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THE NH₂-TERMINAL AMINO ACID SEQUENCE OF THE IMMUNOCHEMICALLY PARTIAL IDENTICAL MAJOR ALLERGENS OF ALDER (*ALNUS GLUTINOSA*) *Aln g I*, BIRCH (*BETULA VERRUCOSA*) *Bet v I*, HORNBEAM (*CARPINUS BETULUS*) *Car b I* AND OAK (*QUERCUS ALBA*) *Que a I* POLLENS

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Abstract—The tree pollen major allergens (*Aln g I*), *Bet v I*, *Car b I* and *Que a I*, were purified to near homogeneity. Rabbit antibodies raised towards the major allergen *Bet v I* react with the major allergens of alder, hazel, hornbeam and oak, although with distinct variation in affinity. Immunochemically the major allergens seem to resemble one another more within than between the botanically established families of the fagale order. The physico-chemical parameters (molecular weight, pI values, amino acid composition and NH₂-terminal amino acid sequence) of the major allergens exhibit a higher degree of consistency than do the immunochemical parameters, indicating that nuances in the allergens' secondary and tertiary structures are likely to govern the immunochemical differences.

INTRODUCTION

Pollens from trees of the Fagales order (alder, birch, hazel, hornbeam and oak) are the main allergen source during spring in the temperate climate zone of the northern hemisphere (Charpin and Surinyach, 1974). There are many indications that patients with allergic symptoms during the pollination season

(February to May) often exhibit reactions towards pollens from more than one species (Dirksen and Østerballe, 1978; Nüchel Petersen *et al.*, 1988; Wihl *et al.*, 1988; Ipsen *et al.*, 1988).

Only a few of the approximately 40 distinct proteins extractable from any of the tree pollens are considered important or major allergens (Belin, 1972; Viander *et al.*, 1979; Borch *et al.*, 1980; Apold *et al.*, 1981; Florvaag *et al.*, 1982; Ipsen and Löwenstein, 1983; Ipsen *et al.*, 1985a, b; Jarolim *et al.*, 1989). Alder and birch pollen extracts each contain one major allergen (Viander *et al.*, 1979; Apold *et al.*, 1981; Florvaag *et al.*, 1982, 1988; Ipsen, 1983, 1985a; Jarolim *et al.*, 1989a, b), whereas hazel (Borch and Skålhegg, 1980; Florvaag *et al.*, 1988; Ipsen *et al.*, 1985a, b), hornbeam and oak pollen extracts each seem to contain two major allergens. Beside the major allergens a limited number of the extractable pollen proteins are considered intermediate and minor allergens (Borch and Skålhegg, 1980; Ipsen *et al.*, 1983, 1985a; Jarolim *et al.*, 1989a, b).

The patients' cross-sensitivity towards pollens from more than one distinct tree pollen species could originate from a consecutive sensitization towards different species. However, a clear immunochemical partial identity (rabbit antibodies) between the major allergens from alder, birch and hazel pollens exists (Ipsen *et al.*, 1985a), and it is possible that IgE raised against identical or partially identical epitopes on the allergens could account for the patients' cross-sensitivity.

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Abbreviations: a-Ag, rabbit anti *Alnus glutinosa* pollen extract antibodies; a-Bet v I, rabbit anti *Bet v I* antibodies; a-Bv, rabbit anti *Betula verrucosa* pollen extract antibodies; a-Ca, rabbit anti *Corylus avellana* pollen extract antibodies; a-Cb, rabbit anti *Carpinus betulus* pollen extract antibodies; a-Qa, rabbit anti *Quercus alba* pollen extract antibodies; Ag, *Alnus glutinosa*; Aln g I, *Alnus glutinosa* pollen major allergen; Bv, *Betula verrucosa*; Bet v I, *Betula verrucosa* pollen major allergen; Ca, *Corylus avellana*; Cor a I, *Corylus avellana* pollen major allergen; Car b I, *Carpinus betulus* pollen major allergen; Cb, *Carpinus betulus*; CC, chelate chromatography; CIE, crossed immunoelectrophoresis; CIEI, crossed immunoelectrophoresis with intermediate gel; CLIE, crossed line immunoelectrophoresis; CRIE, crossed radio immunoelectrophoresis; CRIEI, crossed radio immunoelectrophoresis with intermediate gel; FRIE, fused rocket immunoelectrophoresis; FRRIE, fused radio rocket immunoelectrophoresis; IEC, ion-exchange chromatography; Qa, *Quercus alba*; Que a I, *Quercus alba* pollen major allergen; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecylsulphate-poly acrylamide gel electrophoresis.

