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CHARACTERIZATION AND SENSITIVITY TEST OF THE ALLERGENIC POLLEN PROTEINS FROM *LITCHI CHIMENSIS* PLANT

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Abstract: Pollen of *Litchi chinensis* (Litchi) is a major aeroallergen of Bangladesh. Pollen of this fruits plant was collected from full bloomed flower growing in different places of Rajshahi in Bangladesh. Pollen protein was extracted and partial purified by means of long-term PBS extraction, salting out, dialysis, gel filtrations and DEAE-Cellulose chromatography and the protein was designated as LFPP (Litchi flowers pollen protein). Gel filtration of the purified pollen protein gives two main peaks. The major peak gives four bands on SDS-PAGE. The enzyme (pectate lyase) proteins after gel filtration again re-purified by Ion exchange chromatography, a single band in the protein profile of LFPP, (M.W. 28kDa) 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 prick tests and showed the pectate lyase (Pel) activity. Skin-prick tests also revealed highest degree of sensitivity to the Nawabgang sample giving positive response in 80% of the patients. Skin reactivity ranged between 1^+ and 3^+.

KEYWORDS*: Pollen, Allergen characterization; Litchi chinensis , Skin-prick tests.*

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 (Kjellman, 1993). 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 glycoprotein is now a very challenging task for aero biologists 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, particularly northern parts of Bangladesh, blessed with famous fruits trees like mango, Litchi, banana, black berry etc. We reported earlier, 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 *L. chinensis* tree species has been completely lacking. In view of the extensive distribution of *L. 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 skin prick testing (clinical test) with allergic disorders in the population of different geographic regions (villages) around Rajshahi and partially to characterize its pollen antigen properties.

Materials and Methods

Source of pollen Material

50g of pollen were collected (hung fine net under the litchi trees) separately from the forest ranges of Nawabgang, Thanor, Rajshahi Town and Rajshahi University campus litchi garden in 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 (shape & size) 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 vacuum-dried and stored at 4^0C in airtight containers until further use.

Protein Extraction

The defatted dried pollen powder was extracted at 1:20(w/v) in 0.2M Tris- HCl buffer, pH 7.4 according to the method of Singh et al (1993) with slight modification (Mondal et al. 1997) by continuous stirring at 4^0C for 24 hours using magnetic stirrer. The extract was clarified by centrifugation at 15,000 x g for 20 min at 4^0C. The supernatant was collected and was subjected to fractional precipitation by solid ammonium sulphate. It was made up to 80% saturation by slow addition of the salt and was stored for 4 hours at 4^0C in a freezer. After centrifugation the precipitate was re-suspended in 0.2M Tris-HCl buffer, pH 7.4 and desalted by dialyzing against

distilled water for 48 hrs at 4 -10^{0}C by frequent changes of the 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)

Gel Filtration Chromatography: The crude protein solution (100mg/5ml) was applied to the column (1.5×29.0 cm) of Sephadex-G 75 (Uppsala, Sweden) and was pre-equilibrated with 50 mM phosphate buffer, pH 7.2-7.4, at 4˚C. The column was eluted with the same buffer at a flow rate of 25ml/h and each 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. Figure-1 shows the purification pattern of pollen protein by gel chromatography.

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. After gel filtration protein from peak F-2 (fraction no. 21-35) combines together and reduces its volume to 5ml by freeze drying. Gel filtration protein fractions (5ml volume) containing Pectate lyase activity (around 60mg) were applied to the ion exchange column and washed by about 100 ml of 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 (Fig-2, fraction no. 25-30) were stored in 50% (v/v) glycerol at -20^{0}C. Glycerol was removed by dialysis against Tris-HCl buffer before this enzyme solution is used for further enzyme assay, skin prick test and other allergen research studies.

