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A VARIANT OF RAGWEED ALLERGEN Ra5

by

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ABSTRACT

A variant of short ragweed allergen Ra5 designated as Ra5SII, was isolated from DEAE-fraction C by repetitive column and high performance liquid chromatography.

The preparation was shown to have a high degree of homogeneity by a few physicochemical criteria, including acrylamide gel electrophoresis and high performance liquid chromatography. The molecular weight, though not yet confirmed, was in the range of 5000-6000 daltons and the variant allergen is more cationic than the classical species. The amino acid analysis revealed a distinct composition as the following: asp^{1} thr^{5} ser^{3} glu^{6} pro^{2} cys^{8} gly^{1} ala^{3} val^{2} tyr^{3} phe^{3} lys^{5} arg^{1}.

The RAST-inhibition assay suggests the presence of some shared allergenic determinants in the two forms of Ra5.

ABREGE

Un variant de l'allergène Ra5 du "Ragweed short" designé comme Ra5SII, a été isolé de la fraction C par une grande performance de la chromatographie liquide et une repetition du test sur une colonne de DEAE-25.

La preparation s'est montrée d'un grand degré d'homogenité par des critères physiochimiques tel que l'electrophorese sur gellose acrylamide et la chromatographie liquide. Le poids moleculaire, malgrès qu'il n'est confirmé encore, est situé entre 5,000 et 6,000 daltons. Le variant s'est montre plus cationique que les autres de son espèce. L'analyse des acides amines nous a donné une composition tel qui suit: asp^1 thr^5 ser^3 glu^6 pro^2 cys^8 gly^1 ala^3 val^2 tyr^3 phe^3 lys^5 arg^1.

L'essai par "RAST-inhibition", nous suggere la presence de quelques determinants allergeniques partagés entre les 2 formes de Ra5.

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INTRODUCTION

CHAPTER 1

INTRODUCTION

Early studies by Sela, McDevitt and Benacerraf (1966, 1969, 1972) provided the basis for the current concept that biosynthesis of antibodies is under the control of immune response (Ir) genes located within a major histocompatibility complex, such as HLA in man and H-2 in the mouse. The Ir genes may code for biosynthesis of specific receptors on the surface of T-lymphocytes which interact with carrier regions of the immunogen, providing a signal to B-lymphocytes for production of specific antibodies (Benacerraf, 1981). Studies by Levine and Vaz (1970) on murine IgE antibody response to ovalbumin suggested that biosynthesis of IgE antibodies which mediate allergic reactions is under the same genetic control as that of IgG at both the T and B lymphocyte levels. This finding prompted a search for some association between a specific human IgE antibody response and one or other of the HLA loci (Levine et al., 1972; Marsh et al., 1973). A significant step towards the discovery of HLA-associated immune responsiveness in man came with the isolation of the low molecular weight protein allergen Ra5 from short ragweed pollen (Goodfriend and Lapkoff, 1972; Lapkoff and Goodfriend, 1974).

The isolation as well as chemical and immunological features of Ra5 are summarized in Section 1.1. The present study concerns a molecular variant of Ra5 present in short ragweed pollen. The potential usefulness of such a variant for clarification of the immunoactive sites as well as the HLA-associated activity of Ra5 is indicated in Section

1.2 by reference to similar studies carried out with cytochromes c. These long-range considerations motivated the present study, the immediate aims of which are made explicit in Section 1.3.

1.1 ISOLATION AND PROPERTIES OF RAGWEED ALLERGEN Ra5

1.1.1 Isolation

Utilizing the cationic ragweed allergen Ra3 and anionic ragweed antigen E, Underdown and Goodfriend (1970) extended the 'inverse' antigen-antibody charge relationship (Sela and Mozes, 1966) to the allergen-human IgE class of antibodies. The finding of appreciable overlap in chromatographic distribution of the allergen specific IgE antibodies prompted a search for an allergen more cationic than Ra3. As a result, a new allergen, designated Ra5, was isolated by column chromatography from the start-up DEAE-fraction C (King et al., 1964; Underdown and Goodfriend, 1969) of aqueous ragweed extract. In the final purification step of chromatography on the cation exchanger CM-cellulose (subsequently replaced by CM-Sephadex), Ra5 was eluted following application of a buffer gradient. However, the appearance of a fraction cross-reacting with specific anti-Ra5 antiserum and eluted prior to application of the gradient was an early and persistent observation (L. Goodfriend, unpublished) and formed the basis for the present study. The (major) Ra5 component was purified by Lapkoff and Goodfriend (1974) to molecular size and (cationic) disc electrophoretic homogeneity for studies of its structure and immunological activity.

1.1.2 Structure

Although initially isolated to further examine the inverse antigen-antibody charge relationship, Ra5 was soon considered of interest for study of the structural basis of allergen activity in general owing to its low molecular weight, viz. 5000 daltons. In addition, this protein is carbohydrate-free and has an amino acid composition distinct from those of other purified ragweed pollen allergens (Marsh and Goodfriend, 1979). Of further note with respect to composition is the absence of the amino acids histidine, threonine, phenylalanine and methionine. The most noteworthy feature, however, is the presence of an unusually high content of half-cystine residues.

Determination of the complete amino acid sequence of Ra5 by Mole et al. (1975) established the structure shown in Figure 1. In addition to the absence of some amino acid residues already noted, other features of the primary structure are evident. There exists a markedly basic C-terminal sequence, a feature apparently possessed by many biologically active molecules and essential to their function (Mole et al., 1975). Clarification of this structural feature for Ra5 requires discovery of the as yet unknown plant-physiological role of this protein. In common with other allergens (Goodfriend et al., 1981), amino acid variation has been observed for Ra5, in this case limited to only a single substitution. Thus, about 25% of the Ra5 molecules have leucine in place of valine at the second N-terminal position along the polypeptide chain. As would be expected from the chemical 'equivalence' of these 2 amino acids, the major leu val- and minor leu leu- forms of Ra5 were not resolved by conventional physicochemical techniques such as gel

chromatography or isoelectric focusing. Recently, the leu val- form has been prepared in this laboratory by solid phase peptide synthesis (Choudhury and Goodfriend, 1982, 1983) and found to be equi-active with naturally occurring Ra5 (leu val- plus leu leu- forms) by the radioallergosorbent (RAST) assay for allergenic activity. This would suggest that the 2 forms are allergenically equivalent.

As already noted, a striking feature of the primary structure of Ra5 is the presence of 8 half-cystines out of a total of 45 residues, an unusually high proportion. There being no free sulfhydryl in the protein (Mole et al., 1975), it was inferred that the half-cystines form 4 disulfide bridges and this has been confirmed recently by laser Raman spectroscopy (L. Goodfriend, private communication). Drenth et al. (1980) have drawn attention to the close correspondence between the sequence positions of the half-cystines in Ra5 and the monomer domain of wheat germ agglutinin as well as eurobutoxin and have postulated a 'toxin-agglutin' fold and cys-cys pairing for Ra5. Recent ^{1}H-NMR studies at 500 MHz by Vidusek et al. (1983) have provided a solution confirmation for Ra5 compatible with the Drenth et al. model with modifications. Confirmation and refinement of the solution structure proposed by Vidusek et al. would be facilitated by the availability of homologues of Ra5 with amino acid substitutions appropriate for meaningful NMR comparison.

The high content of cross-links in the Ra5 molecule would be expected to result in a rather rigid tertiary structure and enhance its general immunogenicity. However, extensive skin tests demonstrated that Ra5, although immunogenically active in eliciting human IgE and IgG antibodies, did so in only a small subgroup of the ragweed sensitive population (Marsh et al., 1975; Goodfriend et al., 1977).

