

Structure and Enzymatic Properties of a Two-Domain Family GH19 Chitinase from Japanese Cedar (*Cryptomeria japonica*) Pollen

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ABSTRACT: CJP-4 is an allergen found in pollen of the Japanese cedar *Cryptomeria japonica*. The protein is a two-domain family GH19 (class IV) Chitinase consisting of an N-terminal CBM18 domain and a GH19 catalytic domain. Here, we produced recombinant CJP-4 and CBM18-truncated CJP-4 (CJP-4-Cat) proteins. In addition to solving the crystal structure of CJP-4-Cat by X-ray crystallography, we analyzed the ability of both proteins to hydrolyze chitin oligosaccharides, (GlcNAc)_n, polysaccharide substrates, glycol chitin, and β -chitin nanofiber and examined their inhibitory activity toward fungal growth. Truncation of the CBM18 domain did not significantly affect the mode of (GlcNAc)_n hydrolysis. However, significant effects were observed when we used the polysaccharide substrates. The activity of CJP-4 toward the soluble substrate, glycol chitin, was lower than that of CJP-4-Cat. In contrast, CJP-4 exhibited higher activity toward β -chitin nanofiber, an insoluble substrate, than did CJP-4-Cat. Fungal growth was strongly inhibited by CJP-4 but not by CJP-4-Cat. These results indicate that the CBM18 domain assists the hydrolysis of insoluble substrate and the antifungal action of CJP-4-Cat by binding to chitin. CJP-4-Cat was found to have only two loops (loops I and III), as reported for ChiA, an allergenic class IV Chitinase from maize.

KEYWORDS: *Cryptomeria japonica*, cedar pollen, Chitinase allergen, crystal structure, chitin, antifungal activity

INTRODUCTION

Japanese cedar (*Cryptomeria japonica*) pollen is a major source of aeroallergens in Japan that causes various types of pollinosis including rhinitis and conjunctivitis.¹ Approximately 25 million people in Japan currently suffer from seasonal pollinosis. Among numerous allergens in *C. japonica* pollen, Cry j 1 and Cry j 2 were found to have pectate lyase and polymethylgalacturonase activities, respectively.^{2–8} CJP-6 was also identified as an important allergen from the pollen; this protein has homology to the isoflavone reductase family.⁹ On the other hand, Fujimura et al. identified CJP-4, an allergenic Chitinase from *C. japonica* pollen that has high IgE-binding affinity.¹⁰ Chitinases (EC 3.2.1.14) hydrolyze β -1,4-glycosidic linkages of chitin, a linear homopolymer of N-acetylglucosamine (GlcNAc), and are classified into the GH18 and GH19 families based on the CAZy database (<http://www.cazy.org/Glycoside-Hydrolases.html>).¹¹ According to an independent classification system for plant chitinases, at least five classes (classes I, II, III, IV, and V) have been recognized based on their domain organization and loop deletions.^{12,13} Enzymes belonging to the GH19 family are further subdivided into class I, class II, and class IV. Class I chitinases consist of two domains, an N-terminal hevein domain belonging to the CBM18 family and a GH19 catalytic domain. Class II chitinases have only a catalytic domain homologous to that of the class I enzymes. Class IV chitinases are also two-domain enzymes composed of a CBM18 domain and a GH19 catalytic domain but are smaller due to deletions of four internal loops and a C-terminal loop (Figure 1).

The allergenicity of plant Chitinase was first identified in class I Chitinase from avocado (*Persea americana*).¹⁴ Sub-

sequently, allergenic class I chitinases were identified in several fruits.^{15,16} Due to the close similarity between the N-terminal CBM18 domains of allergenic class I chitinases and hevein, which is a major allergen component in *Hevea brasiliensis* latex,¹⁷ the allergenicity of class I chitinases has been attributed to their CBM18 domains. However, studies of the structure-allergenicity relationship of chestnut class I Chitinase indicated that the GH19 domain also confers allergenic properties.¹⁸ In the early 2000s, class IV chitinases from maize, grapes, and Japanese cedar pollen were shown to bind IgE in the sera of allergic patients.^{10,19,20} Structural and functional analysis of the allergenic plant class IV Chitinase using an enzyme from maize (ChiA) demonstrated that the CBM18 domain is not required for enzymatic activity.²¹ However, there is still controversy regarding which domain of class IV chitinases is responsible for their allergenicity. Furthermore, a direct link between allergenicity and the enzymatic function of class IV chitinases remains uncertain. Previously, we expressed CJP-4 in *E. coli* and showed that the purified protein exhibits Chitinase activity toward glycol chitin and can also bind to di-N-acetyl chitobiose (GlcNAc)₂.²² CJP-4 is an aeroallergen consisting of 247 amino acids and has about 50% sequence identity with class IV chitinases from maize and grape; the latter enzymes have been identified as food allergens. Therefore, we assumed that CJP-4 would be a good model protein that could be used to more

