Antiviral and Hemolytic Activities of Surfactin Isoforms and

Their Methyl Ester Derivatives

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Inactivation of enveloped viruses (VSV, SFV, and SHV-1) by surfactin lipopeptides was dependent on the hydrophobicity, *i.e.* the number of carbon atoms of the fatty acid, and on the charge of the peptide moiety as well as on the virus species. Surfactins with fatty acid chains of 13 carbon atoms showed very low antiviral activity in comparison to C14 and C15 isoforms. C15 surfactin monomethyl ester also inactivated SFV which was resistant to the mixture of surfactin isoforms as produced by *Bacillus subtilis*. In contrast, the dimethyl ester showed no virus-inactivation capacity. Disintegration of viral structures as determined by electron microscopy after inactivation of VSV and SFV was comparable to the titer reduction. The effect of the surfactin isoforms and methyl esters on erythrocyte hemolysis correlated with the virus-inactivation capacity. Surfactins with a fatty acid chain moiety of 15 carbon atoms and one negative charge showed the highest antiviral activity.

Bioactive peptides with diverse structures and biological activities have been isolated from various microorganisms.[1,2] *Bacillus subtilis*, a soil bacterium, produces non-ribosomally the cyclic lipopeptide antibiotic surfactin[2] showing biosurfactant,[3,4] antimicrobial,[5,6] antimycoplasmatic,[7] antiviral,[8~10] antitumor[11] and hemolytic[3,5] properties.

Surfactin consists of an anionic seven-membered peptide cyclo[acyl-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu] and a hydrophobic β-hydroxy fatty acid varying in length and branching of its carbon chain (for a review of these isoforms see ref. 12). The fatty acid is bound via its carboxyl group to the *N*-terminal glutamic acid and the lipopeptide ring is closed by lactone formation between the β-hydroxy group of the fatty acid and the carboxyl group of the *C*-terminal leucine. The number of carbon atoms of its β-hydroxy fatty acid varies from 13 to 15; the main lipid component is 3-hydroxy-13-methylmyristic acid.[13,14]

Several authors[8~10] have shown antiviral properties of surfactin, especially against enveloped viruses.[10] All these studies have been performed with the natural mixture of isoforms produced by certain *B. subtilis* strains. To examine the influence of hydrophobicity of the lipid part of the molecule, we have isolated by preparative reversed-phase HPLC several pure isoforms from the mixture which were characterized by NMR and MS.[12] Variation of the negative charge on the peptide moiety was obtained by preparation of methyl esters.[12,15]

Three enveloped viruses belonging to different virus families[16] were used for virus inactivation studies: vesicular stomatitis virus (VSV, rhabdoviridae), suid herpes virus type 1 (SHV-1, herpesviridae, a model virus for human herpes viruses), and Semliki Forest virus (SFV, Togaviridae, a model virus for the hepatitis C virus).

Materials and Methods

Surfactin, Surfactin Isoforms, and Surfactin Methyl Esters

Growth of organism, preparation of surfactin, syntheses

of esters and separation of isoforms were performed as reported.[12] Briefly, surfactin was purified from culture supernatants of *Bacillus subtilis* OKB 105 by acid precipitation, methanol extraction, charcoal treatment and gel filtration on Pharmacia LH 20. A stock solution of 1 mM surfactin in phosphate-buffered saline (PBS) was autoclaved at 121°C and stored at 4°C.

Surfactin isoforms were separated by preparative reversed-phase HPLC on EnCaPharm 100 RP18TS 5 μm with 10 mM ammonium acetate (A), pH 6.9 and acetonitrile (B), using a gradient of 10~15% B in 0~30 minutes and 15~20% B in 31~90 minutes. The detection wavelength was 220 nm. Three fractions were collected manually and freeze dried; the surfactin isoforms were identified by MALDI-TOF MS in negative mode.[12]

Surfactin monomethyl and dimethyl esters were synthesized by treating surfactin with methanolic HCl for 1~3 days, followed by isolation as above, using a gradient of 35~50% B in 0~30 minutes. Two surfactin monomethyl esters containing fatty acids with 14 and 15 C atoms and one C15 surfactin dimethyl ester were isolated. C15 indicates the number of carbon atoms of the fatty acid chain.

