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Analysis of the Involvement of Chitin-Binding Domain of ChiCW in Antifungal Activity, and Engineering a Novel Chimeric Chitinase with High Enzyme and Antifungal Activities

Huang, Chien-Jui, Shu-Huei Guo, Shu-Chun Chung, Yu-Ju Lin, and Chao-Ying Chen*

Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 10617, Taiwan, Republic of China

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An antifungal chitinase, ChiCW, produced by *Bacillus cereus* 28-9 is effective against conidial germination of *Botrytis elliptica*, the causal agent of lily leaf blight. ChiCW as a modular enzyme consists of a signal peptide, a catalytic domain, a fibronectin type-III-like domain, and a chitin-binding domain. When two C-terminal domains of ChiCW were truncated, ChiCWΔFC (lacking the chitin-binding domain and fibronectin type III-like domain) lost its antifungal activity. Since ChiCWΔC (lacking the chitin-binding domain) could not be expressed in *Escherichia coli* as ChiCWΔFC did, a different strategy based on protein engineering technology was designed to investigate the involvement of the chitin-binding domain of ChiCW ($ChBD_{ChiCW}$) in antifungal activity in this study. Because ChiA1 of *Bacillus circulans* WL-12 is a modular enzyme with a higher hydrolytic activity than ChiCW but not inhibitory to conidial germination of *Bo. elliptica* and the similar domain composition of ChiA1 and ChiCW, the C-terminal truncated derivatives of ChiA1 were generated and used to construct chimeric chitinases with $ChBD_{ChiCW}$. When the chitin-binding domain of ChiA1 was replaced with $ChBD_{ChiCW}$, the chimeric chitinase named ChiAAAW exhibited both high enzyme activity and antifungal activity. The results indicate that $ChBD_{ChiCW}$ may play an important role in the antifungal activity of ChiCW.

Keywords: Chitinase, chitin-binding domain, antifungal activity, protein engineering, hybrid enzyme

An antifungal chitinase, ChiCW, is produced by *Bacillus cereus* 28-9 isolated from the lily rhizosphere and is effective against conidial germination of *Botrytis elliptica*, the causal agent of lily leaf blight [9]. ChiCW, as a modular endochitinase, consists of a signal peptide, a catalytic domain (CatD), a fibronectin type-III-like domain (Fn3D), and a chitin-binding domain (ChBD). Based on peptide sequence similarity of the catalytic domain, ChiCW is categorized as a member of the family 18 chitinases [6, 7, 9]. In previous studies, the ChBD of a family 19 chitinase, ChiC, from *Streptomyces griseus* has been demonstrated to be involved in chitinase and antifungal activities [12, 13]. Hence, we tried to investigate the function of the C-terminal region of ChiCW on enzyme and antifungal activities. However, only ChiCWΔFC (lacking ChBD and Fn3D) was successfully expressed and purified for biochemical characterization, but ChiCWΔC (lacking ChBD) was not [10]. Therefore, an alternative approach was designed to investigate the involvement of $ChBD_{ChiCW}$ in the antifungal activity of ChiCW.

Protein engineering technology has been developed to create a novel protein that possesses an improved or novel property by changing the amino acid sequence of an existing protein and has become an essential tool of basic research for protein biochemistry [1, 19, 21]. One obvious application of this technology is to engineer enzymes with improved properties, which can be achieved by constructing chimeric or hybrid enzymes from pre-existing elements [1, 19, 21]. In this study, an approach based on protein engineering technology was designed to investigate the involvement of $ChBD_{ChiCW}$ in the antifungal activity of ChiCW. As is well known, ChiA1 of *Bacillus circulans* WL-12, as a modular chitinase, consists of a signal peptide, a CatD, two Fn3D, and a ChBD [23]. ChiA1 exhibits higher catalytic activity than ChiCW [8, 24], but ChiA1 was not able to inhibit conidial germination of *Bo. elliptica* in our preliminary study. Thus, the C-terminal truncated derivatives of ChiA1 were used to construct chimeric chitinases with $ChBD_{ChiCW}$. Antifungal activities of constructed

