

Molecular cloning of a class IV chitinase allergen from Japanese cedar (*Cryptomeria japonica*) pollen and competitive inhibition of its immunoglobulin E-binding capacity by latex C-serum

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Summary

Background Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most prevalent allergic diseases in Japan. Only three *C. japonica* allergens, Cry j 1, Cry j 2, and CJP-6, have been characterized. The full IgE-binding spectrum of *C. japonica* pollen allergens demonstrates that many allergens remain to be identified.

Objective The aim of this study was to characterize a novel allergen with a high frequency of IgE binding.

Methods The cDNA coding for a high-frequency IgE-binding protein, designated CJP-4, was cloned from the total mRNA of *C. japonica* pollen. The corresponding native allergen was purified by affinity precipitation with colloidal chitin and gel chromatography. The IgE-binding ability of purified native CJP-4 was characterized by ELISA and ELISA inhibition.

Results The CJP-4 cDNA encoded 281 amino acids with significant sequence homology to class IV chitinases. Purified native CJP-4, migrated as a homogeneous 34-kDa protein on SDS-PAGE, revealed endochitinase activity on native PAGE. The purified protein displayed the ability to bind IgE from all patients tested (31/31) in ELISA, whereas Cry j 1 bound to IgE at a 71% frequency (22/31). Pre-incubation with latex C-serum completely inhibited the reaction of pooled sera IgE from patients with *C. japonica* pollinosis and/or latex allergy to purified CJP-4.

Conclusion We identified CJP-4 as a novel and fourth *C. japonica* chitinase allergen with high IgE-binding frequency. The competitive IgE-binding profile between *C. japonica* chitinase and latex C-serum indicated that *C. japonica* chitinase should be an important pan-allergen in *C. japonica* pollen.

Keywords chitinase, *Cryptomeria japonica* pollinosis, Japanese cedar pollen, latex allergy, pan-allergen

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Introduction

Japanese cedar (*Cryptomeria japonica*) pollen, a major source of aeroallergen in Japan, elicits rhinitis and conjunctivitis in early spring. *C. japonica* pollinosis has become a serious problem; its prevalence has increased steadily, particularly in the younger generation [1]. The existence of numerous allergens in *C. japonica* pollen has been suggested [2, 3], but only a few antigens have been identified. One is Cry j 1, a major allergen [4, 5] with pectate lyase activity [6], which is

homologous to the major short ragweed allergen Amb a 1 family [5]. Another is Cry j 2, a major allergen possessing polymethylgalacturonase activity [7–10]. The third is CJP-6 [3], an additional important allergen homologous to the isoflavone reductase family, which includes birch pollen allergen Bet v 6 [11] and pear allergen Pyr c 5 [12].

In our preceding study [13], we elucidated the full IgE-binding spectrum of *C. japonica* pollen by means of allergenomic analysis. *C. japonica* pollen-allergic patients ($n = 40$) showed distinguishable IgE-binding profiles with 4–86 (mean $SD = 37 \pm 22$) spots in a total of 131 IgE-reactive spots on two-dimensional (2-D) electrophoretograms of a *C. japonica* pollen extract. In addition, 31 unidentified spots had higher IgE-binding frequency than any Cry j 2 isoforms. These important allergens should be characterized, based on the frequency of IgE binding, to establish accurate diagnosis and effective immunotherapy for *C. japonica* pollinosis. Among the tryptic digests of six spots selected from the 31, the

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internal amino acid sequence of spot number 101 (this spot is referred to as CJP-4, with 52.5% (21/40) IgE-binding frequency on 2-D PAGE) was highly homologous to that of plant class IV chitinases.

Plant chitinases play major roles as PR-3 proteins in defence mechanisms against fungal and insect pathogens. Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of the β -1,4-*N*-acetyl-D-glucosamine linkages in chitin polymers. They belong to either family 18 or 19 in the classification of glycosyl hydrolases [14, 15]. Furthermore, family 19 chitinases have been divided into five classes based on their amino acid sequence features [16]. In particular, class I and class IV chitinases are clinically important on account of their high allergenicity. Class I chitinases are proteins of around 30 kDa. Each class I chitinase has an N-terminal hevein-like domain of about 40 amino acids. This domain is predominantly responsible for IgE binding [17], and they each contain a highly conserved catalytic domain [18, 19]. Class I chitinase allergens, Pers a 1 (avocado, *Persea americana*) [20], Mus a 1 (banana, *Musca acuminata*) [21], and Cas s 5 (chestnut, *Castanea sativa*) [22], are well known as major cross-reactive molecules responsible for the latex-fruit syndrome. IgE-mediated hypersensitivity caused by hevein (Hev b 6.02 [23]), prohevein (Hev b 6.01 [24]), class I chitinase (Hev b 11 [25]), and other allergens in latex (*Hevea brasiliensis*) has been recognized as a very important international health problem for health-care workers. Class IV chitinases each contain a similar N-terminal hevein-like domain and a catalytic domain with 41–47% amino acid sequence identity to the catalytic domain of class I chitinase. In the class IV chitinase family, only grape (*Vitis vinifera*) class IV chitinase isoforms have been reported as allergenic enzymes [26]. No information is available yet on the potential for cross-reactivity and/or cross-sensitization between allergenic latex and class IV chitinases.

This study describes the molecular cloning of *C. japonica* class IV chitinase as a fourth important allergen and the IgE-binding activity of the purified native allergen in *C. japonica* pollen. We also present a study that we designed to test the hypothesis that latex C-serum might competitively inhibit the binding of IgE to the purified *C. japonica* class IV chitinase allergen.

Materials and methods

C. japonica pollen

C. japonica pollen was collected in 2003 on Mikado island, Hiroshima, Japan, and was stored at -20°C until use in antigen preparation.