Cell Culture and Virus Propagation

Mink lung cells (ML) or baby hamster kidney cells (BHK-21) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM; ICN, Meckenheim, Germany), supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco, Eggenstein, Germany). Monolayers were infected with VSV, SHV-1 or SFV with a multiplicity of infection of 0.001. The supernatants were harvested when a pronounced cytopathic effect approx. 2~6 days past infection was visible. Aliquots of cell free supernatants were stored at −70°C. Titers of virus stocks for VSV, SHV-1, and SFV on ML cells were 5.6×10^9, 5.6×10^6 and 7.8×10^6 $TCID_{50}/ml$, respectively.

Treatment of Viruses with Surfactin

All incubation steps were performed at 22°C in a water bath. Virus stock was diluted 1 : 10 or 1 : 100 in 10 ml of DMEM without FCS. Lipopeptides were added (final concentration 40 μM) with permanent magnetic stirring. Aliquots were removed at 2, 5, 10, 15 and 20 minutes of incubation and diluted 1 : 3 or 1 : 10 with DMEM supplemented with 5% FCS to stop the effect of the lipopeptide. Then they were immediately titrated. Initial titer and the spontaneous inactivation of virus in medium were determined in parallel experiments. For all inactivation studies using isoforms and derivatives, the surfactin mixture was run as a control in parallel. Virus titers

were determined in a standard assay using a 96-well microtiter plate essentially as described.[10] Briefly, 100 μl of a cell suspension (5×10^4~5×10^5 cells/ml, depending on the virus) were placed in each well. The virus solutions were serially diluted 1 : 3 in DMEM supplemented with 5% FCS. Aliquots of 100 μl per dilution step were added to each of 8 wells of the microtiter plate. After 2~4 days of incubation the cultures were evaluated microscopically. The virus titer ($TCID_{50}/ml$) was calculated according to SPEARMAN and KÄRBER.[17]

Hemolysis of Human Erythrocytes

For hemolysis experiments, 1.4×10^7 freshly prepared human erythrocytes in 0.1 ml 0.15 M NaCl were added to 0.9 ml of serial dilutions of the various surfactin isoforms or derivatives. After incubation for 30 minutes in a water bath at 37°C, the optical density of the supernatant after centrifugation ($15,000 \times g$, 15 minutes) was determined at 540 nm in comparison to the control without lipopeptide added (no lysis). The complete lysis value obtained by adding 0.1 ml erythrocytes to 0.9 ml of distilled water was set as 100%.

