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A NOVEL PROTOPLAST-BURSTING FACTOR (SURFACTIN) OBTAINED FROM *BACILLUS SUBTILIS* IAM 1213

I. THE EFFECTS OF SURFACTIN ON *BACILLUS MEGATERIUM* KM

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SUMMARY

1. Surfactin burst more than 90% of protoplasts prepared from *Bacillus megaterium* KM at a concentration of 14 μg/ml.

2. Surfactin released intracellular ultraviolet-absorbing substances from intact cells without any decrease of their turbidity.

3. Surfactin inhibited glucose oxidation of cells grown in low P_i medium.

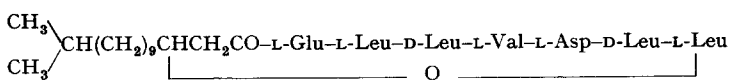
4. Surfactin inhibited the synthesis of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1).

INTRODUCTION

Many workers[1-3] have reported the chemical components of various bacterial membranes, but little is known about the native form of membranes.

In order to clarify the structure of bacterial membranes, it seemed useful to investigate various reagents or treatments that degrade the membrane structure. In surveying active substances which induce the degradation of protoplasts in hypertonic medium, we found the protoplast-bursting factor (P.B. factor) in pig pancreas and reported its physicochemical properties and physiological effects on microorganisms[4,5].

Further screening in more than 100 strains of Gram-positive bacteria led to the discovery of a very strong surface-active substance which was produced by *Bacillus subtilis* IAM 1213 and which burst protoplasts in hypertonic medium. The substance was a peptide lipid which showed the following chemical structure[6]:



and was named surfactin.

The present investigation is an extension of our preliminary studies[7] and describes the physiological effects of surfactin on microorganisms.

METHODS AND MATERIALS

Organisms and cultural conditions

Bacillus megaterium KM was mainly used and grown in nutrient broth. The enzyme alkaline phosphatase is inducible under conditions of phosphate deprivation. The organism was grown at 30° in an L tube with the phosphate-limiting medium of TAKEDA AND TSUGITA[8], which contains 1% Bactopeptone, 1% sodium lactate, 0.01% Tris, 0.3% NaCl, 0.005% $MnSO_4 \cdot n\ H_2O$, 0.03% $MgSO_4 \cdot 7\ H_2O$, 0.01% $CaCl_2 \cdot 2\ H_2O$.

Preparation of protoplasts

B. megaterium KM was cultivated in nutrient broth at 30° for 16 h. After centrifugation, the cells were washed with distilled water and resuspended (100 mg wet wt. per 10 ml) at room temperature in 0.05 M phosphate buffer (pH 7.38) containing 20% sucrose and 5 mM Mg^{2+}. Lysozyme was then added, to a final concentration of 0.1 mg/ml and, the suspension was incubated for 60 min at 37°.

Protoplast-bursting activity

0.5-ml aliquots of solutions containing various concentrations of surfactin, sodium lauryl sulfate and cetyltrimethylammonium bromide solubilized in 0.05 M phosphate buffer (pH 7.38) were added to 2.5-ml aliquots of protoplast suspension. The decrease in absorbance at 600 nm was determined against incubation time at room temperature with a Hitachi–Beckman spectrophotometer.

Determination of leakage of intracellular ultraviolet-absorbing substances

The cells of *B. megaterium* KM, previously grown in nutrient broth at 30° for 20 h, were starved by suspension at 30° for 1 h in 0.05 M phosphate buffer (pH 7.38), with shaking. Surfactin was added to the starved cell suspension at various concentrations. At a given interval the cell suspension was centrifuged and the absorbances at 260 and 280 nm of the supernatant were determined with a Hitachi–Beckman spectrophotometer.

Oxidation of substrates

Conventional manometric techniques were used to measure the oxidation of substrates by *B. megaterium* KM at 30°. The microbe was cultivated either in nutrient broth or in low P_i medium at 30° for 20 h. The cells were then centrifuged and washed once by resuspending in 0.05 M phosphate buffer (pH 7.38). Various concentrations of cells were used. The buffer concentration in the vessels was 0.05 M phosphate buffer (pH 7.38) and the total volume was 2.0 ml.

The assay method for alkaline phosphatase

One drop of toluene was added to 0.5 ml of the culture grown in low P_i medium and the mixture was incubated with 2.0 ml of 0.02% disodium *p*-nitrophenylphosphate in 1 M Tris–HCl buffer (pH 8.0) containing 5 mM Mg^{2+} at 30°. After 20 min, 0.5 ml of 13% K_2HPO_4 was added to stop the reaction. The increase of absorbance at 410 nm resulting from the liberation of *p*-nitrophenol was determined after removal

of the cell debris by centrifugation. One unit of enzyme activity was defined as that giving an increase in absorbance of 1.0 per min.

Protein determination

Protein was determined by the method of LOWRY *et al.*[9].

Chemicals

Lysozyme was obtained from Sigma Chemical Corp.

