

Isolation and characterization of the 18-kDa major apple allergen and comparison with the major birch pollen allergen (*Bet v* I)

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The major allergen from birch pollen, *Bet v* I, and the cross-reacting 18-kDa major allergen from Golden Delicious and Granny Smith apples were isolated by micropreparative SDS–PAGE followed by electroelution. In the case of apples, highly active, low-temperature extracts were used. The purity of the allergens was checked by analytic SDS–PAGE and immunoblotting with allergic patients' sera, as well as by N-terminal amino acid microsequencing, and the allergens were found to be very pure. The strong immunologic activity of the isolates was determined by the enzyme allergosorbent test (EAST) and EAST inhibition assays; this activity was, in the case of *Bet v* I, similar to that of a preparation obtained by monoclonal antibody affinity chromatography. The allergenic potency of *Bet v* I and of the cross-reactive apple allergen was determined by EAST inhibition and dose-related histamine release. With both assay systems, the allergenic reactivity of *Bet v* I was considerably higher than that of the major apple allergen. Furthermore, skin prick tests with the purified allergens and with whole allergenic extracts were performed on a group of 33 patients suffering from birch-pollen and apple hypersensitivity, and on a control group of 10 patients. The frequency of positive prick test results in the allergic patient group ranged from 73% for the major allergen from Golden Delicious apples to 97% with *Bet v* I and whole birch pollen extract, respectively. In contrast to our low-temperature extracts, commercial prick test solutions of four different manufacturers were found to be unreliable for the diagnosis of apple allergy. The skin test results again indicated the strong immunologic activity of the allergen isolates and the predominance of the major allergens in context with birch-pollen and apple hypersensitivity. Taken together, the results support the view that the 18-kDa major allergen represents most of the allergenicity of the apple fruit, and that all allergenic epitopes of the apple proteins are present on *Bet v* I.

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Hay fever caused by birch pollen is very common in northern and central Europe. Together with this pollinosis, IgE-mediated hypersensitivities to many kinds of fresh fruits, nuts, vegetables, and spices occur (3–5, 8, 10, 13, 18, 19, 22, 23, 27, 38). Cross-reactions of allergen-specific IgE antibodies with food allergens and pollen proteins have been considered to be the reason for the clustering of hypersensitivities (1, 3, 7, 11, 12, 21, 29, 31). In this context, many authors found evidence that all of the allergenic epitopes of the fruits occur in the birch pollen extract, but, on the other hand, the fruit proteins have only a few epitopes of the birch pollen

allergens (1, 7, 11, 17, 21, 25, 32). It has been hypothesized that the pollen allergens act as the primary immunogens and that the cross-reactions are caused by epitopes of fruit proteins with a lower affinity to the allergen-specific IgE of the patients (1, 5, 7, 29).

Among the birch pollen-related food hypersensitivities, apple allergy is highly prevalent. From 50 to 93% of birch pollen-allergic patients suffer from apple and hazelnut allergy (5, 7, 8, 13, 22, 23, 27). Apple allergy has therefore been studied as an immunologic model which is typical of the whole phenomenon.

It has recently been recognized that the major

birch pollen allergen, *Bet v I*, and a homologous 18-kDa apple protein are responsible for the majority of allergenic cross-reactions between birch pollen and apples (7, 32). Ebner et al. (7) detected homologous structures on these two allergens at the nucleic acid level by Northern blotting and at the protein level by Western blotting. We were able to confirm these results by immunoblot experiments at the protein level (32). Our ensuing investigations to characterize the apple protein by two-dimensional immunoblotting and N-terminal microsequencing revealed 62% sequence identity of 26 amino acid residues with *Bet v I* (35). Furthermore, it may be assumed that the allergen consists of two isoproteins with nearly identical isoelectric points. In contrast to *Bet v I*, which consists of at least 10–20 isoproteins (14, 15), the apple allergen has a lower microheterogeneity. The 18-kDa allergen has been considered to be a major apple allergen, because more than 60% of patients suffering from apple allergy have IgE against this protein (7, 34, 36). Since the apple allergen had not been purified, previous studies of cross-reactivity to *Bet v I* could be carried out only with the whole apple extract (7, 29, 32). Therefore, the aim of our present study was to isolate the major allergens of birch pollen and apple, and to characterize their allergenic properties further in a comparative study.

Material and methods

Allergenic extracts

Commercial birch pollen extract, containing 1100 µg/ml of protein, was obtained from Allergopharma (Reinbek, Germany).

Apple extracts were prepared from mature, fresh Golden Delicious and Granny Smith apples by a low-temperature method, as previously described (31, 35). Protein concentrations of the apple extracts were determined by a modified Bradford assay (31) using bovine serum albumin as the standard, and ranged typically between 150 and 300 µg/ml (31). The crude protein extracts were stored freeze-dried at -20°C until use.

For skin tests, commercial apple prick test solutions were obtained from Abelló (Bornheim, Germany), ALK (Copenhagen, Denmark), Allergopharma (Reinbek, Germany), and Bencard (Neuss, Germany).

Allergen isolate *Bet v I* (A)

Bet v I isolated from birch pollen by monoclonal antibody affinity chromatography (*Bet v I* (A)) was kindly supplied by Allergopharma (Reinbek, Germany) (37).

