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Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci

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This paper describes the chemical characterization of staphyloferrin B, a new complexone type siderophore isolated from low iron cultures of *Staphylococcus hyicus* DSM 20459. Purification of the very hydrophilic metabolite was achieved by anion exchange high performance liquid chromatography HPLC. Mass spectrometry showed a molecular mass of 448 amu. Hydrolysis with 8 M HCl revealed the presence of L-2,3-diaminopropionic acid, citrate, ethylenediamine and succinic semialdehyde. The connections between the four building blocks were determined by two-dimensional nuclear magnetic resonance measurements. UV/Vis and circular dichroism spectra are consistent with the proposed structure, which could also be confirmed by precursor feeding. The siderophore activity of staphyloferrin B was demonstrated by iron transport measurements.

Keywords: siderophore, iron transport, iron complex, *Staphylococcus hyicus*, staphyloferrin B

Introduction

Iron is an essential element for virtually all living organisms. Although it is the fourth most abundant element in the lithosphere, its bioavailability is limited by its very low solubility at neutral pH. Therefore microorganisms have developed specific uptake systems for iron of which the synthesis and uptake of iron-complexing molecules are essential elements (Neilands 1991). The low molecular weight siderophores have traditionally been divided in two groups, catecholate and hydroxamate siderophores. In recent years, the discovery of more hydrophilic siderophores established a separate class of iron chelators, the polycarboxylate (complexone type) siderophores (Winkelmann 1991), rhizobactin being the first representative (Smith *et al.* 1985). Meiwes *et al.* (1990) and Konetschny-Rapp *et al.* (1990) described the isolation and structure elucidation of a novel siderophore from *Staphylococcus hyicus*, named staphyloferrin A. In 1991 we published the structure elucidation of rhizoferrin (Drechsel *et al.* 1991), discovered in low iron cultures of *Rhizopus microsporus* var. *rhizopodiformis*. Rhizoferrin appeared to be the main siderophore of the Mucorales and Entomophthorales of zygomycetous fungi (Thieken & Winkelmann 1992). Staphyloferrin B, the structural elucidation of which is described here, turned out to be an extremely hydrophilic representative of the polycarboxylate type siderophores. It differs markedly from staphyloferrin A, its companion of staphylococcal origin, and it seems to be similar to vibrioferrin, the components of which have been published recently by Yamamoto *et al.* (1992).

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Materials and methods

Bacteria, media and growth conditions

Strains. For siderophore production we used *S. hyicus* DSM 20459. Further staphylococci strains examined for staphyloferrin B production are described by Haag *et al.* (1993). Biological activity was measured by using *S. epidermidis* ATCC 14990 as the recipient strain in the bioassay.

Growth conditions. Strains were grown on TY medium (8 g tryptone, 5 g yeast extract and 5 g NaCl per liter) or

on nutrient broth medium (8 g nutrient broth and 5 g NaCl per liter). The strains were stored at −70 °C in TY medium with 30% glycerol. A modification of the medium of Cove *et al.* (1980) was used for siderophore production. The medium is detailed in Table 1. Sterile stock solutions of vitamins (1 ml) and trace elements (5 ml) were added (Table 2). Details of the fermentation process, the use of additives for iron removal and the bioassay for siderophore detection are described by Haag *et al.* (1993).

Table 1. Growth medium for *S. hyicus* DSM 20459: components (in mg l^{-1} final concentration)

L-Alanine	200
L-Arginine	400
L-Aspartic acid	20
L-Cysteine	50
L-Cystine	50
L-Glutamic acid	900
L-Glycine	800
L-Histidine	400
L-Hydroxyproline	200
L-Isoleucine	100
L-Leucine	200
L-Lysine	300
L-Methionine	600
L-Phenylalanine	600
L-Proline	200
L-Serine	400
L-Threonine	400
L-Tryptophan	100
L-Tyrosine	200
L-Valine	200
Adenine	20
Guanine	20
Uracil	20
KH_2PO_4	1000
$(NH_4)_2SO_4$	2000
NaCl	1000
TRICINE	17900
Glucose	20000

