Journal of Environmental Biotechnology
Vol. 8, No. 2, 119–121, 2008

Original paper (short paper)

Identification of Inducers for Chitinase B (ChiB) Production in *Bacillus cereus* CH and Estimation of Its Induction Mechanism

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(Received; 14 February, 2008/Accepted; 5 November, 2008)

Chtinolytic *Bacillus cereus* CH produces chitinase A (ChiA) and chitinase B (ChiB) and colloidal chitin and *N*-acetylglucosamine (GlcNAc) oligomers induce their production. Deacetylated chitin products (chitosan 7B, 9B, and 10B) and glucosamine oligomers showed much decrease activity to induce ChiB production campared with that of colloidal chitin and GlcNAc oligomers, indicating that acetylation of chitin and chitin oligosaccharides are essential to their induction activity. Histidine protein kinase inhibitors (closantel and 3,3',4',5-tetrachlorosalicylanilide) inhibited ChiB production even in the presence of an inducer (GlcNAc hexamer). This result suggests that ChiB production in *B. cereus* CH is regulated by two-component regulatory system.

Key words: chitinase B, chitooligosaccharides, two-component regulatory system, *Bacillus cereus*

Chitin is the main component of exoskeleton (cuticle) of crabs. We consume large amounts of crabs and shrimps for food and as a result, enormous amounts of their shell wastes are produced every year. Although chitin is utilized in environmental technology and food medical industries, most of shell wastes are discarded by incineration and burying. Thus, reutilization of shell wastes is an important issue in ecological concern. Cuticles are biopolymers composed of rigid complex of *N*-acetylglucosamine polymers, proteins, and Ca^{2+} and highly insoluble in water and organic solvents. Chitinolytic and non-chitinolytic microorganisms cooperatively degrade cuticles. To understand chitin degradation system, we isolated chitinolytic bacterium *Bacillus cereus* CH and investigated its chitinolytic enzyme system[3,4,7]. *B. cereus* CH produces two chitinases, chitinase A (ChiA) and chitinase B (ChiB) and ChiB is responsible for the major extracellular chitinase activity. ChiA and ChiB production is inducible and colloidal chitin promotes transcription of the *chiA* and *chiB* genes. In this study, we report that the extent of acetylation of chitooligosaccharides affects induction of *chiB* transcription, and that there is the possibility that a two-component regulatory system is involved in the control of *chiB* transcription.

B. cereus CH was cultivated in medium containing 0.2% triptone, 0.1% yeast extract, 0.2% NaCl, 0.025% KH_2PO_4, 0.025% K_2HPO_4, 0.01% calcium acetate, and 0.01% magnesium acetate (pH 7.2) at 33°C. At 12 h after the initiation of cultivation, 0.025% of test compounds were added to induce ChiB production. Extracellular and cytoplasmic ChiB was quantified by enzyme-linked immunosorbent assays (ELISA) with anti-ChiB serum solution as described in the previous report[7]. Quantification of *chiB* mRNA was performed by reverse transcription PCR (RT-PCR) as described previously[7].

In the previous study, we demonstrated that ChiA and ChiB production in *B. cereus* CH is induced by colloidal chitin and *N*-acetylglucosamine oligomers, but not by deacetylated chitin (chitosan 10B)[7]. This result suggests that the extent of acetylation of chitin affects the ability of chitin to induce chitinase genes in *B. cereus* CH. To investigate an effect of the extent of acetylation of chitin, we examined chitin products with different deacetylation ratio, i.e. chitosan 7B, 9B, and 10B for their ability to induce extracellular ChiB production. The deacetylation ration of chitosan 7B, 9B, and 10B is 70, 90, and 100%, respectively. As shown in Fig. 1, colloidal chitin showed the best induction activity and the induction activity decreased with increasing of the deacetylation ratio of chitin. To confirm an effect of acetylation of chitin on induction of ChiB production, we examined the activity of oligomers (dimer to hexamer, [$(GlcN)_2$, $(GlcN)_4$, $(GlcN)_5$, and $(GlcN)_6$]) of glucosamine (GlcN) to induce ChiB production and compared it with that of *N*-acetylglucosamine (GlcNAc) oligomers ($(GlcNAc)_2$ to $(GlcNAc)_6$). As reported in the previous study, GlcNAc oligomers showed remarkable induction activity which increased with residue numbers of the oligomers (Fig. 2).

