

Isolation and immunochemical characterization of the major allergen of birch pollen (*Betula verrucosa*)

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The classification of some of the extractable birch pollen antigens as allergens was established by crossed radioimmuno-electrophoresis (CIE). In CIE the major allergen (antigen 23) exhibited the strongest "radiostaining," and only a few other components of birch pollen extract were visibly radiostained. The major allergen and a preparation containing mainly the minor allergens, antigens 25 and 19, were isolated from a crude aqueous birch pollen extract by a combination of anion-exchange, size-exclusion, and chelate chromatography. Antigen 23 was purified to near homogeneity. The molecular weights and the pIs of antigens 23, 25, and 19 were determined to be 17,000 daltons, pI 5.25 (5.5, 5.0); 25,000 daltons, pI 5.0 (4.9, 5.4); and 29,000 daltons, pI 6.2 (5.4), respectively. The classification of antigen 23 as the major allergen in birch pollen was supported by results of RAST inhibition experiments, RAST screening, and skin prick testing. (J ALLERGY CLIN IMMUNOL 72:150-159, 1983.)

Birch pollen is one of the main allergenic sources in northern Europe and several attempts to identify, characterize, and isolate its major allergenic constituents have been made. Belin^{1, 2} demonstrated that the major allergen was a thermostable component with a molecular weight of 20,000 daltons. This was verified by Viander et al.,³ who, however, found a molecular weight of 10,000 to 17,000 daltons. Recently Apold et al.⁴ and Vik et al.⁵ described immunochemically identical antigens from birch pollen extract with molecular weights of 29,000 and 10,000 daltons that were classified as major allergens. These antigens are apparently different from the thermostable antigen.

The aims of this study were to identify the allergenic components of an aqueous birch pollen extract by means of CIE and to isolate and characterize the major allergenic component(s) and investigate their allergenic significance by independent methods.

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MATERIALS AND METHODS

Birch pollen extract

Pollen from *Betula verrucosa* containing less than 0.5% (w/w) foreign pollen was obtained from Allergon AB, Engelholm, Sweden. The pollen (50 gm) was extracted 1:10 (w/v) in 0.125M ammonium hydrogen carbonate, 0.015M sodium azide, pH 8.3, for 20 hr at 5° C. Particulate matter was removed by centrifugation for 1 hr at 35,000 × g and the supernatant was dialyzed three times against water for 24 hr each and lyophilized. The lyophilized extract (or Bp) was stored at -20° C.

Ion-exchange, size-exclusion, and chelate chromatography

Bp was fractionated by ion-exchange chromatography on a DEAE-Sephacrose CL 6B column, 25 by 5.3 cm² (Pharmacia, Uppsala, Sweden). The antigens were eluted from the column by a salt gradient (0.016M disodium hydrogen phosphate, 0.004M sodium dihydrogen phosphate [pH 7.4, I = 0.05] to 0.016M disodium hydrogen phosphate, 0.004M sodium dihydrogen phosphate, 0.95M sodium chloride [pH 7.4, I = 1.0]). Selected fractions were pooled, dialyzed against water, lyophilized, redissolved in 0.125M ammonium hydrogen carbonate buffer (pH 8.2), and subjected to size-exclusion chromatography on a Sephadex G-75 column, 90 by 5.3 cm² (Pharmacia). The fractions containing the desired antigens were pooled, dialyzed against water, lyophilized, redissolved in 0.029M disodium hydrogen phosphate buffer (pH 7.2, I = 0.1) and subjected to chelate chromatography on an iminodiacetic acid Sepharose CL 4B column, 15 by 0.6 cm² (Pierce Chemical

Abbreviations used

CRIE:	Crossed radioimmuno-electrophoresis
Bp:	Crude birch pollen extract
SDS:	Sodium dodecylsulfate
PAGE:	Polyacrylamide gel electrophoresis
DATD:	<i>N,N'</i> -diallyltartardiamide
DTT:	1,4-dithio-DL-threitol
a-Bv:	Anti-birch pollen extract antibodies
RAST:	Radioallergosorbent test
CIE:	Crossed immunoelectrophoresis
CLIE:	Crossed-line immunoelectrophoresis
SI:	Skin index
I:	Ionic strength
V_t :	Total column volume
EDTA:	Ethylenediamine tetraacetic acid
pI:	Isoelectric point
T:	Acrylamide volume
C:	Bisacrylamide acrylamide

Co., Rockford, Ill.), with the use of Cu as the chelated ion.^{6,7} Chelate chromatography was performed as follows. The column was washed with two V_t of 0.05M EDTA, two times V_t water, two times V_t 0.1M cupric sulfate, and two times V_t water and was equilibrated with phosphate buffer, pH 7.2. The sample was applied to the column and the antigens were eluted from the column by decreasing pH from 7.2 to 4.7 in 0.5 pH unit decrements with appropriate phosphate or acetate buffers ($I = 0.1$). The fractions containing the isolated antigens were pooled, dialyzed against water, and lyophilized.

SDS-PAGE

The molecular weight of the isolated antigens was estimated by gradient gel (T 12%, C 0.48% to T 16%, C 0.64%) SDS-PAGE. The electrophoresis buffer was made up of 0.193M Tris, 0.155M glycine, and 0.15% (w/v) SDS; the stacking gel buffer of 0.75M Tris HCl (pH 7.25); the separating gel buffer of 2.07M Tris HCl (pH 8.8); the stacking gel (T 7%, C 5%) of DATD; and the sample buffer of 0.15M Tris HCl (pH 7.25), 0.65M DTT, 0.1% (w/v) SDS, and 5% (v/v) glycerol.⁸ Electrophoresis was performed in 0.1 by 13 by 14 cm gel slabs at 40 mA for 8 hr with 5 to 50 μ g samples and with the use of the LMW Electrophoresis Calibration kit (Pharmacia) for molecular weight references. Acrylamide and *N,N'*-methylene-bis-acrylamide were from Serva Heidelberg, Germany, DATD was from Bio-Rad Laboratories, Richmond, Calif., and DTT was from Fluka AG, Buchs, Switzerland.

