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Cell-Free Biosynthesis of Surfactin, a Cyclic Lipopeptide Produced by *Bacillus subtilis*†

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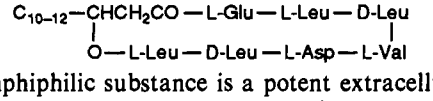
ABSTRACT: The lipopeptide antibiotic surfactin is a potent extracellular biosurfactant produced by various *Bacillus subtilis* strains. Biosynthesis of surfactin was studied in a cell-free system prepared from *B. subtilis* ATCC 21332 and OKB 105, which is a transformant producing surfactin in high yield [Nakano, M. M., Marahiel, M. A., & Zuber, P. (1988) *J. Bacteriol. 170*, 5662–5668]. Cell material was disintegrated by treatment with lysozyme and French press. A cell-free extract was prepared by ammonium sulfate fractionation, which catalyzed the formation of surfactin at the expense of ATP. Lipopeptide biosynthesis required the L-amino acid components of surfactin and D-3-hydroxytetradecanoyl-coenzyme A thioester. D-Leucine which is present in surfactin was not utilized but inhibited the biosynthetic process. The structure of surfactin, synthesized enzymatically in vitro, was confirmed by chromatographic comparison with the authentic compound and by amino acid analyses. An enzyme fraction was prepared by gel permeation chromatography which catalyzed ATP/pyrophosphate exchange reactions dependent on the component amino acids of surfactin. This enzyme fraction was capable of binding substrate amino acids covalently, probably via thioester linkages. The formation of these intermediates was inhibited by various thiol blocking reagents and phenylmethanesulfonyl fluoride. De novo synthesis of the lipopeptide was not observed with this partially purified enzyme fraction most likely due to the lack of an acyltransferase activity required for linking the β-hydroxy fatty acid to the peptide moiety.

Surfactin belongs to a class of cyclic lipopeptide antibiotics produced by various strains of *Bacillus subtilis*. It consists of a peptide moiety containing seven amino acids in combination with a β-hydroxy fatty acid which occurs as a mixture of closely related variants depending on the composition of the fermentation medium (Kakinuma et al., 1969a,b,c,d):



This amphiphilic substance is a potent extracellular biosurfactant with antifungal properties and antitumor activity against Ehrlich ascites carcinoma cells (Kameda et al., 1974). Surfactin inhibits fibrin clot formation and lyses erythrocytes and several bacterial spheroplasts and protoplasts (Arima et

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al., 1968; Bernheimer & Avigad, 1970). It functions as an inhibitor of cyclic-AMP phosphodiesterase (Hosono & Suzuki, 1983a).

Biosynthesis of surfactin has been studied using growing cells of *B. subtilis* ATCC 21332 and was found to be independent of ribosomal protein synthesis, as was demonstrated by product formation in the presence of chloramphenicol (Kluge et al., 1988). Also shown was the in vivo incorporation of ^{14}C-labeled precursors (L-leucine, L-aspartic acid, L-valine, and acetate) into surfactin with optimal rates of product formation in the late exponential phase of bacterial growth (Vater, 1986).

In this paper, we report on the de novo biosynthesis of surfactin in a cell-free extract of *B. subtilis* ATCC 21332 and OKB 105, which is a surfactin-producing derivative of *B. subtilis* 168 (Nakano et al., 1988). Furthermore, the partial purification of an enzyme fraction is described which activates and binds the surfactin-constituting amino acids in thioester linkage.

Materials and Methods

Radioisotopes and Chemicals. ^{14}C-Labeled amino acids, sodium [^{32}P]phosphate, and tetrasodium [^{32}P]pyrophosphate were purchased from Amersham/Buchler (Braunschweig, FRG). Coenzyme A (lithium salt) was from Sigma. All other chemicals purchased were reagent grade.

Syntheses. 3-Hydroxytetradecanoic acid was synthesized in a Reformatzky reaction between laurylaldehyde and ethyl bromoacetate in the presence of active zinc (Frankenfeld & Werner, 1969) and subsequent saponification of the ester with 4% KOH in ethanol/water (9:1 by volume) (Mikkelsen, 1984). Separation of racemic 3-hydroxytetradecanoic acid into its enantiomers was performed by ephedrine resolution.

