Inhibition of Germination of *Bacillus stearothermophilus*
Spores by Sucrose Monoalkylates and
Other Surfactants

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In order to get additional information on antimicrobial action of sucrose monoalkylates,
which are approved as food additives, we studied the effects of the esters on growth of
heat-resistant *Bacillus stearothermophilus* spores, by comparison with those of a variety of
surfactants differing in the chemical structure. Sucrose monoalkylates and several other
surfactants inhibited the growth from spores, by preventing spore germination. The
inhibitory effectiveness of surfactants is closely related to hydrophobicity of surfactants
rather than their intrinsic chemical characteristics. Surfactants acted on spores in a
bacteriostatic manner.

It has been demonstrated that a variety of surfactants act on bacterial spores as anti-
microbial substances\(^1\)\(^-\)\(^8\). Among them sucrose esters of fatty acids are particularly interesting,
because they are approved as food additives. The esters have been used to prevent
microbial spoilage of canned drinks stored in vending machines. Some of the present authors
reported previously that sucrose monoalkylates effectively inhibited the growth of thermophilic
spore-forming bacteria which cause flat sour spoilage of canned milk coffee\(^7\). Thus, the
esters are of potentially validity as food preservatives to inhibit heat-resistant spores
which are hardly sterilized under commercial pasteurization. However, little is known about
details of the inhibitory action of the esters. Questions are: do sucrose esters act on spores
in some specific manner? what process in transformation from spores to vegetative cells
is inhibited by sucrose esters and other surfactants? The present experiment was under-
taken to obtain information on the mode of the inhibitory action of sucrose monoalkylates
on spores, using *Bacillus stearothermophilus*.

**Materials and Methods**

**Materials**

Sucrose monodecanoate (SE 10), sucrose monododecanoate (SE 12) and sucrose monohex-
adecanoate (SE 16) were products of Mitsubishi Kasei Corporation. Sucrose monooctanoate (SE 8)
and sucrose monotetradecanoate (SE 14) were prepared according to the method
described previously\(^9\). 1, 1, 3, 4-Tetramethylbutylphenyl polyoxyethylene (n = 9) glycol (Triton X-100), sodium dodecysulfate (SDS), sodium deoxycholate and sodium cholate were purchased from Wako Chemicals, polyoxyethylene glycol n-dodecylether (C\(_{12}\)E\(_n\)) was from
Nikko Chemicals, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) was from Serva, octyl glucoside was from Pierce, n-dodecyl-N, N-dimethylamine oxide (Ammonyx LO) was from Gunze Sangyo, N-dodecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-10) and N-dodecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-12) were from Calbiochem, sodium octylsulfate, sodium decylsulfate were from Eastman, and sodium taurocholate was from Sigma. Dodecyltrimethylammonium bromide (DTAB), N, N-dimethyl-N-dodecylglycine (DDGly) and nonanoyl-N-methylglucamide (MEGA-9) were synthesized by the methods reported previously\(^\text{10}\). Other reagents were obtained from Wako Chemicals.

Tetradecyl- and cholic acid-conjugated Sepharose 6B gels were prepared according to the procedures described previously\(^\text{11}\,\text{12}\).

Preparation of spores

To prepare Bacillus stearothermophilus IAM 1035 spore suspensions, 0.5 ml portions of a nutrient broth culture that had been incubated at 55°C for 20h were inoculated onto the surface of 100 ml of a sporulation medium (Bacto-peptone 5 g, yeast extract 3 g, NaCl 2 g, MnSO\(_4\) 0.5 g, agar 30 g, H\(_2\)O 1 l, pH 7.2) in culture flasks. The inoculation was spread evenly over the medium surface. The plates were incubated at 55°C for 72h. The spores were harvested and washed three times with deionized water by centrifugation. The crude spores was resuspended (1 g wet cells/ml in 0.15 M NaCl, 0.1 M ethylenediaminetetraacetic acid, pH 8.0) and treated with a mixture of lysozyme (0.1 mg/ml) and trypsin (0.1 mg/ml) at 37°C for 30 min, with a small modification of the procedure of GRECZ \textit{et al}\(^\text{13}\). Final spore suspensions contained more than 95% phase-bright spores, as determined by phase-contrast microscopy. Then spores were collected by centrifugation (6,000 rpm × 30 min) at 4°C and washed sufficiently with deionized water. The spores were stored at −20°C in 1/15 M potassium phosphate buffer, pH 7.0.

