

Identification of *Cannabis* pollens using an allergic patient's immunoglobulin E and purification and characterization of allergens in *Cannabis* pollens

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Abstract

Cannabis pollen allergens were detected using the serum of an allergic patient. The allergens were then purified by sequential column chromatography (including DE52 cellulose and phenyl-Sepharose CL-4B) and preparative HPLC. The molecular weight of the allergens were determined as 10 050 and 13 706 by matrix-assisted laser desorption/ionization time of flight mass spectrometry. We utilised Western blotting and development of an enzyme-linked immunosorbent assay for the detection of *Cannabis* pollen allergens. © 1998 Elsevier Science Ireland Ltd All rights reserved.

Keywords: *Cannabis sativa*; Marihuana; Pollen; Identification; Allergen; Western blotting; ELISA

1. Introduction

A wide variety of methodologies have been investigated for the detection of marihuana samples or *Cannabis* plants (TLC [1–4], HPLC [3–7], GC [2,3,7–10] and GC–MS [2,8,11–13]). Recently, molecular methods, such as random amplified poly-

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morphic DNA have appeared to be good candidates for the identification of varieties in *Cannabis sativa* [14,15]. Furthermore, in the recent development of the molecular biosciences and their biotechnological applications, an immunological approach for assaying quantities of the major active cannabinoid, tetrahydrocannabinol and its metabolites in body fluids and cell preparations has been investigated by many groups [16–19]. We have produced a broad-specificity monoclonal antibody (MAb) against cannabinoids [20] in our continuing investigations of *Cannabis* [21–26] as part of our programme of production of MAbs against naturally occurring bioactive compounds [27–30].

It is well-known that the plant pollens are a major source of allergens [31–39]. Allergies to grass pollens are, however, not of clinical importance in Japan because of the relatively short pollination season and the sporadic distribution of the individual species [40,41]. No antigen of *C. sativa* has been reported in grass pollen allergies due to the limited cultivation in Japan. However, it has been suggested that some allergic patients may have been exposed to *Cannabis* pollen [42]. As an extension of this approach, we discuss an immunoassay system for the detection of *Cannabis* pollen using an allergic patient's immunoglobulin E (IgE) and the purification of *Cannabis* pollen allergens.

2. Materials and methods

2.1. Materials

Pollen was obtained from *C. sativa* (Mexican and CBDA strains) cultivated in the Kyushu University herbal garden in September 1996. Various plant pollens were collected in 1996 in Fukuoka, Japan. Serum was obtained from a patient (Y. Shoyama) allergic to *C. sativa* pollen 3 days after stimulation by *C. sativa* pollen which was determined as an allergen in the Kyushu University Hospital, Fukuoka, Japan [42]. Pooled serum was dialyzed against phosphate-buffered saline (PBS), and stored at -20°C in small aliquots prior to use. The stored serum was used for enzyme-linked immunosorbent assay (ELISA) and Western blot without dilution. Unless indicated otherwise, all extraction and purification procedures were performed at 4°C .

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *C. sativa* pollen using serum of an allergic patient

SDS-PAGE was performed using 15% acrylamide gels (5×70 mm). The gels were stained with Coomassie blue R-250. The marker proteins used as molecular weight references were phosphorylase b (mol. wt. 97 400), bovine serum albumin (mol. wt. 66 000), ovalbumin (mol. wt. 45 000), glyceraldehyde-3-phosphate dehydrogenase (mol. wt. 36 000), carbonic anhydrase (mol. wt. 29 000), trypsinogen (mol. wt. 24 000), trypsin inhibitor (mol. wt. 20 000) and α -lactalbumin (mol. wt. 14 200).

2.3. ELISA for detection of *Cannabis* and various plant pollens

Cannabis and various plant pollens ($10\text{--}20 \cdot 10^2$ /well) which were counted by microscope were added to each well of a 96 well immunoplate (NUNC, Roskilde, Denmark), 100 μl of 0.125 M NaHCO_3 was then added to each well and the plates were incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween 20 (TPBS) and treated with 300 μl PBS containing 0.2% of gelatin (GPBS) for 1 h to reduce nonspecific adsorption. The plates were washed three times with TPBS and reacted with 100 μl of the patient's serum for 1 h. The plates were then washed three times with TPBS, 100 μl of a 1:1000 dilution of peroxidase labeled anti-human IgE (Organon Teknika Cappel Products, West Chester, USA) was added to each well and the plates were incubated for 1 h. After washing the plate three times with TPBS, 100 μl of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.006% H_2O_2 and 0.3 mg/ml 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (Wako, Osaka, Japan), was added to each well and the plates were incubated for 20 min. Absorbance at 405 nm was measured with a microplate reader (Model 450 Microplate Reader, Bio-Rad). All reactions were carried out at 37°C. The reactivity of the patient's IgG to *Cannabis* pollen antigens was determined similarly by ELISA, using peroxidase-labeled anti-human IgG (Organon Teknika Cappel Products).

