

ISOLATION AND PROPERTIES OF A NEW SHORT RAGWEED POLLEN ALLERGEN, Ra6¹

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Two chromatographic forms (A and B) of the rapidly released short ragweed (*Ambrosia elatior*) pollen allergen Ra6 were isolated to antigenic ultrapurity by a combination of membrane filtration and ion exchange and gel filtration chromatography. Both Ra6 forms had a m.w. of 11,500 by gel filtration chromatography and 8000 by SDS-PAGE, and each consisted of two cathodically migrating bands on agarose electrophoresis at pH 8.5. Amino-terminal amino acid sequencing analysis detected two similar sequences (sequence 1 and sequence 2), which were present in different proportions in each of the chromatographic forms of Ra6. Among a ragweed-positive population sample (n = 101), 21% had specific IgE antibody (ab) and 42% specific IgG ab toward Ra6A; among ragweed-negative subjects (n = 260), 10.8% had IgG ab. We were unable to discriminate between Ra6A and Ra6B immunologically with the use of hyperimmune animal antisera or IgE and IgG ab from five Ra6-allergic humans.

It appears that Ra6 is composed of at least four closely related isoallergens of similar size but slightly different charges and amino acid sequences, which appeared to be antigenically and allergenically indistinguishable according to present evidence.

Allergens that are eluted rapidly from pollen grains would be expected to have the greatest relevance in causing allergic symptomatology in view of the short period (6 to 8 min) during which the pollen remains in the nose (1). Sixteen-minute extracts from short ragweed (*Ambrosia elatior*) pollen (RW(16-min))³ contain at least 20 basic and four or five neutral and acidic antigens (2-3). These rapidly released components contribute to a major extent to the biologic activity of conventional (long-term) ragweed extracts (1). A purified, 11,500 dalton basic fraction, G50II, from RW(16-min) ragweed extract caused wheal reactions on skin puncture testing in 17 of 22 ragweed-sensitive subjects (2). The main component (Ra6) of this fraction proved to be highly immunogenic in all rabbits and goats that we immunized with ragweed extracts. A small amount of Ra6 was isolated by using a preliminary procedure (4), and the material was used to raise a specific goat antiserum. We then revised the isolation procedure to produce sufficient quantities of ultra-pure Ra6 for extended chemical and biologic studies, including amino acid sequence determination, epidemiologic studies of antibody (ab) response to this antigen, and future genetic studies. This paper describes the current isolation procedure and the physicochemical and immunochemical analyses of two highly purified Ra6 components, A and B. The IgE and IgG ab responses to Ra6 in humans and the antigenic and allergenic relationship between Ra6A and B are discussed.

MATERIALS AND METHODS

Antisera. An IgG concentrate of antiserum to RW(16-min) extract, anti-RW(16-min), was prepared (5) by hyperimmunizing 11 rabbits with RW(16-min), precipitating the IgG fraction by adding 25 g ammonium sulfate per 100 ml of serum, and concentrating the dialyzed IgG isolate five times relative to the original serum. Anti-RW(16-min) was capable of recognizing at least 20 basic antigens (3). An antiserum highly specific for Ra6 was raised in a goat by using a small amount of Ra6 obtained as described in Reference 2 (with minor modifications consisting of additional recycling Ra6-containing fractions on carboxymethyl-(CM) Sephadex and Sephadex G-50 superfine columns (4)). Injections were given every 3 to 4 wk with approximately 1 mg and 0.2 mg of Ra6, followed by 0.1 mg of Ra6 thereafter. The protein was dissolved in 1 ml saline and emulsified with 1 ml of Freund's adjuvant containing 10 mg of *M. butyricum*. This special Freund's adjuvant was used for the first seven injections; only 1 mg of *M. butyricum* in 1 ml of Freund's adjuvant was used thereafter. The goat was bled 1 wk after each booster injection and the sera with the highest titers were pooled. The availability of this anti-Ra6 serum permitted us to estimate by radial immunodiffusion (RID) analysis the Ra6 contents of several ragweed extracts available from the FDA, and IUIS, as well as extracts prepared in our laboratory.

Preparation of Ra6. The isolation procedure is summarized in Figure 1. Fraction II (Fr. II), an ammonium sulfate precipitate of short ragweed Fr. C, prepared in Dr. T. P. King's laboratory according to published procedures (6), was obtained with the kind assistance of Dr. Daniel Mullally, NIAID. Three-kilogram dry pollen equivalents of Fr. C were dissolved in distilled water and insoluble materials were removed by centrifugation. The solution (3 liters) contained 680 mg of Ra6 by RID (see below). The solution was ultrafiltered sequentially through two Amicon hollow-fiber cartridges with nominal m.w. exclusion limits of 10,000 and 2000. At each step, the retentate was diluted with 3 liters of deionized water and the filtration was resumed. The combined fractions that passed through the first cartridge, but were retained by the second (Fr. II, 470 ml), contained most of the Ra6 and were selected for further purification.

