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Analyses of ChiA and ChiB Production by *Bacillus cereus* CH: Induction, Gene Expression, and Localization of Two Chitinases

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Bacillus cereus CH produces two chitinases (ChiA and ChiB). In the present work different chitin derivatives (chitooligosaccharides) and colloidal chitin were examined for the induction of the genes expression and productivity of enzymes. Enzyme activity in the culture supernatant increased as the length of GlcNAc increased up to six. Gene expression levels of both *chiA* and *chiB* were elevated in the presence of the inducers. However, little amount of ChiA was produced extracellularly while ChiB was secreted normally. It was demonstrated that the ChiB is a dominant extracellular chitinase induced by chitooligosaccharides and colloidal chitin.

Key words: chitinase induction, chitin oligomers, secretion, ELISA, RT-PCR

1. Introduction

Chitin, a homopolymer of β-1,4-linked *N*-acetyl-*D*-glucosamine (GlcNAc), is one of the most abundant carbohydrate polymers in nature. In a portion of fungi and most of nematodes, insects, and crustaceans, this carbohydrate generally occurs in forms of complexes with several unique proteins and other materials. Its structural characteristic is believed to provide rigid and Ca^{2+}-complexed entities with a great advantage for the construction of very robust exoskeletons. In terrestrial biosphere the recycling of chitin seems to be carried out predominantly through co-operative action of chitinolytic and other non-chitinolytic microorganisms. Taking account of a large amount of annual chitin production (about 10^{11} tonnes/year), a variety of chitinolytic enzyme systems may play an important role in recycling of chitin produced in both aquatic and terrestrial biospheres[7]. In the chitin degradation process, at least two distinct enzymes are required for complete degradation of chitin: one is chitinase (EC 3.2.1.14) to depolymerize the polysaccharide structure into dimer or oligomers of GlcNAc and the other is exohydrolytic chitinase or chitobiose-specific *N*-acetylglucosaminidase (chitobiase, EC 3.2.1.14) to hydrolyze a variety of chitin oligomers into monosaccharide GlcNAc. Bacterial species belonging to genera *Alteromonas*[18,19], *Bacillus*[1,11], *Nocardiopsis*[20], *Pseudomonas*[2,3], *Serratia*[4,17], and *Vibrio*[13] have been reported to produce and secrete multiple chitinolytic enzymes. Probably, multiple chitinolytic enzymes provide an effective chitin degradation system through their synergistic action[13]. In order to develop chitin degradation systems, we believe that basic understanding of production mechanisms of chitin producers is important[10,13].

Previous studies on *Bacillus cereus* CH[11,12] showed that colloidal chitin can serve as an inducer in the production of chitinases A and B (ChiA and ChiB) and the respective genes, *chiA* and *chiB*, have been cloned and sequenced. However, detailed mechanisms for their induction, production, and secretion remain to be elucidated. In the present study, several chitin oligomers were tested for inducers of chitin production. We also prepared specific rabbit antisera against recombinant ChiA and ChiB and used for the analysis of ChiA and ChiB production. Moreover, RT-PCR was performed for the analysis of *chiA* and *chiB* transcription levels during induction.

2. Materials and Methods

2.1. Cultivation, induction, and enzyme activity

Bacillus cereus CH was precultivated to exponential phase (6 h) in Luria-Bartini medium (pH 7.2). For chitinase induction, aliquots (1 ml) of the preculture were transferred to each second fresh medium (25 ml, pH 7.2) 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% (w/v) magnesium acetate. After 12 h cultivation, a variety of inducer at a concentration of 0.1% (w/v) was added to the second medium. At indicated time periods, a portion (1 ml) of each induction culture was withdrawn and

centrifuged to separate the culture supernatant and cell pellet. Enzyme activity recovered in each of the supernatants was measured using 1.5 mM *p*-nitrophenyl-β-chitobioside {$(GlcNAc)_2$-*p*NP} as a substrate. Detailed reaction conditions were the same as those described previously[11]. Protein concentration was determined using DC protein assay kit (Bio-Rad, CA, U.S.A.) and bovine serum albumin as standard.