Human sera

C. japonica pollen-allergic sera were obtained from 31 patients selected on the basis of clinical history, seasonal symptoms, and positive value against *C. japonica* pollen-specific IgE (RAST score 2 or higher). Latex-allergic sera were obtained from three subjects selected according to their clinical histories, positive prick tests, and positive RAST. Among them, two latex-allergic patients had RAST scores of

3 against *C. japonica* pollen extract, while the other was not determined. Non-allergic sera from three healthy volunteers were used as a negative control. These sera were stored at -20°C until use. All experiments were approved by the Institutional Review Board of Takanobashi Central Hospital and were described in detail to all participants before they provided their informed consent and were admitted into the study.

cDNA synthesis

Anther (2 g) of *C. japonica* was crushed in liquid nitrogen and extracted with tenfold the volume (v/w) of 2% (w/v) cetyltrimethylammonium bromide, 20 mM ethylenediamine tetraacetic acid (EDTA), and 1.4 M sodium chloride in 0.1 M Tris-HCl buffer (pH 9.5) for 10 min at 65°C . Equal volumes of chloroform:isoamyl alcohol (24:1, v/v) were added to the extract solution and centrifuged at $20630g$ for 10 min at room temperature. After the procedure was repeated, total RNA was recovered by successive precipitation with isopropanol and LiCl [3]. The degenerate primers, 5'-GARATHGCNGCNTTYTTYGC-3' (sense) and 5'-ATNGTNGCNCRAANCCYTG-3' (antisense), were constituted from the amino acid sequences homologous to the class IV chitinases from *Arabidopsis thaliana* (accession number BT010422), *Daucus carota* (U52845–8), *Oryza sativa* (AB054687, AB054811), *V. vinifera* (U97521–2, AF532966), and *Picea abies* (AY270018–9) to obtain a partial fragment of *C. japonica* chitinase cDNA. After the oligo(dT)-containing adapter primer was extended as a template, 3'-RACE was carried out using the nested primers and universal amplification primer according to the manufacturer's instructions for the 3'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Groningen, the Netherlands) [27]. Similarly, cDNA was extended in the 5'-direction using the nested primers and an abridged anchor primer or universal amplification primer in accordance with the instruction manual for the 5'-RACE System Version 2.0 (Invitrogen). Finally, the full length of double-stranded *C. japonica* chitinase cDNA was synthesized by 3'-RACE.

Colloidal chitin

After a chilled chitin suspension (2 g/50 mL) was added to the same volume of chilled sulphuric acid, the mixture was filtered through on glass wool to remove insoluble impurities. The filtrate was dropped into ice-cold water (90 mL), and the resultant colloidal suspension was centrifuged at $2000g$ for 25 min at 4°C . The precipitate was washed several times with distilled water and phosphate-buffered saline (PBS) until the pH was neutral [28].

Purification of CJP-4

C. japonica pollen (80 g) was suspended into 20 mM PBS (pH 7.4) containing 3 mM EDTA for 6 h at 4°C . After centrifugation at $9300g$ for 35 min at 4°C , ammonium sulphate was added to the supernatant until it was 80% saturated. The resultant precipitate was dialysed against 20 mM acetate buffer (pH 6.0) and centrifuged. One gram wet weight of colloidal chitin was added to the supernatant (200 mL). The suspension was stirred overnight at 4°C and then centrifuged.

The resultant precipitate was collected and washed with 20 mM PBS (pH 7.4) containing 0.1% (v/v) Tween-20. After centrifugation, the precipitate was suspended into 100 mM acetate buffer (pH 5.0) to make a final volume of 5 mL. The suspension was incubated at 50 °C for at least 6 h to solubilize the colloidal chitin. It was then recovered by centrifugation and lyophilized. The lyophilizate was referred to as a CJP-4-enriched protein. This protein (about 0.05 mg), in 0.5 mL of 20 mM PBS (pH 7.4), was applied to a Superdex 75 HR 10/30 column (Amersham Biosciences, Buckinghamshire, UK) pre-equilibrated with the same buffer and gel-chromatographed at a flow rate of 0.2 mL/min. Fractions containing CJP-4 were pooled and re-chromatographed on the same column. The purified CJP-4 gave a single band on SDS-PAGE. It was then stored at -20 °C until use.

Detection of chitinase activity on a native polyacrylamide gel

Endochitinase activity was assayed by a modified method of Trudel and Asselin [29]. Briefly, PAGE was performed on an 11.5% slab gel containing 0.01% (w/v) ethyleneglycol chitin (Seikagaku Corp., Tokyo, Japan) under native conditions, and with neither SDS nor dithiothreitol (DTT), by using the discontinuous buffer system of Laemmli [30]. After electrophoresis, the gel was incubated for 2 h at 40 °C with reciprocal shaking in 100 mM acetate buffer (pH 5.0), followed by incubation with 0.01% (w/v) calcofluor white M2R (Sigma-Aldrich, St Louis, MO, USA) in 500 mM Tris-HCl (pH 8.9) for 5 min. The brightener solution was removed and the gels were allowed to stand for 1 h in distilled water. Lytic zones were visualized by a UV transilluminator.

SDS-PAGE and immunoblotting

Protein samples (crude pollen extract, CJP-4-enriched protein, and purified CJP-4) were analysed by SDS-PAGE on a 12.5% slab gel under reduced conditions with 50 mM DTT [30]. Proteins were then detected with PhastGel™ Blue R (Amersham Biosciences) and/or blotted onto a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences). The blot was probed with IgE in sixfold-diluted sera from a *C. japonica*-allergic or a latex-allergic patient. The membrane was overlaid with biotin-conjugated goat anti-human IgE antibody (1:20 000 dilution; Vector Laboratories Inc., Burlingame, CA, USA), followed by reaction with horseradish peroxidase-conjugated streptavidin (1:30 000 dilution; Zymed Laboratories Inc., San Francisco, CA, USA). After immunolabeling, the bands were visualized on an X-ray film (Amersham Biosciences) using an ECL Advance Western Blotting Detection Kit (Amersham Biosciences).

