

Isolation and identification of pollen allergens of *Artemisia scoparia*

Kuldeep S. Jaggi, Ph.D.,* and Sharad V. Gangal, Ph.D. Delhi, India

The allergenic proteins of Artemisia scoparia pollen were separated and identified with ammonium sulfate precipitation, ion-exchange chromatography, gel filtration, and RAST-inhibition techniques. The important allergenic component Artemisia VI b that constitutes 29% of total protein in the extract was purified to homogeneity. It was found to be an acidic protein with isoelectric point 3.8 and molecular weight of 14,300. It was rich in carbohydrate, but the carbohydrate portion did not appear to be important for allergenicity. In the crossed immunoelectrophoresis reference pattern of the whole pollen extract, 37 precipitin lines could be identified on the anodic side, whereas Artemisia VI b could be observed as a single precipitin line. Immunologically, the whole pollen extract of A. scoparia demonstrated shared antigenic and allergenic determinants with Ageratum conyzoides-pollen extract. The use of fast protein liquid chromatography in partial purification of allergenic components is also discussed. (J ALLERGY CLIN IMMUNOL 1987;80:562-72.)

About 400 species of *Artemisia* are known to be abundantly distributed in temperate regions.¹ The pollen of *Artemisia* that belongs to the family Asteraceae is reported to be prevalent in the air in India² and known to be allergenic.³⁻⁵ It has been observed that *A. scoparia* has smaller equatorial and polar axis. The exine wall is thin as compared to other *Artemisia* species such as *A. vulgaris*, *A. absinthium*, etc.⁶ that are prevalent in Western and Scandinavian countries.^{7, 8} Woodhouse⁹ observed extensive cross-reactivity among seven *Artemisia* species, but *A. scoparia* was not included in the study. Recently, Ipsen et al.¹⁰ identified 10 allergens in crude extract of *A. vulgaris* pollen of which one allergen was classified as major, five as intermediate, and four as minor allergens. Paulsen et al.¹¹ observed 18 antigens in *A. vulgaris* extract, of which seven had IgE-binding capacity in CIE. Nilsen and Paulsen¹² further partially purified and identified Ag 7 as the important allergen in *A. vulgaris* extract. The present study has been undertaken to isolate and identify the important allergenic components of *A. scoparia* extract on which

Abbreviations used

Art:	<i>Artemisia</i>
PRU:	Phadebas RAST unit
CIE:	Crossed immunoelectrophoresis
DEAE:	Diethylaminoethyl
PBS:	Phosphate-buffered saline
FPLC:	Fast protein liquid chromatography
PAGE:	Polyacrylamide gel electrophoresis
pI:	Isoelectric point
SDS:	Sodium dodecyl sulphate
TLIEF:	Thin-layer isoelectric focusing
WPE:	Whole pollen extract
FCA:	Freund's complete adjuvant

to standardize the allergen extract used in diagnosis and treatment of allergic patients.

MATERIAL AND METHODS

Preparation of WPE

Pollen grains of *A. scoparia* were collected from the flowering plants from Meerut region (near Delhi) and were dried at room temperature. The highly pure pollen (97.37% as determined according to the method of Cour and Loublier¹³) was defatted with diethyl ether and dried in vacuum. The dried pollen was extracted with PBS (0.1 mol/L, pH 7.8), 1:50 wt/vol, for 24 hours at 4° C. This was followed by centrifugation at 20,000 × g at 4° C for 45 minutes to remove particulate matter and ultrafiltration with Amicon YM5 membrane (Amicon Corp., Danvers, Mass.). The extract was sterilized by passing through 0.22 µm Millipore filter (Millipore Corp., Bedford, Mass.). The large amount of this WPE was dialyzed, lyophilized, and stored at -70° C in sealed vials.

From the Council of Scientific and Industrial Research Center for Biochemicals, Vallabhlal Patel Chest Institute, Delhi University, Delhi, India.

Received for publication Sept. 18, 1985.

Accepted for publication March 24, 1987.

Reprint requests: Sharad V. Gangal, Ph.D., CSIR Center for Biochemicals, V.P. Chest Institute Bldg, Delhi University Campus, Delhi, 110 007, India.

*Supported by Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi, India.

Ion-exchange chromatography and gel filtration

Solid ammonium sulfate was added to fresh *A. scoparia* WPE to 90% saturation level. The precipitated fraction was reconstituted in 0.05 mol/L of phosphate buffer (pH 7.3) and dialyzed against distilled water overnight at 4° C. The dialyzed protein fraction (40 mg of protein) was loaded on 1.5 by 35 cm DEAE Sephadex A-50 column equilibrated with 0.05 mol/L of phosphate buffer (pH 7.3). A linear salt gradient of 0 to 0.5 mol/L of NaCl in 0.05 mol/L of phosphate buffer was applied. The column was finally washed with buffer containing 1 mol/L of NaCl. All fractions of each resolved peak were pooled, dialyzed, and lyophilized. Further fractionation of the potent allergenic component (Art VI) was done on Sephadex G-50 column (2 by 50 cm) with 0.05 mol/L of phosphate buffer as eluent.

Total IgE

Total IgE in serum of patients sensitive to *A. scoparia* pollen was estimated with Phadezym PRIST kit of Pharmacia Diagnostics, Uppsala, Sweden.

Preparation of allergen disks

1 gm of paper disks (6 mm in diameter) punched from Whatman 50 (Whatman Inc., Clifton, N.J.) paper sheets were activated with 2 gm of CNBr (dissolved in dimethyl formamide) at pH 10 for 10 minutes. The disks were washed and dried with 0.1 mol/L of NaHCO₃ and acetone, respectively. The CNBr-activated paper disks³ were subsequently allowed to couple with 200 mg of WPE dissolved in 10 ml of borate-saline buffer (pH 8.0) for 72 hours on a horizontal shaker at 4° C. This was followed by seven to eight washings of disks with borate-saline buffer and at least four washings with RAST diluent (0.1 mol/L of K₂ HPO₄-KH₂ PO₄, 0.2% of bovine serum, albumin, 1% of Tween 20, and 0.1% of NaN₃, pH 7.4). The Art VI allergen disks were prepared similarly by coupling 50 mg of Art VI protein with 250 mg of CNBr-activated disks.

