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Family 19 Chitinase from *Aeromonas* sp. No.10S-24: Role of Chitin-Binding Domain in the Enzymatic Activity

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Family 19 chitinase from *Aeromonas* sp. No.10S-24 (72.6 kDa) is composed of two chitin-binding domains (ChBDs), two proline- and threonine-rich (PT-rich) linkers, and a catalytic domain. The purified enzyme was labile in a standard buffer condition and spontaneously degraded into a 46-kDa fragment upon storage at 4°C. The N-terminal sequence of the 46-kDa fragment was found to correspond to the sequence of the C-terminal region of the second PT-rich linker, indicating that the 46-kDa fragment is produced by truncation of the two ChBDs and the two PT-rich linkers from the mature protein, and consists only of the catalytic domain. The hydrolytic activities toward insoluble and soluble substrates were significantly reduced by the truncation of two ChBDs. In addition, antifungal activity determined from the digestion rate of haustoria of powdery mildew was reduced by the ChBD truncation. Although the profile of the time-course of *N*-acetylglucosamine hexasaccharide [$(GlcNAc)_6$] degradation catalyzed by the ChBD-truncated enzyme was similar to that of the mature enzyme protein, the specific activity of the ChBD-truncated enzyme determined from the rate of hexasaccharide degradation was lower than that of the mature enzyme. The two CBDs appear to be responsible for facilitating the hydrolytic reaction. The sugar residue affinities (binding free energy changes) at the individual subsites, (–2) (–1) (+1) (+2) (+3) (+4), were estimated by modeling the hexasaccharide hydrolysis by the mature and ChBD-truncated enzymes. The truncation of ChBDs was found to strongly affect the affinity at the (–1) site. This situation seems to result in the lower enzymatic activity of the ChBD-truncated enzyme toward the chitinous substrates.

Key words: *Aeromonas* sp., chitin-binding domain, chitin hydrolysis, family 19 chitinase, powdery mildew.

Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_n$, β-1,4–linked oligosaccharide of GlcNAc with a polymerization degree of n; ORF, open reading frame; ChBD, chitin-binding domain; PT-rich, proline- and threonine-rich; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CD, circular dichroism; HPLC, high performance liquid chromatography.

Chitinases are divided into two families (family 18 and family 19) on the basis of their amino acid sequences (*1*). Chitinases of different families do not share similar sequences and have completely different folds in their three-dimensional structures (*2*). Family 18 chitinases have been isolated from bacteria, fungi, viruses, animals, human, and plants (class III and class V chitinases) and are characterized by a TIM barrel structure with some smaller subdomains. Family 19 chitinases have been isolated mostly from plants and can be subdivided into classes I, II, and IV (*3*). These chitinases are composed of two lobes with a high α-helical content. In recent years, family 19 chitinases have also been found in prokaryotic organisms such as *Streptomyces* species (*4–6*) and *Aeromonas* sp. (*7*). It is of interest to examine how the structural and functional properties of such bacterial family 19 chitinases relate to those of plant family 19 chitinases. However, the structural and enzymatic properties of the bacterial family 19 chitinases are not yet fully understood.

In our previous paper (*7*), the family 19 chitinase from *Aeromonas* sp. No. 10S-24 was reported to have relatively large molecular mass of 72.6 kDa. The domain structure was deduced from the amino acid sequence and found to consist of two chitin-binding domains (ChBDs), two proline- and threonine-rich (PT-rich) linkers, and a catalytic domain, as shown in Fig. 1. Most carbohydrolases, which hydrolyze insoluble polysaccharide, have a polysaccharide-binding domain in their molecular structure (*8–10*). The role of this domain in polysaccharide hydrolysis has been intensively studied using the mature and truncated enzymes. For example, ChBDs of family 18 chitinases from *Bacillus circulans* WL-12 and tobacco hornworm, *Manduca sexta*, were examined by comparison of the enzymatic activity or the chitin-binding ability of the mature enzyme and ChBD-truncated derivatives (*11*, *12*). It now appears that ChBDs are important for recog-

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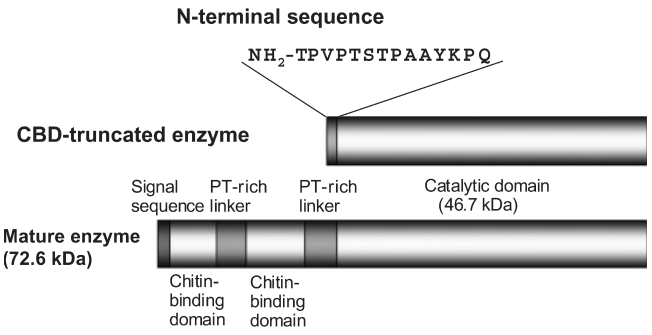

Fig. 1. **Molecular construction of *Aeromonas* family 19 chitinase and the ChBD-truncated enzyme.**

nizing insoluble chitinous substrates. Concerning family 19 chitinases, Itoh *et al.* have conducted a similar study of *Streptomyces griseus* family 19 chitinase (*13*), but more experimental data on the ChBD of bacterial family 19 enzymes are needed in order to understand more precisely the role of ChBD. In particular, study of the effect of ChBD truncation on the substrate affinity at individual subsites of the binding cleft will be valuable. We have been trying to model the hydrolysis of hexasaccharide substrates by chitinase in order to gain an insight into the state of the substrate-binding cleft (*14–17*). This method is expected to provide the binding free energy distribution in the binding cleft of chitinases with or without ChBD, useful information on the role of ChBD.

