

Identification of a 60 kd cross-reactive allergen in pollen and plant-derived food

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Background: Cross-reactive IgE antibodies were found to be responsible for allergic reactions in patients allergic to pollen on ingestion of food (oral allergy syndrome). So far, the major birch pollen allergen Bet v 1 and birch profilin (Bet v 2) were identified as relevant cross-reactive allergens.

Objective: In this study we attempted to identify additional cross-reactive plant allergens, which could be responsible for food intolerance in patients allergic to pollen.

Methods: Monoclonal antibodies specific for the major mugwort pollen allergen, Art v 1, representing a 60 kd glycoprotein, were used to detect cross-reactive allergens in other pollens and plant-derived food. The amino acid compositions of the cross-reactive structures were determined, and their resistance against trypsin treatment was investigated. In addition, IgE immunoblot inhibitions were done with the 60 kd mugwort pollen allergen.

Results: Monoclonal antibodies specific for the major mugwort pollen allergen, Art v 1, cross-reacted with proteins of comparable molecular weight in fruit and vegetables. Preadsorption of patients' sera with the 60 kd mugwort allergen led to a reduction of IgE binding to components of a similar molecular weight present in different pollen (birch, timothy grass), fruit (apple, peanuts), and vegetable (celery) extracts and reduced IgE binding to apple, kiwi, and celery as determined by RAST inhibitions.

Conclusion: A cross-reactive plant panallergen, possibly identical to the major mugwort pollen allergen, Art v 1, is described. The allergen represents a protein of approximately 60 kd present in various pollen and plant foods, which is distinct from Bet v 1 and profilin and hence may represent a novel cross-reactive allergen in the oral allergy syndrome. (*J Allergy Clin Immunol* 1996;98:938-47.)

Key words: Cross-reactive allergens, pollen allergy, food allergy, IgE, IgE inhibition

Allergic reactions to different plants and plant products (pollen, plant-derived food) are frequently observed and may lead to severe anaphylactic events and even death.^{1,2} Cross-reactive allergens present in pollen and plant-derived food

Abbreviation used

SDS-PAGE: Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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have been recognized as major causes of such symptoms.³⁻¹¹

The use of monoclonal antibodies and purified natural or recombinant allergens for IgE inhibition experiments has led to the definition of cross-reactive structures, thus providing a molecular basis for the clinically observed symptoms. Until now, two important pollen allergens, the major birch pollen allergens Bet v 1 and birch profilin, Bet v 2, were identified as cross-reactive structures in pollen and plant-derived food and can presum-

ably function as such because of their conserved biologic functions.¹¹⁻¹⁷ Bet v 1 was found to be highly homologous to pathogenesis-related plant proteins,¹⁸ and Bet v 2¹⁹ was identified as profilin, an actin-binding protein in higher plants.²⁰ Although these two proteins account for numerous IgE-mediated cross-reactivities, other cross-reactive structures have yet to be defined.

In this study we identified a third cross-reactive allergen, of approximately 60 kd, in pollen (mugwort pollen, birch pollen, timothy grass pollen) and fruit and vegetables (peanut, apple, celery) by cross-reactivity of patients' IgE antibodies and monoclonal antibodies and by IgE inhibition experiments.

METHODS

Sera

Sera from patients allergic to mugwort pollen were selected by typical case history, positive skin prick test results, and RAST as previously described.^{21,22} Sera were further characterized for the presence of specific IgE antibodies against the major birch pollen allergen, Bet v 1, birch profilin, timothy grass pollen allergens, Phl p 1, Phl p 2, and Phl p 5 by testing with recombinant allergens. For IgE-inhibition experiments, sera from patients with a polyvalent sensitization to tree, grass, and weed pollens who demonstrated symptoms of allergy on ingestion of plant-derived food (fruits, vegetables) were used.

