

Comparative Studies on Tree-Pollen Allergens

III. A Purified Low Molecular Weight Major Allergen from Birch Pollen (*Betula verrucosa*) Isolated by Gel Permeation Chromatography

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Abstract. Gel permeation chromatography of *Betula verrucosa* (BV) crude pollenextract and pooling of the fractions after their fused rocket immunoelectrophoresis antigenic profile allowed the isolation of a purified major allergen designated BV⁴⁵. This fraction was shown to be a glycoprotein with a tentative molecular weight of 10,000 daltons. The estimated molecular weight differed by approximately factor 2 from the previously reported values of birch pollen major allergens. This fraction was further purified by polyacrylamide gel chromatography (P-10), high performance liquid chromatography (HPLC) silica-base column and high-volt electrophoresis at different dimensions. The purified proteins revealed an immunochemical identity with the initial material. BV⁴⁵ and the previously isolated pI 5.18, though different otherwise, demonstrated a reaction of complete antigenic identity illustrated by tandem-crossed immunoelectrophoresis. Both the amino acid and carbohydrate compositions of BV⁴⁵ were calculated on the basis of the tentative molecular weight obtained. BV⁴⁵ showed a total carbohydrate concentration of 20% of the dry weight, corresponding to 13-14 residues per mol protein. The amino acid composition obtained reflected structural diversity between the BV⁴⁵ and pI 5.18. N-terminal analysis followed by HPLC quantitation of the DNS-amino acids for BV⁴⁵ and the further purification products gave two peaks having retention times corresponding to DNS-Cya and DNS-Asp. Fraction BV⁴⁵ was demonstrated to be a major allergen of birch pollen as assessed by RAST inhibition of 7 out of 10 reaginic sera and the specific radiostaining in crossed radioimmunoelectrophoresis of all omitted nine crossed immunoelectrophoresis plates used. RAST inhibition titration of BV⁴⁵ and BV crude showed qualitative identity of the allergenic determinants of both materials. The question about one or several major allergen(s) in birch pollen extract has so far been unsolved as no structural mapping of BV⁴⁵ and pI 5.18 has been readily available.

Introduction

Previous studies showed that aqueous extract of the nondefatted birch pollen (*Betula verrucosa*) could be eluted by gel permeation chromatography into six separate peaks designated according to their relative elution volumes from the column: BV¹, BV², BV³, BV⁴, BV⁵ and BV⁶. The main allergenic proteins of this preparation could be detected in two peaks with a molecular weight range of 40,000 - 29,000 daltons (BV³ and BV⁴, respectively). The quantitatively dominant fraction (BV⁴) was subjected to isoelectric focusing fractionation resulting in four fractions with the isoelectric points 5.18, 5.42, 5.76 and 5.94. One of these fractions, BV⁴ pI 5.18, was favored for further

characterization on the basis of its high percent of RAST inhibition and relative yield [1]. Preparative isolation of this fraction by isoelectric focusing technique, though yielding a homogeneous protein, has been cumbersome.

The immunochemical reactivity of the different fractions of the gel permeation of BV crude was hence reinvestigated. Results showed that a good deal of the allergenicity was included in the fractions located in the valley between peaks BV⁴ and BV⁵ (fig. 1). However, no antigenic precipitates could be observed in this region as demonstrated by fused rocket immunoelectrophoresis of the eluted fractions. This was an impetus for new trials for rapid and simple alternatives for the isolation of the major allergen of fraction

BV⁴. Therefore, the region corresponding to fractions 49-70 (fig. 1), with the relatively low UV absorption, high scores of reaginic IgE antibody binding and yet apparently no antigenic precipitation, will be the subject of the detailed investigation in the present communication.

Material and Methods

The Crude Extract of Birch Pollen (*B. verrucosa*)

Nondefatted pollen grains, containing less than 0.5% foreign pollen was obtained from Allergon AB, Engelholm, Sweden. Dry pollen (10 g) was suspended into 100 ml NaCl-phosphate buffer, 75 mM at pH 7.2, and gently shaken for 3 h at +4°C. The extraction process was followed by centrifugation in cold for 45 min at 10,000 rpm. The supernatant was filtered to get rid of the rest of the fine debris and the filtrate was dialyzed against two changes of 12 liters of distilled water for a period of 48 h. The dialysate was thereafter lyophilized and the crude BV proteins were stored at -20°C until used.