Electron Microscopy

Virus was propagated in BHK-21 cells in DMEM supplemented with 3% FCS. SFV and VSV were harvested 31 hours and 23 hours past infection, respectively, the supernatant cleared from debris by centrifugation (20 minutes, $200 \times g$). Virus-containing supernatants were incubated with surfactin or C15 surfactin monomethyl ester (40 μM for SFV, 33 μM for VSV) for 1 hour at room temperature. Controls without surfactin were run in parallel. Incubation was stopped by adding 10 ml of cold 0.2 M HEPES buffer (pH 7.2), and the mixture ultracentrifuged through a layer of 4 ml 25% sucrose in PBS (Beckman Optima, rotor SW28; 2 hours, $100,000 \times g$ at 4°C). Pellets were resuspended in 0.5 ml HEPES buffer and aliquots used for determination of infectious virus. After negative staining with 2% phosphotungstic acid (PTA) or uranyl acetate (UAC) on Pioloform-(Wacker Chemie–München)-coated and carbon stabilized 300 mesh grids, the specimens were examined by electron microscopy (Zeiss EM 902, 80 kV).
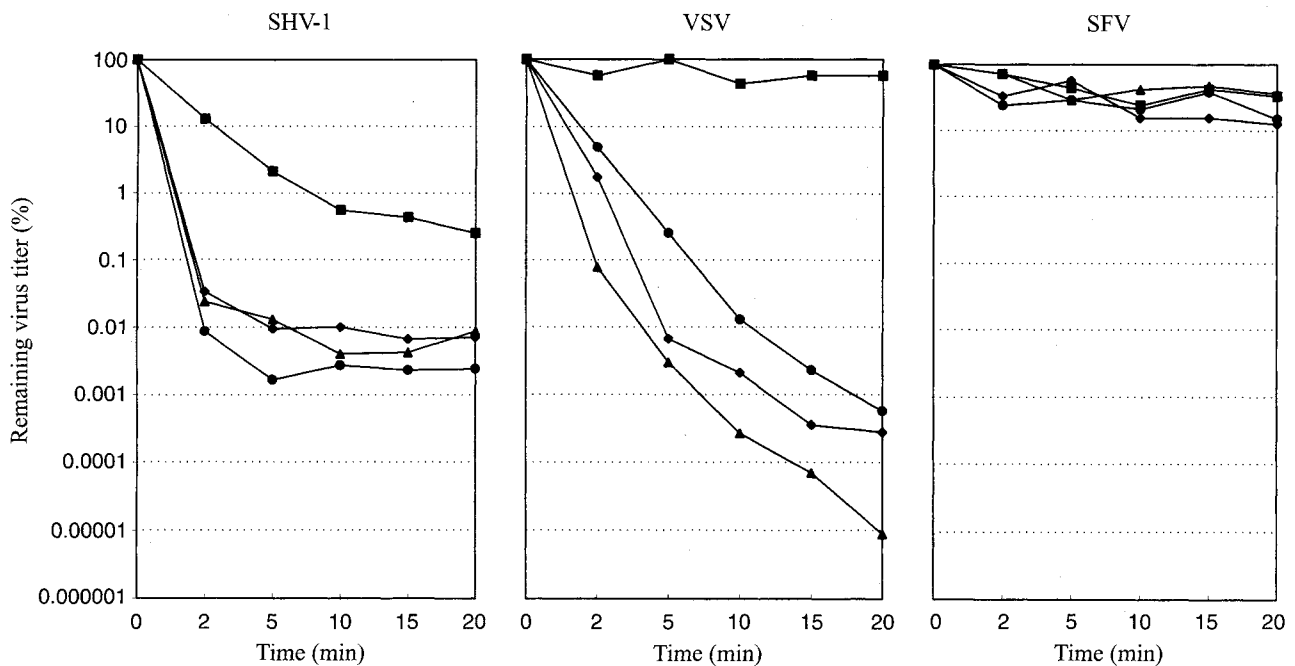
Results

Influence of the Fatty Acid Side Chain and of Esterification of Surfactin on Virus Inactivation Efficiency

The inactivation kinetics of different surfactin isoforms

Fig. 1.   Inactivation kinetics of SFV, VSV and SHV-1 with surfactin isoforms (40 μM at 22°C) in comparison
to surfactin mixture.

■ C13-surfactin, ● C14-, ▲ C15-surfactin, ◆ surfactin mixture.

differing in the fatty acid chain length and esterified surfactin isoforms were analyzed at various incubation times between 0 and 20 minutes at 22°C.

First, the dependence of antiviral activity on the fatty acid chain length of surfactin was investigated (Fig. 1). SHV-1 was inactivated by all three surfactin isoforms. The C14 and C15 isoforms showed an inactivation capacity comparable to that of the surfactin mixture (reduction factor 10^4), the C13 isoform was less active with a reduction factor of 400 after 20 minutes. VSV showed a pronounced dependence on the fatty acid chain length of surfactin. The surfactin mixture (reduction factor 4×10^5) and the C14 isoform showed comparable inactivation kinetics, the C15 species inactivated VSV more efficiently (reduction factor 1.2×10^6), whereas in contrast to SHV-1 the C13 surfactin was inactive with an insignificant reduction factor of <10. In earlier experiments no effect on the inactivation of SFV by the surfactin mixture had been found. This finding was extended by the investigation of the three isoforms which were also inactive (reduction factors <10).