RAST

Direct RAST was performed to screen serum of patients containing allergen-specific IgE with Phadebas RAST isotope of Pharmacia Diagnostics. Aliquots of 50 µl of patient serum was incubated with antigen-coupled paper disks for 24 hours at 4° C. The amount of specific IgE bound to the disk was estimated as percent of total radioactivity bound to the disk after incubation with ¹²⁵I-labeled antihuman IgE for 24 hours at 4° C and three successive washings with normal saline containing 1% Tween 20. The RAST results were also expressed in terms of PRU by comparison of test results with RAST results of reaction of reference birch pollen-allergen disks with sera A, B, C, and D containing specific IgE in concentrations 17.5, 3.50, 0.70, and 0.35 PRU, respectively (procured from Pharmacia Diagnostics). A semilog-arithmetic graph was plotted with PRU versus counts per 120 seconds of each reference serum sample. Based on RAST results, six sera with percent RAST-binding values more than eight to 10 times that of the normal serum were pooled for RAST-inhibition studies.

RAST inhibition

A competitive RAST inhibition was used to check the allergenicity of separated protein peaks after column chromatography; 50 µl of pooled serum of sensitive patients was incubated with various dilutions of protein fractions (1 to 100 µg) for 24 hours at 4° C on a horizontal shaker. The inhibited serum pool was incubated with allergen disk for an additional 24 hours at 4° C. The percent inhibition of binding of specific IgE to allergen disk was calculated after incubation with 50 µl of antihuman ¹²⁵I-labeled IgE. The amount required for 50% inhibition was calculated from the semilogarithmic graph plotted with protein concentration versus percent inhibition.

Rabbit antisera

A group of three healthy male rabbits were immunized with WPE of *A. scoparia* injected during a period of 6 months. The dose schedule was 0.5 mg of WPE protein in FCA injected intradermally biweekly at 10 to 12 sites for 2 weeks, followed by 2 mg of protein in FCA per week intradermally for 2 weeks, and 5 mg of protein in FCA once in 15 days. When antibodies were detected by Ouchterlony immunodiffusion test, booster injections of 10 mg of protein in incomplete Freund's adjuvant were administered by intramuscular injection every month. The animals were bled from ear vein, and CIE was performed with polyvalent sera obtained by mixing an equal volume of serum from all the immunized animals. The antibody titer in the pooled antiserum was determined by quantitative precipitin technique.¹⁴

CIE

CIE was performed according to the procedure described by Axelsen et al.¹⁵ to study the number of antigenic determinants present in WPE by means of rabbit anti-*Artemisia* antibodies. CIE gels were prepared from 1% agarose (Litex, HSA, Denmark) in 0.24 mol/L of barbital buffer (pH 8.6) on 10 by 10 cm glass plates; 100 µg of protein was electrophoresed at 10 V/cm in first dimension for 40 minutes. The second dimensional electrophoresis was performed at 2 V/cm for 20 hours against antibody containing gels on both cathodic and anodic sides. The temperature was maintained at 4° C throughout the process. After the completion of experiment, the agarose gel was fixed and air-dried on the glass plates. The precipitin bands were stained with Coomassie brilliant blue R-250 solution.

TLIEF

The samples were focused in pH gradient 3.5 to 9.5 with commercially available polyacrylamide gels (LKB, Bromma, Sweden). Fifteen microliters of sample was applied, and electric current not exceeding 50 mA and 500 V was applied for 2 hours. Power was adjusted to 20 W. Gel was fixed and stained with Coomassie brilliant blue R-250.

FPLC

FPLC of the WPE was performed on a prepacked anion exchanger Mono Q column of Pharmacia Biotechnology International, Uppsala, Sweden, according to the method

TABLE I. RAST results of patients sensitive to *A. scoparia*

Patients	% RAST binding*	PRU†	Class antibody score‡	Skin test score‡	Total IgE (kU/L)
1	2.71	0.63	1	++	480
2	3.99	1.07	2	++	420
3	4.01	1.07	2	++	470
4	5.23	1.49	2	++	540
5	3.39	0.87	2	++	520
6	10.26	3.64	3	+++	330
7	2.11	0.40	1	++	540
8	22.86	14.97	3	++++	450
9	9.14	3.09	2	++	520
10	2.36	0.50	1	+	540
11	17.00	8.16	3	+++	540
12	4.77	1.33	2	++	430
13	35.08	>17.50	4	+++	895
14§	0.93	—	0	—	68

*Total radioactivity in 50 μ l of ^{125} I-labeled antihuman IgE was 41,201 counts per 120 seconds.

†Phadebas RAST unit (PRU) and class antibody score in patients' sera was determined using reference birch pollen-allergen disks and sera from Pharmacia Diagnostics.

‡Intradermal skin test results were graded as 1+ to 4+ according to the criteria laid down by Shivpuri.²¹

§Nonspecific binding of normal human serum with allergen disk.

described earlier.¹⁶ The allergenic fractions (Art III, Art V, and Art VI/Art VI b) of *A. scoparia* isolated above by ion-exchange and gel-filtration chromatography were also loaded separately on FPLC column with the same gradient program as standardized for WPE to determine their corresponding elution profile with reference to WPE profile.

PAGE

Disk PAGE of isolated and identified important allergenic protein Art VI b (30 μ g) was performed on 5 by 90 mm gel rod according to the method of Davis¹⁷ with 7.7% polyacrylamide separating gel to check its homogeneity. The electrophoresis was performed in 0.05 mol/L of Tris and 0.38 mol/L of glycine buffer (pH 8.3) for about 3 hours at 3 mA per rod. The gel was fixed and stained with Coomassie brilliant blue R-250.

Molecular weight determinations

The molecular weight of the important allergenic component Art VI b was estimated on the calibrated Sephadex G-200 column according to the method of Andrew¹⁸ with β -galactosidase, bovine serum albumin, ovalbumin, trypsin, and lysozyme as marker proteins.

Total protein content was estimated according to the method of Lowry et al.¹⁹ and total carbohydrate by the method of Dubois et al.²⁰

Intradermal skin test

Intradermal skin test was performed on allergic patients according to the criteria laid down by Shivpuri.²¹

Stability studies

The important allergenic component Art VI b was subjected to digestion with immobilized enzymes, trypsin, chymotrypsin, and papain (0.25% in PBS, pH 7.0) at 37° C for 60 minutes. The allergen was also digested with 0.1 N of sulfuric acid and 0.1 N of sodium hydroxide at 4° C for 5 hours. Reduction with β -mercaptoethanol was carried out at 4° C for 2 days. Treatment with denaturing agents, 6 mol/L of urea, SDS, and guanidine hydrochloride was done at 37° C for 25 minutes. The allergenic protein was subjected to oxidative action of sodium metaperiodate (0.037 mol/L) for 7 days at 4° C in the dark. Heat stability of the allergen was checked at 100° C in a boiling-water bath for 10 minutes. The allergenicity remaining after the above enzyme digestions, heat, and other chemical treatments was measured by relative RAST-inhibition test.

