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Molecular cloning of the second major allergen, *Cry j* II, from Japanese cedar pollen

Motoshi Namba*, Mayumi Kurose, Kakuji Torigoe, Katsuhiko Hino, Yoshifumi Taniguchi, Shigeharu Fukuda, Mitsuko Usui, Masashi Kurimoto

Fujisaki Institute, Hayashibara Biochemical Laboratories Inc., 675-1 Fujisaki, Okayama 702, Japan

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Abstract Cloning of a cDNA from *Cry j* II, the second major allergen from Japanese cedar (*Cryptomeria japonica*) pollen, is described. An isolated *Cry j* II cDNA contained an open reading frame coding for 514 amino acid residues. The mature *Cry j* II protein consisted of 388 amino acid residues (R^{46}–S^{433}). According to a homology analysis, no amino acid sequence homology was observed between *Cry j* II and *Cry j* I, another major allergen. But *Cry j* II showed homology with polygalacturonase (PG) derived from tomato (40% identity) at the amino acid level. The sequence information can potentially be used to devise an effective course of immunotherapy for Japanese cedar pollinosis.

Key words: Japanese cedar pollen; *Cry j* II; *Cry j* I; Polygalacturonase; Molecular cloning

1. Introduction

Japanese cedar pollinosis is one of the most important allergic diseases in Japan. Two major allergens, *Cry j* I and *Cry j* II, have now been identified in Japanese cedar pollen. *Cry j* I was first purified and identified in 1983 [1]. *Cry j* II was identified in 1990 [2] as an allergen different from *Cry j* I in molecular weight, antigenicity and N-terminal amino acid sequence (10 residues). *Cry j* I and *Cry j* II are present in a pollen extract at a ratio of about 4:1, and 24 of 25 patients allergic to Japanese cedar pollen showed a positive reaction to *Cry j* I, while 19 of the 25 patients showed a positive reaction to *Cry j* II by a radioallergosorbent test and a skin test [3]. Thus *Cry j* II is also clinically important. Some monoclonal antibodies (mAbs) specific for *Cry j* I or *Cry j* II have been obtained [3], and enzyme-linked immunosorbent assay systems have been established specifically determining *Cry j* I or *Cry j* II in the crude pollen extract [4]. Very recently, *Cry j* I cDNA was obtained [5,6]. However, *Cry j* II cDNA has not been obtained yet.

In this report, the cDNA cloning and the deduced amino acid sequence of a *Cry j* II cDNA clone are described. Partial amino acid sequences determined on the enzyme-digested *Cry j* II peptides were included in the deduced sequence from the cDNA.

2. Materials and methods

2.1. Plant materials

Fresh male flowers were collected from Japanese cedar trees located in Okayama, Japan, and stored at −80°C until used.

2.2. Isolation of Cry j II protein

Cry j II protein was isolated from Japanese cedar pollen extract by affinity chromatography using an mAb specific for *Cry j* II, N26 [7].

*Corresponding author. Fax: (81) (86) 276 6885.

Abbreviations: PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; PG, polygalacturonase; TPCK-trypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin; mAb, monoclonal antibody; CNBr, cyanogen bromide.

2.3. Analysis of the N-terminal amino acid sequence of Cry j II

The *Cry j* II protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (ProBlot, Applied Biosystems, CA, USA) after SDS-PAGE, and was sequenced on a protein sequencer (model 473A, Applied Biosystems).

2.4. Preparation of pyridylethylated Cry j II protein

Pyridylethylated *Cry j* II protein was prepared to analyze the cysteine residues as described previously [8].

2.5. Analysis of enzyme-digested Cry j II

The *Cry j* II protein was digested with V8 protease (from *Staphylococcus*, Sigma, MO, USA), pepsin (from porcine stomach mucosa, Sigma) or lysylendopeptidase (from *Acromobactor*, Seikagaku Corporation, Tokyo, Japan) as described previously [9]. The pyridylethylated *Cry j* II protein was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (TPCK-trypsin) (from bovine pancreas, Sigma; 25 μg per 1 mg *Cry j* II at 37°C for 20 h in 0.1 M sodium bicarbonate) or lysylendopeptidase, or cleaved with cyanogen bromide (CNBr) (Wako Pure Chemical Industries, Osaka, Japan; 6 mg per 1 mg *Cry j* II in 75% trifluoro acetic acid at room temperature for 20 h). The enzyme-digested or CNBr-cleaved peptides of the *Cry j* II protein or the pyridylethylated *Cry j* II protein were separated on a reversed-phase HPLC C18 column (218TP54, Vydac, CA, USA). The amino acid sequences of the peptides were determined on the protein sequencer.