Protein sequencing of purified CJP-4

Purified CJP-4 was electrophoretically transferred onto a Hybond-P membrane. Prior to protein sequencing, the blotted protein was visualized with PhastGel™ Blue R (Amersham Biosciences). The corresponding protein band was excised from the membrane, destained, and enzymatically digested with 10 U of thrombin (Amersham Biosciences) overnight at ambient temperature, because the N-terminal of the purified CJP-4 was blocked. The degradation product was

subject to N-terminal protein sequencing using a protein sequencer (Applied Biosystems model 492, Warrington, UK).

Preparation of latex C-serum

Latex proteins were fractionated according to a modified protocol [31]. Briefly, an ammoniated latex sap (Nishikawa Rubber Co., Ltd, Hiroshima, Japan) was centrifuged at 40 000 *g* for 1 h at 4 °C. A translucent aqueous layer known as C-serum was obtained and was then centrifuged to remove residual latex particles. The pH value of the C-serum was adjusted to 7.0 with acetic acid. The C-serum was then dialysed against water, lyophilized, and stored at -80 °C until use.

Tryptic in-gel digestion of CJP-4 on two-dimensional PAGE gel

In-gel digestion was carried out according to our preceding paper [13]. Briefly, CJP-4 spot was excised from dry 2-D gel and rehydrated in 100 mM ammonium carbonate. The gel piece was destained by several washings with water and a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulphate. After drying in a centrifugal vaporizer, the piece was rehydrated in a digestion buffer containing 50 mM ammonium bicarbonate, 5 mM calcium chloride, and 0.1 mg/mL of modified sequence-grade trypsin (Promega, Madison, WI, USA), and protein was in-gel digested overnight at 37 °C. After the digestion, the resultant peptides were extracted three times with 5% (v/v) trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile. The extract was dried in a centrifugal vaporizer, resuspended in 0.1% (v/v) formic acid in 5% (v/v) methanol, and loaded onto a Ziptip column (Millipore, Bradford, MA, USA). After the column was washed with 0.1% (v/v) TFA and subsequently with 0.1% (v/v) TFA in 5% (v/v) methanol, the adsorbed peptides were eluted with 0.1% (v/v) formic acid in 50% (v/v) methanol.

Electrospray ionization mass spectrometry and de novo sequencing

Peptides loaded in Au/Pd-coated nanoES spray capillary (Protana, Odense, Denmark) were inserted onto nanoflow Z-spray source of a Q-TOF MS (Micromass, Manchester, UK). Mass spectrometry (MS) mode was used to scan tryptic peptides. Subsequently, *de novo* sequencing was carried out to identify the internal amino acid sequences of tryptic fragments.

ELISA and ELISA inhibition test

The IgE-binding frequency of purified Cry j 1 (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) and that of purified native CJP-4 were evaluated by ELISA. In brief, 50 µL of purified Cry j 1 or native CJP-4 solution (200 ng/mL in 100 mM bicarbonate buffer, pH 9.4) was coated onto a 96-well microtitre plate (NUNC-Immuno® Plate Maxisorp F96, Nalge Nunc International, Roskilde, Denmark), and incubated at room temperature for 2 h. After the plate was blocked with blocking buffer (3% (w/v) skim milk, 1% (w/v) bovine serum albumin, and 0.1% (v/v) Tween-20 in PBS) overnight at 4 °C, 50 µL of fiftyfold-diluted sera from patients

allergic to *C. japonica* pollen or latex were supplemented into the wells and incubated for 4 h at room temperature.

Cross-reactivity between CJP-4 and latex C-serum was assessed by ELISA inhibition test. Briefly, purified CJP-4 (20 ng/mL in 100 mM bicarbonate buffer, pH 9.4) was coated onto a 96-well microtitre plate by incubation for 2 h at room temperature, and then incubated overnight at 4 °C with blocking buffer. Prior to the reactions of patients' sera with the coated antigen, pooled sera (diluted 1 : 2800) of the latex-allergic patients ($n = 3$) or pooled sera (diluted 1 : 1600) of the *C. japonica* pollinosis patients ($n = 5$) were pre-incubated with serially diluted competitors (CJP-4-enriched protein, latex C-serum, or OVA) overnight at 4 °C [32]. The pre-absorbed sera were then applied to the purified CJP-4-coated wells and incubated for 4 h at room temperature.

After 4 h incubation with patients' or pre-absorbed patients' sera, 50 µL of biotinylated anti-human IgE (ε chain-specific, Vector Laboratories), diluted 1 : 1000 in the blocking buffer, was added and incubated for 2 h at room temperature, followed by the addition of 50 µL alkaline phosphatase-conjugated streptavidin (Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA) diluted 1 : 1000 in the same buffer for 1 h. AttoPhos[®] substrate solution (Roche, Mannheim, Germany) was added and incubated at room temperature. Fluorescence intensity was measured for each sample well using a Wallac 1420 ARVOsx[™] multilabel counter (Perkin Elmer Life Sciences, Boston, MA, USA).

Results

Molecular cloning and identification of CJP-4 in C. japonica pollen

The internal amino acid sequence from tryptic in-gel digestion of CJP-4 was highly homologous to that of plant class IV chitinases. After a partial nucleotide sequence of CJP-4 was acquired by RT-PCR between the regions highly homologous to plant class IV chitinases (open squares in Fig. 1), the complete length of the CJP-4 nucleotide sequence was obtained by the 3'- and 5'-RACE. The CJP-4 cDNA consisted of 1041 nucleotides containing an 846-bp open reading frame encoding a 281-amino acid polypeptide with a calculated isoelectric point of 4.51 and a molecular weight of 29 286 Da. The deduced amino acid sequence showed significant homology with the class IV chitinases from plants. It includes one chitin-binding motif (boldface in Fig. 1) and a hevein-like domain (a double underline in Fig. 1) at the N-terminal region and two consensus family 19 chitinase signature motifs (boldface in Fig. 1). The sequence MECDG-GNAATVAS, in trypsin-digested fragments of CJP-4 analysed by ESI Q-TOF MS, as well as the N-terminal sequence of thrombin-digested CJP-4, coincided completely with the corresponding regions of the deduced amino acid sequence (underlines in Fig. 1).