Chromatography of Fr. II. The solution of Fr. II was concentrated to 90 ml by using an Amicon UM-2 membrane and was processed through a DEAE-Sephadex A-50 column (5 x 30 cm) equilibrated with 0.001 M Tris-HCl at pH 7.6. The eluates of this and subsequent chromatographic preparations were monitored by absorption at 280 nm, by immunodiffusion against anti-Ra6 serum, and by agarose electrophoresis at pH 8.5. An Ra6-containing fraction (250 ml), which was eluted unretarded from the column, was concentrated to 35 ml. This fraction (Fr. II-DEAE) was applied to a CM-Sephadex C-50 (2.5 x 25 cm) column that was equilibrated with 0.02 M sodium phosphate, pH 7.0. The phosphate buffer concentration was increased in a stepwise fashion to 0.05 M and 0.1 M. Two Ra6-containing fractions (CM-B and CM-A) were pooled separately and each was recycled through a fresh CM-Sephadex C-50 column (2.5 x 25 cm). Fraction CM-B was applied to the column in 0.02 M phosphate. After preliminary elution with 0.02 M phosphate, the first (less cationic) Ra6-containing fraction was eluted with 0.04 M phosphate, pH 7.0. In a second experiment, fraction CM-A was applied in 0.04 M phosphate and the second Ra6-containing fraction was eluted with 0.08 M phosphate, pH 7.0.

Both Ra6-containing fractions from CM-Sephadex chromatography were concentrated to 15 ml and were passed separately through a Biogel P-10 (200 to 400 mesh) column (2.5 x 88 cm). The more cationic Ra6 fraction,

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³ Abbreviations used in this paper: BBS, borate-buffered saline; CIE, crossed immunoelectrophoresis; CM, carboxymethyl; ID, immunodiffusion; IEP, immunoelectrophoresis; RIA, radioimmunoassay; RID, radial immunodiffusion; RW (16-min), 16-min extract of short ragweed pollen.

Ra6A, was recycled through the same column, whereas the less cationic Ra6 fraction, Ra6B, was purified by two passages through a Sephadex G-50 superfine column (1.5 x 90 cm).

Agarose electrophoresis. Analytic agarose electrophoresis at pH 8.5 was performed at 10°C essentially as described by Laurell (7), with the use of 1% agarose (Seakem, Type ME; FMC, Marine Colloids Div., ME) in Tris-barbital buffer (Type C-IV; Sigma Chemical Co., St. Louis, MO) on plastic sheets (Gel Bond; FMC). After completion of the electrophoresis (60 min at 10 V/cm), the protein components were fixed and stained for 1 hr in a solution of 0.9% Coomassie Blue (R-250; Bio-Rad Laboratories, Richmond, CA) in 25% methanol, 10% trichloroacetic acid, 3% sulfosalicylic acid, and 61% H₂O. The slides were destained in a solution of 7.5% acetic acid and 10% methanol in H₂O.

Immunodiffusion (ID) and immunoelectrophoresis (IEP). ID and quantitative RID were carried out essentially as described (8, 9). RID was used to obtain the yields of Ra6 at each fractionation step. Samples of each fraction (Fig. 1) that had been stored at -20°C were analyzed against anti-Ra6 (1/10, w/v) by using the final Ra6A preparation as a standard. For IEP, the samples were first run for 60 min at 10 V/cm with 1% agarose gels in Tris-barbital buffer at pH 8.6. Antiserum was added to troughs cut in the gels, which were then incubated overnight at 23°C. The gels were washed, dried, and then stained with Coomassie Blue. Crossed immunoelectrophoresis (CIE) and tandem CIE were performed at 10°C essentially according to Axelsen *et al.* (10). The first dimension electrophoresis was run at pH 8.5 for 50 to 60 min at 10 V/cm and the second dimension for 14 to 16 hr at 0.5 V/cm.

