

## Chitinases A, B, and C1 of *Serratia marcescens* 2170 Produced by Recombinant *Escherichia coli*: Enzymatic Properties and Synergism on Chitin Degradation

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**To discover the individual roles of the chitinases from *Serratia marcescens* 2170, chitinases A, B, and C1 (ChiA, ChiB, and ChiC1) were produced by *Escherichia coli* and their enzymatic properties as well as synergistic effect on chitin degradation were studied. All three chitinases showed a broad pH optimum and maintained significant chitinolytic activity between pH 4 and 10. ChiA was the most active enzyme toward insoluble chitins, but ChiC1 was the most active toward soluble chitin derivatives among the three chitinases. Although all three chitinases released (GlcNAc)<sub>2</sub> almost exclusively from colloidal chitin, ChiB and ChiC1 split (GlcNAc)<sub>6</sub> to (GlcNAc)<sub>3</sub>, while ChiA exclusively generated (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub>. Clear synergism on the hydrolysis of powdered chitin was observed in the combination between ChiA and either ChiB or ChiC, and the sites attacked by ChiA on the substrate are suggested to be different from those by either ChiB or ChiC1.**

**Key words:** *Serratia marcescens*; chitinase; synergism

Chitinases (EC 3.2.1.14) are the enzymes that hydrolyze  $\beta$ -1,4-linkages in chitin, one of the most abundant biopolymers in nature. In the classification system of glycosyl hydrolases based on amino acid sequence similarity, chitinases are classified into two different families, 18 and 19.<sup>1)</sup> Family 18 chitinases are found in various organisms such as bacteria, fungi, viruses, animals, and higher plants. On the other hand, family 19 chitinases were first found in higher plants and later found in many *Streptomyces* species as well as a limited number of other bacteria.<sup>2–5)</sup> Recently, several genes for family 19 chitinases were found in a nematode, *Caenorhabditis elegans* ([http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf\\_19.html](http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf_19.html)).

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*Serratia marcescens* is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. Two chitinase genes, *chiA* and *chiB*, have been cloned from four different strains of *S. marcescens*, QMB1466,<sup>6,7)</sup> BJL200,<sup>8,9)</sup> KCTC2172,<sup>10,11)</sup> and 2170,<sup>12)</sup> and a third chitinase gene, *chiC*, was identified in the strain 2170 recently.<sup>13)</sup> The *chiA*, *chiB* and *chiC* encode family 18 chitinases, chitinases A (ChiA), B (ChiB), and C1 (ChiC1). The 3-D structures of ChiA from QMB1466 and ChiB from BJL200 have been reported.<sup>14–16)</sup> ChiA consists of an all  $\beta$ -N-terminal domain and a catalytic ( $\beta/\alpha$ )<sub>8</sub>-barrel domain with a small  $\alpha + \beta$  domain inserted between the seventh and eighth  $\beta$ -strands of ( $\beta/\alpha$ )<sub>8</sub>-barrel. ChiB consists of a catalytic domain that has a fold similar to that of ChiA and a small putative chitin-binding domain (ChBD) at its C-terminus. In spite of having the catalytic domains with similar folds, the two chitinases are considered to digest chitin chains in the opposite direction, that is, ChiA is proposed to degrade the chitin chain from the reducing end<sup>17)</sup> and ChiB from the non-reducing end.<sup>15)</sup>

In addition to ChiA, ChiB, and ChiC1, CBP21 and chitinase C2 (ChiC2) have been detected in the culture supernatant of *S. marcescens*. CBP21 is a chitin-binding protein of 21 kDa and production of this protein is cooperatively regulated with three chitinases, although the role of this protein is not known.<sup>18)</sup> ChiC2 is a proteolytic derivative of ChiC1 and correspond to the catalytic domain of this chitinase. ChiC1 is the only *S. marcescens* chitinase which has a subfamily B-type catalytic domain.<sup>13)</sup> As we reported previously, bacterial family 18 chitinases

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; ChiA, chitinase A; ChiB, chitinase B; ChiC1, chitinase C1; ChiC2, chitinase C2; ChBD, chitin-binding domain; FnIII<sub>D</sub>, fibronectin type III domain; HPLC, high-pressure liquid chromatography; WS-chitin, Water-soluble chitin

can be divided into three subfamilies, subfamilies A, B, and C from the amino acid sequence similarity of their catalytic domains.<sup>13)</sup> The major structural difference deduced from the amino acid sequence comparison between subfamilies A and B is that chitinases in subfamily A have an insertion domain between the seventh and eighth  $\beta$ -strands of the  $(\beta/\alpha)_8$ -barrel catalytic domain, that is absent in subfamily B chitinases. ChiC1 has the catalytic domain of subfamily B, while ChiA and ChiB have those of subfamily A. This chitinase also has a fibronectin type III domain (FnIII<sub>D</sub>) that is absent in ChiA and ChiB. Therefore, ChiC1 is structurally very different from the other two chitinases. However, in spite of such interesting structural features, little is known about the enzymatic properties of ChiC1. On the other hand, enzymatic properties of ChiA and ChiB from BJL200 have been studied in detail by Brurberg *et al.* and synergism of the two chitinases have been demonstrated.<sup>19)</sup>

