A Loss of the Antimicrobial Activity of Sucrose Monoesters of Fatty Acids as Caused by Esterase Released from the Germinated Spores and Vegetative Cells of \textit{Bacillus cereus}

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It has been often observed that bacteria seemingly adapt to sucrose monoesters of fatty acids, which are supposed to be effective nontoxic antimicrobial agents. In the present experiment, effects of the esters on the germination, outgrowth and vegetative growth of \textit{Bacillus cereus} were investigated with reference to the adaptation phenomenon. The esters showed inhibitory effect on the development of spores into multiplying cells by preventing outgrowth, in the order of effectiveness of lauryl ester > palmityl ester > stearyl ester. However, the cells of which growth had been once inhibited again developed in the medium. This resumption of growth occurred almost in parallel to a decrease in the concentration of the esters in the medium. Vegetative growth of cells was also prevented by the addition of esters, but the growth-inhibited cells again recovered their ability to grow during further incubation in the medium. Furthermore, it was found that esterase is released from germinated spores and growing cells. Taken together, the results indicated that the loss of antimicrobial activity of the esters observed during cultivation is due to the decomposition of the esters by esterase.

Key words: Sucrose monoalkylates/\textit{Bacillus cereus}/Esterase/Enzymatic degradation/Antimicrobial activity.

INTRODUCTION

Sucrose monoesters of fatty acids, which have been approved as food additives for use as emulsifiers, inhibit the growth of thermophilic spore-forming bacteria (Kato and Shibasaki, 1975; Shibasaki, 1979; Tsuchido et al., 1981, 1987) and are supposed to be effective antimicrobial agents to inhibit heat-resistant spores which are not readily killed under commercial pasteurization procedures. In fact, these esters have been used to prevent microbial spoilage of canned drinks stored in vending machines (Suwa et al., 1986). A problem is, however, that a seeming adaptation of bacterial cells to the esters has often been observed (Kato and Shibasaki, 1975; Tsuchido et al., 1987), which reduces the validity of these esters as a food preservative. The present experiment was undertaken to elucidate the mode of such adaptation, by studying the relationship between the antimicrobial action of the esters on the germination, outgrowth and vegetative growth of \textit{Bacillus cereus} and the degradation of the esters during cultivation of the cells.

MATERIALS AND METHODS

Sucrose monoesters of fatty acids (SEs) and fatty acids

Sucrose monolaurate (SE12), sucrose monopalmitate (SE16) and sucrose monostearate (SE18)
were supplied by Mitsubishi-Kagaku Foods Corporation. [\(^{14}\text{C}\)]-Labeled SEs were prepared according to the method described previously (Moriyama et al., 1996), using \([\text{U-}^{14}\text{C}]\)-sucrose. Lauric acid, palmitic acid and stearic acid were products of Wako Pure Chemical Industries.

**Preparation of dormant and germinated spores**

Spores of \(B.\) cereus IFO 13597 were prepared according to the method described previously (Makino et al., 1994). Dormant spores were heat-activated for 20 min at 70°C before use. Germination was carried out in 0.2 M sodium phosphate, pH 7.0, containing 4 mM adenosine and 10 mM L-alanine for 60 min at 30°C. Dormant spores (>95% phase-bright spores) and germinated spores (>95% phase-dark spores) were sufficiently washed with deionized water and stored at 4°C.

**Development of spores**

Dormant spores and germinated spores were developed in 0.8% (w/v) nutrient broth at 30°C. Development was monitored by changes of optical density at 620 nm (OD\(_{620}\)) with an Erma photo-electric colorimeter (Model AE-11N) during incubation in L-shaped tubes with shaking, 65 strokes per min.

**Lysate of vegetative cells**

Vegetative cells were lysed in 0.2 M sodium phosphate, pH 7.0, by sonication, and lysate was separated from cell debris by centrifugation (12000 x g for 10 min at 4°C).

**Determination of SE concentration in culture medium**

The concentration of SE in the culture medium was determined by using \([\^{14}\text{C}]\)-labeled SEs as follows. Culture medium (100 \(\mu\)l), which contains SE and its decomposed products, was separated from cells by centrifugation (12000 x g for 5 min at 4°C) and mixed with 200 \(\mu\)l of isobutyl alcohol. The mixture was vigorously vortexed and centrifuged (12000 x g for 5 min at 30°C). SE was preferentially partitioned to the alcohol layer (Osipow et al., 1957) and its concentration was determined from the radioactivity in the alcohol layer.

