

Antigens and Allergens in Birch Pollen Extract

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More than 70% of the total allergenic activity of a birch pollen (BP) extract was detected within the first 30 min of extraction. Fractionation of the BP extract by gel filtration and analysis of the eluted antigens by a fused rocket immunoelectrophoresis revealed at least three antigens with molecular weights of about 29 000, and 17 000–10 000, corresponding to antigens Nos. 2, and 7–8, respectively, in crossed-immunoelectrophoresis (CIE) and in crossed-radio-immuno-electrophoresis (CRIE). Gel isoelectrofocusing of the pooled allergenic fractions revealed two major protein bands with pI's around 5.6 and 5.7, probably corresponding to antigens Nos. 7–8 and No. 2, respectively. Antigens Nos. 7–8 were thermoresistant, while antigen No. 2 was thermolabile.

The allergenic activity was determined by prick skin testing and by the RAST inhibition method. More than 90% of the allergenic activity in the fractions was located in the protein peak C (mol. wt. 10 000–17 000) containing antigens 7–8. About 30% of the total allergenic activity of the extract (1:10 w/v) was recovered in the peak C fractions, and only less than 0.5% outside these fractions. Higher allergenic activity was obtained for the peak B fractions (mol. wt. 29 000) by skin prick testing than by the RAST. Peak B contained allergens (antigen 2) distinct from those of peak C by the CRIE and by the RAST. The allergenic material in the low molecular weight fractions of peak D (mol. wt. 2000–5000) was allergenically similar to that of peak C in the RAST. Only weak and even negative skin reactions were observed with the peak D fractions in allergic subjects.

Key words: allergens; antigens; characterization; immunoelectrophoresis; immunoenzyme technics; isoelectric focusing; isolation; pollen, birch; radioallergosorbent test.

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Several antigens and allergens have been recently fractionated and partially characterized from birch pollen extract by using gel chromatography (5, 6, 8) or by isoelectrofocusing (10). The antigens in the fractions have been identified either by crossed immunoelectrophoresis (CIE; 6) or no attempts to identification have been made (8, 10). The allergenic activity of the separated fractions has been determined by skin testing in allergic subjects (5, 6) or by the paper-disc radioimmunosorbent allergen assay (8, 9, 10). According to Belin's studies

the main allergen(s) of birch pollen is thermostable, and of protein nature with a molecular weight of about 20 000 (5, 6, 7). However, no further characterization of either the major or the minor allergens in birch pollen has been carried out.

In this work we have fractionated birch pollen extract by gel filtration and characterized the major and the minor allergens, and antigens in the fractions by skin prick testing, RAST inhibition, CIE, crossed-radio-immunoelectrophoresis (CRIE), and by gel isoelectrofocusing.

MATERIAL AND METHODS

Pollen extracts

Pollen extracts were prepared from *Betula pendula* (*verrucosa*) pollen, lot 464, and from *Alnus glutinosa* pollen, batch 391, kindly supplied by Dome Laboratories, England. The purity of the pollen was 99.8% by microscopical examination. A 1:10 (w/v) extract was prepared by extraction of 1 g of non-defatted pollen in 10 ml of phosphate-buffered saline (equal parts of 0.15 M phosphate buffer, pH 7.2 and of 0.15 M saline) under mild shaking at +4°C for 24 h. After centrifugation at 4°C for 20 min at 22 000 g, the supernatant was sterilized by a 0.45 µm filter, and stored in small portions (1:10 w/v) at -20°C until used. The protein concentration of the birch pollen extract was 7.9 mg/ml, and that of the alder pollen extract 9.0 mg/ml.

The allergenic activity in HEP which is the histamine equivalent in prick testing (2), was used as a biological reference system for the extracts. An allergen extract has an allergen activity of 1 HEP, when inducing a weal of the same size as a histamine HCl solution 1 mg/ml. The 1 HEP concentration for the birch pollen extract (BP extract) was about 20 µg/ml of protein, i.e., about 1/4000 w/v dilution of the extract, and for the alder pollen extract 1 HEP corresponded to about 30 µg/ml (about 1/3000 w/v).

Antiserum

Antibodies against birch pollen were raised by immunization of two rabbits with 0.5 mg of the extract in equal volume of Freund's complete adjuvant (Difco) every 3 to 6 weeks subcutaneously in the subscapular region. After six injections an Ouchterlony double diffusion analysis revealed two clear precipitation lines against birch pollen extract with a titre of 1:32 of the sera. The immunization was completed with one intramuscular injection without adjuvant and the rabbits were exsanguinated 2 weeks after

the last injection. The antiserum was stored in aliquots at -20°C until used.

Gel filtration

Fractionation of the birch pollen extract by gel filtration was performed at +4°C on a column (2.5 cm x 93 cm) of preswollen Sephadex G-100 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) with 10 mM phosphate buffer pH 7.5 containing 0.15 M NaCl in elution. The sample of 5 ml (1:10 w/v birch pollen extract) was applied. A 15 ml/h flow up to down was secured by a peristaltic pump. Fractions of 4.0 ml were collected. Results of three separate runs were identical. The molecular weights were calculated according to Whitaker (26). As a reference, bovine thyroglobulin (mol. wt. 660 000), bovine serum albumin (mol. wt. 69 000) and horse heart cytochrome C (mol. wt. 12 400), all from Sigma, were run through the same column.

Electrophoretic methods

Polyacrylamide gel disc electrophoresis (PAGE) was performed as described by Ornstein (23) and Davis (14). 75 µl of 1:10 (w/v) extract or of pooled Sephadex G-100 fractions were mixed with 75 µl of 40% saccharose and the mixture was applied on the gel in glass tubes (diameter 6 mm, length 70 mm). The tubes contained two gels with different pore size: 225 µl of the large-pore gel (2.5% w/v polyacrylamide in Tris-HCl buffer, pH 6.7) and below that 1.5 ml of the small-pore gel (8% w/v polyacrylamide in Tris-HCl buffer, pH 8.9). The buffer basins contained Tris-glycine buffer, pH 8.3, diluted 1:10 in distilled water. Thus a gradient was formed between the basins and the gel. The electrophoresis was performed to the anodal direction at +0 to +2°C for 1 h at 40 mA and 225-300 V/8 gel tubes. Under these conditions all proteins with isoelectric point less than pH of the Tris-glycine buffer (pH 8.3) moved to the anodal direction. After the run the gels were carefully removed from the

tubes and divided into two longitudinal sections. One of the sections was stained with 0.5% Coomassie Brilliant blue R, and the other half was subjected to crossed immunoelectrophoresis.

