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Influence of Temperature, Oxygen, *m*-Chlorophenylhydrazone, Cerulenin, and Quinacrine on the Production of Extracellular Proteases in *Bacillus cereus*

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Abstract *Bacillus cereus* KCTC 3674 excretes at least two kinds of extracellular proteases into the growth medium. Two major bands of the protease activity with molecular weights of approximately 100 and 38 kDa were obtained after gelatin-SDS-PAGE. The protease with a molecular weight of 38 kDa was identified as an extracellular neutral (metallo-) protease. The neutral protease was quite thermostabile but labile to alkaline pH. On the contrary, the 100-kDa protease was thermolabile but stable to alkaline pH. The production of 38-kDa neutral protease was strongly affected by temperature, oxygen, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) that was defined as a protonophore, and cerulenin which inhibited lipid synthesis and caused changes in the membrane composition. On the other hand, the production of the 100-kDa protease was strongly affected by only temperature and cerulenin. Quinacrine (0.2 mM), which inhibits the penicillinase-releasing protease of *Bacillus licheniformis*, had no effect, whatsoever, on the production of extracellular proteases in *B. cereus* KCTC 3674.

Key words: *Bacillus cereus*, extracellular protease, temperature, oxygen, CCCP, cerulenin, quinacrine

Bacillus cereus is a gram-positive facultative anaerobic spore-forming rod-shaped bacterium [3]. This organism is well known to be responsible for a number of food poisoning cases which are characterized by diarrhea and emesis [6, 10]. A number of studies on the extracellular products of *B. cereus* have been documented [1, 4, 7, 9, 14, 16]. Extracellular products include hemolysins, diarrheogenic enterotoxin, emetic toxin, thermolysin-like neutral protease, and ADP-ribosyltransferase. However, very little is known about the mechanism of how these extracellular products are excreted into the external milieu and also what factors affect their efficient production.

In gram-positive *Bacillus* species, the mechanism of proteins secreted into the growth medium has been of a great interest for a long time to scientists who want to acquire commercially useful enzyme proteins. However, not much is known about the secretion mechanisms of *Bacillus* species due to lack of *in vitro* systems. Most of the research conducted in terms of *Bacillus* species was also primarily geared towards the production of protein rather than towards understanding the secretion mechanism. In this study, in order to understand how the extracellular proteases are excreted into the external milieu and what factors affect their efficient production in *Bacillus cereus*, we examined the type of influences that temperature, oxygen, CCCP, cerulenin, and quinacrine have on the production of extracellular proteases.

The bacterial strain used in this work was *B. cereus* KCTC 3674 (formerly *B. cereus* KYJ 961) [9]. Unless stated otherwise, *B. cereus* KCTC 3674 was grown aerobically at 37°C in a complex medium containing 0.5% polypeptone, 0.5% yeast extract, and 0.5% NaCl in a 50 mM potassium phosphate buffer (pH 6.5).

A preculture grown overnight was used to inoculate the main culture (100 ml) for providing a turbidity of approximately 0.05. Aliquots (1 ml) were withdrawn from the main culture to measure protease activity, and then they were centrifuged at 15,000 rpm for 20 min at 4°C in a micro-centrifuge. The supernatant was saved for a thorough analysis. Extracellualr protease activity was measured by following the procedure as described previously [9]. One unit of extracellular protease activity is defined as the amount of enzyme that increases an absorbancy of 0.1 at 436 nm per 30 min at 45°C.

In the experimental system for the limitation of oxygen or the depletion of H^+ electrochemical potential, concentrated stationary growth phase cultures were used. Cells in 300 ml of the medium were grown by shaking at 37°C

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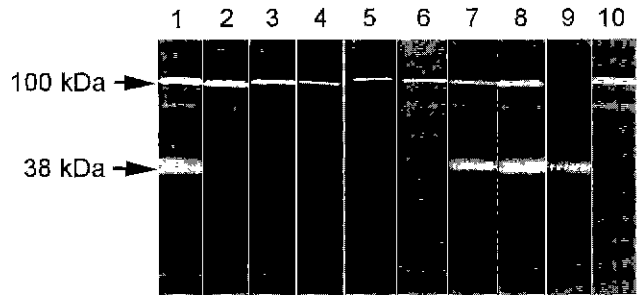
for 4.5 h. The cells were collected by centrifugation at 7,000 ×g at 37°C for 15 min, and resuspended in 200 ml of fresh medium. Cells suspended in fresh medium were divided into two 100-ml samples for the CCCP-induced (or standing culture-induced) and control experiments.