Gel Permeation Chromatography

Swelled Ultrogel AcA-54 (LKB, Bromma, Sweden) was packed into a column (dimensions 100×1.5 cm) using phosphate-saline buffer, 75 mM at pH 7.2. The same buffer was used for the column equilibrium as well as the elution of the BV fractions. In each run, 70 mg BV crude dissolved in 0.5 ml buffer were applied. The resolved peaks were pooled, dialyzed against 12 liters of distilled water for 24 h, lyophilized and stored at -20°C [1].

Several matrices for gel chromatography were tried for achieving optimal resolution at the region between BV⁴ and BV⁵ (designated BV⁴⁵), using guanidin-HCl (500 mM) buffer for disaggregation. A satisfactory degree of purification was obtained by Bio-Rad P-10 fine column (dimensions 40×1.5 cm) using the same buffer. Approximately 30 mg of BV⁴⁵ were applied.

Further purification of Fraction BV⁴⁵

High performance liquid chromatography (HPLC) was performed using Water Associate equipment with a silica-based 'protein-column' I-125 supplied by the same manufacturer. The mobile phase was 0.05 M Na HPO₄-NaH₂PO₄ buffer with pH 7. Approximately 50 µg of fraction BV⁴⁵ were injected. The flow rate was 2 ml·min⁻¹ and the A_{280nm} was registered on a model 440 detector supplied by the manufacturer.

The degree of homogeneity was further examined on analytical thin layer polyacrylamide gel-ampholyte (TLC-PAG Ampholyte, LKB, Bromma, Sweden) isoelectric focusing, at a pH range of 3.5-9.5.

High-Volt Electrophoresis

High-volt electrophoresis was performed by the method described earlier [5].

Molecular Weight Estimation

The molecular weight data of BV crude fractions in the area of the peaks of BV⁴ and BV⁵ was estimated by exclusion chromatography on an Ultrogel AcA-54 column calibrated with molecular

weight marker globular proteins: BSA (67,000 daltons), ovalbumin (45,000 daltons), pepsin (34,000 daltons) and lysozyme (14,300 daltons). The estimated molecular weights were confirmed by HPLC reversed phase column as described above. The marker proteins used for calibration were: trypsinogen (24,000 daltons), β-lactoglobulin (18,400 daltons), lysozyme (14,500 daltons), ribonuclease (12,000 daltons) and insulin (6,000 daltons), respectively.

Amino Acid Analysis and Absorption Spectra

The proteins were hydrolyzed in 6 M HCl in vacuo for 24 h at 110°C and the amino acid composition was calculated by a computer integrator connected to a Kontron Liquimat III automatic amino acid analyser [5].

The UV absorption spectrum was obtained at the wave length range of 220-325 nm and proceeded in the visible region until 580 nm. The proteins were dissolved in 75 mM NaCl/phosphate buffer at pH 7.2 which was also used in the reference cuvette.

Immunization

Antibodies against BV crude were raised in 4 albino rabbits weighing approximately 3 kg each. The preparation of the antigens and the scheme of immunization were as follow: BV crude (2 mg) was dissolved into 0.5 ml NaCl/phosphate buffer, 75 mM, pH 7.2, and 0.5 ml Freund's incomplete adjuvant. Suspensions were thoroughly emulsified prior to injection. The animals were injected on days 0, 14, 28 and 42. The injections were given intradermally in the intrascapular regions; each dose was distributed into nine sites. On the 10th day after the last injection, the animals were bled and sacrificed under anesthesia [11].

Human Sera

Sera were collected from 10 patients allergic to birch pollen (positive clinical history and radioallergosorbent test (RAST) grades 2-4 to birch allergosorbents). None of the selected patients had been through hypsensitization therapy. Individual or pooled sera from these patients were collected and stored at -20°C until used [1]. A control serum from an individual allergic to timothy but tolerating birch under the same criteria was used.

