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Effect of Nisin against *Clostridium botulinum* During Spore-to-Cell Transformation

Yoon-Kyung Chung† and Ahmed E. Yousef

Department of Food Science and Technology, The Ohio State University, Columbus, Ohio 43210-1007, USA

Abstract

It has been proposed that the mode of action of nisin against vegetative cells and spores of *Clostridium botulinum* is different. However, clear explanation is not available. Therefore, nisin action against vegetative cells and spores of *C. botulinum* was investigated in this study. Nisin was added at various stages of spore-to-vegetative cell transition and changes to sensitivity to the bacteriocin were observed. Different nisin preparation (Nisaplin or pure nisin) was compared for their activity against different stages of spore transformation of *C. botulinum* ATCC 25763. Germination was measured by determining loss of heat resistance and observing phase darkening of spores under phase-contrast microscope. Nisin acted bactericidally against vegetative cells, but acted sporostatically against spores of *C. botulinum* under the same concentration. This bactericidal and sporostatic action of nisin was dependent on the concentration of nisin used. Presence of nisin during spore activation by heat increased subsequent phase darkening and germination rates. However, nisin inhibited the germination and the outgrowth, when it was added after heat activation stage. Findings from this study suggest that the time of addition of nisin is very important for the effective control of spores during the heating process of foods. In addition, it may be possible to apply nisin at the stage of processing that coincides with the most sensitive stage of spore transformation.

Key words: *Clostridium botulinum*, nisin, spore transformation

INTRODUCTION

Proteolytic *Clostridium botulinum* such as type A strains produce heat-resistant spores that are of major concern in the processing of low-acid, shelf-stable foods. Because of the severity of botulism, it is essential to prevent the growth of *C. botulinum* producing the potent neurotoxin by an adequate processing. However, it is not always practical to eliminate *C. botulinum* spores during food processing, since harsh treatments may reduce the organoleptic and nutritional quality of foods. Furthermore, the presence of spores in the food does not lead to the toxin production unless a proper environment for germination and growth is provided (1). Therefore, the important consideration for the safety of foods is whether *C. botulinum* spores can germinate, grow and produce toxin. Suitable targets for control of *C. botulinum* toxin production in a food product might be found in the processes involved in the spore-to-cell transition, since this must take place for toxin production to ultimately occur. This transition, from activation and germination through outgrowth to cellular multiplication, would be an ideal target against which to design specific inhibitors that might serve as alternatives to conventional antibotulinal agents, such as nitrite, that are commonly used in foods (2).

Nisin is an antimicrobial peptide, which has been used to preserve foods, such as pasteurized cheese spreads, salad dressings, and low cholesterol liquid eggs in the U. S. (3,4), since it was recognized as a safe and legal biological food additive in 1969 (5). Some studies indicated that nisin acts either sporicidally or as an inhibitor of germination or outgrowth (6-13). These studies show that mode of action of nisin varies with the test organism and the numbers present. Mazzotta and Montville (14) reported that nisin reduced the thermal resistance and increased the germination rate of *C. botulinum* spores. Therefore, effect of nisin on germination of *C. botulinum* spores is currently controversial. In addition, the stage of transformation that is targeted by nisin and its mechanism of action are not known. Therefore, this study was performed to compare the effect of nisin against *C. botulinum* at different stages in spore-to-cell transition and to determine the most susceptible stage of the life cycle of *C. botulinum* to this additive. Detecting and monitoring germination by conventional techniques (e.g., loss of heat resistance measurements) are tedious and time-consuming tasks. This direct approach, however, produces unambiguous results and thus was used in this study.

†Corresponding author. E-mail: Chung.115@osu.edu
Phone: +1-614-247-6831, Fax: +1-614-292-0218