Not only *S. marcescens* but also many other chitinolytic bacteria produce multiple chitinases derived from different genes.<sup>3,13,20-25)</sup> Efficient degradation of chitin is assumed to be done by the combined actions of these multiple chitinases. To discover the individual roles of ChiA, ChiB, and ChiC1 from *S. marcescens* 2170 in chitin degradation, the three chitinases produced by *E. coli* cells were purified, and enzymatic properties and synergistic effects on the chitin degradation were studied.

## Materials and Methods

**Bacterial strains, plasmids, and culture conditions.** *Escherichia coli* DH5 $\alpha$  harboring plasmids pNCA112 carrying the cloned *chiA* gene<sup>18)</sup> and pMCB7 carrying the cloned *chiB* gene<sup>12)</sup> were used for production of ChiA and ChiB, respectively. *E. coli* JM109 harboring plasmid pTCC2 carrying the cloned *chiC*<sup>13)</sup> was used for ChiC1 production. *E. coli* cells harboring these plasmids were grown in Luria-Bertani (LB) medium containing 100  $\mu$ g/ml ampicillin at 30°C.

**Chemicals.** Colloidal chitin and glycol chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo) following the methods described by Jeuniaux,<sup>26)</sup> and Yamada and Imoto,<sup>27)</sup> respectively. Chitin EX (powdered prawn shell chitin) used in the chitin affinity column, chitosan 7B (approximately 30% acetylated), chitosan 8B (approximately 20% acetylated), and chitosan 9B (approximately 10% acetylated) were purchased from Funakoshi Chemical Co. (Tokyo). Regenerated chitin was prepared from chitosan 8B by the method of Molano *et al.*<sup>28)</sup> Chitoooligosaccharides [(GlcNAc)<sub>3</sub>, (GlcNAc)<sub>4</sub>, (GlcNAc)<sub>5</sub>, and (GlcNAc)<sub>6</sub>] were purchased from Seikagaku Corporation (Tokyo, Japan). Water-soluble chitin (WS-chitin) was obtained from

Yaizu Suisan Chemical Co. Ltd. (Shizuoka, Japan). The degree of deacetylation and approximate molecular weight of the WS-chitin were 38.8% and from 200,000 to 300,000, respectively. Restriction enzymes and modification enzymes were purchased from Takara shuzo (Osaka), Toyobo Biochemicals (Osaka), and New England Biolabs (Beverly, MA, USA).

**Production and purification of ChiA, ChiB, and ChiC1.** ChiA produced by *E. coli* DH5 $\alpha$  cells harboring plasmid pNCA112 was purified from the culture supernatant, as described previously with some modifications.<sup>18)</sup> After collecting proteins by ammonium sulfate precipitation, the precipitate was dissolved in a small volume of 1 mM sodium phosphate buffer (pH 6.0) and put on hydroxyapatite column (3.0  $\times$  7.0 cm) previously equilibrated with the same buffer, and eluted with the gradient from 1 to 400 mM sodium phosphate buffer (pH 6.0). The fractions containing ChiA were collected and put on a chitin affinity column for chromatography. ChiC1 produced by *E. coli* JM109 cells harboring plasmid pTCC2 was purified from the soluble fraction of the disrupted cells as described previously.<sup>13)</sup>

ChiB produced by *E. coli* DH5 $\alpha$  cells harboring plasmid pMCB7 carrying the cloned *chiB* gene was purified as follows. *E. coli* cells were grown in LB medium containing 100  $\mu$ g/ml ampicillin and 0.4 mM isopropylthio- $\beta$ -D-galactoside for 24 h, collected by centrifugation, and disrupted by sonication. After unbroken cells and debris were removed by centrifugation, proteins in the soluble fraction were collected by ammonium sulfate precipitation (20–40% saturation). The precipitate was dissolved in a small volume of 1 mM sodium phosphate buffer (pH 6.0) and put on a hydroxyapatite column (3.0  $\times$  7.0 cm) previously equilibrated with the same buffer, and eluted with the same buffer. The unadsorbed protein fractions containing ChiB were collected and lyophilized. Lyophilized ChiB was dissolved in a small volume of 10 mM phosphate buffer (pH 6.0) and purified further by chitin affinity column chromatography as described previously.<sup>18)</sup>

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli.<sup>29)</sup> Detection of chitinase activity after renaturation of enzymes in the polyacrylamide gel was done as described previously.<sup>30)</sup>

