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Crystal structure and biochemical characterization of CJP38, a β-1,3-glucanase and allergen of *Cryptomeria japonica* pollen

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ABSTRACT

A 38 kDa β-1,3-glucanase allergen from *Cryptomeria japonica* pollen (CJP38) was recombinantly produced in *E. coli* and purified to homogeneity with the use of Ni-affinity resin. CJP38 hydrolyzed β-1,3-glucans such as CM-curdlan and laminarioligosaccharides in an endo-splitting manner. The optimum pH and temperature for β-1,3-glucanase activity were approximately 4.5 and 50 °C, respectively. The enzyme was stable at 30–60 °C and pH 4.0–10.5. Furthermore, CJP38 catalyzed a transglycosylation reaction to yield reaction products with a molecular weight higher than those of the starting laminarioligosaccharide substrates. The three-dimensional structure of CJP38 was determined using X-ray crystallography at 1.5 Å resolution. CJP38 exhibited the typical $(\beta/\alpha)_8$ TIM-barrel motif, similar to allergenic β-1,3-glucanases from banana (Mus a 5) and rubber tree latex (Hev b 2). Amino acid sequence alignment of these proteins indicated that the two-consensus IgE epitopes identified on the molecular surfaces of Mus a 5 and Hev b 2 were highly conserved in CJP38. Their conformations and surface locations were quite similar for these proteins. Sequence and structural conservation of these regions suggest that CJP38 is a candidate allergen responsible for the pollen-latex-fruit syndrome relating to Japanese cedar pollinosis.

1. Introduction

Japanese cedar (*Cryptomeria japonica*) pollen has been reported as one of the most prevalent sources of aeroallergens that cause type I allergy in Japan, where the number of patients has been increasing since Japanese cedar pollinosis was first reported in 1964 (Horiguchi and Saito, 1964). Currently, approximately 25 million Japanese individuals suffer from rhinitis and itchy eyes in early spring, bringing about significant economic losses and substantial reductions in their quality of life (Saito, 2014). This pollinosis is now considered a national disease in Japan.

Apart from pollinosis, Tuft et al. reported in 1942 the pollen-food allergy syndrome (PFAS) (Tuft and Blumstein, 1942). PFAS is an IgE-mediated allergy in pollinosis patients, caused by ingestion of raw fruits and vegetables. The allergic reactions occur mainly in the oral cavity and cause mouth and throat itching. Besides PFAS, it has been known that approximately half of latex-allergic patients have IgE antibodies that cross-react with certain fruit and vegetable allergens. This is known as the latex-fruit syndrome (Wagner and Breiteneder, 2002; Wagner et al., 2004). Therefore, the cross-reactivity among pollen, latex, and fruit allergens, the so-called pollen–latex–fruit syndrome, has become a major concern. In fact, Ole e 9 and Ole e 10, olive pollen β-1,3-glucanase and β-1,3-glucan-specific carbohydrate-binding module, respectively, were reported to be strong candidate allergens accounting for the cross-reactivity in the pollen–latex–fruit syndrome (Barral et al., 2004; Palomares et al., 2005; Quiralte et al., 2007). However, there is no direct evidence yet regarding the Japanese cedar pollen allergens that would contribute to this cross-reactivity. In this context, exploring such allergens to subsequently develop therapeutic strategies for the syndrome is required.

Abbreviations: CJP-4, a family GH19 chitinase allergen from *Cryptomeria japonica* pollen; CJP38, a 38 kDa β-1,3-glucanase allergen from *Cryptomeria japonica* pollen; CM-curdlan, carboxymethyl-curdlan; GH, glycosyl hydrolase; Hev b 2, β-1,3-glucanase allergen from *Hevea brasiliensis*; L2-6, laminaribiose to laminarihexaose; Mus a 5, β-1,3-glucanase allergen from banana (*Musa acuminata*); Ole e 9, β-1,3-glucanase allergen from olive (*Olea europaea*) pollen; PFAS, pollen-food allergy syndrome; *p*NP, *p*-nitrophenyl; RMSD, root mean square deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

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As a part of this effort, an allergenome characterization of *C. japonica* proteins was conducted using two-dimensional immunoelectrophoretic analysis. IgE in the sera obtained from patients with Japanese cedar pollinosis was employed in the analysis and a number of major and minor allergens in *C. japonica* pollen were identified (Fujimura et al., 2004; Fujimura and Kawamoto, 2015). However, only two *C. japonica* allergens, Cry j 1 and Cry j 2, which are allergenic proteins with pectate lyase and polymethylgalacturonase activities, respectively, are listed in the WHO/IUIS Allergen Nomenclature Subcommittee database (http://www.allergen.org/) (Sakaguchi et al., 1990; Komiyama et al., 1994; Namba et al., 1994; Sone et al., 1994; Ohtsuki et al., 1995).

