

NMR assignments and ligand-binding studies on a two-domain family GH19 chitinase allergen from Japanese cedar (*Cryptomeria japonica*) pollen

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Abstract A two-domain family GH19 chitinase from Japanese cedar (*Cryptomeria japonica*) pollen, CJP-4, which consists of an N-terminal CBM18 domain and a GH19 catalytic domain, is known to be an important allergen, that causes pollinosis. We report here the resonance assignments of the NMR spectrum of CJP-4. The backbone resonances were almost completely assigned, and the secondary structure was estimated based on the chemical shift values. The addition of a chitin dimer to the enzyme solution perturbed the chemical shifts of the resonances of amino acid residues forming a long extended binding site spanning from the CBM18 domain to the GH19 catalytic domain.

Keywords *Cryptomeria japonica* · Cedar pollen · Two-domain family GH19 chitinase · CBM18 domain · Secondary structure · Ligand binding

Abbreviations

CJP-4	A class IV chitinase allergen from <i>Cryptomeria japonica</i>
GlcNAc	<i>N</i> -acetyl-D-glucosamine
(GlcNAc) _n	β-1,4-Linked oligosaccharide of GlcNAc
NMR	Nuclear magnetic resonance
HSQC	Two-dimensional heteronuclear single quantum correlation

Biological context

Japanese cedar (*Cryptomeria japonica*) pollen is a major source of air-borne allergens in Japan, and it causes pollinosis, including rhinitis and conjunctivitis. One of the important allergens with high IgE-binding ability in *C. japonica* pollen is a chitinase, which was identified and referred to as CJP-4 by Fujimura et al. (2005). Chitinases (EC 3.2.1.14) hydrolyze β-1,4-glycosidic linkages of chitin, a β-1,4-linked polysaccharide of *N*-acetylglucosamine (GlcNAc), and are classified into the GH18 and GH19 families based on their amino acid sequences (<http://www.cazy.org/Glycoside-Hydrolases.html>). Plant GH19 chitinases are further subdivided into at least three classes, class I, class II, and class IV (Arakane et al. 2012). 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 hevein domain and a GH19 catalytic domain, but are smaller due to deletions of four internal loops and a C-terminal loop. The allergic protein CJP-4 described above belongs to class IV chitinases with a smaller molecular mass (25.7 kDa, 247 amino acid residues). Most structural studies on the ligand interaction of multiple-domain chitinases have been conducted using recombinant proteins representing individual domains (Matsumoto et al. 1999; Ikegami et al. 2000; Jee et al. 2002), because crystallization of a full-length protein with a multi-domain structure has been challenging. In this study, to gain insights into the molecular basis of both the enzymatic and allergic functions of the two-domain chitinase, resonances of the ¹H-¹⁵N-HSQC spectrum of CJP-4 were assigned using a three-dimensional NMR approach, and the secondary structures were estimated based on the

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assignment data. Ligand-titration experiments were also conducted to map the binding site.

Methods and experiments

Plasmid construction for expression of the mutant CJP-4, CJP-4(E108Q)

We obtained a plasmid-carrying synthetic DNA fragment encoding the mature region of CJP-4 (Gln1–Cys247), pEX-A-CJP-4, from Eurofins Genomics K.K. (Tokyo, Japan). PCR was carried out using the plasmid as a template with a forward primer, 5'-ATGCACCATCACCATCACCATCAAATTGTGGATGTAATGGACTGTGTTGC-3' and a reverse primer 5'-TTAACATTGAAGATTCGATCC TGTG-3' for cloning into the pETBlue-1 expression vector (Novagen Inc.). A single underline indicates 18 nucleotides encoding a 6× His affinity tag for fusing to the N-terminus of CJP-4. The stop codon is denoted by a double underline. PCR products were purified and ligated into the pETBlue-1 vector by TA-cloning. The resulting plasmid, pETB-CJP-4, was used as a template for site-directed mutagenesis. Sequence alignments of family GH19 (class IV) chitinases revealed that Glu108 is the catalytic acid in CJP-4. To examine the substrate-binding properties of CJP-4, the expression plasmid for a catalytic activity-deficient mutant of CJP-4 was produced by mutating Glu108 to glutamine, and was designated as pETB-CJP-4(E108Q). The mutation was performed by a QuikChange site-directed mutagenesis kit (Agilent Technologies Co.) using primers; 5'-GCTAATGCTGCCCATCAGACTGGAGGGTTTTGC-3' and 5'-GCAAAACCCTCCAGTCTGATGGGCAGCATTAGC-3' (Underlines indicate the mutation sites). The expression plasmids for CJP-4(E108Q) and pLacI were co-introduced into *E. coli* SHuffle T7 (New England Biolabs Japan). *E. coli* cells harboring pETB-CJP-4(E108Q) and pLacI were grown in 1 L of LB-broth with 50 µg/mL ampicillin to OD₆₀₀=0.8 before induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Growth was then continued for 24 h at 16 °C.