**Enzyme and protein assays.** Chitinase activity was measured by a modification of Schales' procedure<sup>31)</sup> with colloidal chitin as the standard assay substrate. The reaction mixture (total 750  $\mu$ l) contained 1 mg of colloidal chitin (dry weight) and enzyme in 0.1 M sodium phosphate buffer, pH 6.0, and the reaction was

done at 37°C for 10 min. One unit of chitinase activity was defined as the amount of enzyme that produces 1  $\mu$ mole of reducing sugar per min. Activities of chitinases at various pHs were measured using 0.1 M sodium citrate (pH 3.5–6.0), sodium phosphate (pH 6.0–7.5), Tris-HCl (pH 7.5–9.0), glycine-NaOH (pH 9.0–10.5), and NaH<sub>2</sub>PO<sub>4</sub>-NaOH (pH 11.0–12.0) buffers.

Protein concentrations were measured by the method of Lowry *et al.*<sup>32)</sup> using bovine serum albumin as the standard.

**Chitin-binding assay.** The binding assay mixture contained 4 mg of powdered prawn shell chitin and 1 M NaCl in 20 mM sodium phosphate buffer, pH 6.0 (total volume 500  $\mu$ l). After various amounts of chitinase protein were added, the mixture was incubated for 1 h on ice, with stirring every 15 min. Supernatant containing unadsorbed protein and powdered chitin was separated by centrifugation (15,000  $\times$  g, 20 min) and the protein concentration in the supernatant was measured.

**N-terminal amino acid sequence analysis.** The purified ChiB on the polyacrylamide gel was electroblotted onto a polyvinylidene difluoride membrane, as described by Matsudaira.<sup>33)</sup> The membrane was briefly stained with Coomassie brilliant blue R-250 to make protein bands visible, and excised chitinase bands were sequenced on a Shimadzu protein sequencer PSQ-21 (Osaka, Japan).

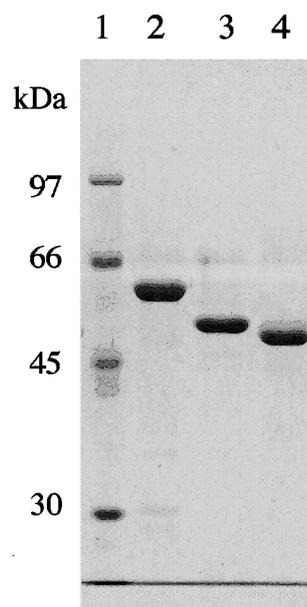
**Analysis of hydrolysis products.** Reaction mixture (final volume, 150  $\mu$ l) containing either 0.2 mg of colloidal chitin and 50 pmole of chitinase or 60  $\mu$ g of chitoooligosaccharide and 5 pmole of chitinase in 0.1 M sodium phosphate buffer, pH 6.0, was incubated at 37°C. The mixture was boiled for 5 min and filtered through a cellulose acetate membrane and analyzed by high-pressure liquid chromatography (HPLC) using an Ultron-NH<sub>2</sub> column (4.6  $\times$  250 mm) in a liquid chromatograph LC-10A system (Shimadzu) with acetonitrile-water (74:26) as the mobile phase. Elution was monitored by measuring UV A<sub>210</sub>.

## Results

### Purification of ChiA, ChiB, and ChiC1

ChiA was produced by *E. coli* carrying the cloned *chiA* gene and purified from the culture supernatant by hydroxyapatite column chromatography and chitin affinity column chromatography.<sup>18)</sup> ChiC1 was produced by *E. coli* carrying the cloned *chiC* gene, extracted from the cells, and purified by chitin affinity column chromatography and gel filtration chromatography.<sup>13)</sup>

ChiB was produced by *E. coli* cells harboring the



**Fig. 1.** SDS-PAGE Analysis of the Purified Chitinases Used in This Study.

All three chitinases were produced in *E. coli* carrying the corresponding gene. Lane 1, size markers; lane 2, ChiA; lane 3, ChiB; lane 4, ChiC1.

plasmid pMCB7 that carries the cloned *chiB* gene. The pMCB7 was constructed by inserting a DNA fragment carrying the *chiB* gene into pUC119 in the same direction of transcription as the *lac* promoter in the vector. Chitinase activity was mainly recovered in the cytoplasmic fraction (approximately 80% of produced chitinase) as expected from the absence of a typical signal sequence at its N-terminus of the deduced polypeptide from the *chiB* gene. The cytoplasmic fraction contained a chitinase protein of a size identical to the ChiB detected in the culture supernatant of *S. marcescens* 2170. The chitinase has an N-terminal amino acid sequence identical to ChiB from *S. marcescens* starting from Ser-2 of the deduced polypeptide. Therefore, we considered that the ChiB produced in *E. coli* is essentially identical to the one produced by *S. marcescens* 2170. ChiB extracted from *E. coli* cells was purified by hydroxyapatite column chromatography and chitin affinity column chromatography. A total 46.8 mg of purified ChiB was obtained from a one-liter culture of *E. coli* DH5 $\alpha$  cells harboring pMCB7.

