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Purification and Characterization of the Major Allergen from Apple and Its Allergenic Cross-Reactivity with Bet v 1

Key Words

Apple allergen

Purification

Monoclonal antibodies

Bet v 1

Cross-reactivity

Abstract

The major allergen from apple extract was concentrated by anion exchange chromatography and further purified by reverse-phase HPLC. A distinct peak with a high degree of homogeneity was obtained. The isolated protein has a MW of 18 kD and specific IgE-binding capacity (immunoblotting, IgE-binding inhibition). N-terminal amino acid analyses of the allergen allowed 37 cleavages and showed 67.6% identity to Bet v 1, the major allergen of birch pollen. Enzyme immunoassay inhibition studies with serum of birch/apple-allergic patients showed that besides cross-reacting structures to Bet v 1, apple-specific IgE antibodies could exist. Monoclonal antibodies (mAbs) were raised against the 18-kD allergen from apple and characterized by means of immunoblotting and ELISA. Only three of the eight studied mAbs reacted with Bet v 1.

Introduction

Allergies due to vegetables are frequently observed in patients with birch pollen allergy. According to previous studies, up to 70% of birch-pollen-allergic patients may be food sensitive, most frequently to apples [1]. The parallel appearance of birch and food allergy can be explained by the occurrence of cross-reactive IgE [2–5] or nonspecific mechanisms [6], like lectin-IgE interactions or unspecific histamine release. Inhibition studies of IgE binding to birch pollen and apple extracts using immunoblotting support the concept that the major birch pollen allergen, Bet v 1 (17 kD), and the 18-kD apple allergen share allergenic epitopes leading to IgE cross-reactivities [7]. However, it was demonstrated that patient serum preincubated with minimal

amounts of birch pollen extract lost their reactivity against apple allergens, but high amounts of apple antigens were only slightly able to inhibit the IgE antibody reaction with birch pollen extract. It has been hypothesized that the birch allergen acts as the primary immunogen and that the cross-reactions are caused by epitopes of apple allergen with a lower affinity to the patients' allergen-specific IgE [1, 4, 7]. To achieve a better understanding of the cross-reactivity between Bet v 1 and the apple equivalent, it is important to isolate and characterize the individual allergen. The purpose of the present study was to purify the 18-kD allergen from apple and to perform an N-terminal sequence analysis. Furthermore, monoclonal antibodies (mAbs) against this allergen were raised and studied with respect to the cross-reactivity between Bet v 1 and apple allergen.

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Materials and Methods

Source Material and Extract Preparation

Extracts from birch pollen were prepared by Allergopharma (Reinbek, Germany) using a conventional buffer extraction procedure followed by diafiltration and lyophilization [8]. Purified Bet v 1, kindly provided by Dr. B. Weber (Allergopharma), was used for cross-reactivity studies [9]. Apple extracts were prepared from mature, fresh Golden Delicious apples by a low-temperature method [10] modified in our laboratory [11]. Briefly, 100-g apples were homogenized with a mixing rod and Ultraturrax and treated with 200 ml diacetone alcohol (4-hydroxy-4-methyl-2-pentanone, Aldrich) for 18 h at -20°C . The solid material (cellulose and proteins) was separated by filtration, dried, and resolubilized with 0.1 M potassium phosphate buffer (pH 7.0) with following additives: 2 mM EDTA, 5 mM diethyldithiocarbamate, 1 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride. Subsequently, the debris was removed by centrifugation at 40,000 g for 30 min. The supernatant containing the apple proteins was dialysed against distilled water. The protein content of the extract was determined by a modified Bradford assay [10, 12] (typical protein content: 0.2–3 mg/ml).

Human Serum

Serum was collected from 32 patients with a positive history of skin tests and a RAST class to birch >4 and to apple >2 [11]. The serum pool had a RAST class to apple of 3.4, indicating high specific IgE levels for apple allergens. The serum pool from nonallergic subjects (RAST class 0) was used as negative control. Three additional sera from patients with an allergy to both birch pollen and apple were studied. The diagnosis was based on anamnesis, RAST and skin tests.

