

## Original article

Aerobiological and immunochemical studies on *Carica papaya* L. pollen: an aeroallergen from India

**Background:** *Carica papaya* L. is a fruit yielding tree, widely grown or cultivated in the tropics and subtropics. Its pollen grain has been reported to be airborne and cause immunoglobulin E (IgE)-mediated hypersensitivity.

**Objective:** To conduct long-term aerobiological study on *Carica* pollen, along with aeroallergenic particles originating from it and to identify vis-à-vis characterize an important IgE-reactive component present in this pollen.

**Methods:** The seasonal and diurnal periodicities of airborne *C. papaya* pollen were recorded in a 5-year survey using a Burkard volumetric sampler. The allergenic potential was studied by skin prick tests, IgE-enzyme-linked immunosorbent assay (ELISA) and also by aeroallergen immunoblotting. The total pollen extract was fractionated by Sephacryl S-200 column, and out of the eluted five fractions, the maximum IgE-reactive fraction (as found in ELISA inhibition) was resolved into five major subfractions in reverse-phase high-performance liquid chromatography (RP-HPLC). The subfraction with optimum IgE reactivity was studied by activity gel, native and nonreducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The homogeneity of the isolated protein fraction was checked by crossed immunoelectrophoresis with rabbit antisera and IgE reactivity was confirmed by ELISA inhibition and immunoblotting using individual patient sera.

**Results:** The *Carica* pollen occurred in the air round the year with peaks during January and September–October. Among a patient population of 1000, skin-test results showed 27.8% +1 level and 5.6% +2/+3 level reactions. In aeroallergen immunoblotting of exposed Burkard tape segments, the detected allergen spots showed a significant correlation with airborne pollen count recorded. The pollen extract elicited loss of IgE reactivity when treated with reducing agent-like  $\beta$ -mercaptoethanol and heat, but showed six IgE-reactive components in nonreducing IgE-immunoblot. The fraction 1 eluted from Sephacryl S-200 column showed highest IgE reactivity and resolved into five major components in RP-HPLC. Out of these, the fraction showing optimum IgE reactivity in IgE-ELISA inhibition and immunoblotting with patient antisera, elicited esterase activity and found to be a homogenous protein of 100 kDa.

**Conclusion:** *Carica papaya* tree contributes significantly to the aeropollen and aeroallergen load of the suburban outskirts of Calcutta metropolis, India. The pollen extract contains an important IgE-reactive protein component of 100 kDa molecular weight with esterase activity.

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Key words: 100 kDa allergen; aeroallergen; *Carica papaya* pollen; esterase activity; immunoglobulin E reactivity.

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*Carica papaya* of family Caricaceae is a fruit-yielding tree and widely distributed in the tropics and subtropics. Although this plant is a native of Central America, it is very common in the Indian subcontinent too. It is cultivated for vegetable, fruit and also as a source of the 23 kDa enzyme 'papain'. The tree flowers round the year having amphiphilous pollination mechanism, i.e. the pollen grains are dispersed both by wind and insects. There are previous reports describing the *C. papaya* pollen and

fruit to be allergenic, where the results were based either on aerobiological (1, 2) or clinical (3, 4) point of view. There are no reports regarding the nature of its immunoglobulin E (IgE)-reactive protein components so far.

The objective of the present study was to evaluate the role of *C. papaya* pollen as a potential aeroallergen and to isolate and characterize an important IgE-reactive component present in the pollen extract used in clinical and diagnostic purposes.

## Materials and methods

### Aerobiological sampling

A continuous air sampling had been conducted at Madhyamgram Experimental Station of Bose Institute, 19 km north from central Calcutta, situated in suburban outskirts. There was a 5-year survey (from 1 July 1994 to 30 June 1999) using a Burkard automatic volumetric sampler placed in the field on a 0.5 m high concrete base. The exposed tapes were microscopically examined according to the guideline of British Aerobiology Federation (5) to record seasonal and diurnal periodicities of the airborne *C. papaya* pollen.