The protocol of Hiramoto et al. (1971) was followed for the synthesis of the *N*-hydroxysuccinimide ester of the β-hydroxy fatty acid. From this compound, the coenzyme A thioester (βHA-CoA)[1] was prepared as described by Blecher (1981). *N*-(3-Hydroxytetradecanoyl)-L-glutamic acid was synthesized by the procedure of Lapidot et al. (1967). βHA-CoA[1] was stored frozen in 25 mM potassium phosphate buffer (pH 5.3). It was stable for several months. Purity was checked by reversed-phase HPLC using the solvent system of Woldegiorgis et al. (1985) and by GC–MS analysis after hydrolysis in methanolic HCl solution. βHA-CoA concentrations were determined spectrophotometrically (Stadtman, 1957) and by HPLC using decanoyl-CoA as a standard.

Bacterial Strains and Growth Media. B. subtilis ATCC 21332 was maintained on 1.5% agar slants of the medium introduced by Landy et al. (1948). Fermentation was performed at 28 °C in a New Brunswick Environmental Shaker G-25 (120 rpm) in 2-L baffled Erlenmeyer flasks containing 500 mL of the Landy medium. A 1% inoculum was provided by a 100-mL culture grown for 14 h. After 27–30 h of fermentation, cells were harvested by centrifugation, and the supernatant was assayed for surfactin. Cells were stored frozen (−30 °C).

B. subtilis OKB 105, which produces surfactin in high yield, was supplied by Dr. P. Zuber (Shreveport, LA). For maintenance and cultivation, the Landy medium was supplemented with 0.1% yeast extract (Difco, Detroit, MI) and L-phenylalanine (2 mg/L).

Surfactin Purification. Surfactin was precipitated from the culture medium by adjusting to pH 2 with HCl, and collected by centrifugation. The precipitate was extracted with ethanol, decolorized on activated charcoal GR (Merck, Darmstadt, FRG), and subjected to gel filtration chromatography on Sephadex LH 20 (Pharmacia/LKB, Freiburg, FRG). Ethanol was used as eluent. Analytical and semipreparative separations of the surfactin mixture were carried out on a Hypersil 5 ODS, 5 μm (250 × 4.6 mm), with a guard column from HPLC Technology Ltd. (Macclesfield, U.K.). Elution was performed with a linear gradient of methanol in 10 mM ammonium acetate, pH 6.9; the flow rate was 0.5 mL/min, and elution was monitored at 220 nm. An LKB HPLC system was used.

Thin-Layer Chromatographic Analyses and Solvents. Silica gel 60 plates (Merck) were used with the following solvent systems: I, $CHCl_3/CH_3OH/H_2O$ (65:25:4); II, $CHCl_3/CH_3OH$/25% NH_4OH (65:25:4); III, BuOH/HOAc/H_2O (4:1:1). For lipopeptide extraction, CH_2Cl_2/EtOAc (1:30, solvent IV) saturated with water was used.

Amino Acid Analysis. Surfactin was hydrolyzed with 6 N HCl (0.1% thioglycolic acid and 0.2% phenol) at 110 °C for 24 h in sealed, evacuated tubes. Amino acid composition and chirality were determined by HPLC analysis of diastereoisomers formed on reaction of the amino acids with N^2-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (Pierce, Rockville, MD) according to Marfey (1984). Chromatographic resolution was achieved with a 250 × 4 mm Hibar column (5 μm, LiChrospher 100 CH-8/II) from Merck with a solvent system as described by Kochhar and Christen (1989).

Configuration Analysis of β-Hydroxy Fatty Acids. Surfactin was hydrolyzed with 12 N HCl/methanol (3:7) at 90 °C for 24 h in sealed tubes. The solution was concentrated and extracted with chloroform. The lipophilic extract was purified by TLC as described by Nishikiori et al. (1986), yielding a mixture of methyl-3-hydroxy fatty acids. Its optical rotation at 589 nm was measured in $CHCl_3$ using a Polartronic D polarimeter (Schmidt/Haensch, Berlin, FRG).