Isoelectric focusing

Column isoelectric focusing (LKB, ampholine column type 8101) was performed in a 0 to 50% (w/v) sucrose gradient containing 1% (v/v) LKB ampholine, pH 3.5 to 10 (LKB, Bromma, Sweden) at 200 V and 2.2 mA for 48 hr.⁹

Rabbit antibodies

Rabbit a-Bv were obtained from Allergologisk Laboratorium, Copenhagen, Denmark.

Human sera

Serum samples from 20 birch pollen-allergic patients were kindly provided by Dr. B. Nüchel-Petersen, The Lung Clinic, County Hospital, Lyngby, Denmark. The patients were selected on the basis of clinical record, results of nasal provocation and skin prick tests, and RAST (Table I). A serum pool was made from equal volumes of the individual sera.

Immuno-electrophoretic methods

CIE, CLIE, fused-rocket immunoelectrophoresis, and CRIE and CRIE classification of the allergens were performed essentially as described by Løwenstein.¹⁰ CRIE inhibition experiments were performed using a serum pool incubated with either heat-treated or native Bp before the incubation with cold-dried CIE plates of Bp, antigen 23, or antigens 25 and 19.

RAST screening and RAST inhibition

RAST experiments were performed as described by Ceska.¹¹ RAST screening and RAST inhibition experiments were all performed with antigen preparations containing the same amount of antigen 23 or antigen 25 as did Bp.

RAST screening was performed with individual patients' sera and two different allergosorbents (antigen 23 and Bp paper discs). Aliquots (0.050 ml) of diluted serum (1:4) were incubated with antigen-coupled paper discs for 24 hr at 25° C, and the amount of IgE absorbed to the discs was estimated as counts per minute bound to the paper discs after incubation with 20 nCi of ¹²⁵I-labeled anti-IgE for 24 hr at 25° C and three successive washings with 0.154M sodium chloride, 1% (v/v) polysorbate (Tween 20; ICI Americas, Inc., Wilmington, Del.). All determinations were made in triplicate.

RAST inhibition experiments were performed with individual allergens and Bp both as inhibitors and allergosorbents. Equal amounts of the serum pool (0.075 ml) and 10-fold dilutions of the inhibitor preparation (0.075 ml) in question were incubated together for 24 hr at 25° C. Two times 0.050 ml of the inhibited serum pool was incubated with antigen-coupled paper discs for 24 hr at 25° C and the amount of IgE absorbed to the discs was estimated as counts per minute bound to the discs after incubation with 20 nCi of ¹²⁵I-anti-IgE for 24 hr at 25° C and three successive washings with 0.154M sodium chloride, 1% (v/v) Tween 20. The fraction of specific IgE bound to the inhibitor, $I(x)$, was calculated as follows:

$$I(x) = \frac{\text{CPM}(0) - \text{CPM}(x)}{\text{CPM}(0) - \text{CPM}(n)} \quad (1)$$

where CPM(0) and CPM(x) are the counts per minute measured with the use of buffer and antigen preparation(s) as

