Original Research

IDENTIFICATION OF MAJOR ALLERGENS OF PAPER MULBERRY (BROUGHNETIA PAPYRIFERA) POLLENS AND PURIFICATION OF NOVEL 40 KDA ALLERGEN PROTEIN

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Keywords | Pollens, Paper Mulberry, Allergens, Purification, Characterisation

ABSTRACT
The pollen of Paper Mulberry (Broussonetia papyrifera) is a major cause of urticaria and severe respiratory symptoms. The composition of allergens of Paper Mulberry pollen has not yet been determined. In the present study, we identified and characterised the potential allergens in Paper Mulberry pollen involved to trigger the allergic reaction in humans and also purified 40 kDa allergen protein of Paper Mulberry. Pollen was collected from Paper Mulberry trees planted in Islamabad. Soluble proteins of pollens were extracted, lyophilised and resolved on SDS-PAGE. Two proteins with molecular weight of 33 kDa and 40 kDa were identified as major allergens by immunoblot analysis using the serum of positive responders against Paper Mulberry. One of these two allergens with molecular mass of 40 kDa was selected for further analysis and confirmed by specific reaction of sera from positive responders to the Paper Mulberry antigen by immunoblot, ELISA and Western blot. The identification and characterisation of potential allergens in Paper Mulberry pollens and the purification 40 kDa allergen of Paper Mulberry pollens will enable specific immunotherapy as well as the production of recombinant allergen for vaccination.

INTRODUCTION

Allergy is an inappropriate immune response to an allergen. An allergen is a nonparasitic antigen that stimulates a type-1 hypersensitivity reaction in atopic individuals. In type-1 hypersensitive response, Immunoglobulin E (IgG E) against the allergen is produced which interacts with tissue mast cells and blood basophils through the Fc receptor resulting in the sensitisation of these cells. Secondary exposure to the same allergen leads to the cross linking of bound IgE on sensitised cells resulting in the degranulation and secretion of histamines, leukotrienes and prostaglandins. These are the active mediators which act on the surrounding tissues and result in smooth muscle contraction and vasodilation.

Allergens are different in nature and include dust particles, pollens, chemicals, milk or animal proteins, foods, venoms and drugs. Some of the allergens, such as dust particles and pollens, are airborne. Localised symptoms appear in those tissues that are in direct contact with air such as the eyes, nose and lungs. Inhaled allergens trigger the increased production of mucus in the lungs and bronchoconstriction which leads to shortness of breath and coughing. Pollen allergens are water-soluble proteins or glycoproteins, capable of inducing IgE-mediated allergic response and they are released from the cytoplasm of pollens through different mechanisms.

Paper Mulberry (Broussonetia Papyrifera) trees were introduced one hundred years ago to the subcontinent. These trees were first introduced to Islamabad (Pakistan) in the 1960s. An appalling rise in Paper Mulberry pollen allergy was seen. This is associated with coughing, asthma and skin rashes and has been observed in the capital of Pakistan and its adjacent areas. Pollen from male Paper Mulberry plants have been reported to be the main cause of respiratory allergy including asthma, in spring. During spring, the pollen count of Paper Mulberry pollen level exceeds 40,000 grains per cubic meter in the air, while the
pollen count for other trees does not exceed one hundred per cubic meter. The incidence of pollen allergy varies at different times of the year, seasons and in different parts of the world.

The diagnosis techniques of allergic diseases are influenced by the heterogeneous nature of allergenic extracts. The allergen extracts are composed of allergens and other non-allergenic, undefined components. Purified allergens can be used for allergen specific immunotherapy and to assess the allergen specific antibodies response after immunotherapy.

Paper Mulberry allergens have not been identified and characterised so far. In the present study, we identified two allergen molecules with molecular weights of 40 kDa and 33 kDa and determined the IgE-binding capacity of these allergens in positive responders to Paper Mulberry pollens. 40 kDa allergen was purified and confirmed as an allergen by using immunodot blot assay, ELISA and Immunoblotting.

