

Multiplicity of cross-reactive epitopes on *Bet v I* as detected with monoclonal antibodies and human IgE

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Six monoclonal antibodies against *Bet v I*, the major cross-reactive allergen of birch pollen (*Betula verrucosa*), were obtained. Four did not react with fruits, but two monoclonal antibodies (mAbs) (5H8 and 9C11) were reactive with apple and other fruits. These two cross-reactive antibodies reacted with identical or overlapping sites, but differed in their relative degree of cross-reactivity toward various fruits and hazelnut. Cross-reactive human IgE antibodies reacted with a nonoverlapping epitope, as indicated by results of a two-site radioimmunoassay (RIA) with the fruit-reactive mAb 9C11. By isoelectric focusing (IEF) in conjunction with immunoblotting, a maximum of seven isoforms could be distinguished. Depletion of birch-pollen extract for *Bet v I* with the most reactive mAb (7F7) removed approximately 95% of the IgE cross-reactivity between birch pollen and apple extract. The remaining 5% cross-reactive material was still capable of inhibiting the binding of IgE to apple allergen completely, and was reactive with mAbs 5H8 and 3C4. By means of IEF/immunoblot, it was shown that these mAbs recognize an isoform of *Bet v I* that is poorly, if at all, recognized by mAb 7F7. These results illustrate the heterogeneity of *Bet v I*, both with respect to the cross-reactive sites as well as to the backbone structure. This type of heterogeneity has possible implications for the use of monoclonal antibodies in allergen standardization.

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Patients with birch pollinosis and food allergy are known to have specific IgE to food-cross-reactive birch proteins (4, 9, 10, 16). Two of these proteins have been identified as the major birch allergen *Bet v I* (8, 11, 13, 17, 19) and the minor allergen profilin (20, 21, 23). *Bet v I* is encoded by a gene which is highly homologous to a pea disease resistance response gene (6). It is cross-reactive with homologous proteins in, e.g., apple and hazelnut. Profilin, a protein controlling actin polymerization in eukaryotic cells, is an even more conserved protein. Profilin is responsible for most of the cross-reactivity between birch pollen and extracts of vegetable foods like carrot, celery, and potato. A third type of cross-reactivity, responsible for cross-reactions between birch pollen and components in phylogenetically even less related vegetables, is due to glycoproteins (2, 7, 22) bearing periodate-susceptible cross-reacting carbohydrate determinants (CCD) on their surface.

The purpose of this investigation was to study the cross-reactivity due to *Bet v I* and *Bet v I* homologous proteins from other vegetable sources. Six monoclonal antibodies to *Bet v I* and sera containing IgE against *Bet v I* were used to identify the cross-reactive epitopes.

Material and methods

Allergens

Allergen extracts were prepared as described previously (4, 7) and coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) using 2.5 mg lyophilized pollen extract or 4 mg lyophilized food allergen extract per 100 mg Sepharose.

Bet v I was purified physicochemically by gel filtration (Sephadex G-75, Pharmacia) and anion-exchange chromatography (FPLC TSK-DEAE, LKB, Bromma, Sweden). Competitive radioimmunoassays (RIAs) were applied to monitor the puri-

fication. Purified *Bet v I*, kindly provided by Dr H. Løwenstein (ALK, Copenhagen, Denmark) was used as reference. Purity was checked by SDS-PAGE, followed by protein staining (Coomassie brilliant blue) or autoradiography (in the case of ^{125}I -labeled *Bet v I*). Both physicochemically purified *Bet v I* and reference *Bet v I* showed one band at 17 kDa.

Sera

Serum PB was obtained by plasmapheresis from a 27-year-old female patient with hay fever. This serum was strongly RAST-positive for both birch pollen and grass pollen (RAST class 5+).

Serum JH was obtained from a 33-year-old male patient with birch pollinosis. SDS-PAGE of birch-pollen extract, followed by immunoblotting (Fig. 1), showed that this serum was exclusively reactive with *Bet v I* (or its counterpart from fruits).