1.1.3. HLA-Associated Activity

Of the ragweed allergic subjects just noted, only 10-20% were found to be sensitive to Ra5. Tissue typing of the test population for the major histocompatibility antigens showed that responsiveness to Ra5 is closely associated with possession of the HLA-B7 cross-reacting group comprising HLA-B7, Bw27, Bw22 and Bw10. This was the first demonstration of a significant association between a specific immune response and a particular HLA locus in man. More recently, with the availability of HLA-D typing, an even higher frequency of association of Ra5 sensitivity with HLA-Dw2 than B7 was observed for the Ra5 positive group (Bias et al., 1979). Indeed, with the use of ultra-pure Ra5 in the sensitivity assays, the association appears to be complete (Marsh et al., 1982).

These HLA findings can be interpreted in light of current concepts of T and B cell directed specificities (Benacerraf, 1982). Thus, B cell specificity is considered to recognize conformation dependent antigenic determinants while T cells likely recognize sequential carrier regions. The HLA-associated activity of Ra5 suggests the latter possesses an immunodominant T cell directed carrier region (Goodfriend, 1975, 1976), i.e., a short sequence stretch of the polypeptide chain capable of interacting with specific T cell receptors coded for by an HLA-associated Ir gene. The interaction may form the basis for the lymphoproliferative activity of Ra5, an activity which survives antigenic and allergenic inactivation of this protein by reduction and alkylation (Choudhury and Goodfriend, 1978). These considerations formed an additional stimulus for undertaking isolation of a putative

homologue of Ra5 in short ragweed pollen. The potential use of such a homologue for defining the carrier region of Ra5 mediating T cell proliferation may be made explicit by reference to similar studies in animal models with the well characterized immunogen, cytochrome c.

1.2. ANALYSIS OF IMMUNOACTIVE REGIONS: THE CYTOCHROME C MODEL

Many globular proteins have been sufficiently purified and characterized such that each is a fully homogeneous material of defined amino acid sequence and tertiary structure. Furthermore, because different species produce variants of a given protein, homologous sets of proteins are available that permit localization of antigenic determinants by comparison of the immunological cross reactivities either of members of the sets or of fragments of these proteins obtained by enzymic or chemical cleavages.

Cytochrome c, a respiratory chain heme protein, has been an especially useful model antigen for studying the specificity of the immune response. This globular protein is composed of a single polypeptide chain of a little over 100 amino acids. The amino acid sequences of over 85 cytochromes c from different species are known (Dayhoff et al., 1972). The structure of these proteins appear to be closely similar (Dickenson and Timkovich, 1975), a feature which has facilitated localization of their antigenic (conformational) determinants (Urbanski and Margoliash, 1977).

In addition, comparative studies with cytochrome c variants (Corradin and Chiller, 1979) have shown that recognitive processes leading to T cell proliferation are discriminatory to the extent that

differences of a single amino acid residue in the primary sequence of proteins or peptides can lead to an all or none effect in the capacity of T cells to be activated.

Thus, in vitro challenge with horse or rabbit cytochromes c of lymph node cells from mice primed with beef cytochrome c induced only minimal proliferation compared to a marked response to challenge with beef cytochrome c. Taking into account the amino acid sequences of these cytochromes, the results suggested that the amino acid at position 89 was critical to the specificity of the proliferative process, a deduction confirmed directly using cyanogen bromide derived peptides of cytochromes c. Thus, peptide fragment 81-104, derived from beef cytochrome c, provoked a proliferative response in mice primed with beef cytochrome c, while horse peptide 81-104 failed to stimulate. The two fragments differ sequentially only by a threonine to glycine interchange at position 89.

Cross-stimulation studies of the murine T lymphocyte proliferative response to pigeon cytochrome c led not only to identification and characterization of the major determinant responsible for the proliferation but also to the finding that recognition of this determinant is under the control of dual major histocompatibility complex (MHC) linked Ir genes (Solinger et al., 1979; Ultee et al., 1980). The proliferative regions were localized by cross-stimulation experiments with species variants and cyanogen bromide cleavage fragments of cytochromes c. The determinant was found to contain three of the seven amino acid residue differences between mouse and pigeon cytochromes c, namely isoleucine-3, glutamine-100 and lysine-104. Tobacco hornworm moth cytochrome c, which shares with pigeon cytochrome

c the glutamine at position 100 but has its carboxyl-terminal lysine at position 103 instead of 104, stimulated a heteroclitic T cell response. The immunodominance of the glutamine and lysine residues was supported by the immunogenicity of pigeon cytochrome c fragment 81-104, which primed T cell clones with similar specificity to those primed by the whole molecule. Finally, mixing experiments using the two cross-stimulating antigens hippopotamus cytochrome c and duck cytochrome c fragment 81-104, each of which contains only one of the two immunodominant residues, demonstrated that the T lymphocytes responding to the proliferative determinant comprised a single family of clones that recognize both amino acids as part of the same determinant.

1.3 AIM OF THE PRESENT STUDY

The cytochrome c model can in principal be applied to any homologous family of proteins: extension of the model to ragweed allergens has been a long term goal of this laboratory. Owing to its size and immunological, particularly immunogenetic properties, interest has centered on allergen Ra5 and the isolation and characterization of homologues of this protein in the pollen of other species, e.g. giant ragweed (Choudhury and Goodfriend, 1978) as well as any variant of Ra5 (hereafter referred to as Ra5SI) which might be present in short ragweed pollen. This thesis describes the isolation of one such variant, Ra5SII. The variant was isolated by ordinary and high performance liquid chromatography from a sub-fraction routinely obtained during purification of Ra5SI. Some chemical and immunological properties of Ra5SII were determined as reported herein.

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1. CHEMICALS

All chemicals were of reagent grade, and were purchased from Fisher Scientific Co., Montreal, unless otherwise indicated. A Mettler H5I analytical balance and a Mettler P1210 top loading balance were used for dry weight measurements. Glass double distilled water was used throughout.

2.2. GELS

Sephadex G-50 (superfine), Sephadex DEAE-A25, and Sephadex CM-C25 were purchased from Pharmacia, Montreal.

Bio-Gel P-6 (200-400 mesh) was purchased from Bio-Rad, Missisauga, Ontario.

2.3. RAGWEED POLLEN

Short ragweed pollen (*Ambrosia elatior*) was purchased from Greer Laboratory, Lenoir, North Carolina, U.S.A.

The pollen was defatted by Soxhlet extraction with anhydrous ether using Whatman cellulose extraction thimbles (Canlab, Montreal). The pollen was air-dried and stored at 4°C until used.

2.4. COLUMN CHROMATOGRAPHY

Sephadex gels and Biogels were allowed to swell overnight in distilled water. Slurries were equilibrated with starting buffers and fines were removed by repeated decantations. Columns (Pharmacia) were poured with the aid of column reservoirs and packed under hydrostatic heads corresponding to 1.5 times operating flow rates. Columns were connected to the LKB Productor peristaltic pumps and equilibrated by passage of three bed volumes of the starting buffer prior to sample application. All samples were Millipore filtered through 0.22 u before application.

Columns were run at room temp. and effluents were monitored at 280 nm in a Uvicord II spectrophotometer (LKB Productor) equipped with a 2 mm flow cell, and connected to an LKB two band bar recorder and LKB fraction collector. Protein concentrations in column effluents were expressed as the number of O.D. (280 nm) units. Where necessary, fractions around the protein peak of a column chromatogram were tested for the presence of antigen by immunodiffusion against specific antisera.

