

Isolation and characterization of two IgE-reactive proteins from *Azadirachta indica* pollen

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Abstract

Two allergenically active components present in the *Azadirachta indica* whole pollen extract have been isolated by sequential ammonium sulfate precipitation (0–90%), DEAE-Sephadex A-50 ion-exchange chromatography followed by gel filtration through Sephadex G-200. The allergenicity of fractionated materials has been tested by skin prick test and ELISA inhibition which reveal that AI_aI and AI_aIVb are the major allergens. Immunoblot confirms the IgE-binding activity of the proteins. Although both fractions are found to be homogeneous by SDS-PAGE, isoelectric focusing produces more than one isoelectric point in AI_aI (pI = 3.15, 3.3 and 3.5) and AI_aIVb (pI = 6.0 and 6.2). Amino acid analyses of the two allergens, the effect of pH on them and cross-reactivity between them have been discussed. (Mol Cell Biochem **131**: 87–96, 1994)

Key words: allergens, *Azadirachta indica* pollen, phadezym RAST

Introduction

Several pollen allergens causing atopic allergies and rhinitis had already been reported and some of them are internationally standardized [1–3] according to the recommendation of World Health Organization for international standard preparations [4]. *Azadirachta indica* (neem) tree being of Indian origin grows wild in the dry forests of the deccan and is now cultivated all over India. It is also widely distributed throughout the Indo-Malayan region and in the open scrub forests of the dry zone of Burma and tropical Africa [5]. The seasonal (March–July) prevalence of neem pollen in the air was documented in the aerobiological survey report [6] and was only 1.5% among the air born pollen. The prelimi-

nary study with *A. indica* antigen whole pollen extract AI_a-WPE) has demonstrated that neem pollen induces antigen-specific IgE-mediated bronchial asthma [7]. In order to standardize the major allergens in AI_a-WPE to obtain an 'in-house' reference preparation of extract, isolation, characterization and identification of the allergens are necessary.

The present study describes the isolation of the allergenic protein from *A. indica* pollen using ion-exchange chromatography and the antigenicity to the serum IgE antibody as allergenicity index. Partial characterization of two allergenic fractions are also included herein.

Materials and methods

DEAE-Sephadex A-50, glutaraldehyde, bovine serum albumin (BSA), benzoylated dialysis tubing, Tween 20, goat-antihuman IgE, horseradish peroxidase Type VI, *o*-phenylenediamine and protein markers were purchased from Sigma Chemical Co., U.S.A. Sephadex G-200 was the product of Pharmacia Fine Chemicals AB, Sweden. Other reagents used were of highest analytical grade. U-bottom 96-well PVC immunoassay microplates were purchased from Flow Lab., U.K.

Isolation and fractionation

Matured flowers of *A. indica* were collected from a local garden, dried in an incubator at 37° C and crushed. Pollens were collected from the resulting mass after sieving through 300, 200 and 100 µm mesh, successively in a sieve-shaker. Purity of the pollen was examined [8] and found to be 98.5%, plant parts 1.2%, foreign pollens 0.1% and foreign particles 0.2%. The pollen (16 g), after defatting with diethyl ether, was extracted with 0.15 M phosphate buffered saline (PBS), pH 7.8 (1:30 w/v) by continuous stirring at 4° C overnight. The extract after being freed from debris by filtration was clarified in a Sorvall RC-5B refrigerated centrifuge at 10,000 rpm for 1 h. The supernatant designated AI_a-WPE was made 90% saturation with solid (NH₄)₂SO₄, stirred for 2 hr and centrifuged at 10,000 rpm for 1 h. The precipitate after being dissolved in PBS was dialyzed against distilled water for 48 hr in cold. The dialyzed material was freeze-dried (259 mg) and stored at – 20° C under desiccation. AI_a-WPE (50 mg in 1 ml 0.05 M Tris-HCl buffer, pH 7.8) was fractionated by ion-exchange chromatography on a DEAE-Sephadex A-50 column (2 × 40 cm), pre-equilibrated with Tris-HCl buffer at 25° C. The protein fractions were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer and finally, the column was washed with the same buffer containing 1 M NaCl. The eluted fractions were monitored by measuring the absorbance at 280 nm. The separated peaks designated AI_I, AI_{II}, . . . AI_V were separately pooled, dialyzed against distilled water and freeze-dried. Two highly SPT-sensitive fractions AI_I and AI_{IV} were separately passed through Sephadex G-200 (2 × 90 cm) column and eluted with Tris-HCl buffer, whereby the later resolved into two fractions, viz., AI_a IVa and AI_a IVb.

Protein estimation

The protein content of the fractions was estimated according to the method of Lowry *et al.* [9] using BSA as the standard.

