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Short Communication

The anti-thrombotic activity of surfactins

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Platelet aggregation was inhibited and the density of platelet-rich plasma (PRP) clots was decreased by the preincubation of PRP with surfactins, an acidic lipopeptide of *Bacillus subtilis* complex BC1212 isolated from soybean paste, in dose-dependent manner. Our findings suggest that surfactins are able to prevent a platelet aggregation leading to an inhibition of additional fibrin clot formation, and to enhance fibrinolysis with facilitated diffusion of fibrinolytic agents.

Key words: anti-platelet aggregation, platelet rich plasma, surfactins

Surfactin isolated from *Bacillus subtilis* has a strong surface tension-lowering activity, together with antiviral, antitumor, fibrinolytic and hypocholesterolemic activities [1,3,11,12]. It is a natural compound of industrial importance and attains increasing biotechnological and pharmaceutical interests [3]. Natural surfactin is a mixture of isofoms which slightly differ in their physiological properties due to a variation in the chain length and branching of its hydroxy fatty acid component as well as substitutions of the amino acid components of the peptide ring [2,5,8,9]. These variations, rather than being genetically determined, depend on the specific *B. subtilis* strain [11,12].

In this paper we report on the preparative separation of the surfactin mixture obtained from the *Bacillus subtilis* complex BC1212 which was isolated from soybean paste and a high producer of surfactin. The surfactin homologos were fractionated by high resolution reversed phase HPLC and characterized by mass spectrometry (electrospray LC/MS). In addition, we investigated that the surfactin inhibited platelet-rich plasma (PRP) clots formation and a platelet aggregation.

Bacillus subtilis complex BC1212 was isolated from soybean paste and *Bacillus subtilis* ATCC 21332, a well-known surfactin producing strain, were grown on a shaking incubator (150 rpm) in Erlenmeyer flasks containing 100 mL of the trypticase soy broth during 30 hr at 28°C.

Cell-free culture was concentration by an Vivaspin20 ultra-filtration cell (Viva science, Germeny) with polyethersulfone membrane of 3,000 molecular weight cut-offs. Ten ml of cell-free culture was centrifuged at approximately 6,000 *g* for 180 min.

The surfactin mixture was purified using a preparative reversed phase HPLC column Watcheres120 ODS-BP (5 ìm, 150 × 4 mm; Daiso, Japan) and a HP1100 series HPLC system. The surfactin homologos were eluted by isocratic runs with a mixture of 0.1% trifluoric aicd-acetonitrile (15 : 85, v/v). The ES-MS analysis was performed on a Hewlett-Packard 5989 electrospray mass spectrometer with a Hewlett-Packard Atmospheric Pressure Ionization (API) interface fitted with a hexapole ion guide. The optimal condition for the analysis of surfactin employed pneumatic nebulization with nitrogen (45 p.s.i.) and a counterflow of nitrogen (9 l/min) heated to 350°C for the nebulization and desolvation of the introduced liquid.

Blood samples were obtained from healthy and drug-free volunteers. Venous blood was collected into a plastic tube containing 1/10 volume of 3.8% sodium citrate. PRP was obtained after centrifugation of whole blood at 1000 rpm for 10 min. Platelet in PRP were counted by the light microscopy. After counting, the number of platelet was adjusted with phosphate buffered saline (PBS) to 1×10^5 platelets/μl. The PRP was incubated with surfactin (final concentrations of 12.5, 25, 50 and 100 μg/ml purified from extracts of *Bacillus subtilis* complex BC1212) for 15 min at the room temperature. Two hundred μl of preincubated PRP and 40 μl of collagen (final concentration 2 μg/ml) were added into siliconized cuvettes. Aggregation was determined with a dual channel aggregometer (NKK HemaTracer 1; SSR Engineering, Japan) at 37°C and stirred at 1,000 rpm. The instrument was calibrated to 100% light transmission with PBS. Aggregation was recorded as the increase in the light transmission.