2.5. ULTRAFILTRATION AND STERILIZATION

All solutions were concentrated at 4°C in Diaflo ultrafiltration cells equipped with UM-2 or UM-05 membranes (Amicon Corporation, Cambridge, Mass. U.S.A.). Solutions were sterilized by passage through 0.22 u Millipore filters (Millipore Corp., Missisauga, Ontario) into sterile glass vials (Hollister-Stier Laboratory, Spokane, Washington, U.S.A.).

2.6. DIALYSIS

Dialysis was carried out at 4°C for periods of 24-48 hr. (with 3 or 4 changes of dialysates), using Spectrophor 3 membrane tubing (Spectrum Medical Industries Inc., Los Angeles, California, U.S.A.) having a molecular weight cut-off of 3500 daltons.

2.7. OPTICAL DENSITY ABSORPTION

Spectrophotometric readings in the ultraviolet (u.v.) and visible regions were obtained manually in a Coleman model 124 Hitachi double beam grating spectrophotometer (Coleman Instruments, Maywool, Illinois, U.S.A.).

2.8. POLYACRYLAMIDE GEL ELECTROPHORESIS

The model 1200 Canalco Research Disc Electrophoresis apparatus, connected to a 500 volt DC power supply (Heathkit) by a safety interlock adaptor (Canalco), was used in these experiments.

Solutions of 15% acrylamide (BDH Chemicals, Montreal) were polymerized in glass tubes (0.5 x 6.5 cm) and electrophoresis was performed at pH 4.3 according to the method of Reisfield et al (1962).

The following stock solutions, each made up to 100 ml with distilled water, were used to prepare the running and stacking gels:

(a) 48 ml 1N potassium hydroxide, 17.2 ml glacial acetic acid, 4.0 ml tetramethylethylenediamine (TEMED) (BDH Chemicals, Montreal);

(b) 58.8 g acrylamide, 1.2 g bisacrylamide (BDH Chemicals, Montreal);

(c) 0.28 g ammonium persulfate;

(d) 48 ml 1N potassium hydroxide, 2.9 ml glacial acetic acid, 0.45 ml TEMED;

(e) 10 g acrylamide, 2.5 g bisacrylamide;

(f) 4 mg riboflavin (BDH Chemicals, Montreal).

In parts by volume, the running gel (pH 4.3) composition was: a1, b2, c4, H_2O 1; the stacking gel: a1, e2, f1, H_2O 4. The electrode buffer was 0.31% B alanine and 0.04% glacial acetic acid,pH 4.5.

Samples were diluted in 66% sucrose containing 0.1% methyl green as tracking dye. Sample loads were 10 to 20 ug in a volume of 20 ul. Electrophoresis was toward the cathode at 2-4 mA per tube.

Protein bands were fixed and stained for 1 hr. in Amido Black 10B (30% methanol, 7% glacial acetic acid, 1% Amido Black (J.T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.)). Background stain was removed by washing in a solution of 7% acetic acid and 30% methanol. Gels were stored in the same solution.

2.9. AMINO ACID ANALYSIS

Amino acid compositions were determined in a Beckman Amino Acid Analyzer (Model 120C) according to the method of Moore, Spackman and Stein (1958) using a single column methodology. Proteins were desalted by dialysis and rotary-evaporated (Buchi-Rotavapor-R, Switzerland). The samples were redissolved in triple distilled 6N HCl and hydrolyzed in hydrolysis tubes (Pierce Chemicals) sealed in vacuo (70 u) for 24 hr. and where necessary, for 48 and 72 hr. at 110°C in a heating module

(Reacti-therm, Pierce Chemicals, Illinois, U.S.A.). Hydrolysates were dried by rotary evaporation and redissolved in 0.5 ml distilled water.

2.10. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography columns I-60 and u-Bondapak C18 and all equipment used were purchased from Waters Associates, Milford, Massachusetts. Columns were connected to a model M-45 and a model 6000A solvent delivery system. The columns were equilibrated by passage of 10-20 x bed vol. of starting buffer. Samples were applied with a Hamilton pos tight blunt needle syringe through a model U6K Universal liquid chromatograph injector. A model 720 system controller was used when a buffer gradient was needed. Columns were run at rm. temp. and effluents monitored at 214 nm in an absorbance detector (model 441) connected to an LKB two channel recorder (model 2210).

2.11. IMMUNODIFFUSION

The tests were done in agar (Hyland Immuno-plate, Costa Mesa, Calif.) with a goat antiserum specific to Ra5 (Lapkoff and Goodfriend, 1974).

2.12. RADIOALLERGOSORBENT (RAST) ASSAY

The assay was performed according to Ceska and Lundkvist (1972).

2.12.1. Activation of paper discs

Filter paper discs, of 6 mm diameter were punched out from Whatman no. 3 filter paper. Four grams of discs were allowed to swell in distilled water for 30 min. and mixed with a solution of cyanogen bromide (4 gm in 150 ml distilled water). The pH was maintained within 10.0 to 10.5 by dropwise addition of 1 M NaOH until approximately 37 ml were consumed. After aspiration, the discs were washed (x 12) in 125 ml of 0.05 M sodium bicarbonate for 2 min. The discs were washed (x 2) with 125 ml distilled water and successively with 25%, 50%, 75% and 100% acetone. They were dried in vacuo overnight at rm. temp. The activated paper discs were flushed with nitrogen and stored at -20°C.

2.12.2. Coupling procedures

A ratio of 6 to 8 ug of allergen per disc was used for coupling. The discs were stirred overnight at 5°C in the allergen solution, 60 to 80 ug/ml, in 0.1M sodium bicarbonate. The discs were washed (x 2) at rm. temp. for 10 min. in 0.1 M sodium bicarbonate (0.2 ml/disc). To block excess reactive groups, the discs were treated with 0.05 M ethanolamine (0.1 ml/disc) in 0.1 M sodium bicarbonate for 3 hr. at rm. temp. They were sequentially washed (x 2) at 0.3 ml/disc with 0.5 M sodium bicarbonate, 0.1 M sodium acetate buffer, pH 4.0, and 0.05 M sodium phosphate buffer ± 0.9% sodium chloride, pH 7.9. The allergen sensitized discs were stored at -20°C in this last buffer.

2.12.3. Assay procedure

The assay was performed in disposable polystyrene test tunes, 5.5 x 1 cm (Pharmacia). Each tube contained one allergen sensitized disc to which was added 50 ul of serum of Ra5 sensitive individuals. For assays of allergenic activity by RAST-inhibition, 50 ul of test allergen in PBS or 50 ul PBS alone (uninhibited control) were added to the tubes along with the allergic serum aliquots. All assays were performed in duplicate. The tubes were covered and incubated for 3 hr at rm. temp. After aspiration, the discs were washed (x 3) with 0.05 M sodium phosphate buffer + 0.9% NaCl, pH 7.9; between each wash the discs were incubated in the buffer for 10 min. After the final aspiration, each disc was incubated overnight at 5°C with 50 ul of ^{125}I-labelled anti-IgE antibody solution (Pharmacia). After aspiration, the discs were washed (x 3) for 10 min. with 0.9% NaCl, and counted for 2 min. in a gamma counter (Packard model 578).

EXPERIMENTS AND RESULTS

CHAPTER 3

EXPERIMENTS AND RESULTS

3.1. ISOLATION AND PURIFICATION OF THE Ra5 VARIANT

In this section, the preparation of the "pre-gradient" fraction and the various stages of purification of Ra5 SII will be described.

