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Immunochemical characterization of *Cocos nucifera* pollen

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The Cocos nucifera pollen, as one of the sources of allergen responsible for immediate hypersensitivity reaction, was confirmed by skin prick test, bronchial provocation test, and RAST. The whole pollen extract (WPE) of C. nucifera was fractionated by combination of gel filtration and ion-exchange columns with fast protein liquid chromatography (Pharmacia, Uppsala, Sweden). Three protein peaks designated Cocos II, Cocos VI, and Cocos VII exhibited allergenic properties, as tested by skin prick test, direct IgE ELISA, bronchial provocation test, and immunoblot analysis. In RAST inhibition, Cocos IIa (a high-molecular-weight protein) obtained by fractionation of Cocos II on Mono Q column (fast protein liquid chromatography) (Pharmacia) was found to be the most potent allergen in Cocos WPE, followed by Cocos VI and Cocos VII, which are low-molecular-weight proteins. The reference patterns of Cocos WPE on crossed immunoelectrophoresis and thin-layer isoelectric focusing were established for future standardization of Cocos WPE to be used in the diagnosis and immunotherapy of allergic patients. (J ALLERGY CLIN IMMUNOL 1989;84:378-85.)

C. nucifera trees, commonly known as coconut trees, are widely cultivated in southern and eastern parts of India, Indonesia, the Phillipines, and throughout the southeast Pacific area. *C. nucifera*-pollen production is very high and is easily airborne. Therefore, it is very likely that a number of patients could become sensitized to this pollen because of continuous ex-

Abbreviations used

BPT:	Bronchial provocation test
CIE:	Crossed immunoelectrophoresis
<i>C. nucifera</i> :	<i>Cocos nucifera</i>
FPLC:	Fast protein liquid chromatography
PBS:	Phosphate-buffered saline
SPT:	Skin prick test
TLIEF:	Thin-layer isoelectric focusing
WPE:	Whole pollen extract
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS:	Tris-buffer saline
pI:	Isoelectric point

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posure in these areas. The prevalence of *C. nucifera* pollen in the air has been reported in aerobiologic survey.¹ However, there is no other study available

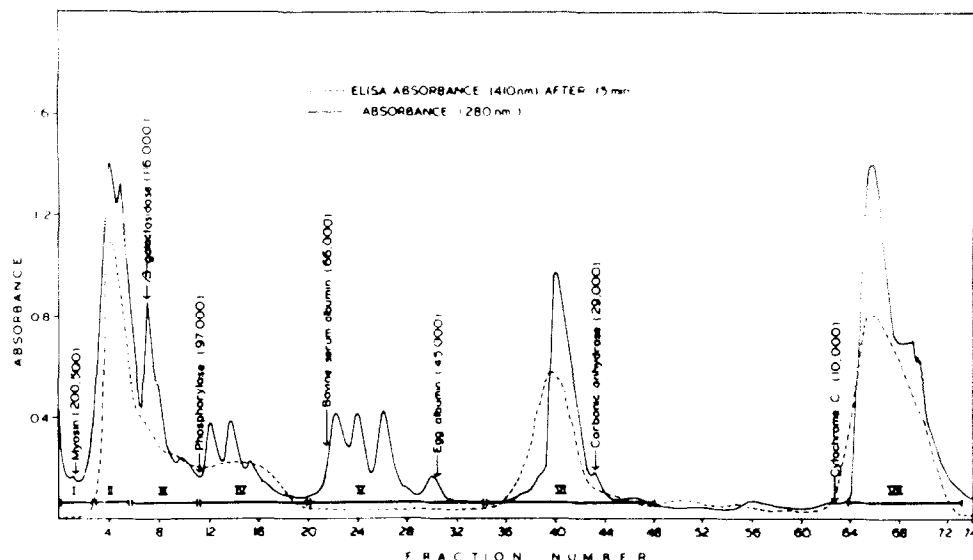


FIG. 1. Purification of *C. nucifera* WPE by FPLC on Superose 12 column with 0.125 mol/L NH_4HCO_3 as eluting buffer. The absorbance (---) of eluted protein fractions was read at 280 nm with the help of an UV monitor. The Superose 12 column was precalibrated with molecular-weight marker proteins. Flow rate, 0.5 ml/min; fraction size, 0.5 ml. The 74 fractions obtained were divided into seven pools, *Cocos* I to *Cocos* VII, as marked above. Dotted line illustrates the IgE binding capacity of 10 μl of each eluted fraction, as determined by direct ELISA.

