SURFACTIN, A CRYSTALLINE PEPTIDELIPID SURFACTANT PRODUCED BY BACILLUS SUBTILIS : ISOLATION, CHARACTERIZATION AND ITS INHIBITION OF FIBRIN CLOT FORMATION

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In a study intending an approach to blood clotting and / or fibrinolytic system(s) from microbiology using microbial products, we have recently found a potent clotting inhibitor produced in the culture fluids of several strains of B. subtilis.

The inhibitor was isolated as white needle crystalls and from its strong surface active nature exceeding that of sodium lauryl sulfate was named "Surfactin". Surfactin is a peptidelipid with the molecular weight of about 1050 and is composed of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine (1:1:1:2:2) and unidentified fatty acids. This bacterial peptidelipid remarkably elongates the time necessary for fibrin clot formation by inhibiting the conversion of fibrin monomer to fibrin polymer.

In this communication we wish to report on the isolation and characterization of surfactin and the determination of its inhibition site in fibrin clot formation.

Further attempts carried out to know some other physiological functions of surfactin have revealed in this peptidelipid several interesting activities, for example, inhibition of protein denaturation, lysis of intact cells of some of both gram positive and gram

negative bacteria with the leakage of cell contents, etc, details of which will appear in succeeding papers.

Isolation and Characterization of Surfactin Surfactin is produced in a fair amount in the culture fluids of several strains of B. subtilis. Especially, B. subtilis IFO 3039, IAM 1069, IAM 1213, IAM 1259, IAM 1260 can accumulate 50 – 100 μg surfactin per ml nutrient broth in 24 h culture.

Surfactin is an acidic substance soluble in alkaline water and in many kinds of organic solvents including methanol, ethanol, acetone, ethylacetate, chloroform, methylene chloride, acetic acid but insoluble in water, petroleum ester, hexane, etc. Addition of $(NH_4)_2SO_4$ to 50 % saturation or of divalent cations in alkaline solution makes surfactin insoluble and results in its precipitation. Using these solubility characteristics, surfactin was crystallized as follows.

B. subtilis IAM 1213 was grown in a nutrient broth at 30° for 24 h on a reciprocal shaker. After the elimination of cells the reaction of filtrate was adjusted to pH 2 with HCl and the resulting precipitate was collected and dissolved in alkaline water, followed by the addition of $CaCl_2$. This Ca-precipitate was collected and resuspended in 0.01 N HCl and newly generated acid-precipitate was extracted with ether. Active material obtained by concentration was dissolved in acetone and decolorized by passing an active charcoal column pretreated with acetone. The clear acetone solution thus obtained was applied to a Sephadex LH 20 column pretreated with acetone and to the eluate containing active fraction, water of about half the volume of acetone was added to make the solution slight turbid. When this solution was preserved in cold for 2 months, surfactin was obtained as crystalline white needles (Figure 1), m.p. 138 – 140°, with the yield of 40 – 50 mg per liter of culture medium.