Table 2. Stock solutions of vitamins and trace elements (mg l^{-1} stock solution)

Vitamins	
folic acid	10
biotin	6
p-aminobenzoic acid	200
thiamine–HCl	1000
pantothenic acid	1200
riboflavine	1000
nicotinic acid	2300
pyridoxine–HCl	12000
cyanocobalamine	100
Trace elements	
$CaCl_2$	1000
$MgSO_4 \cdot 7H_2O$	1000

Biological activity

Iron transport experiments were performed as described by Meiwes *et al.* (1990), with minor modifications as described by Haag *et al.* (1993).

Analytical high-performance liquid chromatography (HPLC) (ion-pair chromatography).

A C18 reversed phase column (Nucleosil-ODS, particle size 5 μm, 125 × 4.6 mm i.d., Grom, Herrenberg, Germany) was used. $FeCl_3$ was added and the samples were separated by gradient elution. Solvent A consisted of 10 mM sodium phosphate buffer (pH 7.3) with 5 mM tetrabutylammonium hydrogensulfate, solvent B was acetonitrile. The gradient was from 15 to 28% B in 3 min, from 28 to 37% in 4 min and raised to 60% in 1 min. The flow rate was 2 ml min^{-1}. Detection was at 340 nm.

Preparative HPLC

Preparative HPLC was performed on a Nucleosil Amin column (200 × 12 mm, 7 μm) using a flow rate of 6 ml min^{-1} and an isocratic elution with doubly distilled water containing 0.2% trifluoroacetic acid. Approximately 5 mg of crude product dissolved in 100 μl gradient grade methanol was injected for each run. Siderophore containing fractions were confirmed by analytical HPLC and Chrome Azurol S activity (Schwyn & Neilands 1987). Lyophilization yielded a colorless, grainy compound.

Capillary electrophoresis

Capillary electrophoresis was performed with an ABI 270A HT capillary electrophoresis system connected to a personal computer with a Kontron A/D card and software (Kontron, München, Germany) for data acquisition and storage. Samples were injected in a 72 cm fused silica capillary coated with 'microcoat' (Applied Biosystems, Weiterstadt, Germany) and run with −15 kV in a buffer of 20 mM citric acid (pH 2.5).

Mass spectra

Pneumatically assisted electrospray mass spectra were recorded on a Sciex API III triple quadrupole mass spectrometer with 2400 Da mass range equipped with an ion spray source (Sciex, Toronto, Canada) as described previously (Drechsel *et al.* 1991). Solutions of staphyloferrin B in methanol containing 1% formic acid were injected with a continuous flow of 2–5 μl min^{-1} and measured in positive mode.

Positive fast atom bombardment (FAB) mass spectra were recorded on a Varian MAT 711 instrument coupled with an SS 200 data system. The FAB spectra were measured from a matrix of glycerol. Temperature of the ion source was 303 K.

Gas chromatography (GC) and GC-mass spectrometry (MS)

Purified staphyloferrin B was hydrolyzed in 8 M HCl for 24 h. The hydrolysate was derivatized to methyl or *n*-propyl esters and pentafluoropropionamides or trifluoroacetamides and analyzed on a dimethylpolysiloxan PS 255 glass capillary with FID detector or on a Chirasil Val glass capillary (Frank *et al.* 1981) with N-selective detection. GC-MS of these derivatives was performed with a Varian MAT 112 S mass spectrometer with electron impact ionization.

Nuclear magnetic resonance (NMR) spectra

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AMX 400 instrument at 400 MHz (^{1}H) in D_2O at 305 K. Assignments were made by comparison with known compounds. Chemical shifts refer to 3-(trimethylsilyl)-2,2,3,3 d_4-sodium salt.