Skin prick test (SPT)

AI_a-WPE and each of the isolated fractions were made solution (1.5 mg protein/ml) in PBS-glycerol (1:1 v/v) and sterilized by Millipore filtration (0.22 µm). The patients were prohibited from using antihistamine, steroid and ephedrin for 48 hr before SPT which was performed *in vivo* by placing 10 µl of each allergen solution 5 cm apart on the volar surface of the forearm of the patients and each site was then pricked with a disposable hypodermic needle (No. 26). The skin reactions were measured after 20 min and graded -ve and 1+ to 4+ as compared with a negative control (PBS-glycerol solution) and a positive control (histamine 1 mg/ml) [10] respectively.

Human sera

The reaginic sera were obtained from 70 neem-positive atopic patients attending at National Institute for Allergy and Asthma Research (NIFAAR), Calcutta, based on clinical history and positive skin reactions to AI_a-WPE. Control sera were collected from two non atopic subjects. The sera were stored at – 50° C in small aliquots prior to use.

Phadezym RAST

The sera were tested for AI_a-WPE specific IgE by phadezym RAST (Pharmacia Diagnostics, Sweden). AI_a-WPE was coupled with CNBr activated paper discs, as described by Ceska and Lundqvist [11]. Briefly, 1 gm of Whatman 50 paper disc (Whatman Ltd., England), 6 mm in diameter, was activated with 2 gm of CNBr and subsequently coupled with AI_a-WPE (100 mg in 2 ml borate buffer, pH 8.5) at 4° C overnight. Individual patient-serum (50 µl) was incubated with AI_a-WPE-coupled paper disc for 24 hr at 4° C. The amount of neem-specific IgE bound to the disc was estimated by enzyme substrate reaction according to Wide *et al.* [12] with phadezym RAST reagents (Pharmacia Diagnostics AB,

Sweden) and the results were scored 0 to 4 + and also as protein RAST unit per milliliter (PRU/ml) according to the manufacturer's instructions.

ELISA

The IgE-binding capacity of isolated fractions was tested by direct ELISA according to the method of Voller *et al.* [13]. Briefly, 10 µl of each fraction was diluted to

Table 1. Clinical characteristics of the investigated patients

Pat	Symptoms ^a	PEFR ^b (%)	SPT						Phadezym RAST ^c	
			AI _s -WPE	AI _s I	AI _s II	AI _s III	AI _s IVa	AI _s IVb	PRU/ml	Class antibody
1	A	73.3	++	++	-	-	-	+++	1.61	2
2	R	86.8	++	+	-	-	-	+++	0.48	1
3	A	68.7	++	++	-	-	-	++	0.51	1
4	A,R	63.8	++	++	-	-	-	+++	0.56	1
5	A	60.1	+++	++	+	-	-	+++	3.58	3
6	A,R	66.1	++	++	-	+	+	++	1.87	2
7	R	63.7	+++	++	+	+	-	+++	2.74	2
8	A,R	68.4	++	++	+	-	+	++	0.59	1
9	A,R	75.5	++	++	+	-	-	++	0.48	1
10	A	80.6	++	-	+	-	-	+++	0.62	1
11	A	64.7	+++	++	-	+	+	+++	2.56	2
12	A	72.6	+++	++	-	-	-	++	3.09	2
13	A,R	75.3	++	++	+	-	+	+++	2.83	2
14	A,R	77.6	++	-	+	+	-	+++	0.64	1
15	R	72.6	++	++	+	-	-	++	2.75	2
16	A	61.3	+++	++	-	+	+	+++	3.82	3
17	A	66.1	+++	++	+	-	-	++	2.88	2
18	A,R	67.7	++	+	+	-	-	+	0.50	1
19	A	64.9	++	++	+	-	+	+++	2.78	2
20	A,R	62.2	++	+	-	-	-	++	0.66	1
21	R	75.3	++	-	-	-	-	++	0.59	1
22	A	59.9	++	++	+	+	+	+++	3.69	3
23	A	68.1	++	++	-	+	-	+++	2.99	2
24	A,R	66.3	+	+	-	-	-	++	0.65	1
25	A	78.7	++	++	+	-	+	+++	0.68	1
26	R	67.5	+	+	+	-	-	++	0.57	1
27	A	64.7	+	+	-	+	-	-	0.58	1
28	A	64.9	++	++	+	-	+	+++	2.81	2
29	A	71.2	++	+	-	-	-	++	0.52	1
30	A	70.6	++	++	-	+	-	+++	2.77	2
31	R	81.9	++	++	-	-	-	+++	0.64	1
32	R	75.3	++	-	-	-	-	++	0.57	1
33	A	71.6	+++	++	+	+	+	+++	2.75	2
34	A	75.3	+	+	-	-	-	++	0.57	1
35	A	72.5	+	+	+	-	+	+	0.59	1
36	A	68.9	+	-	-	-	-	+	0.52	1
37 (NHS) ^d	Nil	97.4	-	-	-	-	-	-	0.27	0
38 (NHS)	Nil	98.5	-	-	-	-	-	-	0.18	0

