J. Microbiol. Biotechnol. (2006), **16**(12), 1897–1903

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Functions of the C-Terminal Region of Chitinase ChiCW from *Bacillus cereus* 28-9 in Substrate-Binding and Hydrolysis of Chitin

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Received: May 20, 2006
Accepted: October 9, 2006

Abstract In order to investigate the functions of the C-terminal region of chitinase ChiCW of *Bacillus cereus* 28-9, a C-terminal truncated enzyme, ChiCWΔFC, was expressed in *Escherichia coli* and purified to homogeneity for biochemical characterization. Compared with ChiCW, ChiCWΔFC exhibited higher chitinase activity at high temperature and pH, but expressed lower hydrolytic and binding activities toward insoluble substrates. In addition, kinetic properties indicated that ChiCWΔFC hydrolyzed oligomeric and polymeric substrates less efficiently than ChiCW. These results suggest that the C-terminal region of ChiCW plays important roles in substrate binding and hydrolysis of chitin. In addition, the biological meaning of C-terminal proteolytic modification of ChiCW is discussed.

Key words: ChiCW, chitin-binding domain, hydrolysis, substrate binding, kinetics

Chitin, an insoluble β-1,4-linked polymer of *N*-acetylglucosamine, is the second most abundant polysaccharide in nature and a major constituent of the cell walls of many fungi, insect exoskeletons, and crustacean shells [3, 4, 15]. Chitinases (E.C. 3.2.1.14), found in bacteria, fungi, virus, and higher plants, catalyze the degradation of chitin [3–5]. Plant chitinases are involved in the defense mechanism against infection by phytopathogenic fungi [5]. Fungal chitinases are required for hyphal growth [16]. Bacterial chitinases primarily digest and utilize chitin as a carbon and nitrogen nutrient [3].

Bacillus cereus 28-9 isolated from the lily rhizosphere can produce two chitinases, ChiCH and ChiCW. The genes encoding these enzymes have been cloned and sequenced, and their biochemical properties have been characterized [6–8]. ChiCH, as an exochitinase consists of a signal peptide followed by a catalytic domain [6, 7]. ChiCW, as a modular endochitinase, consists of a signal peptide, a catalytic domain, a fibronectin type-III domain, and a chitin-binding domain [7, 8].

In our previous report, a proteolytic product of ChiCW was observed in the culture supernatant of *B. cereus* 28-9 [8]. According to the studies of Mabuchi *et al.* [11] and Thamthiankul *et al.* [18], we proposed that a proteolytic product of ChiCW was generated by removal of the C-terminal region, but the biological meaning for this proteolytic modification was unclear. However, other reports have shown that the chitin-binding domains are involved in chitinase activity and substrate binding [13, 20, 21]. Hence, the function of the C-terminal region of ChiCW on its activity and binding of different chitinous substrates was examined in this study.

To approach this goal, a C-terminal truncated enzyme, ChiCWΔFC, was purified by a novel procedure and characterized on its biochemical properties including optimal conditions for enzyme activity, substrate specificities, substrate-binding activities, and kinetics. According to the results, we discussed and proposed the explanations for the functions of proteolytic modification of ChiCW by *B. cereus*.

Materials and Methods

Bacterial Strains, Plasmid, and Media

Chitinolytic *B. cereus* strains 28-9 [6], A3S21, A3S26, and A3S40 [10] were used. For chitinase production, *B. cereus* strains were cultured in M9 broth with 0.5% yeast extract and 0.2% colloidal chitin at 37°C with shaking for 4 days. *Escherichia coli* TOP10F' (Invitrogen) was used as a host for gene cloning. *E. coli* DH5α and pCR2.1-TOPO (Invitrogen) were used to express recombinant protein. All bacterial strains were maintained on Luria-Bertani (LB)

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agar plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) supplemented with appropriate antibiotics.