**MATERIALS AND METHODS**

**PREPARATION OF POLLEN EXTRACT**
Fresh pollen samples were obtained from male flowers of Paper Mulberry trees planted in Shaker Parkan Forest, Islamabad, during peak pollination season. The flowers were sun dried for 48 hours and then the pollen was collected by tipping. Debris was removed by passing through a 100 µm mesh several times. The purity of pollens was checked microscopically, and the collected pollen was stored at -20°C. The pollen extract was prepared, by stirring the pollen samples (10% w/v) with phosphate buffered saline (PBS, pH 7.4) containing 0.05 M NaHCO₃, 3 mM EDTA, caproic acid and 0.3 mM PMSF for 16 hours at 4°C. The suspension was centrifuged at 13000 rpm for one hour at 4°C, the supernatant was dialysed, filtered by 1 µm pore size membrane filter and stored at 4°C. The total protein concentration in pollen extract was estimated by the Bradford assay using bovine serum albumin as a standard. The pollen extract was also analysed on 10% SDS-PAGE as described by Laemmli.

**SERUM SAMPLES**
Blood samples of patients suffering from Paper Mulberry pollen allergy were collected from National Institute of Health, Islamabad. Blood specimens were taken in sterile tubes and were clotted at 37°C for 15 minutes then centrifuged at 3500 rpm for 10 minutes. The supernatant from each tube was collected in a sterile tube and stored at 4°C. Control sera were collected from non-allergic, healthy volunteers with no history of systemic disease and Paper Mulberry pollen allergy.

**IDENTIFICATION OF ALLERGENS IN POLLEN EXTRACT**
To identify the presence of allergen proteins in pollen extract, Immunodot blot, ELISA and Immunoblotting were performed using sera of positive responders to Paper Mulberry and monoclonal anti-human IgE.

**IMMUNODOT BLOT ANALYSIS**
Immunodot blot was performed with slight modifications by using serum of positive responders to Paper Mulberry. Briefly, nitrocellulose membrane (0.45 µm pore size, Sigma) was cut in sizes of 2x2 cm. 5 µl of pollen extract sample was separately spotted on each test and control membranes. Acidic Ponseau S stain (1% Ponseau S in 1% acetic acid solution) was used to confirm the absorption of pollen proteins on the membrane. The membranes were dipped in 5% (w/v) skimmed milk in 1X TBS-T buffer for 1 hour at 4°C to block nonspecific binding sites. After blocking, the test membrane was incubated with the serum of positive responders to Paper Mulberry and the control membrane was dipped in sera from healthy subjects (1:100 dilution in 1X TBS-T) for 1 hour at room temperature. The membranes were washed three times with 1X TBS-T buffer (10 minutes/wash) and incubated in monoclonal anti-human IgE alkaline phosphatase conjugated antibodies (1:5000 diluted in 1X TBS-T) at room temperature for 2 hours. After washing, the colour reaction was developed by incubating the blots in BCIP/NBT substrate solution in carbonate buffer (pH 9.8) containing 0.1M NaHCO₃ and 1 mM MgCl₂. The reaction was stopped by washing the membrane in several changes of water. The membrane was air-dried and analysed.

**ELISA TO DETECT POLLEN ALLERGENS**
The ELISA was performed to detect the Paper Mulberry pollen-specific IgE, by using sera of positive responders to Paper Mulberry. 200 µl (2 µg/well) of pollen proteins extract was used to coat the wells of microtitre plates. The microtitre plates were incubated at 4°C for 1 hour and then...
washed with 1X TBS-T buffer. 200 µl of skimmed milk (5% in 1X TBS-T buffer) was added to each well and incubated for 1 hour at 4°C to block nonspecific binding sites. After blocking, 200 µl of diluted serum from positive responders to Paper Mulberry (PMIgE+) (1:100 dilutions in TBS-T buffer) was added and incubated for 2 hours at room temperature. After three washings (10 minutes/wash) with 1X TBS-T buffer, the wells were incubated with alkaline-phosphatase conjugated mouse anti-human IgE (1:5000 diluted in 1X TBS-T) at room temperature for 2 hours. After washing, colour reaction was developed by adding 200 µl of the substrate solution (p-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.00) to each well and the plates were incubated at 37°C for 30 min.