*Corresponding author
Phone: +886-2-33665207; Fax: +886-2-23657735;
E-mail: cychen@ntu.edu.tw

Present address: Department of Plant Pathology and Microbiology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan, Republic of China.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Characteristics	Source
E. coli strain		
DH5α	F⁻, *supE44*, ϕ80dlacZΔM15, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, Δ(*lacZYA-argF*)*U169*	[5]
XL1-Blue	*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi*, *relA1*, lac⁻, F'[*proAB*, *lacI*^{q}ZΔM15, Tn10 (Tet^r)]	Stratagene
Plasmid		
pBluescript SK$^+$	Cloning vector	Stratagene
pCR2.1-TOPO	Cloning vector	Invitrogen
pNTU110	pCR2.1-TOPO carrying 2.4-kb *chiA* of *B. circulans* WL-12	[3]
pNTU55	pCR2.1-TOPO carrying 2.3-kb *chiCW* of *B. cereus* 28-9	[9]
pGW66	pCR2.1-TOPO carrying the fragment encoding ChiCWΔFC	[10]
pNTU123	pCR2.1-TOPO carrying the fragment encoding ChiA1ΔFC	This study
pNTU124	pCR2.1-TOPO carrying the fragment encoding ChiA1ΔC	This study
pNTU125	pCR2.1-TOPO carrying the fragment encoding ChiA1Δ2FC	This study
pNTU128	pCR2.1-TOPO carrying the fragment encoding ChiAAW (ChiA1ΔFC+ChBD$_{ChiCW}$)	This study
pNTU129	pBluescript SK+ carrying the fragment encoding ChiAAAW (ChiA1ΔC+ChBD$_{ChiCW}$) with *chiA* promoter	This study
pNTU130	pCR2.1-TOPO carrying the fragment encoding ChiAW (ChiA1Δ2FC+ChBD$_{ChiCW}$)	This study

chimeric chitinases were analyzed to investigate the involvement of ChBD$_{ChiCW}$ in the antifungal activity of ChiCW.

Materials and Methods

Strains, Plasmids, and Media

All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains XL1-Blue (Stratagene) and DH5α [5] were used as hosts for gene cloning and expression of recombinant protein, respectively. All bacterial strains were maintained on Luria–Bertani (LB) agar plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) supplemented with appropriate antibiotics. *Bo. elliptica* B061, the causal agent of lily leaf blight, was cultured on V-8 juice agar [20% V-8 juice (Campbell Soup Co.), 0.3% $CaCO_3$, 1.8% agar] at 25°C for sporulation [9].

Construction of Recombinant Plasmids

Six plasmids were constructed for expression of recombinant chitinases. Primers used in this study are listed in Table 2 and a schematic diagram of constructed fragments encoding recombinant chitinases is shown in Fig. 1. DNA fragments were amplified by polymerase chain reaction (PCR) with the primer pairs 215/307, 215/306, and 215/305 from pNTU110. The amplified fragments were ligated into pCR2.1-TOPO (Invitrogen) to construct plasmids pNTU124, pNTU123, and pNTU125 for expression of ChiA1ΔC, ChiA1ΔFC, and ChiA1Δ2FC, respectively.

For expression of chimeric chitinase ChiAAAW, plasmid pNTU129 was constructed. The fragment amplified by PCR with primer pair 44/219 was ligated into pCR2.1-TOPO. This recombinant plasmid was digested with XhoI and KpnI for subcloning the XhoI- and KpnI-digested fragment encoding ChBD$_{ChiCW}$ amplified by PCR with primer pair 220/221.

For expression of chimeric chitinase ChiAAW, plasmid pNTU128 was constructed. The fragment amplified by PCR with primer pair 215/218 was digested with XbaI and XhoI and subcloned into the XbaI and XhoI sites of pBluescript SK+(Stratagene). This recombinant plasmid was digested with XhoI and KpnI for subcloning the XhoI- and KpnI-digested fragment encoding ChBD$_{ChiCW}$ amplified by PCR with primer pair 220/221.