Purification of Apple Allergen

The lyophilized apple extract (1 mg protein) was dissolved in 20 mM Tris-HCl, pH 8.0 (buffer A) and chromatographed on a RESOURCETM Q column (1 ml; Pharmacia, Uppsala, Sweden). The extract was applied to the column and unbound material removed by washing with buffer A. The bound proteins were eluted by a gradient of 0–0.5 M NaCl in 20 mM Tris-HCl, pH 8.0 (buffer B). The main peaks were studied by SDS-PAGE and the fractions which contained the highest amounts of the 18-kD protein (peak 3) were dialysed against distilled water and lyophilized. Subsequently, the lyophilizate was dissolved in water containing 0.1% TFA and loaded on to a RP-C4 column (Vydac, 214TP54) equilibrated with 0.1% TFA. The proteins were eluted with a linear gradient of 0–50% acetonitrile in 0.1% TFA for 30 min and 5 min of 50–80%. Protein maps were obtained by monitoring the absorbance of the eluate at 220 and 280 nm, and the eluted main fraction was manually collected. The solvent was evaporated by vacuum drying and analyzed by SDS-PAGE and immunoblotting. The allergenic activity was measured by ELISA.

Amino Acid Sequencing

Protein was electrotransferred after SDS-PAGE to an Immobilon-P membrane (Millipore). Microsequencing was done by automated Edman degradation in an Applied Biosystems model 476A sequencer, operated in pulsed-liquid mode with an on-line PTH amino acid analyzer.

Production of mAbs to 18-kD Apple Allergen

Female BALB/c mice (8–12 weeks old) were immunized intraperitoneally with 10 μg /mouse of purified 18-kD allergen in complete Freund's adjuvant on day 0 and in incomplete Freund's adjuvant on days 28 and 56. On day 112, 4 days before cell fusion, mice were injected intravenously with 20 μg of 18-kD apple allergen in physiological NaCl solution. Cell fusion and cloning were performed as described elsewhere [13]. Primary screening of hybridoma supernatants was done using an ELISA with solid-phase bound apple extract. The isotype of the mAbs was determined by ELISA using a commercial kit (Boehringer, Mannheim, Germany). The Bet v 1-specific mAb 3B4 was a gift from Dr. Weber (Allergopharma) [14].

SDS-PAGE and Immunoblotting

Apple extract or purified protein was studied by SDS-PAGE (12% PAG) under reducing conditions [15] and detected with silver staining or transferred onto nitrocellulose membranes [16]. The membranes were incubated overnight with hybridoma supernatants adjusted to a mAb content of about 1 $\mu\text{g}/\text{ml}$. After washing with PBS/0.1% (v/v) Tween 20 (TPBS), bound mouse antibody was detected with biotinylated antimouse Ig (Boehringer; diluted 1:1,000 in TPBS) and streptavidin-HRP (Boehringer; diluted 1:2,000 in TPBS). The blot was then washed and stained with 3,3',5,5'-tetramethylbenzidine/dioctyl sodium sulfosuccinate (TMB/DONS) as described by Vieths [17].

To obtain IgE immunoblots, the NC strips were incubated overnight with serum pool diluted 1:6 (v/v) in TPBS, followed by rabbit anti-IgE (1:4,000, 1 h; DAKO, Hamburg, Germany), biotinylated goat anti-rabbit IgG (1:6,000, 1 h; DAKO, streptavidin-HRP (1:20,000, 25 min) and stained as described above. Each incubation step was followed by three washes with TPBS.

ELISA

Direct ELISA. 96-well microtiter plates (Nunc, MaxiSorp) were coated with 50 μl of either apple or birch pollen extract (10 $\mu\text{g}/\text{ml}$) or purified allergens (1 $\mu\text{g}/\text{ml}$) overnight and after that washed and blocked.

For assaying the antibody activity and specificity, 50 μl of hybridoma supernatants (adjusted on similar content of mAbs) were added. The mouse Ig concentration was determined before by capture ELISA as described by Fleming et al. [18]. After washing the plates, bound mouse antibody was detected with biotinylated antimouse Ig and streptavidin-HRP. The substrate was *o*-phenylenediamine (Serva).

