

# Immunoglobulin E Reactivity and Allergenic Potency of *Morus papyrifera* (Paper Mulberry) Pollen

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## ■ Abstract

**Background:** Paper mulberry (*Morus papyrifera*) pollen is considered to be one of the most clinically relevant aeroallergens in Pakistan. To date, the allergenicity of the pollen has not been investigated.

**Objective:** To characterize the sensitization profile of mulberry-allergic patients and the proteins of paper mulberry pollen contributing to pollinosis in the Pakistani population.

**Methods:** Proteins were extracted from mulberry pollen using different protocols. Immunoglobulin (Ig) E binding proteins to mulberry pollen was determined by ImmunoCAP testing and immunoblotting using sera from 29 mulberry pollen-allergic patients with positive skin prick test results to mulberry pollen antigens. The histamine release assay was performed in vitro to determine the allergenic potency of pollen extracts and a partially purified mulberry pollen allergen. The protein was identified using N-terminal sequencing and matrix-assisted laser desorption/ionization–time of flight spectrometry (MALDI-TOF/TOF).

**Results:** IgE sensitization to mulberry pollen was confirmed by positive ImmunoCAP results to pollen from *Morus alba* (white mulberry) in 23 out of 29 mulberry pollen-allergic patients. A 10-kDa protein from the paper mulberry pollen extract was considered a major allergen, along with additional IgE-reactive proteins. Sera from 79% of the patients reacted to the 10-kDa allergen, which showed substantial capacity to trigger histamine release in 3 out of 4 patients. N-terminal sequencing and MALDI-TOF/TOF yielded an amino acid sequence with no homology to known proteins.

**Conclusions:** Mulberry-allergic patients are sensitized to multiple mulberry pollen allergens. We identified a novel 10-kDa protein that was a major allergen and should be further investigated for diagnostic and therapeutic purposes.

**Key words:** Allergen. Histamine. IgE. *Morus papyrifera*. Pollen. Identification. Novel. Immunoblotting. Protein. Mulberry.

## ■ Resumen

**Antecedentes:** El polen de la morera del papel se considera uno de los aeroalérgenos más relevantes en Pakistán, cuyas propiedades alérgicas no han sido estudiadas hasta el momento actual.

**Objetivo:** El objetivo de este estudio fue caracterizar el perfil de sensibilización de los pacientes alérgicos a las proteínas de este polen que contribuye a la polinosis en Pakistán.

**Métodos:** La extracción de las proteínas de este polen fue realizada mediante diferentes protocolos. La unión de la IgE a proteínas del polen de la morera del papel, perteneciente a la familia de las moráceas fue determinada mediante InmunoCAP e Inmunoblotting utilizando suero de 29 pacientes alérgicos a este polen con prueba cutánea positiva. Se realizó test de liberación de histamina in vitro para determinar la potencia alérgica de los extractos de polen y de un alérgeno parcialmente purificado. Se secuenciaron la N-terminal y MALDI-TOF/TOF para identificar la proteína.

**Resultados:** En cuanto a los resultados obtenidos se confirmó la sensibilización a dicho polen mediante ImmunoCAP frente a polen de *Morus alba* en 23 de los 29 pacientes alérgicos al polen de morera del papel. Una proteína a de 10 kDa del extracto de dicho polen se consideró como el alérgeno mayor sobre el resto de las proteínas reactivas a la IgE.

El suero del 79% de los pacientes reaccionó con este alérgeno de 10 kDa, el cual mostró capacidad para liberar histamina in vitro en 3 de 4 pacientes.

La secuenciación N-terminal y MALDI-TOF/TOF arrojó una secuencia de aminoácidos con ausencia de homología con otras proteínas conocidas.

**Conclusiones:** En conclusión, los pacientes alérgicos a polen de morera del papel están sensibilizados a múltiples alérgenos de este polen. Se identifica una nueva proteína de 10 kDa como alérgeno mayoritario que deberá ser investigado con fines diagnósticos y terapéuticos.

**Palabras clave:** Alérgeno. Histamina. IgE. *Morus papyrifera*. Polen. Identificación. Nuevo. Inmunoblotting. Proteína. Morera.

## Introduction

Allergic disorders mediated by immunoglobulin (Ig) E are becoming increasingly frequent worldwide. Study of hypersensitivity reactions shows a strong relationship between airborne pollens and disease symptoms [1,2], and the allergenic pollens eliciting the reactions vary with regional differences in local flora and fauna [3]. Furthermore, as a consequence of climate change, plants are becoming abundant in geographical areas where they did not previously grow.

The *Morus papyrifera* tree (common name: paper mulberry) belongs to the Moraceae family (subfamily Moreae), which is often called the mulberry family or fig family. In Pakistan, paper mulberry pollen is a major cause of respiratory allergy. It is considered to be a high impact invader in this region [4]. Already in the early seventies, mulberry tree pollen was considered to cause respiratory allergy in the US [5], and mulberry pollen counts have been increasing over the last few decades [6]. Studies have shown that mulberry tree pollen is a major aeroallergen in northern regions of India [7,8] and a predominant source of aeroallergens from January to April in Madrid, Spain [9]. Exposure to pollen from white mulberry (*Morus alba*), which belongs to the same family, has been reported to cause asthma, allergic rhinitis, allergic conjunctivitis [10], and symptoms of contact urticaria [11] in Spanish patients. Case reports from Italy have also shown sensitization to mulberry in patients with artichoke allergy [12] and pollinosis [13].

