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

II. THE INTERACTION OF SURFACTIN WITH BACTERIAL MEMBRANES AND LIPIDS

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SUMMARY

1. The protoplast-bursting activity of surfactin was neutralized by membrane fractions which had been solubilized in dilute alkali solution.

2. After digestion of the membranes by trypsin, the residual fraction, containing high contents of lipids, still retained neutralizing activity against surfactin.

3. Phospholipids such as phosphatidylethanolamine and phosphatidylcholine neutralized the activity of surfactin.

INTRODUCTION

Surfactin was a type of surface-active agent and showed various physiological effects on microbes different from those of commercial detergents. It was suggested in the preceding article that these effects might be caused by the interaction between surfactin and some components of the membranes.

As surfactin strongly induced the degradation of protoplasts prepared from *Bacillus megaterium* KM, examinations as to whether the membrane fraction of the microbe had components which neutralized the protoplast-bursting activity of surfactin or not were made.

This report shows that protoplast-bursting activity which was caused by one of the biological effects of surfactin was neutralized by the solubilized membrane and by phospholipids.

METHODS AND MATERIALS

Organism and procedures for preparing protoplasts

Bacillus megaterium KM was cultivated in nutrient broth at 30° for 16 h and protoplasts were prepared in hypertonic medium as described in the previous report[1].

Membrane preparation

The membrane fraction was prepared as described by KABACK[2] using protoplasts

of *B. megaterium* KM cultivated in nutrient broth and low P_i medium[1]. Finally, the pellet containing the membranes was washed by suspending in distilled water and the suspension was then lyophilized.

Solubilization of membrane

Lyophilized membranes were solubilized in hot 0.3 M KOH and then dialyzed against 0.05 M phosphate buffer (pH 7.38) at 4° for 24 h. The dialyzed solution was transparent. When the membrane which had been solubilized in alkaline solution was dialyzed against distilled water, the dialyzed solution was turbid.

Treatment of membranes by trypsin

Trypsin was added to the lyophilized membrane suspended in 0.05 M phosphate buffer (pH 7.38) at a concentration of 1% dry weight of membranes. Incubation was carried out at 37° for 24 h. After incubation, the suspension was centrifuged at 18000 × *g* for 60 min. The sedimented fraction was resolubilized in hot 0.3 M KOH and then dialyzed against 0.05 M phosphate buffer (pH 7.38) at 4° for 24 h.

Neutralization assay of the protoplast-bursting activity of surfactin

When water-soluble substances were assayed, 0.5 ml of a solution containing 200 μg/ml surfactin solubilized in 0.05 M phosphate buffer (pH 7.38) and 0.5 ml of various concentrations of samples in the same buffer were mixed and incubated at 30° for 15 min. 0.5 ml of the mixture was added to 2.5 ml of the protoplast suspension. The decrease in absorbance at 600 nm was determined at room temperature with a Hitachi-Beckman spectrophotometer.

When water-insoluble substances were assayed, 0.1 ml of a solution containing 2 mg/ml surfactin solubilized in ethanol and 0.3 ml of various concentrations of samples in ethanol were mixed and incubated at 30° for 15 min. 0.1 ml of the mixture was added to 2.9 ml of the protoplast suspension. The decrease in absorbance at 600 nm was determined as described in the previous section.

Determination of phosphorus

Samples were digested according to the method of ALLEN[3], and phosphorus contents were determined by the method of NAKAMURA[4].

Determination of protein

Protein contents were determined by the method of LOWRY *et al.*[5].

Chemicals

Lysozyme, phospholipase C (from *Clostridium welchii*), DL-α-lecithin (β,γ-dipalmitoyl-DL-α-lecithin) and DL-α-cephalin (β,γ-dipalmitoyl-DL-α-cephalin) were obtained from Sigma Chemicals Corp. γ-Globulin, lecithin (from egg) and cephalin (from soy bean) were obtained from Tokyo Kasei. Trypsin was obtained from Miles Chemical Co. Phospholipase D was obtained from Boehringer Mannheim.

RESULTS

Neutralization of the protoplast activity of surfactin by the membrane fraction

Two kinds of lyophilized membrane fractions prepared from cells cultivated in nutrient broth and low P_i medium were solubilized completely in hot dilute alkali

solution. These membrane solutions, prepared as described in METHODS, neutralized surfactin equally. When the ratio of the added membranes to the amounts of surfactin was calculated from the protein contents in the membranes, it showed that a value of about 9 was needed to neutralize surfactin.

Trypsin solubilized about 80% of the protein of the membranes. This fraction solubilized by trypsin had no neutralizing activity (Fig. 1). The defatted membrane fraction, which was prepared by extracting lipids from the lyophilized membrane with a chloroform–methanol (2:1, v/v) solution at room temperature, had a small neutralizing activity (Fig. 1). This small activity seemed to be due to the incompleteness of the lipid extraction, because this fraction still contained a small amount of phospholipid material, as shown by the presence of about 0.3% phosphoric acid. The intact membrane contained 0.7–0.8% phosphoric acid by weight. On the other hand, the residual fraction which was not solubilized by trypsin had the neutralizing activity (Fig. 1).