To determine the allergenic activity of purified antigen, a serum pool from patients with an allergy to birch pollen and apple, or non allergic subjects was serially diluted (from 1:4), added to plates and incubated overnight. After washing, specific IgE was determined by sequential incubation with rabbit anti-IgE (1:4,000, 1 h), biotinylated goat antirabbit IgG (1:6,000, 1 h), and streptavidin-HRP (1:20,000, 1 h).

ELI Inhibition. Microtiter plates were coated with purified 18-kD protein of apple or Bet v 1. 25 μl of serum pool or individual serum were incubated with 25 μl of inhibitor solution (concentrations ranging from 100 to 0.01 $\mu\text{g}/\text{ml}$) in tubes overnight and after that added to plates. The following ELISA was performed as described above. For comparison of the allergenic potency of the isolates, the amount of allergen ($\mu\text{g}/\text{ml}$) responsible for a 50% inhibition of the IgE binding to the solid phase (C_{50}) was calculated from the inhibition graphs. The concentration of purified allergens was determined from absorbance at 280 nm using an extinction coefficient of $1.0 (\text{cm}\cdot\text{mg}/\text{ml})^{-1}$.

Results

Purification and Characterization of the 18-kD Allergen of Apple

The separation of apple extract by means of anion exchange chromatography using a RESOURCE[®] Q column resulted in six peaks (fig. 1a). The main peaks were analyzed by SDS-PAGE. No fraction was homogeneous, but peak 3 contained the highest amount of the 18-kD protein besides some contaminants. After rechromatography of this fraction by RP-HPLC gradient elution on a Vydac C-4 column, a single peak was obtained at 50% acetonitrile (fig. 1b). Purity and allergen activity of this preparation were monitored by SDS-PAGE and immunoblot studies. As presented in figure 2, a single major protein was isolated with a MW of 18 kD. The immunoblot analysis of purified protein showed IgE binding in the same range (fig. 2). Furthermore, the allergenicity of the isolated fraction was confirmed by means of direct ELISA (fig. 3). Only serum from patients with allergy to birch pollen/apple showed binding activity to the 18-kD antigen. To assess the contribution of the 18-kD protein from apple to the birch/apple cross-reactivity, inhibition experiments were performed with purified allergens. The resulting inhibition graphs with the serum pool are presented in figure 4. The amounts of allergen which caused a 50% inhibition (C_{50}) of the IgE binding are 60 ng for Bet v 1 and 5,000 ng for the 18-kD apple allergen. The results confirm that Bet v 1 is a more potent inhibitor of IgE binding to the apple allergen. Additional inhibition studies were performed with three individual sera. The results of these experiments are given in figure 5a. The binding of IgE to the 18-kD apple allergen was inhibited with Bet v 1 in each case, however to different degrees. In the case of patient S3, higher amounts of Bet v 1 (C_{50} = 6 μ g) were necessary to inhibit IgE binding to the 18-kD apple protein, in comparison with both S1 and S2. The reverse inhibition experiment confirms that the apple allergen is a more potent inhibitor of IgE binding as Bet v 2 for patient S3 (fig. 5b).

Additionally, the N terminus of the purified 18-kD protein was determined and compared with the published sequence of Bet v 1 [20]:

1	11	21	31
GVYTFENEYT	SEIPPGRLEFK	AFVLDADNLI	PKIAPQA... 18-kD protein (apple)
GVFNYETETT	SVIPAARLEFK	AFILDGDNLF	PKVAPQA... Bet v 1

The small lines between amino acid symbols mark identity. On the basis of the 37 residues, a sequence identity of 67.6% was calculated. Furthermore, 4/37 residues of the two allergens are similar (position 3 and 5: Y/F; positions 23 and 33: I/V).