Construction of Expression Vectors

The fragments encoding C-terminal truncated ChiCW derivatives, ChiCWΔC (lacking chitin-binding domain) and ChiCWΔFC (lacking chitin-binding domain and fibronectin type-III domain), were amplified from the clone carrying the *chiCW* gene [8] by polymerase chain reaction using primer pair 1, cwf (5'-GGAATTTCGAAAGGAGAAATGGC-**ATG**AGGTC-3') and fnIIIr (5'-*CTA*GGTAGGTTGTGATT-TATTTCC-3'), and primer pair 2, cwf and catar (5'-*CTA*CGTTGGTGGCTCAGTATC-3'), respectively. The amplified fragments were cloned into pCR2.1-TOPO to create the recombinant plasmids pGW65 (containing ChiCWΔC) and pGW66 (ChiCWΔFC). The constructs were transformed into *E. coli* TOP10F' and the transformants were screened on LB plate containing 50 μg/ml ampicillin. Constructed plasmids were identified further by restriction enzyme mapping and DNA sequencing.

Bacterial Expression of Truncated Chitinases

The recombinant plasmids were introduced into *E. coli* DH5α for expression of ChiCW derivatives. *E. coli* DH5α (pGW65) and *E. coli* DH5α (pGW66) were cultured in LB broth supplemented with ampicillin on a rotary shaker at 37°C for 20 h to express truncated chitinases. *E. coli* cells were harvested by centrifugation (8,000 ×*g*, 10 min), and the periplasmic proteins of *E. coli* cells were prepared according to the method of Manoil and Beckwith [12]. Chitinase activities of the periplasmic proteins were analyzed to determine the expression levels of the truncated chitinases.

Purification of ChiCWΔFC

All purification steps were performed at 4°C. The periplasmic proteins were precipitated by ammonium sulfate at 70% saturation. The precipitates were dissolved in 25 mM Tris-HCl buffer (pH 8.5) and dialyzed against the same buffer. The dialysate was applied onto a Q-ceramic Hyper-D column (Sigma) equilibrated with 25 mM Tris-HCl buffer (pH 8.5) for anion-exchange chromatography and eluted stepwise by 0.1, 0.3, and 0.5 M NaCl in 25 mM Tris-HCl buffer (pH 8.5). ChiCWΔFC was eluted in the fractions of start-up flow and the fractions with chitinase activity were pooled.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Chitinolytic Zymography Assay

SDS-PAGE and chitinolytic zymography assay were performed using a Tris-Tricine system [14] with modification as described [7]. After electrophoresis, the separating gel was incubated at 37°C in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 for 4 h on an orbital shaker (50 rpm). Then, the gel was stained with 0.01% Calcofluor White M2R (Sigma) in 50 mM Tris-HCl buffer (pH 8.9) for 5 min and destained with distilled water [19]. Chitinolytic zones in the Calcofluor-stained gel were visualized under a UV transilluminator. Separated proteins in the gel were stained with Coomassie Brilliant Blue G-250.

Chitinase Activity Measurement and Protein Concentration Determination

A fluorometric assay was used to determine chitinase activity using 4-methylumbelliferyl-*N,N',N''*-chitotriose [4-MU-$(GlcNAc)_3$, Sigma] as a substrate [8]. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1 μmol of 4-MU per min at 37°C. Protein concentration was determined by Bradford's method [1] using bovine serum albumin as the standard.

Optimal Conditions for Enzyme Activity

Purified ChiCW [7] and ChiCWΔFC were used to determine the effects of pH and temperature on enzyme activities. Glycol chitin was used as a substrate. Chitinase activity was analyzed by the method of Imoto and Yogishita [9]. Hydrolysis reaction of chitinases was performed at 37°C for 30 min in the following buffers of 0.1 M: sodium citrate (pH 3 to 5), potassium phosphate (pH 6 to 7), Tris-HCl (pH 8), and glycine-NaOH (pH 9 to 11) buffers. Temperature effects on chitinase activities of ChiCW and ChiCWΔC were measured in 0.1 M potassium phosphate buffer (pH 6.0) from 20°C to 80°C.