UV spectra

UV spectra were recorded on an Ultrospec III spectrometer (Pharmacia LKB, Freiburg, Germany) connected to a Commodore PC40 computer. Staphyloferrin B was dissolved in doubly distilled water at a concentration of 1.5×10^{-4} M at iron:ligand ratios of 1:1 and measured in quartz cuvettes with a path length of 1 cm at a temperature of 300 K at different pH values.

Circular dichroism (CD) spectra

CD spectra were recorded on a Jasco 720 CD spectrometer (Japan Spectroscopic Co. Ltd, Tokyo, Japan) equipped with a personal computer for data acquisition and processing. Samples and recording conditions were according to those for UV spectroscopy.

Results

Isolation of staphyloferrin B

The production of staphyloferrin B is coupled to the log-phase as to be expected for a siderophore and reaches its maximum after 13 h (Haag *et al.* 1993). After separating the biomass, the supernatant was subjected to alcoholic precipitation. The precipitate was further purified by chromatography on the anion-exchanger Dowex 21K (OH-form) eluted with 0.5N formic acid. The siderophore containing crude material was chromatographed on Biogel P2 (200–400 mesh), equilibrated with 10 mM ammonium acetate buffer, pH 4. The siderophore-containing fractions (activity tested by the Chrome Azurol S assay) were re-chromatographed with 10 mM ammonium acetate buffer, pH 6. The active fractions were chromatographed on Fractogel TSK HW 40, equilibrated with 10% methanol in doubly distilled H_2O. The active fractions were collected, concentrated and lyophilized. For further purification an HPLC anion exchange procedure was employed.

Due to the high hydrophilicity of the compound, interaction with reversed phase material was insufficient for separation of salt-like by-products of the crude material. An anion exchange column packed with Nucleosil Amine gave best separation when eluted with a gradient of increasing ionic strength of phosphate buffer, pH 3.4. Staphyloferrin B eluted at 145 mM phosphate and was well separated from its by-products (Figure 1). The fractions obtained contained about 0.4% w/w organic substance in potassium phosphate and were not suited for desalting. In order to get salt-free material, the elution method was changed to a gradient of pH using the volatile trifluoroacetic acid and finally optimized on an isocratic elution with 0.2% trifluoroacetic acid (Figure 2). The pooled fractions were lyophilized and yielded a colorless, grainy compound.

The general definition of siderophores requires *in vivo* transport of iron into the cells. The time dependent uptake of ^{55}Fe via staphyloferrin B in *S. hyicus* DSM 20459 cells is described by Haag *et al.* (1993). Although the stability constant of staphyloferrin B has not been determined so far, we assume a similar or slightly higher binding constant compared with iron dicitrate. The transport measurements revealed a linear uptake of ^{55}Fe within the range of 1–15 min. The amount of iron taken up was less than in the case of staphyloferrin A.

Structure elucidation

Capillary electrophoresis was carried out in a fused silica capillary (72 cm) coated with 'microcoat' (Applied Biosystems, Weiterstadt, Germany). The samples were dissolved in the analysis buffer (20 mM

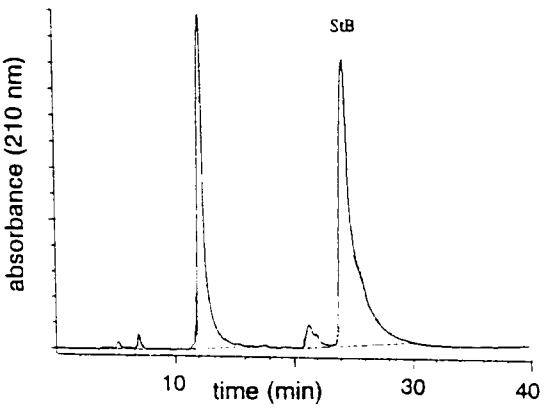

Figure 1. HPLC separation of staphyloferrin B on Nucleosil Amin, 200 × 12 mm, 7 μm, solvent A: doubly distilled H_2O, solvent B: 250 mM KH_2PO_4 pH 3.4, gradient from 5 to 100% within 40 min.