Pectate Lyase (Pel) Activity Assays:

The activity of pectate lyase (Pel)-LFPP 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 dissolved 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 concentrations in the crude extract, as well as in the various eluted enzymatically active fractions, was estimated by the method of Lowry et al. (1951) with a slight modification, as the protein was precipitated with phosphotungestic acid and then dissolved in 2% sodium hydroxide. The experiment was repeated five times within 10% variability, and the mean value was taken for protein concentration. A calibrated solution of lipid-free bovine serum albumin (BSA) was used as a standard.

Protein 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 the method of Laemmli (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 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), albumin (BSA, 67 kDa) [Pharmacia, Uppsala, Sweden]. After electrophoresis, the gel was stained with 0.1% coomassie brilliant blue R-250 and destained with methanol: acetic acid:water (4:1:5) mixture for 12 hours.

Recovery protein from gel

Protein was eluted from the gel following the method of Wilson and Goulding (1986). After electrophoresis, one side of the gel (covering 2 lines) was cut vertically and stained to ascertain the banding positions and only those portions corresponding to the protein bands to be recovered were cut out from the other half of the unstained gel. The gel pieces were transferred to pre-treated dialysis bags filled with electroelution buffer (containing 0.05M Tris and 0.192M Glycine, pH 8.4). These dialysis bags were immersed in the electrophoresis tank of a horizontal gel apparatus containing the same buffer and electroelution was carried out for 3h by passing electric current (120V) through the bags. At the end, the current was reversed for 30seconds in order to release the protein from the wall of the sacs. The buffer containing the eluted proteins were then transferred through a cotton plugged Eppendrof tube and centrifuged for 2 min to remove contaminating gel particles. The protein in each fraction was quantified and each fraction was again electrophoresed to check its homogeneity.

Skin Prick Test

The skin tests were performed on patients suffering from nasobronchial allergy as well as healthy volunteers (120 no. healthy volunteers for 4 different location allergen protein skin tests and 50 no. nasobronchial patients, age between 9 – 60 years; male & female for purified litchi allergen protein) at Rajshahi University Medical center and Rajshahi Medical Collage Hospital, Bangladesh. 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 solution used was the buffer saline in which the allergen was resuspended and the positive control solution 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^+.

Results and discussion

The protein, extracted from the litchi (*Litchi chinensis*) flowers pollen was partially purified by 80% ammonium sulphate salt precipitation and then followed by gel filtration and Ion exchange chromatography and was designated by (LFPP). The protein content and the profile of the pollen allergenic protein of Litchi flower collected from different region (Table-1) showed considerable variation. The concentration of protein varied between 7.6 to 9.2 mg $50g^{-1}$ with the highest concentration observed in the sample collected from Nawabgang town (Table 1). After ion exchange chromatography (Fig-2), two protein peaks were observed at fraction No. 21-35 and fraction. No.54-56. the protein, extracted from the litchi flowers showed pectinolytic activity (Pel) as evidenced in pollen from Japanese cedar *in vitro* test (Taniguchi *et al.*, 1995). Pectate lyases active peak F-2a (fractions no. 25-30) was collected and stored at -80^0C (Taitec-VD-800F Freeze Dryer, Japan) for skin tests and other allergenic research works.
The molecular weight of litchi flower pollen protein (LFPP) was estimated by Sephadex G-75 filtration and by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) 28 kDa (Fig. 3). Salvatore et al., (1984) purified allergen protein from *Parietaria judaica* pollen showed molecular weight of 10.4kDa by HPLC on SDS-PAGE. The SDS-PAGE protein profiles revealed 3 bands (Fig-3) which were designated as IF_1 to IF_3. These bands were present in the molecular weight range of 26kDa (IF_1); 28kDa (IF_2) and 67kDa (IF_3) after gel filtration. Among them, only one band (28 kDa, IF_2) is enzymatically (pectate lyases) active, which was found after DEAE-cellulose column chromatography and also allergenic skin test positive in nature (Table-3).