Although the structure determination of desired allergens is a prerequisite for analyzing their surface exposed areas and for mapping conformational epitopes, the three-dimensional structures of *C. japonica* allergens have been not solved yet, except for the main structure of CJP-4, a glycosyl hydrolase (GH) family 19 chitinase allergen. (Takashima et al., 2018). Among the Japanese cedar pollen allergens, CJP38 was isolated as a 38 kDa allergen that exhibits 42.5–52.5 % IgE-binding frequency in patients allergic to Japanese cedar pollen, which is more reactive than the major allergen Cry j 2 (40%). Analysis of the deduced amino acid sequence of the gene encoding CJP38 revealed that CJP38 consists of 348 amino acids and shows extensive homology with other β-1,3-glucanases, which are enzymes that are ubiquitously distributed among higher plants, fungi, and bacteria. Plant β-1,3-glucanases are thought to be involved in defense against phytopathogens, cell division, pollen development and tube growth (Stintzi et al., 1993; Balasubramanian et al., 2012). To date, two types of allergenic plant β-1,3-glucanases have been reported. One that consists of two functional domains, an N-terminal catalytic domain and a C-terminal carbohydrate binding domain (long β-1,3-glucanase) and other consists of only a catalytic domain (short β-1,3-glucanase). The former (about 45 kDa) is found to be expressed in reproductive organs of higher plants and proposed to be involved in the development of pollens grains (Hird et al., 1993) whereas the latter (about 30 kDa) is widely spread in plants. CJP38 has about 39%, 46% and 43% sequence identity with β-1,3-glucanase allergens from olive pollen (Ole e 9), banana (Mus a 5) and rubber tree latex (Hev b 2), respectively (Huecas et al., 2001; Barre et al., 2009). These enzymes are classified into the GH family 17 in the CAZy database (http://www.cazy.org/) (Henrissat and Davies, 1997). Mus a 5 and Hev b 2 have been reported to be a fruit allergen and latex allergen, respectively (Aleksic et al., 2012; Palosuo et al., 2007). Therefore, we concluded that CJP38 represents a probable Japanese cedar pollen allergen that would be responsible for the cross-reactivity with latex and fruit allergens.

In this study, we recombinantly expressed, purified and enzymatically characterized CJP38. Then, we determined the three-dimensional structure of CJP38 by X-ray crystallography and compared the structures with those of Mus a 5 and Hev b 2. Functional characterization and structural analysis of these allergens may provide us valuable information to understand the structure–allergenicity link of plant β-1,3-glucanases and the molecular basis in the cross-reactivity among pollen, latex and fruit allergens.

2. Materials and methods

2.1. Materials

Laminarioligosaccharides (L2-L6, laminaribiose to laminarihexaose), *p*-nitrophenyl laminarioligosaccharides (*p*NP-L2- *p*NP-4, *p*NP-laminaribiose to *p*NP-laminaritetraose) and CM (Carboxymethyl)-curdlan were purchased from Megazyme (Wicklow, Ireland). *Escherichia coli* strain 10 G cells and pRham N-His Kan vector were purchased from Lucigen (Middleton, WI, USA). Ni-NTA agarose was from Quiagen (Chatsworth, CA, USA) and HiPrep 16/60 Sephacryl S-100 was from GE Healthcare (Tokyo, Japan), respectively. All other reagents were of analytic grade commercially available.

2.2. Plasmid construction for expression of CJP38

GenBank accession number of the gene encoding CJP38 is AB197672. A synthetic gene encoding CJP38 was obtained from Eurofins Genomics K.K. (Tokyo, Japan). Nucleotide sequence of the gene was optimized for better expression in *E. coli* without changing the amino acid sequence of CJP38. The expression vector for CJP38, pRham-CJP38, was constructed by the Expresso(R) Rhamnose Cloning and Expression System, N-His. The primers used for the cloning of CJP38 were 5'-CATCATCACCACCATCACGAACAAATCGGTGTGAACTAT-3' (forward) and 5'-GTGGCGGCCGCTCTATTATTCAGGCTAAATTTCACCGGA -3' (reverse), respectively. Single underlining indicates 18 nucleotides encoding a 6 × His affinity tag for fusing to the N-terminus of CJP38. The stop codon is denoted by double underlining. The PCR product was purified and ligated into pRham N-His Kan expression vector. The expression plasmid for CJP38 was introduced into *E. coli* 10 G. The *E. coli* cells were grown in one liter of LB-broth with 50 μg/mL kanamycin to $OD_{600} = 0.8$ before induction with 0.2% (w/v) rhamnose. Growth was then continued for 24 h at 18 °C.

2.3. Purification of CJP38

Purification of His-tagged CJP38 has been carried out according to the supplier's instruction. Briefly, the cells were harvested by centrifugation, suspended in 20 mM Tris-HCl buffer pH 7.5, and disrupted with sonicator. After centrifugation at 14,000 x *g* for 15 min, the supernatant was applied to a Ni-NTA column equilibrated with 20 mM Tris − HCl buffer pH 7.5 containing 0.1 M NaCl. After washing the column with the same buffer, adsorbed proteins were eluted with 0.3 M imidazole in the same buffer. The fractions with β-1,3-glucanase activity were collected and applied to a gel-filtration column of HiPrep 16/60 Sephacryl S-100 equilibrated with 20 mM Tris − HCl buffer pH 7.5 containing 0.1 M NaCl. The fractions exhibiting a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) were pooled as a purified recombinant CJP38. The protein concentration was determined by measuring absorbance at 280 nm using extinction coefficients for CJP38 (28,880 $M^{-1}\,cm^{-1}$) obtained from the equation proposed by Pace et al. (Pace et al., 1995)

2.4. Assay of β-1,3 glucanase activity

β-1,3 glucanase activity was assayed colorimetrically using CM-curdlan as a substrate. Five microliters of the enzyme solution was added to 250 μl of 0.2% (w/v) CM-curdlan in 20 mM sodium acetate buffer pH 5.0. After incubation at 37 °C for 15 min, the reducing power of the reaction mixture was measured using the ferri-ferrocyanide reagent by the method of Imoto and Yagishita (1971). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute at 37 °C. The optimum pH was determined by measuring the activities at various pHs (2–9) at 37 °C. The optimum temperature was determined by measuring the activities in 20 mM sodium acetate buffer, pH 5.0, at various temperatures (20–70 °C). The pH stability was determined by measuring the residual activities after incubation at various pHs (2–11) at 37 °C for 1 h. The thermal stability was determined by measuring the residual activities after incubation in 20 mM sodium acetate buffer, pH 5.0, at various temperatures for 1 h (30–70 °C). The residual activities were measured at pH 5.0. Measurement of viscosity of CM-curdlan in the reaction mixture was also measured using an Ostwald viscometer. Initial viscosity was measured by adding 20 mM sodium acetate buffer, pH 5.0 instead of enzyme solution, and this was taken to be 1.0 relative activity.