On the other hand, we investigated the influence of esterification of surfactin on the antiviral capacity (Fig. 2). The C14- and the C15 monomethyl ester inactivated SHV-1

and VSV at rates comparable to the surfactin mixture. In contrast to the unesterified surfactin mixture or the purified isoforms of surfactin the monomethyl esters showed a significant inactivation of SFV. The C14 ester inactivated SFV with a reduction factor of 800 (compared to 8 with surfactin mixture). The C15 ester was even more effective against SFV with a reduction factor of 3.8×10^4. The uncharged C15 surfactin dimethyl ester was completely inactive against all three viruses.

Electron Microscopy

To investigate the different effects of esterified and unesterified lipopeptides on the morphology of viruses, VSV and SFV were treated with the surfactin mixture and the C15 surfactin monomethyl ester. After sedimentation, the pellet was resuspended and the specimen negatively stained. In contrast to untreated controls of VSV (data not shown) and SFV (Fig. 3a), surfactin caused a total destruction of VSV (data not shown), but had only a limited effect on the viral envelope and the capsid structure of SFV (Fig. 3b). However, the C15 surfactin monomethyl ester caused an almost total disintegration of the SFV particles

Fig. 2.   Inactivation kinetics of SFV, VSV and SHV-1 with esterified surfactin isoforms (40 μM at 22°C) in comparison to surfactin mixture.

○ C14 monomethyl ester, △ C15 monomethyl ester, + C15 dimethyl ester, ◆ surfactin mixture.
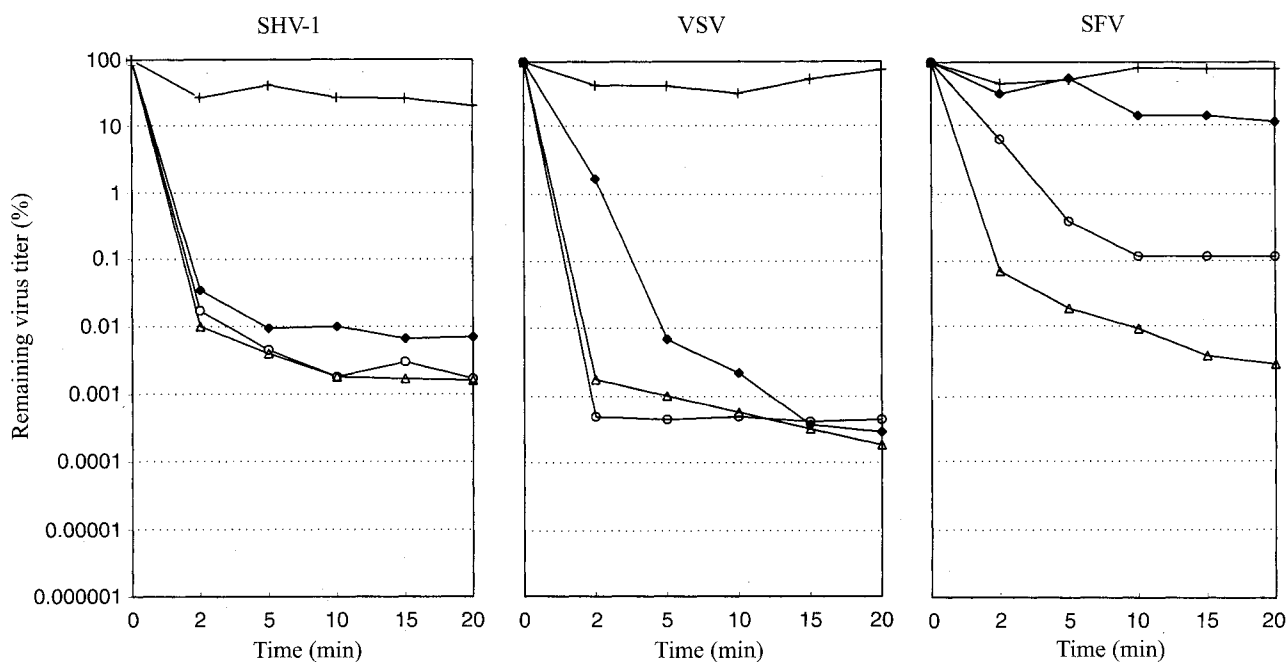
and additionally a partial destruction of the nucleocapsid (Fig. 3c). These effects correlate with the biological activity determined by virus titration.

