

Isolation and characterization of Russian thistle (*Salsola pestifer*) pollen allergens*

Ali Shafiee, Ph.D.,** John W. Yunginger, M.D., and Gerald J. Gleich, M.D.
Rochester, Minn., and Tehran, Iran

*The radioallergosorbent test (RAST) was utilized to identify allergenically active molecules in an extract of Russian thistle (*Salsola pestifer*) pollen. Two glycoproteins (RT₁ and RT₂) were isolated by ion-exchange chromatography, preparative flat-bed electrofocusing, and gel filtration chromatography. These highly purified proteins were similar as judged by discontinuous polyacrylamide gel electrophoresis (PAGE), by PAGE in urea-acetate, and by immunodiffusion analysis. In Ouchterlony double-diffusion, rabbit antiserum to crude pollen extract showed a line of identity between RT₁ and RT₂. Each allergen possessed a single polypeptide chain, with molecular weights of 39,000 and 42,000 for RT₁ and RT₂, respectively. Both allergens contained 9.0% $\pm$ 0.5% carbohydrate and 10.6% $\pm$ 0.2% nitrogen. Both RT₁ and RT₂ eluted from a calibrated Sephadex G-100 column near the ovalbumin marker, but the elution volume of RT₁ was slightly greater than that of RT₂. Amino acid analysis of RT₁ and RT₂ showed that there was no difference in composition between the two proteins. The proteins showed different isoelectric points (RT₁ = 6.7; RT₂ = 6.2). They had similar skin reactivity by Prausnitz-Kustner testing, showed similar potency and identical allergenic qualities as inhibitors in the RAST, and were more potent on a mass basis than crude extract in the inhibition of the reaction between solid-phase crude extract and IgE antibodies in the RAST. RT₁ and RT₂ represent 0.014% and 0.010% by mass, respectively, of the pollen. These results suggest that RT₁ and RT₂ are important allergens in Russian thistle pollen and that these proteins are immunologically identical.*

Plant pollens are the most common cause of seasonal allergic disease, and the major allergenic molecules of ragweed and grass pollen extracts have been isolated and characterized.¹⁻³ Purified allergens have

been useful for standardization of allergy extracts and for investigation of the pathogenesis of immediate hypersensitivity reactions.⁴ In the prior studies of pollen allergens skin testing of allergic individuals was used to identify the allergens. An alternative to skin testing is the radioallergosorbent test (RAST),⁵ which measures the interaction of IgE antibodies with solid-phase allergens and which has been used for identification of allergenic molecules.⁶⁻⁸

Russian thistle (*Salsola pestifer*) pollen is a major cause of allergic rhinitis (hay fever) in Iran⁹ and the western plains of the United States.¹⁰ In these investigations we utilized the RAST to isolate and characterize allergenic molecules from Russian thistle pollen.

EXPERIMENTAL PROCEDURES

Materials

Pollen (lot 23P5-8) and pollen extract were purchased from Greer Laboratories, Lenoir, N.C. Paper discs were from Schleicher and Schuell, Inc., Keene, N.H. Cyanogen bromide and acrylamide were obtained from Eastman Kodak, Rochester, N.Y. Freund's adjuvant was from

From the Departments of Pediatrics, Medicine, and Immunology, the Allergic Diseases Research Laboratory, Mayo Medical School, and Mayo Clinic and Foundation, Rochester, Minn., and from the College of Pharmacy, Tehran University, Tehran, Iran.

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Reprint requests to: Gerald J. Gleich, M.D., Mayo Clinic, Rochester, MN 55905.

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**Presently affiliated with the Department of Medical Biochemistry, University of Missouri, Columbia.

Difco, Detroit, Mich., and Sephadex, blue dextran, and RAST tubes were purchased from Pharmacia AB, Uppsala, Sweden. Diethylaminoethyl (DEAE)-cellulose (DE-52) was obtained from Whatman, Kent, England. Ampholytes (Ampholine) were purchased from LKB, Bromma, Sweden. Bovine serum albumin, ovalbumin, and chymotrypsinogen-A were from Schwarz/Mann, Orangeburg, N.Y. Cytochrome-C and glycyl-L-tyrosine were from Sigma Chemical Co., St. Louis, Mo. Dialysis tubing (Spectropor) and UM-2 membranes were purchased from Spectrum Medical Industries, Los Angeles, Calif., and Amicon, Lexington, Mass., respectively. Urea was from Mallinckrodt, St. Louis, Mo., and all other chemicals were either from Sigma Chemical Co. or Fisher Scientific Co., Pittsburgh, Pa.

Antigenic and allergenic analysis

Direct skin testing and collection of sera. Sera were obtained from nine Russian thistle pollen-hypersensitive individuals in Tehran, Iran, during the spring of 1978. All patients had classic hay fever symptoms during the pollination season of this plant; all showed positive intradermal test reactions to a 1:2000 w/v dilution of pollen extract (0.02 ml); and all had elevated levels of IgE antibodies reactive with allergens in an extract of Russian thistle by RAST (below) as shown in Table I. All patients were skin tested with commercial Russian thistle pollen extract as well as with an extract made from pollen collected in Tehran in the summer of 1977. A serum pool was made by mixing equal volumes of the nine sera and was used to determine allergenic activity.

Prausnitz-Kustner (P-K) testing. The allergic serum pool (0.05 ml) was injected intradermally into several sites on the forearm of a normal individual (A. S.). After 24 hr injected sites were challenged intradermally with different concentrations of purified allergens.

