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# Tuning Micelles of a Bioactive Heptapeptide Biosurfactant via Extrinsically Induced Conformational Transition of Surfactin Assembly

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Abstract: We have studied the effects of extrinsic environmental conditions on the conformation of surfactin, a heptapeptide biosurfactant from *Bacillus subtilis*, in aqueous solutions. It has been made clear that temperature, pH, $Ca^{2+}$ ions and the synthetic nonionic surfactant hepta-ethylene glycol ($C_{12}E_7$) affect the conformation of surfactin in aqueous solutions. The $\beta$-sheet formation reached a maximum at 40°C both in presence and absence of ($C_{12}E_7$) and the nonionic surfactant enhances the $\beta$-sheet formation even at 25°C. $Ca^{2+}$ induced the formation of a-helices and caused this transition at 0.3 mM with surfactin monomers or at 0.5 mM with surfactin micelles, but above these transition concentrations of $Ca^{2+}$ $\beta$-sheets were observed. In micellar solution the $\beta$-sheet structure was stabilized at pH values below 7 or upon addition of $Ca^{2+}$ in concentrations above 0.5 mM. Our results indicated that the bioactive conformation of surfactin is most likely the $\beta$-sheets when the molecules are assembled in micelles. The $\beta$-sheet structure in micelles could be retained by tuning the micelles. Surfactin micelles could be tuned in the bioactive conformation by manipulating pH, temperature, $Ca^{2+}$ or ($C_{12}E_7$) concentrations in surfactin solutions. Our results strongly indicated that $Ca^{2+}$ and other molecules (such as $C_{12}E_7$) may function as directing templates in the assembly and conformation of surfactin in micelles. Thus, we suggest environmental manipulation and template-aided micellation (TAM) as a new approach for preparing predesigned micelles, microemulsions or micro-spheres for specific application purposes. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide surfactant; surfactin; conformation; micelles; biosurfactant

## INTRODUCTION

Surfactin is a lipopeptide produced by *Bacillus subtilis* [1–7] and is abundant in many natural products such as natto (fermented soy beans) [5,8–11]. It was fist produced and characterized by Kakinuma *et al.* [1–3]. Since, many analogues have been produced and studied [10–14]. Surfactin is a biosurfactant with a high industrial and commercial potential because of its superb surface and interfacial activity [1,2,15] and because it has a diversity of bioactive properties [1–3,8–11,16–22]. It forms large micelles at a very low concentration [23] and the cmc of the different analogues is of the order $10^{-5}$ M [4,21,23] or less. Surfactin has also shown ionophoric and sequestering properties [21,24]. The bioactivities of surfactin include: inhibition of blood clotting [1–3], haemolytic activity [21], repression of cAMP phosphodiesterase [14,16–18], hypocholesterolemic action [22], channel formation in membranes [25], synergistic antifungal activity when

Abbreviations: Sf, surfactin; $C_{12}E_7$, heptaethylene glycol.

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combined with iturins [19,20], antibiotic activity [3,26], antitumour action [8,9] and anti HIV effects [11]. These physicochemical and biological activities are most likely related to the mode of molecular assembly of surfactin in micelles as well as the secondary structure and the conformation of the surfactin molecules in the aggregates. The relation of the conformation of peptides and their physicochemical properties to their biological actions have been reported by many authors [27–33]. It has also been observed that peptides adopt multiple conformation in different environments or under distinct conditions in the milieu [34–37].

