

Comparative Studies on Tree Pollen Allergens

II. Isolation of Alder (*Alnus incana*) Pollen Allergens:

Purification and Some Characteristics of the Major Allergen pI 4.78

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Abstract. The aqueous extract of alder (*Alnus incana*) pollen was found to consist of a protein mixture amounting to 3% of the dry pollen weight. Separation by gel permeation chromatography on an AcA-54 column resulted in 6 fractions designated chronologically to their relative elution volumes (AI 1 to AI 6). The immunological reactivity of these fractions was demonstrated in both IgE and IgG test systems. Being both immunochemically and quantitatively dominant fraction AI 4 was selected for further purification by a semipreparative isoelectric focusing column. A well-resolved and apparently homogeneous component with a pI value of 4.78 was eluted from the IEF column. The homogeneity of this fraction was demonstrated by one sharp band in thin layer IEF, one single band in HVE, one distinct sharp peak in HPLC and a symmetrical precipitate in CIE. The molecular weight was estimated to 22,500 daltons. Fraction pI 4.78 was shown to be a major allergen of alder pollen, it elicited a 1.17 histamine wheal reaction in skin prick test at a concentration of $1 \text{ mg} \cdot \text{mL}^{-1}$ and showed a significantly high RAST inhibition in all of the reaginic sera used.

Fraction pI 4.78 was found to be an adequate model allergen of alder pollen well suited for further immunochemical characterization.

Introduction

Tree pollens are major sources of early springtime allergic diseases of the respiratory passages and lungs [Belin, 1972; Viander, 1979]. Birch, alder and hazel are common trees growing densely in greater parts of Scandinavia. Their pollens are dominant ingredients of the seasonal airborne allergenic proteins. Clinical observations as well as systematic studies currently show that tree pollens demonstrate immunological cross-reactivity patterns comprising multiple pollen species [Bernstein et al., 1976; Löwenstein, 1980]. The isolation of homogeneous allergens from different tree pollen extracts should be invaluable for the interpretation of the multiple cross-reactivity patterns in tree pollen hypersensitivity. So far, relatively small efforts have been devoted to the characterization of tree pollen allergens [Herbertson et al., 1958]. Nevertheless, some data on the structural properties of particular components of tree pollen allergens were recently reported [Belin, 1972; Viander, 1979; Borch et al., 1979; Borch and Skålhegg, 1980].

In our laboratory, we recently have partially characterized the major allergen pI 5.18 isolated from a purified extract of birch pollen. This fraction was shown to be homogeneous. Immunochemical examination allowed to conclude that pI 5.18 was a suitable model for further structural characterization [Apold et al., 1981].

The aim of the present study is the isolation of an analogous homogeneous major allergen from alder pollen extract in amounts sufficient for comparative structural and immunological studies.

Material and Methods

Extraction of Pollen Allergens

Alnus incana pollen was supplied by Allergon AB, Engelholm, Sweden, at a given purity of 99%. The extraction procedure was performed using 1:10 w/v of pollen grains to phosphate/NaCl buffer, 0.075 M at pH 7.20. The extraction was allowed to proceed for 3 h at +4°C under gentle shaking and the mixture was then centrifuged for 45 min, 12,000 g. The supernatant was filtered, dialyzed against 12 liters of distilled water and finally lyophilized. The proteins were usually stored at -20°C until used.

Isolation and Purification

Permeation chromatography on an Ultrogel AcA-54 column (Pharmacia, Uppsala, Sweden) was found to give satisfactory separation. A column dimension of 100×1.5 cm was used. Phosphate/NaCl buffer of the same molar concentration and pH as the extraction buffer was utilized for the equilibrium and elution of the fractions. A crude protein load of 60 mg was applied on the column in 1.5 ml of buffer. The resolved peaks were pooled, dialyzed against 2 changes of distilled water, lyophilized and stored at -20°C.

The molecular weights of the isolated fractions were estimated by chromatography on a precalibrated AcA-54 column [Apold and Elsayed, 1979].

Further purification of the allergenic components was initially performed on an analytical scale and later preparatively on an isoelectric focusing column using carrier ampholytes pH 4-6 in a sucrose density gradient including 6 M urea. The electrofocusing proceeded for 48 h at a constant power supply of 2 W. The fractions were eluted and their pI's were measured at 20°C. The pooled fractions were extensively dialyzed and lyophilized. The homogeneity of the fractions was further examined by analytical thin-layer isoelectric focusing on ampholine PAGE plates (LKB, Bromma, Sweden) at a pH range identical to that of the column isoelectric focusing.