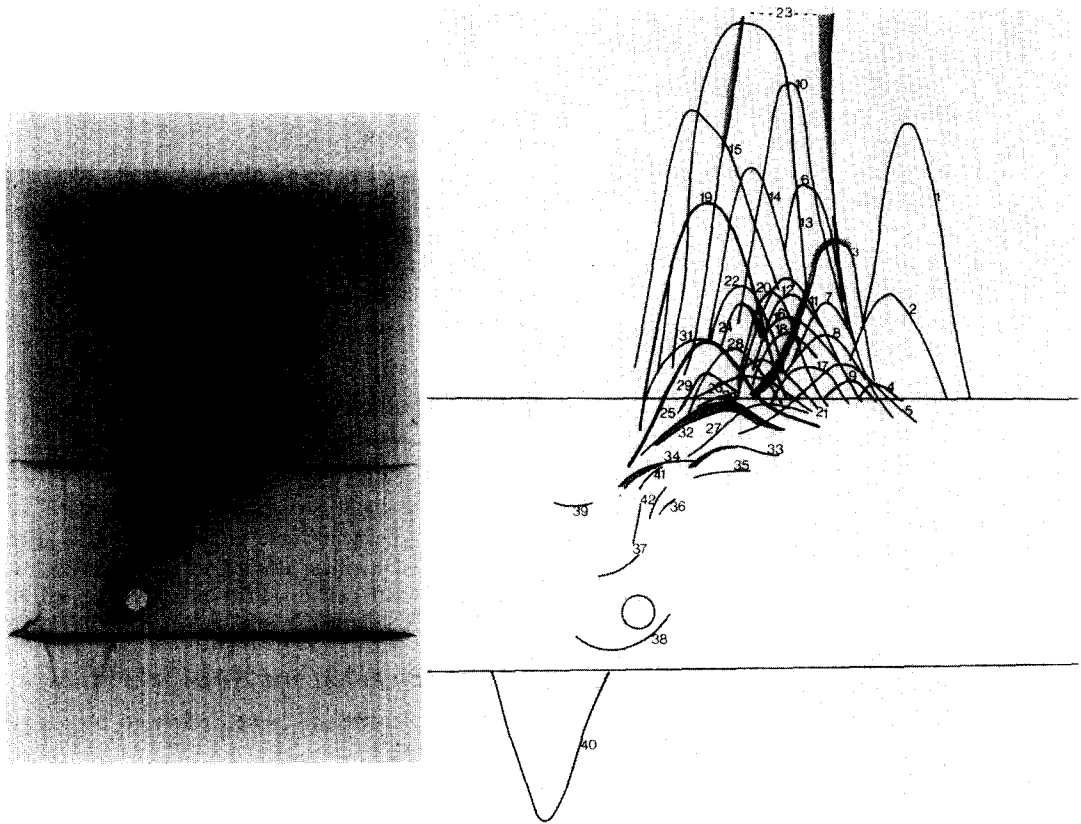


FIG. 1. CIE of Bp and a drawing of the reference precipitation pattern arbitrarily numbered from the anodic to the cathodic side. CIE of 40 μ g of Bp was performed in 1% (w/v) agarose, (0.073M Tris, 0.024M barbital, 0.0006M calcium lactate, pH 8.6) at 15° C. First-dimension electrophoresis: 25 min, 10 V/cm; second-dimension electrophoresis: 16 hr, 2 V/cm; with the use of 17.1 and 13.3 μ l of a-Bv per square centimeter in the anodic- and cathodic antibody-containing gels, respectively.

TABLE I. Selection of birch pollen—allergic patients (only serum from patients who scored >5 was selected for further study)

Score	Clinical record	Positive nasal provocation test* obtained at concentrations (HEP) greater than	Skin prick test* (S.I.† obtained with 1 HEP)	RAST (class)
0	Negative	1.0	<0.5	0-1
1	Doubtful	0.1	0.5-1.0	2
2	Positive	0.01	>1.0	3-4

HEP = Histamine equivalent prick.
*Performed with SQ-standardized Bp for in vivo tests and as allergosorbent.
†Calculated according to equation 2.

inhibitors, respectively. CPM(n) was measured in a 1000-donor normal serum pool instead of the patient serum pool. All determinations were made in duplicate.

Skin prick testing

Skin prick tests were performed on the right and left arm of 10 individual birch pollen—allergic patients with antigen preparations containing the same amount of antigen 23 or

antigen 25 as did Bp. The skin reaction produced by the individual antigen preparations was estimated as the mean of the SI obtained on the two arms. SI was calculated as follows:

$$SI = \frac{D(1) \cdot D(2)}{D(1, his) \cdot D(2, his)} \tag{2}$$

where D(1) and D(2) are the mutual perpendicular diameters

TABLE II. Physicochemical parameters for some of the allergenic molecules of birch pollen

Antigen No.	Molecular weight ^A (daltons)	pI ^A
23	17,000*	5.25, 5.50, 5.00
23	18,000†	
25	25,000*	5.00, 4.90, 5.40
19	29,000*	6.20, 5.40

The pIs of the individual allergens were determined with whole pollen extract. The molecular weights were determined with both whole pollen extract (size-exclusion chromatography) and purified fractions (SDS-PAGE).

*Determined by size-exclusion chromatography (G-75).

†Determined by SDS-PAGE.

of the wheal produced by the antigen preparation, measured in millimeters and D(1,his) and D(2,his) are the mutual perpendicular diameters of the wheal produced by histamine dihydrochloride (1.0 mg/ml), measured in millimeters.

RESULTS

Classification of birch pollen antigens as allergens

A crude aqueous extract of birch pollen contains approximately 40 distinct antigens, as determined by CIE (Fig. 1). The graded classification of some of the antigens as allergens was established by CRIE. Apparently only six antigens may be considered allergens (Fig. 2). The sera from 20 individual birch pollen-allergic patients all contained IgE, which bound to the precipitate of antigen 23, and 95% of the sera exhibited visible "radiostaining" of antigen 23 after 1 day of autoradiography. Antigens 25, 19, and 3 seemed to be intermediate allergens and the remaining two allergens may both be considered minor allergens. The radiostaining observed for antigens 5, 12, 19, and 25 seemed largely to originate from co-precipitated antigen 23, since the radiostaining of these precipitates in nearly all cases was restricted to the part of the precipitation arcs situated beneath the precipitation arc of antigen 23.

Isolation of birch pollen allergens

The major allergen (antigen 23) and a preparation containing mainly the minor allergens (antigens 25 and 19) were isolated from Bp by a combination of ion-exchange, size-exclusion, and chelate chromatography. Fig. 3 shows the fused-rocket immunoelectrophoresis pattern obtained for the individual steps in the purification procedure; the antigens were identified by CLIE performed with appropriate fractions (not shown). Fig. 3, D, shows the CLIE of the isolated allergens (CIE of reference with the isolated allergens in the intermediate gel). The antigen 23