RESULTS

Screening of sensitive patients

Of 13 patients studied with reported sensitivity to *A. scoparia* pollen (based on clinical history combined with positive intradermal skin test score of 2+ or more), sera demonstrated a class 1 antibody score of 0.35 to 0.70 PRU for three patients, a class 2 antibody score of 0.70 to 3.50 PRU for six patients, a class 3 antibody score of 3.50 to 17.50 PRU for three patients, and a class 4 antibody score of >17.5 PRU for one patient.

All patients had high serum total IgE levels. Of the 13 patients tested, nine patients had correlating RAST

and skin test scores. Based on RAST results, sera of six patients (one patient with class 4 antibody score, three with class 3 score, and 2 patients with class 2 score) were pooled and used in RAST-inhibition studies. The pooled patient serum demonstrated 24.20% RAST binding as compared to normal human serum with 0.93% binding (Table I).

Isolation and identification of allergenic components

Multiple pilot experiments were conducted with various gel media to obtain optimal resolution of proteins. The final fractionation scheme involved precipitation of WPE with 90% ammonium sulfate and subsequent fractionation on DEAE Sephadex A-50 column. The proteins were eluted into six peaks. The first five peaks designated Art I to Art V were eluted with a linear salt gradient of 0 to 0.5 mol/L of NaCl in 0.05 mol/L of phosphate buffer (pH 7.3). The last peak Art VI was eluted with buffer containing 1 mol/L of NaCl (Fig. 1, A). About 76.68% of the total protein loaded on the column was recovered in these peaks.

The results of RAST inhibition expressed in a dose-related semilogarithmic graph, illustrated in Fig. 1, B, indicated that allergenicity was associated with Art III, Art V, and VI peaks. Of these three allergenic fractions, Art VI was found to be the most potent inhibitor requiring 24 μ g of protein to produce 50% inhibition of specific IgE binding to WPE-coupled paper disk. The other two proteins, Art III and Art V, required higher protein concentrations for 50% inhibition of RAST. However, WPE of *A. scoparia* required only 2.7 μ g of protein for 50% RAST inhibition.

Art VI was found to be heterogeneous and was further fractionated into Art VI a and Art VI b on Sephadex G-50 column (Fig. 2, A). The RAST-inhibition test with solid-phase Art VI fraction indicated that allergenicity was associated with Art VI b peak (2 μ g of protein was sufficient for 50% inhibition), whereas Art VI a did not demonstrate any significant inhibition. However, WPE required 24 μ g of protein for 50% RAST inhibition against solid-phase Art VI. The Art VI b protein could reach 100% inhibition against solid-phase Art VI. The Art V fraction also demonstrated some inhibition against solid-phase Art VI. However, Art III did not demonstrate any significant inhibition with solid-phase Art VI (Fig. 2, B). The above results suggest Art VI b, Art V, and Art III as the allergenic fractions in *Artemisia* WPE. The allergenicity of these isolated proteins was further confirmed by intradermal skin test on sensitive patients. It was observed that the skin

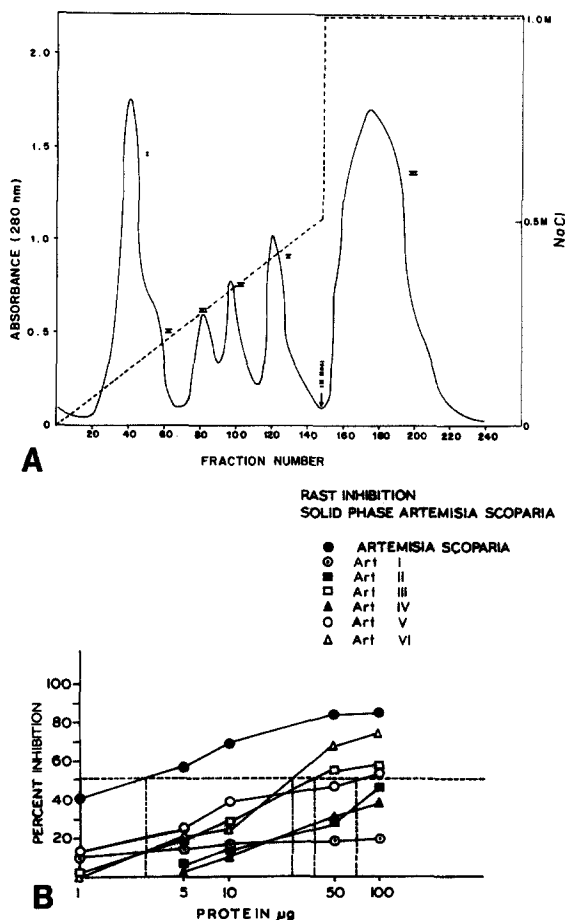


FIG. 1. A, Chromatography of 90% ammonium sulfate precipitable fraction of *Artemisia* WPE on a column of DEAE Sephadex A-50 (1.5 by 35 cm). The fractions were monitored for absorbance at 280 nm; flow rate, 10 ml/hr; fraction size, 2 ml. A linear salt gradient of 0 to 0.5 mol/L of NaCl in 0.05 mol/L of phosphate buffer was applied; 1 mol/L of NaCl was applied at the point indicated above. The ultraviolet absorption of peaks were subsequently designated as Art I to Art VI (— — — indicates gradient molar per liter of NaCl). B, RAST inhibition dose-response curves of Art I to Art VI peaks obtained from DEAE Sephadex A-50 chromatography with *Artemisia* WPE solid phase. The quantity of inhibitor is expressed as microgram of protein.

reactivity of Art VI b and WPE in sensitive patients was almost equivalent (Table II).

CIE

CIE analysis of WPE with rabbit anti-*Artemisia* WPE antibodies is illustrated in Fig. 3, A. Approximately 37 precipitating antigens could be distinguished on anodic side. There was no precipitin line in antibody containing gel on the cathodic side, even on repeated CIE analysis with higher protein concentrations. Fig. 3, C, represents line drawing of CIE