Secondary Ion Mass Spectrometry. Secondary ion mass spectra of surfactin were obtained in a positive and negative ion mode on a time-of-flight mass spectrometer using 10-keV Ar^+ as the primary ion.

Preparation of a Cell-Free Enzyme System (AS-Fraction).[2] *B. subtilis* cells were suspended in buffer A [50 mM Tris-HCl, pH 7.8, 5 mM DTE, 1 mM EDTA, and 20% (w/v) sucrose] and incubated with lysozyme (1 mg/mL) at 30 °C for 15 min. All further operations were carried out at 4 °C. The protoplasts were passed once through a French press at 4000 psi. The cell debris was separated by centrifugation (25000*g*, 20 min). In the supernatant, removal of nucleic acids was achieved by precipitation with 1% streptomycin sulfate and stirring for 25 min. After centrifugation, the supernatant was subjected to ammonium sulfate fractionation. The precipitate formed in the range of 30–70% saturation was dissolved in a minimal volume of buffer B [50 mM Tris-HCl, pH 7.8, 5 mM DTE, 0.25 mM EDTA, and 10% (w/v) sucrose] and extensively dialysed against the same buffer. AS-fraction was kept frozen after addition of half a volume of glycerol without significant loss of activity within 2 weeks.

In Vitro Formation of Surfactin. The reaction mixture contained 10 mM ATP, 10 mM $MgCl_2$, 13 mM DTE, 5 mM each of L-Asp, L-Glu, and L-Val, 0.5 mM L-[^{14}C]Leu (0.5 μCi), 80 mM Tris-HCl (pH 7.8), 0.8 mM DL-3-hydroxytetradecanoyl-CoA, 1.3 mM potassium phosphate, 1 mM

[1] Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; βHA, 3-hydroxytetradecanoic acid; βHA-CoA, CoA thioester of βHA; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

[2] "AS-fraction" refers to cell-free extract prepared by ammonium sulfate fractionation.

EDTA, and AS-fraction. The total volume was 0.5 mL. The samples were incubated at 37 °C for various time intervals. The reaction was stopped by acidification with HCl. After addition of 50 μg of surfactin as a carrier, the reaction mixture was extracted 3 times with 2-mL portions of solvent IV. After evaporation of the combined organic phase, the residue was subjected to TLC in solvent system I. Detection by radioscanning and quantitation was achieved with a Berthold linear analyzer and data system LB 500. A calibration curve was obtained with L-[^{14}C]leucine as a standard. In some cases, surfactin was extracted from the thin-layer plate, purified by HPLC, and subjected to amino acid analysis.

Alternatively, a filter binding test was used. In this case, the reaction was stopped by incubation with 3 M hydroxylamine (pH 7.9) for 20 min at 60 °C, liberating amino acids covalently linked to the enzyme (Gevers et al., 1969). Five milliliters of 5% TCA was added, and the vial was left on ice (30 min). The precipitate was collected on a glass fiber disk (no. 92; Schleicher & Schuell, Dassel, FRG) and washed with 5 mL of 5% TCA, 3 times with 5 mL of 1 mM HCl, and once with water. The filter was dried, and radioactivity was determined in a Packard liquid scintillation analyzer using Quickszint 501 scintillation cocktail (Zinsser Analytic, Frankfurt, FRG). In parallel experiments, the dry filter was extracted with solvent IV and ether, successively, and the radioactive product was cochromatographed with authentic surfactin treated in the same way (R_f = 0.73 in solvent system I).

Gel Permeation Chromatography. Five milliliters of AS-fraction was applied to an Ultrogel AcA-34 column (LKB, 42 × 2.6 cm) previously equilibrated with buffer B. Fractions of 3.5 mL were collected. Active fractions were stored in 30% glycerol at −30 °C.

ATP/PP$_i$ Exchange. Substrate amino acid dependent ATP/PP$_i$ exchange reactions were measured as published by Lee and Lipmann (1975) and Vater et al. (1985). Reaction mixtures (200 μL) contained 100 μL of enzyme, 50 mM Tris-HCl (pH 7.8), 2 mM ATP, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTE, 2 mM amino acid, and 0.5 mM tetrasodium [^{32}P]pyrophosphate. The samples were incubated for 10 min at 37 °C. The reaction was stopped by addition of 1% Norit A charcoal suspension, and radioactive ATP was determined as described.