Table 2. Primers used in this study.

Primer	Sequence[a]	Restriction site
44	AGCGGCTGGAGGCGGCTATACGGC	
215	CCGGTCTAGACAGAGGAGGTTGTGTATTAAATG	XbaI
218	CCGGCTCGAGCAGATTGCCTGCGGCATC	XhoI
219	CCGGCTCGAGCACGTTGCCTGCCGCATC	XhoI
220	CCGGCTCGAGAAATCACAACCTACCGCTC	XhoI
221	CCGGGGTACCCTAGTTTTCGCTAATGACGGT	KpnI
222	CCGGGAGCTCAAATCACAACCTACCGCTC	SacI
305	TTAGAGCTCCCAGAACATCGC	SacI
306	TTACAGATTGCCTGCGGCATC	
307	TTACACGTTGCCTGCCGCATC	

[a] Restriction site is underlined.

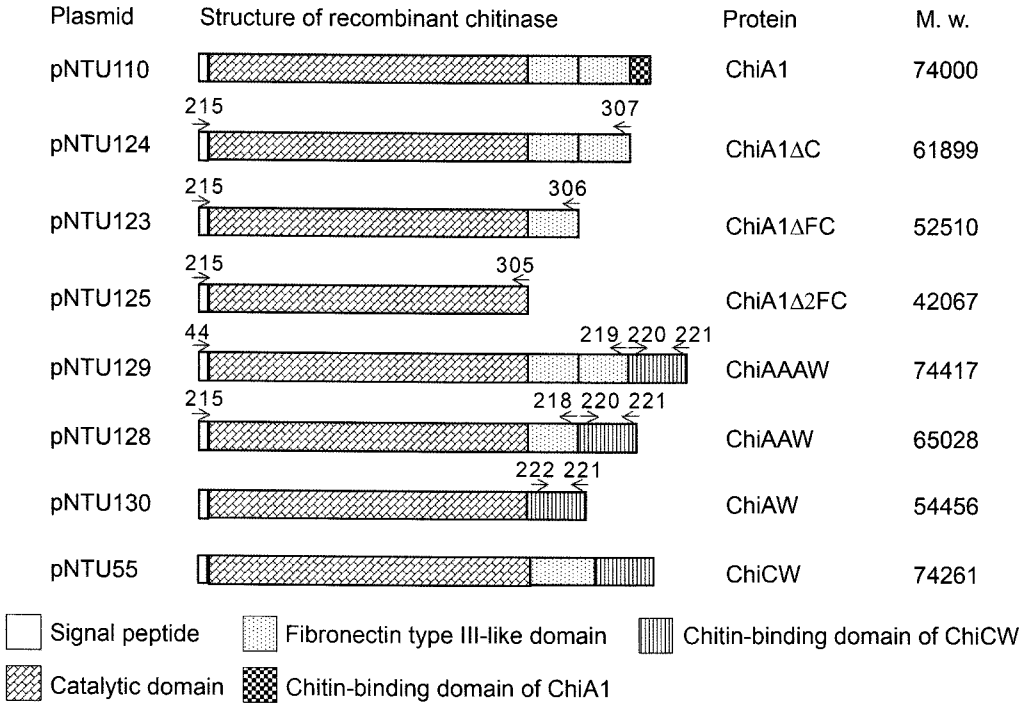

Fig. 1. Schematic diagram of the constructed chimeric chitinases and truncated derivatives used in this study.
Primers used for construction are indicated by arrows and recombinant plasmids used for expression of recombinant chitinases are presented. The designated names and molecular weights of recombinant chitinases are given.

For expression of chimeric chitinase ChiAW, plasmid pNTU130 was constructed. The DNA fragment encoding $ChBD_{ChiCW}$ was amplified by PCR with primer pair 222/221. The amplified fragment was digested with SacI and KpnI and subcloned into the same sites of pNTU110 [3].