2.2. Preparation of rabbit antisera against recombinant ChiA and ChiB

According to the procedure described in our previous report, recombinant ChiA and ChiB (rChiA and rChiB) were produced by *Escherichia coli* harboring pCA-S or pCB-XE and were purified[12]. Both recombinant proteins were used as the immunogens and reference proteins. After dialysis against phosphate-buffer saline (PBS, pH 7.2) and emulsifying with complete Freund adjuvant, each of the purified rChiA and rChiB (0.1 mg) was subcutaneously injected to each group (three) of young female New Zealand white rabbits (7 to 11 weeks, Hokudo, Sapporo, Japan). Each of the same immunogens (0.05 mg) was injected 8 times in intervals of 2 to 3 weeks. At seven days after the final immunization, bloods were collected from the respective groups of immunized rabbits, and anti-sera were prepared in usual procedure and stored at –20°C.

2.3. Western blot analysis and ELISA method

Each sample to be analyzed was denatured by heating (95°C, 3 min) in 0.1% (w/v) SDS and then subjected to 12.5% SDS-PAGE according to the method of Laemmli[9]. After electrotransfer of proteins to PVDF membrane and treatment with blocking solution (KPL, MD, U.S.A.), the membrane was subjected to primary immunoreaction (25°C, 1 h) with adequately diluted anti-rChiA (1 : 500) or anti-rChiB (1 : 8000) serum. After thoroughly washing with 0.05% Tween 20-containing PBS (TPBS), the secondary immunoreaction was carried out with peroxidase-labeled goat anti-rabbit IgG (H+L) (1 : 3000, American Qualex, CA, U.S.A.). Color development by coupled horseradish peroxidase was measured after incubation (30 min, rt) in TPBS containing 0.06% 4-chloro-1-naphtol, 20% ethanol, and 0.1% H_2O_2[6]. For determination of the respective anti-serum titers, an adequate volume of the recombinant ChiA or ChiB solution was added to 96 well plates (Nalgen Nunk, NY, U.S.A.). After standing overnight at 4°C, the unbound protein was removed. The antigen-coated plates were thoroughly washed with 0.05% Tween 20-containing PBS (TPBS), masked with blocking solution (KPL), and then washed with TPBS. To the antigen-coated wells 50 μl of the respective diluted anti-serum solutions was added and incubated at room temperature for 1 h. After thoroughly washing, 50 μl each of diluted secondary antibody and peroxidase-labeled goat anti-rabbit IgG (H+L) (American Qualex) was added. Incubation and washing were carried out as those described in the above. The coupled enzyme activity on formed ternary immunocomplex was measured using TMB peroxidase ELA substrate kit (Bio-Rad) in according to the procedure recommended by the supplier. To determine ranges suitable for quantifing amounts of the ChiA and ChiB, ELISA similar to the above was carried out. For measurement of the ChiA and ChiB amounts in supernatants and fractions, each supernatant from cell collected at the various induction periods and fractions was thoroughly dialyzed against PBS, and a portion of each supernatant protein (anti-rChiA: 64 ng/well and anti-rChiB: 32 ng/well) was subjected to ELISA.

2.4. Preparation of cytosolic, solubilized membrane, and extracellular fractions

To study cellular localization of the ChiA and ChiB proteins, three cell fractions including cytosolic, membrane, and extracellular (culture sup.) were separated as follows. *B. cereus* CH cell was cultivated under similar induction conditions in the presence or absence of 15 mM sodium azide. The harvested cell (5 g, wet weight) was suspended in 10 ml (same volume of culture medium) of 20 mM Tris-HCl buffer (pH 7.2) and sonicated for 1.5 min (15 s intervals for sonication and cooling). After removal of cell debris and wall (13,000 g, 10 min), supernatant was subjected to ultracentrifugation (100,000 g, 30 min). The resulting supernatant and pellet were used as cytosolic and membrane fractions, respectively. The later fraction was further suspended in 1 ml of 20 mM Tris-HCl buffer (pH 7.2) containing 0.6% Triton X-100, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM phenylmethysulfonyl fluoride. The suspension was stirred with magnetic stirrer at 4°C for 30 min and subjected to ultracentrifugation. The obtained supernatant was used as solubilized membrane fraction after dialysis. Chitin oligomers (n=2–6) were purchased from Seikagaku Corp. Tokyo, Japan; other chemicals, Nacalai Tesque, Kyoto, Japan. All experiments in this study were carried out for three times.