Substrate Specificity and Binding Assay

Substrate specificities of ChiCW and ChiCWΔFC were investigated using soluble and insoluble substrates, 4-MU-$(GlcNAc)_3$, glycol chitin, colloidal chitin, unprocessed chitin, and chitosan, purchased from Sigma. On the other hand, insoluble polysaccharides, colloidal chitin, chitin, chitosan, and Avicel (Fluka), were used in the binding assay. The binding assay mixture (total volume as 250 μl) contained 5 μg of purified enzyme and 1 mg of insoluble polysaccharide in 20 mM sodium acetate buffer (pH 5.0). After incubation on ice for 1 h with occasional stirring, the mixture was centrifuged at 12,000 ×*g* for 10 min at 4°C to separate polysaccharide and bound enzyme, and the protein concentration in the supernatant was determined. The amount of enzyme absorbed was calculated from the difference between the amount of enzyme initially added and that recovered in the supernatant.

Kinetic Property Characterization

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined by Lineweaver-Burk double reciprocal plot, and the turnover number (k_{cat}) and catalytic efficiency value (k_{cat}/K_m) were subsequently calculated.

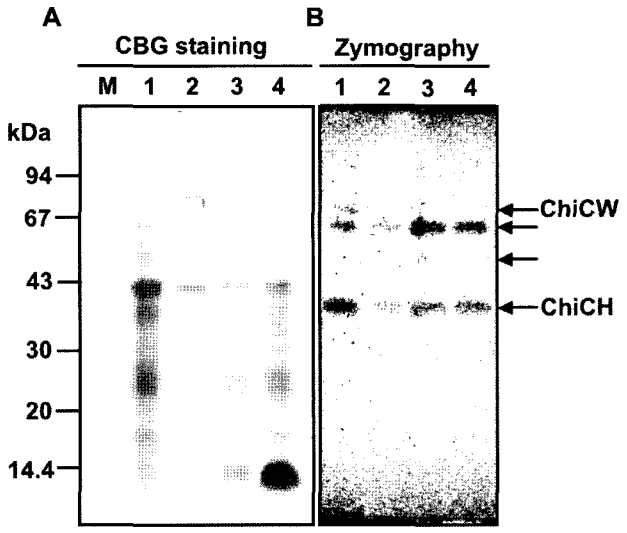

Fig. 1. Chitinolytic pattern of *B. cereus* strains. Culture supernatants of four strains were concentrated by 10% trichloroacetic acid and analyzed by 7.5% Tris-Tricine SDS-PAGE and Coomassie Brilliant Blue G-250 staining (**A**) and chitinolytic zymography (**B**).

B. cereus strains 28-9 (lane 1), A3S21 (lane 2), A3S26 (lane 3), and A3S40 (lane 4) were analyzed. Lane M, low molecular weight protein standards (Amersham Biosciences). Arrows indicated chitinolytic bands detected by zymography.

RESULTS

Chitinolytic Pattern of *B. cereus* Strains

Culture supernatants of four *B. cereus* strains were concentrated and analyzed by SDS-PAGE and chitinolytic zymography (Fig. 1). Four chitinolytic bands were detected and the estimated molecular size of each band was 70, 62, 53, and 37 kDa. In our previous studies [6, 8], 70- and 37-kDa chitinases have been identified as ChiCW and ChiCH, respectively. The other two chitinolytic bands (62 and 53 kDa) should be derived from 70-kDa ChiCW by C-terminal modification according to the studies of Mabuchi *et al.* [11] and Thamthiankul *et al.* [18].

