

Detection of New Antigenic Proteins in Japanese Cedar Pollen

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In Japan, an increasing number of people suffer from pollenosis, a typical atopic disease. Japanese cedar (*Cryptomeria japonica*) pollen is the most common allergen that causes pollenosis. Although Cry j 1 and Cry j 2 are the common allergenic proteins contained in the pollen, there is a small population of patients who exhibit positive skin reactions to the pollen extract but are negative for both Cry j 1 and Cry j 2. This suggests that the pollen may contain other antigenic proteins besides these two molecules. In this study, we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to examine partially purified pollen extract (PPPE) and determine if there were other proteins that reacted to anti-PPPE sera collected from sensitized guinea pigs, mice and rabbits. Subsequently, we detected four bands of proteins with molecular weights around 7, 15, 20 and 31 kDa, which were different from those normally seen for Cry j 1 and Cry j 2. Among these, the 7, 15 and 20 kDa proteins could not be detected when anti-Cry j 1 monoclonal anti-body (mAb) and anti-Cry j 2 mAb were used as anti-bodies for Western blotting. Therefore, these three proteins may differ from Cry j 1 and Cry j 2 not only in molecular weight but also in antigenicity. In conclusion, three new antigenic proteins that are not identical to Cry j 1 and Cry j 2 have been shown to exist in Japanese cedar pollen. Structural analyses of these proteins may be useful in the development of new therapeutic methods, including specific immunotherapy for pollenosis.

Key words cedar pollen; antigen; pollenosis; Cry j 1; Cry j 2; immunoglobulin

Recently, an increasing number of patients in Japan have been found to be suffering from pollenosis, which is a typical atopic disease that includes symptoms such as allergic rhinitis and conjunctivitis. The prevalence of pollenosis is currently estimated to occur in approximately 20% of the general Japanese population. Japanese cedar (*Cryptomeria japonica*) pollen is the most common allergen among various plant-derived pollens in Japan. When the pollen attaches to the nasal and conjunctival mucosal surface, the pollen cracks, which leads to a release of antigenic proteins. This results in the cross-linking of the proteins to antigen-specific IgE antibodies that are bound on the mucosal mast cell surface.

Common antigenic proteins in Japanese cedar pollen are Cry j 1 and Cry j 2. Cry j 1 was isolated by Yasueda *et al.*¹⁾ in 1983, and found to be a 45–50 kDa protein. A few years later, Taniai *et al.*²⁾ and Sakaguchi *et al.*³⁾ identified a second allergenic 37 kDa protein, Cry j 2. Further analyses have documented that patients suffering from Japanese cedar pollenosis possess IgE antibodies against either Cry j 1 or Cry j 2, or even both of these proteins.^{3,4)} However, Sawatani *et al.*⁵⁾ reported that one out of 25 pollenosis patients exhibited no skin reactions to either Cry j 1 or Cry j 2, even though the cutaneous reaction to crude extracts of the cedar pollen is positive. Therefore, it has been hypothesized that Japanese cedar pollen includes other antigenic proteins besides Cry j 1 and Cry j 2. Kawamoto *et al.*⁶⁾ recently identified a new antigenic 30 kDa pollen protein, CJP-6. When derived from Japanese cedar pollenosis patients, 76% of the IgE antibodies reacted to CJP-6. In addition, cDNAs of six Cry j 3 genes have been isolated and characterized,^{7,8)} although it is not clear whether Cry j 3 itself has antigenic properties.

In this study, we tried to detect new antigenic proteins in Japanese cedar pollen using Western blot analysis with anti-sera collected from guinea pigs, mice and rabbits that had been actively sensitized with the pollen extracts. Our results indicate there are three antigenic proteins other than Cry j 1 and Cry j 2 that are present in the pollen.

MATERIALS AND METHODS

Animals For immunization with the pollen extracts, 3-week-old female Hartley guinea pigs weighing 201–250 g, 6-week-old female B10.S mice weighing 16–21 g, and 12-week-old female JW/CSK rabbits (Japan SLC, Hamamatsu, Japan) were used. For measurement of antibody titers by passive cutaneous anaphylaxis (PCA), male Hartley guinea pigs weighing 500–1000 g (Japan SLC, Hamamatsu, Japan) were used. The animals were housed in an air-conditioned room at a temperature of $23 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ humidity with a controlled 12 h light/dark cycle, and fed a standard laboratory diet with water given ad libitum. The Experimental Animal Research Committee at Kyoto Pharmaceutical University approved this animal study.