Extracellular proteases produced by *B. cereus* KCTC 3674 were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin as a copolymerized substrate using a 12.5% gel by the Laemmli method [11]. Samples were heated at 100°C for 2 min prior to electrophoresis. After PAGE, the gel was washed in Triton X-100 (2.5%, v/v) for one hour at room temperature to remove the SDS and to restore enzyme activity. After incubation in a 0.1 M glycine buffer (pH 6.5) containing 4 mM $CaCl_2$ and 50 μM $ZnCl_2$ for 2 h at 45°C, bands of proteases were detected by staining with 0.1% amido black solution in methanol:acetic acid:water (30:10:60), and destaining in methanol:acetic acid:water (30:10:60).

The production of extracellular proteases by *B. cereus* KCTC 3674 occurred during the logarithmic growth phase, and was the highest when cultures reached the stationary growth phase. The extracellular proteases could be renatured and detected *in situ* after gelatin-SDS-PAGE (Fig. 1). Two major bands of protease activity with apparent molecular weights of approximately 100 and 38 kDa were obtained after gelatin-SDS-PAGE. The proteolytic activity of culture supernatant showed its optimum level at pH 6.5 [9] and 45°C (Fig. 2A). The activity was stimulated by Ca^{2+} and Zn^{2+}, and strongly inhibited by either a metal chelator, EDTA, or a zinc-specific chelator, 1,10-phenanthroline [9]. As shown in Fig. 1, the activity of a 38-kDa protease was more affected by Ca^{2+} and Zn^{2+} than that of a 100-kDa protease (lanes 2, 3, and 4), and the activity of the 38 kDa protease was more severely inhibited by EDTA and 1,10-phenanthroline than that of the 100-kDa protease (lanes 5 and 6). Both 38-kDa and 100-kDa protease activities were very resistant to a thiol protease inhibitor, iodoacetic acid (lane 8). However, there were several differences between the 38-kDa and the 100-kDa protease. That is, the activity of the 100-kDa protease was inhibited by a specific serine protease inhibitor of phenylmethylsulfonyl fluoride (PMSF), whereas the activity of the 38-kDa protease was affected only slightly (lane 7). The 38-kDa protease was stable to boiling for 5 min but labile to alkaline pH, whereas the 100-kDa protease was quite unstable to boiling for 5 min but stable to alkaline pH (lanes 9 and 10). Based on the results stated above, the protease with 38 kDa molecular weight must be an extracellular neutral (metallo-) protease. The extracellular neutral protease of *B. cereus* has already been well characterized [14, 16]. The protease with a molecular weight of 100 kDa appears to be an alkaline serine protease. However, it is not yet certain whether a 69-kDa band is a degradation product of 100-kDa protease or a major protease.



Lane	Condition	Band intensity	
		100 kDa	38 kDa
1	Control (pH 6.5)	+++++++	+++++++
2	-50 μM $ZnCl_2$	++++++	+
3	-4 mM $CaCl_2$	++++	-
4	-50 μM $ZnCl_2$ and -4 mM $CaCl_2$	+++	-
5	+4 mM EDTA	++	-
6	+4 mM 1,10-Phenanthroline	++	-
7	+4 mM PMSF	+++	++++++
8	+4 mM Iodoacetic acid	+++++++	+++++++
9	Boiling for 5 min	-	++++++
10	pH 8.5	+++++++	+

Fig. 1. Effects of mineral salts, protease inhibitors, boiling, and pH on the activity of extracellular proteases.
SDS-PAGE process was completed with a 12.5% gel by following the Laemmli method. All samples were heated at 100°C for 2 min before they were added on. In lane 9, the culture supernatant containing enzymes was boiled for 5 min and then centrifuged at 15,000 rpm for 20 min at 4°C in a micro-centrifuge. The sample buffer containing 15 μl of supernatant was heated again at 100°C for 2 min before electrophoresis.

The growth patterns of *B. cereus* KCTC 3674 at 37°C and 45°C were shown to be very similar (data not shown), whereas the production patterns of extracellular proteases at these temperatures were quite different (Figs. 2B and 2C). The production of extracellular proteases, which possess the optimal temperature of proteolytic activity at 45°C (Fig. 2A), was significantly reduced at 45°C (Fig. 2C). A shift of temperature down from 45°C to 37°C reinitiated the production of extracellular proteases (Fig. 2D). As shown in Fig. 2, the productions of the both 38-kDa and 100-kDa proteases were strongly affected by temperature. On the other hand, even though the optimum growth of *B. cereus* KCTC 3674 showed at temperatures ranging from 37°C to 45°C, it was found that higher levels of extracellular proteases were produced at lower temperatures (data not shown). That is, the production of extracellular proteases at 25°C was higher at the stationary growth phase than at 37°C.