Stable isotope-labeling

Cells harboring the expression plasmid for CJP-4(E108Q) were grown in M9 medium containing ¹⁵N-NH₄Cl to produce the ¹⁵N-labeled protein. ¹³C-glucose was also added to the medium, producing a ¹⁵N/¹³C-double-labeled protein. Cultivation was conducted to obtain an OD_{600nm} of 0.6 before induction with 1 mM IPTG. Growth was then continued for 48 h at 18 °C. The cells were harvested by centrifugation, suspended in 20 mM Tris hydrochloride buffer pH 7.5 and disrupted with a sonicator. After

cell debris was removed by centrifugation (10,000×g, 10 min), the supernatant was applied to a Ni-NTA column (1×3 cm) equilibrated with 20 mM Tris hydrochloride buffer pH 7.5. The column was washed with the same buffer and adsorbed proteins were eluted with 0.3 M imidazole in the same buffer. Fractions containing the CJP-4(E108Q) protein were collected and further applied to a Q-Sepharose Fast Flow column equilibrated with 20 mM Tris hydrochloride buffer pH 7.5. The column was washed with the same buffer and adsorbed proteins were eluted with 0.3 M NaCl in the same buffer. The fractions of CJP-4(E108Q) were finally applied to 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. After confirmation of the purity by SDS-PAGE, the fractions were collected as a purified recombinant protein. The protein concentration was determined by measuring absorbance at 280 nm using an extinction coefficient for CJP-4(E108Q) obtained from the equation proposed by Pace et al. (1995).