Reagents used for the preparation of the stock solutions: Acrylamide, Fluka; ammonium persulphate, Merck; glycine (NH_4 -free), Merck; 1 N HCl; *N*, *N*'-methylenebisacrylamide (BIS), Eastman; riboflavin, Biochemica Roche; saccharose, BDH; *N*, *N*', *N*'-tetramethylethylenediamine (TEMED), Eastman; Tris (hydroxymethyl) aminomethane (TRIS), Sigma.

Immunoelectrophoretic methods

Equipment, reagents and methods were essentially as described by Axelsen et al. (4). LKB type 3290 power supply was used. Buffer: 0.075 M barbital buffer, pH 8.6. Gel: 1% (w/v) agarose gel (Agarose A 37, Industrie Biologique Francaise, Geunevilliers) dissolved in the buffer. Unless otherwise indicated, first and second dimension electrophoreses were performed in a 0.15-cm-thick gel at $8\text{--}10\text{ V cm}^{-1}$ for 60 min and in a 0.12-cm-thick gel at $1\text{--}3\text{ V cm}^{-1}$ for 18 h at 15°C , respectively. Glass plates $8 \times 8\text{ cm}$ were used.

Rocket immunoelectrophoresis was used for the quantitation of the thermostable (TS) antigens in the fractions and in the whole extracts. 5 μl samples of heat-treated (15°C , 100°C) extracts diluted or concentrated to correspond to about $100\text{--}200\text{ }\mu\text{g/ml}$ of protein were applied into the wells. Antibody: 2% rabbit anti-birch pollen serum in gel. Electrophoresis was carried out at pH 8.6 with 2 V cm^{-1} in the gel at 15°C overnight (18 h). Standard dilutions of heat-treated whole BP extract (400, 200, 100, 50 $\mu\text{g/ml}$) were used as a reference.

Fused rocket immunoelectrophoresis was used for a semiquantitative identification of the eluted antigens in the fractions after gel filtration. This method is a modified version of the

rocket immunoelectrophoresis. 5 μl samples of the fractions were applied into the wells punched into an antibody-free gel and allowed to diffuse into the gel for 30 min at room temperature. Then electrophoresis was performed into the anodal antibody-containing gel as described above.

Crossed immunoelectrophoresis was performed by using two modifications: separation of the different proteins in the first dimension electrophoresis was achieved either by an agarose gel electrophoresis, or by PAGE (as described above). The sizes of the samples were 5 μl ($40\text{--}400\text{ }\mu\text{g}$ of protein) and 75 μl ($\sim 600\text{ }\mu\text{g}$) in the agarose and polyacrylamide gel electrophoresis, respectively. The second dimension electrophoresis (18 h) was performed in a 0.12-cm-thick gel containing 1–4% of rabbit antibodies to birch pollen, which was poured on an $8 \times 8\text{ cm}$ glass plate with a strip of the first dimension electrophoresis gel on one edge.

After the second electrophoresis the gels were pressed, washed, dried in hot air and stained with Coomassie Brilliant blue R, or left unstained at $+4^\circ\text{C}$ to be used in crossed-radio-immuno-electrophoresis. Attempts to show any antigens moving to the cathodal direction by different placing of the sample were unsuccessful.

Crossed-radio-immuno-electrophoresis (CRIE) was performed as described by Weeke et al. (25). After CIE the unstained gels were incubated with a 1/15 dilution of a patient serum (0.5 ml of serum in 7 ml of phosphate buffer, pH 7.5) overnight at room temperature. After washing the gels 4 times with 0.1 M sodium chloride for 10 min, rabbit ^{125}I -anti-IgE (supplied by Pharmacia AB, Uppsala, Sweden) or sheep ^{125}I -anti-IgG (prepared in this laboratory (30) diluted in incubation buffer (phosphate buffer 0.05 M pH 7.5 containing 0.5% bovine serum albumin, 0.9% sodium chloride, 0.1% sodium azide and 0.1% EDTA) were added in dilutions corresponding to about $30\,000\text{ cpm ml}^{-1}$, and

allowed to react overnight. After repeated washings and drying in hot air the gels were placed on a Kodak RP/S X-Omat (RP/S-14) film in a light-proof box. The exposure time varied from 1 week to several weeks.

No attempts were made to quantitate the amount of antibodies bound to the immunoprecipitates. The specificity of the radiostaining was shown by using sera from atopic and non-atopic subjects with high or negligible amounts of IgE or IgG antibodies to birch pollen antigens by the RAST or by the ELISA (28).

Enzyme-immuno assay (EIA)

An enzyme-immuno assay (EIA) was developed for a semiquantitative detection of the eluted antigens after gel filtration. The assay was performed in polystyrene cuvettes with nine wells constructed for a semi-automatic FP-9 analyzer (Finnpipette-Labsystems, Helsinki, Finland) with a Hewlett-Packard 9815A calculator for recording of the results. The wells were coated with antigens by incubation with 200 μ l of the fractions at 37°C overnight. Thereafter the cuvettes were incubated with PBS containing 5% of normal sheep serum (PBS-NSS) at 37°C for 2 h and washed with PBS. The antigens were then detected by incubation with 150 μ l of the rabbit anti-BP serum (diluted 1:100 in PBS-NSS) at +4°C overnight. After washing (3x) with 0.9% NaCl containing 0.05% of Tween 20 (NaCl-Tween), a second incubation with 150 μ l of alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (Orion Diagnostica, Helsinki, Finland) was performed at +4°C overnight, followed by washing (3x) with NaCl-Tween. The enzymatic activity in the cuvettes was determined by adding 150 μ l of *p*-nitrophenylphosphate (2 mg/ml, Sigma). After incubation at 37°C for 30 min, the reaction was stopped by adding 250 μ l of 1 M sodium hydroxide, and the absorbance was measured at 405 nm (OD_{405}). The specificity of the EIA was shown by incubation with normal rabbit serum in place of the