ATP/P$_i$ Exchange. ATP/phosphate exchange reactions were measured as described by Ghosh et al. (1983).

Assay of Covalent Binding of Substrate Amino Acids by Surfactin Synthetase. The reaction mixture contained, in a total volume of 200 μL, 90 μM unlabeled L-amino acid, 50 mM Tris-HCl (pH 7.8), 2 mM ATP, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTE, 0.5 μCi of ^{14}C-labeled amino acid (specific activities: leucine, 282 Ci/mol; valine, 282 Ci/mol; aspartic acid, 224 Ci/mol; glutamic acid, 290 Ci/mol), and 100 μL of enzyme. Samples were incubated for 10 min at 37 °C. The reaction was stopped by addition of 5 mL of 5% cold TCA. After 30 min on ice, the precipitate was either collected on membrane filters (ME 25, Schleicher & Schuell) or pelleted by centrifugation. Filters were washed once with TCA and 3 times with water; after the filters were dried, the radioactivity was determined by scintillation counting.

Pelleted precipitates were washed, treated with performic acid, and analyzed by TLC as described by Keller (1987).

Results and Discussion

Comparison of the Surfactin Producer Strains. Studies on the biosynthesis of surfactin were initiated by using *B. subtilis* ATCC 21332 as a source of the surfactin-synthesizing enzyme system. They were extended to strain OKB 105, a *B. subtilis* 168 derivative rendered surfactin-producing by transformation with genomic DNA of strain ATCC 21332 (Nakano et al., 1988).

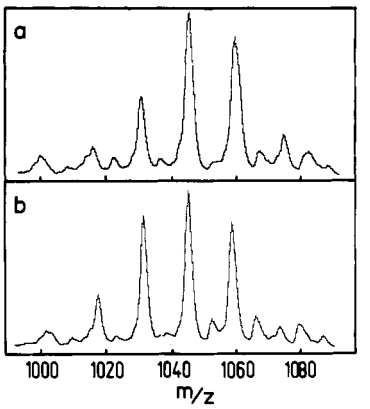

FIGURE 1: Secondary ion mass spectra of the surfactin mixture showing the [M + Na]$^+$ peak region. Surfactin was isolated from (a) *B. subtilis* ATCC 21332 and (b) OKB 105.

Lipopeptide material was isolated from the fermentation media of both strains, purified, and subjected to secondary ion mass spectroscopic analyses and amino acid determination. Surfactin obtained from both sources was identical in terms of total amino acid composition and revealed a very similar pattern of molecular ions on mass spectra indicating a similar distribution of fatty acid homologues in the products (Figure 1). The three main peaks in the [M + Na]$^+$ peak region correspond to surfactin with fatty acid chain lengths of 13, 14, and 15 carbon atoms. A detailed structural analysis of the β-hydroxy fatty acid species present in surfactin was published by Hosono and Suzuki (1983b). These authors detected iso- and anteiso-branched chain fatty acids, which contribute to the complexity of the lipopeptide mixture.

Because of its ability to produce surfactin in higher yield (about twice as much as ATCC 21332), strain OKB 105 was chosen for subsequent studies. Similar results have been obtained with ATCC 21332. In particular, identification of cell-free synthesized product and amino acid activation experiments were performed in parallel with both strains.

Cell-Free Formation of Surfactin. B. subtilis cells grown in the Landy medium were harvested in the late logarithmic phase of growth. A cell-free system (AS-fraction) was prepared by treatment with lysozyme and French press, followed by nucleic acid precipitation and ammonium sulfate fractionation as described under Materials and Methods.