3.1.1. The Pre-gradient Fraction

Crude aqueous extract of short ragweed pollen was prepared by stirring ether-defatted pollen (1 kg) in 5 l water overnight at 5°C. The suspension was filtered by suction through a Whatman No. 3 filter paper in a Buchner funnel and the filtrate clarified by successive filtration through 8 u to 0.22 u Millipore membranes. Proteins in the extract were precipitated by 0.9 saturation in ammonium sulfate and collected by centrifugation at 25,000 rpm for 60 min. in an International B-60 centrifuge (rotor 872). Suspensions of the precipitate were dialyzed for 24-48 hours against several changes of distilled water and clarified by centrifugation. The extract was loaded onto a DEAE-Sephadex A-25 column (5 x 13 cm) equilibrated with 0.025 M Tris buffer, pH 7.6. Elution with the same buffer gave the cationic DEAE-fraction C (Lapkoff and Goodfriend, 1974). Proteins of this fraction were precipitated by 0.9 saturation in ammonium sulfate. After centrifugation (25000 rpm, 60 min), the precipitate was redissolved in 200 ml distilled water and the solution stored frozen at -20°C in 30 ml aliquots until used.

The fraction C aliquots were separately applied to a column of Sephadex G-50 S (5 x 90 cm) and eluted with 0.001 M sodium phosphate buffer pH 7.2, at a flow rate of 60 ml/hour. As shown in Figure 2, three major fractions were obtained, namely AgE-Ra4, cytochrome c-Ra3, and Ra5 which were localized by immunodiffusion against specific antisera.

A pool of Ra5 fractions from the Sephadex G-50 run was loaded onto CM-Sephadex C25 column (2.5 x 13 cm) previously equilibrated with 0.001 M sodium phosphate buffer, pH 7.2. An unabsorbed fraction was obtained during application of the protein sample (Figure 3) which gave a precipitin line on immunodiffusion with anti-Ra5 antiserum. On application of a linear buffer gradient, the Ra5 retained on the column was eluted (legend, Figure 3) and purified as described by Lapkoff and Goodfriend (1974) with minor modifications.

3.1.2. Partial Purification of Ra5 Variant

The unabsorbed Ra5 fraction was dialyzed against several changes of 0.005 M sodium acetate buffer, pH 5.0 and batch chromatographed on DEAE Sephadex A-25 equilibrated with the same buffer in a Buchner funnel (2.5 x 2 cm). The filtrate was applied to a column (1.5 x 10 cm) of CM-Sephadex C25, previously equilibrated with the acetate buffer. Proteins were eluted with a linear gradient composed of 750 ml starting buffer and an equal volume 0.5 M NaCl-acetate at a flow rate of 90 ml/hr. As shown in Figure 4, two poorly separated fractions (I and II) were obtained. Both fractions gave single precipitin lines on immunodiffusion with anti-Ra5 antiserum.

3.1.2.1. Chromatography of Fraction I

Fraction I was dialyzed against several changes of 0.005 M sodium acetate, pH 5.0, concentrated by ultrafiltration to 15 ml, and chromatographed on a Bio-Gel P-6 column (2.5 x 80 cm) pre-equilibrated with the acetate buffer (Figure 5). The major eluate peak obtained cross-reacted with the anti-Ra5 antiserum. It was cycled through a column (1.5 x 13 cm) of CM-Sephadex C25 pre-equilibrated with the same acetate buffer. Application of a linear buffer gradient (100 ml acetate buffer and 100 ml 0.5 M NaCl-acetate) yielded a single almost symmetric eluate peak (Figure 6). No further purification was achieved on recycling this peak through the CM-Sephadex column under identical elution conditions.

3.1.2.2. Chromatography of Fraction II

Fraction II was similarly dialyzed against the acetate buffer, concentrated to 15 ml, and applied to a Bio-Gel P-6 column (2.5 x 80 cm) equilibrated with the acetate buffer. Of the three peaks which were eluted (Figure 7) the major cross-reacted with the anti-Ra5 antiserum. It was concentrated and applied to a CM-Sephadex C25 column (1.5 x 15 cm) previously equilibrated with the acetate buffer. Using a flow rate of 200 ml/hour, a singly broad peak was eluted with a linear gradient of 250 ml 0.4 M NaCl-acetate as the limiting buffer (Figure 8). After dialysis and concentration, the peak fraction was sieved through a Bio-Gel P-6 column, but no further resolution was achieved (Figure 9).

3.1.3. Final Purification of the Ra5 Variant

Polyacrylamide gel disc electrophoresis of the purified fractions I and II revealed the presence of major and minor contaminants (results not shown). These were resolved by reverse phase HPLC.

Purified fraction I (Figure 6) was dialyzed against distilled water and concentrated to 1 ml. Aliquots of 50 ul were injected into a u-Bondapak-C18 column (3.9 x 300 mm) and a linear gradient of 30-40% acetonitrile made in 0.1% triflouroacetic acid was applied over a 20 min. interval at a flow rate of 2 ml/min. As shown in Figure 10, two protein peaks, I-A and I-B were eluted. Repetitive runs were performed and corresponding peak fractions pooled. The corresponding chromatogram for purified fraction II (Figure 11) showed the presence of four distinct components of which the major (II-B) accounted for 80% of the proteins recovered.

Fractions I-B and II-B reacted positively with anti-Ra5 antiserum; their amino acid compositions and disc electrophoretic mobilities were identical with that of Ra5SI (results not shown). Fraction I-A (hereafter referred to as Ra5SII) was analyzed for chemical and immunological properties; fraction II-A appeared to have the same amino acid compositions as I-A but was not further studied.

On PAGE analysis, Ra5SII showed a major band migrating toward the cathode with a higher cationic mobility than Ra5SI (Figure 12). Analysis by HPLC on the sieving column I-60 (7.8 x 300 mm) showed a high degree of molecular size homogeneity, although one or two minor contaminants were evident.

Based on the gel fraction of origin of Ra5SII (Figure 2), the

molecular weight of Ra5SII was estimated to be the same as Ra5SI, approx. 5,000 daltons.

The amino acid composition of Ra5SII is shown in Table I. The composition of the variant was similar to but distinct from that of Ra5SI. Particularly noteworthy is the absence of isoleucine and leucine and the presence of threonine and phenylalanine in the variant molecule.

No reaction was obtained on immunodiffusion of Ra5SII against goat antiserum to Ra5 (Figure 13), even though a higher concentration (100 ug/ml) and longer incubation time (up to 4 days) than for Ra5SI were employed.

The allergenic specificities of the the two forms were assessed by RAST inhibition using Ra5SI-sensitized paper discs and an Ra5SI-allergic human serum (A-48). Figure 14 and Table II shows the % inhibition for varying concentrations of the two forms. While both displayed inhibitory activity, the activity of Ra5SII was 2 or 3 orders of magnitude less than that of Ra5SI. Furthermore, an inhibition of 55% was the maximum achieved by Ra5SII, indicating only partial identity in allergenic specificity of the two forms.

DISCUSSION

CHAPTER 4

DISCUSSION

In the following, I shall make some observations on the isolation, homogeneity and properties of the Ra5SII variant as well as on the Ra5SI component (II-B) isolated concurrently with the variant.

4.1. ISOLATION AND PURIFICATION

The variant, Ra5SII, was isolated from DEAE-fraction C of short ragweed pollen aqueous extract, the same fraction that served as a starting material for the purification of Ra5SI and other cationic protein allergens. The chromatographic procedure consisted essentially of repetitive molecular seiving and charge separation on ion-exchangers until symmetric peaks were obtained.

Despite the repetitive chromatography, the variant preparation was found to be rather contaminated, as judged by disc electrophoresis. A higher degree of purity was attained through the use of high performance liquid chromatography. A major drawback of the overall procedure was the low yield of purified variant, in the range of 25-50 ug/kg pollen, an amount probably much lower than the amount extractable and due to difficulties in purification, repeated chromatography and cuts. The low yield thus presented a severe limitation to the extent of this study.