Sodium dodecyl sulfate-polyacrylamide gradient electrophoresis (SDS-PAGE). SDS-PAGE was performed in slab gels according to the method of Laemmli (11). All chemicals were obtained from Bio-Rad. A linear polyacrylamide gradient from 5 to 20% (prepared with a 100 ml LKB gradient maker; LKB Instruments, Rockville, MD) was used as the separating gel. The separating gel dimensions were 1.5 (thickness) x 115 mm (length), with a 3% stacking gel of 15 mm in length. The average running time was 6 hr at 40 mA and 15°C. Protein loads varied between 15 and 30 µg. The separated proteins were fixed in the gels and stained overnight with the solutions described for agarose electrophoresis. Polyacrylamide gel gradient electrophoresis at pH 4.3 (pH 4.3 PAGE) was essentially the same as SDS-PAGE with the exception of the buffer system. The pH 4.3 buffer system of Reisfeld *et al.* (12) was used in place of the basic system of Laemmli (11).

Amino acid analysis. The amino acid analyses of Ra6A and Ra6B were performed in triplicate after hydrolysis in 6 N HCl *in vacuo* for 22 hr at 110°C. Both Ra6A and Ra6B had been extensively reduced with 0.05 M dithiothreitol in 7 M guanidine-HCl and then radioalkylated with [³H]iodoacetic acid before hydrolysis. Samples were analyzed on a high-pressure liquid chromatography amino acid analyzer with an ion exchange column, with the use of a two-buffer linear gradient elution and ninhydrin detection, as described in detail elsewhere (13).

Amino-terminal amino acid sequencing. Amino-terminal amino acid sequence analysis of Ra6A and Ra6B was performed with an automated sequencer as described elsewhere (14) for a similar analysis of Ra3, except that gas chromatography was not included among the analytic techniques used.

Other physical and chemical analyses. Protein determinations were performed according to the method of Lowry *et al.* (15) with bovine serum albumin (BSA) (Sigma) as a standard. Molecular weight determination by gel filtration was performed according to Andrews (16). A Sephadex G-50 (fine) column (2.5 x 90 cm) was calibrated with the following m.w. markers: ragweed allergen Ra3, cytochrome c, myoglobin (Type I from horse skeletal muscle), soya bean trypsin inhibitor, and chymotrypsinogen. The extinction coefficient $E_{1\%}^{1\text{cm}}$ was calculated from the amino acid composition according to Wetlaufer (17). Total carbohydrate analysis was performed according to Dische (18) with D-arabinose and D-glucose as standards. SDS-PAGE gels were also stained for carbohydrate with the use of the performic acid-Schiff stain.

Study population. Three hundred and sixty-one Caucasian volunteers (271 male, 90 female, aged 39 ± 10 yr) were selected from a larger group of employees of the Westinghouse Electric Corporation in northern Baltimore County (19). Of these, 101 subjects were skin test-positive to short ragweed extract. Additional studies were performed by using a group of 32 Caucasian ragweed-allergic patients participating in various clinical studies of allergy at the Good Samaritan Hospital, Baltimore, MD.

Skin testing. The skin test procedure (19) was a modification of a semi-quantitative puncture method developed by Santilli *et al.* (20). Ragweed positivity was defined by a wheal reaction of >3 mm in diameter to the dialyzed short ragweed 24-hr extract (300 µg/ml) described elsewhere (21).

Double antibody radioimmunoassay (RIA). A direct binding RIA was used to investigate the binding of human IgE and IgG antibodies to Ra6A and Ra6B (3, 5). ¹²⁵I-labeled Ra6A or Ra6B (2.4 ng) in 100 µl of borate-buffered saline (BBS) containing 5% BSA was added to and allowed to incubate (4 hr at 23°C) with human serum (100 µl), diluted 1/2 to 1/5 for the study of IgE ab and 1/10 to 1/50 for the study of IgG ab. For the IgE assay, 100 µl of a mixture containing IgE myeloma serum PS "carrier" (1/200) and a slight excess of goat anti-IgE (ε-chain specific) were added. For the IgG assay, 100 µl of a slight excess of goat anti-human IgG (γ-chain specific) were added. In both types of assay, the immune complexes were allowed to precipitate overnight at 4°C. The precipitates were washed four times with BBS and counted in a gamma counter. The ab contents were computed by comparison against a standard serum of known ab content (estimated by using the procedure of Mulligan *et al.* (22)).

An inhibition RIA was used to study the inhibition of binding of radiolabeled Ra6A to human IgE and IgG ab by cold Ra6A or cold Ra6B (5). The sera of five Ra6-allergic subjects (two treated with short ragweed extracts and three untreated) as well as the serum from a nonallergic control, were used. Unlabeled Ra6A or Ra6B (100 µl, containing 0.3 to 200 ng Ra6/ml in 5% BSA in BBS) was preincubated for 1 hr at room temperature with 100 µl of diluted serum (1/10 to 1/50 dilution in BBS for the IgG assay or 1/2 dilution for the IgE assay). Radiolabeled Ra6A (2.4 ng) in 100 µl was then added and the mixtures were incubated for a further 4 hr at 23°C. Anti-IgE (plus myeloma serum) or anti-IgG was then added, and the resultant immunoprecipitates were washed and counted as in the direct binding assay. The percent of inhibition (%) was calculated with the formula:

$$\%I = \frac{(\text{cpm for test serum without inhibitor}) - (\text{cpm for test serum with inhibitor})}{(\text{cpm for test serum without inhibitor}) - (\text{cpm for control serum})} \times 100$$