### Aeroallergen particle immunoblotting

A number of 48 mm exposed Burkard tape segments (representing each day) of the days of peak and low-pollen count were selected after microscopic examination (without cover slip). The aeroallergen components were transferred (1 h at 25°C) onto a polyvinylidene fluoride (PVDF; NEN Life Science Products, Boston, MA, USA) and detected using chemiluminescence (Boehringer, Mannheim, Germany) (6). The allergen spots were counted microscopically.

### Source material

Fresh pollen samples were collected from male *C. papaya* trees during peak pollination months. The batch contained <1% non-pollen impurities.

### Preparation of extract

The pollen grains were defatted with diethyl ether and extracted in phosphate-buffered saline (PBS; 0.1 M Na-phosphate, 150 mM NaCl, pH 7.2) by continuous stirring for 16 h at 4°C. After centrifugation (12 500 g for 45 min), the supernatant was dialyzed in PBS and passed through 0.22 µm millipore filter (Millipore Corp., Bedford, MA, USA). The filtrate was then lyophilized and stored (–70°C) in sterile vials. The protein content of the whole pollen extract and fractions were determined according to Lowry et al. (7) using bovine serum albumin (BSA) standard.

### Fractionation of *C. papaya* pollen allergen

The whole pollen extract was fractionated in the range of 0–90% saturation of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The salted out fraction was dissolved in PBS and dialyzed (molecular cut-off limit 10 000) to remove traces of ammonia and stored at –70°C.

The concentrated protein (26 mg) solution was subjected to Sephacryl S-200 column (d = 2.4 cm, h = 55.5 cm) equilibrated with 10 mM PBS, pH 7.2 for gel filtration. The fractions were collected in 3 ml aliquots (void volume 83 ml, flow rate 12 ml/h) at 4°C and the elution was monitored at 280 nm using a spectrophotometer.

The optimum IgE-reactive fraction (fraction 1) was then lyophilized and chromatographed by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) using Novapak C18 column (Waters Corp., Milford, MA, USA). The column was eluted (280 nm) at 0.2 ml/min with 14% acetonitrile in 0.1% acetic acid (8).

### Skin prick tests and sera collection

Skin prick tests (SPT) were carried out with *C. papaya* pollen extracts (1 : 50, w/v) on adult subjects with case history of allergic

rhinitis or mild intermittent allergic asthma and having papaya tree/trees growing in their surroundings. All of them were attending the Allergy Clinic of the Institute of Child Health, Calcutta during 1994–1999. The exclusion criteria were perennial or severe asthma, pregnancy or lactation, malignancy or other severe systemic diseases during skin testing or sera collection. To avoid masking of possible symptoms, corticosteroids and anti-histamines were prohibited. The study was approved by the Ethics Committee of the hospital. Histamine phosphate (10 mg/ml) and PBS were used as positive and negative controls respectively. The reaction measurement was performed after 20 min. According to international guidelines, positivity was defined as mean wheal diameter ≥3 mm, compared with negative control (9). Control sera were collected from nonsensitized healthy volunteers [confirmed by negative skin reaction and IgE-enzyme-linked immunosorbent assay (ELISA)] having no history of previous or current episode of any allergic, systemic or joint diseases in them and their families.

### Enzyme-linked immunosorbent assay

The ELISA was performed to determine *C. papaya* pollen-specific IgE levels in individual patient sera using antihuman IgE horse-radish peroxidase conjugate (Sigma-Aldrich, St Louis, MO, USA) in 1 : 1200 dilution and *o*-phenylene diamine substrate (10). The absorbance was measured with an ELISA reader at 492 nm. The individual serum having P/N value (ratio of optical density of patient sera with respect to control) >3.0 were used for ELISA inhibition and immunoblotting (11). Here, the control is the mean value from the panel of 12 normal (control group) sera.

### ELISA inhibition

This analysis was performed by overnight preincubation of patient sera with equal volume of (i) whole extract (100 µg protein/ml), (ii) native protein in reducing sample buffer without any heating and after heating in 100°C for different times and (iii) fractions of *C. papaya* pollen extract with varying protein concentrations at 4°C. The inhibited serum was then used in ELISA with anti-IgE horseradish peroxidase conjugate. Percentage inhibition was calculated as:

$$\text{Inhibition (\%)} = \frac{\text{OD of test sample} - \text{OD of inhibited sample}}{\text{OD of test sample}} \times 100$$

The 0% inhibition was obtained by incubating serum samples with PBS. A dose-response curve was obtained by plotting the percentage inhibition against the log of protein concentrations.