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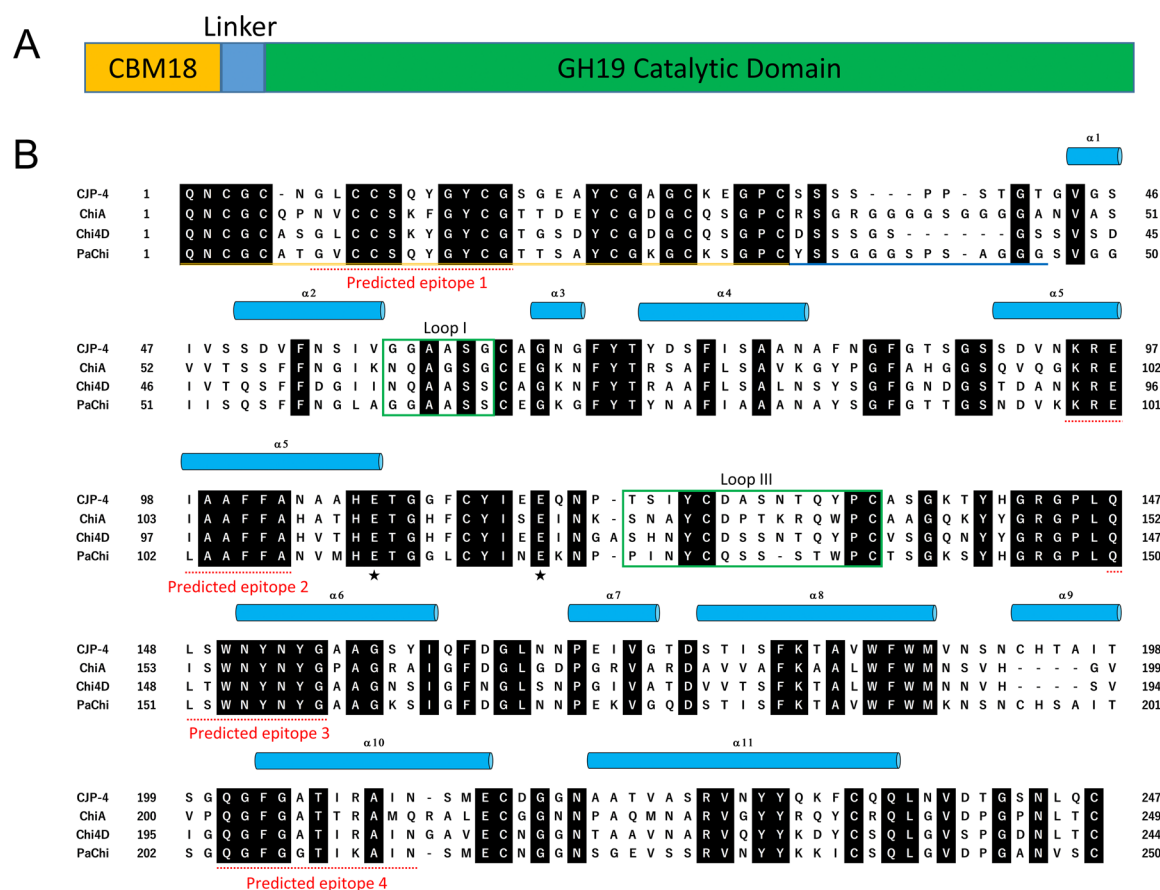


Figure 1. Schematic representation of CJP-4 showing CBM18 domain, linker region, and GH19 domain in yellow, blue, and green, respectively (A). Multiple sequence alignment of class IV chitinases from different plant species, including CJP-4 (*Cryptomeria japonica* class IV chitinase, accession number AB196451), ChiA (*Zea mays* class IV Chitinase, GQ856537), Chi4D (*Vitis vinifera* class IV Chitinase, AF532966), and PaChi (*Picea abies* class IV Chitinase, AY270019) (B). The secondary structural elements of CJP-4-Cat are shown on the top of aligned sequences. Identical amino acid residues are shown in white with a black background. The helices ($\alpha 1$ – $\alpha 11$) are labeled according to the CJP-4-Cat structure. Green-lined boxes indicate the loop structures (loops I and III). The catalytic glutamate residues are indicated by stars. The CBM18 domain and linker region were designated by a double orange line and a single blue line, respectively at the bottom of the alignment. Four predicted epitope regions were designated by dashed red lines at the bottom of the CJP-4 sequence.

clearly understand the structure-allergenicity relationship of plant chitinases.

In this study, we produced recombinant CJP-4 and recombinant CBM18-truncated CJP-4 (CJP-4-Cat) and characterized the individual proteins with respect to their enzymatic and antifungal activities. Furthermore, we determined the crystal structure of CJP-4-Cat by X-ray crystallography and found potential epitopes. Functional and structural properties of CJP-4 were also compared to those of other GH19 chitinases.