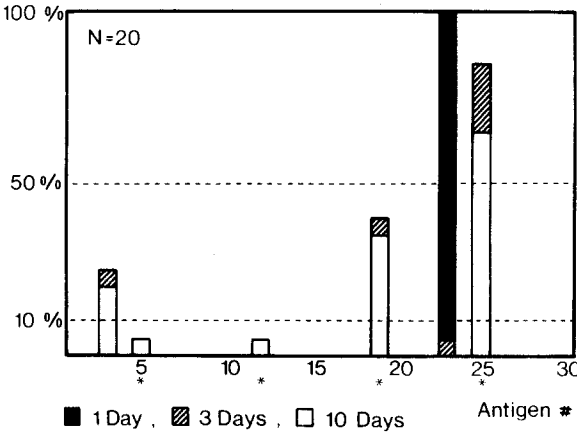


FIG. 2. Classification of Bp antigens as allergens (allergogram). The columns indicate the number of serum samples that, in CRIE, gave rise to visible radiostaining of the antigen after 1, 3, and 10 days of autoradiography. CRIE was performed with sera from 20 individual Birch pollen-allergic patients. Cold-dried CIE plates (as described in Fig. 1) were incubated with 0.5 ml of serum in 8.0 ml of 0.067M phosphate buffer, pH 7.0, for 24 hr at 25° C followed by incubation with approximately 140 nCi of ¹²⁵I-anti-IgE in 8.0 ml of phosphate buffer, pH 7.5, 0.3% (w/v) bovine serum albumin, 0.154M sodium chloride, 0.015M sodium azide, and 0.027M EDTA for 24 hr at 25° C. Plates were washed (0.154M sodium chloride, 1% [v/v] Tween 20) and subjected to autoradiography on Kodak XL-1 x-ray film. Asterisk, Possible co-precipitation.

preparation contained one nonallergenic component, as determined by CIE (Fig. 7, panel 7). The faint bands that were visible with SDS-PAGE (Fig. 4, A, lanes 4 and 5) and the apparent molecular weight of approximately 14,000 daltons seemed to indicate that the antigen 23 preparation may have contained one additional impurity. The amount of antigen 23 in this preparation was approximately 12 times that in Bp, as judged by quantitative immunoelectrophoresis. The antigen 25 and 19 preparation (25-19 preparation), besides some nonallergenic impurities, contained minute amounts of antigen 23. (Results of CIE and CRIE of the isolated allergens are shown in figure 7.)

Physicochemical parameters of the isolated allergens

The molecular weights of antigens 23, 25, and 19 (Table II) were estimated by size-exclusion chromatography (Fig. 3, B) and SDS-PAGE (Fig. 4, A) to be 17,000, 25,000, and 29,000 daltons, respectively. The pIs of the allergens (Table II) were determined by column isoelectric focusing to be 5.25, 5.5, and 5.0 (antigen 23); 5.0, 4.9, and 5.4 (antigen 25); and 6.2 and 5.4 (antigen 19) (Fig. 4, B).

