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# Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332

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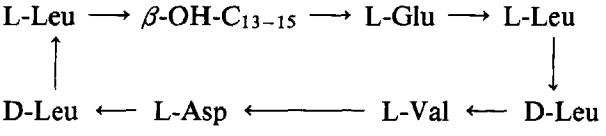
The biosynthesis of the lipopeptide antibiotic surfactin was studied in whole cells of *Bacillus subtilis* ATCC 21332 which incorporate $^{14}C$-labeled precursor amino acids directly into the product. [$^{14}C$]Acetate appeared in the fatty acid portion of surfactin and was also partially converted into leucine. An enzyme was isolated and partially purified from a cell-free extract of the bacillus which catalyzes ATP-$P_i$-exchange reactions which are mediated by the amino acid components of surfactin. This activation pattern is consistent with a peptide synthesizing multienzyme which activates its substrate amino acids simultaneously as reactive aminoacyl phosphates.

Surfactin; Lipopeptide biosynthesis; Precursor incorporation; Substrate activation

## 1. INTRODUCTION

Surfactin which is produced by *Bacillus subtilis* ATCC 21332 is one of the most efficient biosurfactants so far known [1,2]. It is a cyclic lipopeptide consisting of a heptapeptide and a lipid portion which is a mixture of several $\beta$-hydroxy-fatty acids with chain length of 13–15 carbon atoms. The main component is 3-hydroxy-13-methyltetradecanoic acid.




This agent has been isolated and characterized by Arima et al. [1,3,4] as well by Hosono and Suzuki [5,6].

We investigated the biosynthesis of surfactin in whole cells of *B. subtilis* ATCC 21332 by in vivo incorporation of $^{14}C$-labeled precursors and isolated an enzyme which shows ATP-$P_i$-exchange reactions mediated by the amino acid components of surfactin.

## 2. MATERIALS AND METHODS

$^{14}C$-labeled precursor amino acids, [$^{14}C$]acetate and $Na_2H^{32}PO_4$ were purchased from Amersham Buchler (Braunschweig, FRG). Silica Gel DC-60 sheets were obtained from Merck (Darmstadt, FRG). Chloramphenicol was a product of Boehringer (Mannheim, FRG).

### 2.1. *In vivo incorporation of precursors into surfactin*

Cultivation of *B. subtilis* ATCC 21332 and the purification of surfactin was performed, as reported [7]. Precursor incorporation into the lipopeptide was monitored by scintillation counting and thin-layer chromatography in combination with radioscanning. Samples of $^{14}C$-labeled surfactin or hydrolysates of the antibiotic dissolved in ethanol were spotted on Merck silica gel DC-60 sheets (20 × 20 cm). The following solvent mix-

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tures were used as mobile phases: (i) chloroform-methanol-water 65:25:4 (by vol.); (ii) chloroform-methanol-acetic acid 65:25:4 (by vol.); (iii) butanol-acetic acid-water 4:1:1 (by vol.). The $^{14}$C-labeled products were detected using a Berthold Linear Analyzer LB 2832.

2.2. *Amino acid analysis and mass spectrometry of surfactin hydrolysates*

The ring structure of surfactin was opened by hydrolysis of the ester linkage between L-Leu and the $\beta$-hydroxy-fatty acid component with 1 N NaOH at 40–50°C for 4 h. The lipopeptide was hydrolyzed in this form with 6 N HCl at 110°C for 24 h in sealed, evacuated tubes. The amino acid composition of the hydrolysate was monitored qualitatively by thin-layer chromatography using solvent mixture III for separation and ninhydrin for staining. $^{14}$C-labeled precursors were taken as reference compounds. Quantitative automated amino acid analysis was performed using a Durrum analyzer D-500.

Surfactin and its fatty acid portion were analyzed by fast atom bombardment mass spectrometry using a vacuum generator ZAB-3HF mass spectrometer (BEB configuration). For the mass spectroscopical analysis surfactin was dissolved in dimethylsulfoxide and mixed with an equal amount of a 5:1 mixture of dithiothreitol and dithioerythritol. Positive ions were detected. The fatty acid component was taken up in a dilute aqueous solution of ammonia which was mixed with an equal volume of glycerol. In this case negative ions were detected.