Radioallergosorbent test. Allergens were bound to paper discs according to published procedures.¹¹ Briefly, cyanogen bromide (CNBr) (2.5%, 160 ml) was added to 2 gm of washed paper discs; the pH was maintained at 11 for 5 min at room temperature. After repeated washings the CNBr-activated discs were dried for 2 hr at 4° C and kept in a desiccator at -20° C. Isolated fractions of crude Russian thistle extract (below) were coupled to the activated discs according to published procedures.¹² Usually the proportions were 10 mg of lyophilized sample to 100 CNBr-activated discs. The lyophilized sample was dissolved in 0.20M H₃BO₃, 0.04M NaOH, and 0.16M NaCl, pH 8.0, and was added to a small bottle containing the CNBr-activated discs, which were tumbled overnight at 4° C. Coupled discs were washed several times with 0.1M Na₂HPO₄ and KH₂PO₄, pH 7.4, containing 1% polyoxyethylene sorbitan monolaurate (Tween 20) (RAST wash) until the 277 nm absorbance of the wash was <0.005.

In the performance of the RAST the allergic serum pool (0.05 ml) was added to the coupled discs, and after overnight incubation at room temperature the discs were washed thrice with 2.0 ml of 0.15M NaCl. Next, 0.10 ml of

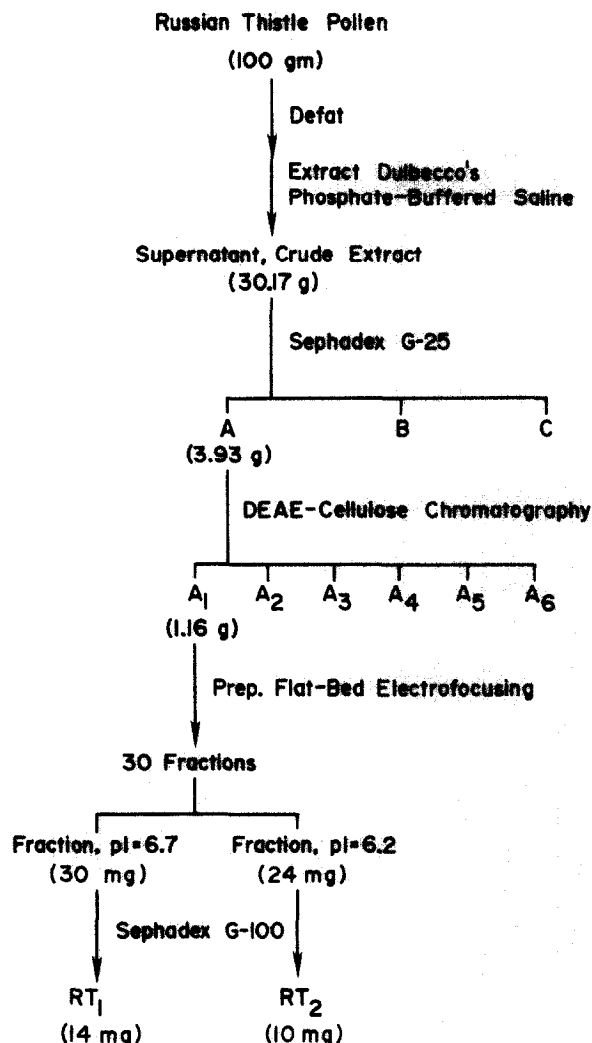


FIG. 1. Flow chart showing the isolation of RT₁ and RT₂. Dry-weight yields at each step of the purification are shown.

radiolabeled affinity chromatography-purified rabbit anti-IgE containing 20 ng of protein was added to the discs. After another overnight incubation at room temperature, the discs were washed again with 0.15M NaCl and counted in a gamma scintillation counter. Results were expressed as the percent of total counts bound to the disc. As a negative control, normal serum never showed over 2.5% binding.

RAST inhibition. RAST inhibition experiments were performed by the method of Gleich and Yunginger¹¹ with modifications. The crude pollen extract was coupled to CNBr-activated discs as above, except 25 mg of lyophilized extract was used to couple with 100 discs. The total absorbance at 277 nm was measured in coupling supernatants and washings; the proportion bound was 56%. For RAST inhibition the allergic serum pool (0.025 ml) and differing volumes of inhibitor were mixed and the final volume was brought to 0.05 ml with 0.1M Na₂HPO₄ and KH₂PO₄, pH 7.4, containing 0.2% bovine serum albumin, 1% Tween 20, and 0.1% Na azide. One allergen disc was added and the

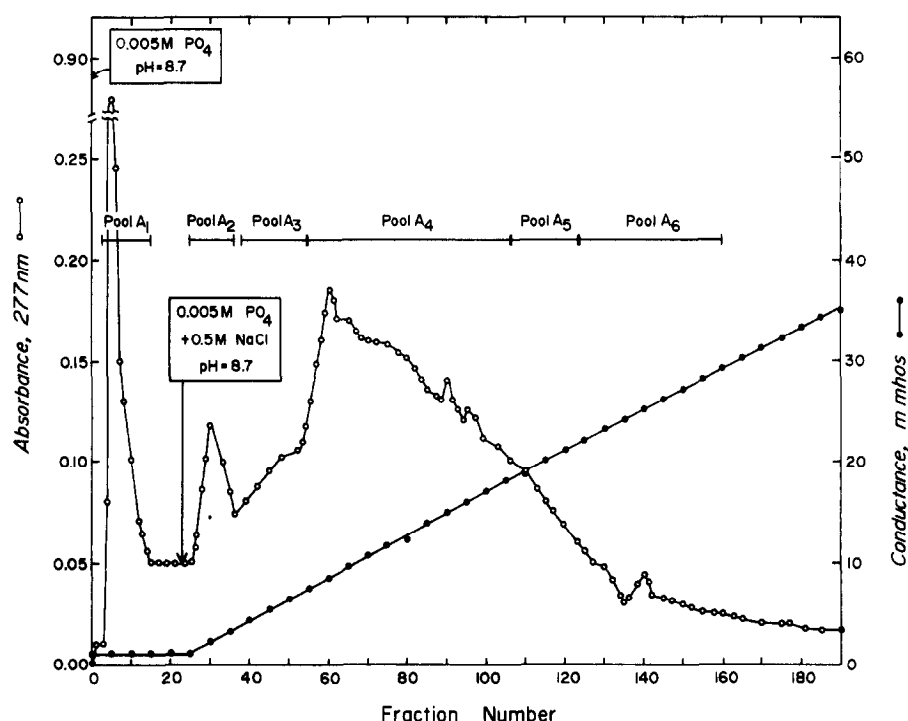


FIG. 2. DEAE-cellulose chromatography of Sephadex G-25 pool A. Horizontal bars indicate pooled fractions; $\circ$, A_{277} ; $\bullet$, conductance.