RESULTS

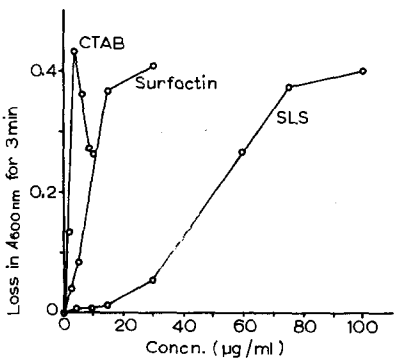
Effects of surfactin and other detergents on protoplasts

Surfactin burst protoplasts instantaneously and a small loss in absorbance at 600 nm was observed even after further incubation. The same phenomena were observed with other detergents, but the extent of protoplast degradation by these materials differed at their effective concentrations. When the loss in $A_{600\,nm}$ for 3 min was plotted against the concentration of reagents, surfactin, sodium lauryl sulfate and cetyltrimethylammonium bromide burst protoplasts by more than 90% at concentrations of 14, 74 and 3 μg/ml, respectively (Fig. 1). The activity of surfactin increased in the presence of K^+ or Na^+

Free carboxyl groups in the peptide part of surfactin were easily esterified by treatment with diazomethane in ether solution. The methylated surfactin thus obtained lost its protoplast-bursting activity, together with its hydrophilic property.

Leakage of intracellular ultraviolet-absorbing substances by surfactin

Surfactin did not inhibit the growth of *B. megaterium* KM in nutrient broth, even at a concentration of 100 μg/ml, but when surfactin was added to the starved cell suspension at the same concentration, intracellular ultraviolet-absorbing substances were released without any decrease of the turbidity of the cell suspension and the viability decreased instantaneously, as shown in Fig. 2.



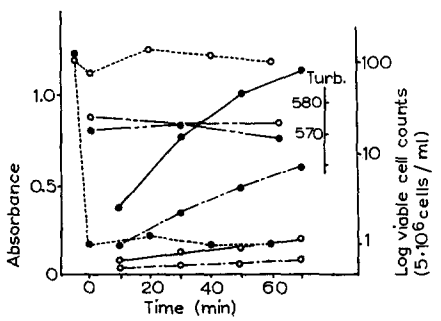


Fig. 1. The protoplast degradation activity of various reagents. The assay method was as described in METHODS. CTAB, cetyltrimethylammonium bromide; SLS, sodium lauryl sulfate.

Fig. 2. Effects of surfactin on the viable cell counts, the leakage of intracellular materials and the turbidity of *B. megaterium* KM. The assay method of the leakage of intracellular materials was as described in METHODS. ○, control; ●, 100 μg/ml surfactin. – – – –, viable cell counts; —·—·—, turbidity; — — —, absorbance at 260 nm; ———, absorbance at 280 nm.

The amount of cellular materials released by the addition of surfactin for 60 min was three times higher than the amount extracted by boiling water. The same phenomenon was observed at a lower concentration of 50 μg/ml. As antagonistic substances to surfactin were not found in nutrient broth, we thought that the membrane structure might have changed during starvation.

On the other hand, surfactin did not affect the viability of starved *Escherichia coli* K 12 or cause the leakage of cellular materials, even at a concentration of 100 μg/ml.

Effect of surfactin on the utilization of glucose by B. megaterium KM

Surfactin inhibited completely the glucose oxidation of microbes grown in low P_i medium at a concentration of 15 μg/ml, but microbes grown in nutrient broth

TABLE I

EFFECTS OF SURFACTIN ON THE OXIDATION OF GLUCOSE BY CELLS GROWN IN LOW P_i MEDIUM AND BOUILLON MEDIUM

0.1 ml of 1% glucose solution was used as a substrate. In the Q_{O_2} values shown, the Q_{O_2} values of endogenous respiration were subtracted.

Medium	*Expt. No.*	*Surfactin (μg/ml)*	*Cell wt. (mg/vessel)*	Q_{O_2} (μl)	%
Cells grown in low P_i medium	1	0	1	12.6	100
		10		8	63.5
		15		0	0
	2	0	2	12	100
		10		10.2	85
		15		0	0
Cells grown in bouillon medium	1	0	1	37.9	100
		10		45.2	119
		15		46.7	123
	2	0	1	40.3	100
		10		53.5	132
		15		40.2	100

TABLE II

EFFECTS OF SURFACTIN ON THE OXIDATION OF VARIOUS AMINO ACIDS BY CELLS GROWN IN LOW P_i MEDIUM

0.1 ml of 0.1 M amino acids solution was used as substrates. The Q_{O_2} values of endogenous respiration were subtracted.