Immunoelectrophoretic Techniques

Crossed immunoelectrophoresis (CIE), crossed-line immunoelectrophoresis (CLIE), tandem-crossed immunoelectrophoresis (tandem-CIE) and fused rocket immunoelectrophoresis were performed as described by Weeke [19], Krøll [13, 14], Svendsen [17], and Axelsen [3].

In crossed radioimmunoelectrophoresis (CRIE), the CIE plates were dried at room temperature and no staining was performed. The glass plates were immersed in the incubation buffer (6.8 ml of 75 mM NaCl/phosphate containing w/v 0.3% sodium azide, 2% Tween 20 and 0.1% EDTA at pH 7.2) and 0.2 ml of the reaginic serum for a period of 24 h. The gels were washed to remove the non-bound proteins, four times at 10-min intervals with 10 ml of physiological saline solution. The preparations were further incubated in 0.5 µCi ¹²⁵I-anti IgE (Pharmacia AB, Uppsala, Sweden) dissolved in 7 ml of the incubation buffer. The preparations were allowed to stand for 24 h. Thereafter the washings proceeded four times for 10 min in 10-ml physiological saline solution followed by a single wash with 10 ml distilled water. Autoradiography was performed using hot air-dried plates placed on an X-ray film in a light-proof chamber. Exposures varied from several days to 4 weeks [2, 15].

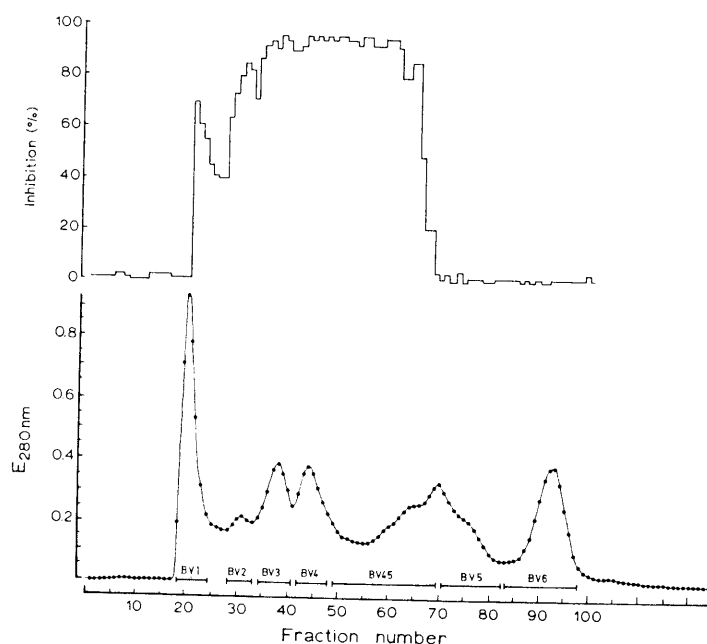


Fig. 1. The elution chromatogram of BV crude aqueous extracts on the Ultrogel AcA-54 column (modified from fig. 2 in ref. 1). Fraction BV⁴⁵ was pooled according to the antigenic profile shown in figure 3. The other fractions, BV¹ - BV⁶, were located as underlined. BV⁴⁵ possessed relatively high allergenic reactivity as illustrated by the RAST inhibition curve.

CRIE was performed on 10 CIE plates of fraction BV⁴⁵. Nine individual sera from patients allergic to *B. verrucosa* and a control serum were tested.

Fused rocket radioimmuno-electrophoresis (FRIE) was based on a combination of the conventional fused rocket immuno-electrophoresis [17] and binding of reaginic IgE antibodies which then could be visualized by autoradiography using ¹²⁵I-anti IgE. In each of the sample wells, 10- to 13- μ l aliquots of the eluted fractions were applied. In the antibody gel 750 μ l anti BV crude were incorporated. Autoradiography was then performed on the fused rocket plates as initially described in this section, except for using larger volumes of the incubation buffer and washing solutions.

RAST and RAST Inhibition

In vitro tests of the allergenic reactivity of the different preparations were essentially performed as described by Yman et al. [21] and Florvaag et al. [7].