IMMUNOBLOT ANALYSIS
Lyophilised pollen extract was resolved on 10% reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by discontinuous buffer system of Laemmli11. Proteins were visualised by Coomassie Brilliant Blue R-250 staining and electrophoretically transferred to nitrocellulose membrane (0.45 µm pore size, Sigma) by using Trans-Blot (Trans-Blot SD Semi-Dry Electrophoresis Transfer Cell, Bio-Rad) at 18 volts for 80 minutes. The membrane was dipped in 5% (w/v) skimmed milk in 1X TBS-T buffer for 2 hours at 4°C to block nonspecific binding sites. After the washing steps, the blot was incubated with primary antibodies, positive sera from Paper Mulberry sensitive patients (1:100 dilution in 1X TBS-T) for 2 hours at room temperature. The blot was washed three times with 1X TBS-T buffer (10 minutes/wash) and the membrane was incubated in mouse anti-human IgE conjugated with alkaline phosphatase (1:5000 diluted in 1X TBS-T) at room temperature for 2 hours. After washing, the colour reaction was developed by incubating the blot in BCIP/NBT substrate solution in carbonate buffer (pH 9.8) containing 0.1 M NaHCO₃ and 1 mM MgCl₂. The reaction was stopped by washing the membrane in several changes of water. Finally, the strip was air dried and then analysed.

PURIFICATION AND CHARACTERISATION OF 40 KDA ALLERGEN PROTEIN
33 kDa and 40 kDa are the main components of pollen associated with allergic reactions. For further study, one of these allergens with a molecular mass of 40 kDa was selected for purification and characterisation. The gel slice containing the 40 kDa protein band was transferred to a clean dialysis tube containing 1.0 ml of 0.2 M Tris/acetate (pH 7.4), 1.0% SDS, 100 mM dithiothreitol per 0.1 gram of wet polyacrylamide gel. The dialysis tubing was placed cross wise in a horizontal electrophoresis chamber and run at 100 volts (about 100 mA). The running buffer was 50 mM Tris/acetate (pH 7.4), 0.1% SDS, and 0.5 mM sodium thioglycollate. After 3 hours one end of the dialysis tubing was opened. The gel bands were removed and stained with Coomassie Brilliant Blue to ensure that the electrophoresis was complete. The tubing was closed again and dialysed with several changes against 0.2 M sodium bicarbonate, 0.02% SDS. After this, the protein solution was removed,
lyophilised and resolved by 10% SDS-PAGE. The characterisation of this purified 40 kDa allergen protein was done by immunodot blot, ELISA and immunoblotting analysis by using the sera of positive responders to Paper Mulberry and monoclonal anti-human IgE.

RESULTS

IDENTIFICATION OF ALLERGEN PROTEINS IN POLLEN EXTRACT

The presence of allergen molecules in pollen extract was confirmed by immunodot blot analysis, ELISA and immunoblotting. It was observed that the components of pollen (allergens), which are involved in eliciting allergic reactions in human, are present in pollen extract. Allergens were identified in pollen extract by using sera of positive responders to Paper Mulberry pollen and monoclonal anti-human IgE. The appearance of a purple spot on the nitrocellulose membrane in the immunodot blot (Figure 1) and a yellow colour in ELISA (Figure 2) indicated the presence of allergens in pollen extract.

