Identification of the Allergenic Proteins of *Litchi chinensis* (Litchi) pollen: Partial Characterization and Sensitivity Test

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Abstract

The pollen of *Litchi chinensis* (Litchi) is an important aeroallergen of Bangladesh. The pollen of this fruits plant was collected from full bloomed flower growing in different places of Rajshahi, Bangladesh. Pollen protein extracted and partial purified by means of long-term PBS extraction, salting out, dialysis, gel filtrations and DEAE-Cellulose chromatography which was designated by LFPP (Litchi flowers pollen protein). Gel filtration of the partial purified pollen protein gives two main peaks. The major peak gives four bands on SDS-PAGE. After gel filtration and Ion exchange chromatography, a single band in the protein profile of LFPP, (M.W. 28 kDa) was the major allergenic component of *Litchi chinensis* (Litchi) flower pollen. The homogeneity and the molecular weight of the protein were estimated by SDS-PAGE, and Gel filtration was 28kDa. The allergenic protein was identified by skin test and shows the pectate lyase (Pel) activity. Skin-prick tests also revealed highest degree of sensitivity to the Nawabgang sample giving positive response in 90% of the patients. Skin reactivity ranged between 1^+ and 3^+.

Key words: Allergen characterization; *Litchi chinensis* (Litchi); Sensitization.

Introduction

It is well known that there has been a gradual increase in the number of people suffering from allergy and other respiratory diseases. About 15-20% of the world's populations are suffering from allergic disorders i.e. allergic rhinitis, bronchial asthma, atopic dermatitis and urticaria (Singh *et al.* 1994). According to a survey by Kjellman (1993), one child out of every three, has a history of allergy or atopic disease before reaching the age of seven to ten years. Among the various aeroallergens, the role of pollen in causing respiratory disorders in sensitive patients has been greatly realized. Pollen allergy is caused by proteins, glycoproteins or even a single peptide which are present in the pollen wall and cytoplasm (Chanda 1994). The soluble proteins have been generally proved to be responsible for causing nasobronchial allergy. Thus the detection of the site of origin, isolation,and characterization of allergy causing proteins or glycoproteins is now a very challenging task for aerobiologists working in the field (Cresti 1992). Pollen samples collected from different source materials, stages of inflorescence, time intervals, years, different geographical places and also periods of storage, show significant variation in their allergenic components (Singh 1993).

The role of pollen as a causative agent of respiratory allergic disorders is very well established, as is evident from the recent increase of reports from across the world (Ortega *et al*., 1992; Singh *et al.* 1994; Amato *et al.* 1998; Arnon *et al*., 1998). Consequently, it provides considerable variation in the quality and quantity of airborne pollen in different ecogeographic regions of the country (Singh *et al*., 1980; Singh 1982; Gupta *et al*., 1984; Nair 1986). Bangladesh is blessed with the richest delicious seasonal fruits flora on the earth. Particularly, northern parts of Bangladesh, blessed with famous fruits trees like mango, *Litchi chinensis* (Litchi), banana, black berry etc. Previously we reported, mango pollen is one of the important components of airborn allergenic pollen (Talukder *et al*., 2012).

The lychee (*Litchi chinensis*, and commonly called leechi, litchi, laichi, lichu, lizhi) is the sole members of the genus *Litchi* in the soapberry family, sapindaceae. It is a tropical and subtropical fruit tree native to

China, and now cultivated in many parts of the world. The fresh fruit has a "delicate, whitish pulp" with a "perfume" flavor that is lost in canning, so the fruit is mostly eaten fresh (Davidson 2006).