Bacterial Expression and Crude Extraction of Recombinant Chitinases

The recombinant plasmids were introduced into *E. coli* DH5α for expression of recombinant chitinases. *E. coli* strains were cultured in 50 ml of LB broth supplemented with ampicillin (50 ppm) on a rotary shaker at 37°C for 14 h to express recombinant chitinases. *E. coli* cells were harvested by centrifugation (8000 ×*g*, 10 min at 4°C) and periplasmic proteins of *E. coli* cells were prepared according to the method of Manoil and Beckwith [17]. Periplasmic proteins were used to assay chitinase and antifungal activities.

Chitinase Activity Measurements 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 [10]. When colloidal chitin was used as a substrate, chitinase activity was measured by using the method of Imoto and Yogishita [11]. Protein concentration was determined by the Bradford method [2] using bovine serum albumin as a standard.

Bioassay of Antifungal Activity

Antifungal activity was assayed by the method of Huang *et al.* [9] with a slight modification. Assay mixtures contained 10 μl of a conidial suspension of *Bo. elliptica* B061 (1×10^5 conidia/ml) and an equal volume of an enzyme solution. In the control, sterile distilled water was used instead of a test solution. Conidial germination was examined under a light microscope and the percentage of inhibition was calculated after incubation of the prepared assay mixtures at 25°C for 12 h. Each assay was triplicated.

Purification of ChiAAAW and Partial Purification of ChiA1ΔC

All purification steps were performed at 4°C. To purify ChiAAAW, the periplasmic proteins of *E. coli* DH5α(pNTU129) were precipitated by ammonium sulfate at 70% saturation. The precipitate was dissolved in 25 mM potassium phosphate buffer (KPB, pH 6.0) and dialyzed against the same buffer. The dialysate was applied onto a Q-ceramic Hyper-D column (Sigma) equilibrated with 25 mM KPB (pH 6.0) for anion-exchange chromatography and eluted stepwise by 0.1, 0.3, and 0.5 M NaCl in 25 mM KPB (pH 6.0). The fractions with chitinase activities were pooled and dialyzed against 25 mM sodium citrate buffer (SCB, pH 4.0). The dialysate was applied onto a S-ceramic Hyper-D column (Sigma) equilibrated with 25 mM SCB (pH 4.0) for cation-exchange chromatography and eluted stepwise by 0.1, 0.3, and 0.5 M NaCl in 25 mM SCB (pH 4.0). ChiAAAW was eluted by 0.3 M NaCl in 25 mM SCB (pH 4.0) and the fractions with chitinase activities were pooled.

On the other hand, ChiA1ΔC was partially purified from the periplasmic proteins of *E. coli* DH5α(pNTU124). The periplasmic proteins of *E. coli* DH5α(pNTU124) were precipitated by ammonium sulfate at 70% saturation. The precipitate was dissolved in 25 mM KPB (pH 6.0) and dialyzed against the same buffer. The dialysate was applied onto a Q-ceramic Hyper-D column (Sigma) equilibrated with 25 mM KPB (pH 6.0) for anion-exchange chromatography and eluted stepwise by 0.1, 0.3, and 0.5 M NaCl in 25 mM KPB (pH 6.0).

Table 3. Chitinase and antifungal activities of crude extracts of ChiCW, ChiCWDFC, and ChiA1.

Extract of *E. coli* strain[a]	Expressed chitinase	Chitinase specific activity (U/mg)[c]	Inhibition of conidial germination (%)[d]
DH5α(pNTU55)	ChiCW	2.2×10^{-1}	58±1
DH5α(pGW66)	ChiCWΔFC	2.42	0±0
DH5α(pNTU110)	ChiA1	7.6×10^{-1}	16±4
DH5α(pCR2.1-TOPO)[b]	-	1.6×10^{-3}	0±0

[a] Crude periplasmic extracts of *E. coli* strains expressing different chitinases were prepared to analyze chitinase and antifungal activities.
[b] *E. coli* DH5α(pCR2.1-TOPO) as the control without expression of recombinant chitinase.
[c] One unit=1 μmole of 4-methylumbelliferone released per minute at 37°C.
[d] Each treatment included 10 μl of a crude extract and 10 μl of a conidial suspension.