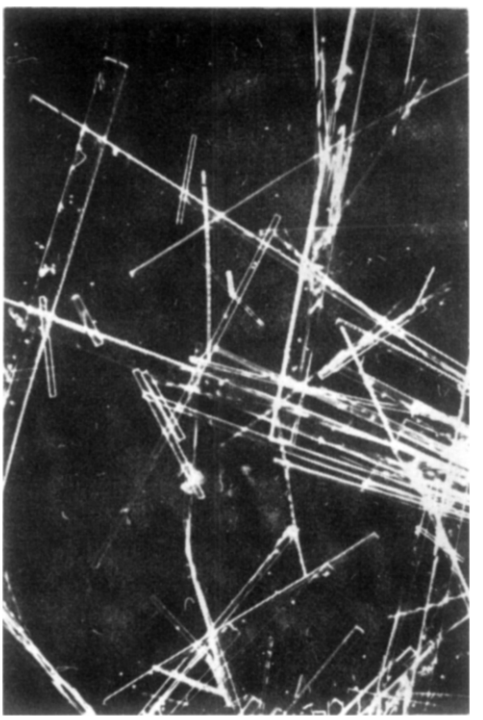

Figure 1. Micrograph of crystalls of surfactin

Chemical or physicochemical data of surfactin were as follows. C 59.6 %, H 9.0 %, N 9.1 %. $[\alpha]_D^{27}$ +40 (1 %, in chloroform), -39° (1 %, in methanol). By vapor pressure method, the molecular weight was calculated to be about 1050. UV absorption spectrum showed the absence of absorption maximum in the range from 230 mμ to 400 mμ. On the other hand, presence of peptide bonds was cleary demonstrable from IR spectrum (Figure 2). Biuret reaction is positive, ninhydrin reaction is negative. When surfactin was subjected to acid hydrolysis in 6 N HCl in a sealed tube at 110° for 20 h, L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine were detected by an amino acid analyzer and by microbioassay in the molecular ratio of 1:1:1:2:2, always accompanied

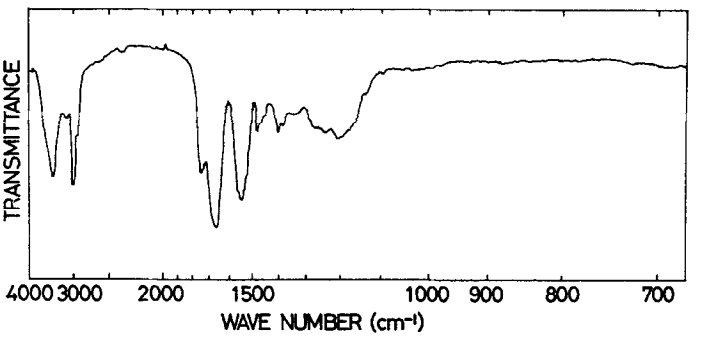

Figure 2. IR spectrum of surfactin (KBr)

by a slight amount of L-isoleucine. And simultaneously, yellowish brown-colored HCl-insoluble oily material was liberated with the recovery of 20 – 25 % original surfactin. This oily material, after purification by repeated transfer between ether and alkaline water, did not contain N and showed in IR spectrum typical features characteristic to fatty acids.

From these data, surfactin is confirmed to be a peptidelipid, with the molecular weight aound 1050, composed of amino acids and fatty acids. The identification of fatty acids are now in progress and will be reported in a forthcoming paper.

Surfactin was proved to have a potent surface activity by far stronger than that of sodium lauryl sulfate (TABLE 1). It might be the first time that such a strong surfactant was isolated from microbial origins. The role that surfactin may play in the physiology of parent bacteria has not yet been sufficiently clarified and remains for us as a tempting problem in the future.

Inhibition of Fibrin Clot Formation When surfactin was added to the thrombin-fibrinogen system, the formation of fibrin clot was dramatically inhibited and a remarkable elongation of clotting time and

TABLE 1 Surface activity of surfactin

	Surface Tension[a] (dyne/cm)
Distilled water	71.98
0.1 M $NaHCO_3$ containing	
———	71.57
0.005 % SLS[b]	56.56
0.05 % SLS	31.25
0.005 % surfactin	27.90
0.05 % surfactin	27.00

(a) Determined by drop weight method using a stalagometer.
(b) Sodium lauryl sulfate.

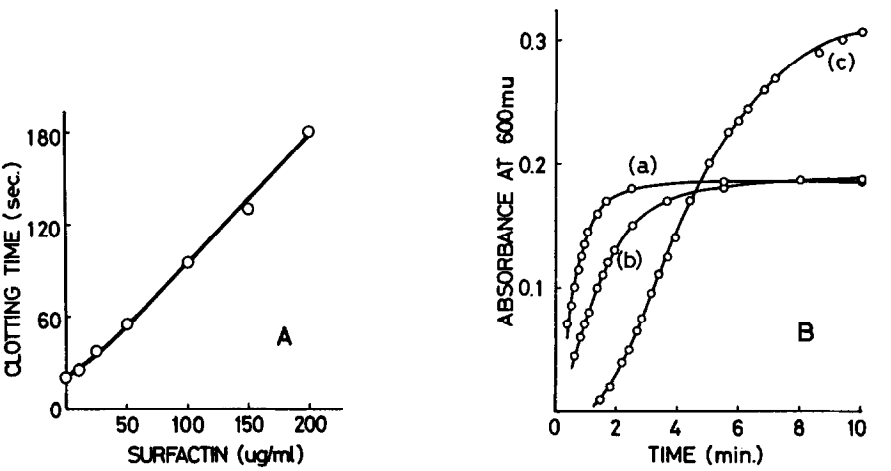

Figure 3 Inhibition of fibrin clot formation by surfactin

A. Elongation of clotting time Clotting system : fibrinogen 0.15 %, (Armour Bovine Fibrinogen Fraction I containing approximately 30 % clottable protein was used throughout these experiments), surfactin (dissolved and neutralized with NaOH) as indicated, thrombin 5 u./ml, 0.01 M tris-0.073 M NaCl (pH 7.4). 37°.