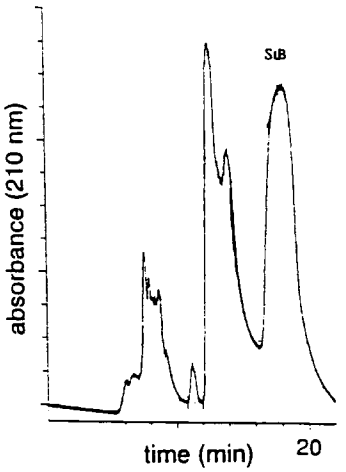

Figure 2. HPLC separation of staphyloferrin B on Nucleosil Amin 200 × 12 mm, 7 μm, isocratic elution with doubly distilled H_2O containing 0.2% TFA.

citric acid, pH 2.5), injected by vacuum and run with 15 kV, polarity negative at inlet side. The coating of the inner surface of the capillary produces an electro-osmotic flow, which will flush even uncharged components past the detector. The electric field of the applied polarity will then slow down positive and accelerate negative analytes. Referencing against the neutral marker mesityl oxide, staphyloferrin B exhibited a small net negative charge (Figure 3A). Its initial purity was approximately 90%, the impurity having opposite charge. After several days at room temperature in citric acid buffer the siderophore showed strong decomposition into at least three additional compounds and fragments (Figure 3B).

FAB mass spectrometric measurements showed the compound to have a molecular mass of 448 amu (Figure 4). Pneumatically assisted electrospray MS additionally indicated free carboxy groups by adducts of sodium and potassium ions, and the presence of hydroxy groups by water elimination from the molecular ion (Figure 5).

The purified siderophore was dissolved in D_2O and its one- and two-dimensional NMR spectra were recorded. ^1H spectra excluded the presence of methyl groups and unsaturated or aromatic residues. Constituent building blocks of the molecule were identified by $^1H/^1H$ and $^{13}C/^1H$ coupling as well as nuclear overhauser experiments (NOE) (data not shown). From the results of these NMR experiments the non-functionalized carbon skeleton could be derived. (Figure 6).

Based on this information the presumed hydrolysis products were compared with reference compounds using GC and GC-MS. Finally the hydrolysis

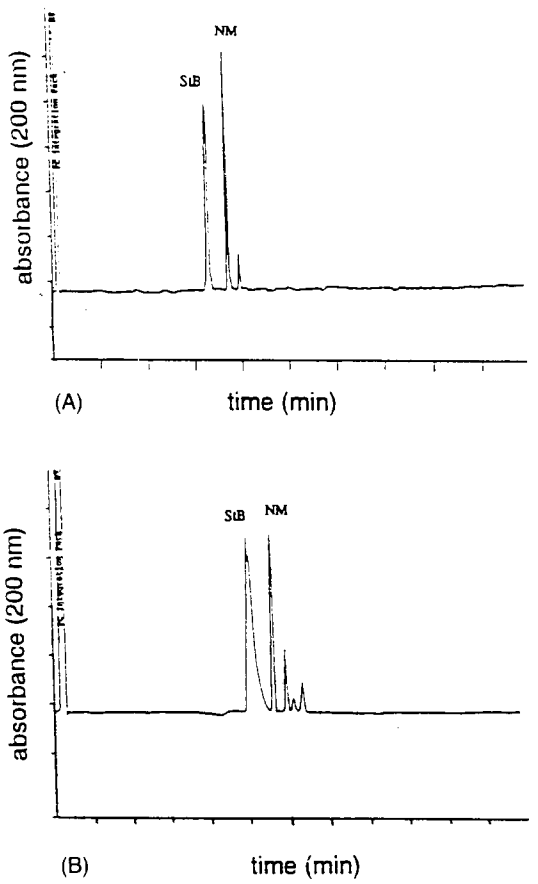

Figure 3. Capillary electrophoresis of HPLC purified staphyloferrin B. Capillarry fused silica, coated inside with microcoat (trademark of Aplied Biosystems), length 72 cm, voltage −15 kV, polarity negative at inlet side. Buffer 20 mM citric acid, pH 2.5, detector wavelength 200 nm. NM, neutral marker; StB, staphyloferrin B. (**A**) Fresh sample. (**B**) Sample after 3 days in 20 mM citric acid buffer, pH 2.5.