The allergenic activity of the isolated allergens was

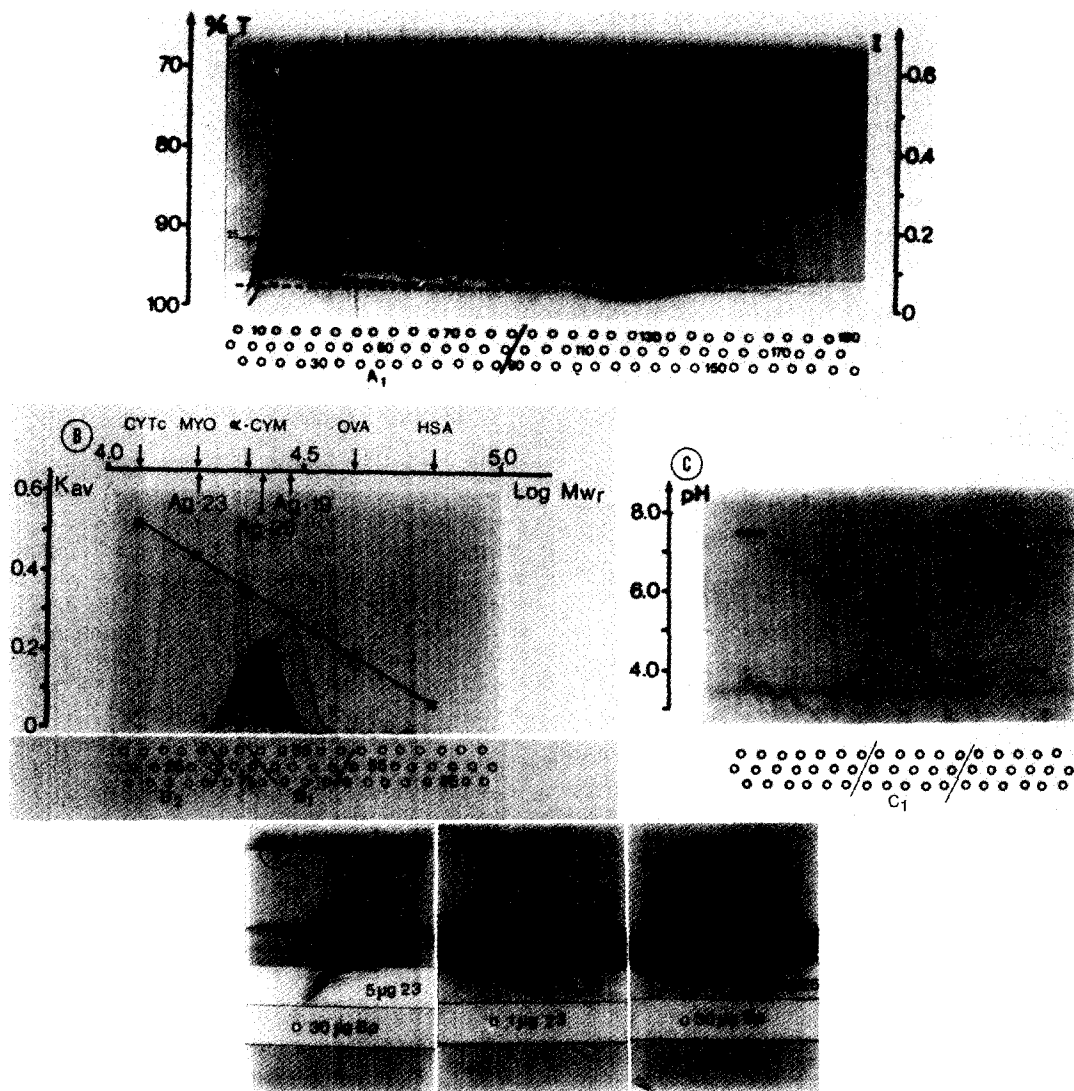


FIG. 3. Isolation of the major allergen (antigen 23) from birch pollen. **A**, Fused-rocket immunoelectrophoresis pattern obtained after ion-exchange chromatography of 100 mg of Bp on a DEAE-Sepharose CL 6B column. *Dashed line*, I of the elution buffer; *solid line*, ultraviolet transmission (%T, 280 nm). The fractions [A(1)] were pooled and lyophilized. **B**, Fused-rocket immunoelectrophoresis of the fractions obtained after size-exclusion chromatography of A(1) (88 mg including salt) on a Sephadex G-75 column. The molecular weights of antigens 23, 25, and 19 were estimated from the individual retention volumes as follows: K_{av} —antigen 23 = 0.427, antigen 25 = 0.318, antigen 19 = 0.290; human serum albumin (HSA), ovalbumin (OVA), chymotrypsinogen (α -CYM), myoglobin (MYO), and cytochrome c (CYTc) used as molecular weight references. The fractions B(1) and B(2) were each pooled and lyophilized. **C**, Fused-rocket immunoelectrophoresis of the fractions obtained after Cu-chelated chromatography of B(2) (10 mg) on an iminodiacetic acid-Sepharose CL 4B column. The horizontal bars indicate the pH of the buffers used for elution. The fractions containing the isolated antigen 23 [pH 5.0 to 4.0, C(1)] were pooled and lyophilized (3.2 mg). A preparation containing mainly a mixture of antigens 25 and 19 was isolated from Bp by repeated size-exclusion chromatography (G-75 not shown) of B(1). **D**, Identification of the isolated antigens by means of CLIE.

investigated by RAST inhibition, RAST screening, and skin prick testing. The RAST inhibitory effect of antigen 23 was determined to be 90% to 95%, indicating that antigen 23 was able to abolish the binding of 90% to 95% of the total amount of the specific IgE to

Bp discs. The inhibition curves obtained with antigen 23 as inhibitor were indistinguishable from those obtained with Bp as inhibitor (Fig. 5, A and B). The inhibitory capacity of the antigen 25-19 preparation was largely due to the minute amount of antigen 23 in