Antisera comprised goat antimouse IgG antibodies (GaM) (M1201, CLB, Amsterdam, The Netherlands), rat antimouse kappa monoclonal antibodies (RaM) (M1482, CLB), and sheep antihuman IgE antibodies (SaHIgE) (SH25-1-p7, CLB; affinity-purified before iodination).

Production of mAbs against *Bet v I*

BALB/c mice were immunized subcutaneously with purified *Bet v I* in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant on day 28. On days 38 and 59, mice with the highest serum antibody titer against birch pollen, as tested by RAST, were boosted with purified *Bet v I*. On day 115, 3 days before spleen removal, mice were injected intravenously with the same amount of antigen. Myeloma cell line SP-2 was used for the cell fusion, as described by Köhler & Milstein (14).

Six monoclonal antibodies (mAbs) (7F7, 5H8, 9C11, 9B5, 3C4, and 11E12) were selected on the basis of reactivity with *Bet v I*.

The mAbs were all of the IgG1 subclass, as demonstrated by a mouse monoclonal antibody isotyp-

ing kit (Gibco BRL, Gaithersburg, MD, USA). Analysis of the six mAbs by SDS-PAGE/immunoblotting demonstrated that all mAbs recognized exclusively the 17-kDa *Bet v I* band in birch-pollen extract (Fig. 1). mAbs were purified from ascites (7F7) or hybridoma supernatant with protein A Sepharose (Pharmacia).

Solid-phase immunoassays

RAST. Experiments were performed as described previously (2). In short, Sepharose (0.5 mg Sepharose/test in case of pollen allergen, 1.5 mg Sepharose/test in case of food allergen)-coupled allergen extract was incubated with 50 μl human serum or 50- μl dilutions of protein A purified ascites or hybridoma supernatants in a final volume of 300 μl . Unbound material was removed by washing, followed by immunodetection with either ^{125}I -labeled SaHIgE, ^{125}I -labeled GaM, or ^{125}I -labeled RaM.

RAST inhibition. A volume of 50 μl serum JH was incubated for 2 h with 50 μl of twofold serial dilutions of birch-pollen extract prior to addition of 250 μl Sepharose-coupled apple extract (1.5 mg Sepharose/test in a final volume of 350 μl). Immunodetection was performed as described above.

Two-site RIA. mAb 7F7 ascites (200 μl) and culture supernatant (2 ml) of mAbs 5H8, 9C11, 9B5, 3C4, and 11E12 were coupled to 200 mg CNBr-activated Sepharose 4B. Sepharose-coupled mAbs (0.5 mg Sepharose/test) were incubated with 50- μl dilutions of birch pollen or apple extract. After washing, the resulting solid phase was incubated with 50 μl ^{125}I -labeled mAbs 7F7, 9C11, and 5H8, or 50 μl serum (IgE antibodies bound were detected as described above). As specificity control, a nonrelated monoclonal antibody (reactive to yellow-jacket antigen 5) was coupled to CNBr-activated Sepharose and incubated with birch-pollen extract. Nonspecific binding corresponded to less than 1% of added radioactivity.

Allergen-binding RIA. Solid-phase-coupled antibodies, as described above, were used as capturing antibodies for ^{125}I -labeled *Bet v I*.

For all solid-phase immunoassays, the results were expressed as percent binding of total counts added.

Isoelectric focusing (IEF)

IEF of 600 μg birch-pollen protein was performed, according to the manufacturer's instructions, on an Ampholine PAGplate, pH range 4–6.5 (Pharmacia).



Fig. 1. SDS-PAGE of birch-pollen extract, followed by immunoblotting with six mAbs (100 μg /strip) and 150 μl serum JH. Lane 1: 7F7; 2: 5H8; 3: 9C11; 4: 3C4; 5: 11E12; 6: 9B5; 7: JH. Antibodies bound were detected with either ^{125}I -labeled antimouse IgG or radiolabeled anti-IgE.

A calibration kit (Low pI, pH 2.5–6.5, Pharmacia) was used to determine the pI of *Bet v I* isoforms.