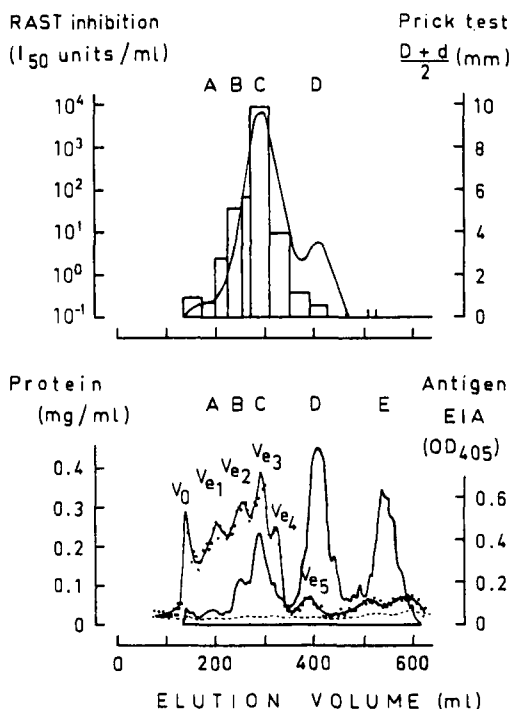


Fig. 1. Sephadex G-100 gel filtration of birch pollen extract (5 ml, 10^{-1} w/v). Protein concentrations of the fractions (peaks A, B, C, D and E), are shown by solid line (—). Binding of rabbit antibodies to the different fractions (EIA, absorbancy at 405 nm = OD_{405} ; peaks V_0 , V_1 , V_2 , V_3 , V_4 and V_5) is shown by dots connected by a line (—). Background binding of antibodies from a normal rabbit serum is indicated by dotted line (---).

The upper part of the Fig. shows results of prick tests (open columns, mean of 10 individuals) and of RAST inhibition (solid line) using pooled fractions corresponding to the various protein peaks. A mixture of sera from 20 allergic subjects was used for the RAST inhibition.

immune serum (Fig. 1), and with absorption experiments using allergen-coated cuvettes or those coated with NSS for absorption. The detection limit for the EIA using the rabbit anti-BP-serum (1:100 dilution) was about 100 ng of protein of the BP extract or of the pooled fractions from gel filtration on Sephadex G-100.

Isoelectric focusing

Analytical thin-layer gel electrofocusing in polyacrylamide gel was performed by using

an LKB 2117 Multiphor apparatus and Ampholine solutions as carrier ampholytes (LKB-Produkter AB, Bromma, Sweden). The procedure has been described elsewhere (15). For details see Fig. 3.

Skin tests

Duplicate skin prick tests were performed with Sephadex G-100 fractions of the birch pollen extract in 10 pollen sensitive subjects and in non-atopic controls.

The allergenic activity of the fractions based on skin prick tests was expressed as per cent of that of the whole extract (1:10 w/v) in each individual (5). An approximately linear relationship between the mean weal diameter (%) and the $^{10}\log$ allergen dilution was found when testing 10 individuals with five successive dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} w/v) of the whole extract, although a slight upward deviation was found with the highest dilutions. The corresponding allergenic activities (%) were 100, 10, 1, 0.1 and 0.01, respectively.

RAST (Radio-Allergo-Sorbent-Test)

Birch pollen extract or its pooled fractions in optimal concentrations (to achieve a plateau) were coupled to paper discs activated by cyanogen bromide (BrCN) exactly as described by Ceska et al. (9). These allergen-discs were used in the RAST to determine the inhibition by allergens. In brief: after incubation in birch pollen allergic serum the paper-birch-IgE complex was determined by addition of ^{125}I -labelled rabbit anti-human IgE (kindly supplied by Pharmacia AB, Uppsala, Sweden) and subsequent counting.

RAST inhibition experiments were carried out essentially as described by Yman et al. (32), using the allergen-discs mentioned above. To measure the inhibitory capacity of the extract, serial dilutions of the extract in phosphate buffer (PBS) were allowed to react with

a mixture of 20 allergic patient sera (20 μl of serum + 30 μl of allergen dilution) first 3 h at room temperature, and then overnight at $+4^\circ\text{C}$. These mixtures were then used in duplicate for RAST determinations on birch pollen extract coupled to paper discs. 20 μl of serum + 30 μl of PBS was used to obtain the uninhibited RAST result with allergen discs ($=\text{cpm}_{100}$), or the background RAST result with paper discs without antigen ($=\text{cpm}_0$).

The allergenic activity was based on the counts obtained in RAST. The inhibition % was calculated according to the following formula:

$$\text{Inhibition\%} = \frac{(\text{cpm}_{100} - \text{cpm}_0) - (\text{cpm}_x - \text{cpm}_0)}{\text{cpm}_{100} - \text{cpm}_0} \times 100\%$$

cpm_{100} = uninhibited

cpm_0 = background counts bound to discs without antigen

cpm_x = inhibited

The inhibition curves were plotted on a semilog paper, and the amount of allergen (ng of protein) or the allergen concentration producing a 50% inhibition (I_{50}) was read. This allergen concentration contained 1 I_{50} unit/ml. (See Fig. 1 and Fig. 5).

Protein determinations

Protein contents of the extracts were determined according to Lowry et al. (19) with bovine serum albumin as reference.

The amount of peptides was measured by using the Folin-Ciocalteu method and the ninhydrin method after removing TCA precipitable proteins as described by Fräki (16).

RESULTS

Gel filtration of birch pollen extract

Gel filtration on Sephadex G-100 column of birch pollen extract (1:10 w/v) gave five protein peaks (A, B, C, D and E; Fig. 1). The results of three separate runs were identical.