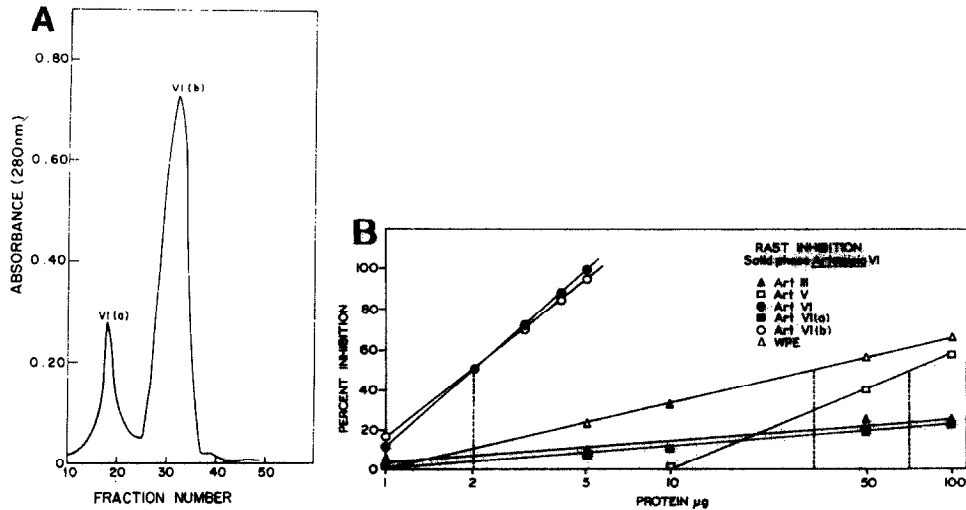


FIG. 2. A, Purification of Art VI preparation on Sephadex G-50 column (2 by 50 cm) eluted with 0.05 mol/L of phosphate buffer (pH 7.3); flow rate, 15 ml/hr; fraction size, 2.5 ml. The protein elution was measured by absorbance at 280 nm. The ultraviolet absorption peaks were designated as Art VI a and Art VI b. B, RAST inhibition assay of allergenic components with solid-phase Art VI.

TABLE II. Results of intradermal skin test of *A. scoparia* allergenic fractions in sensitive patients

Patients	Art III	Art V	Art VI b	WPE
B. P.	+	++	+++	+++
A. D.	++	+	+++	+++
L. D.	++	+	+++	++++
L. A.	++	++	++++	++++
L. M.	++	+++	+++	++++

reference pattern illustrating 37 precipitin lines. CIE of Art VI b protein demonstrated a single precipitin line against anti-*Artemisia* WPE antibodies (Fig. 3, B).

In TLIEF pattern of WPE, 24 Coomassie blue stained bands were visible between pI 3.5 to 6.8. The Art VI b protein appeared as a single band with pI 3.8 (Fig. 4). On disk PAGE, Art VI b moved as a single homogeneous band.

Molecular weight, protein, and carbohydrate determinations

The elution volume of Art VI b on Sephadex G-200 column when it is plotted on the molecular weight calibration curve indicated that Art VI b is eluted close to lysozyme. The molecular weight was estimated to be about 14,300 daltons. It has protein and carbohydrate ratio of 5.8:1.

FPLC

The FPLC elution profile of *Artemisia* WPE had six major and few minor peaks. The allergenic com-

ponents (Art III/Art V/Art VI), isolated as above by DEAE Sephadex chromatography, when they were loaded individually on Mono Q column under the same conditions as for WPE, could be eluted at the positions marked in the chromatogram (Fig. 5). The important allergenic proteins Art VI/Art VI b became strongly bound to the ion exchanger and could be eluted only when 100% of the limiting buffer was passed through the column. Its position on the elution profile corresponded to the last peak of *Artemisia* WPE chromatogram.

Stability studies

The important allergenic component Art VI b was found to be resistant to digestion by trypsin, denaturing agents (urea, SDS, and guanidine hydrochloride) and periodate oxidation. Digestion with chymotrypsin resulted in moderate loss of allergenicity. Papain, which had a broad specificity, did not alter allergenicity significantly. The allergen was slightly susceptible to acid, alkali, and β -mercaptoethanol. Boiling in water bath for 10 minutes did not result in

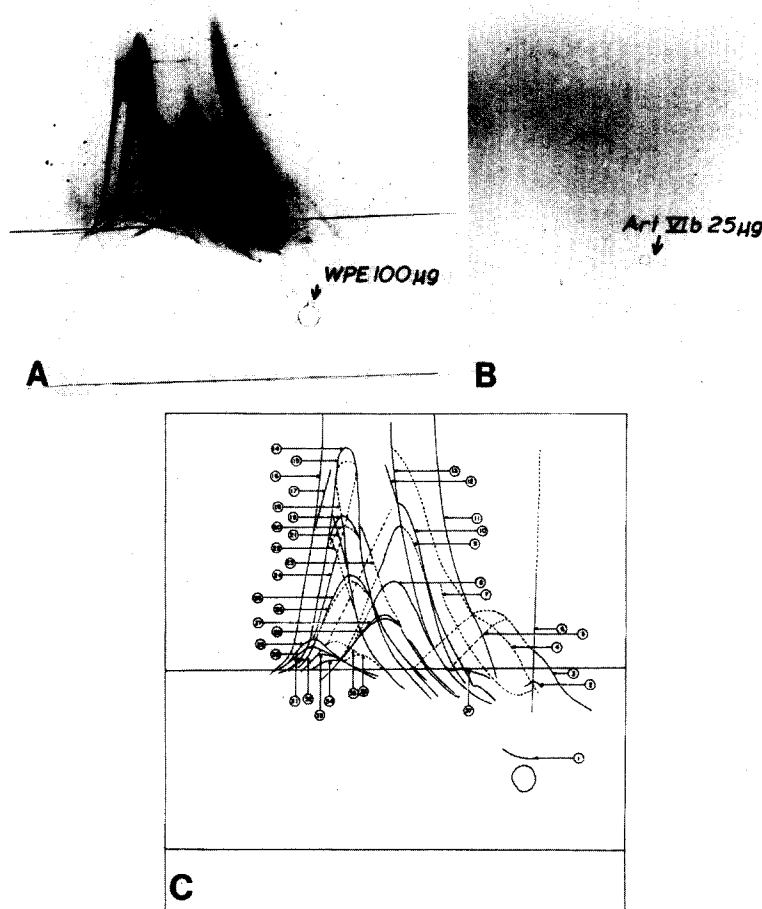


FIG. 3. Antigen antibody two-dimensional CIE pattern. **A**, *Artemisia* WPE 100 µg. **B**, Art VI b, 25 µg. **C**, Schematic drawing of the precipitates of CIE reference pattern. The precipitates were arbitrarily numbered. Dotted lines represent faintly visible precipitin lines. Anode is to the left in first dimension and to the top in second dimensional electrophoresis.

marked change in allergenic activity of Art VI b (<10% reduction in RAST inhibition) (Table III).

Cross-reactivity studies

RAST inhibition of related pollen allergens, i.e., *Xanthium strumarium* and *Ageratum conyzoides* with solid-phase *A. scoparia* WPE, was performed to check cross-reactivity among these members of the Asteraceae family. The results demonstrated that *Xanthium*- and *Ageratum*-pollen extracts produced inhibition to solid-phase *Artemisia* WPE but were 28 and 14 times less potent inhibitors, respectively, as compared to *Artemisia* WPE (Fig. 6, A).