SDS-PAGE analysis of the purified preparations of the three chitinases used in this study is shown in Fig. 1.

### Effects of temperature and pH on the activities of three chitinases

Effects of temperature and pH on the activities of ChiA, ChiB, and ChiC1 were studied using colloidal chitin as the assay substrate. Maximum activities of ChiA and ChiB measured at pH 6.0 were observed at

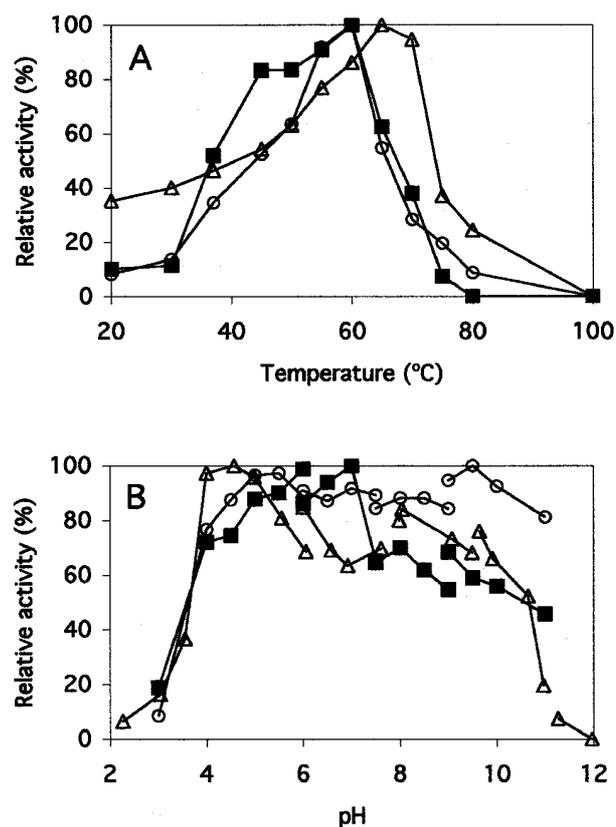


Fig. 2. Effects of Temperature (A) and pH (B) on the Activities of ChiA, ChiB, and ChiC1.

Chitinase activity was measured using colloidal chitin as the assay substrate. Open circles, ChiA; closed squares, ChiB; open triangles, ChiC1.

60°C as shown in Fig. 2(A). Activities of these chitinases were impaired at temperatures over 60°C. On the other hand, the optimum temperature for ChiC1 was a little higher than for the other two chitinases. ChiC1 showed maximum activity 65 and 70°C. In addition, ChiC1 retained higher relative activity at lower temperatures than the other two chitinases. Approximately 40% of the maximum activity remained at temperatures below 30°C, while the other chitinases had only 10% of their maximum activity.

All three chitinases maintained significant hydrolyzing activity in a broad pH range from pH 4 to 10 as shown in Fig. 2(B), although ChiB and ChiC1 had relatively higher activity at lower pH than at higher pH. On the other hand, ChiA maintained the activity higher than 80% of its maximum between pH 4 and 11.

#### Hydrolyzing activities against various chitinous substrates

Hydrolyzing activities of ChiA, ChiB, and ChiC1 against various chitinous substrates were compared (Fig. 3). The substrates used include powdered chitin, colloidal chitin, glycol chitin, WS-chitin, 10%

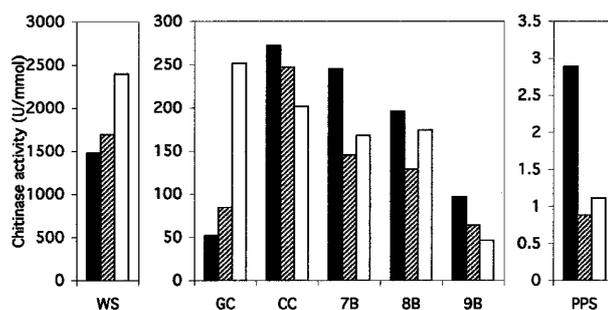


Fig. 3. Hydrolyzing Activities of ChiA, ChiB, and ChiC1 against Various Chitinous Substrates.

Closed boxes, ChiA; shaded boxes, ChiB; open boxes, ChiC1. WS, Water-soluble chitin; GC, glycol chitin; CC, colloidal chitin; 7B, 30% acetylated chitosan; 8B, 20% acetylated chitosan; 9B, 10% acetylated chitosan; PPS, powdered prawn shell chitin.

acetylated chitosan, 20% acetylated chitosan, and 30% acetylated chitosan.