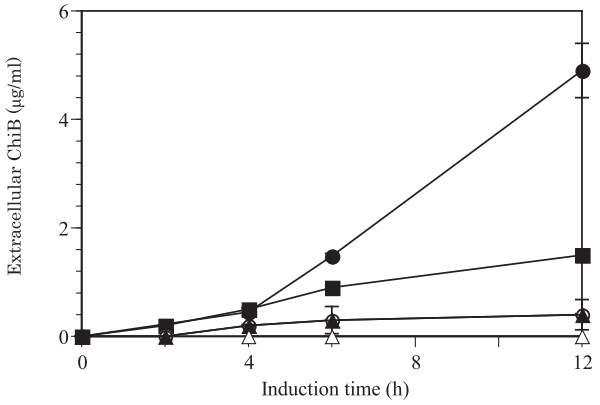

Fig. 1. Induction of extracellular ChiB production in *B. cereus* CH by colloidal chitin and chitosan products.
B. cereus CH was cultivated at 33°C and at 12 h after the initiation of cultivation, 0.025% (w/v) colloidal chitin (closed circles), chitosan 7B (closed squares), chitosan 9B (open circles), and chitosan 10B (closed triangles) were added to medium. Culture broth was withdrawn at each time point and subjected to quantification of extracellular ChiB by ELISA. Open triangles represent extracellular ChiB production in the negative control (no addition of inducer).

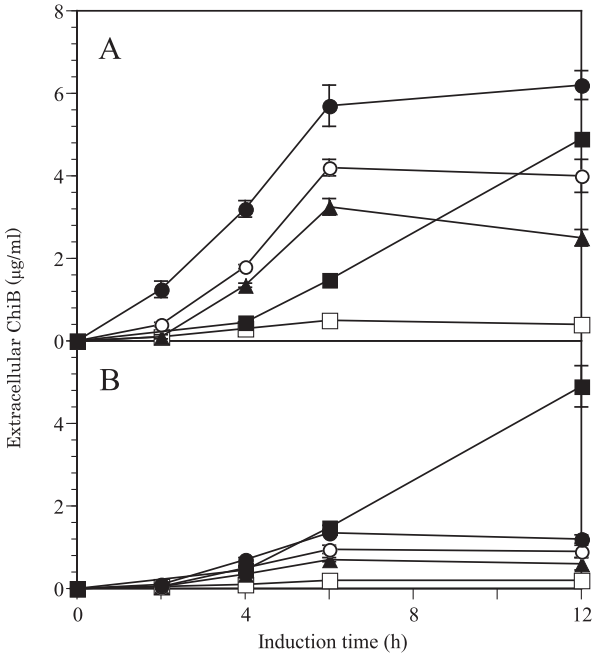

Fig. 2. Induction of extracellular ChiB production in *B. cereus* CH by GlcNAc and GlcN oligomers.
Cultivation and induction of *B. cereus* CH, and quantification of extracellular ChiB protein were performed as described in Fig. 1. (A) 0.025% (w/v) $(GlcNAc)_2$ (open squares), $(GlcNAc)_4$ (closed triangles), $(GlcNAc)_5$ (open circles), $(GlcNAc)_6$ (closed circles), and colloidal chitin (closed squares) were added to medium as inducers. (B) 0.025% (w/v) $(GlcN)_2$ (open squares), $(GlcN)_4$ (closed triangles), $(GlcN)_5$ (open circles), $(GlcN)_6$ (closed circles), and colloidal chitin (closed squares) were added to medium as inducers.

Although the induction activity of GlcN oligomers also increased with residue numbers, they showed much decreased activity compared with that of GlcNAc oligomers. These results clearly demonstrate that the extent of acetylation of chitin and chitooligosaccharides affects their activity to induce ChiB production. It is interesting to mention that $(GlcNAc)_6$ induced higher ChiB production than colloidal chitin with short induction time (~12 h) (Fig. 2) although colloidal chitin induced higher ChiB production than $(GlcNAc)_6$ with longer induction time (48-72 h)[7]. It suggests that acetylated chitin oligosaccharides (degradation products of chitin), rather than chitin itself, are inducers.