2.4. ELISA for detection of *Cannabis* pollen allergens

The reactivity of the patient's IgE to allergen was determined by ELISA. Each well of a 96 well immunoplate (NUNC) was coated with 100 μl of allergen and treated with 300 μl GPBS for 1 h to reduce nonspecific adsorption. The plates were washed three times with TPBS and reacted with 100 μl of the patient's serum for 1 h. The plates were then washed three times with TPBS, 100 μl of a 1:1000 dilution of peroxidase-labeled anti-human IgE (Organon Teknika Cappel Products) was added to each well and the plates were incubated for 1 h. After washing the plates three times with TPBS, 100 μl of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.006% H_2O_2 and 0.3 mg/ml 2,2-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (Wako), was added to each well and the plates were incubated for 20 min. Absorbance at 405 nm was measured with a microplate reader (Model 450 Microplate Reader, Bio-Rad). All reactions were carried out at 37°C.

2.5. Purification of *Cannabis* pollen allergens

Pollen (19.9 g) was extracted with 0.125 M NaHCO_3 (150 ml) for 5 h and then centrifuged $950 \times g$ rpm for 10 min. The supernatant was partitioned with ether three times, dialyzed against H_2O , and then lyophilized. The lyophilized residue was redissolved in 10 mM Tris-HCl buffer and applied to a 2.5×18 cm column of DE52 cellulose (Whatman) that had been preequilibrated with 10 mM Tris-HCl buffer (pH 8). After the column was washed with the same buffer, bound proteins were eluted with a 600 ml linear NaCl gradient (0 M to 1.0 M) at a flow-rate of 1.0 ml/min. Elution was monitored photometrically at 280 nm and by ELISA (see below) and fractions of 16 ml

were collected. The ELISA-positive fractions (Fr.A: fractions 6–13, Fr.B: fractions 24 and 25) were pooled and dialyzed overnight against three changes of water. The Fr.A dialysate (85 mg) was applied to a 1.1×21 cm phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column preequilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 1.7 M ammonium sulfate. The column was rinsed with three column volumes of the same buffer, and bound proteins were eluted with a 400 ml linear gradient of 50 mM sodium phosphate buffer containing 1.7 M to 0 M ammonium sulfate at a flow-rate of 0.8 ml/min. The ELISA-positive fractions (16–26) were dialyzed against three changes of water, and lyophilized to yield the pure allergen, CS-1 (8 mg). The same procedure was applied to the Fr.B dialysate (40 mg). The ELISA-positive fractions (37–43) were dialyzed against three changes of water. The dialysate was further purified by HPLC (Model LC-10AD, Shimadzu, Kyoto, Japan), using a pump connected to a Cosmosil 5Ph-AR-300 (4.6×150 mm, Nacalai Tesque, Kyoto, Japan) equipped with a variable wave length detector (set at 215 nm). The mobile phase was an acetonitrile gradient of 20% to 60% containing 0.05% of trifluoroacetic acid, at a flow-rate of 1.0 ml/min. The positive fractions were dialyzed against three changes of water, and lyophilized to yield the pure allergen, CS-2 (5 mg).

2.6. Analysis of allergen by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry

A small amount (1–10 pM) of *Cannabis* allergen was mixed with a 10^3 -fold molar excess of sinapinic acid in an aqueous solution containing 0.10% trifluoroacetic acid. The mixture was placed inside a JMS-ELITE MALDI-TOF mass spectrometer (Jeol, Tokyo, Japan) and irradiated with a N_2 laser (337 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20 kV potential into a 2.0 m evacuated tube and detected using a microchannel plate detector with a Micron Millennia plus computer as previously reported [21,43,44].

2.7. Western blotting of Cannabis allergens

The polyvinylidene difluoride (PVDF) membrane blotted from SDS-PAGE was incubated with PBS at room temperature for 10 min, then blocked with GPBS solution at room temperature overnight. The membrane was washed with TPBS twice, then with distilled water, and incubated with the patient's serum at 37°C for 3 h. Following two washes with TPBS and one with distilled water, biotin-labeled anti-human IgE was added and the membrane was incubated at 37°C for 1 h. After washing with TPBS twice and once with water, the membrane was treated with peroxidase-labeled streptavidin at 37°C for 1 h, washed with TPBS twice and finally exposed to 1 mg/ml 4-chloro-1-naphthol–0.03% H_2O_2 in PBS solution at room temperature. The reaction was stopped by washing with water.

