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Purification and Identification of 72 kDa and 15 kDa Allergens from *Broussonetia papyrifera* Pollen

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ABSTRACT

"*Broussonetia papyrifera*" (Chinese mulberry) pollen is an important source of allergens in regions surrounding Shanghai, China. To identify and purify major allergens from "*B. papyrifera*" pollen that reacted with serum antibodies from sensitized patients, "*B. papyrifera*" pollen was defatted, dried, and extracted proteins were separated using SP cationic exchange or Q anionic exchange columns.

Serum samples from 29 allergic patients and 4 healthy controls were collected. Allergens in eluted fractions were identified by Western blot and enzyme-linked immunosorbent assays (ELISA) using serum samples. An inhibitory assay was used to verify allergen-specific antiserum specificity.

Serum IgE of 2 patients reacted with a 15 kDa protein band. The protein was eluted with 0.1M NaCl from a SP cationic exchange column. Serum samples from the same patients positively reacted with ELISA plate coated with partially purified 15 kDa protein. Serum IgE of 11 patients reacted with a 72 kDa protein band. The protein was eluted with 0.3M NaCl from a Q anionic exchange column. Serum samples from five patients positively reacted with ELISA coated with partially purified 72 kDa protein.

Our preliminary purifications identified two proteins of 72 kDa and 15 kDa as allergens derived from "*B. papyrifera*" pollen, which reacted with allergic patients' serum IgE.

Keywords: Allergens; *Broussonetia papyrifera*; Chinese mulberry tree; Pollen

INTRODUCTION

"Broussonetia papyrifera" (Family Moraceae), also known as Chinese mulberry or Paper mulberry, is a deciduous, dioecious tree.

It has exceptionally strong adaptability and strong resistance.[1] It can be used as an ornamental tree in wastelands, remote areas, and areas with severe pollution, and can also be used as a street tree. Since the time of the Eastern Han Dynasty," *Broussonetia*" and

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hemp have been used as raw materials for making cloth and paper. "*Broussonetia*" also has good medicinal value and is a good ingredient for livestock feed.

This species is distributed in provinces in the basins of the Yellow River, Yangtze River, and Pearl River in China, and it is especially common in the southern areas. Its flowers are unisexual, although it is dioecious. The male inflorescence spicate is catkin-like with a shape like that of a caterpillar. Inflorescences appear from May to June. A single male inflorescence produces about 6×10^8 pollen grains.[2] At specific temperature and humidity, the emitted pollen can form a visible, fine mist of white colour. Thus, "*B. papyrifera*" is known as the "smoke tree". "*B. papyrifera*" is wind-pollinated and these plants are widely distributed. They produce large amounts of pollen during pollination, which becomes an important source of allergens.

The Allergy Research Lab, Department of Respiratory Diseases, Huashan Hospital began to conduct surveys on the common allergens that afflicted patients with asthma in Shanghai and East China in the 1950s. Statistical data from 1962-1965 showed that the wind-borne pollens in the Shanghai area were primarily from "*B. papyrifera*", "*Morus alba*", and "*Humulus scandens*".[3] In subsequent decades, the allergenicity of these pollens has been further confirmed by skin prick tests and specific immunoassays.[1] Although "*B. papyrifera*" is native to East Asia, it has spread to other parts of the world. In 2004, Zanforlin and Incorvaia[4] reported a case of "*B. papyrifera*" pollen allergy in Padua in northeastern Italy. The database of the Royal Botanic Gardens, Kew, describes that this pollen is a major source of allergens in the Islamabad region (http://apps.kew.org/trees/? page_id=113). Halonen et al.[5] found that mulberry tree ("*Morus alba*", Family Moraceae), also known as the white mulberry, was one of the major risk factors for asthma at both age 6 and age 11 among the children living in Tucson, Arizona.[5] For the results of skin test, 8.6% and 14% of children at age 6 and 11, respectively, sensitive to the allergen of mulberry tree.[6]

Because of its wide distribution and large quantities of pollen, "*B. papyrifera*" is an important source of pollen allergens. Therefore, we sought to identify and purify the major allergen(s) of "*B. papyrifera*" pollen. We used standard methods for pollen protein purification[7] and characterization.[8] Our results indicate that there are two allergens in the pollen of "*B. papyrifera*" that interact with IgE antibodies from patients with pollen allergy.

