Published in final edited form as:
Allergy. 2007 May ; 62(5): 547–553. doi:10.1111/j.1398-9995.2007.01331.x.

Isolation and characterization of native Cry j 3 from Japanese cedar (*Cryptomeria japonica*) pollen

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Abstract

Background—Japanese cedar (*Cryptomeria japonica*) pollinosis is the most prevalent allergy in Japan. Recently, the Japanese cedar pollen allergen Cry j 3 was cloned as a homologue of Jun a 3, which is a major allergen from mountain cedar (*Juniperus ashei*) pollen. However, native Cry j 3 has not been isolated and there are no reports on its allergenic activity. The aims of this study were to isolate native Cry j 3 and assess its immunoglobulin E (IgE)-binding capacity in patients with Japanese cedar pollinosis.

Methods—Native Cry j 3 was purified from Japanese cedar pollen by multidimensional chromatography. We assessed the IgE-binding capacity using sera from patients allergic to Japanese cedar pollen by immunoblot analysis and ELISA. Moreover, we assayed the capacity of Cry j 3 to induce histamine release from the patient's leukocytes. We cloned cDNA corresponding to purified Cry j 3 from a cDNA library of Japanese cedar pollen.

Results—We isolated native Cry j 3 as a 27-kDa protein. The IgE-binding frequency of Cry j 3 from the sera of patients allergic to Japanese cedar pollen was estimated as 27% (27/100) by ELISA. Cry j 3 induced the release of histamine from leukocytes. We cloned the cDNA and named it Cry j 3.8. Cry j 3.8 cDNA encoded 225 amino acids and had significant homology with thaumatin-like proteins.

Conclusions—Cry j 3 is a causative allergen in Japanese cedar pollinosis and may play crucial roles in the cross-reactivity with oral allergy syndrome.

Keywords

Japanese cedar; oral allergy syndrome; pathogenesis-related protein; pollinosis; thaumatin-like protein

Japanese cedar (*Cryptomeria japonica*) pollen is one of the most prevalent allergens in Japan. A nationwide survey using a cross-sectional random sampling method estimated that at least

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13% of the Japanese population is suffering from Japanese cedar pollinosis (1). Because of the steady increase in this allergy, especially among school children, Japanese cedar pollinosis has become a severe social problem.

Two major allergens from Japanese cedar pollen, Cry j 1 and Cry j 2, have been shown to cause Japanese cedar pollinosis (2,3). Cry j 1 was identified as a 41–46 kDa allergen with pectate lyase enzyme activity (2,4,5), while Cry j 2 was identified as a 45 kDa allergen with polymethylgalacturonase enzyme activity (3,6–8). Both Cry j 1 and Cry j 2 have several isoforms that differ in their primary structures, post-translational modifications, or reactivity with antibodies (9–11).

Recently, we cloned seven isoforms of cDNA encoding Cry j 3 as homologues of Jun a 3 (12,13). Jun a 3 is a major allergen from mountain cedar (*Juniperus ashei*) pollen and has been reported as a pathogenesis-related-5 group family (PR-5) protein allergen with an immunoglobulin E (IgE)-binding reactivity of 42.9% (6/14) in patients allergic to mountain cedar pollen. Moreover, Jun a 3 has been reported to cross-react in pollinosis patients allergic to Japanese cedar pollen at a rate of 33.3% (12/36) (14). This suggests that Cry j 3 has IgE-binding capacity in Japanese cedar pollinosis patients and cross-reacts with Jun a 3. However, the native protein of Cry j 3 has not been isolated, and thus its IgE-binding capacity has not been elucidated despite its importance as a PR-5 family allergen. Therefore, we isolated native Cry j 3 from Japanese cedar pollen and assessed its IgE-binding capacity.

In the present study, we determined the IgE-binding capacity of native Cry j 3 in patients allergic to Japanese cedar pollen using immunoblot, ELISA and histamine-release assays. We also cloned the cDNA corresponding to the isolated novel isoform of Cry j 3, and named it Cry j 3.8.