Materials In 1998, we personally collected Japanese cedar pollens in the Japanese prefectures of Gifu and Shiga. $\text{Al}(\text{OH})_3$ gels were prepared with 0.5 N NaOH and 0.5 N $\text{Al}_2(\text{SO}_4)_3$, as previously described.⁹⁾ This method is a modification of the method of Levine and Vaz.¹⁰⁾

The following reagents were obtained from the indicated commercial sources: complete Freund's adjuvant (CFA, Difco Lab., Detroit, MI, U.S.A.), bovine serum albumin (BSA) and trichloroacetic acid (TCA) (Wako Pure Chem., Osaka, Japan), deoxycholic acid sodium salt (DOC), phenol reagent and skim milk (Nacalai Tesque, Kyoto, Japan), purified Cry j 1, purified Cry j 2, anti-Cry j 1 monoclonal antibody (mAb) (clone 013 and 026), anti-Cry j 2 mAb (clone T27), horseradish peroxidase (HRP)-conjugated anti-Cry j 1 mAb (clone 053), HRP-conjugated anti-Cry j 2 polyclonal antibody and anti-Cry j 1 rabbit IgG fraction (Seikagaku Corp., Tokyo, Japan), Evans blue (Merck, Darmstadt, Germany), fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, U.S.A.), biotin-conjugated rat anti-mouse IgG₁, biotin-conjugated mouse anti-rabbit IgG and avidin-HRP conjugate (BD Pharmingen, San Jose, CA, U.S.A.), 3,3',5,5'-tetramethylbenzidine (TMB, Sigma Chem., St. Louis, MO,

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U.S.A.), HRP-conjugated anti-guinea pig IgG, HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA, U.S.A.), and ECLTM Western blotting detection reagents (ECL, Amersham Biosciences, Buckinghamshire, U.K.).

Partial Purification of Pollen Extracts Pollens were suspended in phosphate buffered saline (PBS, pH 7.4) at 100 g/l, vigorously stirred at 4 °C for 72 h, and then centrifuged (31000×g, 4 °C, 30 min), after which the supernatant was collected. The resultant pollen pellet was resuspended in 1 mM phosphate buffer (pH 6.5), stirred for 1 h and centrifuged again. After combining two supernatants and filtration through a 1.2 µm filter, the combined supernatant was treated with 80% saturated ammonium sulfate in ice for 1 h while stirring. Following centrifugation (31000×g, 4 °C, 30 min), the obtained precipitation was dissolved in distilled water. The solution was chilled in an ethanol bath at 0 °C, followed by addition of distilled ethanol, which had been chilled to -3 to -6 °C with ethanol-dry ice, in order to achieve a final 75% ethanol solution. This solution was stirred for 30 min, and centrifuged (8000×g, 4 °C, 30 min), after which the precipitation was collected. The 75% ethanol treatment was repeated 2 more times, and precipitations obtained was combined, and then dried under a reduced pressure. The residue was dissolved in distilled water, and used as a partially purified pollen extract (PPPE).

Total protein amount in PPPE was estimated by the method of Bensadoun and Weinstein¹¹⁾ who modified an earlier method reported by Lowry *et al.*¹²⁾

Amounts of Cry j 1 and Cry j 2 in PPPE were measured by an enzyme-linked immunosorbent assay (ELISA). Wells of an ELISA plate, (Immulon4 HBX, Thermo LabSystems, Beverly, MA, U.S.A.) were coated with 1 µg/ml of either anti-Cry j 1 mAb (clone 013) or anti-Cry j 2 mAb (clone T27) by incubation at 4 °C for 12–18 h. After washing the wells 3 times with 0.05% Tween 20-containing PBS (T-PBS), wells were treated with a blocking buffer, 3% BSA-containing T-PBS at 37 °C for 2 h. After washing 3 times, PPPE, or purified Cry j 1 or Cry j 2, which was appropriately diluted with the blocking buffer, was added. After allowing the wells to stand for 12–18 h at 4 °C, they were washed 3 times, followed by the addition of HRP-conjugated anti-Cry j 1 mAb (clone 053, 0.5 µg/ml) or HRP-conjugated anti-Cry j 2 polyclonal antibody (0.25 µg/ml). Wells were incubated at room temperature for 1 h, and then washed 6 times. Afterwards, TMB solution was added followed by development of the enzyme reaction for 30 min. The reaction was stopped by addition of 1 M sulfuric acid. A microplate reader was used to measure the absorbance at 450 nm, and amounts of Cry j 1 and Cry j 2 in the PPPE were calculated from respective standard curves of purified Cry j 1 and Cry j 2.

