

Solid-Phase Synthesis of Ragweed Allergen Ra5

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A two-stage strategy was used to synthesize the 45-residue, four-disulfide-bonded ragweed protein allergen Ra5: (i) the protected linear chain was prepared by solid-phase, stepwise synthesis according to the sequence of the fully reduced protein; (ii) after removal from the resin and deprotection by hydrogen fluoride, the reduced chain was purified by gel filtration on Sephadex G-50 and allowed to fold "spontaneously" in the presence of oxidized dithiothreitol to form the disulfide links. The product (synthetic Ra5) was purified to disc-electrophoretic homogeneity by cycling through Sephadex G-50 and CM-Sephadex (overall yield: 6% theoretical). Synthetic Ra5 was closely similar in structure to the natural protein by amino acid analysis, polyacrylamide gel electrophoresis, tryptic peptide mapping, and circular dichroism spectra, as well as in vitro and in vivo immunoassays of antigenic/allergenic activities.

KEY WORDS: peptide synthesis; ragweed allergen Ra5; structure; antigenic/allergenic activity.

1. INTRODUCTION

Short ragweed pollen allergen Ra5 (Lapkoff and Goodfriend, 1974) is a single-chain, 5000-D protein, sensitivity to which is associated with possession of one or two loci within the major histocompatibility complex (Marsh *et al.*, 1975, 1982; Bias *et al.*, 1979). Following determination of its amino acid sequence (Mole *et al.*, 1975) and in light of progress in this laboratory in delineating lymphoproliferative sites along the Ra5 peptide chain (Choudhury and Goodfriend, 1978a; and unpublished results), we undertook synthesis of the allergen preparatory to synthesis of allergenically intact analogues modified at one or two amino acid sites critical to native Ra5-driven T-cell proliferation: such analogues might serve as useful reagents for suppressing Ra5-specific IgE antibody formation by blockade of allergen-specific B-cell receptors of the allergic host.

Of the constituent 45 amino acid residues in Ra5 (Fig. 1), eight are half-cystines forming four intrachain Cys-Cys bridges of as yet unspecified pairing. However, renaturation studies (Choudhury and Goodfriend, 1978b) with fully reduced Ra5 suggested the feasibility of preparing the allergen by synthesis of the reduced chain for subsequent "spontaneous" folding and Cys-Cys bond formation. In our initial approach, we used solution-phase methodology to synthesize consecutive fragments

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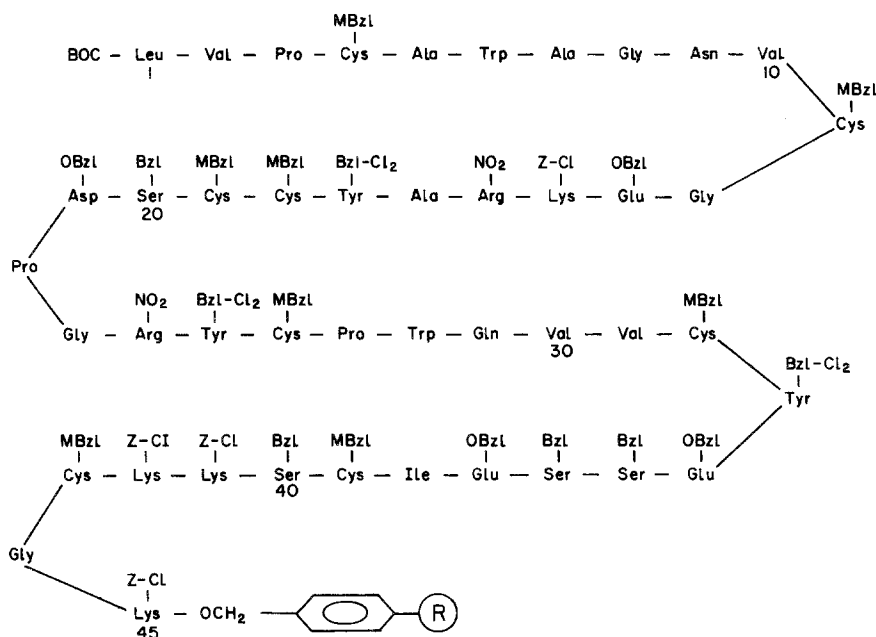


Fig. 1. Structure of the protected Ra5 peptide-resin.

of the reduced Ra5 chain for subsequent condensation. This had to be abandoned owing to solubility problems encountered with some of the fragments. We therefore chose solid-phase, stepwise synthesis (Merrifield, 1963) of the complete (reduced) chain, incorporating some methodological features introduced by Tan and Kaiser (1976) in their synthesis of a 58-residue, three-disulfide-bonded trypsin inhibitor of high purity and specific activity. A preliminary account of this study has been published (Choudhury and Goodfriend, 1982).

2. EXPERIMENTAL PROCEDURES AND RESULTS¹

Solvents were purchased from British Drug House (Montreal) and Fisher Scientific (Montreal) and were redistilled. Chloromethyl-copoly(styrene-1% divinylbenzene)-resin was purchased from Pierce Chemical Co. (Rockford, Illinois). The Boc amino acid derivatives were prepared (Schnabel, 1967) from L-amino acids (Sigma Chemical Co., St. Louis, Missouri); their purity was checked by melting point and thin-layer chromatography (Eastman Chromagram sheets 6061).