MATERIALS AND METHODS

Case Selection and Sample Collection

During the spring of 2010, 29 patients with asthma were recruited in the outpatient clinic of the Department of Respiratory Diseases, Huashan Hospital. Patients' medical histories and other relevant information were recorded. After patients discontinued their anti-allergy medications for 3 days, their serum total IgE levels were determined by Huashan Hospital laboratory. A non-coagulated venous blood sample (5ml) was obtained, and serum was collected after centrifugation at 2000rpm. Serum was frozen at -18ºC. In addition, serum samples of 13 healthy volunteers with no personal or family history of allergies were collected and used as negative controls. This study was reviewed and approved by the Institutional Review Board (IRB) of Huashan Hospital. Signed informed consents were obtained from all patients and healthy controls.

Preparation of Pollen Extract

Male inflorescences of "*B. papyrifera*" in full bloom were harvested and pollen was collected, sieved, and stored in dry containers. Pollen was defatted using ethylether,[9] and then soaked for 48 hours at a ratio of 1:10 (w/v) in Coca solution: NaCl (5g), sodium bicarbonate (2.75g), phenol (4 ml), with deionized water added for a final volume of 1000 ml.[10] The supernatant was collected via suction filtration under negative pressure, and then dialyzed until the liquid was colorless using dialysis tubing with a molecular weight cutoff (MWCO) of 3.5kDa (Union Carbide Corp, New York, NY). Finally, the sample was concentrated with a Vivaspin sample concentrator tube with a MWCO of 3-30kDa (GE Healthcare UK LTD, Buckinghamshire, UK).

SDS-PAGE

We used the procedures modified from Laemmli's method for SDS-PAGE.[11] Samples were first pre-treated. A sample of crude extract (12.5µl, 300µg/ml) was mixed with 2×SDS gel loading buffer (12.5µl), and then placed in a water bath at 100ºC. The separating gel was 12% PAGE-Tris-HCl. The protein samples and pre-stained protein markers (Fermentas, Inc., Ontario,

Canada) were loaded. The electrophoresis was performed using a vertical plate electrophoresis tank (BioRad, Berkeley, CA, USA), and EPS-200A electrophoresis equipment (Shanghai Tianneng Technology Co., Ltd., Shanghai, China).

Western Blotting

After SDS-PAGE was complete, the separated proteins were electrophoretically transferred to an nitrocellulose filter membrane. Each nitrocellulose membrane was stained with Ponceau S and washed out with PBS before immunological reaction with patient's serum. Ponceau S insured the equal amount of protein loading and the quality of the transblotting. The menbrane was carefully cut into slices along with each line of the proteins and soaked each slice in blocking solution (TBS +5% skim milk powder) at 4ºC overnight. Serum samples (primary antibody) from patients were diluted 1:6 in blocking solution, and each NC membrane slice was incubated with one serum sample at 37ºC for 1.5 h. After rinsing the membrane, a secondary antibody, horseradish peroxidase-labeled goat anti-human IgE (diluted at 1:1000; Serotec, Raleigh, NC, USA) was added and the membrane was incubated at 37ºC for 1.5h. After rinsing the membrane, ECL Western blotting reagent (Pierce, Rockford, IL, USA) was added and optical density was quantified with a Luminescent Image Analyzer (LAS4000, Fuji, Tokyo, Japan).

Inhibitory Assay

An inhibitory assay was used to verify that the Western blot results were due to specific binding between pollen allergens and serum antibodies. Prior to Western blot analysis, the sera reacting with 15kDa (serum from Patient B9) and the 72kDa (serum from Patient A5) antigens were incubated with a crude pollen extract in a blocking solution (TBS +5% skim milk powder). The volume ratio of serum: pollen crude extract: blocking solution was 1:1:5. Three above-mentioned NC membrane slices were incubated with three types of samples, separately, at 37ºC for 1.5h. Three types of samples included: (1) diluted patient serum without incubating with crude pollen extract; (2) diluted patient serum incubated with crude pollen extract; (3) buffer solution (blank control). After the incubation, the membranes were treated as described in Western blot protocol.