Surfactin (μg/ml):	*0*		*10*		*15*	
	Q_{O_2} (μl)	%	Q_{O_2} (μl)	%	Q_{O_2} (μl)	%
L-Serine	109	100	94	86	88	81
L-Glutamic acid	177	100	181	102	174	98.4
DL-Alanine	86	100	93	109	90	105
L-Aspartic acid	173	100	177	102	153	89.4
L-Arginine	148	100	111	75	122	83
L-Valine	14	100	10.5	77	12	88

oxidized glucose normally in the presence of surfactin (Table I). On the contrary, the oxidation of various amino acids such as glutamic acid, aspartic acid, serine and alanine was not inhibited by surfactin even in cells grown in low P_i medium (Table II). Since these amino acids were oxidized through the tricarboxylic acid cycle, it can be presumed at least that surfactin did not inhibit the enzymes of the tricarboxylic acid cycle. Sodium lauryl sulfate did not inhibit glucose oxidation by cells grown in low P_i medium (Table III).

TABLE III

EFFECTS OF SODIUM LAURYL SULFATE ON THE OXIDATION OF GLUCOSE BY CELLS GROWN IN LOW P_i MEDIUM AND BOUILLON MEDIUM

0.1 ml of 1 % glucose solution was used as a substrate.

	Low P_i medium				*Bouillon medium*			
	Endogenous respiration	*Substrate respiration*	*Sodium lauryl sulfate (μg/ml)*		*Endogenous respiration*	*Substrate respiration*	*Sodium lauryl sulfate (μg/ml)*	
			50	*100*			*50*	*100*
Q_{O_2} (μl)	18.4	28.4	28.2	30.9	92.8	142.9	140.5	133.5
	—	10.3	9.8	12.5	—	50.1	47.7	40.7
%	—	100	95.4	118.5	—	100	95.1	81.2

Glucose was thought to be oxidized during the process of incorporation into cells mediated mainly by hexokinase. Surfactin, however, did not inhibit the [^{14}C]-glucose uptake by cells grown in low P_i medium or the hexokinase activity of yeasts.

When the contents of lipids in two kinds of membrane fractions prepared from cells grown both in nutrient broth and low P_i medium were compared, the former contained 0.7 % more phospholipids and 3.1 % more neutral lipids than did the latter, but their components were the same. So it may be attributed to the difference in their neutral lipids contents that the effects of surfactin are noticeably different.

Effects of surfactin on the synthesis of alkaline phosphatase of B. megaterium KM

When the effect of surfactin on alkaline phosphatase synthesis, which was believed to be carried out at the cell membrane, was examined, surfactin inhibited the synthesis of the enzyme at a concentration of 10 μg/ml, which did not affect the growth but induced the degradation of protoplasts in hypertonic medium. As shown in Fig. 3, the activity of alkaline phosphatase without surfactin increased with incubation time, but no further increase in alkaline phosphatase activity was observed in the presence of surfactin. When the increase in the amount of the cellular protein was plotted against incubation time, surfactin did not affect the rate of protein synthesis by the cells.

On the other hand, surfactin did not inhibit the activity of alkaline phosphatase of *B. megaterium* KM, even at the high concentration of 40 μg/ml.

The effects of surfactin on the synthesis of the alkaline phosphatase of other microorganisms, *e.g. B. subtilis*, *Bacillus cereus*, *E. coli*, were not as marked as in the case of *B. megaterium* KM. Surfactin just delayed the commencement of the enzyme synthesis by 30 min, even when present in higher concentrations.

On the other hand, sodium lauryl sulfate did not affect significantly the growth or the synthesis of alkaline phosphatase of *B. megaterium* KM in low P_i medium, even at a concentration of 100 μg/ml. Tween 20 showed no effects on growth or alkaline phosphatase synthesis, as was the case with sodium lauryl sulfate.

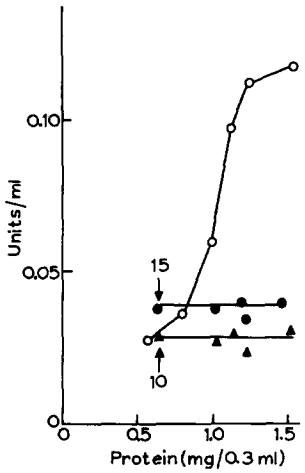


Fig. 3. Differential synthesis of alkaline phosphatase plotted in the presence (●—●, ▲—▲) or absence (○—○) of surfactin. The assay method was as described in METHODS. Surfactin was added (see arrow) at the concentrations shown (μg/ml).

DISCUSSION

A few substances of bacterial origins were reported to have the protoplast-bursting activity, *e.g.* "autolysin"[10], "bacteriolytic principle"[11] and "protoplast dissolving factor"[12], but these substances were very crude. Their effects on microorganisms were not studied in detail. A strong surface-active substance from the culture filtrate of *B. subtilis* IAM 1213 was purified and named surfactin.

Surfactin induced not only a rapid degradation of protoplasts in hypertonic medium but also a remarkable leakage of ultraviolet-absorbing materials from starved cells of *B. megaterium* KM, concomitantly with loss of its viability. These data suggest that surfactin causes some damage to the membrane structure and that this damage is fatal to the starved cell.

Surfactin also inhibited glucose oxidation and the synthesis of alkaline phosphatase by *B. megaterium* KM grown in low P_i medium. As these processes are thought to be involved in various functions of bacterial membranes, these inhibitions appear to be the results of particular interactions between surfactin and some components of the cell membrane.

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