4.2. HOMOGENEITY

The final preparation of Ra5SII was considered to have a high degree of homogeneity by a number of criteria. Thus ordinary and high performance liquid chromatography yielded single major symmetrical peaks, although the latter showed the presence of one or two minor contaminants. In addition, disc electrophoresis of Ra5SII showed a single band and only at very high sample loads could a slight degree of contamination be detected (observed as diffusion).

It would have been advantageous to form an antiserum to Ra5SII but this was precluded by the low yield of the purified protein.

4.3. CHEMICAL AND IMMUNOLOGICAL PROPERTIES

Gel filtration through Bio-Gel P-6 indicated that the variant as well as the Ra5SI component (II-B) had molecular weights in the range of 5000-6000 daltons. Within experimental error, the amino acid composition of II-B was identical to that of RA5SI. Furthermore, no difference between the (cationic) disc electrophoretic mobilities of the two proteins was observed . On the other hand, the composition of Ra5SII was clearly distinct from that of Ra5SI. In addition, Ra5SII displayed a higher cationic disc electrophoretic mobility than Ra5SI. This finding was surprising in view of the CM-Sephadex fraction of origin of Ra5SII. It would seem that purification may have removed some (positively) charged-neutralizing component(s) bound to Ra5SII in the impure state.

Apparently, the amino acid compositional differences between Ra5SII

and Ra5SI were such as to impart major differences in immunological specificity of the two proteins. Thus, Ra5SII failed to react with the anti-Ra5 antiserum, and had clear differences in composition and antigenic specificity. The results of the RAST-inhibition assay suggest the presence of only some shared allergenic determinants in Ra5SII and Ra5SI.

Recently, Roebber et al. (1982) have isolated two species of Ra5 from short ragweed, denoted Ra5A and Ra5B. Analyses revealed that Ra5A was chemically and immunologically identical to conventional Ra5. While Ra5B was more anionic than Ra5A, both forms were antigenically indistinguishable when tested by immunoelectrophoresis with goat anti-Ra5 serum. Amino acid compositions of the two forms were not available but sequencing data indicated no differences between the two forms through the first 30 amino-terminal residues. The authors speculated that charge or immunological differences may have arisen within the 15 carboxy-terminal residues.

While the findings of Roebber et al. appear to indicate the absence of any 'real' variant of Ra5SI in short ragweed pollen extract, the results of our study suggest the contrary. Current studies in this laboratory are directed to a fuller chemical and immunological characterization of the variant protein, Ra5SII.

SUMMARY AND CLAIMS TO ORIGINALITY

1. A variant of short ragweed allergen Ra5, designated as Ra5SII, was isolated from DEAE-fraction C of short ragweed pollen aqueous extract. The techniques used included essentially repetitive molecular sieving, charge separation on ion-exchangers and HPLC.
2. The Ra5SII preparation was considered to be highly homogeneous by gel filtration on Bio-Gel P-6, disc electrophoresis at pH 4.3 and HPLC.
3. Amino acid composition of Ra5SII showed a marked difference from Ra5SI, notably the absence of isoleucine and leucine and the presence of phenylalanine and threonine.
4. Preliminary evidence for the allergenicity of Ra5SII was obtained by RAST-inhibition assays, indicating the presence of some shared allergenic determinants in Ra5SI and Ra5SII.

TABLE I

AMINO ACID COMPOSITION OF Ra5SII

Amino Acid	nmoles	Number of Residues Found	Assumed	
Aspartic acid	14.74	1.27*	1	(2)**
Threonine	60.33	5.20	5	(0)
Serine	31.47	2.71	3	(4)
Glutamic acid	69.23	5.96	6	(4)
Proline	22.63	1.94	2	(3)
Cystine/2	39.51	6.80	8	(8)
Glycine	8.22	0.72	1	(4)
Alanine	29.10	2.51	3	(3)
Isoleucine	0.00	0.00	0	(1)
Leucine	0.00	0.00	0	(1)
Valine	18.25	1.58	2	(4)
Tyrosine	35.04	3.02	3	(3)
Phenylalanine	29.43	2.54	3	(0)
Lysine	56.24	4.84	5	(4)
Arginine	12.58	1.08	1	(2)
Tryptophan	-	-	ND	(2)

* Calculated by assuming Ra5SII contains 6 Glutamic acid residues. Averages of duplicates of 24 and 48 hr hydrolysis were used.

**Corresponding numbers of amino acid residues of Ra5SI are shown in parenthesis.

TABLE II

RAST ASSAY OF Ra5SI AND Ra5SII
USING ALLERGIC SERUM A-48

1. RAST-Inhibition by Ra5SI

concentration, ug per ml.	% Inhibition				Average
	Assay 1		Assay 2		
nil	-	(1990)*	-	(2117)	-
0.001	0	(2401)	0	(2571)	0
0.01	10	(1784)	9	(1933)	9.5
0.1	48	(1040)	47	(1115)	47.5
1.0	65	(697)	66	(719)	65.5
5.0	79	(410)	81	(400)	80.0
25.0	94	(117)	96	(92)	95.0

2. RAST-Inhibition by Ra5SII

concentration, ug per ml.	% Inhibition				Average
	Assay 1		Assay 2		
nil	-	(1734)*	-	(1750)	-
0.1	0	(1975)	0	(1990)	0
1.0	0	(1787)	0	(1786)	0
5.0	20	(1389)	21	(1390)	20.5
25.0	42	(1013)	42	(1010)	42.0
50.0	55	(782)	55	(792)	55.0
100.0	55	(783)	55	(789)	55.0

* Counts (shown in parenthesis) are net after subtraction of background; the assays were done in duplicate and the results averaged.

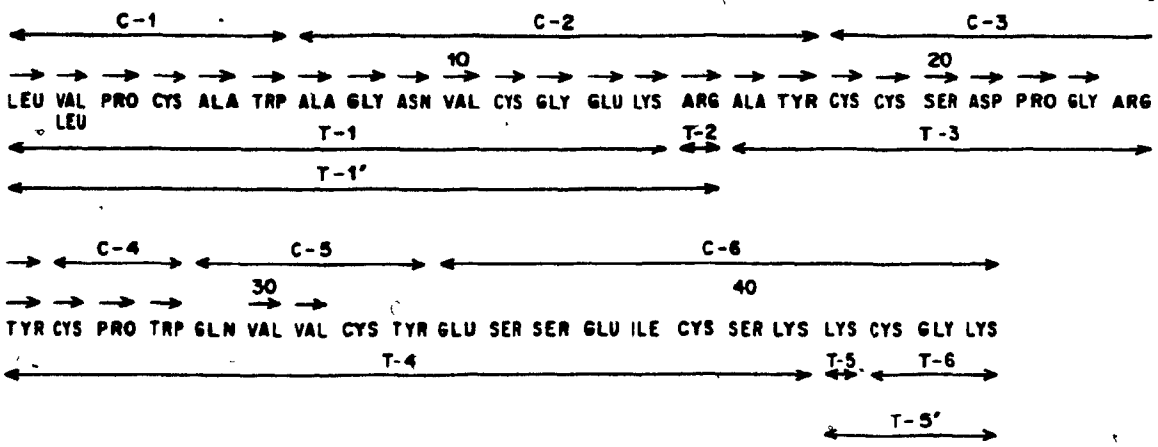

Figure 1. The complete amino acid sequence of ragweed allergen Ra5. Arrows to the right above each residue indicate the sequence obtained on the whole protein. The location of each tryptic (T) and chymotryptic (C) peptide is indicated as well.