MATERIALS AND METHODS

Bacterial strains and preparation of spore suspensions

Proteolytic *C. botulinum* Type A ATCC 25763 was obtained from the American Type Culture Collection (Manassas, VA). Culture was grown in Tryptone-Peptone-Glucose-Yeast extract (TPGY) broth and kept frozen at -80°C with 20% (v/v) sterile 80%-glycerol (Sigma Chemical Co., St. Louis, MO) added to the broth. TPGY broth consists of 50 g tryptone (Difco), 5 g peptone (Difco), 4 g dextrose (Difco), 20 g yeast extract (Difco), and 1 g L-cysteine hydrochloride (Sigma) per liter preparation (10). Spores of *C. botulinum* were formed by growing the bacterium on TPGY agar plate. An overnight culture was spread onto TPGY agar and incubated anaerobically at 37°C until more than 90% of population were sporulated, which took 7 to 10 days. Spores were harvested by adding 10 mL of sterile cold deionized water, releasing the colonies containing spores from the surface of the agar with the use of a sterile disposable inoculating loop. Suspensions were centrifuged at 3,000 $\times g$ for 10 min at 4°C and the resulting pellet was washed in sterile cold deionized water, and centrifuged again at 10,000$\times g$ for 10 min at 4°C. The washing procedure was repeated three times. Spore suspensions were then enzymatically cleaned by the methods of Billon et al. (15). Lysozyme solution (200 μg/mL of lysozyme in 0.05 mol/L potassium phosphate buffer, pH 8.1) was added to the spore pellets and incubated at 45°C for 30 min. Then, trypsin solution (100 μg/mL of trypsin in 0.05 mol/L potassium phosphate buffer, pH 8.1) were added and incubated at 45°C for 2 hours. Spore crops, then, were washed six times in sterile deionized water by centrifugation at 10,000$\times g$ for 10 min at 4°C. The final pellets were resuspended in sterile cold deionized water and kept at 4°C. Microscopic observations were made to confirm the purity of spore suspensions. No vegetative cells were observed and spores were all refractile under phase-contrast microscope.

Preparation of nisin

Nisaplin® (10^6 IU of nisin/g), a commercial nisin concentrate composed of 2.5% nisin in denatured milk solids and sodium chloride, or pure nisin was obtained from Aplin and Barrette Ltd. (Trowbridge, England). Stock solutions of nisin containing 10^5 IU/mL in 0.02 N HCl solution were prepared as recommended by Scott and Taylor (10). The pH was adjusted to 2～3 with HCl solution and the mixture was autoclaved (10 min at 121°C) and stored at 4°C. Activity of nisin levels in these autoclaved stock solutions (prepared as 10^5 IU/mL) were measured in Arbitrary Units (AU)/mL with the spot-on-the-lawn assay employing *Lactobacillus leichmannii* ATCC 4797 as an indicator microorganism. The conversion between the two units (AU and IU) of bacteriocin activity was calculated as follows: 10^5 IU/mL of commercial nisin produced the activity of 3.2×10^6 AU/mL against *L. leichmannii* ATCC 4797. Therefore, 1 IU is approximately equivalent to 32 AU against this indicator microorganism.

Comparison of sensitivity of vegetative and spore forms of *C. botulinum* to nisin

Sensitivity of vegetative and spore forms of *C. botulinum* to nisin in TPGY broth medium was examined. Overnight (18 hr) culture of vegetative cells was diluted to appropriate concentrations in 0.1% peptone water. Spore suspensions were also diluted to suitable concentrations in 0.1% peptone water. The diluted suspensions were heat-shocked at 80°C for 10 min. The heat-shocked suspensions were inoculated into TPGY broth containing nisin. The final vegetative cell and spore levels in the inoculated broth were approximately 10^3 to 10^4 CFU/mL TPGY broth. Duplicate TPGY tubes were inoculated for each set of conditions. Tubes without nisin served as controls. The nisin levels used were 10, 100, and 1000 IU/mL (0.25, 2.5, and 25 μg/mL, respectively). Plate counts were obtained after incubated at 37°C for predetermined periods.

In all of the experiments, *C. botulinum* manipulation and incubation were done in an anaerobic chamber containing an atmosphere of 5% H_2, 10% CO_2, and 85% N_2 (Forma Scientific Inc., Marietta, OH). All media and reagents were prepared at least 24 hr before use and kept at 22～25°C in the anaerobic chamber (14).

Effect of nisin on activation stage

Optimal heat activation (85°C for 90 min) condition for this *C. botulinum* strain was chosen from previous experiments (16). Nisaplin® or pure nisin was added to the spore suspension to achieve a concentration of 250 μg/mL. The addition of nisin preparation to the spore suspension lowered the pH of the activation medium (i.e., mixture of nisin stock solution and spore in deionized water). The pH values of activation medium were 2.8 and 3.4, for Nisaplin® and pure nisin-containing solution, respectively. Peptone water adjusted pH to 2.8 and 3.4 was used as control, to differentiate the effect of pH and nisin. The pH value of the spore suspension without nisin preparation was 4.9. Phase change of spores was examined before and after heat activation treatment in the presence of nisin. In addition, spore counts were obtained at 0, 30, and 90 min during heat activation treatment.