3. Results and discussion

C. sativa plants are mainly classified into two phenotypes such that one group contains Δ^1 -tetrahydrocannabinolic acid (THCA) as a major cannabinoid, and the other

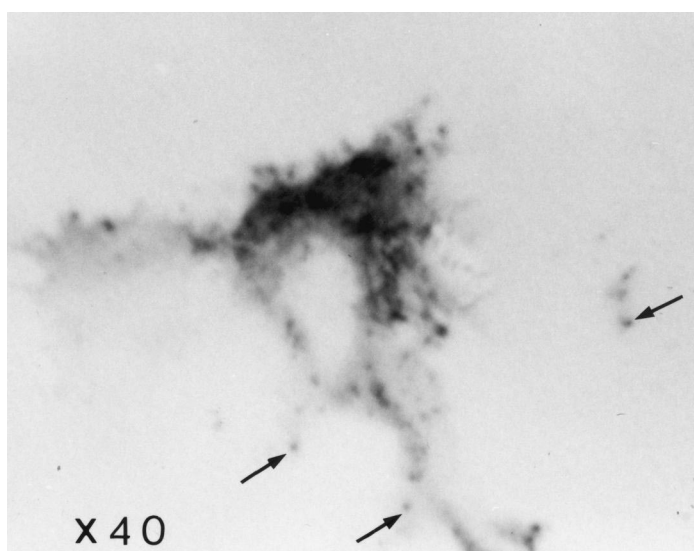


Fig. 1. Micrograph for the Western blot of *Cannabis* pollen collected from the dried *Cannabis* leaves. Arrow shows a single pollen stained by the Western blot.

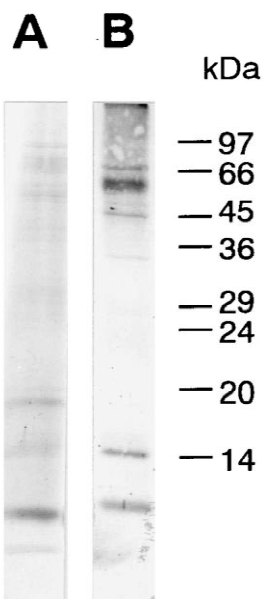


Fig. 2. SDS-PAGE and Western blot of crude extracts of *Cannabis* pollen. (A) SDS-PAGE, (B) Western blot. The pollens of THCA strain were used.

Table 1

Reactivity of allergic patient's IgE against various plant pollen extractives by ELISA

