

Identification and characterization of the major allergen of the *Humulus japonicus* pollen

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Summary

Background Pollen of *Humulus japonicus* has been known as one of the important causes of pollinosis in Korea and China. To date, the major allergen of *H. japonicus* has not been determined.

Objective To identify the major allergen of *H. japonicus* pollen and characterize its biochemical properties.

Methods With the sera of 29 patients reactive to *H. japonicus*, the major allergen of *H. japonicus* was determined from the results of IgE immunoblotting and ELISA inhibition. The biochemical properties of the major allergen of *H. japonicus* were evaluated by lectin blotting assay and 2-dimensional PAGE blot. N-terminal amino acid sequences were determined by the Edman degradation method. The suggested major allergen was purified by DEAE anion exchange and gel filtration chromatography.

Results Twenty-nine sera contained IgE bound to the 10, 16, 20, 29 and 42 kDa proteins of *H. japonicus* in immunoblot analysis. A protein of 10 kDa was the most prevalent allergen in the sera of *H. japonicus*-reactive patients (72%). The ELISA optical density of *H. japonicus*-specific IgE was not inhibited by pollen extracts of birch, oak, rye grass and mugwort. The 10-kDa allergen was neither stained with PAS nor bound with ConA and five other lectins. The isoelectric point of the 10-kDa allergen was approximately pH 5.1. We sequenced the N-terminal amino acids of the 10-kDa allergen, which was not homologous with any previously characterized allergen. The 10-kDa allergen could be purified with DEAE anion exchange and gel filtration chromatography. Maximum inhibitions of *H. japonicus*-specific IgE ELISA by whole extract of *H. japonicus* and purified 10-kDa allergen were more than 97 and 88%, respectively, while the 50% inhibitory concentration of the whole extract of *H. japonicus* and purified 10 kDa were 38 and 20 ng/mL, respectively.

Conclusion The 10-kDa peptide could be a major allergen of *H. japonicus*. Its isoelectric point was 5.1 and it did not bind with lectins. The N-terminal amino acid sequence of the 10-kDa major allergen was also determined.

Keywords: characterization, *Humulus japonicus*, major allergen

Clinical and Experimental Allergy, Vol. 29, pp. 1080–1086. Submitted 16 June 1998; re-revised 1 December 1998; accepted 23 January 1999.

Introduction

Humulus, which belongs to the *Cannabis* family, is a widespread weed in Korea and China [1–4]. It is wind-borne and has a flowering period that lasts from August to October in

these regions. According to aerobiological studies in Korea and near Beijing in China, the *Humulus* pollen count is greater than mugwort or ragweed and accounts for about 18% of total pollen during the pollination period [1,3,4]. Although numerous people suffer from *Humulus* pollinosis, only a few reports on this allergy have been published [4–7]. Recently Park *et al.* [8] reported the clinical evidence of *Humulus* pollinosis with skin test, IgE immunoblotting and bronchial provocation test. They also showed that

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H. japonicus is not cross-reactive to other weed pollens with ELISA inhibition. However, the characterization of the major allergens of *Humulus* has not yet been done. In this study, the major allergen of *H. japonicus* was identified by IgE immunoblot analyses with *H. japonicus*-reactive patient sera. We also purified the major allergen and characterized the major allergen of *H. japonicus* with 2-dimensional PAGE blot, lectin blotting assay and by sequencing the N-terminus amino acids.

Methods

Human sera

Serum samples were obtained from 29 subjects who had lived in Seoul area, where the *H. japonicus* is regarded as the species mainly responsible for *Humulus* pollinosis. These subjects, all of whom had never received specific immunotherapy, were diagnosed as allergic to *H. japonicus* on the basis of clinical history and skin prick test (SPT) which was done with commercial *H. japonicus* extract (Torii Co., Tokyo, Japan). The size of weal produced by *H. japonicus* was at least as large as that of the histamine-positive control (1 mg/mL) in all encased patients. All of them had been diagnosed as allergic rhinitis and/or asthma. Their mean age was 29.1 years and the male : female ratio was 16 : 13.