According to Thommen (1931), an essential requirement for pollen to cause an allergic reaction in human beings, is that it should come from a wind pollinated plant. It is also known that the abundance of particular taxa in a particular area could be responsible for the predominance of the pollen of those taxa in the atmosphere of that region. Although studies on the allergenic properties of airborne pollen from various species have been carried out by several workers (Shivpuri *et al.,* 1979; Singh *et al.* 1987; Singh *et al.,* 1993; Mondal *et al.,* 1997) information on allergy to aerial pollen from *Litchi chinensis* tree species has been completely lacking. In view of the extensive distribution of *Litchi chinensis* trees in the Rajshahi area and the prevalence of its pollen in the atmosphere, we decided to study the allergenic properties of its pollen with particular reference to respiratory allergic disorders in the population of different geographic regions (villages) around Rajshahi and partially to characterize its pollen antigen.

Materials and Methods

Source Material

The pollen was collected from the ranges Nawabgang, Thanor, Rajshahi Town and Rajshahi University campus of the Rajshahi region, Bangladesh during the pollination period in March to April. These pollen grains were then processed for >95% purity by sieving through different grades of sieves (100, 200, and 300 mesh/cm^2). All the samples were analyzed under the microscope which revealed pollen purity varying from 85% to 90%. To remove lipids and irritants of low molecular mass, the pollen sample was defatted with diethyl ether by repeated changes, until the ether become colorless. The defatted pollen powder was then completely dried and stored at 4^{0}C in airtight containers until further use.

Protein Extraction

The defatted pollen was then used for protein extraction. Proteins were extracted in 0.2M Tris- HCl buffer, pH 7.4 by continuous stirring at 4^{0}C for 24 hours. The extract was clarified by centrifugation at 15,000 x g for 20 min at 4^{0}C. The supernatant was collected and was subjected to fractional precipitation by solid ammonium sulphate. It was made upto 80% saturation by slow addition of the salt at 4^{0}C. After centrifugation the precipitate was resuspended in 0.1M Tris HCl buffer, pH 7.4 and desalted by dialyzing against distilled water for 48 hrs at 4^{0}C by frequent changes of the distilled water using dialysis sacs (MW cut off 9 kDa). Finally the supernatant was passed through a Millipore filter membrane (0.45µm), lyophilized in small aliquots, and stored at -20^{0}C until further use.

Purification of Litchi Flowers Pollen Protein (LFPP)

Sephadex-G75 Gel Filtration Chromatography: The crude protein solution (100mg/5ml) was applied to the column (1.5×29.0 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.2-7.4, at 4˚C. The column was eluted at a flow rate of 25ml/h and 2.5 ml fractions were collected. The absorbance of the column effluent was monitored at 280 nm using an Auto UV-Visible sprectophotometer, UV-280, Japan.

Ion Exchange Chromatography on DEAE cellulose: A column (2.0X26.0cm) of DEAE-Cellulose was equilibrated with 10 mM Tris-HCl buffer, pH 8.4, and operated at a flow rate of 25ml/h. Gel filtration fractions containing Pectate lyase (Pel) activity were applied to the ion exchange column followed by about 100ml 10mM Tris-HCl buffer. Enzyme protein was eluted with 0.2 M NaCl in 10mM Tris-HCl buffer pH 8.3. Following ion-exchange chromatography, the combined enzyme active fractions were stored in 50% (v/v) glycerol at -20^{0}C. Glycerol was removed by dialysis against buffer before use of this enzyme solution in further studies.

Pectate Lyase (Pel) Activity Assays:

The activity of Pel-LFPP (pectate lyase Litchi flower-pollen protein) was determined by monitoring the absorbance increase at 232 nm of a 1-ml reaction mixture containing sodium polypectate at 22˚C. To start the reaction 5 µl of appropriately diluted protein were mixed with 870 µl of 50 mM Bis-Tris-propane (BTP), pH 9.5, containing the optimal $CaCl_2$ concentration and 125 µl of 1% sodium polypectate. [For preparation of stock solutions of 1% sodium polypectate, polygalacturonic acid (85-90% purity, Sigma) was dissolve in deionized water, boiled for 5 minutes, and centrifuged at 10,000×g for 10 minutes to remove sedimented materials. 50mM BTP, pH 9.5, containing the optimal $CaCl_2$ concentration was prepared just prior to assays.] One unit of pectate lyase activity was defined as described (Wellhausen *et al,*. 1996). 1 µmol of unsaturated product formed/min, which equaled 1.73 absorbance unit's min^{-1} and the specific activity, was expressed as unit's mg^{-1} protein. Protein concentration was determined by the method of Bradford (Bradford 1976).