Patient sera

Sera Bo 44 and Bo 46 from patients with allergy to birch pollen and apple were obtained from Hospital Borkum Riff, Borkum, Germany (Dr H. Aulepp). The diagnosis was based on anamnesis, RAST, and skin tests. In addition, the two pool sera Wn 13/32 ($n = 7$, RAST classes: birch pollen 3, Golden Delicious apple 2) and Zie 39/32 ($n = 12$, RAST classes: birch pollen 3, Golden Delicious apple 2) were kindly supplied by Allergopharma (Reinbek, Germany). We selected these sera on the basis of RAST results, but the patient's clinical history of food allergy was unknown. As negative control, the serum Zie from a nonatopic individual was used.

Monoclonal antibodies

"3F8", a mouse monoclonal antibody (mAb) raised against the 18-kDa apple allergen, was kindly supplied by Dr B. Fahlbusch, Department of Clinical Immunology, University of Jena, Germany (9). The *Bet v I*-specific mouse mAb "BIP 1" was a gift from the research group of Prof. Dr D. Kraft, Institute of Experimental Pathology, Vienna, Austria (16). Hybridoma supernatants of both antibodies were used.

SDS-PAGE

Discontinuous SDS-PAGE was performed according to Laemmli (20). As previously described, 5% (w/v) acrylamide stacking gels and 13% (w/v) resolving gels were used (30). The cross-linker concentration was 0.13% (w/v) in the stacking gels and 0.35% (w/v) in the resolving gels.

For allergen isolation, 200 µg of total protein from birch pollen extract, 225 µg of protein from Golden Delicious extract, and 300 µg of protein from Granny Smith extract were loaded on the gels (55-mm preparative slots). After electrophoresis, proteins were stained with copper(II) chloride as described by Tessmer & Dernick (26).

For purity control of the isolated proteins, analytic SDS-PAGE, followed by silver staining, was applied (30).

Electroelution

Protein bands of the 18-kDa apple allergen and of *Bet v I* made visible by copper staining were cut out to form gel slices equal in size (width of 2 mm). Destaining of the gel slices was carried out as described by Tessmer & Dernick (26). Electroelution was performed in the "Centrilutor" apparatus (Amicon, Witten, Germany). Elution buffer (stock solution: 0.025 M Tris/0.192 M Glycin/0.0017 M SDS) was used in a twofold dilution. Proteins were elec-

troeluted at 200 V and 20 mA at room temperature for 3.5 h into Centricon microconcentrators (cutoff: 3 kDa, Amicon, Witten, Germany). Bubbles developing at the bottom of the microconcentrators during elution were removed with a 45° bend pipette. Afterward, proteins were washed twice with 300 µl 0.01 M phosphate buffer (0.01 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.4) and concentrated in the Centricon units already used for elution at 4°C. For quantitative removal of the isolated proteins, the microconcentrators were rinsed out with 50 µl 0.01 M phosphate buffer, and the proteins were freeze-dried. The amount of the isolated proteins was determined as described for allergenic extracts.

Microsequencing analysis

Microsequencing of the N-terminal amino acids by Edman degradation was performed with a 473A pulsed liquid protein sequencer with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

Enzyme allergosorbent test and inhibition assays

Isolated proteins and whole protein extracts were coupled to cyanogen bromide activated filter paper disks, as described previously (31), using protein concentrations of 1.5 µg/ml (isolated proteins) and 12 µg/ml (protein extracts) in the coupling solution. Specific IgE was determined by a commercially available enzyme allergosorbent test (EAST) according to the manufacturer's instructions (Allergopharma, Reinbek, Germany). The EAST system used a monoclonal anti-IgE alkaline phosphatase conjugate and *p*-nitrophenylphosphate as the substrate. Absorbances were measured at 405 nm, and the reference system of the manufacturer was used for quantification. The results were expressed in EAST classes and in units per ml (U/ml, according to Phadebas RAST units).

Competitive EAST inhibition assays were carried out in homologous and heterologous systems as described (29, 33), using 50 µl of diluted patient serum and 50 µl of inhibitor solution. Ovalbumin was used as the control inhibitor. For comparison of the allergenic potency of the isolates, the absolute amounts of protein causing 50% inhibition of the IgE-binding to the solid phase (c_{50}) were calculated from the inhibition graphs.

All determinations were run in duplicate.

Immunoblot experiments

For preparative SDS-PAGE, 6 µg of isolated proteins and 80 µg of total protein from the allergenic extracts were applied. Protein transfer was carried

out by semidry blotting as described (30). The dried nitrocellulose membranes were blocked twice (15 min) with blocking buffer (0.3% (v/v) Tween 20 in 0.01 M potassium phosphate buffer, pH 7.4 M sodium chloride (PBS)).

Immunodetection was performed with patient sera and mAbs. The cut nitrocellulose strips were incubated overnight with 100 µl of patient serum in 900 µl of incubation buffer or 1 ml of mAb diluted 1:100 in incubation buffer. The following incubation steps were carried out as previously described (30, 33).

Histamine-release assays

The quantitative dose-related histamine release was performed as described by Kleine-Tebbe et al. (17) and Vieths et al. (34). Peripheral blood samples (20 ml) from two donors with birch pollinosis and apple hypersensitivity and from a nonallergic person were used. The isolated and washed leukocytes of each blood sample were adjusted to 3×10^7 cells/ml. A volume of 250 µl of this cell suspension was incubated with 250 µl of 10-fold serial dilution (1:100 to 1:100 000) of the isolated proteins (initial protein concentration: 44 µg/ml) and the whole allergenic extracts (initial protein concentration: 160 µg/ml of Golden Delicious extract and 220 µg/ml of birch pollen extract). In addition, the cells were incubated with anti-IgE (400 U/ml) to determine the non-specific histamine release. Buffer blanks and the total histamine content of the leukocytes were assayed as previously described (34).