Materials and methods

Purification of Cry j 3

Crude extracts were obtained from Japanese cedar pollen as described previously (2). Briefly, Japanese cedar pollen was suspended in 125 mM sodium bicarbonate buffer (pH 8.0) containing 3 mM ethylenediamine tetraacetic acid for 4 h at 4°C, and the suspension was centrifuged at 9300 ***g*** for 35 min at 4°C. Ammonium sulfate was added to the supernatant until 80% saturation. The resultant precipitate was dialyzed against 5 mM phosphate buffer (pH 6.8) and then centrifuged at 10 000 ***g*** for 15 min at 4°C. The supernatant was filtered through a 1.0 μm pore membrane filter (Toyo Roshi Ltd, Tokyo, Japan) to obtain crude Japanese cedar pollen extract. The crude extract was applied directly to a DEAE-Cellulofine column (Seikagaku Corporation, Tokyo, Japan). The unadsorbed fraction was applied to a Micro-Prep® Ceramic Hydroxyapatite type I column (BioRad Laboratories Inc., Hercules, CA, USA), and then ammonium sulfate was added to the unadsorbed fraction until 80% saturation. The resultant precipitate was dialyzed against 20 mM acetate buffer (pH 5.0). The dialyzed solution was applied to a Hiload 26/10 SP Sepharose HP column (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA). The obtained unadsorbed fraction was dialyzed against 0.065% trifluoroacetic acid in 2% acetonitrile, and then applied to a Resource™ RPC column (GE Healthcare Bio-Sciences Corporation). The adsorbed fraction was obtained by gradient elution with 0.05% trifluoroacetic acid in 80% acetonitrile. The fractions containing native Cry j 3 were pooled and phosphate-buffered saline (PBS) was used as an exchange buffer in a PD-10 desalting column (GE Healthcare Bio-Sciences Corporation). The resultant single protein was named Cry j 3. N-terminal amino acid sequencing of purified Cry j 3 was performed by Toray Research Center Inc., Tokyo, Japan.

SDS-PAGE and immunoblotting

Protein samples (crude pollen extract and purified Cry j 3) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% slab gel under reducing conditions with 50 mM dithiothreitol using the discontinuous buffer system of Laemmli (15). Proteins were then detected with Phast- Gel™ Blue R (GE Healthcare Bio-Sciences Corporation) or blotted onto a Hybond-P membrane (GE Healthcare Bio-Sciences Corporation) at 1 mA/cm^2 for 1.5 h. The blot was probed with primary antibody (anti-Jun a 3 rabbit serum IgG, or sera from Japanese cedar pollinosis patients or healthy individuals). To detect rabbit IgG, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA, USA). In the case of human IgE, the blot was overlaid with biotinylated anti-human IgE (Vector Laboratories Inc., Burlingame, CA, USA), followed by reaction with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories). After immunolabeling, the positive bands were visualized on the membrane using 3,3′,5,5′-tetramethylbenzidine or on a chemiluminescence imager (Atto Corp., Tokyo, Japan) or an X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan) using an ECL-Plus Western blotting detection kit (GE Healthcare Bio-Sciences Corporation).

Analysis of glycosylation on Cry j 3

Five micrograms of protein samples (Cry j 1, Cry j 3, horseradish peroxidase as a positive control and soybean trypsin inhibitor as a negative control) were fractionated by SDS-PAGE, followed by blotting onto polyvinylidene difluoride (PVDF) membrane as described above. Glycoprotein was visualized by using a GelCode™ Glycoprotein staining kit (PIERCE Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instruction. Briefly, gel was incubated with oxidizing solution and then washed three times by gently agitating with 3% acetic acid. The gel was submerged in glycoprotein staining reagent, followed by reaction with reducing solution with gentle agitation. The gel was washed with 3% acetic acid, followed by ultrapure water.

ELISA for specific IgE to pollen allergens

Specific IgE to pollen allergens was measured by fluorometric ELISA. Briefly, purified antigen solutions (500 ng/ml of Cry j 1, Cry j 2 or Cry j 3) were applied to a 96-well microtiter plate (NUNC-Immuno® Plate Maxisorp F96; NalgeNunc International, Roskilde, Denmark) and incubated at 4°C overnight. After the plate was blocked with 1% (w/v) bovine serum albumin in PBS for 2 h at 37°C, 10-fold diluted patient's sera were added and incubated for 4 h at room temperature. Diluted (1 : 10) β-galactosidase- conjugated anti-human IgE monoclonal antibody (Pharmacia Diagnostics AB, Uppsala, Sweden) was then added, followed by incubation at 4° C overnight. For enzymatic reaction, 0.2 mM 4-methylumbelliferyl β-D -galactopyranoside (Sigma Aldrich Corp., St Louis, MO, USA) was added, followed by incubation at 37°C for 2 h. The fluorescence intensity was measured using a fluorometric microplate reader (Fluoroscan; Flow Laboratories, McLean, VA, USA).