Ion Exchange Chromatography

A Hitrap SP (1 ml) column (a strong cation exchanger) and a Hitrap Q (1 ml) column (a strong anion exchanger) (GE, Uppsala, Sweden) were used for allergenic protein purification. Hitrap SP column was first equilibrated with a pH 6.5 wash buffer (20 mmol/L K_2HPO_4, 20 mmol/L KH_2PO_4, pH6.5), and Hitrap Q column was equilibrated with a pH 7.8 wash buffer (20 mmol/L K_2HPO_4, 20 mmol/L KH_2PO_4, pH7.8). To purify specific allergens from pollen, a crude pollen extract was first loaded onto the ionic exchange column and the column was washed using 2-3 bed volumes of washing buffer after loading. Bound proteins were eluted using phosphate buffers containing 0.1 M, 0.3 M, or 0.5 M NaCl, respectively. An Ultraviolet spectrophotometer (UV-2 dual-channel UV protein detector, GE, USA) was used for protein detection, and proteins eluted by different NaCl concentrations were collected. The Bradford method[12] was used to measure the protein concentrations in each of the separated samples, and samples were stored at -20 ºC after dialysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

A coating buffer (0.1M phosphate buffer, pH=9.6) was used to dilute the chromatography elution fractions. Partially purified allergens were coated onto a microtiter plate (100µl/well) and incubated at 4ºC overnight. The wells were washed, blocking solution was added (PBS containing 5% fetal bovine serum, pH=7.4), and the plates were incubated again at 4ºC overnight. The plates were washed and 1:10 diluted serum samples were added. The plates were incubated at 37ºC for 1 hour, washed, and a biotinylated goat anti-human IgE antibody (secondary antibody; diluted 1:1000, Serotec, Raleigh, NC, USA) was added. The plates were washed, avidin-horseradish peroxidase conjugated antibody was added (Avidin-HRP; diluted 1:1000, Boster, Wuhan, China), and the plates were incubated at 37ºC for 1 hour. After washing, OPD-H_2O_2 substrate was added and the plates were incubated at 37ºC for 30 min. To terminate the reaction, a sulfuric acid solution (2 M) was added to wells (50µl/well). Optical density in 492nm (OD_{492}) values were read immediately using a microplate reader (Wellscan MK-3 microplate reader, Labsystems Dragon, Finland). OD_{492} values>0.1 were considered positive.

Statistical Analysis

Results for continuous variables are given as medians (interquartile ranges) and results for categorical variables are given as numbers (percentages). Because of the small sample sizes, comparisons among 3 groups (Healthy Controls; Asthma Only; Asthma + rhinitis) used the Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses used SPSS 15.0 statistics software (SPSS Inc, Chicago, IL).

RESULTS

Patient Demographics

Table 1 shows the demographic and clinical characteristics of the three groups: Healthy Controls; Asthma Only; and Asthma + Rhinitis. Among the 29 participants, 23 patients suffered from both seasonal rhinitis and asthma, and 6 patients had asthma only. There were no significant differences for age and gender among the 3 groups (p>0.05) but there were significant differences in total serum IgE levels among three groups (p=0.031). Total serum IgE level of Asthma Only group was significantly higher than that of healthy control (p=0.006).

Identifying Patients Who Produced Antibodies against B. Papyrifera Pollen Protein Extracts

Pollen extracts were used for Western blot to detect pollen antigen-specific IgE antibodies in the subjects' sera (Figure 1). The outcomes of serum samples from 29 patients and 4 healthy controls randomly selected from 13 healthy volunteers were shown in Figure 1. Among them, serum samples from 5 patients (A4, A5, A6, A9, B7) were further 1:5 titrated to confirm the results (refer to the figure legend). IgE antibodies in 12 serum samples (A4, A5, A7, A10, A12, A13, B7, B8, B11, C5, C6, C11) reacted with the protein of 72 kDa with different extent (Figure 1). B11 was a repeat of A5. Thus, sera from 11 patients reacted with a protein with a molecular weight of about 72 kDa. One serum sample (B9) reacted with a protein with a molecular weight of about 15 kDa. In addition, C6 also weakly reacted with a protein of 15 kDa protein.