The three chitinases hydrolyzed WS-chitin most efficiently and, on the other hand, powdered chitin was the hardest substrate for hydrolysis by all three chitinases. Hydrolyzing activities against powdered chitin of the three chitinases were less than 1/100 of those against colloidal chitin. ChiA had the highest hydrolyzing activity among three chitinases against all insoluble substrates. Activity of ChiA against powdered chitin was approximately three times higher than those of ChiC1 and ChiB. On the other hand, ChiC1 had highest activity among three chitinases against soluble chitin, such as glycol chitin and WS-chitin. The activity against glycol chitin was three to five times higher than the other chitinases. Therefore, it seems that ChiA is the enzyme that should be adopted to hydrolyze insoluble chitin, and ChiC1 is for hydrolysis of soluble forms. ChiB had activities closer to ChiC1 than ChiA against powdered chitin, and to ChiA than ChiC1 against soluble chitins.

Hydrolyzing activity of all three chitinases against chitosan decreased along with the decrease in degree of acetylation.

#### Binding of the chitinases to insoluble chitin

ChiA had the highest hydrolyzing activity against all insoluble chitins as described above, but on the other hand ChiC1 showed highest activity on soluble substrates. To see whether the highest activity of ChiA in the hydrolysis of insoluble chitins is somehow related to the binding activity of these chitinases, binding of three chitinases to powdered chitin were compared. Various amounts of chitinases were mixed with powdered chitin and incubated for 1 h on ice and unadsorbed protein concentration in the supernatant was measured after centrifugation. The amount of adsorbed protein was estimated from the difference between the initial protein concentration and the unadsorbed protein concentration. As

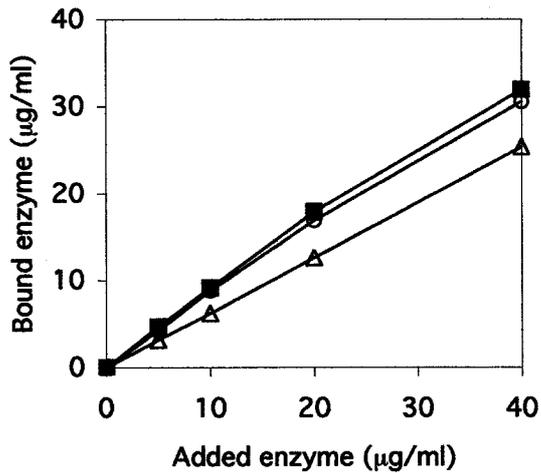


Fig. 4. Binding Activities of ChiA, ChiB, and ChiC1 to Powdered Chitin.

Binding assay mixtures contained 4 mg (dry weight) of powdered chitin and 2.5–20 µg of each chitinase in 0.5 ml of 20 mM sodium phosphate buffer (pH 6.0) containing 1 M NaCl. Open circle, ChiA; closed square, ChiB; open triangle, ChiC1.

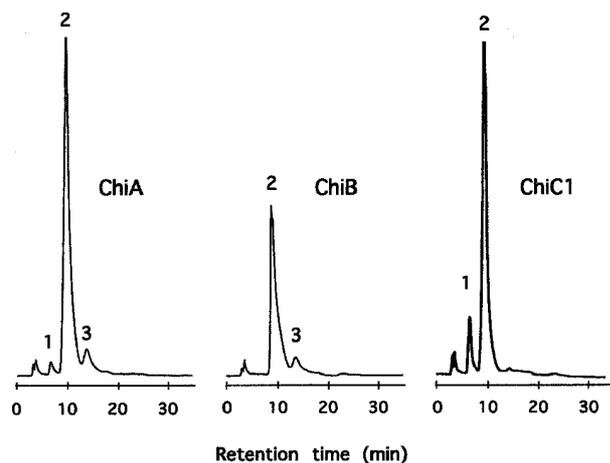


Fig. 5. HPLC Analyses of Chitoooligosaccharides Produced from Colloidal Chitin by ChiA, ChiB, and ChiC1.

Reaction mixture (final volume, 150 µl) containing 0.2 mg of colloidal chitin and 50 pmole of chitinase was incubated at 37°C for 12 h. 1, GlcNAc; 2, (GlcNAc)<sub>2</sub>; 3, (GlcNAc)<sub>3</sub>.

shown in Fig. 4, while ChiC1 bound a little less efficiently to powdered chitin than the other two chitinases, ChiB and ChiA bound almost equally to powdered chitin. These results demonstrated that differences in the hydrolyzing activity observed among three chitinases are not solely due to the differences in the binding activities of these chitinases and are rather due to the structure and properties of the catalytic domains of the chitinases.