High performance liquid chromatography (HPLC) was performed on Waters Associate Equipment (USA) using J-125 protein silica-based column supplied by the same manufacturer. The mobile phase was 0.5 M Na₂HPO₄/NaH₂PO₄ buffer with pH 7.0. Approximately 50 µg of the proteins were injected. The flow rate was 2 ml·min⁻¹ and the A₂₈₀ nm was registered on model 440 detector supplied by the same manufacturer. The molecular weight estimation of fraction pI 4.78 was repeated on a HPLC protein column using a diagram of the elution volume against the logarithm of the molecular weights of the marker proteins BSA (67,000 daltons), egg albumin (45,000 daltons), myoglobin (17,000 daltons), ribonuclease (13,700 daltons) and cytochrome c (12,500 daltons).

Two direction high volt electrophoresis (HVE) was performed for 90 min as described in earlier papers [Elsayed et al., 1974; Elsayed and Bennich, 1975].

Amino Acid Analysis

Samples were hydrolyzed in 6 M HCl in vacuo for 24 h at 110°C and the composition was obtained from a computer integrator connected to a Kontron Liquamat III automatic analyzer [Elsayed et al., 1974].

Absorption Spectrum

The UV absorption spectrum was obtained in the region of 220-340 nm using phosphate/NaCl buffer in both the sample and the reference cuvettes.

Rabbit and Human Sera

Antibodies against *A. incana* were raised in rabbits. An emulsion was obtained by mixing 2 mg of the crude extract in 0.5 ml phosphate/NaCl buffer and 0.5 ml of incomplete Freund's adjuvant. After 4 intradermal injections in the interscapular region with 2-week intervals the animals were bled 10 days after the last injection. Additional serum was collected after 2 booster injections, 2 and 4 months later.

Human sera were collected from 10 individuals with a clinical history of tree pollen allergy and 2-3 RAST grades to alder. When-

ever needed either individual or pooled sera were utilized. Serum from a timothy-sensitive patient tolerating alder was used as negative control.

Immunoelectrophoretic Techniques

Crossed immunoelectrophoresis (CIE), crossed line immunoelectrophoresis (CLIE), tandem-crossed immunoelectrophoresis (tandem-CIE) and fused rocket immunoelectrophoresis were essentially performed as described earlier [Axelsen et al., 1973; Lowenstein, 1978; Apold et al., 1979]. Crossed radioimmunoelectrophoresis (CIE) was essentially performed as described by Lowenstein et al. [1977].

Allergenic Reactivity Tests

In vitro testing of the allergenicity was performed using RAST techniques. Detailed procedures for the activation of the paper discs by CNBr, the coupling procedure and measurement of specific IgE uptake are outlined elsewhere [Yman et al., 1975]. Radiolabelled anti-IgE and *A. incana* allergen discs were obtained from Pharmacia AB.

In direct RAST, the allergosorbents were prepared using 200-µl aliquots of allergens in serial 2-fold dilutions or 200-µl samples from gel chromatography or isoelectric focusing fractions for one CNBr-activated paper disc.

RAST inhibition experiments were essentially carried out as described earlier [Apold and Elsayed, 1980] using *A. incana* allergosorbents. Preincubation was performed using 50 µl of (1+1) diluted pooled human serum together with 25-µl aliquots of the crude *A. incana* extract or protein fractions. The inhibition percentage was calculated by the formula:

$$1 - \frac{\text{Counts per min of inhibited serum}}{\text{Counts per min of noninhibited serum}} \cdot 100.$$

In vivo testing of the crude AI extract, fractions AI 1 to AI 6 and pI 4.78 was performed by skin prick tests (SPT) at a concentration of 1 mg·ml⁻¹ in 1 alder-sensitive individual [Elsayed et al., 1976]. SPT in 1 nonallergic individual was performed as control.

Results

Extraction and Gel Filtration

The extraction procedure of the nondefatted dry pollen resulted in a protein yield of 25-30 mg·g⁻¹. Permeation chromatography of the crude *A. incana* extract on the Ultrogel AcA-54 column allowed a separation of 6 protein peaks designated AI 1 to AI 6 as demonstrated by the elution profile in figure 1A.

