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Proteomic Identification of Allergenic Proteins of *Morus alba* L. Pollen

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Abstract

Background: Tree pollens are well-known aeroallergens all over the world. Little is known about the allergenicity of *Morus alba* (white mulberry) pollen.

Objective: We aimed to explore the potential allergens of this pollen and its clinical relevance in tree pollen allergic patients living in Istanbul, Turkey.

Methods: Twenty three seasonal allergic rhinitis patients with a confirmed tree pollen allergy and 5 healthy control subjects underwent skin prick and nasal provocation tests with *M.alba* pollen extract. The pollen extract was then resolved by gel electrophoresis, and immunoblotted with sera from patients/control individuals to detect the potential allergenic proteins. The prevalent IgE binding proteins from 1D-gel were analyzed by MALDI-TOF/TOF.

Results: Eleven out of 23 patients were reactive to the extract with skin prick tests. Seven of those patients also reacted positively to the nasal provocation tests. The most common IgE-binding pollen proteins were detected between 55-100 kDa, and also at molecular weights lower than 30 kDa for some patients. Mass spectrometry analyses revealed that the principal IgE-binding protein was methionine synthase (5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase), which is then proposed as a novel allergen in *M.alba* pollen.

Conclusion: This study provides the first detailed information for the potential allergens of *Morus alba* pollen of Istanbul. Methionine synthase with an apparent molecular weight of 80 to 85 kDa has been recognized as one of the allergens in *Morus alba* pollen for the first time.

Key words: IgE-binding proteins, methionine synthase (MetE), Moraceae, *Morus alba*, pollen allergy, white mulberry

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Introduction

Tree pollens are well-known aeroallergens all over the world. When they are released into the atmosphere in sufficient amounts, they can cause various allergic diseases such as asthma, rhinitis and conjunctivitis in sensitive individuals, especially in their pollen spreading periods.[1,2] Pollen proteins and glycoproteins with molecular weights of 5-150 kDa are primarily responsible for allergenicity.[3] Proteomic studies represent a quick and efficient way of identifying protein(s) of interest and characterizing complex allergen sources. The combination of

immunoblotting with 1-D or 2-D gel electrophoresis provides an opportunity to detect Ig-E binding proteins in more detail after protein separation depending on their isoelectric points and/or molecular weights. After the detection of IgE binding proteins, they can be identified efficiently by mass spectrometry (MS).[4] Such an effort may help to extend the efficiency of both diagnostic and therapeutic tools for allergic diseases.[5] The protein family distribution of pollen allergens is regarded as up to 29 families[6] while tree pollen allergens are mostly found in pathogenesis-related group 10 (PR-10 or Bet v I-related) proteins, profilins, calcium binding proteins (polcalcins), expansins and pectate lyases.[7]

Mulberry (*Morus*) is a genus of the Moraceae family, which comprises native or cultivated trees in mild regions of the world. The fruits are consumed by humans as food and as traditional medicine, and the leaves are used as animal feed for both silkworms in silk production and for farm animals.[8] The *Morus* genus contains widespread species such as *M. nigra* (black mulberry), *M. rubra* (red mulberry), *M. microphylla* (Texas mulberry), *M. papyrifera* (paper mulberry) and *M. alba* (white mulberry). However, there are a limited number of studies on the allergenic proteins of these species. A non-specific lipid transfer protein (ns-LTP), Mor n 3 from the black mulberry with a molecular mass of 9246 Da, the first isolated and completely characterized fruit allergen was shown to cross-react with other plant-derived LTPs.[9] More recently a 10-kDa protein has been proposed by Micheal et al. as an unidentified pollen allergen from the paper mulberry.[10] The authors suggested that paper mulberry pollen allergens show no homology with nsLTPs or birch pollen allergens.

Some clinical studies indicate that *M.alba* pollen induces allergic diseases, such as asthma, allergic rhinitis, allergic conjunctivitis and urticaria, especially in pollen-spreading periods (April-May).[11-13] Although white mulberry pollen is regarded as an important aeroallergen, there are a limited number of reports on its allergenicity and allergenic proteins. Navarro and coworkers demonstrated that IgE antibodies were produced against 10- and 18-kDa allergens from white mulberry fruit in a 46-year-old female patient.[13] The latter allergen (18 kDa) is also present in white mulberry pollen and leaves, and has been found to cross-react with birch pollen.

The present study aimed to investigate the allergenicity of white mulberry pollen extract and to identify its allergenic proteins using the immunoproteomic approach called Serological Proteomic Analysis (SERPA). As this study was the first clinical report on Turkish population, our results have been expected to contribute both to clinical data and to pollen proteomics.

Methods

Pollen collection

Pollen samples from *Morus alba* L. were collected from the garden of Faculty of Forestry, Istanbul University (Bahçeköy -Istanbul/Turkey) during the pollen-spreading period (April/ 2012). The plants were identified by means of rigorous botanical criteria, and pollen was collected from the mature flowering plants by using, at a close distance, a filter-equipped vacuum device to avoid contamination. Pollen purity (> 99%) was assessed by microscopic analysis performed by a well-trained specialist. Pollen grains were separated with different pore size (250, 180 and 90 μm) sieves, dried at room temperature, and kept at -80ºC until protein extraction.

Preparation of pollen extract

Pollen extraction was performed with some modifications according to Iacovacci et al.[14] Ten grams of dried pollen was suspended in 125 mM NH_4HCO_3 at a ratio of 1:12 (w/v) for 12 hours at 4ºC with constant stirring. Insoluble materials were removed by centrifugation at 13000x*g* for 1 hour at 4ºC. Afterwards the extract was filtered through a Whatman No. 1 filter paper with 0.45 μm pore size and 125 mm Whatman filter paper with a Millipore vacuum filtration system. The filtrate was dialyzed for 24 hours at 4ºC with distilled water using 43 mm dialysis tubing. The final dialysate was lyophilized and stored at -80ºC.