### 11% native and SDS-PAGE

Native-polyacrylamide gel electrophoresis (PAGE) analysis of whole pollen extract and fractions, were performed. Sodium dodecyl sulphate (SDS)-PAGE was performed for specific fraction according to Laemmli (12) both in reducing and nonreducing conditions. The protein components were observed by silver staining.

### Determination of esterase activity

The esterase activity was determined using α-naphthyl acetate as substrate (13).

## Crossed immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed using rabbit antisera against *C. papaya* pollen extract (5 mg/ml) for checking the homogeneity of the isolated protein component according to Weeke (14).

## IgE-specific immunoblotting

The IgE-immunoblotting was performed onto PVDF membrane according to Sambrook et al. (15) for whole pollen extract and isolated fractions. Serum was diluted in 1 : 5 (v/v) ratio in PBS. The IgE-reactive bands were detected with diaminobenzidine as substrate.

## Results

In a 5-year long aerobiological survey at Madhyamgram Experimental Station, *C. papaya* pollen grains were found to be perennial (Fig. 1A). The pollen count attained the peak in the month of January and occurred in a lower degree in September–October. During the peak months, the pollen grains contributed 10–18% of total aeropollen load on an average. The airborne pollen showed a typical

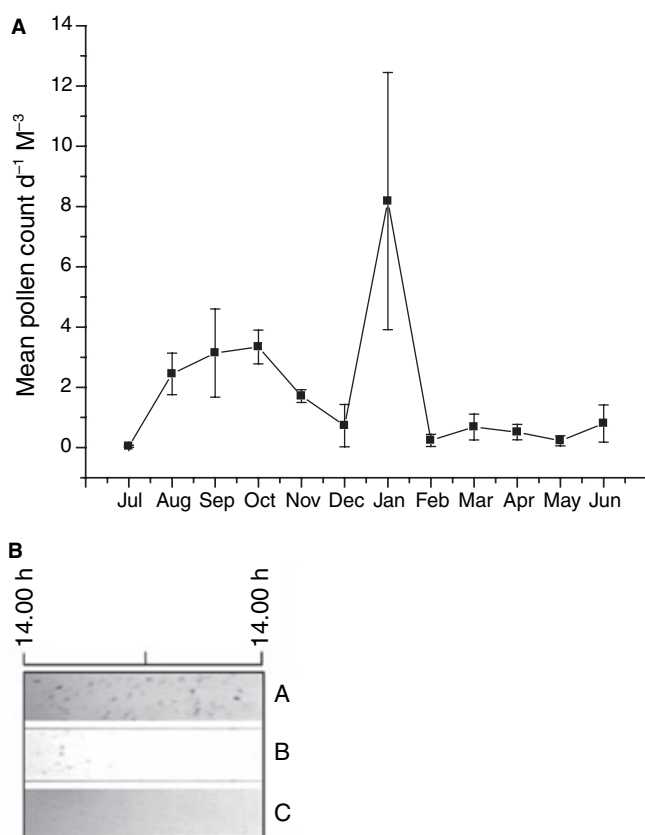


Figure 1. (A) Seasonal mean periodicity of airborne *Carica papaya* pollen (1994–1999). (B) Aeroallergen particle immunoblotting for *C. papaya* pollen. Exposed Burkard tape segments for (A) peak day, (B) day of lower count and (C) negative control.

afternoon pattern in the context of diurnal periodicity (Figure not shown). Its concentration reached the maxima in between 15.00 and 19.00 hours during dry days.

Skin prick tests were performed with *C. papaya* pollen extract since 1994. In a population of 1000 respiratory allergic patients [mean age 34.36 year (age range: 18–17), M/F = 1.2/1.0], 27.8% showed +1 and 5.6% showed +2/3 level of reaction. On the basis of positive SPT and high titre of *C. papaya* pollen-specific IgE-ELISA results, 63 patients (M/F = 29/34, mean age 36.4 years) with allergic rhinitis or bronchial asthma (or combination of both) were selected for sera collection. Among them 15 showed IgE reactivity to the isolated 100 kDa allergen (Table 1), which has been described later. They were found to be sensitive to other pollen types, e.g. *Eucalyptus*, *Catharanthus* and palm members-like *Areca*, *Borassus*, *Cocos* and *Phoenix*. In case of aeroallergen immunoblotting, to avoid cross-reaction, the exposed tape segments were selected for such days when these pollen types were absent in air.