2.5. RNA extraction and RT-PCR

B. cereus CH was cultivated for 12 h in the second medium and then each inducer was added at a concentration of 0.025%. At indicated periods, aliquots (0.5–0.7 ml) of the respective culture were well mixed with the same volume of bacterial RNA reagent (Qiagen, CA, U.S.A.). The resulting cell mixture was centrifuged. After removal of the supernatant, wet weight of cell pellet was prepared in the same weight. Extraction and purification of total RNA were carried out according to the procedure of Gilman and Chamberlin[5]. The pellet was dissolved in a mixture of lysing matrix B (Q-BIO gene, CA, U.S.A.) and a solution containing 0.6% CTAB and 1 mM DTT. Purification of total RNA was carried out by phenol-chloroform extraction, ethanol precipitation, and sequential digestion with DNase I (Takara Bio Inc, Otsu, Japan). Each of the resulting RNAs was resuspended in 0.1 ml DEPC-treated water and stored at –80°C until it was used for RT-PCR. To determine the expression levels of *chiA* and *chiB* mRNAs, RT-PCR was

carried out using the same concentration of prepared total RNA. On the basis of nucleotide sequences previously reported for *chiA* and *chiB* genes[12], most suitable primer pairs were constructed as follows: forward and reverse primers for *chiA* were 5'-GTGGACAAAATGGAGTCGTTT-3' (corresponding to 520–540 sequence of *chiA* gene) and 5'-TAGGCGGTGAACCTCGACGGC-3' (1057–1077), respectively; those for *chiB*, 5'-AAAGCAGGTGCTGAAGATGG-3' (786–805 sequence of *chiB* gene) and 5'-ACCTGAACATGCCGTTA-GAG-3' (1408–1427), respectively. The first strands of cDNAs against the respective total RNAs (1 μg each) were prepared by using reverse transcriptase (Invitrogen Corp., CA, U.S.A.) and random hexamers (50 μg) as primers. The reaction mixtures for RT-PCR contained 1 mM dNTPs, 5 mM $MgCl_2$, and 20 mM DTT. After preincubation (rt, 5 min), reverse transcriptase (20 U) was added to the mixture. Annealing (25°C, 10 min) and extension (42°C, 50 min) were carried out and the reaction was terminated by heating (70°C, 15 min). A primer pair of 16S rRNA sequences (341F and 926R)[21] were used as an external standard. Reaction mixture for PCR contained 50 ng template DNA or cDNA, 12.5 μM primer pair, 200 μM dNTPs, and 125 mU Taq Ex polymerase (Takara) in a total volume of 12.5 μM. The extension was initiated by hot start method. The PCR reaction was done in following program: 94°C for 1 min, at 54°C for 30 s, and at 74°C for 2 min. The RT-PCR product was electrophoresed on 1.2% agarose (Takara) gel for 1 h at 100 V with 1×TAE running buffer. The gel was stained with ethidium bromide, and visualized by UV light and documented.

3. Results and Discussion

3.1. Extracellular chitinolytic activities induced by various chitin oligomers

As shown in our previous paper[11], colloidal chitin served as a good inducer for production of extracellular chitinolytic enzymes. However, colloidal chitin is a water-insoluble large molecular weight compound and hard to interact directly with a cell surface receptor and transfer extracellular signal to promote transcription of *chi* genes. Previously, Yu *et al.* suggested a possibility that in *Vibrio furnissii* the induction of chitinase may arise from any physical contact with solid particles such as chitin-containing substance[22,23]. We tested this possibility using a chitin derivative, fully de-*N*-acetylated chitin, chitosan 10B. The level of extracellular chitinolytic activity induced by chitosan 10B was very low (less than 1.5 mU/ml) as well as in the absence of any inducer. This result suggests that physical contact of solid form polysaccharides (chitosan 10B) is not enough to induce chitinase production of the strain. Since it is known that chitin oligomers could serve as inducers of other chitinases, monomer to hexamer of GlcNAc was tested for *B. cereus* CH[15]. As shown in Fig. 1, enzyme activity increased as the chain length of chitin oligomer increased. In all of these cases, the extracellular chitinolytic activity reached a maximal level within short induction periods (less than 12 h) without lag period. In contrast, there was a significant lag period in the case for induction by colloidal chitin. This may be due to that reaction products (chitin oligomers) of chitinase from colloidal chitin function as autonomous inducers.