products could be confirmed as: L-2,3-diaminopropionic acid, 1,2-diaminoethane, citric acid and succinic semialdehyde. This analysis indicated that staphyloferrin B seems to have a similar structure to vibrioferrin, which consists of alanine, ethanolamine, citric acid and 2-ketoglutaric acid (Yamamoto *et al.* 1992). The configuration of 2,3-diaminopropionic acid in staphyloferrin B was determined unequivocally by GC on Chirasil Val to be the L form. 2,3-Diaminopropionic acid was found to be highly susceptible to racemization during hydrolysis. Time dependent measurement of nitrogen-containing hydrolysis products revealed an initially faster release of L-2,3-diaminopropionic acid, consistent with its terminal position and finally showed a precise 1:1 ratio with diaminoethane (Table 3).

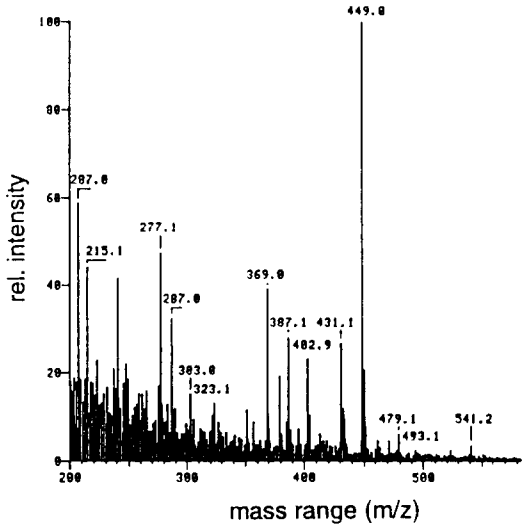

Figure 4. FAB mass spectrum of staphyloferrrin B in glycerol matrix, positive mode. Temperature of ion source 303 K.

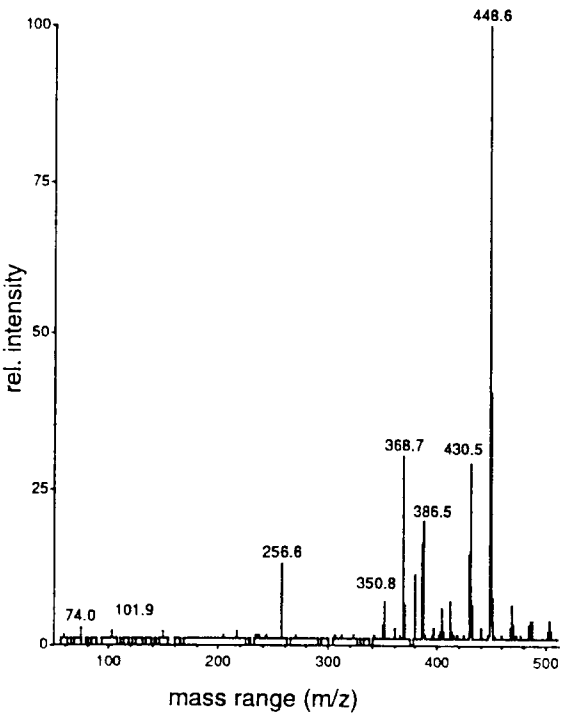

Figure 5. Atmospheric pressure ion spray mass spectrum of staphyloferrin B, positive mode; direct injection from a solution in MeOH, containing 1% formic acid.

Taking into consideration the molecular mass of 448 amu, we propose the structure given in Figure 7.