Pat, Patient

^a Symptoms associated with the patients on neem pollen exposure; A, asthma; R, allergic rhinitis;

^b PEFR of the patients in lit/min; % of the predicted value was calculated from the standard PEFR values of the respective subjects;

^c Phadezym RAST unit per milliliter (PRU/ml) and class antibody in patient serum was determined using reference birch pollen sera and allergen discs (Pharmacia Diagnostics, Sweden) per manufacturer's directions; antibody score for class 1 (0.35–0.70 PRU/ml); class 2 (0.70–3.50 PRU/ml) and class 3 (3.50–17.5 PRU/ml);

^d Non-specific binding of normal human serum (NHS) with allergen disc.

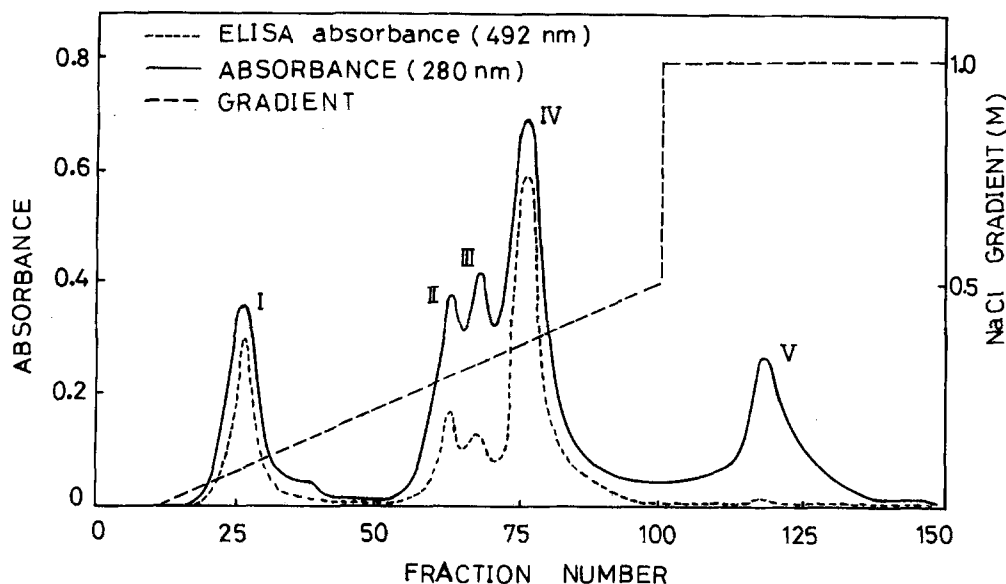


Fig. 1. Isolation of allergens by ion-exchange chromatography. Ninety percent $(\text{NH}_4)_2\text{SO}_4$ fraction of Al_a -WPE was loaded on a DEAE-Sephadex A-50 (2×40 cm) column and eluted with 0.05 M Tris/HCl. The fractions (12 ml/h; 4.5 ml each) were monitored for absorbance at 280 nm (—). A linear salt gradient of 0–0.5 M NaCl and then 1 M NaCl were applied (---). The peaks were designated as Al_a , Al_aII , ... Al_aV . Dotted line (---) illustrates the IgE binding capacity determined by direct ELISA.

100 μl with 10 mM carbonate buffer (pH 9.6) was coated on PVC plate wells by overnight incubation at 4° C. After washing with PBS – T (PBS containing 0.05% Tween 20, pH 7.4) followed by 1% BSA at 37° C for 1 h, 100 μl of reaginic sera (diluted 1:10) were added to each well and incubated overnight at 4° C. The wells after washing were incubated with 10 μl of goat-antihuman IgE (1:100 dilution with PBS containing 0.05% BSA) coupled with horseradish peroxidase [14] for 3 hr and finally, with 100 μl of *o*-phenylene-diamine (1 mg/ml in 0.05 M ci-

trate – phosphate buffer, pH 5.0) for 30 min in the dark at 25° C and the absorbance was measured at 492 nm in a Titertek Multiskan Plus MK II ELISA reader (Flow Lab., UK). Each incubation step was followed by 4×5 min washing with PBS – T.