2.6. Analysis of the C-terminal amino acid sequence of Cry j II

The lysylendopeptidase-digested *Cry j* II protein was applied to an anhydrotrypsin-agarose column (Takara Shuzo, Kyoto, Japan). This column has the ability to catch peptide fragments, the C-terminal ends of which are either a lysine residue, an arginine residue or an aminoethyl derivative of a cysteine residue [10]. The fraction, being passed through the column, was collected and separated on a C18 column for amino acid sequencing.

2.7. Isolation of RNA

Japanese cedar male flower RNA was isolated according to [11], and further purified on a hydroxyapatite (Gigapite, Toa Gosei, Tokyo, Japan) column equilibrated with 10 mM sodium phosphate buffer (pH 6.8), then the RNA was eluted with 300 mM sodium phosphate buffer (pH 6.8).

2.8. Amplification of Cry j II cDNA

The primers used for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized on a DNA synthesizer (model 394, Applied Biosystems). Primer-specific DNAs were amplified from 1 μg RNA using a Gene Amp RNA PCR kit (Takara Shuzo) as described previously [12]. Cloning of the cDNA terminal regions (3′ or 5′ end) was

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performed by rapid amplification of the cDNA ends (RACE) method as described previously [13].

2.9. Screening and sequencing of PCR products

The PCR products were examined by Southern blotting. The DNAs showing a positive signal were ligated into a sequencing vector, pT7Blue (NoVagen, WI, USA). Positive clones were sequenced on a DNA sequencer (model 373A, Applied Biosystems).

3. Results

Before cloning *Cry j* II cDNA, the partial amino acid sequences of *Cry j* II were determined. First, the N-terminal amino acid sequence of *Cry j* II was determined as RK-VEHSRHDAINIFNVEKYGAVGDGKH. Second, the *Cry j* II protein or the pyridylethylated *Cry j* II protein was digested with several enzymes, and then the amino acid sequences of the resulting peptides were determined. Based on these sequences, we synthesized some degenerate sense and antisense oligonucleotides, and tried RT-PCR on cedar male flower RNA using combinations of the synthesized primers (Table 1). Five clones having partial *Cry j* II cDNA were obtained and designated CJII-1, CJII-2, CJII-3, CJII-4 and CJII-5 as shown in Fig. 1A. By combination and rearrangement of the nucleotide sequences of the five clones, we obtained a complete sequence of *Cry j* II cDNA having 1790 bp. Considering the amino acid sequence analysis, we determined that the open reading frame of *Cry j* II begins from the start codon at position 126–128 and ends at the stop codon at position 1668–1670, as shown in boxes in Fig. 1A. Thus the protein coding region of the *Cry j* II cDNA consisted of 1542 bp coding 514 amino acid residues. As shown in Fig. 1B, three possible N-linked glycosylation sites having the characteristic N-X-S/T motif were observed at the 429-, 460- and 472-asparagine residues in the amino acid sequence deduced from *Cry j* II cDNA. The amino acid sequences of the 26 fragments analyzed are shown underlined as amino acid residues. They were completely compatible with the corresponding amino acid sequences deduced from the cDNA. In Fig. 1B, ATL-5, the peptide fragment obtained by C-terminal amino acid sequence analysis was located in the last position of the all analyzed sequences. We therefore decided that the C-terminal amino acid sequence of *Cry j* II must be NLSPS. From these results, we decided that the *Cry j* II protein is constructed from 388 amino acid residues (Arg^{46}–Ser^{433}).