¹ Abbreviations used; Boc, *tert*-butoxycarbonyl; Bzl-Cl₂, 2,6-dichlorobenzyl; CD, circular dichroism; DCCI, dicyclohexylcarbodiimide; DMF, dimethylformamide; DTNB, 5,5'-dithio-bis 2-nitrobenzoic acid; DTT, dithiothreitol; Gu, guanidinium; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MBzl, *p*-methylbenzyl; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline (0.02 M sodium phosphate buffer, pH 7.2, in 0.13 M NaCl); PK, Prausnitz-Kustner; RAST, radioallergosorbent test; TFA, trifluoroacetic acid; Z-Cl, 2-chlorobenzylloxycarbonyl.

Allergen Ra5 was isolated from short ragweed pollen (Greer Lab., Lenoire, North Carolina) and purified to size and charge homogeneity as previously described (Lapkoff and Goodfriend, 1974) with minor modifications. Allergen Ra3, isolated from the same pollen source, contained the major and minor forms of the protein (Goodfriend *et al.*, 1981). Protein concentrations of natural and synthetic Ra5 were based on optical density measurements at 280 nm and an $E_{1\text{ cm}, 280\text{ nm}}^{1\text{ mg/ml}} = 2.50$; concentrations of Ra3 were obtained using an $E_{1\text{ cm}, 280\text{ nm}}^{1\text{ mg/ml}} = 1.1$.

2.1. Synthesis of the Protected Peptide

Synthesis was initiated (Fig. 1) by esterification (Gisin, 1973) of *N*-Boc-Lys(2-Cl-Z)OH to 0.5 g chloromethyl-copoly(styrene-1% divinylbenzene)-resin pre-washed with strong Lewis acid to remove any free hydroxymethyl sites (Merrifield *et al.*, 1976). Amino acid analysis of the esterified resin showed an incorporation of 0.21 mM Lys/0.5 g. Synthesis was carried out manually and stepwise except for Pro₂₂-Gly₂₃, which was incorporated as dipeptide owing to its availability as highly pure Boc-Pro-Gly. With solvent/resin ratios of 40–50 ml/0.5 g and solvent washes timed for 5 min, each step was carried out according to the protocol: (1) deprotect with TFA-CH₂Cl₂ (1:1 v/v) containing 3% 2-mercaptoethanol for 45 min; (2) CH₂Cl₂ × 3 wash; (3) neutralize with 5% diisopropylethylamine in CH₂Cl₂ for 5 min; (4) CH₂Cl₂ wash; (5) repeat steps 3 and 4; (6) isopropanol × 3 wash; (7) DMF × 2 wash; (8) CH₂Cl₂ × 4 wash; (9) add 5 equiv Boc-amino acid in DMF, mixing and cooling in ice bath for 10 min; add 5 equiv DCCI and, after 30 min, allow reaction to continue at room temperature for 4–6 h; (10) DMF × 1 and CH₂Cl₂ × 2 wash; (11) repeat step 9 in presence of 5 equiv HOBt for 4–6 h; (12) DMF × 2 and CH₂Cl₂ × 4 wash; (13) *N*-acetylate with acetic anhydride (2 ml) and *N*-methylmorpholine (1 ml) in DMF for 1 h; (14) repeat steps 7 and 8. Addition of each residue was monitored by ninhydrin test (Kaiser *et al.*, 1970); in addition, aliquots of peptide-resin were routinely hydrolyzed in 12 N HCl-propionic acid (1:1) for 3 h at 140°C (Westall *et al.*, 1972) for amino acid analysis in a Beckman Model 120C. In the case of Asn and Gln, the initial coupling (step 9) was done in the presence of HOBt as in step 11 and repeated two additional times to achieve a negative ninhydrin test. After incorporation of Trp₂₈ (17th coupling), 0.2% indole was added to the deprotection reagent (step 1) to inhibit alkylation of the indole ring of Trp (Wünsch *et al.*, 1977); all subsequent steps were carried out under N₂. Based on incorporation of Leu (45th residue) as determined by amino acid analysis, the yield of the protected 45-residue peptide coupled to the resin was ~75 mg (0.015 mM)/0.5 g.

2.2. Preparation and Partial Purification of the Reduced Peptide

The peptide-resin (~0.6 g) was dried *in vacuo* and treated in an HF apparatus (Sakakibara *et al.*, 1967) with anhydrous HF (10 ml) for 1 h at 0°C in the presence of anisole (0.5 ml), ethylmethyl sulfide (0.5 ml), and skatole (0.1 g). After removal of HF *in vacuo* at 0°C and separately washing with ethylacetate and ether (3 × 15 ml

each), the (deprotected) peptide mixture was extracted with deaerated 10% acetic acid (15 ml) and fractionated anaerobically (Repaske, 1971; Tan and Kaiser, 1976) on a column (2.5 × 80 cm) of Sephadex G-50 superfine in 10% acetic acid. The eluate peaks monitored (here and throughout the purification steps) at 280 nm are shown in Fig. 2A. The synthetic Ra5 peptide was eluted in an eluate peak with the same holdup volume (denoted by the arrow in the figure) as natural Ra5. Attempts to recover additional Ra5 peptide by treatment of the earliest (major) peak fraction with excess DTT in 7 M GuCl (pH 8.6) yielded only negligible amounts. The Ra5 peptide fraction was lyophilized (yield 38 mg) and, to ensure maximum uniformity in reduced molecular species, treated with 0.5 M DTT in 7 M GuCl–0.5 M Tris-HCl (pH 8.6) at 37°C under N₂. After 18 h, the reduced peptide fraction (29 mg) was isolated by anaerobic chromatography on the Sephadex G-50 column preequilibrated with 0.01 N HCl (Fig. 2B).