RESULTS

Ra6 content of short ragweed pollen. By using the anti-Ra6 serum prepared previously (4) in RID experiments, we found that the Ra6 contents of four 24-hr reference short ragweed (1/10, w/v) extracts from the FDA to be 123 µg ± 8 Ra6/ml (range, 110 to 130 µg/ml). The proposed IUIS (1/10, w/v) short ragweed reference contained 110 µg ± 6 Ra6/ml. A 1/10 (w/v) 8-min extract of short ragweed prepared at 23°C in our laboratory contained 122 µg ± 9 Ra6/ml and a 24-hr 1/10 (w/v) extract prepared at 4°C from the same batch of pollen contained 119 µg ± 3 Ra6/ml. These values are equivalent to Ra6 contents of 1.1 to 1.3 mg/g of dry pollen. The rapid extraction of Ra6 from the pollen (within 8 min) confirms previous data (2). Similar antigen levels in dry ragweed pollen were reported for AgK (1.2 mg/g) (23), with significantly lower antigen contents for Ra3 and Ra5 (0.8 and 0.3 mg/g, respectively) (24).

Preparation of Ra6A and Ra6B. In the Amicon hollow-fiber

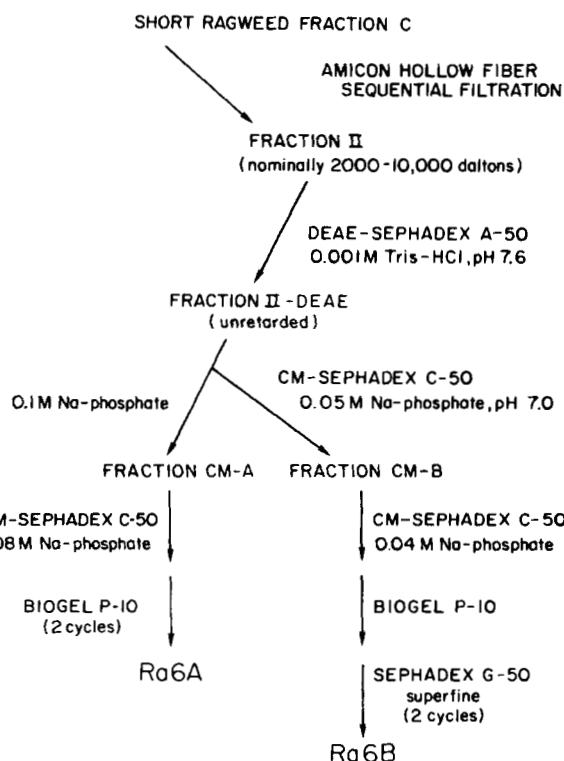


Figure 1. Schematic outline of the isolation procedures for Ra6A and Ra6B.

filtration step, 90% of Ra6 (600 mg by RID assay) passed through the H1P10 hollow-fiber cartridge and 30% of this material also passed through the H1P2 cartridge, leaving 400 mg of Ra6 in Fr. II (Fig. 1, Table I). This fraction was further separated into unretarded and retarded fractions by DEAE-Sephadex column chromatography with 0.001 M Tris-HCl buffer at pH 7.6. The unretarded fraction (Fr. II-DEAE) that contained all of the Ra6 was eluted just before allergen Ra3, which was weakly absorbed to the DEAE ion exchanger (25).

Fr. II-DEAE (350 mg) was further purified by CM-Sephadex chromatography (Fig. 2). Two widely separated Ra6-containing fractions were identified by ID assay. The less cationic Fr. CM-B was eluted with the first buffer step (0.05 M phosphate) as part of a complex series of protein peaks, and the more cationic Fr. CM-A was eluted with twice the buffer concentration. The recycling of each Ra6-containing fraction separately, with slightly lower phosphate concentrations (0.04 M and 0.08 M, respectively), achieved further purification depending on the charge. The Ra6 contents of the resultant two fractions were 110 and 120 mg for Ra6B and A, respectively.

Final purification of Ra6A and B was achieved by gel filtration in Biogel P-10. Ra6A was located in the main peak in Figure 3A. Only fractions found to contain Ra6 components by agarose electrophoresis at pH 8.5 were selected for recycling on the same column (not shown), yielding 40 mg of Ra6A.