TABLE I. Elevated levels of IgE antibodies to crude Russian thistle pollen extract in nine hypersensitive patients

Patient No.									
1	2	3	4	5	6	7	8	9	
RAST*	6.3	17.5	19.1	9.7	23.2	12.1	5.2	6.8	13.4

*RAST results are expressed as percent of total counts bound; normal sera produced binding of 1.5% to 2.5%.

tube was incubated overnight at room temperature. The rest of the procedure was carried out as described above. Percent inhibition was calculated according to the following formula:

$$\frac{\% \text{ counts bound without inhibitor} - \% \text{ counts bound with inhibitor}}{\% \text{ counts bound without inhibitor}} \times 100\%$$

The quantities of the inhibitor needed to obtain between 20% to 70% inhibition were determined. Percent inhibition was plotted on semilog paper with the log of inhibitor quantities on the abscissa. The slopes of the lines were compared by linear regression analysis; a programmable Hewlett-Packard 9810A calculator was used for the statistical evaluation.

Rabbit antiserum to crude Russian thistle pollen extract. New Zealand white rabbits were injected with antigen emulsified in Freund's complete adjuvant every 2 wk for 6 wk. The first injection contained 1.1 mg of lyophilized crude Russian thistle pollen extract dissolved in 0.5 ml of

TABLE II. Allergenic activity of DEAE-cellulose pools as measured by the RAST*

	DEAE-cellulose pools					
	A ₁	A ₂	A ₃	A ₄	A ₅	A ₆
RAST	18.8	8.4	7.2	6.0	4.2	3.4

*A sample of each pool, which contained the same absorbance at 277 nm, was bound to CNBr-activated paper discs. After washing, the solid-phase allergen was reacted with 0.50 ml of the serum pool containing IgE antibodies to Russian thistle pollen. The rest of the procedure is described in the text. Normal sera produced binding < 1.9%.

0.15M NaCl and 0.5 ml of adjuvant. This emulsion was injected into the footpads of each rabbit. The second and third injections each contained 2.85 mg of crude extract, respectively, and were administered subcutaneously on four different sites in each rabbit. The fourth injection contained 2.85 mg of antigen dissolved in 0.15M NaCl and was given intravenously. Sera were obtained 1 wk after a fifth and sixth biweekly subcutaneous injection of 10 mg of antigen emulsified in Freund's complete adjuvant.

Immunodiffusion analyses. In Ouchterlony double-diffusion analysis, 0.01 ml of antigen and antibody were added to wells, and precipitin bands were observed after 48 hr. In immunoelectrophoresis the wells were filled with antigen, and after 20 min of electrophoresis at 250 V and 5 mA, antiserum was added to the trough. Precipitin bands were observed after 48 hr.

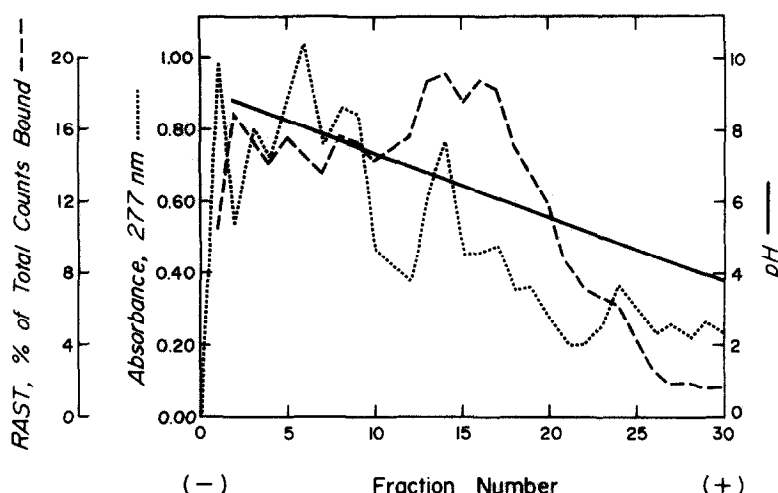


FIG. 3. Preparative flat-bed electrofocusing of DEAE-cellulose pool A₁. The absorbance (....), pH (—), and RAST activity (----) profiles are given. Fraction numbers are shown on abscissa with (–) and (+) indicating the cathode and anode of the gel bed.