Test for Patients' Serum Specificity

The outcome of the inhibitory assay was shown in Figure 2. In Lane 1, the patient sera from patient B9 and A5 without incubating with crude pollen extract clearly reacted with 15 kDa (Figure 2A) and the 72 kDa (Figure 2B) protein, respectively. In Lane 2, no band was found on membrane, suggesting that the allergen-specific antibodies were neutralized by the allergens in crude pollen extract during incubation. In Lane 3, no band was found on membrane for the blank control. These results demonstrated that the IgE antibodies in patients' sera were allergen-specific.

SP Cationic Exchange Chromatography and Identification of a 15 kDa Pollen Allergen

Four peaks appeared during chromatography (Figure 3A). The fractions eluted with 0.1M NaCl and 0.3M NaCl were collected, separated by SDS-PAGE, and stained with Coomassie blue to examine protein purity (Figure 3B).

Table 1. Demographic and clinical characteristics of the study subjects.

	Healthy (n=13)	Asthma only (n=6)	Asthma + Rhinitis (n=23)	*P*-value
Age (years) [a]	33.5 (28.0, 45.5)	53.0 (38.0, 57.0)	36.0 (28.0, 41.0)	0.178
Gender, n (%)[b]				0.308
Male	6 (50.0)	2 (20.0)	6 (30.0)	
Female	6 (50.0)	8 (80.0)	14 (70.0)	
IgE (ng/ml)[a]	168.5 (103.7, 202.5)	381.6 (216.0, 981.0) †	255.0 (122.0, 550.0)	0.031*
SP 0.1M[c]	0 (0.0)	0 (0.0)	2 (10.0)	0.489
Q 0.3M[c]	0 (0.0)	2 (20.0)	3 (15.0)	0.345

[a] Results are medians (interquartile ranges); [b] Results are Numbers (percentages) of patients

[c] Numbers (percentages) of patients whose serum samples were positive in ELISA coating with either proteins eluted with 0.1 M NaCl from Hitrap SP column (SP 0.1 M) or proteins eluted with 0.3 M NaCl from Hitrap Q column (Q 0.3M)

* indicates significant difference among 3 groups using Kruskal-Wallis test ($p < 0.05$).

† indicates a significant difference compared with the healthy group (p=0.006)