2.5. TLC analysis of laminarioligosaccharide degradation

Reaction mixture containing 5 mM laminariorigosaccharide (L2-L6) or *p*NP- laminariorigosaccharide (*p*NP-L2- *p*NP-L4) and 2 μM CJP38 in 20 mM sodium acetate buffer pH 5.0 was incubated at 25 °C for 2, 5, 10, 20, 30, 60 min. The reaction was terminated by boiling the mixture for 10 min. For product analysis, each reaction mixture was applied to a silica TLC plate (6 × 10 cm), and then chromatographed three times (1 h each) in a mobile phase containing *n*-BuOH:AcOH:H2O (3:1:1) (v/v/v). The reaction products were visualized under UV light at 254 nm for detection of *p*NP-laminarioligosaccharides or sprayed with MeOH:H_2SO_4 (19:1) (v/v) and baked at 180 °C for 3 min for detection of both *p*NP-laminarioligosaccharides and laminarioligosaccharides.

2.6. Crystallization and data collection

Crystallization conditions for CJP38 were screened using the sparse matrix sampling method by sitting drop vapor diffusion at 20 °C. Under optimized crystallization conditions, 1 μl of protein solution (6.3 mg/ml in water) was mixed with 1 μl of reservoir solution containing 0.2 M calcium chloride, 20% polyethylene glycol 3350. Rectangular prism crystals grew within 2 weeks under all conditions. For data collection, the crystals were transferred into the cryoprotectant solution containing 0.2 M calcium chloride, 20% polyethylene glycol 3350, and 20% ethylene glycol, and then flash-cooled in a nitrogen stream at 95 K. Diffraction data were collected at the beam-line BL-17A of the Photon Factory (Ibaraki, Japan) using an ADSC Q270 CCD detector at a cryogenic temperature (95 K). Data were integrated and scaled with HKL2000 (Otwinowski and Minor, 1997). The crystals belong to the tetragonal space group $P4_1$, with unit cell dimensions of, a =71.4 Å, b =71.4 Å, c =63.4 Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 90^\circ$. The processing statistics are summarized in Table 1.

2.7. Structural determination and refinement

The structure of CJP38 was solved by the molecular replacement method using the program MOLREP (Vagin and Teplyakov, 2010), where the structure of a GH17 glucanase from *Musa acuminata*, PDB code 2CYG, served as a search model (Receveur-Bréchot et al., 2006). Single protein molecule was located in the crystallographic asymmetric unit. The model was improved by several rounds of refinement with REFMAC5 (Murshudov et al., 1997; Vagin et al., 2004) and manual rebuilding with COOT (Emsley and Cowtan, 2004). The structure of CJP38 was refined to an R_{work}/R_{free} of 0.148/0.168 at a resolution of 1.50 Å. The final model contains single protein molecule that include residues 29-348, two ethylene glycol molecules, and 317 water molecules. The stereochemistry of the model was verified using PROCHECK (Laskowski et al., 1993), showing 91.2%, 8.8%, 0.0%, and 0.0% of protein residues in the most favored, additionally allowed, generously allowed, and disallowed regions of the Ramachandran plot, respectively. Molecular graphics were illustrated with PyMol (http://www.pymol.org/).

The atomic coordinates and structure factor of CJP38 were deposited in the Protein Data Bank under the PDB code 6JMS.

Table 1
Data collection and refinement statistics[a].

Data collection	
Space Group	$P4_1$
Cell dimensions	
a, *b*, *c* (Å)	71.4, 71.4, 63.5
α, *β*, *γ* (°)	90.0, 90.0, 90.0
Wavelength (Å)	0.98000
Resolution (Å)	71.42 - 1.50 (1.53-1.50)[a]
R_{merge}	0.057 (0.402)
$< I/\sigma I >$	35.5 (4.7)
Completeness (%)	99.4 (99.0)
Redundancy	13.3(12.6)
Refinement	
Resolution	35.71 – 1.50
No. reflections	48,087
R_{work}[b]/R_{free}[c]	0.148 / 0.168
No. of atoms	
Protein	2444
Water	317
Average B-factors (Å^2)	
Protein	17.6
Water	25.8
RMS deviations	
Bond lengths(Å)	0.010
Bond angles (°)	1.527

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

3. Results and discussion

3.1. Amino acid sequence of CJP38

Shigeta et al. identified CJP38, a 38 kDa allergenic β-1,3-glucanase from *C. japonica* pollen that shows a high-binding affinity with IgE in the sera obtained from patients allergic to Japanese cedar pollen (Shigeta et al., 2011). β-1,3-glucanases (EC 3.2.1.39) hydrolyze β-1,3-glycosidic linkages of laminarin, a linear homopolymer of glucose, and are classified into the GH family 17 on the CAZy database. Fig. 1 shows a comparison of amino acid sequences between four allergenic β-1,3-glucanases. Hev b 2 is a major IgE-binding allergen with β-1,3-glucanase activity in natural rubber latex (Palosuo et al., 2007). Mus a 5 is a banana β-1,3-glucanase allergen which was found to bind to 74% IgE in the sera obtained from patients sensitized to banana fruit (Aleksic et al., 2012). Ole e 9 is a β-1,3-glucanase allergen from olive pollen, which is one of the principal causes of allergy in Mediterranean countries (Palomares et al., 2006). Previously, Barre et al. identified surface-exposed two-consensus epitopes, xSEVXXLYKxxNIXRMRXYDPNQA (epitope 1, x and X indicate non-conserved and conserved/semi-conserved amino acid residue, respectively) and NLIXHVxxGTPXRPx (epitope 2), that bind to IgE and account for the cross-reactivity between Mus a 5 and Hev b 2 using IgE from latex allergic patients as a probe (Barre et al., 2009). Since these two epitopes have similar conformations on their three-dimensional structures and their amino acid sequences are well conserved in β-1,3-glucanases from other allergenic fruits and vegetables, it is expected that these two epitopes played a role in the latex-food syndrome. These two sequences are also well conserved in the corresponding regions of CJP38 (sequence identity ranging from 46% to 67%). Therefore, at least these two epitope regions seem to act as the IgE-binding cross-reactive epitopes of β-1,3-glucanases obtained from Japanese cedar pollen, banana and latex.