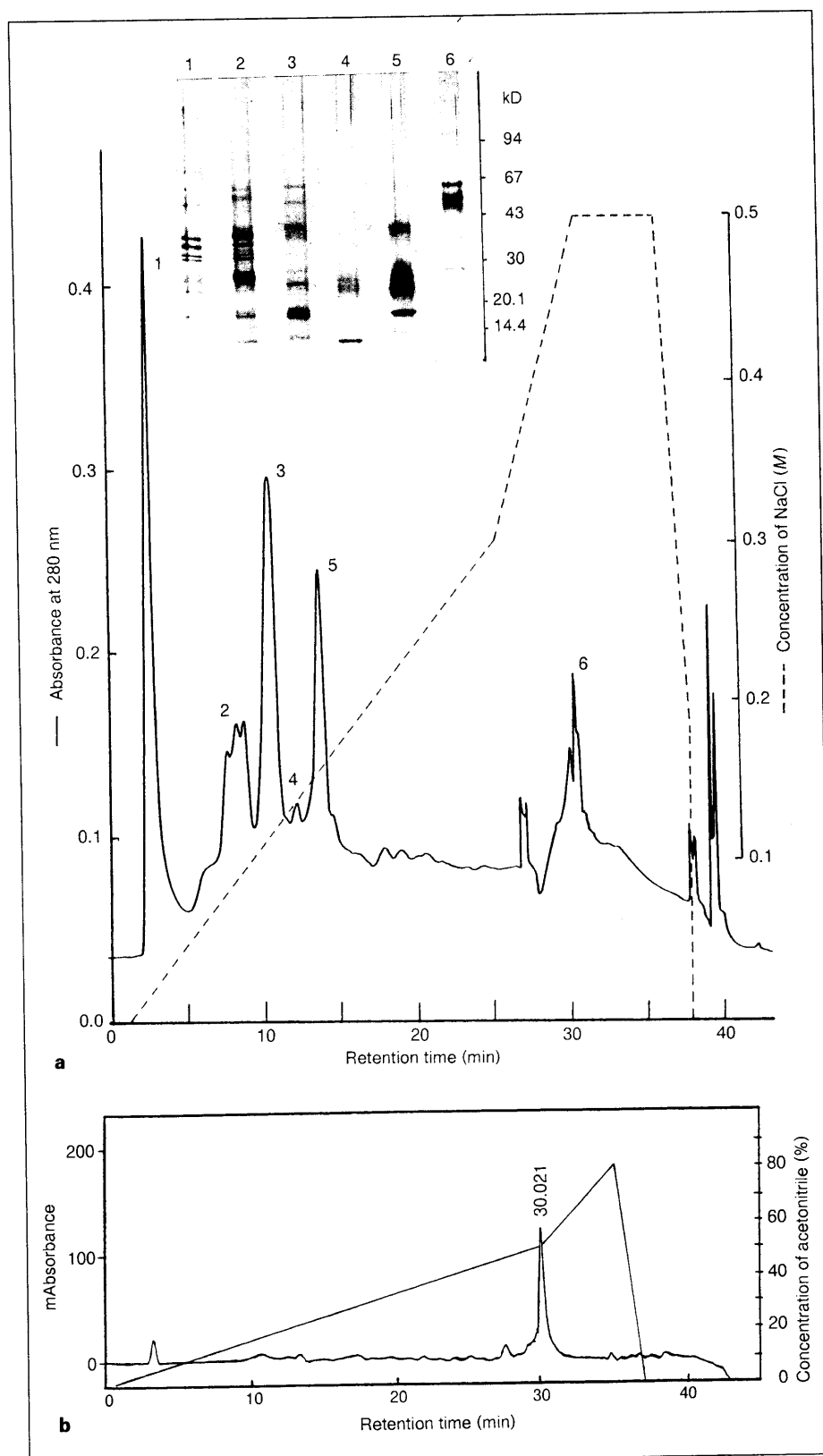
Characterization of mAbs

Of 14 positive clones producing mAbs against the 18-kD apple protein, eight clones were selected for further studies. All eight mAbs belong to the IgG1 subclass. To evaluate the binding behavior to birch pollen and apple extract, a direct ELISA was performed with serial dilutions of mAbs (fig. 6a). The affinity of mAbs to apple decreased in the following order: 2B2>3F8>2C9>3E1>ID6>3H1>3E7>3E8. Under the same experimental conditions only three mAbs (2B2>2C9>3F8) bound to microtiter plates coated with birch pollen extract (fig. 6b). Similar results were obtained with the purified allergens on the solid phase (fig. 6c). All antibodies reacted strongly with purified apple allergen and three mAbs (2B2, 2C9, 3F8) also reacted with Bet v 1. Furthermore, the specificity of antibodies was detected by immunoblot studies with apple extract (fig. 7). Surprisingly, only mAbs 2B2, 2C9, 3F8, 3E7, and ID6 recognized the 18-kD apple allergen although all mAbs were adjusted to a concentration of 1 μ g/ml allowing binding under saturating conditions (see fig. 6a). We could not detect any binding of mAbs to blots of birch pollen extract.

