



PURIFICATION, CHARACTERIZATION AND MOLECULAR CLONING OF Cha o 1, A MAJOR ALLERGEN OF *CHAMAECYPARIS OBTUSA* (JAPANESE CYPRESS) POLLEN§

MOTOHIKO SUZUKI,*¶ NAOKI KOMIYAMA,† MAKOTO ITOH,‡
HIROTAKA ITOH,* TOSHIO SONE,† KOHSUKE KINO,† IPPEI TAKAGI,*
and NOBUO OHTA‡

*Department of Otorhinolaryngology, Nagoya City University Medical School, Nagoya, Japan;
†Meiji Institute of Health Science, Odawara, Japan; ‡Department of Medical Zoology, Nagoya City
University Medical School, Nagoya, Japan

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Abstract—Pollen of *Chamaecyparis obtusa* (Japanese cypress) is one of the causes of allergic pollinosis in Japan. A major allergen of the pollen designated Cha o 1, was purified by two-step ion exchange chromatography. Cha o 1 was separated into four components with molecular masses of 48.5 kDa and 52.0 kDa, each with pIs of 6.77 and 6.82. The 23-residue N-terminal sequence of Cha o 1 was determined and shown to have high identity with that of Cry j 1, a major allergen of *Cryptomeria japonica* pollen. cDNA coding for Cha o 1 was cloned by hybridization screening using Cry j 1 cDNA as a probe. One of the cDNA clones, pCHA-1 was sequenced and found to code for a putative 21-residue signal peptide and a 354-residue native protein with a derived molecular mass of 38.1 kDa. The deduced amino acid sequence of Cha o 1 showed 79–80% identity with those of Cry j 1. These findings were consistent with observations of a close crossreaction between the two allergens. Homology analyses revealed that Cha o 1 had 46–49% identity with Amb a 1 families and Amb a 2, the major allergens of short ragweed. Cry j 1 has pectate lyase enzyme activity, suggesting that Cha o 1 may have the same enzyme activity as Cry j 1. Copyright © 1996 Elsevier Science Ltd.

Key words: *Chamaecyparis obtusa*, Cha o 1, cDNA cloning, major allergen.

INTRODUCTION

Allergic diseases are the most prevalent of human disorders, affecting almost 15% of the population in developed countries. Pollen of species of the Cupressaceae family are one of the most important causes of respiratory allergies in North America (Gross *et al.*, 1978; Ramirez, 1984; Yoo *et al.*, 1975), Australia (Ford *et al.*, 1991), Japan (Itoh *et al.*, 1995; Suzuki *et al.*, 1995) and some countries of the Mediterranean area including France (Bousquet *et al.*, 1984; Panzani *et al.*, 1986a) and Italy (Caiaffa *et al.*, 1993), and among these species, pollen of *Chamaecyparis obtusa* (Japanese cypress, Hinoki) is most often responsible for the respiratory allergies seen in Japan. Of the various airborne allergens, pollen of *Cryptomeria japonica* (Japanese cedar, Sugi) and *C. obtusa* are a yearly source of distress for many allergic people in this country. Over the past 13 years in Japan, a larger area has been reforested with *C. obtusa* trees than with *C. japonica*. Therefore, pollinosis caused by *C. obtusa* should be given close attention.

Clinical studies have shown that in Japan many patients with pollinosis have specific IgE antibodies not only to *C. japonica* but also to *C. obtusa* (Itoh *et al.*, 1995; Suzuki *et al.*, 1995). Sensitization to the two pollens may occur independently or, alternately, a close cross-reactivity may exist between the two so that sensitization to one would confer sensitization to the other. Many clinicians think that the latter situation may be the case and our recent findings regarding competitive inhibition between *C. japonica* and *C. obtusa* also support this view (Itoh *et al.*, 1995).

Two allergen molecules of *C. japonica* have been purified; SBP or Cry j 1 and Cry j 2, which have molecular masses of 41–45 kDa and 37 kDa, respectively (King *et al.*, 1994; Sakaguchi *et al.*, 1990; Yasueda *et al.*, 1983). Furthermore, the complete amino acid sequences of Cry j 1 and Cry j 2 were recently reported (Komiyama *et al.*, 1994; Sone *et al.*, 1994). However, in the case of *C. obtusa*, molecular analysis of the allergens remains to be carried out. In the present study, we purified a new major allergen of *C. obtusa*, designated Cha o 1 according to WHO nomenclature (King *et al.*, 1994), and the predicted primary structure of Cha o 1 was determined by sequencing its cDNA. Based on our observations, the probable biological properties of Cha o 1 and cross-reactivity of Cry j 1 and Cha o 1 allergens are discussed.

§The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number D45404.

¶ Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Plant materials

Pollen was collected in Japan from trees of *C. japonica* and *C. obtusa*. All materials were stored at -70°C until use.