The molecular weights of peak A, B and C were found to be in reference runs about 79 000, 29 000 and 17 000, respectively. The most prominent peak D appeared to be of low molecular weight (about 2000–5000). Peak E was found to contain the visible yellow pigment with a maximal absorbancy at 380 nm.

Immunochemical identification of antigens in birch pollen extract and its fractions

The eluted antigens in gel filtration on Sephadex G-100 column were demonstrated by EIA using the rabbit anti-BP antiserum (Fig. 1). Six different peaks binding rabbit antibodies were revealed, three of them (Ve_1 , Ve_2 and Ve_3) corresponding well to the main protein peaks A, B and C. Ve_4 had a molecular weight around 10 000 and Ve_5 around 5000, which both differed slightly from the main protein peaks.

CIE studies of birch pollen extract (1:10 w/v) revealed nine antigens numbered in the order of R_f s as seen in Fig. 2a. Twelve protein bands were seen after the first dimension run in polyacrylamide gel. The strongest bands with R_f s 0.69 and 0.38, also formed the strongest immunoprecipitates (antigens Nos. 7–8, and antigen No. 2, respectively). Antigens Nos. 7 and 8, which shared a partial identity, appeared to be thermoresistant, while antigen No. 2 was thermolabile (Fig. 2b). In CIE studies using agarose gel in both steps no cathodal protein bands or immunoprecipitates could be shown.

Fused rocket immunoelectrophoresis of the fractions obtained from Sephadex G-100 gel filtration demonstrated three precipitin lines; one with peak B fractions and two with partial identity with peak C fractions (Fig. 2e). No antigens could be found in the unconcentrated peak A, peak D or peak E fractions. Heating of the fractions at 100°C for 15 min destroyed the thermolabile antigens in peak B, while only slight decrease was found in the immunoreactivity of the heat-stable antigens in peak C.

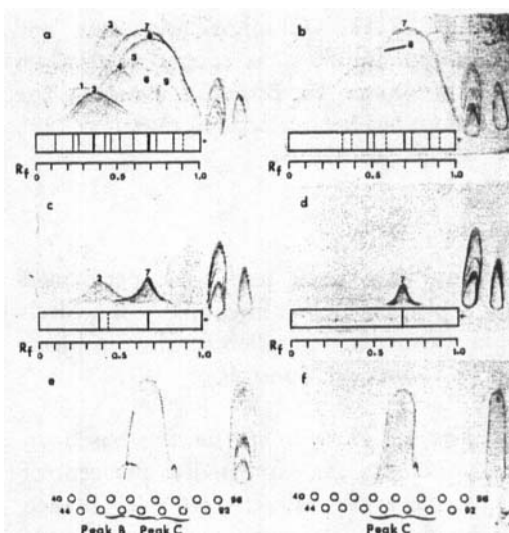


Fig. 2. Crossed immunoelectrophoretic (CIE) patterns of whole birch pollen extract (10^{-1} w/v) (a); heat-treated extract (100°C for 15 min) (b); pooled peak B + peak C Sephadex G-100 fractions (c), and heat-treated peak B + peak C fractions (d). The first dimensions electrophoresis was performed in polyacrylamide gel (PAGE; 75 μ l samples) at +0 – +2°C (1 h) at 40 mA and 225–300 V/8 gel tubes. R_f values for the different protein bands are given. Antigen: the amount of protein applied in PAGE: 590 μ g (a); 560 μ g (b); 7.5 μ g (c); 7 μ g (d). Antibodies: a 1:50 dilution of the rabbit anti-birch pollen (BP) serum in 1% agarose in barbital buffer, pH 8.6. Second dimension electrophoresis: overnight (18 h), 1–3 V/cm, 15°C anode at the top. Staining: 0.5% Coomassie Brilliant blue R.

Fused rocket immunoelectrophoresis (e, f). Antigen: 5 μ l fractions of gel filtration after Sephadex G-100, either as such (e), or heat-treated (100°C for 15 min) (f). Antibodies and electrophoresis as in CIE, except that 1:100 dilution of the anti-BP antiserum was used.

In all experiments, 5 μ l samples of either heat-treated or native birch pollen extract (1:10 w/v) were used as controls during the second dimension electrophoresis.

Pooled fractions of peak B and peak C were further separated by CIE, in which two non-cross-reacting antigens could be shown (Fig. 2c). One of these had R_f 0.67 and was heat-stable, and the other with R_f 0.38 was heat-labile.

The antigens of peak B and peak C were also studied by gel isoelectrofocusing (Fig. 3). Separation of the whole birch pollen extract revealed several protein bands on the anodal side, but only one on the cathodal side (pI

