Partial Purification of Antigen E from Mixed Stems and Leaves of Short Ragweed Plant

A. SHAFIEE, E. J. STABA▲, and Y. J. ABUL-HAJJ

Abstract □ Short ragweed leaves-stems, roots, and seeds were examined for the presence of the most potent pollen allergen, antigen E. Soluble proteins were precipitated by ammonium sulfate and partially purified by a combination of dialysis and anion exchange and gel filtration chromatography. Immunodiffusion, disk electrophoresis, and skin testing of short ragweed pollen-sensitive individuals indicated that antigen E was present in leaves-stems portion of this plant as well as the pollen.

Keyphrases □ Ragweed leaves-stems, roots, and seeds—distribution and isolation of antigen E □ Antigen E, short ragweed—distribution in leaves-stems, roots, and seeds, isolation from leaves-stems □ Allergen distribution—antigen E in short ragweed leaves-stems, roots, and seeds

Wind-borne plant particles other than pollen can cause allergic symptoms in persons showing pollen hypersensitivity (1). Rebhun *et al.* (2), by direct skin testing of ragweed-sensitive patients, demonstrated that reagin reacting antigens were present in all parts of the giant ragweed plant. Other researchers reported the purification of antigens E and K (3–5), of Ra. 3 (6), and of a basic protein from short ragweed pollen (7). The distribution of short ragweed pollen antigen E in other portions of this plant was investigated in this study.

EXPERIMENTAL

Plant Materials and Antigen E Source—In 1971, short ragweed plants[1] (*Ambrosia elatior* L.) were grown and collected shortly before pollination in the medicinal plant garden and were also received from a commercial source[2]. The collected plant materials were air dried, milled, and stored in an airtight container at room temperature. Plant materials used for extraction did not contain pollen when examined microscopically both before and after staining with Calberla's solution (8).

Antigen E from short ragweed pollen[3] was prepared according to a reported procedure (3, 4). The prepared antigen E (Sephadex G-100, Fraction IV) had a similar disk electrophoresis pattern (Fig. 1), and a complete line of identity with National Institutes of Health (NIH) antigen E (NIH antigen E) when evaluated with antiantigen E and antipollen crude extract serums by the Ouchterlony immunodiffusion test.

Allergen Isolation Procedure—A modification of the literature procedure (3, 4) was used to isolate the allergens from short ragweed leaves-stems, roots, and seeds.

Leaves-Stems—Plant samples collected from the medicinal plant garden and from the commercial source (100 g. leaves-stems) were separately defatted and depigmented with peroxide-free ether for 24 hr. in a soxhlet apparatus. The defatted, air-dried plant material was extracted with 800 ml. of 0.005 *M* iced phosphate buffer (pH 7.4) in a 2-l. erlenmeyer flask on a gyrotory shaker[4] at 200 r.p.m. for 24 hr. at 22°. The extract was filtered through glass wool at 4°[5], and the filtrate (500 ml., pH 6.5) was adjusted to pH 7.4 with 3 *N* ammonium hydroxide. The filtrate was further clarified by centrifugation at 1230×*g* for 20 min. in a centrifuge[6]. The precipitate was washed with extracting buffer until the wash buffer reacted negatively against antipollen crude extract rabbit serum in the immunodiffusion test. The combined filtrate (510 ml., pH 7.2) was concentrated to approximately 51 ml. with an ultrafiltration cell[7] equipped with a membrane[8] and adjusted to 0.9 saturation with 31.7 g. ammonium sulfate[9].