In aeroallergen studies of exposed tapes, a number of recognizable spots were detected both for the days of higher and lower concentrations of *C. papaya* pollen (Fig. 1B). The detected allergen spot count was always greater than the microscopic pollen count of the same exposed tape and their diameter varied from 5 to 200  $\mu$ m. Allergen spot count showed significant correlation (correlation coefficient '*r*' ranged from 2.67 to 2.98, which was significant at <2% level in '*t*' test) with airborne pollen count.

Results from IgE-ELISA inhibition (Fig. 2A) showed that the treatment of *C. papaya* pollen extract with sample buffer containing  $\beta$ -mercaptoethanol as reducing agent and subsequent heating in 100°C leads to remarkable decrease of IgE-binding activity in ELISA inhibition. The study was conducted with 100  $\mu$ g/ml protein concentration which was found to be optimum in standardization using 15 patient sera of group B (Table 1) individually and the mean was plotted (Fig. 2A). Even when reducing buffer was added without any heating, it showed >30% inhibition in average (15 patients). When the extract was heated for 5, 10 and 30 s separately at 100°C with reducing buffer, it showed gradual loss of IgE-binding capacity, which was almost lost after 30 s of heating. In case of treatment with nonreducing sample buffer and 30 s heating in 100°C, more than 95% IgE reactivity was retained. In SDS-PAGE also (Fig. 2B), we found gradual denaturation of protein components in reducing condition and heat treatment. In contrast, *C. papaya* pollen extract showed a number of protein components in nonreducing SDS-PAGE (30 s heating), among which six (molecular weight ranging from 14 to 189 kDa) were found to bind with patient IgE in immunoblotting (Fig. 3). As a result the further steps of this experiment were carried out in nonreducing condition.

Table 1. Characterization of patients selected for study

| Number  | Sex | Age (years) | Symptoms* | Reaction in SPT | FEV <sub>1</sub> (percentage predictive value) | IgE-ELISA P/N value | Adverse reactions to other pollen/food       |
|---------|-----|-------------|-----------|-----------------|------------------------------------------------|---------------------|----------------------------------------------|
| Group A |     |             |           |                 |                                                |                     |                                              |
| 1       | M   | 22          | AR + BA   | +1              | 88.0                                           | 3.81                | <i>Phoenix</i> , papaya                      |
| 2       | M   | 32          | As above  | +2              | 83.6                                           | 4.65                | <i>Borassus</i> , <i>Phoenix</i>             |
| 3       | F   | 45          | AR        | +2              | 91.8                                           | 5.18                | <i>Areca</i> , <i>Cocos</i> , house dust     |
| Group B |     |             |           |                 |                                                |                     |                                              |
| 1       | F   | 22          | AR + BA   | +3              | 83.2                                           | 4.31                | <i>Areca</i> , <i>Eucalyptus</i>             |
| 2       | F   | 26          | AR        | +2              | 87.3                                           | 3.87                | <i>Eucalyptus</i> , house dust               |
| 3       | M   | 36          | As above  | +1              | 90.4                                           | 3.78                | <i>Cocos</i> , house dust, papaya            |
| 4       | F   | 40          | AR + BA   | +1              | 82.3                                           | 3.60                | <i>Areca</i> , <i>Catharanthus</i>           |
| 5       | M   | 42          | As above  | +2              | 79.9                                           | 3.86                | <i>Areca</i> , <i>Cocos</i> , house dust     |
| 6       | M   | 20          | BA        | +2              | 83.5                                           | 4.12                | <i>Areca</i> , <i>Cocos</i> , <i>Phoenix</i> |
| 7       | M   | 33          | AR        | +3              | 78.0                                           | 4.33                | <i>Cocos</i> , wheat                         |
| 8       | F   | 28          | AR + BA   | +2              | 91.3                                           | 5.38                | <i>Saccharum</i> , house dust                |
| 9       | M   | 51          | BA        | +2              | 76.7                                           | 3.80                | House dust, papaya                           |
| 10      | F   | 48          | AR+BA     | +3              | 88.6                                           | 4.79                | <i>Cocos</i> , spinach                       |
| 11      | F   | 33          | AR        | +2              | 89.6                                           | 5.89                | <i>Areca</i> , rohu fish                     |
| 12      | F   | 27          | As above  | +1              | 93.3                                           | 5.22                | <i>Phoenix</i> , <i>Eucalyptus</i>           |
| 13      | M   | 42          | As above  | +3              | 86.8                                           | 4.38                | House dust, wheat                            |
| 14      | M   | 41          | AR + BA   | +3              | 83.6                                           | 4.79                | <i>Cocos</i> , spinach, wheat                |
| 15      | F   | 33          | AR        | +2              | 86.6                                           | 5.89                | <i>Areca</i> , <i>Eucalyptus</i>             |