B. Retardation of Opacity Development Clotting system : fibrinogen 0.15 %, surfactin 0 µg/ml (a), 50 µg/ml (b), 500 µg/ml (c), thrombin 2.5 u./ml, 0.02 M tris-0.145 M NaCl (pH 7.4) (Yachnin, 1964). Opacity development after thrombin addition was followed at 20° by reading absorbance at 600 mµ in cuvettes with 1 cm light path.

a retardation of opacity development were observed (Figure 3A, Figure 3B). Calculated from the relationship between thrombin concentration and clotting time, the concentration of surfactin needed to bring about 50 % inhibition of thrombin activity was about 30 µg/ml.

The inhibition site of surfactin was determined by several methods including sedimentation analyses with an ultracentrifuge. In Figure 4, which is a sedimentation diagram of surfactin-inhibited clotting system containing fibrinogen, surfactin and thrombin, nearly complete disappearance of fibrinogen and appearance of a much heavier component are cleary indicated. This new component is comparable with those intermediary polymers which appeared in thrombin-fibrinogen systems inhibited by hexamethylene glycol (Ferry and Shulman, 1949), by urea (Ehrlich et al, 1952), and by 1 M NaBr at pH 6 (Donnelly et al, 1955). The fact that no clotting occurred even after the proteolytic action of thrombin has completed with the generation of polymerization intermediates shows that the inhibition site of surfactin in this clotting

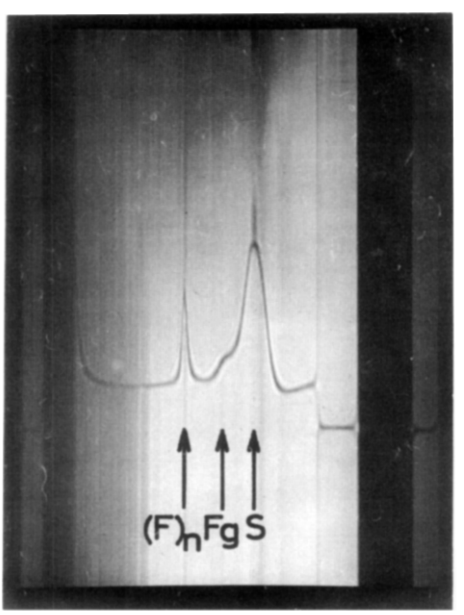

Figure 4 Sedimantation diagram of surfactin-inhibited clotting system
Clotting system : fibrinogen 0.3 %, surfactin 1.0 %, thrombin 0.5 u./ml, 0.01 M tris-0.073 M NaCl (pH 7.4). Reaction : 20°, 30 min. Sedimented from right to left in a Hitachi Ultra-centrifuge Type UCA-1A at 55,430 rpm and at 11°, using a synthetic boundary cell. Photograph at 15 min. after attaining top speed. Bar angle 70°. Abbrivations in photograph : S, surfactin; F, fibrinogen; $(F)_n$, intermediates in polymerization from fibrin monomer to fibrin polymer.

system is the polymerization step from fibrin monomer to fibrin polymer. This conclusion was strongly supported by both the facts that esterase activity of thrombin against tosyl arginine methyl ester was not inhibited at all by surfactin of the concentration used in experiments of Figure 3 and that fibrin monomer prepared in 1 M NaBr, pH 5.3 (Donnelly et al, 1955) did not clot in the presense of surfactin even after dilution with tris-NaCl, pH 7.4.

Several surfactants like sodium lauryl sulfate are known to disaggregate and solubilize the fibrin polymer (Loewy et al, 1961). The ability of surfactin to inhibit clot formation may derive from its strong surface active nature.

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