Despite the growing frequency of allergy due to paper mulberry pollen, the causative allergens have yet to be identified. A single protein with an apparent molecular weight of 35–50 kDa detected in mulberry silk extract bound immunoglobulin (Ig) E from 41% of patients with mulberry silk allergy [14]. A limited number of studies have addressed the allergenicity of edible fruits of the Moraceae family. Jackfruit (*Artocarpus integrifolia*), a tropical fruit from the breadfruit tree, which belongs to the subfamily Artocapeae, has been reported to cause oral allergy syndrome. IgE reactivity to jackfruit was due to allergens that did not cross-react with the birch pollen allergens Bet v 1 and Bet v 2 [15]. Allergic reactions to fruits of the Moraceae family are also associated with the latex-fruit syndrome. IgE cross-reactivity was observed between *Ficus benjamina* latex and fig (*Ficus carica*) fruits (members of the Moraceae subfamily Ficeae), and both were shown to be allergenic for patients with fig allergy [16]. However, although 2 IgE-binding proteins of 22 kDa and 28–34 kDa were detected in *Ficus benjamina* latex, no corresponding protein was found in figs. Clinical cross-reactivity between members of the Moraceae family was confirmed by a report of 3 fig-allergic Italian patients with an associated allergy to mulberry fruit (*Morus nigra* and *Morus alba*) [17]. Recently, Mor n 3, a nonspecific lipid transfer protein (nsLTP) from *Morus nigra* fruits, was included in the allergen database of the International Union of Immunological Societies. Despite their importance as aeroallergens, no mulberry pollen allergens have been identified to date.

The present study was conducted to investigate the sensitization profile of patients who are allergic to mulberry pollen and to characterize allergens of the paper mulberry pollen responsible for the increasing frequency of mulberry allergy in Pakistani patients.

## Methods

### Extraction of Pollen Proteins

Paper mulberry pollen defatted with diethyl ether was either purchased from the company GREER or collected in Pakistan. The purity of the pollen was verified using scanning electron microscopy. Proteins were extracted using 3 extraction buffers to compare the total protein pattern: lithium chloride (LiCl) extraction buffer, isoelectric focusing (IEF) buffer, and phosphate-buffered saline (PBS). LiCl-based extraction was performed as described by Segura et al [18]. In brief, 2 g of pollen was suspended in 8 mL of Tris-EDTA buffer (100 mM Tris, 10 mM EDTA, pH 7.5), stirred for 1 hour at 4°C, and centrifuged at 2200g for 15 minutes at 4°C. The pellet was washed with water twice and dissolved in 5 mL of LiCl buffer (1.5 M), stirred overnight at 4°C, and subsequently discarded by centrifugation. The supernatant was dialyzed against water (Slide-A-Lyzer, 3.5K MWCO [Pierce]) and filtered (Minisart, pore sizes 5–0.2 µm). For IEF buffer and PBS extraction, 4 g of pollen was suspended in 20 mL of ice-cold acetone, before being stirred and centrifuged as described above. The pellet was resuspended in a 5-mL mixture of ethanol/acetone (1:3 vol/vol) followed by centrifugation and air-drying. For extraction, the pellet was divided into 2 parts; one part was dissolved in 20 mL of IEF buffer composed of 7 M urea, 2 M thiourea, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% Bio-Lyte 3–10, and 1% dithiothreitol (DTT), and the other part was suspended in 10 mL of PBS buffer (20 mM phosphate, 150 mM NaCl, pH 7.1), stirred for 3 hours, centrifuged, and filtered. Total protein content was determined using the Bradford method [19].

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10 µg protein/cm/slot) was performed according to Laemmli [20] using 15% polyacrylamide gels. The LiCl and IEF extracts were reduced by adding 1 M DTT (1 µL per 30-µL sample) before and after heating the samples (diluted in 40 mM Tris/HCl, 4 mM EDTA, 4% SDS, 50% glycerin, 0.04% bromphenol blue, pH 6.8), whereas the PBS extract was separated under nonreducing conditions. Proteins were stained with GelCode Blue solution (Pierce), and the apparent molecular mass of proteins was determined by SeeBlue Plus2 protein markers (Invitrogen).