Plant name	Family	Relative absorbance (%) ^a
Dicotyledoneae		
<i>Cannabis sativa</i> (THCA strain)	Moraceae	100.0±30.4
<i>Cannabis sativa</i> (CBDA strain)	Moraceae	92.6±7.3
<i>Humulus japonicus</i>	Moraceae	4.5±1.3
<i>Eupatorium chinense</i> L. var. <i>simplicifolium</i>	Compositae	4.7±3.8
<i>Ambrosia artemisifolia</i> L. var. <i>elatior</i>	Compositae	14.1±2.3
<i>Achillea millefolium</i>	Compositae	2.1±0.4
<i>Coreopsis drummondii</i>	Compositae	3.7±0.9
<i>Luffa cylindrica</i>	Cucurbitaceae	15.7±13.1
<i>Abelia grandiflora</i>	Caprifoliaceae	1.4±0.7
<i>Lonicera japonica</i>	Caprifoliaceae	–
<i>Weigela hortensis</i>	Caprifoliaceae	0.6±0.5
<i>Campsis chinensis</i>	Bignoniaceae	–
<i>Verbascum thapsus</i>	Scrophulariaceae	1.0±1.2
<i>Rhemantha glutinosa</i> Libosch. forma <i>hueichingensis</i>	Scrophulariaceae	2.3±0.7
<i>Datura innoxia</i>	Solanaceae	19.8±4.0
<i>Salvia ranzaniana</i>	Labiatae	–
<i>Amsonia elliptica</i>	Apocynaceae	2.9±2.9
<i>Ligustrum japonicum</i>	Oleaceae	1.7±0.2
<i>Rhododendron indicum</i>	Ericaceae	0.2±1.9
<i>Angelica acutiloba</i>	Umbelliferae	2.1±0.7
<i>Oenothera parviflora</i>	Oenotheraceae	2.7±2.2
<i>Pyracantha angustifolia</i>	Myrtaceae	–
<i>Callistemon rigidus</i>	Myrtaceae	0.3±0.1
<i>Hibiscus syriacus</i>	Malvaceae	9.4±4.5
<i>Citrus unshiu</i>	Rutaceae	17.5±4.0
<i>Geranium graveolens</i>	Geraniaceae	0.6±0.6
<i>Oxalis corymbosa</i>	Oxalidaceae	–
<i>Baptisia caerulea</i>	Leguminosae	1.2±1.4
<i>Rhaphiolepis umbellata</i>	Rosaceae	–
<i>Agrimonia pilosa</i>	Rosaceae	3.9±2.2
<i>Hydrangea macrophylla</i>	Saxifragaceae	33.3±14.6
<i>Philadelphus satsumi</i>	Saxifragaceae	3.9±0.7
<i>Cardiandra alternifolia</i>	Saxifragaceae	3.8±1.2
<i>Lagerstroemia indica</i>	Sarraceniaceae	1.2±1.7
<i>Chelidonium majus</i>	Papaveraceae	2.3±0.5
<i>Papaver somniferum</i>	Papaveraceae	1.3±0.5
<i>Paeonia albiflora</i>	Paeoniaceae	–
<i>Clematis terniflora</i>	Ranunculaceae	5.8±1.1
<i>Clematis japonica</i>	Ranunculaceae	5.2±0.7
<i>Ranunculus japonicus</i>	Ranunculaceae	0.6±0.6
<i>Phytolacca americana</i>	Phytolaccaceae	1.8±0.5
<i>Houttuynia cordata</i>	Saururaceae	3.9±0.7
Monocotyledoneae		
<i>Dioscorea tokoro</i>	Dioscoreaceae	15.8±3.2
<i>Hemerocallis longituba</i>	Liliaceae	–
<i>Liriope platyphylla</i>	Liliaceae	5.3±5.0
<i>Tradescantia reflexa</i>	Commelinaceae	4.1±1.2
Gymnospermae		
<i>Cryptomeria japonica</i>	Taxodiaceae	1.8±0.7
<i>Cedrus deodara</i>	Pinaceae	1.9±1.1

^a Relative absorbance values as % of the absorbance value obtained with *C. sativa* (THCA strain).

contains cannabidiolic acid (CBDA) as a major cannabinoid. Furthermore, we distinguish them by the alkyl group in the molecule named as pentyl-, propyl- and methyl-cannabinoids. There is a clear morphological similarity between the two. Also, phenotypes related to the alkyl group cannot be distinguished (data not shown) which means it is impossible to identify *Cannabis* phenotypes by the examination of scanning electron micrograph for *Cannabis* pollens, as previously reported [45].

The patient's serum reacted only with *Cannabis* pollen allergens, and no immunostaining was observed when pollens of other plant species were used. Using serum from a nonallergic person as a negative control showed no staining (data not shown). The

Pollens of *Cannabis sativa* (19.9 g)