3.2. Expression and purification of CJP38

Analysis of the amino acid sequence of CJP38 with the SignalP program (Bendtsen et al., 2004) indicated that it contained a signal sequence consisting of 28 amino acid residues at the N-terminus. Thus, an expression system for the predicted mature protein consisting of 320 amino acid residues (29–348) fused to an N-terminal 6 × His tag was created and the recombinant CJP38 protein was produced in *Escherichia coli*. The CJP38 protein was successfully purified to homogeneity, showing a single band on SDS-PAGE corresponding to the molecular mass calculated for the amino acid sequence of 6 × His-tagged CJP38 fusion protein with N-terminal methionine (M_r = 35739.3), as shown in Fig. 2. The yield of recombinant CJP38 was approximately 30 mg from one liter of induced culture.

Fig. 1. Amino acid sequence alignment of β-1,3-glucanase allergens.
CJP38, a β-1,3-glucanase allergen from *Cryptomeria japonica*; Hev b 2, a β-1,3-glucanase allergen from *Hevea brasiliensis*; Mus a 5, a β-1,3-glucanase allergen from *Musa acuminate*; Ole e 9, an N-terminal domain of β-1,3-glucanase allergen from *Olea europaea*. The alignment was obtained with the ClustalW program. Asterisks indicate identical residues; colons and periods indicate conserved and semiconserved substitutions, respectively. Dashes indicate gaps. Secondary structure elements of CJP38 were shown at the top of the sequence alignment. α-Helices are represented as solid cylinders, 3_{10} helices as grey shaded cylinders and β-strands as arrows. The α-helices and β-strands of CJP38 are named as α1, α2,…, and as β1, β2, β3,…, from the N-terminus, respectively. 3_{10} helices are also named as η1, η2, …in the same way. Two boxes correspond to two consensus epitopes (epitope 1 and 2) accounting for the cross-reactivity between Mus a 5 and Hev b 2.

3.3. Enzymatic characterization of CJP38

The specific activity of the recombinant CJP38 was determined to be 6.26 U/mg, using CM-curdlan as a substrate. The optimum pH for β-1,3-glucanase activity is approximately 4.5 and the optimum temperature is at 50 °C (Fig. 3A and B). CJP38 was stable between pH 4.0 and 10.5, but unstable below pH 3.0 and above pH 10.5 (Fig. 3C). Regarding the temperature, CJP38 was stable up to 60 °C and unstable above 65 °C (Fig. 3D). In order to determine whether CJP38 is endo- or exo-type, a viscometric study was carried out using CM-curdlan as a substrate. Since endo-type GH enzyme splits the internal glycosidic bonds of the polysaccharide chain randomly, a few breaks in the chain would rapidly reduce the viscosity of aqueous solutions of polysaccharides. As shown in Fig. 4, CJP38 rapidly reduced the viscosity of CM-curdlan solution (within 30 min), while it linearly increased the reduced sugar concentration. The results obtained here indicate that CJP38 is an endo-type β-1,3-glucanase. The degradation and transglycosylation products of laminarioligosaccharides produced by CJP38 were analyzed by TLC (Fig. 5A-E). Laminaribiose (L2) was not hydrolyzed by the enzyme, while laminaritriose (L3) was converted to glucose (G) and L2. When laminarioligosaccharides with a degree of polymerization greater than 4 were used as substrate, in addition to glucose and shorter laminarioligosaccharides than the starting substrates, longer ones were observed, indicating that CJP38 catalyzes transglycosylation reactions in addition to hydrolysis. In order to clarify the splitting sites in laminarioligosaccharides, *p*NP-laminarioligosaccharides, in which the reducing end is modified with *p*-nitrophenol (*p*NP), were used as the substrates (Fig. 6A-C). *p*NP-laminaribiose (*p*NP-L2) was slightly hydrolyzed into *p*NP and L2. When *p*NP-laminaritriose (*p*NP-L3) and *p*NP-laminaritetraose (*p*NP-L4) were used as the substrates, the second and third glycosidic bonds from the non-reducing ends of the substrates were mainly cleaved, producing *p*NP-glycoside (*p*NP-G) and L2 or L3, respectively (Fig. 6B-C). In these cases, transglycosylation products with and without *p*NP at their reducing ends were also detected. For example, *p*NP-laminaripentaose (*p*NP-L5) and *p*NP-laminarihexaose (*p*NP-L6) appeared to be synthesized from initial substrates *p*NP-L3 or *p*NP-L4 and the hydrolyzed product L2, respectively. These results indicate that CJP38 has a strong preference for hydrolysis of the internal glycosidic linkages of laminarioligosaccharides. CJP38 did not hydrolyzed gentiooligosaccharides (β-1,6-linked glucooligosaccharides) and cellooligosaccharides (β-1,4-linked glucooligosaccharides), indicating that this enzyme specifically hydrolyzed β-1,3-glucosidic linkage (data not shown). Unfortunately, enzymatic properties of the other two major allergens, Mus a 5 and Hev b 2, are not well characterized yet. Therefore, we were unable to thoroughly compare the enzymatic properties of these proteins. However, it remains of interest to understand the enzymatic functions of these proteins.