MATERIALS AND METHODS

Pollen extracts

Pollens from alder (*Alnus glutinosa*), birch (*Betula verrucosa*), hazel (*Corylus avellana*), hornbeam (*Carpinus betulus*) and oak (*Quercus alba*) each, according to the manufacturer's certificate, containing less than 2% (w/w) non-pollen debris and less than 0.5% (w/w) foreign pollens, were obtained from Allergon AB (Engelholm, Sweden).

The pollens, 50 g of each species, were extracted 1:10 (w/v) in 0.125 M NH₄HCO₃, 0.015 M NaN₃, pH 8.3 for 24 hr at 5°C. Particulate matter was removed by centrifugation (35,000 g, 1 hr, 15°C) and the supernatants were dialysed three times against 0.005 M NH₄CO₃, pH 8.3, 5°C each and lyophilized. In general, 1–1.5 g lyophilized material containing approx. 80% (w/w) protein (Lowry *et al.*, 1951; Ipsen *et al.*, 1985a) were obtained. The extracts and working solutions (10 mg extract per ml of 0.030 M NH₄HCO₃, 0.086 M NaCl, pH 8.3, 50% (v/v) glycerol) of the extracts were stored at –20°C.

Rabbit antibodies

Rabbit anti alder (a-Ag), anti birch (a-Bv), anti hazel (a-Ca) and anti oak (a-Qa) pollen extract antibodies were raised by immunizing rabbits bi-weekly with 500 µg pollen extract in 100 µl Freund's incomplete adjuvant; each of the extracts was injected into three rabbits. Antibodies reacting with the purified major allergens (a-Bet v I, a-Car b I and a-Que a I) were raised similarly, using 5–10 µg allergen per injection. The immunoglobulins were isolated and concentrated (six times) essentially as described by Harboe and Ingild (1983). The immunoglobulins obtained from three animals and several (at least ten) bi-weekly bleedings were pooled and stored at 5°C.

Patient sera

Serum samples were obtained from non-treated patients (N = 38) allergic to tree pollens. The selection of the patients is described in detail elsewhere (Nüchel Petersen *et al.*, 1988). A serum pool was made by mixing equal volumes of each of the sera. The sera were stored at –20°C.

Immunoelectrophoretic methods

Crossed immunoelectrophoresis (CIE) (Høiby and Axelsson, 1983) was performed in 1% (w/v) agarose (type HSA, Litex, Denmark) in 0.073 M Tris, 0.024 M barbital, 0.0006 M calcium lactate, pH 8.6, 10–15°C, moulded on 5 × 7 cm glass plates. First-dimension electrophoresis: 40 µg of the crude pollen extracts were electrophoresed for 25 min, 10 V/cm at 10–15°C. Second-dimension electrophoresis: electrophoresis for 16 hr perpendicular to the first dimension into antibody-containing anodic and cathodic gels using appropriate amounts of antibodies (antibody, volume in anodic gel, volume in cathodic gel: a-Ag,

8.7 µl/cm², 6.7 µl/cm²; a-Bv, 20 µl/cm², 13.3 µl/cm²; a-Ca, 10 µl/cm², 8 µl/cm²; a-Qa, 13.3 µl/cm², 6.7 µl/cm²). The CIE plates were stained with Coomassie Brilliant Blue as described by Svendsen *et al.* (1983).

Crossed radio immunoelectrophoresis (CRIE) (Weeke *et al.*, 1983) was performed using cold-dried CIE plates incubated with patient serum pool diluted 1:32 (v/v) in 0.067 M phosphate buffer, pH 7.0 for 24 hr at 20°C, followed by incubation with ¹²⁵I-anti-IgE (Pharmacia, Sweden) in the same buffer containing 0.3% (w/v) bovine serum albumin, 0.154 M NaCl, 0.015 M NaN₃, 0.027 M EDTA for 24 hr at 20°C. The plates were washed three times in 0.154 M NaCl, 1% (v/v) Tween 20, dried and subjected to autoradiography on Kodak XL-1 X-ray film at –20°C for 1, 4 and 10 days.

Crossed immunoelectrophoresis with antibodies in the intermediate gel (CIEI) was performed as described by Axelsen (1983). CIEI experiments were performed with all the pollen extracts and their corresponding antibodies in the anodic and cathodic gels, using a-Bet v I in the intermediate gel. Electrophoretic conditions were the same as those described for CIE.