In this study, the CBD-truncated enzyme of family 19 chitinase from the *Aeromonas* strain, which had been formed spontaneously, probably as the result of cleavage by an unknown protease, was examined for hydrolytic activities using oligosaccharide and polysaccharide substrates. The antifungal activity was also examined using barley coleoptile tissue invaded by powdery mildew by microscopically observing the chitinase digestion. The role of CBDs in the enzymatic activity was discussed based on a comparison of the enzymatic properties between the mature *Aeromonas* family 19 chitinase and its ChBD-truncated derivative.

MATERIALS AND METHODS

Materials, Bacterial Strains, Plasmids, and Media—*Aeromonas* sp. No. 10S-24 was used as a DNA donor. *Escherichia coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1. λ^-, Δ (*lac-proAB*), [F′, *proAB*, *lac1*^{q}ZΔM15, *traD*36)] was used as host strain. A plasmid containing the family 19 chitinase gene, pRYA1, was used to express the chitinase gene (*7*). The *E. coli* strain was cultured and maintained appropriately in LB or 2× YT supplemented with ampicillin (100 μg/ml) if necessary. Solid crystalline chitin was the product of Nakarai Chemical Co. Glycol chitin was prepared by the method of Yamada and Imoto (*18*). Colloidal chitin was prepared by the method of Shimahara and Takiguchi (*19*). $(GlcNAc)_6$ was purchased from Seikagaku Kogyo Co. The strain of powdery mildew, *Blumeria graminis* f. sp. *hordei* race I, was used for enzymatic digestion of the haustorial cell wall.

Expression and Purification of Recombinant Family 19 Chitinase—The family 19 chitinase was produced by the method described in the previous paper (*7*). *E. coli* cells containing the *Aeromonas* chitinase gene were cultured for 20 h in 2× YT and harvested by centrifugation (10,000 rpm, 5 min, and 4°C). The periplasmic proteins were isolated by the method of Iriarte and Cornelis (*20*) with a minor modification. The cells were suspended in 10 mM Tris-HCl buffer, pH 7.0, containing 25% sucrose, washed with the same buffer, and spun down. The pellet was resuspended 10 mM Tris-HCl buffer, pH 7.0, containing 25% sucrose and 1 mM EDTA, gently shaken for 10 min, and spun down again. The pellet was osmotically shocked by resuspending in ice-cold water and shaking for 40 min on ice. The cells were precipitated, and the periplasmic proteins in the supernatant were applied onto a DEAE-Toyopearl 650M column previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The unadsorbed fractions were pooled, and the enzyme protein was precipitated by adding solid ammonium sulfate to 80% saturation. After centrifugation, the pellet was dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.0) and the solution was put onto a Sephadex G-75 column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.2 M NaCl. After elution with the same buffer, active fractions were pooled and stored in a mixed solution of glycerol and 20 mM Tris-HCl buffer pH 7.0 (4:6) at 4°C.

Purification of the CBD-Truncated Enzyme—As described below, the purified enzyme preparation spontaneously degraded into a smaller protein fragment upon storage at 4°C for several weeks. The degraded protein fragment was then purified by Sephadex G-75 under the same condition as described above and used for protein chemical and enzymatic characterizations.

N-Terminal Amino Acid Sequence Determination—Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was washed extensively with water, stained with 0.25% Coomassie Brilliant Blue R-250 in 5% aqueous methanol and 7.5% acetic acid for 5 min, and destained with 90% aqueous methanol for 10 min. A portion of the membrane containing the desired protein band was cut out, and the N-terminal amino acid sequence was determined by use of an automated pulseliquid protein sequencer (model 492, Applied Biosystems).

Enzyme Activity—A reaction mixture containing 0.2 ml of 0.3% ethylene glycol chitin, 0.4 ml of 0.1 M Tris-HCl buffer (pH 7.0), and 0.2 ml of enzyme solution was incubated for 15 min at 37°C. As insoluble substrates, identical concentrations of colloidal chitin and crystalline chitin suspensions were used instead of the ethylene glycol chitin. In these cases, after mixing with the enzyme, the hydrolytic reactions were conducted with constant stirring. The amount of reducing sugars produced was determined by the modified Schales' method (*21*).

Protein Concentration—Protein concentrations were determined by ultraviolet absorption at 280 nm using the extinction coefficients calculated from the equation reported by Pace *et al.* (*22*).