**Esterase activity assay**

Esterase activity was detected according to the method of Guilbault and Kramer (1964), using fluorescein diacetate (Wako Pure Chemicals) as a substrate. Decomposition of SE by esterase was evaluated by measuring the concentration of undecomposed SE partitioned to isobutyl alcohol layer as described above, after SE was incubated with cell lysate, culture medium or spores for 24 h at 30°C. One unit of esterase activity for SE was defined as a decomposition of 1 nmole SE/h \cdot 10^{10} cells.

**RESULTS**

**Effects of sucrose monoalkylates on spore germination and on development of spores into multiplying cells**

The effects of SE12, SE16 and SE18 on spore germination are shown in Fig. 1. The concentration of the esters used in the experiments, 1.6 mM, has been known to be enough to inhibit development of \(B.\) cereus spores into multiplying cells over 10 h at 30°C (Moriyama et al., 1996). Spore germination, which was monitored by a decrease in OD\(_{620}\) and microscopic observation, was not eventually suppressed in the presence of SE. Thus, SE was indicated to inhibit \(B.\) cereus cells in stages other than that of germination.

Figure 2 shows the development of spores into multiplying cells in nutrient broth containing SEs (0.4 mM each) at 30°C. Decreases in OD\(_{620}\) caused by germination were observed at the early stage of cultivation and, then, the effect of the esters was seen as a lag in the onset of vegetative growth, with the order of
FIG. 2. Effects of sucrose monoalkylates on the development of *B. cereus* spores into multiplying cells. Heat-activated spores (5.0 x 10^7 spores/ml) were incubated at 30°C in 0.8% nutrient broth in the absence or presence of SEs (0.4 mM each). The concentrations of SEs (shaded symbols) and OD_{620} (unshaded symbols) was measured at the indicated times. Symbols: △, control; □, SE12; ◊ and ●, SE16; ○ and ●, SE18.

Effectiveness of SE12 > SE16 > SE18. This means that SEs prevent the growth of cells at the stage of outgrowth, but that the inhibited cells again recover ability to grow. A possible factor causing the resumption of growth is a lowering of the SE concentration in the medium which may have occurred during incubation. To verify this, we measured the amount of SE remaining in the medium. As shown in Fig. 2, SE concentration of the medium was reduced during cultivation, which took place in a nearly parallel manner to the loss of the inhibitory effect of SE. In addition, it was indicated by thin layer chromatographic analysis (Moriyama et al., 1996) that the decrease in SE concentration is caused by degradation of the ester to sucrose and fatty acid, not the incorporation of the ester into cells (data not shown), suggesting involvement of esterase in the degradation of SE. The esters also inhibited vegetative growth when added to exponentially growing cells, but the growth-inhibited cells again acquired potency to grow during further incubation (Fig. 3).

Esterase activity released from *B. cereus* cells

Cell lysate and culture medium of *B. cereus* in the stage of exponential growth had ability to lyse fluorescein diacetate, a synthetic substrate for esterases, which unequivocally indicates the release of esterase from cells into the medium. As an example, the activity in the culture medium of cells reached the stationary phase is shown in Fig. 4. In spores, the exudate of germinated spores was found to also contain esterase, but the enzyme was not exuded from

FIG. 3. Effects of sucrose monoalkylates on the vegetative growth of *B. cereus*. Heat-activated spores (5.0 x 10^7 spores/ml) were incubated at 30°C in 0.8% nutrient broth, and SEs (0.4 mM each) were added to exponentially growing cells at the time (3 h) indicated by arrow. Control cells are also indicated. Symbols: △, control; □, SE12; ◊ and ●, SE16; ○ and ●, SE18.