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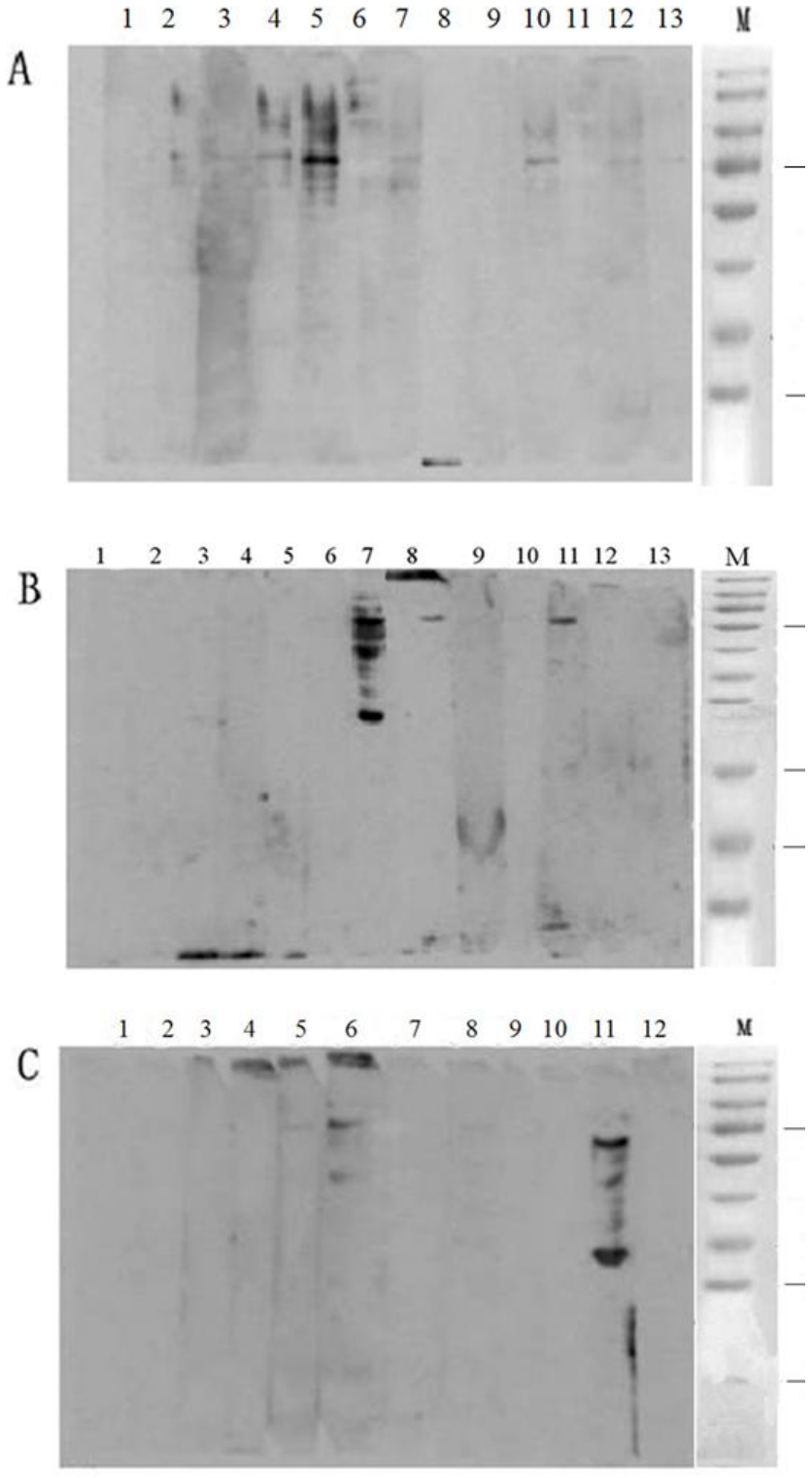

Figure 1. Detection of *B. papyrifera* pollen allergens using sera from allergic patients.

Crude pollen proteins were separated and transferred to nitrocellulose membranes and blotted with sera from allergic patients (n=29), which were run in membranes A, B and C. B12 was a repeat of A4, B11 was a repeat of A5, C4 was a repeat of A6, C3 was a repeat of A9, and C11 was a repeat of B7. Those sera were further 1:5 titrated to confirm the results. A1, C7, C10 and C12 were sera from healthy control (n=4).

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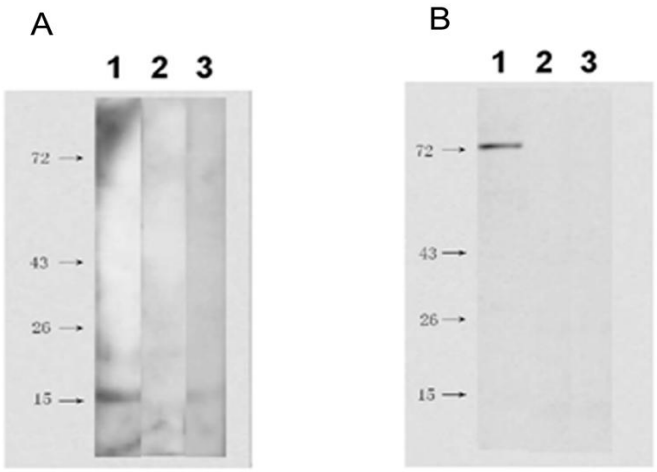

Figure 2. Blocking anti-allergen antibodies using pollen crude extract.
The specificity of allergen-binding antibodies was examined by incubating patients' sera with crude pollen extract before use in immunoblotting assay. (A) Serum from patient B9. (B) Serum from patient A5. Lane 1: Immunoblotting assay with diluted patient serum without incubating with crude pollen extract; Lane 2: Immunoblotting assay with diluted patient serum incubated with crude pollen extract; Lane 3: Immunoblotting assay with buffer solution (blank control).