Skin tests

Skin tests were performed by the prick test method on the forearms, using the proteins isolated by electroelution (5 µg/ml), commercial birch pollen extract (total protein: 20 µg/ml), and low-temperature extracts of Golden Delicious (protein content: 42 µg/ml) and Granny Smith (protein content: 46 µg/ml). The four commercial apple prick test solutions were also used. All tests were carried out according to the recommendations of the European Academy of Allergy and Clinical Immunology (6), as described previously (34, 36). Histamine (0.1%) and sodium chloride (0.15 M) served as positive and negative controls.

Skin reactivity was determined by measuring the wheal diameter. The results were evaluated as follows: 0: <4 mm, (+): 4–5 mm, +: 5–7 mm, ++: 7–9 mm, +++: 9–12 mm, ++++: >12 mm. As a minimum, a 5–7 mm reaction was taken as positive.

The positive patients group with hypersensitivity to birch pollen and apples consisted of 33 individuals. The mean age of the 26 women was 32 years,

and that of the seven male patients was 40 years. Most of the apple-allergic patients were also sensitive to other fruits and vegetables and to hazelnuts. All patients suffered from oropharyngeal food-allergy symptoms and also had atopic dermatitis. The negative control group consisted of 10 persons (six women, mean age 34 years; four men, mean age 33 years). All control subjects tolerated fresh apples. Five were nonatopic. Two participants suffered from isolated grass pollinosis. One was allergic to fish protein, and one had recurrent urticaria without food intolerance. One control patient was sensitized to birch pollen, but tolerated apples.

Informed consent was obtained from all participants. The skin tests were performed at the Hospital Borkum Riff (Borkum, Germany).

Since only intensity classes of skin reactions were determined in the applied standard procedure for skin testing, no further quantitative evaluation of the prick test results was carried out, and only frequencies of positive results were compared. For statistical analysis, the one-sided and two-sided chi-square tests were used at the $P = 0.05$ and $P = 0.01$ levels (24).

Results

Isolation of the 18-kDa apple allergen and of Bet v I

Allergenic extracts of Golden Delicious, Granny Smith, and birch pollen were separated by preparative SDS-PAGE. The resulting gels were copper stained. The proteins appeared as transparent bands in the semipaque gels.

After cutting out the protein bands of the apple allergen at a molecular mass of 18 kDa and of *Bet v I*, the allergens were electroeluted. Analytic gels were run and silver stained to control the purity of each fraction (see characterization section). The results of the protein determination revealed that 350 µg of *Bet v I* and 480 µg of the 18-kDa allergen from each apple strain could successfully be iso-

lated. In the following sections, the protein fractions are designated as follows: "18-kDa (GD)": apple allergen isolated from Golden Delicious extract, "18-kDa (GS)": apple allergen isolated from Granny Smith extract, "*Bet v I* (EE)": *Bet v I* isolated from birch pollen by electroelution. To compare the immunologic activity to that of *Bet v I* (EE) in some experiments, "*Bet v I* (A)" isolated by monoclonal antibody affinity chromatography was used simultaneously.

Characterization of the homologous allergens

SDS-PAGE. SDS-PAGE followed by silver staining was performed with the isolates 18-kDa (GD), 18-kDa (GS), *Bet v I* (EE), and *Bet v I* (A). For comparison, the whole allergenic extracts of both apple strains and of birch pollen were also analyzed. One resulting gel is presented in Fig. 1. The isolated proteins appeared as sharp and individual bands, and no impurities were detected.

Microsequencing analyses. For further purity control, 10 N-terminal amino acid residues of the isolated allergens 18-kDa (GD), 18-kDa (GS), and *Bet v I* (EE) were analyzed by microsequencing. The partial sequences obtained were identical with data presented for *Bet v I* by Breiteneder et al. (2) and with our previous microsequencing results for the apple allergen (35).

EAST. IgE binding to the three isolated proteins, *Bet v I* (A), and the corresponding whole allergenic extracts was examined for the two individual sera of patients allergic to birch pollen and apples (Bo 44 and Bo 46), the two RAST-positive pool sera (Wn 13/32 and Zie 39/32), and the nonallergic control serum (Zie) (Table 1).

The four "positive" sera revealed EAST classes of 2 and 3 (2.1–10.8 U/ml) to all of the isolates and the allergenic extracts, indicating immunologic activity

Table 1. Results of EAST to 18-kDa apple allergen, *Bet v I*, and corresponding allergenic extracts for two individual sera Bo 46 and Bo 44 of patients allergic to birch and apple pollen, two birch/apple-positive pool sera Zie 39/32 and Wn 13/32, and negative control individual serum Zie

Allergen/allergenic extract	Patient serum									
	EAST class					PRU/ml				
	Bo 46	Bo 44	Zie 39/32	Wn 13/32	Zie	Bo 46	Bo 44	Zie 39/32	Wn 13/32	Zie
18-kDa (GD)	3	3	2	2	0	3.6	3.5	2.1	2.1	0.0
18-kDa (GS)	3	2	2	2	0	5.2	2.7	2.5	2.9	0.0
<i>Bet v I</i> (EE)	3	3	2	3	0	5.7	7.9	2.8	10.3	0.0
<i>Bet v I</i> (A)	3	3	3	2	0	5.2	7.6	5.6	2.2	0.0
Golden Delicious	3	3	2	2	0	3.3	3.7	2.1	2.6	0.0
Granny Smith	3	2	2	2	0	2.1	2.9	4.1	3.7	0.0
Birch pollen	3	3	3	3	0	6.0	7.4	10.8	9.6	0.0