Tandem-crossed immunoelectrophoresis (TCIE) was performed essentially as described by Krøll (1983); utilizing monospecific rabbit antibodies (a-Bet v I) in the anodic and cathodic antibody containing gels. Electrophoretic conditions were identical to those described for CIE.

Fused rocket immunoelectrophoresis (FRIE) was performed essentially as described by Harboe and Ingild (1983) and Harboe and Svendsen (1983). The samples applied (10–15 µl) in the wells diffused at 10–15°C 1 hr before the electrophoresis (2 V/cm), 16 hr, 10–15°C into the antibody containing gel (the amounts of antibodies and the buffer were the same as the CIE). Fused radio rocket immunoelectrophoresis (FRRIE) was performed as CRIE using cold-dried FRIE plates.

Size exclusion, ion-exchange and chelate-chromatography

Size exclusion chromatography (SEC) of 150 mg (dry wt) of the pollen extracts (Ag, Bv, Cb and Qa) was performed on a G-75 Superfine (Pharmacia, Sweden) 90 cm × 2 cm² (K15/100 Pharmacia, Sweden) column, equilibrated with 0.125 M NH₄HCO₃, pH 8.3. The fractions containing the major allergen(s) (assayed by FRIE and FRRIE) were pooled and lyophilized. Typically 15–30 mg dry wt material was obtained.

Ion-exchange chromatography (IEC) of approx. 20 mg SEC fractionated material was performed on CM-Sepharose and DEAE-Sepharose (Pharmacia) columns connected in series, each containing 2 ml of ion-exchange matrix and equilibrated with 0.0095 M NaH₂PO₄/0.0134 M Na₂HPO₄ phosphate buffer I = 0.05, pH 7.0. The proteins not retained by

the columns (assayed by FRIE and FRRIE) were pooled and subjected to chelate chromatography.

Chelate chromatography (CC) was performed on 5 ml Chelating-Sepharose (Pharmacia) charged with Cu²⁺ and equilibrated with phosphate buffer I = 0.05, pH 7.0. The pooled IEC fractions were applied on the column without further processing. The proteins were eluted from the column by a stepwise change of pH (I: 0.0095 M NaH₂PO₄/0.0134 M Na₂HPO₄ phosphate buffer I = 0.05, pH 7.0; II: 0.0351 M NaH₂PO₄/0.0050 M Na₂HPO₄ phosphate buffer I = 0.05, pH 6.0; III: 0.050 M CH₃COONa/CH₃COOH acetate buffer I = 0.05, pH 4.0; IV: 0.050 M EDTA, 0.4 M NaCl, V: 0.0095 M NaH₂PO₄/0.0134 M Na₂HPO₄ phosphate buffer I = 0.05, pH 7.0). The fractions containing the purified allergen (assayed by FRIE and FRRIE) were pooled and dialysed extensively against 0.005 M NH₄HCO₃ and lyophilized.

SDS-poly acrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE and immunoblotting experiments were performed with the crude extracts and the purified allergens as described (Ipsen *et al.*, 1985b). The pollen proteins blotted onto nitro cellulose (NC) or Immobilon (PVDF) membranes (Millipore) were visualized with colloidal gold (Moeremans *et al.*, 1985), Coomassie Brilliant Blue (Matsudaira, 1987) or with alkaline phosphatase conjugated swine anti-rabbit Ig (D306 DAKO, Denmark) after incubation with rabbit antibodies (a-Bet v I).

Amino acid composition and sequence analysis

Amino acid analysis of *Aln g I*, *Bet v I* and *Car b I* was performed by hydrolysing 0.1–0.35 nmol protein in 6.0 N HCl, 0.05% phenol in evacuated, sealed glass ampules for 18, 44 or 68 hr (Blackburn, 1978; Welinder, 1988). The individual amino acids were identified and quantified on a Waters HPLC system, using post-column derivatization with *o*-phthalaldehyde (Welinder, 1988).

NH₂-terminal amino acid sequence analysis was performed on a gas-phase microsequencer (Applied Biosystems Inc. model 470A) (Hewick *et al.*, 1981), all reagents and solvents were obtained from Applied Biosystems Inc. (Foster City, CA). Each analysis was carried out using 2–4 nmol of protein. The released phenylthiohydantoin (PTH) amino acid derivatives were identified by reversed phase HPLC (Waters) chromatography (Spherisorb ODS II 125 × 4.6 mm) at 254 and 313 nm. Separation of the 20 PTH-derivatives and sequencer artefacts was achieved eluting the column with a gradient of 16% (v/v) methanol to 38% (v/v) ethanol in 0.02–0.05 M CH₃COONH₄, pH 4.2. Serine and threonine were partially detected as their dehydro forms which absorb at 313 nm. The average repetitive yields of the proteins sequenced were 95–96%.