Effect of nisin on germination stage

Germination was monitored in the presence of nisin when it was added before or after heat activation treatment (i.e., pre-activation or post-activation treatment, respectively). For pre-activation treatment, 250 μg/mL nisin (Nisaplin® or pure nisin) was added to the spore suspension [nisin solution : spore suspension = 1:9 (vol/vol)] and the mixture was heated at 85°C for 90 min. After heating, the heated mixture was transferred to the germination medium [heated mixture : germination medium = 1:9 (vol/vol)] and incubated at 37°C. TPGY broth containing 200 mM L-alanine was selected as a germination medium based on the previous experiments (16). For post-activation treatment, spore suspension was heated at 85°C for 90 min, and heated spores were transferred to the germination medium containing 250 μg/mL nisin (Nisaplin® or pure nisin). The volume ratio of transferred heated spore suspension in germination medium was 1:9 (vol/vol).

Monitoring spore germination

Spore germination was measured by determining loss of heat resistance (i.e., decrease in plate count upon heating) and observing the increase in phase darkening of spores. The general scheme of monitoring germination is shown in Fig. 1. At predetermined intervals during germination incubation, samples were withdrawn and examined under phase contrast microscope (Bausch and Lomb, Rochester, NY). Between 100 and 200 spores were counted for each sample, and the numbers of phase-bright (ungerminated) spores and phase-dark (germinated) spores were recorded. Samples of the incubated mixture were also enumerated using TPGY agar medium without heat treatment or plated on TPGY agar after heat treatment (80°C for 30 min).

Effect of nisin on the outgrowth stage

Spore suspension was heat-activated at 85°C for 90 min, transferred to the germination medium (TPGY broth containing 200 mM L-alanine) and incubated anaerobically at 37°C. After 3 hr germination incubation, nisin (250 μg/mL) was added to the germination medium. At appropriate intervals, samples were withdrawn and phase-bright/phase-dark spores were counted under a phase-contrast microscope (Bausch and Lomb, Rochester, NY).

RESULTS AND DISCUSSION

Comparison of sensitivity of vegetative cells and spores of *C. botulinum* to nisin

Sensitivities of spores and vegetative cells of *C. botulinum* were compared in TPGY broth using three different concentrations of commercial nisin (Fig. 2). Population of *C. botulinum* vegetative cells decreased >2 logs in mixtures containing 25 μg/mL nisin. The bacterium remained undetectable for 36 hr incubation. Spore outgrowth was prevented for about 15 hr by the same concentration of nisin, and then they grew and reached to 10^8 CFU/mL at 28 hr period (Fig. 2a). When 2.5 μg/mL nisin was used, population of vegetative cells decreased >2.5 logs and remained undetectable (<10 CFU/mL) for 15 hr. A similar concentration of nisin prevented spore outgrowth for 9 hr, and then the count increased to 10^7 CFU/mL after 30 hr incubation (Fig. 2b). When nisin concentration was lowered to 0.25 μg/mL, vegetative cells were inhibited for 3 hr and then started to grow and reached to 10^8 CFU/mL. However, spores were not affected with the same concentration of nisin (Fig. 2c).

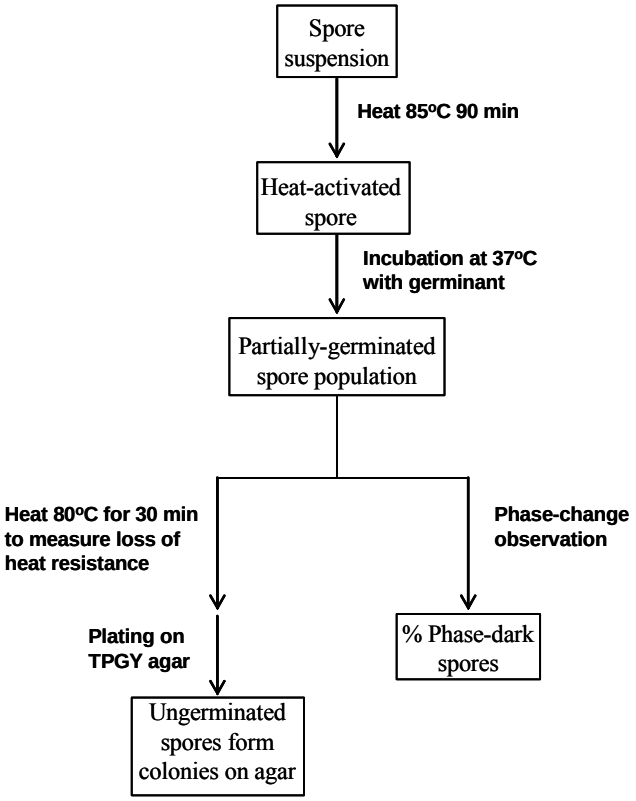

Fig. 1. Monitoring germination by determining loss of heat resistance and observing phase-darkening of spores.