Multiple sequence alignment showed high degrees of identity (43.1–65.6%) between CJP-4 and the class IV chitinases previously described in other plant species (Fig. 2). The deduced CJP-4 polypeptide sequence also displayed deletions in the catalytic domain characteristic of the class IV

chitinases. These data supported the hypothesis that CJP-4 cDNA might correspond to a *C. japonica* class IV chitinase gene. CJP-4 had a 38.6% and 61.9% sequence identity with corresponding hevein-like domain and chitin-binding domain of latex hevein (Hev b 6.02), respectively. CJP-4 also had a high sequence identity with class I chitinase allergens such as Hev b 11 (38.4% identity), Pers a 1 (38.2%), Mus a 1 (41.5%), and Cas s 5 (36.1%) (Fig. 2). The sequence identities increased to 44.2–47.4% when the comparison was carried out in the hevein-like domains conserved at the N-terminals of respective allergens that have been considered to play crucial roles in the corresponding allergies.

Identification of native CJP-4 and determination of CJP-4-specific IgE levels in the sera of patients allergic to C. japonica pollen

Native CJP-4 was purified by affinity precipitation with colloidal chitin and gel filtration chromatography. A homogeneous band was obtained at 34 kDa on SDS-PAGE under reduced conditions (Fig. 3a). The purified CJP-4 on a blot was recognized with sera IgE from patients allergic to *C. japonica* pollen, but not with that from non-allergic subjects (Fig. 3b). We also examined whether or not the native CJP-4 had an endochitinase activity. A clear lytic band was observed by activity staining with ethyleneglycol chitin after native PAGE (arrowhead in Fig. 3c). Thus, the purified CJP-4 was confirmed to be a *C. japonica* class IV chitinase. Some minor lytic bands were also detected below the major lytic band, parallel to the silver staining of the corresponding protein bands. The discrepancy in the electrophoretic mobilities (single band on SDS-PAGE and plural bands on native PAGE) may reflect the occurrence of endochitinase isozymes.

ELISA was performed using the sera of the 31 *C. japonica*-allergic patients to analyse IgE-binding frequency and specific IgE levels of CJP-4. All of these *C. japonica* pollinosis patients (31/31) exhibited an IgE-binding ability against CJP-4, while a positive reaction to Cry j 1 was detected in the sera of 22 of the 31 patients (71%) (Fig. 4). CJP-4-specific IgE levels in the patients' sera were comparable with or slightly higher than Cry j 1-specific IgE levels, except for the number of seven sera.

Cross-reactivity of sera from latex-allergic patients with C. japonica class IV chitinase

As *C. japonica* class IV chitinases each possess a hevein-like domain highly homologous to that of class I chitinases, we assessed the cross-reactivity of *C. japonica* class IV chitinase with latex C-serum using pooled IgE from patients with *C. japonica* pollinosis or latex allergy in competitive ELISA. Pre-incubation with latex C-serum completely inhibited the IgE reaction in pooled *C. japonica*-allergic patients' sera to the purified *C. japonica* class IV chitinase (Fig. 5a). Similarly, pre-incubation with latex C-serum completely inhibited the IgE binding in the pooled sera of latex-allergic and *C. japonica*-allergic patients to the purified *C. japonica* class IV chitinase (Fig. 5b). These results support the possibility that *C. japonica* class IV chitinase-specific IgE in both pools of sera could cross-react with latex C-serum. The results also suggest that *C. japonica* class IV chitinase should be a pan-allergen among patients with *C. japonica* pollinosis and latex allergy,

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1 ATAGAATGCAAATCATGGCTACACAAAATAGTAAGAGCAACATCTTCTGGTCCTCTTCAG 60
  M Q I M A T Q N S K S N I F W S S S A 19

CATCTGTAGTGCTTGTTTTACTGCTTGTAGATGTGGGAGTGTGTCAAAATTGTGGAT 120
  S V V L V L L L L V D V G V C Q N C G C 39

GTAATGGACTGTGTTGCAGTCAGTATGGATACTGTGGAAGTGAGAGGCCTACTGTGGGG 180
  N G L C C S Q Y G Y C G S G E A Y C G A 59

CTGGTTGTAAAGAAGGTCCCTGTTCTTCTTCTTCTCCCTTCTACTGGCACTGGAGTGG 240
  G C K E G P C S S S S P P S T G T G V G 79

GCTCTATAGTTTCTCTGATGTTTTTAACCTCTATAGTTGGAGGAGCTGCCAGTGGGTGTG 300
  S I V S S D V F N S I V G G A A S G C A 99

CAGGAAATGGGTTTTATACATACGATTCCTTTATTAGCGCTGCTAATGCTTTCAATGGCT 360
  G N G F Y T Y D S F I S A A N A F N G F 119

TTGGGACATCTGGGTCTTCTGATGTTAATAAGAGAGAAATCGCCGCCTTCTTTGCTAATG 420
  G T S G S S D V N K R E I A A F F A N A 139

CTGCCCATGAGACTGGAGGGTTTTGCTACATCGAAGAGCAGAATCCAACCAGCATATACT 480
  A H E T G G F C Y I E E Q N P T S I Y C 159

GCGATGCCTCCAACACTCAGTATCCCTGCGCTCCGGCAAGACTTATCATGGCAGAGGCC 540
  D A S N T Q Y P C A S G K T Y H G R G P 179

CTCTGCAGCTCTCATGGAACATAAATACTACGGGCGGCAGGAAGCTACATTCAATTTGATG 600
  L Q L S W N Y N Y G A A G S Y I Q F D G 199

GGCTGAACAATCCAGAGATTGTGGGCACTGATTTCGACCATCTCATTTAAGACGGCGGTGT 660
  L N N P E I V G T D S T I S F K T A V W 219

GGTTTTGGATGGTGAACAGCAACTGTCATACAGCAATCACATCTGGGCAAGGCTTTGGAG 720
  F W M V N S N C H T A I T S G Q G F G A 239

CCACGATTAGAGCTATCAATAGCATGGAATGCGACGGTGGAATGCTGCAACTGTGGCCA 780
  T I R A I N S M E C D G G N A A T V A S 259

GCAGAGTCAATTATTATCAGAAGTTTGGCAGCAACTCAACGTCGACACAGGATCGAATC 840
  R V N Y Y Q K F C Q Q L N V D T G S N L 279

TTCAATGTTAATTTGCCTTTCCTTCGGTCGAGTATTGTCAATTCCTAGAATAAGAGAGCA 900
  Q C * 281

ATGCCAATAATGAACAAAATAGTCCGTAGGCAGTTGCCAATTCACATCACCTCCATCTCA 960
  GTACTTTACGAGACGGATAATACAATAAGTGTTCTGATTTTCATACAGAGATCAGTTTTTT 1020
  AGATAAAAAAAAAAAAAAAAAA 1041