Allergen extract

H. japonicus pollen was obtained in fields around Seoul in September 1996. Five grams of pollen was defatted with ethylether and it was then extracted in 100 mL of carbonate buffer (0.125 mol/L NH_4HCO_3 –0.015 mol/L NaN_3 , pH 7.5) for 24 h at 4 °C under constant stirring. The extract was centrifuged at 50 000 *g* for 1 h at 4 °C and the supernatant was dialysed (the cutoff molecular weight was 3.5 kDa; Spectrum, Houston, TX, USA) against distilled water for 48 h. The dialysed supernatant was lyophilized and stored at –28 °C until use. The mugwort and rye grass pollens were purchased from the Pharmacia & Upjohn Allergon AB (Ängelholm, Sweden), and the birch and oak pollens were obtained in the field. Aqueous extracts of these pollens were prepared by the same methods as described in the preparation of *H. japonicus* pollen extract. Recombinant birch profilin was kindly provided by Dr Valenta [9].

SDS-PAGE of *H. japonicus*

SDS-PAGE was carried out by the methods of Laemmli [10] under reducing conditions. For allergen isolation, 2 mg/mL of *H. japonicus* crude antigen was dissolved in sample buffer (12.5 mmol/L TRIS-HCl, pH 6.8, 5% glycerol,

0.4% SDS, 1% 2-mercaptoethanol, 0.02% bromophenol blue) and boiled at 100 °C for 5 min. For a preparative sheet, 200 µg of *H. japonicus* antigen was separated in 16% polyacrylamide gels with a 4% polyacrylamide stacking gel (Mighty Small electrophoresis unit; Hoeffer, San Francisco, CA, USA) at 50 V for 30 min and 100 V for 2 h.

2-dimensional PAGE

H. japonicus extract was dissolved in the isoelectric focusing sample buffer (20 mmol/L arginine, 20 mmol/L lysine, 15% glycerol) and 40 µg of *H. japonicus* extract was applied per lane of precasted native IEF gel (pH range 3–10, Novex, San Diego, CA, USA) and focused at 100 V for 60 min, 200 V for 60 min and 500 V for 30 min. After focusing, each lane of the IEF gel was cut into 4-mm widths and incubated in equilibration buffer (5% 2-mercaptoethanol, 62.5 mmol/L TRIS-HCl, 2.3% SDS, and 10% glycerol) for 30 min at room temperature and applied onto polyacrylamide stacking gel. Then SDS-PAGE was carried out.

IgE immunoblotting

Transfer of the proteins onto nitrocellulose membranes (pore size 0.45 µm; Amersham, UK) was performed on a Transpor TE 42 Unit (Hoeffer) in transfer buffer (25 mmol/L TRIS, 192 mmol/L glycine, and 20% methanol, pH 8.3) at 350 mA for 1 h. The membrane was sliced into 4-mm widths, if required. The sliced membranes were blocked by 5% non-fat dried milk in TBS-T (50 mmol/L TRIS with 0.1% Tween, pH 7.5) and then incubated with patient sera (1 : 5 diluted in TBS-T, 0.02% NaN_3) at 4 °C for 18 h and washed with TBS-T. Goat antihuman IgE conjugated with alkaline phosphatase (Sigma, St Louis, MO, USA) was diluted 1 : 2000 in TBS-T before incubation with the blots. After washing with TBS-T, bound goat antibodies were detected with a BCIP/NBT system (Promega, Madison, WI, USA).

ELISA inhibition tests with *H. japonicus* and other pollens

Polystyrene microplates (Costar, Cambridge, MA, USA) were coated with 50 µL of 20 µg/mL of *H. japonicus* extract in 0.05 mol/L carbonate buffer (pH 9.6) for 2 h at room temperature and overnight at 4 °C. The non-diluted pooled sera from 10 atopic patients was preincubated with *H. japonicus*, rye grass, mugwort, ragweed, birch and oak extracts, respectively, overnight at 4 °C. After washing with PBS-T, each well of microtitre plate was blocked by incubation with 200 µL of 1% bovine serum albumin in PBS-T (137 mmol/L NaCl, 1.8 mmol/L KH_2PO_4 , 10 mmol/L Na_2HPO_4 , 27 mmol/L KCl, 0.1% Tween-20, pH 7.4) for 1 h at room