2.3. Preparation and Purification of the Oxidized Peptide

A solution of 28 mg reduced peptide (1 mg/15 ml) in 0.1 M Tris-Cl–0.2 M KCl–1 mM EDTA (pH 8.7) was made 5 mM in oxidized DTT (Creighton, 1974) and stirred for 5 days under N₂ in the dark. After clearing by millipore filtration, the reaction mixture was concentrated to ~5 ml in an Amicon cell over a UM-2 membrane and the oxidized Ra5 peptide fraction isolated by gel filtration (Fig. 2C) on the Sephadex G50 column preequilibrated with 0.025 M Tris-HCl (pH 7.6). Purification of the oxidized Ra5 peptide was achieved as follows. The peptide fraction (pooled as shown in Fig. 2C) was equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, by gel filtration on the Sephadex G-50 column and cycled through a 1 × 10 cm column of DEAE-Sephadex A25 in series with a 1 × 5 cm column of CM-Sephadex C25, both columns having been preequilibrated with the sodium phosphate buffer. The DEAE-Sephadex column served to retain some slightly yellow pigmented material; after elution of an initial peak from the CM-Sephadex column, application of a linear gradient to 0.1 M sodium phosphate buffer (75 ml 0.1 M into 75 ml 0.01 M) directly to the CM-Sephadex column eluted several additional peaks, of which the major contained the Ra5 peptide (Fig. 2D). A cut was made as shown in the figure, the pooled Ra5 fraction reequilibrated with startup sodium phosphate buffer on the Sephadex G-50 column (which served also to remove some minor contaminants), and was recycled through the CM-Sephadex column as previously. On application of the buffer gradient, the Ra5 peptide emerged as a single, fairly homogeneous peak (Fig. 2E). No further purification of the peptide was achieved in two additional chromatographic steps: gel filtration on a 2.5 × 80 cm column of Sephadex G-50 superfine preequilibrated with 5% acetic acid and ionexchange on a 1 × 5 cm column of CM-Sephadex C25 under a linear gradient of 0–1.0 M NaCl in 0.01 M sodium acetate buffer, pH 5.0 (results not shown). Finally, the Ra5 peptide was equilibrated in 5% acetic acid (by gel filtration as above) and stored frozen at –20°C. For all subsequent analyses, aliquots of this solution were dried by rotary evaporation and redissolved in appropriate solvents. In addition to chromatographic criteria, the oxidized peptide (synthetic Ra5) was homogeneous