Estimation of Protein

The protein concentration in the extract, as well as in the various eluted fractions, was estimated by the modified method of Lowry (Lowry et al. 1951). A calibrated solution of bovine serum albumin was used as a standard.

Gel Electrophoresis

The protein sample was heated with an equal amount of sample buffer [0.06M Tris HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% Bromophenol blue] at 100^0C for 3 min. 10µl of the sample containing 85µg of protein was loaded in the well of a 12% T mini-gel (8 x7 cm gel) Mini-Protean II slab gel apparatus (Bio-Rad, Hercules, CA, USA) and the gel was run using Laemmli buffer system (1971) [0.05M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature for 2 hours 30 min, at 70 V. The molecular mass of the fractions was calculated by calibrating with standard marker protein, lysozyme (14,000 kDa), trypsin inhibitor (20,000 kDa), carbonic anhydrase (29,000 kDa), ovalbumin (45,000 kDa), albumin (BSA, 67,000 kDa) (Pharmacia, Uppsala, Sweden). After electrophoresis, the gel stained with 0.1% Coomassie Brilliant Blue R-250 and destained with methanol: acetic acid:water (4:1:5) mixture.

Skin Prick Test

Each patient was tested by placing 10µl of each allergen; at least 5 cm apart on the volar surface of his/ her forearm and each site was then pricked with a disposable hypodermic needle. Negative and positive controls were also performed. The negative control was the buffer saline in which the allergen was resuspended and the positive control was histamine acid phosphate injection diluted with buffered saline to 1:10,000 ie 1µg of histamine acid phosphate. The patients were prohibited from using antihistamine, steroid and ephedrine for 48 hrs before the skin prick tests. The skin reactions were read after 15 to 20 min from the commencement of the test. The test was quantified on the basis of the wheel diameter and graded 1^+ to 3^+. The skin tests were conducted at Rajshahi University Medical center and Rajshahi Medical Collage Hospital, Bangladesh. The patients were selected on the basis of their suffering from respiratory allergic disorders.

Skin Tests

A diluted extract of each kind of pollen is applied to a scratch or puncture made on the patient's arm or back or injected under the patient's skin. With a positive reaction, a small, raised, reddened area with a surrounding flush (called a wheal and flare) will appear at the test site. The size of the wheal can provide the physician with an important reaction diagnostic clue. Skin testing remains the most sensitive and least costly diagnostic tool.

Results and Discussion

An attempt was made to extract and purify *Litchi chinensis* flower pollen protein (LFPP). The protein content and the profile of the pollen of Litchi flower collected from different region showed considerable variation. The concentration of protein varied between 7.6 to 9.2 mg ml^{-1} with the highest concentration observed in the sample collected from Nawabgang town (Table-1).

Protein Purification

The protein, extracted from the litchi (*Litchi chinensis*) flowers pollen (procedure as described in materials methods), was partial purified by ammonium sulphate precipitation and then followed by gel filtration and Ion exchange chromatography respectively. Fig-1 and 2 shows the purification pattern of pollen protein by gel and Ion exchange chromatography. After gel filtration protein from peak F-2 (fraction no. 21-35) combines together and reduces its volume to 5ml by freeze drying. Then the concentrated protein solution loaded on a previously equilibrated (10 mM Tris-HCl buffer, pH 8.4,) Ion exchange column (DEAE-cellulose), then eluted with the sane buffer with salt gradient (0.1 - 0.2M NaCl). Two protein picks observed at fraction No. 21-35 and fraction. No.54-56. Pectate lyases active peak F-2a (fraction no. 25-30) collected together and stored at -80^0C (Taitec-VD-800F Freeze Dryer, Japan) for further research works.