ELISA – inhibition

Sera of 5 patients with high PRU/ml values (from phade-

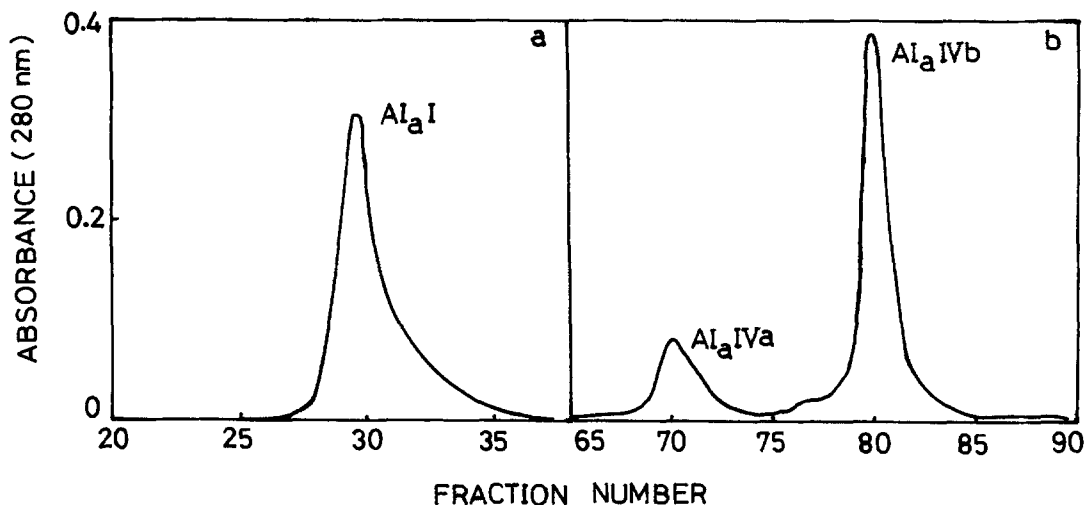


Fig. 2. Separation of allergens Al_aI (a) and Al_aIV (b) on Sephadex G-200 column (2×90 cm) eluted with 0.05 M Tris/HCl buffer (pH 7.8, 10 ml/h; 5 ml each). The fractions were measured at 280 nm.

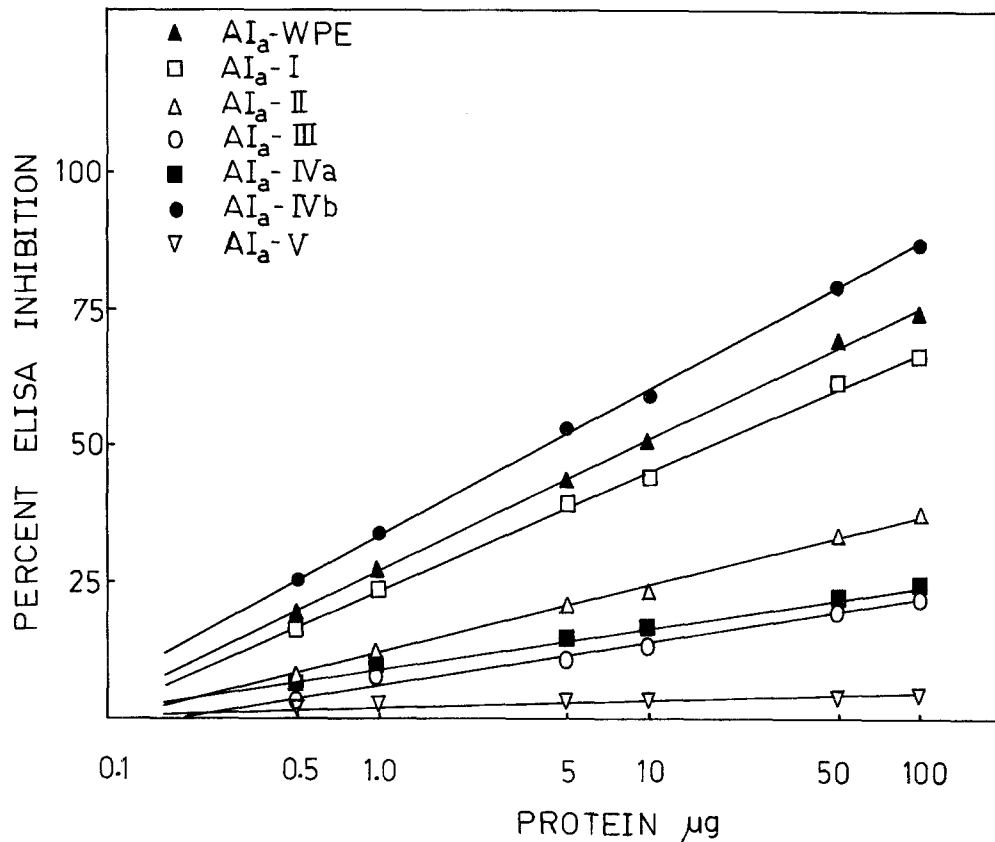


Fig. 3. ELISA-inhibition studies of the allergen fractions on specific IgE binding to solid-phase AI_a-WPE. Each point is the mean of four determinations.

zym RAST) were pooled for ELISA inhibition study. 50 µl reagenic serum pool was incubated separately with 0.5 to 100 µg of isolated fractions and was kept for 24 h at 4° C prior to incubation with AI_a-WPE coated wells of PVC plate. The successive steps were same as those described for ELISA. For ELISA-reference values instead of inhibitors only PBS-T was used. ELISA inhibition values were expressed as percent of ELISA reference value. The amount of allergenic protein required for ELISA inhibition was calculated from semilogarithmic graph plotted with percent inhibition versus protein concentration.