CIE of *Ageratum* extract against anti-*Artemisia* WPE antibodies revealed 12 precipitin lines, whereas only one precipitin line was detected in CIE of *Xanthium* extract under similar conditions (Fig. 6, B and C).

DISCUSSION

The extraction of proteins from *A. scoparia* was performed in PBS. A preliminary study²² suggested that PBS extract not only contained more protein and carbohydrate but it also had more biologic activity, as compared to Tris HCl, NH_4HCO_3 , Coca's solution, and distilled water extracts. The studies by Paulsen et al.¹¹ also suggested that extraction of *A. vulgaris* pollen with PBS resulted in higher yield of extract. The WPE of *A. scoparia*, like *A. vulgaris*,¹¹ was found to contain substantial quantity of yellow-brown pigments that were too difficult to remove by treatment with various organic solvents. The addition of polyvinyl pyrrolidone²³ and thiourea^{11, 12} in extraction medium did not significantly remove the coloring matter. It was considered important to precipitate WPE of *A. scoparia* with 90% ammonium sulfate before loading on DEAE Sephadex column, since initial exper-

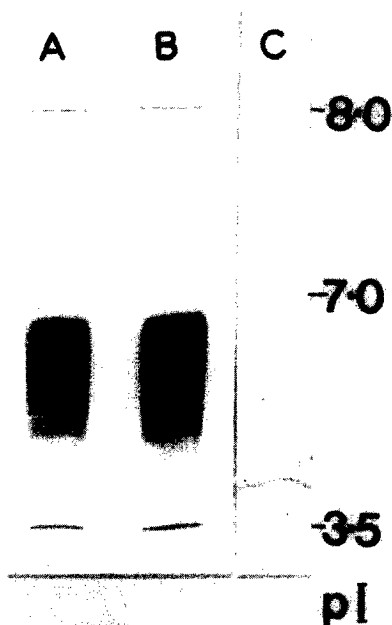


FIG. 4. A-B, TLIEF analysis of WPE, 80 μ g. C, Art VI b, 25 μ g, in 5% polyacrylamide gels. The anode is at the top; pI gradient 3.5 to 9.5 was determined by pH surface microelectrode.

iments conducted by loading WPE directly on DEAE Sephadex resulted in poor resolution of proteins. This was probably caused by high amount of pigmented material in the extract that might have disturbed the separation of proteins by binding strongly to the gel matrix. The precipitation of WPE with 90% ammonium sulfate not only improved the resolution on DEAE Sephadex but also decreased the pigmented material to some extent. The chromatography of dialyzed 90% ammonium sulfate precipitable fraction on DEAE Sephadex A-50 resulted in elution of six peaks (Art I to Art VI), of which Art III, Art V, and Art VI were found to be allergenic (Fig. 2, A and B). The small amount of fraction containing yellow-brown pigments remained strongly bound to the gel and could be eluted to a large extent with 7% acetic acid in phosphate buffer. However, it was not found to be allergenic when it was tested by RAST inhibition. Of the three identified allergenic fractions (Art III, Art V, and Art VI), Art VI fraction was demonstrated to be the most potent. In RAST-inhibition studies with solid-phase WPE, 24 μ g of Art VI protein was required for 50% inhibition whereas only 2.7 μ g of WPE protein produced 50% inhibition (Fig. 2, B). Similar results were reported in case of ragweed²⁴ and ryegrass^{25, 26} pollen in which purified allergenic components had less biologic ac-

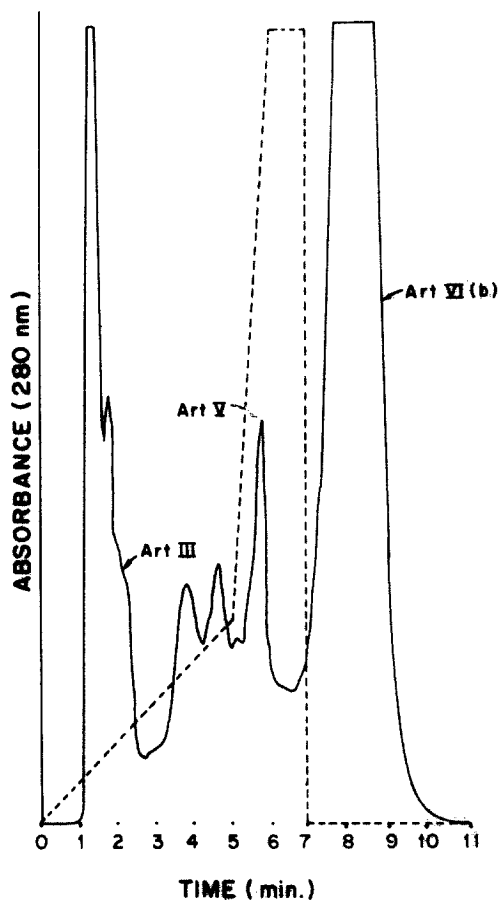


FIG. 5. Chromatography of *Artemisia* WPE purified on Mono Q HR 5/5 column; starting buffer, 20 mmol/L of Tris (pH 7.5); limiting buffer, 20 mmol/L of Tris containing 0.7 mol/L of sodium acetate (pH 7.5); flow rate, 1 ml/min; fraction size, 1 ml. Thick solid line illustrates absorbance of eluted fraction at 280 nm; broken line illustrates gradient as percent of limiting buffer.

tivity as compared to the WPEs. The lower biologic activity of the purified fraction, as observed in the RAST-inhibition activity, could be due to partial denaturation of the activity during extraction or due to removal of specificity on purification.²⁶ Insufficient representation of the purified fraction on the solid phase cannot be ruled out.

In the present study, repeated chromatography of WPE on DEAE Sephadex under well-defined temperature conditions revealed similar results. The Art VI fraction was found to be heterogeneous and could be further resolved on Sephadex G-50 into two component Art VI a and Art VI b.

The results of RAST inhibition expressed in a dose-related semilogarithmic graph illustrated in Fig. 2, B, indicated that allergenicity was mainly associated with Art VI b, whereas Art VI a did not have any inhibition

against solid-phase Art VI. It was also observed that in this case WPE was about 12 times less potent an inhibitor as compared to Art VI/Art VI b.