2.3. *Preparation of a cell-free system from* B. subtilis

*B. subtilis* cells were harvested in the late exponential phase of the growth. 40 g of the cell material was suspended in 200 ml 50 mM Tris-HCl buffer, pH 7.8, 1 mM EDTA and 1 mM dithioerythritol (buffer A). Protoplasts were generated by incubation with lysozyme (1 mg/ml; 20 min at 30°C) and disintegrated by French press treatment (4000 lb/in$^2$). The cell fragments were removed by centrifugation (25000 × *g*; 20 min). In the supernatant nucleic acids were precipitated with 1% streptomycin sulfate. In the crude extract an ammonium sulfate fractionation in steps of 10% each was performed between 40 and 80% saturation of the precipitant. The resulting pellets were dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.8; 0.5 mM EDTA; 1 mM dithioerythritol (buffer B) and dialyzed against 4 × 1 l of buffer B. These fractions were screened for the activation of the amino acid components of surfactin using (i) the thioester formation assay [8,9], and (ii) the ATP-$P_i$-exchange technique [10,11].

## 3. RESULTS AND DISCUSSION

It has been shown previously that the biosynthesis of surfactin appears in the exponential phase of the bacterial growth of *B. subtilis* and continues over a wide range of the cell cycle [12]. Optimal rates of lipopeptide formation were observed at the end of the logarithmic phase.

Biosynthesis of surfactin was monitored by in vivo incorporation of the $^{14}$C-labeled precursor amino acids and [$^{14}$C]acetate which were efficiently incorporated into the lipopeptide, as demonstrated in table 1. Labeled surfactin was isolated from the culture medium and detected by scintillation counting as well as by thin-layer chromatography in combination with radioscanning. Three solvent mixtures have been used as mobile phases which are specified in section 2. The following $R_f$ data were obtained for surfactin: $R_f$ (i) = 0.65; $R_f$ (ii) = 0.78 and $R_f$ (iii) = 0.85.

Precursor incorporation into the lipopeptide was controlled by hydrolysis (6 N HCl at 110°C for 24 h). The hydrolysates were analyzed by thin-layer chromatography and radioscanning as well as by high resolution cation exchange chromatography on Aminex Q 15 S. The amino acid components of surfactin were directly incorporated into the lipopeptide. They were identified in the hydrolysates by cochromatography with the authentic $^{14}$C-labeled precursor amino acids and additionally by amino acid analysis. For example, if L-[$^{14}$C]leucine was used as tracer, this amino acid appeared in labeled form in the product.

If, however, [$^{14}$C]acetate was added to the growing culture of *B. subtilis* two radioactive components were detected after hydrolysis of surfactin. One of them has been identified as [$^{14}$C]leucine ($R_f$ (iii) = 0.46–0.48). Obviously acetate is partially converted by the bacillus into leucine presumably via acetyl-CoA which is condensed with $\alpha$-ketoisovaleric acid in the leucine pathway. The other compound showed a $R_f$ (iii) = 0.90. It was excluded from the Aminex column. Obviously it is negatively charged. This substance was extracted from the hydrolysates with chloroform. It was identified as the fatty acid portion of surfactin by fast atom bombardment mass spectrometry. Mass numbers $[M-H]^-$ of 243 and 257 were detected in a ratio of ~4:5 corresponding

Table 1

Incorporation of $^{14}$C-labeled precursors into surfactin

| Precursor | Incorporation of precursor (cpm) |
|---|---|
| L-[$^{14}$C]Leu | 907140 |
| L-[$^{14}$C]Val | 631080 |
| L-[$^{14}$C]Asp | 160950 |
| U-[$^{14}$C]acetate | 440060 |

to the $C_{14}$ and $C_{15}$ components of the fatty acid moiety of surfactin. Using our hydrolysis procedure a part of these fatty acids appeared in a dehydrated form ($[M-H]^-$: 239 and 225) which presumably represents the $\alpha,\beta$-unsaturated compounds. Again a ratio of 4:5 was obtained for the $C_{14}$ and $C_{15}$ fatty acids. These results were corroborated by FAB mass spectrometry of the complete lipopeptide. For example, three mass numbers $[M+Na]^+$ of 1031, 1045 and 1059 were measured in a ratio of 1:4:5 which were assigned to the surfactin variants containing either a $C_{13}$, $C_{14}$ or $C_{15}$ hydroxy fatty acid as structural element. These data are in agreement with previous reports [4–6] which characterized surfactin as a mixture of closely related species which vary in their fatty acid portion.