Discussion

Several investigations have demonstrated that birch pollen allergens induce cross-reactivity with apple antigens [19]. Many attempts have been made to identify and characterize these allergenic constituents [5]. In the meantime, there is evidence(s) for cross-reactivity of Bet v 1 (17 kD) and the 18-kD apple equivalent. Ebner et al. [7] showed binding activity of monoclonal anti-Bet v 1 antibody to the corresponding apple allergen. This was verified by Vieths et al. [20]. Furthermore, a labelled Bet v 1 probe hybridized to the RNA extracted from apples in Northern blots [7]. How-

Fig. 1. a Ion exchange chromatography of apple extract on RESOURCE™ Q using FPLC. 1 mg protein was loaded on a column equilibrated with 20 mM TRIS-HCl, pH 8.0. Elution was performed using a salt gradient with buffer containing 0.5 M NaCl. *Inset*: SDS-PAGE analysis of designed peaks in lanes 1–6. **b** Rechromatography of peak 3 on RP-C4 column (Vydac 214TP54). Protein was eluted using an acetonitrile gradient as indicated.



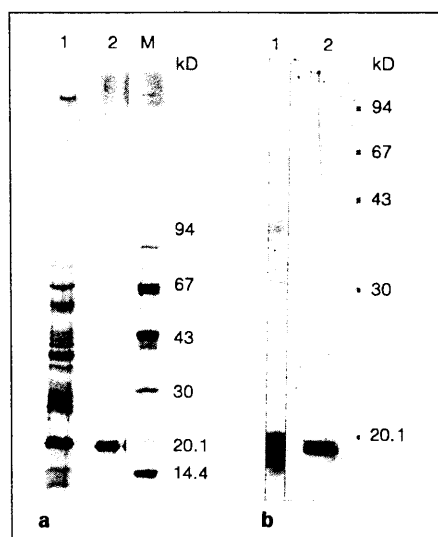


Fig. 2. Analysis of apple extract and the purified 18-kD protein by SDS-PAGE and immunoblotting. **a** Silver-stained gel. Lane M = MW standards; 1 = apple extract; 2 = 18-kD protein purified by FPLC and reverse-phase HPLC. **b** In a second run the proteins (lanes 1 and 2) were separated, transferred to nitrocellulose and incubated with pooled sera. Bound IgE was detected as described in Materials and Methods.

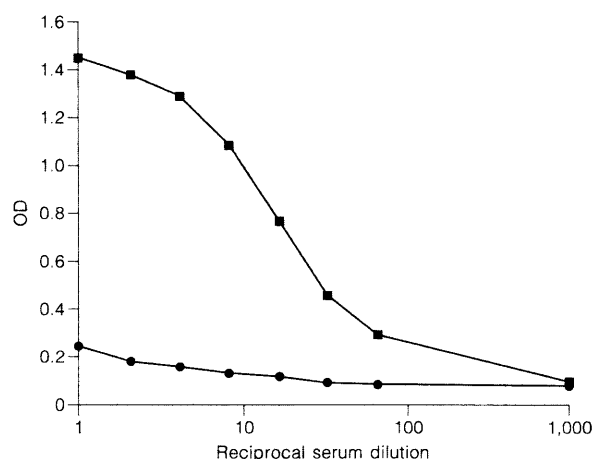


Fig. 3. Examination of the allergenic activity of the 18-kD apple protein. IgE binding was detected by direct ELISA using the purified 18-kD protein on solid phase. Serial dilutions of pooled serum from patients with birch/apple allergy (■) were tested as was control serum (●).