about the incidence of *C. nucifera* pollen in the atmosphere or its allergenicity. Preliminary studies with crude *C. nucifera*-pollen extract tested in a group of patients with asthma and rhinitis, selected on the basis of history, demonstrated increased nasal bronchial and cutaneous reactivity. The present study was undertaken to confirm the allergenicity of *C. nucifera* pollen and to identify and isolate the allergenic proteins in *C. nucifera*-pollen extract responsible for immediate hypersensitivity reactions in sensitive patients.

MATERIAL AND METHODS

Preparation of WPE

Pollen grains of *C. nucifera* were collected from flowering trees and dried at room temperature. The pollen was defatted with peroxide-free diethyl ether and dried in vacuum. WPE was prepared in 0.01 mol/L NH_4HCO_3 at a concentration of 1:10 (wt/vol) by continuous stirring overnight at 4°C. The pollen debris was removed by centrifugation at 20,000 g and dialyzed through Amicon Ym 5 membrane (Amicon Laboratories, Inc., Nashville, Tenn.) (cutoff volume, 5000 daltons). The extract was filtered through 0.22 μm Millipore filter (Millipore Corp., Bedford, Mass.) and lyophilized in aliquots of known volume. The lyophilized aliquots were reconstituted as required with PBS-glycerol to the original concentration (1:10) (wt/vol) or with PBS buffer to a concentration of 1:1000 (wt/vol) for RPT.

RAST

Specific IgE to *C. nucifera*-pollen allergen was estimated by RAST assay. *C. nucifera* WPE was coupled to CNBr-

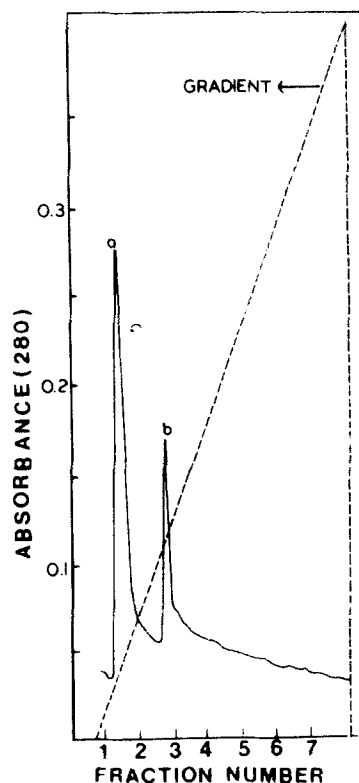


FIG. 2. Purification of *Cocos* II on Mono Q HR column, 50 by 5 mm inner diameter, starting buffer 20 mmol/L of Tris (pH 7.5); limiting buffer 20 mmol/L of Tris containing 0.7 mol/L of sodium acetate (pH 7.5); flow rate, 1 ml/min; fraction size, 1 ml. Thick solid line illustrates absorbance of eluted protein fractions at 280 nm; broken line illustrates gradient as percent of limiting buffer.

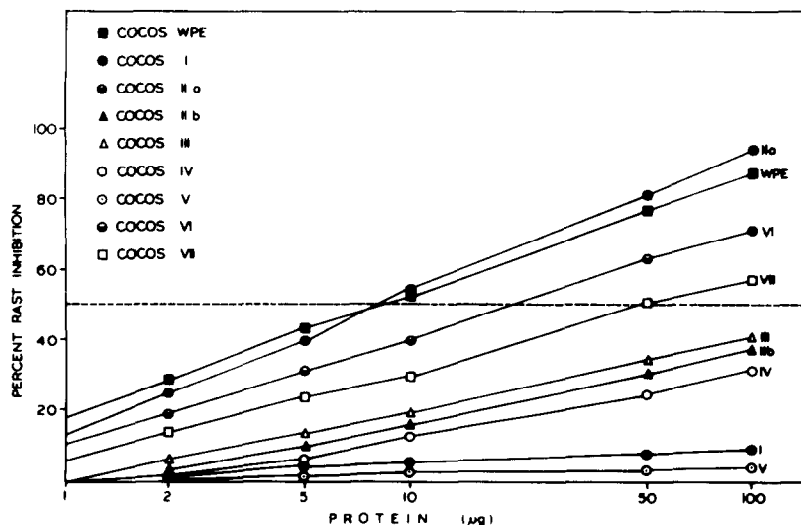


FIG. 3. RAST-inhibition studies (on fractions *Cocos* I to *Cocos* VII) of specific IgE binding to solid-phase *Cocos* WPE. Significant inhibition was achieved by WPE; *Cocos* IIa, VI, and VII fractions.