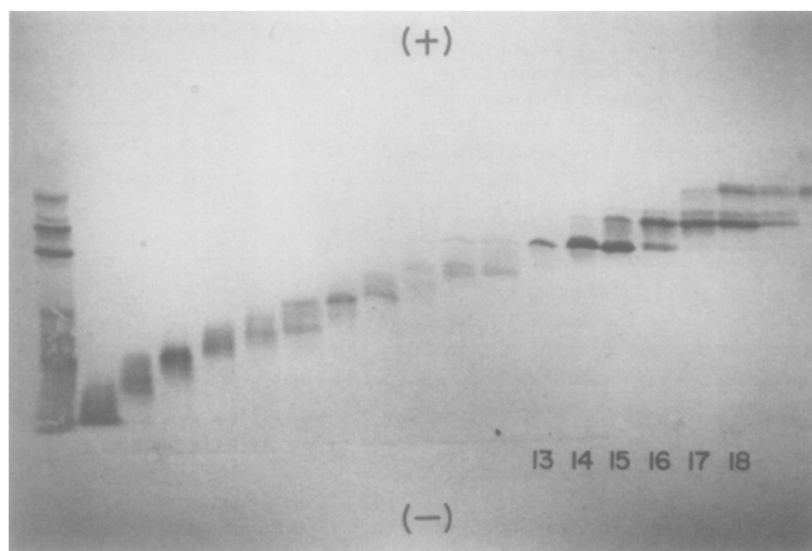


FIG. 4. Thin-layer electrofocusing of DEAE-cellulose fraction A₁ (far left) and fractions 2 to 20 from preparative flat-bed electrofocusing in 5.0% polyacrylamide gel with pH gradient 3.5 to 9.5. Cathode (–) and anode (+) are on the bottom and top, respectively. Fractions 13, 14, and 15 (pI 6.7), and fractions 16, 17, and 18 (pI 6.2), numbering from the left, showed the highest reactivity with IgE antibodies by the RAST.

Allergen isolation and analyses

Crude pollen extract and Sephadex G-25 chromatography. Russian thistle pollen (100 gm) was defatted with four 500 ml portions of anhydrous diethyl ether by stirring and decantation. The pollen suspension was filtered through a Buchner funnel and the pollen was air-dried for 48 hr. The defatted pollen was extracted with 1 L Dulbecco's phosphate-buffered saline (pH 7.4, 2.0 gm of KCl, 11.4 gm of Na₂HPO₄, 2.0 gm of KH₂PO₄, and 80.0 gm of NaCl per 10 L H₂O) for 24 hr. During the first 5 hr the pH of the suspension was monitored hourly and adjusted to 7.4 with

4.0M NaOH. The extract was centrifuged at 2000 rpm at 4° C for 15 min and the supernatant (crude extract) was collected. The precipitate was washed twice with extraction buffer and centrifuged, and the supernatants were combined. The crude extract was concentrated by ultrafiltration through a UM-2 membrane at 4° C. The retentate was dialyzed against 0.1M Tris HCl and 0.1M NaCl, pH 7.5, and was fractionated on a calibrated Sephadex G-25 column (10 by 75 cm). The flow rate was 200 ml/hr; 15 ml fractions were collected and the absorbance at 277 nm was monitored.

Ion exchange chromatography. Allergenic fractions were

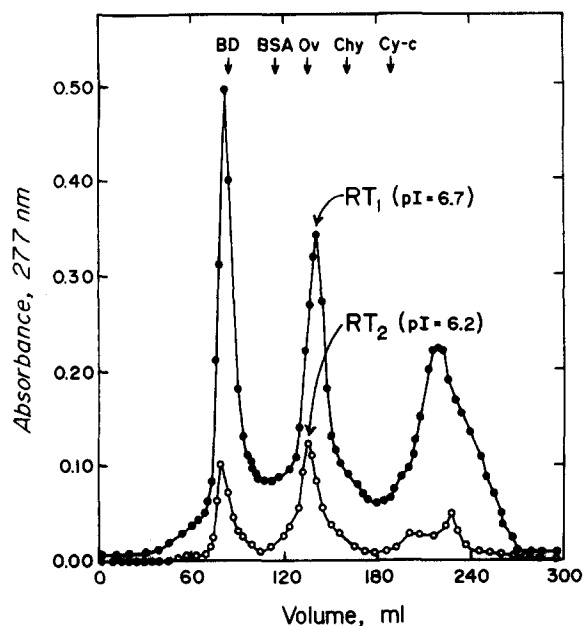


FIG. 5. Sephadex G-100 column (1.2 by 200 cm) chromatography of fractions with pI 6.7 (●) and pI 6.2 (○) from preparative flat-bed electrofocusing. Arrows indicate the elution volumes of the following markers: blue dextran (BD, 2×10^6); bovine serum albumin (BSA, 6.9×10^4); ovalbumin (OV, 4.5×10^4); chymotrypsinogen-A (Chy, 2.4×10^4); and cytochrome-C (Cy-c, 1.2×10^4).

exhaustively dialyzed against 0.005M K_2HPO_4 and KH_2PO_4 buffer (pH 8.7, conductivity = 1.2 mmhos) with molecular weight 1000 cutoff casing and were chromatographed on a DEAE-cellulose column (4 by 36 cm) equilibrated with the same buffer. The column was eluted at 50 ml/hr with the same buffer at room temperature; 13 ml fractions were collected. When the 277 nm absorbance dropped to 0.005, a linear NaCl gradient (0 to 0.5M, 1.2 to 36 mmhos) in starting buffer was applied. Six different pools (A_1 to A_6) were collected. Finally, the column was washed with the starting buffer containing 2M NaCl. All pools were concentrated through UM-2 membranes, dialyzed against 0.05M NH_4HCO_3 , and lyophilized.