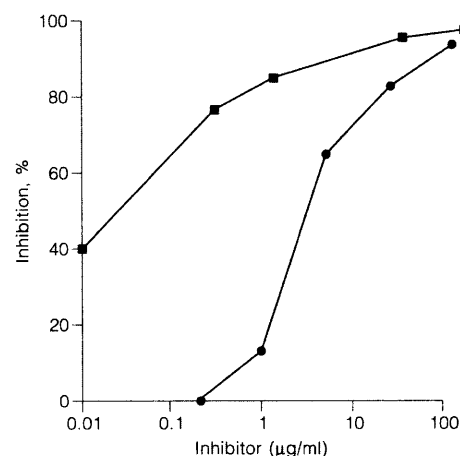


Fig. 4. EIA inhibition of IgE binding to the 18-kD protein by Bet v 1 (■) or 18-kD apple allergen (●) using a serum pool.

ever, so far none of the allergenic components has been isolated and purified from apple.

Since apple allergens are highly labile, extracts with high allergenic activity first had to be prepared as described by Rudeschko et al. [11]. These extracts are excellent for both in vitro and in vivo diagnostic studies. Using such concentrated apple extracts, the isolation of the major allergen in an active form was successful using anion exchange chromatography followed by RP-HPLC (fig. 1, 2). Interestingly, 18-kD proteins were found in fractions 3 and 5 (fig. 1). This might be explained by the presence of isoforms of the 18-kD protein as recently described by Vieths [20]. Our own studies using a two-dimensional PAGE immunoblot of apple and our mAbs support this assumption (results not shown). From the sequence data presented, it can be seen that the analysed fraction of the 18-kD apple protein was pure enough to determine unambiguously, the N-terminal sequence of 37 amino acids. The same sequence has been published recently by Vieths et al. [19] based on 26 amino acids, with one exception. We could not confirm the uncommon sequence of three consecutive proline residues in positions 14–16. Comparing the 18-kD protein from apple with Bet v 1 [20] reveals 67.7% identity (78.4% similarity) for the amino acid sequence 1–37. This partial identity confirms, furthermore, the existence of identical as well as nonrelated structures on both allergens. Subsequent comparison of the 18-kD apple protein with protein sequences residing in the Swiss Prot data base revealed additional sequence similar-

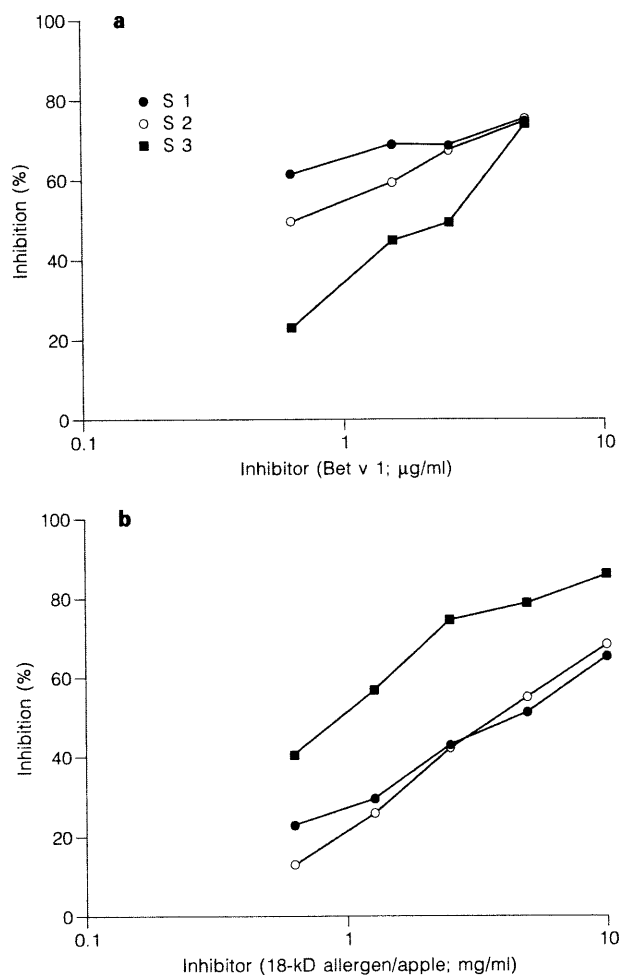


Fig. 5. EIA inhibition of IgE binding with purified allergens using three individual sera(s) from patients with birch/apple allergy. **a** Inhibition of 18-kD allergen with Bet v 1. **b** Inhibition of Bet v 1 with 18-kD protein.

ities to other major allergens (Aln g 1, Car b 1, Que a 1 [21], and Cor a 1 [22]) ranging from 58 to 65%.