\*AR, allergic rhinitis; BA, bronchial asthma.

Sera from group A was used for aeroallergen immunoblotting and group B for IgE-ELISA inhibition and immunoblotting.

SPT, skin prick test; IgE-ELISA, immunoglobulin E enzyme-linked immunosorbent assay.

In Sephacryl S-200 column chromatography five fractions (Fig. 4A) were eluted from total salted out pollen protein. In IgE-ELISA inhibition, the dose-response curve (Fig. 4B) showed the 'fraction 1' to have an optimum IgE-binding activity with respect to the crude extract.

Fraction 1 showed the presence of few protein components in native-PAGE, out of which one was found to be an esterase in activity gel (Fig. 5). Here, in crude extract, there were two components with esterase activity, out of which the one having high-molecular weight was present in the fraction 1.

The fraction was then studied by RP-HPLC (Fig. 6A) and separated five distinct peaks (peaks 3 and 4 seemed not to be separated in native-PAGE). The peak 2 contained the fraction with esterase activity. In IgE-ELISA inhibition (Fig. 6B) of the fractions (fractions 1a-e) respective to each peak, the fraction 1b (i.e. with esterase activity) showed remarkable IgE reactivity. In nonreducing SDS-PAGE of this fraction, the presence of a single component of 100 kDa molecular weight (Fig. 7A) was recorded. The isolated component was found to be homogenous in CIE where the second dimension of 1% agarose gel contained rabbit antisera against *C. papaya* pollen (Fig. 7B).

Finally, IgE-immunoblotting with 15 individual sera from a population of 63 *Carica*-sensitive patient population of Calcutta city (33.81%) with P/N value for specific IgE > 3.5 (group B of Table 1) revealed the 100 kDa component of *C. papaya* pollen to be potentially IgE reactive (Fig. 7C).

## Discussion

In this study, we had described the role of *C. papaya* pollen grain as a potential aeroallergen and isolated a 100 kDa as a IgE-reactive protein with esterase activity. *Carica papaya* tree flowers throughout the year, as found in the results of aerobiological survey. During the periods of maximum concentration, *C. papaya* pollen contributed a high amount in the total aeropollen load. The IgE-reactive spots were found to be greater in number in the same exposed surface when compared with the number of airborne *C. papaya* pollen trapped during the air sampling. A large number of aerodynamic particles of varying sizes (may differ from the size of the intact pollen) are produced, which were detected as spots in particle immunoblotting. The spot counts showed a significant correlation with the pollen count. These micronic particles may easily be trapped in the lower airways to trigger allergenic reaction.

The results of SPT on 1000 patients along with specific IgE-ELISA demonstrated the allergenic potential of *C. papaya* pollen in a large population. The ELISA inhibition and PAGE confirmed the loss of IgE reactivity and protein denaturation during the steps involved in the treatment of *C. papaya* pollen extract with reducing agent-like  $\beta$ -mercaptoethanol. The effect becomes more deleterious with heating. A detail study on *Ficus* fruit described that treatment with dithiothreitol (DTT), another reducing agent, lead to a substantial loss of IgE reactivity and identified two (22 and 28–34 kDa) allergens by nonreducing SDS-PAGE (16). So, in the