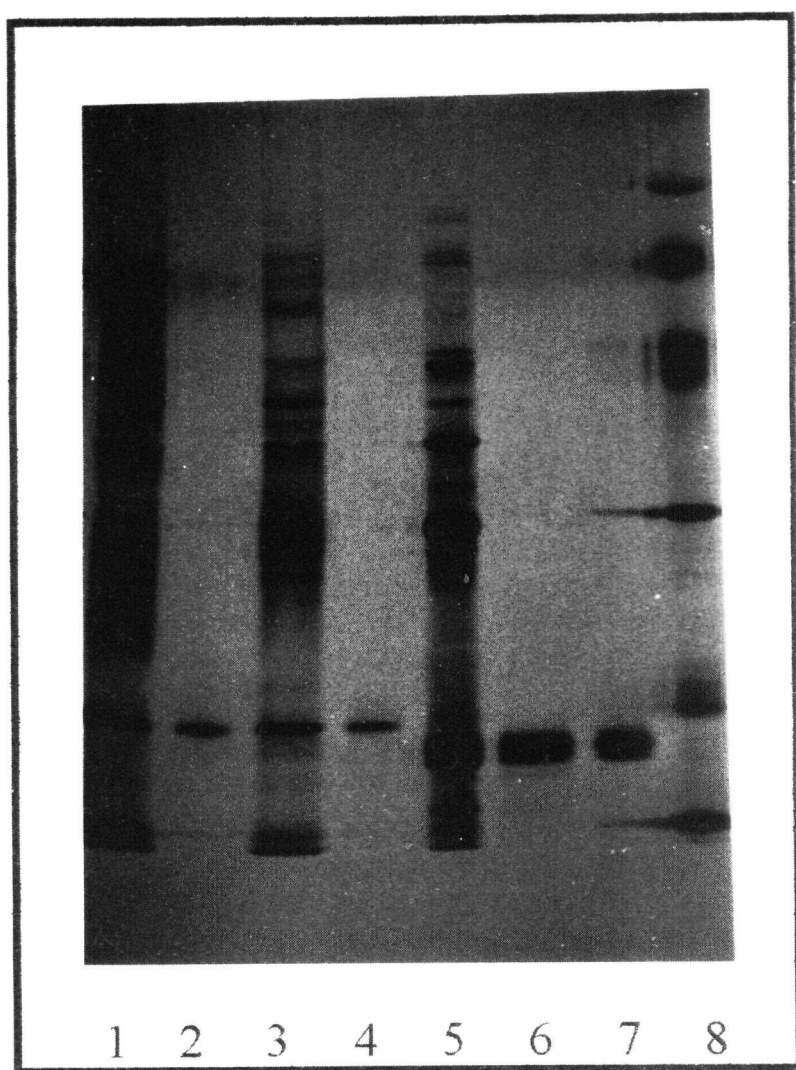


Fig. 1. SDS-PAGE analyses of 18-kDa and *Bet v I* isolates. Protein bands were made visible by silver staining. Lane 1: Golden Delicious extract, lane 2: 18-kDa (GD), lane 3: Granny Smith extract, lane 4: 18-kDa (GS), lane 5: birch pollen extract, lane 6: *Bet v I* (EE), lane 7: *Bet v I* (A), lane 8: low molecular weight marker proteins.

for all four preparations. The IgE antibody reactivity to the *Bet v I* isolates/birch pollen was somewhat higher than to the 18-kDa apple allergens/apple extracts (see data in U/ml). The strongest IgE binding was found with birch pollen extract (6.0–10.8 U/ml). Interestingly, small differences in the antibody binding of both pool sera to *Bet v I* (EE) and *Bet v I* (A) occurred. No IgE binding was observed with the negative control serum Zie.

Immunoblot experiments. To confirm the EAST results and detect IgE binding impurities of the isolates, immunoblot experiments were performed. Immunodetection was carried out with allergic human sera and allergen-specific mAbs, using nitrocellulose strips loaded with all four isolates and the corresponding whole allergenic extracts. The strips were incubated with the serum of the allergic patient Bo 46, the serum pool Zie 39/32 (both 1:10), and 1:100 dilution of the mAbs BIP 1 (raised against *Bet v I*) and 3F8 (raised against the 18-kDa apple allergen). The resulting immunoblots after incubation with human sera are shown in Fig. 2A and B. The IgE