The lyophilized pollen extract was solubilized in distilled water and the protein concentration was determined by using a Bicinchoninic Acid (BCA) Protein Assay Kit. The final absorbance of the assay mixture was measured by VarioScan Flash Image System (Bio-Rad) at a 562 nm wavelength.

Clinical Studies

Patient Selection: After receiving ethical approval from the Ethics Committee of Istanbul Faculty of Medicine, Ethical Committee and written informed consents from the subjects, 23 seasonal allergic rhinitis patients (16 female, 7 male; 21-56 year old) who displayed positive prick test results for common tree pollens and 5 healthy control subjects were included into the study. A pollen allergy was established by means of positive skin prick test (SPT) and nasal provocation test (NPT) results.

Skin Prick Test (SPT): A skin prick test was performed with the commercial allergen extracts from different tree pollens (*Betula verrucosa*, *Platanus acerifolia*, *Quercus ilex*, *Cupressus arizonica*, *Cupressus sempervirens*, *Corylus avellana*, *Alnus glutinosa*, *Fagus sylvatica*, *Quercus robur*), as well as with the prepared *M.alba* pollen extract. The prepared *M.alba* pollen extract was used for skin prick-testing in four different concentrations starting with a 1/1000 diluted suspension to 1/1 undiluted (5 mg/mL lyophilized powder) raw extract. The test was repeated with a tenfold increase in the extract concentration if the previous test was found negative. A positive response was defined as a wheal measuring at least 3 mm in diameter when compared with serum physiologic that was used as a negative control.

Nasal Provocation Test (NPT):Each nasal cavity was evaluated separately. Airflow was measured under 150 Pa pressure and resistance was calculated using an anterior rhinomanometer (Jaeger brand Masterscope Rhino Carefusion, Germany). Patients were challenged first with 2 puffs (100 μL) of saline in each nostril to exclude nasal hyperreactivity. If no reaction to the physiological saline solution occurred, NPT was initiated with increasing concentrations of *M.alba* pollen extract in 15-minute intervals. Two puffs (100 μL) of the solution at room temperature were applied to each nostril. If a positive reaction did not occur with the previous concentration, the concentration of pollen extract was incrementally increased until the final concentration of a 1/1 undiluted form. Symptom scores and nasal resistance with anterior rhinometry were recorded before and after each provocation. Positivity criteria

in the nasal provocation test consisted of both symptom score positivity according to the Lebel symptom score scale and changes in the measurement of rhinometry, which included a fall in peak inspiratory flow (PIF) of ≥ 40% post-NPT and/or increase in airflow resistance by 100%.[15-17]

Electrophoresis

SDS- PAGE for Western blotting was carried out as described earlier with a slight modification in sample buffer.[18] Lyophilized pollen extract in distilled water was mixed with sample buffer containing 200 mM Tris-HCl, 8% SDS, 40% glycerol and 0.04% bromophenol blue in a proper ratio, heated in a hot plate at 95°C for 5 minutes and 20 microgram of each sample were loaded onto the electrophoresis system (Mini -PROTEAN 3 Cell, Bio-Rad).

Protein samples were migrated on a discontinuous gel consisting of a stacking part (5% acrylamide) and a resolving part (10% acrylamide) under 200 V until the dye front reached the bottom of the gel. Proteins were visualized with Imperial Protein Stain (Thermo Scientific) based on Coomassie R-250 dye. The SDS-PAGE gel was scanned by a Chemidoc TM XRS+System (Bio-Rad).

Western blotting

Separated proteins on 1D-gel were transferred onto a PVDF membrane by a semidry blotting system (Bio-Rad) at 0.5 mA/gel and 25 V for 90 minutes. The membrane was blocked with 5% skimmed milk in phosphate buffered saline (PBS) containing 0.5% Tween 20 for 1 hour. After washing with PBS-0.5% Tween 20, the membrane was incubated overnight with a 1:4 dilution of sera from patients or healthy control subjects at 4°C. IgE-binding proteins were detected using a 1:1000 dilution of HRP (horse radish peroxidase)-conjugated mouse anti-human IgE (Fc) antibody (Southern Biotech). Revelation was carried out using ECL Western Blotting Detection Reagents (GE-Healthcare), and the membrane was scanned by the Chemidoc ™ XRS+System (Bio-Rad).

Proteomic Analysis

In Gel Digestion: Common IgE-binding protein bands from 1D-gel were excised into small pieces and destained in 100 mM ammonium bicarbonate and in 100% acetonitrile (ACN), alternatively, and then dried at 37°C. In-gel digestion was performed as following: the dried gel pieces were reduced with 65 mM DTT for 1hour at 37°C and alkylated with 135 mM iodoacetamide (IAA) for 30 minutes at room temperature in the dark. After removing the solution, the gel pieces were washed with 100 mM ammonium bicarbonate; and an equal volume of 100% ACN was added and incubated for 10 minutes, and then dried at 37°C. For gel digestion, MS grade trypsin (Trypsin Gold, Promega) was added to the gel pieces at 125 ng in 0.01% surfactant (ProteaseMAX™ Surfactant, Trypsin Enhancer, Promega) and incubated for 2 hours at 37°C. The digestion was stopped by adding 0.4 µL of 10% trifluoroacetic acid (TFA). The resulting peptides were concentrated by vacuum centrifuge and maintained at -20°C until further analysis.

Mass spectrometric analysis: In order to remove salt and contaminants from the peptide mixture, it was purified and condensed with Zip Tip C18 tips (Millipore) and mixed with α-cyano-4 hydroxy-cinnamic acid (Sigma-Aldrich) and spotted onto target MALDI plates. The peptides were identified by the 4800 MALDI TOF/TOF mass spectrometer (ABSciex, Les Ulis, France). Data acquisition was carried out using 4000 Series Explorer software, V3.5.3 (ABSCiex) in positive reflector ion mode for both MS and MS/MS analyses. The mass spectrometer was calibrated before each analysis with Peptide Calibration Standard II (Bruker Daltonics, Bremen, Germany). MS analyses were performed within a range of *m/z* 700 - *m/z* 4000. MS/MS experiments were performed on the 30 most abundant ions with a threshold of S/N higher than 30 by using CID (Collision Induced Dissociation) activation mode.