3.4. Crystal structure of CJP38

The crystallography study showed that CJP38 has $(\beta/\alpha)_8$ TIM barrel

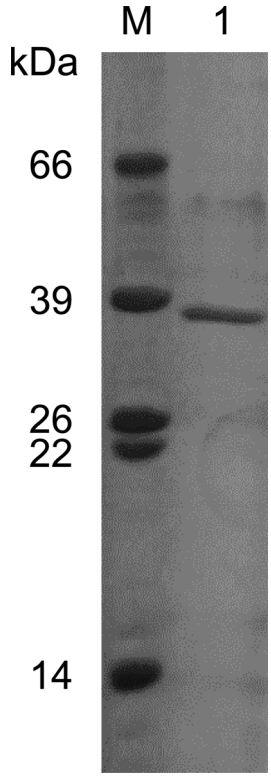

Fig. 2. SDS-PAGE of the purified CJP38 expressed in *E. coli*.
Lane M indicates molecular weight markers. Lane 1, purified CJP38.

structure composed of α-helices and β-strands, which is a typical structure of GH17 family members (Fig. 7A). To date, only two crystal structures of allergenic β-1,3-glucanase, Mus a 5 and Hev b 2, have been solved. Fig. 7B shows the superimposition of the CJP38 structure with those of Mus a 5 (PDB ID 2CYG) and Hev b 2 (PDB ID 4HPG) (Receveur-Bréchot et al., 2006; Rodríguez-Romero et al., 2014). The structure of CJP38 was very similar to those of Mus a 5 and Hev b 2, with RMSDs of 0.706 and 0.709 Å for superimposition of the corresponding 249 and 243 Cα atoms, respectively. It was found that there is a long substrate-binding cleft spanning across the molecular surface of

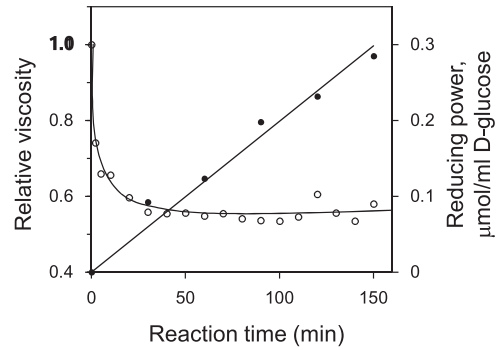

Fig. 4. Viscosity loss of CM-curdlan and release of reducing sugars by CJP38. The reaction mixture contained 0.2% CM-curdlan and 0.8 μM CJP38 in 20 mM sodium acetate buffer, pH 5.0. The enzyme reaction was started by the addition of CJP38 and decreases of viscosity were measured by an Ostwald viscometer at the indicated intervals. Initial viscosity was measured by adding 20 mM sodium acetate buffer, pH 5.0 instead of enzyme solution, and this was taken to be 1.0. Increases of reducing power were also measured at 30 min intervals. In both cases, the reaction was carried out at 25 °C. Open and closed circles indicate the relative viscosity and the reducing power, respectively.

CJP38 as there are in Mus a 5 and Hev b 2, accounting for its endo mode of action (Fig. 8 bottom). The active site of family GH17 β-1,3-glucanases consist of two glutamates that act as proton donor and nucleophilic residues, respectively. These glutamate residues are strictly conserved in the active site of the allergenic glucanases and correspond to E96 (proton donor residue) and E240 (nucleophile residue) in CJP38. Furthermore, Rodríguez-Romero et al. suggested that several aromatic residues in Hev b 2 directly interact with laminarioligosaccharides based on the three-dimensional structure of potato endo-β-1,3-glucanase in complexed with the oligosaccharides (Rodríguez-Romero et al., 2014; Wojtkowiak et al., 2013). These aromatic residues are all conserved in the allergenic glucanases: Y34, Y178, F284 and F300 in Hev b 2 (corresponding to Y33, Y174, F281 and F297 in Mus a 5, respectively; corresponding to F35, Y179, F286 and F303 in CJP38, respectively) (Fig. 7B left). From these results, we assume that these allergenic glucanases share similar enzymatic properties and substrate specificity. Two amino acid sequence regions, 17-AGDVVSLMKKNNIGKMRIFGP-NAD-40 and 261-NLIKHVLSNAGTPKRPG-277 that correspond to those of surface-exposed two-consensus IgE-binding epitopes (epitope 1 and

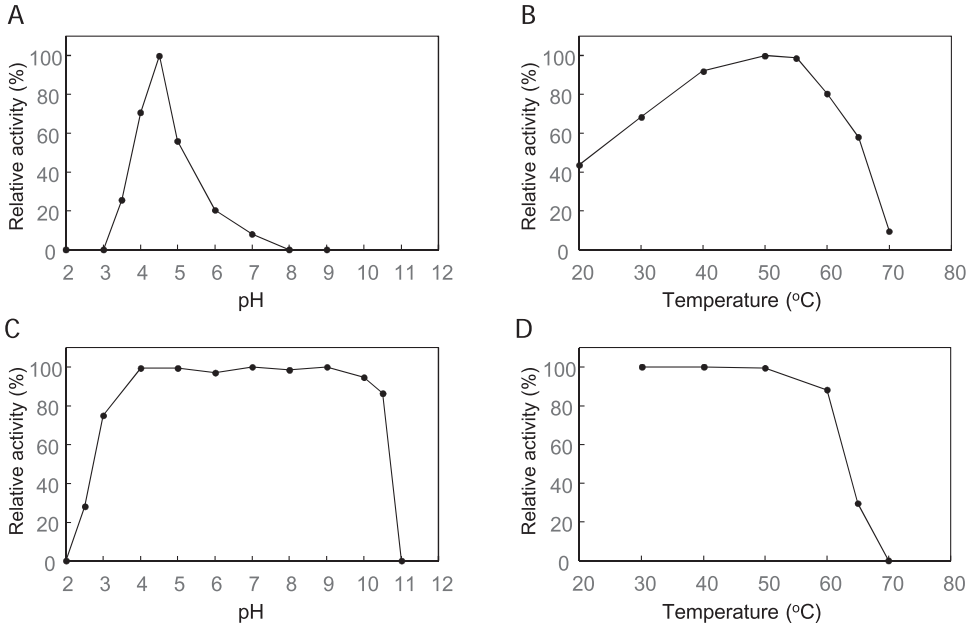

Fig. 3. Effect of pH and temperature on β-1,3-glucanase activity and stability of CJP38.
A, The optimum pH was determined by measuring the activities at various pHs at 37 °C. B, The optimum temperature was determined by measuring the activities in 20 mM sodium acetate buffer, pH 5.0, at various temperatures. C, The pH stability was determined by measuring the residual activities after incubation at various pHs at 37 °C for 1 h. D, The thermal stability was determined by measuring the residual activities after incubation in 20 mM sodium acetate buffer, pH 5.0, at various temperatures for 1 h. The residual activities were measured at pH 5.0.