MATERIALS AND METHODS

Materials. Chitin oligosaccharides (GlcNAc)_n with different degrees of polymerization ($n = 1$ – 6) were prepared by partial acid hydrolysis of chitin,²³ followed by gel filtration on Cellufine GcL-25m (JNC Co., Tokyo, Japan). Ni-NTA, Q-Sepharose Fast Flow, and HiPrep 16/60 Sephacryl S-100 were GE Healthcare products (Tokyo, Japan). The competent *E. coli* strain SHuffle T7 (New England Biolabs, Tokyo, Japan) was used as the host for expression of CJP-4 and CJP-4-Cat. All other chemicals and reagents were of analytical grade.

Plasmid Construction for Expression of CJP-4, CJP-4-Cat, and Their Inactive Mutants. The recombinant CJP-4 was prepared using pETB-CJP-4 as an expression vector, as described previously.²² The gene only encoding the catalytic domain of CJP-4 (CJP-4-Cat)

was obtained by PCR amplification with pETB-CJP-4 as a template. Sequences of primers used for cloning of CJP-4-Cat were 5'-ATGCATCACCATCACCATCACCAAAATTGTGGATGTAATGG-ACTG-3' (forward) and 5'-TTAACATTGAAGATTTCGATCCTGT-3' (reverse). The purified PCR product was ligated into pETBlue-1 expression vector (Novagen, Madison, WI) and designated as pETB-CJP-4-Cat. Since Glu108 was defined as a catalytic amino acid of CJP-4,²² the E108Q mutation, which abolishes the Chitinase activity, was introduced into pETB-CJP-4 and pETB-CJP-4-Cat using a Quik-Change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) with primers, 5'-GCTAATGCTGCCCATCAGACTGGA-GGGTTTTGC-3' and 5'-GCAAAACCCCTCCAGTCTGATGGGC-AGCATTAGC-3' (underlines indicate the mutation sites), and designated as pETB-CJP-4(E108Q) and pETB-CJP-4(E108Q)-Cat, respectively. The expression plasmid for CJP-4, CJP-4-Cat, CJP-4(E108Q), or CJP-4(E108Q)-Cat was cointroduced together with pLacI into *E. coli* SHuffle T7. Expression of these proteins was carried out according to the manufacturer's procedure.

Purification of CJP-4, CJP-4-Cat, and Their Inactive Mutants. Harvested cell pellets were resuspended in 20 mM Tris hydrochloride buffer pH 7.5 and lysed by ultrasonication. The cell debris was removed by centrifugation at 10,000 × g for 10 min. The supernatant was loaded onto a Ni-NTA column (1 × 3 cm) equilibrated with 20 mM Tris hydrochloride buffer pH 7.5. The bound proteins were eluted with 0.3 M imidazole in the same buffer, and the fractions containing the CJP-4 protein were collected and then applied to a Q-

Sephacryl Fast Flow column equilibrated with 20 mM Tris hydrochloride buffer pH 7.5. After washing the column with the same buffer, the bound proteins were eluted with 0.3 M NaCl in the same buffer. The protein fractions were finally loaded onto a gel-filtration column of HiPrep 16/60 Sephacryl S-100 equilibrated with 10 mM Tris hydrochloride buffer pH 7.5 containing 0.1 M NaCl. Purity of the eluted fractions was analyzed by 12.5% SDS-PAGE,²⁴ and pure fractions were pooled as a purified recombinant protein. The protein concentration was determined by measuring absorbance at 280 nm using extinction coefficients for individual proteins obtained from the equation proposed by Pace et al.²⁵

Anomeric Form of the Enzymatic Products. To investigate the splitting mode of CJP-4 and CJP-4-Cat, the anomeric forms of the hydrolytic products from (GlcNAc)₆ (4.5 mM) were determined using an isocratic HPLC method. The enzymatic reaction was performed in 20 mM sodium acetate buffer pH 5.0 at 25 °C with an enzyme concentration of 0.04 μM. After incubation for 30 min, an aliquot of the reaction solution was immediately loaded onto a TSK Amide 80 column (Tosoh) and eluted with acetonitrile–water (7:3 v/v) at a flow rate of 0.8 mL/min at 25 °C, to separate the (GlcNAc)_n anomers. The substrate and the enzymatic products were detected by absorbance at 220 nm. The splitting mode of the oligosaccharide substrates was estimated from the product distribution and the anomer ratio (α/β) of the individual oligosaccharide products.²⁶ The inverting enzymes CJP-4 and CJP-4-Cat give α -anomer at the newly produced reducing ends, hence the products which are rich in α -form were regarded as the glycon side of the substrate.

HPLC-Based Determination of the Reaction Time-Course.

For determination of time-courses of the substrate degradation and product formation, the reaction products from the Chitinase-catalyzed hydrolysis of (GlcNAc)_n ($n = 4, 5$, or 6) (4.5 mM each) were analyzed by gel-filtration HPLC, which gives quantitative data. The enzymatic reaction was done in 20 mM sodium acetate buffer, pH 5.0, at 40 °C with an enzyme concentration of 0.04 μM. The enzymatic reaction was terminated by adding 0.1 M NaOH solution, and immediately frozen in liquid nitrogen. The resultant solution was applied onto a gel-filtration column of TSK-GEL G2000PW (Tosoh, Tokyo). Elution was done with distilled water at a flow rate of 0.3 mL/min. Oligosaccharides were detected by ultraviolet absorption at 220 nm. Peak areas obtained for individual oligosaccharides were converted to molar concentrations, which were then plotted against reaction time to obtain the reaction time-course.