### Hemolysis of Human Erythrocytes by Surfactin Isoforms and Their Methyl Esters

A rapid lysis of erythrocytes was observed with the surfactin mixture at 40 μM, which is the concentration used for virus-inactivation studies: After 5 minutes >80% of the erythrocytes were lysed. The concentration dependency of hemolysis with various surfactins was measured after 30 minutes at 37°C and the 50% effective dose (ED_{50}) calculated (Table 1).

The obtained results show a good correlation with the virus-inactivation studies. C13 surfactin had the lowest activity of the fatty acid chain isoforms. The hemolytic activity increased with their hydrophobicity. Again, the C15 surfactin monomethyl ester showed the highest efficiency, while the diester was almost ineffective (2% hemolysis at 100 μM for 30 minutes).

Discussion

The number of carbon atoms of the fatty acid chain of surfactin is one of the important factors for virus inactivation by this lipopeptide. C13 surfactins showed either no (VSV) or a lower activity (SHV-1) than the surfactin mixture. With increasing fatty acid hydrophobicity the virus-inactivation capacity of the isoforms increased. For VSV the C15 surfactin showed the highest activity, i.e. reduction factor was six times higher compared to the surfactin mixture. Surfactin mixture, C14 and C15 isoforms induced similar reduction factors against SHV-1. None of the purified isoforms showed significant inactivation of SFV. Comparing these results obtained with SFV, VSV and SHV-1, which were grown on the same cells, specific differences in inactivation capacity of the surfactin isoforms were observed. Since the virion's lipid composition reflects the composition of the host plasma membrane and the protein composition is determined by the viral genome,[18] it has to be postulated that the mechanism of virus inactivation with lipopeptides depends on the lipid composition of the viral envelope as well as on the virus

Fig. 3. Effects of esterified and unesterified lipopeptides on the morphology of SFV.
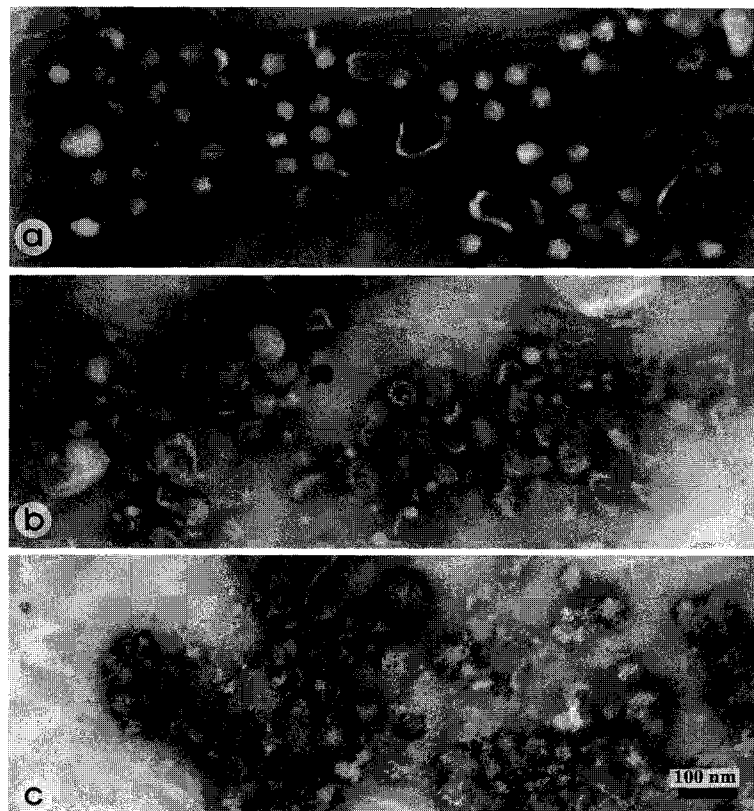
Magnification: ×125,000.