Assay of histamine release from human leukocytes

Histamine release experiments from washed leukocytes were conducted using the same method as described previously (16). Washed leukocytes were obtained from the venous blood of donors and suspended in PIPES buffer (25 mM piperazine-*N*,*N*′-bis-2-ethane-sulfonic acid, 110 mM sodium chloride and 5 nM potassium chloride) containing 1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.03% (w/v) human serum albumin (pH 7.4). Allergen preparations diluted in the same PIPES buffer were mixed at a series of concentrations with washed leukocytes at 37°C for 45 min. The histamine released into the supernatant was measured by automated fluorometry (17).

Molecular cloning of Cry j 3.8 from a cDNA library

We constructed a full-length cDNA library from male flowers and pollen of Japanese cedar at several stages during the development (N. Futamura et al., unpublished data). In addition, the 5′ ends of cDNA sequences from approximately 20 000 clones obtained from the cDNA library were analyzed. We obtained a clone in which the 5′-end sequence coincided completely with the sequence deduced from the N-terminal amino acid sequence of purified native Cry j 3. Thus, we determined the complete cDNA sequence from the clone and named it Cry j 3.8.

Results

Purification of Cry j 3 from Japanese cedar pollen

As in the many cases of purification of thaumatin-like proteins (TLPs), we isolated a single protein after gradient elution with acetonitrile on reverse phase chromatography following anion exchange, hydroxyapatite and cation exchange chromatography. The purified protein migrated at 27.3 kDa under reducing conditions on SDS-PAGE (Fig. 1A). The homologous purified protein reacted with anti-Jun a 3 polyclonal antibody on Western blotting (Fig. 1B). To confirm that this positive protein was Cry j 3, we analyzed the N-terminal amino acid sequence, which was found to coincide completely with that of Jun a 3. Therefore, we concluded that the purified protein was native Cry j 3 (Fig. 2).

Determination of Cry j 3-specific IgE levels in the sera of patients allergic to Japanese cedar pollen

Purified Cry j 3 on a blot was reactive with the sera from patients allergic to Japanese cedar pollen but not with the sera from nonallergic individuals (Fig. 3A). ELISA was performed using the sera of 100 Japanese cedar pollinosis patients to analyze the IgE-binding frequency and specific IgE levels against Cry j 3. Twenty-seven percent (27/100) of these patients exhibited significantly greater IgE-binding ability against Cry j 3 compared with healthy volunteers (Fig. 3B). The IgE-binding intensity of Cry j 3 in the patient's sera was almost one 14th and one 10th of the IgE-binding intensities of Cry j 1 and Cry j 2, respectively (the mean fluorescence intensities with patient's IgE against Cry j 1, Cry j 2 and Cry j 3 were 5800, 3900 and 410, respectively; Fig. 3C).

Purified Cry j 3 induced histamine release from leukocytes obtained from Cry j 3-positive patients in a dose-dependent manner, but not from Cry j 3-negative patients (Fig. 4). The sensitivity (the dose of allergen that induced 25% histamine release) was one 15th that of Cry j 1 (32 ng/ml for Cry j 1 and 480 ng/ml for Cry j 3; Fig. 4A). These data were consistent with the difference in IgE-binding intensity between Cry j 3 and Cry j 1 (1 : 14).

Molecular cloning of Cry j 3.8 from Japanese cedar pollen

The N-terminal sequence of the purified protein did not coincide with the deduced amino acid sequences from any of the previously cloned Cry j 3 isoforms, Cry j 3.1 to Cry j 3.6 (12,13) and Cry j 3.7 (accession number AB212218) (Fig. 2). Therefore, we again cloned the cDNA from the cDNA library of Japanese cedar and named the novel isoform Cry j 3.8 (accession number AB254807).

Sequence analysis revealed that the Cry j 3.8 cDNA consisted of 943 nucleotides containing a 678 bp open reading frame (ORF). The ORF encoded a 225 amino acid polypeptide. In addition, we found two potential N-glycosylation sites, one consensus pattern of the TLP family, and 16 consensus cysteine residues in the ORF (18,19) (Fig. 5). By glycoprotein staining of Cry j 3, we detected a faint positive band on the blot, indicating that native Cry j 3 was actually glycosylated (data not shown). The deduced amino acid sequence of whole Cry j 3.8 had 45–76% identity with other Cry j 3 isoforms and 86% identity with Jun a 3. Multiple

sequence alignment showed a high degree of identity between Cry j 3.8 and other TLPs, especially with previously described TLP allergens from other plant species (Fig. 6). The amino acid identities between Cry j 3.8 and the other TLP allergens, Jun r 3.1 (*J. rigida*), Jun r 3.2, Jun a 3, Cup s 3.2 (*Cupressus sempervirens*) and Cup s 3.3 were 86.2%, 85.8%, 85.8%, 84.9% and 84.9%, respectively.