Fraction CM-B was passed once through the same P-10 column (Fig. 3B), but because the agarose electrophoretic pattern indicated that impurities were not readily eliminated, two cycles through a Sephadex G-50 (superfine) column were performed for final purification, yielding 40 mg of Ra6B. The first of these two G-50 runs is shown in Figure 3C.

Homogeneity and antigenic relatedness. When carried out

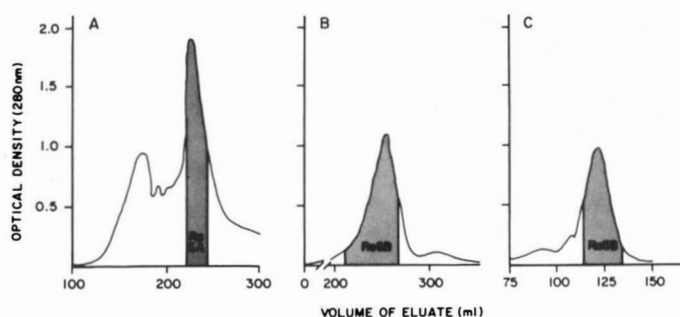


Figure 3. Purification of recycled CM-Sephadex fractions CM-A and CM-B on a Biogel P-10 column (2.5 x 88 cm) equilibrated with 0.05 M Tris buffer pH 7.6, yielding partially purified Ra6A (A) and Ra6B (B). C, further purification of Ra6B with a Sephadex G-50 (superfine) column (1.5 x 90 cm). This fraction was subsequently recycled through the same column.

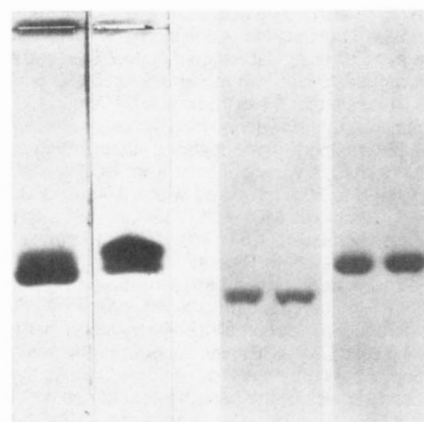


Figure 4. Electrophoretic analyses of the purified chromatographic forms of Ra6. A, agarose electrophoresis of Ra6A and Ra6B (4 μ g each) at pH 8.5 for 60 min at 10 V/cm. B, SDS-PAGE analysis of Ra6A and Ra6B, (25 μ g) for 6 hr at 40 mA with a 5 to 20% acrylamide gradient. C, pH 4.3 PAGE of Ra6A and Ra6B with the analogous conditions for SDS-PAGE.

Fraction	Ra6 Component(s)	Ra6 Content (mg)	Percent Yield
Short ragweed			
Fraction C	A + B	680*	100
Fraction II	A + B	400	59
Fraction II, DEAE	A + B	350	51
CM-A	A	120	18
CM-B	B	110	16
Ra6A (final)	A	40	6
Ra6B (final)	B	40	6

* These data represent 19% of the total Ra6 in 3 kg of dry pollen used to prepare Fr. C.

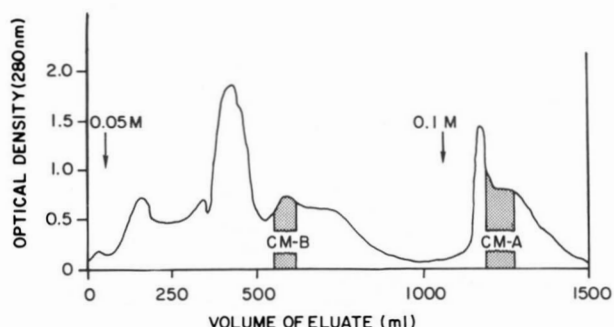


Figure 2. Separation of Ra6-containing fractions by CM-Sephadex column chromatography. Fr. II-DEAE in 0.02 M sodium phosphate, pH 7.0 was applied to a CM-Sephadex C-50 column (2.5 x 25 cm) that was equilibrated with the same buffer. A second buffer step (0.05 M phosphate, pH 7.0), following immediately after complete adsorption of the applied proteins, eluted an Ra6-containing fraction, Fr. CM-B. After passage of 1000 ml of this buffer, the final buffer step (0.1 M) was used to elute the second Ra6-containing fraction, Fr. CM-A. Fractions CM-B and CM-A were recycled separately through a second CM-Sephadex column (2.5 x 25 cm) with additional optimal buffer concentrations of 0.04 M and 0.08 M, respectively.