### Patients

We recruited 29 Pakistani mulberry pollen-allergic patients from the National Institute of Health, Islamabad (Table 1). Patients were diagnosed on the basis of presence of allergic reactions and allergy tests that included skin prick test (SPT) with paper mulberry pollen extract prepared in coca solution (0.5% sodium chloride, 0.275% sodium bicarbonate, and 0.4% phenol) along with PBS as a negative control. Patients with a wheal and flare diameter greater than 3 mm were selected for further analysis. Human serum samples were stored at –20°C until processing. The study was approved by the local

Table 1. ImmunoCAP Measurements With *Morus alba* Pollen Extract

Patient No.	Age	Gender	Province	Positive SPT Results	Symptoms	Smoking	Total IgE, kU <sub>A</sub> /L	CAP Class	Specific IgE, kU <sub>A</sub> /L
1	34	Male	NWFP	6 mm	Asthma	Yes	2685	0	0.20
2	32	Female	NWFP	>7 mm	Asthma + AR	No	280	0	0.21
3	25	Male	NWFP	>7 mm	AR	No	704	1	0.52
4	20	Male	NWFP	>7 mm	Asthma	No	909	0	0.06
5	35	Male	Punjab	>7 mm	AR	No	3220	3	7.85
6	37	Male	NWFP	>7 mm	AR	Yes	432	0	0.00
7	39	Male	Punjab	>7mm	AR	No	2267	0	0.14
8	40	Male	NWFP	>7 mm	AR	No	2205	0	0.00
9	40	Male	Punjab	>7 mm	AR	Yes	1569	3	7.04
10	35	Male	NWFP	>7 mm	Asthma + AR	No	1116	2	2.80
11	18	Male	NWFP	6.5 mm	AR	No	758	4	42.75
12	53	Male	Federal	5.5 mm	AR	No	391	3	9.38
13	38	Female	AJK	5.5 mm	Asthma	Yes	718	4	49.14
14	23	Female	Federal	6 mm	AR	No	803	4	27.87
15	42	Female	Punjab	>7 mm	AR	Yes	567	3	8.36
16	23	Female	Federal	>7 mm	Asthma	Yes	511	5	66.81
17	33	Male	Federal	6 mm	Asthma + AR	Yes	1032	4	22.88
18	35	Female	Punjab	6 mm	Asthma + AR	Yes	934	5	57.68
19	40	Male	AJK	6 mm	Asthma	No	1045	4	33.60
20	45	Female	Federal	5.5 mm	AR	No	917	1	0.49
21	35	Female	Punjab	>7 mm	Asthma + AR	No	2588	5	85.12
22	27	Female	Punjab	>7 mm	Asthma + AR	Yes	1647	5	88.60
23	27	Male	NWFP	6 mm	AR	No	2565	6	232.04 (max)
24	40	Male	NWFP	>7 mm	Asthma	Yes	1503	6	232.04 (max)
25	55	Female	NWFP	6 mm	AR + edema	No	657	5	60.80
26	37	Male	Punjab	6 mm	AR	No	886	4	31.24
27	28	Female	Punjab	>7 mm	Asthma	Yes	813	4	21.24
28	32	Male	NWFP	6 mm	Asthma	No	883	3	6.11
29	26	Male	Punjab	>7 mm	AR	No	807	5	88.76

Abbreviations: AJK, Azad Jammu Kashmir; AR, allergic rhinitis; Ig, immunoglobulin; NWFP, North-West Frontier Province; SPT, skin prick test.

ethics committee, and informed consent was obtained from all patients and controls.

#### Total and Specific IgE Measurements

Total serum IgE levels were determined using a solid phase enzyme-linked immunosorbent assay (BioCheck, Inc). As no CAPs are commercially available for determination of specific IgE levels (sIgE) to mulberry pollen, specific IgE levels were measured using the ImmunoCAP 250 system (Phadia) with cross-reactive white mulberry pollen extract CAPs. sIgE values are expressed as CAP classes: class 0,  $\leq 0.35$  kU<sub>A</sub>/L; class 1,  $\leq 0.7$  kU<sub>A</sub>/L; class 2,  $\leq 3.5$  kU<sub>A</sub>/L; class 3,  $\leq 17.5$  kU<sub>A</sub>/L; class 4,  $\leq 50$  kU<sub>A</sub>/L; class 5,  $\leq 100$  kU<sub>A</sub>/L; class 6,  $> 100$  kU<sub>A</sub>/L. Serum samples from nonatopic volunteers were included as negative controls.

#### Western Blot and Immunodetection

Protein bands obtained by SDS-PAGE were transferred by either semidry blotting (45 minutes, 0.8 mA/cm<sup>2</sup>) or tank blotting (1 hour, 150 V) onto 0.2- $\mu$ m nitrocellulose membranes (Schleicher & Schuell). In order to detect paper mulberry-specific IgE, the nitrocellulose membranes were cut into

strips, washed with 0.05% Tris-buffered saline-Tween buffer (TBST: 50 mM Tris, 150 mM NaCl pH 7.4, 0.05% Tween20) for 5 minutes at room temperature, blocked twice with 0.3% TBST for 30 minutes, washed again, and incubated overnight in a 1:10 dilution of sera from patients (n=29) and controls. Strips were subsequently washed 4 times with 0.05% TBST buffer and incubated with a 1:750 dilution of mouse antihuman IgE-alkaline phosphatase (AP) (catalog number A555894, Pharmingen) for 3 hours. Antibody binding was detected by staining with the chromogenic substrate nitro-blue tetrazolium, 5-bromo-4-chloro-3'-indolylphosphate (Sigma-Aldrich).