The resulting suspension was stirred overnight at 4° and centrifuged at 14,350×*g* for 40 min. The precipitate was then dissolved in 25 ml. of 0.1 *M* tromethamine-hydrochloric acid buffer (pH 7.9) to form a dark-brown solution. This solution was desalted and further depigmented on an anionic exchange column[10] (50 × 4 cm.), which was equilibrated and eluted with 0.025 *M* tromethamine-hydrochloric acid buffer (pH 7.9). The flow rate was adjusted to 60 ml./hr., and 15-ml. fractions were collected at 22° and monitored by their absorption at 280 nm. The first peak, Fraction A, was concentrated to 20 ml. at a pressure differential of 700 mm. Hg using size 8 dialysis tubing[11]. Since loss of allergen through dialysis tubing was detected, the dialysate was concentrated in the ultrafiltration cell as before and combined with the dialysis tubing retentate. The second peak, Fraction B, containing the pigments and ammonium salt was discarded. The combined concentrate Fraction A (22 ml., 6 mg. dry wt./ml.) was exhaustively dialyzed against 0.025 *M* tromethamine hydrochloric acid (pH 7.9) and applied on diethylaminoethyl-cellulose (medium mesh, 0.88 meq./g.) column[12] (25 × 2.7 cm.) previously equilibrated and eluted with the 0.025 *M* tromethamine-hydrochloric acid buffer (pH 7.9). The flow rate was adjusted to 180 ml./hr., and 13-ml. fractions were collected and monitored as previously described. The first diethylaminoethyl-cellulose peak, Fraction C, was collected. The column was then eluted with 0.05 *M* tromethamine-hydrochloric acid–0.2 *M* NaCl buffer (pH 7.9) to obtain a second peak, Fraction D. Fraction D was concentrated to 1.5 ml. and contained 0.8 mg. protein/ml. Protein was determined by the method of Lowry *et al.* (9) with crystalline bovine albumin as standard.

Roots and Seeds—Short ragweed roots were extracted and partially purified for antigen E as described for plant leaves-stems. However, the allergen isolation procedure for the seeds was slightly different from that already described. Seed samples (100 g.) were ground into fine pieces with a mortar and pestle and were defatted. The defatted seed was extracted with 500 ml. of 0.005 *M* phosphate buffer (pH 7.4), and the resulting suspension was expressed through four layers of muslin cloth. The residue was mixed with enough buffer to form a uniform suspension and sonified[13] for 2 min. with the power source set at 90 w. The sonified suspension was also expressed through four layers of muslin cloth, and the filtrates of the two extracts were mixed. Seed Fraction D was obtained as previously described for leaves-stems. Seed Fraction D was concentrated to 10 ml. by use of the ultrafiltration cell. The concentrated Fraction D (10 mg. protein/ml.) was chromatographed on a resin column[14] (195 × 2.5 cm.). Prior to the application of the sample to the column, the concentrate was equilibrated by dialysis with the column buffer [0.1 *M* tromethamine-hydrochloric acid–0.2 *M* ammonium sulfate (pH 7.9)]. The flow rate was adjusted to 20 ml./hr., and 6.2-ml. fractions were collected and monitored as previously described.

[1] Voucher specimen deposited in Department of Pharmacognosy, University of Minnesota.
[2] Greer Labs., Inc., Lenoir, N. C.
[3] 1970 Collection, Greer Labs., Inc.
[4] NBS model G-10, New Brunswick, N. J.
[5] All filtrations, centrifugations, and dialyses were carried out at 4°.
[6] Sorvall SS-1, Newton, Conn.
[7] Diaflo.
[8] UM-2, Amicon Corp. Cambridge, Mass.
[9] Ultra-pure, Schwartz/Mann, Orangeburg, N. Y.
[10] Sephadex G-25 (medium), Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[11] Union Carbide Co., Chicago, Ill.
[12] Sigma Chemical Co., St. Louis, Mo.
[13] S-12 Branson Sonifier, Danbury, Conn.
[14] Sephadex G-100, 40–120 μ.