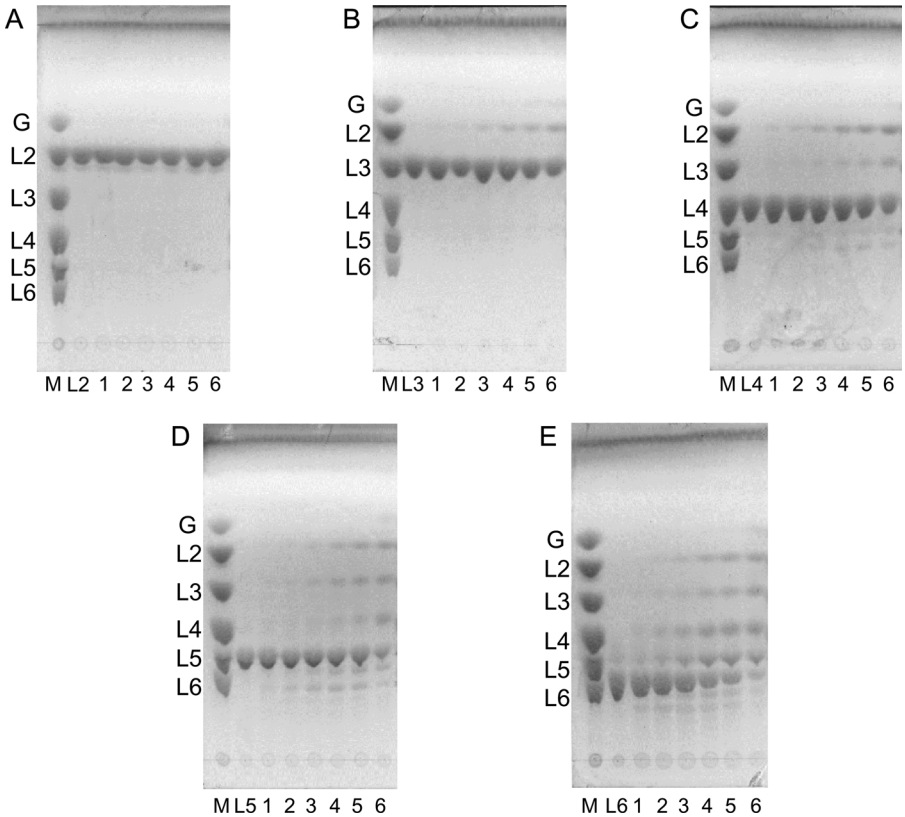

Fig. 5. Time-courses of laminarioligosaccharide hydrolysis of CJP38 as analyzed by TLC.
A reaction mixture containing 2 μM CJP38 and 5 mM of laminariorigosaccharide in 20 mM sodium acetate buffer, pH 5.0, was incubated at various times at 25 °C, and then analyzed by TLC. Sugar products were detected with methanol containing sulfuric acid. A, substrate laminaribiose (L2). B, substrate laminaritriose (L3). C, substrate laminaritetraose (L4). D, substrate laminaripentaose (L5). E, substrate laminarihexaose (L6). Lane M, marker; Lanes 1–6, incubation at 2, 5, 10, 20, 30, 60 min, respectively; G, glucose.