#### Purification of IgE-Reactive Paper Mulberry Pollen Protein

In order to separate the proteins, the LiCl extract underwent preparative SDS-PAGE (Model 491 Prep Cell, Bio-Rad), which was performed under reducing conditions. The process involved loading 1.3 mg of total protein onto a 17% gel with a length of 5 cm and collecting 2-mL fractions for analysis by SDS-PAGE and silver staining. Fractions containing the 10-kDa protein were pooled, and IgE-reactivity was

determined by immunoblotting with sera from mulberry pollen-allergic patients, as described above.

### N-Terminal Sequencing

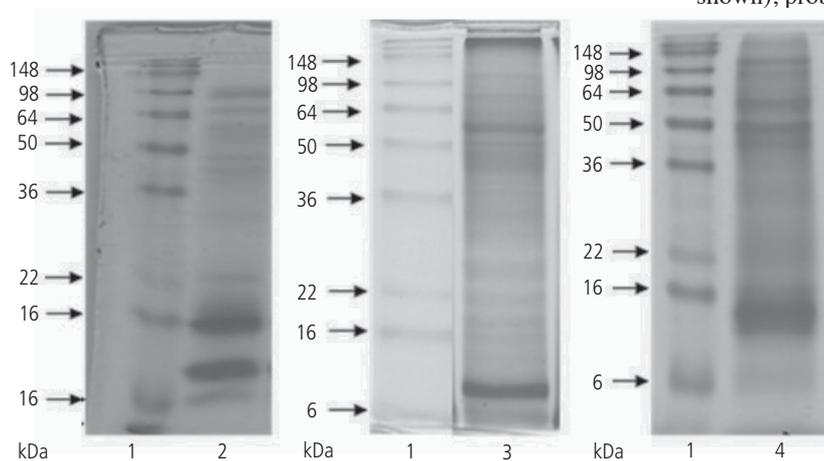
The partially purified 10-kDa protein bands from LiCl and IEF protein extracts (paper mulberry pollen from the United States) were excised and processed using N-terminal sequencing (Proteome Factory).

### Dimensional Gel Electrophoresis and Mass Spectrometry

The partially purified 10-kDa protein (prepared from paper mulberry extract obtained with LiCl buffer) underwent 2D electrophoresis. PROTEAN IEF Cell (Bio-Rad Inc) was used for IEF with a pI range of 5-8. SDS-PAGE was then performed using 15% polyacrylamide gel. The 10-kDa band was cut from the 2D electrophoresis gel, and mass spectrometry was performed using a commercial device (4800 ABI MALDI TOF/TOF, Nanyang Technological University).

### Basophil Histamine Release Assay

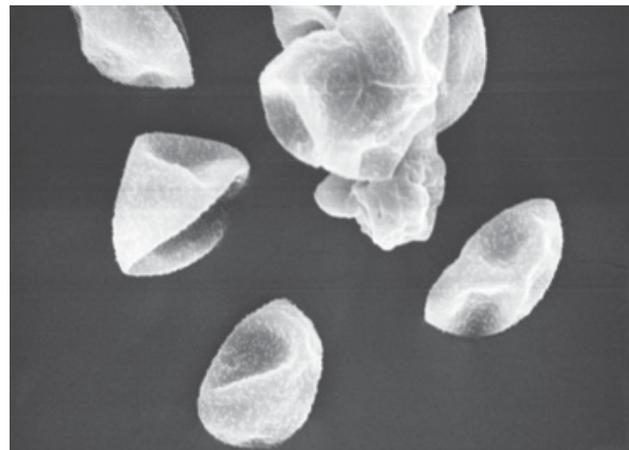
The histamine release assay was performed with sera from 4 patients as described by Foetisch et al [21]. Patients were selected according to responsiveness to SPT, sensitization to mulberry pollen confirmed by immunoblot and ImmunoCAP testing, and availability of serum samples. Passively sensitized basophils were stimulated with 10-fold dilutions of paper mulberry pollen extracts (LiCl) from US pollen and from a partially purified 10-kDa protein (LiCl extract) in the range of 10  $\mu\text{g/mL}$  to 0.1  $\text{ng/mL}$ , respectively. Birch pollen extract and bovine serum albumin were used as negative controls. All experiments were performed in duplicate. Histamine release of more than 10% was considered to be positive.



**Figure 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (15%) and Coomassie staining of crude paper mulberry extracts prepared from US pollens. Lane 1, molecular marker; lane 2, lithium chloride extract reduced with dithiothreitol (9  $\mu\text{g/lane}$ ); lane 3, isoelectric focusing extract (unknown amount); lane 4, phosphate-buffered saline extract (10  $\mu\text{g/lane}$ ).