Proteins were transferred to nitrocellulose on an LKB Bromma 2117 Multiphor II electrophoresis unit according to the manufacturer's instructions (nitrocellulose: pore size 0.45 μ M, Schleicher & Schuell, Dassel, Germany). Blots were blocked by incubation for at least 1 h at room temperature with phosphate-buffered saline (PBS) containing 0.01 M EDTA, 4.6 mM NaN_3 , 0.45% bovine serum albumin, and 0.1% Tween 20. Nitrocellulose sheets were cut into 2.5-mm strips and separately incubated overnight with the six mAbs described (100 μ g antibody per strip) or 150 μ l serum JH, in a final volume of 3 ml of the buffer described above without Tween 20. Blot strips were extensively washed with PBS/0.1% Tween. Next, the strips were incubated overnight with 100 000 cpm of ^{125}I -labeled RaM in 3 ml of bovine/sheep serum (= 9 parts bovine serum + 1 part sheep serum). The strips were extensively washed again and air-dried. Autoradiography was performed at -70°C for approximately 60 h with Kodak X-OMAT-AR films (Kodak, France), using an intensifying screen.

Radiolabeling

An amount of 50 μ g *Bet v I* was radiolabeled with 37 MBq ^{125}I (Amersham, Amersham, UK) by the chloramine T method. mAbs (50 μ g) were labeled with 37 MBq by the iodogen method. In both cases, BSA was used as carrier protein. Unbound ^{125}I was separated from the labeled material by gel filtration on an ACA-54 column (IBF, Villeneuve-la-Garenne, France).

Bet v I depletion from birch-pollen extract

Depletion experiments were performed with 5 mg purified mAb 7F7 which was coupled to 300 mg CNBr-activated Sepharose 4B. Birch-pollen extract in a volume of 4 ml (protein content: 1230 μ g/ml, *Bet v I* content 100 μ g/ml) was incubated overnight with 300 mg 7F7 anti-*Bet v I* Sepharose. Supernatant (2.4 ml) was subjected to a second depletion for *Bet v I* with a fresh batch of 300 mg 7F7-Sepharose. Dilution of the birch-pollen extract during the depletion procedure was determined by measuring the profilin content in starting material and depleted extract. Profilin was detected by indirect RAST. This test is based on the specific binding of profilin to poly-L-proline (15). After incubation of Sepharose-coupled poly-L-proline with twofold serial dilutions of birch-pollen extract, serum pf. 40 was added to the solid phase. This serum contained a high-titer IgE to profilin from vegetable sources, such as pollen, celery, and potato (23). Binding of

IgE to Sepharose-bound profilin was detected with radiolabeled anti-IgE.

The efficiency of the depletion was monitored by two competitive RIAs.

Protein determination

Food and tree pollen protein was measured with Pierce BCA Protein Assay Reagent at 37°C (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. BSA was used as protein standard. Protein content of the mAbs was determined by measuring the absorbance at 280 nm ($\epsilon = 1.4$).

Results

Six monoclonal antibodies against *Bet v I* (7F7, 5H8, 3C4, 9B5, 9C11, and 11E12) were tested by RAST with Sepharose-coupled birch-pollen extract (Fig. 2). A low Sepharose concentration (0.1 mg/ml) was used in order to allow binding of antibodies under saturating conditions (i.e., increasing the concentrations of mAb did not result in more binding of monoclonal antibody). The highest binding was observed for 7F7 and 5H8. The other four mAbs (3C4, 9C11, 11E12, and 9B5) demonstrated considerably lower binding. By means of an allergen-binding assay performed with Sepharose-coupled mAbs and radiolabeled *Bet v I* (Table 1) and by quantitative inhibition assays, it was found that 7F7 and 5H8 were the most avid *Bet v I* binders.