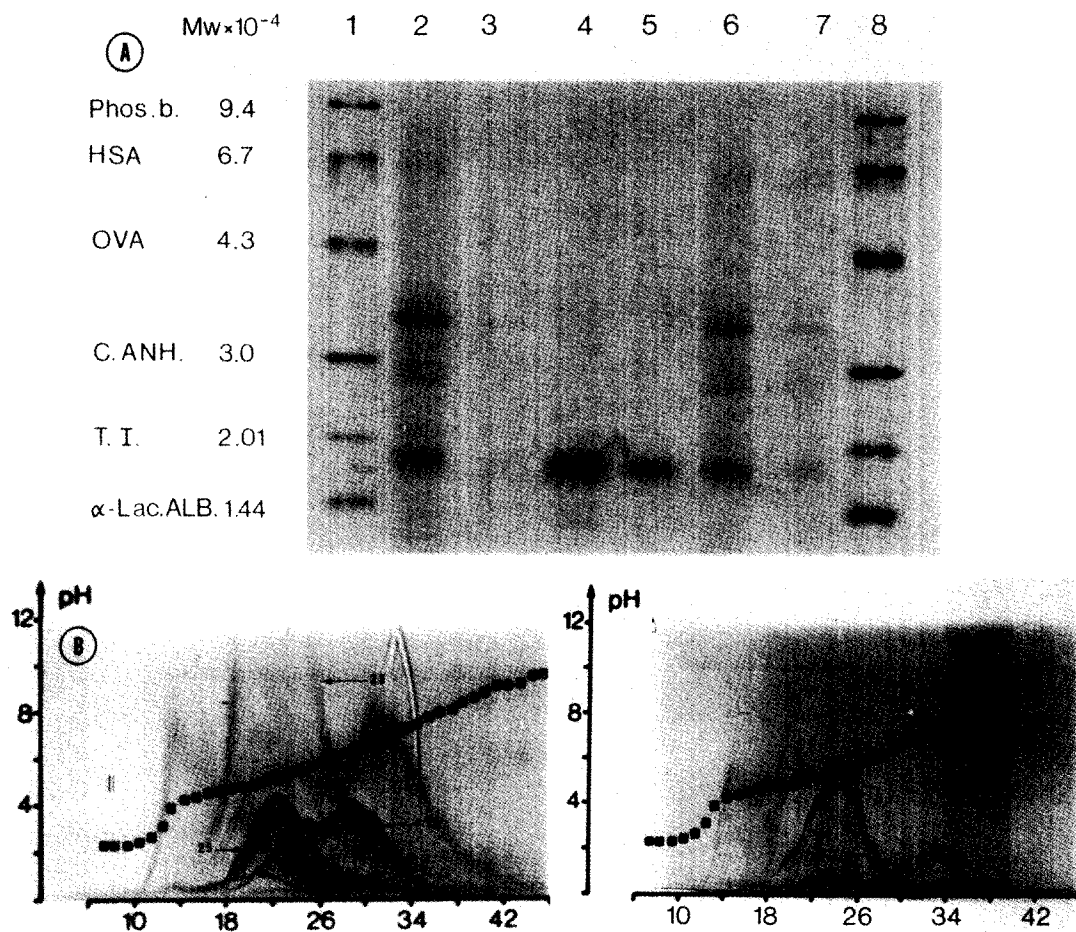


FIG. 4. A, Determination of the molecular weight (M_w) of antigen 23 by SDS-PAGE. Lanes 1, 8, Molecular weight references; lanes 2, 3, 6, and 7, 75, 10, 50, and 25 μ g of Bp; lanes 4 and 5, 15 and 5 μ g of antigen 23. The molecular weight references used were phosphorylase b (*Phos.b.*), human serum albumin (*HSA*), ovalbumin (*OVA*), carbonic anhydrase (*C.ANH.*), trypsin inhibitor (*T.I.*), and α -lactalbumin (α -*Lac.ALB.*). B, The pI determination of individual antigens by column isoelectric focusing of 50 mg Bp; fused-rocket immunoelectrophoresis was performed with either (left) 15 or (right) 5 μ l of each fraction eluted from the column.

this preparation (Fig. 7). The amount of Bp and antigen 23 preparation used in the inhibition experiments contained approximately 60 times as much antigen 23 as did the antigen 25-19 preparation; the inhibitory potency [I(50%) of Bp or antigen 23/I(50%) of antigen 25-19] of the antigen 25-19 preparation was 60 to 100 times less than that of either Bp or the antigen 23 preparation (Fig. 5, A and B). The inhibitory effects of antigens 25-19 corresponded to 10% to 20% of the inhibitory effect of Bp, since the antigen 23 preparation inhibited the IgE binding to antigen 25-19 discs by approximately 80% (Fig. 5, C). Fig. 6, A shows that IgE from individual Bp-sensitive patients was bound to antigen 23 discs to approximately the same extent that it was bound to Bp discs.

Results of skin prick testing (Fig. 6, B) showed that preparations of Bp and antigen 23 containing the same

amount of this allergen elicited approximately the same response in individual birch pollen-allergic patients whereas antigen 25-19 preparation elicited small responses if any at all.

Thermolabile and thermostable antigens of Bp

The boiling of a Bp solution for 15 min destroyed the antigenicity of all antigens but Nos. 23 and 13, as determined by CIE (Fig. 7). CRIE inhibition experiments performed with native and heat-treated Bp solutions as inhibitors showed that antigen 23 was the only allergen that retained most of its IgE binding capacity, since heat-treated Bp abolished the radiostaining of the antigen 23 precipitate to the same extent as did native Bp (Fig. 7). The radiostaining of the antigen 25-19 precipitates was completely abolished