Hydropathy, secondary structure estimation and homology searches

The hydropathy profiles of the NH₂-terminal sequences were calculated using the method of Kyte and Doolittle (1982), a moving average of 5–11 residues was utilized. The secondary structure propensities were estimated according to the semi-empirical rules provided by Chou and Fasman (1978). Homology searches were performed with Genepro version 4.2 (Riverside Scientific Enterprises, Seattle, WA), using PIR release no. 20.

RESULTS

The pollen extracts each contain approx. 40 apparently distinct antigens as judged by CIE (Fig. 1). Of these antigens only a limited number bind IgE in CRIE. Alder and birch pollen extracts each appear to contain one major allergen, whereas hazel, hornbeam and oak pollen extracts appear to contain two major allergens (Fig. 1A). The major allergens are all anodic migrating proteins at pH 8.6.

SDS-PAGE of the different pollen extracts, stained by Coomassie Brilliant Blue (Fig. 2A) or colloidal gold (Fig. 2B), visualizes a number of bands which correspond to the number of antigens detected by CIE. a-Bet v I visualized bands in the 17–20 kDa range in all the extracts (Fig. 2C). The allergens, except *Que a I*, seem to consist of isoforms with slightly variable (±1.0 kDa) apparent molecular weights (Fig. 2C).

The major allergens from the different pollen species were purified by the same combination of size exclusion chromatography (SEC), ion-exchange chromatography (IEC) and chelate chromatography (CC). FRIE and FRRIE were used to identify the antigens and the allergens after each step in the purification procedure (Fig. 3A SEC; Fig. 3B IEC; Fig. 3C CC), illustrated here by the isolation of *Que a I*. All isolated allergens, except *Aln g I*, appeared to be homogenous when assayed by CIE using antibodies raised against the crude extracts (not shown); however Coomassie Brilliant Blue stained electrophoretic blots (PVDF membrane) of *Bet v I* and *Car b I* (Fig. 2A, lanes 10 and 14) apart from a number of bands in the 17–20 kDa region revealed extremely weak band(s) in the 45 kDa region. *Que a I* (Fig. 2A, lane 16) seems to be homogenous since only one band is detectable in the 17–20 kDa region. The gold-stained electrophoretic blots (nitrocellulose membrane) visualized a number of additional weakly stained bands (Fig. 2B, lanes 6, 8, 10) in the purified allergen preparations in the region below 17 kDa and around 30 kDa, the amount of which obviously are below the detection limit of the Coomassie staining procedure. The allergens visualized by a-Bet v I (Fig. 2C) all appear in the 17–20 kDa region. Typical yields of each major allergen were approximately 1.0 mg dry wt material.

Most of the antigens in any of the tree pollen extracts seem to have an identical or partial identical

antigen counterpart in the extracts of the related species when assayed by CIE (not shown) and SDS-PAGE immunoblotting incubated with the antibodies raised towards the crude extracts (not shown). The major allergens clearly exhibit a partial immunochemical identity with one another (Figs 1 and 4). Antibodies raised towards the purified major allergens, e.g. *a-Bet v I*, react in CIEI and CRIEI with the precipitates of the major allergens of the related species (Fig. 1C,D). In fact, the monospecific antibodies *a-Bet v I* seem to react with the two major allergens of both *Ca* and *Cb*, indicating that the major allergens from these pollens (*Ca* or *Cb*) may

share similar epitopes, although the proteins seem to be distinct when analysed with antibodies raised against the crude extracts (Fig. 1B,C). The TCIE experiments (Fig. 4) indicate that the major allergens from the botanical established families (*Ag/Bv*, *Ca/Cb*, *Qa/(Fs)*) seem to be more alike than the allergens from different families, since the precipitates of the major allergens from different botanically families exhibit pronounced "spurs" (Fig. 4G-N), whereas the "spurs" are weak between major allergens from the same botanical family (Fig. 4E,F).