The ^{13}C NMR signals recorded from staphyloferrin B are in good accordance with literature data of its components (Table 4). The differences are consistent with the shifts associated with acylation of amines and amidation of the free acids. The hydration of α-keto acids has been described by Cooper & Redfield (1975). The data on 2,3-diaminopropionic acid, citric acid and 1,2-diaminoethane have been taken from Kalinowski *et al.* (1984). The data on 2-oxoglutaric acid has been measured in D_2O, pH 3.

2-Oxoglutaric acid could not yet be found in the hydrolyzed and derivatized samples. This may be due to its reported lability within certain pH ranges (Yamamoto *et al.* 1992), its tendency to form hydroxylactones, which might hydrolyze with decarboxylation, or due to trace contamination with d-block elements (Kalnitsky *et al.* 1953, Rackis *et al.* 1957). We are presently working on the synthesis of suitable amides of 2-oxoglutaric acid for comparison of hydrolysis behavior and NMR data.

UV spectra were recorded from mixtures of $FeCl_3 \cdot 6H_2O$ in doubly distilled H_2O and a stock solution of staphyloferrin B with a stoichiometric ratio of 1:1 and a final concentration of 1.5×10^{-4} M. The pH was varied from 3.0 to 9.5. UV spectra of desferri-staphyloferrin B demonstrate the absence of chromophores by having a single maximum at 202 nm. On addition of iron on an iron:ligand ratio of approximately 1:1, a charge transfer band at 330 nm appeared (Figure 8). The molar extinction coefficient could be calculated as $\varepsilon = 2600\ M^{-1}\ cm^{-1}$ (330 nm, pH 4.8). This UV absorption band as well as its pH dependency are very similar to the other carboxylate siderophores such as staphyloferrin A (Konetschny-Rapp *et al.* 1990), rhizoferrin, imidorhizoferrin (Drechsel *et al.* 1992) and analogs of rhizoferrin (unpublished results).

The CD spectrum of the iron-free ligand showed a

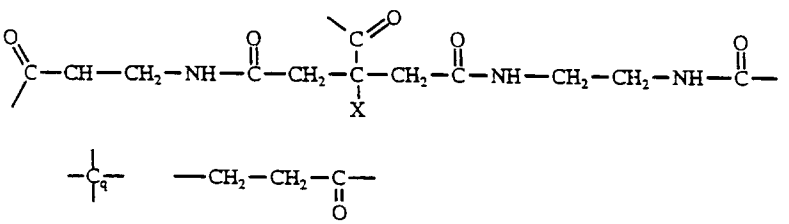

Figure 6. Non-functionalized carbon skeleton for staphyloferrin B.

Table 3. Time dependent hydrolysis of staphyloferrin B (mol mol siderophore^{-1})

Time of hydrolysis (h)	1,2-Diaminoethane	2,3-Diaminopropionic acid
4.5	0.49	0.79
21	0.87	0.85
37	0.86	0.84

single Cotton effect at 219 nm, with a molar ellipticity of 8307 deg cm^2 M^{-1}, indicating the presence of at least one chiral center in an unsymmetric molecule (Figure 9). CD spectra of the iron complexes were recorded in a series of increasing pH and constant iron:ligand ratio of approximately 1:1. A concentration of ligand of 1.5×10^{-4} M was chosen. By comparison with the iron-free ligand, a broad CD band stretching from 275 to 345 nm with a maximum of 2495 deg cm^2 M^{-1} at 296.0 nm, and a negative extremum of -2685 deg cm^2 M^{-1} at 238.2 nm are iron-dependent (Figure 9). At approximately pH 5 the CD spectra showed an irregular behavior associated with the breakdown of the ferric complex due to complexation of Fe^{3+} by hydroxide ions. This was observed visually by the appearance of turbidity due to iron oxide formation within several hours after preparation of the solutions.