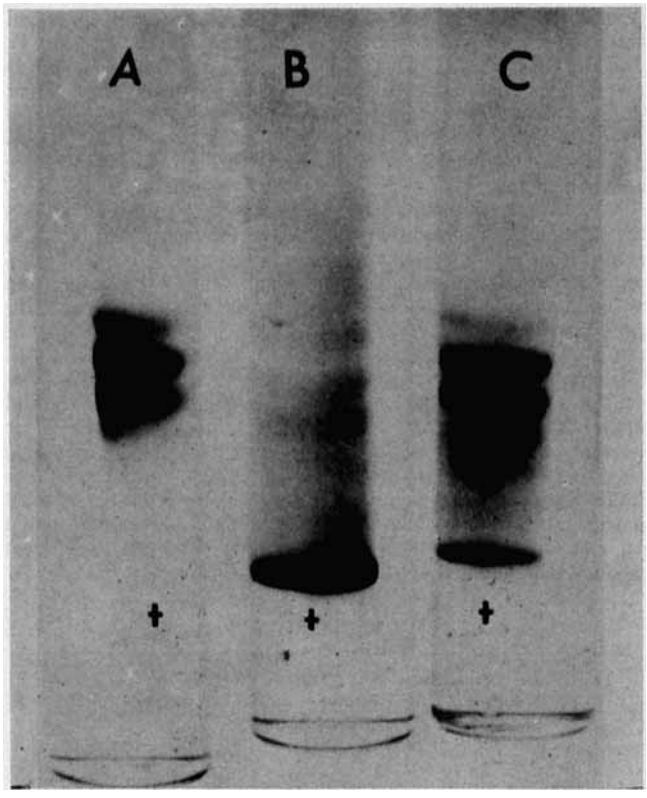

Figure 1—*Polyacrylamide gel electrophoresis patterns at pH 8.9. Key: A, NIH antigen E; B, leaves–stems Fraction D; and C, pollen antigen E (Sephadex G-100, Fraction IV). The fastest moving anodic band in B and C is bromphenol blue marker dye and it is barely seen in A.*

Rabbit Antiserum Preparation—Antipollen crude extract serum was prepared by injecting male rabbits (New Zealand White) at four weekly intervals subcutaneously with an emulsion of 0.5 ml. of 0.1 *M* tromethamine–hydrochloric acid (pH 7.9) buffer solution of 0.9 saturation ammonium sulfate precipitate (81 mg./ml. dry weight) and 0.5 ml. of complete Freund's adjuvant[15] (6). The animals were bled from the marginal ear vein 10 days after the last injection. The serum from each animal was separately tested against pollen crude extract and NIH antigen E by the Ouchterlony immunodiffusion test (10). Positively reacting serum was pooled and stored at −10° in 1-ml. portions until used.

Antiantigen E serum was prepared by injecting each rabbit subcutaneously at four different sites on the back and neck areas at weekly intervals with an emulsion of 0.5 ml. (0.52 mg. protein/ml.) antigen E (Sephadex G-100, Fraction IV) and 1 ml. of complete Freund's adjuvant. The antiantigen E serum was tested and stored as described for antipollen crude extract serum.

Disk Electrophoresis—The reagents and apparatus used for disk electrophoresis were purchased[16]. Disk electrophoresis of Fraction D (leaves–stems), pollen antigen E (Sephadex G-100, Fraction IV), and NIH antigen E was performed at a running gel pH of 9.5 with the discontinuous buffer system of Davis and Ornstein (11). A sample load [0.1–0.2 mg. in 0.2 ml. of 0.025 *M* tromethamine–hydrochloric acid (pH 7.0)] was added to the top of the spacer gel, and larger pore solution was added to fill the electrophoresis tube. Bromphenol blue was used as a marker dye. The gels were stained[17] after being run at 4-mamp./tube current for 45 min.

Immunological Studies—Ouchterlony immunodiffusion tests were carried out in 60-mm. plastic petri dishes containing 5 ml. of 1% Noble agar[18] and 0.1% sodium azide (10). The antiserum was placed in the center well and antigens were placed in the outer wells (3-mm. diameter). After 48–72 hr., the precipitin line positions were observed and photographed. Antigen E concentrations in leaves–stems were estimated by the double-diffusion technique with antipollen crude extract serum (12). The standard curve was established by using purified antigen E in a concentration range of 0.125–0.015 mg./ml. The precipitin line positions on negative films were measured with a shadow graph[19].

[15] Difco, Detroit, Mich.
[16] Canalco Co., Rockville, Md.
[17] Amido Schwarz.
[18] Difco.

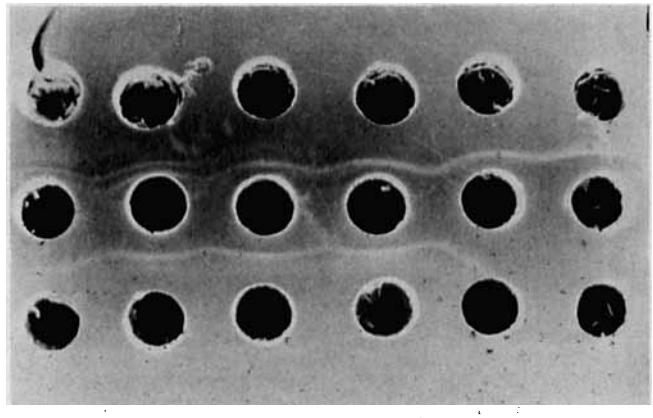

Figure 2—*Antigen E quantitative immunodiffusion analysis. The center wells were filled with antipollen crude extract serum. The upper wells were filled with NIH antigen E (left to right: 0.5, 0.25, 0.125, 0.062, 0.031, and 0.015 mg. protein/ml.), and the lower wells were filled with leaves–stems Fraction D (left to right: 0.1, 0.2, 0.4, and 0.8 mg. protein/ml.). The lower last two wells were filled with buffer and are controls.*