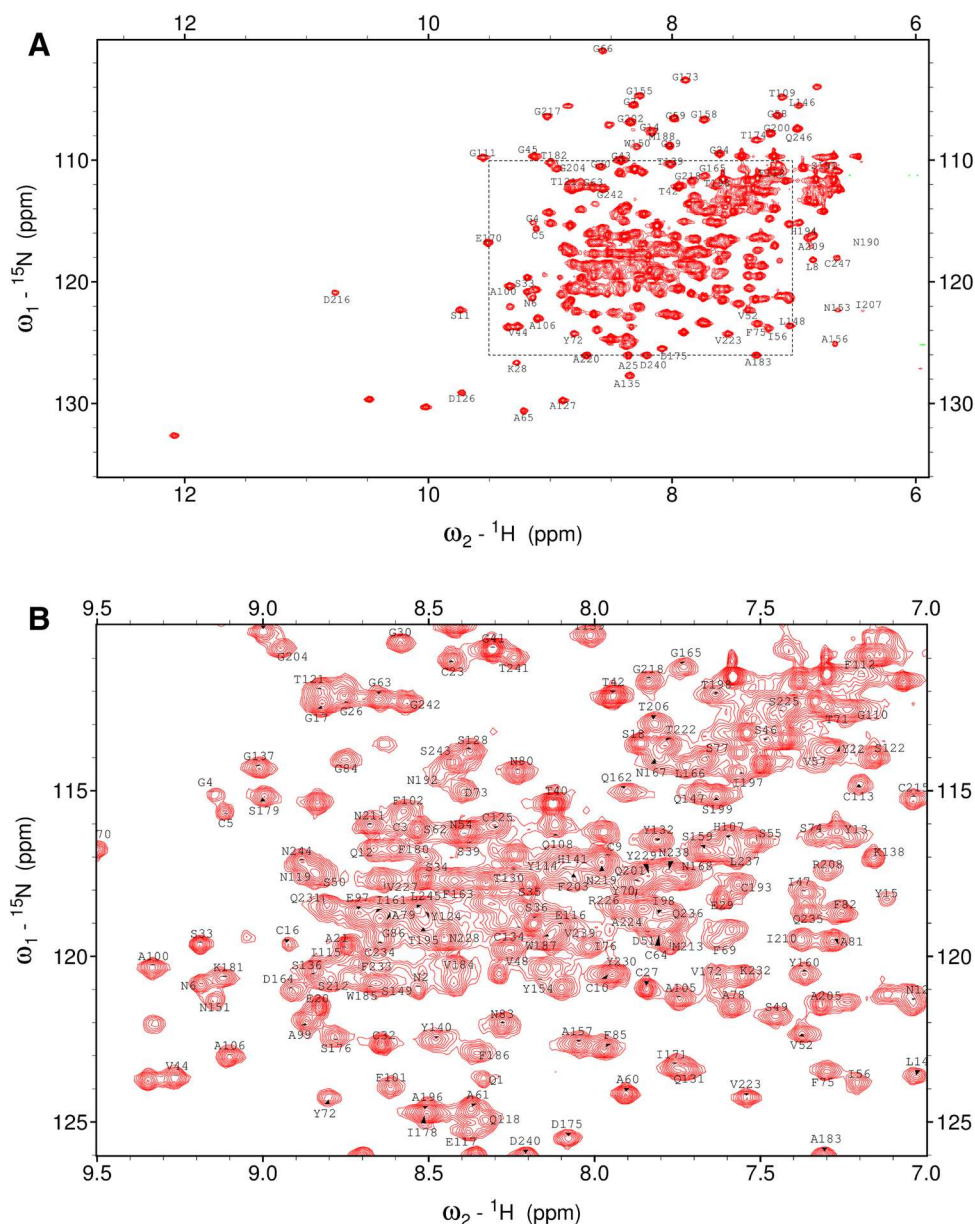
Chitinase activity

The soluble substrate, glycol chitin, was synthesized from crab chitin by the addition of ethylene chlorohydrin to an alkaline chitin solution (Yamada and Imoto 1981). Chitinase activity toward glycol chitin was evaluated from the production of reducing sugars by the method of Imoto and Yagishita (1971).

Sequential assignments and secondary structure of CJP-4(E108Q)

NMR samples contained 0.3 mM protein in 50 mM sodium acetate buffer pH 5.0 (90% H₂O/10% D₂O). All NMR spectra were acquired at 300 K using a Bruker AV500 spectrometer controlled with TopSpin 3.0 software and equipped with a triple-resonance pulsed-field-gradient cryoprobe head. ¹H chemical shifts were referenced to HDO (4.64 ppm at 30 °C) relative to trimethylsilyl propionate. ¹⁵N and ¹³C chemical shifts were indirectly calibrated from each gyromagnetic ratio (Wishart et al. 1995). Sequential assignments were performed using ¹⁵N/¹³C-double-labeled CJP-4(E108Q) from two-dimensional ¹H-¹⁵N HSQC experiments and from three-dimensional HNCACB, CBCA(CO)NH, HNCACO and HNCO experiments. All spectra were processed using the software NMRPipe (Delaglio et al. 1995) and analyzed using Sparky software (Godard and Kneller 2008). The secondary structure of CJP-4(E108Q) was estimated from the chemical shift data using TALOS+ software (Shen et al. 2009).