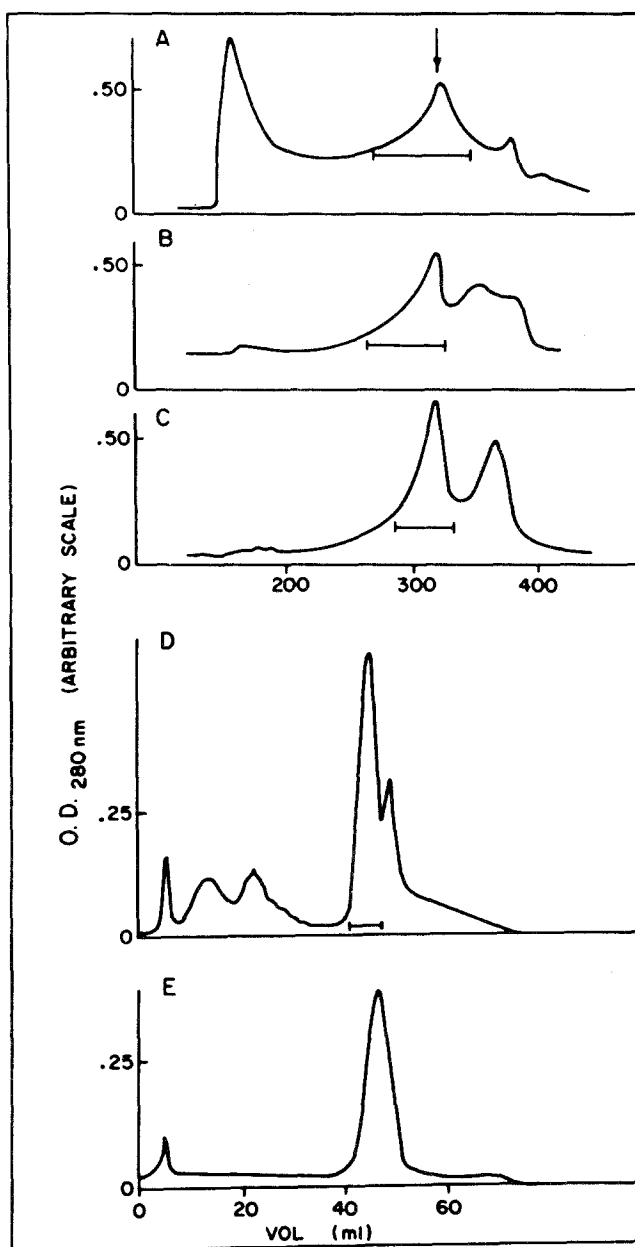


Fig. 2. Stages in column chromatographic purification of the synthetic Ra5 peptide. All chromatographic eluates were monitored continuously at 280 nm. (A) Anaerobic chromatography of Sephadex G-50 superfine of peptide mixture from deprotection and cleavage of the peptide-resin. The synthetic Ra5 peak was localized using natural Ra5 as marker (arrow) and the peak fraction (denoted here and throughout by the horizontal bar) reduced with dithiothreitol. (B) Anaerobic chromatography (as in A) of the reduced Ra5 peak fraction. (C) Gel filtration on Sephadex G-50 superfine of the oxidized Ra5 peptide. (D/E) CM-Sephadex chromatography/rechromatography of the oxidized Ra5 peptide.

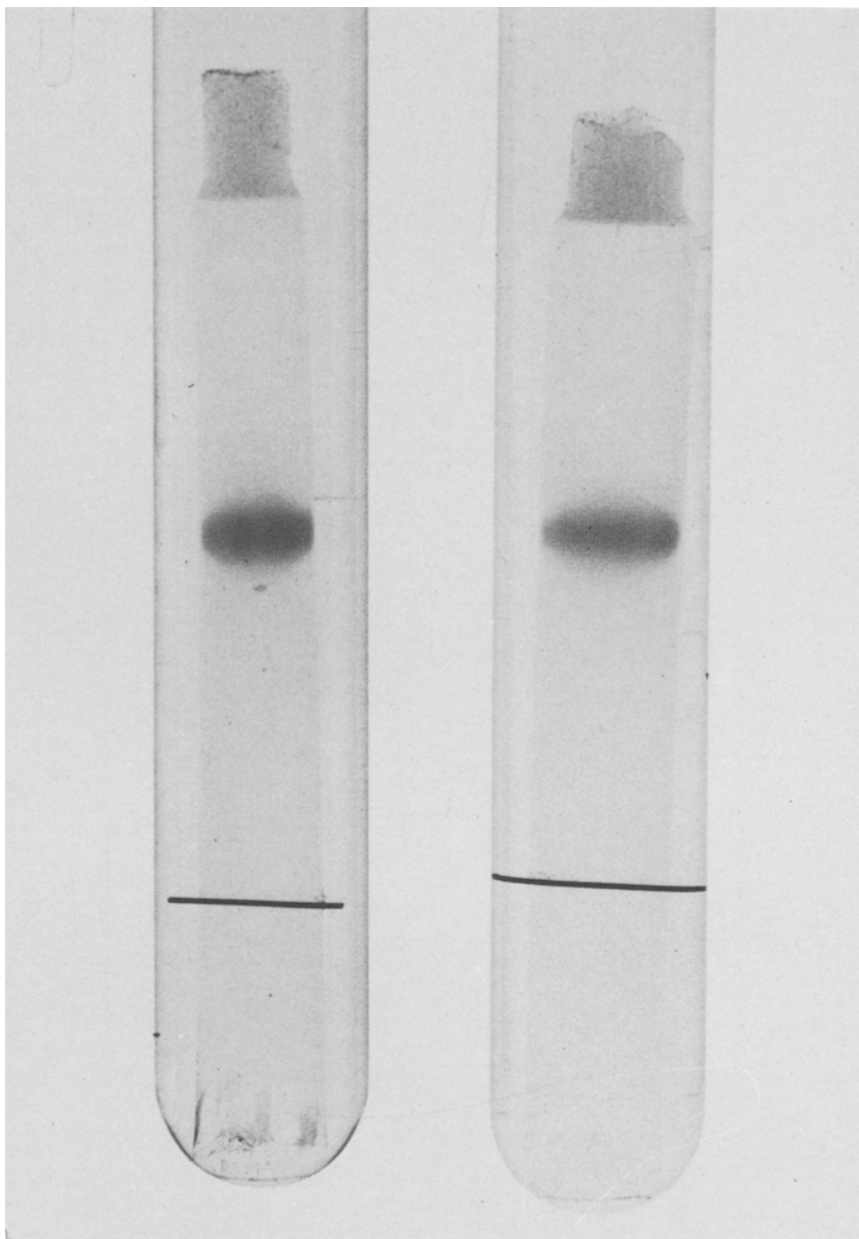


Fig. 3. PAGE at pH 4.3 of natural Ra5 (left) and synthetic Ra5 (right). Horizontal lines are needles through the dye marker.

by PAGE and HPLC (see Figs. 3 and 4 and below); based on amino acid analysis, it was obtained in a yield of 4.5 mg ($0.9 \mu\text{M}$; 6% theoretical).

2.4. Chemical Properties of Synthetic and Natural Ra5

The molecular weights of synthetic and natural Ra5 were estimated by Sephadex G-50 gel filtration to be closely similar (Fig. 2). For charge comparison, samples ($100 \mu\text{g}/\text{ml}$) were examined by PAGE at pH 4.3 in 15% gels (Lapkoﬀ and Goodfriend, 1974). After fixing and staining with Coomassie blue, the gels showed single bands of identical cationic mobilities (Fig. 3).