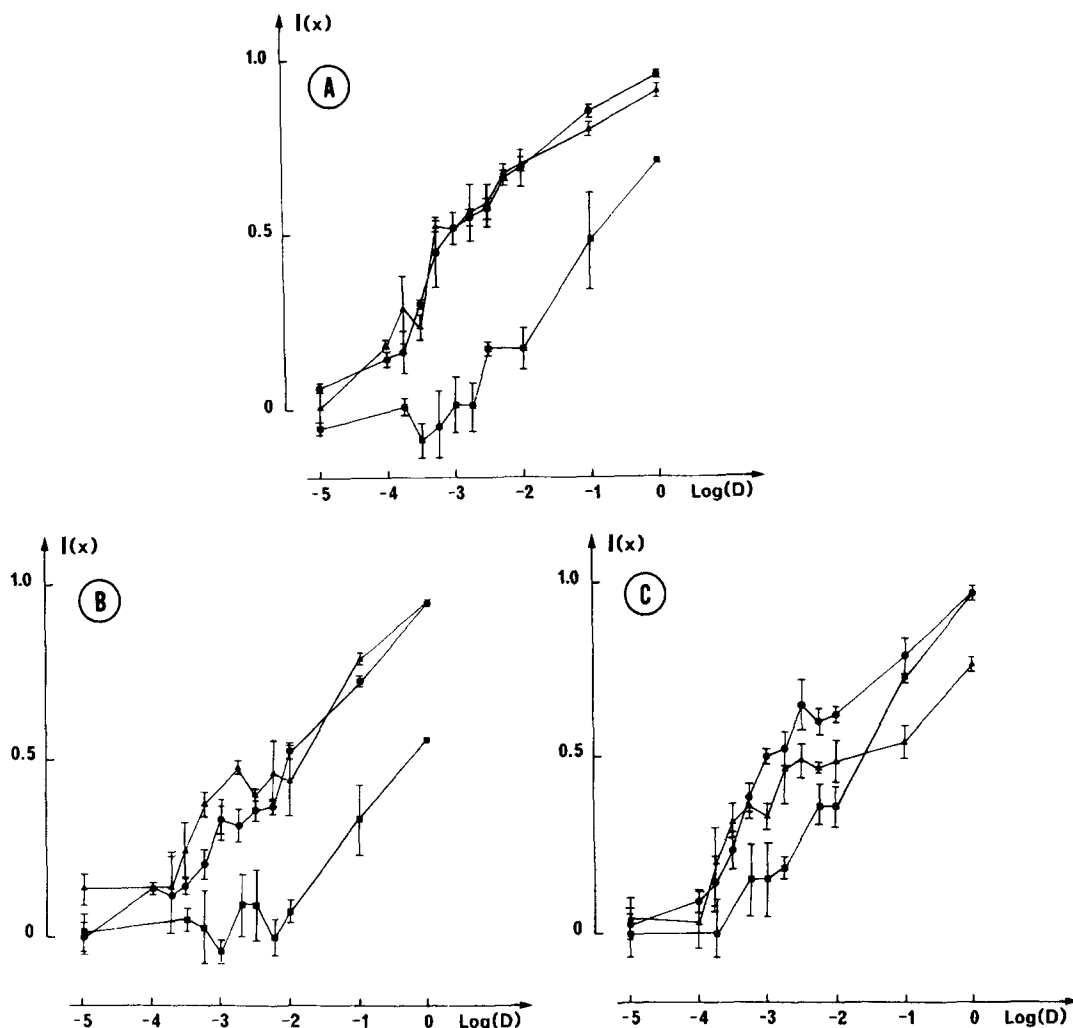


FIG. 5. RAST inhibition curves. Allergosorbents; **(A)** Bp (coupling concentration 40 $\mu\text{g}/\text{disc}$); **(B)** antigen 23 (coupling concentration 3.2 $\mu\text{g}/\text{disc}$); **(C)** antigen 25-19 (coupling concentration 40 $\mu\text{g}/\text{disc}$). Inhibitors: (●) Bp (undiluted inhibitor solution 10 mg/ml); (▲) antigen 23 (undiluted inhibitor solution 0.8 mg/ml); (■) antigen 25-19 (undiluted inhibitor solution 10 mg/ml). Allergosorbents and inhibitor preparations both contained the same amounts of antigen 23 or antigen 25 as did Bp. D , Dilution of the inhibitor solution; vertical bars, S.D. of duplicate determinations.

by native Bp, whereas heat-treated Bp only partly blocked the radiostaining of these precipitates.

DISCUSSION

An aqueous extract of birch pollen contains, as determined by CRIE, six allergens, but only one (antigen 23) fulfills the requirements for a major allergen.¹⁰ Antigen 23 seems to account for most of the allergenic activity of Birch pollen extract. The classification of antigens as allergens by CRIE is subject to the following limitations. (1) Only antigens actually precipitated in CIE may be revealed as allergens. (2) The rabbit a-Bv may to some extent pre-

vent the binding of IgE to the precipitate(s). (3) The human sera may contain so-called "blocking IgG," which can compete with IgE for the binding to particular precipitates. (4) Allergens may co-precipitate in nonallergen precipitates, which may give rise to misleading interpretation. Therefore the CRIE classification system should be used with protocols designed to reduce or rule out these pitfalls. The CRIE classification of antigen 23 as the major allergen, and the one that is by far the most important of birch pollen extract, is substantiated by results of RAST inhibition experiments, RAST screening, and skin prick testing.

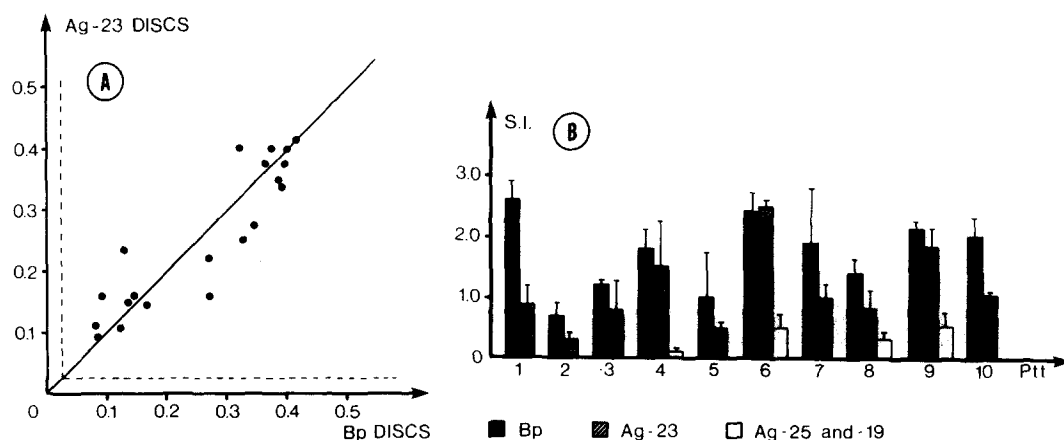


FIG. 6. **A**, RAST screening performed with serum samples from individual birch pollen-allergic patients ($n = 20$). Points, Fraction [cpm(bound)/cpm(added)] of ^{125}I -anti-IgE bound to the allergosorbent; solid line, expected relationship between the RAST experiments if antigen 23 is responsible for most of the allergenic activity of Bp; broken lines, nonspecific binding measured in a 1000-donor serum pool instead of the serum from allergic patients. **B**, Results of skin prick testing in 10 individual birch pollen-allergic patients. Tests were performed with 1.0 mg/ml Bp, 0.08 mg/ml antigen 23, and antigen 1.0 mg/ml 25-19. Bars, S.D. of the mean SI obtained.

The inhibitory effect of antigen 23 is 90% to 95% and the inhibitory capacity of Bp seems to be a function of the amount of antigen 23 in the extract, since preparations of Bp and antigen 23 containing equal amounts of this allergen exhibit indistinguishable inhibition curves and since the inhibitory capacity of the antigen 25-19 preparation partly corresponds to the minute amount of antigen 23 in this preparation.

RAST screening of the sera from 20 birch pollen-allergic patients shows that Bp and antigen 23 discs bind comparable amounts of specific IgE and that matched preparations of Bp and antigen 23 elicit similar responses in skin prick testing, indicating that antigen 23 accounts for most of the allergenic activity of Bp.