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Fig. 1. Nucleotide sequence of CJP-4 cDNA and its deduced amino acid sequence. Open squares indicate highly homologous amino acid sequences of plant class IV chitinases for construction of PCR primers. A chitin-binding motif (residues 37–57), C-X(4, 5)-C-C-S-X(2)-G-X-C-G-X(4)-[FYW]-C, is shown by boldface and a hevein-like domain (residues 35–63) is underlined with a double line. Two consensus family 19 chitinase signature motifs (residues 98–120 and 212–222), C-X(4,5)-F-Y-[ST]-X(3)-[FY]-[LIVMF]-X-A-X(3)-[YF]-X(2)-F-[GSA] and [LIVM]-[GSA]-F-X-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM], are indicated by boldface. Internal amino acid sequences (residues 247–259 and 178–186) of CJP-4 from tryptic digest and thrombin digest, respectively, are underlined with a single line.

especially for patients who react potently to *C. japonica* class IV chitinase.

Discussion

The selection of patients' sera is the most important process in the assessment of cross-reactivity between *C. japonica*

pollen and latex. The *C. japonica* pollen-allergic sera chosen for use in this study were selected if they met all four of the following criteria: (1) the symptoms of pollinosis emerged in parallel with the scattering of *C. japonica* pollen in early spring, (2) the infiltration of eosinophils occurred in rhinorrhea, (3) the level of anti-*C. japonica* pollen-specific IgE antibody was estimated to have a RAST score 2 or higher, and (4) patients who might be sensitized to latex were

CJP-4	MQIMATQNSKSNIFWSSASVVLVLLLVLDVGVQCNCN-GL CCS QYGYCGSGEAY CGA
Pa Chia4-Pa2	-----MGSIIIDKSVMLVLVLLLVGVSVNAQNC GCATGVCCS QYGYCGTTSAY CGK
Vv Chi4D	-----MAAKLLTVLLVGLFGAAVAQNC GCASGLCCS QYGYCGTGS SDYCGD
Pv	-----MGNKLVVLVAVALVMGPKNVSAQNC CAEGLCCS QYGYCGTGEDY CGT
Hev b 11	-----EQCGRQAGGALCPGG LCCS QYGWCA NTPEYCGS
Pers a 1	--MVYCTASLPLLLLLLVGLLAGEAF AEQCGRQAGGALCPGG L CCS Q FGWCGSTSDYCGP
Cas s 5	-----MKLFSLLLFLAFL LGTSAEQCGRQAGGAACANNLCCS Q FGWCGNTAEYCGA
CJP-4	GCKEGPCSSS-----SPSTGTGVGSIVSSDV FNS IVGGAASG-CAGNG FYTYDSFI
Pa Chia4-Pa2	GCKSGPCYSSGGG-----SPSAGGGSVGGIISQ SF FNGLAGGAASS-CEGKG FYTYNAFI
Vv Chi4D	GCQSGPCDSSS-----GSGSSVSDIVTQ SF FDGIINQAASS-CAGKN FYTRAAFL
Pv	GCQQGPCTTASPP-----PS-NNVNVADIVTAD FNG IIDQADSG-CAGKN FYTRDAFL
Hev b 11	GCQS-QC--DGGVGGEGG-----CVDLGSII S RS T FE EM LK HR NNAACPAK GFYTYDAFI
Pers a 1	TCQS-QC--GG--VT--PSP-GGGVASLISQSV FN QMLK HR NDAA CQAKGFYTYNAFI
Cas s 5	GCQS-QCSSPTTTTSSPTASSGGG GDV GLISAS LFD QMLK YR NDPRCK SN GF FYTYNAFI
CJP-4	SAANAFNGFGTSGSSDV NKREIA FAFFANA HET -GG-----FCYIE EQNP -TS
Pa Chia4-Pa2	AAANAYS GF TTGSNDV KKRELA FAFFAN VMHET -GG-----LCYIN EKNP -PI
Vv Chi4D	SALNSYSGFGNDGSTDANK REIA FAFFAHV THET -GH-----FCYIE EINGASH
Pv	SALNSYTD FR GVGS EDDSKREIA FAFA H TH ET -GH-----DGPYAWGY CH -EELNQASK
Hev b 11	SAAKAFPA FG TTGDVDT CKREIA AF FG QT SHAT TGGWPTAP-----FCYIE EIDGASK
Pers a 1	AAANSFNGFASVGD TATRKREIA FLAQ TS HE TT GGWATAPDGPYAWGY CF L KEQ -GNPP
Cas s 5	AAARSFNG FG TTGDVTT TRKRELA FLAQ TS HE TT GGWATAPDGPYAWGY CF VM ENNK --Q
CJP-4	IYCDASN-TQYPCASGKTY HGRGPIQL SWNY NYGA AGSYIQFDGLNN PE IVGTDST ISFK
Pa Chia4-Pa2	NYCQSS--STW PCT SGKSY HGRGPIQL SWNY NYGA AGKSIGFDGLNN PE KVGQDST ISFK
Vv Chi4D	NYCDSSN-TQYPCVSGQNY YGRGPIQL TWNY NYGA AGNSIGFNGLSN PG IVATDVVT SFK
Pv	SYCSPS--PAYPCAPGKKY YGRGPIQL SWNY NYG QCGSANNFDGLGAPETVSN DVVVSFK
Hev b 11	DYCDSESAQYPCSSSKY HGRGPIQL SWN FN YGPAGQALGLDLLNN PD L VATDRVISFK
Pers a 1	DYCVPT--AQWPCAPGKKY YGRGPIQ ISYNY NYG PAGRAIGYDLINN PD AVATDP VISFK
Cas s 5	TYCT-S--KSWPCVFGKQY YGRGPIQL THNY NYG QAGKAIGADLIN PD L VATNP T ISFK
CJP-4	TAVWFWMVNS----NCHTAITS-----G--QGFGATIRAIN-SMECDGGNAA-T
Pa Chia4-Pa2	TAVWFWMKNS----NCHSAITS-----G--QGFGGTIKAIN-SMECNGGNSG-E
Vv Chi4D	TALWFWMNN-----VHSVI-----G--QGFGATIRAIN GA VE C NGGNTA-A
Pv	TALWYWMQH-----VRPVIN-----QGFGATIRAIN GA LE C DGANPT-T
Hev b 11	AAIWFWMT PQ FPKPSCHDVITGQWSPTGHDISAGRAPGYGVITNI INGGLE CGS-GWDAR
Pers a 1	TALWFWM T PQSPKPSCHNVITGRWTPSAADRAAGRLPGYGVITNI INGGIE CGK-GFNDK
Cas s 5	TAIWFWMT PQ ANKPSSHDV I IGNWRPSAADTSAGRVPSYGVITNI INGGLE CGHGSDD-R
CJP-4	VASRVNYYQKFCQQLNVD TGS N LQC -----
Pa Chia4-Pa2	VSSRVNYYKKIC S QLGVDPGANVSC-----
Vv Chi4D	VNARVQYKYD S QLGVSPGD NLTC -----
Pv	VQARVNYYTEYCRQLGVATGD NLTC -----
Hev b 11	VEDRIGFYKRYCDMFGVGYGS N LD CYN QTPFGLG-----
Pers a 1	VADRIGFYKRYCDLLGVSYGS N LD CYN Q RS FGVSTN PLA ASS
Cas s 5	VANRIGFYKRYCDTLGVSY G NNLD CYN Q K PF A -----