Recently, Paulsen et al.¹¹ also fractionated *A. vulgaris*-pollen extract on DEAE Sephadex A-25 column and could detect the presence of at least three allergenic proteins in fractions eluted with the starting buffer, 0.5 mol/L of NaCl and 1 mol/L of NaCl, respectively. The fractions eluted with 0.5 mol/L and 1 mol/L of NaCl may probably correspond to Art V and Art VI proteins eluted in the present study from DEAE Sephadex A-50 column. The Art III protein was eluted from DEAE Sephadex column in the present study with approximately 0.25 mol/L of NaCl. The fraction that could be eluted with starting buffer (Art I) was, however, not found to be allergenic (Fig. 1, A, and B). It was also interesting to observe from the findings of Paulsen et al.¹¹ that there are at least two antigenic proteins in the fraction eluted with 1 mol/L of NaCl, of which one had the IgE-binding capacity as observed in rocket radioimmuno-electrophoresis that may correspond with Art VI b protein isolated in the present study. It was also reported to be heat stable like Art VI b.¹¹ The Art V protein fraction had RAST inhibition against solid-phase Art VI, suggesting the presence of common allergenic determinants between the two proteins. The overall results of identified allergenic proteins (Art III, Art V, and Art VI b), as determined by RAST inhibition and skin test on sensitive patients, suggested Art VI b as the important allergenic protein in *A. scoparia*-pollen extract, contributing 29% of total protein in the extract.

The homogeneity of isolated Art VI b protein was tested by various analytical procedures and was found to be homogeneous by PAGE, CIE, and TLIEF (Figs. 3, B, and 4, C). It was found to be a nondialyzable protein of molecular weight 14,300 daltons and to be a glycoprotein containing approximately 17% carbohydrate. Nilsen and Paulsen¹² described Ag 7 as the important allergenic protein from *A. vulgaris* extract. It was reported to be a heat-stable glycoprotein with pI 4.3, demonstrating microheterogeneity in the carbohydrate moiety. The characteristics of Ag 7 are in close agreement to characteristics of Art VI b protein in the present study that was also a heat-stable glycoprotein with pI 3.8. The Art VI b protein was also found to be resistant to denaturing agents. Similar results have also been observed for cocoon allergen,²⁷ allergen C of Kentucky bluegrass,²⁸ and a few other grass pollens.²⁹ The digestion of Art VI b with enzyme chymotrypsin resulted in moderate loss of allergenicity, suggesting that aromatic

TABLE III. Characterization of *Artemisia* VI b

Protein: carbohydrate ratio	5.80:1
Molecular weight	14,300
Relative RAST inhibition*	
after treatment with	
Nil	100
Trypsin	100
Chymotrypsin	74
Papain	95
Urea	100
SDS	100
Guanidine hydrochloride	100
β -Mercaptoethanol	81
Acid	82
Alkali	78
Sodium metaperiodate	100
Heat 100° C, 10 minutes	93

*Relative RAST inhibition was calculated as percentage of the inhibitory ability of *Artemisia* VI b (with solid-phase Art VI) that was considered as 100%.

amino acid residues might be involved to some extent in the integrity of determinants responsible for allergenic activity. The allergen was resistant to trypsin and papain digestions. The Kentucky bluegrass allergen C has also been reported to be resistant to papain.²⁸ The Art VI b protein was, however, slightly susceptible to acid, alkali, and reduction with β -mercaptoethanol. Recently, Bennett and Howden³⁰ reported isolation of a peanut allergen stable in both acidic and alkaline pH. The periodate oxidation of Art VI b did not result in any loss of allergenicity, indicating that carbohydrate moiety might not be important for allergenicity of this protein. Similar results have been reported for peanut allergen.³⁰

The CIE reference pattern of WPE constructed by varying the concentration of antigen and antiserum had 37 anodic migrating antigens. No precipitin line was visible in the antibody containing gel on the cathodic side. Ipsen et al.¹⁰ could detect 42 distinct antigens in the reference extract of *A. vulgaris*, of which 24 could be detected on anodic side and 18 on cathodic side. Paulsen et al.¹¹ observed 18 anodic migrating antigens in dialyzed extract of *A. vulgaris*. They also observed that when the extract was dialyzed, cathodic migrating antigens/allergens are lost. Ingemann et al.³¹ also could not detect cathodic migrating proteins in two commercial extracts of *A. vulgaris* pollen. The absence of cathodic migrating proteins in *A. scoparia* extract in both CIE and TLIEF analysis could be either due to dialysis of the extract or due to head-crushing method used in the present study for