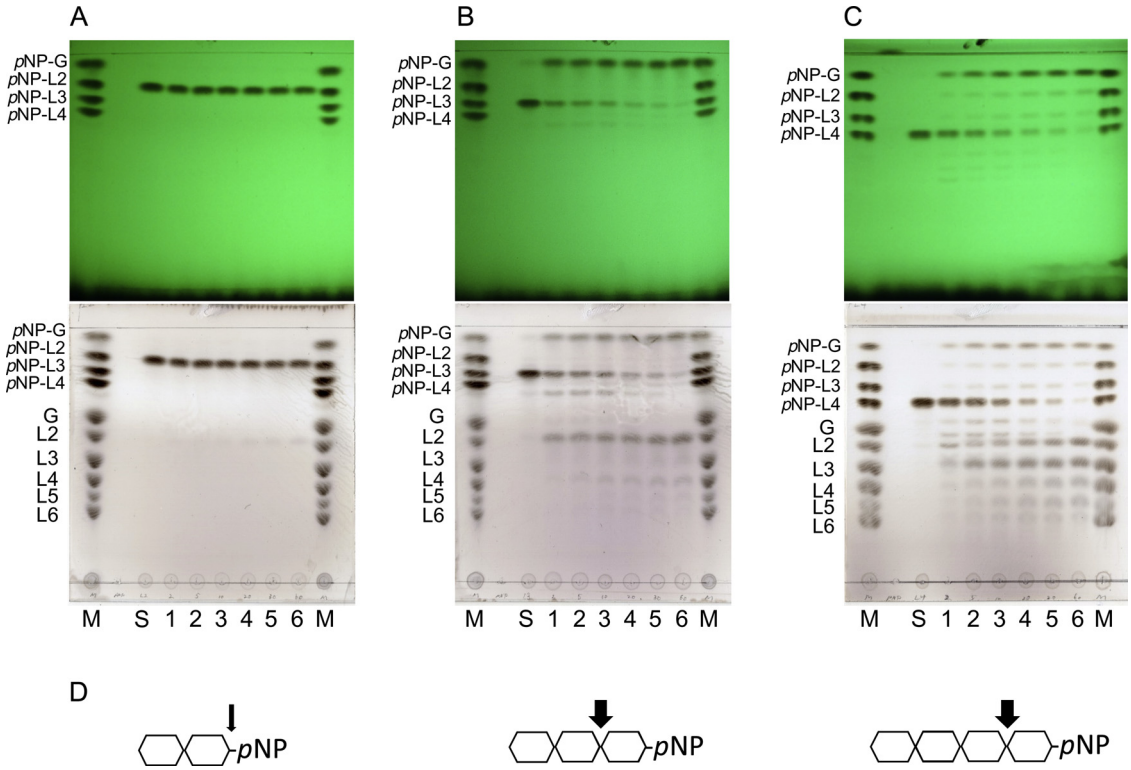

Fig. 6. Time-courses of *p*NP-laminarioligosaccharide hydrolysis of CJP38 as analyzed by TLC.
A reaction mixture containing 2 μM CJP38 and 5 mM of *p*NP-laminariorigosaccharide in 20 mM sodium acetate buffer, pH 5.0 was incubated at various times at 25 °C, and then analyzed by TLC. Sugar products were visualized under UV light at 254 nm (upper panels) and detected with methanol containing sulfuric acid (lower panels). A, substrate *p*NP-laminaribiose (*p*NP-L2). B, substrate *p*NP-laminaritriose (*p*NP-L3). C, substrate *p*NP-laminaritetraose (*p*NP-L4). Lane M, marker; S, substrate; Lanes 1–6, incubation at 2, 5, 10, 20, 30, 60 min, respectively; *p*NP-G, *p*NP-glucose. D, Schematic representation of the main cleavage sites of *p*NP-laminarioligosaccharides by CJP38. Arrow size represents the relative hydrolytic rate. White hexagons represent the glucose unit; *p*NP indicates the *p*-nitrophenyl group.

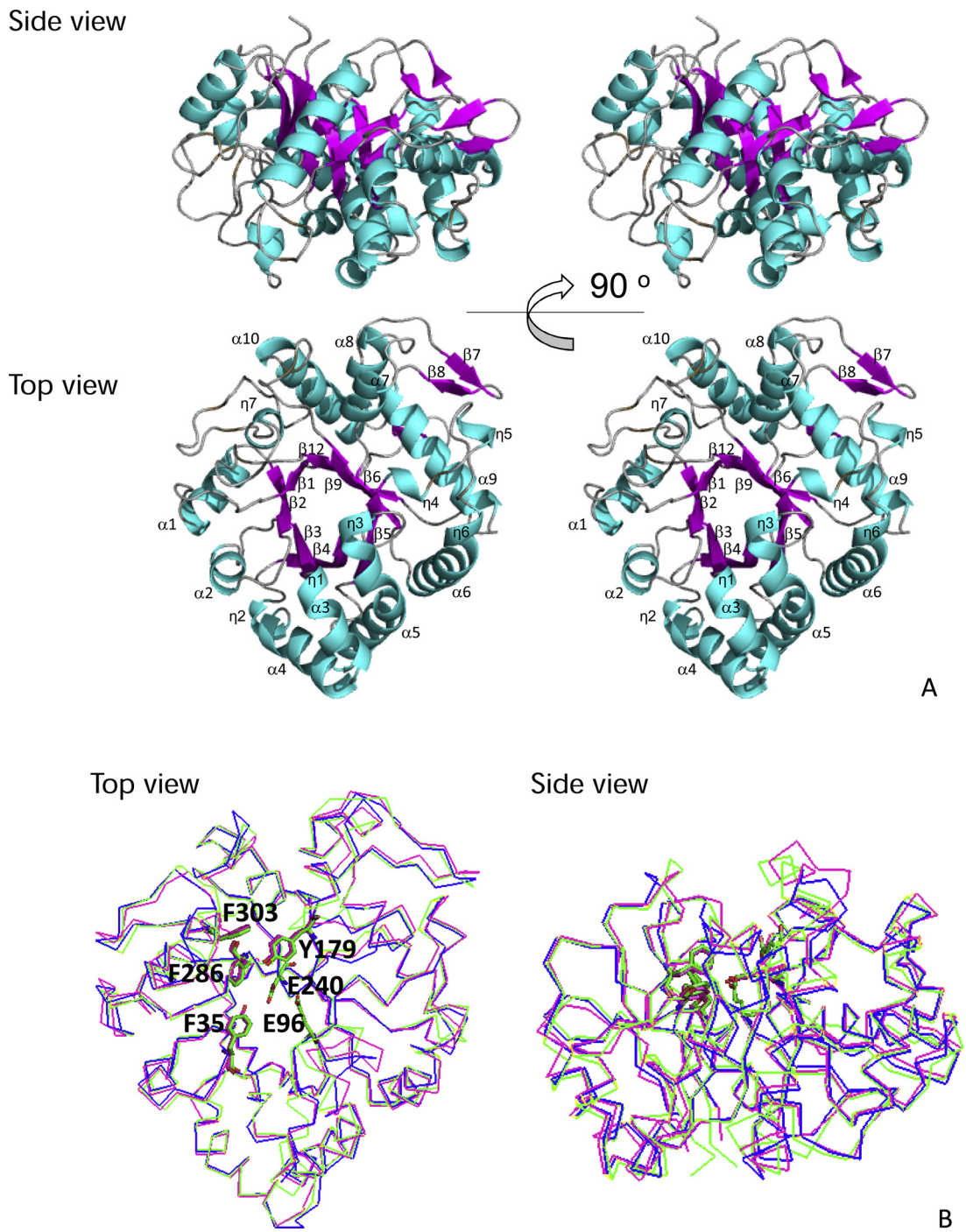

Fig. 7. Three-dimensional structure of CJP38.
A, Stereo view of the overall fold of CJP38 shown by ribbon model. The secondary structure elements are numbered and colored magenta (β-sheets) and cyan (α- and 3_{10} helices), respectively. Side view, side view orientation (top). Top view, top orientation (bottom). B, Superimposition of Cα traces of Hev b 2 (magenta; PDB ID 34HPG), Mus a 5 (blue; PDB ID 2CYG) and CJP38 (green). Top view, top view orientation (left). Side view, side view orientation (right). Amino acid residues involved in catalysis (E96 and E240) and substrate binding (F35, Y179, F286 and F303) in CJP38 were illustrated by a stick model and labeled.
Aromatic amino acid residues involved in substrate binding in Mus a 5 and Hev b 2 were also illustrated by a stick model.