Fig. 2. Multiple sequence alignment of CJP-4 polypeptide with family 19 chitinase members, including Pa Chia4-Pa2 (*Picea abies* class IV chitinase, accession number AY270019), Vv Chi4D (*Vitis vinifera* class IV chitinase, AF532966), Pv (*Phaseolus vulgaris* chitinase, X57187), Hev b 11 (*Hevea brasiliensis* class I chitinase, AJ431363), Pers a 1 (*Persea americana* class I chitinase, Z78202), and Cas s 5 (*Castanea sativa* class I chitinase, X95610). Identical amino acid residues are indicated by boldface.

excluded to the furthest extent possible. As to the fourth criterion, we selected *C. japonica* pollen-allergic patients who had no opportunity to touch large quantities of latex in daily life and who were not engaged in work relevant to health care.

By the internal amino acid sequencing from the tryptic digest of a highly IgE-reactive CJP-4 on 2-D SDS-PAGE, we inferred with high probability that the spot might correspond to a member of the class IV chitinase family. The full length of the nucleotide sequence was determined after the partial cDNA encoding CJP-4 was amplified by using two highly homologous regions in class IV chitinases as primers for RT-PCR and then was expanded by 3'- and 5'-RACE. The deduced amino acid sequence of CJP-4 contained a hevein-

like domain at the N-terminal and displayed deletions and two consensus motifs in the catalytic domain. These features are common characteristics of class IV chitinases in family 19 chitinases. The equivalence between the deduced amino acid sequence of the cloned cDNA and the purified chitinase was confirmed by TOF-MS analysis of tryptic fragments and protein sequence analysis of thrombin digest. Furthermore, after CJP-4 was co-precipitated with colloidal chitin, the precipitate was significantly solubilized when incubated at 50 °C for 6 h. After native PAGE, CJP-4 generated a lytic band on an ethyleneglycol chitin gel by activity staining. Thus, CJP-4 was concluded to be an endochitinase in a *C. japonica* pollen.

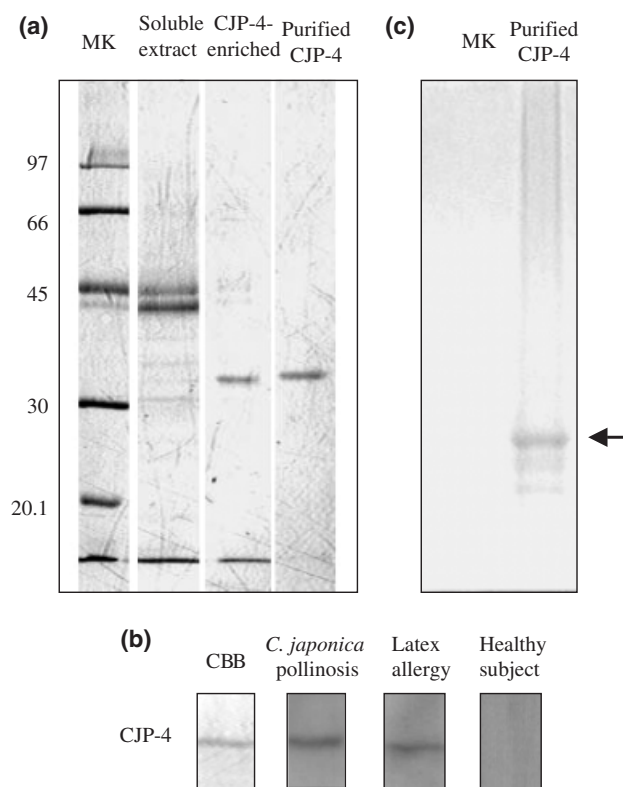


Fig. 3. Purification of native CJP-4 from a crude *Cryptomeria japonica* pollen extract (a), immunoblotting analysis (b), and activity staining (c) of the purified CJP-4. (a) After purification of CJP-4 by chitin-affinity precipitation and gel filtration chromatography, a single band of CJP-4 was obtained around 34 kDa. Molecular weight marker (lane 1), crude soluble extract (lane 2), CJP-4-enriched protein (lane 3), and purified CJP-4 (lane 4) were separated by SDS-PAGE. (b) Purified CJP-4 was subjected to SDS-PAGE and transferred onto a Hybond-P membrane. The blots were stained with serum IgE from a *C. japonica* pollen-sensitized patient, a latex-allergic patient, or a healthy subject as a control. (c) After electrophoresis on an ethyleneglycol chitin gel, a lytic band was visualized by calcofluor white M2R as shown by the arrowhead.