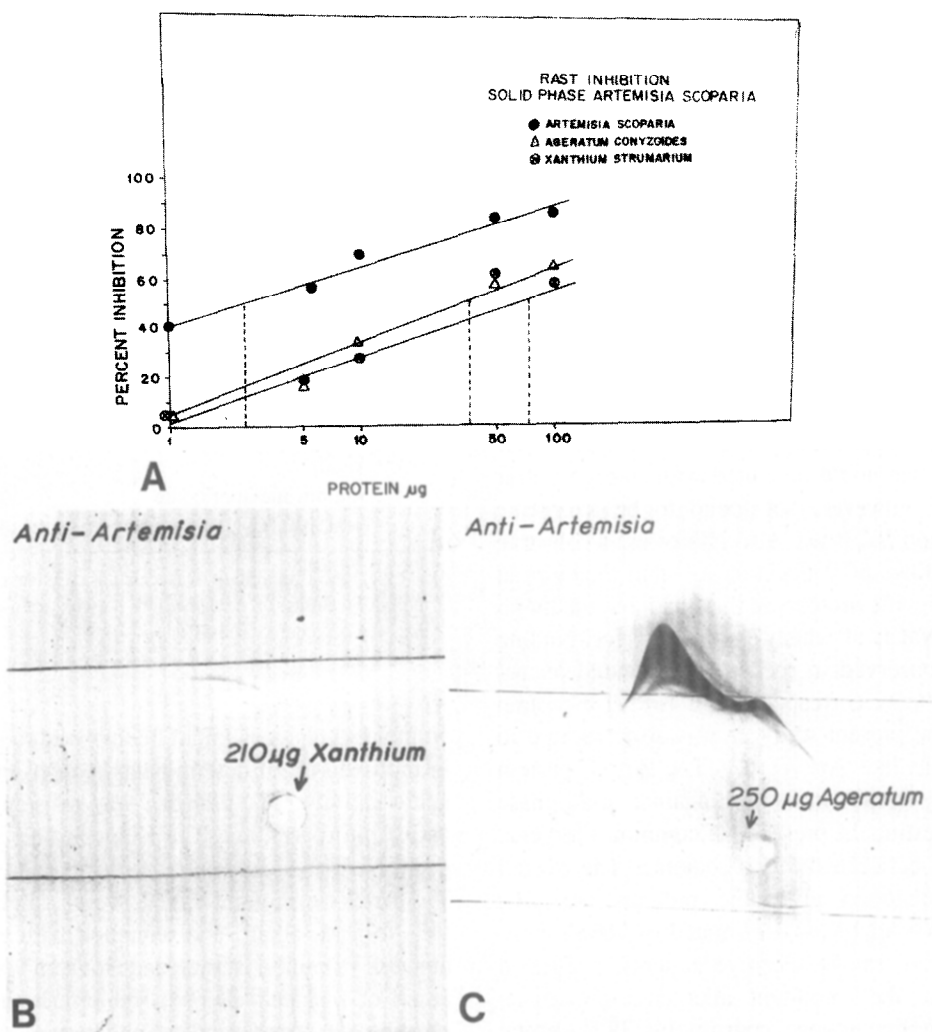


FIG. 6. A, RAST-inhibition curves with *X. strumarium* and *A. conyzoides* WPE with solid-phase *A. scoparia* WPE. B, CIE analysis of *X. strumarium*, 210 μ g. C, *A. conyzoides*, 250 μ g, against anti-*Artemisia* rabbit antibodies.

pollen collection.³² It has been reported that the extract of pollen collected by heat-crushing method loses cathodic migrating proteins, whereas extracts of pollen collected by vacuum-suction method retain cathodic migrating proteins.³² The CIE reference pattern of anodic migrating antigens of *A. scoparia* and *A. vulgaris* extracts appeared apparently similar.^{10, 11} The 37 precipitin lines detected in the present study on the anodic side might have been caused by difference in titer of the antiserum or caused by difference in species. Studies by Ipsen et al.¹⁰ and Nilsen and Paulsen¹² suggest Ag 20 and Ag 7 in CIE as the major and important allergenic component, respectively, in *A. vulgaris* extract. It was interesting to observe that the precipitin lines of Ag 20,^{10, 31} Ag 7,^{11, 12} and Art VI b in the present study appear almost at the same position in CIE pattern, i.e., at the demarcation line between interme-

diatate gel and antibody-containing gel on the anodic side.

In order to isolate the allergenic components present in *A. scoparia* WPE in less time, FPLC was carried out with a strong anion-exchanger Mono Q column under the conditions illustrated in Fig. 5. The WPE could be resolved in 11 minutes into six major and few minor peaks. The FPLC elution profile of the extract resembled the elution profile observed with DEAE Sephadex column. The important allergenic proteins Art VI/ Art VI b, when proteins were loaded on Mono Q column, were firmly bound to the gel medium and could be eluted only when 100% of the limiting buffer was passed through the column. The last fraction eluted on FPLC corresponding to Art VI on DEAE Sephadex was also found to elicit positive skin reaction in sensitive patients (results not

presented). The Art VI b can be purified from this fraction by passing through Sephadex G-50 column. Although good resolution of extract on FPLC was observed, it was associated with increased back pressure caused by clogging of the column by strong binding of pigments to gel matrix after repeated use and required extensive washing with acetic acid and methanol. Similar observations were made by Haavik et al.³³ for purification of timothy pollen extract on Mono Q column. They suggested partial purification of the extract before ion-exchange chromatography on FPLC to avoid clogging of the column. Ten milligrams of protein of *A. scoparia* WPE could be run through two subsequent runs without much difficulty. FPLC thus could be used in partial purification of allergens from *A. scoparia* pollen extract. However, incorporation of a preliminary purification step before loading the WPE can certainly be advantageous to increase the efficiency of column.

In the present study, the pollen extract of *A. scoparia* had shared antigenic and allergenic determinants with *Ageratum conyzoides* as determined by CIE and RAST inhibition, respectively. The cross-reactivity among *Xanthium*, *Artemisia*, and ragweed pollen has been reported by other workers.³⁴⁻³⁶ The antigenic/allergenic cross-reactivity between the pollen extracts of *A. vulgaris* and extracts of plantain,³⁷ birch,³⁸ and chrysanthemum³⁹ pollen and also with some fruits and vegetables^{40, 41} has been reported. The closer antigenic/allergenic composition of *A. scoparia* and *Ageratum* observed in the present study need to be explored further by crossed-line immunoelectrophoresis technique and to detect if these cross-reacting antigens are allergenic as well.

The present study suggests similar allergenic and antigenic composition of proteins in the extracts of *A. scoparia* and *A. vulgaris* pollen. The allergenic proteins detected in *A. vulgaris*¹¹ also appear to resemble the allergenic proteins identified in the present study in *A. scoparia*-pollen extract. However, cross-reactivity studies may provide a clear understanding of the similar antigenic/allergenic composition of these two important species of *Artemisia*.

We thank Drs. Dharam Parkash, S. N. Gaur, and P. U. Rao of Clinical Research Center, Vallabhshai Patel Chest Institute, Delhi, for providing the clinical help.