3.2. Quality of anti-ChiA and anti-ChiB serum

B. cereus CH was known to produce two different chitinase, ChiA and ChiB. In order to analyze the productivity of each enzyme, we adopted ELISA method by using specific anti-rChiA and anti-rChiB sera prepared from rabbit. In order to verify this method, ELISA was carried out in combination of antigen (rChiA or rChiB)-coated plates (250 ng/well) and serially diluted anti-sera (1 : 250 to 1 : 512,000). Up to the dilution folds 1 : 8000 and 1 : 128,000 for anti-rChiA and anti-rChiB sera, respectively, well linearity was obtained in the range 0–64 ng/well in rChiA and 0–32 ng/well in rChiB (Fig. 2). Specificity of anti-rChiA and anti-rChiB sera was ascertained by Western blot analysis (Fig. 3). It was clearly shown that anti-rChiA and anti-rChiB sera

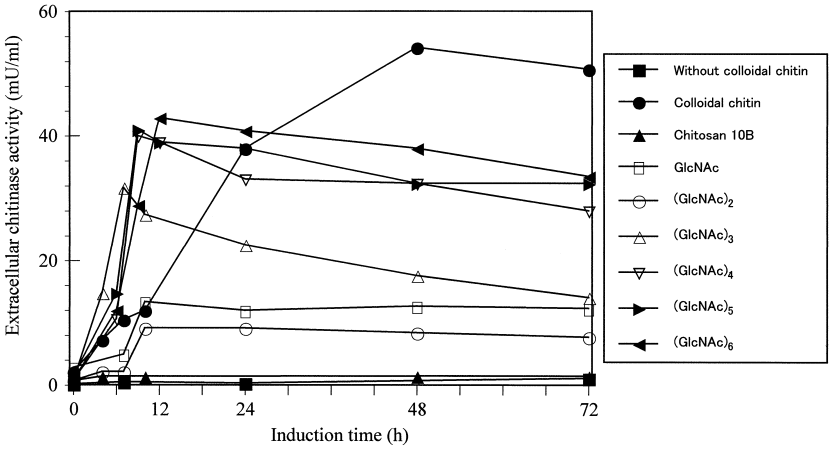

Fig. 1. Chitinolytic activity in the culture supernatants induced by various inducers.
The used inducer was presented in the next to the above figure, and 0.1% (w/v) inducer was applied to culture medium. All induction conditions were also the same.

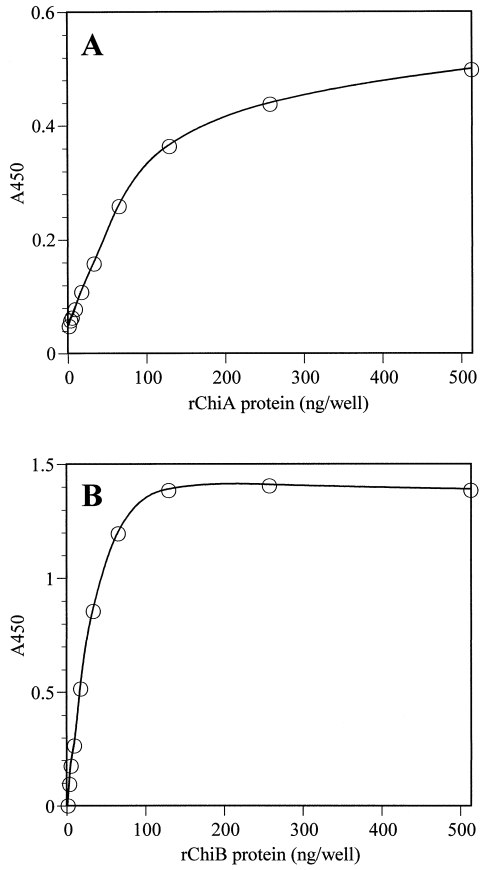

Fig. 2. Quantitativeness of ELISA method.
Each well of immunoassay plate was coated with various amounts (0–512 ng/well) of rChiA or rChiB in a series of two-fold dilutions, and then, immunostained by sequential addition of anti-ChiA (panel A) or ani-ChiB (panel B) serum followed by crossreaction with peroxidase-coupled anti-rabbit serum and developing reagent mixture. After incubation, absorbance at 450 nm was measured.