ChiA1ΔC was eluted by 0.3 M NaCl in 25 mM KPB (pH 6.0) and the fractions with chitinase activities were pooled.

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

SDS–PAGE and chitinolytic zymography assay were performed using Laemmli's method with modification as previously described [9].

Results

Chitinase and Antifungal Activities of ChiCW, ChiCWΔFC, and ChiA1

As shown in Table 3, the chitinase and antifungal activities of crude extracts of ChiCW, ChiCWΔFC, and ChiA1 against *Bo. elliptica* were analyzed. Compared with ChiCW, ChiCWΔFC did not inhibit conidial germination of *Bo. elliptica,* although the crude extract of ChiCWΔFC had a higher specific activity than that of ChiCW. Thus, ChiCW lost its antifungal activity when its C-terminal region was removed. This result indicates that the C-terminal region of ChiCW may play an important role in antifungal activity.

The effect of crude extract of ChiA1 against conidial germination of *Bo. elliptica* was also investigated (Table 3). The crude extract of ChiA1 had a higher specific activity than that of ChiCW, but ChiA1 exhibited much less effect to inhibit conidial germination of *Bo. elliptica* than ChiCW. Afterwards, ChiA1 was used to construct chimeric chitinases with $ChBD_{ChiCW}$ to investigate whether $ChBD_{ChiCW}$ was involved in the antifungal activity of ChiCW.

Vector Construction and Expression of Recombinant Chitinases in *E. coli*

Fig. 1 shows schematic diagrams, designated names, and calculated molecular weights of recombinant chitinases. In this study, six recombinant plasmids were constructed to express three C-terminal domain-truncated derivatives of ChiA1 (ChiA1ΔC, ChiA1ΔFC, ChiA1Δ2FC) and three chimeric chitinases (ChiAAAW, ChiAAW, ChiAW). ChiAAAW was engineered by replacing ChBD of ChiA1 with $ChBD_{ChiCW}$. ChiAAW and ChiAW were engineered by grafting $ChBD_{ChiCW}$ to ChiA1ΔFC and ChiA1Δ2FC.

The expressions of the three C-terminal domain-truncated derivatives of ChiA1 and three chimeric chitinases in *E. coli* DH5α cells were investigated (Table 4). Comparing the specific activities of the crude extracts, ChiA1ΔC was moderately expressed in *E. coli* DH5α cells but ChiA1ΔFC and ChiA1Δ2FC were lowly expressed. When $ChBD_{ChiCW}$ was fused with the three derivatives of ChiA1, chimeric chitinases ChiAAAW and ChiAAW were highly expressed

Table 4. Chitinase and antifungal activities of crude extracts of parental, mutant, and chimeric chitinases.

Extract of *E. coli* strain[a]	Expressed chitinase	Specific activity (U/mg)[c]	Inhibition of conidial germination (%)[d]
DH5α(pNTU110)	ChiA1	7.6×10^{-1}	16±4
DH5α(pNTU124)	ChiA1ΔC	1.3×10^{-1}	16±4
DH5α(pNTU123)	ChiA1ΔFC	5.4×10^{-3}	4±3
DH5α(pNTU125)	ChiA1Δ2FC	3.8×10^{-4}	16±6
DH5α(pNTU129)	ChiAAAW	1.2	40±1
DH5α(pNTU128)	ChiAAW	1.9×10^{-1}	39±3
DH5α(pNTU130)	ChiAW	1.0×10^{-3}	61±10
DH5α(pNTU55)	ChiCW	2.2×10^{-1}	58±1
DH5α(pCR2.1-TOPO)[b]	-	1.6×10^{-3}	0±0

[a] Crude periplasmic extracts of *E. coli* strains expressing parental and chimeric chitinases were prepared to analyze chitinase and antifungal activities.
[b] *E. coli* DH5α(pCR2.1-TOPO) as the control without expression of recombinant chitinase.
[c] One unit=1 μmole of 4-methylumbelliferone released per minute at 37°C.
[d] Each treatment included 10 μl of a crude extract and 10 μl of a conidial suspension.