temperature. Specific IgE detection was carried out by incubating 50 μ L of preincubated pooled sera for 1 h at room temperature. After washing three times with PBS-T, 50 μ L of 1:1000 biotin-labelled goat antihuman IgE (Vector, Burlingame, CA, USA) was added to the each of wells and incubated for 1 h. After washing, the wells were incubated with 1:1000 v/v streptavidin-peroxidase (Sigma) for 30 min before another washing step. Colourimetric reaction was developed with 50 μ L of ABTS solution (25 mg of 2,2-azino-bis-3-ethylbenzthiazoline-sulfonic acid in 50 mmol/L citrate phosphate buffer, 50 μ L of 30% H_2O_2) for 5 min. The reaction was stopped by the addition of 50 μ L of 2 mmol/L NaN_3 , and the optical densities were determined at 410 nm of UV by an automated spectrophotometer for microplate (Dynatec, Alexandria, CA, USA). All assays were performed in duplicate.

PAS staining and lectin blotting assay

PAS staining of blotted *H. japonicus* protein was done by Thornton's method [11]. The specific binding of lectins to the allergenic component of *H. japonicus* was evaluated by Glycan Differentiation Kit (Boehringer-Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's guidelines. The blotted nitrocellulose strips were incubated with the following digoxigenin-labelled lectins (galanthus nivalis [GNA, 1:1000], sambucus nigra [SNA, 1:1000], datura stramonium [DSA, 1:1000], maackia amurensis [MAA, 1:200], peanut agglutinin [PNA, 1:100]) in TBS buffer (50 mmol/L TRIS, 1 mmol/L $CaCl_2$, 1 mmol/L $MnCl_2$, 1 mmol/L $MgCl_2$, pH 7.5) for 1 h at room temperature. After washing with TBS, the strips were incubated in 10 U/mL of alkaline phosphatase-conjugated antidigoxigenin antibody. The colourimetric reaction was developed with a BCIP/NBT system.

N-terminal sequencing analysis

For N-terminal sequencing, 2-dimensional PAGE was blotted to a Polyvinylidene difluoride (PVDF) membrane (pore size $\sim 0.45 \mu m$; MSI, Westboro, MA, USA). The PVDF membrane was stained with 0.1% Coomassie blue

in 50% methanol, destained in 50% methanol, and air-dried. The protein spot was excised and microsequencing was performed by using Procise 476 A protein sequencer (Applied Biosystems, Foster, CA, USA).

Anion exchange and gel filtration chromatography

One hundred milligrams of lyophilized whole *H. japonicus* extract were resuspended in 20 mmol/L TRIS-HCl (pH 8.0) and applied to a diethylaminoethyl (DEAE) cellulose column (10×200 mm). The bound proteins were eluted with 0.1, 0.2, 0.3, 0.4 and 0.5 mol/L NaCl and the fractions were pooled, dialysed, and lyophilized. Four milligrams of lyophilized unbound fraction from the DEAE anion exchange chromatography was fractionated by Sepacryl-200 column (20×50 cm; Pharmacia, Upsala, Sweden). The low rate was 0.4 mL/min and the fraction volume was 2 mL. The protein contents of each fraction were determined by Bio-Rad protein assay kit (Hercules, CA, USA) and were analysed by SDS-PAGE and IgE immunoblotting.

Results

IgE reactivity of sera from patients to *H. japonicus*

Figure 1 shows the binding of specific IgE in the sera of 29 *H. japonicus*-reactive patients. Twenty-two sera (72%) showed IgE reactivity to proteins of 10 kDa and the density IgE binding band of the 10-kDa allergen was more intense than other molecular weight bands. Other allergenic components of *H. japonicus* such as 16 kDa (five sera), 20 kDa (six sera), 29 kDa (four sera) and 42 kDa (five sera) were also identified.