Fig. 1 ^1H - ^{15}N -HSQC spectrum and the backbone signal assignment of CJP-4(E108Q). **a** the entire spectrum. The protein was dissolved in 50 mM sodium acetate buffer pH 5.0 containing 10% D_2O to obtain a 0.3 mM CJP-4(E108Q) solution. The backbone resonances were assigned in a sequential manner as labeled nearby the individual resonances. **b** an enlargement of the boxed region in the entire spectrum



Ligand-binding experiments

Two-dimensional ^1H - ^{15}N HSQC spectra were recorded for 75 μM ^{15}N -labeled CJP-4(E108Q) in 50 mM sodium acetate buffer pH 5.0 (90% H_2O /10% D_2O), in the presence of various concentrations of $(\text{GlcNAc})_2$, which was purified from the acid hydrolyzate of crab chitin (Rupley 1964). Chemical shift changes induced by the oligosaccharide binding ($\Delta\delta$) were calculated using the equation,

$$\Delta\delta = \{(\Delta\text{NH}^2 + \Delta\text{N}^2/25)/2\}^{1/2}$$

where ΔNH and ΔN represent the observed shifts in the ^1H -axis and ^{15}N -axis, respectively. Amino acid residues whose main-chain resonances were affected by the addition

of $(\text{GlcNAc})_2$ ($\Delta\delta > 0.02$) were highlighted on the modeled structure of CJP-4(E108Q), which was obtained by homology-modeling using the SWISS-MODEL (Peitsch 1995). The templates used for the modeling were the crystal structure of a class IV chitinase from the Norway spruce, *Picea abies* (PaChi(1), PDB code, 3HBD; sequence similarity, 72%; Ubhayasekera et al. 2009) for the catalytic domain, and a corresponding part of the crystal structure of wheat germ agglutinin in complex with *N*-acetylglucosamine (PDB code, 2UVO; amino acid residues 133–171; sequence homology, 57%; Schwefel et al. 2010) for the CBM18 domain. The protein structure was drawn using the software Pymol.

Assignments and data deposition

^1H - ^{15}N HSQC spectra of the CJP-4 inactive mutant

Crystal structures of catalytic domains of family GH19 chitinases from various plant species and bacteria revealed that the enzymes comprised two lobes, both of which are rich in α -helices (Ohnuma et al. 2012, 2013, 2014; Ubhayasekera et al. 2007, 2009; Kezuka 2006; Hoell 2006). Similarly, CJP-4(E108Q) appears to be predominantly α -helical,

because the chemical shifts were not fully dispersed in the ^1H - ^{15}N HSQC spectrum, especially in the region from 8.0 to 9.0 ppm of the ^1H -axis and from 115 to 120 ppm of the ^{15}N -axis, as shown in Fig. 1. Nevertheless, the backbone resonances were sequentially assigned by means of a combination of three-dimensional NMR experiments using $^{15}\text{N}/^{13}\text{C}$ -labeled CJP-4(E108Q). These assignments are given in Fig. 1. A total of 90% of the backbone $^1\text{H}^{\text{N}}$ and ^{15}N , 94% of the $^{13}\text{C}\alpha$, 95% of the $^{13}\text{C}\beta$, and 94% of the $^{13}\text{C}'$ resonances were assigned; the missing assignments were

Fig. 2 TALOS-derived ϕ (red circles) and ψ (black circles) dihedral angle predictions as a function of the residue sequence. The secondary structure outlined on top corresponds to the predictions obtained from TALOS+ (Cornilescu et al. 1999). The secondary structure predicted for BcChi-A (Shinya et al. 2012) is also aligned for comparison. The secondary structure elements were numbered referring to those reported for BcChi-A. Arrowed regions at the top of the figure correspond to a CBM18 hevin domain (red), a linker (green), and a GH19 catalytic domain (orange). Arrowed regions (blue) at the bottom of the figure indicate the missing assignments