2, respectively), identified in Mus a 5 and Hev b 2 (Fig. 1), were found on the molecular surface of CJP38. The first sequence region is localized in α-helix 1, β-strand 2, N-terminal part of α-helix 2 and two loops connecting these three structural elements. The second region is localized in the C-terminal part of α-helix 10 and the following loop structure containing two β-strands (β-strands 10 and 11). These two regions not only share sequence similarity, but also show conformations very similar to those of Mus a 5 and Hev b 2 (Fig. 8). Therefore, these two regions may be involved in the cross-reactivity among CJP38, Mus a 5 and Hev b 2 that contribute to the pollen–latex–fruit syndrome. However, since these two epitopes are identified by using the overlapping linear peptides which cover entire amino acid sequences of Mus a 5 and Hev b 2, discontinuous conformational epitopes on the allergen proteins might be overlooked.

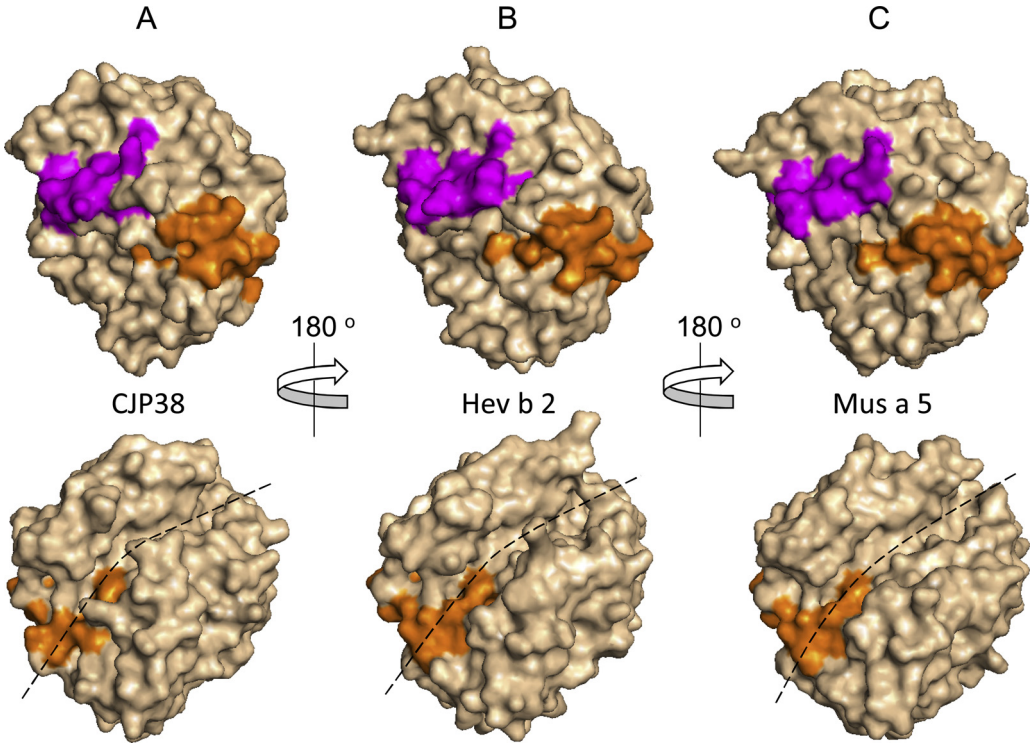

Fig. 8. Structural mapping of the two putative consensus epitopes on the molecular surfaces of β-1,3-glucanase allergens. Epitope 1 and 2 regions were colored **orange** and magenta, respectively. A, CJP38. B, Hev b 2. C, Mus a 5. Dashed lines indicate the substrate-binding clefts.

4. Conclusion

Palomares et al. showed that sera from patients allergic to olive pollen allergen recognize the N-terminal part (β-1,3-glucanase domain) of Ole e 9 (Palomares et al., 2005). They also detected homologous counterpart proteins to Ole e 9 not only in extracts of ash and birch pollen, but also in tomato, pepper, banana, and hevea latex. From these results, they implicated the allergenic potential of β-1,3-glucanases from different allergenic sources to cause the pollen–latex–fruit syndrome. In this study, a recombinant form of CJP38 from *C. japonica* pollen was expressed, purified, and characterized. We determined the three-dimensional structure of CJP38 and compared it to those of the allergens Mus a 5 and Hev b 2. As a result, two regions, which are sequentially and conformationally similar to two-consensus IgE-epitopes accounting for the cross-reactivity between Mus a 5 and Hev b 2, were found on the molecular surface of CJP38. Therefore, we suggest that CJP38 is a candidate allergen responsible for the pollen–latex–fruit syndrome related to Japanese cedar pollinosis. To investigate this possibility, we are planning to conduct quantitative IgE-inhibition experiments with Mus a 5 and Hev b 2 and measure the inhibition of CJP38-specific IgE binding. Identification of precise continuous and discontinuous IgE epitopes in CJP38 is necessary in order to develop therapeutic strategies for this syndrome.

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Declaration of Competing Interest

The authors declare no competing financial interest.

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