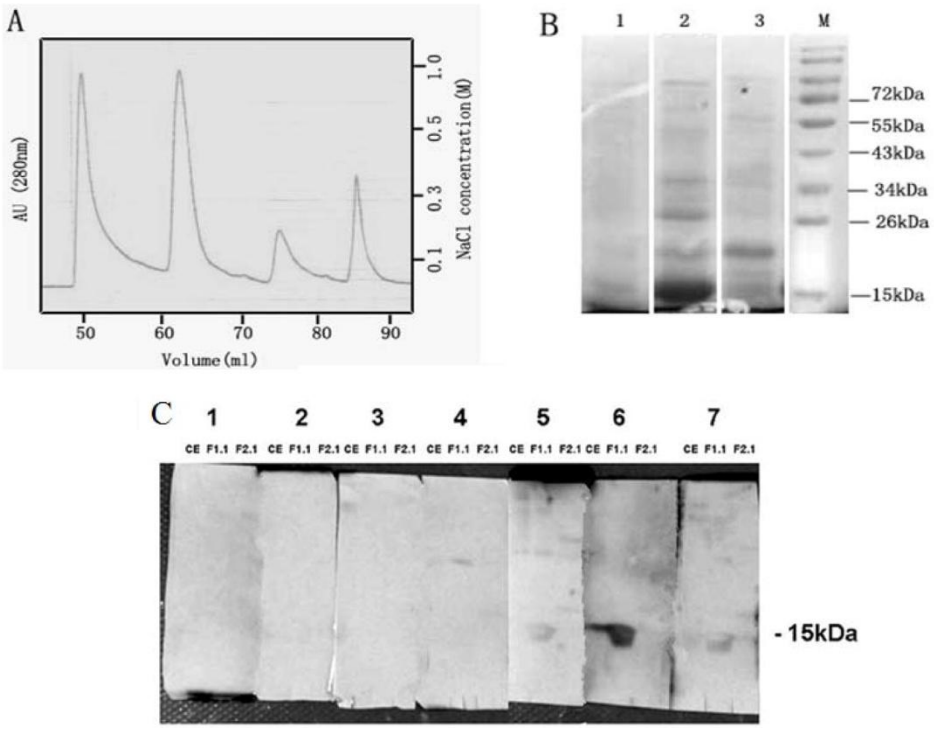

Figure 3. Purification of *B. papyrifera* pollen allergen using a Hitrap SP column.
(A) Profile of proteins eluted from a Hitrap SP column using elution buffers with different concentrations of NaCl. (B) The purity of proteins eluted from Hitrap SP column was examined by SDS-PAGE, followed by Coomassie blue staining. Lane 1: diluted crude protein extract; Lane 2: fraction eluted with 0.1M NaCl; Lane 3: fraction eluted with 0.3M NaCl. (C) Pollen allergens in crude protein extract (CE), fraction eluted with 0.1M NaCl (F1.1) and fraction eluted with 0.3M NaCl (F2.1) were detected by immunoblotting assay using sera from 7 different patients: Sample No. 1: A12; Sample No. 2: C10; Sample No. 3: C12; Sample No. 4: C9; Sample No. 5: A10; Sample No.6: B9; Sample No. 7: B13.