N-Terminal Analysis

End-group analysis was done by the dansyl-Edman method described by Gray [9]. The dansyl amino acids were identified and quantitated by reversed phase HPLC on a single column. A spectrofluorometric detector, SFM-23 Kontron, was connected to the HPLC outfit. The degraded DNS-amino acids were investigated at emission and excitation bands of 533 and 361 nm [6, 20].

Carbohydrate Analysis

Samples of 1.5 mg were subjected to methanolysis in 1 M HCl in CH₃OH for 24 h at 80°C, after the addition of 50 μ g of mannitol as internal standard. The methanolysate was treated as described previously [16] and the resulting methylglycoside was converted to the corresponding trimethylsilyl ethers (TMS-derivatives). The mixtures were subjected to analysis by gas chromatography and gas chromatography coupled to mass spectrometry. Qualitative and quantitative analyses were performed on a Carbo Erba 4200 gas chromatograph fitted with 25 m SE-30 glass capillary column, using H₂ as carrier gas and flame ionization detector (FID). The injection temperature was 70°C, followed by a rapid increase to 150°C, then 2° per minute up to 200°C. The identity of the monosaccharides was also confirmed by gas chromatography coupled to mass spectrometry. The gas chromatograph was coupled with a Micro-mass 7070 F mass spectrometer with Vg system (2200 data system).

Results

Production of BV Crude

A yield of approximately 1.5% w/w was obtained in each run of BV crude extraction of the pollen. For

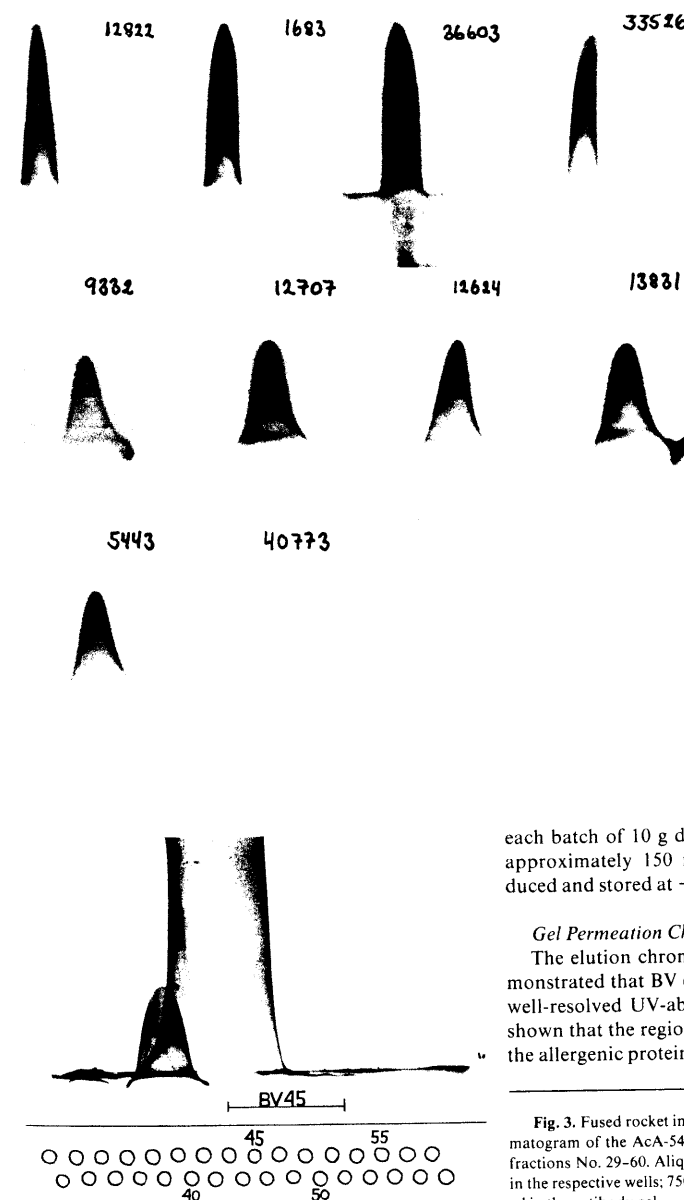


Fig. 2. CRIE preparations of nine CIE plates of fraction BV⁴⁵ with nine birch pollen reaginic sera. The experiment was performed at different exposure times; the radiostaining illustrated was intensified by longer exposure of the X-ray plates (4 weeks). The specificity of the blacking was ascertained by control serum No. 40773 from a patient allergic to timothy but tolerating birch under the same criteria.

each batch of 10 g dry nondefatted grains a yield of approximately 150 mg BV crude was usually produced and stored at -20°C until utilized.