ELISA inhibition of *H. japonicus*-specific IgE

The *H. japonicus*-specific IgE could be completely inhibited with 1 $\mu g/mL$ of *H. japonicus* antigens. However, the pollen extracts of ragweed, oak, birch and ryegrass did not inhibit the *H. japonicus*-specific IgE. Mugwort extract could reach a maximum of 18% inhibition at the concentration of 100 $\mu g/mL$ (Fig. 2).

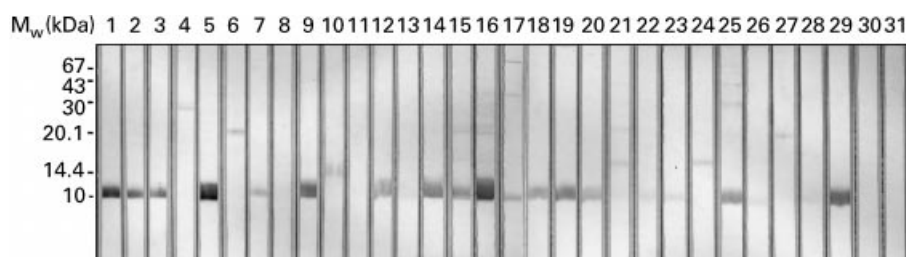


Fig. 1. IgE Immunoblotting patterns of *H. japonicus*-reactive patient sera (lanes 1–29), nonatopic serum (lane 30), buffer control (lane 31).

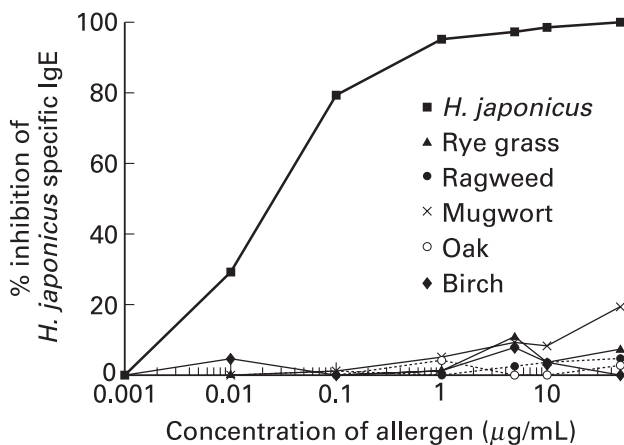


Fig. 2. ELISA inhibition of *H. japonicus*-specific IgE by the pollen extracts of *H. japonicus*, rye grass, ragweed, mugwort, oak and birch.

PAS and lectin blotting assay

The presence of carbohydrate moieties in the allergenic component of *H. japonicus* were evaluated by PAS staining and lectin blotting assay with GNA, SNA, DSA, MAA, PNA and ConA. About 14 different bands reacted with the lectins tested, but the 10-kDa allergenic component did not bind to six different lectins, including ConA, nor stained with PAS.

2-dimensional PAGE blot

Through the 2-dimensional PAGE blot with pooled sera from 10 atopic patients, the molecular weight and isoelectric points of two allergens could be identified. The isoelectric focusing point of the 10-kDa allergen and the split allergen of 29 kDa were approximately pI5.1 (Fig. 3).

N-terminal amino acid sequencing of 10-kDa allergen

We excised the corresponding 10-kDa protein from the Coomassie blue-stained PVDF membrane and micro-sequenced its N-terminus (Fig. 4). Although we compared

the N-terminal sequence of the 10-kDa allergen with the National Center for Biological Information's database (BLASTP, SWISS-PROT database), we could not find any homologous pollen or vegetable allergens.

Purification of 10 kDa allergen of *H. japonicus*

The 10-kDa allergen was partially purified by DEAE anion exchange chromatography and Sephacryl-200 gel filtration chromatography. With IgE immunoblotting we could identify the 10-kDa allergen in an unbound fraction of DEAE anion exchange chromatography (Fig. 5). We lyophilized the unbound fraction of the DEAE column and then performed gel filtration chromatography. With protein staining of SDS-PAGE, we could identify the 10-kDa protein band at the 32nd fraction of gel filtration chromatography (Fig. 6).