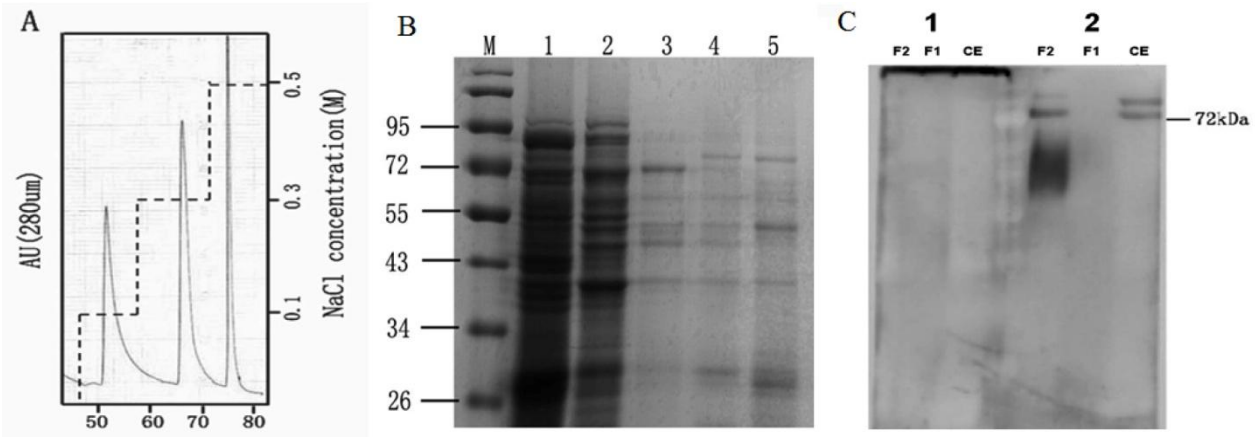

Figure 4. Purification of *B. papyrifera* pollen allergen using a Hitrap Q column.
The profile of proteins eluted from a Hitrap Q column using elution buffers with different concentrations of NaCl. (B) The purity of proteins eluted from Hitrap Q column was examined by SDS-PAGE, followed by Coommassie blue staining. M: molecular weight markers; Lane 1: crude protein extract (undiluted); Lane 2: fraction eluted with 0.1M NaCl (F1.1); Lane 3: fraction eluted with 0.3M NaCl (F2.1); Lane 4: fraction eluted with 0.3M NaCl (F2.2); Lane 5: fraction eluted with 0.5M NaCl (F3.1). (C) Detection of *B. papyrifera* pollen allergens using sera from patient A7 (sample 1; negative control) and patient A5 (sample 2). CE: crude protein extract; F1: fraction eluted with 0.1M NaCl; F2: fraction eluted with 0.3M NaCl.

The fraction eluted with 0.1M NaCl (F1.1) and the fraction eluted with 0.3M NaCl (F2.1) were used for Western blotting to detect pollen antigens associated with allergy. Sera from 7 different patients were used for Western blotting to detect pollen allergens in the fractions eluted from the SP cationic exchange column (Figure 3C).

Serum IgE from B9 (No. 6) clearly detected a protein with a molecular weight of about 15 kDa in the F1.1 fraction (0.1M NaCl) and crude extract. A10 (No. 5) and B13 (No. 7) slightly reacted with 15 kDa protein as well. The 72 kDa pollen allergen was not detected in the fractions eluted from the SP cationic exchange column (Figure 3C).

Q-Anionic Exchange Chromatography

We used a Q anionic exchange column because the SP cation exchange column fractions could not detect the 72 kDa pollen allergen. Three peaks appeared during chromatography (Figure 4A). The fractions eluted with 0.1M, 0.3M and 0.5M NaCl were collected, separated by SDS-PAGE, followed by Coommassie blue staining (Figure 4B). The pollen allergen was detected by Western blot. Two patients' sera (A5, A10) were used for immunoblotting with pollen allergen eluted from the Q column. These results showed that a protein with a molecular weight of about 72 kDa, which strongly reacted with serum sample from A5 patient, was eluted by 0.3 M NaCl in pH 7.8 washing buffer (Figure 4C).

ELISA Results

The pollen components separated by the SP cationic exchange column were used to coat microtiter plates. Among the serum samples from 33 patients, only 2 serum samples (B9 and C6) showed positive results with the fraction eluted with 0.1M NaCl (Table 1, Table 2). The flow-through, washing solution, and the fractions eluted with 0.3 and 0.5 M NaCl were also used to coat microtiter plates; no immunological reactivity was detected with these preparations (Table 2). Thus, the major allergen (15 kDa protein detected by B9 serum) was in the fraction eluted with 0.1M NaCl. Compared with the crude pollen extract, the detected signals (OD_{492} values) in the wells coated with the fractions eluted with 0.1M NaCl were markedly enhanced, which indicated that SP cationic chromatography had purified and concentrated this allergen.

When the pollen components separated by the Q-anionic exchange chromatography were used to coat the microtiter plates, five serum samples including serum from A5, B6, B7, B8, C6 showed positive results to the fraction eluted with 0.3M NaCl (Table 1, Table 3).

