

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

Kei Arima, Atsushi Kakinuma, and Gakuzo Tamura

Department of Agricultural Chemistry
University of Tokyo, Tokyo, Japan

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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 crystals 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 $(\text{NH}_4)_2\text{SO}_4$ 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.

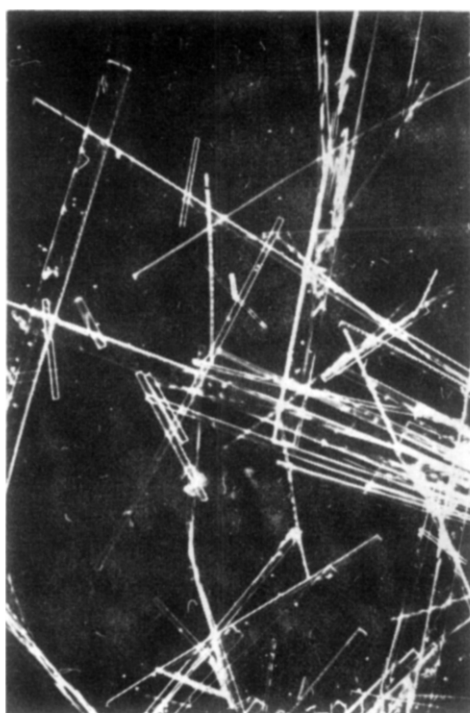


Figure 1. Micrograph of crystals of surfactin

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 m μ to 400 m μ . On the other hand, presence of peptide bonds was clearly 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

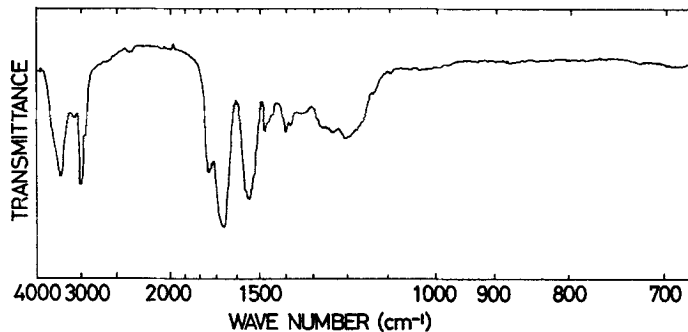


Figure 2. IR spectrum of surfactin (KBr)

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 around 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	
0.005 % SLS ^(b)	71.57
0.05 % SLS	56.56
0.005 % surfactin	27.90
0.05 % surfactin	27.00

(a) Determined by drop weight method using a stalagometer.

(b) Sodium lauryl sulfate.

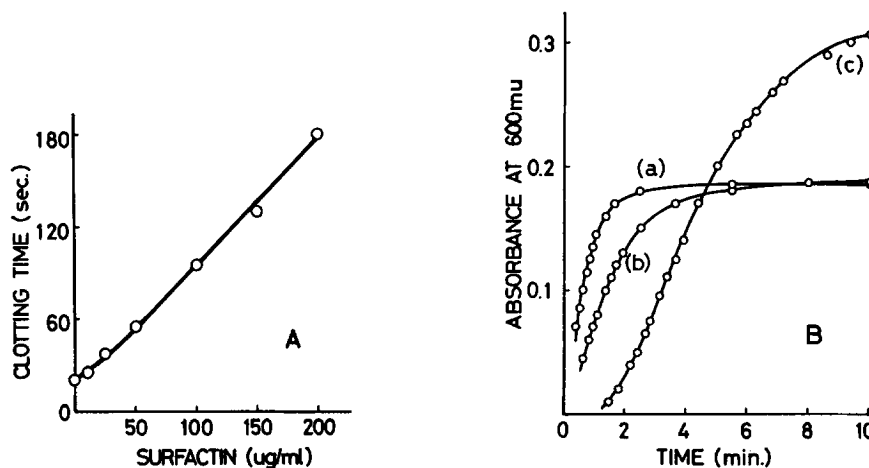


Figure 3 Inhibition of fibrin clot formation by surfactin

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 μ g/ml (a), 50 μ g/ml (b), 500 μ g/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 μ in cuvettes with 1 cm light path.

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