Protein Identification: Post-analysis data processing was performed using Protein Pilot 4.5 software with Mascot search engine and the protein database of National Center for Biotechnology Information (NCBI - February 2015). The sequence query searching was set up using the following parameters: carbamidomethyl (C) as fixed modification, deamidated (NQ), oxidation (HW) and oxidation (M) as variable modifications with one missed cleavage and a *m/z* tolerance of 50 ppm for the precursor ion and a *m/z* tolerance of 0.1 Da for the product ions. Protein identification was based on taxonomic similarities with *M. notabilis* since corresponding proteins in *M. alba* have not yet been sequenced. Only protein Mascot scores greater than 70 are significant ($p < 0.05$) for protein identification.

IgE Measurement

Allergen specific IgE was measured with an ELISA kit (Allercoat™ 6 Microplate ELISA, Euroimmun, Germany). Sera samples were applied to the microplate wells, which were assembled with the rings coated with commercial *M. alba* pollen extract and incubated for 60 minutes at 37°C. After washing the microplate wells with wash buffer, a component of the kit, alkaline phosphatase labelled anti-human IgE antibody was added and incubated for 60 minutes at 37°C. Substrate solution was added into each well and incubated for 30 minutes at 37°C. After washing, bound conjugate was detected with p-nitrophenyl phosphate (PNPP) by incubating for 30 minutes at 37°C. The reaction was stopped with 1 M NaOH and read at 405 nm on an ELISA reader. ELISA for prepared *M.alba* pollen extract could not be performed due to a lack of availability of the relevant allergen ring coated with this extract.

Results

Skin prick tests

Eleven of 23 patients who were sensitized to one or more standardized tree pollens (ALK-Abello, Spain) reacted to *Morus alba* L. pollen extract in different concentrations (1 patient with 1/100 dilution, 3 patients with 1/10 dilution, and 7 patients with the undiluted extract). None of the healthy control subjects reacted to the SPT. The skin prick test results of 11 patients (A-K) with *M. alba* pollen and other standard tree pollens are presented in **Table 1**. The most prevalent tree pollen reactivities were against *Cupressus arizonica* (9 patients) and *Platanus acerifolia* (7 patients).

Table 1. Skin prick test results for *Morus alba* L. pollen extract and some other commercial tree pollen extracts.

Patient	Mora	Commercial pollen extracts									
		Betv	Plaa	Quei	Cupa	Cups	Cora	Alng	Fags	Pins	Quer
A	4 mm	4 mm	4 mm	–	4 mm	5 mm	–	5 mm	5 mm	–	5 mm
B	5 mm	–	–	–	–	–	–	–	–	–	–
C	4 mm	–	–	–	–	–	4 mm	4mm	–	–	–
D	5 mm	4 mm	5 mm	–	5 mm	–	–	–	–	–	–
E	4 mm	–	–	4 mm	4 mm	4 mm	4 mm	4 mm	4 mm	–	–
F	4 mm	4 mm	5 mm	4 mm	4 mm	4 mm	–	–	–	–	+
G	5 mm	–	4 mm	–	5 mm	–	–	–	–	–	–
H	5 mm	–	–	–	5 mm	–	–	–	–	–	–
I	5 mm	–	4 mm	–	6 mm	–	4 mm	–	–	–	–
J	4 mm	–	4 mm	–	4 mm	5 mm	5 mm	4 mm	–	–	–
K	4 mm	4 mm	4 mm	4 mm	4 mm	4 mm	–	4 mm	4 mm	4 mm	5 mm

Mora, *Morus alba*; Betv, *Betula verrucosa*; Plaa, *Platanus acerifolia*; Quei, *Quercus ilex*; Cupa, *Cupressus arizonica*; Cups, *Cupressus sempervirens*; Cora, *Corylus avellana*; Alng, *Alnus glutinosa*; Fags, *Fagus sylvatica*; Pins, *Pinus sylvestris*; Quer, *Quercus robur*; (+) indicates positive response, (–) indicates no response to the pollen sample in patient.

Nasal provocation tests

Nasal provocation with *M. alba* pollen extract was conducted with 23 patients. Seven out of 11 SPT (+) patients (A-G) were also NPT (+) whereas 2 (H and I) were negative and 2 (J and K) were hyperreactive. Five of the remaining 12 SPT (–) patients were hyperreactive while 4 patients reacted to different concentrations of pollen extract in the NPT. Three of the SPT (–) patients were also NPT (–). None of the healthy control subjects reacted to the NPT.

Detection of IgE-binding proteins by 1D-SDS PAGE and immunoblotting

The SDS-PAGE of the *M. alba* pollen extract indicated at least 18 proteins (**Figure 1a**). These proteins were then transferred to a PVDF membrane without staining for the detection of IgE-binding capacity using Western blotting. Each blot was individually incubated with the sera of the 23 seasonal allergic rhinitis patients and 5 healthy control subjects. Specific IgEs against *M.alba* polypeptides were detected in 11 out of 23 patients' sera (**Figure 1b**). The results have been evaluated with both SPT and NPT results. The 1D-immunoblotting profile resulting from interaction between specific IgEs of patient F and *M. alba* pollen proteins is presented in **Figure 1c**. IgE antibodies of the remaining 12 patients and of the control individuals did not react with the pollen proteins.

Immunoblots showed that *M. alba* pollen contained common IgE-binding polypeptides between 55-100 kDa. One protein with a molecular weight of 80 kDa produced a significant reaction in 5 patients (A, C, E, F and K). IgE-binding protein(s) with a molecular weight of < 30 kDa were also detected. These small proteins were predominant in one of the hyperreactive patients (J) whereas the 80 kDa protein was predominant in hyperreactive patient (K). Polypeptide bands corresponding to the identified allergens were excised from the polyacrylamide gel and analyzed by MS/MS analysis.