The peptide conformation is not directed solely by the primary structure of the peptide or its intrinsic properties but it is also controlled by the constituents of the extrinsic environment [34–45]. Peptide may form ion channels, proteins or undergo conformational transitions depending on extrinsic templates [39], ion types [38,44], hydrophobicity/hydrophilicity of the environment [43], redox properties of the environment [40], types of applied solvents [37,42] influence of pH [4] and temperature [45]. The importance of the conformation of peptides in general and the peptide-biosurfactant systems in particular is due to the relation of the conformation to the system stability as well as the specificity and ability of the peptides to bind to either ligands or receptors, and the resulting induced biological actions. Although many reports have addressed these questions, there are no reports regarding the influence of such environmental factors on the secondary structure, the conformation and the bioactivity of micelles formed by peptide biosurfactants. The investigation of the conformation of lipopeptides, particularly of peptide biosurfactants, is of crucial importance for the understanding of their potential applications. Systems based on biosurfactants have been suggested for use in drug targeting, controlled drug release, DDS (drug delivery systems), intelligent liposomes, transdermal absorbtion treatments and biosensors [46]. The effectiveness of such systems may depend totally or partly on the correct conformation of the peptide biosurfactant molecules in the surfactant systems, such as liposomes, micelles, microemulsions and lipopeptide-microspheres. Therefore understanding the behaviour and the structural transitions in relation to the bioactive conformation of the lipopeptides in these surfactant systems, is crucial for designing suitable, stable and effective peptide biosurfactant systems.

We have recently reported on the transition of $\alpha$-helix structure to $\beta$-sheets in linear surfactin [47] and we have also reported on the formation of large micelles of cyclic surfactin by $\beta$-sheet formation [23].

In the present paper we present our results regarding the effects of the manipulation of the extrinsic environment on the conformation of surfactin molecules and we discuss the stabilization of the secondary structures as well as the induction of conformational transitions which are favoured for bioactivity. We finally show the possibility of using this approach to tune micelles or prepare other micro-structures in surfactant systems of peptide biosurfactants where stability and biological efficacy can be retained at an optimum level.

## MATERIALS AND METHODS

Surfactin was purchased from Wako Pure Chem Industries Ltd., Japan. The chemical structure of cyclic surfactin samples was ascertained by fast atom bombardment mass spectroscopy (FAB-MS) reported elsewhere [47]. The base peak, 1036.8 corresponded exactly to the molecular weight of cyclic surfactin. The cyclic structure was further confirmed by Furier-transformed Infrared spectroscopic (FTIR) measurements, the 1730 $cm^{-1}$ band indicated the cyclic structure of the cyclic surfactin.

All other chemicals were of reagent grade. The water used in the measurements was purified by passing through an ion exchange resin column, followed by distillation in an all quartz distillation column filled by tipped glass tubes.

Phosphate buffered saline (PBS buffer) had the following formulation: NaCl 8.0 g, $KH_2PO_4$ 0.2 g, $Na_2HPO_4$ 1.15 g, KCl 0.2 g, per litre of distilled water. The pH was adjusted by 0.1 N HCl or 0.1 N NaOH to the desired pH.

FAB mass spectra were taken by a JEOL HX-100 double focusing mass spectrometer operated at a resolving power of 2000 (10% valley definition). About 1 μg of sample was dissolved in 2 μl of glycerol/$H_2O$ mixture (glycerol:$H_2O$ = 1:10 v/v) on a stainless-steel plate. A small amount of 1 N HCl was added for the measurement of positive ion spectra. Samples were bombarded with a xenon atomic beam of 6 keV. Data acquisition and processing were performed using JEOL JMADA 5000 system.

FT-JR spectroscopy was performed on surfactin using KBr disks. The FT-JR spectra were measured using a JASCO FT-IR-5000 spectrophotometer and data were acquired and processed using JASCO-5000 software, version 2.2.

The CD spectra were measured using a JASCO J 600 polarimeter and the data were acquired and processed using the J-600 software, version 2a. The spectra obtained were reasonably reproducible by repeated measurement under the forced experimental conditions.

The effect of temperature on the conformation of surfactin micelles both in the presence and absence of the nonionic surfactant Heptaethylene glycol ($C_{12}E_7$) were performed in 0.1 M $NaHCO_3$ solutions having a pH of 8.7. In absence of $C_{12}E_7$ the surfactin concentrations ranged from 1 to 3 mM and in the presence of $C_{12}E_7$ the surfactin concentrations ranged from 0.25 to 1 mM.

The effect of temperature and $C_{12}E_7$ addition on weight-average ($M_w$) of surfactin micelles and the aggregation number was obtained in the manner described elsewhere [23].