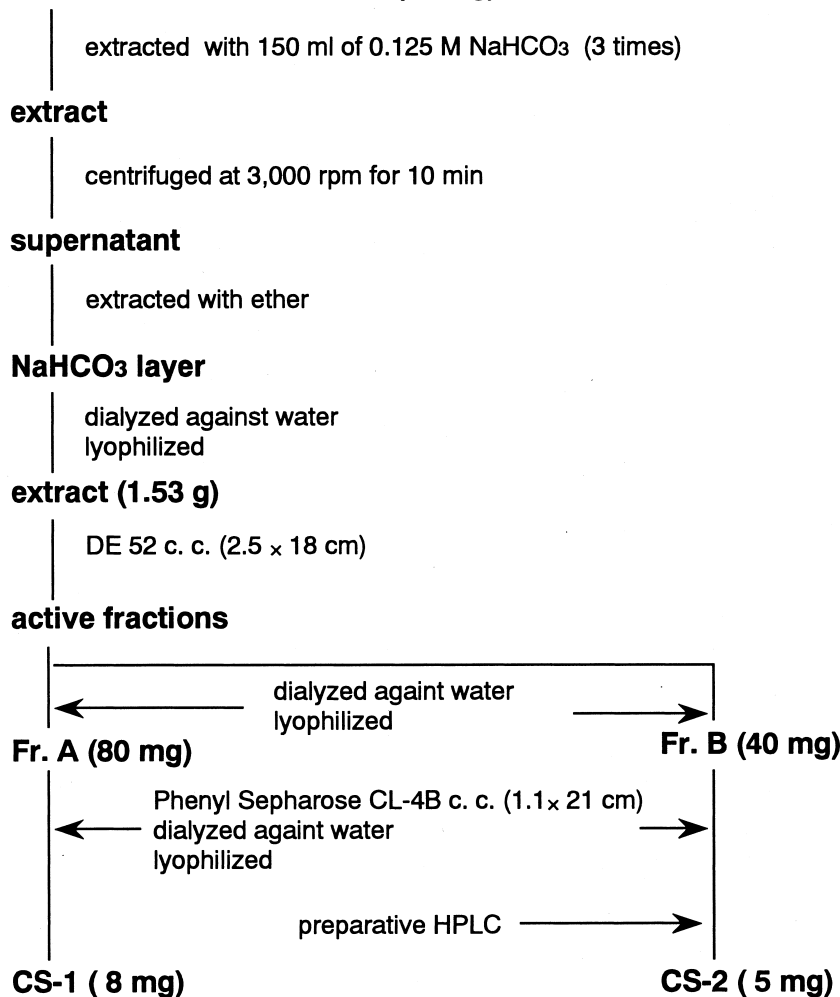


Fig. 3. Flowchart for isolation of allergens from *Cannabis* pollen.

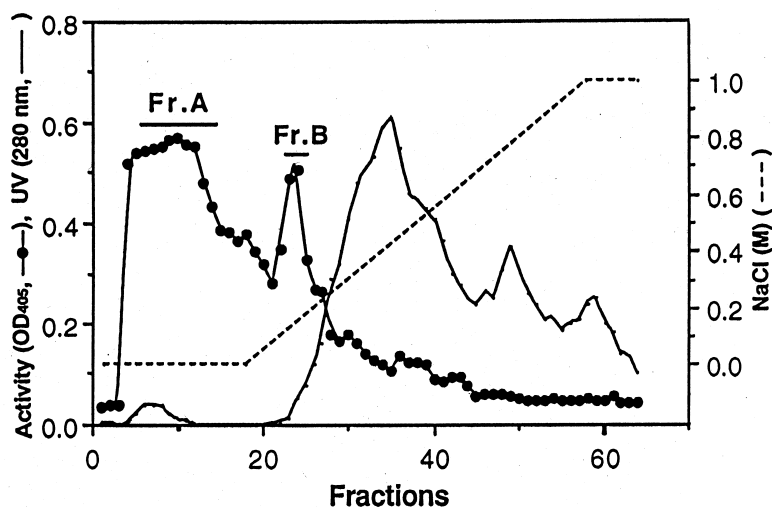


Fig. 4. Gel chromatography of crude *Cannabis* pollen allergens. A lyophilized pollen extract was applied to a DE52 cellulose column and eluted with a NaCl gradient. The experimental conditions are described Section 2. Flow-rate, 1 ml/min; fraction volume, 16 ml; (—●—), ELISA activity; (—), A_{280} . Fr.A and Fr.B were pooled separately and further purified as indicated in Figs. 5 and 6, respectively.

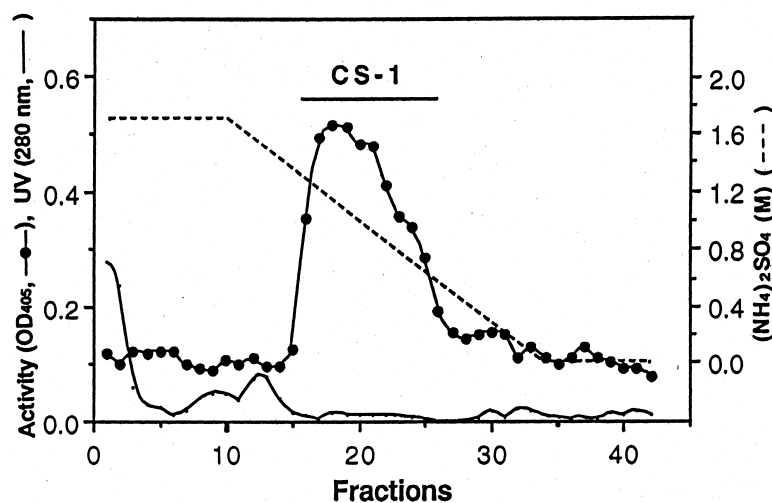


Fig. 5. Purification of CS-1 on phenyl-Sepharose CL-4B using an (NH₄)₂SO₄ gradient. Flow-rate, 0.8 ml/min; fraction volume, 10 ml; (—●—), ELISA activity; (—), A_{280} .