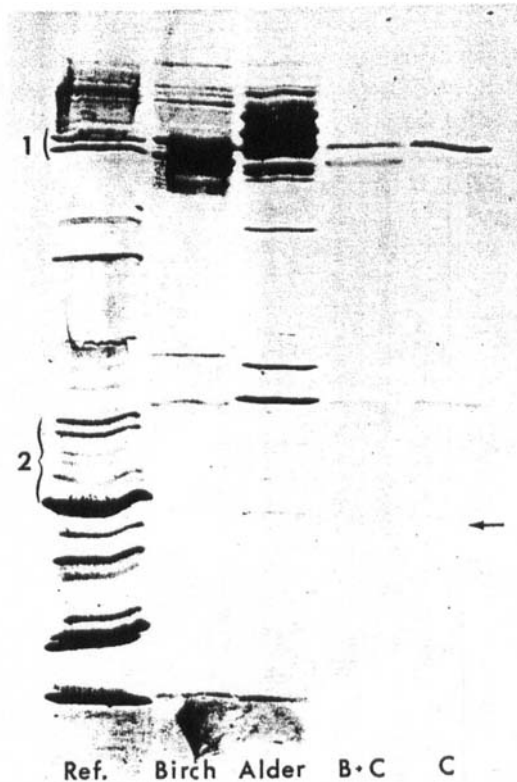


Fig. 3. Analytical thin-layer electrofocusing in polyacrylamide gel. Fractionation of birch (7.9 mg/ml) and alder pollen (9 mg/ml) extracts and of pooled Sephadex G-100 fractions from peaks B+C (100 µg/ml) or from peak C (131 µg/ml) alone 10x concentrated. Samples were applied about 20 mm from the cathode in 20 µl aliquots absorbed in Whatman 3 MM filter paper applicators (arrow). A pH range of 3.8 to 8.9 was achieved by using Ampholine of pH 3.5–9.5 at a final concentration of 4% v/v and focusing for 2 h at +2°C in a gel with a total monomere concentration $T = 5\%$ and a crosslinking $C = 3\%$ under influence of 30 W constant power. Staining of total proteins was done with 0.1% w/v Coomassie Brilliant blue R-250 (Serva Feinbiochemica, Heidelberg, Fed. Rep. Germany). As a reference, Serva Reference Solution 8 was introduced to provide direct side-by-side comparison: (1) lactoglobulin (pI 5.4–5.7), (2) horse myoglobin (pI 7.3–7.7).

8.9). The most prominent bands had pI's 5.6 and 5.7. These two distinct bands were also seen in concentrated (10x), pooled peak B+C fractions; in addition, four faint bands with pI's 5.9, 6.0, 6.3 and 7.2 were found in these fractions. The weak anodal band with pI 6.0 seemed to reside only in peak B fractions,

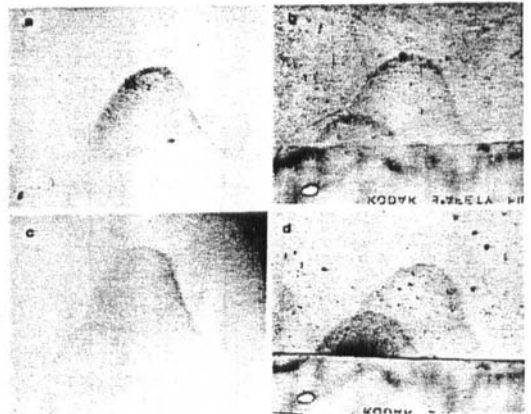


Fig. 4. Crossed-radio-immuno-electrophoretic patterns of birch pollen extract (1:10 w/v). Binding of IgE (a, c) from sera obtained before, and of IgG (b, d) from sera obtained after hyposensitization of two allergic subjects are shown. After CIE of 5 µl of BP extract on 1% agarose the gels were incubated with a 1:15 dilution of patients' sera (0.5 ml of serum in 7 ml of phosphate buffer, pH 7.5) overnight (18 h) at room temperature, followed by a second incubation with ^{125}I -labelled anti-human-IgE (Pharmacia AB, Uppsala, Sweden) or ^{125}I -labelled anti-human-IgG antibodies (both in dilutions corresponding to about 30 000 cpm ml $^{-1}$), respectively. Autoradiographs on Kodak RP/S X-Omat film with exposure time for 1 week are shown.

while those with pI's 5.9, 6.3 and 7.2 were also found in peak C fractions. Only one band with pI 5.6 was clearly visible when concentrated peak C fractions alone were separated. Several anodal protein bands including those with pI's 5.6 and 7.2 were also found in alder pollen extract (1:10 w/v) prepared in the same way as the birch pollen extract.

Allergenic activity measured by CRIE

The results of CRIE using sera obtained from two allergic patients both before and after hyposensitization therapy are shown in Fig. 4. Binding of both IgE and IgG antibodies to the thermostable (TS) antigens Nos. 7/8 was evident. On the other hand, only weak binding of IgE antibodies and moderate binding of IgG antibodies to the thermolabile antigen No. 2 was found.

The specificity of the binding of IgE

Table 1
Allergenic activities and concentrations of the thermostable (TS) antigen of pooled Sephadex G-100 fractions compared to those of the whole pollen extract

	Protein µg/ml	Antigen ^a %	Allergenic activity %		Specific activity ^d		Recovery ^e %	
			Prick test ^b	RAST inh. ^c	Prick test	RAST inh.	Prick test	RAST inh.
Whole extract (10 ⁻¹ w/v) (5 ml)	7900	100	100	100	1	1	100	100
Peak A (fr. 37-51)	13	0	0.01	0.0001	0.05	0.001	0.16	0.0025
Peak B (fr. 58-64)	55	0	0.19	0.004	0.45	0.006	0.09	0.028
Peak C (fr. 69-77)	131	10.9	3.3	3.04	4.5	1.84	30.1	27.8
Peak D (fr. 97-104)	330	0	0.01	0.003	0.004	0.0007	0.1	0.027

^a The concentration of thermostable (TS) antigen measured by rocket immunoelectrophoresis (% of whole extract, 10⁻¹ w/v).

^b Mean allergenic activity (%) relative to that of the whole extract (10⁻¹ w/v) measured by skin prick testing in 10 allergic individuals.

^c Expressed as $\frac{I_{50} \text{ units/ml of the fractions}}{I_{50} \text{ units/ml of the whole extract}} \times 100$ (%). Mean results of two separate determinations varied less than 5%. A mixture of sera from 20 allergic subjects was used.

^d Allergenic activities relative to the amount of protein of the fractions compared to that of the whole extract; the activity of the whole extract was arbitrarily chosen to be 1.

^e Recovery of the total allergenic activity of the various protein peaks as percentages of that of the whole extract.

antibodies in CRIE was shown by negative results with sera from non-atopic subjects or atopic subjects with negative RAST results. Negative results were also observed with a serum from a patient who had a negative RAST, but high levels of total IgE immunoglobulins by PRIST and high concentrations of IgG antibodies to birch pollen by ELISA (28).

The specificity of the binding of IgG antibodies in CRIE was demonstrated by using sera from two non-atopic subjects with low amounts of IgG antibodies to birch pollen by ELISA (28). Only weak radiostaining corresponding to antigen 2 was observed with these sera. A more intense radiostaining was observed in the same region with two sera (one atopic and one non-atopic) containing high amounts of IgG antibodies to birch pollen by ELISA. None of the sera from individuals not given immunotherapy with birch pollen extract contained detectable amounts of IgG antibodies to the main allergens of birch pollen (antigens 7/8) in CRIE.