REFERENCES

1. Babu CR. Herbaceous flora of Dehradun. New Delhi: Publications and Information Directorate, Council of Scientific & Industrial Research, 1977:240.
2. Singh AB, Babu CR. Survey of atmospheric pollen allergens in Delhi: seasonal periodicity. *Ann Allergy* 1982;48:115.
3. Shivpuri DN. Clinically important pollen, fungal, and insect allergens of nasobronchial allergy patients in India. *Asp Allergy App Immunol* 1980;13:19.
4. Agashe SN, Anand P. Immediate type hypersensitivity to common pollen and molds in Bangalore City. *Asp Allergy App Immunol* 1982;15:49.
5. Gupta MC, Mittal OP, Katiyar SK. Pollen allergy in Kanpur. *Asp Allergy App Immunol* 1984;17:99.
6. Gurdip Singh, Joshi RD. Pollen morphology of some Eurasian species of *Artemisia*. *Grana Palynologica* 1969;9:1.
7. Lewis WH. Pollen allergy. In: Korenblat PE, Wedners HJ, eds. *Allergy, theory and practice*. New York: Grune & Stratton, 1984:353.
8. Strandhede SO, Wihl JA. Comparison of pollen counts in Copenhagen and Malmo. *Grana* 1981;20:187.
9. Woodhouse RP. Patterns of allergic sensitization. *Ann Allergy* 1948;6:358.
10. Ipsen H, Formgren H, Lowenstein H, Ingemann L. Immunochemical and biological characterization of a mugwort (*Artemisia vulgaris*) pollen extract. *Allergy* 1985;40:289.
11. Paulsen BS, Flo L, Nesje G, Wold JK. Allergens in pollen from mugwort (*Artemisia vulgaris* L). I. Partial characterization of allergen preparation from mugwort pollen with emphasis on the carbohydrate moiety. *Int Arch Allergy Appl Immunol* 1985;78:206.
12. Nilsen BM, Paulsen BS. Allergens in pollen from mugwort (*Artemisia vulgaris* L). II. Characterization of a crude and a partly purified extract of mugwort pollen with particular emphasis on the glycoprotein allergen Ag 7. *Int Arch Allergy Appl Immunol* 1986;80:111.
13. Cour P, Loublier Y. Sampling and preparation of pollens for microscopic analysis. *Rev Fr Allergol* 1980;20:197.
14. Harboe NMB, Ingild A. Immunization, isolation of immunoglobulin, and antibody titer determination. *Scand J Immunol* 1983;17:345.
15. Axelsen NH, Kroll J, Weeke B. A manual of quantitative immunoelectrophoresis: methods and applications. *Scand J Immunol* 1973;2(suppl 2):49.
16. Jaggi KS, Gangal SV. Fast protein liquid chromatography for identification of pollen allergens. *Asp Allergy Appl Immunol* 1984;17:121.
17. Davis BJ. Disc electrophoresis. II. Methods and application to human serum proteins. *Ann NY Acad Sci* 1964;121:404.
18. Andrews P. The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem J* 1965;96:595.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951;193:265.
20. Dubois M, Gilles KA, Hamilton JK, Robers RA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350.
21. Shivpuri DN. Comparative evaluation of sensitivity of common methods of diagnostic antigen tests in patients of respiratory allergy. *Indian J Chest Dis Allied Sci* 1969;4:102.
22. Jaggi KS. Biochemical studies on allergenic pollen [Thesis]. Delhi, India: University of Delhi, 1985.
23. Bjorksten F, Halmepuro L, Hannuksela M, Lahti A. Extraction and properties of apple allergens. *Allergy* 1980;35:671.
24. Gleich GJ, Larson JB, Jones RT, Baer H. Measurement of the potency of allergy extracts by their inhibitory capacities in radioallergosorbent test. *J ALLERGY CLIN IMMUNOL* 1974;53:158.
25. Smart IJ, Knox RB. Rapid batch fractionation of ryegrass pollen allergens. *Int Arch Allergy Appl Immunol* 1980;62:179.
26. Cottam GP, Moran DM, Standing P. Physiocochemical and

- immunochemical characterization of allergenic proteins from ryegrass (*Lolium perenne*) pollen prepared by a rapid and efficient purification method. *Biochem J* 1986;234:305.
27. Subba Rao PV, Mangala A, Harindranath N. Mouse model for purification and standardization of inhalant allergens. *Asp Allergy Appl Immunol* 1985;18:135.
 28. Chakrabarty S, Ekramoddoullah AKM, Kishil FT, Sehon AH. Isolation and partial characterization of allergen C from Kentucky bluegrass pollen. *Int Arch Allergy Appl Immunol* 1981;65:377.
 29. Marsh DG. Allergens and genetics of allergy. In: Sela M, ed. *The antigens*. New York: Academic Press, 1975:271.
 30. Bernett D, Howden MEH. Partial characterization of an allergenic glycoprotein from peanut (*Arachis hypogaea* L). *Biochem Biophys Acta* 1986;882:97.
 31. Ingemann L, Formgren H, Lowenstein H, Ipsen H. The use of reference allergenic extract in the evaluation of allergen products. *Allergy* 1985;40:273.
 32. Anderson MC. The effect of quality of source materials on reference preparations. In: *Proceedings of the Fourth International Paul Ehrlich seminar on the regulatory control and standardization of allergenic extract*. Bethesda, Md.: 1985:17.
 33. Haavik S, Paulsen BS, Wold JK. Purification of a basic glycoprotein allergen from pollen of timothy by high performance liquid chromatography. *J Chromatogr* 1985;321:199.
 34. Dumn JF, Zarte O. *Artemisia verticillata* L as a cause of pollinosis in city of La Plata. *Rev Assoc Med Argentina* 1944;58:590.
 35. Yunginger JW, Gleich GJ. Measurement of ragweed antigen E by double antibody radioimmunoassay. *J ALLERGY CLIN IMMUNOL* 1972;50:326.
 36. Leiferman KM, Gleich GJ, Jones RT. The cross-reactivity of IgE antibodies with pollen allergens. II. Analysis of various species of ragweed and other fall weed pollens. *J ALLERGY CLIN IMMUNOL* 1976;58:140.
 37. Baldo BA, Chensee QJ, Howden MEH, Sharp PJ. Allergens from plantain (*Plantago lanceolata*). *Int Arch Allergy Appl Immunol* 1982;68:295.
 38. Halmepuro L, Vuontela K, Kalimo K, Bjorksten F. Cross-reactivity of IgE antibodies with allergens in birch pollen, fruits, and vegetables. *Int Arch Allergy Appl Immunol* 1984;74:235.
 39. Lowenstein H. Cross-reactions among pollen antigens. *Allergy* 1980;35:198.
 40. Pauli G, Bessot JC, Molard AD, Braun PA, Thierry R. Celery sensitivity: clinical and immunological correlation with pollen allergy. *Clin Allergy* 1985;15:273.
 41. Halmepuro L, Lowenstein H. Immunological investigation of possible structural similarities between pollen antigens and antigens in apple, carrot, and celery tuber. *Allergy* 1985;40:264.

AVAILABLE NOW! The PROCEEDINGS OF THE INTERNATIONAL CONGRESS OF ALLERGOLOGY AND CLINICAL IMMUNOLOGY can be purchased from the Publisher. This collection of "state-of-the-art" presentations from the XII Congress held October 20-25, 1985, in Washington, D.C., brings together the current advances in basic and applied aspects of allergy and allergic diseases. It includes 528 pages covering such topics as IgE, roles of the different cell types and their products, clinical problems, asthma, rhinitis, and reactions to foods and drugs and occupational agents, collected and reviewed by Editor Charles E. Reed, M.D. (U.S.A.) and Associate Editors Joseph Bellanti, M.D. (U.S.A.), Robert J. Davies, M.D. (U.K.), Sidney Friedlaender, M.D. (U.S.A.), Albert Oehling, M.D. (Spain), and Raymond G. Slavin, M.D. (U.S.A.).

To purchase, call or write: The C. V. Mosby Company, 11830 Westline Industrial Dr., St. Louis, MO 63146, or telephone FREE 1-800-325-4177, Journal Fulfillment, ext. 531 (in Missouri call collect at 314-872-8370, Journal Fulfillment, ext. 531). Prepayment required. Make checks payable to The C. V. Mosby Company. (All payments must be in U.S. funds drawn on a U.S. bank.) Price: \$36.50 in the U.S., \$40.50 in Canada, and \$41.50 international (surface shipping charges included).