In our preceding study, denatured CJP-4 possessed 52.5% (21/40) of IgE-binding frequency on 2-D immunoblotting [13]. In ELISA, native CJP-4 reacted to patients' IgE with *C. japonica* pollinosis at a 100% (31/31) frequency. These IgE-binding frequencies of CJP-4 on immunoblotting and ELISA were relatively higher than with Cry j 1 (51% (35/68) and 85.7% (12/14)), Cry j 2 (47% (32/68) and 71% (10/14)), and CJP-6 (67% (10/15) and 76% (19/25)), respectively [3, 8, 33]. These results suggested that CJP-4 should be a clinically important allergen.

Cas s 5, Der f 15 [34] and Der f 18w [35] (mite, *Dermatophagoides farinae*), Pers a 1, and Hev b 11 have been registered as chitinase allergens with the Allergen Nomenclature, Allergen Nomenclature Sub-Committee, International Union of Immunological Society (as of 30 October 2003). Other chitinase allergens from banana, kidney bean (*Phaseolus vulgaris*) [36], and grape have been reported but are not registered yet. From the viewpoint of sequence similarity, the chitinase allergens described above were categorized into the family 19 chitinases, except that Der f 15 and Der f 18w belong to the family 18 chitinases. Family 19 chitinases possess two consensus family 19 signature motifs in the catalytic domain in addition to the chitin-

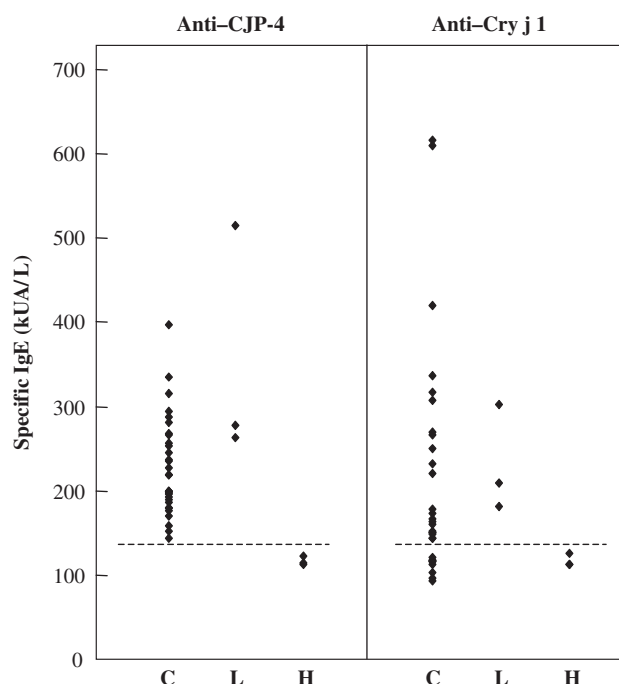


Fig. 4. IgE-binding frequencies and IgE levels to Cry j 1 and CJP-4. ELISA was performed using Cry j 1- and CJP-4-specific IgE in the respective sera from 31 patients with *Cryptomeria japonica* pollinosis (C), three latex-allergic patients (L), and three healthy subjects (H). The mean + 3 SD of the control values was chosen as the threshold value for a positive result. Respective cut-off levels for a positive response are indicated by the horizontal broken lines. A UA is defined as the amount equivalent to 1 ng of human IgE standard (Athens Research and Technology Inc., Athens, GA, USA).

binding motif in the hevein-like domain. These motifs were highly conserved in the CJP-4 molecule.

Plant class I chitinases had an N-terminal hevein-like domain that shares 65–70% sequence identity with the latex hevein that is predominantly responsible for IgE binding [17]. N-terminal hevein-like domains in class I chitinase allergens, Pers a 1, Mus a 1, and Cas s 5, are well known as major cross-reactive domains responsible for the latex-fruit syndrome [37, 38] and are also ubiquitous protein structures in class IV chitinases. CJP-4 shows high degrees of identity among class I chitinase allergens such as Hev b 11 (38.4% identity; hevein-like domain, 47.4% identity; chitin-binding motif, 54.5% identity), Pers a 1 (38.2%, 47.4%, 59.0%), Mus a 1 (41.5%, 47.4%, 59.0%), and Cas s 5 (36.1%, 44.2%, 50.0%) and among class IV chitinases from Norway spruce (*P. abies*, 65.6%, 70.5%, 72.7%), wine grape (*V. vinifera* Chi4D, 56.7%, 65.1%, 72.7%), cowpea (*Vigna unguiculata*, 50.2%, 77.8%, 81.8%), kidney bean (*P. vulgaris*, 54.9%, 72.2%, 81.8%), rape (*Brassica napus*, 51.0%, 71.4%, 72.7%), carrot (*Daucus carota* EP3B/E6, 48.1%, 58.3%, 59.1%), sugar beet (*Beta vulgaris*, 51.4%, 67.6%, 63.6%), maize (*Zea mays*, 48.5%, 62.9%, 59.1%), and thalecress (*A. thaliana*, 52.8%, 63.6%, 59.1%). The lack of allergenic activity of the homologous class II chitinases without hevein-like domain [18, 22] and a minor role of hevamine, which is a class III chitinase without a hevein-like domain, as a latex allergen [39] seemed to imply the significance of hevein-like domain for their allergenicity. Two linear amino acid regions, 19–24 and 25–37 [40] or 13–24 and 29–36 [41], which are located mainly