Discussion

The IgE-binding frequency of Cry j 3 in pollinosis patients allergic to Japanese cedar pollen was estimated as 27%. The IgE-binding capacity was estimated as one 15th when compared with Cry j 1 by ELISA and histamine-release assay from leukocytes, and one 10th when compared with Cry j 2 by ELISA. In one patient, the specific IgE titer against Cry j 3 was comparable to that against Cry j 2 (filled circles in Fig. 3C). This suggests that Cry j 3 is an important allergen in some patients. It has been reported that the expression level and allergenic activity of TLPs vary under different environmental conditions (20). Therefore, the importance of Cry j 3 as a Japanese cedar allergen may differ among years and locations.

Cry j 1 cross-reacts with Jun a 1, and the two allergens share some linear and probable conformational epitopes (21). Moreover, cross-reactivity between Cry j 3 and Jun a 3 has also been predicted. Indeed, in the present study the anti-serum raised against Jun a 3 recognized Cry j 3, and it has been reported that 33% (12/36) of a group of patients allergic to Japanese cedar pollen showed cross-reaction with Jun a 3 (22). In Japanese cedar pollinosis patients, the IgE-binding frequency against Jun a 3 is comparable with that against Cry j 3 (33% and 27%, respectively). Furthermore, three sequential IgE epitopes of Jun a 3 (amino acids 120–131, 132–145 and 152–165) are highly conserved in Cry j 3.8, at 92% (120– 130), 100% (132–145) and 86% (152–153, 155–162 and 164–165) (23). These data strongly imply cross-reactivity between Cry j 3 and Jun a 3.

Cross-reactivity between allergens from Japanese cedar pollen and tomato have been reported in dogs and humans (24,25), although the causative allergens remain unknown. Because two isoforms of TLP have been isolated from tomato fruit and their amounts shown to increase with ripening (26), Cry j 3 may be one of the causative allergens of the cross-reactivity. TLPs are reported to be contained in many fruits, as is chitinase. TLPs have significant amino acid sequence identity with thaumatin and are categorized in the PR-5 family (27). The PR-5 family comprises thaumatin, osmotin and zeamatin, and proteins highly homologous to them. PR-5 family proteins are well known as allergens from pollen and sweet fruits. Mal d 2 from apple (*Malus domestica*) was the first allergen described as a TLP (28). Following the identification of Mal d 2, several fruit allergens, including Pru av 2 from cherry (*Prunus avium*), Act c 2 and Act d 2 from kiwi (*Actinidia chinensis* and *A. deliciosa*, respectively), Cap a 1 from bell pepper (*Capsicum annuum*) and thaumatin from grape (*Vitis vinifera*), were reported as allergens belonging to the PR5 family (19,29–32). Recently, PR-5 allergens from pollen have been identified as Jun a 3 from mountain cedar (*J. ashei*), Jun v 3 from eastern red cedar (*J. virginiana*) and Cup a 3 from *C. arizonica* (20,22,33). The PR proteins are considered important pan-allergens responsible for pollinosis and oral allergy syndrome (14). A recently identified allergen from Japanese cedar pollen, CJP-4, also belongs to the PR family. CJP-4 has been identified as a 34 kDa protein with endochitinase activity that cross-reacts with latex allergens (34). Therefore, both Cry j 3 and CJP-4 may act as causative allergens of the cross-reactivity in Japanese cedar pollinosis and oral allergy syndrome.

We purified native Cry j 3 from Japanese cedar pollen. The N-terminal sequence of purified Cry j 3 did not completely coincide with any previously reported sequences of Cry j 3, but did coincide with that of Jun a 3. Cry j 3.8 has the highest amino acid identity with Jun a 3 (85.8%) among the Cry j 3 isoforms (Cry j 3.1–3.6, 42.5– 54.8%; Cry j 3.7, 73.6%) (12,13). However,

it remains to be elucidated whether the other Cry j 3 isoforms exist in mature Japanese cedar pollen.