Identification of potential allergenic proteins

Common IgE-binding protein bands (Band 1-5 from 1D-gel in **Figure 1a**) were excised, digested by trypsin and analyzed using MALDI TOF/TOF mass spectrometer for protein identification. To check the accuracy of this experiment, a 50 kDa marker protein was also analyzed and the protein was identified with a high score (431). Only the proteins identified with significant Mascot scores were summarized in **Table 2** (for detailed information, see **Tables S1-5**). It should be mentioned that the database research was conducted by similitudes to *M. notabilis*, a recently sequenced *Morus* species as the complete sequences of proteins in *M. alba* are still not well understood.[19,20] Band 1 matched with two isoforms of methionine synthase(5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase) with a Mascot protein scores of 353 and 215. Two isoforms of L-ascorbate oxidase-like protein were also identified from band 1, but with much lower scores (173 and 87). One isoform of L-ascorbate oxidase-like protein was also identified from band 2, which was very close to band 1. The calculated masses from the primary sequences of both L-ascorbate oxidase-like protein isoforms is around 60-62 kDa, a value lower than the observed masses for band 1 and 2 of 82 and 79 kDa, respectively. This difference could be explained by a glycosylation, well described for several ascorbate oxidase enzymes in pollens.[21]

A phosphoglucomutase and the subtilisin-like protease SDD1 were both identified in band 3 (observed mass of 68 kDa). Band 4 and band 5 also gave positive results with the identified proteins hypothetical protein L484_006703, a protein from the glycosyl hydrolase family 9 and hypothetical protein L484_025194 with a conserved domain found in a variety of structurally related metalloproteins like glyoxalase I or dioxygenases. However, these proteins are less significant with mascot scores of 71 and 73, respectively, close to the threshold score of 60 used for the validation of Mascot identifications.

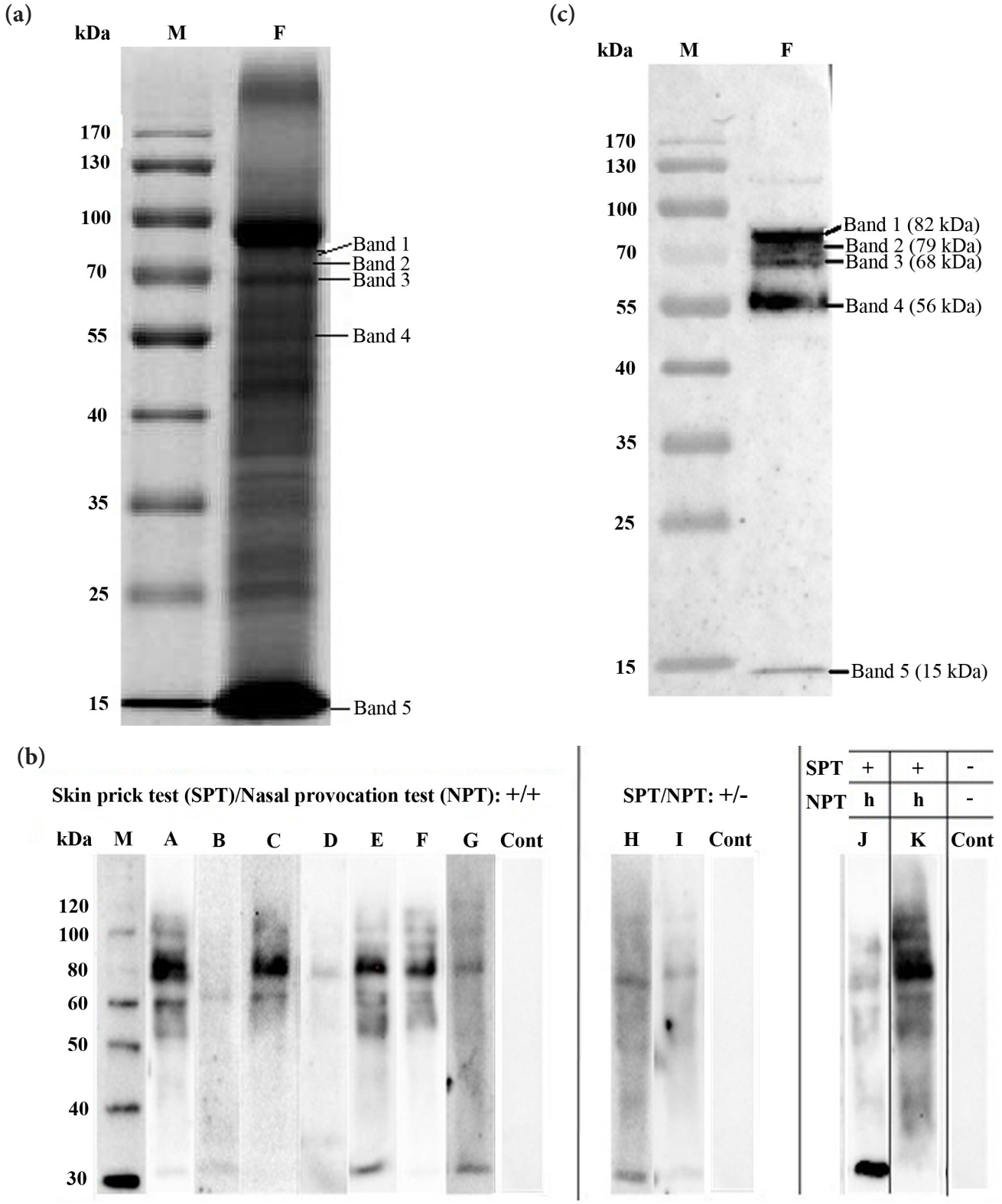

Figure 1. (a) 1-D Coomassie blue stained protein profile of *Morus alba* L. pollen; (b) IgE immunoblotting analysis of the sera of 11 patients (1-11) with *Morus alba* L. pollen extract. Patients were presented in three classes of clinical response; (c) 1D-immunoblotting result from patient F serum. M, molecular weight markers (PageRuler Prestained Protein Ladder (Thermo Scientific) in (a) and MagicMark XP Western Protein Standard (Life Technologies) in (b) and (c); Cont., serum sample from healthy control subjects; h, hypersensitive; Mora: *Morus alba*.