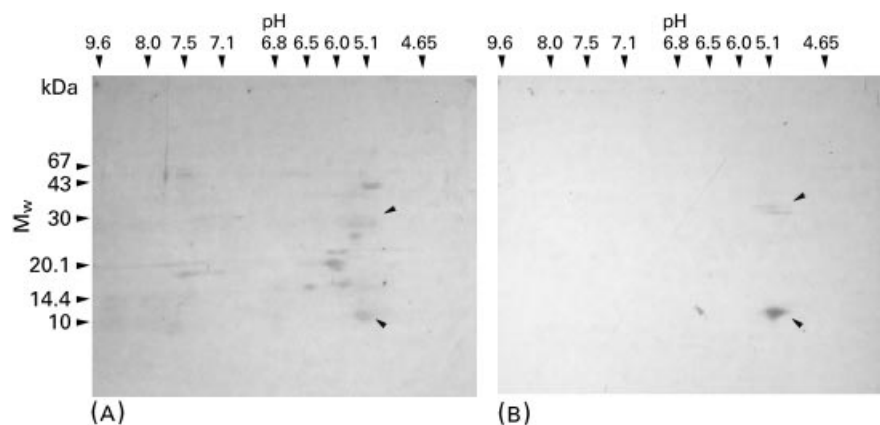
ELISA inhibition of *H. japonicus*-specific IgE with whole extract of *H. japonicus* and purified 10-kDa allergen

The whole extract of *H. japonicus* reached a maximum of 97% inhibition and the purified 10-kDa allergen could inhibit the *H. japonicus*-specific IgE by 88%. The 50% inhibition concentration of purified 10-kDa allergen and whole extract of *H. japonicus* was 20 and 38 ng/mL, respectively (Fig. 7).

IgE Immunoblotting of purified 10-kDa protein and recombinant birch profilin

IgE immunoblotting to purified 10-kDa allergen and recombinant birch profilin was done with the pooled sera from five atopics which were reactive to 10-kDa protein. The pooled sera showed strong IgE reactivity to purified 10-kDa allergen but it did not have IgE reactivity to recombinant birch profilin (Fig. 8).

Fig. 3. 2-dimensional PAGE blot of *H. japonicus*. (a) Amido-black staining of 2-dimensional PAGE blot. (b) Immunoblotting features of 2-dimensional PAGE with the reactive pooled sera. The isoelectric points of both the 10- and 29-kDa allergens were approximately 5.1.



Sequences

Asp-Asn-X-Phe-Glu-Asn-Gly-Met-Lys-Ala-
X-Thr-Ser-Leu-Tyr-Asp-X-Lys-Tyr-Gln

X; not identified

Fig. 4. N-terminal amino acid sequence of the 10-kDa allergen of *H. japonicus*.

Discussion

The *Humulus* pollen is one of the most prevalent weed pollens in the environment of Korea and China [1–4], but its clinical significance has not yet been well recognized. Recently, some clinical studies have emphasized the allergenic roles of *Humulus* pollens during the autumn in Korea and China [4–8]. However, to our knowledge, this study is the first trial to determine and characterize the major allergen of the *Humulus* species. Since *Humulus japonicus* is the most prevalent species of *Humulus* in Korea [12], we studied it.

The immunoblotting pattern of *H. japonicus* in reduced status is more apparent than in non-reduced status (data not shown). Consequently, we evaluated the allergenicity of *H. japonicus* with reduced SDS-PAGE. Immunoblotting recognized five allergic components. Of these bands, the 10-kDa allergen was predominant both in the intensity of its reaction and in the frequency of its recognition by human allergic sera. With the results of immunoblotting and ELISA inhibition with purified 10-kDa allergen, we considered the 10-kDa allergen as the major allergen of *H. japonicus*. Park *et al.* [8] noted IgE binding bands in 13, 74 and 88 kDa on immunoblotting of *H. japonicus* with eight atopic sera. In our study, we ascertained the 10 kDa components of *H. japonicus* as the major allergen, but our immunoblotting data did not sufficiently reveal the higher molecular weight allergenic components of *H. japonicus*.