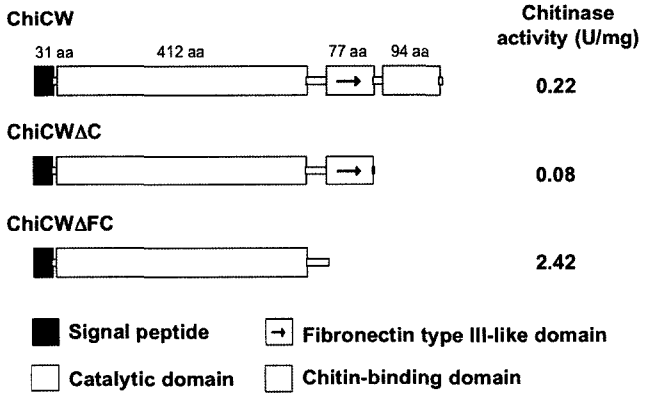

Fig. 2. Schematic diagram of the domain structures of ChiCW and two derivatives.

Production of chitinases from *E. coli* transformants was analyzed by measuring the chitinase specific activity of the periplasmic proteins, and 4-MU-$(GlcNAc)_3$ was used as a substrate.

Expression of ChiCW Derivatives

Fig. 2 shows that two recombinant plasmids were constructed for expression of ChiCW derivatives, ChiCWΔC and ChiCWΔFC. The molecular size and p*I* value of each truncated enzyme without the signal peptide were calculated based on its peptide sequence. ChiCWΔC is a 58.763-kDa protein with p*I* of 5.79 and ChiCWΔFC is a 50.573-kDa protein with p*I* of 5.75. Expression of the truncated chitinases in *E. coli* DH5α cells was analyzed (Fig. 2). ChiCWΔFC was highly expressed in *E. coli* DH5α cells, but ChiCWΔC was weakly expressed. Therefore, we focused on the characterization of ChiCWΔFC.

Purification of ChiCWΔFC

ChiCWΔFC was purified by ammonium sulfate precipitation and anion-exchange chromatography from the periplasmic fraction of *E. coli* DH5α (pGW66). Fig. 3 shows the elution profile of ChiCWΔFC on anion-exchange chromatography. ChiCWΔFC was almost eluted in the fractions of start-up flow. By SDS-PAGE analysis and chitinolytic zymography assay (Fig. 4), ChiCWΔFC was purified to apparent homogeneity and the molecular size of ChiCWΔFC was estimated to be 53 kDa, which closely corresponded to the value calculated from the peptide sequence of mature ChiCWΔFC (50.573 kDa). The result of purification of ChiCWΔFC is summarized in Table 1.

Optimal Conditions for Enzyme Activity

As shown in Fig. 5, optimal temperatures for ChiCW and ChiCWΔFC activities were determined. Although ChiCW and ChiCWΔFC both had maximal enzyme activities at 40°C, the profiles of ChiCW and ChiCWΔFC activities between 10 and 80°C were different. ChiCW retained at

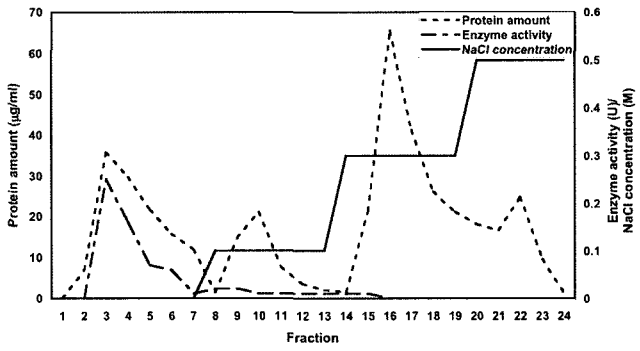

Fig. 3. Elution profile of ChiCWΔFC purification by anion-exchange chromatography. Most of ChiCWΔFC was eluted in the fractions of start-up flow.