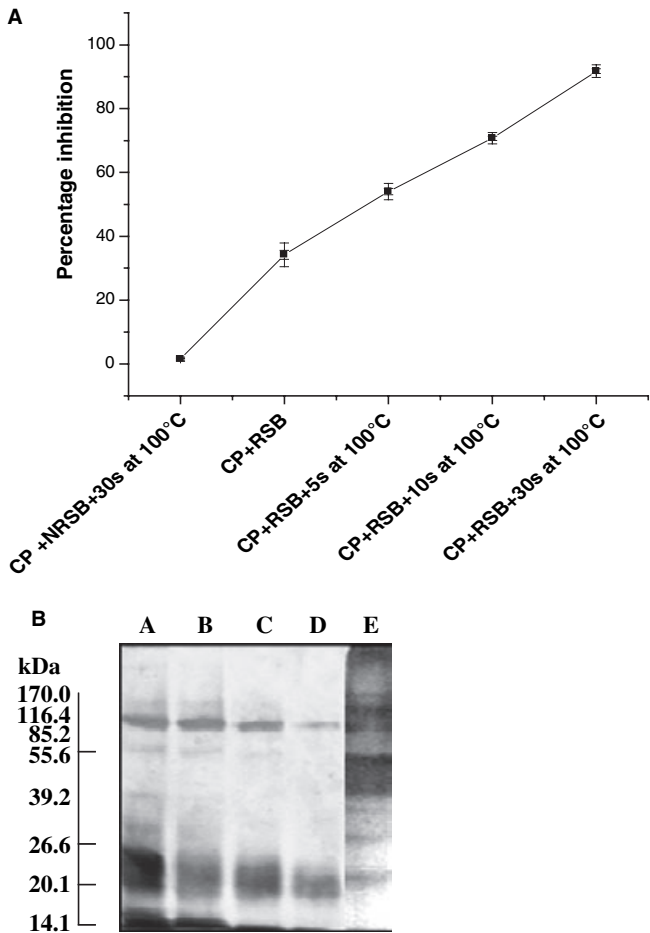


Figure 2. (A) Immunoglobulin E enzyme-linked immunosorbent assay (IgE-ELISA) inhibition of *Carica papaya* pollen-sensitive sera. The inhibitor proteins were used at a final concentration of 100 µg/ml and separately pretreated as follows: 1, *Carica* pollen extract + nonreducing sample buffer (NRSB) + 30 s heating at 100°C; 2, *Carica* pollen extract + RSB without heating; 3, *Carica* pollen extract + RSB + 5 s heating at 100°C; 4, *Carica* pollen extract + RSB + 10 s heating at 100°C; 5, *Carica* pollen extract + RSB + 30 s heating at 100°C; (B) 11% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *C. papaya* pollen extract. The extracts in different lanes were pretreated as follows: A, *Carica* pollen extract + RSB without heating; B, *Carica* pollen extract + RSB + 5 s heating at 100°C; C, *Carica* pollen extract + RSB + 10 s heating at 100°C; D, *Carica* pollen extract + RSB + 30 s heating at 100°C; E, *Carica* pollen extract + nonreducing buffer + 30 s heating at 100°C.

present study, the presence of six IgE-reactive components in *C. papaya* pollen extract was confirmed by IgE-immunoblotting in nonreducing condition to avoid the loss of IgE reactivity on chemical reduction and heating. The major allergenic fraction of the relevant pollen (as found by IgE-ELISA inhibition) showed the presence of five major subfractions in RP-HPLC, among them one elicited esterase activity. This fraction contained a

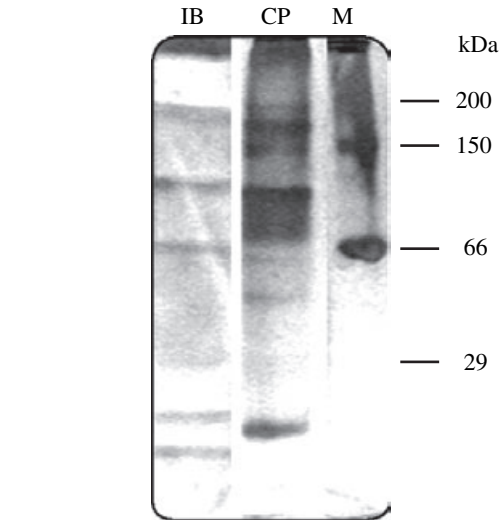


Figure 3. Immunoglobulin E (IgE)-immunoblotting of *Carica papaya* pollen extract. M, Marker; *C. papaya*, whole pollen extract in nonreducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); IB, immunoblotting of whole extract with pooled sera.