Peak expiratory flow rate (PEFR)

PEFR of each patient was measured (lit/min) with a Peak Flow Meter (Health Scan Products, Inc. N.J., U.S.A., during the first visit of the patients and was then calculated as % of the predicted value of the respective patient.

Immunoblotting

SDS-PAGE of AI_a-WPE, AI_aI and AI_aIVb as being found highly sensitive by SPT was performed in 12% polyacrylamide gel using alkaline buffer (pH 8.9) for 4 hr at 30 mA [15]. The protein bands were visualized by staining with coomassie brilliant blue. In a parallel experiment the separated protein bands were electrophoretically transferred from the gel to nitrocellulose membrane (NCM) at a constant current of 190 mA for 18 hr with help of a Bio-Rad transblot cell (Bio-Rad Lab, Richmond, Calif.) [16]. NCM was incubated with 1% BSA in TBS-T (20 mM Tris containing 0.5 M NaCl, pH 7.5) at 37° C for 1 h, followed by allergic serum pool (1:1 dilution with 1% BSA in TBS) overnight at room temperature in a horizontal shaker and subsequently washing with TBS-T was incubated for 3 hr at 37° C with goat antihuman IgE horse-radish peroxidase conjugate [14] diluted 50-fold with TBS containing 1% gelatin. Binding of the enzyme conjugate to allergenic protein bands was observed after addition of 4, chloronaphthol and hydrogen peroxide.

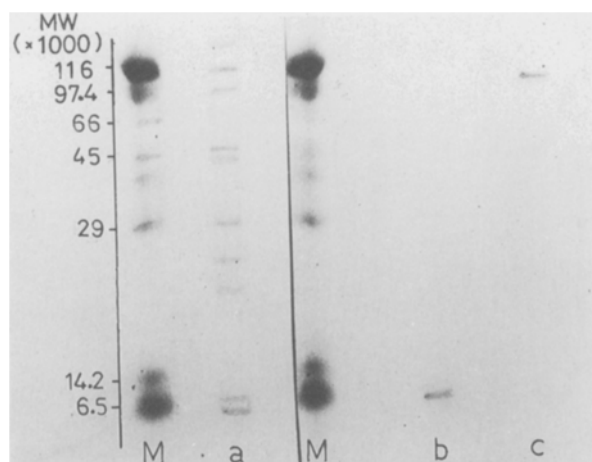


Fig. 4. SDS-PAGE of the allergens. (a) AI_a -WPE, (b) AI_a IVb and (c) AI_a I; Protein markers (M): β -galactosidase (M_r , 116,000), phosphorylase b (97,400), BSA (66,000), egg albumin (45,000), carbonic anhydrase (29,000), α -lactalbumin (14,200), Aprotinin (6,500).

Molecular weight

The molecular weight of AI_a I and AI_a IVb was determined according to Andrews [17] on Sephadex G-200 column (2×90 cm) calibrated with standard proteins of known molecular weights; urease (480,000), β -amylase

(200,000), alcohol dehydrogenase (150,000), BSA (66,000), carbonic anhydrase (29,000), cytochrome C (12,400) and aprotinin (6,500).

Isoelectric focusing (IEF)

IEF was performed according to the method of Malik and Berrie [18] using 5% polyacrylamide gel containing 2% carrier ampholytes (pH 3–10).

Cross reactivity

Allergenic cross reactivity between AI_a I and AI_a IVb was measured by ELISA-inhibition. AI_a IVb coated plate was used as the immobilized allergen and varying concentrations of AI_a I (0.1 to 50 μ g/ml) were used as inhibitors.

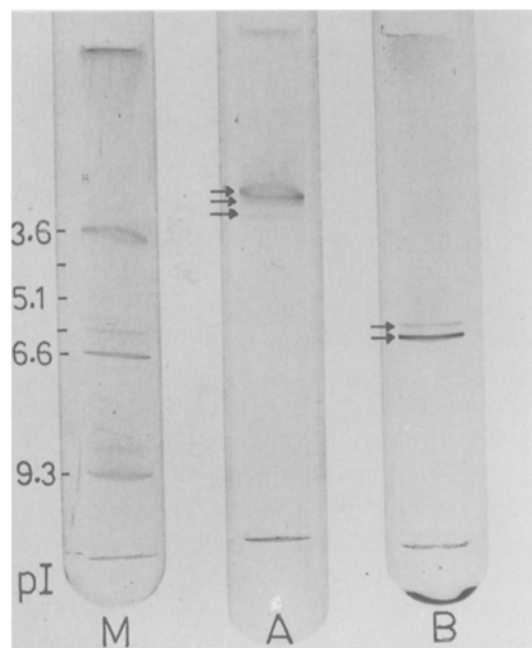


Fig. 5. IEF (pI gradient 3–10) of AI_a I (A) and AI_a IVb (B); Marker (M): amyloglycosidase (pI 3.6), trypsin inhibitor (4.6), β -lactoglobulin (5.1), carbonic anhydrase II (5.9), Carbonic anhydrase I (6.6) and trypsinogen (9.3).