Acknowledgements

We thank Professor Kazuhisa Ono (Hiroshima University, Hiroshima, Japan) for providing the Japanese cedar pollen. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan and by an EPA STAR research grant (no. 83-0399) (to R.M.G).

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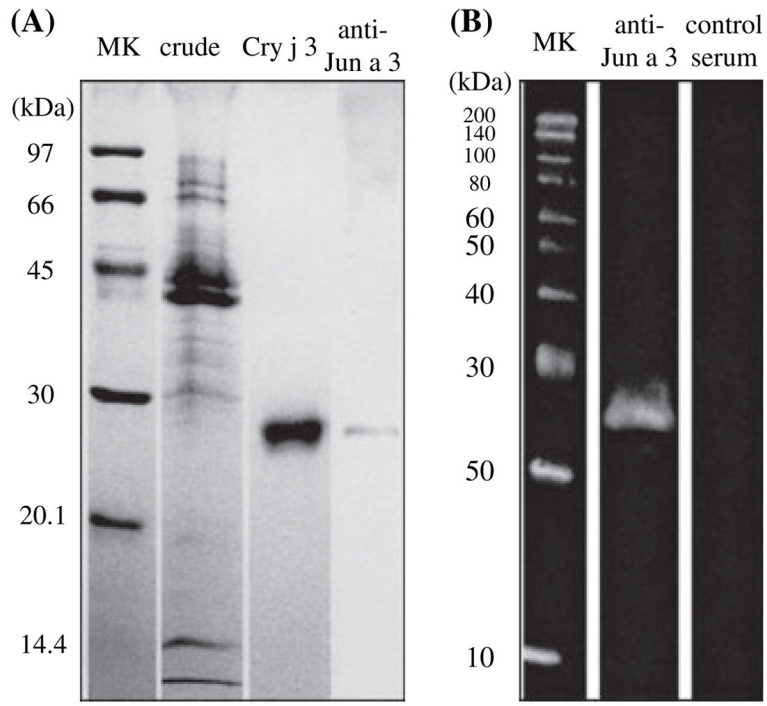

Figure 1.

Purification of native Cry j 3 from crude Japanese cedar pollen extract. Crude extract from Japanese cedar pollen and purified Cry j 3 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight marker (lane 1), crude soluble extract (lane 2) and purified Cry j 3 (lane 3) were separated. Crude soluble extract was then transferred onto polyvinylidene difluoride (PVDF) membrane, followed by staining with anti-Jun a 3 rabbit serum (lane 4) (A). Cry j 3.8 was subjected to SDS-PAGE and transferred onto a PVDF membrane. The blots were stained with anti-Jun a 3 rabbit serum or control rabbit serum (B).

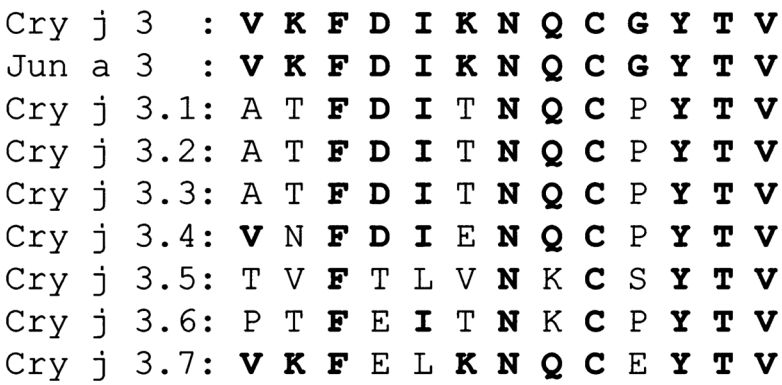

Figure 2.
Alignment of N-terminal amino acid sequences from Cry j 3 isoforms and Jun a 3.