Table 2. The potential allergenic proteins of *Morus alba* L. pollen.

	Protein names	Accession number	Calculated Molecular Mass (Da)	Observed Molecular Mass (Da)	Mascot Score	Matches (# Peptides)	Protein sequence coverage (%)
Band 1	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010097930	84904	82000	353	19	18
	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010103482	83389	82000	215	17	17
	L-ascorbate oxidase-like protein	XP_010093150	60499	82000	173	26	23
	L-ascorbate oxidase-like protein	XP_010093149	62333	82000	87	18	21
Band 2	L-ascorbate oxidase-like protein	XP_010093150	60499	79000	103	22	23
Band 3	Subtilisin-like protease SDD1	XP_010108074	85546	68000	115	19	20
	Phosphoglucomutase	XP_010101975	63757	68000	107	22	27
Band 4	hypothetical protein L484_006703	XP_010112623	53747	56000	71	6	9
Band 5	hypothetical protein L484_025194	XP_010106438	16003	15000	73	9	48

Table S1. Protein identification of band 1 (82 kDa), 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, protein score 353 (A), 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, protein score 215 (B), L-ascorbate oxidase-like protein, protein score 173 (C), L-ascorbate oxidase-like protein, protein score 87 (D).

A.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010097930.1	84904	353	6.42	19	18

Protein sequence coverage (Matched peptides shown in **bold red**):

1	**MASHIVGYPR**	VGPKRELK**FA**	**LESFWDGK**SS	AEDLQKVAAD	LRASIWKQMS
51	EAGIKYIPSN	TFSYYDQVLD	TTAMLGAVPP	RYGWNGGEIG	FDVYFSMARG
101	NASVPAMEMT	KWFDTNYHYI	VPELSPEIKF	SYASHKAVEE	YKEAKALGVE
151	TVPVLVGPVS	YLLLSKPAKG	VEKSFSLLSL	IGSILPVYKE	VLAELKAAGA
201	TWVQFDEPTL	VKDLDAHQLQ	AFTHAYSELE	SSLSGLNVVI	ETYFADVTAE
251	AFKTLTGLK**G**	**VTGYGFDLVR**	**GTK**TLDLIKG	GFPSGK**YLFA**	**GVVDGR**NIWA
301	NDLASSLSTL	EALEGIVGKD	KLVVSTSCSL	LHTAVDLVNE	TKLDKEIK**SW**
351	**LAFAAQK**VVE	VNALAK**ALAG**	**QKDEAFFTAN**	**AGAQASR**RSS	PRVTNEAVQK
401	AAAALKGSDH	RRATNVSSRL	DAQQKKLNLP	ALPTTTIGSF	PQTLELRRVR
451	REYKAKKISE	EEYVNAIKEE	IKKVVK**LQEE**	**LDIDVLVHGE**	**PER**NDMVEYF
501	GEQLSGFAFT	VNGWVQSYGS	RCVKPPIIYG	DVSRPKPMTV	FWSSFAQSTT
551	KRPMKGMLTG	PVTILNWSFV	RNDQPRFETC	YQIALAIKDE	VEDLEK**AGIT**
601	**VIQIDEAALR**	EGLPLRK**SEE**	**AFYLNWAVHS**	**FR**ITNCGVQD	TTQIHTHMCY
651	SNFNDIIHSI	IDMDADVITI	ENSRSDEK**LL**	**SVFR**EGVK**YG**	**AGIGPGVYDI**
701	**HSPR**IPSREE	IADRINKMLA	VLESNILWVN	PDCGLKTRKY	SEVKPALSNM
751	VAAAKLLRSQ	LASAK			

B.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	XP_010103482.1	83389	215	5.96	17	17

Protein sequence coverage (Matched peptides shown in **bold red**):

1	**MASHIVGYPR**	MGPERELK**FA**	**LESFWDGK**SS	ADDFKK**FSMA**	**RGNSSVPAME**
51	**MTK**WFDTNYH	FIIPELGPNV	TFSYASHKAV	DEYKEAKSIG	VDTVPVLIGP
101	VSYLLLSKPA	KSIGNTFSFL	SLLDKILPIY	KEVISELKVA	GVTWIHFDEP
151	TLVLDLDSHK	YEAFKNAYAA	LESTLSGLNV	LVETYFTDVT	AEAYKTISEL
201	K**GVTAYGFDF**	**VR**GTNTIDLV	KESTLSGLNV	LVETYFTDVT	AEAYKTISEL
251	K**GVTAYGFDF**	**VR**GTTTIDLV	KGGFPHGK**YL**	**FAGVVDGR**NI	WTNDLDASLS
301	TLKSLEGIVG	KDKLVVSTSC	SLLHTAVDLV	NETKLDKEIK	**SWLAFAAQK**I
351	VEFNALANAL	AGQKDEAYFS	NNAAAQASRR	SSPRVTNEAV	QKAAAALRGS
401	DHRRATNVST	RLDAQQKKLN	LPILPTTTIG	CKVGVNFSLR	IGTVLLRISE
451	DVYVKAIKVE	ISKVVK**LQEE**	**LDIDVLVHGE**	**PER**NDMVEYF	GEQLSGFAFT
501	VNGWVQSYGS	RCVKPPIIYG	DVSRPNLMTV	FWSSAAQSFT	ARPMKGMLTG
551	PVTILNWSFV	RNDQPRFETC	DQIALAIKDE	VEDLEK**AGIN**	**VIQIDEAALR**
601	EGLPLRK**SEQ**	**AFYLDWAVHS**	**FR**ITNYGVQD	TTQIHTHMCY	SNFNDIIHSI
651	IDMDADVITI	ENSRSNEK**LL**	**SVFR**EGVKYD	AGIGPGVYDI	HSPRIPSTKE
701	IADRINKMLA	VLETNILWVN	PNCGLKTRKY	SEVKPALKNM	VDAAKLLRT