The two proteins were compared by reverse phase HPLC. Aliquots ($15 \mu\text{l}$) of aqueous solutions of the proteins ($\sim 100 \mu\text{g}/\text{ml}$) were applied to an analytical C18 uBondapak column of a Waters Associates HPLC system (Waters Scientific, Mississauga, Ontario, Canada). The column was eluted at a flow rate of 1.0 ml/min over a 30 min period with a linear gradient of 5–35% aqueous acetonitrile containing 0.1% TFA and the eluate monitored continuously by absorbance at 214 nm. As shown in Fig. 4, synthetic and natural Ra5 gave absorbance peaks of identical elution times.

The amino acid composition of the oxidized peptide was determined on aliquots (10 nM) hydrolyzed in 6 N HCl for 24, 48, and 72 h at 110° ; Cys/2 was determined as cysteic acid following performic acid oxidation of the intact protein (Hirs, 1967), and Trp spectrophotometrically (Beaven and Holiday, 1952) assuming 3 Tyr/mole. The composition was reasonably close to theoretical (values in parentheses): Cys 7.70 (8), Asp 2.15 (2), Ser 3.55 (4), Glu 4.00 (4), Pro 3.30 (3), Gly 3.80 (4), Ala 2.91 (3), Val 3.30 (4), Ile 1.02 (1), Leu 1.11 (1), Tyr 2.88 (3), Lys 3.89 (4), Arg 1.86 (2), Trp 2.07 (2). In addition, as previously reported for Ra5 (Mole *et al.*, 1975), no free sulfhydryl was detected for the oxidized peptide on titration with DTNB (Pierce Chemical Co., Rockford, Illinois) in the presence of 6 M GuCl (Ellman, 1959).

Evidence for congruence in the amino acid sequences of synthetic and natural Ra5 was obtained by peptide mapping. Solutions ($100 \mu\text{g}/0.1 \text{ ml}$) of the corresponding *S*-acetamidomethyl derivatives (Choudhury and Goodfriend, 1978a) in 1 mM NaHCO_3 (pH 8.3) saturated with toluene were separately digested for 48 h at 34°C with TPCK trypsin (Sigma Chemical Co., St. Louis, Missouri) at an enzyme–substrate ratio of 1 : 5. After neutralizing with dilute acetic acid, the digests were dried by rotary evaporation and redissolved in H_2O for analysis by reverse phase chromatography on the C18 uBondapak column. Peptides were eluted at 0.5 ml/min under a linear gradient of 5–35% acetonitrile containing 0.1% TFA. As shown in Fig. 5, except for differences in yield of some corresponding peptides (due to differences in extent of proteolysis), synthetic and natural Ra5 gave essentially identical peptide maps.

The CD spectra of synthetic and natural Ra5 ($0.2 \mu\text{M}/\text{ml}$) in 0.1 M Tris-HCl (pH 7.6) were obtained in a Carey-Varian dichrometer (model G1) equipped with a quartz cell (1.0 cm path) thermostatted at 37.5°C ; molar ellipticities were calculated using a mean residue molecular weight of 121.8. The spectra (Fig. 6) were closely similar, although some divergence was apparent in both the far and near UV.

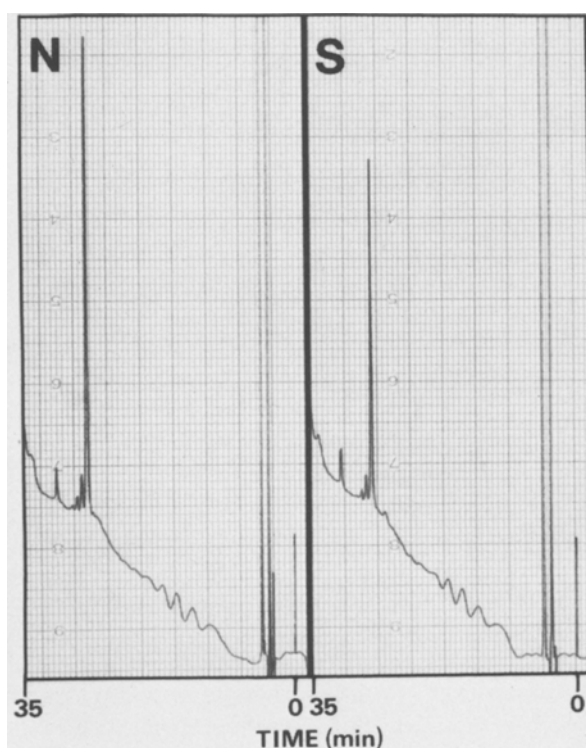


Fig. 4. Reversed phase HPLC of natural (N) and synthetic (S) Ra5. The ordinate represents absorbance at 214 nm.

2.5. Immunoassays of Synthetic and Natural Ra5

Immunodiffusion of the two proteins at 0.1 mg/ml against a goat anti-Ra5 antiserum (Lapkoff and Goodfriend, 1974) gave single precipitin arcs in a reaction of complete antigenic identity (Fig. 7).