## Results

The purity of paper mulberry tree pollen from the US and Pakistan was compared using scanning electron microscopy. In contrast to paper mulberry pollen collected in Pakistan, pollen from the US was homogeneous, with a purity of approximately 90% (Figure 1). The protein pattern of paper mulberry pollen obtained using the different extraction methods (LiCl protein quantity, 781  $\mu\text{g/mL/2 g}$ , IEF [concentration was not detectable due to interfering compounds], and PBS [514  $\mu\text{g/mL/2 g}$ ]) revealed a panel of proteins in a wide molecular range (from 6 kDa to at least 100 kDa), indicating the proteolytic stability of the protein extracts. The most prominent bands displayed

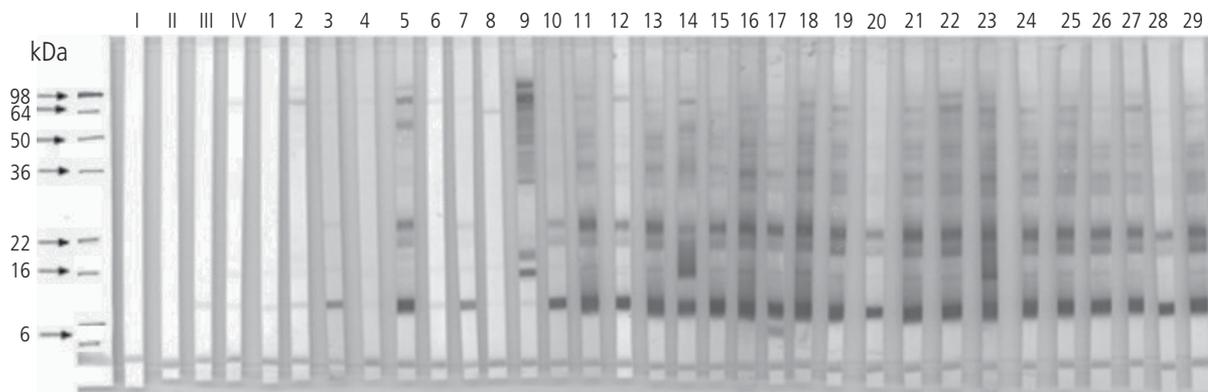


**Figure 1.** Scanning electron micrograph of paper mulberry pollen (US).

proteins of approximately 16 kDa and 10 kDa (LiCl extract), 10 kDa (IEF extract), and 12 kDa (PBS extract) (Figure 2). Since protein extraction was impaired in pollen from Pakistan (not shown), probably owing to contaminating plant constituents, mulberry pollen purchased from the US was further analyzed.

Overall, 29 paper mulberry pollen-allergic patients with a positive SPT reaction were included in the study. Total and white mulberry pollen-specific IgE levels were determined from the sera of all the patients (Table 1). Sensitization to white mulberry pollen was confirmed for 23 of the 29 patients. Specific IgE values for most of the patients who were sensitized to mulberry pollen were high (class 3 and higher,  $n=20$ ). Asthma was detected in 14 patients, allergic rhinitis in 21, and edema in 1. No correlations were detected between white mulberry-specific IgE values and total IgE values, demographic data (age, gender, and habitation), or severity of symptoms.

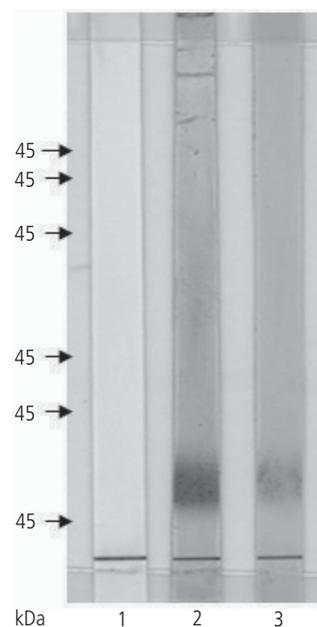
The IgE sensitization profile was studied using immunoblot experiments based on protein preparations (LiCl extraction) from



**Figure 3.** Immunoglobulin E immunoblot of mulberry pollen extract (US) with sera from patients with confirmed allergy to paper mulberry pollen (lanes 1 to 29) and controls. LiCl buffer extract: I, buffer control; II, second antibody control (mouse antihuman immunoglobulin E–alkaline phosphatase [Pharmingen]); III, serum from a nonallergic donor; and IV, serum from a Bet v 1–positive patient.

paper mulberry (Figure 3). Of note, the sensitization pattern for each extract was almost similar for all the patients tested.