under optimal conditions, agarose electrophoresis at pH 8.5 consistently showed double bands for both the A and B forms of Ra6 at each purification step (e.g., Fig. 4A). Another feature of these patterns is the similarity in the electrophoretic mobilities of Ra6A and Ra6B at pH 8.5, which was not expected in view of the difference in their adsorption to CM-Sephadex (Fig. 2). With SDS-PAGE (Fig. 4B) and pH 4.3 PAGE (Fig. 4C) with acrylamide gradients of 5 to 20%, Ra6A and Ra6B both showed single bands having identical migration rates. Contaminants were not visible at protein loads up to 25 μ g, although irregular bands were produced at protein loads exceeding 15 μ g and extensive band spreading occurred with loads over 25 μ g.

The antigenic purity of the Ra6A and Ra6B preparations was shown by CIE analysis with the use of our hyperimmune rabbit serum IgG concentrate, anti-RW(16-min). Characteristic sharp peaks of intense immunoprecipitation were observed (Fig. 5A and B). Complete antigenic identity of the A and the B forms of Ra6 was shown by tandem CIE with Ra6A applied in the well to the left of Ra6B (Fig. 5C).

Physical and chemical properties. Ra6A and B had identical elution volumes from gel filtration columns. Both Ra6 forms were found to have a m.w. of $11,500 \pm 1000$ by Sephadex G-50 (fine) gel filtration and 8000 by SDS-PAGE. The $E_{280}^{1\%}$ for Ra6A was 3.0 with use of the Lowry procedure to determine protein concentration. This is in agreement with the value of 2.9 determined from the amino acid compositions of Ra6A and Ra6B. Carbohydrate analysis showed that less than 1% carbohydrate was present both in Ra6A and Ra6B.

Amino acid composition. The amino acid compositions for

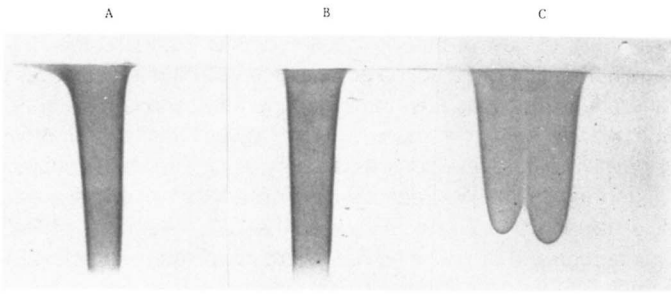


Figure 5. CIE analysis of A, purified Ra6A, 2.5 μ g and B, Ra6B, 2.5 μ g at pH 8.5. Electrophoresis was for 1 hr at 10 V/cm in the first dimension and for 16 hr at 0.5 V/cm in the second. The second dimension gel contained 20 μ l/cm² of IgG concentrate of anti-RW(16-min). C, tandem CIE analysis showing antigenic identity of Ra6A (left) and Ra6B. The antigens were applied to two wells 0.5 cm apart for first dimension electrophoresis; all other conditions were the same as in A and B. In all cases, the anode is to the right in the first dimension and to the top in the second.

TABLE II
Amino acid composition of allergens Ra6A and Ra6B

Amino Acids	Ra6A			Ra6B		
	Mol. per cent		No. residues ^a	Mol. per cent		No. residues ^a
CYS	7.48	7.7	8	6.23	6.3	6
ASX	11.20	11.4	11	9.83	9.8	10
THR	6.88	7.1	7	6.34	6.3	6
SER	5.47	5.6	6	5.43	5.4	6
GLX	4.91	5.1	5	4.96	5.0	5
PRO	9.82	10.0	10	10.00	10.0	10
GLY	5.41	5.6	6	5.86	5.9	6
ALA	9.43	9.7	10	9.30	9.3	9
VAL	7.59	7.8	8	7.93	7.9	8
MET	1.44	1.5	1	1.62	1.6	1
ILE	3.78	4.5	5	3.97	4.0	4
LEU	9.08	9.3	9	9.18	9.2	9
TYR	1.40	1.4	2	1.63	1.6	2
PHE	3.90	4.0	4	4.13	4.1	4
HIS	0.48	0.49	0	1.10	1.1	1
LYS	10.20	10.5	11	12.00	12.0	12
ARG	3.90	4.6	5	4.07	4.1	4
TRP	—	0	0	—	0	0
Total number of residues	108			103		

^a The number of residues was calculated on the basis of the m.w. obtained by gel filtration (11,500). The second column provides our estimate of the integral values.