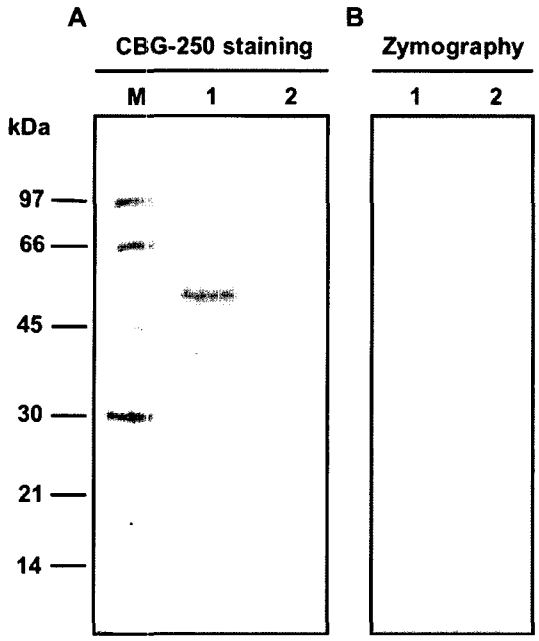

Fig. 4. SDS-PAGE and zymography assay of ChiCW and ChiCWΔFC.
Purified ChiCWΔFC (lane 1) and ChiCW (lane 2) were analyzed by 7.5% Tris-Tricine SDS-PAGE and Coomassie Brilliant Blue G-250 staining (**A**) and chitinolytic zymography (**B**). Lane M, low molecular weight protein marker (Amersham Biosciences).

least 80% of its optimal activity between 20 and 50°C but lost its catalytic activity at temperatures 70°C and above. On the other hand, ChiCWΔFC retained only 40% of the optimal activity between 20 and 50°C and maintained above 10% of the optimal activity between 60 and 80°C.

Investigation of optimal pH values of ChiCW and ChiCWΔFC showed that both enzymes had maximum activities at pH 5 (Fig. 6). ChiCW exhibited at least 50% of the optimal activity between pH 3 and 7 and lost its activity above pH 7. Although ChiCWΔFC had lower activities than ChiCW between pH 3 and 4, it retained at least 40% of the optimal activity between pH 8 and 11.

Substrate Specificity and Binding Activity

Table 2 shows the substrate specificities of ChiCW and ChiCWΔFC. ChiCW was more effective than ChiCWΔFC in hydrolyzing all tested substrates, especially unprocessed polymeric ones.

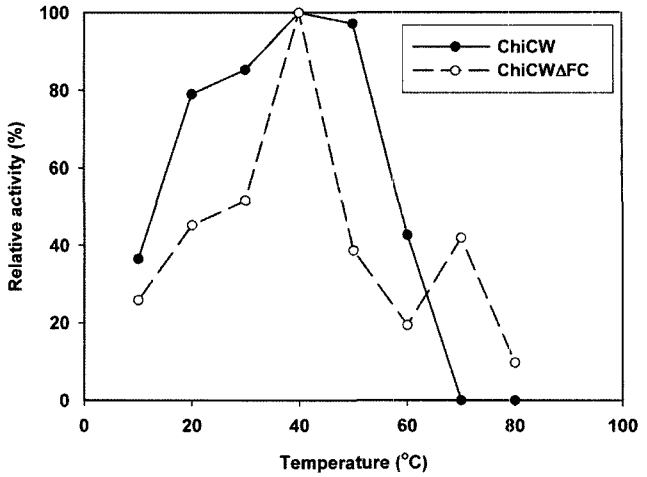

Fig. 5. Effect of temperature on the chitinase activity of ChiCW and ChiCWΔFC.
Purified ChiCW (solid circle) and ChiCWΔFC (open circle) were assayed for the effect of temperature on enzyme activity. Values for the assay represent one of the similar results of three independent experiments.

As shown in Fig. 7, ChiCW bound well onto colloidal chitin and chitin, and deletion of the C-terminal region (ChiCWΔFC) markedly reduced chitin-binding activity. However, both enzymes bound to chitosan to a small extent and did not show apparent difference in binding activities. In addition to the strong binding to chitin and colloidal chitin, ChiCW exhibited an affinity to cellulosic substrate, Avicel, but deletion of the C-terminal region of ChiCW also caused a significant decrease in binding activity to Avicel.