Development from spores

Spores were activated by heating in 1/15 M potassium phosphate buffer, pH 7.0, at 95°C for 10 min. Then spores were inoculated into 1.8% nutrient broth and developed in L-shaped tubes at 55°C with shaking (Taiyo Mono-IIA, 120 strokes/min). In the experiment of spore germination, 1/15 M potassium phosphate buffer, pH 7.0, was used as a germination medium. Vegetative propagation and germination were monitored by changes of optical density at 620 nm (Erma photo-electric calorimeter model FE-1 E) during incubation. Initial spore concentration was approximately \(2 \times 10^6\) spores/ml for propagation experiments and approximately \(3 \times 10^8\) spores/ml for germination experiments, respectively.

Fig. 1 Effect of sucrose monoalkylates on development from spores of Bacillus stearothermophilus IAM 1035

Heat-activated spores were incubated at 55°C, 120 strokes per min, in a nutrient broth medium; \(<\text{A}>\) Inhibitory action of SE 12. control (○), 10 μM (△), 20 μM (●), 50 μM (□), 100 μM (●); \(<\text{B}>\) Inhibitory action of sucrose monoalkylates at their concentration of 20 μM of SE 8 (△), SE 10 (▲), SE 12 (□), SE 14 (●), SE 16 (○), control (○).
Results

Effect of sucrose monoalkylates and other surfactants on propagation of spores

Fig. 1 shows the inhibitory effects of sucrose monoalkylates on propagation of Bacillus stearothermophilus spores. As shown in Fig. 1-A, SE12 displayed its inhibitory effect at 10\(\mu\)M, which appeared as a lag of vegetative growth, and completely inhibited propagation of spores at concentrations more than 50 \(\mu\)M. The inhibitory ability of sucrose monoalkylates examined here was apparently potentiated with the increase of hydrocarbon chain length constituting the ester (Fig. 1-B).

We further examined the effects of various surfactants on propagation of Bacillus stearothermophilus spores at a surfactant concentration of 0.3 mM. The results were summarized in Table 1, with critical micell concentrations (CMC)\(^{14-18}\) of surfactants, in which the inhibitory effects of surfactants were graded as follows; no inhibition, a partial inhibition and a complete inhibition. Coincident with the inhibitory potency found in the case of sucrose monoalkylates, surfactants displayed an increased inhibitory action with the increase of hydrocarbon chain length of surfactants, as seen in series of alkylsulfates and Zwittergents. Among surfactants which effectively inhibit spores, SDS and DTAB are typical denaturants for proteins, while sucrose monoalkylates, C\(_{12}\)E\(_9\), Triton X-100 and deoxycholate have no denaturing action\(^{10}\). These results strongly suggest that the antimicrobial activity of surfactants is not primarily related to either

<table>
<thead>
<tr>
<th>Surfactant(^b)</th>
<th>Inhibition of development (0.3 mM)</th>
<th>Inhibition of germination (0.5 mM)</th>
<th>CMC(^c) (mM)</th>
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\(^a\) -, no inhibition ; +, a partial inhibition ; ++, a complete inhibition ; ND, not determined.

\(^b\) See Materials and Methods for the names of surfactants.

\(^c\) Refer to the literatures\(^{14-18}\) for the values of CMC.
intrinsic chemical characteristic or physical property of individual surfactants. A significant factor which inhibits spores is hydrophobicity of surfactants. Further, a number of surfactants inactivated at the concentration less than the CMC, indicating that a monomeric form of the surfactants, not a micellar form, plays an important role to inhibit spores.

Fig. 2 shows that spores do not grow in the presence of tetradecyl- or cholic acid-conjugated Sepharose 6B gel, whereas Sepharose 6B gel itself does not affect propagation of spores. This implies that surfactants do not need to be in a free state to exert their antimicrobial activity. It was found by microscopic observation that spores uniformly dispersed in a medium without an association with ligand-conjugated gels.

**Effect of sucrose monoalkylates and other surfactants on spore germination**

In order to clarify what process of development from spores is inhibited by surfactants, we examined an action of SE 16 on *Bacillus stearothermophilus* in different stages. The growth from bacterial spores is roughly divided into three stages; germination, outgrowth and vegetative growth\(^{19-20}\). SE 16 could exert inhibitory effects on the bacterium in respective stages (data not shown). Thus, the action of surfactants observed in Fig. 1 and the column 2 of Table 1 is recognized as a sum of the inhibitory effects exerted on *Bacillus stearo-

**Fig. 2** Inhibition of development from *Bacillus stearothermophilus* IAM 1035 spores by hydrophobic ligand-conjugated Sepharose 6B gels

Spores were incubated at 55°C in a medium containing Sepharose 6B gel (△), tetradecyl-conjugated Sepharose 6B gel (▲), or cholic acid-conjugated hexyl-Sepharose 6B gel (□). Control experiment is also indicated (○). The amount of gel added was 70 mg of wet gel/ml of spores suspension. In the case of cholic acid-conjugated gel, this amount corresponds to roughly 0.2 mM as a concentration of cholic acid\(^{12}\).