Immunization with PPPE PPPE solution was mixed with CFA at a ratio of 4.5:5.5, and then the mixture was emulsified. The emulsion (protein concentration: 158 µg/ml) was repeatedly administered to guinea pigs, mice and rabbits at an interval of 2 weeks for a total of 5 times. In guinea pigs and rabbits, the emulsion was subcutaneously administered to footpads at volumes of 0.125 (guinea pig) and 0.25 (rabbits) ml/animal/time at the 1st immunization, and then intradermally injected in the back skin at 0.5 (guinea pig) and 1 (rabbits) ml/animal/time at the 2nd–5th immunizations. In

mice, the emulsion was subcutaneously administered in the back at 0.1 ml/animal/time at the 1st–5th immunizations.

Ten days after the respective 2nd–4th immunizations, blood was drawn from the vein of a forepaw (guinea pig), the ear vein (rabbit) or the orbital veniplex (mouse). The infraorbital puncture in mice was performed under ether anesthesia. All animals were sacrificed 10 d after the 5th immunization by bleeding under anesthesia with pentobarbital (guinea pig), ether (mouse) or urethane (rabbit). Sera were isolated from collected blood.

Measurements of Antibody Titers Pollen-specific γ_1 antibody titer in the serum collected from the sensitized guinea pig was estimated by a 4-h passive cutaneous anaphylaxis (PCA) reaction in guinea pigs according to the methods of Levine *et al.*¹³⁾ and Ovary *et al.*¹⁴⁾ In brief, the shaved back skin of a guinea pig was passively sensitized by intradermal injection with serially diluted sera obtained from the sensitized guinea pig. The passively sensitized guinea pig was challenged 4 h after the sensitization by intravenous injection with a solution of pollen extract and Evans blue. The guinea pig was sacrificed 30 min after the challenge followed by isolation of the back skin. γ_1 antibody titer was estimated by macroscopic observation at the reaction sites.

ELISA was used to measure Cry j 1-specific IgG₁ and IgG antibody amounts in sera from the sensitized mouse and rabbit. Wells of an ELISA plate (Immulon4 HBX) were coated with 2 µg/ml of Cry j 1 by incubation at 4 °C for 12–18 h. After washing the wells 3 times with T-PBS, wells were treated with a blocking buffer, which consisted of 10% FBS-containing PBS, at room temperature for 2 h. After washing 3 times, diluted sample sera were added and then allowed to stand at 4 °C. The following day, the plate was washed 3 times, and then either biotin-conjugated rat anti-mouse IgG₁ or biotin-conjugated mouse anti-rabbit IgG was added at 2 µg/ml. One hour after the addition, avidin-HRP (1:1000) was added after washing 6 times, and then the plate was allowed to stand at room temperature for 30 min. After 6 more washings, the enzyme reaction was initiated by addition of the substrate, TMB. The reaction was terminated by addition of sulfuric acid solution 30 min after the start of the reaction. A microplate reader was used to measure absorbance at 450 nm, and amounts of Cry j 1-specific mouse IgG₁ (arbitrary unit (au)/ml) or Cry j 1-specific rabbit IgG antibody (µg/ml) were calculated from each standard curve of a pooled anti-Cry j 1 mouse serum or anti-Cry j 1 rabbit IgG fraction.