C.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
L-ascorbate oxidase-like protein	XP_010093150.1	60499	173	9.09	26	23

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MARAEDPYLF	FTWNVTYGTI	SPLGVPQQGI	LINGQFPGPN	INSTTNNNIV
51	LNVFNNIDEP	ILFTWLGIQQ	RKNSWQDGVL	GTNCPILPGT	NFTYRFQVKD
101	QIGSYFYYPT	TAIHR**AAGGF**	**GGLR**VNSRLL	IPVPYADPED	DYTILIGDWY
151	TESHSTLRK**F**	**LDIGRSLGRP**	**DGVLINGK**SA	KSDGSDEPLF	TMKPGKIYKY
201	RICNVGLK**NS**	**LNFR**IQGHPL	KLVEMEGSHT	VQNTYESLDV	HVGQCFAVLV
251	TADKAPK**DYL**	**VVASTR**FTKN	VLTGKGIIRY	TNAKPAPPSP	DVVEAPVGWA
301	WSLNQFRSFR	**WNLTSSAAR**P	NPQGSYHYGK	INITRTIKLV	NSAVRVQGKL
351	RYAINGVSHV	DPYTPLK**LAE**	**YYQVADK**VFK	YDIISDEPPA	NAGDKITVAT
401	NVVNQTFR**NF**	**VEIIFENHEK**	SLQTWHLDGY	SFFAVAIEPG	RWSPDKR**SRY**
451	**NLLDAVSR**HT	IQVFPKSWAA	IMLTFDNAGM	WNLR**SELTER**	R**YLGQQLYIS**
501	**VQSPARSLRD**	**EYNMPDNALL**	**CGVVK**DLPWP	PPYSI	

D.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
L-ascorbate oxidase-like protein	XP_010093149.1		87	9.61	18	21

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MRRVIFVTWL	VLLSAVVQLR	AEDPYLFFTW	NVTYGTISPL	GVPQQGILIN
51	GQFPGPNINS	TTNNNIVLNV	LNNLDEPFLL	TWTGVQHR**KN**	**SWQDGVVGTN**
101	**CPIPPGKNFT**	**YHFQVK**DQIG	SYIYYPTTAV	HRAAGAFGGL	RVNSRLLIPV
151	PYADPEDDYT	VLIGDWYVKS	HKTLKNFLDS	GR**SLGRPDGV**	**LINGK**SGNDK
201	KPLFTMKPGK	**TYKYRICNVG**	**LK**DSLNFRIQ	DHPMKLVEME	GSHTVQNTYE
251	SLDVHVGQCF	SVLVTADK**AP**	**KEYYMVASTR**	**FTK**TVLTGKA	IIRYTNGRKG
301	SASLKNIPEA	PVGWAWSLNQ	FRSFR**WNLTA**	**SAAR**PNPQGS	YHYGKIPITR
351	TIKIVNSASR	VKGKLRYGIN	GVSHVNPVTP	LKLAEYYGVA	DKVFKYDLIK
401	DEPPKKLSGE	VTLAPNVVNQ	TFR**NFVEIIF**	**ENHEK**SLQSW	HLAGYSFFAV
451	SIEPGRWRPE	KR**KNYNLLDA**	**VSR**HTIQVYP	KSYAAVLLTF	DNAGMWNLRS
501	ELTENRYLGQ	QLYISVLSPA	RSLR**DEYNMP**	**DNALLCGIVK**	DLPKPHPYTI

Table S2. Protein identification of band 2 (79 kDa), L-ascorbate oxidase-like protein, protein score 103 (A).

A.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
L-ascorbate oxidase-like protein	XP_010093150.1	60499	103	9.09	22	23

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MARAEDPYLF	FTWNVTYGTI	SPLGVPQQGI	LINGQFPGPN	INSTTNNNIV
51	LNVFNNIDEP	ILFTWLGIQQ	RKNSWQDGVL	GTNCPILPGT	NFTYRFQVKD
101	QIGSYFYYPT	TAIHR**AAGGF**	**GGLR**VNSRLL	IPVPYADPED	DYTILIGDWY
151	TESHSTLRK**F**	**LDIGRSLGRP**	**DGVLINGK**SA	KSDGSDEPLF	TMKPGKIYKY
201	RICNVGLK**NS**	**LNFR**IQGHPL	KLVEMEGSHT	VQNTYESLDV	HVGQCFAVLV
251	TADKAPK**DYL**	**VVASTR**FTKN	VLTGKGIIRY	TNAKPAPPSP	DVVEAPVGWA
301	WSLNQFRSFR	WNLTSSAARP	NPQGSYHYGK	INITRTIKLV	NSAVRVQGKL
351	R**YAINGVSHV**	**DPYTPLKLAE**	**YYQVADK**VFK	YDIISDEPPA	NAGDKITVAT
401	NVVNQTFR**NF**	**VEIIFENHEK**	SLQTWHLDGY	SFFAVAIEPG	RWSPDKR**SRY**
451	**NLLDAVSR**HT	IQVFPKSWAA	IMLTFDNAGM	WNLRSELTER	R**YLGQQLYIS**
501	**VQSPARSLRD**	**EYNMPDNALL**	**CGVVK**DLPWP	PPYSI	

Table S3. Protein identification of band 3 (68 kDa), Subtilisin-like protease SDD1, protein score 115 (A), Phosphoglucomutase, protein score 107 (B).