#### Hydrolysis products from chitin and chitoooligosaccharides

Chitinases were incubated with colloidal chitin at 37°C for 12 h and hydrolysis products generated

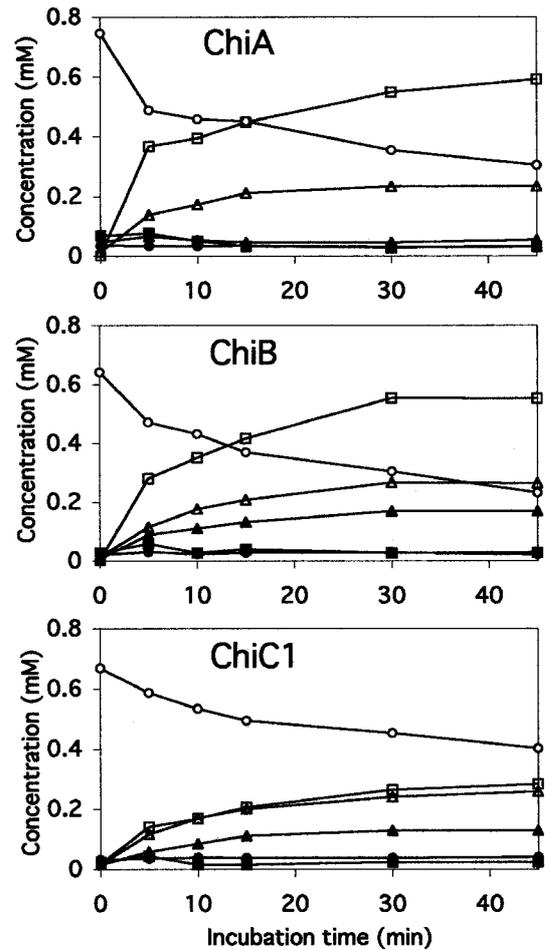


Fig. 6. Time Course of (GlcNAc)<sub>6</sub> Hydrolysis by ChiA, ChiB, and ChiC1.

Open circles, (GlcNAc)<sub>6</sub>; closed circle, (GlcNAc)<sub>5</sub>; open triangles, (GlcNAc)<sub>4</sub>; closed triangles, (GlcNAc)<sub>3</sub>; open squares, (GlcNAc)<sub>2</sub>; closed squares, GlcNAc.

were analyzed by HPLC. As shown in Fig. 5, all three chitinases predominantly produced (GlcNAc)<sub>2</sub> from colloidal chitin. However, differences were observed in generation of minor products. ChiA produced both GlcNAc and (GlcNAc)<sub>3</sub> as the minor products, on the other hand, ChiB produced only (GlcNAc)<sub>3</sub> and ChiC1 produced only GlcNAc in addition to a large amount of (GlcNAc)<sub>2</sub>.

Hydrolysis of chitoooligosaccharides by three chitinases was also examined. Figure 6 shows the courses of (GlcNAc)<sub>6</sub> hydrolysis. ChiA produced (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub> almost exclusively. On the other hand, ChiB and ChiC1 produced significant amounts of (GlcNAc)<sub>3</sub> in addition to (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub>. Therefore, endo-typic action in oligomer hydrolysis was demonstrated, although the two chitinases both produced (GlcNAc)<sub>2</sub> almost exclusively from colloidal chitin. Brurberg *et al.* also detected chitotriosidase activity of ChiB from *S. marcescens* BJL200.<sup>19</sup> From the 3-D structural analysis of BJL200 ChiB by van Aalten *et al.*, the

chitotriosidase activity of this enzyme was explained by the observation that binding of chitin oligomers was blocked beyond the  $-3$  subsite.<sup>15)</sup>

Both ChiA and ChiB produced twice as much (GlcNAc)<sub>2</sub> as (GlcNAc)<sub>4</sub>, while ChiC1 produced almost equal amount of (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub>. These results suggest that ChiA and ChiB successively hydrolyze (GlcNAc)<sub>4</sub> generated by first cleavage of (GlcNAc)<sub>6</sub> and, on the other hand, hydrolysis of (GlcNAc)<sub>4</sub> generated by first cleavage proceed very slowly in the case of ChiC1. Indeed, when hydrolysis of (GlcNAc)<sub>4</sub> was examined, ChiC1 hydrolyzed it much more slowly than the other two chitinases (data not shown).

#### Synergistic effects on chitin degradation

To examine whether the three chitinases produced by *S. marcescens* 2170 act synergistically on chitin degradation, the synergistic effect was measured in two different ways. Figure 7 shows released reducing

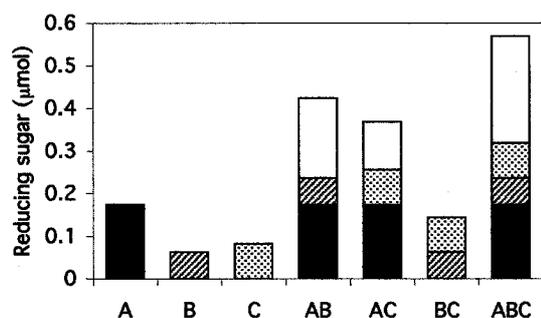


Fig. 7. Synergistic Effects of ChiA, ChiB, and ChiC1 on the Degradation of Powdered Chitin.