Semliki Forest virus (SFV), propagated in BHK-21 cells and isolated from culture supernatant (a) or treated with 40 μM surfactin (b) or C15 surfactin monomethyl ester (c) for 1 hour at room temperature.

For negative staining electron microscopy 2% PTA on carbonstabilized Cu-grids were used. Surfactin caused a moderate virus membrane and capsid damage of SFV (b), but treatment with C15 monomethyl ester showed a total destruction of the virus particles (c).

encoded glycoproteins.

The influence of the negative charges of the peptide moiety of surfactin was examined with monomethyl and dimethyl ester derivatives. In contrast to the strong inactivation capacity of C15 surfactin against VSV and SHV-1, the uncharged C15 dimethyl ester was completely ineffective. The activity of the two surfactin monomethyl ester isoforms against VSV and SHV-1 was comparable to that of the surfactin mixture. In contrast to all other surfactin isoforms, the two compounds, especially the C15 monomethyl ester, showed a high inactivation capacity against SFV. Differences in inactivation kinetics of SFV with single esterified and unesterified surfactin isoforms could be explained by the structure of the virion. The nucleocapsid of SFV is enveloped by a lipid bilayer, which contains the viral spike proteins. In contrast to SHV-1 and VSV, the

Table 1. Hemolytic activity of surfactin and its derivatives.

Surfactin isoforms/esters	ED_{50} for erythrocyte hemolysis
Surfactin mixture	13 μM
C13 surfactin	70 μM
C14 surfactin	17 μM
C15 surfactin	13 μM
C14 surfactin monomethyl ester	9 μM
C15 surfactin monomethyl ester	2 μM
C15 surfactin dimethyl ester	≫100 μM

lipid membrane of SFV is tightly bound to the nucleocapsid structure. Biochemical data support the existence of an interaction between the spike glycoproteins and the nucleocapsid.[19] During inactivation the surfactin-mixture and the isoforms incorporate into the lipid bilayer inducing a complete disintegration of the envelope which contains the viral proteins involved in virus adsorption and penetration. Its loss accounts for the losing of virus infectivity. Due to the interaction between nucleocapsid and glycoproteins, SFV keeps its glycoproteins and therefore maintains its infectivity and only diminishes the lipid bilayer under treatment with the surfactin mixture and isoforms. During inactivation with the monomethyl ester the molecules seem to interact with the virus subunits inducing a loss of infectivity of SFV due to the disintegration of viral proteins and lipid bilayer.

The effect of treating viruses with surfactin mixture or C15 surfactin monomethyl ester was visualized by electron microscopy. Destruction of virus particles (Fig. 3b and 3c) is correlated with reduction of infectivity titers after treatment with lipopeptides.

The sensitivity of human erythrocytes to the lipopeptides correlates to some degree with the results obtained with virus inactivation. Therefore, the determination of this effect can be used for preselection of active antiviral compounds for virus inactivation.

Summarizing the results, inactivation of enveloped viruses by surfactin lipopeptides depends on the hydrophobicity of the fatty acid moiety, on the charge of the peptide moiety as well as on the virus species. Higher numbers of carbon atoms in the fatty acid chain and one negative charge of the peptide ring result in more active compounds.

Therefore, addition of the lipopeptides to cell cultures used in laboratories and biotechnological production can be a useful tool for virus inactivation and protection against virus infection. Due to the hemolytic activity, the clinical application is limited to local treatment of viral infections or to the prevention of virus transmission.

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