The amino acid composition of *Aln g I*, *Bet v I* and *Car b I* are strongly correlated (Table 1). None of

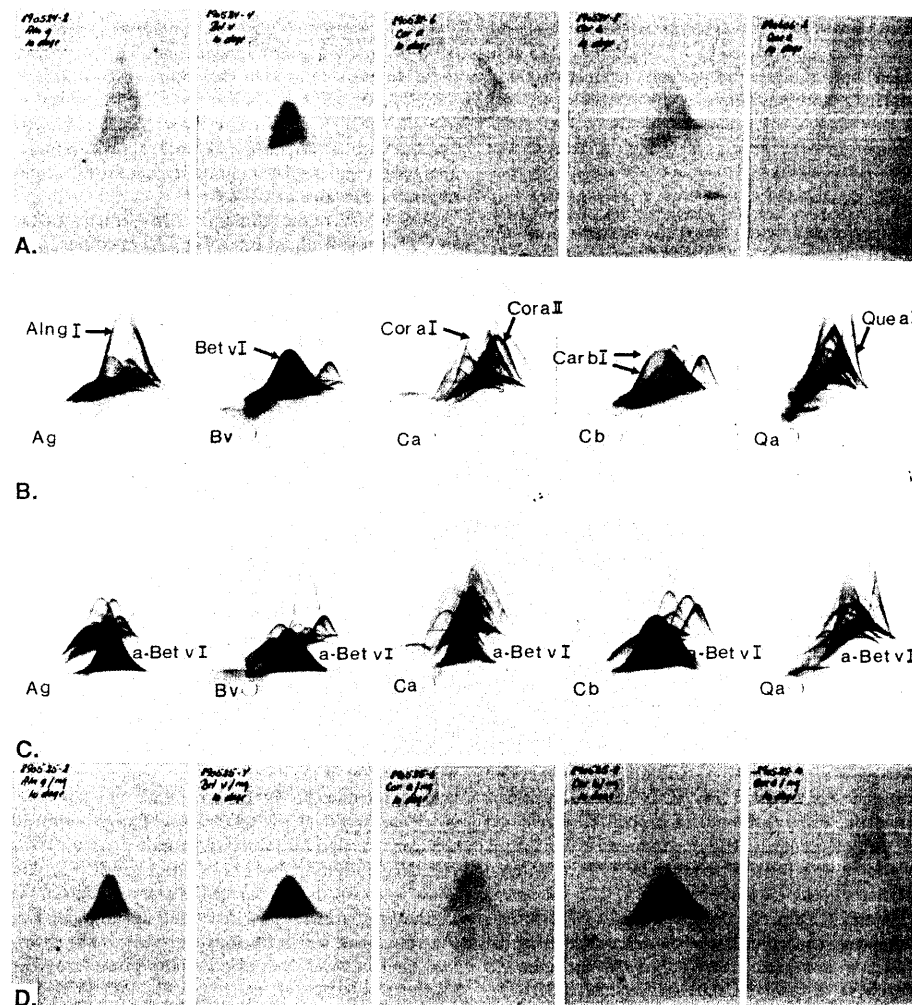


Fig. 1. CRIE autoradiographs (A), CIE (B), CIEI (C) and CRIEI autoradiographs of the crude pollen extracts of alder (*Ag*), birch (*Bv*), hazel (*Ca*), hornbeam (*Cb*) and oak (*Qa*). CIEI (C) was performed with *a-Bet v I* included in the intermediate gel. The autoradiographs (10 days) were acquired with a serum pool from allergic individuals.