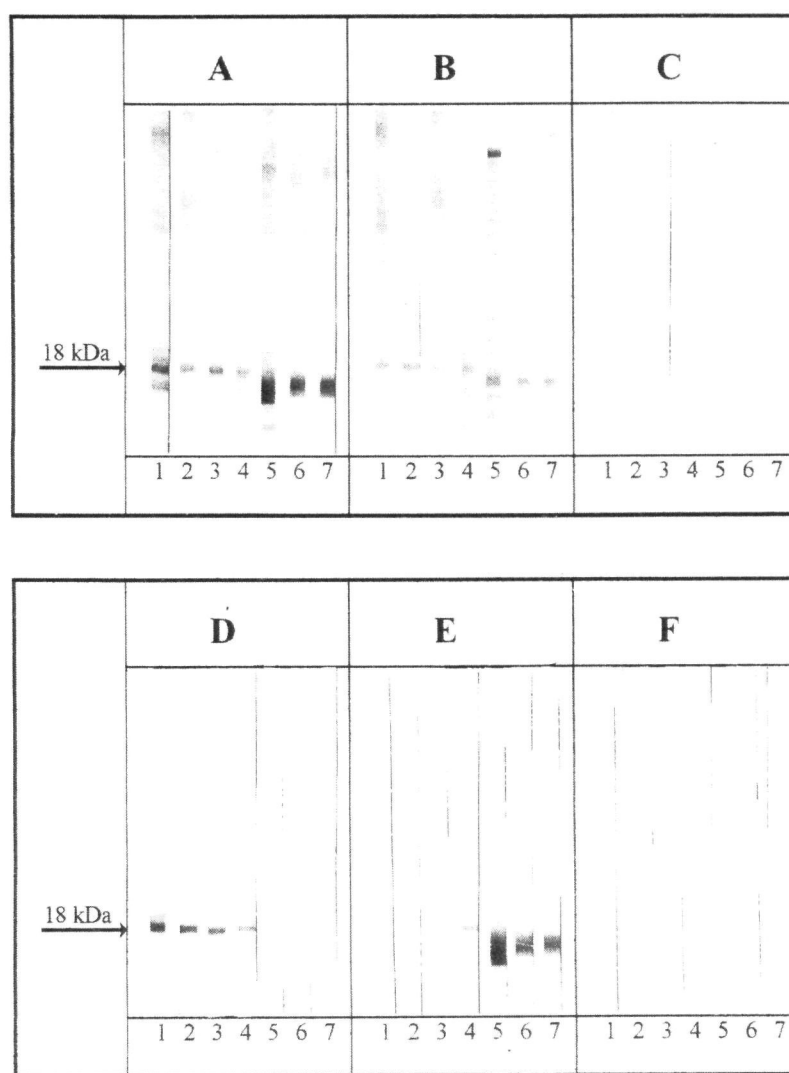


Fig. 2. Immunoblot detection of immunologic activity and purity of 18-kDa and *Bet v I* isolates. *Blot strips*: lane 1: Golden Delicious extract, lane 2: 18-kDa (GD), lane 3: Granny Smith extract, lane 4: 18-kDa (GS), lane 5: birch pollen extract, lane 6: *Bet v I* (EE), lane 7: *Bet v I* (A). *Detection*: human sera – A) serum of birch pollen- and apple-allergic patient (Bo 46), B) pool serum (Zie 39/32), C) nonspecific binding (buffer control); mAbs – D) 3F8 (raised against 18-kDa apple allergen), E) BIP 1 (raised against *Bet v I*), F) nonspecific binding (buffer control).

antibodies of both human sera clearly recognized *Bet v I* and the 18-kDa allergen, again indicating the strong immunologic activity of the isolates. Very weak allergen bands of higher molecular masses were obtained with the isolates and the serum Bo 46. This serum showed additional binding to the 16-kDa allergen of the Golden Delicious extract, and low amounts of this allergen were also detected in the 18-kDa (GD) preparation.

Strong antibody binding was also obtained with the mAb 3F8 and the apple extracts/isolates, as well as with BIP 1 and birch pollen extract, *Bet v I* (EE), and *Bet v I* (A) (Fig. 2D and E). In addition, BIP 1 recognized a *Bet v I*-related epitope on the apple allergen isolated from Granny Smith.

Nonspecific binding was checked by appropriate reagent controls (incubation of immunoblots with all immunochemicals but without the human sera/mAbs). All gave negative results (Fig. 2C and F).

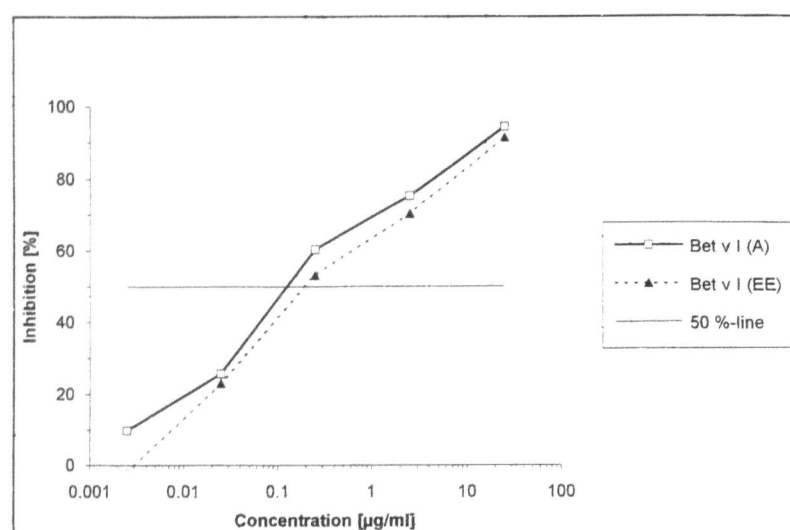


Fig. 3. EAST inhibition experiments with *Bet v I* (A) allergen disks and serum pool Zie 39/32 (1:2.5), performed to compare allergenic potency of different *Bet v I* isolates.

EAST inhibition experiments. Inhibition assays were carried out in order to determine and compare the allergenic potency of the apple allergens with *Bet v I*. Allergen disks of *Bet v I* (A), *Bet v I* (EE), and 18-kDa (GD) were incubated with a 1:2.5 dilution of the serum pool Zie 39/32 and 50 µl of inhibitor solution. Dose-related inhibition graphs were recorded with all isolates as the inhibitors. As an example, the results with *Bet v I* (A) allergen disks are presented in Fig. 3.