The topography of the binding sites recognized by the six mAbs was investigated with a two-site assay with Sepharose-coupled mAbs and radiolabeled mAbs (Fig. 3). Three apparently nonoverlapping sites were distinguished: one recognized by 7F7 and 3C4; one by 5H8, 11E12, and 9C11; and one by 9B5. The failure of 5H8/11E12 and 9C11 to bind

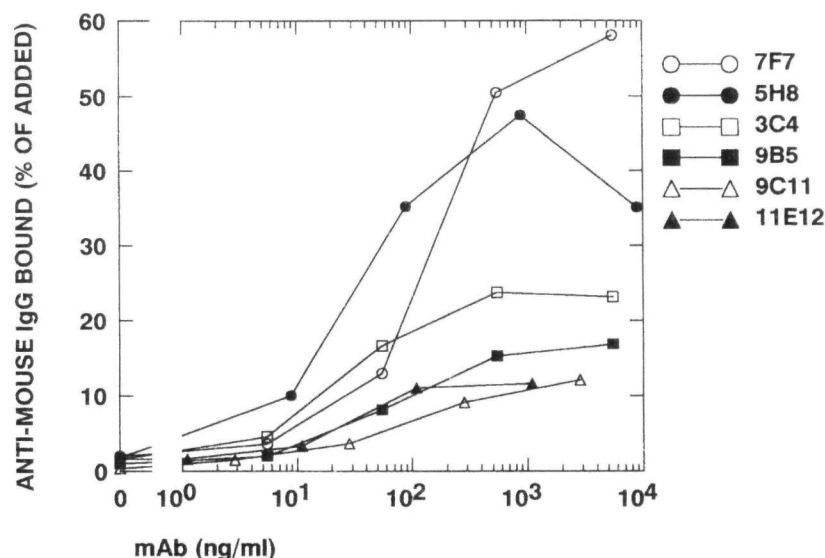


Fig. 2. Tenfold serial dilutions of mAbs were incubated overnight with birch pollen coupled to CNBr-Sepharose (0.1 mg Sepharose/test).

Table 1. Allergen-binding assay. Sepharose-coupled antibodies were used as capturing antibodies for radiolabeled *Bet v I*

mAb	¹²⁵ I-labeled <i>Bet v I</i> bound (% of added)
7F7	52.2
5H8	58.7
9B5	10.0
3C4	7.2
9C11	7.0
11E12	7.0

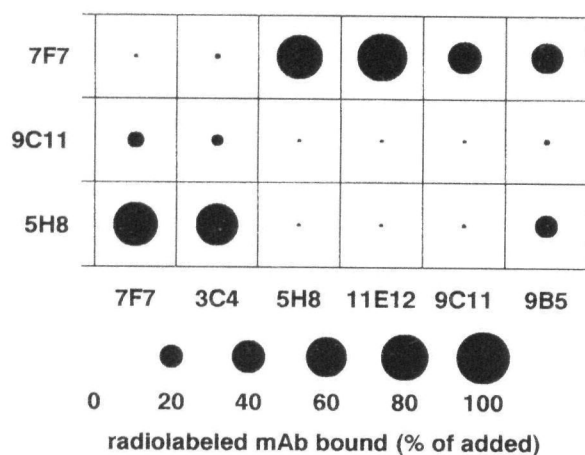


Fig. 3. Two-site RIA. Sepharose-coupled mAbs were incubated with birch-pollen extract, followed by incubation with ¹²⁵I-labeled mAbs 7F7, 9C11, or 5H8.

simultaneously to *Bet v I* suggested that they recognize a related site.

The fine specificity of the six mAbs was further analyzed in a RAST with Sepharose-coupled tree pollen allergens and vegetable food allergens (Table 2). mAbs were allowed to bind under saturating conditions. mAbs 7F7 and 3C4 were highly cross-reactive with alder pollen, but not with any of the tested vegetable foods. The other four mAbs showed only weak binding to alder (<5%). mAbs 5H8 and 9C11 were cross-reactive with apple, pear, and cherry. In addition, 5H8 was cross-reactive with

Table 2. Six mAbs were incubated with Sepharose-coupled tree pollen, and hazelnut, and fruit allergen. Sepharose-coupled amino acid glycine was used as control for non-specific binding. Following mAb concentrations were used: 7F7: 5.5 µg/ml; 3C4: 0.55 µg/ml; 5H8: 0.89 µg/ml; 11E12: 11.0 µg/ml; 9C11: 2.86 µg/ml; and 9B5: 0.55 µg/ml.