Precursor feeding

Addition of D/L- or L-2,3-diaminopropionic acid to the growth medium resulted in a marked increase in the production of staphyloferrin B up to 2.5-fold compared with the control. Maximum increase was reached with concentrations of 3 mM L- or 5 mM D/L-diaminopropionic acid, as expected for the 1:1 racemic mixture (Table 5). This indicates that only the L-enantiomere is incorporated into staphyloferrin B. Higher amounts of 2,3-diaminopropionic acid led to a decrease in the production of staphyloferrin B. The reason why the inhibitory action of pure L-2,3-diaminopropionic acid is stronger than that of the racemic mixture is unclear. 2,3-Diaminopropionic acid is a very unusual amino acid. It is not known whether there are further compounds in *S. hyicus* which contain 2,3-diaminopropionic acid. Addition of 1,2-diaminoethane had only little effect

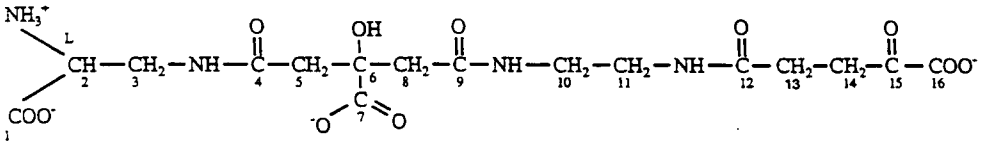

Figure 7. Proposed structure for staphyloferrin B.

Table 4. Comparison of ^{13}C chemical shifts between staphyloferrin B and its constituents

Staphyloferrin B D_2O, pH 3		Reference compound	
carbon atom (see structural formula)	chemical shift (p.p.m.)	carbon atom (IUPAC)	chemical shift (p.p.m.)
1	171.0	2,3-diaminopropionic acid, C-1	172.3
2	53.7	C-2	51.7
3	39.0	C-3	40.3
4	173.3	citric acid, C-1	174.2
5	44.0	C-2 (methylene)	44.1
6	73.5	C-3 (quaternary)	74.2
7	177.3	C-4 (COOH bound to C-3)	177.5
8	44.3	C-2	44.1
9	172.0	C-1	174.2
10	37.5	1,2-diaminoethane, C-1	44.3
11	39.1	1,2-diaminoethane, C-2	44.3
12	179.2	2-oxoglutaric acid, C-1	180.0
13	31.5	C-3	30.9
14	28.5	C-4	30.3
15	91.5	C-2	96.4 (hydrated)
16	175.0	C-5	176.8

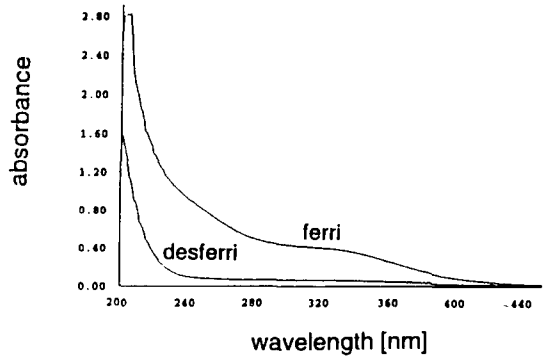

Figure 8. UV spectra of ferric staphyloferrin B in doubly distilled H_2O, $c = 1.5 \times 10^{-4}$ M; quartz cuvettes, optical pathlength 1 cm, pH 4.8. Lowest curve, desferri form of staphyloferrin B, pH 3.3.

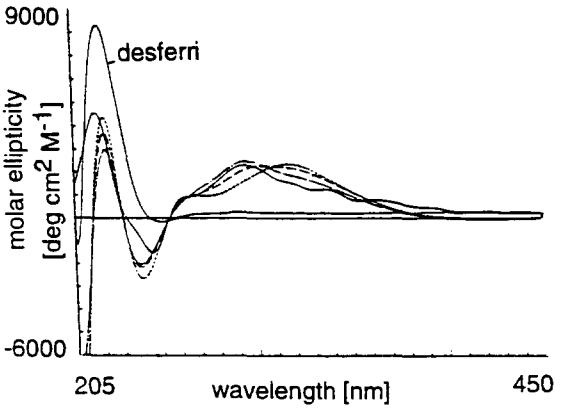

Figure 9. CD spectra of staphyloferrin B in doubly distilled H_2O, $c = 1.5 \times 10^{-4}$ M, quartz cuvettes, optical pathlength 1 cm, pH 3.6 (· · · ·), 4.35 (– – –), 4.8 (– · –), 5.5 (——); desferri form of staphyloferrin B, pH 5.3.