Unstained electrophoresis gels loaded with Fraction D (leaves–stems) were aligned with stained pollen antigen E and gel cuts made in the region of the pollen antigen E. The gel cuts were eluted with 1 ml. of 0.025 *M* tromethamine–hydrochloric acid buffer (pH 7.9) at 4° overnight. The eluate was lyophilized, and the appropriate concentration was made by redissolving the lyophilized eluate in the buffer prior to the Ouchterlony immunodiffusion test.

Allergenic activity of column fractions and NIH antigen E was determined by direct skin testing on patients with known short ragweed pollen sensitivity. Solutions were sterilized by filtration[20] (0.22-μ pore size). Serial 10-fold dilutions of the sterilized solutions were made immediately prior to the injections with sterile phosphate-buffered saline[21]. Intradermal injections (0.02 ml.) were made into the forearm of the patients, and the wheal and flare reactions were read within 20 min. Nonallergic individuals served as controls and were injected with high concentrations of column fractions and NIH antigen E solutions. As suggested by Berrens (13), the allergenic activities of leaves–stems Fraction D and pollen antigen E are reported in terms of specific activity (units per milligram protein). In this system, one unit of activity represents the minimum quantity of the fraction (milligrams protein) eliciting a minimum reaction in specifically sensitive patients.

RESULTS AND DISCUSSION

The literature procedure (3, 4) was successfully used to isolate antigen E (Sephadex G-100, Fraction IV) from short ragweed pollen (1.3 mg. protein/g. pollen). Leaves–stems Fraction D (0.1–0.8 mg. protein/ml.) extracted from commercially supplied plants and NIH antigen E (0.015–0.125 mg. protein/ml.) gave a single precipitin line against antipollen crude extract serum (Fig. 2) and antiantigen E serum. However, when high concentrations of NIH antigen E (>0.125 mg. protein/ml.) were used, a contaminating antigen was detected (Fig. 2, first three upper wells from left). To prevent any nonspecific precipitin line formation, 0.062 mg. protein/ml. of NIH antigen E was used in all comparative immunodiffusion studies shown in Fig. 3.

Figure 3A illustrates the formation of lines of identity when NIH antigen E (Well 2) and leaves–stems Fraction D (Well 3) were placed in adjacent wells against antiantigen E serum. The material eluted from the unstained polyacrylamide cut gels in the region of standard antigen E (Fig. 3B, Wells 3 and 5) formed a common line

[19] Nikon model 6C.
[20] Millipore Corp., Bedford, Mass.
[21] Hollister-Stier Lab., Downers Grove, Ill.