The graphs with both *Bet v I* isolates were nearly identical, and the antibody binding was completely inhibited at an inhibitor concentration of 25 µg/ml. With *Bet v I* (EE) allergen disks, an identical inhibition potency was obtained with both isolates from birch pollen. Since only a weak inhibition was expected for the apple isolates, only the highest inhibitor concentration of 25 µg/ml was applied when *Bet v I* (A) allergen disks were used. The inhibition results with *Bet v I* (A) allergen disks and the apple allergen were 14.1% for 18-kDa (GD) and 17.4% for 18-kDa (GS), respectively. Increasing the apple allergen concentration to 100 µg/ml resulted in an increase to 35.5% inhibition with 18-kDa (GD) and 30.5% inhibition with 18-kDa (GS). The absolute amounts of protein responsible for 50% inhibition (c_{50}) of the IgE binding to the solid phase were calculated from the inhibition graphs with *Bet v I* (A) and 18-kDa (GD) allergen disks and are summa-

Table 2. EAST inhibition experiments with serum pool Zie 39/32-pool (1:2.5). Presentation of absolute protein amount responsible for 50% inhibition (µg)

Inhibitor	18-kDa (GD)	18-kDa (GS)	<i>Bet v I</i> (EE)	<i>Bet v I</i> (A)
Allergen disk				
18-kDa (GD)	9.0×10^{-2}	1.3×10^{-2}	2.0×10^{-4}	7.5×10^{-4}
<i>Bet v I</i> (A)	>5	>5	1.0×10^{-2}	6.5×10^{-3}

Table 3. EAST inhibition experiments with serum pool Zie 39/32 (1:2.5) and 18-kDa and *Bet v I* isolates as inhibitors. Inhibitor concentration: 25 µg/ml. Results are presented in percent inhibition

Inhibitor	18-kDa (GD)	18-kDa (GS)	<i>Bet v I</i> (EE)	<i>Bet v I</i> (A)
Allergen disk				
Golden Delicious extract	90	86	100	77
18-kDa (GD)	93	116	110	85

rized for all four inhibitors in Table 2. Compared to the apple allergen, the allergenic potency of the *Bet v I* was clearly higher in all experiments (at least 17 times when apple allergen disks were used and at least 500 times when *Bet v I* allergen disks were used).

Additional experiments were performed with whole Golden Delicious extract at the solid phase and the four isolates as the inhibitors at a protein concentration of 25 µg/ml. The results are presented in Table 3. For comparison, the inhibition data with 18-kDa (GD) allergen disks are also shown. The IgE binding to the whole Golden Delicious proteins was almost completely inhibited by the isolates, indicating that the major apple allergen represents most of the IgE-binding potency of Golden Delicious for the serum used.

In all experiments, the control inhibitions with ovalbumin were negative.

In vitro histamine release

Blood samples were taken from two atopic donors: patients Scho and Bia, both allergic to birch pollen, apples, nuts, and several kinds of fruit, and from one nonallergic individual (Tsch). The dose-related histamine-release graphs of the blood basophils from

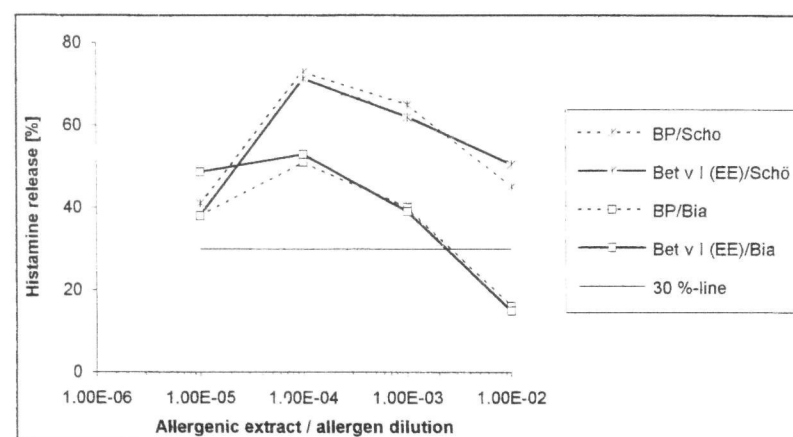


Fig. 4. Histamine release induced by birch pollen extract and *Bet v I* (EE) for patients Scho and Bia. Initial protein concentration: 220 µg/ml of birch pollen extract, 44 µg/ml of *Bet v I* (EE). Protein concentrations responsible for 30% histamine release: $< 2.2 \times 10^{-3}$ µg/ml for birch pollen and $< 4.4 \times 10^{-4}$ µg/ml for *Bet v I* (EE) in both patients. Maximal histamine release: 73% (patient Scho) and 51% (patient Bia) for birch pollen, 71% (patient Scho) and 53% (patient Bia) for *Bet v I* (EE).

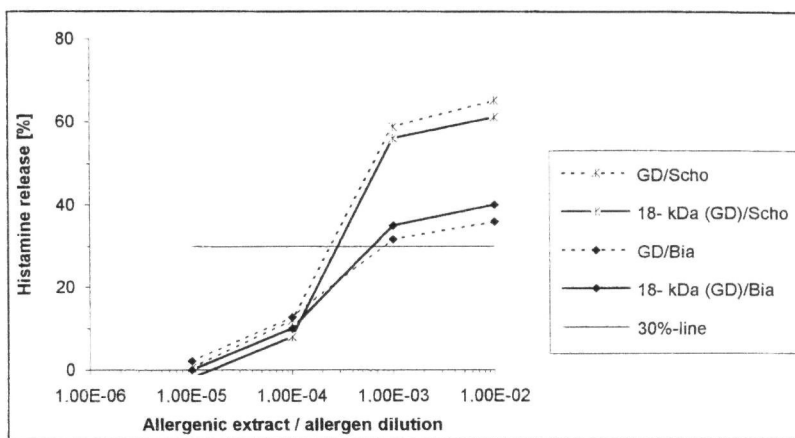


Fig. 5. Histamine release induced by Golden Delicious extract and 18-kDa (GD) for patients Scho and Bia. Initial protein concentration: 160 µg/ml of Golden Delicious extract, 44 µg/ml of 18-kDa (GD). Protein concentrations responsible for 30% histamine release: 4.0×10^{-2} µg/ml (patient Scho) and 1.3×10^{-1} µg/ml (patient Bia) for Golden Delicious extract, 1.3×10^{-2} µg/ml (patient Scho) and 2.9×10^{-2} µg/ml (patient Bia) for 18-kDa (GD). Maximal histamine release: 65% (patient Scho) and 36% (patient Bia) for Golden Delicious extract, and 61% (patient Scho) and 40% (patient Bia) for 18-kDa apple allergen.