Electrophoresis of Protein—SDS–polyacrylamide gel electrophoresis was done by the method of Laemmli (*23*) using the molecular weight marker Daiichi II (Daiichi

Pure Chemicals) as a standard. Protein bands were detected by staining with Coomassie Brilliant Blue R-250.

Inoculation of Barley Coleoptile Tissues with Barley Powdery Mildew—Seeds of barley (*Hordeum vulgare* L. cv. Kobinkatagi) were surface-sterilized and germinated at 20°C on water-moistened filter paper in a Petri dish. Germinating seeds were transferred to agar-solidified plates supplied with sterile chemical fertilizer and incubated at 20°C under continuous illumination of 4,000 lx. After 10 days of incubation, coleoptiles were inoculated with conidiospores of the powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei* race I. After 1–2 weeks of incubation (20°C, 4,000 lx), coleoptiles were excised from primary leaves, and the detached epidermal cells were boiled with lactophenol solution to fix the pathogen. Fixed epidermis was rinsed 10 times with sterilized water and stored at 4°C in 50 mM phosphate buffer (pH 7.0).

Enzyme Digestion of Haustoria in Powdery Mildew-Infected Coleoptiles—Enzyme digestion was done by dipping the epidermis in individual chitinase solutions (20 μM). On removal from the enzyme solution, the epidermis was observed by light microscopy (*24*). To monitor the enzymatic digestion of haustoria in powdery mildew-infected coleoptile tissue, the degradation of haustoria was divided into three levels (Fig. 4), and the number of haustoria at each level was counted by microscopic observation. The percentages of individual degradation levels to the total number of haustoria counted were plotted against the incubation time to obtain the time-course of haustorial digestion. Then, the digestion rates of mature and CBD-truncated enzymes were compared.

HPLC Analysis of Enzymatic Products—Hexasaccharide substrate was dissolved in 50 mM sodium acetate buffer, pH 5.0, and several microliters of the chitinase solution dialyzed against the same buffer were added to 200 μl of the substrate solution. In analyzing the α/β ratio of the hydrolytic products, the reaction mixture was incubated at the lower temperature (25°C) to suppress the mutarotation. After a given incubation period, a portion of the reaction mixture was withdrawn and immediately applied onto a column of TSK-GEL Amide-80 (4.6 × 250 mm, Tosoh). The column was eluted with 70% acetonitrile at a flow rate of 0.7 ml/min, and the substrate and products were monitored by ultraviolet absorption at 220 nm.

To quantitatively determine the time-course of substrate degradation and product formation, the reaction mixture was incubated at 40°C for an appropriate period. A portion of the reaction mixture was withdrawn and mixed with the same volume of 0.5 M sodium hydroxide solution to completely terminate the enzymatic reaction, and the resultant solution was applied onto a gel-filtration column of TSK-GEL G2000PW (7.5 × 600 mm, Tosoh). Elution was conducted with distilled water at a flow rate of 0.3 ml/min, and the eluate was monitored as described above. From the peak area obtained by the HPLC, oligosaccharide concentrations at each reaction time were calculated using the standard curve obtained with the authentic saccharide solutions.

Modeling of the Enzymatic Hydrolysis—Theoretical analysis of the reaction time-course was carried out using the reaction model reported for hen egg white lysozyme (*25*). A slight modification was introduced into the subsite model. Based on the splitting mode of the substrate estimated from HPLC profiles (*7*), the *Aeromonas* chitinases were assumed to have a (–2)(–1)(+1)(+2)(+3)(+4)-type binding cleft. To estimate the affinity (binding free energy) at each subsite, an optimization technique based on the modified Powell method (*26*) was employed using the cost function,

$$F = \sum_i \sum_n [(\mathrm{GlcNAc})^c_{n,i} - (\mathrm{GlcNAc})^e_{n,i}]^2 \quad (1)$$

where e and c represent the experimental and calculated values, n is the size of the oligosaccharides and i the reaction time. Optimization was conducted by changing the binding free energy values of the individual subsites, (–2)–(+4), while fixing the estimated rate constant values. The rate constant value of k_{+1} (for cleavage of glycosidic linkage) was assumed to be dependent upon the substrate size (*14–16*), and estimated from the initial velocity data (0.5 s^{-1} for hexasaccharide hydrolysis, 0.6 s^{-1} for pentasaccharide hydrolysis, and 1.7 s^{-1} for tetrasaccharide). The value of k_{-1} (rate constant for transglycosylation) was estimated to be zero, because no transglycosylation product was detected in HPLC profile. A constant value of 200.0 s^{-1} was tentatively allocated to k_{+2} (*16*).

Thermal Stability Experiments—The chitinase preparations were dialyzed against 20 mM Tris-HCl buffer, pH 7.0. The thermal unfolding curves of the chitinases were obtained by monitoring the CD value at 222 nm using a Jasco J-720 spectropolarimeter (cell length, 0.1 cm), as the solution temperature was raised at a rate of 1°C/min. Solution temperature was measured directly with a model DP-500 thermocouple set in the cell (Rikagaku Kogyo). To facilitate comparison between the unfolding curves obtained, the experimental data were normalized as follows. The fraction of unfolded protein at each temperature was calculated from the CD value by linearly extrapolating the pre- and post-transition baselines into the transition zone, and the value obtained was plotted against temperature. Assays were performed in duplicate. Thermodynamic parameters could not be obtained, because of the poor reversibility of the unfolding transition.