**Fig. 3** Effect of SE 16 on germination of *Bacillus stearothermophilus* IAM 1035 spores

Heat-activated spores were incubated at 55°C in a 1/15M phosphate buffer, pH 7.0. A> SE 16 was added before inoculation at the concentrations of 0 μM (○), 2 μM (●), 10 μM (△), 100 μM (▲). B> SE 16 (final concentration of 50 μM) was added before inoculation (●), and at 10 min (△) or at 30 min (▲) after inoculation. After spores were incubated for 2 h in phosphate buffer containing SE 16 of 50 μM, the amphiphile was removed by centrifugation and spores were resuspended in a fresh germination buffer (□). Spores treated with no amphiphile (○) were also indicated.
thermophilus in different stages. There is no doubt, however, that the inhibitory action on germination process is a crucial step to prevent development from Bacillus stearothermophilus spores, and this prompted us to characterize the effect of surfactants on spore germination.

Fig. 3-A shows the effect of SE 16 on spore germination. Decrease of optical density, a measure of spore germination, was suppressed with increasing concentrations of SE 16 added. As shown in Fig. 3-B, the addition of SE 16 to germinating spores inhibited germination without any time lag. On the contrary, germination took place promptly when the chemical was removed from the spore suspension. This reversibility means that SE 16 inhibits spore germination in a bacteriostatic manner. In fact, we have observed previously that Bacillus stearothermophilus spores still retain the ability to develop even after exposure of spores to SE16 during a month.

We examined the inhibitory action of a number of surfactants on germination process, and the results are summarized in the column 3 of Table 1 at their concentration of 0.5 mM. The effect of DTAB was not determined because of the spore aggregation. The effectiveness of other surfactants on germination agreed well with that on vegetative propagation.

Discussion

Using Bacillus stearothermophilus spores, we studied here the effects of surfactants on growth from spores, with special attention to those of food approved antimicrobial agents, sucrose monoalkylates. The present results unequivocally indicate that a diversity of surfactants inhibits growth from spores and that the inhibitory effectiveness is closely related to their hydrophobicity, rather than their chemical characteristics or their ability to denature proteins. It was further suggested that sucrose monoalkylates and probably other surfactants prevented development from spores by inhibiting spore germination in a bacteriostatic fashion. In sucrose monoalkylates examined here, SE16 is proved to be a promising food preservative, which inhibits heat-resistant spores at a concentration less than 0.1 mM.

The present studies may afford an insight into the mode of inhibitory action of surfactants on spore germination. As shown in Fig. 3-B, a removal of SE16 from spore suspension in a bacteriostatic state gave rise promptly to germinate, suggesting that the chemical does not penetrate into spores. Furthermore, it is of interest to note that hydrophobic ligand-conjugated Sepharose 6 B gels also inhibit development from spores. Sepharose 6 B gels exclude particles larger than the size of 200 nm from the gel matrix. This indicates that Bacillus stearothermophilus spores, the diameter of 700–1000 nm, are virtually unable to directly contact with hydrophobic ligands attached to gels. It seems less likely, therefore, that surfactants directly attack spores to disturb biochemical events in cells, resulting in the inhibition of spore germination. The present results appear to rather suggest that surfactants may interact with germination-specific enzymes or metabolites which are released from germinating spores into medium; for instance, inactivation of spore-lytic enzyme, which plays a key role as a crucial primary germination event, by association with hydrophobic ligands. Regardless of whether this speculation proved to be correct, studies on the action of surfactants on spore germination should be help for understanding in molecular level of a germination process which is still poorly understood.

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桝醤脂肪酸エステル及び各種界面活性剤による Bacillus stearothermophilus 胞子の発芽抑制

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食品添加物である桝醤脂肪酸エステルの抗菌作用に関する知見を得るために、耐熱性を有する Bacillus stearothermophilus 胞子の生育への影響を化学構造の異なる種々の界面活性剤において比較検討した。桝醤脂肪酸エステルおよび数種の界面活性剤が、発芽を阻害することによって生育を阻害した。界面活性剤の阻害効果は、それらの化学的特性よりもむしろ藻水性との間に相関が認められ、その作用は静電的なものであった。