both atopic patients induced by *Bet v* I (EE) and birch pollen, as well as 18-kDa (GD) and Golden Delicious, are presented in Figs. 4 and 5. No histamine release occurred after allergen stimulation of the leukocyte fraction from the control patient Tsch.

The total histamine release resulting from an incubation with anti-IgE (400 U/ml) was calculated as 33.9% for patient Scho, 19.6% for patient Bia, and 46.1% for patient Tsch. For all three patients, the buffer control (blank) was negative. The protein concentrations responsible for a 30% histamine release (c_{30} , cell sensitivity) were clearly higher with *Bet v* I (EE) and the birch pollen extract, when compared to 18-kDa (GD) and Golden Delicious apple extract (see legends of Figs. 4 and 5). In accordance with the EAST inhibition results, a distance of 1–2 orders of magnitude between the allergenic activity of the apple isolate and *Bet v* I (EE) was obtained. The cell sensitivity of *Bet v* I was at least 29-fold below that of the major apple allergen.

The histamine-release graphs obtained with the whole extracts and the corresponding isolates presented in Figs. 4 and 5 were almost identical. Compared to the whole birch pollen extract, an applica-

tion of approx. 20% of *Bet v* I induced the same histamine release in both patients. With the Golden Delicious extract and the 18-kDa allergen, a value of 30% was calculated.

Skin prick test

All apple-allergic patients and all control persons were prick tested with whole allergenic extracts from birch pollen, Golden Delicious apples, and the allergen isolates *Bet v* I (EE), 18-kDa (GD), and 18-kDa (GS), as well as with commercial apple prick test solutions from four different manufacturers. Furthermore, 20 allergic patients and all controls were tested with whole Granny Smith extract. All prick tests were negative in the control group, with the exception of the patient suffering from isolated birch pollinosis, who reacted to birch pollen extract and *Bet v* I, but not to the apple extracts and the pure apple allergens. In the allergic-patient group, only two positive tests were obtained with two of the commercial apple prick test solutions, a result which again underlines their known unreliability for the diagnosis of apple allergy (34, 36). The results with commercial birch pollen extract, the low-temperature apple extracts, and the purified major allergens are summarized in Table 4.

High sensitivities were generally obtained with all six test solutions and were between 91% and 97% for birch pollen extract, *Bet v* I, and the whole apple extracts. Somewhat lower sensitivities of 73% and 76% were obtained with the major allergens of Golden Delicious and Granny Smith, respectively. Statistical analysis by the one-sided chi-square test revealed no significant differences between the number of positive test results with whole birch pollen extract, *Bet v* I, and the whole apple extracts ($P = 0.05$). However, compared to the corresponding apple extracts, the sensitivities with the major allergen isolates 18-kDa (GD) and 18-kDa (GS) were significantly lower ($P = 0.05$). In addition, the two-sided chi-square test revealed that the positivity rates obtained with the major apple allergens were also significantly lower when compared to *Bet v* I ($P = 0.01$).

Table 4. Skin prick test results in patients allergic to birch pollen and apples, using whole allergenic extracts and major allergens isolated by electroelution

Prick test solution	Protein (µg/ml)	No. of patients (n)	Frequency of positive prick tests (n)	% of positive prick tests
Birch pollen extract	20	33	32	97
<i>Bet v</i> I (EE)	5	33	32	97
Golden Delicious extract	42	33	30	91
18-kDa (GD)	5	33	24	73
Granny Smith extract	46	20	19	95
18-kDa (GS)	5	33	25	76

Discussion

In order to compare the allergenic activity of the major apple allergen with *Bet v I* in patients with birch pollen-related food hypersensitivities, the two cross-reactive major allergens were isolated and their allergenic properties were investigated in a comparative study. *Bet v I* can be conveniently isolated by monoclonal antibody affinity chromatography, but attempts to use the mAbs which have recently been raised against the major apple allergen (9) for that purpose were not successful.

In contrast, electroelution according to Tessmer & Dernick (26) was found to be suitable for micro-preparative purification of the 18-kDa apple allergen as well as of *Bet v I*. The purity and identity of the isolated allergens was checked by analytic SDS-PAGE, immunoblotting, and N-terminal micro-sequencing analyses. Only the highly sensitive immunoblotting procedure detected low amounts of some minor impurities. From these results, the purity was estimated to be above 95%; therefore, the allergens were used in further characterization experiments without any additional purification. Despite the denaturing conditions of SDS-PAGE, characterization by EAST, immunoblotting, histamine release, and skin testing revealed strong immunologic activity of the allergens in all four methods. This could be due to the predominance of sequence epitopes or to renaturation of the protein tertiary structure. Since *Bet v I* is known to have no cysteine residues, the latter explanation is probable. In addition, EAST and immunoblotting revealed only small differences in IgE binding to *Bet v I* (EE) and monoclonal antibody affinity chromatography isolated *Bet v I* (A), indicating that the immunologic activity was retained by both isolation procedures.