Purification of Cha o 1 and Cry j 1

Cry j 1 was purified as described by Yasueda *et al.* (1983). The same method was used for Cha o 1 purification by monitoring the presence of Cha o 1 in each step using the enzyme immunoassay described below. Briefly, 4.2 g of pollen from *C. obtusa* were defatted three times with 40 ml of diethyl ether and dried overnight at room temperature. The pollen was homogenized with a teflon pestle at a maximum speed of 20 strokes in 25 ml of 10 mM Tris-HCl, pH 7.8, at 4°C . The homogenate was stirred in 60 ml of the same buffer for 1 h at 4°C . It was then centrifuged at $12,000g$ for 20 min and the supernatant was adjusted to pH 7.8 with 1 M Tris-HCl. Fifty millilitres of the supernatant were then applied to a DE52 anion-exchange cellulose column (1.1×5.5 cm; Whatman, Maidstone, U.K.) equilibrated with 10 mM Tris-HCl, pH 7.8. One hundred millilitres of unabsorbed fractions monitored at 280 nm were collected and dialysed against 5 l of 10 mM sodium acetate, pH 5.0, at 4°C overnight. The solution was then applied to a CM52 cation-exchange cellulose column (1.1×5.5 cm; Whatman) equilibrated with 10 mM sodium acetate, pH 5.0. After washing the column with the buffer, the Cha o 1 fraction was eluted with 0.5 M NaCl, 10 mM Tris-HCl, pH 7.8. Concentrations of Cha o 1 in the samples were measured by an enzyme immunoassay using biotinylated anti-Cry j 1 rabbit IgG which was prepared by DE52 anion-exchange chromatography from sera of rabbits immunized with Cry j 1. In order to determine the amino acid sequence, further purification of Cha o 1 was carried out with reversed-phase HPLC (high performance liquid chromatography) using a C4 column ($5 \mu\text{m}$, 0.46×25 cm; Vydac, Hesperia, CA) with a 30–40% acetonitrile linear gradient, in 15 min, containing 0.1% trifluoroacetic acid and the flow rate was set at 1 ml/min.

Protein concentration determination

The protein concentration was determined using a BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol.

Sequence analysis of purified Cha o 1 protein

Automated gas-phase Edman degradation was performed on a 470A gas-phase protein sequencer (Applied Biosystems Inc., Foster City, CA) using the manufacturer's standard programme. Polybrene was used as an inert support and 1 nmol of Cha o 1 was applied. The PTH-amino acids (phenylthiohydantoin amino acid) were analysed using an on-line PTH-Analyzer 120A 5 (Applied Biosystems).

Electrophoresis and immunoblotting

Gel electrophoresis in the presence of SDS (sodium dodecyl sulphate) was carried out in 8% polyacrylamide slab gels according to the method of Laemmli (1970). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) with slight modifications. Briefly, 10 μg of Cha o 1 were loaded onto the top of a gel containing 4% Ampholine (pH 3.5–10, Pharmacia, Uppsala, Sweden), 2% NP-40, and 8 M urea and electrofocused according to the following schedule: (1) 100 volts for $\frac{1}{4}$ hr; (2) 200 volts for $\frac{1}{2}$ hr; (3) 300 volts for $\frac{1}{2}$ hr and (4) 400 volts for 15 hr. The gel was placed on a slab of 8% acrylamide gel containing SDS and electrophoresed for 6 hr at 20 mA. The gels were stained with Coomassie brilliant blue R-250 or, in some cases, using the silver-staining method described by Oakley *et al.* (1980).

Cha o 1 bands separated by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) were identified by immunoblotting using anti-Cry j 1 rabbit IgG. A crude extract of *C. obtusa* pollen was subjected to 8% acrylamide SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane (TEF Corporation, Nagano, Japan) by the method of Towbin *et al.* (1979). The membrane was blocked with Block Ace (Dainippon Pharmaceutical Co., Tokyo, Japan) overnight at 4°C , then incubated with biotin labelled anti-Cry j 1 rabbit IgG (10 $\mu\text{g}/\text{ml}$) for 1 hr at 37°C . After washing five times with a washing buffer (0.05% Tween 20, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5), the membrane was incubated with streptavidin-peroxidase conjugate ($\times 1000$ diluted; Serotec, Oxford, U.K.) for 0.5 hr at 37°C . The membrane was washed again and incubated with 4-chloro-1-naphthol (Bio-Rad, Hercules, CA) solution.

Enzyme immunoassay of Cha o 1

The relative concentration of Cha o 1 in samples obtained at each purification step was measured by an enzyme immunoassay. Each sample was coated onto a 96-well plate (100 μl of 10 $\mu\text{g}/\text{ml}$ protein/well; ICN Pharmaceutical Inc., Costa Mesa, CA) overnight at 4°C and the wells were blocked with Block Ace for 2 hr at 37°C . The plate was incubated with biotin labelled anti-Cry j 1 rabbit IgG (100 μl of 10 $\mu\text{g}/\text{ml}$ protein/well) for 1 hr at 37°C . After washing five times, the plate was incubated with 100 $\mu\text{l}/\text{well}$ of streptavidin-peroxidase conjugate ($\times 1000$ diluted; Serotec) for 0.5 hr at 37°C . The plate was washed again and incubated with a substrate solution containing O-phenylenediamine (Sigma, St Louis, MO) for 15 min at room temperature. The enzyme reaction was stopped by addition of 3 N sulphuric acid (100 $\mu\text{l}/\text{well}$) and the optical density of each well was measured at 492 nm with a reference at 600 nm using a micro plate reader (Tosoh, Tokyo, Japan). The relative concentration of Cha o 1 was expressed as units/ml using Cry j 1 as a standard, with 1 unit/ml of Cha o 1 corresponding to 1 ng/ml of Cry j 1.