With the lectin blotting assay, we identified the 10-kDa allergen bound to none of the six different lectins which selectively bound to oligosaccharides, or stained with PAS. In addition, incubation of the blotted nitrocellulose membrane with periodate did not influence the reactivity of specific IgE bindings (data not shown). These results suggested that the 10-kDa allergen is less likely to be the glycoprotein.

We also confirmed that *H. japonicus* had minimal cross-reactivity with other extracts of pollens such as mugwort, ragweed, rye and oak pollen and this result was similar to the other study. Park *et al.* [8] reported that about

50% of patients who showed a positive response to the *H. japonicus* bronchial challenge test were only sensitized to *H. japonicus* pollen. With the ELISA inhibition test, they also showed little cross-reactivity of *H. japonicus* with other weed pollens such as mugwort and ragweed. We had identified the 16 N-terminal amino acid sequences

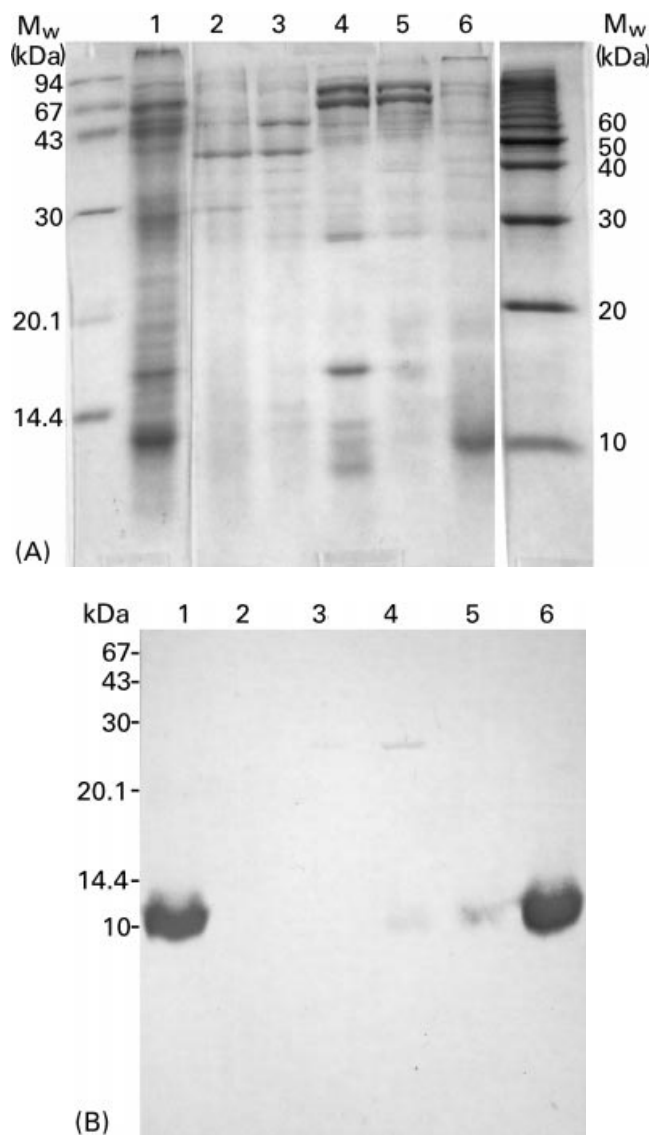


Fig. 5. SDS-PAGE (a) and IgE immunoblotting (b) of *H. japonicus* antigens eluted from the DEAE anion exchange chromatography. The contents of loaded protein were 10 µg for each lane. The proteins in SDS-PAGE were stained with Coomassie blue. Immunoblotting was done with atopic pooled sera. Lane 1, whole extracts of *H. japonicus*; lane 2, eluate by 0.4 mol/L NaCl; lane 3, eluate by 0.3 mol/L NaCl; lane 4, eluate by 0.2 mol/L NaCl; lane 5, eluate by 0.1 mol/L NaCl; lane 6, unbound fraction of *H. japonicus* from DEAE gel.