The SDS-PAGE protein profile of the extracted pollen protein after gel filtration & Ion exchange chromatography shows in Fig-3. The bands are present between 26 kDa to 67 kDa after gel filtration. Among them only one band (28 kDa) is enzymatically (pectate lyases) active, which was found after DEAE-cellulose column chromatography and also allergenic in nature (Table-3). The molecular weight of which was estimated by Sephadex G-75 filtration (28kDa) by plotting the logarithms of the known molecular weights of proteins versus the relative mobility of the extracted protein. The relative mobility of the antigens of unknown molecular weight was then fitted to this curve to determine the molecular weight (Fig. 4). The molecular weight of the purified Litchi flowers pollen protein (LFPP) as determined by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) to be 28,000 also (Fig. 3). Whereas, Salvatore Feo et al purified allergen protein from *Parietaria judaica* pollen having molecular weight 10,400 by HPLC on SDS-PAGE (Salvatore *et al*., 1984). Extracellularly secreted pectate lyases share resigns of sequence similarity with fungal pectin lyases (Gysler *et al.,* 1990; Kusters-Van Someren *et al*., 1991; Van Someren *et al*., 1992] and certain plant pollen and style specific proteins (Budelier *et al*., 1980; Rafinar *et al.,* 1991; Rogers *et al.,* 1992; Wing *et al.,* 1989). Our protein shows pectinolytic activity. This result is supported by pollen specific Pel-homologue from Japanese cedar was shown to exhibit in vitro pectinolytic activity (Taniguchi *et al.,* 1995).

Variation in skin sensitivity was also observed in the different samples (Table-2). Skin prick tests (plate-1) with the extracts of pollen sampled in different locations were performed in 30 patients already suffering from respiratory allergic disorders, with the ages ranging between 12-65 years. The highest degree of skin reactivity was observed in the case of the Nawabgang town sample with 28(93%) showing positive reactions, of whom 4 (13.3%) showed 1^+ reaction, 6 (20%) showed 2^+ reaction , while 3^+ reaction was obtained in 18 patients (60%). The lowest degree of skin reactivity was observed in case of the Rajshahi University campus sample with 21 patients (70%) reacting positively and only 3(10%) showing 3^+ reaction. The pollen of Litchi flower proves to be one of the important aeroallergens of Rajshahi Regions capable of inducing respiratory allergic diseases in Bangladesh. We reported earlier (G. Talukder *et al*., 2012) a new allergen from mango flower pollen, the M.W 27 kDa. The differences in the protein profiles, as well as the protein content in the Litchi flower pollen samples collected from the various regions of Rajshahi, could be due to variable climatic conditions prevailing in these areas and soil conditions, as has been earlier suggested by Singh *et al*. (1993). The highest rate of skin sensitivity in the sample collected from Nawabgang town may be due to the higher protein content as well as the additional protein bands, resulting in sensitivity to some patients due to these proteins fractions. Another factor for increasing the allergenicity of pollen in air pollution. This is effected by changing the kind and proportions of exinic mineral elements (Amato *et al.* 1998 ; Newmark *et al.,* 1967) or by affecting pollen morphology or protein profile (Arnon *et al*., 1998 ; Shivpuri *et al.,* 1979). Thus , the atmosphere of Rajshahi (Nawabjang town) , being the most

polluted due to brick , rice mills, industrialization and dust particle in the air from the dry Padma river by wind, may be responsible for the increased rate of allergenicity of the pollen grains.