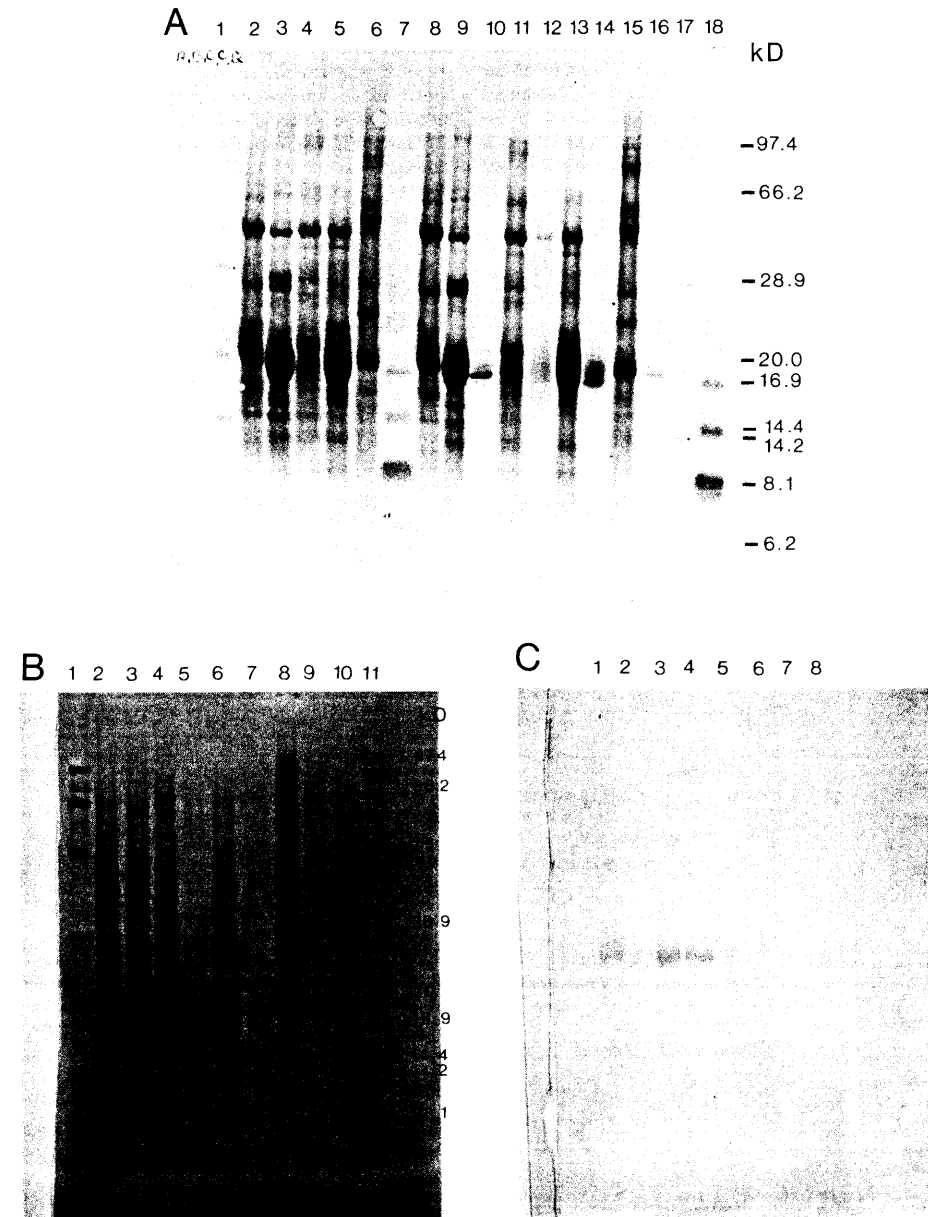


Fig. 2. Electrophoretograms of crude pollen extracts and purified allergens separated by SDS-PAGE. (A) PVDF membrane stained with Coomassie Brilliant Blue; lanes 1,17: mol. wt standards (Bio-Rad) 14.2–97.4 kDa; lanes 7,18: mol. wt standards (LKB) 4.1–16.9 kDa; lanes 2,8: 75 μ g *Ag*; lanes 3,9: 75 μ g *Bv*; lanes 4,11: 75 μ g *Ca*; lanes 5,13: 75 μ g *Cb*; lanes 6,15: 75 μ g *Qa*; lane 10: 1.0 μ g *Bet v I*; lane 12: *Cor a I*; lane 14: 1.0 μ g *Car b I*; lane 16: 0.5 μ g *Que a I*. (B) Nitrocellulose membrane stained with colloidal gold; lanes 1,11: mol. wt. standards (Bio-rad) 14.2–97.4 kDa; lane 10: mol. wt standards (LKB) 4.1–16.9 kDa; lane 2: 30 μ g *Ag*; lane 3: 30 μ g *Ca*; lane 4: 30 μ g *Bv*; lane 5: 0.3 μ g *Bet v I*; lane 6: 30 μ g *Cb*; lane 7: 0.3 μ g *Car b I*; lane 8: 30 μ g *Qa*; lane 9: 0.1 μ g *Que a I*. (C) Nitrocellulose membranes incubated with *a-Bet v I* (diluted 1:500,000 lanes 1–6 or 1:50,000 lanes 7–8) and developed with alkaline phosphatase swine anti-rabbit IgG conjugate. Lane 1: 30 μ g *Ag*; lane 2: 30 μ g *Ca*; lane 3: 30 μ g *Bv*; lane 4: 1.0 μ g *Bet v I*; lane 5: 30 μ g *Cb*; lane 6: 1.0 μ g *Car b I*; lane 7: 30 μ g *Qa*; lane 8: 0.5 μ g *Que a I*.

these allergens seem to contain cysteine residue(s), and no "unusual" amino acids (e.g. methylated histidine, hydroxy-proline etc.) were detected in any of the molecules. Tryptophane residues were not determined.