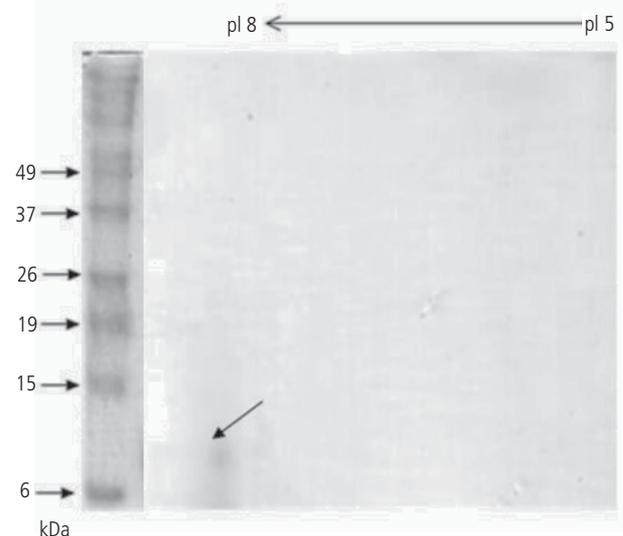
No IgE reactivity was detected with the negative controls (buffer control, second antibody control [mouse anti-human IgE-AP; Pharmingen], serum from a nonallergic donor, and serum from a Bet v 1–positive patient). Using LiCl extract, 23 of the 29 sera (79%) recognized a predominant 10-kDa protein, whereas the second predominant 16-kDa protein was IgE-reactive for only 3 sera (nos. 9, 14, and 23). Moreover, IgE from most sera bound to proteins in the range of 22 kDa to 24 kDa. The reactivity of sera in the negative white mulberry ImmunoCAP (Table 1: patients 1, 2, 4, 6, 7, and 8) was consistent with that observed with immunoblot, namely,



**Figure 4.** Immunoglobulin E immunodetection of lithium chloride extract fractions with serum from paper mulberry–allergic patients. Lane 1, antibody negative control; lane 2, serum no. 23; lane 3, serum no. 24.

no sensitization to paper mulberry proteins prepared by LiCl extraction (n=5). Mulberry-allergic patients were sensitized to multiple paper mulberry pollen allergens. In addition to other proteins, the predominant 10-kDa protein from the LiCl extract was identified as a major paper mulberry pollen allergen.

The 10-kDa IgE-reactive protein from the LiCl extract was further characterized using N-terminal amino acid sequence analysis (Proteome Factory AG). The corresponding 10-kDa protein from the IEF extract was further analyzed using N-terminal sequencing to confirm its identity in both extracts. The N-terminal sequences obtained were D(G)SHSNIQPL<sub>10</sub> (LiCl extract) and D(G/E/A)SxS(E)NIIQPL<sub>10</sub> (IEF extract). A databank homology search was unable to identify the corresponding protein. Preparative SDS-PAGE purification of protein from the LiCl extract resulted in a semipurified 10-kDa fraction. The IgE reactivity of the protein was demonstrated by immunoblotting using sera selected on the basis of strong reactivity to mulberry with CAP classes 5 and 6 (Figure 4).



**Figure 5.** Coomassie staining of 2D electrophoresis of partially purified 10-kDa allergen from *Morus papyrifera*.

Table 2. Sequence Search for 10-kDa/pI 5.5 Partially Purified Allergen With Mascot

Matching	GenBank	Molecular Weight/pI	Sequence	
Hypothetical protein BGP_6374 ( <i>Beggiatoa</i> species PS)	gil153879852	3861.9	<sup>2</sup> HQGNHKGLPLQTFDDGRGK <sup>20</sup> <sup>21</sup> SFMVALNDYFLR <sup>32</sup>	63
Glycine-rich RNA binding protein 1 ( <i>Pelargonium × hortorum</i> )	gil2267567	16814.6	<sup>50</sup> GFGFVTFSENEK <sup>60</sup> <sup>78</sup> NITVNEAQRS <sup>87</sup> <sup>155</sup> YSRDSGDNWR <sup>165</sup>	62
Hypothetical protein BURPS305_0108 ( <i>Burkholderia pseudomallei</i> 305]	gil134284281	3863.9	<sup>1</sup> MDGAHWLVVNMGKAACGPAR <sup>20</sup> <sup>22</sup> ASGPQAAPSVSEMAGRA <sup>38</sup>	52
Conserved hypothetical protein ( <i>Streptomyces</i> species Mg1)	gil194345746	8952.6	<sup>2</sup> NPGAEVSTDQESRPGRGPR <sup>20</sup> <sup>35</sup> RRATGAGGSPAR <sup>46</sup> <sup>54</sup> AAAGEIRSGR <sup>63</sup>	48
Hypothetical protein Bm1_21300 ( <i>Brugia malayi</i> )	gil170581510	19340.9	<sup>30</sup> DDDLQKFMHTVDDIQRR <sup>46</sup> <sup>47</sup> MQCCGFAGNHTWQMCK <sup>63</sup> <sup>63</sup> KIIHYDYPVK <sup>72</sup> <sup>102</sup> LQIEDELK <sup>109</sup> <sup>110</sup> IMLDQADIWK <sup>119</sup> <sup>127</sup> MNSASLSCSNTKRNCSK <sup>144</sup>	48
ORF2 [TT virus]	gil15011982	3166.5	<sup>10</sup> SGSTTEGDHRVPRAGAGGEFT <sup>30</sup>	45
Putative AC4 protein (tomato mottle leaf curl virus)	gil19033368	11114.3	<sup>2</sup> GTFISTCSSSK <sup>13</sup> <sup>20</sup> TTDSLTSYPQPDQHSIR <sup>37</sup> <sup>44</sup> ALQMSNHTWR <sup>53</sup> <sup>66</sup> STADQLEEVSNLPTTRMPR <sup>84</sup>	45

2D electrophoresis of the 10-kDa protein revealed that it was a single polypeptide with an approximate pI of 7.5 (Figure 5). Mass spectrometry followed by peptide searches using Mascot did not reveal sequence similarity with any proteins in the NCBI database (Table 2).