Preparative and analytical electrofocusing. The most allergenic pool from DEAE-cellulose was fractionated by preparative flat-bed electrofocusing in Sephadex G-75 containing 2.5% ampholyte with a pH range of 3.5 to 9.5.¹³ Electrofocusing was carried out for 18 hr with a constant power of 8 W. After completion the Sephadex gel bed was sliced into 30 portions. A small amount of each portion was eluted in H_2O for measurement of allergenic activity and pH, and the remainder was eluted with 0.1M NH_4HCO_3 . Analytical electrofocusing was performed on a portion of each fraction in a thin layer of 5% polyacrylamide on a glass plate (12 by 25 cm) containing 2.2% ampholytes with a pH range between 3.5 to 9.5.¹⁴

Sephadex G-100 chromatography. Two fractions from the preparative electrofocusing experiment, with isoelectric points (pI) of 6.7 and 6.2, were separately gel-filtered on a

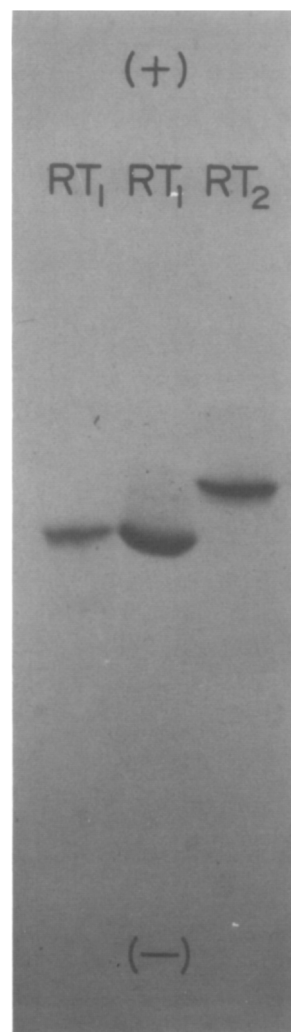


FIG. 6. Thin-layer electrofocusing (pH 3.5 to 9.5) of Sephadex G-100 fractions RT₁ (2.5 mg/ml and 5.0 mg/ml) and RT₂ (3.0 mg/ml).

calibrated Sephadex G-100 column (1.2 by 200 cm). After dialysis against 0.1M Na_2HPO_4 , KH_2PO_4 , and 0.1M NaCl, pH 7.4, each sample was chromatographed in the same buffer with a flow rate of 10 ml/hr. Three-milliliter fractions were collected and monitored at 277 nm.

Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Davis and Ornstein¹⁵ with a 0.1M Tris-borate buffer, pH 8.3, as modified by Nesheim* in a 7.5% polyacrylamide gel. The method of Fairbanks et al.¹⁶ was used for PAGE in 1% sodium dodecyl sulfate (SDS) with an 8% polyacrylamide gel; all samples were run in the presence and absence of 0.04M dithiothreitol. Acetate-urea PAGE was carried out in 15% polyacrylamide gels in 6.25M urea at a final pH of 3.2 as suggested by Panyim and Chalkley.¹⁷ In all the PAGE systems the gels were stained

*Personal communication.

with Coomassie blue and destained in several changes of 10% acetic acid.

Molecular weight estimation. Molecular weights of purified fractions were estimated by chromatography on a calibrated Sephadex G-100 column (1.2 by 200 cm) and by SDS-PAGE.

Chemical analyses

Protein, nitrogen, and carbohydrate content, dry weight, and amino acid analysis. Protein, nitrogen, and carbohydrate contents were determined by biuret,¹⁸ micro-Kjeldahl test,¹⁷ and the method of DuBois et al.,¹⁹ respectively. Bovine serum albumin and glucose were used as standards in the biuret and DuBois methods, respectively. For dry weight determinations, 1-ml aliquots of sample solution and dialysate were separately dried in tared platinum shells at 110°C until constant weights were obtained. The difference was taken as the dry weight of the sample. The dry weights of preparative electrofocusing fractions were determined after exhaustive dialysis in molecular weight 12,000 to 14,000 cutoff casing to exclude ampholytes. Amino acid analyses were performed as described by Spackman et al.²⁰ with a Beckman Model 119 automatic amino acid analyzer.

Extinction coefficient. The absorption spectra of the purified allergenic molecules in 0.005M NH_4HCO_3 , pH 8.3, were obtained in the wavelength range between 250 to 600 nm, and the wavelengths of the maximum absorption peaks were determined. The extinction coefficients of the allergens were obtained by determination of the absorbance at the 277-nm absorbance maxima with a 1.0 cm pathlength, with the dry weight of the samples already established.

RESULTS

Isolation and purification

A flow chart with the yields of various fractions in the isolation of Russian thistle pollen allergens (RT₁ and RT₂) is shown in Fig. 1. Crude pollen extract was concentrated by ultrafiltration with a UM-2 membrane. The retentate and filtrate were separately collected, and the retentate was dialyzed against RAST wash solution with molecular weight 1000 cutoff casing. The dialysate was desalted on a Sephadex G-10 column in RAST wash. An aliquot of each fraction that contained an equal mass of solid, as determined by dry weight, was coupled to CNBr-activated discs and examined for reactivity with IgE antibodies by the RAST. The retentate showed reactivity with IgE antibodies; no reactivity with IgE antibodies was detected in the dialysate. The retentate was fractionated on a Sephadex G-25 column, and three absorbance peaks at 277 nm were observed. Each peak was tested for allergenic activity by the RAST, but only the void volume peak (peak A) showed allergenic activity. Fraction A was then applied to a DEAE-cellulose column. Six pools (A₁ to A₆), as shown in Fig. 2, were

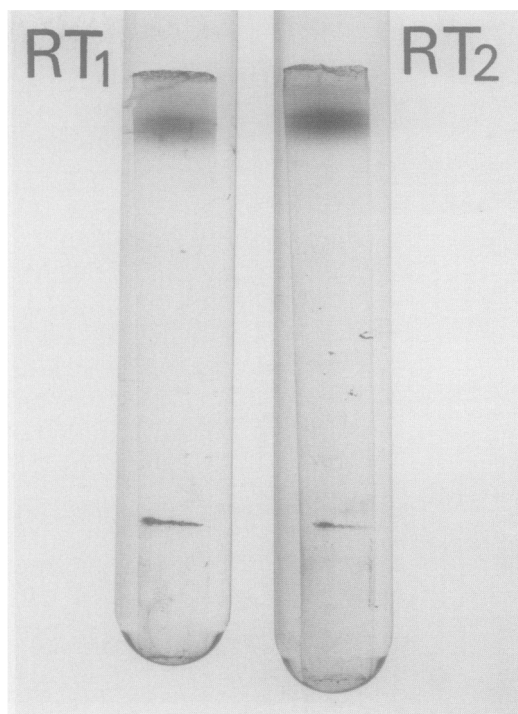


FIG. 7. Discontinuous PAGE analysis of 20 μg of RT₁ and RT₂ in Tris-borate buffer, pH 8.3. For both RT₁ and RT₂ two bands appear in the gel.