The CD spectra were obtained under strictly controlled temperatures in the range of $\pm 0.1$°C. The CD spectra were taken in the temperatures range 25–55°C. The effect of $C_{12}E_7$ on the conformation at different temperatures was measured in surfactin solutions having a concentration in the range 0.25–1 mM and the ratios of surfactin/$C_{12}E_7$ were either 50:50 or 25:75.

The molar elipticity ($\theta$) values whenever necessary were obtained directly from the CD spectra processed by the computer using the software mentioned above.

Effects of pH on the conformation were performed on a series of surfactin solutions in PBS buffer where pH values ranged from 6 to 9. A concentration of $1 \times 10^{-6}$ M, which is 1/10 of surfactin's cmc, was used to examine the effects of pH on the conformation of surfactin in monomeric form. A concentration of $2 \times 10^{-5}$ M, which is twice the cmc of surfactin, was used to examine the effects of pH on the conformation of surfactin molecules in the micellar form. Effects of pH on conformation were measured at 25°C.

The effect of $Ca^{2+}$ on the conformation was performed on a series of surfactin solutions in Tris buffer having a pH of 7.5. The concentrations of $Ca^{2+}$ ranged from 0.1 0.7 mM.

## RESULTS AND DISCUSSION

### β-Turns and the Secondary Structure of Cyclic Peptides

Cyclic peptides have been often used as a model for studying β-turn conformation [48]. Since surfactin is a cyclic peptide and CD spectra given later on may be confused with β-turn configuration, we do feel it necessary to address the question of β-turn. The β-turn configuration is also an important constituent of many proteins, where the polypeptide chain abides a relatively reversal turn in direction [49,50]. The spectrum of most common β-turn structures have a negative band near 225 nm, a very strong negative band at 180–190 nm and a strong positive maximum at 200–205 nm. There is noticeable difference of 5–10 nm red shift between the maxima of β-turn and β-sheet spectra. However, in some special cases resemblance to α-helix have been observed, where the spectrum has negative bands near 220 nm and 210 nm and a positive band near 190 nm. The deviation in such cases is of few nanometres and is not very distinct. The theoretical calculations have demonstrated that no single CD spectra could be assigned to the structural conformation called β-turn [51]. In cyclic peptides, the β-turn conformation may look like either a β-sheet or α-helix configuration. However, X-ray diffraction and NMR are used to substantiate such structures in cyclic peptides [52,53]. In addition the configuration in the cyclic structure is restricted by the structural bonds and any possible β-turn is very specific and can not be altered unless the cyclic structure itself is destructed.

The explanatory remarks mentioned above could be summarized in the following points:

- No single CD spectra could be attributed to the conformation designate β-turn [51].
- Possible β-turn configuration in cyclic peptides such as surfactin need X-ray diffraction and NMR for corroboration [52,53].
- Any possible β-turn in a cyclic peptide is definite and will not transform unless the cyclic structure is wrecked.

Since the cyclic structure of the surfactin molecule was maintained all the time under the experimental conditions, then the observed gradual alterations in the CD spectra of surfactin that are described later on in this paper are related to the aggregational behaviour of surfactin and could not be associated with the possible β-turn configuration attributed to the cyclic structure of the surfactin molecule.

### Effect of the Nonionic Surfactant ($C_{12}E_7$) on the Assembly, Conformation and Micellation of Surfactin Molecules

Reports by other authors suggest that the addition of synthetic surfactants to a peptide solution induce conformational transitions in their secondary structure [34,54,55]. However, little is known about the synthetic surfactants as enhancers or stabilisers of defined micellar conformation. Since nonionic surfactants are used in a wide range of applications, we have therefore tested the effect of the nonionic surfactant heptaethylene glycol ($C_{12}E_7$) as a model for this group of surfactants on the secondary structure of surfactin micelles. Contrary to our anticipation, the micellar aggregation number became much higher than expected which indicated an enhancement of the assembly of surfactin (Sf) molecules in $\beta$-sheets and an increase in micelle formation. The values of micellar weight-average and aggregation number were calculated by the extrapolation of the Zimm plots obtained from the static light scattering measurements [23]. Figure 1 shows the changes in micellar weight and the aggregation number at different Sf/$C_{12}E_7$ ratios.