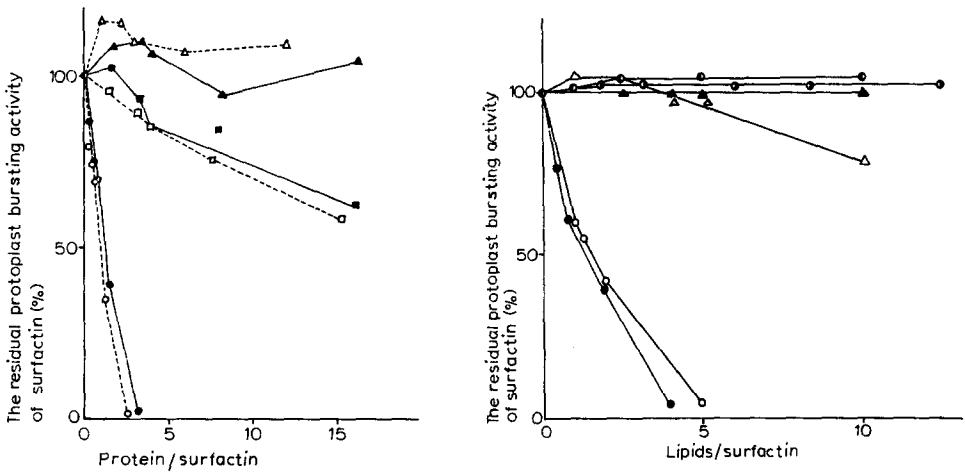

Fig. 1. The neutralizing activity of defatted membranes, digestible fraction with trypsin, non-digestible fraction with trypsin. The axis shows the ratios of the contents of proteins in three samples to the amount (17 μg/ml) of surfactin. – – –, cells grown in nutrient broth; ———, cells grown in low P_i medium. △ and ▲, defatted membranes; □ and ■, digestible fraction by trypsin; ○ and ●, non-digestible fraction by trypsin.

Fig. 2. The neutralizing activity of various lipids. The axis shows the ratios of the contents of lipids to the amounts (17 μg/ml) of surfactin. ◐, monoethanolamine; ◑, choline; ▲, *O*-phosphorylethanolamine; △, triglyceride extracted from beef liver; ○, phosphatidylethanolamine; ●, phosphatidylcholine.

Though this residual fraction was treated by phospholipase C, the neutralizing activity of the fraction did not decrease. The same phenomena were observed in two kinds of membranes prepared from cells grown in nutrient broth and low P_i medium.

Neutralization effects of various lipids on surfactin

As shown in Fig. 2, phospholipids such as phosphatidylcholine and phosphatidylethanolamine neutralized the protoplast-bursting activity of surfactin, but neutral lipids such as monoethanolamine, choline, *O*-phosphorylethanolamine and tryglyceride had no neutralizing activity.

When synthetic phospholipids such as DL-lecithin and DL-cephalin were used, DL-lecithin neutralized the protoplast-bursting activity of surfactin as did natural lecithin, but DL-cephalin seemed to activate surfactin. Under the same conditions, DL-cephalin itself did not induce the degradation of protoplasts. This phenomenon might be attributed to its amphoteric properties.

When natural lecithin was treated with phospholipase C and D, various kinds of lipids were obtained. In these lipids, only the acidic phospholipid fraction which was fractionated by DEAE-cellulose (acetate form) column chromatography[6] after treatment with phospholipase D had neutralizing activity.

DISCUSSION

Surfactin was an acidic peptide lipid and had a very strong protoplast-bursting activity. This activity was neutralized when the membrane fraction was solubilized in dilute alkali solution. This result seems to support our suggestion in the previous report that the physiological effects of surfactin on microbes were caused by the interaction between surfactin and the membranes.

As membranes have a very complex structure, it was necessary to examine which components of the membranes showed an affinity for surfactin.

Protein parts seemed not to exert any neutralization effects, since trypsin digestion of the membranes did not cause any decrease in the neutralizing activity and defatted membranes had only a slight neutralizing activity. These data suggest that surfactin had a higher affinity for lipids in the membranes than for protein.

This deduction was confirmed further. When the neutralization effects of many kinds of proteins were tested, bovine serum albumin and lysozyme were found to neutralize surfactin. However, these neutralizations seemed not to be specific, because bovine serum albumin and lysozyme were known to bind with fatty acids[7] and anionic substances[8], respectively. Other proteins, for example, γ-globulin and papain, did not show any neutralizing activity.

On the other hand, phospholipids such as phosphatidylethanolamine and phosphatidylcholine showed neutralizing activity, but other neutral lipids such as triglyceride, *O*-phosphorylethanolamine, monoethanolamine and choline did not have any neutralizing activities.

Further, in lipids which were prepared from lecithin by treatment with phospholipase C and D, only the acidic phospholipids fraction neutralized surfactin and the diglyceride fraction had no neutralizing activity. These data suggest that the presence of a phosphoric acid moiety linked to the diglyceride is needed for neutralization of surfactin.

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