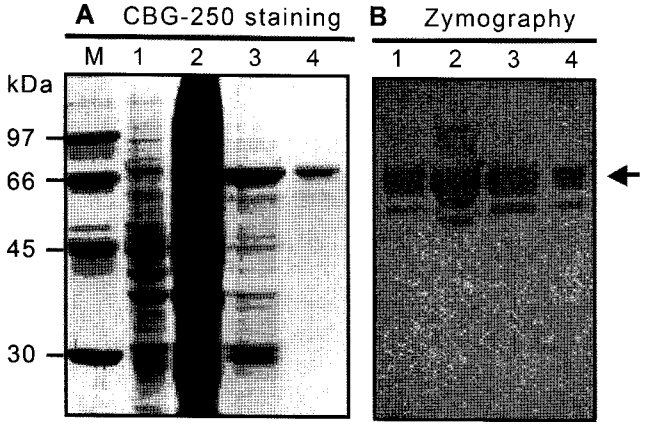

Fig. 2. Purification of ChiAAAW. Sample from each step was analyzed by SDS–PAGE and Coomassie blue G-250 staining (**A**) and chitinolyic zymography (**B**).
Lane 1, crude periplasmic extract of *E. coli* DH5α(pNTU129); lane 2, ammonium sulfate precipitated proteins; lane 3, fraction of anion-exchange chromatography; lane 4, fraction of cation-exchange chromatography; lane M, low molecular weight protein marker (GE Healthcare).

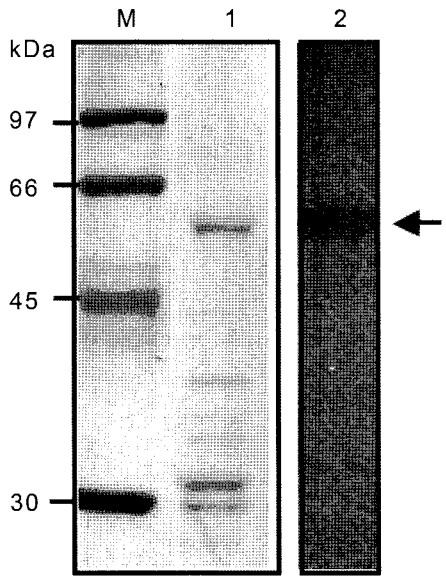

Fig. 3. SDS–PAGE and zymography assay of partially purified ChiA1ΔC.
Partially purified ChiA1ΔC was analyzed by SDS–PAGE and Coomassie blue G-250 staining (lane 1) and chitinolytic zymography (lane 2). Lane M, low molecular weight protein marker (GE Healthcare).

in *E. coli* DH5α cells, especially ChiAAAW, but ChiAW was lowly expressed. Similar results were observed by SDS–PAGE and chitinolytic zymography (data not shown).

Antifungal Activities of C-Terminal Domain-Truncated Derivatives of ChiA1 and Three Chimeric Chitinases

As shown in Table 4, the antifungal activities of crude extracts of the three C-terminal domain-truncated derivatives of ChiA1 and the three chimeric chitinases were investigated. The C-terminal domain-truncated derivatives of ChiA1 did not exhibit antifungal activities to inhibit conidial germination of *Bo. elliptica*. Surprisingly, when $ChBD_{ChiCW}$ was used to replace ChBD of ChiA1 or to fuse to ChiA1ΔFC and ChiA1Δ2FC, the three chimeric chitinases exhibited higher antifungal activities than that of ChiA1, ChiA1ΔFC, and ChiA1Δ2FC. The results implicate that $ChBD_{ChiCW}$ is involved in the antifungal activity of chitinase and strongly suggest that $ChBD_{ChiCW}$ contributes to the antifungal activity of ChiCW.

