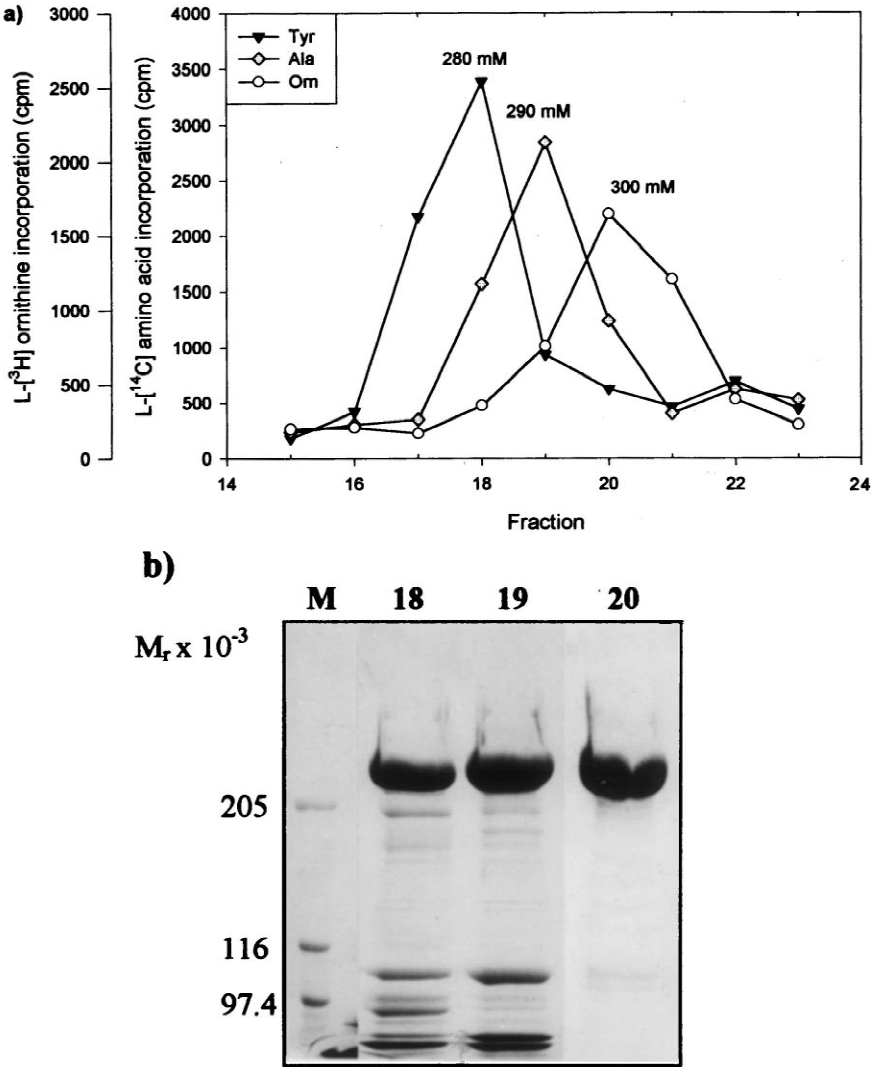


Fig. 4. Separation of the three two-module enzymes of fraction II by Smart MonoQ. These enzymes were eluted with a linear gradient of 200–500 m*M* NaCl (10 m*M* min^{-1}) in 50 m*M* Tris–HCl buffer pH 7.5 containing 5 m*M* DTE. The flow-rate was 100 μl min^{-1}. Fraction size 100 μl. (a) The enzymes were tested for thioester formation with L-[^{14}C]Tyr, L-[^{14}C]Thr, L-[^{14}C]Val, L-[^{14}C]Ala, L-[^{14}C]Glu, L-[^{3}H]Orn (here only thioester formation with L-[^{14}C]Tyr, L-[^{14}C]Ala and L-[^{3}H]Orn is shown). (b) Coomassie-stained 5% SDS-polyacrylamide slab gel of the fractions showing maximal thioester binding activities with L-[^{14}C]Tyr, L-[^{14}C]Ala and L-[^{3}H]Orn (marker proteins: myosin, 205 000; β-galactosidase, 116 000; phosphorylase b, 97 400; bovine albumin, 66 000).

3.3. Purification of fraction III

The L-Ile activating component of fengycin synthetase detected in fraction III of the initial gel filtration was further purified in four steps, first by anion-exchange FPLC on ResourceQ, then by size exclusion chromatography on Sephacryl S-200, followed by anion-exchange FPLC on MonoQ and finally on a Smart MonoQ under the high resolution conditions of the Pharmacia Smart system. This enzyme eluted from the MonoQ column at a relatively low NaCl concentration of about 190 m*M*. It forms a thioester only with L-Ile, as demonstrated in Fig. 5 which shows the L-[^{14}C]Ile incorporation profile and an autoradiogram of a polyacrylamide gel of the L-[^{14}C]Ile-labeled enzyme in fraction 31.