A.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
Subtilisin-like protease SDD1	XP_010108074.1	85546	115	5.56	19	20

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MEWKALNLVY	LFVSLFIILN	CSDLVGADYQ	KMKLVFEKSA	KIEADHDHHD
51	RISSLKTYIV	HVKKPQISGV	LSVSDQDLKA	WYQTFLPSTT	PTIATTRSSH
101	YPRLVHAYKN	VVSGFAAR**LT**	**ADEVKAMEKK**	**DGFVSAR**EEK	IYSLHTTHTP
151	K**FLGLFQGLG**	**LWNDSR**LGEG	VIVGLLDTGI	WPDHPSFSDE	GLPPPPAKWR
201	GKCDFTGTEC	NNKLIGARDF	VTSTKSTGTK	SPSGQPPFDL	EGHGTHTSST
251	AAGNFVSGAN	AFGMANGTAA	GIAPR**AHLAM**	**YR**QENEEYLQ	YLCPVCAEGC
301	SEADILAALD	AAIEDGVDVL	SLSLGGGSAP	FYFDSIAIGA	FAAIQKGIIV
351	SCSAGNEGPD	YFTLSNEAPW	ILTVGASTVD	RKIKADAILG	NGEVLEGESL
401	NQLAPFDSSK	PYPLIYPGAS	GNESVK**YCAP**	**GSLQSLDVK**G	KIVACDRGGG
451	IARIDKGTEV	KSAGGIAMIL	MNEKIDGFST	LADAHVLPAT	HVSFAASLKI
501	KAYIKSSSSP	LATILFK**GTV**	**IGDSHAPVVT**	**SFSSR**GPSEA	SIGILKPDII
551	GPGVSILAAW	PVSVDNSTTS	GKATFNMISG	TSMSCPHLSG	VAALLK**SSHP**
601	**EWSPATIKSA**	**ILTTADVSNL**	**GGGAILDEKA**	**SPADVFATGA**	**GHVNPSK**ANN
651	PGLIYDIEPE	DYIPYLCGLN	YTDDQVSTIT	QTTVK**CSEVQ**	**SIPESQLNYP**
701	**TFTVLLGNER**	LSYTRTVTNV	GEANSEYTLD	VYPPVGTGIN	VTPNKLTFTE
751	VNQKAEYKIE	IIQVSGPGR**S**	**TNPFEQGYLV**	**WK**SDKYSVRS	QITAIFAV

B.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
Phosphoglucomutase	XP_010101975.1	63757	107	5.65	22	27

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MVVFNVSK**VE**	**TTPFDGQKPG**	**TSGLR**KKVKV	FVQPHYLQNF	VQSTFNALSG
51	EKVRGATLVV	SGDGRYYSKD	AIQIIIMMAA	ANGVRR**IWVG**	**QNGLLSTPAV**
101	**SAVIR**ERTGV	DGSRASGAFI	LTASHNPGGP	HEDFGIK**YNM**	**ENGGPAPEAI**
151	**TDK**IYENTKT	IKEYLIADLP	DVDISTIGLT	SFNGPEGQFD	VEVFDSASDY
201	IKLMK**SIFDF**	**ESIRK**LLTSP	KFTFCFDALH	GVAGAYAKRI	FVEELGAQES
251	SLLNCTPKED	FGGGHPDPNL	TYAKELVARM	GLGK**SDTQEE**	**PPEFGAAADG**
301	**DADRNMILGK**	RFFVTPSDSV	AIIAANAVDA	IPYFSAGLKG	VARSMPTSAA
351	LDVVAKHLNL	KFFEVPTGWK	FFGNLMDAGL	CSICGEESFG	TGSDHIREKD
401	GIWAVLAWLS	ILAHKNKENL	GGEKLVTVEK	**IVRQHWATYG**	**RHYYIRYDYE**
451	**NVDAGAAK**EL	MAYLVKLQSS	LPEVNEIVHG	ACPDVSK**VVH**	**GDEFEYKDSV**
501	**DGSISK**HQGI	R**YLFEDGSR**L	VFRLSGTGSE	GATIR**LYIEQ**	**YEKDPSK**TGR
551	DSQEALAPLV	EVAIKLSK**MQ**	**EFTGR**SAPTV	IT	

Table S4. Protein identification of band 4 (56 kDa), hypothetical protein L484_006703, protein score 71 (A).

A.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
hypothetical protein L484_006703	XP_010112623.1	53747	71	6.01	6	9

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MGHCGGVVLV	TLALFCFFVS	VKGEANFEDD	FLFSLAANHD	YKDALGK**GIL**
51	**FFEGQR**SGKL	PSSQRVTWR**G**	**DSALSDGKPE**	**GANLVGGYYD**	**AGDNVK**FVWP
101	MAFSVCLLSW	AAVEYQQEIS	SANQLKHLRD	AIRWGADFIL	EAHTSPTTLY
151	TQVGDGNSDH	QCWERPEDMD	TSRALFKITS	NSPGSEAAAE	AAAALAAASI
201	VFKGVDSNYS	SRLLRNSEST	NIEDLTKVLV	HSTAHTLAIS	CLILQTNIED
251	LTKVLVHSIA	HTLAISETPS	QEFYGGKKDL	EKYKNDIESF	ICAVMPGSSS
301	VQIR**TTPGGL**	**LYTR**DSSNLQ	YVTTVTMALL	IHSKTISAAQ	SGGVQCGSAK
351	FSASQIRAFA	KSQVDYILGN	NPMKTSYMVG	FGSKYPTQLH	HRGASIPSIR
401	VHPTKVGCNE	GQNLYFSSTK	PNPNIHVGAL	VGGPNSNDQF	NDVRSDYSHL
451	EPTSYINAAF	VGSVAAFLAE	NNENYLQLSR	VKTTAELYTA	NI

Table S5. Protein identification of band 5 (15 kDa), hypothetical protein L484_025194, protein score 73 (A).