Skin prick tests were performed with the total extract (protein purified by gel filtration) on 30 healthy volunteers and 50 patients already suffering from nasobronchial allergy (Table-1I).Variation in skin sensitivity was also observed in the different samples (Table-2), as well as the various eluted fractions (IF_1 to IF_3) of pollen sampled in different locations performed in 50 patients already suffering from respiratory allergic disorders with their ages ranging between 9-65 years (Table-3). The highest degree (3^+) of skin reactivity was observed in the case of the Nawabgang town sample with 28 patients (93%) showing positive reactions, of whom 4 patients (13.3%) showed 1^+ reaction, 6 patients (20%) showed 2^+ reactions, while 3^+ reactions was obtained in 18 patients (60%). The lowest degree (1^+) of skin reactivity was observed in case of the Rajshahi University campus sample with 21 patients (70%) reacting positively and only 3 person (10%) showing 3^+ reaction. The pollen of Litchi flower proves to be one of the aeroallergens of Rajshahi Regions capable of inducing respiratory allergic diseases in Bangladesh. A new allergen protein M.W 27 kDa, from mango pollen, collected from Rajshahi zone has been reported earlier (Talukder *et al.*, 2012). The differences in the protein profiles, as well as the protein content in the Litchi flower pollen samples collected from the various locations of Rajshahi, likely to be associated with 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 allergenic components, resulting in sensitivity to some patients due to these proteins fractions. Another factor for increasing the allergenicity of pollen is air pollution. This is effected by changing the kind and proportions of exinic mineral elements, which was reported earlier by (Newmark *et al.*, 1967; Amato *et al.* 1998) or by affecting pollen morphology or protein profile (Shivpuri *et al.*, 1979; Arnon *et al.*, 1998). Thus, the highest air-

allergenic atmosphere of Nawabjang town, being the most polluted due to bricks making industries, rice mills, pulp & paper industry, Jute mills (industrialization) and dust particle carried by wind, may be responsible for the increased rate of allergenicity of the pollen grains.

Conclusion

The present study reveals that litchi flower pollen is capable of inducing hypersensitivity is one of the aeroallergens and should be considered to be a cause of respiratory allergic causes in Bangladesh. Skin tests are the major diagnostic tool for allergy and reveal the pollen of litchi to be a potent allergenic offender. Out of three proteins IF_1, IF_2, IF_3 only IF_2 (28 KDa) showed positive response in all the 50 patients with total pollen extract, can thus be said to be the major allergenic band 28kDa being other minor contributors. Pollen extracts contain complex mixtures of proteins, complex carbohydrates, lipids, enzymes, lectins etc (Mondal & Mondal, 1997, Mondal et al. 1997 a, b, 1998; Parui & Mondal 1998, Parui et al. 1998, 1999) and the crude antigenic extracts usually contain several other components other than the proteins to which the patients may show some allergic reaction. The method followed in this investigation enable us to isolate particular protein bands from a mixture of total proteins and to test with present day knowledge of epitope mapping and molecular cloning, the present study will contribute to the design of immunotherapic vaccines and the production of unlimited quantities of defined allergens. 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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Table 1:
Protein content of pollen extracts of Litchi flower
from different location at Rajshahi District in Bangladesh.

Place of Collection	Protein (mg/50g of dry litchi pollen)
Nawabgang Sadder	9.2
Thanor Sadder	8.4
Rajshahi University	7.6
Rajshahi Town	8.6

Table 2:
Results of skin tests with the active protein of Litchi flower pollen from different location at Rajshahi Division in Bangladesh.

Place of Collection	Total number of Test persons	Negative	1^+	2^+	3^+
Nawabgang sadder	30	2 (6.7%)	4 (13.3%)	18 (60%)	6 (20%)
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:
Result of skin tests against purified protein after gel filtration and individual antigenic (electrophoresis band proteins 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; 1-5mm: 1^+; 6-10mm: 2^+; 11-15mm: 3^+. (Total protein = purified protein from gel filtration and IF = Electrophoresis band fraction proteins).