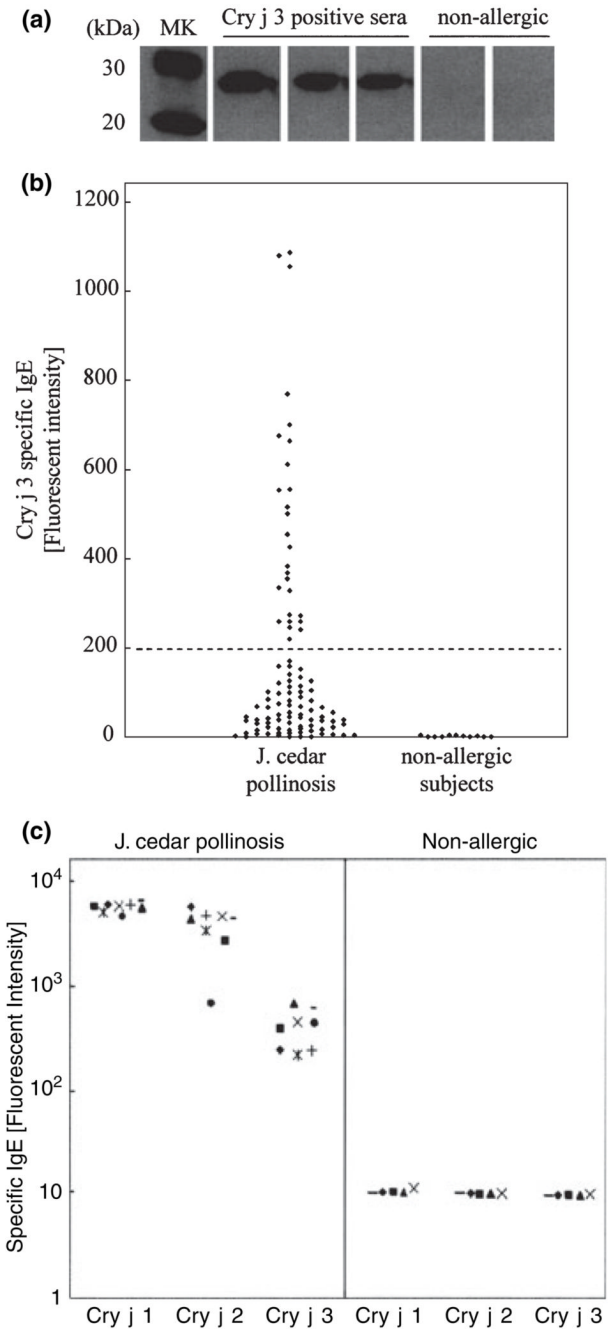

Figure 3.

Immunoglobulin E (IgE)-binding ability of Cry j 3 in patients allergic to Japanese cedar pollen. Purified Cry j 3 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were stained with serum IgE from Japanese cedar pollen-sensitized patients or healthy subjects as controls (A). ELISA was performed using IgE in the sera from 100 patients with Japanese cedar pollinosis and 11 healthy subjects. The cut line shows the cut-off value for a positive result (B). IgE-binding capacity was compared among Cry j 1, Cry j 2 and Cry j 3 (C). The specific IgE level against each allergen was assayed by ELISA using sera from eight Cry j 3.8-

positive allergic patients with Japanese cedar pollinosis and five nonallergic subjects as controls. Identical symbols show IgE titers from the same patient or healthy subject.