The AS-fraction was able to catalyze total surfactin synthesis if supplied with ATP, Mg^{2+}, and the appropiate precursors. The requirements of surfactin formation are shown from the experiments listed in Table I. The hydroxy fatty acid component had to be present in the reaction mixture in the form of coenzyme A thioester. The enzyme system accepted neither the free fatty acid (in addition to CoASH) nor the synthetic *N*-(3-hydroxytetradecanoyl)-L-glutamic acid (not shown). The latter was offered as a possible intermediate in surfactin synthesis. All constituent amino acid components were accepted in the L form and incorporated into surfactin in the relative amounts of 4.3 (Leu), 0.9 (Asp), 1.0 (Val), and 1.0 (Glu), as determined by application of the respective ^{14}C-labeled substrate amino acid in the reaction mixture, which contained the amino acids in equimolar amounts. Under such conditions, leucine and valine were approximately 4:1. These data are in good agreement with the surfactin amino acid composition.

Table I: Requirements for Cell-Free Synthesis of Surfactin[a]

conditions	radioactivity incorporated into surfactin (cpm)
complete assay	75400
minus ATP	1000
minus βHA-CoA	900
minus Mg^{2+}	22000
minus L-Glu	9600
chloramphenicol added (50 μg/mL)	73600
D-Leu added (5 mM)	35900

[a] Experiments were done in duplicate (averaged values) as described under Materials and Methods. Incubation time was 90 min. Surfactin formation was determined by TLC and radioscanning.

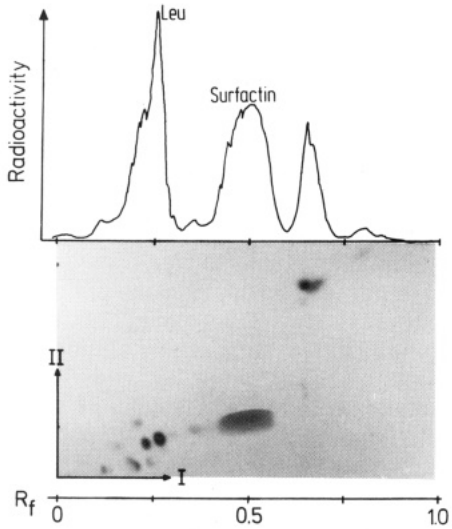

FIGURE 2: In vitro biosynthesis of surfactin by AS-fraction prepared from *B. subtilis* ATCC 21332 as monitored by [^{14}C]leucine incorporation. Incubation was as described under Materials and Methods. Lipopeptide material extracted from the reaction mixture was analyzed by two-dimensional TLC in solvent systems I and II. Radioactivity was detected by radioscanning (upper graph) and autoradiography. Exposure of the chromatogram to X-ray film was for 7 days. Compounds other than surfactin and leucine were not identified.

If D-leucine was added to the standard reaction mixture, incorporation of radioactivity into surfactin decreased. This was due to inhibition of the overall biosynthetic process, since amino acid analysis of recovered lipopeptide yielded D-[^{14}C]leucine and L-[^{14}C]leucine in a constant ratio (1.2), irrespective of the applied D-leucine concentration. This indicated that only the L enantiomer was accepted and subsequently racemized by the enzyme protein. The ratio of 1.2 observed in the case of the standard reaction mixture (10-fold excess of L-valine compared with the tracer L-[^{14}C]leucine) decreased to 1.0 if the concentration of L-valine in the reaction mixture was lowered to 0.5 mM, equal to that of the radiolabel L-leucine. The last finding suggested that L-valine might compete with L-leucine for incorporation into surfactin in amino acid position 2 or 7 of the peptide moiety, which is shown below.

Omitting sucrose from the buffers for cell lysis and extract preparation resulted in complete loss of biosynthetic activity.

Identification of in Vitro Synthesized Lipopeptide. The identity of the in vitro synthesized product with surfactin was demonstrated by TLC in various solvent systems and by reversed-phase HPLC in combination with amino acid analysis. In each case, highly purified surfactin synthesized in vivo by whole *B. subtilis* cells was used as the reference.

In Figure 2, the radioactively labeled lipopeptide extracted from the reaction mixture was analyzed by two-dimensional TLC in solvent systems I and II. It was detected by autoradiography and radioscanning. The position of the nonlabeled authentic carrier surfactin, determined by spraying the thin-layer plate with H_2SO_4 and charring, showed complete coincidence with the radioactive spot at $R_{f,I} = 0.5$, $R_{f,II} = 0.2$ (not shown).