Detection of IgE specific to Cha o 1

Serum IgE specific for the purified Cha o 1 was measured by the method of Kato *et al.* (1976). One hundred microlitres of purified Cha o 1 (10 µg/ml) were coated onto a 96-well black plate (Dainippon Pharmaceutical) and incubated at 4°C for 16 hr. After blocking the wells with Block Ace at 37°C for 2 hr, 100 µl of each human serum ($\times 3.3$ diluted with Block Ace) were added to wells and incubated at 37°C for 4 hr. IgE antibodies which bound to the solid-phase antigens were detected by β -galactosidase-conjugated anti-human IgE monoclonal antibody (Pharmacia). 4-Methylumbelliferyl- β -D-galactopyranoside was used as the enzyme substrate and the fluorescence obtained was measured using a Titertek Fluoroskan II (ICN Pharmaceutical). We tentatively set cutoff lines determining positive and negative IgE binding at a relative fluorescence unit = 25.

Isolation of RNA

Total RNA was isolated from *C. obtusa* pollen by the phenol extraction method established to isolate the total RNA from *C. japonica* (Sone *et al.*, 1994). Poly (A)⁺ RNA was obtained using oligo(dT)-cellulose (Clone-Tech Lab., Palo Alto, CA)

Northern blotting

Total RNA and mRNA were electrophoresed in 1.0% formaldehyde-agarose gels under denaturing conditions (Alwine *et al.*, 1977), and blotted onto a Hybond-N filter (Amersham, Buckinghamshire, U.K.). The filters were prehybridized at 65°C for 1 hr in a solution consisting of 10 $\times$ Denhardt's solution (where 50 $\times$ Denhardt's is 1.0% (w/v) Ficoll, 1.0% polyvinyl pyrrolidone, and 1.0% bovine serum albumin (Pentex Fraction V)) containing 4 $\times$ SSC (where 20 $\times$ SSC is 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0). Hybridization was performed at 30°C for 18 hr in a solution (4 $\times$ SSC, 10 $\times$ Denhardt's solution) containing a ³²P-labelled clone of Cry j 1 as a probe (5 $\times 10^7$ cpm/10⁷ ml). The ³²P-labelled Cry j 1 clone used in this study was pCCI2-2 ([³²P] pCCI2-2) which has previously been described (Sone *et al.*, 1994). The filters were washed at 30°C for 30 min in 2 $\times$ SSC, 0.1% SDS and for 30 min in 1 $\times$ SSC, 0.1% SDS. The filters were then exposed to X-Omat film (Kodak, Rochester, NY) at -80°C for 14 hr and the bands were detected on the film after development.

cDNA library screening

The λ gt 10 cDNA library, constructed by the same method described previously (Sone *et al.*, 1994), was screened using [³²P]pCCI2-2 as a probe. Plaques (100 000) were lifted onto Hybond-N filters (Amersham), and were denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min. They were then neutralized in 0.5 M Tris, pH 8.0, 1.5 M NaCl for 5 min and rinsed with 2 $\times$ SSC. Following air drying for 15 min, the filters were baked at 80°C for 2 hr immersed in 2 $\times$ SSC and washed for 1 hr with a solution of 5 $\times$ SSC, 0.5% SDS, 1 mM EDTA (ethy-

lenediaminetetracetic acid). The filters were then prehybridized at 65°C for 1 hr in a solution consisting of 10 $\times$ Denhardt's solution containing 4 $\times$ SSC and 10 mg/ml salmon sperm DNA. Hybridization was performed at 65°C for 12 hr in the hybridizing solution (4 $\times$ SSC, 20 $\times$ Denhardt's solution) containing [³²]pCCI2-2 (1 ng). The filters were washed twice in 2 $\times$ SSC, 0.1% SDS at room temperature for 20 min and once in 2 $\times$ SSC, 0.1% SDS at 65°C for 30 min. Filters were autoradiographed at -80°C for 3 hr. Positive clones were picked up and rescreened.

Nucleotide sequencing

The cDNA insert obtained by EcoRI digestion of λ gt10 vector was subcloned into the EcoRI site of the plasmid vector, pUC119. The DNA sequence was determined by means of fluorescence-based dideoxy sequencing using a 370A DNA Sequencer (Applied Biosystems) (Gibbs *et al.*, 1989). Both strands of the cDNA clone were sequenced completely.

Human sera

Sera from five allergic patients and five non-allergic individuals were used. The patients were diagnosed on the basis of case history and the presence of allergen-specific IgE as measured by AlaSTAT[®] (Diagnostic Products Corporation, Los Angeles, CA). All patients had IgE specific for *C. japonica* and *C. obtusa* pollen extracts at concentrations greater than 1.5 IU/ml, whereas concentrations in all healthy sera were less than 0.35 IU/ml (Table 2).

RESULTS

Purification and characterization of Cha o 1

We first attempted to determine whether a Cry j 1-like allergen exists in *C. obtusa* pollen using anti-Cry j 1 IgG prepared from rabbits immunized with purified Cry j 1, a major allergen of *C. japonica* pollen. Immunoblotting analysis of *C. obtusa* pollen showed that there were crossreactive components to anti-Cry j 1 IgG in the extract and these had molecular masses of 48.5 and 52.0 kDa, respectively. We tentatively designated these proteins Cha o 1 (Fig. 1). By monitoring the binding activity with anti-Cry j 1 IgG, Cha o 1 proteins were purified by the method developed for Cry j 1 purification (Yasueda *et al.*, 1983). Elution profiles of the homogenates of *C. obtusa* pollen obtained by DE52 and CM52 ion-exchange gel chromatography are shown in Fig. 2A and Fig. 2B. The anti-Cry j 1 IgG reacted to Cha o 1 proteins which did not bind to the DE52 column but were absorbed to CM52 (Table 1). As compared with crude extracts, Cha o 1 was purified with a 25-fold increase in specific activity. From 4.2 g of *C. obtusa* pollen, we obtained 1.23 mg of Cha o 1, and this value was comparable to 25 mg of Cry j 1/100 g of *C. japonica* pollen (Yasueda *et al.*, 1983). Cha o 1 purified by column chromatography had identical molecular masses to those