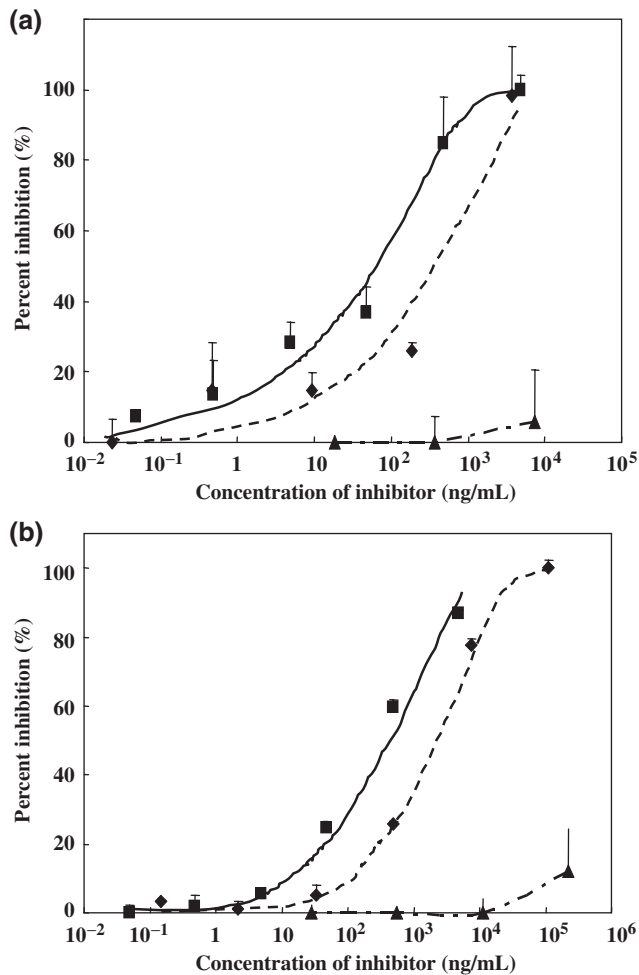


Fig. 5. ELISA inhibition of CJP-4 with latex C-serum. Purified CJP-4 was coated onto 96-well microtitre plates. After pooled sera from five patients with *Cryptomeria japonica* pollinosis (a) or three patients with latex allergy (b) were pre-incubated with serial-diluted inhibitor solution (CJP-4-enriched protein (closed square), latex C-serum (closed diamond), and ovalbumin (OVA) (closed triangle)), the pre-absorbed sera were used to measure the residual IgE-binding activity in ELISA. CJP-4-enriched protein or OVA was used as a positive or a negative control, respectively.

in the chitin-binding motif in the hevein, have been suggested to be directly involved in IgE binding. In spite of all the evidence mentioned, the possibility that a specified region of a primary amino acid sequence in a chitin-binding motif or a hevein-like domain might contribute to their allergenicity could be excluded, because we observed no obvious relationship between allergenicity and primary amino acid sequence in chitin-binding motifs or hevein-like domains by homology analysis. This is also supported from the result that wheat germ agglutinin, which includes four hevein-like sequences in its primary structure, was not immunodetected with the pool of sera from patients allergic to fruit [22]. Competitive ELISA inhibition with latex C-serum against the reaction of purified CJP-4 to *C. japonica* allergic patients' IgE indicated that all specific IgE antibodies against linear and/or conformational IgE epitopes on the class IV chitinase molecule could be completely pre-absorbed with hevein, prohevein, class I chitinase, and relevant proteins in latex C-serum. Joint consideration of the data discussed above suggests that conformational IgE epitopes involving the chitin-binding

motif or hevein-like domain together with other parts of the molecules would be commonly present in these class I and class IV chitinase allergens. In addition to participation of the catalytic domain of fruit class I chitinases in IgE binding [38], involvement of the N-terminal and C-terminal regions [42], potential IgE-interacting residues, R⁵, K¹⁰, E²⁹, Y³⁰, H³⁵, and Q³⁸ [43], of hevein for the formation of viable IgE-binding hevein epitopes has been reported.

Whereas avocado class I chitinase is involved with the latex-fruit syndrome, class I chitinase in green bean is not. The allergenicity of both chitinases was reported to be lost by heating [36]. This fact could explain why plant foods containing these putative allergens that are consumed after cooking are not usually associated with the latex-fruit syndrome. On the other hand, the IgE-binding capacity of CJP-4 diminished, although not significantly, by heating (10 min at 100 °C) in ELISA (data not shown). The difference in heat stability would reflect slight variations among hevein-like domains in conformation based on disulphide bonds. This distinction about the conformational structure should explain the shift to higher concentration of the inhibitor (latex C-serum) in the cross-reactivity between *C. japonica* class IV chitinase and latex C-serum. Similar shifts were also observed between avocado or chestnut class I chitinase and natural latex extract [18].

Cross-reactivity among plant pollen and some oral allergy syndromes has been reported [44, 45]. However, few reports have demonstrated the cross-reactivity of allergens in *C. japonica* pollen with other food or latex allergens, except for *C. japonica* pollen and tomato (*Lycopersicon esculentum*) in humans and dogs [46, 47]. Judging from the IgE-binding frequency and the probable cross-reactivity to latex C-serum, we concluded that *C. japonica* class IV chitinase is an important allergen in *C. japonica* pollinosis. This is the first report in which a certain chitinase in plant pollen was proved to be an allergen. CJP-4 may be one of the key allergens of *C. japonica* pollinosis triggering latex allergy or oral allergy, and may have potential in eliciting co-sensitization and cross-reactivity to various plants containing class I and class IV chitinases. Recently, endogenous acidic mammalian chitinase was reported to exacerbate T-helper type 2 inflammation and airway hyper-responsiveness, in part by accelerating IL-13 pathway activation and chemokine induction [48]. Exogenous pollinic chitinase is also seemed to have the potential for participation in these physiological functions.

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