RESULTS

Purification of CBD-Truncated Enzyme—When the homogeneity of the recombinant enzyme was assessed by SDS-PAGE immediately after its purification, a protein band was found at 72 kDa as reported in the previous paper (Fig. 2, Lane 2). However, the enzyme protein was found to be degraded into a 46-kDa fragment upon storage at 4°C for several weeks (Lane 3). Protein fragments other than the 46-kDa product could not be observed, probably because their molecular masses are too small to be detected. Subsequently, the addition of 40% glycerol was found to stabilize the protein (Lane 4). Some chitinases that belong to glycosyl hydrolase family 18 were reported to be easily cleaved at the linker regions (*27*, *28*). The *Aeromonas* chitinase is the first example of such a labile chitinase belonging to family 19.

To identify the degradation product, the 46-kDa fragment was purified by gel-filtration and subjected to N-

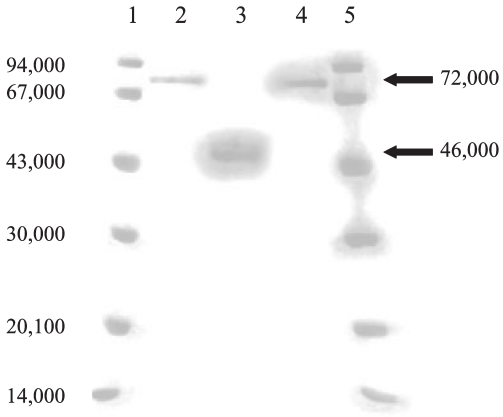

Fig. 2. **SDS-PAGE of the purified *Aeromonas* family 19 chitinase and the ChBD-truncated enzyme.** Lanes 1 and 5, molecular weight markers. Lane 2, the freshly purified enzyme preparation. Lane 3, the purified enzyme preparation stored at 4°C in Tris buffer for 3 weeks. Lane 4, the purified enzyme preparation stored at 4°C in the buffer containing 40% glycerol for 3 weeks.

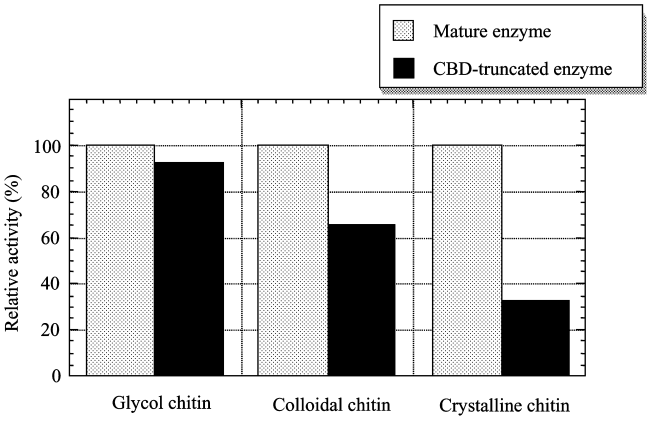

Fig. 3. **Enzymatic activities of *Aeromonas* family 19 chitinase toward polysaccharide substrates, glycol chitin, colloidal chitin, and crystalline chitin.** The reaction mixture was incubated in 50 mM sodium acetate buffer, pH 5.0, at 40°C. The enzymatic reaction was monitored by reducing sugar determination using the modified Schales' method (*21*).

terminal sequence analysis. The sequence was found to be N-TPVPTSTPAAYKPQ, which corresponds to part of the sequence of the PT rich linker regions as shown in Fig. 1. The molecular mass of 46 kDa, estimated from SDS-PAGE, corresponds to that of the catalytic domain. Thus, the two chitin-binding domains and two PT-rich linkers were truncated by spontaneous degradation, and the 46-kDa fragment was found to consist only of the catalytic domain. The 46-kDa fragment, namely, the ChBD-truncated enzyme, was purified by Sephadex G-75 and used in the following experiments to examine the role of ChBDs in the enzyme action.