	7F7	3C4	5H8	11E12	9C11	9B5
Birch	33.5	23.6	37.2	13.6	11.0	14.6
Alder	15.2	25.1	4.5	3.1	4.2	1.0
Hazelnut	5.2	0.9	8.3	1.0	1.1	1.1
Apple	3.7	0.5	32.9	1.9	29.5	1.4
Cherry	4.8	0.8	20.7	3.2	8.3	1.7
Pear	5.0	0.6	15.5	1.3	20.9	1.3
Peach	4.8	0.8	8.8	1.1	2.6	0.9
Glycine	3.8	0.4	0.7	1.7	0.7	0.8

hazelnut and peach, confirming that 5H8 and 9C11 do indeed have different specificity. Surprisingly, 9C11 demonstrated higher binding to pear than to birch pollen, in contrast to what was observed for 5H8. Although mAb 11E12 showed the same pattern as 5H8/9C11 in the two-site assay, the fine specificities were different.

A two-site RIA with serum JH and Sepharose-coupled 7F7 and 9C11 (9C11 as capturing antibody for *Bet v I* and its counterpart from apple) was performed (Fig. 4). IgE binding was demonstrated to both *Bet v I* and the cross-reactive allergen from apple, showing that a second birch–apple cross-reactive site exists beside the one recognized by 9C11 (and 5H8).

In order to assess the contribution of *Bet v I* to the birch–apple cross-reactivity of serum JH, a depletion experiment was performed with Sepharose-coupled 7F7. The efficiency of the depletion of birch-pollen extract with this solid phase was ≥98%, as judged by a two-site assay with mAb 7F7 (Fig. 5). This depletion resulted in a 20-fold decrease of inhibitory potency of the birch-pollen extract in an apple RAST inhibition with serum JH. Nevertheless, at higher concentrations, complete inhibition was still observed (Fig. 6), suggesting that a *Bet v I* isoform was recognized by cross-reactive IgE, but not by 7F7. In a two-site RIA with Sepharose-coupled 3C4 and serum PB, 8% residual activity was found after 7F7 depletion, confirming that this activity was due to *Bet v I*.

IEF of birch-pollen extract followed by immunoblotting showed seven different isoforms with pI values of 5.0, 4.8, 4.6, 4.5, 4.4, 4.3, and 4.2, respectively (Fig. 7). mAb 7F7 failed to recognize the *Bet v I* isoform with pI 4.6. mAb 5H8 and IgE of serum JH demonstrated binding to all the isoforms described above.

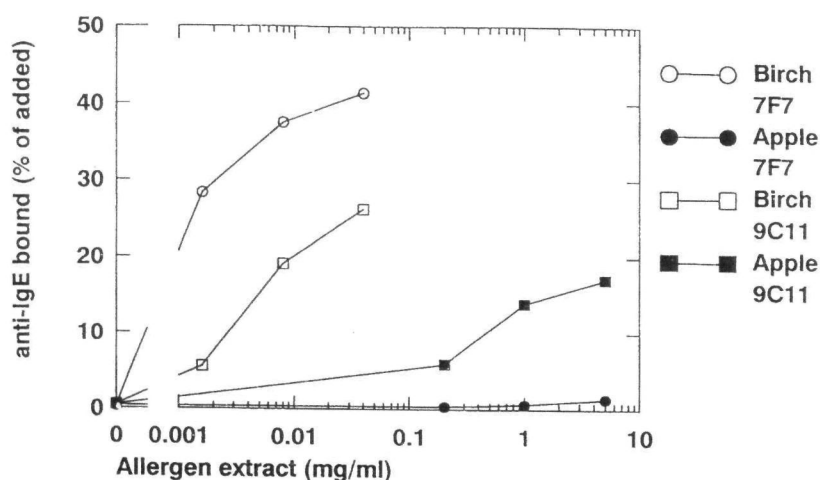


Fig. 4. Two-site RIA. Sepharose-coupled mAbs 7F7 and 9C11 were used as capturing antibodies. After incubations with different concentrations of either birch pollen or apple extract, non-bound components were washed away, and serum JH (containing IgE against *Bet v I*) was added.