As is shown the aggregation number was increased at the Sf/$C_{12}E_7$ molar ratio of 25:75 and the aggregation number raised to 144, indicating enhancement of micellation. Taking the $\theta$ values for the molar elipticity shown in Table 1 into consideration it becomes clear that the $C_{12}E_7$ enhanced the formation of micelles by promoting the assembly of surfactin molecules in $\beta$-sheets even at very low surfactin concentrations of 0.75 mM.

Table 1 shows the increase of the molar elipticity value $\theta$ at the single minima of 218 obtained from the CD spectra measurement in the presence and absence of $C_{12}E_7$ at different temperatures.

Surprisingly, the synthetic surfactant $C_{12}E_7$ did not induce any conformational transitions but to the contrary, it enhanced the formation of $\beta$-sheets. Table 1 shows that at 25°C the $\theta$ value is $1.62 \times 10^{-7}$ deg cm$^2$ dmol$^{-1}$ in the absence of $C_{12}E_7$ for the 3 mM surfactin solution, but this value increased to $1.71 \times 10^{-7}$ deg cm$^2$ dmol$^{-1}$ at the lower surfactin concentration (1 mM) when $C_{12}E_7$ was added in a 50:50 Sf/$C_{12}E_7$ ratio The $\theta$ value increased even further to $1.85 \times 10^{-7}$ deg cm$^2$ dmol$^{-1}$, when surfactin concentration was reduced to 0 75 mM and $C_{12}E_7$ was added in a 25:75 Sf/$C_{12}E_7$ ratio. This indicates that the $\beta$-sheets are formed even at very low surfactin concentration due to the enhancement caused by the addition of the synthetic surfactant $C_{12}E_7$. These observations may be explained by possible intercalation of the nonionic surfactant between surfactin molecules in the micelle, where they possibly function as a template that directs the assembly of surfactin in $\beta$-sheet micelles and thus enhances the $\beta$-sheet formation.

### Effect of Temperature on the Conformation and Assembly of Surfactin Molecules

We further studied the effects of temperature elevation on the piling of surfactin molecules in the micelles, the secondary structure and the formation of $\beta$-sheet-micelles, both in presence and in absence of $C_{12}E_7$. Our observations clearly indicated that increasing the temperature enhanced the formation of $\beta$-sheets both in the absence and in the presence of the synthetic surfactant. It also showed an increase in micelles aggregation as well as in their stability.

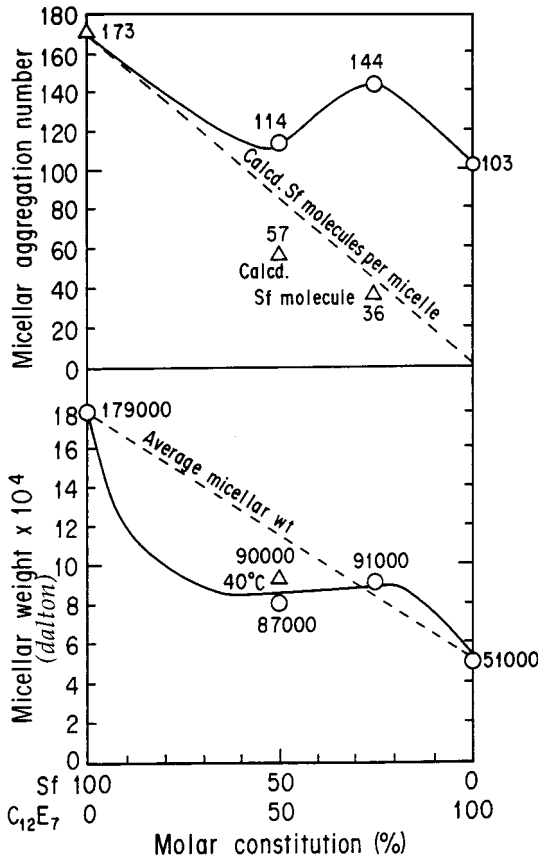


Figure 1 The changes in micellar weight and the aggregation number of surfactin at different Sf/$C_{12}E_7$ ratios and the enhancement of $\beta$-sheet micellation.