The PRP was incubated with surfactin (yielding final concentrations of 2.5, 25, 125 and 250 μg/ml) purified from

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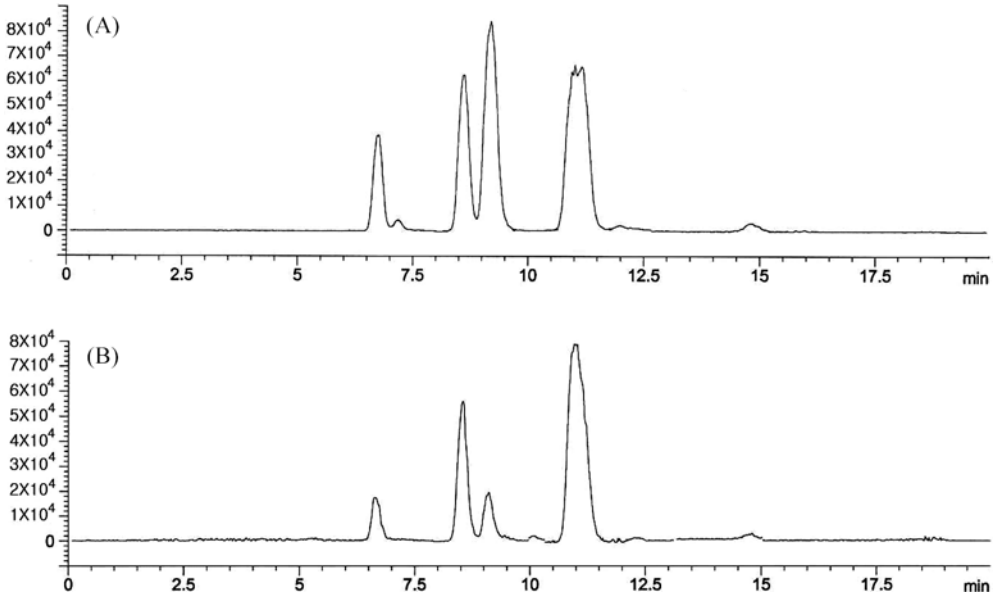

Fig. 1. Separation of surfactin isoforms isolated from *Bacillus subtilis* ATCC 21332 (A) and *Bacillus subtilis* complex BC1212 (B) with a mobile phase consisting of 0.1% trifluoracetic acid-acetonitrile. The compositions of surfactin isoforms showed a significant difference from the surfactin produced by *Bacillus subtilis* ATCC21332 and the surfactin produced by *Bacillus subtilis* complex BC1212.

extracts of *Bacillus subtilis* complex BC1212 for 15 min at the room temperature. Clots were formed in 96-well flat bottom micro-well plates. First, 120 μl of preincubated PRP were poured into well, and then 20 μl of thrombin (5 NIH units/ml)-$CaCl_2$ (25 mM) solution was adequately mixed with reagents and clot formation began immediately. The value of lysis was estimated as the changes of turbidity of the clots. During the experiment, the turbidity was monitored as the optical densities (OD) at 405 nm at 5-min intervals using a microphotometer (Thermo-max; Molecular Device, USA).

An ultrafiltration of medium was concentrated and chromatogrphed on a reversed phase column with a mixture of 0.1% trifluoric aicd-acetonitrile (15 : 85, v/v) as the eluent. MS analysis of surfactin isoforms revealed that it was mixture with *quasi*-molecular ions at *m/z* = 1008, 1022, 1036, 1050 ($[M+H]^+$), and *m/z* = 1030, 1044, 1058, 1072 ($[M+Na]^+$). By reversed-phase HPLC, surfactin was found to be a mixture of at least 5 compounds on the basis of their molecular weight (Fig.1). Surfactin isolated from *Bacillus subtilis* complex BC1212 was composed of sufactin A (8.94 ± 2.65% based on the relative amount), B_1 (23.87 ± 0.14), B_2 (10.61 ± 2.56%), C (56.57 ± 5.32%) and D (0.10 ± 0.02%). These compositions showed a significant difference from the surfactin isolated from *Bacillus subtilis* ATCC21332 (surfactin A, 11.15 ± 1.09%; B_1, 19.02 ± 0.26%; B_2, 30.08 ± 0.03%; C, 39.04 ± 1.37%; D, 0.13 ± 0.03%). The surfactin analogs were found to be produced by different bacilli strains. Originally isolated from soil strain *Bacillus subtilis*, standard surfactin is a macrolide containing the heptapeptide