No amino acid sequence homology was observed between

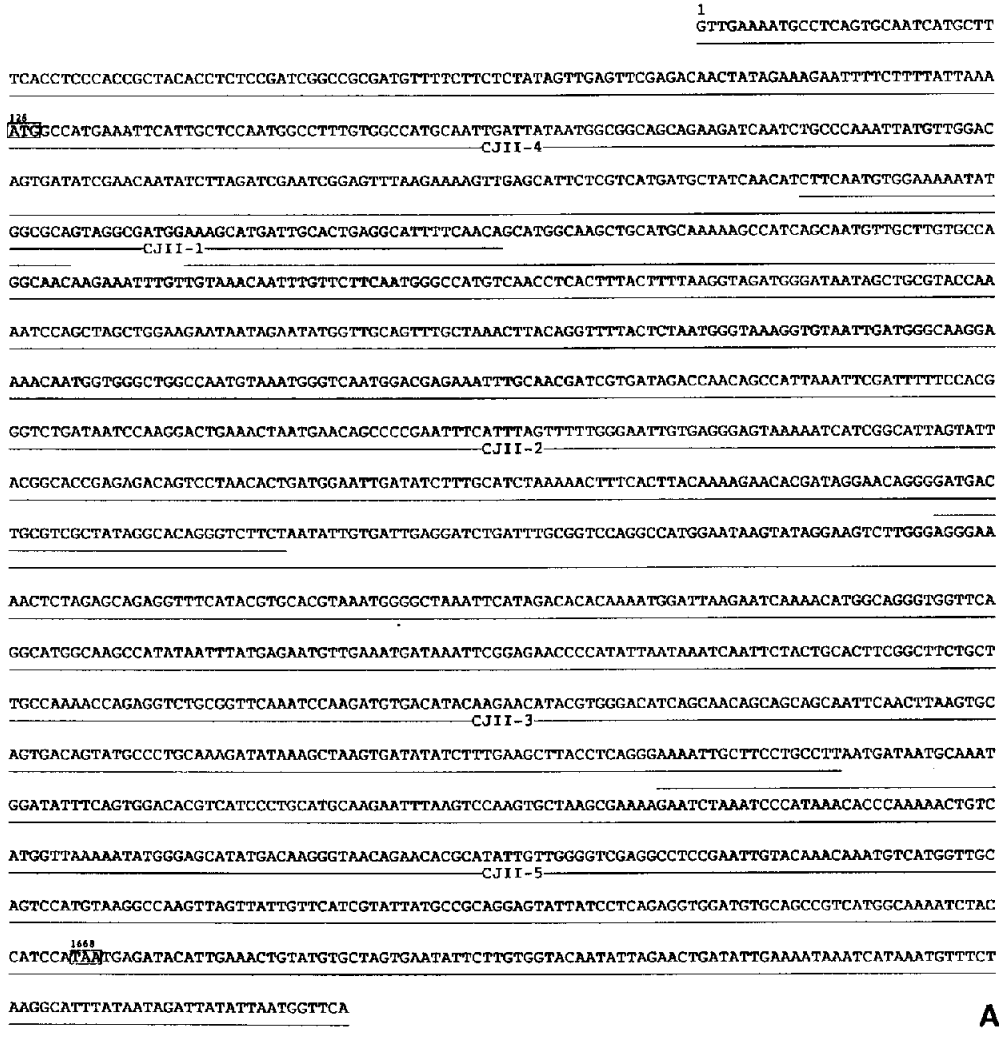

Fig. 1. (A) Nucleotide sequence of *Cry j* II cDNA. Translation initiation codon and stop codon are shown in boxes. Nucleotide sequences of five clones obtained separately by RT-PCR and RACE methods are underlined.