In vitro histamine release assay was performed with paper mulberry pollen extracts and the partially purified paper mulberry 10-kDa protein with birch pollen extract (Paul-Ehrlich-Institut) and BSA as negative controls. Three out of the 4 patient sera tested showed strong reactivity to the paper mulberry pollen extracts, triggering up to 100% histamine release (Figure 6). The release induced by the partially purified 10-kDa protein from paper mulberry was 100- to 10 000-fold weaker (considering the HR60% values), but clearly dose-dependent. In contrast, release of histamine from serum S9 was only triggered at the highest concentration of the crude pollen extract, whereas no reactivity was observed with the partially purified fraction. Of note, this patient was not sensitized to the 10-kDa protein present in the LiCl extract (Figure 3). In all cases, the negative controls (birch pollen extract and BSA) did not induce histamine release at the concentrations tested.

## Discussion

Mulberry tree pollen is highly allergenic in urbanized and rural communities where this tree is planted. The mulberry tree is found throughout the world, including Asia, Eastern and Midwestern US, and Southern Europe. In Islamabad, Pakistan, paper mulberry trees are the leading cause of pollen allergy. Although clinical cross-reactivity between Moraceae fruits and birch pollen has been reported and the LTP Mor n 3 has been described as a fruit allergen from black mulberry (*Morus nigra*), data on mulberry pollen proteins are scarce.

In the present study, we used commercially available paper mulberry pollen from the US that showed the same morphology but higher purity than pollen from Pakistan. Different methods were applied to optimize protein extraction from pollen. In contrast to LiCl extraction, IEF and PBS extraction were performed with defatted pollen. Proteins obtained after LiCl and IEF extraction were separated under reducing conditions, whereas proteins obtained after PBS extraction were separated under nonreducing conditions. Both protein extracts separated under reducing conditions showed a common predominant