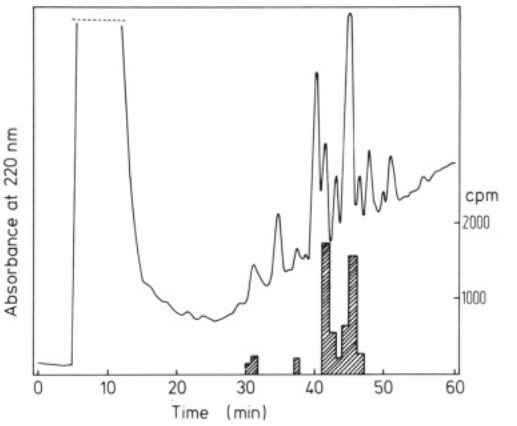

FIGURE 3: Identification of radiolabeled reaction products by HPLC. Reaction conditions were as given under Materials and Methods; [^{14}C]glutamate was used as the label. The surfactin mixture consisted of the radiolabeled enzymatically formed reaction products and the in vivo synthesized carrier surfactin. It was separated on a Hypersil 5 ODS column using a linear gradient of 70–88% methanol in 10 mM ammonium acetate within 59 min. Details are given under Materials and Methods. The dashed area represents radioactivity eluted.

In the HPLC experiment (Figure 3), the labeled reaction products coeluted with the reference surfactin. The HPLC analysis shows the pronounced microheterogeneity of surfactin, which is partially resolved into at least nine species showing hemolytic activity on blood agar plates. Essentially two labeled products appeared in the in vitro synthesized lipopeptide material although only one acyl-CoA species, β-hydroxytetradecanoyl-CoA, was used in the reaction mixture. This phenomenon was observed irrespective of the ^{14}C-labeled substrate used in the assay, provided that L-valine was present in 10-fold excess compared with L-leucine. In another experiment, L-[^{14}C]leucine and L-[^{14}C]valine were supplied simultaneously. The two reaction products were separated as demonstrated in Figure 3 and subjected to [^{14}C]amino acid analysis. The product eluting at $t = 41$ min contained labeled L-Val (1), L-Leu (2), and D-Leu (2), whereas the species at $t = 44$ min contained L-Val (2), L-Leu (1), and D-Leu (2). In neither case could D-[^{14}C]valine be detected. Thus, the influence L-valine exerts on the ratio of the leucine enantiomers found in surfactin produced in vitro can be explained by partial substitution in one L-Leu position of the surfactin molecule. This is corroborated by recent studies of surfactin structure and conformation using two-dimensional NMR methodology which revealed the presence of two congeners of surfactin in the lipopeptide material produced by *B. subtilis* ATCC 21332 as well as by OKB 105. The sequence of these species was determined by using rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY). A surfactin analogue was detected which contained valine as carboxy-terminal amino acid (Val_7) instead of leucine (F. Baumgart, B. Kluge, C. Ullrich, J. Vater, and D. Ziessow, unpublished experiments). It is interesting to notice that esperin, produced by *Bacillus mesentericus*, also contains a variable carboxy-terminal amino acid, being leucine (70%) or valine (30%). This antibiotic differs from surfactin only in such a way that cyclization occurs between the β-hydroxy group of the fatty acid and the β-carboxyl of the aspartate (Asp_4) residue (Thomas & Ito, 1969).