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Table 2. The patients whose serum positively reacted with fraction eluted from ionic-exchange column in ELISA: SP-cationic exchange column[a].

Patient No.	Crude extract	0.1M NaCl (F1.1)	0.1M NaCl (F1.2)	0.3M NaCl (F2.1)	0.5M NaCl (F3.1)
B9	0.053	0.132	0.088	0.011	0.011
C6	0.067	0.115	0.007	0.032	0.037
C12[b]	0.010	0.014	0.026	0.027	0.001

[a] Data were absorbance at 492nm (OD_{492}).

[b] C12 is a negative control.

Table 3. The patients whose serum positively reacted with fraction eluted from ionic-exchange column in ELISA: Q-anionic exchange column[a].

Patient No.	Crude extract	0.1M NaCl (F1.1)	0.3M NaCl (F2.1)	0.5 M NaCl (F3.1)
A5	0.085	0.051	0.361	0.024
B6	0.047	0.026	0.149	0.026
B7	0.076	0.039	0.156	0.092
B8	0.065	0.057	0.238	0.032
C6	0.071	0.039	0.121	0.059
B9[b]	0.044	0.013	0.044	0.026

[a] Data were absorbance at 492nm (OD_{492}).

[b] B9 is a negative control.

Thus, the major allergen (72 kDa protein detected by A5 serum) was eluted with 0.3M NaCl, and it was concentrated after the allergy was purified by Q-anionic exchange chromatography.

DISCUSSION

In this study, we detected 2 different proteins in a "*B. papyrifera*" pollen extract that could bind specifically to human serum IgE antibodies. Based on this, we explored preliminary purification methods for these two proteins. Because the isoelectric points of these proteins were different, different purification media and experimental conditions were adopted to make purification more efficient. The purified proteins retained their original immune reactivities.

Because the protein contents of the crude pollen extracts were low (300 μg/ml), purification was difficult, and the protein electrophoresis map was not very clear. Although ultrasound can be used to crush the pollen wall and increase the protein concentration of an extract, the dissolution of other pollen components may make purification more difficult. Finding means to effectively extract and further purify active proteins will be the direction used for our next steps. Determining the physical and chemical properties of allergens and sequencing them can only be accomplished if an allergen of a certain purity can be obtained. However, the preliminary purification of allergens may facilitate the production of allergy diagnostic and treatment products with regional characteristics.

We are not yet able to provide sequence data for the putative allergens that we have isolated. We cut out the bands obtained by SDS-PAGE and used in-gel digestion for the 72 kDa and 15 kDa allergens of "*B. papyrifera*" pollen. We performed multiple peptide fingerprinting and tandem mass spectrometry, but obtained no high-score reliable results. A single band from one-dimensional electrophoresis may contain more than one protein, and limited purification methods cannot separate these completely, which makes the final search results difficult to interpret. Two-dimensional electrophoresis (2-D gel) can separate components in two dimensions, by isoelectric point and molecular weight. Therefore, the combined use of 2-D gel and mass spectrometry may be more effective than conventional SDS-PAGE for isolating and indentifying allergenic proteins.[13] We are currently purifying more allergenic samples and running on the 2-D gel to improve the purity of allergens.

Since the late 1990s, skin prick reagents made from

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natural pollen crude extracts and specific immunotherapy products have been expressly prohibited due to the potential risk of immediate hypersensitivity reactions. In the absence of certified commercially available *B. papyrifera* allergen products, this study did not include *in vivo* skin prick test results. Instead, we selected patients with clear histories of seasonal allergy during the corresponding season. In the future, we will continue to collect cases of *B. papyrifera* pollen allergy and make more informed judgments regarding the allergenicity of this pollen and specific IgE response to the major allergens found in this study.

To our knowledge, this is the first report that attempted to identify allergens in the pollen from the Chinese mulberry. Our preliminary investigation identified two putative allergenic proteins in the pollen extracts from *B. papyrifera*; a major allergen of 72 kDa and a minor allergen of 15 kDa. Our future investigations will focus on methods to improve the protein yields from *B. papyrifera* pollen extracts and make more definitive characterizations of the physical, chemical, and biological properties of these allergens.

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