The fengycin synthetase components obtained in purified form from fractions I–III were blotted from

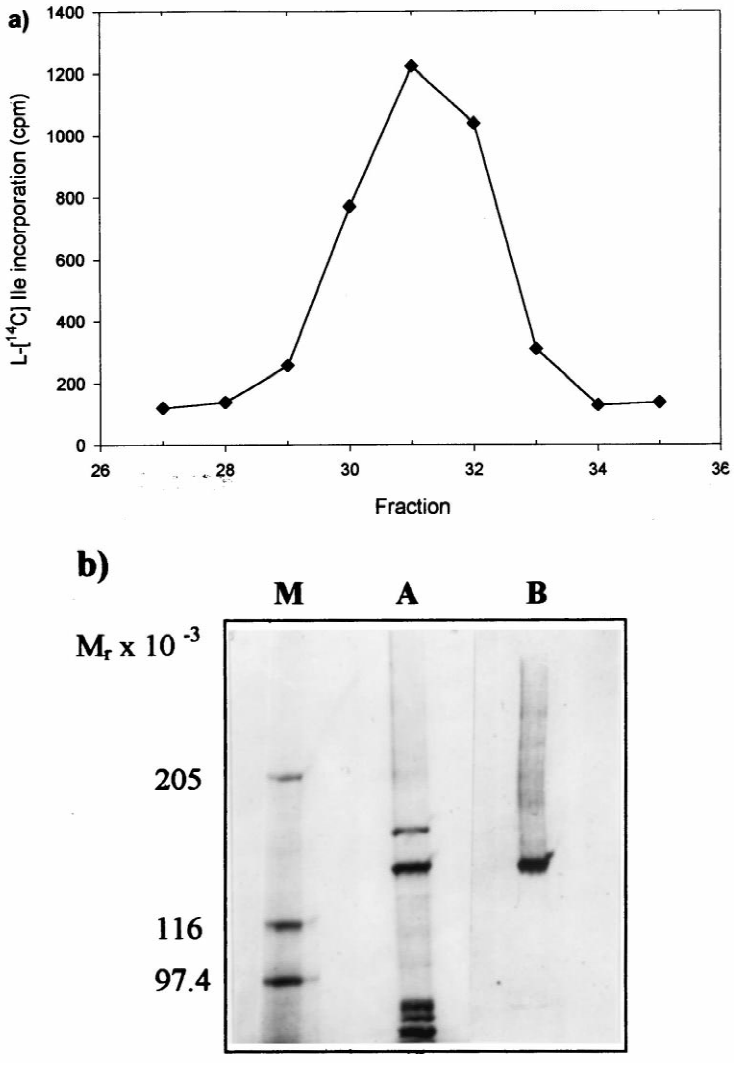


Fig. 5. Anion-exchange FPLC of the fengycin synthetase component in fraction III on Pharmacia MonoQ. (a) Thioester incorporation of L-[^{14}C]Ile. (b) 5% Coomassie-stained SDS-polyarylamide slab gel showing in lane A the protein composition of fraction 31 with maximal thioester formation of isoleucine and in lane B an autoradiogram of this gel containing the L-[^{14}C]Ile labeled enzyme (marker proteins: myosin, 205 000; β-galactosidase, 116 000; phosphorylase b, 97 400; bovine albumin, 66 000).

Table 2
Characterisation of the fengycin synthetase components

Gene product	$M_r \times 10^{-3}$ [a]	Number of modules	Activated amino acid	Amino-terminal-sequence
Fen 1	288.9	two	L-glutamic acid, L-ornithine	MENTVYSLTHAQ
Fen 2	289.8	two	L-tyrosine, L-threonine	TQATEIQDIY
Fen 3	287.2	two	L-glutamic acid, L-alanine or L-valine	QQPEIQDIYPLSFMQ
Fen 4	406.4	three	L-tyrosine, L-glutamine, L-proline	TKKNAIQDIY
Fen 5	143.8	one	L-isoleucine	MDKTKNIQNIYP

[a] The relative molecular masses M_r were calculated from the amino acid sequences of these proteins as obtained from the gene structures.

preparative SDS-polyacrylamide gels onto polyvinylidene difluoride membranes and subjected to N-terminal sequence analysis by Edmann degradation. In this way these enzymes could be attributed to the corresponding genes in the *pps (fen)* operons. They were characterized by their amino acid activation patterns. The obtained data are summarized in Table 2.

Their N-terminal protein sequences are in perfect agreement with those derived from the gene sequences of the five open reading frames in the *fen*-operon in the genome of *B. subtilis* A 1/3. On the other hand homologies between 58 and 100% were observed in relation to the sequences derived from the corresponding genes in the *pps*-operon of *B. subtilis* 168. Obviously, there is an appreciable sequence variety in the gene products of various fengycin producing *B. subtilis* strains. From the amino acid activation pattern the functional organisation of the fengycin synthetase could be derived. The five open reading frames of the *pps*- and *fen*-operon comprise ten amino acid activating modules which cooperate in fengycin biosynthesis. The first three genes encode two-module enzymes which in turn activate amino acid components 1–6 of fengycin: Fen 1 activates glutamate and ornithine which form the side chain of this lipopeptide. Fen 2 thiolates tyrosine and allothreonine, whereas Fen 3 incorporates glutamate and valine or alanine. The fen 1-3 genes are followed in the *pps* (*fen*)-operon by a larger ORF coding for Fen 4, a three-module enzyme that activates proline, glutamine and tyrosine. Fen 5 is encoded by the final and smallest ORF. It activates isoleucine and is presumably involved in closing the fengycin ring by facilitating lactone formation between the carboxyl group of L-Ile and the hydroxyl group of L-tyrosine in position 3 of the fengycin ring system. Our results are in agreement with the colinearity rule which postulates that the amino acid activating modules of a peptide synthetase are arranged in the same sequence as the amino acid components in the peptide product. Research is in progress to investigate the mechanism of fengycin biosynthesis in detail. This work is part of a comprehensive investigation of the peptide cell factories in *Bacillus subtilis* to utilize their lipopeptide products in pharmaceutical and food industries and for plant protection.

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