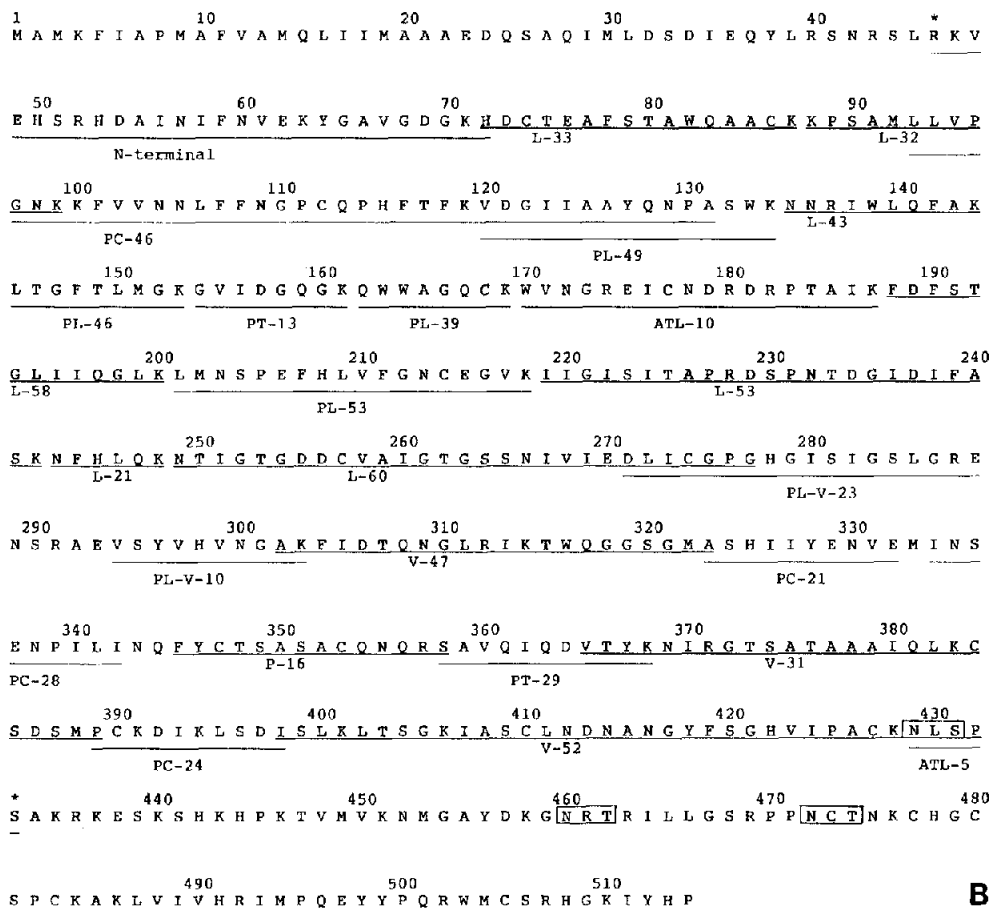

Fig. 1 (B) Amino acid sequence deduced from *Cry j* II cDNA. The amino acid sequences underlined were analyzed on an amino acid sequencer after being treated as follows. N-terminal, PVDF blotting after SDS-PAGE; ATL, lysylendopeptidase digestion followed by fractionation with anhydrotrypsin-agarose column; P, pepsin digestion; L, lysylendopeptidase digestion; V, V8 protease digestion; PT, TPCK-trypsin digestion of pyridylethylated *Cry j* II protein; PC, CNBr cleavage of pyridylethylated *Cry j* II protein; PL, lysylendpeptidase digestion of pyridylethylated *Cry j* II protein, and PL-V, lysylendpeptidase followed by V8 protease digestion of pyridylethylated *Cry j* II protein. *The N-terminal (R-46) and the C-terminal (S-433) amino acids of the *Cry j* II protein are indicated with asterisks. The enzyme used and the peptide fraction number are shown together underlined. N-linked glycosylation sites are shown in boxes.

Table 1
Oligonucleotides used to obtain clones of *Cry j* II cDNA by RT-PCR and RACE methods

Clones	Primers or probes[a]	Sequences[b]	Nucleotide positions
CJII-1	SP1	5′ CAYGAYGCNATHAAYAT	282–298
	AP1	5′ GCNGCYTGCCANGC	366–379
(299–365)	P1	5′ TTYAAYGTNGARAARTAYGG	300–319
CJII-2	SP2	5′ ATATGGCGCAGTAGGCGATGG	314–334
	AP2	5′ TCDATNACDATRTT	921–934
(335–920)	P2	5′ TGTTGAAAATGCCTCAGTGC	346–365
CJII-3	SP3	5′ AAGAACACGATAGGAACAGGG	867–887
	AP3	5′ TANCCRTTNGCRTTRTCRTT	1359–1378
(888–1358)	P3	5′ GTGCCTATAGCGACGCAGTC	890–910
CJII-4	$(dT)_{17}$-adaptor[c]	5′ GACTCGAGTCGACATCGA(T)17	
	AP4	5′ TCATGCTTTCCATCGCCTAC	324–343
(1–323)	P4	5′ TGTGGAAAAATATGGCGCAG	305–314
CJII-5	SP4	5′ ATCTTTGAAGCTTACCTCAGGG	1319–1340
	$(dT)_{17}$-adaptor	5′ GACTCGAGTCGACATCGA(T) 17	
(1341–1790)	P5	5′ AAGGCAGGAAGCAATTTT	1341–1358

[a]SP, sense primer; AP, antisense primer; P, probe. [b]Genetic codes used are: N, AGCT; Y, CT; R, AG; H, ATC; D, AGT c) primer for RACE method, annealing with a poly(A) tail.