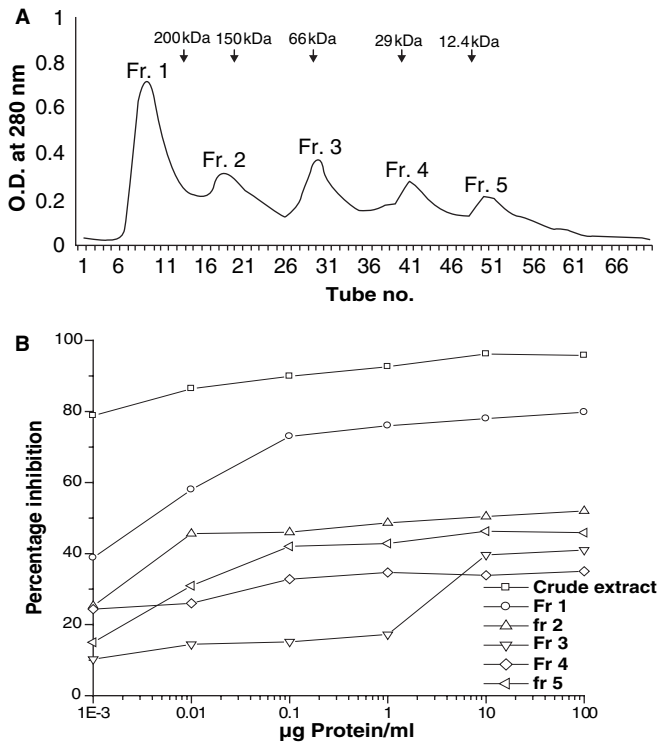


Figure 4. (A) Gel filtration of whole pollen extract of *Carica papaya* pollen in Sephacryl S-200 column. (B) Immunoglobulin E enzyme-linked immunosorbent assay (IgE-ELISA) inhibition with pooled patient sera using different fractions of *C. papaya* pollen in comparison with crude extract.

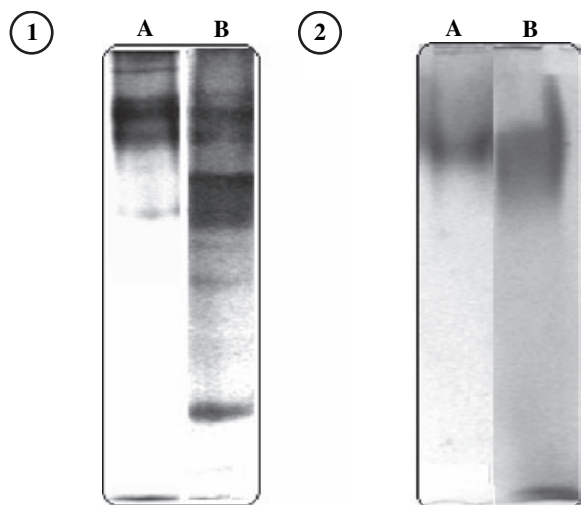


Figure 5. 1, native-polyacrylamide gel electrophoresis (PAGE); 2, activity gel for esterase activity of (A): fraction 1 of *Carica papaya* pollen and (B) whole pollen extract.