Purification of ChiAAAW and Partial Purification of ChiA1DC

According to the results of expression and antifungal effects of chimeric chitinases (Table 4), the expression of ChiAAAW in *E. coli* DH5α cells was highest among the three chimeric enzymes, and ChiAAAW effectively inhibited conidial germination of *Bo. elliptica*. Therefore, we focused on further study of ChiAAAW.

ChiAAAW was purified by ammonium sulfate precipitation, anion-exchange chromatography, and cation-exchange chromatography from the periplasmic fraction of *E. coli* DH5α(pNTU129). By SDS–PAGE analysis and chitinolytic zymography assay, ChiAAAW was purified to apparent homogeneity (Fig. 2). The molecular size of ChiAAAW was estimated to be 74 kDa, which closely corresponded to the value calculated from the peptide sequence of mature ChiAAAW (74.416 kDa). From a 1,200 ml culture, 0.06 mg of ChiAAAW was purified. The result of purification of ChiAAAW is summarized in Table 5.

In addition, ChiA1ΔC was partially purified for further assays. Although the specific activity of crude ChiA1ΔC extract was relatively high (Table 4), the amount of ChiA1ΔC expressed in *E. coli* cells was not as high as expected (data not shown). Therefore, ChiA1ΔC was

Table 5. Purification of ChiAAAW.

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Recovery rate (%)	Purification fold
Crude extract	1.44	3.15	2.19	100	1
Ammonium sulfate precipitation	0.36	2.20	6.11	70	3
Anion-exchange chromatography	0.18	1.64	9.11	52	4
Cation-exchange chromatography	0.06	2.31	38.50	73	18

[a]One unit=1 μmole of 4-methylumbelliferone released per minute at 37°C.

Table 6. Chitinase and antifungal activities of four chitinases.

Chitinase[a]	Specific activity (U/mg)[b]	Inhibition of conidial germination (%)[c]
ChiA1	1.22	0±0
ChiA1ΔC	0.69	6±5
ChiAAAW	1.16	42±6
ChiCW	0.36	35±3

[a]Purified ChiA1, ChiAAAW, and ChiCW, and partially purified ChiA1ΔC were used in determination of chitinase and antifungal activities.
[b]Colloidal chitin was used as a substrate to determine the specific activity of chitinase and 1 U of chitinase activity was defined as the amount of enzyme required to release one mmole of *N*-acetylglucosamine per minute at 37°C.
[c]All chitinases were adjusted to the same enzyme activity as 5 μU/μl for bioassay of antifungal activity.

hardly purified and 0.02 mg of partially purified ChiA1ΔC was obtained from a 1,200 ml culture. By SDS–PAGE analysis and chitinolytic zymography assay (Fig. 3), the molecular size of ChiA1ΔC was estimated to be 62 kDa, which closely corresponded to the value calculated from the peptide sequence of mature ChiA1ΔC (61.899 kDa).

Hydrolysis of Colloidal Chitin and Antifungal Activity of Purified Chitinase

Table 6 shows the hydrolytic activities towards colloidal chitin and the antifungal activities of purified ChiA1, ChiAAAW, ChiCW, and partially purified ChiA1ΔC. In accordance with the previous study [24], ChiA1 exhibited excellent hydrolytic activity towards colloidal chitin, and ChiA1ΔC exhibited less hydrolytic activity than ChiA1. In addition, ChiCW had lower hydrolytic activity than ChiA1 but the chimeric chitinase ChiAAAW exhibited similar hydrolytic activity to ChiA1.

