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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 β-sheet formation reached a maximum at 40°C both in presence and absence of ($C_{12}E_7$) and the nonionic surfactant enhances the β-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+} β-sheets were observed. In micellar solution the β-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 β-sheets when the molecules are assembled in micelles. The β-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 α-helix structure to β-sheets in linear surfactin [47] and we have also reported on the formation of large micelles of cyclic surfactin by β-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 ± 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 (θ) 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 1×10^{-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 2×10^{-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 β-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 θ 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 β-sheets even at very low surfactin concentrations of 0.75 mM.

Table 1 shows the increase of the molar elipticity value θ 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 β-sheets. Table 1 shows that at 25°C the θ value is 1.62×10^{-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 1.71×10^{-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 θ value increased even further to 1.85×10^{-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 β-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 β-sheet micelles and thus enhances the β-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 β-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 β-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.

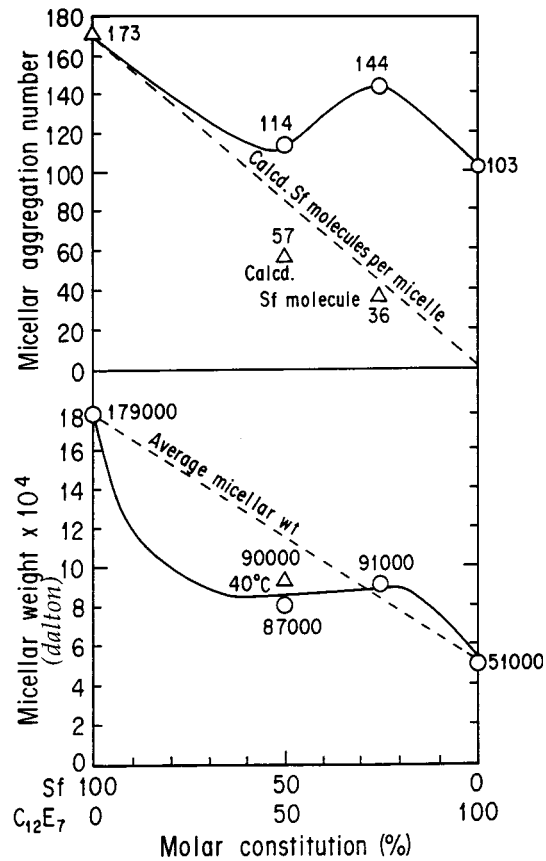

Figure 1 The changes in micellar weight and the aggregation number of surfactin at different Sf/$C_{12}E_7$ ratios and the enhancement of β-sheet micellation.

Table 1 Molar Elipticity Changes of Surfactin

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

From the results shown in Table 1 we estimated maximal increase of 13% in the β-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 β-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 1×10^{-6} M, which corresponds to 1/10 cmc of surfactin, and the micelles in solutions of 2×10^{-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 β-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 α-helical conformation, with two minima at 203 and a stronger minimal value at 222 and a maximal value at about 190 nm.