Reproduced from Mole et al. (1975).

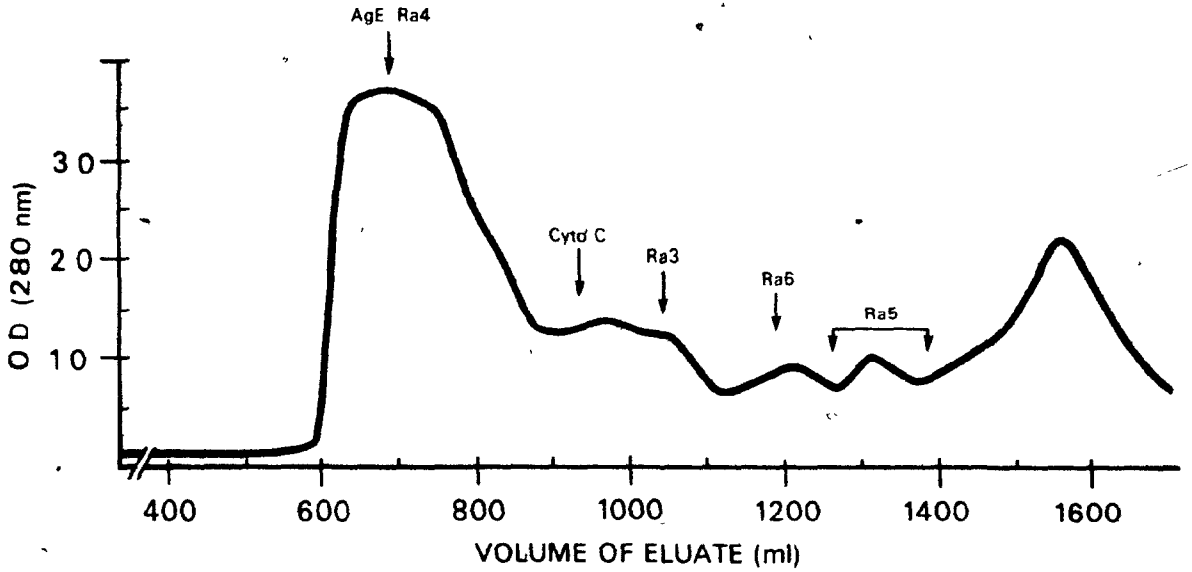

Figure 2. Chromatography of DEAE-fraction C on Sephadex-G50S

Bed: 5 x 90 cm; buffer: 0.001M sodium phosphate, pH 7.2; flow: 60 ml/hr.

Cuts were made as shown by the arrows.

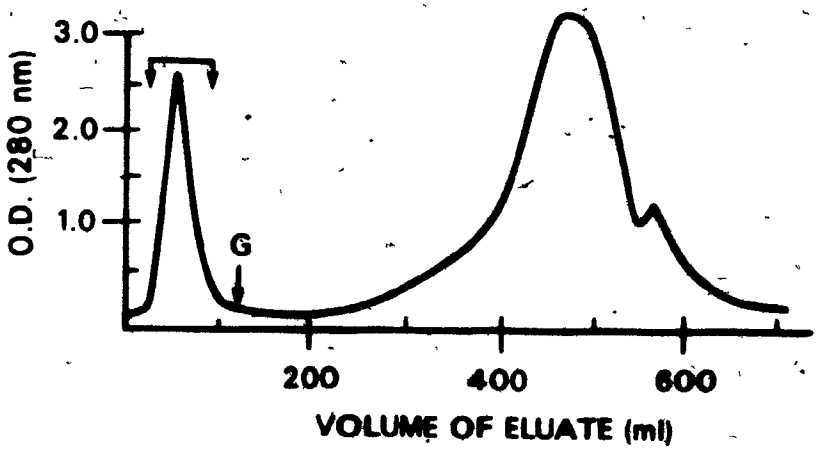

Figure 3. Chromatograhpy of pooled Ra5 fractions on CM-Sephadex C25.

Bed: 2.5 x 13 cm; buffer gradient: 0.001M phosphate (250 ml) into 0.5M NaCl-phosphate (250 ml), pH 7.2; flow: 60 ml/hr.

Cuts were made as shown by the arrows.

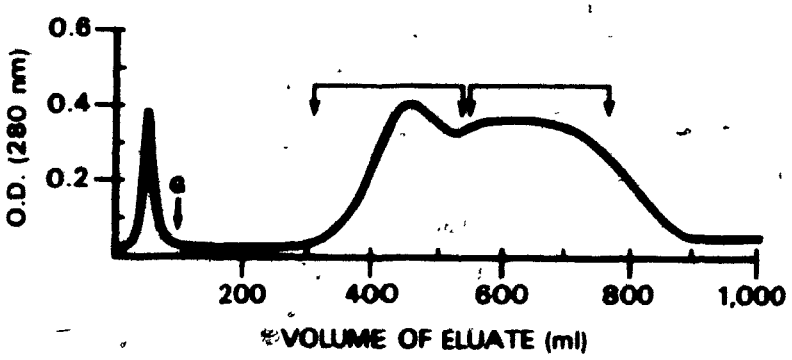

Figure 4. Chromatography of pre-gradient fraction on CM-Sephadex C25.

Two major peaks were eluted: I followed by II. Bed: 1.5 x 10 cm; buffer gradient: 0.005M sodium acetate (750 ml) into 0.5M NaCl-acetate (750 ml), pH 5.0; flow: 90 ml/hr.

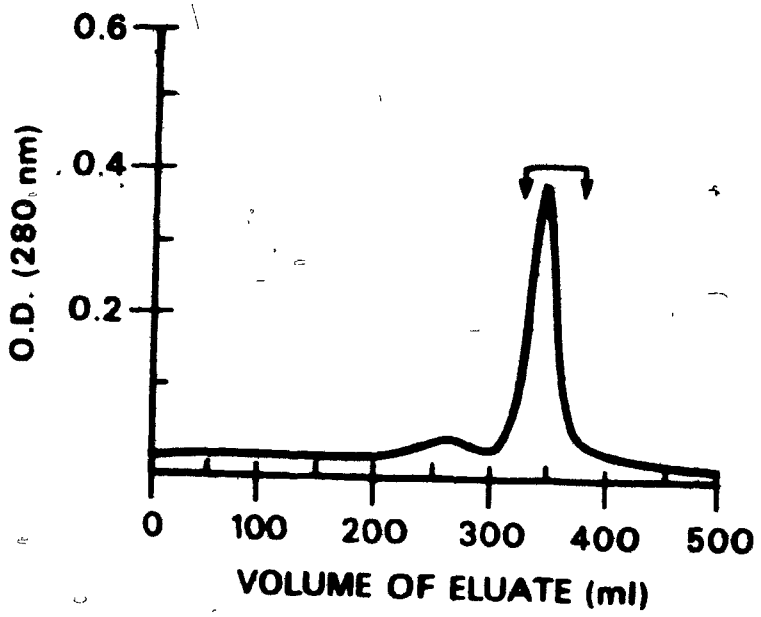

Figure 5. Chromatography of Fraction I on Bio-Gel P-6.
Bed: 2.5 x 80 cm; buffer: 0.005M acetate, pH 5.0; flow: 30 ml/hr.

