Chapter-3

MATERIALS AND METHODS

3. Materials and methods

3.1 Pollen collection

Pollen samples were collected in large volume from the plants of *Datura* sp. growing in South Kolkata, West Bengal, India under the supervision of Dr. Sanjukta Mondal (Parui). Three species of the plants of *Datura* i.e. *Datura metel, Datura stramonium* ans *Datura inoxia* were selectively collected for the study. During collection, selection was made depending upon maturity of flowers i.e. two different types of pollen samples were collected - one from the mature buds and other from full bloomed flowers which had finished blooming on the same day as well as different days of different seasons i.e. during autumn and winter months of September to December and also in summer from March to May. For collection of pollen from anthers different sizes of the meshes (100, 200 and 300 µm) were used corresponding to the size of pollens. Microscopic analysis was performed to assume 90%-95% of pollen purity.

Fig. 1: *Datura metel* in full bloom

Fig. 2 : *Datura stramonium* in full bloom

Fig 3: Flower of *Datura stramonium*

Fig 4: Flower of *Datura inoxia*

3.2 Plant identification

The diagnostic features of the plants were studied to produce an accurate and precise means for species identification with the help of available literature. The specimens were collected from the field and spread out between the folds of old newspapers or blotting sheets avoiding overlapping of parts. The specimens were allowed to dry and the dried specimens were mounted on herbarium sheets of standard size (41 x 29 cm). Mounting was done with the help of adhesive and cello-tape. Herbarium sheets of the plants prepared were submitted to Prof. Amal Kumar Mondal, Department of Botany and Forestry, Vidyasagar University, Midnapore along with photographs of plants for identification.

Fig. 5: Herbarium of *Datura metel*

Fig. 6: Herbarium of ***Datura inoxia***

Fig.7: Herbarium of *Datura stramonium*

3.3 Ultrastructural analysis of pollen of *Datura* sp.

3.3.1 Light microscope study

The materials were prepared for microscopic analysis by the method of acetolysis by Erdtman (1960) and the improved method by Faegari and Iversen (1964).

For light microscopic analysis, the previously sieved and dried pollen sample was washed with deionized water and centrifuged for 5 minutes. Glacial acetic acid was added to the pellet residue in the bottom and centrifuged again. The residue was then catalysed by adding a mixture of acetic anhydride and concentrated H_2SO_4. Tubes were heated to 110^0C for five minutes in a metal heat block. This process removed unwanted organic debris, leaving just the clear outer shell of the pollen grain. After further centrifugation, the waste acid was decanted and the sample was washed several times with deionised water. The pollen samples were mounted for L.M study using glycerine jelly.

3.3.2 SEM study

For SEM analysis, properly dried samples were required to be ready and mounted on specimen stubs and sputter coated with approximately 200 Å thick coating of gold. For observation a Zeiss SEM was used at 5 k.V. at the Instrumentation Centre of IIT, Kharagpur. The other measurements like diameters of pollen grains were performed using ocular and stage micrometer under compound microscope (100X).

3.3.3 TEM study

For TEM analysis sample preparation was done following the method of Diethart et al. (2007). For study under Transmission Electron Microscope, the properly dried pollens of *Datura metel, Datura stramonium* and *Datura inoxia* were fixed for 6 hours in 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). Pollen grains were then washed using buffer and distilled water and post fixed in 2% osmium tetroxide (OsO_4) and 0.8% potassium hexacyanoferrate ($K_4\ Fe(CN)_6.3H_20$) overnight at 4^0C.

After fixation was completed, the samples were washed with buffer and distilled water and then dehydrated in 2, 2-dimethoxypropane (DMP) and acetone. Embedding was done in Spurr's low viscosity epoxy resin (Spurr, 1969). For sectioning, (about 70 – 90 nm) a Reichert-Jung Ultracut S microtome was used with a diamond knife. Finally the micro sections were shifted on copper and gold grids. Sections of the 3 types of pollen of *Datura* were generally stained with heavy metal uranyl acetate followed by lead citrate and a modified Thièry test. For this Thièry test, the method of Weber and Frosch (1995) was followed. According to this method, pollen sections were dissolved in 0.2% Thiocarbohydrazide (TCH) for 15 minutes, 1% silver proteinate for 10 minutes and Periodic Acid for additional 10 minutes. Additional test (PA for 30 minutes, TCH for 15 hours, Sp for 30 minutes) (Thièry, 1967) and lipid-tests (TCH for 15 hours, Sp for 30 minutes) (Rowley and Dahl, 1977) was carried out when satisfied result was not obtained. Furthermore 1% potassium permanganate (KMnO4, 7 minutes) showed good abilities to stain the endexine in particular. The analysis of the section of pollen was done by Transmission Electron Microscope (Model- FEI Philips Morgagni 268D) used at 200 kv, at the All India Institute of Medical Sciences, New Delhi.