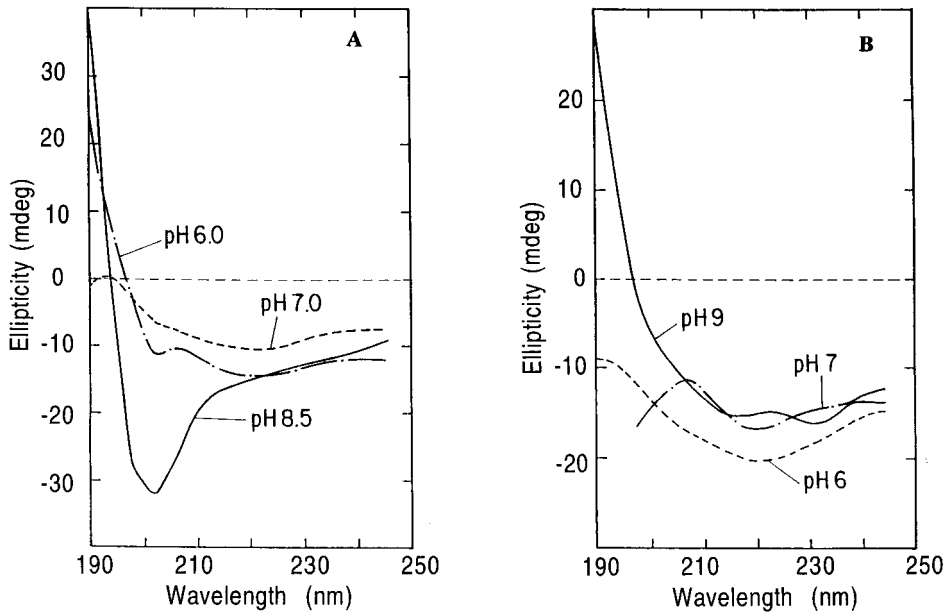

Figure 2 Effect of pH on the conformation of surfactin molecules in an aqueous solution, as determined by CD. A, surfactin monomers; and B, surfactin micelles.

However, different transient conformations were observed between pH values shown in Figure 2A. This indicated a gradual change from one conformation to another. The minima around 203 nm and 222 nm in the CD spectra of these transient structures indicated that the surfactin molecules adapted a mixed conformation of both β-sheets and α-helices where the percentages of each form was pH dependent. Thus, the change was gradual and a sudden shifting from one form to another was never observed. This gradual alteration was most likely due to the formation of surfactin clusters (dimers, trimers, etc.) by the gradual reduction in pH value.

Above the cmc the pH effect was quite different. At an alkaline pH of 9 or more α-helices were formed. These were characterized by a CD spectra having two minima at 212 nm and 228 nm as well as shoulder minima at 206 and a maxima close to 190 nm. While at lower pH values of less than 9 and as low as pH 6 β-sheets were formed. The CD spectra had typically a single minima at 220 and a maxima about 195–200 nm. This indicated that normally the surfactin micelles would have a β-sheet structure when pH conditions are similar to those obtained under the physiological condition. Figure 2B shows the CD spectra of surfactin micelles at different pH values.

Since surfactin has been shown to be most bioactive at physiological pH values [8,9,21] and has adopted the β-sheet conformation under the same pH values according to the above results, thus it may be concluded that the most favourable bioactivity would be attained when surfactin micelles retain the β-sheet structure. However, this conformation may be affected by the presence of metal ions, where the type and concentration of metal ions may enhance or dwindle the assembly in β-sheet conformation.

It may be worth while mentioning that at pH values above 9 the cyclic lactone ring of surfactin may be cleaved to form linear surfactin in the solutions stored for relatively longer periods of time (data not published), and therefore observations above pH 9 may be related to linear surfactin rather than to cyclic surfactin [47].

Effect of Ca^{2+} on Inducing Conformational Transitions in Surfactin

Effects of Ca^{2+} and other ions on the restoration or repression of the biological activity of surfactin have been reported [17,20], and the role of ions in the formation of surfactin micelles have also been reported in the literature [20]. Hosono *et.al.* [17] observed an increase in the inhibitory activity of the deactivated-surfactin on cAMP phosphodiesterase by Ca^{2+}. Thimon *et al.* [20] observed that the formation of surfactin micelles increased the antifungal activity of iturins, but excessive amounts of