FIG. 4. Decomposition of fluorescein diacetate by culture medium of cells reached the stationary phase. After 5 h of cultivation in nutrient broth, the culture medium was separated from cells by centrifugation (8000 x g for 10 min at 4°C). One ml of the culture medium was mixed with 1 ml of 0.2 M sodium phosphate, pH 7.0, containing 20 μM of fluorescein diacetate. Production of fluorescein was measured at 30°C at the indicated times. A control experiment was performed with the culture medium boiled for 20 min. Symbols: ○, heat-untreated culture medium; □, heat-treated culture medium.
FIG. 5. Hydrolysis of sucrose monoalkylates by esterase released from *B. cereus* cells. A, Release of esterase from dormant and germinated *B. cereus* spores. Dormant and germinated spores (final 4.0 x 10^8 spores/ml each) were incubated in 5 ml of 0.2 M sodium phosphate, pH 7.0, containing 0.4 mM SEs for 24 h at 30°C. After removal of spores by centrifugation, esterase activity for SEs of the supernatants was evaluated as described in Materials and Methods. B, Esterase activity of exponentially growing cells. Cells reaching the stationary phase (5 h cultivation, 8 x 10^8 cells/ml) were separated from culture medium by centrifugation (8000 g for 10 min at 4°C). To the culture medium (5 ml) and cell lysate (5 ml) made from 4.0 x 10^8 cells/ml in 0.2 M sodium phosphate, pH 7.0, SEs (final 0.4 mM each) were added. After incubation for 24 h at 30°C, esterase activity was evaluated as Fig. 5A.

dormant spores. The susceptibility of SEs to the enzyme liberated from germinated spores was in the order of SE18>SE16>SE12 (Fig. 5A). On the other hand, esterase detected in lysate and the culture medium of exponentially growing cells decomposed SEs in the order of SE16>SE12>SE18 (Fig. 5B). The reason why esterases differing in the lytic ability for SEs are present was not explored. However, the results shown in Fig. 5B indicate that roughly 10% of total amount of the enzyme exudes into the medium when cultivation reached the stationary phase.

Effects of fatty acids on outgrowth and vegetative growth

It has been known that fatty acids themselves possess antimicrobial action (Kato and Shibasaki, 1975; Shibasaki, 1979; Tsuchido et al., 1985). Hence, effects of lauric acid, palmitic acid and stearic acid, which are degradation products from SEs used here, on germination, outgrowth and vegetative growth of *B. cereus* were examined. While these fatty acids (0.2 mM) had no effect on cells at the stage of exponential phase (data not shown), lauric acid and palmitic acid revealed inhibitory effect on cells at the stage of outgrowth, with lauric acid more effective than palmitic acid (Fig. 6). Stearic acid had eventually no effect on cell propagation under the conditions used here.

FIG. 6. Effects of fatty acids on the outgrowth of *B. cereus* cells. Germinated spores (5.0 x 10^7 spores/ml) were incubated at 30°C in 0.8% nutrient broth in the absence or presence of fatty acids (0.2 mM each) and OD_{620} was measured. Symbols: △, control; □, lauric acid; ◇, palmitic acid; ○, stearic acid.
DISCUSSION

Sucrose monoalkylates had eventually no effect on germination of B. cereus, which is in marked contrast to the observation that the reagents reveal bacteriostatic action on the germination of B. stearothermophilus (Tomida et al., 1991). The chemicals exerted inhibitory action on B. cereus cells at the stages of outgrowth and vegetative development, with the order of effectiveness of SE12 > SE16 > SE18. It seems that the inhibition is caused by cell lysis, as well as the case of B. subtilis evidenced by Tsuchido et al. (1987).

Under the conditions employed here (high density of cells, 0.4 mM SE, 30°C), the inhibitory action of SE which had been once exerted disappeared as revealed by resumption of cell growth (Figs. 2 and 3). Though the possibility that surviving cells acquire tolerance to the esters cannot be excluded, it was indicated that the loss of antimicrobial activity of SE is rather due to the decrease of SE concentration in the culture medium which results from degradation of SE by esterase released from cells. Furthermore, the present results suggested that antimicrobial action of fatty acid, a degradation product of SE, may compensate to some degree the loss of inhibitory effect of SE by hydrolysis.

It has been known that the antimicrobial action of amphiphilic substances and alcohols is closely related to the hydrophobic nature (Tomida et al., 1991; Tsuchido et al., 1983; Yasuda-Yasaki et al., 1978). However, the dependence of the inhibitory effect of SEs on hydrocarbon chain length has often shown that SE16 has the most potent inhibitory ability, with decreased potency in the esters having shorter and longer hydrocarbon chains (Suwa et al., 1986; Tsuchido et al., 1987). This was also the case for the present experiment. As suggested in this paper, it is most likely that this phenomenon is caused by complicated synergistic effects of many variables of actions such as the effectiveness of the inhibitory action of SEs and their degradation products, fatty acids, on bacterial growth, the possible presence of esterases differing in degradative potency for SEs and so on. In conclusion, it must be stressed that effectiveness of the esters as antimicrobial agents may be in some cases reduced by the action of esterases released from microorganisms and that this is a problem if occurs in foods.

REFERENCES