This direct comparison between *C. botulinum* vegetative cells and spores in sensitivity to nisin illustrates the mode of action of nisin against this pathogen. Nisin acted bactericidally against vegetative cells, but acted sporostatically against spores of *C. botulinum* under the same concentration (Fig. 2). Sensitivity of vegetative cells and spores to nisin varies between genera and even between strains of the same species (17), but direct comparisons

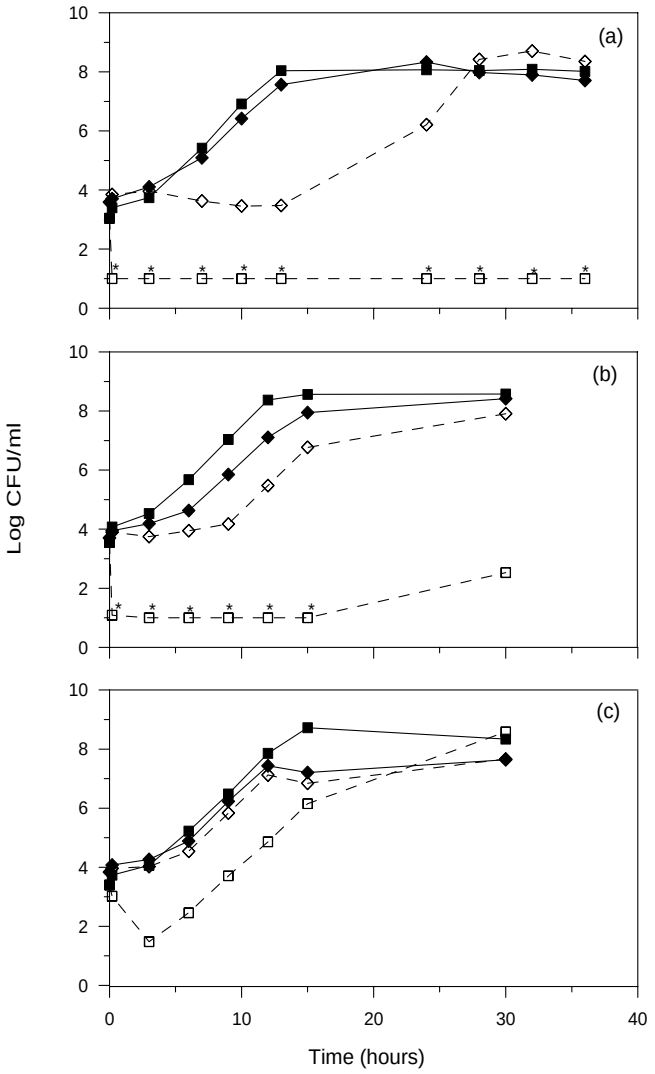

Fig. 2. Behavior of vegetative cells and spores of *Clostridium botulinum* in TPGY broth at 37°C in the presence of nisin. (a) 25 μg/mL; (b) 2.5 μg/mL; (c) 0.25 μg/mL. (■) vegetative cell; (□) vegetative cell + nisin; (◆) spore; (◇) spore + nisin. * indicates less than 10 CFU/mL (detection level). Data points shown are average values of 2 individual experiments.

between vegetative and spore forms were not tested earlier. The bactericidal and sporostatic action of nisin was dependent on the concentration of nisin used. The lag in germination of *C. botulinum* spores was related proportionally with the concentration of nisin used (Fig. 3). It has been known that nisin works in a concentration-dependent fashion both in terms of amount of nisin applied and the number of vegetative cells or spores that needs to be inhibited (17). In addition, the lag period of vegetative cells was much longer than the lag time of growth from spore inocula with the same concentration of nisin. Therefore, vegetative cells were more sensitive to nisin than were the spores of *C. botulinum*.