Gel Permeation Chromatography of Fraction BV⁴⁵

The elution chromatogram showed in figure 1 demonstrated that BV crude could be separated into six well-resolved UV-absorbing peaks. RAST data have shown that the region between BV² and BV⁵ included the allergenic proteins with the maximal IgE antibody

Fig. 3. Fused rocket immuno-electrophoresis of the elution chromatogram of the AcA-54 column, shown in figure 1, representing fractions No. 29-60. Aliquots of 13 μ l of the fractions were applied in the respective wells; 750 μ l rabbit anti-BV crude were incorporated in the antibody gel.

binding. Fractions in the region of BV⁴⁵ of the elution chromatogram were pooled. The UV absorption spectrum of these fractions scanned at 220–325 nm showed a typical spectral profile of proteins with a peak at 280 nm. No UV absorption was observed in the region of expected absorption of the carbohydrates.

CIE plates of fraction BV⁴⁵ showed a single immunoprecipitation line. All the nine CRIE preparations of the BV⁴⁵ gave a high degree of specific radiostaining of the films. The blacking of the films was moderate after 1 week and became intense with longer exposures.

The control plate incubated with the serum from a patient allergic to timothy but tolerating birch gave no blacking of the film, ascertaining the immunological specificity of the radiostain (fig. 2).

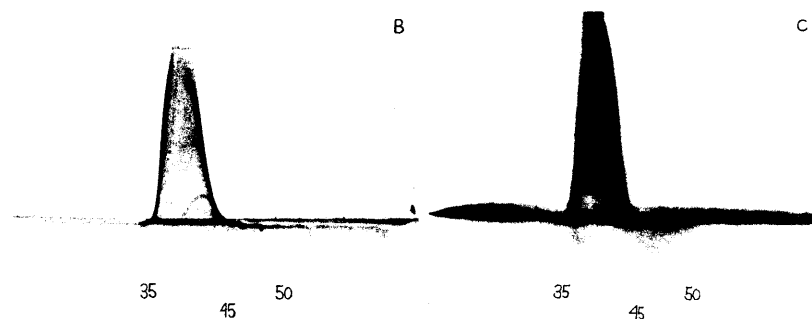
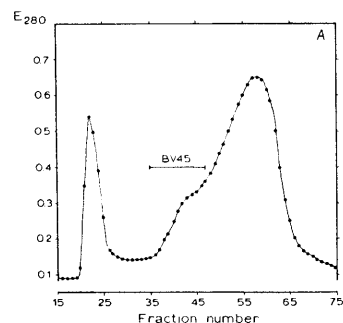


Fig. 4. Rechromatography of BV⁴⁵ on the Ultrogel AcA-54 column showing the location of BV⁴⁵ (A). Aliquots of the elution fractions underwent fused rocket immunoelectrophoresis and the antigenic pattern was illustrated in B. The plate was further incubated

Fused rocket immunoelectrophoresis of the fractions of BV crude from the AcA-54 column gave precipitation lines for all the fractions starting with the BV² peak and proceeding to the BV⁵ peak. One precipitation line, the most dominant in the region of BV⁴–BV⁵, was found to be a common antigen covering both BV⁴ and BV⁵ (fig. 3).

Rechromatography of fraction BV⁴⁵ using the same AcA-54 column gave two distinct peaks. A fused rocket immunoelectrophoresis of these fractions gave a single precipitation line located in the region of the second peak having the same relative elution volume as the beginning of peak BV⁵. Autoradiography of this preparation (FRIE) showed that this line of precipitation specifically bound reaginic IgE antibodies (fig. 4).

Further Purification of Fraction BV⁴⁵

Rechromatography of BV⁴⁵ on a Biorad P-10 column allowed the isolation of two peaks designated BV⁴⁵¹ and BV⁴⁵². UV spectral scanning of the void volume peak showed an identical protein pattern to that of the initial BV⁴⁵. Random and weak absorption was obtained when scanning the second peak.