Column Isoelectric Focusing Fractionation (IEF)

Fraction AI 4 was selected for further purification by IEF as justified by its immunochemical reactivity, molecular size and yield. AI 4 could be separated into 4 major peaks designated pI 4.78, pI 5.35, pI 5.67 and pI 6.08 after their pI values measured at the fraction of max A₂₈₀ nm as shown in figure 2. Fraction pI 4.78

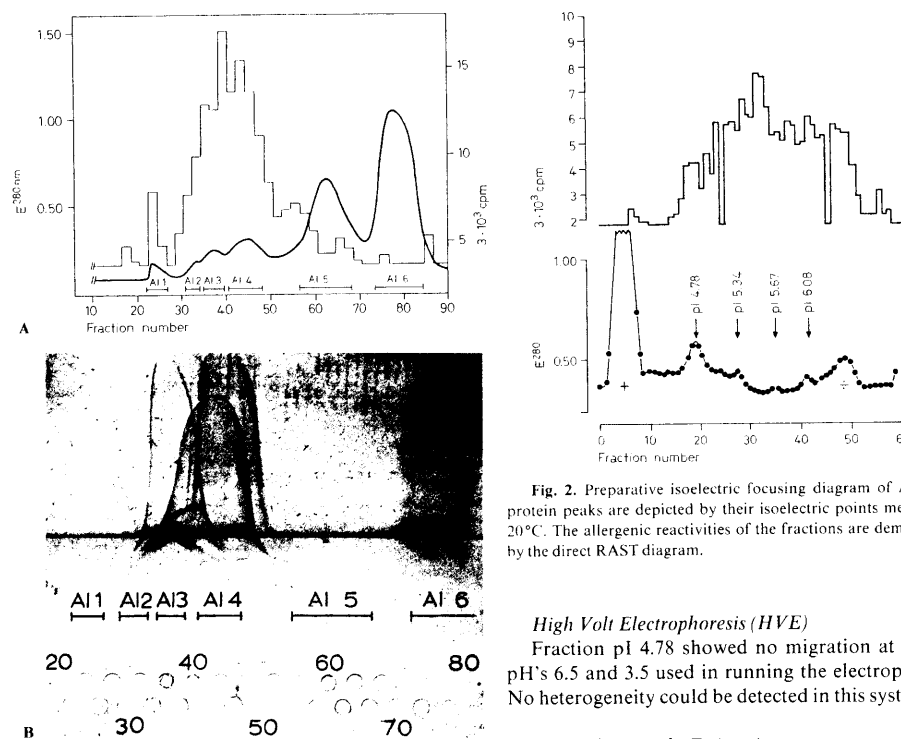


Fig. 1. A Elution diagram of the crude *A. incana* extract from the Ultrogel AcA-54 gel filtration column. The allergenic reactivities of the fractions are indicated by the direct RAST diagram. B Fused rocket immunoelectrophoresis with 15 µl of AI fractions 16-80 from the Ultrogel AcA-54 column in the wells and 800 µl polyspecific rabbit antiserum in the antibody gel.

was quantitatively dominant, it forms approximately 15% of the weight of fraction AI 4 applied on the column. It was well separated from the other fractions and could be eluted from the column with minimal heterogeneity as shown by the purity criteria.

Analytical Thin-Layer Isoelectric Focusing

Figure 3 shows the isoelectric fractionation patterns of AI crude, AI 4 and fraction pI 4.78. AI crude as well as AI 4 gave multiple bands covering the total pH range of the plate. Fraction pI 4.78 was homogeneous giving one band located at pH similar to that of the column IEF.

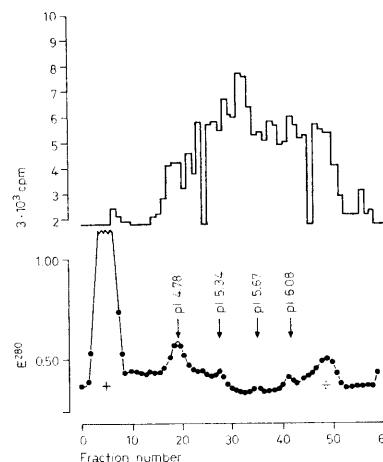


Fig. 2. Preparative isoelectric focusing diagram of AI 4. The protein peaks are depicted by their isoelectric points measured at 20°C. The allergenic reactivities of the fractions are demonstrated by the direct RAST diagram.