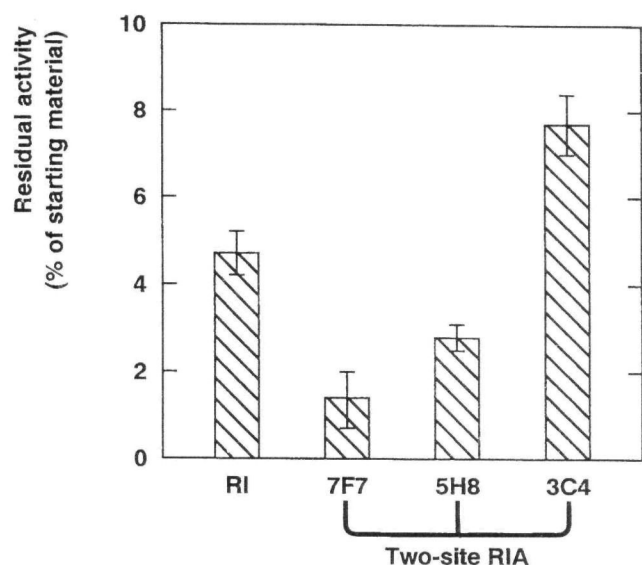


Fig. 5. Measurement of residual *Bet v I* activity in birch-pollen extract after depletion with mAb 7F7 (expressed as percentage of total activity). *RI*: Apple RAST inhibition of patient JH with mAb 7F7 depleted birch-pollen extract. *Two-site RIA*: Sepharose-coupled mAbs 7F7, 5H8, and 3C4 were incubated with birch-pollen extract. Allergens bound were detected by adding serum PB. Error bars indicate range of reproducibility of assay result.

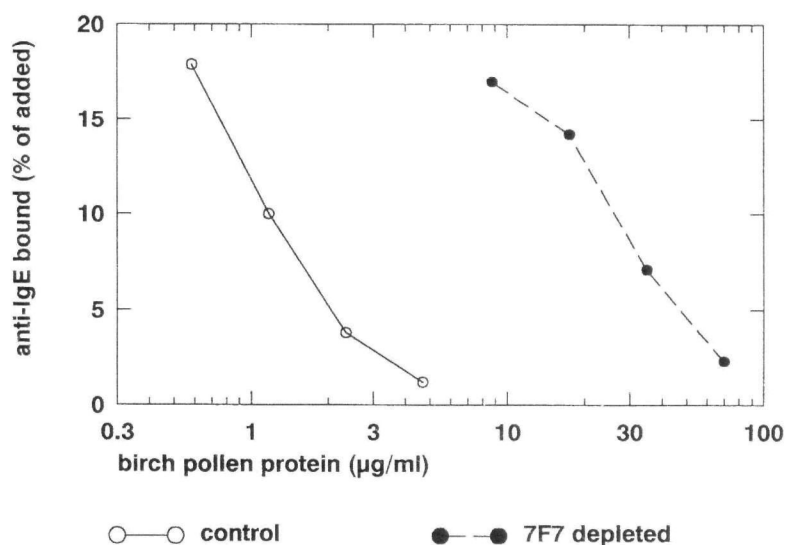


Fig. 6. Apple RAST inhibition of patient JH. Birch-pollen and 7F7-depleted birch-pollen extract was used as inhibitor.