Furthermore, our EIA inhibition experiments with purified allergens and serum pool show that, indeed, Bet v 1 is a more potent inhibitor of IgE binding to the apple allergen. Compared to the apple allergen, the allergenic potency of Bet v 1 was nearly 100-fold higher (fig. 4). Interestingly, the IgE binding using a serum pool was inhibited at a lower Bet v 1 concentration in comparison with the individual sera (fig. 5a). One explanation for this phenomenon could be a higher diversity of IgE specificities in pooled sera, recognizing a broader spectrum of IgE epitopes on the Bet v 1. Considering the data shown with the individual sera, we suggest

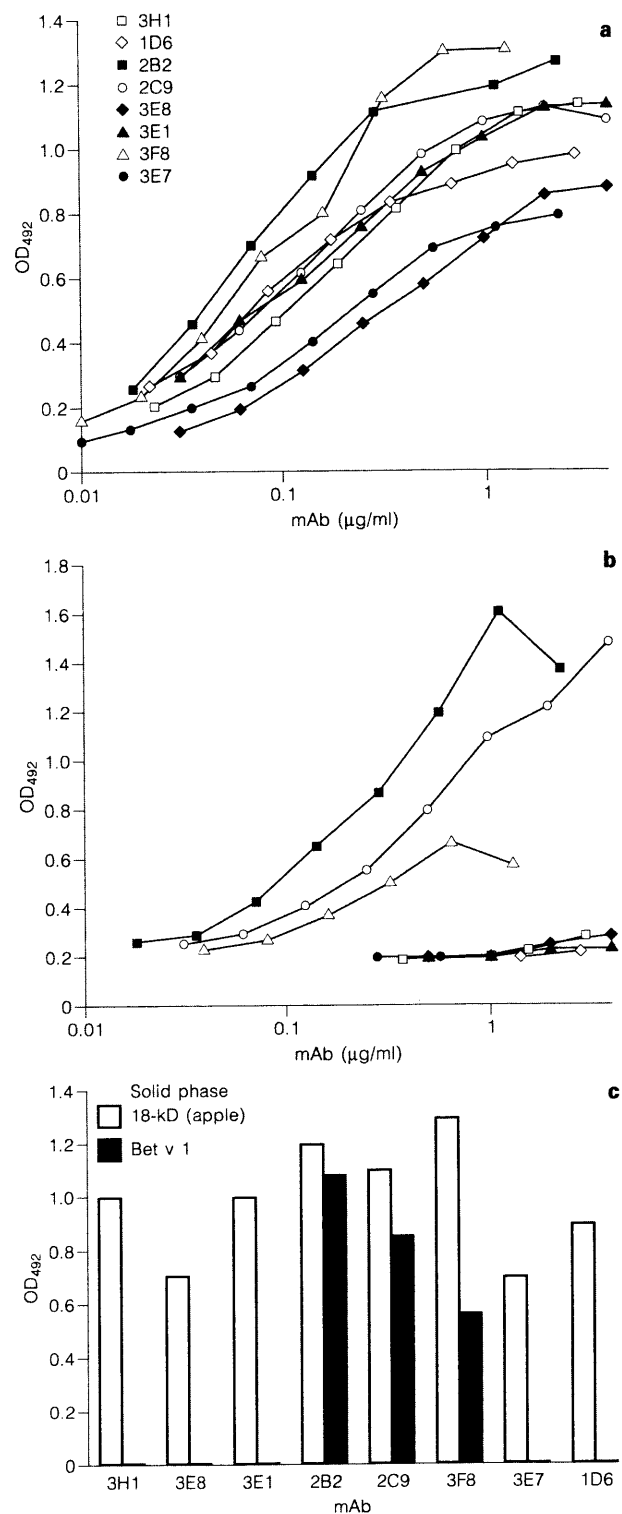


Fig. 6. Detection of mAb affinity by direct ELISA. Microtiter plates were coated with apple extract (**a**) or birch pollen (**b**) and incubated with serial dilutions of mAb. The mAb specificity was studied with purified 18-kD protein or Bet v 1 on the solid phase (**c**) and mAb concentrations of $>1 \mu\text{g/ml}$.

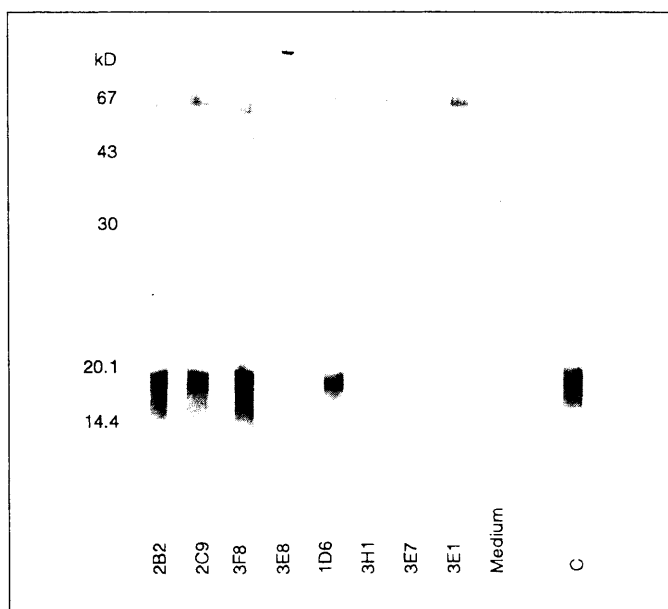


Fig. 7. Reactivities of mAbs after Western blotting of apple extract. Lane C is the control immunoblot of birch pollen extract with monoclonal anti-Bet v 1 antibody 3B4 [22].

furthermore that beside cross-reacting structures to Bet v 1, apple-specific IgE antibodies may also exist (see patient S3, fig. 5). Unfortunately, we do not have any serum from patients only with apple allergy, i.e. without birch pollen allergy.

To obtain more information on the structure similarity of the allergens, we raised mAbs against the 18-kD apple com-