Enzymatic Activity toward Polymer Substrates—We next examined enzymatic activities of the mature and ChBD-truncated enzymes toward three polymer substrates; ethylene glycol chitin, colloidal chitin, and crystalline chitin. The results are shown in Fig. 3, in which the relative activities are given in percentage relative to

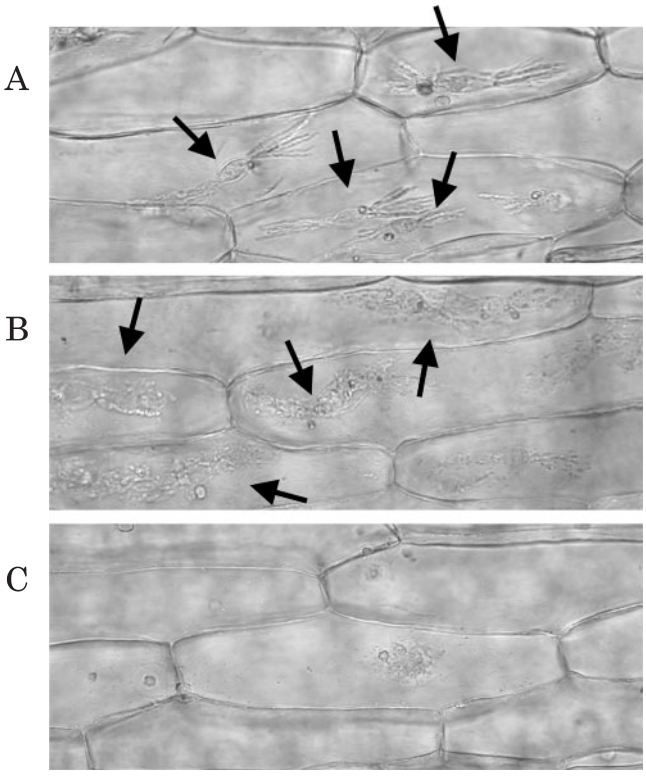

Fig. 4. **Chitinase digestion of haustoria of powdery mildew in infected coleoptile tissue.** Three levels of degradation were distinguished; non-digested (A), partially digested (B), and completely digested (C).

mature enzyme activities for individual substrates to facilitate comparison between the mature and ChBD-truncated enzymes. With the soluble substrate, ethylene glycol chitin, a small but significant decrease was found in the chitinase activity of the ChBD-truncated enzyme. On the other hand, the relative activity of the ChBD-truncated enzyme toward colloidal chitin decreased by about 30% from that of the mature enzyme, while that toward crystalline chitin decreased by about 65%. Thus, ChBDs appear to facilitate the hydrolysis of polymeric substrates.

Enzymatic Digestion of Haustoria of Powdery Mildew in Barley Coleoptile Tissue—In the microscopic observation of powdery mildew in barley coleoptile tissue, haustoria exhibit a specific morphology and are distinct from other fungal organs. Thus, we focused on the haustoria for monitoring enzymatic digestion of the fungal cell wall. The digestion process could be successfully monitored by light microscopy as shown in Fig. 4.

Before the chitinase treatment, a haustorium in the infected coleoptile tissue exhibited a specific crab-shape having some lobes (Fig. 4A, non-digested). After an appropriate period of chitinase treatment, however, the specific morphology collapsed as a result of enzymatic digestion of the cell wall (Fig. 4B, partially digested). Further chitinase treatment resulted in disappearance of the haustorial morphology (Fig. 4C, completely digested). The three levels of degradation are clearly distinguishable under the microscope. After counting the number of haustoria at each level, the percentage of haustoria at

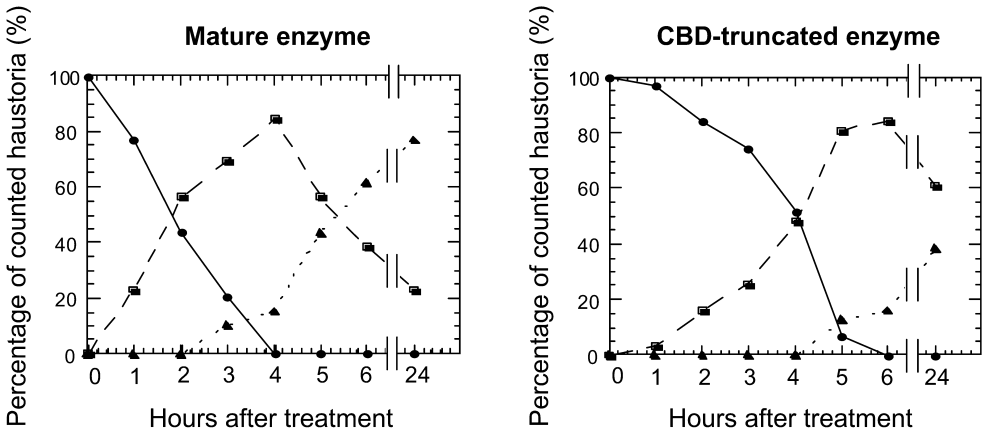

Fig. 5. **Time-course of chitinase digestion of haustoria of powdery mildew in infected coleoptile tissue.** Left panel, the mature family 19 chitinase from *Aeromonas* sp. No10S-24; right panel, the ChBD-truncated enzyme. Solid lines with closed circles, non-digested stage; broken lines with closed squares, partially digested stage; dotted lines with closed triangles, completely digested stage.

each level relative to the total number counted was plotted against the period of chitinase treatment to obtain the time-course of haustorial digestion. The results are shown in Fig. 5. When the infected coleoptile tissues were dipped in the mature *Aeromonas* chitinase solution, the percentage of non-digested haustoria fell into zero within 4 h of incubation. The percentage of partially digested haustoria increased to a maximum at 4 h, while that of completely digested haustoria gradually increased from 2 h and reached 80% of all haustoria counted after 24 h of incubation. On the other hand, when the ChBD-truncated enzyme was used instead of the mature enzyme, the digestion was significantly delayed; the percentage of partially digested haustoria attained the maximum at 6 h, and that of completely digested haustoria increased from 4 h and reached only about 40% after 24 h of incubation. These results suggest that ChBDs enhance the digestion of fungal cell wall.