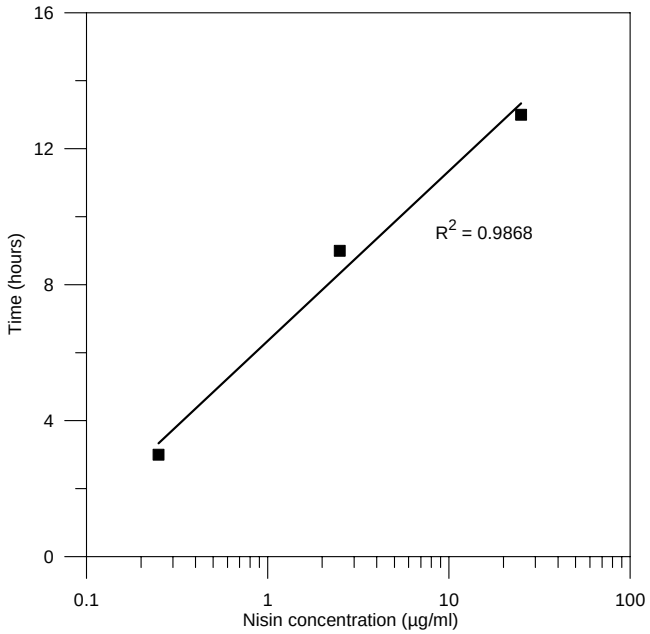

Fig. 3. Lag time of growth from spore inocula, produced by increasing nisin concentrations in the growth media.

Effect of nisin on different stages of spore-to-vegetative cell transition

This experiment was done to determine the stages of spore-to-cell transition that are affected most by nisin. Therefore, nisin was added at different stages of spore transformation and its action on the transforming spores was examined. Nisin, as a form of Nisaplin, was added to the dormant spore suspension (pre-activation treatment), spores were heat activated at 85°C for 90 min, and then subsequent germination was monitored by observing phase contrast change (Fig. 4a) and by determining loss of heat resistance at 80°C for 30 min (Fig. 4c). Presence of nisin during heat activation of spores enhanced phase-change after activation treatment and subsequent germination incubation as measured microscopically (Fig. 4a). In terms of the germination incubation period only, the number of phase-dark spores increased by 20～30% after the initial phase change during heat activation, regardless of presence of nisin in the germination medium. Therefore, germination was dependent on the activation condition (Fig. 4a). Determination for the loss of heat resistance showed that presence of nisin shortened the heating time required for spore activation (Fig. 4c). Maximum culturability was observed when spores were heated, in the presence of nisin, for 30 min at 85°C. However, after extended heating during activation (90 min), the spore count decreased. This observation may be resulted from sensitization of nisin-treated spores to heat. Therefore, sublethal heating intended to only activate spores became lethal when ap-