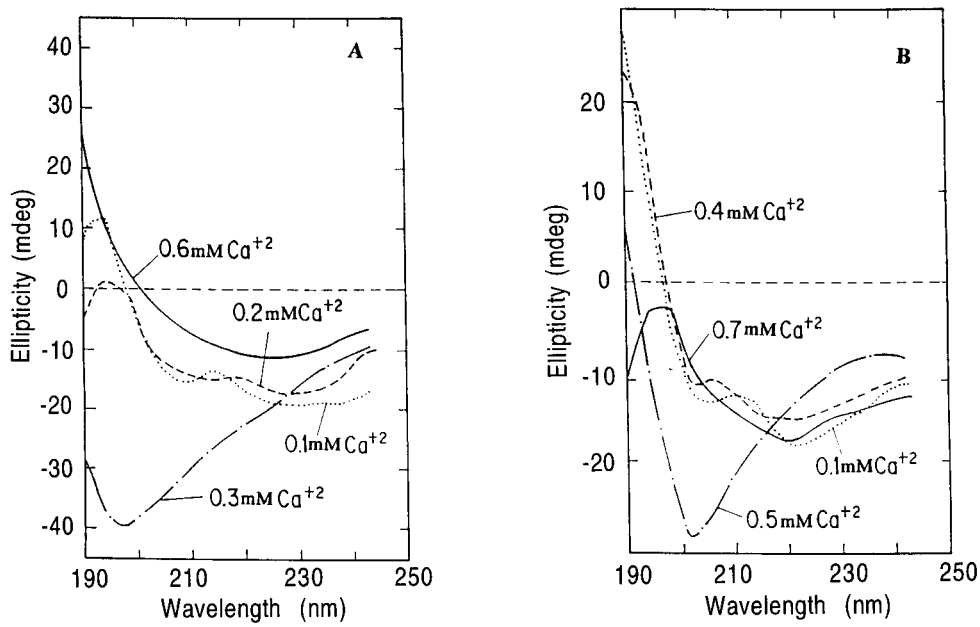

Figure 3 CD spectra for surfactin monomers and micelles in aqueous solution at different Ca^{2+} concentrations and at pH of 7.5. A, surfactin monomers; and B, surfactin micelles.

metal ions decreased the synergism of surfactin interacted with iturins [20]. Yet possible relationship between ions and the preferred bioconformation in surfactin were not examined We have investigated the effects of Ca^{2+} on the β-sheet conformation of surfactin in both monomeric form (1×10^{-6} M solutions) and micellar form (2×10^{-5} M solutions) in aqueous solutions of pH 7.5.

The transitions induced in the monomers were dependent on the concentration of Ca^{2+}. When the Ca^{2+} concentration was below 0.3 mM, α-helices were formed with two minima at 205 and 225 nm, and a maximum around 195 nm. At concentration of 0.3 mM the CD showed a strong minimal value at 198 nm indicating an unordered structure. Such strong minima also means that a transition from one conformation to another is about to take place. At concentrations above 0.3 mM the CD spectra of monomeric solution showed a β-sheet conformation with a minimal value at 220 nm and a maximal value around 190 nm. Figure 3A shows the CD spectra for surfactin monomers at the different Ca^{2+} concentration.

The transitions observed are likely due to the binding of surfactin to Ca^{2+} and formation of surfactin clusters.

The transitions induced by Ca^{2+} in surfactin micelles were totally different from those we observed in the monomers. At concentrations below 0.5 mM α-helical structures were observed, with characteristic CD spectra having two minima at 202–206 nm and at 222 nm, and a maximal value at 190 nm. At a concentration of 0.5 mM Ca^{2+} the CD spectra showed a strong minimum at 202 nm, indicating an unordered structure. This minimum also evidenced the start of transition from α-helix to β-sheet structure. At concentrations above 0.5 mM, the Ca^{2+} ions stabilised the formation of β-sheets with characteristic CD spectra having a single minimum at 220 nm and a maximum at 195 nm. Figure 3B shows the CD spectra of surfactin micelles at different Ca^{2+} concentrations.

These observations show that the Ca^{2+} induces a-helical structure both in monomers and in micelles of surfactin, but at critical concentration above 0.3 mM for monomers and above 0.5 mM for micelles, Ca^{2+} causes a transition to β-sheet structure.

The above observations indicated that calcium ions not only affect the conformation of surfactin both in monomeric and micellar form, but they also induce concentration-dependent transitions. It is most likely, that Ca^{2+} ions in aqueous solutions of surfactin function as templates that direct the clustering, micellation, aggregation or assembly as well as conformation of surfactin molecules, and this function is quite dependent on the Ca^{2+} concentration.