Closed, shaded, and dotted boxes correspond to the amount of reducing sugar produced by the actions of ChiA, ChiB, and ChiC1, respectively. Open boxes indicate the amount of reducing sugar increased by the synergistic effect of the chitinases. A, ChiA; B, ChiB; C, ChiC1; AB, ChiA plus ChiB; BC, ChiB plus ChiC1; AC, ChiA plus ChiC1; ABC, ChiA plus ChiB plus ChiC1.

sugar measured after treatment of powdered chitin with various combinations of ChiA, ChiB, and ChiC1. Reaction mixtures contained 0.2% (w/v) powdered chitin and either one of the three chitinases or various combinations (5 μg of each chitinase). Released reducing sugar in the reaction mixture was measured after 4 h of incubation at 37°C. As shown in the figure, a clear synergistic effect was observed when powdered chitin was treated with ChiA plus ChiB or ChiA plus ChiC1. On the other hand, the combination of ChiB and ChiC1 did not show any synergistic effect. Combination of ChiA and ChiB, and ChiA and ChiC1, increased reducing sugar by approximately 80% and 45%, respectively. When powdered chitin was treated with a mixture of three chitinases, a nearly 100% increase was observed.

Figure 8 shows the effects of addition of three chitinases on the hydrolysis of powdered chitin during the treatment with one of the three chitinases. After the treatment with the first chitinase for 24 h, one of the three chitinases was added to each reaction mixture. Addition of ChiA after treatment with ChiB and vice versa greatly increased the degradation of powdered chitin. Addition of ChiC1 after treatment of ChiA and vice versa enhanced the degradation moderately. Addition of ChiB after treatment with ChiC1 and vice versa increased degradation only very slightly. Addition of the same chitinase did not increase the degradation significantly in any case. These results suggest that the sites on the substrate attacked by ChiA are different from those by ChiB or ChiC1.

#### Discussion

Brurberg *et al.* described the purification and enzymatic properties of ChiA and ChiB from *S. marcescens* BJL200 as well as their synergistic effects on colloidal chitin degradation.<sup>19)</sup> ChiA and ChiB of BJL200 and those of strain 2170 characterized in this

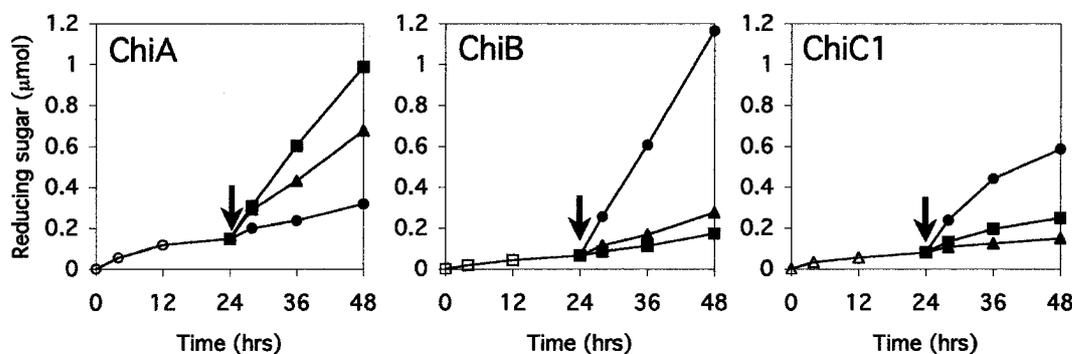


Fig. 8. Effects of Addition of Other Chitinases on the Degradation of Powdered Chitin That Had Been Already Treated with One of the Chitinases.

The added chitinases were added at the times (24 h) indicated by arrows and the reaction lasted 24 h more. Open symbols indicate amounts of reducing sugar generated before addition of another chitinases. Closed symbols indicate those after the addition of chitinases. Circle, ChiA; square, ChiB; triangle, ChiC1.