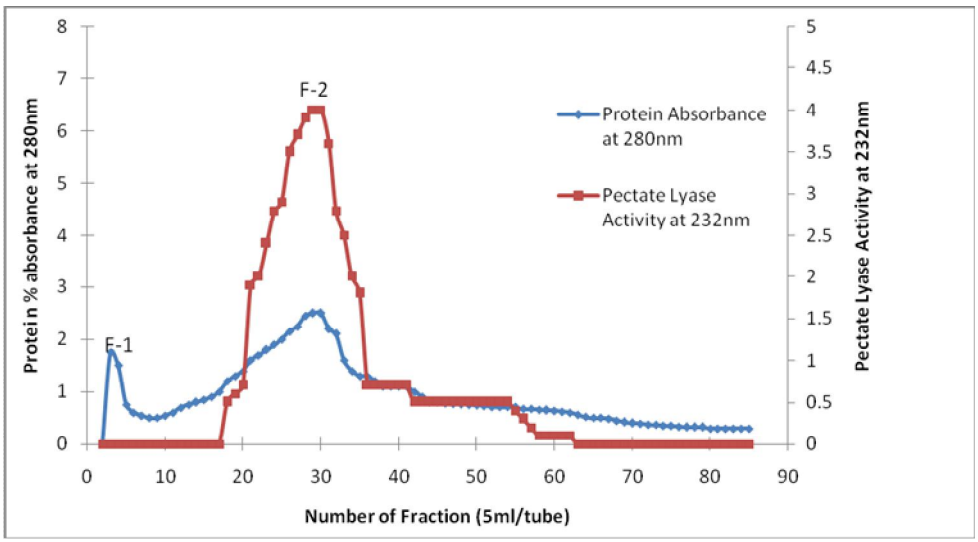

Fig.1: Gel Filtration of 40% ammonium sulphate saturated crude extract on Sephadex G-75. The crude protein solution (100 mg) was supplied to the column (1.0x 29.0 cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.2-7.4, at 4^0C and developed with the same buffer. Flow rate: 25 ml/hour.