made. No absorbance or reactivity with IgE antibodies by the RAST was observed when the column was finally washed with column buffer containing 2M NaCl. For the RAST, equal absorbances of each pool were allowed to react with CNBr-activated discs. Pool A₁ showed the highest RAST reactivity with IgE antibodies (Table II) and was further purified by preparative flat-bed electrofocusing. Thirty fractions were obtained, and an equal absorbance of every fraction was allowed to react with CNBr-activated discs and was tested for RAST reactivity with IgE antibodies. Fig. 3 shows the absorbance, RAST results, and pH profile of fractions obtained from preparative electrofocusing. Fractions 13 to 18 contained the peak RAST reactivity with IgE antibodies. Fig. 4 shows the analytical electrofocusing results of fractions 2 to 20. Two distinct bands appeared in fractions 13 to 15 and 16 to 18. Fractions 13 to 15 with a pI of 6.7 were pooled, dialyzed, and concentrated; fractions 16 to 18 with a pI of 6.2 were also pooled, dialyzed, and concentrated. Each pool was passed over a Sephadex G-100 column to further purify the fractions (Fig. 5). Each preparative electrofocusing pool was resolved into three absorbance peaks, which were pooled separately; equal absorbances of each peak were used to examine RAST reactivity with IgE

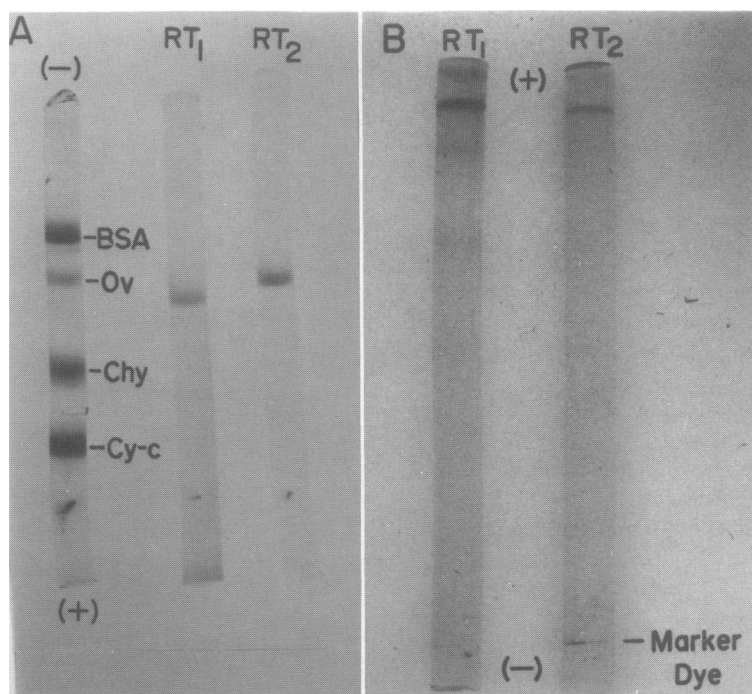


FIG. 8. Analysis by PAGE of RT₁ and RT₂ in gels containing 1% SDS (A) and acetate-urea (B). In the SDS-PAGE analysis the relative mobilities of the markers to that of marker dye were plotted against the log of the molecular weights of the standards. The regression line for the standards had a correlation coefficient of -0.995 . The molecular-weight marker proteins are the same as those in Fig. 5. RT₁, RT₂, and the molecular-weight marker proteins were treated with 0.04M dithiothreitol prior to electrophoresis.

TABLE III. Amino acid compositions of RT₁ and RT₂*

Residue	Moles amino acid/mole protein of RT ₁ †		Moles amino acid/mole protein of RT ₂ ‡	
	To nearest integer	Found	To nearest integer	Found
Aspartic	33	33.5 ± 0.6§	30	29.7 ± 0.7
Threonine	30	30.3 ± 0.4	33	32.6 ± 0.7
Serine	18	18.3 ± 0.3	19	18.8 ± 0.5
Glutamine	39	38.7 ± 0.7	41	40.7 ± 0.9
Proline	21	20.8 ± 1.1	30	29.7 ± 4.3
Glycine	34	33.7 ± 0.6	37	37.1 ± 0.8
Alanine	37	36.8 ± 0.7	42	42.1 ± 1.2
Valine	26	26.3 ± 0.3	29	29.4 ± 0.8
Methionine	5	5.2 ± 0.1	5	4.6 ± 0.5
Isoleucine	19	18.8 ± 0.2	19	18.9 ± 1.6
Leucine	18	18.4 ± 0.04	18	17.7 ± 1.3
Tyrosine	14	13.6 ± 0.1	14	14.1 ± 1.3
Phenylalanine	18	18.2 ± 0.3	20	19.7 ± 1.2
Histidine	6	5.7 ± 0.02	7	6.5 ± 0.9
Lysine	27	27.5 ± 2.6	32	32.0 ± 1.6
Arginine	14	14.2 ± 0.4	16	16.2 ± 0.6

*Samples were hydrolyzed in vacuo for 24 hr in 6M HCl; cysteine and tryptophan were destroyed by hydrolysis; duplicate determinations were made of RT₁ and RT₂.