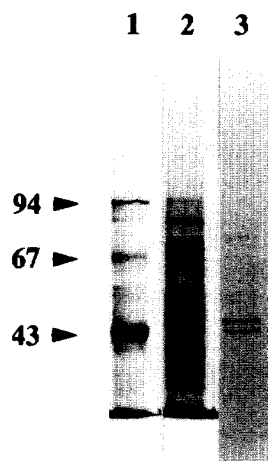


Fig. 1. Immunoblotting of Cha o 1. A crude extract of *C. obtusa* pollen was subjected to SDS-polyacrylamide gel (8%) electrophoresis, then transferred to a nitrocellulose membrane. Lane 1, molecular markers (Pharmacia): phosphorylase b (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa); lane 2, 9 μ g of total protein of crude extract; lane 3, bands of crude extracts detected by biotin-labelled anti-Cryj 1 rabbit IgG and peroxidase-labelled streptavidin.

observed in the crude extracts with anti-Cryj 1 IgG. The molecular masses of Cha o 1 were apparently different from those of Cryj 1 (Fig. 3A). Using two dimensional electrophoresis, the two components of Cha o 1 detected on SDS-PAGE were further separated into two components with different isoelectric points of 6.77 and 6.82. However, the 48.5 and 52.0 kDa components with a pI of 6.77 appeared faint on electrophoresis (Fig. 3B).

The allergenicity of Cha o 1 was confirmed by testing its reactivity with human IgE. Only sera obtained from donors who were hypersensitive to pollen of both *C. obtusa* and *C. japonica* possessed IgE specific to Cha o 1, whereas sera from healthy individuals showed no detectable reaction. Sera from four out of five patients reacted with Cha o 1 (Table 2).

The primary amino acid sequence of purified Cha o 1 was partly determined. The 23 N-terminal residues were Asp-Asn-Pro-Ile-Asp-Ser-X-Trp-Arg-Gly-Asp-Ala-Asn-Trp-Asp-Gln-Asn-Arg-Met-Lys-Leu-Ala-Asp, and this sequence showed high conformity with that of Cryj 1 except for three amino acids: an undetermined amino acid at position 7 and two different amino acids at positions 12 (Ser to Ala) and 15 (Ala to Asp).

Northern blotting

To confirm the feasibility of using Cryj 1 cDNA as a probe for cloning the Cha o 1 gene, we determined whether it was possible to detect Cha o 1 mRNA from *C. obtusa* by means of Northern blotting analysis using a ^{32} P-labelled pCCI-2-2 encoding Cryj 1. The probe was hybridized with a gene coding for a Cryj 1 homologue in *C. obtusa* of approximately 1.3 kb in length (Fig. 4).

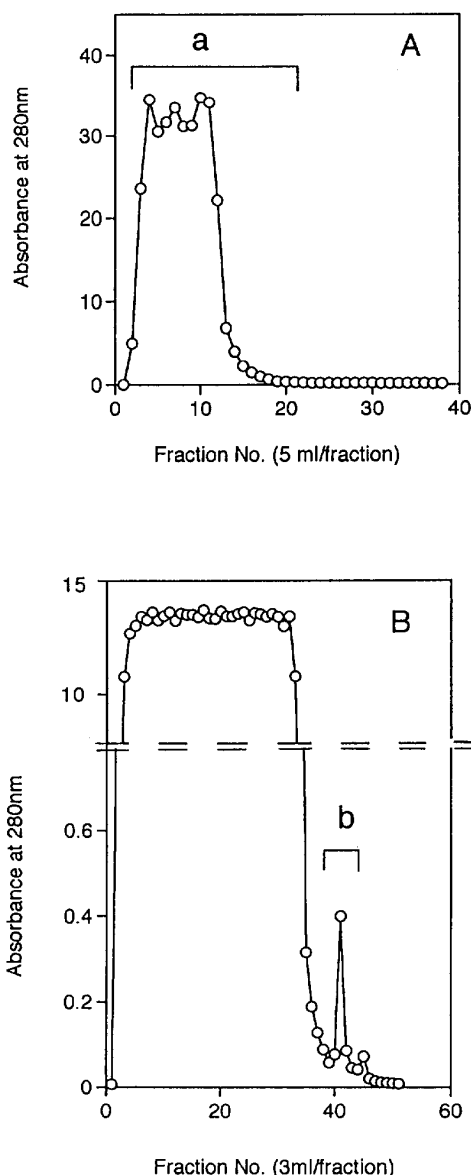


Fig. 2. Purification of Cha o 1 by ion-exchange chromatography. A, Elution profile of Cha o 1 obtained using a DE52 anion-exchange cellulose column (1.1 $\times$ 5.5 cm). Defatted and dried *C. obtusa* pollen was homogenized in 60 ml of 10 mM Tris-HCl, pH 7.8, at 4°C and centrifuged at 12 000 $\times g$ for 20 min. The supernatant, adjusted to pH 7.8 with 1 M Tris-HCl was applied to a column equilibrated with 10 mM Tris-HCl, pH 7.8, and eluted with the same buffer (5 ml/fraction). Unabsorbed fractions (a) were collected. B, elution profile of Cha o 1 obtained using a CM52 cation-exchange cellulose column (1.1 $\times$ 5.5 cm). The unabsorbed fraction (a) was dialysed against 10 mM sodium acetate, pH 5.0, at 4°C overnight and was applied to a column equilibrated with the same buffer. After washing the column with this buffer (3 ml/fraction), the Cha o 1 fraction (b) was eluted with 0.5 mM NaCl, 10 mM Tris-HCl, pH 7.8. In both fractions a and b, the presence of Cha o 1 was determined by enzyme immunoassay.