activated paper disks, as described earlier.^{2,3} Briefly, 1 gm of Whatman paper disk (Whatman, Inc., Clifton, N.J.) (6 mm in diameter) was activated with 2 gm of cyanogen bromide and subsequently allowed to couple with 200 mg of *C. nucifera* WPE in borate buffer. RAST results were expressed as Phadebas RAST units, and percent RAST binding was calculated by dividing counts per minute bound by the patient's serum IgE by counts per minute of total ¹²⁵I-labeled antihuman IgE added, multiplied by 100. Serum samples of patients with percent RAST binding values of more than eight to 10 times that of the control serum (non-allergic serum) were pooled for use in RAST-inhibition studies.

Purification of allergenic proteins

The WPE of *C. nucifera* was fractionated by gel filtration on Superose 12 column with the help of FPLC with 0.125 mol/L of NH₄HCO₃ as eluent. The eluted fractions were pooled into seven fractions named *Cocos* I to *Cocos* VII, as illustrated in Fig. 1. Molecular-weight standards were also run on the same column under similar conditions. Each pool was lyophilized and checked for its allergenic activity by RAST-inhibition test. *Cocos* II was further purified on anion-exchange Mono Q column by FPLC into *Cocos* IIa and *Cocos* IIb with 20 mol/L of Tris (pH 7.5) as a starting buffer and 20 mmol/L of Tris containing 0.7 mol/L of sodium acetate as a limiting buffer (Fig. 2).

ELISA

The IgE binding capacity of fractions obtained from Superose 12 column was determined by direct ELISA (Fig. 1), according to the procedure of Voller et al.⁴; 10 µl of each fraction was mixed with 100 µl of carbonate buffer (pH 9.6) and coated on microtiter-plate wells by overnight incubation at 4° C. After wells were washed with PBS-Tween, the free sites were blocked with 3% gelatin solution

at 37° C for 1 hour. This was followed by an overnight incubation with 100 µl of allergic serum pool (diluted 1:2). After wells were washed with PBS-Tween mixture, they were incubated for 3 hours with goat antihuman IgE conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) (1:1000 dilution with PBS containing 0.05% bovine serum albumin). Each well was washed at least three times with PBS-Tween solution and incubated with the enzyme substrate, p-nitrophenyl phosphate at a concentration of 1 mg/ml in diethanolamine buffer (pH 9.8). The absorbance of each well was measured at 410 nm with a Nunc ELISA reader (Nunc GmbH, Roskilde, Denmark) after 45 minutes.

RAST inhibition

The competitive RAST-inhibition test was performed with a serum pool of five allergic patients to check the allergenic activity of fractionated proteins, according to the procedure described earlier.² Briefly, different concentrations (2 to 100 µg) of fractionated proteins (*Cocos* I to *Cocos* VII pools) (Fig. 3) were incubated with 50 µl of allergic serum pool for 24 hours at 4° C before incubating with *Cocos* WPE allergen disk. The percent of RAST inhibition was calculated from the decrease in counts per minute of ¹²⁵I-labeled antihuman IgE bound to the allergen disk. The amount of protein required to produce 50% RAST inhibition was calculated from semilogarithmic graph plotted with percent inhibition versus protein concentration.