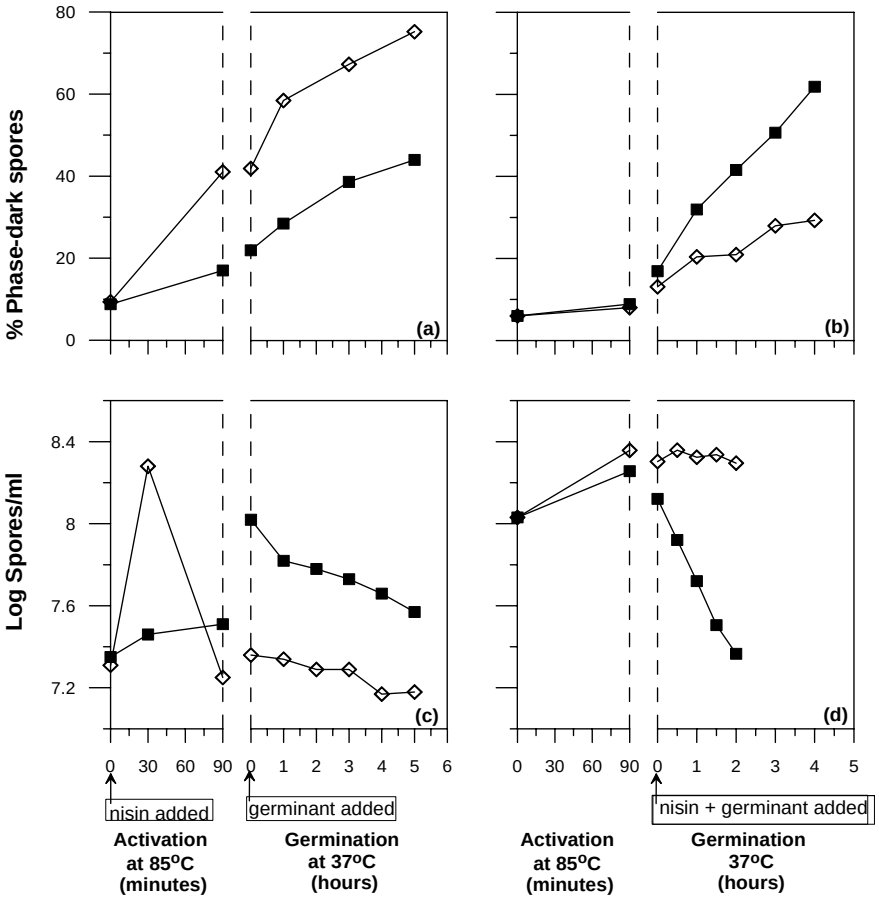

Fig. 4. Monitoring germination of *Clostridium botulinum* spores in the absence (■) or the presence (◇) of nisin. (a), (c) Spore suspensions were heat activated at 85°C for 90 min at pH 2.8 in the presence of Nisaplin (250 μg/mL). Activated spores were transferred to the germination medium and incubated at 37°C. (b), (d) Spore suspensions were heat activated at 85°C for 90 min. Activated spores were transferred to the germination medium containing Nisaplin and incubated at 37°C. Each data points indicate the average values of at least two individual experiments.

plied for an extended period. Alternatively, extended heating with nisin only activated spores rapidly, it also led to spore germination during the extended heating step. Considering the phase-change observation, as a parallel experiment, the latter explanation is supported. In other words, nisin enhanced activation and the subsequent germination, resulted in decreased spore count as the germination proceeded. Studies indicated that germination, due to the changes in the cortex structure, could be induced by drastic means such as surface active agents (18), or heating (19), in addition to the use of compounds that promote physiological germination such as L-alanine.

To investigate the effect of nisin on the germination stage *per se*, Nisaplin was added into the germination medium after heat activation treatment. Nisaplin delayed phase darkening of spores (Fig. 4b). Therefore, opposite effect of nisin on the phase-change of spores was observed depending on the time of nisin addition. When added before heat activation, nisin enhanced phase darkening (germination) (Fig. 4a). On the other hand, nisin delayed phase darkening when it was added after heat activation (Fig. 4b). Phase darkening of spores is thought to correspond with an increase in spore hydration after peptidoglycan layers in cortex have been ruptured (20, 21).

No decrease in spore counts was observed in 2-hr germination period in the presence of Nisaplin (Fig. 4d). This may indicate that NaCl in Nisaplin prevents germination, because commercial nisin contains 77.5% NaCl as a carrier material (Aplin & Barrette Ltd.). The amount of nisin solution in the germination medium was 10% (vol/vol), so the amount of NaCl in germination medium was 7.75%. Warth and Strominger (22) proposed the contractile cortex theory explaining the inhibition of germination in the presence of ionic solute, such as NaCl. Spore peptidoglycan is lightly cross-linked and carries a net negative charge resulting from free carboxyl groups. If these were neutralized by the cations, cortex might be expected to contract and the permeability of

spore might be changed as well (23). These events could interfere with the germination process.

Therefore, pure nisin was used to investigate the effect of nisin *per se*, due to the presence of other ingredients such as NaCl and milk solids in Nisaplin. Germination monitored by phase change observation (Fig. 5a and 5b) indicated the similar results as those obtained with Nisaplin (Fig. 4a and 4b); nisin enhanced phase darkening (germination) when added before heat activation, and delayed phase darkening when added after heat activation. In addition, enhanced culturability was obtained when nisin was added before activation (Fig. 5c). This activating effect of nisin was greater when the pH of the heat-activated spore suspension was lower (Fig. 4c and 5c). The pH values were 2.8 and 3.4, for Nisaplin- and pure nisin-containing solutions, respectively. However, pH effect during germination was not of concern, because the germination buffer containing activated spores was neutral (pH 6.8).