†Numbers of moles of amino acid were calculated with a molecular weight of 39,000 for RT₁ (from the SDS-PAGE determination).

‡Numbers of moles of amino acid were calculated with a molecular weight of 42,000 for RT₂ (from the SDS-PAGE determination).

§Mean ± SD.

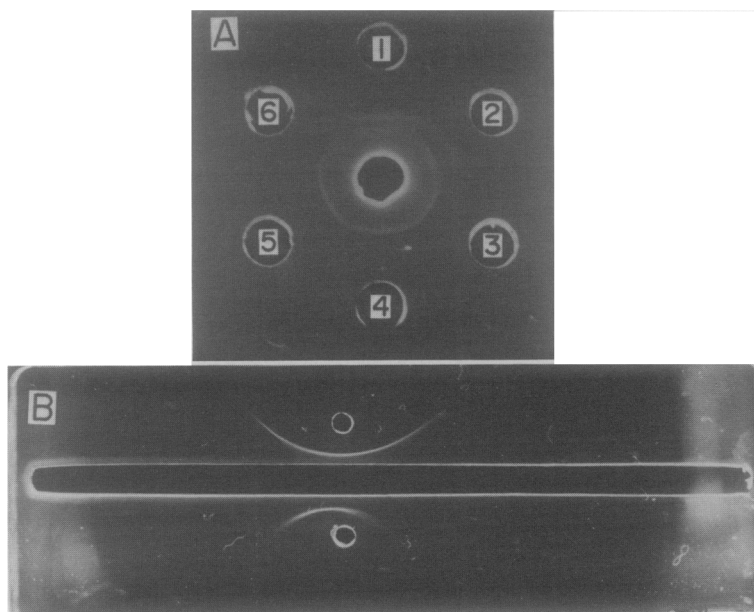


FIG. 9. Analysis by Ouchterlony double-diffusion (A) and immunoelectrophoresis (B) of RT₁ and RT₂ against rabbit antiserum to crude Russian thistle pollen. In Ouchterlony immunodiffusion we used 0.010 ml of RT₁ (1.45 mg/ml, wells 3 and 5), RT₂ (1.11 mg/ml, wells 4 and 6), and preparative electrofocusing fraction 14 (2.00 mg/ml, wells 1 and 2). Rabbit antiserum (0.010 ml) was added to the middle well. In immunoelectrophoresis (B) the top and bottom wells were filled with RT₁ (1.45 mg/ml) and RT₂ (0.5 mg/ml), respectively, and antiserum was added to the trough after antigen electrophoresis. In both cases precipitin bands were photographed after 48 hr.

antibodies. In both cases the fractions between the elution volumes of ovalbumin and chymotrypsinogen-A had the highest reactivity with IgE antibodies. The protein with a pI of 6.7 was designated RT₁ and that with a pI of 6.2 was designated RT₂. Rechromatography of RT₁ and RT₂ on G-100 showed single peaks with the same elution volumes as those in Fig. 5 (results not shown). Note that RT₂ eluted from the G-100 column with a slightly smaller elution volume than that of RT₁.

Analyses of homogeneity

The purified allergens were analyzed for homogeneity (1) by electrofocusing, (2) by gel electrophoresis, and (3) by immunologic analyses. Fig. 6 shows the electrofocusing results: RT₁ and RT₂ each gave a single band with pI of 6.7 and 6.2, respectively. Fig. 7 shows the results from discontinuous PAGE: RT₁ and RT₂ both showed two bands. Fig. 8, A, shows the results from SDS-PAGE, and Fig. 8, B, shows acetate-urea PAGE results. In both procedures RT₂ gave a single band; RT₁ gave a single band that electrophoresed further than RT₂ by SDS-PAGE. However, in acetate-urea PAGE, RT₁ gave a major band and at least two faint bands. The purity and antigenicity of RT₁ and RT₂ were examined by

Ouchterlony double-diffusion against rabbit antiserum to crude Russian thistle pollen (Fig. 9, A) and by immunoelectrophoresis (Fig. 9, B). Both allergens formed single precipitin bands with antiserum to crude extract as opposed to multiple bands, which formed when crude extract was tested with the same antibody (results not shown). A strong band of identity formed between RT₁ and RT₂ by Ouchterlony immunodiffusion, and there was also a second faint precipitin line for RT₁ in Fig. 9, A (opposite well 3). In immunoelectrophoresis RT₁ and RT₂ migrated equal distances.

Protein, nitrogen, and carbohydrate content, extinction coefficient, and amino acid analyses

The protein contents of RT₁ and RT₂ were determined by biuret; on this basis the percentages of nitrogen and carbohydrate were calculated. The results indicate $10.6\% \pm 0.2\%$ nitrogen and $9\% \pm 0.5\%$ carbohydrate in both RT₁ and RT₂. The absorbance spectra of RT₁ (0.70 mg/ml) and RT₂ (0.45 mg/ml) solutions in 250- to 600-nm wavelength range showed absorbance maxima at 277 nm, with no other absorbance peaks. The extinction coefficients ($E_{1\%}^{1\text{cm}}$) for RT₁ and RT₂ at 277 nm were calculated as $10.5 \pm$