A comparison of our present results with those of other authors [17,20] suggests that the preferred bioactive conformation of surfactin is likely to be the β-sheets. This is because surfactin micelles retain the β-sheet structure when calcium is added in sufficient amounts to reach concentration ratios similar to those reported [17] and because the bioactivity also was retained when the overall reported experimental conditions [17,20] were also comparable with our forced conditions.

To conclude this discussion, it should be noted that since β-sheet enhancement reached a maximum at 40°C, the physiological temperature of 37.5 would be perfect for the induction of bioactively conformed surfactin. And because optimum pH values for β-sheet formation is close to the physiological one (pH 7) and due to the possibility of directing, stabilising and enhancing the β-sheet formation by other molecule such as Ca^{2+} or $C_{12}E_7$. Thus, the surfactin micelles could be tuned to maintain the most biologically active conformation by inducing and stabilising the β-sheet as a secondary structure. Since pH and temperature under the physiological conditions are fairly stable, then micelles, microspheres or microemulsions of surfactin or other peptide biosurfactants could be probably designed and preconformed in a bioactive form in advance, using Ca^{2+} or other assembly directing molecules. Otherwise the tuning for non-physiological purposes could be controlled by pH manipulation, exploitation of directing molecules such as Ca^{2+}, temperature or a combination of these.

According to the above presented results, discussion and remarks both Ca^{2+} and $C_{12}E_7$ are most likely functioning as assembly templates that direct the micellation, surfactin micelles could be assumed as biologically active protein, structured by template-aided aggregation or template-aided molecular assembly. And consequently we may suggest the use of ions or other suitable templates to direct the micellation, preparation of micro-emulsions, or micro-sphere devices. Such a template-assembled micelles (TAM) approach could be used to direct the formation of not only micelles but also microemulsions, micro-spheres (of specific size, dimensions and function) and other molecular devices of potential industrial application such as drug delivery systems (DDS), drug targeting, micellar catalysis and in many industrial applications. In such process metal ions could be used as ionic templates in an ion-aided micellation (IAM), but other molecules specially designed as templates to aid formation of micelles or micro emulsions could be used in a template-aided micellation (TAM) approach.

CONCLUSIONS

It has been made clear that the peptide biosurfactant surfactin does not follow the normal kinetics of surfactant aggregation because the micellation was enhanced by increasing the temperature. Another deviation from the normal kinetics was the enhancement of aggregation by the synthetic surfactants. Comparing our results regarding the conformation of surfactin with the conditions used by other authors to test the bioactivity of surfactin it became clear that the β-sheet micelles were most likely the best bioactive form of surfactin. The β-sheet conformation could be easily retained by manipulation of extrinsic environmental factors such as temperature, pH or Ca^{2+}. There were also strong indications that substances such as Ca^{2+} and $C_{12}E_7$ direct the micellation and function as assembly templates for the aggregation process. These indications were confirmed by the enhancing β-sheet micellation as well as micelle stabilization using such substances. Due to these observations the tuning of micelles either by environmental manipulation or by template-aide aggregation (TAM) was suggested. This approach could make possible the preparation of micelles, microspheres or microemulsions with specific dimension, function, stability and bioactivity.

REFERENCES

1. K. Arima, A. Kakinuma and G. Tamura (1968). Surfactin, a crystalline surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun. 31*, 488–494.
2. A. Kakinuma, G. Tamura and K. Arima (1968). Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. *Separatum Exp. 24*, 1120–1121.
3. A. Kakinuma and K. Arima (1969). Surfactin, a novel peptidelipid surfactant produced by *Bacillus subtilis* in: *Annual Report of the Takeda Research Laboratories 28*, p. 140–193.
4. D.G. Cooper, C.R. McDonald, S.J.B. Duff and N. Kosaric, (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation addition. *Appl. Environ. Microbiol. 42*, 408–412.