High Volt Electrophoresis (HVE)

Fraction pI 4.78 showed no migration at the two pH's 6.5 and 3.5 used in running the electrophoresis. No heterogeneity could be detected in this system.

Molecular Weight Estimation

The relative elution volumes as well as the approximate molecular weights of the different gel filtration fractions are shown in table I. The result of the HPLC molecular weight determination gave a linear function from which the molecular weight of fraction pI 4.78 could be deduced to 22,500 daltons.

Fraction pI 4.78 gave one single, symmetrical and sharp peak in the HPLC system utilized, together with some minor contaminant proteins with slightly higher molecular weights.

Amino Acid Composition

The molecular weight of fraction pI 4.78 (22,500 daltons) was used for the calculation of the amino acid composition shown in table II. A tentative number of 133-138 amino acid residues per mole protein was shown. The amino acid pool included 11 residues of lysine, 3-4 residues of arginine, 2-3 residues of methionine and a single half cystine residue.

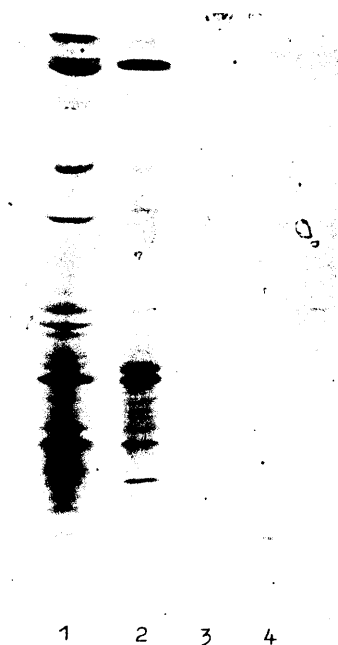


Fig. 3. Analytical thin-layer isoelectric focusing on PAGE plate on 15- μ l samples of AI crude (1), AI 4 (2) and two productions of pI 4.78 (3 and 4) with the latter being the most homogeneous protein.

Absorption Spectrum

The UV absorption spectrum showed the usual pattern of proteins with a peak at 280 nm.

Antigenic Reactivity

The CIE pattern obtained by the crude extract against a polyspecific rabbit antiserum showed at least 11 distinct precipitating lines. Only lines with a strong color intensity were numerated as shown in figure 4A and B. The antigenic line No. 10 is apparently the most distinct. Figure 4C shows the CIE pattern of fraction AI 4 against the same rabbit antiserum with six distinct lines of precipitation including the line corresponding to precipitate No. 10 of the crude pattern.

Fraction pI 4.78 gave one single distinct precipitate against the same rabbit antibodies, though a slight he-

Table 1. Molecular weights of the gel filtration fractions estimated by permeation chromatography

Fraction	Ve/Vt	Tentative MW, daltons
AI 1	0.38	>70,000
AI 2	0.47	>70,000
AI 3	0.64	37,000
AI 4	0.77	22,500
AI 5	1.08	6,600

Table II. The amino acid composition of fraction pI 4.78 isolated by isoelectric focusing of AI 4 from the *A. incana* pollen extract: the protein was hydrolyzed at 110°C in vacuo for 24 h using 6 M HCl including norleucine as internal standard

Amino acid	mol/mol protein	Integral number of residues/mol
Lysine	11.10	11
Histidine	0	
Arginine	3.57	3-4
Aspartic acid	16.51	16-17
Threonine	7.99	8
Serine	10.20	10
Glutamic acid	19.70	20
Glycine	15.01	15
Alanine	14.95	15
Half cysteine	1.30	1
Valine	11.24	11
Methionine	2.50	2-3
Isoleucine	6.70	6-7
Leucine	8.78	8-9
Tyrosine	2.01	2
Phenylalanine	4.61	4-5
Tryptophane	ND	
Residues		133-138

ND = Not determined.

terogeneity was visualized by a faint line (fig. 4D). The identity of fraction pI 4.78 was examined by tandem-CIE and CLIE. In tandem-CIE, pI 4.78 showed a complete immunological reaction of identity with antigen No. 10 of the crude CIE pattern as shown in figure 5B. Similarly pI 4.78 showed a complete reaction of identity with the most distinct line of AI 4 as shown in figure 5E.