The NH₂-terminal amino acid sequences of the major allergens *Aln g* I, *Car b* I and *Que a* I were determined at least twice. The sequence of *Bet v* I and

Car b I were determined three times using two different batches of purified material. The *Aln g* I sequence was determined using a preparation which contained a contaminating protein, however no ambiguities were detected and the sequence obtained correlates with those for the other allergens. This indicates that the contaminating protein was either

present in a very low concentration or that its NH₂-terminal amino acid was blocked. Table 2 shows the aligned NH₂-terminal amino acid sequences of *Aln g* I, *Bet v* I, *Cor a* I (Borch, 1979; Borch *et al.*, 1987), *Car b* I and *Que a* I. The NH₂-terminal amino acid sequence of the major allergens from the birch and hazel families exhibit

a high degree of sequence identity, 79–87%, whereas only approximately half of the *Que a* I residues match any of the other sequences. The NH₂-terminal sequence of *Bet v* I is in complete accordance with the amino acid sequence obtained from the translated cDNA sequence (Breiteneder *et al.*, 1989).

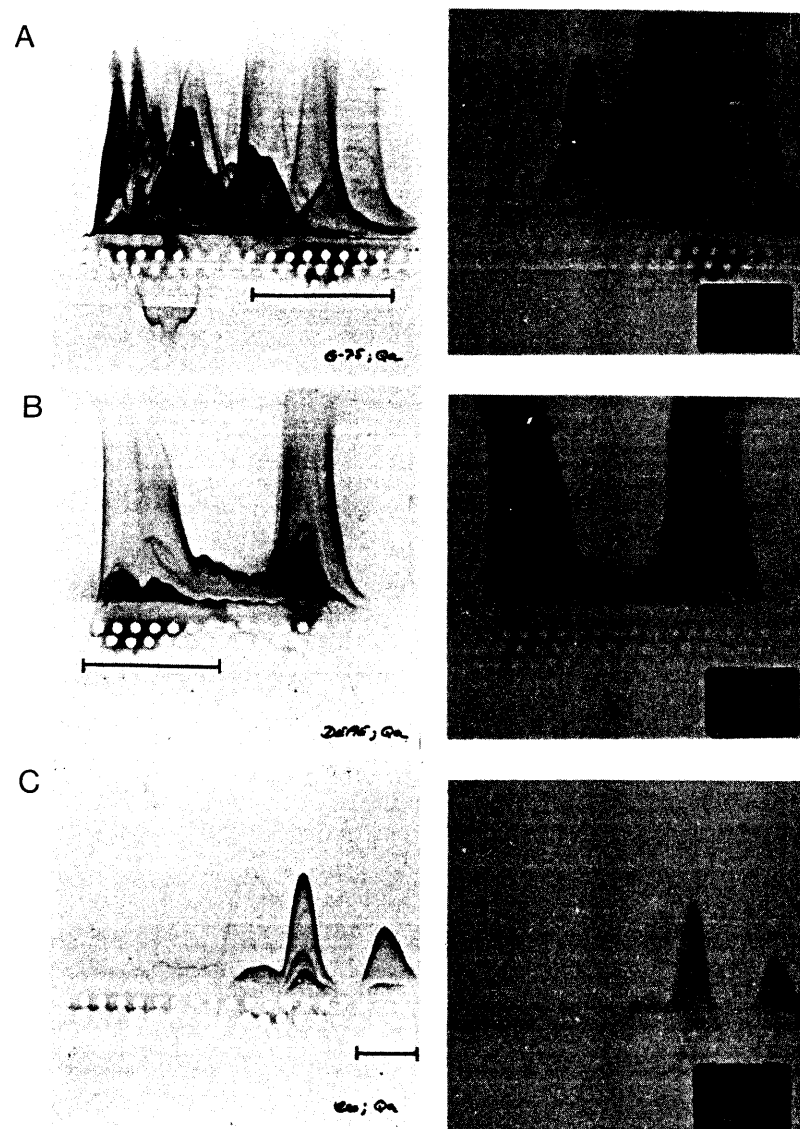


Fig. 3. Purification of *Que a* I from a crude oak pollen extract. (A) FRIE and FRRIE of the fractions (5 μ l of each) obtained after SEC of 150 mg *Qa*. (B) FRIE and FRRIE of the fractions (5 μ l of each) obtained after IEC of approximately 30 mg SEC fractionated material. (C) FRIE and FRRIE of the fractions (5 μ l of each) obtained after CC. The fractions pooled for further processing after each chromatographic step are indicated by a bar.