The CRIE investigation of Bp classifies antigens 25 and 19 as intermediate allergens, but this classification is uncertain because most of the radiostaining of these precipitates in CRIE presumably originates from co-precipitated antigen 23. CRIE experiments performed with the antigen 25-19 preparation show that these antigen precipitates exhibit far less radiostaining than the same precipitates in the Bp CRIE experiments, indicating that antigen 25 and 19 should be considered minor allergens. This classification is supported by results of RAST inhibition experiments and skin prick testing. Antigens 25 and 19 contribute only little to the allergenic activity of birch pollen extracts.

Antigen 23 is most probably identical to the thermostable allergen described by Belin^{1, 2} and Viander et al.,³ since it is the only thermostable allergen of Bp and since the molecular weight determined for this

antigen corresponds to the weights found by both of these authors. The allergen isolated by Apold et al.⁴ (mol wt = 29,000 daltons) and Vik et al.⁵ (mol wt = 10,000 daltons) seems to be different from antigen 23 because the molecular weights diverge considerably and the RAST inhibition potency reported for the 29,000-dalton allergen⁴ is much too low for a major allergen.

We would like to point out that classification of antigens as allergens should be performed with great caution and the classification should be based on as many methods as possible in order to avoid misleading conclusions.

Currently, further physicochemical characterization of antigen 23 is being undertaken in our laboratory in order to investigate the partial identity of antigen 23 and the major allergen from alder pollen (*Alnus glutinosa*) and one of the major allergens from hazel pollen (*Corylus avellana*).

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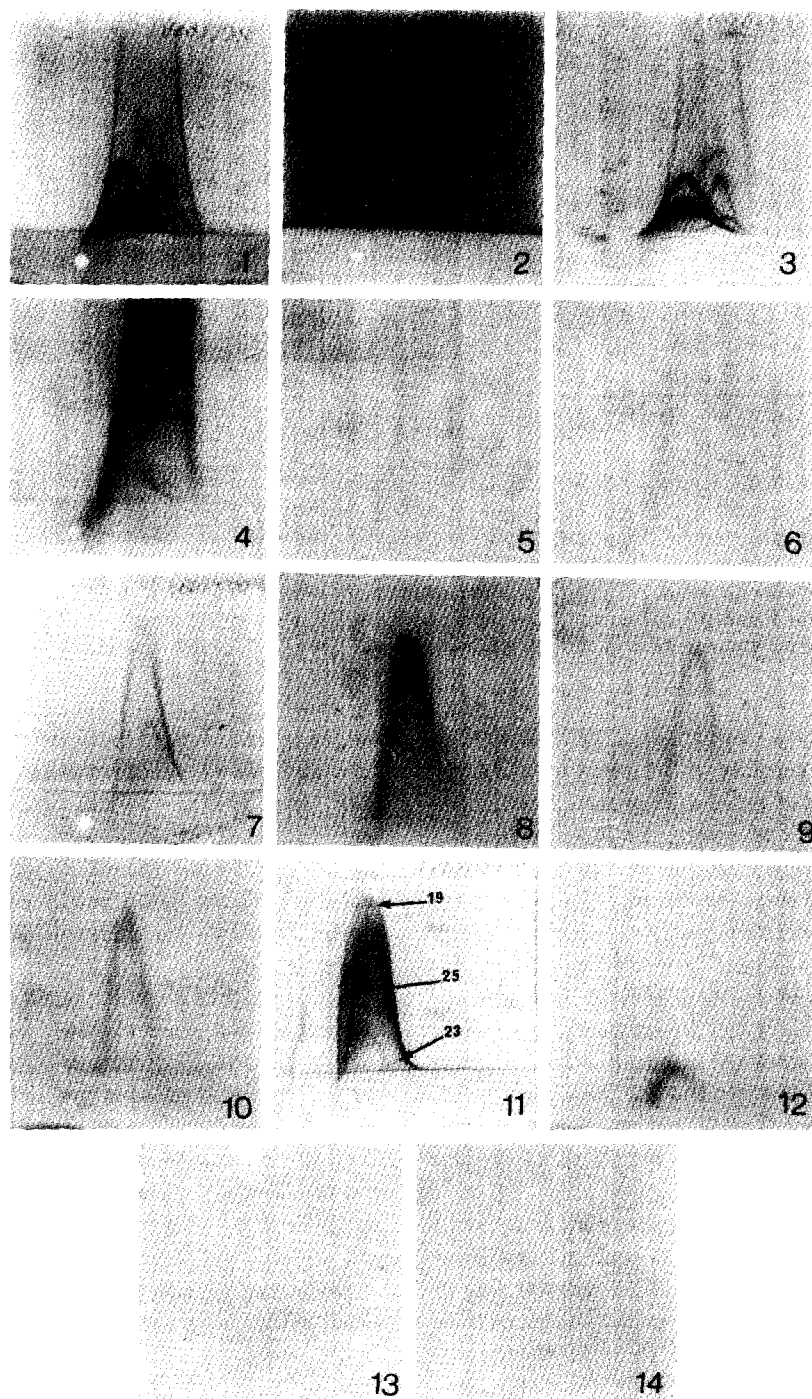


FIG. 7. Thermostability of Bp antigens. CIE performed with 30 μ g of native Bp (1) and 30 μ g of heat-treated Bp (2) (100° C, 15 min). CRIE autoradiographs (10 days) performed with CIE of Bp (3), antigen 23 (7), and antigen 19-25 (11) with the use of a serum pool incubated with buffer (4, 8, 12), native Bp (5, 9, 13), or heat-treated Bp (6, 10, 14).

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