The antifungal activities of purified ChiA1, ChiAAAW, ChiCW, and partially purified ChiA1ΔC were examined (Table 6). Although ChiA1 exhibited excellent hydrolytic activity towards colloidal chitin, ChiA1 did not inhibit conidial germination of *Bo. elliptica*. When ChBD of ChiA1 was deleted, ChiA1ΔC as well as ChiA1 did not show inhibitory effect to *Bo. elliptica*. On the other hand, ChiCW exhibited antifungal activity against *Bo. elliptica*. When $ChBD_{ChiCW}$ replaced ChBD of ChiA1, ChiAAAW exhibited antifungal activity against *Bo. elliptica* and the antifungal activity of ChiAAAW was slightly higher than that of ChiCW.

Discussion

ChBDs involved in enzyme activity and substrate binding have been demonstrated in bacterial modular chitinases of family 18 and 19 [12, 13, 18, 22, 24]. However, correlation between ChBD and antifungal activity of bacterial chitinase has only been investigated in ChiC of *S. griseus* HUT6037, a member of family 19 chitinases, by deletion and site-directed mutagenesis [12, 13, 20]. ChBD of ChiC can enhance not only the hydrolytic activity but also the antifungal activity of CatD of ChiC [12, 13]. On the contrary, correlation between ChBD and antifungal activity of bacterial family 18 chitinases had not been reported. Although ChiCWΔC was not successfully expressed in *E. coli* cells [10], we still tried to investigate the correlation between ChBD and antifungal activity of ChiCW, a member of the family 18 chitinase. Thus, we used protein engineering technology as an alternative approach to examine the importance of $ChBD_{ChiCW}$ in the antifungal activity of ChiCW.

According to our results, purified ChiA1 and its ChBD-truncated derivative did not inhibit conidial germination of *Bo. elliptica* (Table 6). In addition, the DNA fragment encoding $ChBD_{ChiCW}$ was subcloned into the pQE30 vector for expression and the purified recombinant $ChBD_{ChiCW}$ did not inhibit conidial germination of *Bo. elliptica* (unpublished data). When ChBD of ChiA1 was replaced with $ChBD_{ChiCW}$ to generate ChiAAAW, this chimeric chitinase showed antifungal activity (Table 6). Compared with ChiA1 and ChiCW, ChiAAAW exhibited high hydrolytic activity towards colloidal chitin and high antifungal activity (Table 6). This result indicates that using $ChBD_{ChiCW}$ to replace ChBD of ChiA1 could engineer a new chitinase with high enzyme and antifungal activities. Thus, we suggest that $ChBD_{ChiCW}$ plays an important role in the antifungal activity of ChiCW, and using $ChBD_{ChiCW}$ to engineer chitinases probably can obtain new enzymes with enhanced antifungal activity.

Engineering enzymes to improve their properties is one of the obvious goals of biotechnology [1, 19, 21]. Two chitinases, Chit33 and Chit42, from *Trichoderma harzianum* CECT 2413 are considered to play an important role in the biocontrol activity of this strain against plant pathogens, and both Chit33 and Chit42 lack a chitin-binding domain [4, 14]. These two enzymes were engineered by addition of substrate-binding domains to increase their substrate-binding capacity and specific activity [15, 16]. According to these studies, the substrate-binding and chitinase specific activities of these chimeric chitinases were increased, but the antifungal activities of these chimeric chitinases were not reported [15, 16]. In this study, a new chimeric chitinase, ChiAAAW, was engineered by replacing ChBD of ChiA1 with $ChBD_{ChiCW}$ and the purified ChiAAAW was demonstrated to exhibit not only high enzyme activity but also high antifungal activity. In our previous study [9], peptide sequences of ChBDs of ChiCW and ChiA1 were compared and very low similarity between the two ChBDs was observed. The result of this study shows that chitinases with improved properties can be engineered by manipulating those substrate-binding domains.

In this study, we designed an approach based on protein engineering technology to demonstrate that $ChBD_{ChiCW}$ plays an important role in the antifungal activity of ChiCW,

and a new chimeric chitinase, ChiAAAW, with high enzyme and antifungal activities was created. This study may be helpful to understand the relationship between ChBD and the antifungal activity of the family 18 chitinase.

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