This finding was confirmed by CLIE where fraction pI 4.78 also demonstrated a complete reaction of identity with the dominating antigenic line No. 10 of the crude CIE pattern. An additional faint reaction of identity with antigen No. 11 was also shown (fig. 5C).

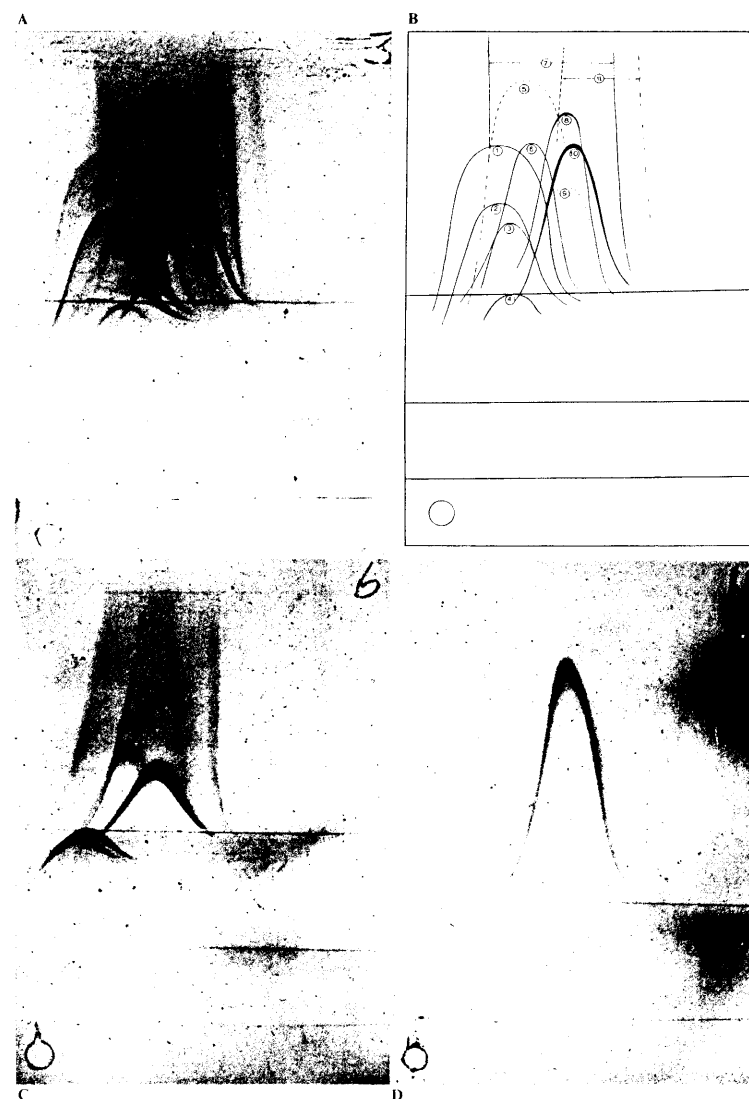


Fig. 4. A The CIE reference pattern of the crude *A. incana* extract with 7 μ l of the crude extract (10 mg $\cdot$ ml $^{-1}$) in the well and 400 μ l of a polyspecific rabbit antiserum in the antibody gel. Staining by Coomassie brilliant blue. B Schematic representation of the major immunoprecipitates of the crude extract with arbitrary numera-

tion. C The CIE pattern of AI 4 with 6 major precipitates produced by 10 μ l of AI 4 (2.2 mg $\cdot$ ml $^{-1}$) in the well and 400 μ l of a polyspecific rabbit antiserum in the antibody gel. D The CIE result produced by 10 μ l of fraction pI 4.78 (60 μ g $\cdot$ ml $^{-1}$) in the well and 400 μ l rabbit antiserum in the antibody gel.