Fig. 6. Immunoblot of allergens. Fractions that bind IgE in allergic serum pool were detected by enzyme-immuno reaction, as described in Materials and methods. Normal serum (A); IgE-binding from allergic serum pool (B); (a) AI_a -WPE, (b) AI_a I and (c) AI_a -IVb.

Effect of pH

AI_aI and AI_aIVb (100 µg/ml) were made into solutions at different pH (0.2 M citrate-phosphate buffer, pH 3.5–7.0; 0.2 M Tris-HCl buffer, pH 7.5–9.0 and glycine-NaOH buffer pH 9.5–10.5). ELISA was performed to ascertain their allergenicity at different pH.

Amino acid analysis

Amino acid analyses were performed by separately hydrolyzing AI_aI (4 mg) and AI_aIVb (4.5 mg) with 6 M HCl for 24 h and analyzing the hydrolyzates by h.p.l.c. on a PICO-TAG C₁₈ reverse phase column [19] as their isothiocyanate derivatives. Tryptophan was determined separately on unhydrolyzed allergens by spectro-photometric method [20].

Results

Occurrence of neem sensitivity

The clinical characteristics of the investigated patients were shown in Table 1. The initial screening of patients

Table 2. Amino acid composition of AI_aI and AI_aIVb

Amino acid	Allergens			
	AI _a I		AI _a IVb	
	Wt %	Mole %	Wt %	Mole %
Asp	6.22	9.57	5.34	8.01
Glu	11.26	16.08	6.36	9.52
Ser	5.43	6.98	8.12	12.44
Gly	7.99	8.89	6.99	10.56
Hist	0.34	0.40	0.48	0.75
Arg	5.17	7.56	1.59	2.64
Thr	4.11	6.60	4.43	6.51
Ala	7.55	7.37	9.39	12.82
Pro	4.68	5.36	3.18	3.77
Tyr	2.38	2.58	1.93	2.45
Val	3.98	4.88	5.84	7.83
Met	1.47	1.72	1.86	2.26
Cys	0.69	0.69	1.07	1.04
Ile	3.39	4.69	1.71	2.36
Leu	4.19	5.45	3.74	5.09
Phe	3.27	4.21	1.47	1.79
Lys	4.13	2.77	5.91	4.53
Trp*	5.99	4.18	7.89	5.63

* Determined separately according to Goodwin and Morton [20].

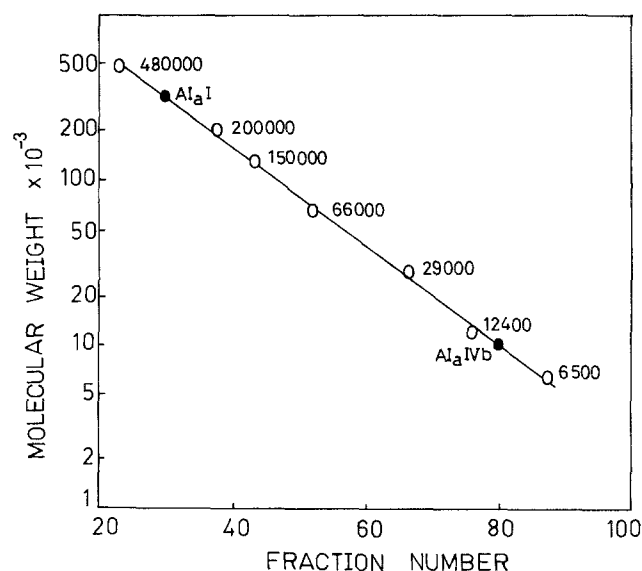


Fig. 7. Determination of the molecular weight of AI_aI and AI_aIVb. Each allergen (0.5 ml, 12 mg/ml in the eluent) was applied separately to Sephadex G-200 and eluted with 0.05 M Tris/HCl bufer (pH 7.8), 10 ml/h, 5 ml each). Elution volumes were determined from the position of maxima of the elution profiles.

was done on the basis of clinical history and the results of SPT and phadezym RAST. Out of 70 selected atopic patients (skin positive to neem) only 36 (51.4%) were found to have neem – specific serum IgE as shown in Table 1. The rest with phadezym RAST class antibody zero were not shown in the Table. Among those 36 patients 20 were found to have phadezym RAST class 1 antibody, 13 have class 2 antibody and the remaining 3 have class 3 antibody. PEFR of those patients were found to be from 59.85% to 86.76% of their respective predicted values. Among those, 20 were found to suffer from asthma, 7 from allergic rhinitis and 9 from both.