The CIE Coomassie blue-stained plate of BV⁴⁵ showed identical single immunoprecipitate to that of BV⁴⁵². No lines of precipitation could be seen in CIE from BV⁴⁵². Autoradiography of one of the CIE plates of BV⁴⁵¹ (CRIE) gave a single radiostain of the film of few days' exposure. The blacking became deeper on longer exposure times.

with a reaginic serum, the IgE-antibodies were visualized by the autoradiography shown in C. The exposure time of the FRIE plate was 4 weeks.

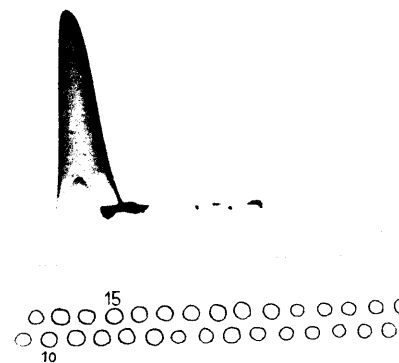
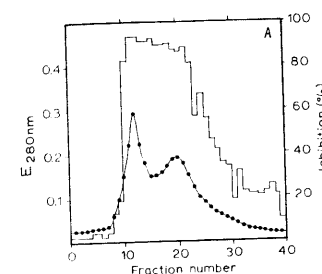


Fig. 5. Further purification of BV⁴⁵ on the polyacrylamide agarose column (P-10). The void volume peak represented BV⁴⁵¹. The location was demonstrated by the RAST inhibition diagram in A and supported by the fused rocket immunoelectrophoresis antigenic pattern shown in B.

Fused rocket immunoelectrophoresis of the fractions eluted from the P-10 column gave one dominant precipitate corresponding to the first void volume peak. The same immunoprecipitate showed a remarkable affinity of binding specific IgE from birch pollen-allergic patients' serum-pool used in FRIE (fig. 5).

Homogeneity of BV⁴⁵

The results of HPLC 'protein column' used for further purification of BV⁴⁵ showed that this fraction could be separated into one main symmetrical peak and other minor contaminating ones (fig. 6). These contaminants were not detected on BV⁴⁵¹ chromatograms.

The results of the N-terminal analysis of fraction

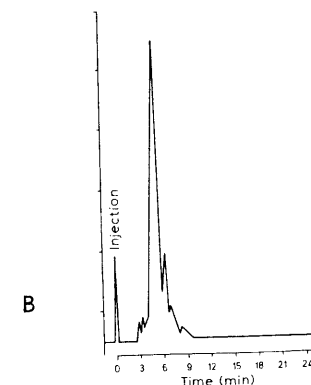


Fig. 6. The elution chromatogram of BV⁴⁵ from a silica-based 'protein column' [25] of HPLC. Solvent = 0.05 M Na₂HPO₄-NaH₂PO₄ buffer at pH 7.0; flow rate = 2 ml·min⁻¹; detection = A_{280 nm} 0.1 AUFS; sample size = 100 µl.

BV⁴⁵ subjected to dansyl-Edman degradation prior to HPLC analyses of the DNS-amino acids, showed two peaks with retention times corresponding to DNS-Cys and DNS Asp. Considering an approximately similar response of the two amino acids, the concentration was calculated to be almost equal. Analysis of the BV⁴⁵ showed lower concentration of DNS-Asp. A similar tendency was also found for the HPLC-purified BV⁴⁵. However, some of the later analyses showed nearly equal concentrations of both amino acids.

High-volt electrophoresis of BV⁴⁵ showed one major fluorescent band together with some faint ones. In addition, slight nonmigrating protein could be seen at the application line. The main band could be eluted from the paper and the immunological reactivity could be reproduced.

TLC PAG-Ampholine plates illustrated a similar homogeneity: one band for BV⁴⁵ together with some slight contaminants.

In conclusion, fraction BV⁴⁵¹ and HPLC-purified BV⁴⁵ were of higher degree of purity than the initial BV⁴⁵.