Chitinase Activity toward Polymeric Substrates. The enzyme activities toward polymeric substrates, glycol chitin and β -chitin nanofiber, were measured by the colorimetry method. Glycol chitin, a water-soluble chitin derivative, was prepared as described by Yamada and Imoto.²⁷ β -Chitin nanofibers were prepared from squid pen by simple mechanical treatment under acidic conditions.²⁸ Those are generally defined as fibers with a diameter of less than 100 nm and an aspect ratio of more than 100. Five microliters of the enzyme solution was added to 500 μL of 0.4% (w/v) glycol chitin or β -chitin nanofiber (OD₅₄₀ = 1.0) suspension in a 50 mM sodium acetate buffer, pH 5.0. After incubation of the reaction mixture at 37 °C for a given period, the reducing sugars produced were determined using ferri-ferrocyanide reagent by the method of Imoto and Yagishita.²⁹

Antifungal Activity toward *Trichoderma viride*. The antifungal activity assay was conducted according to the method of Schlumbaum et al.³⁰ with minor modifications. An agar disk (6 mm in diameter) with the fungus *Trichoderma viride*, which was derived from the fungus in an actively growing state previously cultured on PDA, a potato dextrose broth with 1.5% (w/v) agar, was placed in the center of a Petri dish containing PDA. The plate was incubated at room temperature for 12 h. Wells were subsequently punched into the agar at a distance of 15 mm from the center of the plate. The samples to be tested were placed into the wells containing 10 μL of sterile dH₂O. The plate was incubated for 24 h at room temperature and then photographed.

Crystallization and Data Collection. Prior to crystallization, the protein was concentrated to 5 mg/mL. Crystallization was achieved by screening with commercially available crystallization kits from

Hampton Research using the sitting-drop vapor-diffusion method. Sitting drops were prepared by mixing 1 μL of protein solution (5 mg/mL in water) with 1 μL of reservoir solution containing 0.2 M BIS-TRIS, pH 6.5, 15% polyethylene glycol 3350. Quadrangular prism crystals grew within 2 weeks under all conditions. For data collection, the crystals were cryoprotected in a solution consisting of 0.2 M BIS-TRIS, pH 6.5, 15% polyethylene glycol 3350, and 20% glycerol and then flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected at the beamline BL-17A of the Photon Factory (Ibaraki, Japan) using an ADSC Q270 CCD detector at a cryogenic temperature (95 K). Diffraction data were integrated and scaled with HKL2000.³¹ The crystals belong to the monoclinic space group $P2_1$, with unit cell dimensions of $a = 33.0$ Å, $b = 74.3$ Å, $c = 35.8$ Å, $\alpha = 90^\circ$, $\beta = 99.9^\circ$, and $\gamma = 90^\circ$. The processing statistics are summarized in Table 1.

Table 1. Data Collection and Refinement Statistics^a

parameter	value
data collection	
space group	$P2_1$
cell dimensions	
a, b, c (Å)	33.01, 74.30, 35.81
α, β, γ (deg)	90.0, 99.9, 90.0
wavelength (Å)	0.98
resolution (Å)	50–1.19 (1.21–1.19)
R_{merge}	0.059 (0.156)
$\langle I/\sigma I \rangle$	54.1 (20.5)
completeness (%)	93.8 (83.4)
redundancy	7.7 (7.3)
refinement	
resolution (Å)	37.15–1.19
no. of reflections	48394
$R_{\text{work}}^b/R_{\text{free}}^c$	0.146/0.164
no. of atoms	
protein	1600
water	183
average B-factors (Å ²)	
protein	6.92
water	17.5
RMS deviations	
bond lengths (Å)	0.007
bond angles (deg)	1.155

^aThe values in parentheses are for the outermost shell. ^b $R_{\text{work}} = \Sigma |F_o - F_c|/\Sigma F_o$ for reflections of working set. ^c $R_{\text{free}} = \Sigma |F_o - F_c|/\Sigma F_o$ for reflections of test set (5.0% of total reflections).

Structural Determination and Refinement. The three-dimensional structure of CJP-4-Cat was determined by molecular replacement with the program MOLREP,³² using PaChi(s) (PDB code 3HBD³³) as a search model. One protein molecule was located in the crystallographic asymmetric unit. The model was improved by iterative cycles of refinement with REFMAC5^{34,35} and manual rebuilding with COOT.³⁶ The structure of CJP-4-Cat was refined to an $R_{\text{work}}/R_{\text{free}}$ of 0.146/0.164 at a resolution of 1.19 Å. The final model contains a single protein molecule that include residues 43–247 and 183 water molecules. The stereochemistry of the model was verified using PROCHECK,³⁷ showing 85.7%, 13.7%, 0.0%, and 0.6% of protein residues in the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran plot, respectively. Molecular graphics were prepared using PyMol (<http://www.pymol.org/>). The atomic coordinates and structure factor of CJP-4-Cat were deposited in the Protein Data Bank under the PDB code 5H7T.