Oxygen and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) also affected the production of extracellular proteases. Shaking aerated cultures grew at a rate faster than standing cultures, but the aeration of concentrated late logarithmic growth phase cultures slightly affected the

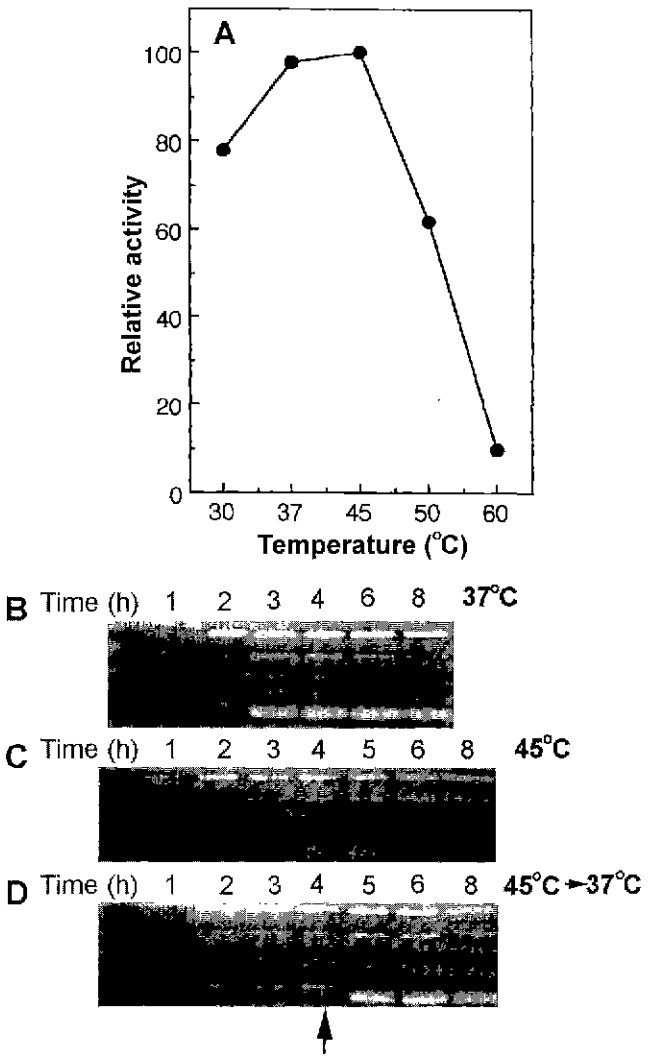


Fig. 2. Effect of temperature on the extracellular proteolytic activity (A) and on the production of extracellular proteases (B, C, D).

(A) The proteolytic activity of culture supernatants was assayed at various ranges of temperature in a 100 mM MES-NaOH buffer (pH 6.5). (B, C, D) For the production of extracellular proteases, cultures were incubated at 37°C, 45°C, and initially at 45°C but shifted down to 37°C at 4 h. The arrow indicates the time of a temperature shift from 45°C to 37°C. Protease activity of culture supernatants was detected *in situ* after gelatin-SDS-PAGE. A 12.5% gel was used to analyze 15 μl of culture supernatant.

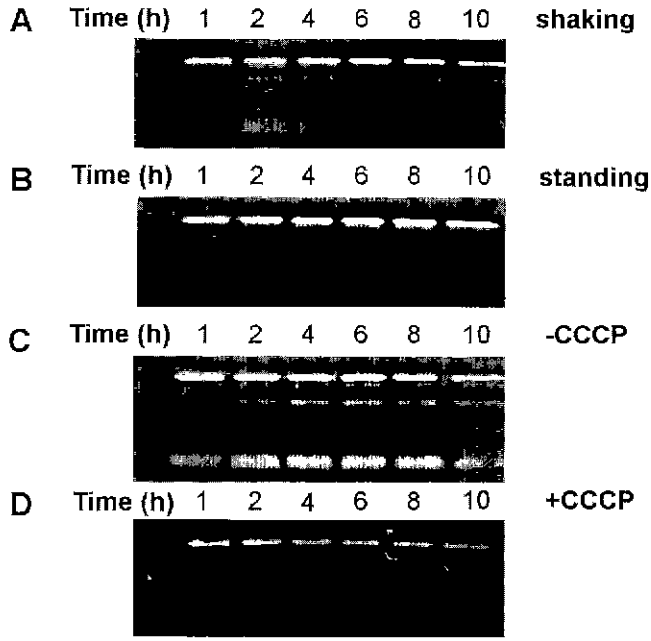


Fig. 3. Effects of aeration and CCCP on the production of extracellular proteases.

Extracellular proteolytic activity was measured with (A) and without (B) aeration, and it was also measured without (C) and with (D) 8 μM CCCP. Proteolytic activity of culture supernatants was detected *in situ* after gelatin-SDS-PAGE. A 12.5% gel was used to analyze 15 μl of the culture supernatant.

enhancement of cell growth (data not shown). Interestingly, the production of the 38-kDa neutral protease was severely inhibited by the decrease of aeration; on the contrary, the 100-kDa protease was only slightly inhibited (Figs. 3A and 3B).