Enzymatic Activity toward Oligosaccharide Substrate—HPLC analysis of hydrolytic products formed from $(GlcNAc)_6$ substrate by the CBD-truncated enzyme was first performed with a partition column, TSK-GEL Amide-80, which can separate α- and β-anomers of each oligosaccharide. The result was completely identical to that obtained with the mature enzyme (7). The CBD-truncated enzyme produced α-anomer of $(GlcNAc)_2$, whereas the product $(GlcNAc)_4$ was in mutarotation equilibrium (data not shown). This indicates that the enzyme splits the second glycosidic linkage from the non-reducing end of $(GlcNAc)_6$ through an inverting mechanism.

When the products were analyzed by gel-filtration column, which can give more quantitative data, the distri-

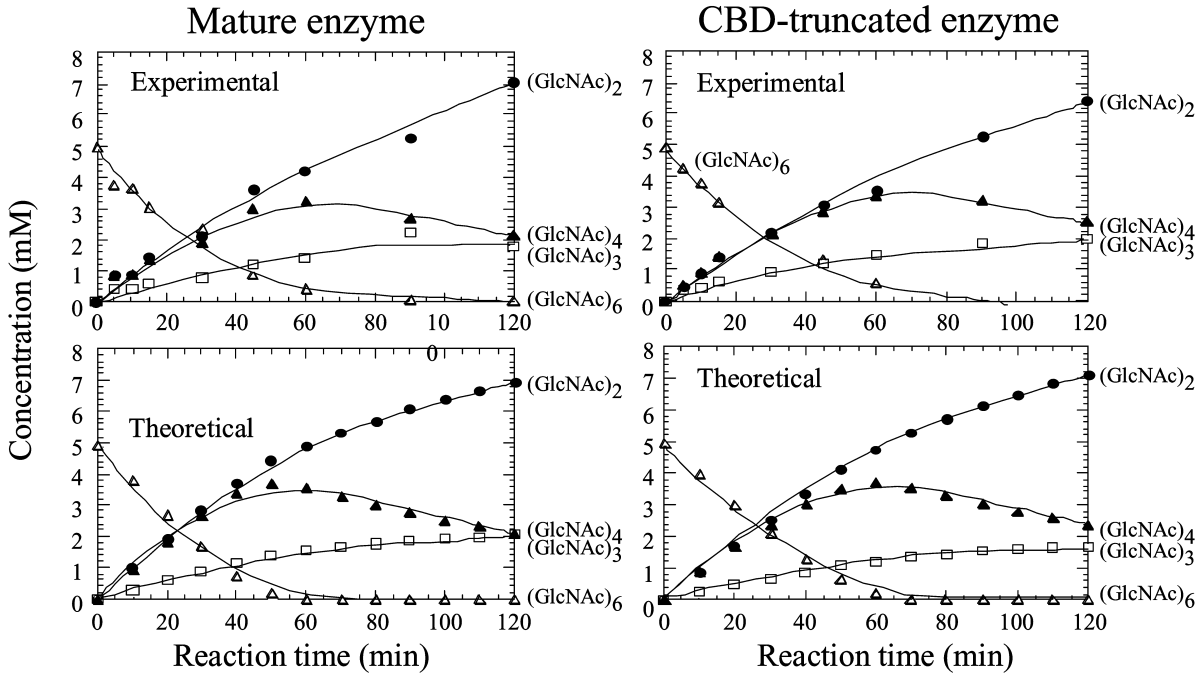

Fig. 6. **Upper two figures: Experimental time-courses of $(GlcNAc)_6$ hydrolysis catalyzed by the mature and ChBD-truncated family 19 chitinase from *Aeromomas* sp. No.10S-24.** The reactions were carried out in 50 mM sodium acetate buffer, pH 5.0, at 40°C. Enzyme concentrations were 1.9 μM for the mature enzyme and 3.7 μM for the ChBD-truncated enzyme. Solid circles, $(GlcNAc)_2$; open squares, $(GlcNAc)_3$; silid triangles, $(GlcNAc)_4$; open triangles, $(GlcNAc)_6$. Lower figures: Theoretical time-courses best fitted to the experimental time-courses. The rate constant values and binding free energy values used in these calculations are listed in the text and Table 1, respectively.