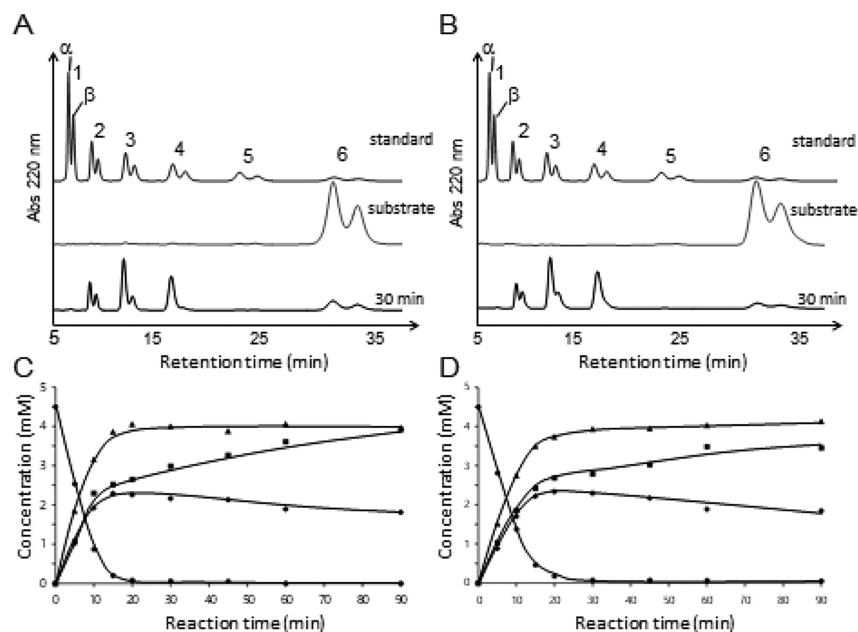


Figure 2. Time-dependent HPLC profiles showing the hydrolysis of $(\text{GlcNAc})_6$ by CJP-4 (A) and CJP-4-Cat (B). Numerals in the figure represent the degree of polymerization. Experimental time-courses of $(\text{GlcNAc})_6$ degradation by CJP-4 (C) and CJP-4-Cat (D). The presented curves for individual $(\text{GlcNAc})_n$ ($n = 2-6$) were obtained by visual estimation of the best fit to the experimental data points. Symbols: square, $(\text{GlcNAc})_2$; triangle, $(\text{GlcNAc})_3$; diamond, $(\text{GlcNAc})_4$; closed circle, $(\text{GlcNAc})_6$.

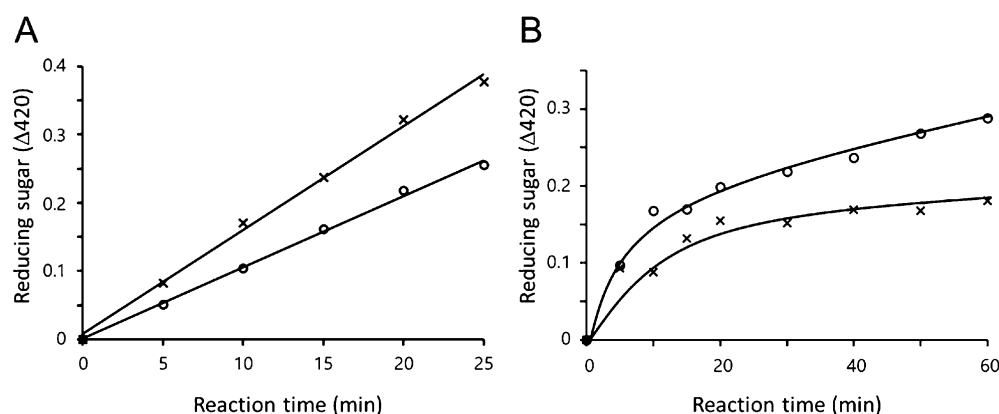


Figure 3. Hydrolysis of glycol chitin (A) and chitin nanofiber (B) by CJP-4 and CJP-4-Cat. Reactions were done at 37 °C, and the amount of reducing sugar generated was monitored. Symbols: open circle, CJP-4; cross, CJP-4-Cat.

RESULTS AND DISCUSSION

Production and Purification of CJP-4 and CJP-4-Cat.

CJP-4, CJP-4-Cat, CJP-4(E108Q), and CJP-4(E108Q)-Cat were successfully produced and purified by the method described above. The yields of purified CJP-4, CJP-4-Cat, CJP-4(E108Q), and CJP-4(E108Q)-Cat were in the range of 10–16 mg from one liter of induced culture, respectively. On SDS-PAGE, the recombinant proteins CJP-4, CJP-4-Cat, CJP-4(E108Q), and CJP-4(E108Q)-Cat exhibited a single protein band with molecular masses of 28.5, 25.5, 28.5, and 25.5 kDa, respectively; these masses correspond to those calculated from the amino acid sequences of the individual proteins (data not shown).