The Antigenic Identity of Fractions pI 5.18 and BV⁴⁵

Tandem-CIE results of fraction pI 5.18 [1] and BV⁴⁵ showed a reaction of complete identity between

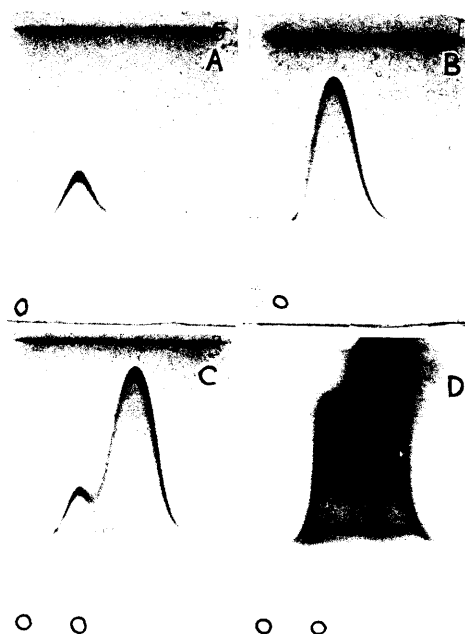


Fig. 7. A CIE preparation of BV pI 5.18 [1]. B CIE of BV⁴⁵ indicating a similar antigenic homogeneity to fraction pI 5.18. C Tandem-CIE of fractions pI 5.18 and BV⁴⁵ in the first and second wells, respectively, emphasizing a reaction of complete antigenic identity between the two proteins. D Autoradiography of a tandem-CIE plate of a different run than that shown in C. The figure demonstrated identical allergenic determinants in both fractions. The amounts of the proteins applied were 25 µg per sample.

the antigenic lines of these two antigens. CRIE of the tandem-CIE plate of these antigens illustrated that both precipitates could interact with specific IgE antibodies at the same grade (fig. 7).

Molecular Weight Estimation, Amino Acid Analyses and Carbohydrate Composition

Both results of the AcA-54 gel filtration and the 'protein column'-HPLC gave a value of approximately 10,000 daltons for the molecular weight of BV⁴⁵.

The tentative molecular weight of 10,000 daltons including the carbohydrate part of the molecule was used for the calculation of the amino acid composition depicted in table I.

Table I. The amino acid composition of fraction BV⁴⁵ isolated by gel permeation chromatography of BV-crude.

Amino acid	Concentration µmol/l	R/100 R	Nearest integer
Aspartic acid	167.9	8.61	(8-9)
Threonine	78.6	4.01	(4)
Serine	103.0	5.27	(5)
Glutamic acid	250.8	12.86	(13)
Glycine	249.1	12.77	(13)
Alanine	156.6	8.03	(8)
Half cystine	49.2	2.52	(2-3)
Valine	110.5	5.66	(5-6)
Methionine	28.4	1.45	(1-2)
Isoleucine	91.7	4.70	(5)
Leucine	87.2	4.47	(4-5)
Tyrosine	33.6	1.71	(1-2)
Phenylalanine	44.9	2.29	(2)
Lysine	143.8	7.37	(7)
Histidine	28.5	1.46	(1-2)
Arginine	34.1	1.74	(2)
Tryptophan	ND	-	-
Total		84.92	(81-88)

The protein was hydrolysed in 6 M HCl *in vacuo* at 110°C in sealed glass tubes for 24 h. The composition was calculated on a tentative molecular weight basis of 10,000 including the carbohydrate.

ND = Not done.

Table II. The carbohydrate content of BV⁴⁵ determined by gas chromatography

Carbohydrate	Weight percent	Residue per mol
Arabinose	6.0	4.5 (4-5)
Rhamnose	2.4	1.7 (1-2)
Fucose	1.0	0.7 (1)
Xylose	1.2	0.9 (1)
Galactose	7.6	4.7 (4-5)
Glucose	1.7	1.1 (1)
Total	19.9	13.6 (13-14)

BV⁴⁵ was shown to be a glycoprotein containing about 20% w/w carbohydrate. Arabinose and galactose were the dominating monosaccharides.

The carbohydrate composition is given in table II. It shows that the total carbohydrate concentration of BV⁴⁵ was approximately 20% of the allergen dry weight. The amount of pentoses and hexoses corresponds to 13-14 residues per mol BV⁴⁵.