Although eightfold lower protein concentrations of the purified allergens were used in the coupling solutions for allergen disk preparation, similar EAST results were obtained with the isolated proteins and the whole allergen extracts (Table 1). Furthermore, an almost complete inhibition of the IgE binding to whole apple extract allergen disks was obtained when the major allergens from apples and birch pollen were used as the inhibitors. From both results, it may be concluded that the 18-kDa apple allergen (and *Bet v I*) represent most of the allergenic potency of apple fruit.

Many studies have shown a higher allergenic activity for birch pollen extracts when compared to apples and other fruits in patients with birch pollen-related food allergies, e.g., by RAST or EAST inhibition (1, 11, 21, 29, 32) or histamine-release experiments (17). Our present results clearly indicate that in the case of apples this phenomenon is due to differences in the allergenic activity of *Bet v I* and the

major fruit allergen. In the EAST inhibition experiments, the c_{50} amount of *Bet v I* was at least 17-fold lower with allergen disks loaded with the purified Golden Delicious apple allergen and at least 500-fold lower with *Bet v I* (A) allergen disks, when compared to the major apple allergen.

The cell sensitivities (c_{30}) of *Bet v I* (EE) determined by the dose-related histamine release were 29- and 65-fold below that of the 18-kDa apple protein for the two patients investigated. In addition, the c_{30} values of the whole Golden Delicious apple extract were at least 18-fold higher when compared to the birch pollen extract. The maximum responses of 53% and 71% histamine release obtained in our study after leukocyte stimulation with *Bet v I* (EE) are in accordance with the higher values reported by Valenta et al. (28). The authors investigated a group of 21 patients with samples of native and recombinant *Bet v I* and determined a maximal histamine release of 85%. However, somewhat lower maximum responses of 36% and 61% were obtained with the cross-reactive apple allergen in our investigation. Since the amount of the purified major allergens was limited, histamine-release assays could be performed only with two allergic patients, both suffering from typical birch pollen related food hypersensitivity pattern. However, the results coincided well with all other test methods applied in this study and therefore further supported our general conclusions.

In addition, very interesting results were obtained in the skin prick test study. Firstly, the highly allergenic activity of the purified major allergens was clearly confirmed by *in vivo* testing. Secondly, the sensitivity with purified *Bet v I* was 97% and almost identical with that obtained with whole birch pollen extract. This is in full accordance with the frequency of sensitization to *Bet v I* determined in the immunoblot studies of Jarolim et al. (15) and Ebner et al. (7), who found IgE to *Bet v I* in 96.6% and 97.6% of birch pollen-allergic patients, respectively. Furthermore, birch pollen extract, *Bet v I*, and the low-temperature whole apple extracts were found to be very useful tools for skin test diagnosis of apple and birch pollen allergy in patients with a clear anamnesis, and there were no statistically significant differences among the obtained sensitivities. However, we have not studied the rate of latent sensitizations to apples in birch pollen-allergic patients without apple hypersensitivity.

The problems of fruit extract preparation have been known for many years (1, 18). Enzymatically oxidized plant phenolics interact with the apple allergens and lead to a rapid denaturation of the allergenic proteins when apple tissue is crushed in standard extraction buffers. The problem can be circumvented by adding enzyme inhibitors (1) or by our

low-temperature acetone powder method, by which the plant phenolics are quickly separated from the polyphenoloxidases (31, 35). Since commercial apple extracts were again found to be unreliable for apple-allergy diagnosis, it is obvious that up to the present the problem has not been considered by the manufacturers.

However, when compared to our low-temperature apple extracts, the sensitivities obtained with the pure major apple allergens were somewhat lower, and the differences were found to be statistically significant at the applied protein concentrations. In contrast to birch pollen allergy, these differences may be due to a higher sensitization rate of the allergic patients to minor apple allergens. Another probable explanation is that the major allergen concentration of 5 µg/ml which was applied for skin testing was below the major allergen content of the whole extracts used in the same procedure. Finally, attention has to be focused on the fact that the prick test sensitivities determined with the major apple allergens were significantly lower ($P = 0.01$) when compared to *Bet v I* at a standardized protein concentration of 5 µg/ml. In view of the high degree of cross-reactivity between these major allergens, a lower allergenic potency of the major apple allergens must also be stated from the results of the skin test study.

From the EAST inhibition results, it has to be further concluded that all epitopes of the major apple allergen are present on *Bet v I*. Since a very low inhibition potency of the 18-kDa apple allergen was determined by EAST inhibition with *Bet v I* allergen disks, our results support the view that the *Bet v I*-specific IgE antibodies have a lower association constant to the cross-reacting epitope(s) of the major apple allergen. Additional evidence for this explanation is given by the results of the skin prick tests with the isolated major allergens.

Since even a four-fold excess (100 µg/ml) of the pure apple allergen produced only weak inhibition of the IgE binding to *Bet v I* allergen disks (maximum 35.5%), the results may also be due to the existence of non-cross-reactive allergenic epitopes on the birch pollen allergen. This explanation has previously been suggested for whole birch pollen extract (11). Unfortunately, the amount of purified major apple allergen was limited; therefore, drastically higher inhibitor concentrations could not be applied to confirm this hypothesis. Further work is in progress to identify the epitope regions of the apple allergen and compare them with the known sequence of *Bet v I*.

Taken together, our results greatly strengthen the hypothesis that birch pollen *Bet v I* acts as the primary immunogen and is the essential cause of the patients' food hypersensitivities which are due to

low-affinity cross-reactions of *Bet v I*-specific IgE antibodies.

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