The allergenic activities of synthetic and natural Ra5 were assessed *in vitro* by RAST inhibition of solid-phase Ra5 and (for control of specificity) Ra3 (Goodfriend *et al.*, 1981). The assay incorporated human allergic sera R.C. and A.S. [having closely similar (uninhibited) RAST activities] and paper discs separately conjugated with each protein according to Aas and Lundkvist (1973). Duplicate aliquots (100 μ l) of varying concentrations (0–1 μ g/50 μ l) of test inhibitor in PBS were added to the allergen-coupled discs prior to addition of 50- μ l aliquots of allergic serum. After standing for 3 h at room temperature, the discs were washed with PBS and 100- μ l aliquots of ^{125} I-labeled rabbit antiserum to human IgE (Pharmacia-Canada, Montreal) were added. After overnight incubation at 4°C, the PBS-washed discs were counted (2 min) in a Packard gamma scintillation spectrometer (model 578). The percentages inhibition relative to uninhibited (PBS) controls were averaged for least square plots (Fig. 8). For both allergic sera, natural and synthetic Ra5 had close,

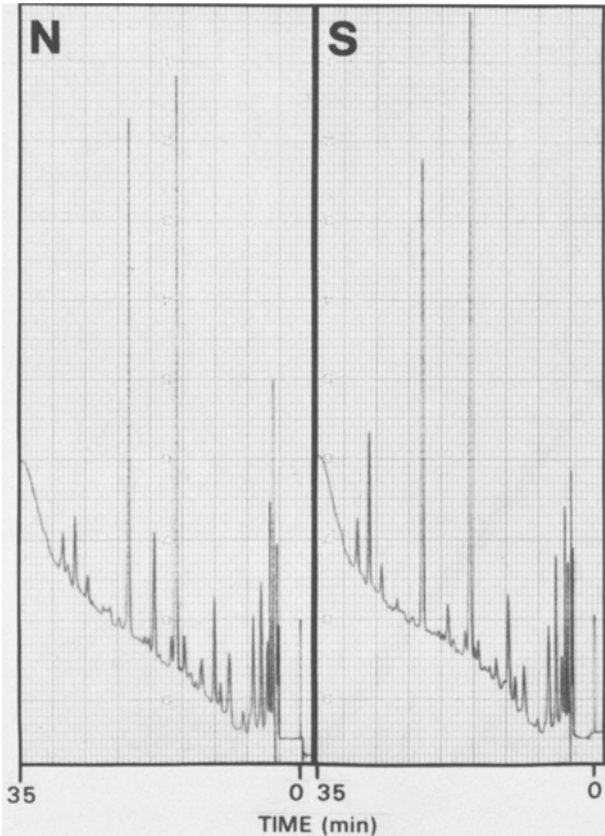


Fig. 5. Separation by reversed phase HPLC of peptides generated by tryptic digestion of natural (N) and synthetic (S) Ra5. The ordinate represents absorbance at 214 nm.

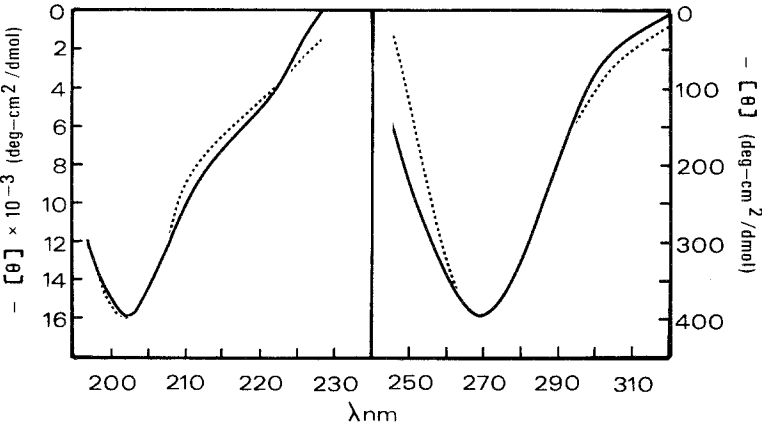


Fig. 6. Circular dichroism spectrum of natural (—) and synthetic (···) Ra5.

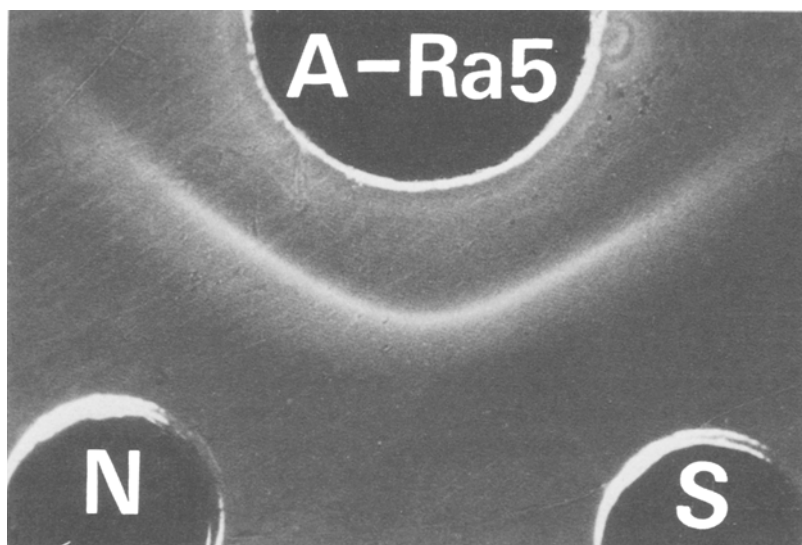


Fig. 7. Immunodiffusion in agar of natural (N) and synthetic (S) Ra5 against goat antiserum to natural Ra5 (A-Ra5).

if not equal, inhibitory activity in the homologous, solid-phase natural Ra5 system (lines a, a¹). To exclude the possibility of nonspecific inhibition by synthetic Ra5, serum A.S., containing IgE antibodies to Ra3 as well as Ra5, was employed in RAST inhibition of solid-phase Ra3 with Ra3 and synthetic Ra5. As shown in the figure, in contrast to Ra3, synthetic Ra5 showed only baseline activity in the heterologous system (lines b, b¹).