Kinetics of ChiCW and ChiCWΔFC

As shown in Table 3, kinetic properties of ChiCWΔFC were determined and kinetic constants were estimated. Compared with kinetic properties of ChiCW [7], ChiCWΔFC exhibited higher K_m, V_{max}, and k_{cat} and lower k_{cat}/K_m toward 4-MU-$(GlcNAc)_3$ than did ChiCW. In addition, the four kinetic parameters of ChiCWΔFC toward glycol chitin and colloidal chitin were lower than those of ChiCW.

DISCUSSION

ChiCW of *B. cereus* 28-9 is a modular chitinase consisting of a signal peptide and three domains [8]. In this report,

Table 1. Purification of ChiCWΔFC.

Purification step	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification fold	Recovery rate (%)
Crude extract	3.64	8.81	2.42	1.0	100
Ammonium sulfate precipitation	1.27	3.38	2.66	1.1	38
Anion-exchange chromatography	0.12	0.55	4.58	1.89	6

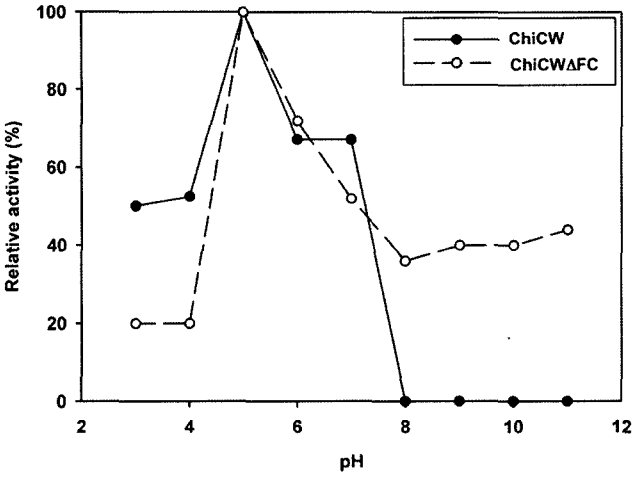

Fig. 6. Effect of pH on the chitinase activity of ChiCW and ChiCWΔFC.
Purified ChiCW (solid circle) and ChiCWΔFC (open circle) were assayed for the effect of pH on enzyme activity. Values for the assay represent one of the similar results of three independent experiments.

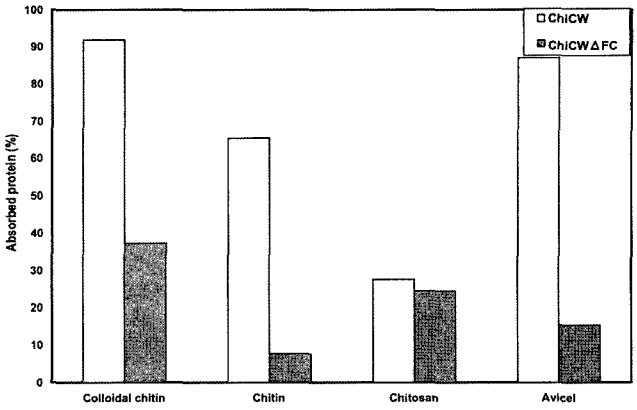

Fig. 7. Insoluble substrate-binding ability of ChiCW and ChiCWΔFC.
Each protein (5 μg) was mixed with various insoluble polysaccharides and allowed to absorbed for 1 h on ice before centrifugation. The amount of enzyme absorbed was calculated from the difference between the amount of enzyme initially added and that recovered in the supernatant.

proteolytic products of ChiCW were observed in the culture supernatant of *B. cereus* 28-9. Similar phenomenon of C-terminal proteolytic modification of chitinase has been found in many chitinases from *Bacillus* species [11, 17, 18, 22] and the other *B. cereus* strains in our laboratory, but the reasons for this proteolytic modification have not been understood. According to the studies of Mabuchi *et al.* [11] and Thamthiankul *et al.* [18], we proposed that proteolytic modification of ChiCW removes the C-terminal region. On the other hand, the functions of the C-terminal region on ChiCW activity are interesting to investigate. Hence, we tried to express, purify, and characterize ChiCWΔC and ChiCWΔFC. However, high-level expression of ChiCWΔC could not be successful in *E. coli* cells but ChiCWΔFC could. Therefore, ChiCWΔFC was heterologously expressed and purified to study the functions of the C-terminal region on ChiCW and to propose possible explanations for *B. cereus* 28-9 to produce the proteolytic product of ChiCW.