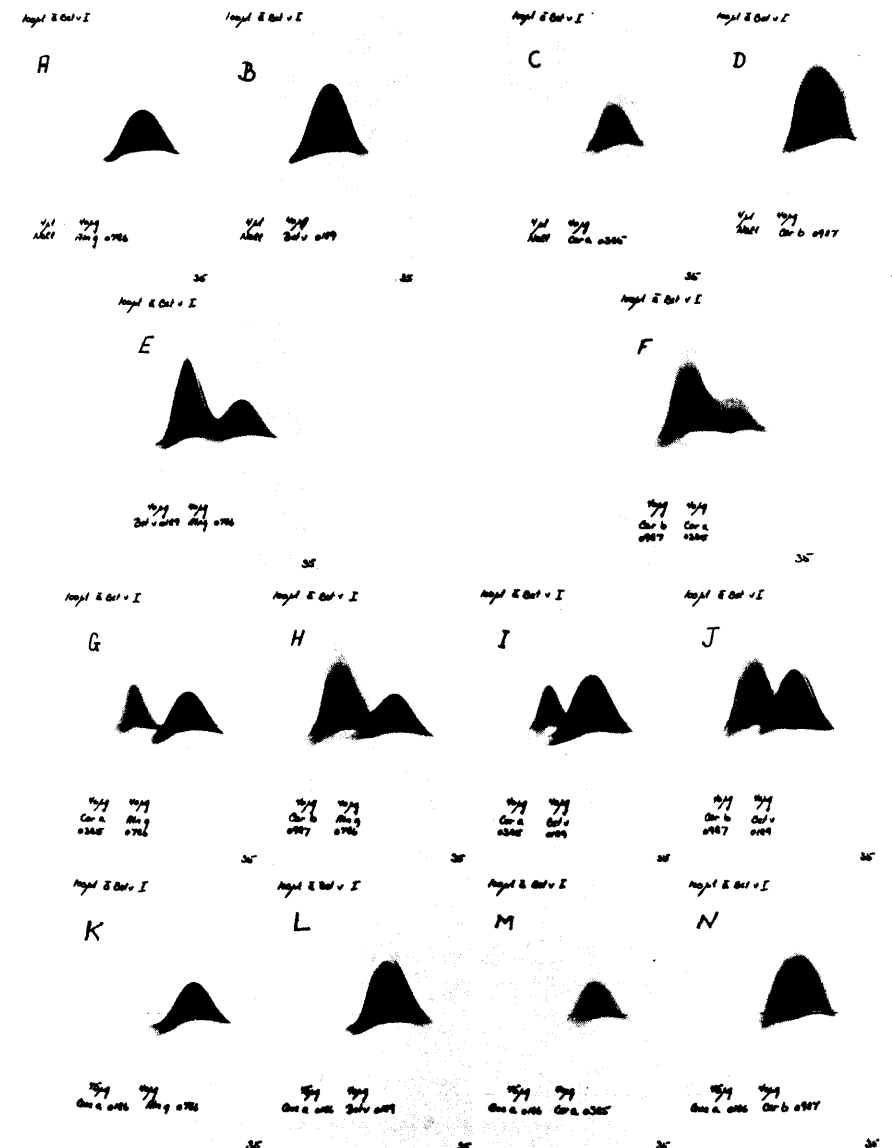


Fig. 4. Tandem-crossed immunoelectrophoresis (TCIE) of the crude pollen extracts precipitated with a-*Bet v* I. (A) NaCl vs Ag. (B) NaCl vs Bv. (C) NaCl vs Ca. (D) NaCl vs Cb. (E) Ag vs Bv. (F) Ca vs Cb. (G) Ca vs Ag. (H) Cb vs Ag. (I) Ca vs Bv. (J) Cb vs Bv. (K) *Qa* vs Ag. (L) *Qa* vs Bv. (M) *Qa* vs Ca. (N) *Qa* vs Cb. 40 μ g of each extract was used in each TCIE experiment.

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