5. J.D. Sheppard and C.N. Mulligan (1987). The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. *Appl. Microbiol. Biotechnol. 27*, 110–116.
6. C.N. Mulligan and B.F. Gibbs (1990). Recovery of biosurfactants by ultra filtration. *J. Chem. Tech. Biotechnol. 47*, 23–29.
7. C. Ullrich, B. Kluge, Z. Palacz and J. Vater (1991). Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by Bacillus subtilis. *Biochemistry 30*, 6503–6508.
8. Y. Kameda, K. Matsui, H. Kato, T. Yamada and H. Sagai (1972). Antitumor activity of *Bacillus natto*. III. Isolation and characterization of cytolic substance on Ehrlich Ascites Carcinoma cells in culture medium of *Bacillus natto* KMD 1126. *Chem. Pharm. Bull. 20*, 1551–1557.
9. Y. Kameda, S. Ouhira, K. Matsui, S. Kanatomo, T. Hase, and T. Atsusaka (1974). Antitumour activity of *Bacillus natto*. V. Isolation and characterization of surfactin in the culture medium of *Bacillus natto* KMD 2311. *Chem. Pharm. Bull. 20*, 938–944.
10. K. Oka, T. Hirano, M. Homma, H. Ishii, K. Murakami, S. Mogami, A. Motizuki, H. Morita, K. Takeya and H. Itokawa (1993). Satisfactory separation and Ms-Ms spectrometry of six surfactants isolated from *Bacillus subtilis natto*. *Chem. Pharm. Bull. 41*, 1000–1002.
11. H. Itokawa, T. Miyashita, H. Morita, K. Takeya, T. Hirano, M. Homma and K. Oka (1994). Structural and conformational studies of [Ile^7] and [Leu^7] surfactins from *Bacillus subtilis natto*. *Chem. Pharm. Bull. 42*, 604–607.
12. F. Peypoux, J.M. Bonmatin, H. Labbe, B.C. Das, M. Ptak and G. Michel (1991). Isolation and characterisation of new variant of surfactin, the [Val^7] surfactin. *Eur. J. Biochem. 202*, 101–106.
13. F. Baumgart, B. Kluge, C. Ulrich, J. Vater, and D. Ziessow (1991). Identification of amino acid substitutions in the lipopeptide surfactin using 2D NMR spectroscopy. *Biochem. Biophys. Res. Commun. 177*, 998–1005.
14. K. Hosono and H. Suzuki (1983). Acylpeptides, the inhibitors of cyclic adenosine 3', 5'-Monophosphate phosphodiesterase. II. Amino acid sequence and location of lactone linkage. *J. Antibiot. 36*, 674–678.
15. R. Marget-Dana and M. Ptak (1992). Interfacial properties of surfactin. *J. Colloid Interface Sci. 153*, 285–291.
16. K. Hosono and H. Suzuki (1983). Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphte phsphodiesterase. I. Purification, physicochemical properties and structures of fatty acid residues. *J. Antibiot. 36*, 667–673.
17. K. Hosono and H. Suzuki (1983). Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphte phsphodiesterase. III. Inhibition of cyclic AMP phosphodiesterase. *J. Antibiot. 36*, 679–683.
18. K. Hosono and H. Suzuki (1985). Morphological transformation of Chinese hamster cells by acylpeptides, inhibitors of cAMP phosphodiesterase, produced by *Bacillus subtilis*. *J. Biol. Chem. 260*, 11252–11255.