Western blot can be used for the analysis of *Cannabis* pollen by microscope. Fig. 1 shows the micrograph for the Western blot of *Cannabis* pollen collected from the dried *Cannabis* leaves. From this result it became clear that a single *Cannabis* pollen can be determined by the newly established Western blot hence forth becoming an important tool in the field of forensic science since the morphological determination of *Cannabis* pollen by microscopy is impossible [45].

In order to expand the applications of patient's IgE for the detection of *Cannabis* pollens, various plant pollens together with those of THCA and CBDA strains were analyzed. The newly established ELISA was specific for both *Cannabis* pollens, indicating that the reactivity of THCA strain was 100% and that of CBDA was about 93%. The patient serum cannot distinguish between the two strains of *Cannabis*. The pollens of other plants, including that of another member of the Moraceae family, *Humulus japonicus*, were also tested. As can be seen in Table 1, the allergic patient's IgE showed no affinity for *H. japonicus* or for other plants.

Since it is well known that *Cannabis* can be classified according to two phenotypes as described above, pollens of both strains were analyzed by SDS-PAGE. The gels of the soluble allergens revealed three small molecular bands (10, 14 and 20 kiloDaltons [kDa]) and three higher molecular bands (45, around 60 and 68 kDa) (Fig. 2A). On the other hand, upon Western blotting, using the patient's serum IgE and antiIgE antibody, five bands of 10, 14, 45, 60 and 68 kDa, respectively appeared in both *Cannabis* strains. This suggests that *C. sativa* pollen contains at least five allergens (Fig. 2B).

Since the complete identification of *Cannabis* pollens using IgE needs specific allergens, we purified the antigens. The schematic diagram of the procedure used for the purification of *Cannabis* pollen allergens is depicted in Fig. 3. A three-step procedure was developed that resulted in the purification of the allergens to apparent homogeneity.

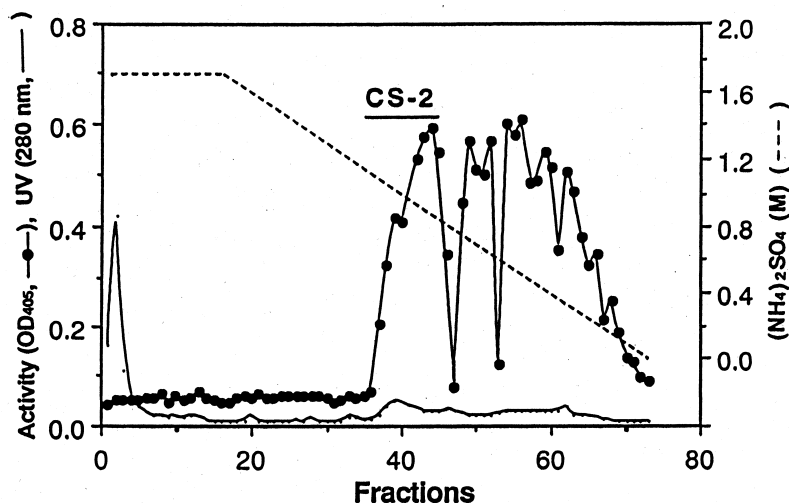


Fig. 6. Purification of CS-2 on phenyl-Sepharose CL-4B using an $(\text{NH}_4)_2\text{SO}_4$ gradient. Flow-rate, 0.8 ml/min; fraction volume, 10 ml; (—●—), ELISA activity; (—), A_{280} .

As a first step, the solubilized crude allergen fraction was applied to a DEAE-cellulose (DE52) column, where most of the ELISA-positive fraction was not bound to the matrix. Two fractions, A and B, were obtained with a gradient of sodium chloride from 0 to 1 M. This procedure separated most of the ELISA-negative protein, as indicated in Fig. 4. Fraction A was further purified using phenyl-Sepharose CL-4B. The bound allergens were eluted with a descending gradient of ammonium sulfate (1.7 M to 0 M). The most