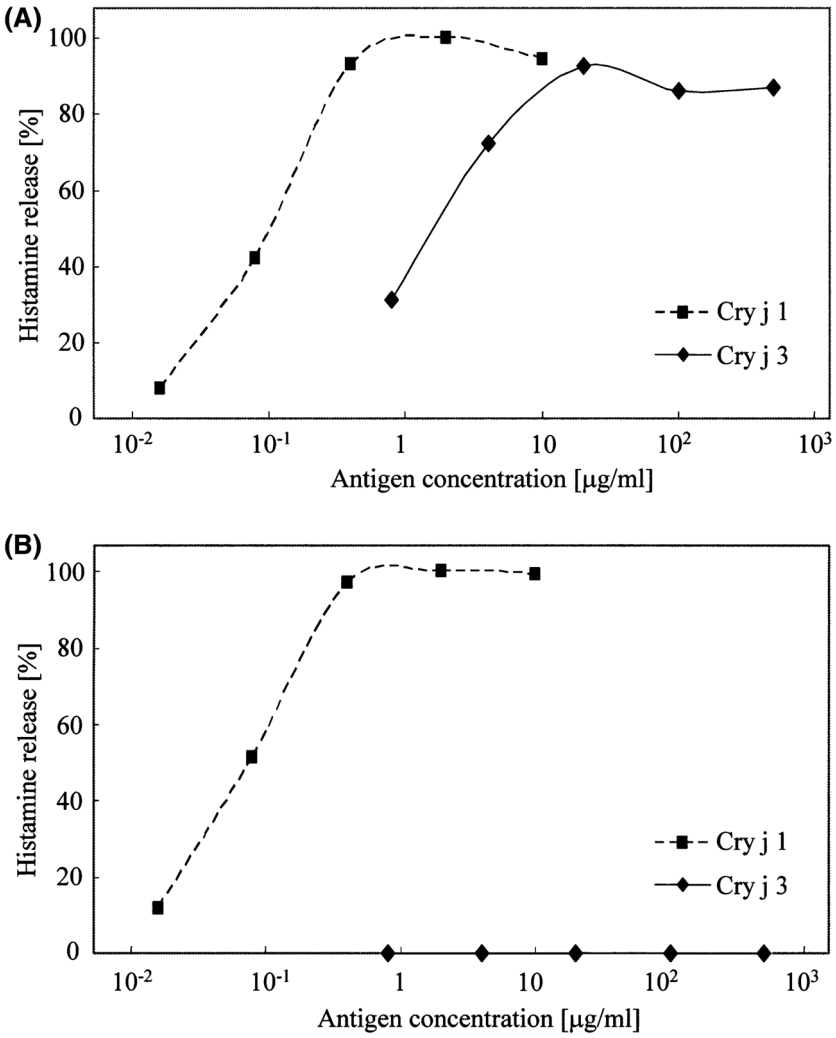

Figure 4.

The amount of histamine released into the culture supernatant from leukocytes obtained from Cry j 3-positive (A) and Cry j 3-negative (B) Japanese cedar pollinosis patients stimulated with Cry j 1 or Cry j 3 was assayed by automated fluorometry.

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GATTCAGTCT TTGCTGTATG CGATATAGAT ACTAGTTATA ATGGCAAAAG TATCAGATCT  60
                                            M  A  K  V   S  D  L
TGCGCTTCTT CTTGTGGCTG GAATGGCCAT ATCTCTTTAT ATTCAAGAGA CGGGAGCAGT 120
 A  L  L   L  V  A  G   M  A  I   S  L  Y   I  Q  E  T   G  A  V
TAAGTTTGAT ATAAAGAACC AGTGCGGGTA CACAGTCTGG GCGGCGGGAC TACCCGGAGG 180
 K  F  D   I  K  N  Q   C* G  Y   T  V  W   A  A  G  L   P  G  G
AGGGCAGCAG CTGACCCAGG GTCAGACATG GACGGTTAAT TTGGCGGCAG GGACACAGTC 240
 G  Q  Q   L  T  Q  G   Q  T  W   T  V  N   L  A  A  G   T  Q  S
GGCAAGATTC TGGGGTCGAA CCGGCTGCTC TTTCGATGCG AGCGGCAAAG GGACCTGTCA 300
A  R  F   W  G  R  T   G  C* S   F  D  A   S  G  K  G   T  C* Q
AACCGGTGAC TGCGGTGGCC AACTGAGCTG CACAGTTTCG GGAGCTGTTC CCGCCACGCT 360
 T  G  D   C* G  G  Q   L  S  C*  T  V  S   G  A  V  P   A  T  L
GGCCGAGTAC ACCCAGAGCG ACCAGGACTA CTACGACGTC TCCCTGGTGG ACGGCTTCAA 420
 A  E  Y   T  Q  S  D   Q  D  Y   Y  D  V   S  L  V  D   G  F  N
CATTCCTCTT TCCATCAACC CTACCAATGC ACAGTGTACC GCCCCTGCAT GCAAAGCAGA 480
 I  P  L   S  I  N  P   T  N  A   Q  C* T   A  P  A  C*  K  A  D
CGTGAACGCT GTGTGCCCTG CTGAACTGAA GGTGGATGGG GGATGCAAGA GTGCCTGCGC 540
 V  N  A   V  C* P  A   E  L  K   V  D  G   G  C* K  S   A  C* A
TGCCTTTCAA ACTGACCAGT ACTGCTGCAC TGGCACCTAT GCCAATAGCT GCCCTGCCAC 600
 A  F  Q   T  D  Q  Y   C* C* T   G  T  Y   A  N  S  C*  P  A  T
AAACTACTCG ATGATATTCA AGAACCAGTG CCCCCAGGCC TACAGTTATC CCAAGGACGA 660
 N  Y  S   M  I  F  K   N  Q  C*  P  Q  A   Y  S  Y  P   K  D  D
TACAGCCACA TTCGCTTGCC CCTCTGGTAC AGACTACAGT ATTGTATTCT GTCCCTAGAT 720
 T  A  T   F  A  C* P   S  G  T   D  Y  S   I  V  F  C*  P  *