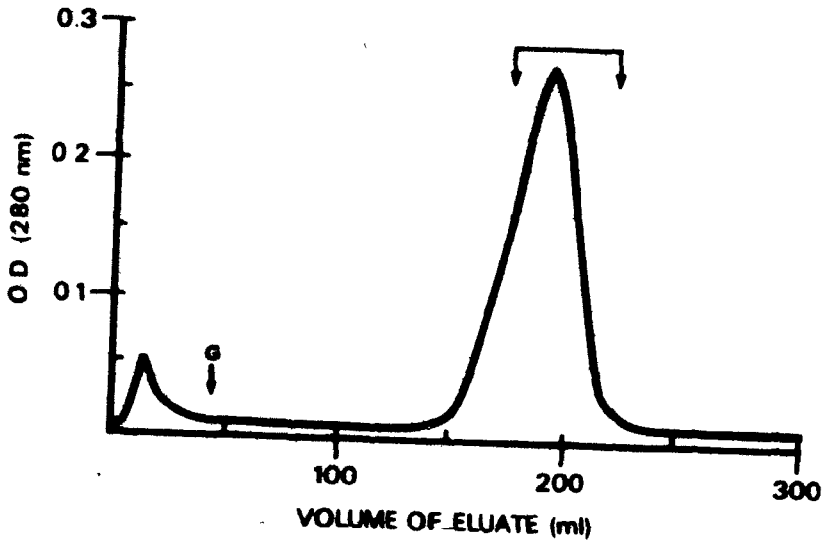

Figure 6. Chromatography of Fraction I from Bio-Gel P-6 on CM-Sephadex C25.

Bed: 1.5 x 13 cm; buffer gradient: 0.005M acetate (100 ml) into 0.5M NaCl-acetate (100 ml), pH 5.0; flow: 60 ml/hr.

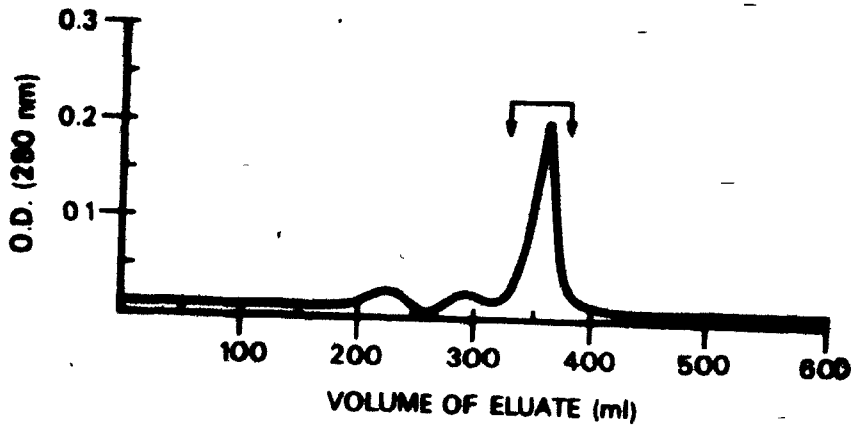

Figure 7. Chromatography of Fraction II on Bio-Gel P-6.
Bed: 2.5 x 80 cm; buffer: 0.005M acetate, pH 5.0; flow: 30 ml/hr.

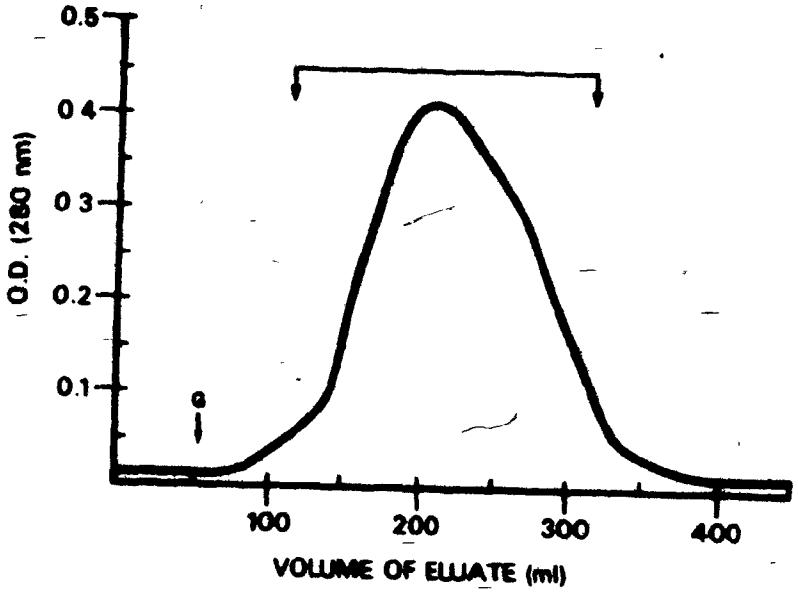

Figure 8. Chromatography of Fraction II from Bio-Gel P-6 on CM-Sephadex C25.

Bed: 1.5 x 15 cm; buffer gradient: 0.005M acetate (250 ml) into 0.4M NaCl-acetate (250 ml); pH 5.0; flow: 60 ml/hr.

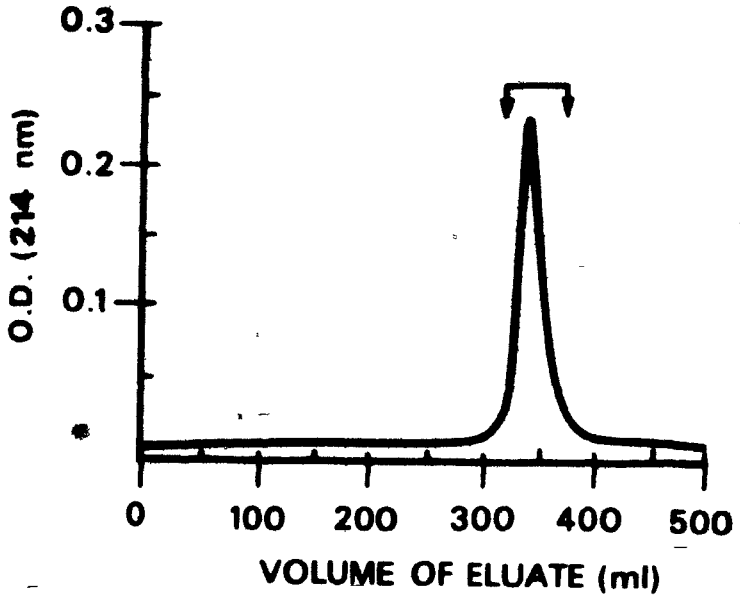

Figure 9. Chromatography of Fraction II on Bio-Gel P-6.

Bed: 2.5 x 80 cm; buffer: 0.005M acetate; pH 5.0; flow: 30 ml/hr.

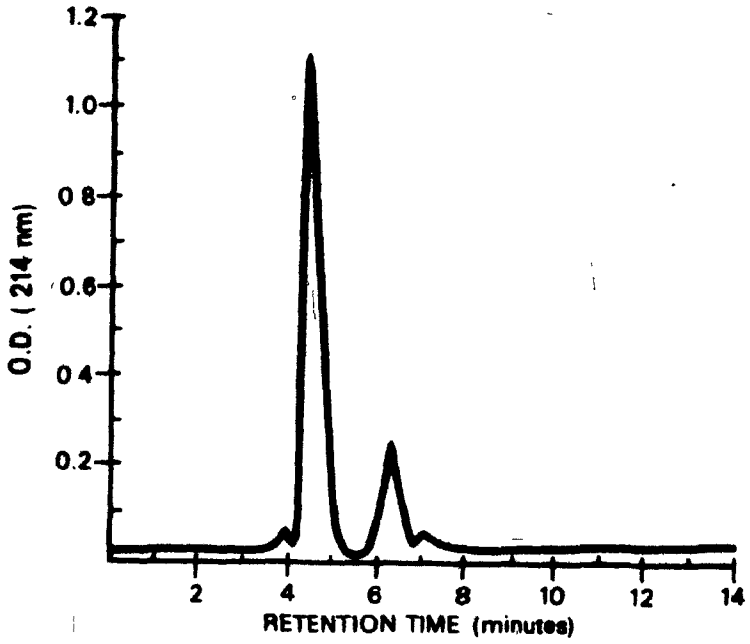

Figure 10. Chromatography of Fraction I on HPLC (u-Bondapak-C18).