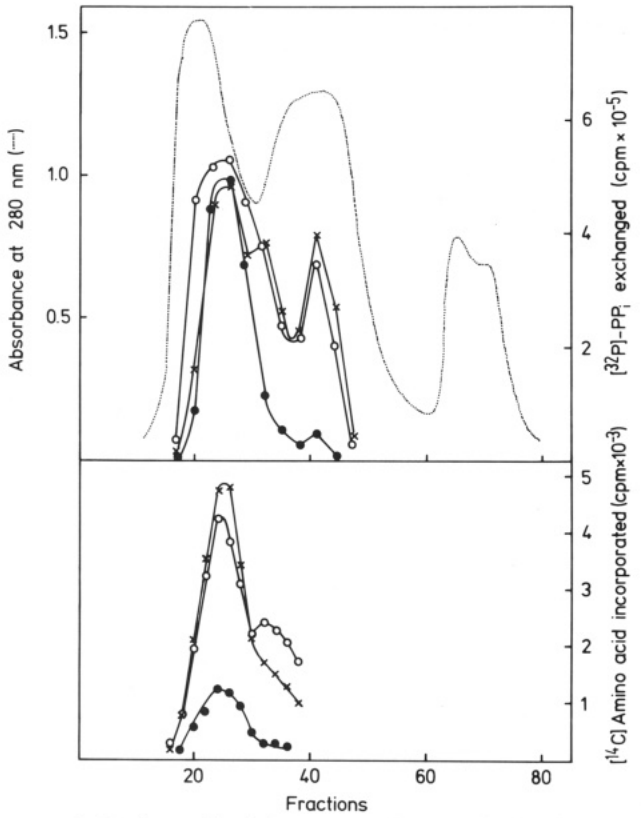

FIGURE 4: Elution profile of the AcA-34 column. Individual fractions of the AS-fraction chromatographed on AcA-34 were tested (a) for their ability to catalyze amino acid dependent ATP/[^{32}P]pyrophosphate exchanges and (b) for their capacity to bind the constitutive amino acids of surfactin as thioesters. Assay procedures are described under Materials and Methods. Values were corrected with blanks from incubations (a) without amino acid and (b) with boiled enzyme. Activation patterns are shown for L-leucine (○), L-valine (×), and L-aspartic acid (●). Effects similar to aspartic acid have been observed for L-glutamic acid (data not shown).

C-3 Configuration of the βHA-CoA Substrate. The influence of the C–3 configuration of the βHA–CoA substrate on the biosynthesis of surfactin was studied in order to establish which enantiomer is preferentially used by surfactin synthetase. Surfactin production was measured at 25 °C in the presence of βHA-CoA synthesized from racemic and enantiomerically enriched β-hydroxytetradecanoate. Three βHA-CoA mixtures were applied in a modified assay containing (a) 93% D/7% L, (b) 50% D/50% L, and (c) 30% D/70% L. The total βHA-CoA concentration was 0.16 mM. Samples were incubated for 30 min. Under these conditions, the following amounts of surfactin have been obtained: (a) 53 nmol; (b) 36 nmol; and (c) 31 nmol. Obviously, the D(−) form is favored as substrate by surfactin synthetase. This is consistent with a configurational analysis of the fatty acid components of in vivo produced surfactin. The mixture of the methyl esters of 3-hydroxy fatty acids obtained by hydrolysis of the product in methanol/HCl solution showed a negative optical rotation ($[\alpha]^{22}_{589} = -11°$) indicating D configuration at the C-3 position (Nishikiori et al., 1986).

For example, other lipopeptides from bacterial sources, like viscosin from *Pseudomonas viscosa* and plipastatins from *Bacillus cereus*, also contain D-β-hydroxy fatty acids (Hiramoto et al., 1970; Nishikiori et al., 1986).

Detection of an Enzyme Fraction Activating the Surfactin Amino Acid Components. The AS-fraction was loaded onto an AcA-34 gel permeation column and eluted with buffer B. The eluate was screened for the activation of the amino acid

Table II: Requirements for Enzyme–Leucine Complex Formation[a]

conditions[b]	relative [^{14}C]leucine binding activity (%)
complete assay	100
minus ATP	0
minus Mg^{2+}	7
modified assay	107
10 μM NEM added	11
10 μM DTNB added	22
1 mM iodoacetamide added	21
100 μM PMSF added	27

[a] The enzyme fraction was prepared by AcA-34 chromatography. Buffer B without DTE was used. [b] Complete reaction mixture is described under Materials and Methods. The enzyme fraction was preincubated with DTE for 10 min at 37 °C. The modified assay was performed in the absence of DTE. The enzyme fraction was preincubated with inhibitors for 10 min at 37 °C before reactants were added.