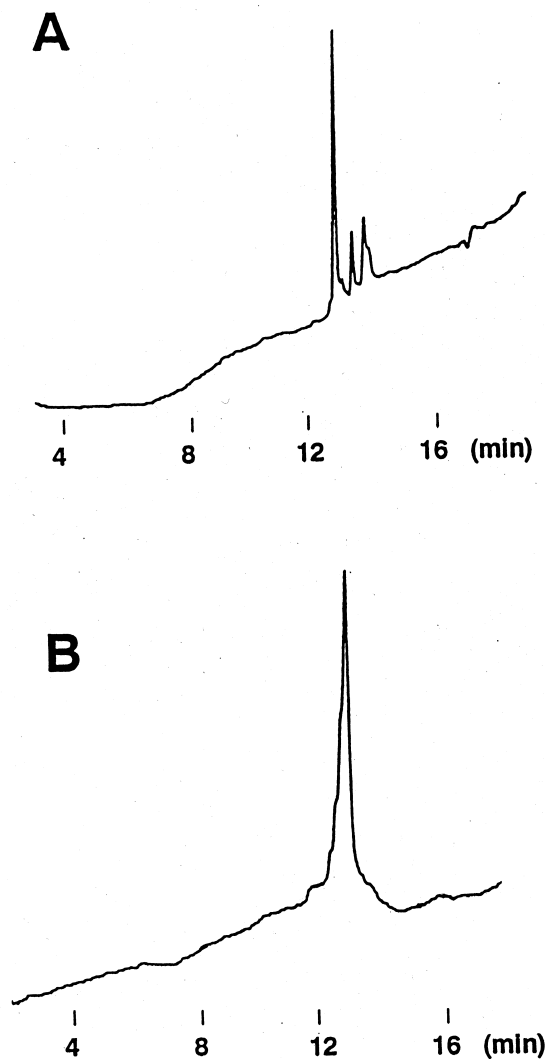


Fig. 7. HPLC profiles of CS-2 fraction and purified CS-2. (A) Shows the CS-2 fraction purified by phenyl-Sepharose CL-4B as described in Fig. 6. (B) Shows the CS-2 purified by HPLC, as described in Section 3.

active and nearly homogeneous peak (fractions 16–26), designated allergen CS-1, was separated as indicated in Fig. 5.

Fraction B was further purified using phenyl-Sepharose CL-4B as above. The most active and nearly homogeneous peak (fractions 37–43), was designated allergen CS-2. The additional peaks were composed of several ELISA-positive proteins (Fig. 6). The HPLC profile of the partially purified CS-2 fraction showed a major peak in addition to three minor contaminations (Fig. 7A). Therefore, we applied preparative HPLC to the fraction, and obtained a single peak, as indicated in Fig. 7B. To confirm the homogeneity of the purified allergen, SDS-PAGE and Western blotting were performed. Fig. 8 shows that the purification by HPLC was satisfactory, with a single band at 14 kDa appearing in the Western blot (Fig. 8 2).

The purity and molecular weight of allergens were confirmed by SDS-PAGE (Fig. 8) and MALDI-TOF mass spectrometry (Fig. 9). Molecular weights of 10 050 and 13 706 were obtained for CS-1 and CS-2, respectively. Since only one main molecular peak, with some small contaminations, was observed for each, it is evident that the purification method was satisfactory. Moreover, the newly purified allergens reacted specifically with the IgE of the allergic patient as previously indicated in Fig. 8.

We previously reported that the pollen of various *Cannabis* strains was reactive against anti-cannabinoid MAb [20]. The use of the anti-cannabinoid MAb in conjunction with our newly established ELISA for the detection of *Cannabis* pollen allergens makes it possible to distinguish *Cannabis* pollen from that of other species. The purified allergens are now being sequenced, and could be used for the identification of *Cannabis*

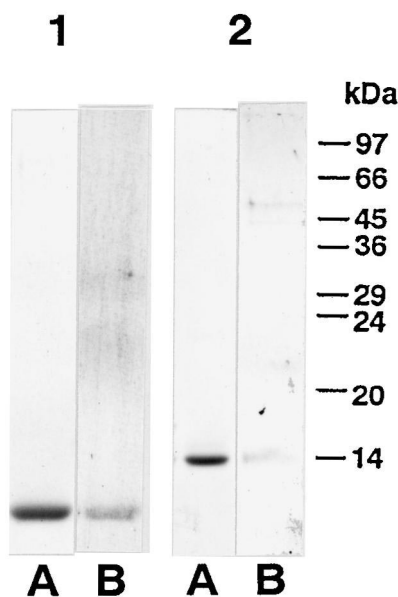


Fig. 8. SDS-PAGE and Western blot of *Cannabis* pollen allergens. (1) CS-1, (2) CS-2, (A) SDS-PAGE, (B) Western blot.