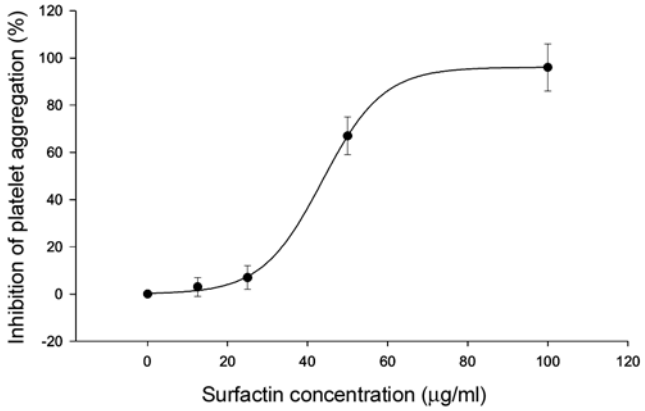

Fig. 2. Inhibition of the platelet aggregation by preincubation of platelet-rich plasma with surfactins. Aggregation was determined with a dual channel aggregometer at 37°C and stirred at 1000 rpm. Aggregation was recorded as the increase in the light transmission. Data were presented as mean ± SD.

sequenced Glu-Leu-Leu-Val-Asp-Leu-Leu and a lipid portion which is a mixture of several â-hydroxy-fatty acids with chain length of 13-15 carbon atoms [1,5,8,10]. Nagai *et al.* [8] revealed that *Bacillus natto* KMD 2311 contained at least 8 homologus depsipeptides with a *n*-, *iso*, *anteiso*-β-hydroxy-fatty acids of carbon numbers 13 to 16 as part of the ring system.

Platelet activation and the resulting aggregation are associated with various pathologic conditions including cardiovascular and cerebrovascular thromboembolic disorders. It is well known that platelet-rich thrombi are resistant to

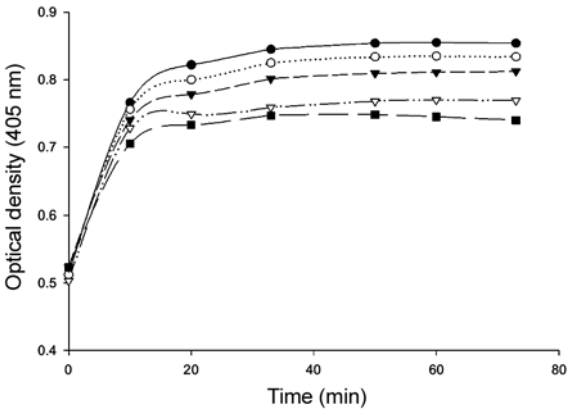

Fig. 3. Effect of surfactins, on density of PRP clot. PRP clots were formed in 96-well flat-bottom microwell plate which was treated with various concentration of surfactin, 0 (●), 2.5 μg/ml (○), 25 μg/ml (▼), 125 μg/ml (▽) and 250 μg/ml (□). The density of PRP clot evaluated as the changes of turbidity at 405 nm. Each point presents the mean of three separated triplicate experiments.

lysis by plasminogen activators [4,13]. Surfactin has been previously reported to increase plasmin-catalyzed *in vitro* fibrin dissolution in a fibrin plate assay [1,6]. However, the influence of surfactin on platelet aggregation was not fully investigated as compared to its fibrinolytic activity. In this study, platelet aggregation which was induced by collagen was inhibited by surfactins. This inhibition was dependent on the concentrations of surfactin (Fig. 2). EC_{50} (corresponding to the surfactin concentration giving a 50% inhibition of platelet aggregation) was obtained at 42.45 ± 6.23 mg/ml.