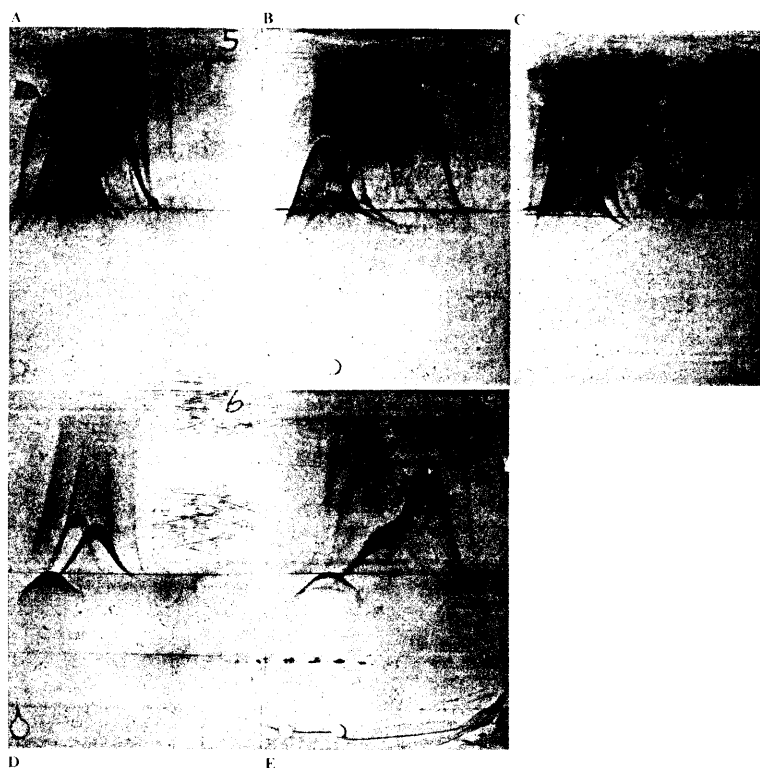


Fig. 5. A CIE pattern of the crude *A. incana* extract. B Tandem-CIE result with 7 μ l of the crude extract ($10 \text{ mg} \cdot \text{ml}^{-1}$) in the first well and 10 μ l of fraction pI 4.78 ($60 \mu\text{g} \cdot \text{ml}^{-1}$) in the second well. C CLIE result with 7 μ l of the crude extract ($10 \text{ mg} \cdot \text{ml}^{-1}$) in the well, 10 μ l of fraction pI 4.78 ($60 \mu\text{g} \cdot \text{ml}^{-1}$) in the intermediate

gel next to the first dimension gel and 400 μ l rabbit antiserum in the antibody gel. D CIE pattern of AI 4 ($2.2 \text{ mg} \cdot \text{ml}^{-1}$). E Tandem result with 10 μ l of fraction AI 4 ($2.2 \text{ mg} \cdot \text{ml}^{-1}$) in the first well and 10 μ l of fraction pI 4.78 ($60 \mu\text{g} \cdot \text{ml}^{-1}$) in the second well.

Fused rocket immunoelectrophoresis run with the gel filtration fractions showed 7–8 precipitates corresponding to the fractions AI 2 to AI 4 (fig. 1B), whereas no precipitates could be seen in AI 1, AI 5 or AI 6.

Allergenic Reactivity

Allergosorbents of the fractions obtained by gel filtration (AI 1 to AI 6) tested by the serum pool in direct RAST showed the highest uptake of ^{125}I -anti-IgE in the region of AI 3 and AI 4 of the elution profile in figure 1A. The allergenic reactivity of the isoelectric focusing fractions of AI 4 showed a moderately high reactivity for pI 4.78 as measured by direct RAST.

The maximum uptake was in the region of pI 5.34, pI 5.67 and near the cathode (fig. 2).

Fraction pI 4.78 elicited a high degree of inhibition in RAST using 10 individual sera from patients with tree pollen hypersensitivity and 2–3 RAST grades to alder pollen (fig. 6). RAST inhibition titration curves, using pooled serum, showed parallel slopes and approximately equal maximum inhibition for AI crude and pI 4.78 (results not shown).

CRIE demonstrated a moderate degree of radiostaining of one precipitate corresponding to that of pI 4.78. In addition a slight radiostaining of a second precipitate could be seen (fig. 7).