3.4 Protein extraction, isolation and characterization

3.4.1 Protein extraction

Proteins from the pollen of the three species of *Datura* was extracted according to the method of Singh et al. (1993). A slightly modified method of Mondal and Parui (1997) was used for actual extraction. The pollen was first defatted and then proteins from the pollen were extracted in 0.2 M Tris-HCl (pH 7.4) buffer. The process was done by continuous stirring at 4^0C for 20 h. The extract was centrifuged at 12000xg at 4^0C for 5 minutes. The sample was stored at -20^0C for further use.

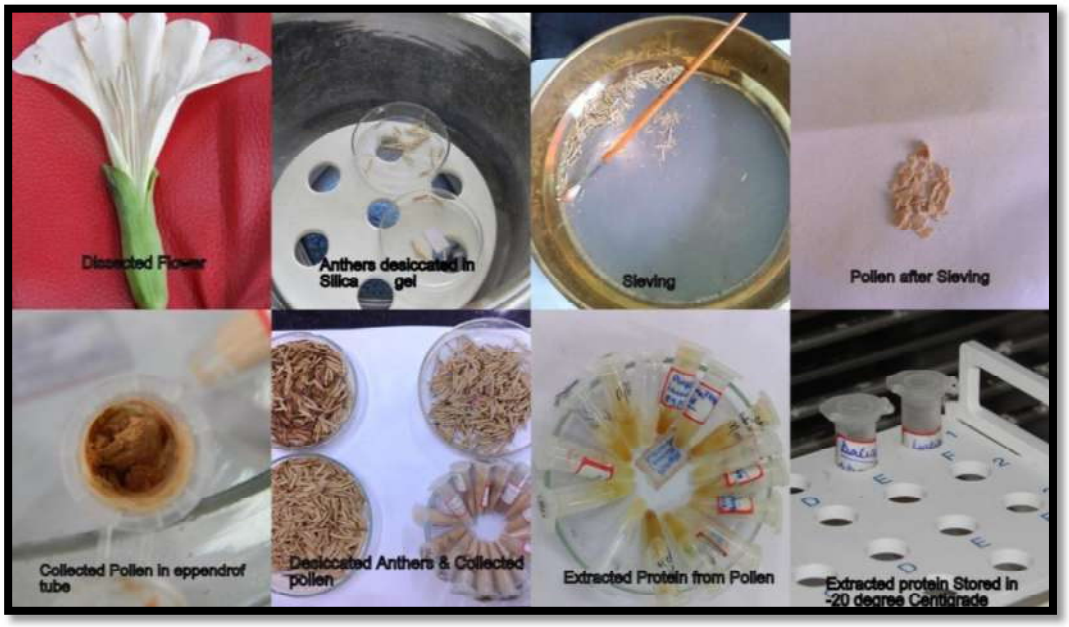

Fig. 8: Pollen collection, sieving, storage and extraction

3.4.2 Estimation of proteins

The protein concentration in the extract was estimated by the modified method of Lowry (Lowry et al., 1951). A calibrated solution of bovine serum albumin was used as a standard. The standard curve was prepared using a working standard containing 200µg of BSA per ml of the solution. 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1 ml of the working standard containing 40 µg , 80 µg , 120 µg , 160 µg and 200 µg of protein respectively was pipetted out into a series of test tubes and the volume in each test tube was made up to 1ml with distilled water. 5ml of alkaline copper sulphate solution was added to each test tube followed by 0.5ml of Folin-ciocalteau reagent. After incubation in the dark for 20 minutes, the absorbance was read at 660 nm using a UV

Spectrophotometer (Shimadzu). The Optical Density at 660 nm was plotted against the concentration of the known protein standards to get the standard curve. For unknown sample 1ml of sample was taken to which 5ml of alkaline copper sulphate solution was added followed by 0.5ml of Folin-ciocalteau reagent. After incubation in the dark for 20 minutes, the absorbance was read at the same wavelength. The amount of protein in the unknown sample was found by plotting the OD on the standard graph.

3.4.3 Gel electrophoresis

The protein sample from pollen extract was subjected to SDS-PAGE (SDS-Polyacrylamide gel electrophoresis) according to the method of Laemmli (1970). The protein samples were mixed with equal volume of sample buffer made up with 0.06M Tris HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% ß-mercaptoethanol, 0.01% Bromophenol blue and then heated at 100^0C for 3 minutes. 10% T mini gel (8x7 cm gel) was prepared and the samples were loaded in the wells of this gel. Gel was run at room temperature for 2 hrs and 35 min at 70 V using Laemmli Buffer System (1970) (0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4). A marker protein mixture consisting of Myosin, Rabbit Muscle (205 kDa), Phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (29 kDa) and Soyabean Trypsin Inhibitor (20.1 kDa) was use for the calibration of the gel. After electrophoresis the gel was stained with 0.1% Coomassie Brilliant Blue R 250 for 1 hr and destained with methanol:acetic acid:water (4:1:5) mixture later on.