Fractionation and characterization of allergens

The AI_a-WPE was fractionated by ion-exchange chromatography on DEAE-Sephadex A-50 column whereby five fractions were obtained. The first four peaks designated AI_aI to AI_aIV were eluted with a linear salt gradient of 0–0.5 M NaCl in 0.05 M Tris buffer and the last peak, AI_aV, was eluted with buffer containing 1 M NaCl.

The direct IgE ELISA performed on the isolated fractions indicated that AI_aI and AI_aIV had strong IgE binding reactivity whereas AI_aV did not show any allergenic activity (Fig. 1). When AI_aI and AI_aIV were further passed through Sephadex G-200 separately, the former

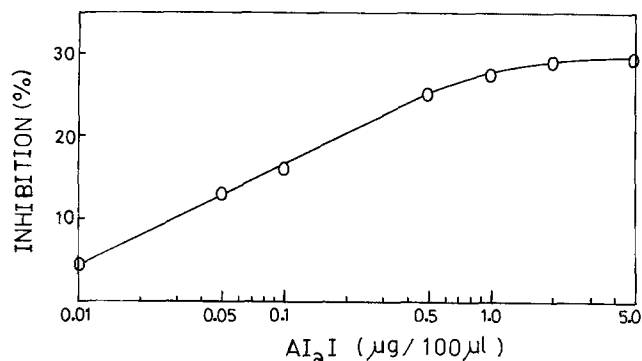


Fig. 8. Allergic cross-reactivity between AI_aI and AI_aIVb. ELISA-inhibition was performed with AI_aI as inhibitor and wells coated with AI_aIVb. Each point is the mean of four determinations.

resolved into a single peak (Fig. 2a) whereas the later into two – AI_aVIa and AI_aIVb (Fig. 2b).

The results of ELISA inhibition revealed that both AI_aI and AI_aIVb had higher inhibitory capacity and required 16.3 and 4.25 µg protein respectively, to produce 50% ELISA inhibition (Fig. 3). The other fractions could not show 50% inhibition even with 100 µg protein.

SPT performed on 36 neem-sensitive patients revealed that, 31 (86.11%) were skin positive to AI_aI, 17 (47.22%) to AI_aII, 10 (27.78%) to AI_aIII, 11 (30.56%) to AI_aIVa and 35 (97.22%) to AI_aIVb. Among two normal human subjects none was found to be skin sensitive to the above fractions.

The SDS-PAGE of AI_a-WPE demonstrated a number of bands (Fig. 4), whereas AI_aI and AI_aIVb resulted a single band in each case and were found to be homogeneous (Fig. 4). However on IEF AI_aI produced three bands of pI values 3.15, 3.30 and 3.50 respectively and AI_aIVb yielded two bands of pI 6.0 and 6.2 respectively (Fig. 5). After transfer of the separated proteins to NCM and subsequent immunoblot with serum pool, only 5 protein bands were observed to possess IgE binding capacity (Fig. 6). A negative control immunoblot with normal human serum pool did not demonstrate any band. AI_aI and AI_aIVb were found to have molecular weights of 330 kD and 10 kD respectively, as determined by gel filtration (Fig. 7).

Amino acid analysis of AI_aI was characterized by a high content of acidic amino acids, serine, glycine, alanine, proline, valine and leucine with a little amount of histidine and relatively moderate amount of other amino acids. AI_aIVb contains high amount of serine, glycine, alanine and valine; relatively moderate amount of acidic amino acids, threonine, proline, leucine, lysine, tryptophan and a low amount of tyrosine, methionine, cysteine, isoleucine and phenylalanine (Table 2).

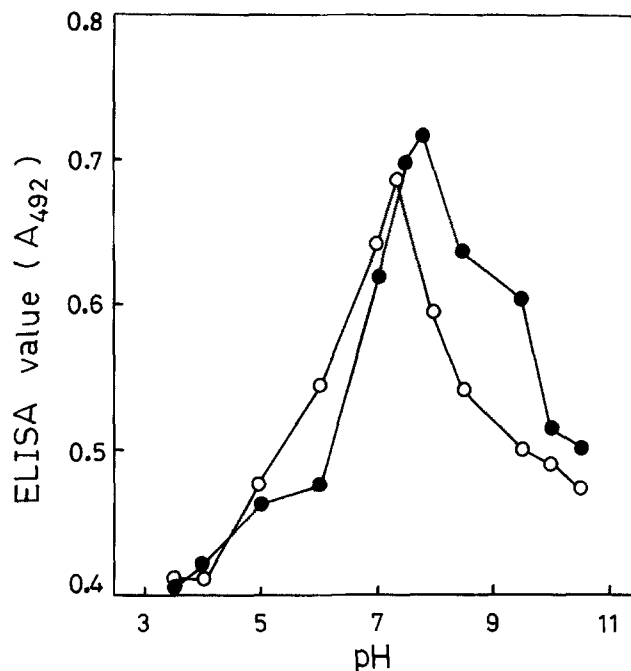


Fig. 9. Effect of pH on AI_aI (●) and AI_aIVb (○).