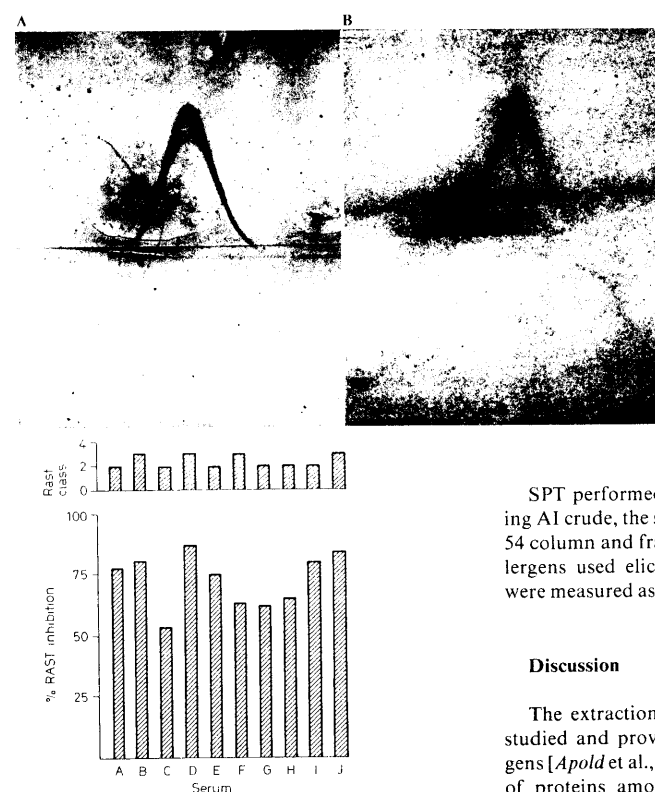


Fig. 6. RAST inhibition percentages elicited by pI 4.78 in 10 individual patient sera. The vertical bars A–J represent the ten individual sera and their respective inhibition percentages. The bars at the top indicate RAST classes to alder.

Table III. Results from SPT in duplicate in 1 alder-sensitive individual: all preparations were tested in concentrations of $1 \text{ mg} \cdot \text{ml}^{-1}$; the wheal reactions were given in mean $\frac{10-0}{2}$ mm and in % of the histamine reaction

Preparation	Mean diameter, mm	% histamine
AI crude	5.0	106
AI 1	3.5	74
AI 2	3.5	74
AI 3	3.5	74
AI 4	5.5	117
AI 5	5.2	110
AI 6	2.0	42
pI 4.78	5.5	117

Fig. 7. Crossed radioimmuno-electrophoresis. A A CIE preparation with 10 μ l pI 4.78 ($60 \mu\text{g} \cdot \text{ml}^{-1}$) in the well and 400 μ l polyspecific rabbit antiserum in the antibody gel. B The CRIE result after 4 weeks exposure on a Kodak X-Omat S film.

SPT performed in 1 alder-sensitive individual using AI crude, the six fractions obtained from the AcA-54 column and fraction pI 4.78 showed that all the allergens used elicited a positive SPT. The reactions were measured as shown in table III.

Discussion

The extraction procedure utilized was previously studied and proved adequate for birch pollen allergens [Apold et al., 1981]. The process yielded a mixture of proteins amounting to 3% relative to the total weight of pollen used. Gel filtration and subsequent isoelectric focusing fractionation allowed the isolation of the allergenic fraction pI 4.78. This fraction was apparently homogeneous as indicated by a well-isolated peak in column isoelectric focusing and one sharp band on thin-layer IEF. Further evidence on the homogeneity of pI 4.78 could be derived from the single band in HVE and HPLC, and the symmetrical single precipitate in CIE. However, through careful examination of some of the plates, a slight antigenic heterogeneity in the latter system could be observed.

Fraction pI 4.78 is a major allergen, it gave a significantly high RAST inhibition in all of the 10 reaginic sera used. The RAST inhibition titration curve obtained showed that the allergenic determinants incorporated in the crude extract were represented in fraction pI 4.78 though at a lower concentration.

Accordingly, fraction pI 4.78 was considered a suitable major representative of the *A. incana* allergens, adequate for further comparative structural studies between different tree pollen allergens. The conclusion was justified by the recovery and the moderately high uptake of ^{125}I -anti-IgE in the direct RAST system.

On comparing the amino acid composition of this protein and the recently reported pI 5.18 from birch pollen, a similar number of lysine, arginine, threonine, cysteine, methionine, leucine and phenylalanine residues could be observed [Apold et al., 1981]. Further studies should answer the question about eventual structural and immunological homologies between the two allergens.

Acknowledgements

This study was partly supported by the Research Fund of the Norwegian Asthma and Allergy Federation and the Norwegian Research Council for Science and Humanities.

The skilled technical assistance of Mrs. Gunn Nævdal is acknowledged.

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Received: April 7, 1981

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