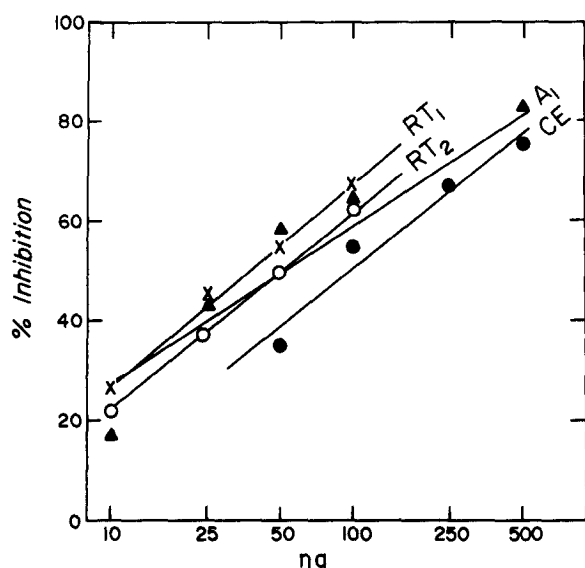


FIG. 10. Inhibition curves showing allergenic potencies and cross-reactivity of RT₁ (X), RT₂ (o), crude pollen extract (CE) (●), and fraction A₁ from DEAE-cellulose chromatography (▲) with solid-phase crude pollen extract. The logarithm of the mass of inhibitor is plotted on the abscissa, and percent inhibition is plotted on the ordinate. The regression lines relating percent inhibition and mass inhibitor for RT₁ and RT₂ had correlation coefficients > 0.99. By regression analysis the slopes of the inhibition lines for RT₁:RT₂ ($F_{1,4} = 0.096$, $p > 0.75$) and RT₁:RT₂:crude extract ($F_{2,6} = 0.014$, $p > 0.975$) were not statistically different.

0.4 and 10.2 ± 0.4 , respectively. Comparison of the amino acid compositions of RT₁ and RT₂ (Table III) revealed that RT₁ and RT₂ had similar compositions.

Molecular weight estimation

The molecular weights of RT₁ and RT₂ were also estimated by gel filtration and SDS-PAGE. As shown in Fig. 5, both RT₁ and RT₂ emerged from the Sephadex G-100 column between the OV (4.5×10^4 daltons) and Chy (2.4×10^4 daltons) markers; the elution volume for RT₁ was slightly larger than that of RT₂. The calculated molecular weights for RT₁ and RT₂ from SDS-PAGE (Fig. 8, A) were 39,000 and 42,000, respectively.

Allergenic analyses

The reactivity of RT₁ and RT₂ with IgE antibodies was examined by P-K testing on a normal individual, by the RAST, and by RAST inhibition. In the P-K test, 0.8 and 0.9 ng of RT₁ and RT₂, respectively, induced skin reactions with 13 by 10 mm wheal and 40 by 30 mm flare, compared to a 5 by 5 mm wheal in the control site. In the RAST, direct coupling of column fractions of the two allergens always gave a per-

cent of total counts bound of at least ten times that of the control serum. The allergenic potencies of RT₁ and RT₂ were compared to that of crude extract by RAST inhibition. In this analysis, solid-phase crude Russian thistle extract was reacted with the allergic serum pool, and quantities of various materials needed for 50% inhibition were determined as shown in Fig. 10. Comparison of the slopes of the inhibition curves for crude pollen extract, RT₁, and RT₂ did not show a difference. Furthermore, comparison of the slopes of the inhibition lines for RT₁ and RT₂ did not show a difference, suggesting that these preparations were allergenically qualitatively the same. On a quantitative basis, 37 ng of RT₁ were required for 50% inhibition, and 51 ng of RT₂ were required for 50% inhibition. Remarkably, both RT₁ and RT₂ were more potent than crude extract as inhibitors in the RAST.

DISCUSSION

Previously, the principal assay system for allergen purification has been direct skin testing of human subjects. Recently, however, numerous studies^{6-8,11} have shown that the RAST, introduced by Wide et al.,⁵ can be used to detect the interaction of IgE antibodies with allergens and thus for assay of allergens. Because the RAST is more convenient than skin testing for the detection of allergens, we used it to isolate allergens from Russian thistle pollen. We realized that using the RAST to isolate allergenic activity in column effluents may be limited because the allergenic site(s) on a protein could be altered, sterically hindered, or not coupled at all in the solid-phase procedure. Two glycoprotein allergens, RT₁ and RT₂, were purified and analyzed. They had identical immunologic reactivity with human IgE antibodies in the RAST. With rabbit antiserum to crude Russian thistle extract they had identical immunologic reactions with the exception of a faint extra band seen with RT₁ in one instance. Also, the physicochemical properties of RT₁ and RT₂ were similar; they had identical patterns by PAGE at alkaline pH, the same nitrogen and carbohydrate contents, and similar amino acid contents. They differed in their isoelectric points; RT₁ had a pI of 6.7 and RT₂ had a pI of 6.2. The closely spaced double bands seen for both RT₁ and RT₂ in PAGE at alkaline pH may reflect inhomogeneity at these particular conditions. In PAGE at acid pH both RT₁ and RT₂ showed a strong major band, but RT₁ also had two minor bands, perhaps reflecting a trace impurity. They also differed slightly in their molecular weights, RT₂ being larger than RT₁, as shown by the elution volumes from Sephadex G-100 and by their mobility on SDS-PAGE.

In a previous report²¹ RAST inhibition was used to examine allergenic properties and cross-reactivity of purified allergens. Therefore this technique was used to study the allergenic properties and cross-reactivity of RT₁ and RT₂ with crude Russian thistle extract and with the DEAE fraction A₁. Fig. 10 shows that RT₁ and RT₂ were more potent on a mass basis in producing 50% inhibition than the crude extract or fraction A₁, and the slopes of the regression lines for RT₁, RT₂, and crude extract were not statistically different, indicating the allergenic similarity of these molecules to the crude extract. Thus these proteins, which constitute only 0.025% by mass of the dry pollen, are important allergens of Russian thistle pollen. However, as shown in the results from DEAE chromatography and from preparative electrofocusing, other allergens are undoubtedly present in the crude pollen extract.

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