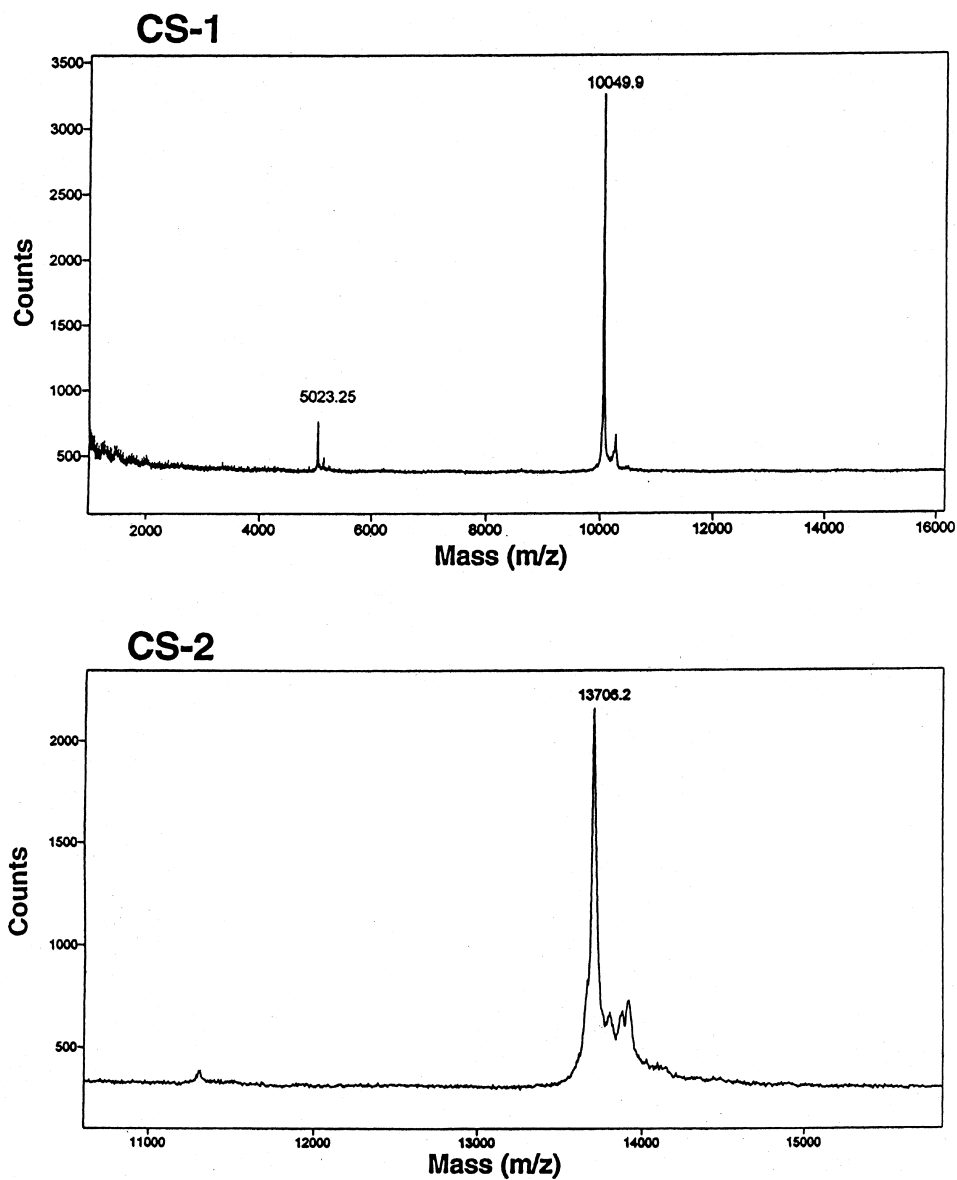


Fig. 9. MALDI-TOF mass spectra of allergens CS-1 and CS-2.

pollens as standards, for clinical diagnosis, immunological and physicochemical characterization of the individual allergens and for genetic studies on the immune response to *Cannabis* allergens.

To our knowledge, this is the first report on the isolation of the allergens contained in *Cannabis* pollen and their detection by a reliable immunoassay.

4. Notation

MAB, monoclonal antibody
THCA, Δ^1 -tetrahydrocannabinolic acid
CBDA, cannabidiolic acid
ELISA, enzyme-linked immunosorbent assay
MALDI, matrix-assisted laser desorption/ionization
PBS, phosphate buffered saline
GPBS, PBS containing 0.2% of gelatin
TPBS, PBS containing 0.05% Tween 20
HPLC, high-performance liquid chromatography
PVDF, polyvinylidene difluoride

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