Detection of Antigenic Proteins by Western Blotting Analysis PPPE (87.5, 175 and 350 µg/ml), purified Cry j 1 (6.25 and 12.5 µg/ml) and purified Cry j 2 (50 and 100 µg/ml) in PBS, were diluted 2-fold with sample buffer, 0.1 M Tris-HCl buffer (pH 6.8) containing 4% sodium dodecyl sulfate (SDS) and 0.01% bromophenol blue. The sample (15 µl) combined with a size marker (5 µl) (Kaleidoscope prestained standards, Bio-Rad, Hercules, CA, U.S.A.) was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically (100 V, 60 min) transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was treated with a blocking buffer, 5% skim milk-containing Tris buffered saline, at 4 °C for 12–18 h. Following washing with 0.1% Tween 20-containing Tris buffered saline, the membrane was treated with anti-PPPE sera, or anti-Cry j 1 mAb (clone 026) or anti-Cry j 2 mAb (Clone T27), which

were diluted with 1% skim milk-containing Tris buffered saline. The membrane was incubated for 2 h, washed, and then HRP-conjugated anti-mouse IgG (1:2000 or 1:8000), HRP-conjugated anti-guinea pig IgG (1:2000), or HRP-conjugated anti-rabbit IgG (1:40000) was added. One hour after the addition, the membrane was washed, and then treated with ECL for 5 min. Detection of the bands was performed after exposure to an X-ray film (Fuji Film, Tokyo, Japan).

RESULTS

Amounts of Total Protein, Cry j 1 and Cry j 2 in PPPE

As shown in Table 1, total protein concentration in PPPE was 350 $\mu\text{g}/\text{ml}$, and amount of total protein derived from 100 g cedar pollen was 74 mg. Ratios of Cry j 1 and Cry j 2 in total protein were 80.3 and 3.7%, respectively.

Time Course of Changes in Antigen-Specific Antibodies in Sera of Sensitized Animals Figure 1 represents time-course changes in pollen-specific γ_1 , Cry j 1-specific IgG₁ and Cry j 1-specific IgG antibodies in sera from sensitized guinea pigs, mice and rabbits, respectively, 10 d after the 2nd—5th immunizations. Sera from all species were positive for the allergen 10 d after the 2nd immunization. Antibody titers continued to be high on day 10 after the 5th immunization. Therefore, in order to detect antigenic proteins by the Western blotting analyses described below, sera collected 10 d after the 5th immunization were used.

Detection of Cry j 1 and Cry j 2 in PPPE by Western Blotting PPPE was separated by SDS-PAGE, and then Cry j 1 and Cry j 2 were detected using anti-Cry j 1 mAb and anti-Cry j 2 mAb (Figs. 2A, B). Consistent with previous reports,^{1,2)} proteins were detected in PPPE that corresponded to Cry j 1 (40—45 kDa) and Cry j 2 (approximately 37 kDa). Faint bands were also detected around 30 and 32 kDa when anti-Cry j 1 mAb and anti-Cry j 2 mAb were used, respectively.

Detection of New Antigenic Proteins in PPPE by Western Blotting To detect other antigenic proteins besides Cry j 1 and Cry j 2, anti-PPPE sera obtained from sensitized guinea pigs, mice and rabbits were used as detection antibodies in the Western blotting analyses. As shown in Figs. 3A—C, both Cry j 1 and Cry j 2 bands were clearly detected in all species. Interestingly, four bands besides Cry j 1 and Cry j 2 were also detected around 31, 20, 15 and 7 kDa in all experiments (Figs. 3A—C). On the other hand, no bands were observed for normal sera collected from non-sensitized guinea pigs, mice and rabbits (data not shown).

DISCUSSION

Although Cry j 1¹⁾ and Cry j 2^{2,3)} are common allergenic proteins contained in Japanese cedar pollen, there are small populations of patients who suffer from cedar pollenosis even though they have negative skin reactions to Cry j 1 and Cry j 2.⁵⁾ Thus, it has been suggested that the pollen contains other antigenic proteins besides these two molecules. In this study, we evaluated whether other proteins in PPPE separated by SDS-PAGE can react to anti-PPPE sera collected from sensitized guinea pigs, mice and rabbits. Subsequently, four other antigenic proteins with molecular weights around 7, 15, 20 and 31 kDa were detected. The band of proteins in PPPE that exists around 31 kDa was also observed when the anti-