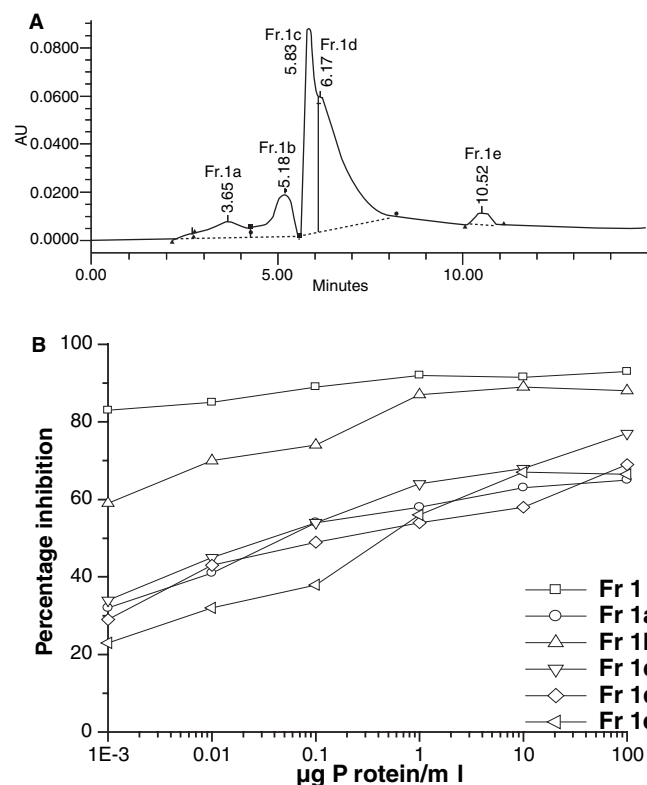


Figure 6. (A) Reverse phase high-performance liquid chromatography (RP-HPLC) analysis of fraction 1 in Novapak C-18 column. (B) Immunoglobulin enzyme-linked immunosorbent assay (IgE-ELISA) inhibition with pooled patient sera using different subfractions of fraction 1 eluted in RP-HPLC, in comparison with fraction 1.

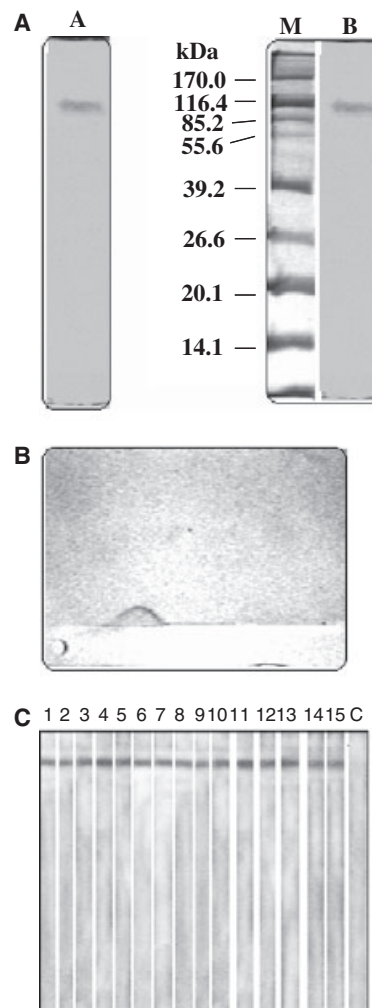


Figure 7. Native (A) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (B) of fraction 1b of *Carica* pollen. M, marker. (B) Crossed immunoelectrophoresis of fraction 1b using rabbit antisera. (C) Immunoglobulin E (IgE)-immunoblotting with isolated fraction 1b using 15 individual patient sera. C, Pooled sera from control group.

100 kDa component which was isolated, and found to be homogenous in CIE. Lastly, by IgE-ELISA inhibition and immunoblotting with 15 sensitive patient sera, from a population of 63 *Carica*-sensitive patients, the component was evident as IgE reactive.

Allergenic components may have some biological functions in addition to their IgE-binding capacity. Different reports on aeroallergens showed that major allergens of dust mite (Der p 1), (17) fungal spores like *Penicillium* (Pen ch 13) and *Aspergillus* (Asp fl 13), (18, 19) pollen grains of ragweed (20) and grasses (21) are proteases. Similarly, Phl p 5b from timothy grass pollen is a ribonuclease (22), Bet v 5 from birch pollen is an isoflavone reductase (23). The most important study showed that Bet v 8, a 65 kDa cross-reactive allergen from *Betula* pollen, is a pectin esterase (24).

In papaya, other enzymes-like papain (25, 26) and chymopapain (27) had been found to cause allergy in occupational environment. In this respect, the evidence of an allergenic component of 100 kDa with esterase activity is recorded for *C. papaya* pollen for the first time.

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