Discussion

Distinct cross-reactive sites could be distinguished. mAbs 7F7 and 3C4 recognized a site cross-reactive with alder pollen. The birch–apple cross-reactive mAbs 9C11 and 5H8 weakly bound to a second birch–alder cross-reactive site, which was different from the previous one (Fig. 3). In addition to the epitope for 9C11 and 5H8, another birch–apple cross-reactive determinant was recognized by human IgE. mAbs 7F7/3C4 did not react with fruits. This suggests (but does not fully prove) that the birch-pollen epitope recognized by this pair of mAbs is distinct from the second cross-reactive IgE epitope. A difference in specificity of mAbs 9C11, 5H8, and 11E12 was demonstrated by the different pattern of cross-reactivity with various fruit allergens. Hazel-nut and peach were recognized only by 5H8, whereas

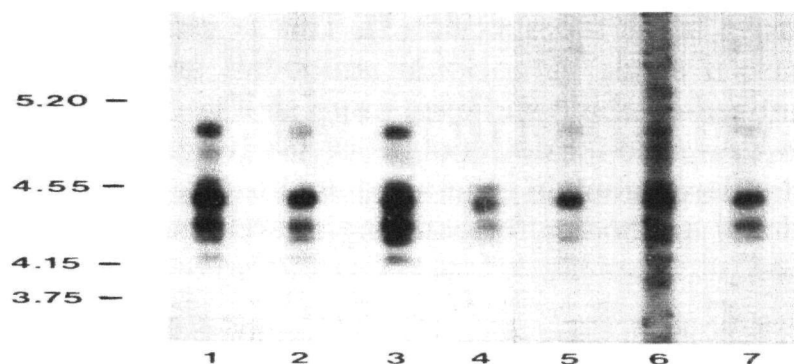


Fig. 7. IEF of birch-pollen extract, followed by immunoblotting with serum JH and six mAbs. Lane 1: serum JH; 2: 7F7; 3: 5H8; 4: 3C4; 5: 9C11; 6: 9B5; 7: 11E12.

9C11 demonstrated a much more pronounced binding to pear. In view of the results presented in Fig. 3, it is possible that the three mAbs (9C11, 5H8, and 11E12) might react with partially overlapping epitopes, the overlapping area being a conserved structure.

In view of the monomeric nature of the *Bet v I* molecule (and therefore presumably also of its equivalents in fruits), and assuming that reactivity in mast-cell or basophil assays depends on the binding of at least two IgE antibodies, the existence of at least two cross-reactive epitopes was to be expected. It will be interesting to see whether fruit cross-reactive monoclonal antibodies to epitopes (other than the one(s) defined by 9C11/5H8 and 11E12) will be found.

By means of IEF/immunoblotting, the existence of at least seven isoforms, recognized by IgE and the majority of the monoclonals, was confirmed (12, 18). This heterogeneity presumably explains why mAb 7F7 failed to remove all *Bet v I* activity from birch-pollen extract, because 7F7 did not recognize the isoform of *Bet v I* with pI 4.6. This isoform was capable of completely inhibiting the apple RAST of a birch–apple cross-reactive serum and was also reactive with antibirch mAbs (other than 7F7).

In view of this “deficiency” of 7F7, it was unexpected to find (Fig. 2) that this mAb showed a much *higher* maximum binding under saturating conditions than four other (on the basis of IEF/immunoblotting, more “complete”) mAbs. The most likely explanation is that the results shown in Fig. 1 were markedly affected by differences in avidity. Presumably, the four low-avidity antibodies 3C4, 9B5, 9C11, and 11E12 were partially released from the allergosorbent during the second incubation step of the test. This resulted in partial consumption of the iodinated detecting reagent and thus in too low test results.

mAbs directed to an allergenic protein are often used to standardize extracts (1, 3). The existence of different isoforms (5, 12, 18), combined with the finding that these can be distinguished by some mAbs, indicates that this approach may sometimes

prove to be too specific (3). This is especially the case if single monoclonal antibodies are used for antigen-catching and even more so if another single mAb is used for detection. It is clearly advisable, for standardization in general, to use mixtures of monoclonal antibodies for catching as well as for detection.

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