Table 1. Purification of Cha o 1 by DES2 and CM52 ion-exchange chromatography

Purification step	Total protein (mg)	Specific activity ^a (units/mg)	Relative specific activity	Total activity (units) ^b	Yield (%)
Extract	132.00	8.98	1.0	1185	100
DES2	45.30	18.60	2.1	844	71
CM52	1.23	227.00	25.0	280	24

^aThe activity of Cha o 1 was determined by enzyme immunoassay using rabbit anti-Cty j I IgG and Cry j 1 as a standard control.

^bOne unit of Cha o 1 corresponds to 1 ng of Cry j 1.

Cloning of Cha o 1

The λ gt10 cDNA library was screened with a ³²P-labelled cDNA (pCCI-2-2) coding for Cry j 1 as a probe and three positive clones were obtained. Both strands of the longest cDNA clone were sequenced completely and designated pCHA-1. This clone was found to consist of 1260 nucleotides (Fig. 5). Forty-nine of these nucleotides precede the initiator ATG start codon and the clone terminates with a TGA stop codon at position 1177. The sequence has an open reading frame of 1125 nucleotides coding for a protein of 375 amino acids. The 21 *N*-terminal amino acids are probably the signal peptide because these amino acids are not found in the native protein and are hydrophobic, as has been noted for other signal peptides. A 23-residue *N*-terminal sequence of the purified Cha o 1 was determined. The native protein was found to contain 354 residues with four potential *N*-linked glycosylation sites corresponding to the consensus sequence N-X-S/T (X except for D and P).

Sequence comparison

The amino acid sequence of Cha o 1 showed 79–80% identity with those of Cry j 1. The nucleotide sequence and deduced amino acid sequence of Cha o 1 were compared to sequences in GenBank (R81.0, Feb. 1994), EMBL-GDB (R38.0, Mar. 1994) and SWISS-PROT (R28.0, Jan. 1994). The amino acid sequence of Cha o 1 showed 46–49% identity with those of the Amb a 1 family (Amb a 1.01–1.04, isoforms), 49% with Amb a 2 (both are allergens of short ragweed) (Griffith *et al.*, 1991; Rafnar *et al.*, 1991; Rogers *et al.*, 1991), 45% with LAT59 (pollen protein in tomato), 9612 (pistil-expressed gene in tomato) (McCormick, 1991) and Zm58.2 (pollen protein in maize) (Turcich *et al.*, 1993), and 44% with LAT56 (pollen protein in tomato) (McCormick, 1991) (Fig. 6). Short ragweed (*Ambrosia artemisiifolia*) pollen is an important airborne allergen in North America, and the Amb a 1 family and Amb a 2 are the major allergens of patients with pollinosis in that country. Cha o 1 has sequence

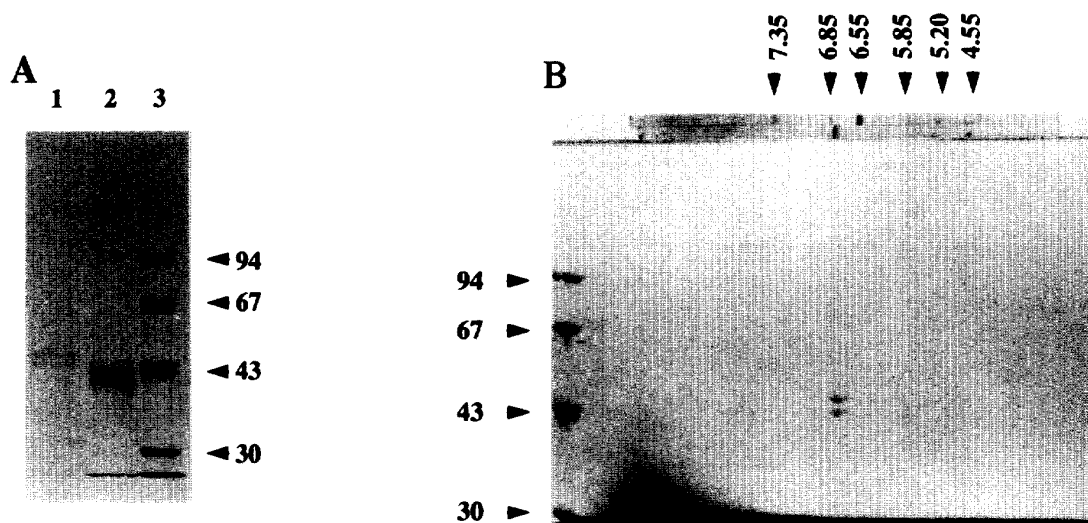


Fig. 3. Electrophoresis of Cha o 1. A, SDS-PAGE profiles of Cha o 1 and Cry j 1 using the silver staining method. Cha o 1 (4 μ g) and Cry j 1 (7 μ g) purified by means of DES2 and CM52 ion-exchange chromatography were subjected to SDS-polyacrylamide gel (8%) electrophoresis. Lane 1, Cha o 1; lane 2, Cry j 1; lane 3, molecular markers (listed in the legend of Fig. 1). B, two-dimensional gel electrophoresis profile. Purified Cha o 1 (10 μ g) was subjected to isoelectric focusing (right to left) followed by 8% SDS-polyacrylamide gel electrophoresis (top to bottom). pI Markers (Pharmacia) used were soybean trypsin inhibitor (pI 5.20), bovine and human carbonic anhydrase B (pIs 5.85 and 6.55), and horse myoglobin (pIs 6.85 and 7.35). The gel was stained with Coomassie brilliant blue R-250.