3.4.4 Isolation of individual protein fractions by gel filtration

Proteins were separated according to their size or molecular weight by SDS-PAGE analysis and Gel filtration chromatography. A Pasteur pipette was plugged with a small amount of cotton. Solution of silica gel adsorbent, 230-400 mesh, with a nominal pore size of 300 Å and 1000 Å, giving a linear calibration range from 100 to 50,000 dalton, was prepared by dissolving the gel in Tris-HCL buffer (pH-7.4). The column had a flow rate range of 0.2–1.0 mL/min. The column was prepared by pouring the gel into it. The filled column was clamped securely to a ring stand using a small three-pronged clamp. Firstly, the column was pre-eluted with the Tris-HCL buffer (pH-7.4). Then samples were separated according to their size by using this column technique and collected for further analysis.

Fig. 9: **Gel filtration of *Datura* pollen extract**

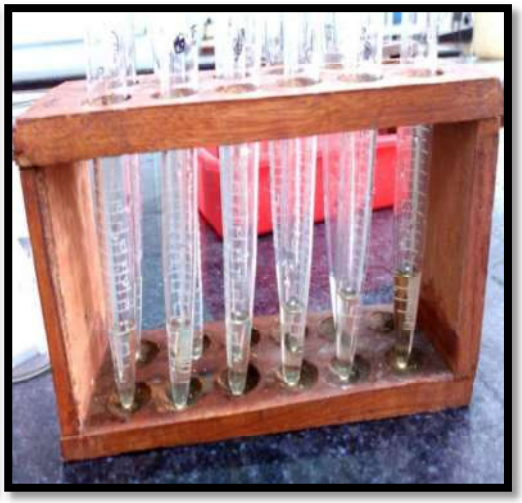

Fig. 10: Fractions of pollen protein after gel filtration

3.4.5 Analysis of protein fractions by PAGE

The fractions were then analyzed by PAGE to confirm the molecular weights of the individual proteins. The protein samples were heated with an equal amount of sample buffer [0.06M Tris HCl (pH 6.8), 10% sucrose, 0.5% β-mercaptoethanol, 0.01% Bromophenol blue] at 100˚C for 3 min. The samples were loaded in the wells of a 10% T mini-gel (8x7 cm gel) and the gel was run using Laemmli buffer system (1970) [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature for 2hrs 35 min at 70V. The gel was calibrated using total protein extract as mentioned earlier. 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

3.5 Identification of antigenic fractions and sequencing

3.5.1 Preparation of antigenic extracts from total protein

The method of Sheldon et al. (1967) was followed for the preparation of total antigenic extract of pollen. Pollen grains were first defatted by soaking pollen in differential volumes of diethyl ether and kept at 4^{o}C for 12-18 hours with periodical shaking and stirring. Frequent changes with fresh solvent of diethyl ether were given to the extract to confirm complete defatting until the supernatant became colourless. The sample was then filtered through a filter paper and the particulate matter allowed to dry at room temperature. The defatted pollen was then desiccated over anhydrous calcium chloride and stored in airtight vials at 4^{o}C. Active allergenic substances were

then extracted from the defatted pollen grains using buffered saline [0.5% sodium chloride, 0.036% monobasic potassium phosphate, 0.7% dibasic sodium phosphate (anhydrous), 0.4% phenol crystals; pH 8.0] in the ratio of 1:50 w/v i.e. 1gram of pollen was suspended in 50ml of buffered saline. It was kept for 72 hours with periodical shakings using a rotary shaker. The suspension was then filtered and subjected to centrifugation at 4000 rpm for 20 min at 4^0C. The supernatant was separated by discarding the solution and finally filtered through a Millipore Filter Paper [0.22 (m)]. Before testing this antigenic extract on patients, the sterility was checked for microorganism by transferring a few drops of the extract to LB (Leuria Broth) agar plate. The plates were incubated at 37^0C for 24 hrs. The extracts were stored in sterile vaccine vials at 4^0C.

3.5.2 Preparation of antiserum

Skin prick tests were performed with the commercially available total antigenic extract of the pollen at Allergy & Asthma Treatment Centre, Kolkata – 29 an allergy clinic. Blood samples were collected from the patients showing positive response to the total pollen extracts and who volunteered to donate the blood samples for further experimentation. The blood samples were allowed to clot standing for 3-4 hrs at room temperature according to the method of Sadasivam and Manickam (1992). The serum was then separated from the clot by low speed centrifugation and stored at -20^{o}C. 0.1% Sodium azide was used as antibacterial agent.