As platelets aggregate in clot formation, the clot retracts to a small fraction of its original volume, and the density of the clot increases [3]. When the PRP clots were completely formed and retracted, the density of the clots reached the max OD. The max OD value was significantly decreased by pretreatment of PRP with surfactin. This effect of surfactin was seen to be dose-dependent. These result indicated that the preincubation of PRP with surfactin inhibited the retraction of the PRP clot without retraction abolished the platelet-induced inhibition of lysis. Therefore, it is possible that the PRP clot which has been reacted with surfactin is easy to dissolve. The effect of surfactin was concluded to be attributable to facilitated diffusion of plasmin on the PRP clot.

In conclusion, the density of PRP clots was decreased and platelet aggregation was inhibited by the preincubation of PRP with surfactin in dose-dependent manner. Our findings suggest that surfactin is able to prevent a platelet aggregation leading to an inhibition of additional fibrin clot formation, and to enhance fibrinolysis with facilitated diffusion of fibrinolytic agents. The use of surfactin in combination with thromlytic agents may efficiently protect thromboembolic disorders.

Acknowledgments

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References

1. **Arima K, Kakinuma A, Tamura G.** Surfactin, acrystalline peptidelipid surfactant produced by Bacillus subtilis: isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun 1968, **31**, 488-494.
2. **Baumgart F, Kluge B, Ullrich C, Vater J, Ziessow D.** Identification of amino acid substitutions in the lipopeptide surfactin using 2D NMR spectroscopy. Biochem Biophys Res Commun. 1991, **77**, 998-1005
3. **Desai JD, Banat IM.** Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 1997, **61**, 47-64.
4. **Flank E.** Dynamics in thrombus formation. Ann N Y Acad Sci 1992, **667**, 204-223.
5. **Kanatomo S, Nagai S, Ohki K, Yasuda Y.** Study on surfactin, a cyclic depsipeptide. I. Isolation and structure of eight surfuctin analogs produced by Bacillus natto KMD 2311 Yakugaku Zasshi. 1995, **115**, 756-764.
6. **Kikuchi T, Hasumi K.** Enhancement of plasminogen activation by surfactin C: augmentation of fibrinolysis in vitro and in vivo. Biochim Biophys Acta 2002, **1596**, 234-245.
7. **Itokawa H, Miyashita T, Morita H, Takeya K, Hirano T, Homma M, Oka K.** Structural and conformational studies of [Ile7] and [Leu7]surfactins from *Bacillus subtilis natto*. Chem Pharm Bull 1994, **42**, 604-607.
8. **Nagai S, Ohki K, Yasuda Y.** Study on surfactin, a cyclic depsipeptide, I: isolation and structure of eight surfactin analogs produced by *Bacillus natto* KMD 2311. Yakugaku Zasshi 1995, **115**, 756-764.
9. **Kowall M, Kluge B, Stein T, Franke P, Ziessow D.** Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB-105. J Colloid Interphase Sci 1998, **204**, 1-8.
10. **Oka K, Hirano T, Homma M, Ishii H, Murakami K, Mogami S, Motizuki A, Morita H, Takeya K, Itokawa H.** Satisfactory separation and MS-MS spectrometry of six surfactants isolated from *Bacillus subtilis natto*. Chem Pharm Bull 1993, **41**, 1000-1002.
11. **Singer ME.** Molecular genetics of biosurfactnats production. Curr Opin Microbiol 1985, **9**, 263-269.
12. **Thimon L, Peypoux F, Maget-Dana R, Roux B, Michel G.** Interactions of bioactive lipopeptides, iturin A and surfactin from *Bacillus subtilis*. Biotechnol Appl Biochem 1992, **16**, 144-151.
13. **Vargafting BB, Chiganard M, Benvensite J.** Present concepts on the mechanisms of platelet aggregation. Biochem Pharmacol 1981, **30**, 263-271.