Spore count during germination incubation in the presence of pure nisin decreased slightly faster than that in the control sample when it was added after heat activation (Fig. 5d). Therefore, when nisin was added after heat activation stage, the discrepancy between the plate counting results from the experiments using different nisin preparation (Nisaplin and pure nisin), was probably attributed to the presence of NaCl (Fig. 4d and 5d). Furthermore, these plate counting results (Fig. 5d), apparently, were not consistent with those obtained from phase contrast observation, which indicated slower phase darkening in the presence of nisin than its absence (Fig. 5b). However, the difference between the absence and presence of nisin may not be considerable. On the other hand, it may possibly suggest that nisin inhibited the step between the stage of loss of heat resistance and the stage of phase darkening during the transition of spores. Hitchins et al. (24) indicated that nisin prevented post-germination swelling and subsequent spore outgrowth. Several studies supported that measurable events during spore germination do not occur simultaneously, but rath-

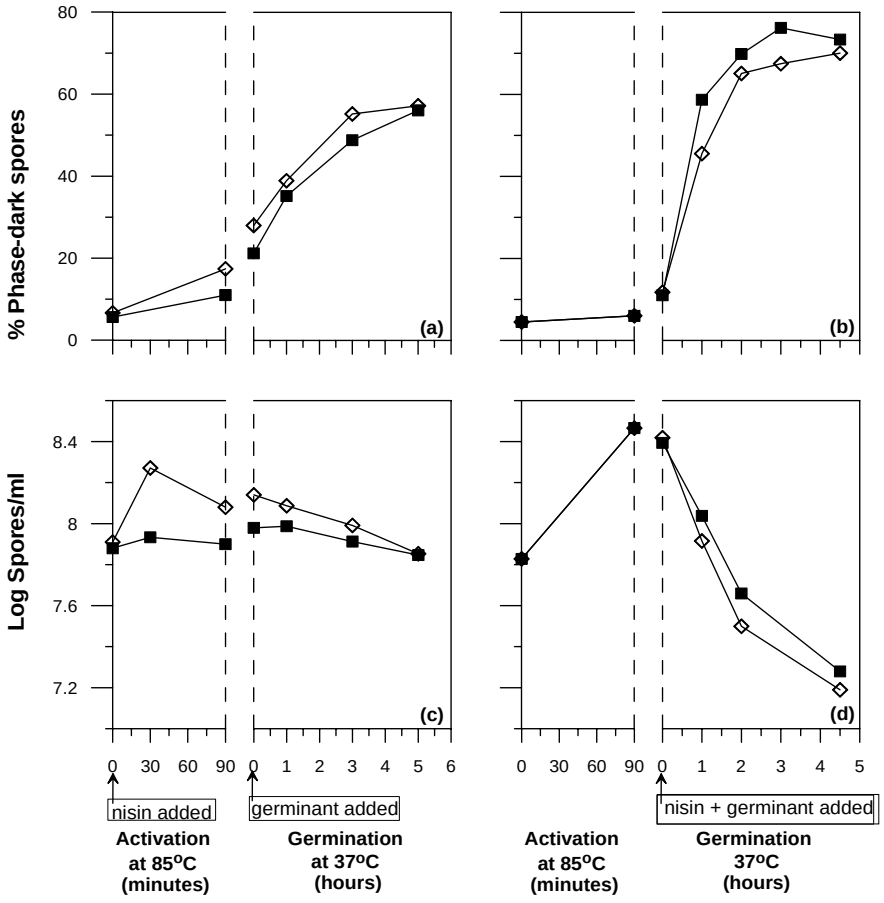

Fig. 5. Monitoring germination of *Clostridium botulinum* spores in the absence (■) or the presence (◇) of nisin. (a), (c) Spore suspensions were heat activated at 85°C for 90 min at pH 3.4 in the presence of pure nisin (250 μg/mL). Activated spores were transferred to the germination medium and incubated at 37°C. (b), (d) Spore suspensions were heat activated at 85°C for 90 min. Activated spores were transferred to the germination medium containing pure nisin and incubated at 37°C. Each data points indicate the average values of at least two individual experiments.

er constitute a time-ordered sequence (25-29). The observable changes during germination of *B. megaterium* QM BI55I spores occurred in the following sequence: loss of resistance to heat, release of dipicolinic acid (DPA), onset of stainability, darkening of individual spores under phase contrast microscope and decrease in turbidity of spore suspension (26). Uehara and Frank (27) also indicated that specific changes occurred in two distinct regions of spores, i.e., one located at the outer layer (spore coats) and the second at the core membrane. During the initial stage of germination, DPA is released, and the cortex disintegrates rapidly until it is completely destroyed (25). In addition, researchers agreed that heat resistance is lost early during germination, even before all DPA has been released (26-28).