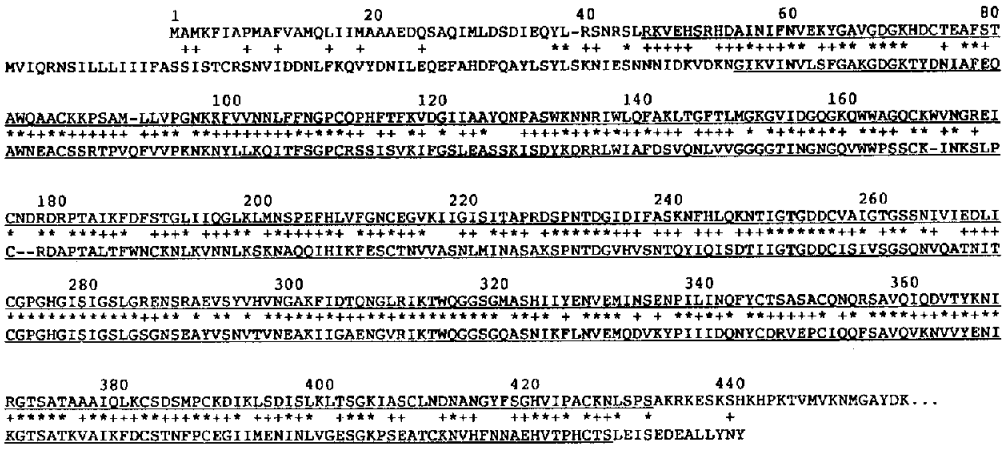

Fig. 2. Comparison of the deduced amino acid sequences of *Cry j* II (upper) and *Lycopersicon esculentum* PG (lower). The amino acid residues underlined indicate a mature protein region. *Identical amino acid. +Conservative substitution.

Cry j II and *Cry j* I. However, searching the SWISS-PROT database revealed that the *Cry j* II amino acid sequence shares a significant homology with polygalacturonases (PGs), especially that derived from tomato, *Lycopersicon esculentum* (40% identity, Fig. 2) [14,15], from *Zea mays* (34% identity) [16,17], and that from *Oenothera organensis* (34% identity) [18].

4. Discussion

As a result of molecular cloning and amino acid sequence analysis of *Cry j* II, we have isolated a cDNA clone coding 514 amino acid residues. The mature *Cry j* II protein consisted of 388 amino acid residues. It was reported that the N-terminal amino acid sequence of *Cry j* II was AINIFNVEKY [2]. However, our results showed that the N-terminal amino acid residue begins at arginine, located 9 residues before this sequence. This difference may be due to different preparation methods. Calculating the molecular weight from the deduced amino acid sequence, that of the mature protein (388 residues) was 42.2 kDa and that of the precursor protein (514 residues) was 56.6 kDa. By SDS-PAGE analysis under reducing conditions, the estimated molecular weight of the mature protein was 42 kDa, which was found to agree with the calculated molecular weight. It is likely, therefore, that both the 45 N-terminal amino acid residues and the 81 C-terminal amino acid residues are cleaved from the precursor protein. It has been reported that *Cry j* II is located in the amyloplast in Japanese cedar pollen [19]. The rye-grass allergen, *Lol p* I was also reported to be located in the amyloplast [20]. This allergen is thought to be synthesized in the cytosol as a precursor containing a transit peptide (25 amino acid residues) that is cleaved after transport of the protein to the amyloplast. A similar cleavage may occur after the transport of precursor *Cry j* II protein to the amyloplast.

Because *Cry j* II has a homology with PGs, we assessed the PG activity of the *Cry j* II protein. We found that it has enzymatic activity that hydrolyzes pectin (Otsuki, manuscript in preparation), although the physiological role of *Cry j* II in the amyloplast is still unknown.

PGs of plant origin have not been reported to cause pollinosis, although the PG from *Aspergillus niger* has been reported to cause allergic asthma [21]. It would be interesting to examine the immunoreactive sites of *Cry j* II and compare them with the active sites in the PGs.

In many clinical reports [22], a majority of patients sensitive to Japanese cedar pollen also react to pollen allergens from plants botanically related to the Japanese cedar. When the primary structures of those allergens are determined, the sequence similarities between the allergens and *Cry j* I or *Cry j* II will be clarified. A good knowledge of the immunoreactive sites responsible for the cross reactions will be beneficial in the treatment of allergic diseases. The *Cry j* II sequence information revealed in this report can potentially be used to devise an effective course of immunotherapy for Japanese cedar pollinosis.

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