SPT

The SPT with *Cocos* WPE and semipurified allergenic proteins, *Cocos* IIa, *Cocos* VI, and *Cocos* VII, was performed on sensitive patients. The allergenic proteins were dissolved in PBS-glycerol solution, and PBS-glycerol was used as a negative control. Histamine (10 mg/ml) was used as a positive control. Any reaction eliciting a 3 mm wheal

TABLE II. RAST results of patients sensitive to *C. nucifera*-pollen extract

Pat No.	SPT	PRU	Class antibody	% RAST binding
1	++	1.96*	2	9.39
2	+	0.65	1	2.98
3	++	0.65	1	3.01
4	++	1.96*	2	9.39
5	++	2.33*	2	10.88
6	++	1.76	2	8.51
7	+++	2.65*	2	12.05
8	++	2.06*	2	9.79
9	++	0.79	2	3.73
10	++	1.08†	2	5.33
11	+	0.41	1	2.02
12	++	1.12†	2	5.33
13	+++	0.42	1	2.07
14	++	0.49	1	2.42
15	+	0.39	1	1.92
16	++	0.58	1	2.86
17	+++	0.46	1	2.27
18	++	0.47	1	2.32
19	++	0.76†	2	3.75
20	+	0.82†	2	4.05
21	+++	0.63†	1	3.11
22	+	0.38	1	1.86
23	+	0.40	1	1.37
24	++	0.42	1	2.07
25	+	0.42	1	2.07
26	+++	0.50	1	2.47
27 (NHS)	—	—	0	0.25

Pat, Patient; PRU, Phadebas RAST unit; NHS, normal human serum.

PRU and class antibody scores were calculated with RAST reference unit of Pharmacia diagnostics per manufacturer's directions.

*Serum pool used in RAST-inhibition.

†Serum pool used for immunoblot studies.

was considered a positive reaction. Reaction eliciting wheal size equivalent to that of the positive control was considered as 2+. Reaction eliciting a wheal size >5 mm with pseudopodia was considered as 3+ and 4+ and was labeled only to massive positive reaction double the size of the positive control.

BPT

The BPT with *Cocos* WPE and purified allergenic proteins was performed on sensitive patients with the DeVilbiss nebulizer (DeVilbiss Co., Somerset, Pa.) with normal saline as a control and as a diluent. Only qualitative change in the response was noted with concentrations of allergenic extract starting from 1:10,000 to 1:1000 (wt/vol).

Rabbit antiserum and CIE

Rabbit anti-*C. nucifera* serum was obtained by immunizing rabbits with *Cocos* WPE emulsified with Freund's complete adjuvant. The immunizing schedule was the same as that described earlier.² CIE was performed in 1% agarose

gel on a 10 by 10 cm glass plate, according to the method of Axelsen et al.⁵ Briefly, 100 µg protein of WPE was electrophoresed in first dimension for 40 minutes at 10 V/cm. The second dimensional electrophoresis was performed at 2 V/cm for 20 hours against rabbit antibody containing gels on both cathodic and anodic sides. The precipitin lines obtained were visualized by staining CIE plates with Coomassie brilliant blue solution.

Western blotting

SDS-PAGE of *C. nucifera* WPE was performed in 12% polyacrylamide gel by the Laemmli buffer system.⁶ The separated protein bands were visualized by staining with Coomassie brilliant blue solution. In a parallel experiment, for Western blotting analysis, the separated protein bands were electrophoretically transferred from the gel on to nitrocellulose membrane at a constant current of 500 mA^{7,8} for 5 hours with the help of Bio-Rad transblot cell (Bio-Rad Laboratories, Richmond, Calif.). The unreacted sites on nitrocellulose membrane were blocked by incubation of

TABLE II. Skin test results of allergenic components of *C. nucifera* on 48 sensitive patients

Pat No.	Skin test	WPE	WPE II	WPE VI	WPE VII
1	++	++	+	+	++
2	+	+	+	-	-
3	++	+	++	-	-
4	+	-	-	-	-
5	+	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	++	-	++	-	-
9	++	++	++	-	-
10	+	-	-	-	-
11	+++	++	++	-	-
12	++	++	++	-	-
13	+	-	-	-	-
14	+	-	-	-	-
15	+	-	-	-	-
16	+	+	-	-	-
17	++	+	-	+	+
18	+	-	-	-	-
19	+	-	-	-	-
20	+	-	-	-	-
21	+	-	-	-	-
22	++	+	-	-	+
23	++	-	-	-	-
24	+	-	-	-	-
25	++	++	-	++	-
26	++	++	++	++	-
27	++	-	-	-	-
28	++	++	-	++	++
29	++	++	-	+	+
30	++	-	-	-	-
31	++	++	+	-	-
32	+	-	-	-	-
33	++	+	+	-	+
34	+	+	-	-	-
35	++	+	-	-	-
36	++	-	+	-	+
37	+	-	-	-	-
38	+	-	-	-	-
39	+++	++	++	++	++
40	+	-	-	-	-
41	++	++	++	-	++
42	+	-	-	-	-
43	++	+	+	-	+
44	+	-	-	-	++
45	+	-	-	-	-
46	+	-	-	-	-
47	++	+	+	-	+
48	++	-	-	-	-