Conclusion

In summary, the protein extracted from Litchi (*Litchi chinensis*) flowers pollen cause the seasonal allergy at the time of flowering. It might be designated as allergen protein. One of the future aims of this study is to unravel the amino acid sequences, 3-dimensional structure of these antigenic proteins and to elucidate the reaction between antigenic proteins and antibody which might help to understand the molecular basis of hypersensitivity. This approach of isolating particular proteins from a mixture of total proteins for the detection of allergenic proteins is reported here for the first time.

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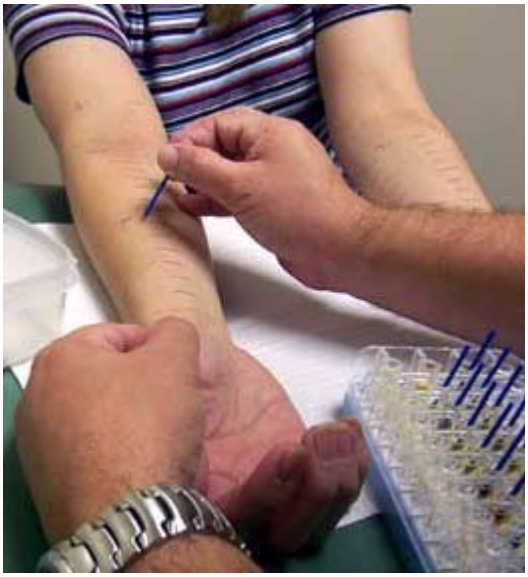


Plate1: Skin prick test

Table 1:
Name: Protein content of pollen extracts of Litchi flower from different places

Place of Collection	Protein (mg ml^{-1})
Nawabgang Sadder	9.2
Thanor Sadder	8.4
Rajshahi University	7.6
Rajshahi Town	8.6

Table 2:
Name: Results of skin tests with the antigen of Litchi flower pollen from different places.

Place of Collection	Total no. of Test	Negative	1^+	2^+	3^+
Nawabgang sadder	30	2 (6.7%)	4 (13.3%)	6 (20%)	18 (60%)
Thanor Sadder	30	12 (40%)	9 (30%)	6 (20%)	3 (10%)
Rajshahi University	30	11 (36.7%)	9 (30%)	7 (23.3%)	3 (10%)
Rajshahi Town	30	9 (30%)	11(36.7%)	5 (16.7%)	5 (16.6%)

Table 3:
Name: Result of skin tests against total extract and individual antigenic fraction of litchi Pollen sensitive patients.

No. of Patients	Ages (yrs)	Sex	Total Protein	IF_1	IF_2	IF_3
5	09-12	F	2^+	-ve	3^+	1^+
8	21-27	F	1^+	1^+	3^+	-ve
5	50-60	F	3^+	1^+	3^+	-ve
6	31-45	M	2^+	-ve	3^+	-ve
12	55	M	3^+	1+	2^+	1^+
14	20-26	M	2^+	-ve	3^+	-ve

Diameter of erythema (mm): 0: no reaction; 1-5: 1^+; 6-10: 2^+; 11-15: 3^+.

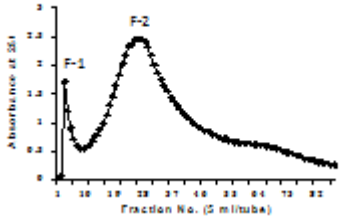


Fig.1: Gel Filtration of 40% ammonium sulphate saturated crude extract on Sephadex G-75. The crude protein solution (100 mg) was supplied to the column (1.0x 29.0 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.2-7.4, at 40C and developed with the same buffer. Flow rate: 25 ml/hour.