Table 2. Detection of IgE specific to purified Cha o 1 in human sera

Sera	AlaSTAT® (IU/ml) ^a		Purified allergen ^b	
	<i>C. obtusa</i>	<i>C. japonica</i>	Cha o 1	Cry j 1
Allergic patients				
#1	3.0–14.9	3.0–14.9	248	3000 ^c
#2	3.0–14.9	> 15	99	1683
#3	1.5–2.99	> 15	96	2280
#4	1.5–2.99	> 15	48	2461
#5	1.5–2.99	> 15	11	1934
Non-allergic control				
#1	<0.35	<0.35	4	22
#2	<0.35	<0.35	4	18
#3	<0.35	<0.35	4	4
#4	<0.35	<0.35	3	4
#5	<0.35	<0.35	4	4

^aIgE specific to pollen of *C. obtusa* and *C. japonica* in patients and control sera was measured by AlaSTAT®.

^bIgE specific to the purified Cha o 1 and Cry j 1 in the sera was measured by enzyme immunoassay.

^cThe reactivity of the serum of patient #1 was tentatively assigned 3000 and values are relative fluorescence units compared with the reference reactivity.

identities to limited regions of pectate lyases A and E (42% of 113 amino acids and 30% of 175 amino acids, respectively) of *Erwinia chrysanthemi* (a bacterial plant pathogen) (Keen and Tamaki, 1986; Tamaki *et al.*, 1988), and pectate lyases A and B (44% of 39 amino acids and 25% of 51 amino acids, respectively) of *Erwinia carotovora* (Lei *et al.*, 1987, 1988).

DISCUSSION

More than 85 kinds of cDNA coding for important allergens have been isolated, sequenced, and their primary structures deduced. However, in spite of its clinical

importance, the allergen of *C. obtusa* pollen has not as yet been isolated. The present report describes the biochemical properties of the purified allergen, Cha o 1, as well as the predicted amino acid sequence obtained by sequencing its cDNA.

To purify and clone Cha o 1, we used an anti-Cry j 1 IgG and a Cry j 1 cDNA clone. We used Cry j 1 as a reference for Cha o 1 since a cross-allergenicity exists between *C. japonica* and *C. obtusa* (Itoh *et al.*, 1995), whose allergen molecules have a similar molecular mass, and because people hypersensitive to *C. obtusa* have been shown to possess IgE to Cry j 1 with few exceptions. Our results clearly show that Cha o 1 is recognized by anti-Cry j 1 IgG and that the mRNA derived from *C. obtusa* reacted with a Cry j 1 cDNA clone. Together with these findings, we showed that employing Cry j 1 in the cloning of Cha o 1 was a useful strategy.

The sera from four out of five patients reacted with purified Cha o 1 (Table 2), and 22 out of 25 patients had IgE specific to Cha o 1 (submitted for publication). These results suggest that Cha o 1 is a major allergen of *C. obtusa*. Purified Cha o 1 of 48.5–52.0 kDa has strong binding activity to anti-Cry j 1 IgG. N-terminal amino acid sequencing of Cha o 1 revealed a high level of conformity with the same region of Cry j 1. By screening a cDNA library with a Cry j 1 probe, clones coding for Cha o 1 were obtained and the entire sequence of Cha o 1 was derived. The complete amino acid sequence of Cha o 1 showed significant conformity with those of Cry j 1. Taken together, *C. obtusa* possesses an allergen molecule highly homologous to Cry j 1, although *C. obtusa* and *C. japonica* do not belong to the same family.

The molecular mass of Cha o 1 deduced from its DNA sequence is calculated to be 38.1 kDa, whereas in its native form Cha o 1 is estimated to be 48.5 and 52.0 kDa (Fig. 3). Similar observations were made with respect to the purified and native forms of Cry j 1 (Sone *et al.*, 1994; Yasueda *et al.*, 1983) and it was recently reported that the glycosylation of two Asn residues at positions 170 and 333 in the Cry j 1 sequence causes a difference in the molecular mass between the predicted amino acid sequence and the native form of the Cry j 1 molecule (Hino *et al.*, 1995). Four possible N-linked glycosylation sites have been found in the Cha o 1 sequence. Therefore, one or more Asn residues at positions 89, 127, 157 and 331 in Cha o 1 may be glycosylated.

Cha o 1 was shown to migrate to two different positions with isoelectric points of 6.77 and 6.82 on the two-dimensional gel electrophoresis profile (Fig. 3B), whereas Cry j 1 has basic proteins with isoelectric points of 8.9 and 9.2 (Yasueda *et al.*, 1983). The difference in isoelectric points between Cha o 1 and Cry j 1 may be due to a difference in the number of charged amino acids, such as Arg, Lys (positively charged residues), Asp and Glu (negatively charged residues) in each allergen. That is, Cha o 1 contains 28 Arg plus Lys residues and 32 Asp plus Glu residues, whereas Cry j 1 contains 30 and 28, respectively.