To examine the effect of nisin on the outgrowth stage of *C. botulinum* spores, nisin was added into the germination medium after 3-hr germination incubation period (usually, when 50～75% of spores have germinated). Outgrowth occurred in control sample, and some vegetative cells were observed in 4-hr germination period. However, nisin prevented the outgrowth of germinated spores, and no vegetative cells were observed for 14 days from phase-contrast microscopic observation (Fig. 6). This clearly indicates that nisin prevents the outgrowth of germinated spores of *C. botulinum*. Nisin effectively inhibits *B. cereus* and *C. botulinum* spore outgrowth by inactivating the membrane sulfhydryl groups in newly germinated spores (10-13). It has been reported that the activity of nisin is directly related to the presence of dehydroalanine residues, which can act as acceptors for electrophilic reagents and readily react with sulfhydryl groups (26-28).

The mechanism of action of nisin against spores is not clear and research in this area has been inconclusive. Early studies suggested that nisin acts either sporicidally or as an inhibitor of germination or outgrowth (6-9). Even though the main purpose of this study was to investigate the action of nisin during spore transformation, the other factors, such as pH or NaCl, were also considered due to the characteristics of commercial nisin preparation. Therefore, our results show the compounding effects of three factors (e.g. pH, NaCl, and nisin). Two major effects of nisin preparations were observed during the spore transition. First, nisin activated spores during heat treatment and the effect of nisin on the activation was greater under low pH conditions. Secondly, nisin prevented the outgrowth of germinated spores. On the other hand, the effect of nisin on the germination stage of spores, was slightly different depending on the time of addition. When nisin was added before heat activation, germination proceeded slightly faster in the presence of nisin. Addition of nisin after heat activation decreased phase-darkening. Therefore, under combined treatment of heat and nisin, nisin activates spores, triggers initiation of germination, and enhances the subsequent germination. Nisin, on the other hand, delays germination in the absence of heat treatment. In addition, nisin probably prevents the late event of germination stage (after loss of heat resistance and before phase darkening, i.e., pre-emergent swelling) in the absence of simultaneous heat treatment.

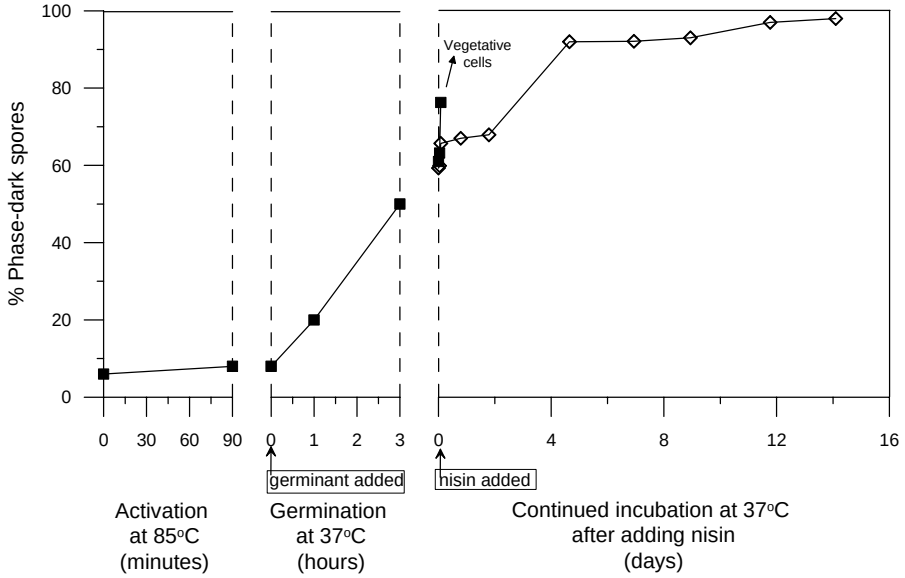

Fig. 6. Effect of nisin (250 μg/mL) on outgrowth stage of *Clostridium botulinum* spores when added after 3-hr germination incubation at 37°C. (■) no nisin added; (◇) nisin added.

It is suggested that the adding time of nisin is very important for the effective control of spores during the heating process of foods. The action of nisin, combined effect of other factors such as pH and NaCl in food systems, should also be considered during the process. In addition, it may be possible to apply nisin at the stage of processing that corresponded with the most sensitive stage of spore transformation.

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