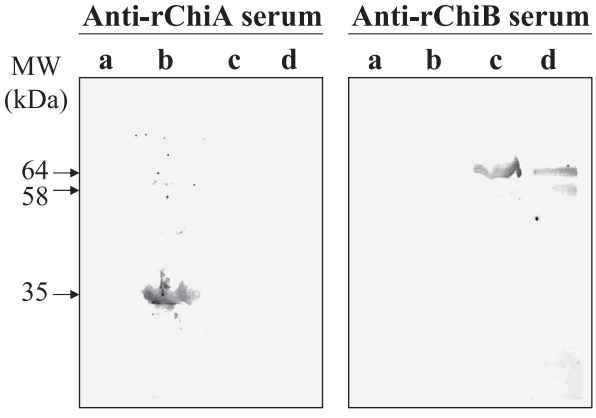

Fig. 3. Immuno-specificity of anti-rChiA and anti-rChiB sera.
Lane a, 5 μg protein marker; lane b, 5 μg rChiA; lane c, 5 μg rChiB; lane d, 30 μg of culture supernatant after induction for 72 h were subjected to 12.5% SDS-PAGE respectively, and then proteins were electrotransferred to PVDF membrane. The membrane was treated with TPBS containing anti-rChiA sera (left panel, 1 : 500) or anti-rChiB sera (right panel, 1 : 8000).

specifically bound to rChiA and rChiB, respectively (Fig. 3a, b, c). When the culture supernatant at 72 h was used, only anti-rChiB serum showed bands (Fig. 3d, right). This should be because the amount of ChiA in the supernatant was too low to be detected by anti-rChiA serum (Fig. 3d, left). Upper and lower bands would be intact ChiB and a proteolytic product derived from ChiB, respectively (Fig. 3d, right). Above results demonstrate that anti-rChiA and anti-rChiB sera were sensitive and specific enough for detection and quantification of ChiA and ChiB, respectively, in the culture or cell lysate.

The linearity of ELISA method and specificity of each antiserum enabled us to determine extracellular and intracellular amounts of ChiA and ChiB under various induction conditions. We used inducers at 0.025% in further experiments because the extracellular enzyme activity induced was almost the same at 0.1%. As shown in Fig. 4, only a small amount of extracellular ChiA (less than 0.65 μg/ml) was