Pat, Patient.

the membrane with 3% gelatin in TBS (20 mmol/L of Tris with 0.5 mol/L of NaCl, pH 7.5) for 1 hour at 37° C. After the blocking step, the membrane was subsequently washed with TBS-Tween (1%) and incubated with allergic serum pool (1:5 dilution with 1% gelatin in TBS) overnight at

room temperature on a horizontal shaker. The membrane was further washed at least three times with TBS-Tween and incubated for another 24 hours at room temperature with goat antihuman IgE horseradish peroxidase conjugate (Tago, Inc., Burlingame, Calif.) diluted 200-fold with TBS

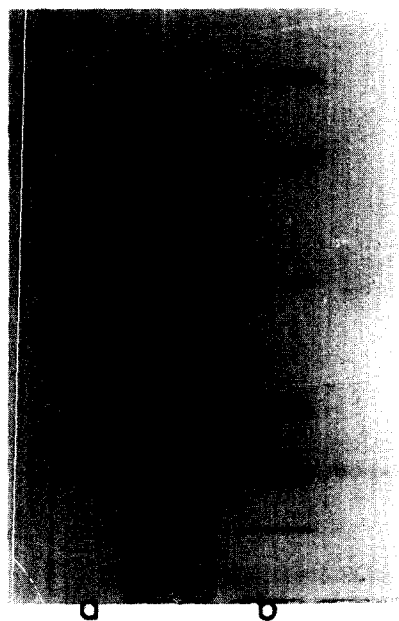


FIG. 4. SDS-PAGE analysis of *C. nucifera* WPE (a); molecular-weight markers (b). Protein bands were stained with Coomassie brilliant blue.

containing 1% gelatin. Binding of the enzyme conjugate to SDS-PAGE-separated allergenic protein bands was observed after addition of enzyme substrate 4, chloronaphthol and hydrogen peroxide on the membrane.

TLIEF

Isoelectric focusing was performed in commercially available polyacrylamide gel plates (LKB, Bromma, Sweden) in a pI gradient 3.5 to 9.5. The protein samples (125 μ g/10 μ l) were focused at 500 V, 50 mA, and at a constant power of 20 W for 2 hours. The gels were fixed with trichloroacetic acid and stained with Coomassie brilliant blue R250.

Protein estimation

Protein content was estimated according to the method of Lowry et al.⁹ with bovine serum albumin as standard.

RESULTS

Screening of sensitive patients

The initial screening of patients with reported sensitivity to *C. nucifera* pollen was done on the basis of skin test combined with clinical history. This was followed by RAST with WPE coupled to CNBr-activated paper disks as solid support. Of 41 selected patients, 26 patients (63.41%) were found to have specific serum IgE to *C. nucifera*-pollen extract (Table I).

Identification, isolation, and characterization of allergenic components

The FPLC was used in the present study to isolate the allergenic proteins from *C. nucifera* WPE. The



FIG. 5. Binding of human IgE binding from pooled sera to an immunoblot prepared from SDS-PAGE gel of *C. nucifera* WPE. Components that bind IgE in allergic serum pool were detected by enzyme-immuno reaction, as described in Material and Methods; negative control with normal serum (a); IgE binding from allergic serum pool (b).

WPE was loaded on gel-filtration column (Superose 12) under the conditions illustrated in Fig. 1. The eluted protein aliquots of the individual peak were pooled to get seven fractions, *Cocos* I to *Cocos* VII. About 77% of the total protein loaded on the column was recovered in seven pooled fractions. Since the *Cocos* II pool consisted of two closely associated protein peaks, it was further fractionated on anion-exchange Mono Q column (Pharmacia, Uppsala, Sweden) by FPLC into *Cocos* IIa and *Cocos* IIb (Fig. 2).

The direct IgE ELISA performed on the fractions eluted from Superose 12 column indicated that peaks II, VI, and VII contained IgE binding activity.