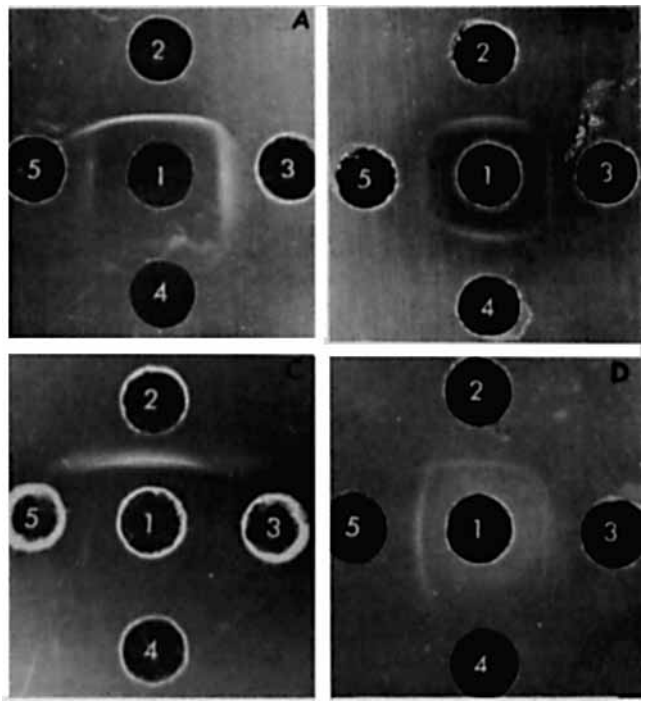

Figure 3 *Comparative immunodiffusion analysis of short ragweed leaves-stems and root Fraction D with NIH antigen E against antipollen crude extract and antiantigen E serum. (A) Leaves-stems Fraction D and NIH antigen E against antiantigen E serum: 1, antiantigen E serum; 2, NIH antigen E (0.062 mg./ml.); 3, leaves-stems Fraction D (0.8 mg./ml.); 4, buffer control; and 5, leaves-stems Fraction D (0.2 mg./ml.). (B) Leaves-stems Fraction D polyacrylamide gel eluate and NIH antigen E against antipollen crude extract serum: 1, antipollen crude extract serum; 2, 4, NIH antigen E (0.062 mg./ml.); and 3, 5, leaves-stems polyacrylamide gel eluate. (C) Root Fraction D and NIH antigen E against antiantigen E serum: 1, antiantigen E serum; 2, NIH antigen E (0.062 mg./ml.); and 3, 4, 5, root Fraction D (1.6, 0.8, and 0.4 mg./ml., respectively). (D) Root Fraction D and NIH antigen E against antipollen crude extract serum: 1, antipollen crude extract serum; 2, 3, root Fraction D (1.6 and 0.4 mg./ml., respectively); 4, buffer control; and 5, NIH antigen E (0.062 mg./ml.).*

of identity with NIH antigen E (Fig. 3B, Wells 2 and 4) against antipollen crude extract serum. Similar results were obtained when antiantigen E serum was used in place of antipollen crude extract serum. Further evidence for the presence of antigen E in short ragweed leaves-stems was obtained by disk electrophoresis and direct skin testing of sensitive individuals. Disk electrophoresis of NIH antigen E, leaves-stems Fraction D, and pollen antigen E (Sephadex G-100, Fraction IV) shows a similar pattern (Fig. 1). However, purified NIH antigen E had a slower anodic movement, which possibly resulted from the 50% glycerol present as a stabilizer. Leaves-stems Fraction D was tested on the skin of two short ragweed pollen-sensitive individuals. The specific activity of this fraction was established to be 10^7 (units/mg. protein) as compared to 10^9 (units/mg. protein) for NIH antigen E on the same patients.

The protein content of the commercially supplied leaves-stems in Fraction D was found to be 0.012 mg./g. while the antigen E content was estimated to be 0.0022 mg. protein/g. when quantitated from the standard curve of the logarithm of the NIH antigen E concentrations *versus* precipitin line positions (Fig. 2). Twice as much protein was obtained from leaves-stems that had been collected from the medicinal plant garden.

Figures 3C and 3D illustrate the reaction of root Fraction D and NIH antigen E against antiantigen E and antipollen crude extract serums, respectively. Precipitin lines between various concentrations of root Fraction D and antiantigen E serum did not appear (Fig. 3C, Wells 3, 4, and 5). A complete line of nonidentity developed between NIH antigen E, root Fraction D (Fig. 3D, Wells 2 and 5), and antipollen crude extract serum. Short ragweed seed Fraction D was obtained in a similar manner as that for pollen antigen E and was further fractionated by chromatography[14]. Five peaks eluted from the column were separately subjected to immunodiffusion and disk electrophoresis testing as described for leaves-stems; none contained antigen E.

In conclusion, immunodiffusion, disk electrophoresis, and skin testing evidence showed that the allergen, antigen E, is present in leaves-stems of short ragweed plant. Although the results of immunodiffusion and disk electrophoretic analysis indicated that antigen E was absent from roots and seeds of short ragweed, the possibility that other pollen allergens or their modification may be present in other parts of this plant cannot be excluded. In fact, this assumption is supported by immunofluorescent antibody studies substantiating that surface materials of ragweed pollen (exine and intine) are derived from the diploid tapetum and not from the haploid pollen (14). Results also suggest that the antigen E present in short ragweed leaves-stems may have been a major cause of hay fever symptoms, as recently suggested (1) for wind-borne plant particles. It is also concluded that short ragweed leaves-stems might serve as a source of antigen E, although the concentration present is much less than that of the pollen.

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▲ To whom inquiries should be directed.