ATATATAGGA TTATGTTTGT GTGGAATAAT ATATTAATAA GTGCTGTCAT CTTTGTGTTG 780
ACGCTGTGGT GTAGCTGCAT AGTTGCAACT CTCACCACTT ATTATCGACC AATAAAACAT 840
GTCTGTCATC TCACCACTAA TGACAGAAGC GTAATTGACA CGCAATACAC TGTTATGAGA 900
GAATATTAAT AATAAATATA TATTTTGAAA AAAAAAAAAA AAA                   943
```

Figure 5.

Nucleotide sequence of Cry j 3.8 cDNA and its deduced amino acid sequence. A consensus pattern of TLRs (residues 82– 97), G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-G-x(2,3)-C, is shown in boldface, and conserved cysteine residues are indicated as C*. Two potential N-glycosylation sites (residues 133 and 188) are double-underlined, and the N-terminal sequence determined is single-underlined.

```
Cry j 3.8       : MAKVSDLALLLVAGMAISLYIQETGAVKFDIKNQCGYTVWAAGLPGGGQQ  50
Jun r 3.1       : MARVSELALLLVATLAISLHMQEAGAVKFDIKNQCGYTVWAAGLPGGGKR  50
Jun a 3         : MARVSELAFLLAATLAISLHMQEAGVVKFDIKNQCGYTVWAAGLPGGGKR  50
Cup s 3.2       : MARVSELALLLVATLAISLHMQEAGAVKFDIKNQCGYTVWAAGLPGGGKR  50
T. occidentalis : MATVSDLALLLVAGLAISLCTQEAGAVKFNIRNQCGYTVWAAGLPGGGKQ  50

Cry j 3.8       : LTQGQTWTVNLAAGTQSARFWGRTGCSFDASGKGTCQTGDCGGQLSCTVS 100
Jun r 3.1       : LDQGQTWTLNLAAGTASARFWGRTGCTFDASGKGSCKTGDCGGQLSCTVS 100
Jun a 3         : LDQGQTWTVNLAAGTASARFWGRTGCTFDASGKGSCQTGDCGGQLSCTVS 100
Cup s 3.2       : LDQGQTWTVNLAAGTASARFWGRTGCTFDASGKGSCRSGDCGGQLSCTVS 100
T. occidentalis : LDQGQTWSVDVAAGTKGARFWGRTGCSFDGSGRGSCQTGDCGGQLSCTLS 100

Cry j 3.8       : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLSINPTNAQCTAPACKAD 147
Jun r 3.1       : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLAINPTNAQCTAPACKAD 147
Jun a 3         : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLAINPTNAQCTAPACKAD 147
Cup s 3.2       : GAVPATLAEYT---QSDKDYYDVSLVDGFNIPLAINPTNTKCTAPACKAD 147
T. occidentalis : GAVPATLAEYSLNGNDNKDFYDVSLVDGFNVPLSINPTNTGCTAPACKAD 150

Cry j 3.8       : VNAVCPAELKVDGGCKSACAAFQTDQYCCTGTYANSCPATNYSMIFKNQC 197
Jun r 3.1       : INAVCPSELKVEGGCNSACNVFQTDQYCCRNAYVDNCPATNYSKIFKNQC 197
Jun a 3         : INAVCPSELKVDGGCNSACNVFKTDQYCCRNAYVDNCPATNYSKIFKNQC 197
Cup s 3.2       : INAVCPSELKVDGGCNSACNVLQTDQYCCRNAYVDNCPATNYSKIFKNQC 197
T. occidentalis : VNAACPAELKVNSGCNSACNVFQTDQYCCRGANVDNCPATNYSMIFKNQC 200

Cry j 3.8       : PQAYSYPKDDTA-TFACPSGT-DYSIVFCP 225
Jun r 3.1       : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
Jun a 3         : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
Cup s 3.2       : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
T. occidentalis : PQAYSYAKDDTSSTFTCPSGTTDYSIVFCP 230
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Figure 6.

Multiple sequence alignment of Cry j 3.8 with the TLPs, Jun r 3.1 (*Juniperus rigida*, accession number AY353703), Jun a 3 (*J. ashei*, AF121776), Cup s 3.2 (*Cupressus sempervirens*, AY353706), and *T. occidentalis* (*Thuja occidentalis*, AY795850). Identical amino acid residues are indicated in boldface.