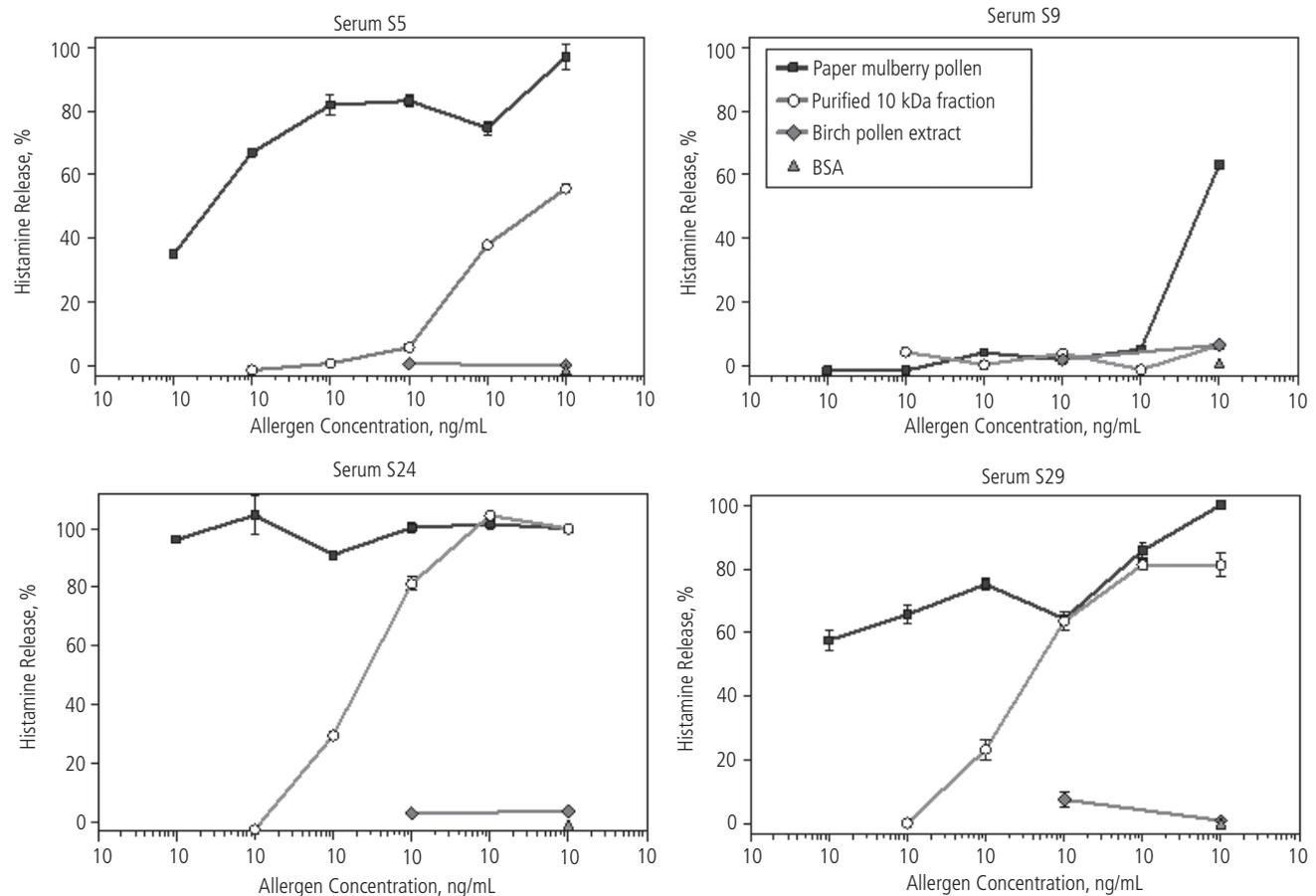


Figure 6. In vitro histamine release from human basophils passively sensitized with 4 different sera (S5, S9, S24, S29) from mulberry pollen-allergic patients using paper mulberry pollen extract, the purified 10-kDa fraction, birch pollen extract, and bovine serum albumin as stimuli.

10-kDa protein; otherwise, the protein pattern was different (data not shown).

To evaluate the sensitization profile, extracts from paper mulberry pollen underwent immunoblotting using 29 sera from mulberry-allergic patients. Positive IgE-reactivity to paper mulberry pollen was consistent with sensitization to white mulberry pollen, as determined by ImmunoCAP testing. All patients with IgE reactivity to white mulberry pollen were sensitized to paper mulberry pollen, whereas only 1 patient (no. 7) was sensitized to paper mulberry pollen, but not to white mulberry pollen. Although not confirmed by experimental testing, the results provide some evidence about cross-reactivity between the 2 pollen species. Since white mulberry and paper mulberry belong to the subfamily Moreae, expression of homologous allergens in both genotypes is likely. Therefore, ImmunoCAP analysis with white mulberry pollen can be used as a diagnostic tool to monitor sensitization to paper mulberry pollen.

Of note, despite the presence of multiple IgE-reactive proteins, the sensitization pattern was highly similar for all the sera tested. Furthermore, a 10-kDa protein was identified as a major allergen with an IgE prevalence of 79% using extract from paper mulberry pollen. The IgE binding of the 10-kDa

allergen is not affected by treatment with DTT, probably because of the lack of intramolecular disulfide bonds or because of epitopes not defined by the tertiary structure. The IgE-reactive 10-kDa protein was attributed to a predominant protein with an apparent molecular mass of 10 kDa according to the corresponding Coomassie-stained SDS-PAGE. Therefore, we further characterized this allergen. As the nsLTP Mor n 3 has been reported to be a mulberry fruit allergen, IgE inhibition experiments were performed using the potentially cross-reactive nsLTPs Pru p 3 (peach) and Cor a 8 (hazelnut) [22]. Since neither nsLTP competed with IgE binding to mulberry pollen proteins, we can probably exclude expression of a cross-reactive nsLTP (data not shown). Moreover, even the major birch pollen allergen Bet v 1 showed no cross-reactivity, and only 2 out of 29 sera from mulberry-allergic patients recognized a 6-kDa protein in the birch pollen extract (data not shown). Therefore, we conclude that paper mulberry pollen allergens show no homology with nsLTPs or birch pollen allergens. Analysis of the N-terminal sequence and mass spectrometry of the 10-kDa allergen provided no evidence of the identity of the protein. Finally, the results indicate that the 10-kDa allergen may be an as yet unidentified protein.

In vitro histamine release assay using basophils passively

sensitized with sera from allergic donors was performed to evaluate the allergenic potency of the partially purified 10-kDa protein from paper mulberry pollen extracts. Paper mulberry pollen extract displayed greater allergenicity than the enriched 10-kDa protein. Since the patients were sensitized to additional mulberry proteins, we conclude that the 10-kDa protein contributed only partially to overall allergenicity. Although the 10-kDa protein was not completely purified, it was sufficiently allergenic to induce dose-dependent histamine release. The biological activity of the allergen was further substantiated by abrogated histamine release using sera from patient 9, who was not sensitized to this allergen.

In conclusion, mulberry pollen-allergic patients show an almost uniform sensitization pattern. In addition to an as yet unidentified 10-kDa protein, several mulberry pollen allergens probably contribute to allergenicity. Further studies are required to identify the 10-kDa protein. Immunoscreening of a cDNA expression library using patient sera is an effective technique for identifying allergens. Component-resolved diagnosis requires almost all mulberry pollen allergens to be identified. Sensitization to white mulberry pollen can be used as a diagnostic marker to identify reactivity to mulberry pollen.

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