Figure 8 demonstrated that AI_aI and AI_aIVb showed 28% allergic cross-reactivity. From Fig. 9 the highest IgE-specific reactivity of AI_aI was found to be at pH 7.8 whereas that for AI_aIVb was at pH 7.4.

Discussion

In the present study, out of 70 selected patients 36 (51.4%) demonstrated the presence of *A. indica* – pollen allergen – specific IgE, as tested by phadezym RAST, and most of them were under class 1 or class 2 antibody. These patients also exhibited a positive SPT, much lower PEFR than their respective predicted values (59.85% to 86.76%) and suffered either from asthma or allergic rhinitis or from both. These results suggest IgE-mediated sensitization is due to *A. indica* pollen.

To maximize the yield of AI_a-WPE, multiple pilot experiments were conducted for extraction, by using small quantities of the pollen, with different buffers in different proportions with variation of time. Keeping in view the maximum yield of AI_a-WPE on the basis of protein, the *A. indica* pollen was extracted overnight with 0.15 M PBS, pH 7.8. This could allow the proteolytic digestion of the naturally occurring high molecular weight allergens by the enzymes solubilized from the same pollen membranes and resulted in low molecular weight protein molecules [21]. Hence, PMSF, a proteolytic inhib-

itor, that prevented the formation of such small molecules was used.

To identify and characterize the proteins responsible for allergenicity in *A. indica* pollen, the AI_a -WPE was fractionated by combination of ion-exchange on DEAE Sephadex and gel filtration on G-200. AI_a -WPE resolved into five peaks on DEAE Sephadex. The first four were eluted with a linear salt gradient of 0–0.05 M NaCl in 0.05 M Tris/HCl buffer. One additional peak was eluted with buffer containing 1 M NaCl (Fig. 1) and contained a lot of pigment. There was much shrinkage of gel at this high salt concentration. The brown pigmented material in the extract was firmly bound to the gel and disturbed the separation of proteins. Hence it was considered important to precipitate AI_a -WPE with 90% ammonium sulfate before to DEAE Sephadex A-50 column. This is not only reduced the pigments in the extract but also improved the resolution of proteins on DEAE Sephadex A-50 column. The brown pigments which still remained strongly bound to the gel could be eluted to a great extent with 7% acetic acid in Tris/HCl buffer. However, they were not found to possess any allergenic activity.

The two important allergenic peaks AI_aI and AI_aIVb (Fig. 1) were further purified by gel-filtration. The AI_aI was resolved into a single peak and AI_aIV into two peaks viz., AI_aIVa and AI_aIVb , the later having (Fig. 2) the major allergenic activity. The significant inhibition (> 50%) was achieved by AI_a -WPE, AI_aI and AI_aIVb whereas other fractions had poor inhibitory capacity (Fig. 3). The allergenicity of the isolated fractions was further judged by SPT whereby AI_aI and AI_aIVb were found to be more sensitive than the other fractions (Table 1).

In general, the above results showed that AI_aI and AI_aIVb are the significant allergenic proteins in AI_a -WPE and are homogeneous by SDS-PAGE (Fig. 4). AI_aI is a high molecular-weight protein ($\approx$ 330 kD) whereas AI_aIVb is of low molecular weight ($\approx$ 10 kD) (Fig. 7). On IEF analysis, AI_aI demonstrated three protein bands between pI 3.15–3.50 and AI_aIVb showed two bands between pI 6.0–6.2 (Fig. 5). Twentyeight percent allergenic cross-reactivity between AI_aI and AI_aIVb (Fig. 8) indicates that AI_aI and AI_aIVb despite being different proteins, have 28% common allergenic determinants or epitopes. Such cross-reactivity among different fractions from the same species had also been reported earlier [22, 23]. The two fractions show the highest IgE-specific reactivity nearly at same pH (Fig. 9).

Like many other allergens [23–26] amino acid analysis

of both AI_aI and AI_aIVb were characterized by a high or moderate amount of acidic amino acids, serine, glycine, lysine, threonine, alanine, proline, valine and leucine. Arginine, isoleucine and phenylalanine were also found to be present in AI_aI . Both contained a little amount of histidine, tyrosine, methionine and cystine.

In conclusion, this study demonstrated the isolation and partial characterization of two major allergens, AI_aI and AI_aIVb from *A. indica* pollen.

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