The allergenic activities were also evaluated by PK tests performed as described elsewhere (Lapkoff and Goodfriend, 1974). Duplicate skin test sites of a nonallergic volunteer were injected intradermally with 0.02-ml aliquots of Ra5-allergic serum R.C. and 24 h later challenged with intradermal injections of 0.02 ml synthetic or natural Ra5 in physiological saline (Hollister-Stier). As shown in Table I, both proteins elicited essentially identical wheal and erythema reactions 15 min postinjection. It should be noted that control (non-serum-sensitized) sites were completely negative to synthetic Ra5 at the highest concentration (1.0 μ g/ml) used for challenge.

3. DISCUSSION

From the yields of carboxy-terminal lysine (0.21 mM) and completed (protected) peptide (0.015 mM) attached to resin, an average coupling efficiency of only 94% was achieved during synthesis in spite of multiple couplings and prolonged coupling reaction times. Consecutive amino acid analyses did not reveal any precipitous reduction in yield of peptide-resin complex at any point in the synthesis and it is not clear, therefore, why a higher coupling efficiency was not achieved.

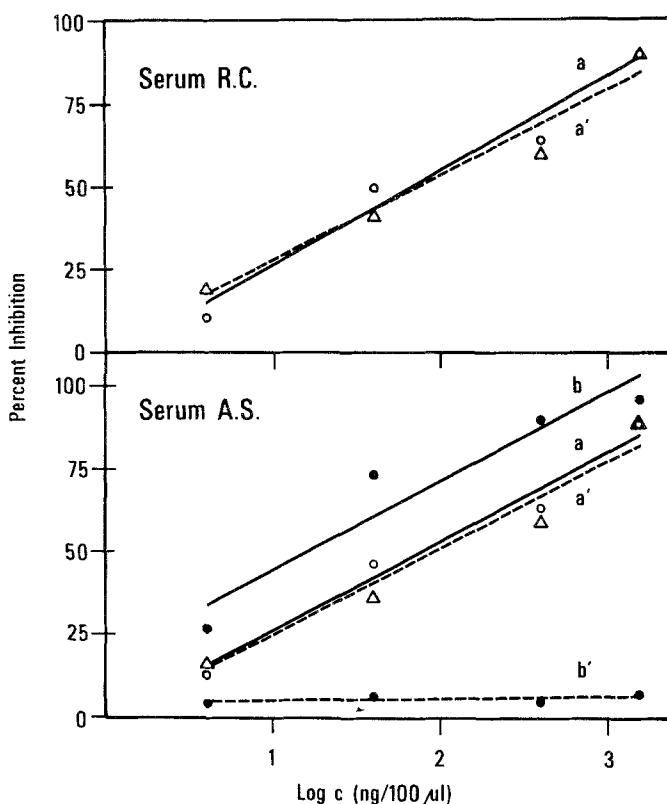


Fig. 8. Least squares plot of percent RAST inhibition vs. log concentration c of solution-phase natural Ra5 (a), Ra3 (b), and synthetic Ra5 (a^1 , b^1). Lines a and a^1 were obtained using solid-phase natural Ra5, lines b and b^1 using solid-phase Ra3.

After deprotection and cleavage of the completed chain from resin, initial attempts to purify the reduced chain by gel filtration under conditions in which atmospheric oxygen was not excluded led to extremely complex products. In contrast, adoption of anaerobic chromatographic conditions led to the relatively simple elution profile of Fig. 2 corresponding to an Ra5 as well as higher and lower molecular size fractions. This size distribution was qualitatively similar to that obtained by Tan and Kaiser (1976) in their synthesis of the trypsin inhibitor. In the latter case, the higher molecular size fraction was likely formed by interchain disulfide linkage since appreciable amounts of monomer chain were recovered by reduction in 6 M GuCl. In the Ra5 synthesis, other mechanisms were likely operative since the same treatment of the higher molecular size fraction yielded only negligible amounts of monomer.

Oxidation of the reduced peptide fraction was effected by the mixed disulfide-sulfhydryl exchange reaction with oxidized dithiothreitol (Creighton, 1974). The reaction was accompanied by some precipitate formation as found in our earlier

Table I. Allergenic Activity of Natural and Synthetic Ra5 by PK Test^a

Concentration, $\mu\text{g/ml}$	Diameter of wheal (W) and erythema (E), mm				
		Natural Ra 5		Synthetic Ra5	
1.0 ^b	W	10×7	8.6	13×8	12×8
	E	40	35	40	40
0.1 ^b	W	8×6	8×6	9×8	8×9
	E	30	40	45	40
0.01	W	8×6	8×5	6×4	7×5
	E	25	25	30	40

^a See text for experimental procedure. The horizontal and vertical diameters are given for wheal, the longest diameter for erythema reactions.