Purified Cry j 1 was classified into two groups according to the difference in isoelectric points. This difference may reflect the existence of isoallergens in which charged

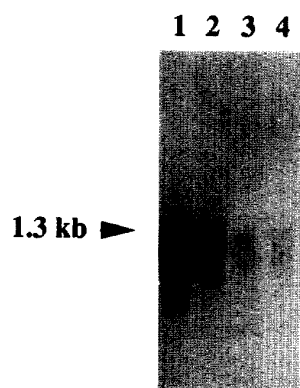


Fig. 4. Northern blot of RNA from *C. obtusa* pollen. Total RNA and mRNA from *C. japonica* and *C. obtusa* was electrophoresed in 1.0% formaldehyde-agarose gels, transferred to a Hybond-N filter, and probed with radiolabelled cDNA coding for Cry j 1 (pCCI-2-2). Lane 1, total RNA of *C. japonica*; lane 2, total RNA of *C. obtusa*; lane 3, m-RNA of *C. japonica*; lane 4, m-RNA of *C. obtusa*.

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ATG	GCT	TCC	TGT	ACC	TTA	TTA	GCA	GTC	CTT	GTT	TTC	CTT	TGT	GCA	ATT	GTA	TCT	TGT	TTT	TCT																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															</

Fig. 5. Nucleotide sequence and deduced amino acid sequence of Cha o 1. The deduced amino acid sequence is numbered by designating Asp the first amino acid of the mature protein. Underlined amino acids were identified by amino acid sequencing of the purified Cha o 1. Four possible *N*-linked glycosylation sites are denoted by dotted underlines. A TGA stop codon is indicated by an asterisk.

A.	Cha o 1	DNPIDSCWRG	DANWDQNRMK	LADCAVGFGS	SAMGGKGGA	F YTVTS*SDDD	PVNPA	PGTLR	59	
B.	Cry j 1 (pCCI2-2)	-----	-S--A----	-----	-T-----	DL ---N*----	-----		59	
C.	Cry j 1 (pCCI-15)	-----	-S--A----	-----	-T-----	DL ---N*----	-----		59	
D.	Amb a 1.03	L-I--K----	K-D-EN--QA	----	Q--AK	GTY--W-DV	-----	NL--- VA--KE----	60	
E.	Amb a 2	H-I--K---C	KPD-AE--QA	-G---Q---K	ATH--W-DI	-M---DQ---	V---KE----		60	
A.	YGATRERSLW	IIFSKNLNIK	LNMPYIAGN	KTIDGRGAEV	HIGNGG	PCLF	MRTVSHVILH	GLNIHGCNTS	VSGNVLISEA	139
B.	-----D-P--	-----G-M---	-K--M---Y	--F-----Q-	Y-----V-	IKR--N--I-	--HLY--S--	-L-----N-S		139
C.	-----D-P--	-----G-M---	-K--M---Y	--F-----Q-	Y-----V-	IKR--N--I-	--YLY--S--	-L-----N-S		139
D.	FA-AQN-P--	---KNDMV-N	--QE-VVNSD	-----VK-	E-I---LT-*	-N*-KNI-I-	NI---DVKVL	PG-MIK*-ND		138
E.	F---QD-P--	---QRDMI-Y	-QQEMVVTSD	-----K-	ELVY--IT-*	-N*-KN--I-	NID--DVRVL	PG-RIK*-NG		138
A.	SGVVPVHAQD	GDAITMRNVT	DVWIDHNSLS	DSSDGLVDVT	LASTGVTISN	NHFFNHHKVM	LLGHSDIYSD	DKSMKVTVAF		219
B.	F--E---P--	---L-L-TA-	NI-----F-	N-----	-S-----	-L-----	---D-A--	-----		219
C.	F--E---P--	---L-L-TA-	NI-----F-	N-----	-T-----	-L-----	S---D-A--	-----		219
D.	GPPILRQ-S-	--T-NVAGSS	QI---C---	K-F-----	-G--H-----	CK-TQQS-AI	---AD-THVQ	--G-LA----		217
E.	GPAI-R-QS-	---HVTGSS	-I---CT--	K-F-----N	WG-----	CK-TH-E-AV	---A--THFQ	-LK-H--L-Y		217
A.	NQFGPNAGQR	MPRARYGLIH	VANNNYDFWS	IYAIGGSSNP	TILSEGSFT	APNDSKKEV	TRRVGCESPS	TCANWVWRST		299
B.	-----C---	-----V-	-----T	-----	-----	---E-Y--Q-	-I-I--KTS-	S-S---Q--		299
C.	-----C---	-----V-	-----T	-----	-----	---E-Y--Q-	-I-I--KTS-	S-S---Q--		299
D.	-M-TD-VD--	---C-F-FFQ	-V-----R-G	T-----A-	---CQ--R-L	--D-QI--N-	LA-T-TGAEE	SM-*N---D		296
E.	-I-TNTVHE-	---C-F-FFQ	IV--F--R-D	K-----	---Q--K-V	--DFIY--N-	CL-T-AQE-E	WM*T-N--TQ		296
									Amino Acid	Identity
A.	QDSFNNGAYF	VSSGKNEGTV	*IYNNNEAFK	VENGSAAPQL	TKNAGVLTCT	LSKPCS	354			
B.	--V-Y-----	-----Y--G-	*--TKK---N	---N-T--	-----S	---R-*	353	80 %		
C.	--V-Y-----	-----Y--G-	*--TKK---N	---N-T-H-	-Q-----S	---R-*	353	79 %		
D.	K-LLE---I-	-T--SDPVL	T*PVQSAGMIP	A-P-E--IK-	-SS----S-R	PGA--*	350	49 %		
E.	N-VLE---I-	-A--SDPVL	T*AEQ-AGMMQ	A-P-DMV---	-M-----S	PGA--*	350	49 %		