A.

Protein name	Accession number	Mass	Score	pI	Matches	Protein sequence coverage (%)
hypothetical protein L484_025194	XP_010106438.1	16003	73	5.66	9	48

Protein sequence coverage (Matched peptides shown in **bold red**):

1	MASK**LSPEFA**	**YTVVYVK**DVA	RCVEFYK**NAF**	**GFSVRRLDES**	**HR**WGELESGQ
51	TTIAFTPLHQ	HETDDLTGSV	QTPEYARDR**A**	**PVEVCFVYSD**	**VDAAYKKAVE**
101	**NGAVPVSEPE**	**QK**EWGQKVGY	LR**DLNGIVVR**	IGSHVHPPKH	D

ELISA results

Specific IgE antibodies were only detected in two sera samples (patients E and K) with standardized commercial *M. alba* pollen extract possibly due to absence or inadequate concentration of allergenic protein(s) in this extract.

Discussion

In this study, a 1D-immunoproteomics approach provided the first study of the allergenic protein profile of the *M. alba* (white mulberry) pollen extract, which was confirmed as an allergen for 11 patients living in Istanbul, Turkey. It was found that *M. alba* pollen contains many allergenic proteins between 15-100 kDa. The most prominent bands are proteins of approximately 55-100 kDa in the majority of patients.

Until now, only two distinct Ig-E binding proteins around 10- and 18 kDa have been reported for *M. alba* in a case report.[13] The 18 kDa protein was proposed to be in accordance with the Bet v1 allergen and its homologs, however, identification of

this protein has not been achieved. Our study revealed that the allergenic proteins of *M. alba* pollen have a molecular weight of 82, 79, 68, 56 and 15 kDa. Thus it can be suggested that *M. alba* pollen contains allergenic proteins with higher molecular weight than known allergenic proteins with low molecular weight as in the other *Morus* species and common tree pollens.[13] In fact, some IgE reactive proteins between 36-98 kDa have also been detected in paper mulberry (*M. papyrifera*) grown in Pakistan, however in this study, the authors focused only on a 10 kDa protein.[10]

After MS/MS analyses of the major protein bands in 1D-gel, methionine synthase (MetE) (Band 1) showed the highest protein score (353) among all identified IgE-binding proteins. This protein with a MW of 85 kDa belongs to the vitamin-B12 independent methionine synthase (MetE) family and catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate (N^5-MeTHF) to homocysteine resulting in methionine formation.[22] Two reports were published on allergenic MetEs among plants, but never in *M. alba*. In the first study Chardin et al. showed that the amino acid sequence of a high molecular weight allergenic protein (approximately 80 kDa) from the oilseed rape (*Brassica napus*) pollen was very similar to that of the cobalamin-independent MetE of *Arabidopsis thaliana* (AtMetE).[23] The authors demonstrated that this 80 kDa protein represented an allergen from the oilseed rape pollen. In 2011, a study from Iran identified the cobalamin-independent MetE as a new allergen of *Salsola kali* pollen.[22] This study showed that *S. kali* MetE shares a high degree of amino acid sequence homology with the MetE from other plants including *Beta vulgaris* (Amaranthaceae) (91%), *Solanum tuberosum* (89%), and *Arabidopsis thaliana* (88%). The new allergen was designated Sal k 3 by the WHO/IUIS Allergen Nomenclature Subcommittee. Our study is the first report on allergenic MetE in *M. alba*. Although we propose MetE as the major allergen of *M. alba* we have no data regarding the cross-reactivity to Amaranthaceae pollens in our patients.

Our results are also partly correlated with the findings of Erler et al. for birch pollen.[24] These researchers evaluated the profile of allergenic and non-allergenic proteins in extracts of birch pollen from different origins by MS-based proteomics, and they detected MetEs with high scores. Thus, *M. alba* pollen might be expected to display cross-reactivity with birch pollen through these proteins. However, we detected skin test positivity for birch pollen in only 4 patients (**Table 1**).

Six other proteins were also detected in MS analyses with lower protein scores. The allergenicity of L-ascorbate oxidase -like protein (Band 1, 2) could be explained by the carbohydrate epitope in the glycan moiety of this protein as in the previous studies with Cupressaceae pollens[21] and olive pollens.[25] Subtilisine-like protease and phosphoglucomutase (Band 3) were already described in other species as potential allergenic proteins.[24,26-29]

Band 4 and band 5 also allowed the identification of potential allergenic proteins: hypothetical protein L484_006703, a protein from the glycosyl hydrolase family 9, and hypothetical protein L484_025194 with a conserved domain found in a variety of structurally related metalloproteins like glyoxalase I or dioxygenases. There are 10 records related to glycosyl hydrolases in the allergome database (http://www.allergome.org/script/search_step2.php). Two belong to olive tree-derived allergens, Ole e 10 and Ole e 10.0101. A novel allergenic glyoxalase has been demonstrated with rice, and the role of indoleamine 2,3-dioxygenase (IDO), an initiator of tryptophan catabolism, on allergic inflammation has been explored.[30-31]

Although this study provides new data regarding the allergenic proteins in *M. alba* pollen, it contains some limitations because ELISA results did not concur with the immunoproteomic results. The discrepancy might be explained by the failure to detect low antibody levels. Further studies on sIgE detection in patients' sera are needed. In addition, the results should be supported by other diagnostic tests such as a basophil activation test.

In conclusion, IgE-binding proteins detected in our study are relatively different than those reported earlier, probably as a result of the region where the pollen samples were collected as it is well known that the pollen content and the allergenicity are affected by the climate and other environmental conditions.[32] Methionine synthase is a potential allergenic protein in *Morus alba* pollen. Further studies such as 2D-gel electrophoresis and other MS techniques, ELISA testing and extending the clinical data are in progress for a better understanding of the allergy mechanism of mulberries.

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Conflict of interest

The authors declare no conflict of interest.

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