RAST and RAST Inhibition

The results of RAST inhibition performed on the elution fractions of BV crude chromatographed on the AcA-54 column showed values varying from 75 to 95% inhibition (fig. 1). When using the same allergosorbents and inhibiting by elution of BV⁴⁵ on the P-10 column, a value of 85-95% was shown in the region of the void volume peak (BV⁴⁵¹). The percentage of inhibition was strongly reduced in the region of BV⁴⁵².

RAST inhibition titration curves of fraction BV⁴⁵ and BV crude at initial equal concentrations showed qualitatively similar grades of inhibition. They ran parallel and showed similar maximum inhibition indicating qualitative allergenic identity.

Direct RAST using BV⁴⁵ allergosorbents showed that 7 out of 10 reaginic sera gave high binding to the paper discs.

Discussion

Recent studies on the isolation of major allergen from birch pollen using a combination of gel permeation chromatography and isoelectric focusing resulted in the isolation of an allergenic protein pI 5.18 with a molecular weight of 29,000 daltons [1]. Correlation of the A_{280 nm} of the fractions BV⁴ and BV⁵ in this study with their antigenicity and allergenicity urged to extend the search for a more adequate isolation of major allergen(s). Fused rocket immunoelectrophoresis of the fractions eluted from the AcA-54 column recognized more antigenic homogeneity at the tail of peak BV⁴ rather than at the fractions of maximum absorption. Irrespective of the relatively low A_{280 nm} of the fractions located between BV⁴ and BV⁵, they certainly gave high bindings of reaginic IgE antibodies. Nevertheless, control CIE plates of the six pooled BV fractions, occasionally showed distinct homogeneous antigenic precipitation in the region of BV⁵.

In view of these observations, the implication of isoelectric focusing, with its practical disadvantages, was disputed. The new rational pooling of fractions marked BV⁴⁵ in figure 1, hence served to warrant an optimal selection for an allergen with a satisfactory grade of purity.

Further purification of fraction BV⁴⁵ on P-10 column gave a main void volume peak BV⁴⁵¹ and a retarded diffused peak of contaminants BV⁴⁵². The antigenic and allergenic reactivity of BV⁴⁵ were demonstrated to be merely related to the main peak as as-

sessed by CIE and CRIE respectively. No immunological reactivity was contributed by BV⁴⁵².

HPLC and high-volt electrophoresis purification of BV⁴⁵, respectively, gave one main fraction and minor heterogeneous low molecular weight contaminants. The initial fraction BV⁴⁵ isolated by gel filtration and pooled according to the antigenic profile provided by fused rocket immunoelectrophoresis, seemed to be of a fair grade of homogeneity.

Fraction BV⁴⁵ gave a reaction of complete antigenic identity to pI 5.18, in spite of the structural diversity reflected by their amino acid compositions (table I) [1].

A detailed study on the carbohydrate part of the molecule is still lacking. No information has been available as to the length of the oligosaccharide chains, the mode of their linkage to the protein core or influence on the molecular size and conformation. However, interesting similarities were found in the carbohydrate composition of this allergen with that of the carbohydrate present in allergen preparations from other pollen sources, under investigations in our laboratories [7, 10, 18, 8].

BV⁴⁵ was clearly demonstrated to be a major allergen with a molecular weight differing by 'at least' factor 2 from the values reported for allergens from birch pollen [1, 4, 12]. It could bind specifically reaginic IgE antibodies of 7 out of 10 sera selected from birch pollen allergic patients, with remarkably high affinity. Similar evidence was revealed by the autoradiography of all the nine CIE plates of BV⁴⁵ used. The specificity of the radiostaining was ascertained by a control serum from a patient allergic to timothy but tolerating birch, giving no blacking of the X-ray film.

RAST inhibition titration curves of fractions BV⁴⁵ and BV crude illustrated similar maximum inhibition and the two curves were parallel, indicating qualitative allergenic identity.

An ultimate evaluation of the presence of one or several major allergen(s) in BV crude, could not be settled so far, awaiting future developments on the structural relationship between the two major allergens isolated in our laboratory.

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