Table 1. Concentrations of Total Protein, Cry j 1 and Cry j 2 in Partially Purified Pollen Extracts (PPPE) and Their Amounts in Japanese Cedar Pollen

	Concentration in PPPE ($\mu\text{g}/\text{ml}$)	Amount in the pollen (mg/100 g pollen)	% of total protein
Total protein	350	74.0	—
Cry j 1	281	59.4	80.3
Cry j 2	13	2.8	3.7

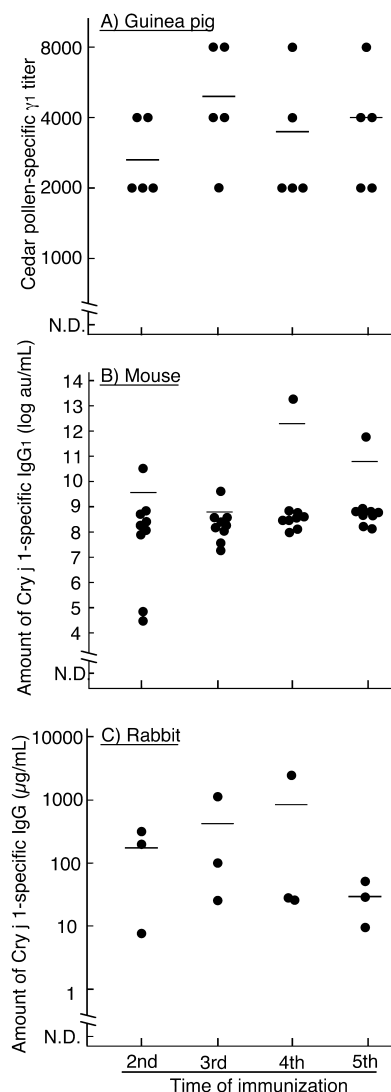


Fig. 1. Time-Course Changes in Titers of Pollen-Specific γ_1 Antibody, Amounts of Cry j 1-Specific IgG₁ and Cry j 1-Specific IgG Antibodies in Sera from Guinea Pigs, Mice and Rabbits, Respectively, during the 2nd—5th Immunizations with Partially Purified Extracts of Japanese Cedar Pollen (PPPE) Emulsified with Complete Freund's Adjuvant

Animals were bled 10 d after respective immunizations. Each bar represents the mean of 5 (guinea pigs), 9 (mice) and 3 (rabbits) animals. N.D.: Not detectable.

Cry j 1 mAb and anti-Cry j 2 mAb were used, which suggests that these proteins may possess identical epitopes to Cry j 1 or Cry j 2. As reported by Kawamoto *et al.*,⁶⁾ the proteins around 31 kDa are speculated to include CJP-6. However, the other relatively low molecular weight proteins found at 7, 15 and 20 kDa proteins were not detected when anti-Cry j 1 mAb and anti-Cry j 2 mAb were used as antibodies for West-

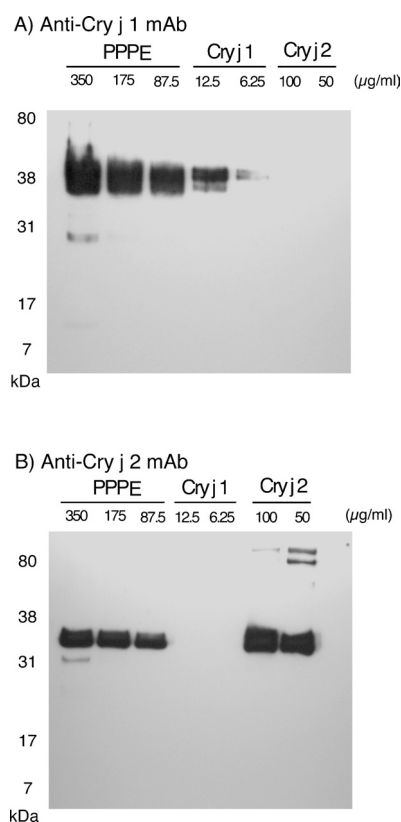


Fig. 2. Western Blotting Analyses of Partially Purified Extracts of Japanese Cedar Pollen (PPPE) Using Anti-Cry j 1 mAb (A) or Anti-Cry j 2 mAb (B) as a Detection Antibody