The lyophilised pollen protein sample was subjected to SDS-PAGE analysis (Figure 3) and electro-transferred onto a nitrocellulose membrane for the immunoblot analysis. The pollen extract proteins transferred onto the nitrocellulose membrane were treated with the sera of the positive responders to Paper Mulberry pollen and then treated with the monoclonal anti-human IgE (as secondary antibody). Two bands developed on the membrane with molecular weights of 33 kDa and 40 kDa (Figure 3). The specific reaction of the pollen allergens of 33 kDa and 40 kDa with anti-allergen antibodies present in the serum of allergy patients indicated that these two components of Paper Mulberry pollen are major allergens in human. It was observed that when the proteins that had been transferred onto the nitrocellulose membrane were treated with sera from the positive Paper Mulberry responders were treated with alkaline phosphatase conjugated monoclonal anti-human IgG (as secondary antibody), the reaction was developed on the whole strip, which indicated the exposure of whole pollen proteins to the individual (Figure 3).

CONFIRMATION OF ALLERGENICITY OF IDENTIFIED ALLERGENS

Allergen molecules were characterised by immunoblotting, ELISA and immunodot blot analysis by using 10 sera of patients suffering from Paper Mulberry pollen allergy and 10 normal serum samples as primary antibodies and by using alkaline phosphatase conjugated monoclonal anti-human IgE as secondary antibody. With all 10 sera of patients suffering from Paper Mulberry pollen allergy, the specific reaction of the pollen allergens of 33 kDa and 40 kDa was observed. Conversely, in all 10 control serum samples no reaction against pollen allergens of 33 kDa and 40 kDa was observed. The specific immunological reaction of the pollen allergens with the sera of the 10 patients sensitised to Paper Mulberry was also confirmed by ELISA (Figure 4) and immunodot blot (Figure 6).

PURIFICATION AND CHARACTERISATION OF 40 KDA ALLERGEN PROTEIN

Preparative SDS-PAGE gels were run and gel strips having single bands were cut and electro eluted lyophilised and then dialysed. After dialysis concentration of purified proteins was determined and then samples were run on 10% SDS-PAGE, the appearance of single bands on the gel indicated the purified pollen proteins. The appearance of a purple blue colour on the membrane, after the immunodot blot of purified 40 kDa pollen allergen with patients’ serum by using alkaline phosphate conjugated mouse anti-human IgE antibodies as secondary antibodies, confirmed the purification of 40 kDa pollen allergen and no spot appeared in the control membrane. The appearance of a yellow colour in well 1 after the ELISA with purified 40 kDa pollen allergen with patients’ serum by using alkaline phosphate conjugated mouse anti-human IgG (as secondary antibody), the reaction was developed on the whole strip, which indicated the exposure of whole pollen proteins to the individual (Figure 3).

Figure 4: ELISA: It was observed that yellow colour was shown in wells 1-10 which indicated the specific reaction of the pollen allergens with all ten patients sensitised to Paper Mulberry pollen but no colour was developed in the control well. Immunodot blot: A purple blue colour spot was shown on test strips from 1-10 which indicated the specific reaction of the pollen allergens with the ten patients sensitised to Paper Mulberry but no colour was developed on the control strip (c)
IgE monoclonal antibodies as secondary antibodies, confirmed the purification of 40 kDa pollen allergen and no colour appeared in the control well. The purified pollen protein of molecular weight 40 kDa was transferred onto a nitrocellulose membrane after 10% SDS-PAGE, and the membrane was treated with the sera of patients sensitised to Paper Mulberry pollen. It was further treated with alkaline phosphatase conjugated mouse anti-human IgE secondary antibodies. A single band was developed on the membrane having molecular weight 40 kDa indicating the purified pollen allergen.