RAST-inhibition test performed on *Cocos* IIa, VI, and VII pooled fractions demonstrated that *Cocos* IIa was the most potent allergenic fraction followed by *Cocos* VI and *Cocos* VII (Fig. 3).

Of SPTs performed on 21 sensitive patients, 15 SPTs were found to be positive to *Cocos* II and seven

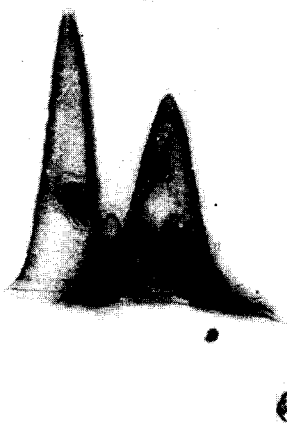


FIG. 6. CIE of *Cocos* WPE against rabbit anti-*C. nucifera* serum. Coomassie brilliant blue-stained CIE plate.

to *Cocos* VI. Eleven patients elicited positive reaction to *Cocos* VII as well (Table II).

The allergenicity of proteins present in *C. nucifera* WPE was further confirmed by Western blotting and immunoblot analysis. The SDS-PAGE of WPE demonstrated 21 Coomassie brilliant blue-stained bands (Fig. 4). After transfer of the separated proteins to nitrocellulose membrane and subsequent immunoblot with serum pool, only three protein bands were observed to possess IgE binding capacity (Fig. 5, b). A negative control immunoblot with normal human serum pool did not demonstrate any bands (Fig. 5, a).

CIE

A total of 29 precipitin lines could be demonstrated by CIE analysis of WPE with anti-*C. nucifera* rabbit antibodies (Fig. 6). All precipitins were on the anodic side. When the fractions were tested individually, the fraction *Cocos* IIa exhibited eight precipitin lines, whereas *Cocos* VI exhibited only one precipitin band. No band was visible corresponding to the *Cocos* VII fraction (results not presented).

TLIEF

With TLIEF analysis (Fig. 7), a total of 18 Coomassie brilliant blue-stained bands were visible in pI range 3.5 to 7.00. The fraction *Cocos* IIa demonstrated five bands, whereas *Cocos* VI demonstrated three bands and *Cocos* VII did not demonstrate any visible protein band at the concentration tested.

DISCUSSION

During the last two decades, much attention has been focused toward pollen-allergen extracts to gain better knowledge about their composition and im-

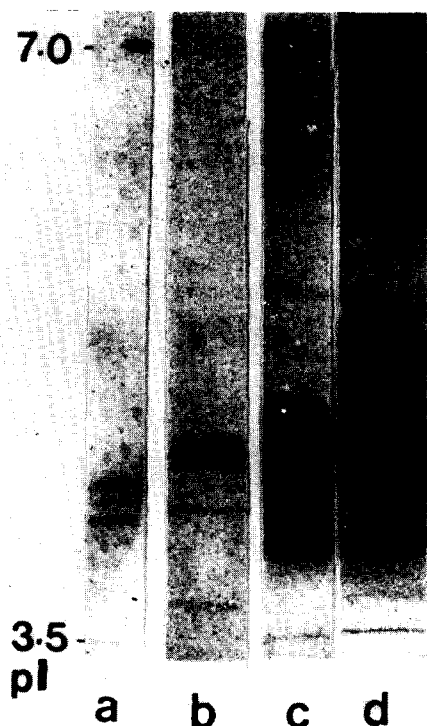


FIG. 7. TLIEF of protein in 5% polyacrylamide gel slabs (pI gradient, 3.5 to 9.5); *Cocos* IIa (a); *Cocos* VI (b); *Cocos* II (c); WPE (d).

munologic competence. Identification of these allergenic proteins is a prerequisite to the goal of allergen standardization.