As the p*I* of ChiCWFC (5.75) is close to the p*I* of ChiCW (5.77), we designed the procedure to purify ChiCWΔFC according to that for the purification of ChiCW [8]. Anion-exchange chromatography was performed in the second step to purify ChiCWΔFC. In the procedure, ChiCWΔFC was presumed to be eluted with 0.1 or 0.3 M NaCl in 25 mM Tris-HCl buffer (pH 8.5). However, the elution profile of ChiCWΔFC on anion-exchange chromatography (Fig. 3) was surprising, since ChiCWΔFC was eluted in the fractions of start-up flow. Fortunately, the eluted ChiCWΔFC was apparently homogeneous as demonstrated by SDS-PAGE analysis; therefore, a unique and efficient procedure to purify ChiCWΔFC was established. This procedure had two advantages. First, ChiCWΔFC could be easily purified by this two-step procedure. Second, ChiCWΔFC eluted in the fractions of start-up flow did not contain NaCl to interfere subsequent experiments, thus making an additional step to remove NaCl (such as dialysis or ultracentrifugation) unnecessary. Therefore, the target protein eluted in the fractions of start-up flow could be a good strategy for protein purification.

Table 2. Substrate specificity of ChiCW and ChiCWΔFC.

Substrates	Hydrolysis (U/mg) with indicated chitinase			
	ChiCW		ChiCWΔFC	
Glycol chitin	0.45	(100)[a]	0.19	(42)
Colloidal chitin	0.50	(100)	0.40	(80)
Chitin	0.32	(100)	0.01	(3)
Chitosan	0.39	(100)	0.01	(3)

[a]Values in parentheses represent percent relative specific activities.

Both ChiCW and ChiCWΔFC were characterized on optimal pH and temperature for enzyme activities. Interestingly, profiles of ChiCW and ChiCWΔFC activities at the tested ranges of pH and temperature were different, although both enzymes exhibited maximum activity at the same pH and temperature (Figs. 5, 6). ChiCW retained higher activities at lower pHs and lower temperatures, whereas ChiCWΔFC retained higher activities at higher pHs and higher temperatures. These results indicate that deletion of the C-terminal region from ChiCW could enhance enzyme activities at broad ranges. Hence, we suggest that *B. cereus* 28-9 proteolytically modifying ChiCW to ChiCWΔFC could improve efficiency of chitin degradation in certain environments.

Deletion of the C-terminal region from ChiCW decreased the enzyme activity of ChiCW toward oligomeric and polymeric substrates. Hydrolysis of glycol chitin and colloidal chitin

Table 3. Kinetics of ChiCW[a] and ChiCWΔFC.

Substrate	Kinetic parameters							
	K_m[b]		V_{max}[c]		K_{cat}[d]		k_{cat}/K_m[e]	
	ChiCW	ChiCWΔFC	ChiCW	ChiCWΔFC	ChiCW	ChiCWΔFC	ChiCW	ChiCWΔFC
4-MU-$(GlcNAc)_3$	25.3	55.3	0.52	0.91	0.6	0.9	2.5×10^{-2}	1.6×10^{-2}
Glycol chitin	4.1	1.9	2.99	0.09	3.6	7.5×10^{-2}	9.0×10^{-1}	3.9×10^{-2}
Colloidal chitin	43.6	13.7	1.6×10^{-2}	8×10^{-3}	1.1	4.7×10^{-2}	2.6×10^{-2}	3.4×10^{-3}