on the production of staphyloferrin B (data not shown). The experiments will be continued with ^{13}C and/or ^{15}N-labelled D/L-2,3-diaminopropionic acid to find out whether 1,2-diaminoethane is generated by decarboxylation of 2,3-diaminopropionic acid and whether this occurs before or after coupling to citric acid.

Discussion

Using the ion-pair HPLC system for the detection of staphyloferrin A (Meiwes *et al.* 1990), we found a further strong iron regulated compound, which we called staphyloferrin B. Extremely hydrophilic and anionic, staphyloferrin B turned out to be yet another representative of the polycarboxylate (complexone type) class of siderophores. UV spectra of the desferri form were very similar to staphyloferrin A and rhizoferrin (Konetschny-Rapp *et al.* 1990, Drechsel *et al.* 1991). UV spectra of the ferri form confirmed the absence of catecholate and hydroxamate groups by having a maximum at 330 nm and lacking charge transfer bands between 420 and 500 nm (Figure 8). The chemical tests for catechol and hydroxamate groups (Arnow 1937, Csaky 1948, Rioux *et al.* 1983) were both negative.

Isolation of the active compound was monitored using the CAS assay and the biological growth stimulation test. After alcoholic precipitation, staphyloferrin B could be chromatographed on the weak anion exchanger Dowex 21K. Size exclusion chromatography was performed in a similar manner as previously for the purification of staphyloferrin A

Table 5. Production of staphyloferrin B on addition of L- and D/L-2,3-diaminopropionic acid to the growth medium

Concentration of L-2,3-diaminopropionic acid (mM)	Production of staphyloferrin B (1000 integration units)	Concentration of D/L-2,3-diaminopropionic acid (mM)	Production of staphyloferrin B (1000 integration units)
0	1251	0	1251
1	2436	1	1609
2	2669	2	1819
3	3490	3	2110
4	992	4	2596
5	786	5	3505
6	668	6	3330
7	732	7	3085
8	695	8	2629
9		9	2487
10		10	2179
20		20	861
100		100	490

(Meiwes *et al*. 1990). Salts and several siderophore-inactive organic contaminants could be removed successfully by preparative HPLC with an anion-exchange column and acidified doubly distilled H_2O as the mobile phase.

Transport measurements revealed a linear uptake of ^{55}Fe within the range of 1–15 min. The amount of iron taken up was less than in the case of staphyloferrin A.

Structural elucidation was based complementarily on NMR experiments and GC-MS identification. Two dimensional NMR revealed the carbon–carbon skeleton without most of the functional groups. The siderophore was hydrolyzed and the constituent building blocks were measured on GC-MS. Their electron impact fragmentation pattern was compared with the presumed components compatible with the NMR data and confirmed by GC and GC-MS measurements of reference substances. The NMR data finally proved the sequence and the positions of the links of the constituent molecules. Chiral separation on Chirasil Val revealed the L-configuration of 2,3-diaminopropionic acid. The chirality inherent in the asymmetric substitution of citric acid requires isotopically labelled precursors and will be undertaken in the context of precursor feeding currently in progress to elucidate the pathways of biosynthesis. Further characterization of the siderophore was done with capillary electrophoresis, quantitative UV and CD spectroscopy.

The proposed structure of staphyloferrin B fits well to the complexone type. Lacking the symmetry of staphyloferrin A and rhizoferrin, it is a less potent chelator of iron. The similarity to staphyloferrin A, which had been presumed earlier, is limited to the common constituent citric acid. It has, however, a high degree of similarity to vibrioferrin, produced by *Vibrio parahaemolyticus* (Yamamoto *et al*. 1992), which still awaits complete structural elucidation.

Acknowledgments

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