Of the 41 selected patients in the present study, 26 patients (63.41%) demonstrated the presence of *C. nucifera*-pollen, allergen-specific IgE, as tested by RAST. These patients also exhibited a positive SPT and BPT. (BPT was carried out only in 14 patients.) These results suggest IgE-mediated sensitization to *C. nucifera* pollen. However, it was observed that percent RAST binding was comparatively low; most patients demonstrating sensitivity to *Cocos* WPE illustrated either class 2 or class 1 antibody by RAST. The history and the habitat of the patients did indicate that sensitization could be due to *C. nucifera* pollen. Bernstein et al.¹⁰ have reported a comparative study of RAST analysis of various tree pollens and concluded that rank order among tree allergens coincided with the ecology of the tree flora (where the patients were living). It may be possible that addition of these allergens to the immunotherapy extract could possibly improve the therapy of hyposensitization in the patients.

To identify and characterize the proteins responsible for allergenicity in *C. nucifera* pollen, the WPE of *C. nucifera* pollen was fractionated by a combination of gel filtration and ion-exchange on FPLC.

C. nucifera pollen WPE resolved into 10 peaks on gel filtration. The aliquots were pooled in seven frac-

tions. Most pigmented material passed down to the fractions VI and VII.

Reference patterns obtained on CIE exhibited 29 precipitin lines. Precipitin lines against *Cocos* VII were not visible, probably because it did not produce antibody in rabbit because of its low molecular weight. This fraction contained a lot of pigment and was not stained by Coomassie brilliant blue in TLIEF gels. The fractionation of *Cocos* WPE by gel filtration and subsequent analysis of fractionated proteins by IgE ELISA and RAST-inhibition test revealed the presence of at least three allergenic proteins. The *Cocos* IIa is a high-molecular-weight protein (between 116,000 to 200,500 daltons) (Fig. 1). *Cocos* VI is a low-molecular-weight protein (approximately 29,000 daltons), and *Cocos* VII is a very low-molecular-weight protein of <10,000 daltons (Fig. 1). These three fractions also elicited positive skin test response in sensitive patients. In RAST inhibition, 8.3, 21, and 54 μ g of *Cocos* IIa, VI, and VII, respectively, were required for 50% inhibition, whereas 8.5 μ g of WPE produced 50% inhibition when WPE was bound to solid support.

The fraction *Cocos* IIa demonstrated almost the same inhibition as WPE when WPE is used to bind on solid support. Similar studies were reported with *Artemisia scoparia*, ragweed, and rye-grass pollen.² This finding might have been due to partial denaturation or due to removal of specificity. Insufficient representation of purified fraction on solid phase could also contribute to the low potency.

The presence of three allergenic proteins in *Cocos* WPE was further confirmed by immunoblot results. A high-molecular-weight protein band and two low-molecular-weight protein bands demonstrated IgE binding capacity in immunoblot analysis. However, this does not rule out the possibility of more minor allergenic proteins in *C. nucifera*-pollen extract. The results of the SPT indicate that *Cocos* II can be considered a major allergenic protein, followed by *Cocos* VII and *Cocos* VI, respectively. There was individual variation in the skin test to the various fractions, and the best material for the skin testing and for RAST would be WPE.

The characteristic pattern of WPE obtained by gel filtration on Superose 12 column can help in internal standardization, for checking batch-to-batch variation in preparations of *C. nucifera*-pollen extract for use in diagnosis, and immunotherapy. In the present study, a reference CIE pattern of *Cocos* WPE was established by varying the concentration of antigen and rabbit antibody demonstrating a maximum total of 29 Coomassie brilliant blue-stained bands. However, CIE of *Cocos* IIa and *Cocos* VI against rabbit anti-*C. nucifera* serum demonstrated eight and one antigenic components, respectively, whereas *Cocos* VII was unable to elicit any

precipitin band. This finding could be due to low molecular weight of *Cocos* VII (<10,000 daltons). The fraction was immunogenic in man, as observed by ELISA. Possibly the lack of precipitating activity with rabbit antibody might have been the limited number of rabbits immunized or because of insufficient duration of immunization. Increased concentrations of fraction used did not exhibit visible precipitating lines. Also on TLIEF analysis, *Cocos* VII did not demonstrate any visible protein band. The exact reason cannot be explained. It could also be due to the high amount of pigmented material present.

In conclusion, the study demonstrated *C. nucifera* as one of the aeroallergens that could trigger IgE-mediated hypersensitivity reaction. The allergenic proteins contributing to allergenicity of *C. nucifera*-pollen extract have been identified and partially purified. The study revealed *Cocos* II (*Cocos* IIa), VI, and VII as the important allergenic proteins in *C. nucifera*-pollen extract.

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