It has been reported that the ion electrochemical potential affects the production of an extracellular amylase in *Vibrio alginolyticus* [8]. In order to examine the correlation between a H^+ electrochemical potential and the production of extracellular proteases in *B. cereus* KCTC 3674, we used a protonophore, CCCP. The growth of *B. cereus* KCTC 3674 at pH 6.5 was stopped completely by CCCP at 10 μM concentration (data not shown). This result indicates that *B. cereus* KCTC 3674 can not survive under conditions where the H^+ electrochemical potential is disrupted. Thus, in an experiment to elucidate whether the H^+ electrochemical potential affected the production of extracellular proteases, we used concentrated late logarithmic growth phase cultures to minimize the inhibition of cell growth by CCCP. As shown in Figs. 3C and 3D, the production of the 38-kDa neutral protease was strongly inhibited by 8 μM CCCP, whereas the 100-kDa protease was far less inhibited.

Quinacrine has been reported to interfere with the action of the *Bacillus licheniformis* penicillinase-releasing protease [15] and inhibit levansucrase release from *Bacillus subtilis* [2]. Therefore, if the extracellular proteases of *B. cereus* KCTC 3674 were also released by a similar process like *B. licheniformis* and *B. subtilis*, it is expected that quinacrine can prevent the release of extracellular proteases. However, as shown in Figs. 4A and 4B, the release of the 38-kDa and 100-kDa proteases in *B. cereus* KCTC 3674 was not inhibited by 0.2 mM quinacrine at all. Thus, it was concluded that the secretion of extracellular proteases in *B. cereus* KCTC 3674 was not affected at all by exoprotein-releasing factors similar to *B. licheniformis* or *B. subtilis*. The antibiotic cerulenin which inhibits fatty acid synthesis [12] is well known as an inhibitor of the secretion of

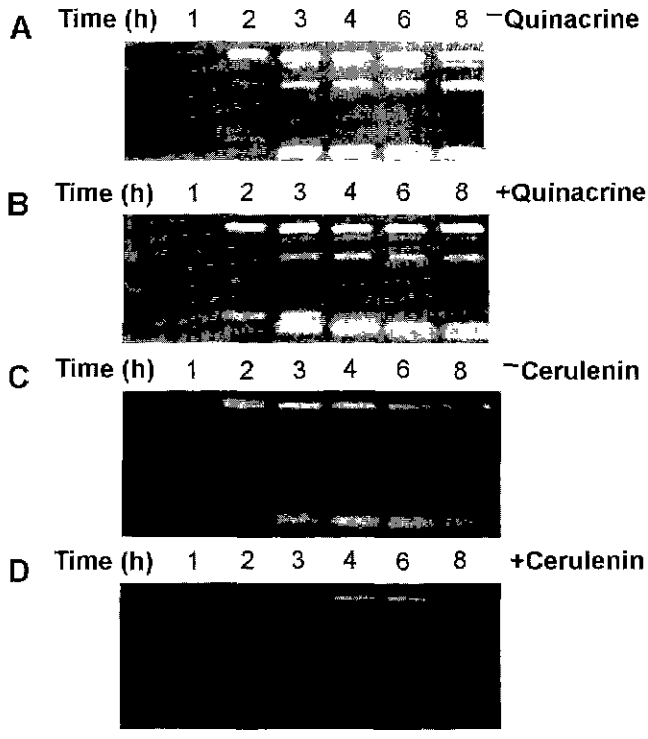


Fig. 4. Effects of quinacrine and cerulenin on the production of extracellular proteases.

Extracellular proteolytic activity was measured without (A) and with (B) 0.2 mM quinacrine and was also measured with (C) and without (D) cerulenin (5 μg/ml). Proteolytic activity of culture supernatants was detected *in situ* after gelatin-SDS-PAGE. A 12.5% gel was used to analyze 15 μl of the culture supernatant.

levansucrase [2], α-amylase, protease [13], and penicillinase [5] in bacilli; however, its effects on general protein synthesis at low concentrations (5 to 10 μg/ml) is marginal [12]. In our study, cerulenin (5 μg/ml) slightly affected the growth of *B. cereus* KCTC 3674 (data not shown), whereas the production of extracellular proteases was strongly inhibited. As shown in Figs. 4C and 4D, the production of the 38-kDa and 100-kDa proteases was severely inhibited by cerulenin (5 μg/ml). The result of cerulenin led us to propose that the secretion of extracellular proteases in *B. cereus* KCTC 3674 was closely linked with the membrane and could be reduced by membrane modifications which had little or no effect on the synthesis of other proteins. Based on these findings, we concluded that the production of the 38-kDa neutral protease and the 100-kDa protease in *B. cereus* KCTC 3674 was regulated differently.

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