SEQUENCE 1: 1 ALA PRO THR CYS ASP THR VAL GLN ASN ASN LEU
5
SEQUENCE 2: SER PRO THR CYS ASP ASP VAL GLN ASN ILE LEU ALA
10

Figure 6. Amino-terminal sequences of Ra6A and Ra6B. Two slightly different sequences (1 and 2) were found for the first 12 NH₂-terminal residues in both Ra6A and Ra6B. The sequences are similar and clearly related, assuming a proline deletion at position 3 in sequence 2. Ra6A had 90% of sequence 1 and 10% of sequence 2. Ra6B had 50% of sequence 1 and 50% of sequence 2.

Ra6A and Ra6B were computed assuming a m.w. of 11,500 (Table II).⁴ As expected, the compositions are virtually identical. Of the commonly found amino acids, tryptophan is absent.

Amino-terminal amino acid sequence. Twelve NH₂-terminal amino acid residues for Ra6A and Ra6B are shown in Figure 6. There were two slightly different sequences (1 and 2) in each of the two chromatographic forms of Ra6. These sequences could be readily distinguished by the deletion of a proline residue at position three in sequence 2, so that otherwise similar sequences were shifted by one position. Ra6A consisted of 90% sequence 1 and 10% sequence 2 and Ra6B consisted of 50% sequence 1 and 50% sequence 2. Assuming a proline deletion in sequence 2, three further differences were found at positions 1, 7, and 11

⁴ The value of approximately 11,500 daltons obtained by column chromatography is supported by preliminary amino acid sequence data, which indicates about 100 residues (to be reported in a subsequent paper).

(Fig. 6). As part of another study in progress, the individual NH₂-terminal peptides of Ra6B have been isolated and sequenced; these data fully confirm the sequences deduced by calculating yields of PTH amino acids from analyses of the mixture of molecules in both Ra6A and Ra6B.

Antigenic and allergenic activity. Human IgE and IgG ab responses toward Ra6A were studied in 361 Caucasian volunteers from the Westinghouse Electric Corporation by RIA of their sera (Table III). Among the 101 ragweed-sensitive subjects, 42% had detectable IgG ab and 21% had detectable IgE ab. Among 260 subjects who were skin test-negative to ragweed, 10.8% had detectable IgG ab and 0.4% had detectable IgE ab to Ra6. The correlation coefficient for log (IgE ab) vs log (IgG ab) for the 42 persons who were ragweed-sensitive and had Ra6-specific IgG ab was $r = 0.86$ ($p < 0.001$).

Antigenic and allergenic identity of the Ra6 variants in the human system was shown by double antibody inhibition RIA in five Ra6-allergic patients. Three representative curves for each of the IgG ab and the IgE ab systems are shown in Figures 7 and 8. Identical inhibition curves were obtained for the five sera tested irrespective of whether the A or B form was used as an inhibitor. Further evidence for the allergenic identity was obtained by puncture skin testing of 32 highly ragweed-allergic clinic

TABLE III
Immunoglobulin G and E ab toward Ra6A

Ab	Total Group (n = 361)	Ragweed-sensitive ^a (n = 101)	Ragweed-insensitive (n = 260)
IgG	70	42	28
IgE	22	21	1

^a Ragweed sensitivity was determined by puncture skin testing with dialyzed short ragweed extract (300 μ g/ml) (see Reference 19).

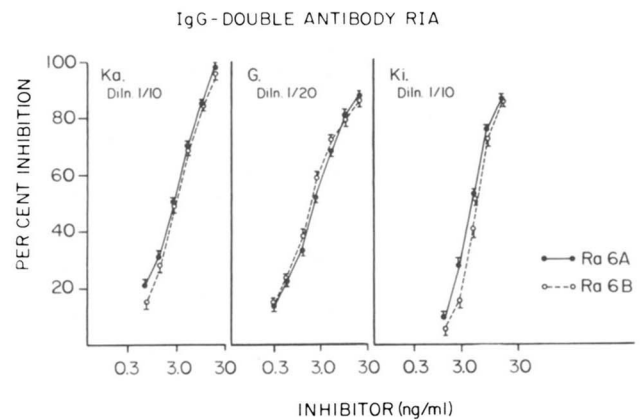


Figure 7. Inhibition RIA comparing the relative abilities of Ra6A and Ra6B to inhibit the binding of ¹²⁵I-Ra6A to the IgG ab in one pretreatment serum (Ka.) and two post-treatment sera.