[a]Kinetic parameters of ChiCW from Ref. [7].
[b]Unit of K_m: μM toward 4-MU-$(GlcNAc)_3$; mg/ml toward glycol chitin and colloidal chitin.
[c]Unit of V_{max}: μmol/min/mg toward 4-MU-$(GlcNAc)_3$, and glycol chitin; μmol/h/mg toward colloidal chitin.
[d]Unit of k_{cat}: s^{-1} toward 4-MU-$(GlcNAc)_3$, and glycol chitin; h^{-1} toward colloidal chitin.
[e]Unit of k_{cat}/K_m: $s^{-1}\mu M^{-1}$ toward 4-MU-$(GlcNAc)_3$; $s^{-1}mg/ml^{-1}$ toward glycol chitin; $h^{-1}mg/ml^{-1}$ toward colloidal chitin.

with ChiCWΔFC decreased about 50% and 20%, respectively, compared with that with ChiCW (Table 2). Furthermore, ChiCWΔFC showed only 3% of ChiCW enzyme activity toward unprocessed chitin. In contrast, removal of the C-terminal region from ChiCW increased enzyme activity toward small synthetic substrates (Fig. 2). A similar phenomenon was reported in ChiB of *Clostridium paraputrificum* [13]. Hence, these results indicate that the C-terminal region plays an important role in ChiCW hydrolysis of oligomeric and polymeric substrates.

ChiCW showed binding affinity toward colloidal chitin, unprocessed chitin, and Avicel (crystalline cellulose) (Fig. 7); similar results were reported for ChiB of *C. paraputrificum* [13] and for Chi1 of *Aeromonas caviae* [20]. Deletion of the C-terminal region from ChiCW significantly reduced the binding affinity of ChiCW toward these substrates. ChiCWΔFC exhibited about 40% and 10% of ChiCW binding affinity toward colloidal chitin and unprocessed chitin, respectively. Although the structures of native chitin is not exactly known, it is assumed that the crystallinity of unprocessed chitin is higher than colloidal chitin. Therefore, the results indicate that the C-terminal region plays an important role in ChiCW to bind insoluble substrate, especially higher crystalline substrates.

In this study, we investigated the kinetics of ChiCW and ChiCWΔFC toward three kinds of substrates and determined K_m, V_{max}, k_{cat}, and k_{cat}/K_m (Table 3). ChiCWΔFC exhibited higher K_m toward the tetrameric substrate, 4-MU-$(GlcNAc)_3$, than did ChiCW. These results reveal that the catalytic affinity of ChiCW toward 4-MU-$(GlcNAc)_3$ is better than that of ChiCWΔFC. Furthermore, K_m of ChiCWΔFC toward the oligomeric and polymeric substrates, glycol chitin and colloidal chitin, was lower than that of ChiCW, suggesting that the C-terminal region had a negative effect on the catalytically relevant binding of ChiCW on oligomeric and polymeric substrates.

According to the kinetic data of the two enzymes toward 4-MU-$(GlcNAc)_3$, ChiCW had a higher catalytic efficiency value and was more efficient in hydrolyzing this substrate than ChiCWΔFC, although the maximum velocity and turnover number of ChiCW were lower than those of ChiCWΔFC. Additionally, V_{max}, k_{cat}, and k_{cat}/K_m of ChiCW toward glycol chitin and colloidal chitin were higher than ChiCWΔFC, indicating that ChiCW hydrolyzes oligomeric and polymeric substrates more efficiently than ChiCWΔFC.

Based on our results, we propose that ChiCW is responsible for degrading polymeric chitinous substrate to smaller or oligomeric substrates, whereas both ChiCW and the C-terminal proteolytic form contribute to the further degradation of smaller and oligomeric substrates. In addition, the C-terminal proteolytic modification of ChiCW may confer a fitness advantage on chitin degradation and utilization by *B. cereus* in certain environments.

Acknowledgment

This study was financially supported by the National Science Council, Taiwan, Republic of China.

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