The formation of surfactin occurs nonribosomally similar to numerous other peptide antibiotics [13–18], as demonstrated in the presence of inhibitors for protein biosynthesis [13–15]. If, for example, chloramphenicol was added to growing cultures of *B. subtilis* in the exponential phase at concentrations of 0.1–0.5 mg/ml, the growth of the microorganism and the incorporation of $^{14}C$-labeled amino acids into cellular proteins were strongly inhibited. In contrast lipopeptide biosynthesis proceeded at a high rate.

Two mechanisms for substrate amino acid activation in nonribosomal biosynthesis of bioactive peptides have been reported [16,18]. Several multienzymes, such as gramicidin S synthetase, have been characterized in detail [13–17] which activate their substrates in a two-step mechanism involving aminoacyl adenylate and thioester formation (thiotemplate mechanism). Well-known examples for the second activation mechanism are the synthetases for glutathione [19] or mycobacillin [20], which obviously activate their substrate amino acids in one step as aminoacyl phosphates.

We prepared a cell-free system from *B. subtilis* in order to clarify the mode of substrate activation in surfactin biosynthesis. Protein fractions obtained by ammonium sulfate fractionation of the crude cell extract were screened for activation reactions using the thioester formation and the ATP-$P_i$-exchange assays. In none of these fractions could

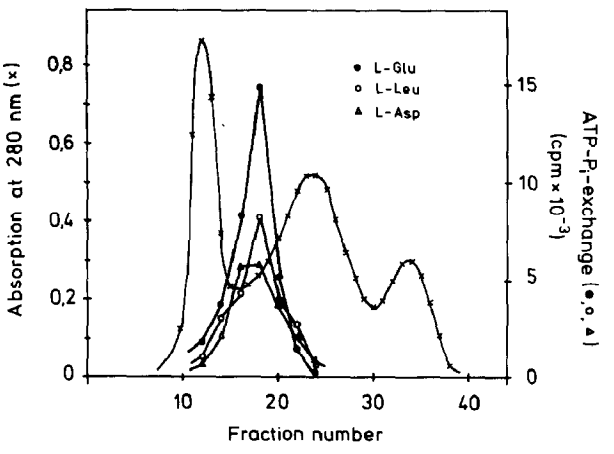


Fig.1. AcA34 gel filtration of an enzyme which catalyzes ATP-$P_i$-exchange reactions mediated by the amino acid components of surfactin. The precipitate obtained by ammonium sulfate fractionation in the range of 40–50% saturation of the precipitant was dissolved in a minimum volume of buffer B and loaded on the AcA34 column (53 × 2 cm). Elution was performed with the same buffer. Flow rate: 100 ml/h. Temperature: 4°C.

Table 2

Detection of an enzyme fraction after AcA34 gel filtration of a crude extract of *B. subtilis* ATCC 21332 which catalyzes ATP-$P_i$-exchange reactions mediated by the amino acid components of surfactin

| Amino acid | ATP-$P_i$-exchange (cpm) |
|---|---|
| L-Glu | 14297 |
| L-Leu | 10535 |
| D-Leu | 7661 |
| L-Val | 5317 |
| L-Asp | 5319 |
| L-Gly | – |
| L-Ala | – |
| L-Pro | – |
| L-Ile | – |
| L-Gln | – |
| L-Asn | – |
| L-Ser | – |
| L-Thr | – |
| L-Phe | – |
| L-Tyr | – |
| L-Lys | – |

The ATP-$P_i$-exchange reactions were measured at 37°C for 10 min. Composition of the reaction mixture: 100 mM Tris-HCl buffer, pH 7.8; 3 mM ATP; 5 mM amino acid; 5 mM $MgCl_2$; ~10 $\mu$M $^{32}P_i$ (1 $\mu$Ci/assay); 2 mM dithioerythritol; 10 mM NaF. Exchange reactions were stopped by the addition of 1 ml Norit A mixture. $^{32}P$-labeled ATP was detected by scintillation counting

thioester incorporation of amino acids into protein be detected. In the protein portion precipitated in the range of 40–50% ammonium sulfate, however, ATP-$P_i$-exchange reactions were observed which are mediated specifically by the surfactin components (L-Leu, D-Leu, L-Val, L-Asp, L-Glu). After AcA34 gel filtration (fig.1) these activities appeared at the same elution volume (table 2). These results are consistent with a peptide synthesizing system which activates its substrate amino acids simultaneously as aminoacyl phosphates. This activation pattern is different from the reaction sequence shown for glutathione or mycobacillin synthetase [19,20] which form reactive aminoacyl phosphates only with the start amino acid followed by a sequential activation of the peptide intermediates formed in the elongation process. The purification and characterization of surfactin synthetase is in preparation.

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