19. R. Marget-Dana, L. Thimon, F. Peypoux and M. Ptak (1992). Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochemie 74*, 1047–1051.
20. L. Thimon, F. Peypoux, R. Marget-Dana, B. Roux and G. Michel (1992). Interaction of bioactive lipopeptides, iturin A and Surfactin from *Bacillus subtilis*. *Biotech. Appl. Biochem. 16*, 144–151.
21. L. Thimon, F. Peypoux and G. Michel (1992). Interaction of surfactin, a biosurfactant from *Bacillus subtilis*, with inorganic cations. *Biotech. Lett. 14*, 713–718.
22. Y. Imai, H. Sugino, T. Fujita and A. Kakinuma (1971). Hypocholesterolemic effects of surfactin, a novel bacterial peptidelipid. *J. Takeda Res. Lab. 30*, 728–734.
23. Y. Ishigami, M. Osman, H. Nakahara, Y. Sano, R. Ishiguro and M. Matsumoto (1995). Significance of β-sheet formation for micellization and surface adsorption of surfactin. *Coll. Surfaces, B: Biosurfactants 4*, 341–348.
24. L. Thimon, F. Peypoux, J. Wallach and G. Michel (1993). Ionophorous and sequestring properties of surfactin, a biosurfactant from Bacillus subtilis. *Coll. Surfaces. B: Biosurfactants 1*, 57–62.
25. J.D. Sheppard, C. Jumarie, D.G. Cooper and R. Laprade (1991). Ionic channels induced by surfactin in planer lipid bilayer membranes. *Biochem. Biophys. Acta 1064*, 13–23.
26. J. Vater (1986). Lipopeptides, an attractive class of microbial surfactants. *Progr. Colloid Polym. Sci. 72*, 12–18.
27. R.M. Freidinger and D.F. Veber (1984). Design of novel cyclic hexapeptide somatostatin analogs from a model of the bioactive conformation, in: *Conformationally Directed Drug Design. Peptides and Nuclic Acids as Templets or Targets 1984*, J.A. Vida and M. Gordon, Eds, p. 169–187, ACS Symposium Series 251, USA.
28. E.T. Kaiser and F.J. Kézdy (1984). Amphiphilic secondary structure: design of peptide hormones. *Science 223*, 249–255.
29. S.R. LaBrenz and J.W. Kelly (1995). Peptidomimetic host that binds a peptide guest affording a β-sheet structure that subsequently self-assembles. A simple receptor mimic. *J. Am. Chem. Soc. 117*, 1655–1656.
30. D.F. Veber, R.M. Freidinger. D.S. Perlow, W.J. Paleveda Jr, F.W. Holly, R.G. Strachan, R.F. Nutt, B.H. Arison, C. Homnick, W.C. Randall, M.S. Glitzer, R. Saperstein and R. Hirschmann (1981). A potent cyclic hexapeptide analogue of somatostatin. *Nature 292*, 55–58.
31. R.M. Freidinger, D.F. Veber and D.S. Perlow (1980). Bioactive conformation of luteinizing hormone-releasing hormone: Evidence from a conformationally constrained analog. *Science 210*, 656–658.
32. P.W. Schiller and J. DiMaio (1982). Opiate receptor subclasses differ in their conformational requirements. *Nature 297*, 74–76.