Table 1. **Binding free energies of individual subsites of the mature and ChBD-truncated chitinases from *Aeromonas* sp. 10S-24.**

Enzymes	Binding free energies (kcal/mol)					
	(–2)	(–1)	(+1)	(+2)	(+3)	(+4)
Mature	–2.0	–1.8	0.0	–1.2	–1.5	–1.2
ChBD-truncated	–2.5	–1.0	0.0	–1.0	–1.6	–1.3

bution of hydrolysis products generated from $(GlcNAc)_6$ by the ChBD-truncated enzyme was $(GlcNAc)_2$ > $(GlcNAc)_4$ > $(GlcNAc)_3$, similar to that of the mature enzyme (*7*), as shown in Fig. 6. However, the reaction rates of the enzymes were somewhat different. Although the mature enzyme concentration used in the hexasaccharide hydrolysis was lower than that of the ChBD-truncated enzyme, the rate of substrate degradation is slightly higher in the mature enzyme reaction. The specific activities determined from the rate of substrate degradation were calculated to be 87 min^{-1} for the mature enzyme and 30 min^{-1} for the ChBD-truncated enzyme.

By modeling the enzymatic hydrolysis of the hexasaccharide substrate, theoretical time-courses satisfactorily fitted to the experimental ones were obtained for the mature and ChBD-truncated enzymes, as shown in Fig. 6. The affinities (binding free energies) of the individual subsites, (–2)–(+4), of both enzymes could be successfully estimated by the data-fitting as listed in Table 1. A significant affinity, –1.2 kcal/mol, was found in subsite (+4) of the mature enzyme. This is one of the most important differences between the *Aeromonas* family 19 enzyme and the plant family 19 enzymes from rice and barley, which exhibit the subsite structure of (–3)–(+3) (*14*, *17*). On the other hand, the subsite affinities of ChBD-truncated enzyme were somewhat different from those estimated for the mature enzyme (Table 1). The affinity of (–1) site was most strongly affected by the truncation of ChBDs, and the lower specific activity of the ChBD-truncated enzyme probably results from the lower affinity at this site. Although the affinity at (–2) site of the ChBD-truncated enzyme was higher than that of the mature enzyme, the affinity change at the upper end-most site (–2) is unlikely to significantly affect the overall specific activity.

Thermal Stabilities of the Mature and the CBD-Truncated Enzymes—When the thermal stability was examined by CD spectroscopy, the transition temperature of the thermal unfolding of the mature enzyme was found to be 60.3°C, while that of the CBD-truncated enzyme was 58.6°C. ChBDs are unlikely to participate in the protein structure stabilization.

DISCUSSION

After purification of the recombinant *Aeromonas* family 19 chitinase, we found that this enzyme is susceptible to spontaneous degradation and the degradation is suppressed by addition of glycerol (Fig. 2). N-terminal sequence analysis indicated that the proteolysis takes place at the C-terminal region of the second PT-rich linker (Fig. 1). Several proteolytic sites probably occur in the region containing the two ChBDs and two linkers, because proteolytic products other than the 46-kDa fragment have too small molecular masses for their protein bands to be found in the SDS-PAGE profile (Fig. 2, Lane 3). It is recognized that the PT-rich sequence is susceptible to proteolytic cleavage (*29*). So the purified enzyme preparation is thought to contain a trace amount of a protease that brings about the degradation of the *Aeromonas* family 19 chitinase. It now appears that the PT-rich sequence has often attached carbohydrate moieties (*30*), which stabilize the structure of the linker region. In fact, in other chitinases purified from *Aeromonas* sp. No.10S-24, we found certain amounts of the carbohydrates (*31*, *32*). The recombinant enzyme produced by the *E. coli* strain, however, does not have any carbohydrate moieties. The absence of carbohydrate might destabilize the PT-rich linker region, making it susceptible to proteolytic cleavage. The addition of glycerol stabilizes the structure of the linker region, and this would protect it from proteolytic attack. Further experiments are needed to clearly explain the mechanistic details of the protein degradation. Anyway, the protein degradation fortunately gave us an opportunity to examine the role of the two ChBDs of the *Aeromonas* family 19 chitinase.

Several studies on the ChBD-truncated chitinases have been already conducted, and the role of ChBD has been discussed from the enzymological viewpoint. One example is chitinase A1 (family 18) from *Bacillus circulans* WL-12, which comprises a ChBD, two type III modules, and a catalytic domain. The mature chitinase A1 specifically binds to insoluble chitin, whereas the ChBD-truncated chitinases lose much of this binding ability. However, the activity toward soluble substrate was not affected by the ChBD truncation. Thus, it was concluded that the ChBD is responsible for interacting with insoluble chitin but not with soluble substrates (*11*, *33*). A similar result was obtained for the family 18 chitinase from *Manduca sexta* (tobacco hornworm), which comprises a catalytic domain, a serine/threonine-rich region, and a ChBD (*12*). The ChBD of family 19 chitinase from *Streptomyces griseus* HUT6037 was also studied by Itoh *et al.* (*13*) using similar experimental techniques. They reported that truncation of ChBD from the *S. griseus*