Table 1 Molar Elipticity Changes of Surfactin

| Substance | Concentration of surfactin in the solution (mM) | Temperature (°C) | Molar ellipticity [$\theta$] deg cm$^2$ dmol$^{-1}$ at 218 nm minima |
|---|---|---|---|
| Surfactin (Sf) | 3 | 25 | $1.62 \times 10^7$ |
| | 3 | 40 | $1.71 \times 10^7$ |
| | 3 | 55 | $1.83 \times 10^7$ |
| | 2 | 25 | $2.04 \times 10^7$ |
| | 2 | 40 | $2.08 \times 10^7$ |
| | 1 | 25 | $1.80 \times 10^7$ |
| | 1 | 40 | $1.86 \times 10^7$ |
| Sf/$C_{12}E_7$ (molar ratio 50:50) | 1 | 25 | $1.71 \times 10^7$ |
| | 1 | 40 | $1.73 \times 10^7$ |
| | 0.5 | 25 | $1.63 \times 10^7$ |
| | 0.5 | 40 | $1.73 \times 107$ |
| Sf/$C_{12}E_7$ (molar ratio 25:75) | 0.75 | 25 | $1.85 \times 10^7$ |
| | 0.75 | 40 | $1.99 \times 10^7$ |
| | 0.5 | 25 | $1.32 \times 10^7$ |
| | 0.5 | 40 | $1.32 \times 10^7$ |
| | 0.25 | 25 | $1.63 \times 10^7$ |
| | 0.25 | 40 | $1.19 \times 10^7$ |

From the results shown in Table 1 we estimated maximal increase of 13% in the $\beta$-sheet formation due to temperature raise. The maximum enhancement caused by heating of the surfactin system was reached at surfactin concentration of 2 mM and at temperature of 40°C in the absence of $C_{12}E_7$. The maximum temperature effect on enhancing $\beta$-sheet formation in presence of $C_{12}E_7$ was reached when surfactin concentration was 0.75mM, the Sf/$C_{12}E_7$ ratio was 25:75 and the temperature was 40°C.

This micellation enhancement induced by increased temperature is in contradiction to what is known in the case of normal surfactants. In surfactant systems employing normal synthetic surfactants, heating of the surfactant in the aqueous solution leads to the de-micellation of aggregated surfactant molecules and increase of the dissociation of surfactant molecules and hence formation of larger number of monomers in the solution.

The enhancement of micellation by increased temperature for this peptide biosurfactant indicates strongly that the micellation/de-micellation (association/dissociation) of peptide surfactants is most likely ruled by different kinetics from those conventionally comprehended and applied in the case of normal surfactants. It also clearly indicates that the theoretical approaches traditionally applied to explain the behaviour of normal surfactant systems may have feasible difficulties in explaining phenomena such as the enhancement of micellation by heating the surfactant in aqueous solutions.

### Effects of pH on the Induction of Conformational Transitions

Variation in pH is an easy tool for changing properties, structure and behaviour of surfactant systems. We have examined the effects of pH on the conformation of surfactin in both micellar and non-micellar solutions, as it is depicted in Figure 2A. The monomers were examined in solutions of $1 \times 10^{-6}$ M, which corresponds to 1/10 cmc of surfactin, and the micelles in solutions of $2 \times 10^{-5}$ M, which corresponds to about twice the cmc value. Below the cmc the surfactin monomers have an unordered conformation in alkaline solutions when pH is 8.5 or more. The unordered structure is distinguished by a CD spectra having a strong minima at 202 nm and a maxima around 190 nm. At neutral pH the conformation changed to $\beta$-sheets with a single minima at 220 nm and a maxima at 194 nm. In slightly acidic pH (pH 6) the surfactin monomers have an $\alpha$-helical conformation, with two minima at 203 and a stronger minimal value at 222 and a maximal value at about 190 nm.

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