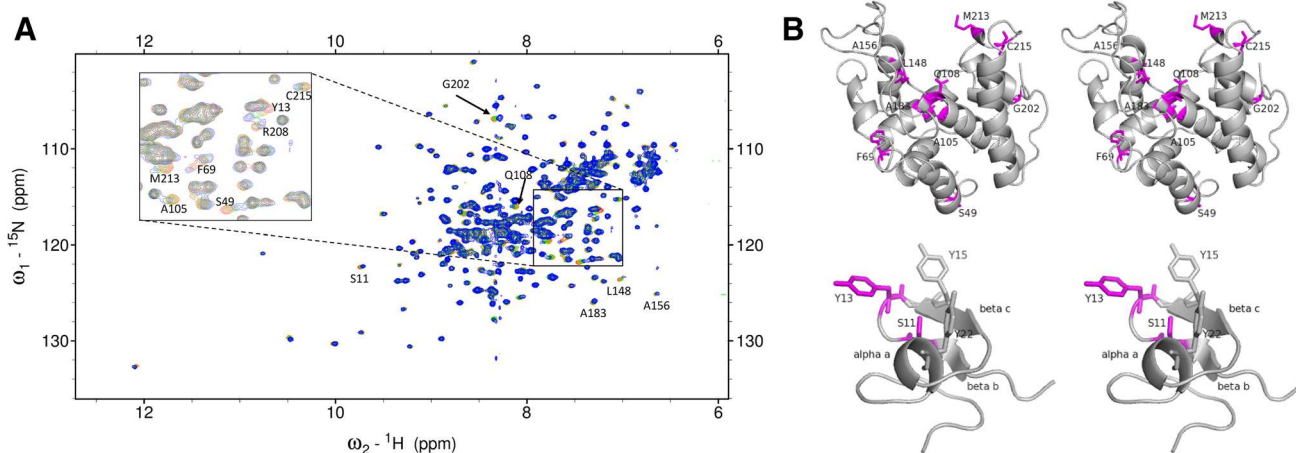
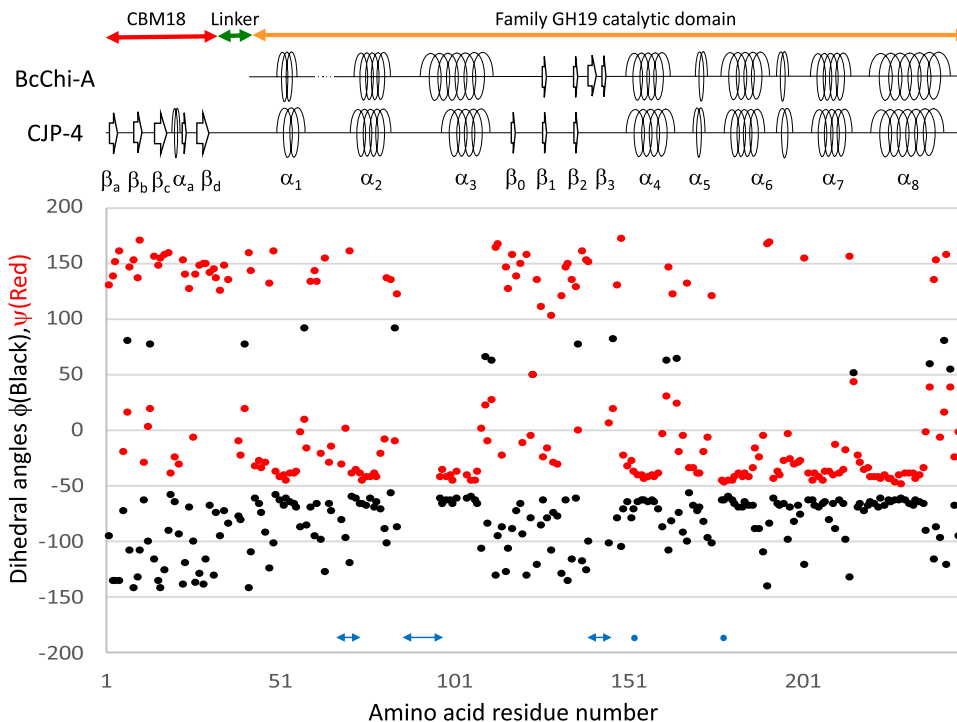


Fig. 3 **a** ^1H - ^{15}N -HSQC spectra of CJP-4(E108Q) in the absence or presence of various concentrations of $(\text{GlcNAc})_2$. Molar ratios of the enzyme to the ligand are 1:0 (red), 1:10 (orange), 1:35 (green), and 1:75 (blue). The titration was conducted using 75 μM CJP-4(E108Q) solution in 50 mM sodium acetate buffer pH 5.0 containing 10%