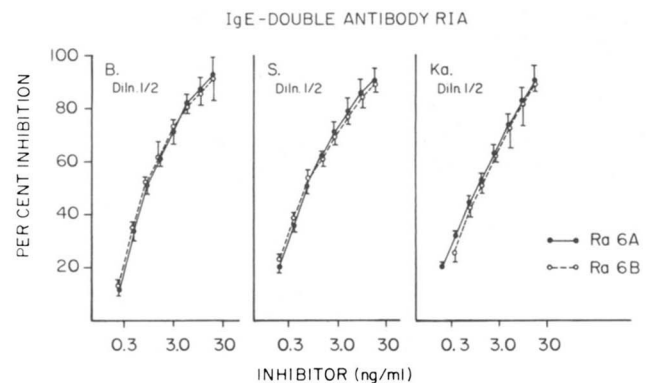


Figure 8. Inhibition RIA comparing the relative abilities of Ra6A and Ra6B to inhibit the binding of ¹²⁵I-Ra6A to the IgE ab in three pretreatment sera.

patients. Eight patients were similarly sensitive to both forms of Ra6 and the remainder was insensitive to both.

DISCUSSION

The main goal of this study—to purify and characterize Ra6, a recently isolated, rapidly released short ragweed pollen allergen—has been achieved. The Ra6 content of ragweed pollen (about 1.2 mg/g) represents 1.0 and 5.0% of the nondialyzable components present in 4-day and 16-min extracts, respectively (1). Allergen Ra6 was isolated in two chromatographic forms, Ra6A and B, which were indistinguishable antigenically and allergenically. Both forms were isolated in sufficient amounts of antigenically ultrapure material (40 mg) to permit physical, chemical, and immunologic characterization. The yields of Ra6A and B (6% each of the total Ra6 in the starting material, Fr.C.; Table I) are reasonable in view of our goal to sacrifice quantity for purity.

Ra6A and B are both small, basic, carbohydrate-free proteins. The m.w. of both (11,500), computed from gel filtration data, appear to be more in line with the correct value than the 8000 m.w. value obtained by SDS-PAGE, according to recent amino acid sequencing data.⁴ Interestingly, two protein-staining bands of slightly differing charges were detected by agarose electrophoresis at pH 8.5 in both Ra6A and Ra6B (although no such charge difference was detectable by pH 4.3 PAGE). This suggests the existence of at least four variants of Ra6, because both Ra6A and Ra6B were well separated by CM-Sephadex chromatography. The existence of (at least) two forms in both the A and B fractions was confirmed by amino acid sequence determination of 12 NH₂-terminal residues (Fig. 6). In both cases, two equivalent sequences were found that differed by a proline deletion at position 3 and at three other residues. These two sequences were present in different proportions in Ra6A and Ra6B.

Rabbit and goat antisera to Ra6 and to crude ragweed extracts did not detect antigenic differences; tandem CIE assays of Ra6A and Ra6B showed complete joining of the precipitin arcs (Fig. 5C). Immunologic identity of Ra6A and Ra6B was also shown with the use of inhibition RIA with IgE and IgG ab from five different Ra6-allergic humans. Identical inhibition curves were obtained irrespective of the Ra6 form used to inhibit binding of radiolabeled Ra6A to these human IgG and IgE ab. Thus, immunologic differences between the two Ra6 fractions (if they exist) have not yet been detected by this highly sensitive assay (5) in a relatively small number of allergic subjects tested at this time.

Among a ragweed-sensitive population ($n = 101$), 21% had IgE ab and 42% had IgG ab to Ra6A. This classifies Ra6 as a "minor" allergen when compared with a frequency of skin test sensitivity to AgE (25) of 91% in the same population (unpublished data). Among the same ragweed-sensitive population, 9% were found to have IgE ab to the 5000 dalton allergen Ra5 (3), and 18% to the 12,000 dalton allergen Ra3 (unpublished observations). The twofold higher prevalence rate of IgG ab vs IgE ab response to Ra6, and the presence of IgG ab in 10.8% of nonragweed-allergic subjects, is somewhat surprising, although a similar situation exists for Ra3 (unpublished data). A possible explanation in both cases could be antigenic cross-reactivity with other environmental antigens.

It would appear that Ra6 is composed of at least four closely related "isoallergens" (27), toward which we have not yet discov-

ered any differential immunologic response with our highly sensitive RIA. In view of this finding, it seems unlikely that the NH₂-terminal portion of 12 amino acids, where we have found several differences, contains any major immunodeterminants. The reaction of the human immune system toward these and other chemically different regions would provide an interesting subject for further study. We recently determined that a single amino acid deletion of a lysine residue at the COOH-terminal of Ra5 was recognized by 8% of an Ra5-allergic population sample (28), a finding that pointed to the COOH-terminus as being an immunodeterminant of this allergen molecule. Completion of the amino acid sequence of Ra6A and Ra6B, together with the availability of fragments of known primary structure, will enable us to pursue this investigation further.

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