HPLC Analysis of the Products from CJP-4- and CJP-4-Cat-Dependent $(\text{GlcNAc})_6$ Degradation. After a hydrolytic reaction catalyzed by CJP-4, enzymatic digests from $(\text{GlcNAc})_6$ were applied to an HPLC column, which enabled the anomer separations for individual oligosaccharides, as shown in Figure

2A. $(\text{GlcNAc})_6$ appeared to be split into $(\text{GlcNAc})_3 + (\text{GlcNAc})_3$ and $(\text{GlcNAc})_4 + (\text{GlcNAc})_2$. Consistent with a report that GH19 enzymes (including CJP-4) hydrolysis of substrates is associated with anomer inversion,¹¹ α -anomer was predominant in the products, $(\text{GlcNAc})_3$ and $(\text{GlcNAc})_4$. Production of the $(\text{GlcNAc})_4$ α -anomer was more pronounced than that of $(\text{GlcNAc})_3$ (Figure 2A). The $(\text{GlcNAc})_2$ product was almost at equilibrium in mutarotation between α - and β -forms. These results indicate that $(\text{GlcNAc})_4$ was derived from the glycon moiety of the $(\text{GlcNAc})_6$ substrate, while $(\text{GlcNAc})_2$ was derived from the aglycon moiety. The reducing ends of the $(\text{GlcNAc})_3$ product appeared to be the sum of the newly produced reducing ends and the reducing ends from the original substrate, $(\text{GlcNAc})_6$. Thus, CJP-4 hydrolyzed $(\text{GlcNAc})_6$ at the second glycosidic linkage from the reducing end in addition to the middle linkage. The mode of $(\text{GlcNAc})_6$ hydrolysis by CJP-4 appears to be identical to that of NaCHIT1, a class IV Chitinase from the pitcher of

the carnivorous plant *Nepenthes alata*.³⁸ Similar product distribution and anomer distributions were observed for CJP-4-Cat, as shown in Figure 2B, indicating that the CBM18 domain (which is attached to the N-terminus of the GH19 domain) is not involved in the hydrolysis of (GlcNAc)₆.

Time-courses of the enzymatic degradation of (GlcNAc)₆ are shown in Figure 2C and 2D. (GlcNAc)₆ was hydrolyzed to (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄ by CJP-4, which was further hydrolyzed to (GlcNAc)₂. The initial velocity of (GlcNAc)₆ degradation catalyzed by CJP-4 was similar to that catalyzed by CJP-4-Cat, and the profile of the time-course of CJP-4 was almost identical to that of CJP-4-Cat. The CBM18 domain and the linker region did not affect the enzymatic activity of CJP-4. The data shown in Figure 2 are fully consistent with the NMR spectra previously reported for CJP-4 and CJP-4-Cat.²² Superimposition of ¹H–¹⁵N HSQC spectra revealed that the resonances for the GH19 domain of CJP-4 almost completely overlapped with those of CJP-4-Cat, indicating that the GH19 domain does not interact with the CBM18 domain in the two-domain CJP-4. This likely explains the almost identical reaction profiles between CJP-4 and CJP-4-Cat (Figure 2); that is, the enzymatic reaction of CJP-4 toward (GlcNAc)₆ takes place at the GH19 binding site independently and does not depend on the state of the CBM18 domain and the linker region. Sasaki et al. previously showed that the CBM18 domain of class I rice Chitinase (OsChia1c) does not significantly participate in hydrolysis of (GlcNAc)₆.³⁹ Although the CBM18 and GH19 domains of class IV Chitinase are shorter than those of class I chitinases, preferential binding of chitin oligosaccharide substrates to the catalytic domain may be a common feature of the two-domain GH19 chitinases.

Enzymatic Activities toward Polymeric Substrates.

Enzymatic properties of CJP-4 and CJP-4-Cat were also investigated using the polymeric substrates, soluble glycol chitin and insoluble β -chitin nanofiber. As shown in Figure 3, truncation of the CBM18 domain and the linker region significantly enhanced activity toward the soluble substrate but suppressed activity toward the insoluble substrate. Thus, the presence of the CBM18 domain may facilitate enzymatic activity toward insoluble β -chitin nanofibers, while it is less important or even a disadvantage for the hydrolysis of soluble glycol chitin. Class I chitinases have relatively higher activity toward insoluble substrates compared to those lacking the CBM18 and class II Chitinase consisting of only the GH19 domain; that is due to the presence of the CBM18 domain linked to the GH19 domain by a linker.^{40,41} These results indicate that the CBM18 domains of both class I and IV two-domain family GH19 chitinases are essential for chitin-binding ability and increase the efficiency of hydrolysis of insoluble substrates by the GH19 domain. Previously, we suggested that the linker region between the CBM18 and GH19 domains in CJP-4 allows these domains to fold independently and that no domain–domain interactions occur.²² Additionally, we demonstrated the importance of the CBM18 domain for recognition of insoluble chitin based on the NMR binding data obtained for full-length CJP-4.⁴² This current study, in what we believe is the first published report, has demonstrated the contribution of the CBM18 domain of class IV Chitinase to the hydrolysis of an insoluble substrate.