33. Z. Szewczuk, K.L. Rebholz and D.H. Rich (1992). Synthesis and biological activity of new conformationally restricted analogues of pepstatin. *Int. J. Peptide Protein Res. 40*, 233–242.
34. L. Zhong and W.C. Johnson Jr (1992). Environment affects amino acid preference for secondary structure. *Proc. Natl. Acad. Sci. USA 89*, 4462–4465.
35. Shun-Cheng Li and C. M. Deber (1992). Influence of glycine residue on peptide conformation in membrane environments. *Int. J. Peptide Protein Res. 40*, 243–248.
36. Shun-Cheng Li and C. M. Deber (1993). Peptide environment specifies conformation. *J. Biol. Chem. 268*, 22975–22978.
37. S.N. Timasheff, R. Townend and L. Mescanti (1966). The optical rotary dispersion of the β-lactoglobulins. *J. Biol. Chem. 241*, 1863–1870.
38. M.R. Ghadiri, C. Soares and C. Choi (1992). A convergent approach to protein design. Metal ion-assisted spontaneous self-assembly of polypeptide into a triple-helix bundle protein. *J. Am. Chem. Soc. 114*, 825–831.
39. A. Grove, M. Mutter, J.E. Rivier and M. Montal (1993). Templet-assembled synthetic proteins designed to adopt a globular, four-helix bundle conformation from ionic channels in lipid bilayers. *J. Am. Chem. Soc. 115*, 5919–5924.
40. G.P. Dado and S.H. Gellman (1993). Redox control of secondary structure i a desigened peptide. *J. Am. Chem. Soc. 115*, 12609–12610.
41. M. Mutter, R. Gassmann, U. Buttkus and K.-H Altmann (1991). Switch peptides: pH-induced α-helix to β-sheet transitions of bis-amphiphilic oligopeptides. *Angew. Chem. Int. Ed. Engl. 30*, 1514–1516.
42. M. Mutter and R. Hersperger (1990). Peptides as conformational Switch: Medium-induced conformational transitions of designed peptides. *Angew. Chem. Int. Ed. Engl. 29*, 185–187.
43. E.T. Kaiser and F.J. Kezdy (1983). Secondary structure of proteins and peptides in amphiphilic environments (a review). *Proc. Natl. Acad. Sci. USA 80*, 1137–1143.
44. M.R. Ghadiri and C. Choi (1990). Secondary structure neucleation in peptides. Transition metal ion stabilised α-helices. *J. Am. Chem. Soc. 112*, 1630–1632.
45. C.M. Deber, A.R. Khan, Z. Li, C. Joensson, M. Glibowicka and J. Wang (1993). Val→Ala mutations selectively alter helix packing in the transmembrane segment of phage M13 coat protein. *Proc. Natl. Acad. Sci. USA 90*, 11648–11652.
46. Y. Ishigami (1993). Biosurfactants face increasing interest. *Inform 4*, 1156–1164.
47. M. Osman, Y. Ishigami, K. Ishikawa, Y. Ishizuka and H. Holmsen (1994). Dynamic transition of α-helix to β-sheet structure in linear surfactin correlating to critical micelle concentration. *Biotechnol. Lett. 16*, 913–918.
48. J.A. Smith and L.G. Pease (1980). Reverse turns in peptides and proteins. *CRC Crit. Rev. Biochem. 8*, 315–399.
49. W. Kabsch and C. Sander (1983). Dictionary of protein secondary structure: pattern recognition of hydrogen-bond and geometrical features. *Biopolymers 22*, 2577–2637.
50. G.D. Rose, L.M. Gierasch and J.A. Smith (1985). Turns in peptides and proteins. *Adv. Protein Chem. 37*, 1–109.
51. R.W. Woody (1974). Theoretical circular dichroism of polypeptides. Contribution of β-turn, in: *Peptides, Polypeptides and Proteins 1974*, E.R. Blout, F.A. Bovey, M. Goodman and N. Laton, Eds, p. 338–3350, John Wiley and Sons, New York.
52. J.H. Dyson, M. Rance, R.A. Houghton, R.A. Learner and P.E. Wright (1988). Folding of immunogenic peptide fragments of proteins in water solution I. Sequence requirements for the formation of a reverse turn. *J. Mol. Biol. 201*, 161–200.
53. B. Imperiali, S.L. Fisher, R.A. Moats and T.J. Prins (1992). A conformational study of peptides with the general structure Ac-L-Xaa-Pro-D-Xaa-L-Xaa-NH_2: Spectroscopic evidence for a peptide with significant β-turn character in water and in dimethyl sulfoxide. *J. Am. Chem. Soc. 114*, 3182–3188.
54. K. Takeda, A. Iba and EC. Shirahama (1981). Conformational change of poly (L-lysine) by sodium octyl sulfate as studied by stopped-flow circular dichroism method. *Bull. Chem. Soc. Jpn. 54*, 1793–1796.
55. K. Takeda (1985). Conformation analysis of poly (L-lysine) induced by various surfactants. *Bull. Chem. Soc. Jpn. 58*, 1210–1214.