Fig. 6. Sequence comparison among Cha o 1, Cry j 1 (pCCI-2-2 and pCCI-15), Amb a 1.03, and Amb a 2. Amb a 1.03 and Amb a 2 are pollen allergens of short ragweed. Horizontal bars denote identical amino acids. The asterisk indicates a blank that has been inserted to maximize homology.

amino acids are exchanged or a microheterogeneity of charged sugars, such as neuraminic acid attached to a carbohydrate side chain. Four oligosaccharide structures bound to Asn residues of Cry j 1 were recently determined and the carbohydrate side chains were found not to contain charged sugars (Hino *et al.*, 1995). Furthermore, the existence of two isoforms of Cry j 1 has been reported (Sone *et al.*, 1994). The difference in isoelectric points found in Cry j 1 may therefore indicate the existence of the isoform. The cDNA of allergens such as Amb a 1, Lol p 1 (rye grass) (Perez *et al.*, 1990), Poa p 9 (Kentucky

blue grass) (Silvanovich *et al.*, 1991), Bet v 1 (birch) (Swoboda *et al.*, 1995), Ole e 1 (olive) (Villalba *et al.*, 1994) from weed, grass and tree pollen has been cloned and the presence of isoforms in each allergen was demonstrated. Thus, the discovery of isoforms in Cha o 1 would not be unexpected.

It is well known that IgE from patients with pollinosis tends to be cross-reactive with allergens of pollen from grasses and trees belonging to the same order. For example, birch, hornbeam, hazel and alder belong to the order *Fagales* and allergens of their respective tree pollen, such

as Bet v 1, Car b 1, Cor a 1 and Aln g 1, are mutually cross-reactive. The cDNA encoding these allergens has been cloned and their amino acid sequences have been deduced from the nucleotide sequences (Breiteneder *et al.*, 1992, 1993; Larsen *et al.*, 1992). Comparison of their amino acid sequences revealed identities of 72–88%, supporting the crossreactivity of IgE to those allergens. Cross-reactivity between allergens of pollen of the Taxodiaceae and Cupressaceae families, both of which belong to the order *Pinales*, has been shown at the IgE level (Panzani *et al.*, 1986b; Taniai *et al.*, 1993). In this study, we clearly demonstrated that the major allergen of the Taxodiaceae (Cry j 1) and Cupressaceae (Cha o 1) have similar amino acid sequences (80% identity). The major allergens of *Cupressus sempervirens* (common cypress, Italian cypress), *Cupressus arizonica* (Arizona cypress), and *Callitris glaucophylla* (white cypress pine), all of which belong to the same family (the Cupressaceae family), have molecular masses of 42, 43 and 46, and 43 and 50 kDa, respectively, and the primary structures of those allergens are expected to have similar sequences (Di-Felice *et al.*, 1994; Ford *et al.*, 1991; Pham *et al.*, 1994).

The allergenic activity of Cry j 1 is the strongest found in whole *C. japonica* pollen. Homology of the amino acid sequence of Cha o 1 with that of Cry j 1 would account for a crossreactive sensitivity to both *C. japonica* and *C. obtusa*. There are, however, some individuals with high titres of *C. obtusa*-specific IgE but low titres of *C. japonica*-specific IgE, and vice versa. It seems likely that a difference of approximately 20% in amino acid sequences between Cry j 1 and Cha o 1 might be responsible for the above phenomena. Therefore, investigation of this difference may help improve immunotherapy of pollinosis caused by *C. japonica* and/or *C. obtusa*. One must consider, however, the possibility of other major allergens specific for *C. obtusa*.

The amino acid sequence of Cha o 1 also showed homology with those of the Amb a 1 family (Amb a 1.01–1.04) and Amb a 2 of short ragweed. The amino acid sequences of allergens from tree pollen showed homology with those of grass pollen. These similarities are interesting in terms of the mechanisms involved in the onset of pollinosis. It is known that profilins from trees and grasses have structural homologues and that all these proteins are allergens (Valenta *et al.*, 1994).

Recently, it has been reported that Cry j 1 has pectate lyase enzyme activity (Taniguchi *et al.*, 1995). Cha o 1 as well as allergens and proteins such as Amb a 1, Amb a 2, LAT59, LAT56, and Zm58.2, all of which are homologous to Cry j 1, may therefore have the same enzyme activity. The biological function of these pollen proteins remains to be clarified, however it is possible that they may be associated with pollen tube penetration and expansion into pistils.

Our present results should prove to be useful for the development of a new immunotherapy since T and B lymphocyte epitopes of Cha o 1 could be isolated based on the primary sequence of the allergen molecule determined. Furthermore, the results presented here provide new information for improving the diagnosis and immu-

notherapy of pollinosis and for understanding the immunopathogenesis of this disorder.

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