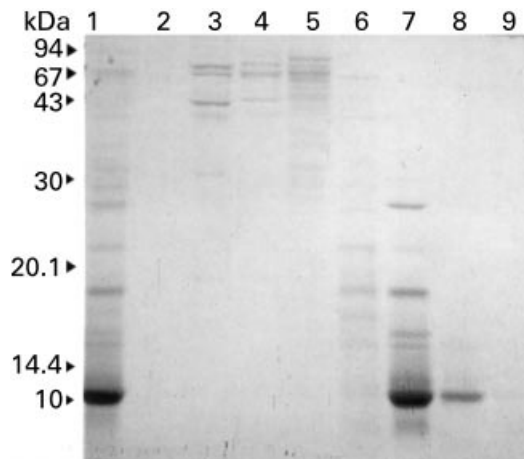


Fig. 6. Coomassie blue protein staining of the fractions from the gel filtration chromatography. Lane 1, unbound component from DEAE chromatography; lane 2, fraction 14; lane 3, fraction 16; lane 4, fraction 18; lane 5, fraction 21; lane 6, fraction 25; lane 7, fraction 28; lane 8, fraction 32; lane 9, fraction 38.

of the 10-kDa allergen. Although we compared the N-terminal sequence of the 10-kDa allergen with the National Center for Biological Information's database, we could not find any vegetable allergens with a similar N-terminal sequence. These results suggested the 10-kDa protein of *H. japonicus* may be a new and unique allergenic material.

In this study, we revealed that the 10-kDa protein could be the major allergen of *H. japonicus*. Its isoelectric point was approximately 5.1 and it did not bind with six different lectins. We also determined the N-terminal amino acid sequences of the 10-kDa allergen.

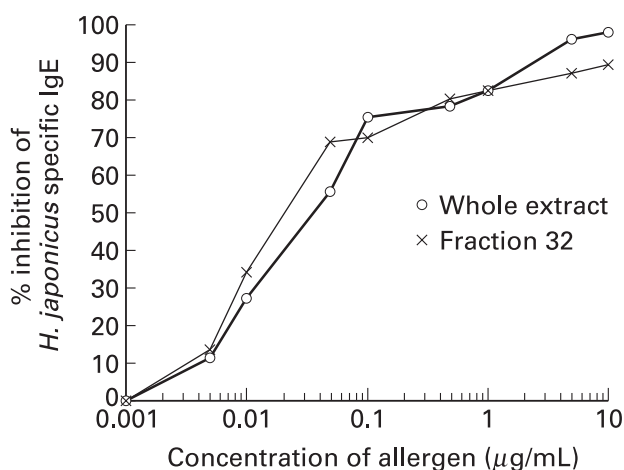


Fig. 7. ELISA inhibition of *H. japonicus*-specific IgE with whole extract of *H. japonicus* and purified 10-kDa allergen.

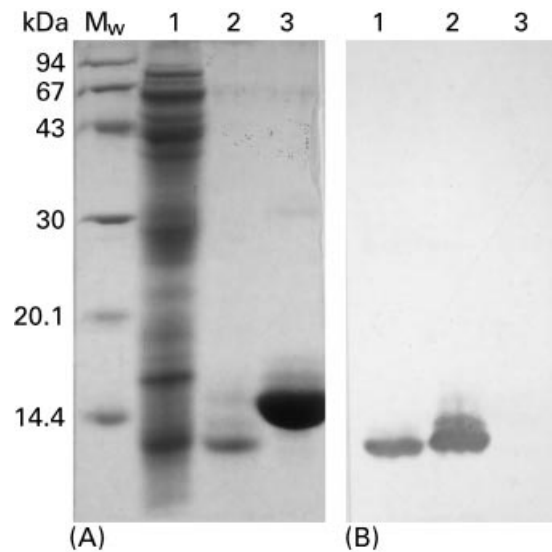


Fig. 8. SDS-PAGE and immunoblotting of whole *H. japonicus* antigen, purified 10-kDa protein and recombinant birch profilin. (a) SDS-PAGE. The proteins were stained with Coomassie blue. (b) IgE immunoblotting with atopic pooled sera. Lane 1, 10 µg of whole *H. japonicus* antigen; lane 2, 1 µg of purified 10 kDa; lane 3, 10 µg of purified recombinant birch profilin.

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