Bed: 3.9 x 300 mm; buffer gradient: 30–40% acetonitrile in 0.1% triflouroacetic acid for 20 minutes; flow: 2 ml/min.

I-A (left) and I-B (right) were collected.

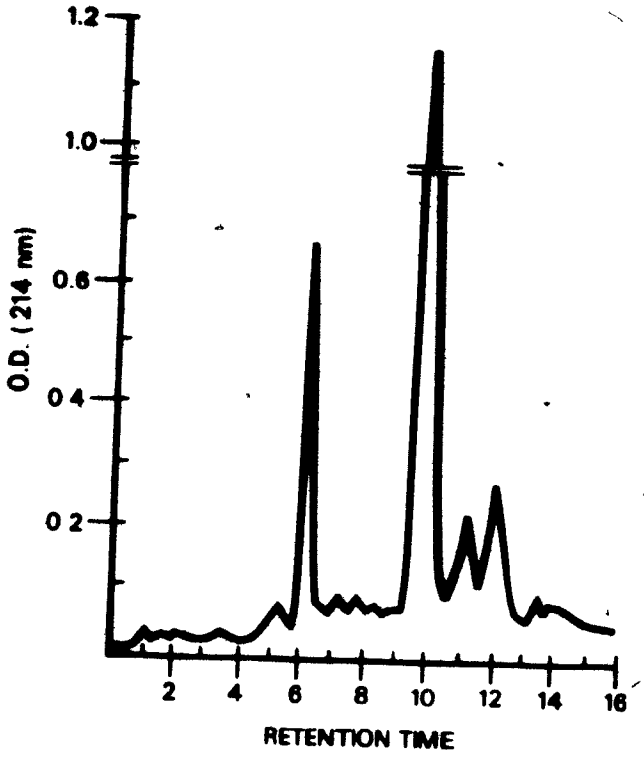

Figure 11. Chromatography of Fraction II on HPLC (u-Bondapak-C18).

Bed: 3.9 x 300 mm; buffer gradient: 30-40% acetonitrile in 0.1% triflouro-acetic acid for 20 minutes; flow: 2 ml/min.

II-A (left) and II-B (right) were collected.

Figure 12. Polyacrylamide gel disc electrophoresis, pH 4.3, of 10 ug Ra5SI (left) and 10 ug Ra5SII (right).

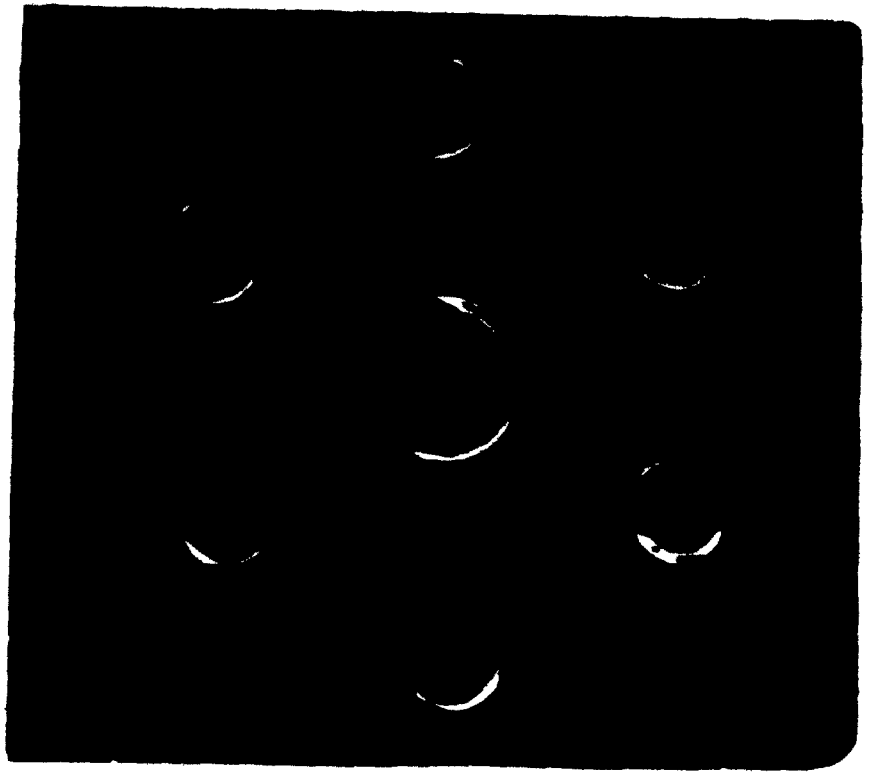

Figure 13. Immunodiffusion of Ra58II (II) and Ra58I (I) against anti-Ra5 goat antiserum.

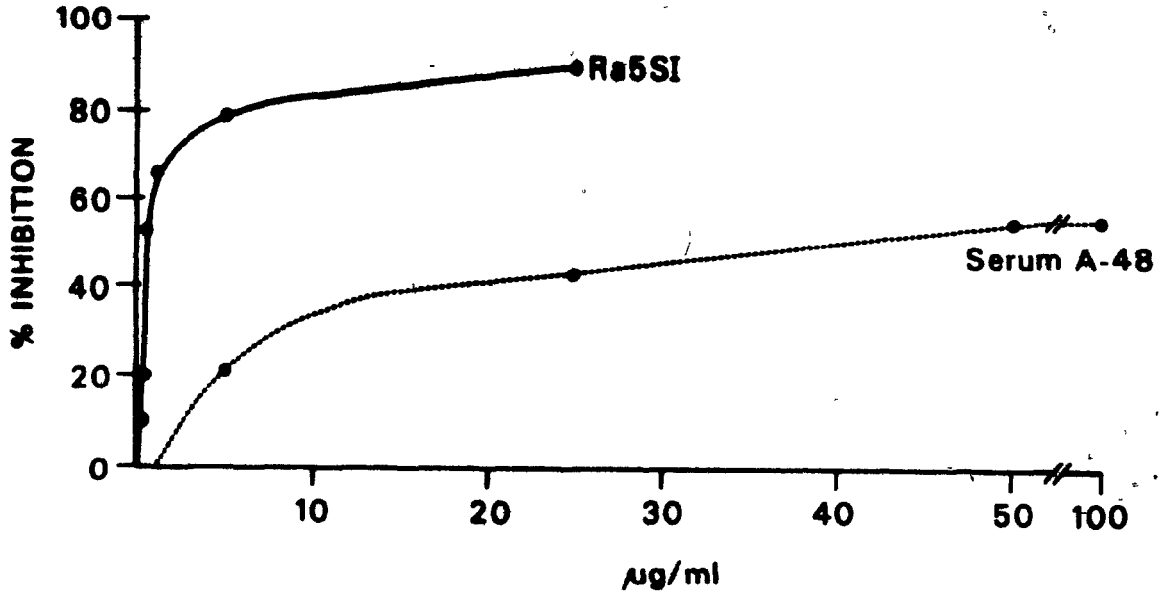

Figure 14. Titration of Ra5SII and Ra5SI by RAST-inhibition using allergic serum (A-48).

——— Ra5SI ------ Ra5SII

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