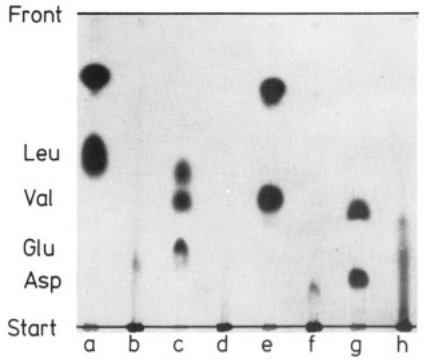

FIGURE 5: Liberation of covalently bound amino acids by treatment with performic acid after labeling of the enzyme with the respective [^{14}C]amino acid substrate: leucine (lane a), glutamic acid (lane c), valine (lane e), aspartic acid (lane g). Lanes b, d, f, and h represent control experiments with formic acid instead of performic acid using the labeled enzyme. TLC was carried out in solvent III. Exposure of the chromatogram to X-ray film was for 14 days.

components of surfactin by measuring ATP/pyrophosphate and ATP/phosphate exchange reactions, thus testing the two commonly encountered amino acid activation mechanisms in nonribosomal peptide synthesis (Kleinkauf & von Döhren, 1987). By use of the ATP/PP_i exchange technique, several enzyme activities were detected which are depicted in Figure 4. An enzyme fraction which appeared near the exclusion volume of the AcA-34 column catalyzed ATP/PP_i exchange reactions specifically mediated by the four component amino acids of surfactin. By use of a Sephacryl S-300 column of the same size, which affords a higher resolution in the upper molecular mass range, a molecular mass of 600 kDa was estimated for this multienzyme. It also shows covalent binding of the substrate amino acids, as demonstrated in Figure 4. The latter reactions were inhibited by sulfhydryl-specific blocking reagents as listed in Table II. A second activity with a molecular mass of approximately 280 kDa, dependent on leucine or valine, resided nearby the main peak with a slightly larger elution volume (fractions 30–35). This suggests the presence of more than one polypeptide in surfactin synthetase. The activities that appeared at higher elution volumes (fractions 38–50) were attributed to aminoacyl-tRNA synthetases.

The nature of the linkage between the multienzyme and its substrate amino acids was investigated. The complex was destroyed by treatment with dilute NaOH (pH 9–10), 3 M hydroxylamine (pH 7.9), and performic acid at elevated temperatures. The effect of performic acid is shown in Figure 5. In addition to the released amino acids, spots were observed, which presumably represent the corresponding formylated amino acids as established for *N*-formylvaline by TLC comparison (not shown). Thus, these covalent complexes behaved

like thioesters as characterized by Gevers et al. (1969).

The pattern of amino acid activation obtained with the partially purified surfactin synthetase fraction is consistent with the thiotemplate mechanism of nonribosomal peptide synthesis (Lipmann, 1971; Laland & Zimmer, 1973; Kurahashi, 1974). Several multifunctional peptide synthetases, such as gramicidin S synthetase [reviewed by Vater (1990)], follow this scheme by activating their substrates in a two-step mechanism involving aminoacyl adenylate and thioester formation. The ATP/P_i exchange reactions observed earlier (Kluge et al., 1988) turned out to be specific for surfactin amino acid components but varied significantly with different preparations. Most likely, these reactions do not represent intrinsic catalytic properties of the surfactin-synthesizing enzyme system but are due to unrelated enzyme activities channelling inorganic phosphate into the ATP/PP_i exchanges.

Only limited information is yet available on the biosynthesis of lipopeptides. More advanced studies of this kind have been performed for polymyxin (Komura & Kurahashi, 1980) and cyclosporin A (Billich & Zocher, 1987; Lawen & Zocher, 1990). The multienzymes, that are involved in the biosynthesis of these compounds, activate their substrate amino acids via adenylation and thiolation reactions (thiotemplate mechanism). For polymyxin and cyclosporin, complete in vitro biosynthesis has been obtained. However, knowledge is still lacking about the structural and functional organization of these multifunctional enzymes. In particular, it has to be clarified how the fatty acid component is attached to the peptide moiety. We expect an important contribution to the solution of these questions from a detailed characterization of surfactin synthetase.

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