DISCUSSION
An allergy is an exaggerated immune response of our body to foreign substances also known as allergens. Allergens are variable and include pollen, drugs, chemicals, dust mites, food and animal danders. The pollen grains are produced during the life cycle of the flowering plant. Its biological function is to fertilize the female gametophyte. In addition to their biological function, they also cause human respiratory allergic disorders. Allergenic components of the pollen are released from the cytoplasm by different mechanisms, which trigger symptoms in sensitised individuals. When an individual is exposed to the airborne pollen grain, the allergens are released onto the surface of the upper airway mucosa. Meteorological factors affect the release of pollen grains into the atmosphere. Amongst different allergy causing pollens, Paper Mulberry pollen is found to be allergenic in different areas of Pakistan.

Previous studies have shown that pollen allergens are water-soluble proteins or glycoproteins. In the present study, we identified and characterised two components of Paper Mulberry pollens with the molecular weights of 33 kDa and 40 kDa involved in triggering an allergic reaction by using the serum of patients sensitised to Paper Mulberry. In addition, the allergenic protein with the molecular weight of 40 kDa was also purified. These 33 kDa and 40 kDa components were identified as proteins by staining with Coomassie Brilliant Blue stain. These two pollen allergens were identified by monoclonal antibody specificity of mouse anti-human IgE.

Pollen samples were collected from Paper Mulberry trees planted in Shakar Parian, Islamabad. The pollen extract was prepared and the presence of allergenic molecules in the pollen extract was confirmed by immunodot blot analysis and ELISA. Both ELISA and immunodot blot analysis were performed by using the sera of patients sensitised to Paper Mulberry as well as the sera of non-responders as controls. When the serum of the Paper Mulberry pollen allergy patients was used as the primary antibody and alkaline phosphatase conjugated mouse anti-human IgE antibody as the secondary antibody, both ELISA and immunodot blot analysis were found to be positive. When the serum of non-responders was used as the primary antibody and alkaline phosphatase conjugated mouse anti-human IgE antibody as the secondary antibody, both the ELISA and immunodot blot analysis were found to be negative.
The lyophilised pollen extract sample was then analysed by SDS-PAGE. Multiple proteins were identified by SDS-PAGE with Coomassie Brilliant Blue staining. Proteins were then electro-transferred onto nitrocellulose membrane and developed by using serum of positive responders to Paper Mulberry and monoclonal mouse anti-human IgE as the secondary antibody. The specific reaction of the pollen allergens of 33 kDa and 40 kDa with the serum of patients suffering from Paper Mulberry pollen allergy and monoclonal mouse anti-human IgE indicated that these two allergens of 33 kDa and 40 kDa are involved in triggering the allergic reaction in human.

Pollen allergens of 33 kDa and 40 kDa were also confirmed by demonstrating the antigen-antibody reactions of the serum samples of the test and control sera. In the sera from sensitised patients, the specific reaction of the pollen allergens of 33 kDa and 40 kDa were observed. But in the 10 control serum samples no reaction of the pollen allergens of 33 kDa and 40 kDa were observed.

The allergen with the molecular mass of 40 kDa was selected for further purification and characterisation. The 40 kDa protein band was excised from preparative SDS-PAGE, purified by electro elution and confirmed by specific reaction of sera from paper mulberry sensitive patients by immunoblot, ELISA and immunoblot analysis by using mouse anti-human IgE monoclonal antibodies. The identification, purification and characterisation of 40 kDa allergen of Paper Mulberry pollens will be of value in selecting patients for specific immunotherapy as well as forming a basis for research into recombinant allergen for vaccination.

There is a need for further experimental confirmation of the class of human antibodies produced through in vitro immunisation. In addition, the purified pollen allergen can be sequenced for the production of recombinant Paper Mulberry pollen allergens for the development of allergen specific immunotherapy and diagnostic assays.

DECLARATION OF INTEREST
This research work was supported by the Institute of Biochemistry and Biotechnology, where the complete research work was carried out. This research work was not supported or funded by any company. Author and co-authors also agreed to publish this paper in Current Allergy & Clinical Immunology and we do not have any conflict of interest.

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