Indicated concentrations of PPPE, purified Cry j 1, or purified Cry j 2 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transference to a polyvinylidene difluoride membrane. Subsequently, the membrane was stained with either anti-Cry j 1 mAb or anti-Cry j 2 mAb.

ern blotting. This strongly suggests that these three proteins are different from Cry j 1 and Cry j 2 not only in molecular weight, but also in antigenicity. On the other hand, a faint band was also detected around 15 kDa when the purified Cry j 1 was separated by SDS-PAGE and stained with anti-PPPE sera from sensitized guinea pigs and mice. Thus, we can not deny a possibility that the 15 kDa protein(s) may be decomposed product(s) of Cry j 1.

Although the intensities of the bands around 7, 15 and 20 kDa were different among guinea pigs, mice and rabbits, it appears that all of these species produce IgG class antibodies against these three proteins in PPPE after immunization. Since the intensities of these bands are weaker than those for Cry j 1 and Cry j 2, there may only be minor amounts of these relatively low molecular antigenic proteins. However, the lack of species difference suggests that the 7, 15 and 20 kDa proteins could show antigenicity for humans. Therefore, not only Cry j 1 and Cry j 2 but also these three antigen proteins may be involved in inductions of nasal or conjunctival symptoms in cedar pollenosis patients. To confirm this hypothesis, future experiments will need to be done using sera collected from pollenosis patients. Additionally, although we evaluated IgG class antibody production against the pollen in a preliminary study, further examination of whether there is production of IgE antibodies specific for the 7, 15 and 20 kDa proteins after immunization with PPPE needs to be undertaken.

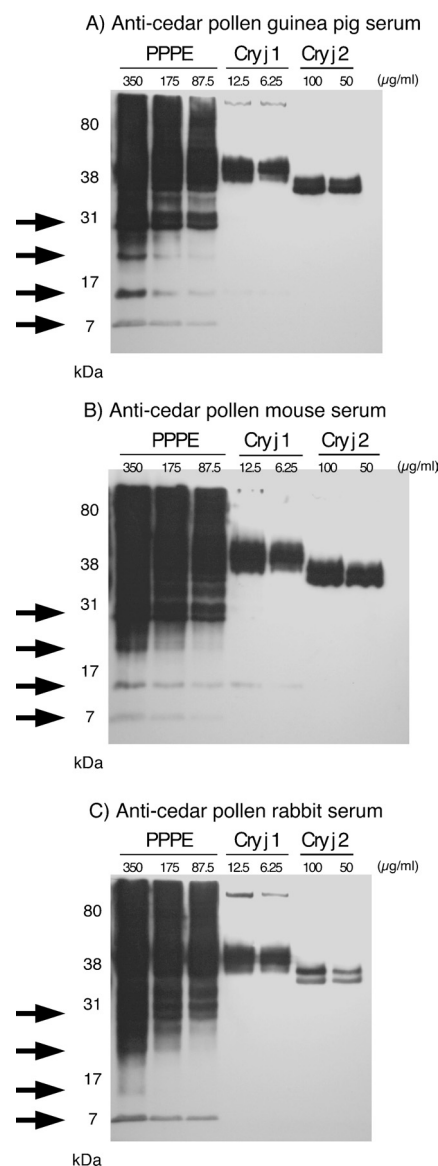


Fig. 3. Western Blotting Analyses of Partially Purified Extracts of Japanese Cedar Pollen (PPPE) using Anti-Pollen Sera from Sensitized Guinea Pigs (A), Mice (B) or Rabbits (C) as a Detection Antibody

Indicated concentrations of PPPE, purified Cry j 1, or purified Cry j 2 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transference to a polyvinylidene difluoride membrane. Subsequently, the membrane was stained with anti-pollen sera from 3 species of sensitized animals. Arrows indicate antigenic proteins existing around 31, 20, 15 and 7 kDa.

In conclusion, our results indicate that there are three additional antigenic proteins in Japanese cedar pollen that have different molecular weights from Cry j 1, Cry j 2 and CJP-6. Structural analyses of these proteins may be useful in the development of new therapeutic methods, including specific immunotherapy for pollenosis.

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