study are very similar to each other. ChiAs from the two strains share 99.3% and ChiBs share 98.4% amino acid identity. As expected from this high sequence similarity, enzymatic properties of ChiAs as well as ChiBs from the two strains are also very similar. ChiAs and ChiBs were active in a broad pH range and the optimum temperatures were between 50 and 60°C. ChiAs as well as ChiBs both produced (GlcNAc)<sub>2</sub> as the major products from colloidal chitin. ChiAs from the two strains produced (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub> almost exclusively from (GlcNAc)<sub>6</sub>. On the other hand, ChiBs also produced (GlcNAc)<sub>2</sub> and (GlcNAc)<sub>4</sub> mainly, but had significant activity to split (GlcNAc)<sub>6</sub> to (GlcNAc)<sub>3</sub>. In spite of striking similarity between ChiAs and between ChiBs from the two strains, we were aware of the interesting difference between ChiBs from 2170 and BJL200 in the localization of cloned gene products in *E. coli*. Cloned ChiB from 2170 was detected in the cytoplasm and recovered from the soluble protein fraction prepared from the disrupted *E. coli* cells. On the other hand, Brurberg *et al.* detected cloned BJL200 ChiB in the periplasmic space of *E. coli* cells and recovered it from the cells by cold osmotic shock treatment, which is the standard procedure to extract periplasmic proteins.<sup>8,19</sup> This difference in localization could be attributable to the difference in the host strain, vector plasmids, and sizes of the cloned DNA fragments used for cloning of the *chiB* genes as well as cultivation conditions used. However, it has been reported that BJL200 ChiB is almost exclusively directed towards periplasm in exponential and early stationary phases of the culture even in the original *S. marcescens* cells.<sup>8</sup> On the other hand, in the case of 2170, ChiB was detected in the culture supernatant and the amount of ChiB associated with the 2170 cells was not significant throughout the cultivation (data not shown). Therefore, it seems more likely that the difference in the localization (or secretion) between 2170 ChiB and BJL200 ChiB reflects difference in the properties of the chitinase proteins themselves, although their primary structure and enzymatic properties are very similar.

In contrast to ChiA and ChiB, enzymatic properties of ChiC1 have not been described in detail. As demonstrated in this study, ChiC1 showed characteristic properties different from the other two chitinases. The optimum pH of ChiC1 was lower and optimum temperature was higher than the other two chitinases. ChiC1 was most active toward soluble chitin derivatives and had the lowest binding to powdered chitin among the three chitinases. ChiC1 is structurally unique because this chitinase is the only *S. marcescens* chitinase which has a subfamily B-type catalytic domain and an FnIID.<sup>13</sup> The catalytic domains of all family 18 chitinases are expected to have the ( $\beta/\alpha$ )<sub>8</sub>-barrel fold. Subfamily A-type catalytic domains such as those of ChiA and ChiB are

suggested to contain insertion domains between the seventh and eighth  $\beta$ -strands which possibly provide the deep catalytic cleft.<sup>13</sup> This is consistent with the 3D-structures of ChiA and ChiB determined by Perakis *et al.* and van Aalten *et al.*, respectively.<sup>14,15</sup> On the other hand, the catalytic clefts of subfamily B-type chitinases are assumed to be shallower than those of subfamily A chitinases. Characteristic features of the enzymatic properties of ChiC1 which are distinct from the other two *S. marcescens* chitinases may be attributable to the unique structure of this chitinase.

Synergistic effects on the hydrolysis of powdered chitin between ChiA and ChiB as well as ChiA and ChiC1 were demonstrated. Brurberg *et al.* also described synergism between ChiA and ChiB from BJL200 on the hydrolysis of colloidal chitin.<sup>19</sup> The results shown in Fig. 8 imply that ChiA attacks different sites on the substrate from those by ChiB or ChiC1, and the observation could be interpreted as follows. Treatment with only ChiA (or ChiB) soon runs out the sites degradable by this chitinase and when ChiB (or ChiA, if the first enzyme was ChiB) was added to the reaction mixture, it attacks and hydrolyzes new sites which are not accessible to ChiA, and this action of ChiB creates new degradable sites for ChiA. Although the synergistic effect is less significant, similar mechanisms of increasing of chitin degradation could be illustrated for the synergism between ChiA and ChiC1. As for the combination of ChiB and ChiC1, there was no synergistic effect in spite of the characteristic difference in the enzymatic properties between the two enzymes. It has been proposed that ChiA hydrolyze crystalline chitin processively from the reducing end of the chitin chain.<sup>17</sup> Exposed aromatic residues on the surface of N-terminal domain and chitin-binding domain are proposed to play a vital role for the hydrolysis of crystalline chitin. On the other hand, van Aalten *et al.* suggested from the structural study of ChiB that this chitinase has a tunnel-like catalytic cleft, which is often observed in exo-enzymes, and it hydrolyzes the chitin chain from the non-reducing end.<sup>15</sup> ChiA has a more groove-like character than ChiB and this may confer endo-type activity to ChiA to some extent. van Aalten *et al.* suggested that the synergy between ChiA and ChiB likely to be attributable to the endo-activity displayed by the former enzyme only. This could be a possible explanation for the synergistic effect between ChiA and ChiB (and may be between ChiA and ChiC1). However, further structural studies on both chitinase proteins and their substrate chitin together with the biochemical efforts must be necessary to understand mechanisms for efficient degradation of chitin by microorganisms.

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