^b Control (duplicate non-serum-sensitized) sites were completely negative (zero wheal and erythema) to either protein at these concentrations.

study on renaturation of reduced natural Ra5 (Choudhury and Goodfriend, 1978b). Thus, the conditions of folding and oxidation employed by Creighton (1974) for reduced trypsin inhibitor chain, particularly that of protein concentration, may not have been optimal for the reduced Ra5 chain with its higher sulfhydryl content. Nevertheless, after allowing for precipitate, these conditions led to a predominantly single size molecular species, which was readily purified to PAGE and HPLC homogeneity (Figs. 3 and 4) for an overall yield of 6%.

Chemical and immunological assays provided evidence for close similarity in structure of the synthetic product with Ra5. The corresponding amino acid compositions (Sec. 2.4) and tryptic peptide maps (Fig. 8) were in good agreement for corresponding primary structures. In addition to common chromatographic properties (Figs. 2 and 4), the proteins had essentially identical cationic disc electrophoretic mobilities (Fig. 3), reflecting similarity in their net charge, size, and shape. Some differences in secondary structure were detected by CD spectral comparison (Fig. 6). Analysis of the far-UV CD spectra has indicated a 6% higher β -sheet content for the synthetic protein, apparently derived at the expense of two or three of an estimated 17 residues in natural Ra5 in the helical or random coil conformations (L. Goodfriend, R. E. Williams, and A. M. Choudhury, unpublished data). Deviations that this may have caused away from native tertiary structure and surface topography did not, in any event, lead to appreciable differences between the antigenic/allergenic determinant structures of the two proteins, since the corresponding activities were essentially identical *in vitro* (Figs. 7 and 8) and *in vivo* (Table I).

From the above characterization, it seems fair to conclude that the two-step strategy used by Tan and Kaiser (1976) for synthesis of trypsin inhibitor was efficacious also for synthesis of a protein closely similar in structure and immunological activity to ragweed allergen Ra5. The strategy would appear to be of general applicability to synthesis of proteins or protein domains of diverse biological activities that are highly disulfide-bonded and in the molecular weight range of 5000–10,000 D.

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REFERENCES

- Aas, K., and Lundkvist, U. (1973). *Clin. Allergy* **3**, 225.
- Beaven, G. H., and Holiday, E. R. (1952). *Adv. Protein Chem.* **7**, 319.
- Bias, W. B., Hsu, S. H., Meyers, D. A., Goodfriend, L., and Marsh, D. G. (1979). *Transplant. Proc.* **11**, 1853.
- Choudhury, A. M., and Goodfriend, L. (1978a). *Fed. Proc.* **37**, 1553.
- Choudhury, A. M., and Goodfriend, L. (1978b). *Proc. Can. Fed. Biol. Soc.* **21**, 31.
- Choudhury, A. M., and Goodfriend, L. (1982). *Fed. Proc.* **41**, 826.
- Creighton, T. E. (1974). *J. Mol. Biol.* **87**, 563.
- Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
- Gisin, B. F. (1973). *Helv. Chim. Acta* **56**, 1476.
- Goodfriend, L., Roebber, M., Lundkvist, U., and Choudhury, A. M. (1981). *J. Allergy Clin. Immunol.* **67**, 299.
- Hirs, C. H. W. (1967). In Colowick, S. P., and Kaplan, N. O. (eds.), *Methods in Enzymology*, Vol. 11, Academic Press, New York, p. 59.
- Kaiser, E., Colescott, R. L., Bodsinger, C. D., and Cook, P. I. (1970). *Anal. Biochem.* **34**, 595.
- Kent, S. B. H., and Merrifield, R. B. (1979). In Gross, E., and Meinjofer, J. (eds.), *Peptides: Structure and Biological Function* (Proceedings of the 6th American Peptide Symposium), Pierce Chemical Co., Rockford, Illinois, p. 345.
- Lapkoff, C. B., and Goodfriend, L. (1974). *Int. Arch. Allergy* **46**, 215.
- Marsh, D. G., Bias, W. B., Santilli, J., Schacter, B., and Goodfriend, L. (1975). *Immunochemistry* **12**, 539.
- Marsh, D. G., Hsu, S. H., Roebber, M., Ehrlich-Kautzky, E., Friedhoff, L. R., Meyers, D. A., Pollard, M. D., and Bias, W. B. (1982). *J. Exp. Med.* **155**, 1439.
- Merrifield, R. B. (1963). *J. Am. Chem. Soc.* **85**, 2149.
- Mole, L. E., Goodfriend, L., Lapkoff, C. B., Kehoe, J. M., and Capra, J. D. (1975). *Biochemistry* **14**, 1216.
- Repaske, R. (1971). In *Methods in Enzymology*, Vol. 22, Academic Press, New York, p. 322.
- Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967), *Bull. Chem. Soc. (Jpn.)* **40**, 2164.
- Schabel, E. (1967). In *Liebigs Ann. Chem.* **702**, 188.
- Tan, H. H., and Kaiser, E. T. (1976). *J. Org. Chem.* **41**, 2787.
- Westall, F. C., Scotchler, J., and Robinson, A. B. (1972). *J. Org. Chem.* **37**, 3363.
- Wünsch, E., Jaeger, E., Kisfaludy, L., and Low, M. (1977). *Angew. Chem. Int.* **16**, 317.