Antigen contents and allergenic activities of pooled Sephadex G-100 fractions relative to those of the whole extract

Table 1 shows the amount of TS-antigen and the allergenic activity in the whole birch pollen extract (1:10 w/v; 24 h extraction) and its Sephadex G-100 fractions.

The allergenic activity was demonstrated by skin prick testing and by the RAST inhibition (Fig. 1; Fig. 5). Comparable results were obtained by both methods with pooled peak C fractions, while higher allergenic activity was observed by prick tests than by the RAST inhibition in fractions of peak A, peak B and peak D (Table 1). Pooled peak C fractions containing the main allergenic activity showed about 5-fold increase in the concentration of the TS-antigen and about 2- to 5-fold increase in the specific allergenic activity (per μg of protein), when compared to the whole extract. Peak C contained about

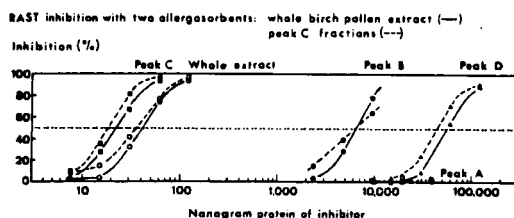


Fig. 5. Inhibition curves showing the relative allergenic activity and allergenic cross-reactivity of the whole birch pollen extract and of the pooled fractions after Sephadex gel filtration. Paper discs coupled with either the whole BP extract (solid line —) or with pooled peak C fractions (dotted line ----) were used. The log-linear plots provided curves which were linear between 25% and 75% of inhibition. Results of two separate determinations gave less than 5% variation of the relative allergenic activity. In the absence of inhibitor the serum pool IgE antibody (from 20 allergic subjects) produced 39.8% of the total counts added bound to the discs coupled with the whole extract, and 34.6% bound to those coupled with pooled C fractions. Only 0.8% of the total counts were bound to discs without allergens in the presence of the serum pool.

10–15% of the total proteins and more than 90% of the total allergenic activity of the extract. About 28–30% of the total allergenic activity was recovered in the peak C fractions, and only less than 0.5% in the fractions outside peak C.

The effect of the extraction time on the appearance of the thermostable (TS) antigen and the total allergenic activity in the extract was studied by using freshly collected pollen of different origin (17) than that used in the previous experiments (Table 2). About 70% of the total allergenic activity appeared in less than 30 min and a maximum was reached in 3 h. From 3 h to 24 h a considerable decrease (about 30%) of the total allergenic activity was observed. However, the allergenic activity in protein peak C and the amount of the TS-antigen were increased during the 24 h extraction. The long extraction time also resulted in a 2-fold increase in the amount of small peptides in the extract. Likewise the amount of the total proteins was doubled during this longer extraction.

Table 2

Effect of extraction time on the amount of total proteins and small peptides, the thermostable (TS) antigen and allergenic activity in birch pollen extract and its main allergenic fractions after gel filtration on Sephadex G-100

Extraction ^a time	Proteins ^b g/l	Peptides ^c		Antigen (% of reference) ^d		Allergenic activity (I ₅₀ U/ml ^e)	
		F-C mmol/l	Ninh. mmol/l	Whole extract	Peak C fractions	Whole extract	Peak C fractions
15 min	2.9	0.51	8.7	18	0.08	33 900	30
30 min	3.3	0.56	8.9	26	0.38	56 500	130
3 h	3.6	0.57	12.9	33	0.92	80 000	770
24 h	7.4	1.15	20.9	34	1.18	53 600	930

^a Birch pollen (1/10 w/v) in 0.15 M PBS (slow rotation at +4°C). Freshly collected fertile pollen (17) was used.

^b Concentration of total proteins in the whole extract according to Lowry et al. (19).

^c Concentration of peptides in the whole extract after removal of the proteins by TCA precipitation; F-C = Folin-Ciocalteu; Ninh. = ninhydrin method, performed as described in (16).

^d Concentration of thermostable (TS) antigen measured by rocket immunoelectrophoresis using heat-treated samples and heat-treated (100°C for 15 min) birch pollen extract (BP extract, 1/10 w/v; see material and methods) as a standard.

^e Total allergenic activity in the whole extract and in the pooled peak C fractions after gel filtration of the extract. The mean values of two separate determinations varied less than 5%.

Allergenic cross-reactivity between the separated fractions

The inhibition curves obtained by the pooled fractions of peaks A, B, C and D are seen in Fig. 5. Rather similar inhibition curves are observed by using either the whole birch pollen extract or the pooled peak C fractions coupled to paper discs as a solid-phase allergen, indicating that peak C contains the major allergen in the whole extract. A 50% inhibition (I₅₀) was obtained with about 20 ng protein of the peak C fractions, about 40 ng of the whole extract, about 6300 ng of the peak B and about 55 000 ng of the peak D fractions. No inhibition was observed with concentrated peak A fractions (38 000 ng of protein).

By inspection of the slopes of the curves it may be suggested that the whole extract, the peak C fractions and the peak D fractions, which have parallel slopes, are for the most part allergenically identical (3).

Peak C fractions gave parallel inhibition curves with the two allergosorbents; those coated with the whole extract and those with the peak C fractions. The same was observed

with peak D fractions, while divergent inhibition curves were obtained by the whole extract and by the peak B fractions when using these different allergen-discs. These findings indicate that although the peak C fractions contain the major allergens of birch pollen, there are allergens with RAST inhibiting capacity outside these fractions. It seems evident that the antigens 7/8 in peak C and the antigen 2 in peak B differ both antigenically and allergenically from each other, although some allergenic cross-reactivity between these antigens has not been totally excluded.

DISCUSSION

A birch pollen extract (1:10 w/v) was prepared by simple extraction in phosphate buffered saline. The immunologic specificity of the undialysed whole extract was shown in atopic allergic and non-allergic subjects and in non-atopic ones (28, 29). Thus the undialysed extract was used in the study of the different allergenic components in birch pollen, since by dialysis some low molecular weight components are lost.