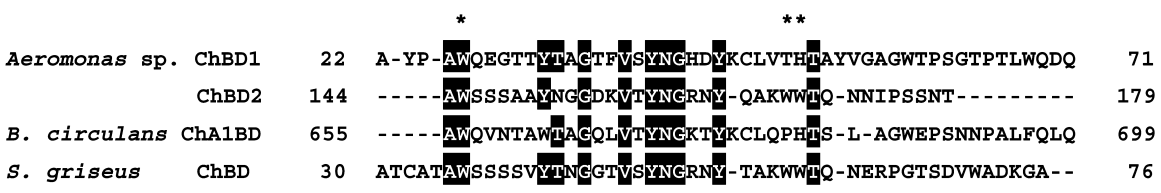

Fig. 7. **Amino acid sequence alignment of ChBDs of chitinases from *Aeromonas* sp. No.10S-24 (family 19), *Streptomyces griseus* HUT6037 (family 19), and *Bacillus circulans* WL-12 (family 18).** Identical amino acids are shown by white letters on black. Asterisks indicate the three conserved tryptophan residues in ChBDs.

enzyme reduced the activities toward insoluble and soluble substrates by about 50% and 20%, respectively. The antifungal activity of the *S. griseus* enzyme was also evaluated from its inhibitory action toward hyphal extension of *Trichoderma reesei* on an agar plate, and found to be abolished by the ChBD truncation.

To elucidate the role of ChBDs of *Aeromonas* family 19 chitinase, the enzymatic properties of the mature and the ChBD-truncated enzymes were examined. Relative to the mature enzyme, the ChBD-truncated enzyme showed reduced activity toward insoluble substrates (colloidal and crystalline chitins) (Fig. 3). A similar result was obtained when these two enzyme preparations were tested for antifungal effect using barley powdery mildew (Fig. 5). The activity decrease in the truncated enzyme was also found when the soluble substrates, glycol chitin and chitin hexasaccharide, were used in the activity determination (Figs. 3 and 6). No significant difference was observed between thermal stabilities of the mature and ChBD-truncated enzymes. ChBDs are unlikely to participate in the enzyme structure stabilization. From the results overall, we concluded that ChBDs of *Aeromonas* family 19 chitinase are responsible for enhancing the rate of hydrolytic reaction. ChBDs enhance the hydrolytic reaction of soluble substrates as well as insoluble substrates. This enzymatic property is similar to that of *S. griseus* family 19 chitinase.

We also noted that *Aeromonas* family 19 chitinase is composed of two different ChBDs in addition to the catalytic domain. Fig. 7 shows the sequence alignment of ChBDs of bacterial chitinases. The sequence of the second ChBD (ChBD2) of *Aeromonas* family 19 chitinase has 62.9% identity to the ChBD of *S. griseus* family 19 chitinase. This ChBD appears to share sequence similarity with family V carbohydrate–binding modules (*8*, *34*). The family V modules possess three tryptophan residues, which are aligned and exposed on the molecular surface to allow hydrophobic interaction with the glucose moiety of cellulose. The three tryptophan residues are conserved in ChBD2 of *Aeromonas* family 19 chitinase at corresponding positions (W145, W168, and W169, designated by asterisks in Fig. 7). ChBD2 of *Aeromonas* family 19 chitinase might have a similar function to the *S. griseus* enzyme. In contrast, the sequence of the first ChBD (ChBD1) of *Aeromonas* family 19 chitinase has 55.6% identity to ChBD of *B. circulans* WL-12 chitinase, which interacts with insoluble chitin but not with soluble substrates (*33*). The function of ChBD1 might be similar to that of ChBD of *B. circulans* chitinase. Thus, *Aeromonas* family 19 chitinase has two different types of ChBD; and their overall function might be more complicated than expected. This complexity might underlie several unaccountable observations, as follows.

It is commonly accepted that the truncation of a binding module has a greater effect on the activity toward a polymeric substrate than that toward an oligosaccharide. In our *Aeromonas* family 19 enzyme, however, the activity of the ChBD-truncated enzyme toward glycol chitin decreased only by 8%, while the activity toward hexasaccharide substrate decreased by 66%. This might be derived from complicated participation of the two contiguous ChBDs of *Aeromonas* family 19 chitinase in the enzymatic activity. In this study, we estimated the affinities at individual subsites of the mature and ChBD-truncated enzymes. Interestingly, the truncation of ChBD was found to affect the affinities at (–2) and (–1) sites. In particular, the decrease in the affinity at (–1) site appears to be a direct cause of the lower specific activity of the ChBD-truncated enzyme. The three-dimensional structure of the mature enzyme is not available at present, but these domains might be positioned in an appropriate orientation by interacting with each other, because the two ChBDs and the catalytic domain are connected by PT-rich linkers, which seem to be flexible. If this is the case, the truncation of ChBDs might affect the conformation of the catalytic center, resulting in the lower affinity at (–1) site. Further studies are needed to precisely understand the role of the individual ChBDs of this unique type of bacterial family 19 chitinase.

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