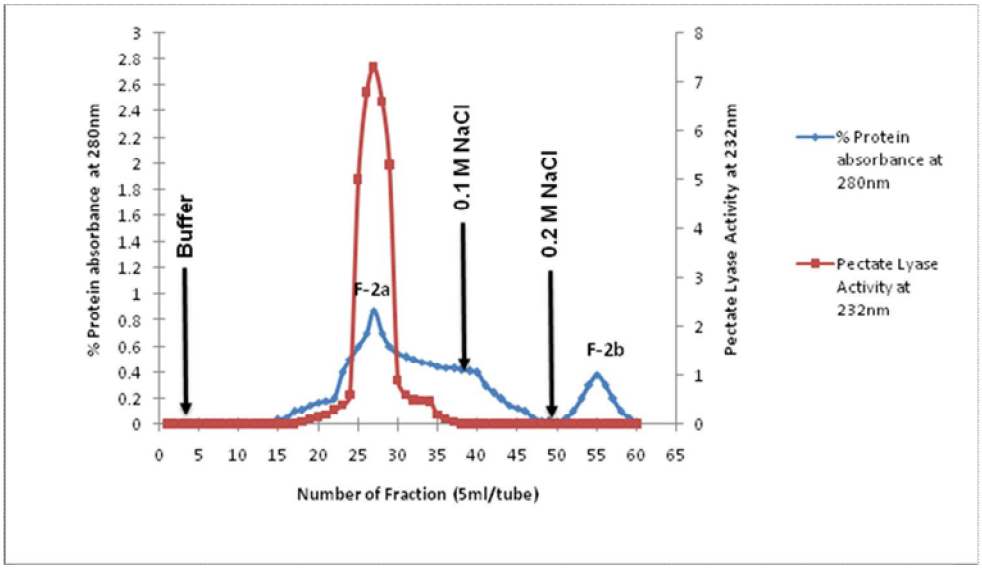

Fig. 2: Ion exchange chromatography of F-2 fraction (60mg) on DEAE cellulose, obtain by gel filtration, was applied to the column (1.0 x26.0 cm) pre-washed with 10 mM Tris-HCl buffer, pH 8.4, at 4^0C and eluted by stepwise increase of NaCl concentration in the same buffer. Flow rate: 25 ml/hour.

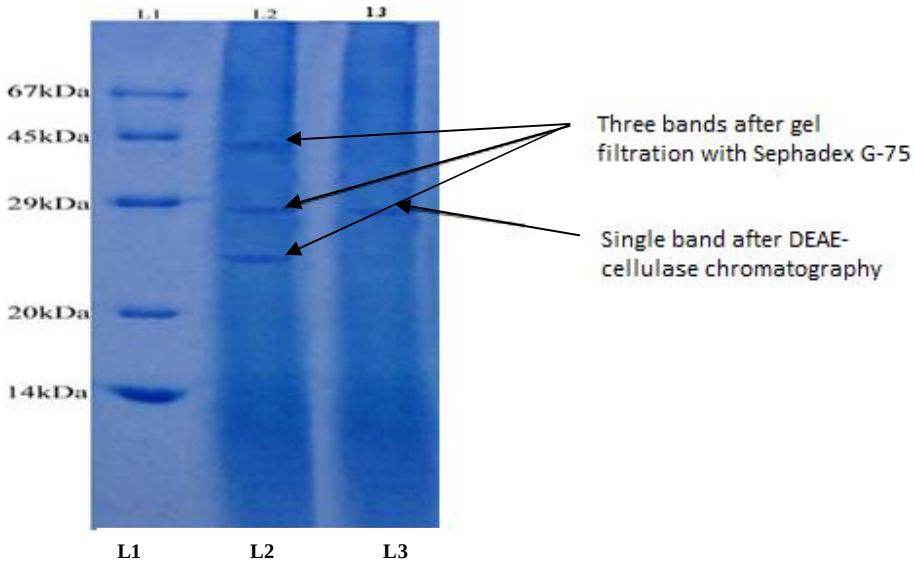

Fig3.Photographic representation of sodium dodecyl sulfate polyacrylamide slab gel electrophoretic pattern of the purified protein. L-1: Marker protein solution containing lysozyme (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), albumin (BSA, 67kDa). L-2: Fraction from gel filtration column. L-3: Fraction from DEAE-Cellulose column. (Line-1.1: marker proteins, line-1.2: proteins after gel filtration and line-1.3: purified protein after DEAE chromatogram).

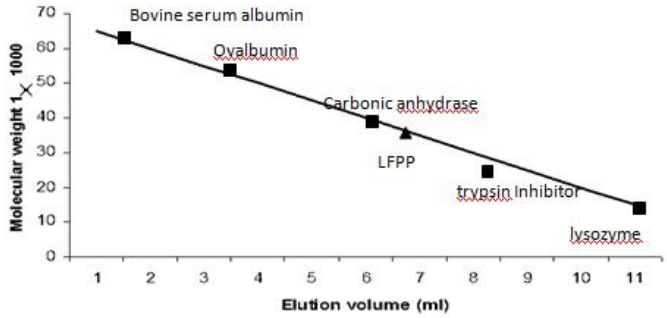

Fig.4: Molecular weight determination by gel filtration.

The molecular weight of the protein was also determined by gel filtration on Sephadex G-75 using lysozyme (14 kDa), trypsin inhibitor (20kDa), carbonic anhydrase (29kDa), ovalbumin (45kDa), albumin (BSA, 67kDa) 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 27kDa for purified LFPP.