Antifungal Activity. The antifungal activities of CJP-4 proteins were determined by using the hyphal extension inhibition assay on agar plate with *Trichoderma viride* as the test fungus (Figure 4). CJP-4 inhibited hyphal extension, whereas

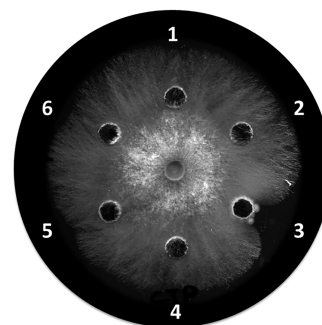


Figure 4. Antifungal activity of CJP-4 against *Trichoderma viride* on culture medium. The samples to be tested were placed into the wells in 10 μ L of distilled water with 500 pmol of purified chitinases. 1, control (distilled water); 2, heat-denatured CJP-4; 3, CJP-4; 4, CJP-4 (E108Q); 5, CJP-4-Cat; 6, CJP-4-Cat (E108Q).

heat-denatured CJP-4 did not. Inactive CJP-4 mutant E108Q, CJP-4-Cat, and inactive CJP-4-Cat mutant E108Q showed faint inhibition against the test fungus. These results indicate that the CBM18 domain contributes significantly to the antifungal activity of CJP-4 and that the catalytic activity of CJP-4 appears essential for antifungal activity. The contribution of the CBM18 domain to antifungal activity has also been observed in class I chitinases. For example, class I chitinases from tobacco and rye inhibited fungal growth more effectively compared to their CBM18 deletion mutant counterparts.^{40,43} Therefore, chitin microfibrils in fungal hyphae are possible physiological substrates for GH19 chitinases that contain a CBM18 domain.

Crystal Structure of CJP-4-Cat. Obtaining the three-dimensional structures of allergens is important in order to understand the molecular basis of allergenicity. Furthermore, knowledge of structure in relation to the information on B-cell and T-cell epitopes is also necessary to develop allergen-specific immunotherapy. During the current study, we attempted to determine the three-dimensional structure of CJP-4. However, we could not obtain crystals of CJP-4, perhaps due to the flexibility of the linker region connecting the N-terminal CBM18 and C-terminal GH19 domains. Hence, we crystallized CJP-4-Cat, and the structure was successfully solved as described in the Methods section. The crystal structure of CJP-4-Cat was bilobal with 11 distinct α -helices (Figure 5A), which is a typical structure of GH19 family members. To date, only two crystal structures of the catalytic domain of class IV Chitinase have been solved: PaChi(s) from Norway spruce³³ and ChitA from maize.²¹ The overall structure of CJP-4-Cat was similar to that of PaChi(s) (PDB code 3HBD) over the corresponding 192 C α atoms, with an RMSD of 1.166 Å. Similarities to ChitA (PDB code 4MCK) were also found over 186 C α atoms with an RMSD of 1.823 Å (Figure 5B). Crystallization of full-length class IV chitinases has been unsuccessful. However, this is a critical gap in our knowledge, and further investigation is required to elucidate whether the structural conservation of these class IV chitinases brings about immunological cross-reactivity among these proteins. This is especially important, given that the class IV chitinases endochitinase 4A, ChitA, and CJP-4 are all reported to be allergenic.^{10,19,20} ADFS (Allergen Database for Food Safety) is a Web server database system which is comprised of allergenic proteins for food safety and allergenicity prediction tools. The database contains allergens classified into 8 categories (pollen, mite, animal, fungus, insect, food, latex, and others) and

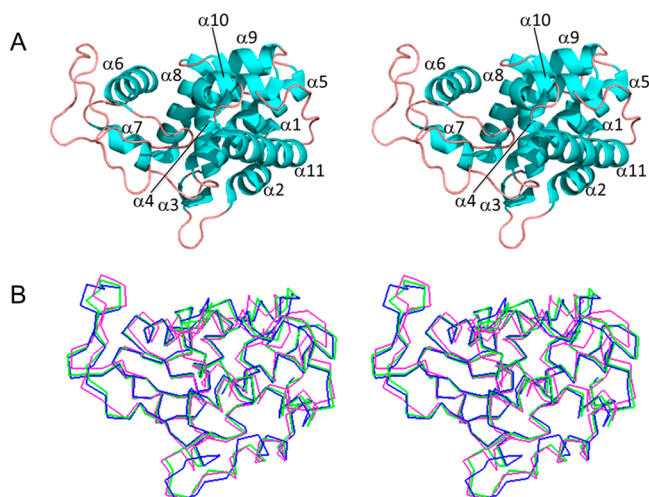


Figure 5. Stereo view of a ribbon representation of the main chain structure of CJP-4-Cat (A). Superimposition of α traces of CJP-4-Cat (blue; PDB code 5H7T), PaChi(s) (green; PDB code 3HBD), and ChitA (magenta; PDB code 4MCK) (B).

identified at least four potential allergenic epitopes in CJP-4 (Figure 1). These epitopes are well conserved among class IV chitinases. Of these, predicted epitope 1 is present in the CBM18 domain, and the other three epitopes are in the GH19 domain.