D_2O . **b** Stereo view of the modeled structures of GH19 catalytic domain (upper panel) and CBM18 domain (lower panel) of CJP-4(E108Q). The amino acid residues with resonances affected by the ligand binding are highlighted by stick models colored in magenta

the amino acid residues, 67, 68, 87–96, 142–145, 152, and 177, in addition to the seven proline residues. Most amino acid residues with unassigned resonances are located in the flexible loop regions, which often adopt multiple conformations. This is one of the possible reasons for missing assignments. The missing assignments may possibly be derived from solvent exchanges on an intermediate NMR time scale. The assignment data have been deposited in BioMagResBank (<http://www.bmrb.wisc.edu>) under the accession code, 12,000. Figure 2 shows the secondary structure of CJP-4(E108Q) estimated from the chemical shift data using the software TALOS+ (Shen et al. 2009). The secondary structure of the catalytic domain of CJP-4(E108Q) was almost consistent with that of a family GH19 chitinase from the fern, *Bryum coronatum*, (BcChi-A) estimated from the NMR assignment data (Shinya et al. 2012), as shown in Fig. 2. However, differences were found in arrangements of β -hairpins between $\alpha 3$ and $\alpha 4$. In most family GH19 chitinases, the catalytic glutamic acid is located at the end of $\alpha 3$ (Monzingo et al. 1996). Thus, Glu108 was identified as a catalytic acid of CJP-4. In fact, the chitinase activity was found to be completely eliminated by the E108Q mutation of CJP-4. The N-terminal CBM18 domain was found to adopt an irregular β -sheet formed by four strands. The secondary structure arrangement of the central part of the CBM18 domain including β_b , β_c and α_a was similar to that previously deduced by NMR for hevein in complex with a chitin oligosaccharide ligand (Asensio et al. 1995, 1998, 2000; Aboitiz et al. 2004).

Ligand binding and chemical shift perturbations

Chemical shift perturbation induced by ligand binding to CJP-4(E108Q) was examined using an *N*-acetylglucosamine dimer (GlcNAc)₂ as a ligand. Chemical shifts of several resonances, which were assigned to the main chain NH of Ser11, Tyr13, Ser49, Phe69, Ala105, Gln108, Leu148, Ala156, Ala183, Gly202, Arg208, Met213, and Cys215, were perturbed by the addition of (GlcNAc)₂, as shown in Fig. 3a. The transition between the free and bound states was found to be fast with respect to the NMR time-scale. The amino acid residues whose resonances were perturbed were highlighted in the modeled structure of CJP-4(E108Q), as shown in Fig. 3b. Most amino acids sensitive to the (GlcNAc)₂ binding were found to be localized to the putative binding cleft. It should be noted that amino acid residues, Ser49 and Phe69, localized to the hinge between the two lobes were also sensitive to the ligand binding. Domain motion induced by the ligand binding appeared to affect the local conformation of the hinge region, as reported previously for plant GH19 chitinases from rye seeds and fern (Ohnuma et al. 2013, 2014).

Ser11 and Tyr13 are located in the CBM18 domain, and their chemical shifts were also perturbed by the ligand binding. In the CBM18 members, the three conserved aromatic amino acids are important for chitin oligosaccharide binding (Aboitiz et al. 2004), and correspond to Tyr13, Tyr15, and Tyr22 in the CBM18 domain of CJP-4. Although one of the three aromatic residues was only responsive to the (GlcNAc)₂ binding, the binding mode to the CBM18 domain may be similar to those reported previously for the other CBM18 proteins. A couple of (GlcNAc)₂ molecules may simultaneously bind to the long-extended binding site spanning from the CBM18 domain to the GH19 catalytic domain of CJP-4(E108Q). These NMR assignments will enable comprehensive analysis of the interaction of a two-domain chitinase allergen CJP-4 with substrate and/or antibody in solution.

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