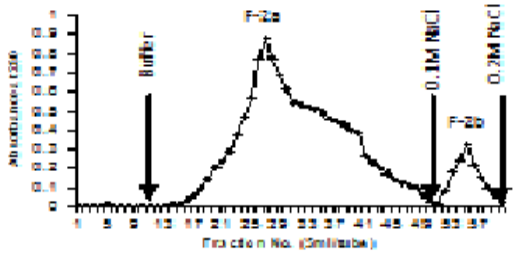
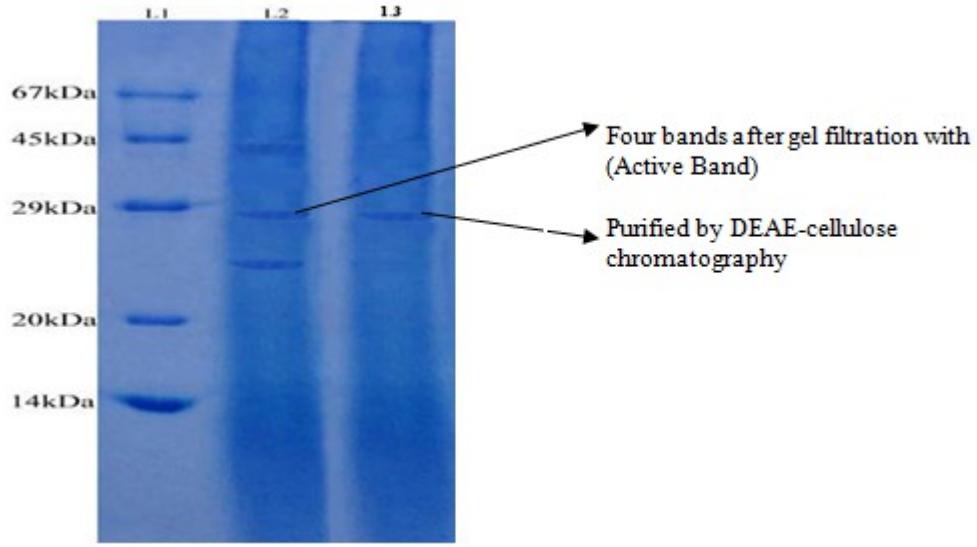


Fig-2. Ion exchange chromatography of F-2 fraction (60mg) on DEAE cellulose, obtain by gel filtration, was applied to the column (1.0x26.0 cm) pre-washed with 10 mM Tris-HCL buffer, pH 8.4, at 40C and eluted by stepwise increase of NaCL concentration in the same buffer. Flow rate: 25 ml/hour.



(1.1: marker proteins, 1.2: proteins after gel filtration and 1.3: purified protein after DEAE)

Fig3.Photographic representation of sodium dodecyl sulfate polyacrylamide slab gel electrophoretic pattern of the purified protein. L-1: Marker protein solution containing lysozyme (14,000), trypsin inhibitor (20,000), carbonic anhydrase (29,000), ovalbumin (45,000), albumin (BSA, 67,000). L-2: Fraction from gel filtration column. L-3: Fraction from DEAE-Cellulose column.

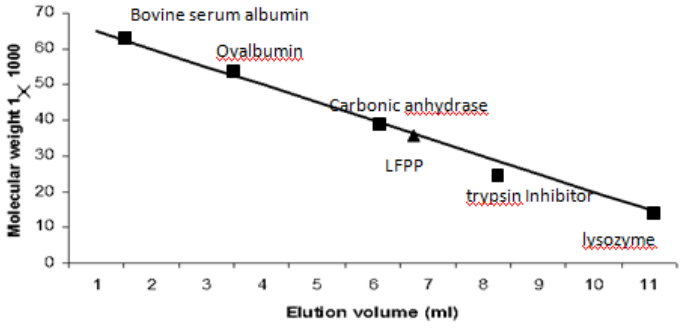


Fig.4: Molecular weight determination by gel filtration.

The molecular weight of the protein was also determined by gel filtration on Sephadex G-75 using lysozyme (14,000), trypsin inhibitor (20,000), carbonic anhydrase (29,000), ovalbumin (45,000), albumin (BSA, 67,000) as standard proteins. The molecular weight was calculated from the standard curve of reference proteins, which was constructed by plotting log of molecular weight against elution volume on gel filtration (Fig.-4) and estimated to be 27,000 for purified LFPP.