ponent. All mAbs studied reacted with solid-phase bound apple extract or purified 18-kD protein. Three mAbs (2B2, 2C9, 3F8) also recognized birch pollen allergen (Bet v 1) on solid phase (fig. 6). We concluded from this finding that these three mAbs recognize cross-reacting structures on apple and birch pollen allergen. To confirm this suggestion, we studied Western blots of apple and birch extracts with all antibodies (fig. 7). Although the antibodies were adjusted to saturating conditions (1 µg/ml, see fig. 6a), only the antibodies 2B2, 2C9, 3F8, 3E7, and 1D6 reacted with the 18-kD apple protein, whereas the mAbs 3H1, 3E8, and 3E1 showed no reaction to any protein. Similar binding patterns were obtained with our antibodies by Vieths et al. [23]. Since all mAbs reacted with the native apple extract or the purified 18-kD allergen in a direct ELISA, we suggest that the mAbs 3H1, 3E8, and 3E1 recognize conformational epitope(s). Differences were found in the binding pattern of mAbs 2B2, 2C9, and 3F8 to solid-phase bound native and immunoblotted birch pollen allergen. No antibody reacted with birch pollen protein bound to nitrocellulose membrane. Possible explanations could be the denaturation of epitopes by SDS-PAGE and the blot technique or different affinities of antibodies to solid-phase bound antigen. Therefore, it will be interesting to perform inhibition experiments with native proteins.

Since our antibodies recognize different allergen sites, we will study whether the antibodies are suitable for quantification of the allergen in apple extracts. Immunoblot studies using our mAbs pointed to a different 18-kD protein content in different apple strains [23].

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