Fractionation of a birch pollen extract by gel filtration followed by a fused rocket immunoelectrophoresis revealed three antigens with molecular weights of about 29 000, 17 000 and 10 000. These were shown by CIE to correspond to antigens Nos. 2 and antigens 7/8, respectively. Antigens Nos. 7/8 were thermostable (TS) and antigenically cross-reacting thus corresponding to the main antigens and allergens of birch pollen described by Belin (5, 6). Strong binding of the IgE antibodies from allergic sera to the TS-antigens, but only weak binding to the thermolabile antigen No. 2 was found in CRIE. Thus direct evidence was obtained for the TS-antigens being the main allergens of birch pollen. When the binding of IgG antibodies to these antigens was studied by using sera obtained from the same patients after hyposensitization, quite a similar pattern was found. On the other hand, none of the non-hyposensitized subjects, either allergic or non-atopic, had developed IgG antibodies to the TS-antigens, although some of them had IgG antibodies to the antigen No. 2. Our results thus indicate that high amounts of IgG antibodies against the main allergens of birch pollen are formed only after hyposensitization therapy. This is in agreement with the findings obtained by measuring with the ELISA technique the amount of IgG antibodies bound to the different fractions of birch pollen extract (27).

Fractionation of birch pollen extract by gel isoelectric focusing revealed several protein bands on the anodal side, but only one weak band on the cathodal side. By using this method, pooled Sephadex-fractions containing the main antigens of birch pollen were separated into two major protein bands with pI's 5.6 and 5.7, and into four weak bands with pI's from 5.9 to 7.2. Since even homogeneous, purified proteins may be separated into fractions with different isoelectric points due to removal of amide groups from the molecule (31), the presence of several proteins with different pI's in the

pooled fractions was to be expected. A distinct protein band with pI 5.6 was also found in alder pollen extract. Our data suggest that this band contains the allergenic thermostable antigen(s) common for both alder and birch pollen (5). This is in agreement with the results of Ceska et al. (10), who found the main allergenic activity of birch pollen (*Betula lenta*) within pI range 4.8–5.7.

In the present study the allergenic activity of the main allergenic fractions measured by the RAST inhibition method was comparable to that obtained by skin prick testing. However, a markedly higher allergenic activity was obtained by prick testing than by the RAST inhibition method for the fractions outside peak C. The reason for this discrepancy between the biologic and the *in vitro* allergenic activity in these fractions is not known. A possible explanation for this phenomenon might be a selective binding of some allergens to the paper discs used in the RAST (9). A preferential binding of the main allergens (peak C) to the paper discs would give an misrepresentatively high allergenic activity for the allergens in peak C in relation to the allergens outside these fractions. Our data (Table 1, Fig. 5) support this view. More than 90% of the total activity was recovered in the peak C fractions. In addition, rather similar inhibition curves were obtained by using paper discs coupled with the whole extract or dialysed whole extract and those coupled with the peak C fractions, indicating that the major part of the allergens in the discs coated with the whole extract were of peak C origin.

The relative allergenic activity of the peak B fractions was 10–300 times lower than that of the peak C fractions measured by skin prick testing and by the RAST inhibition method, respectively. The non-parallelity of the inhibition curves obtained by the peak B fractions and those of peak C indicates that peak B contains allergens distinct from those of peak C (3). Although antigenically distinct as shown by CRIE, some cross-reactivity

between the allergens of the peak B and those of peak C cannot be excluded. It is also evident that peak B contains some of the peak C allergens as contaminants not totally separated from the peak B allergens by simple gel filtration.

The fractions of peak D showed about 10–100 times lower specific allergenic activity than those of peak B in the RAST inhibition and by skin prick testing, respectively. Such small peptides of a low molecular weight (mol. wt. 2000–5000) could not be studied by CRIE, since no precipitation was obtained by rabbit anti-birch pollen serum. However, RAST inhibition studies with the peak D fractions clearly showed parallel inhibition curves with those of the peak C fractions indicating qualitative allergenic similarity between these fractions (3).

Our data do not allow a clear distinction of the origin of the allergenically active components of the peak D fractions. The amount of peptides was doubled during the long extraction (3–24 h, at +4°C) suggesting that also the active components might be degradation products of the allergens by proteolysis. A number of enzymes are liberated into the extract during the extraction, some of them already during the first minutes of extraction. However, leucine aminopeptidase, the only proteinase studied, could not be shown in the extract, although it was shown after disintegration of the pollen grains (17). The presence of proteinases, especially pronase, in the extract would destroy all the allergenic activity of birch pollen extract (11, 12). On the other hand, the main allergens of birch pollen are relatively resistant to many carbohydrases and to some proteinases, e.g., trypsin and pepsin. Our results show that even if some proteolysis might have occurred during the long extraction, it did not markedly destroy the main allergens of birch pollen. Although the total allergenic activity of the extract was about 30% decreased, the allergenic activity in the peak C fractions and the amount of the TS-antigen in the extract were increased

during this extraction (Table 2). Our findings are also in agreement with those of Belin (5), who demonstrated the extraction of the major part of the main antigens and allergens of birch pollen during the first 45 min under similar conditions as described here.

Our results suggest that the allergenic components in the peak D fractions, even if products of enzymatic degradation of the larger molecular weight allergens of birch pollen, still may be biologically active. This is an interesting finding, since proteolytic activity in nasal secretions causes a rapid destruction of the allergens of timothy pollen and the loss of allergenic activity (20).

Further studies are needed to solve the importance of the small molecular weight allergens in the fractions of peak D. Preliminary results suggest that allergic subjects are not equally sensitive to these allergens (27). Similar observations have been made with small molecular weight allergens isolated from timothy pollen (13, 21) and from ragweed pollen (18, 24). Due to the genetic requirements only individuals with an inherited liability to immune responses to these antigens with few antigenic and allergenic determinants will be sensitized (1, 22).