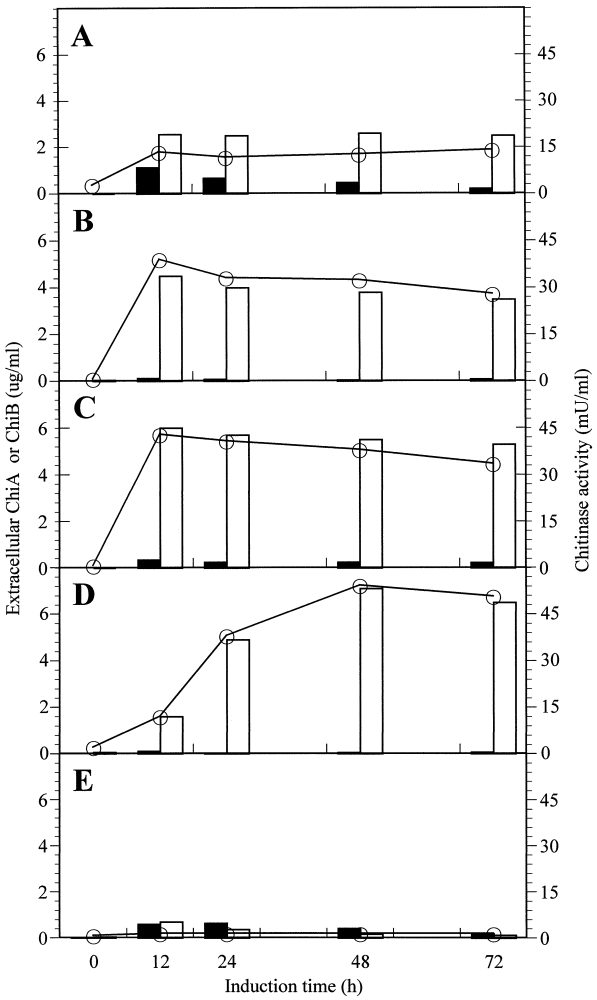

Fig. 4. Amounts of ChiA, ChiB, and chitinolytic activity in the culture supernatant.
Inducers used was 0.025% each of GlcNAc, A; $(GlcNAc)_4$, B; $(GlcNAc)_6$, C; colloidal chitin, D; chitosan 10B, E. At indicated time periods, an adequate volume was withdrawn and chitinolytic activity (white circle) and amounts of extracellular ChiA (black bar) and ChiB (white bar) in the culture supernatant were measured.

produced under the conditions except for GlcNAc monomer (Fig. 4A). We do not know the reason why the production level of ChiA is higher when GlcNAc monomer was used as an inducer. On the contrary, a large increment of extracellular ChiB (more than 15 μg/ml) was observed under the every condition, and amount of extracellular ChiB correlates with chitinolytic activity. These results clearly demonstrated that ChiB is a dominant extracellular chitinase produced by *B. cereus* CH. Furthermore, hexamer of GlcNAc induced production of extracellular ChiB in a short time as compared with that of colloidal chitin. Therefore, we judged that hexamer of GlcNAc was the best inducer among tested.

3.3. Expression levels of *chiA* and *chiB* mRNAs

We assumed that the lower production of ChiA than ChiB would attribute to less amount of *chiA* mRNA than that of *chiB*. In order to compare the amount of mRNA of *chiA* and *chiB*, we adopted RT-PCR method by using specific primer pairs to *chiA* and *chiB* genes. Expected sizes of RT-PCR products were 585 bp (16S rRNA), 557 bp (*chiA*), and 641 bp (*chiB*), respectively, and 16S rRNA product was used as an external standard to standardize the amount of total mRNA used. As shown in Fig. 5A, amounts of *chiA* and *chiB* mRNAs were similarly elevated within 12 h after addition of monomer, tetramer, or hexamer of GlcNAc or colloidal chitin. A contradiction between high level of *chiA* transcription and low level of ChiA production may attribute to its low translation or secretion efficiency. It is worth to note that even in the absence of inducer, mRNA of *chiA* and *chiB* were detectable. This result supports an idea that this constitutively transcribed and translated ChiA and ChiB degraded colloidal chitin, and the product chitin oligomers functioned as autonomous inducer (Fig. 1).

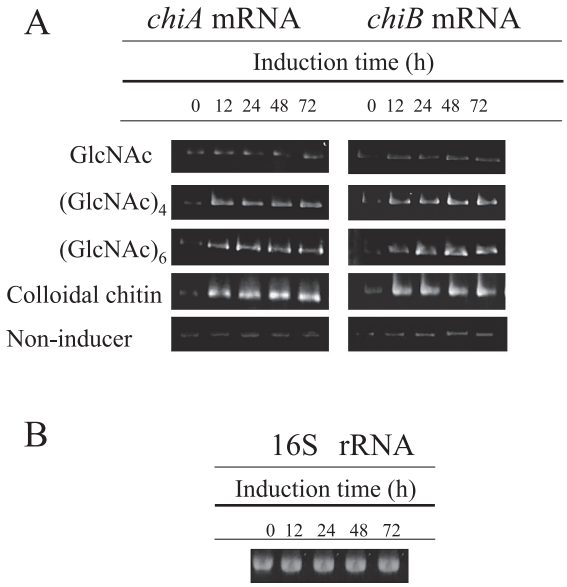

Fig. 5. RT-PCR analyses for expression levels of *chiA* and *chiB*. In this experiment a lowered concentration (0.025%) of monomer, tetramer, or hexamer of GlcNAc or colloidal chitin were used. At indicated induction periods, an adequate volume was withdrawn from the respective cultures and centrifuged. Total RNA was extracted from the resulting cell pellet and subjected to RT-PCR. Panel A, expression levels of *chiA* and *chiB* mRNAs. Panel B, an example of external standard of 16S rRNA.