The catalytic GH19 domains consist of a conserved α -helical core-region and a variable number (1–6) of loop structures, located at both ends of the substrate-binding groove. CJP-4-Cat was found to have only two loops (loops I and III), as reported for PaChi(s) and ChiA (two-loop type). In addition to these structures, three-dimensional structures of the six-loop type (loops I to V and C-terminal one) and one-loop type (only loop III) GH19 domains in complex with their substrate have been determined (Figure 6).^{44,45} RSC-c (rye seed chitinase-c), a six-loop type GH19 Chitinase from *Secale cereale*, possesses an extended substrate-binding groove consisting of eight subsites; -4 , -3 , -2 , -1 , $+1$, $+2$, $+3$, and $+4$. On the other hand, only four sugar binding subsites (-2 to $+2$) have been identified in BcChi-A, a small GH19 Chitinase from *Bryum coronatum*; this is likely because the enzyme is a GH19 type with only one loop. As we could not obtain crystals of the CJP-4-Cat–substrate complex, the number of sugar binding sites in CJP-4-Cat remains unknown. Ubhayasekera et al. have suggested that lacking the loops (loops II, IV, V and C-terminal one) from six-

loop type GH19 makes the substrate-binding cleft of class IV Chitinase shorter.³³ In fact, $(\text{GlcNAc})_4$ was further hydrolyzed into $(\text{GlcNAc})_2$ by CJP-4-Cat (Figure 2). Such degradation was hardly observed in the time-course of $(\text{GlcNAc})_6$ degradation catalyzed by the GH19 domain with six loop structures.³⁹ CJP-4 and CJP-4-Cat hydrolyzed $(\text{GlcNAc})_4$ into mainly $(\text{GlcNAc})_2$ + $(\text{GlcNAc})_2$; this pattern was also observed with BcChi-A⁴⁶ (Figure 2). As they lack the loop structures forming the glycon and aglycon binding sites, class IV chitinases may have a smaller number of substrate-binding subsites in the cleft compared to those of six-loop type GH19 enzymes.

Recently, a class II Chitinase has been identified as an allergen in wheat flour.⁴⁸ Additionally, two class II chitinases (DDBJ accession numbers Q05539 and Q7Y0S1) were found registered in the Allergome (<http://www.allergome.org/>), a platform for allergen knowledge. Since a number of conserved residues are found in the GH19 domains of allergenic class I, II, and IV chitinases, IgE-binding epitopes responsible for cross-reactivity among GH19 enzymes may be present in this domain. This should be investigated in future studies.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CJP-4, a class IV Chitinase from cedar pollen with allergic activity; CJP-4-Cat, catalytic domain of CJP-4; CJP-4(E108Q), an inactive mutant of CJP-4 in which Glu108 was mutated to Gln; CJP-4(E108Q)-Cat, an inactive mutant of CJP-4-Cat in which Glu108 was mutated to Gln; GlcNAc, N-acetylglucosamine; $(\text{GlcNAc})_n$, β -1,4-linked oligosaccharide of GlcNAc with polymerization degree of n ; HPLC, high performance liquid chromatography; RSC-c, rye seed Chitinase-c; BcChi-A, *Bryum coronatum* Chitinase-A

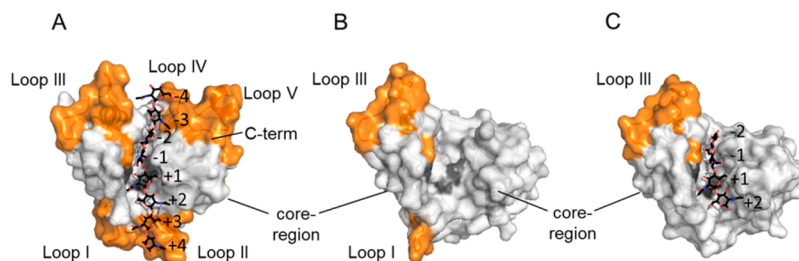


Figure 6. Three-dimensional structures of the GH19 Chitinase family. Six-loop type GH19 Chitinase from *Secale cereale* (RSC-c) in complex with two molecules of $(\text{GlcNAc})_4$ (PDB code 4JOL) (A). Two-loop type GH19 Chitinase from *Cryptomeria japonica* (CJP-4-Cat) (PDB code 5H7T) (B). One-loop type GH19 Chitinase from *Bryum coronatum* in complex with $(\text{GlcNAc})_4$ (PDB code 3WH1) (C). GH19 enzymes are represented as surface models. Loop structures are labeled Loop I, II, III, IV, V, and C-term and highlighted in orange. The conserved core-regions are also labeled and indicated in light gray. The catalytic acids and bases are highlighted in dark gray. $(\text{GlcNAc})_4$ molecules are represented by stick models colored in black. Individual binding subsites are numbered according to the nomenclature suggested by Biely et al.⁴⁷

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