3.5.3 Ouchterlony - Double immunodiffusion

Double immunodiffusion Ouchterlony immunodiffusion was also performed according to the method of Johnstone and Thorpe (1982). Equal amount of protein loading of each fractions of *Datura* sp. were loaded into outer wells whereas 25µl of the mixture of antiserum containing 150 gm of total protein was loaded in the central well. All the plates were then incubated for 48 hrs at 25^{o}C. After incubation, the plates were washed several times with 0.9% (w/v) NaCl (saline) and finally dried to observe precipitation arcs.

3.5.4 Production of species specific antibody in Rat

Male LOBUND –Wistar rats (weight approximate 150 gms) between the ages of 6 – 20 weeks were used for the study. Here separate rats were used for three species of

Datura sp. Intramuscular (IM) injections were used for the initial injection and subsequent boosts. The maximum total volume (antigen + adjuvant) for IM injection was 100 microliters. It was divided equally between the hind leg quadriceps. Test bleeds were required prior to boosting at 21 days to 28 days post immunization. This test bleed could indicate whether a boost was needed or if blood collection for antibody harvest could begin. It was usually a sterile saline solution containing only the antigen. A final boost was given intravenously. The IM injections used were free of debris and contaminants. Maximum IM injection volume was 200 microliters.

3.5.5 Collection of blood serum from rat

Blood samples, collected from rats, were allowed to clot standing for 3-4 hrs at room temperature according to the method described by Sadasivam and Manickam (1992). The serum was then separated from the clot by low speed centrifugation and stored at -20^{o}C. 0.1% sodium azide was used as antibacterial agent.

Fig. 11: White Rat being injected with pollen extract

Fig. 12: Collection of blood serum from rat (by heart puncturing method)

3.5.6 Identify the allergenic protein fractions and measure the total and specific immunoglobulin in serum by ELISA-inhibition.

ELISA was performed with the different isolated protein fractions to identify the antigenic fractions in the total protein extract according to the method of Sadasivam and Manickam (1996).

ELISA was performed with the different isolated protein fractions to identify the antigenic fractions in the total protein extract. The protein fractions were diluted with Coating Buffer (1XPBS Buffer containing 8.5 g NaCl, 1.4 g Na_2HPO_4, 0.2 g NaH_2PO_4, 1000 ml ddH_2O, adjusted to pH 7.4 and store at 4°C) and the wells of ELISA plate were coated by using 100 µl of diluted antigen solution. The concentration of the antigens used to coat the wells were ranged from 1- 10 µg/ml and then the plate was covered by an adhesive plastic and incubated at 37°C for 2 hours. Then the plate was washed with 200 µl of Washing Buffer (0.5 ml Tween 20 in 1000 ml PBS Buffer stored at 4°C) for three times. 200 µl of Blocking Buffer consisting of 1 g of BSA in 100 ml Washing Buffer, stored at 4°C, was added. Blocking buffer generally blocks any non-specific binding sites in the coated wells. The plate was then covered once again with an adhesive plastic and incubated at 37°C for 1 hour and then the plate was washed with 200 µl of Washing Buffer for three times. The HRP (enzyme horseradish peroxidase)-conjugated antibody (antiserum of *Datura* pollen sensitive patients containing the primary antibody) was diluted with Blocking Buffer and 100 µl of the diluted antibody was added to each well of the plate. The plate was covered with an adhesive plastic and incubated at 37°C for 30 minutes. The plate was then washed with 200 µl of Washing Buffer for five times. 100 µl of a chromogenic substrate TMB Reagent (3,3',5,5'-Tetramethylbenzidine GenScript Cat.No M00078) was added per well with a multichannel pipette. After sufficient color development, 100 µl of Stop Buffer (8.3 ml 12 mol/L HCl, 91.7 ml ddH_2O, stored at 4°C) was added to the wells. The absorbance of each well was then read at 450 nm.

3.5.7 Study the cross-reactivities

Ouchterlony immunodiffusion was performed according to the method described by Johnstone and Thorpe (1982) to check the cross reactivity reactions of whole protein extract and the individual protein fractions among these three species. ELISA was also performed to confirm the cross reactivity reactions according to the method of Sadasivam and Manickam (1992).

3.5.8 Epitope mapping

MALDI was used for the identification of allergenic proteins isolated at Xcelris Labs Limited, Navrangpura, Ahmedabad, Gujarat. MALDI TOF/TOF mass spectrometer was used to reveal the amino acid sequence of the peptides using post-source decay or high energy collision – induced dissociation. Cross-linking coupled Mass Spectrometry was used for epitope mapping. In this the antibody and the antigen were first bound with a labeled cross-linker. High mass MALDI detected the complex formation. After confirming the detection, binding location was then identified. Detection was performed using high resolution mass spectrometry or MS/MS techniques. It identifies the peptides bound and the labeled cross-linkers amino acid locations.