3.4. Effects of sodium azide on the production of ChiA and ChiB

In order to get further insight into the low-level production of extracellular ChiA irrespective of high-level of transcription, cytosolic, membrane, and extracellular fraction were prepared and analyzed for localization of ChiA (Fig. 6A). It was found that production level of ChiA was significantly lower than ChiB and most of ChiA was produced in the cytosolic and membrane fractions. While, more than half of ChiB was produced extracellularly. These results show that both translation and secretion efficiency of ChiA are lower than ChiB. Total amount of enzyme remained low level over the time, about 0.8 μg/ml for ChiA and 2.0 μg/ml for ChiB, respectively, in the absence of inducer.

In a separate experiment, cytosolic chitinase was found to be enzymatically inactive (data not shown). This is probably

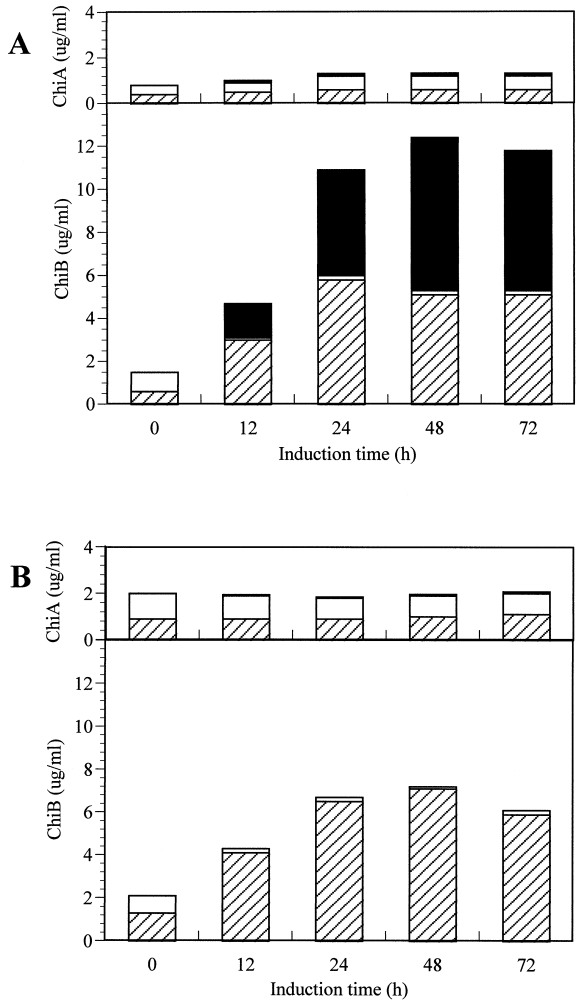

Fig. 6. Localizations of ChiA and ChiB and effect of sodium azide. Amounts of ChiA and ChiB were measured by ELISA method. Cytosolic (zebra bar), solubilized membrane (white bar), and extracellular fractions (black bar) were prepared from cells cultivated in the presence of 0.025% colloidal chitin, panel A. In panel B, 15 mM sodium azide was added to the culture at 0 h.

due to an improper folding of the enzyme with N-terimnal signal peptide. Homology search to accessible genomic DNA sequences, several nucleotide sequences involving in the regulation of Sec secretion pathway were found in our cloning *chiB* gene[12]. Furthermore, a previous study for *Vibrio cholerae* showed that mRNAs of chitinase and *secA* were similarly induced[13]. It is known that sodium azide inhibits the activity of SecA-related ATPases[8,14,24]. We were interested in testing the effect of sodium azide, at 15 mM, on the secretion of ChiA and ChiB by *B. cereus* CH. Cell grew normally in the presence of sodium azide at this concentration. It was found that secretion of ChiB was completely impaired and the amount of ChiB in the cytosolic fraction slightly increased in the presence of 15 mM sodium azide in the culture (Fig. 6B). Effect of sodium azide on the secretion of ChiA was not clear because secretion level of original culture without sodium azide was very low (Fig. 6A). In contrast to ChiB, the amounts of ChiA produced in the cytosolic and membrane fractions increased by two folds.

In this report, we demonstrated that ChiB is a dominant chitinase produced by *B. cereus* CH, is excreted from the cell by way of general Sec-pathway. Gradual increase in the production of chitinase upon addition of colloidal chitin was suggested to be attributed to the gradual accumulation of chitin oligomers produced by constitutive and inducible chitinase activities. Analyses of translation and secretion mechanism of ChiA remain to be elucidated on the molecular level.

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