It was reported that two-component regulatory system is involved in induction of chitinase production by chitin oligosaccharides in *Streptmyces thermoviolaceus*, *Vibrio* spp., and *Vibrio cholerae*[2,6,9]. We investigated whether ChiB production is regulated by two-component regulatory system using inhibitors for autophosphorylation of histidine protein kinases of two-component regulatory systems. Closantel and 3,3',4',5-tetrachlorosalicylanilide (TCSA) were shown to act as general inhibitors of autophosphorylation of histidine protein kinases[1,5,8]. Both 15 mM closantel and TCSA inhibited induction of extracellular ChiB production by $(GlcNAc)_6$ (Fig. 3). Closantel and TCSA rarely affected growth of *B. cereus* CH (data not shown). In our previous study, we demonstrated that sodium azide inhibits secretion of ChiB protein but not ChiB production[7]. ChiB protein was accumulated inside of cells in the presence of inducer (colloidal chitin) and sodium azide. Therefore, there is the possibility that closantel and TCSA also inhibit secretion of ChiB but do not affect ChiB production. To rule out this possibility, cytoplasmic ChiB was measured for cells simultaneously exposed to $(GlcNAc)_6$ (inducer) and inhibitors (closantel, TCSA, and sodium azide) by ELISA. Cells exposed to $(GlcNAc)_6$ and sodium azide accumulated significant amounts of cytoplasmic ChiB, whereas virtually no cytoplasmic ChiB was detected with cells exposed to $(GlcNAc)_6$ and histidine protein kinase inhibitors (Fig. 3). To further confirm that closantel inhibits ChiB production, RT-PCR was performed to measure *chiB* mRNA. Significant amounts of PCR product derived from *chiB* mRNA was detected when cells were incubated with $(GlcNAc)_6$, no PCR product was detected with cells exposed to $(GlcNAc)_6$ and closantel. Thus, it was clearly demonstrated that closantel and TCSA inhibit ChiB production in *B. cereus* CH. These results suggest that ChiB production in *B. cereus* CH is regulated by two-component regulatory system. Further molecular biological research is needed to elucidate the induction mechanism of chitinase production in *B. cereus* CH.

Acknowledgments

This work was partly supported by the 21st century COE program of prediction and voidance of an abrupt change in the bio-geosphere system at the Graduate School of Environmental Earth Science, Hokkaido University. The authors deeply thank members of the COE program for employment as a research assistant in the school and education by the project.

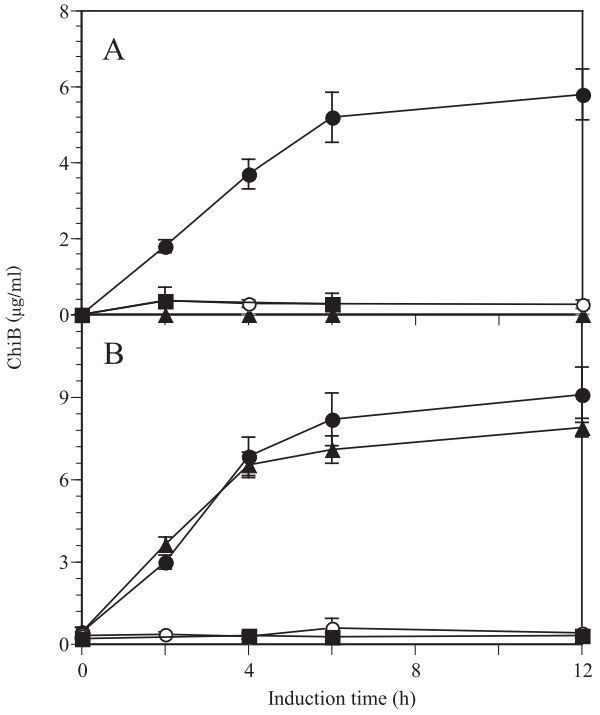

Fig. 3. Inhibition of extracellular ChiB production (A) and cytoplasmic ChiB production (B) by closantel, TCSA and sodium azide.
B. cereus CH was cultivated at 33°C and at 12 h after the initiation of cultivation, 15 mM closantel (open circles), TCSA (closed squares), and sodium azide (closed triangles) together with 0.025% (w/v) $(GlcNAc)_6$ were added to medium. Culture broth was withdrawn at each time point and subjected to quantification of extracellular and cytoplasmic ChiB by ELISA. Closed circles represent ChiB concentrations in positive control (no addition of inhibitors).

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