We have confirmed the findings that birch pollen allergens are rapidly diffused from pollen grains during extraction in physiological saline. Using a simple gel filtration combined with gel isoelectric focusing the main allergens of birch pollen were obtained in a relatively pure form. The isolation and characterization of the different major and minor allergens of birch pollen allergens of birch pollen by the methods described here will allow the use of purified birch pollen antigens in immunological studies of pollen allergy.

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REFERENCES

1. Aas, K.: What makes an allergen an allergen. *Allergy* 33, 3–14, 1978.
2. Aas, K. & Belin, L.: Standardization of diagnostic work in allergy. *Acta Allergol.* 27, 439–469, 1972.
3. Adolphson, C., Goodfriend, L. & Gleich, G. J.: Reactivity of ragweed allergens with IgE antibodies. Analyses by leukocyte histamine release and the radioallergosorbent test and determination of cross-reactivity. *J. Allergy Clin. Immunol.* 62, 197–210, 1978.
4. Axelsen, N. H., Krøll, J. & Weeke, B.: A manual of quantitative immunoelectrophoresis. Methods and applications. *Scand. J. Immunol.* 2, Suppl. 1, 1973.
5. Belin, L.: Immunological analyses of birch pollen antigens, with special reference to the allergenic components. *Int. Arch. Allergy* 42, 300–322, 1972.
6. Belin, L.: Separation and characterization of birch pollen antigens with special reference to the allergenic components. *Int. Arch. Allergy* 42, 329–342, 1972.
7. Belin, L. & Rowley, J. R.: Demonstration of pollen allergens from isolated pollen grains using immunofluorescence and a single radial immunodiffusion technique. *Int. Arch. Allergy* 40, 754–769, 1971.
8. Ceska, M.: Column chromatography of allergens and assessment of their activities by the paper disk radioallergosorbent method. *Int. Arch. Allergy* 45, 405–416, 1973.
9. Ceska, M., Eriksson, R. & Varga, J. M.: Radio-immunosorbent assay of allergens. *J. Allergy Clin. Immunol.* 49, 1–9, 1972.
10. Ceska, M., Eriksson, R. & Varga, J. M.: Characterization of allergen extracts by polyacrylamide gel isoelectrofocusing and radio-immunosorbent allergen assay. *Int. Arch. Allergy* 42, 430–437, 1972.
11. Ceska, M. & Ponterius, G.: Study of nature of binding sites of some allergens interacting with IgE. *Int. Arch. Allergy* 45, 819–827, 1973.
12. Ceska, M., Ponterius, G. & Soininen, K.: Enzymatic hydrolysis of allergens and assessment of the remaining allergenic activities by the paper disk radioallergosorbent method. *Int. Arch. Allergy* 45, 417–428, 1973.
13. Cramer-Kleinert, B. & Schneider, C. H.: Studies of antigens from timothy pollen. *Dev. Biol. Stand.*, vol. 29, pp. 188–196, Karger, Basel, 1975.
14. Davis, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* 121, 404–427, 1964.
15. Djupsund, B. M.: Proteintaxonomical studies of whitelish and tapeworms with thin layer electrofocusing. Application Note 243, LKB-Produkter AB, Bromma, 1976.
16. Fräki, J.: Human skin proteases, p. 29. M.D. thesis, University of Turku, Turku 1976.
17. Laine, S.: Diffusion of proteins by intact birch pollen grains: enzymic and antigenic activity. *Grana* 16, 187–189, 1977.
18. Lapkoff, C. B. & Goodfriend, L.: Isolation of a low molecular weight ragweed pollen allergen: Ra 5. *Int. Arch. Allergy* 46, 215–229, 1974.
19. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275, 1951.
20. Löwenstein, H. & Mygind, N.: Extraction and degradation of timothy pollen allergen during simulated *in vivo* conditions. *Allergy* 33, 238–240, 1978.
21. Malley, A., Campbell, D. H. & Heimlich, E. M.: Isolation and immunochemical properties of haptenic material from timothy pollen. *J. Immunol.* 93, 420–425, 1964.
22. Marsh, D. G.: Allergens and the genetics of allergy. In Sela, M. (ed.): *The Antigens*, vol. III, pp. 271–359, Academic Press, New York, 1975.
23. Ornstein, L.: Disc electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.* 121, 321–349, 1964.
24. Schon, A. H.: Heterogeneity of allergenic extracts and of the antibody response in allergic patients. *Dev. Biol. Stand.* vol. 29, pp. 1–25, Karger, Basel, 1975.
25. Weeke, B., Löwenstein, H. & Nielsen, L.: Allergens in timothy pollen identified by crossed-radio-immuno-electrophoresis (CRIE). *Acta Allergol.* 29, 402–417, 1974.
26. Whitaker, J. R.: Determination of molecular weight of proteins by gel filtration on Sephadex. *Analyt. Chem.* 35, 1950–1959, 1963.
27. Viander, M.: Humoral and cell-mediated immune responses to fractions of birch pollen extract (to be published).
28. Viander, M. & Eskola, J.: *In vitro* lymphocyte transformation by birch pollen antigens: a comparison to specific IgE and IgG antibodies and nasal allergy. *J. Clin. Lab. Immunol.* 1979 (in press).
29. Viander, M. & Koivikko, A.: The seasonal symptoms of hyposensitized and untreated hay fever patients in relation to birch pollen counts: correlations with nasal sensitivity, prick tests and RAST. *Clin. Allergy* 8, 387–396, 1978.

30. Viljanen, M. K., Granfors, K. & Toivanen, P.: Radioimmunoassay of class-specific antibodies (RIACA): chicken antibodies to bovine serum albumin. *Immunochemistry* 12, 699-705, 1975.
31. Williamson, A. R., Salaman, M. R. & Kreth, H. W.: Microheterogeneity and allomorphism of proteins. *Ann. N.Y. Acad. Sci.* 209, 210-224, 1973.
32. Yman, L., Ponterius, G. & Brandt, R.: RAST-based allergen assay methods. *Dev. Biol. Stand.* vol. 29, pp. 151-165, Karger, Basel, 1975.

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