Characterization of monoclonal antibodies

The mouse monoclonal antibodies 2B3, 2G12, 3A4, and 4D1 were produced by immunization of female BALB/c mice (8 to 12 weeks old) with the 60 kd major mugwort allergen purified as described by De la Hoz et al.²³ Mice were immunized intraperitoneally with complete Freund's adjuvant (Sigma, St. Louis, Mo.) together with 5 µg of purified allergen, followed by two booster injections with incomplete Freund's adjuvant after 4 and 8 weeks. Ten micrograms of purified allergen was injected intravenously in the mice 4 days before cell fusion. Hybridomas were selected by ELISA with solid-phase-bound total mugwort pollen extract, as well as the purified mugwort pollen allergen. Monoclonal antibodies 2B3, 2G12, 3A4, and 4D1 could be identified as IgM antibodies by using a typing kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Protein extracts

Pollen from birch (*Betula verrucosa*), mugwort (*Artemisia vulgaris*), and timothy grass (*Phleum pratense*) was purchased from Allergon (Välinge, Sweden). One gram of pollen was homogenized in 20 ml of distilled water with an Ultraturrax (IKA, Heidelberg, Germany) and incubated at room temperature for 30 minutes under continuous shaking. Fruits and vegetables were pur-

chased at a local retail shop. Protein extracts of nuts, fruits, and vegetables were prepared as previously described.^{11,14} Extracts were then centrifuged for 30 minutes at 10,000 g at 4° C to remove insoluble particles. The supernatants were lyophilized, and the protein content was checked for quantity and quality by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)²⁴ and Coomassie Brilliant Blue (Bio-Rad, Richmond, Calif.) staining.²⁵

Immunoblotting

Pollen, fruit, and vegetable proteins were separated by SDS-PAGE and transferred to nitrocellulose.²⁶ Nitrocellulose blots were incubated overnight at 4° C with 1:5 diluted hybridoma supernatants. Dilutions and incubations were made with buffer A: 50 mmol/L sodium phosphate (pH 7.5), 0.5% vol/vol Tween-20, 0.5% wt/vol bovine serum albumin, and 0.05% NaN₃. Bound monoclonal antibodies were detected with a 1:500 diluted iodine 125-labeled sheep anti-mouse anti-serum (Amersham, Buckinghamshire, U.K.) as previously described.¹⁴

For detection of bound serum IgE, nitrocellulose blots were incubated overnight with 1:10 diluted sera.¹⁵ Bound IgE was detected with 1:10 diluted ¹²⁵I-labeled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden).

Analyses of amino acid compositions of cross-reactive allergens: Peptide sequencing of the 60 kd mugwort allergen

Birch pollen, mugwort pollen, timothy grass pollen, peanut, apple, and celery protein extracts were separated by SDS-PAGE and blotted onto Immobilon-P transfer membranes (Millipore Corp., Bedford, Mass.) according to the manufacturer's advice. The protein bands that had been identified as IgE-binding components at 60 kd were indicated. The amino acid compositions of these bands were determined at the W. M. Keck Foundation Biotechnology Resource Laboratory in New Haven, Connecticut, with a Beckman Analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Mugwort pollen extract was separated by SDS-PAGE. After Coomassie Brilliant Blue staining of the gel, the 60 kd band was excised, and pieces of gel containing approximately 5 µg protein/cm gel were frozen on dry ice and shipped to the W. M. Keck Foundation Biotechnology Resource Laboratory. Proteins were digested in the gel matrix with trypsin and endoproteinase Lys-C and then eluted from the gel. A piece of gel without protein was used as a control. Peptides were fractionated and purified by high-pressure liquid chromatography. The peptide samples were analyzed by laser desorption mass spectrometry, and samples containing a single species were subjected to amino acid sequencing with an Applied Biosystems sequencer (Applied Biosystems, Division of Perkin-Elmer Corp., Foster City, Calif.). The obtained amino acid sequence was compared with the National Center for Biological Information's databases

TABLE I. Inhibition of IgE binding to apple, kiwi, and celery CAPs after preincubation of sera with the 60 kd cross-reactive mugwort allergen

Patient No.	Apple 0 (KUA/L)	Apple + (KUA/L)	Percent reduction	Kiwi 0 (KUA/L)	Kiwi + (KUA/L)	Percent reduction	Celery 0 (KUA/L)	Celery + (KUA/L)	Percent reduction
1	5.2	4.9	14.13	3.0	2.3	23.46	7.4	6.0	14.88
2	15.7	14.8	3.71	4.9	4.7	2.94	12.4	12.0	2.03
3	3.5	2.2	35.92	2.4	0.8	61.55	3.5	2.0	40.45
4	0	0	0	0	0	0	0	0	0

Sera from three patients (nos. 1 to 3) with allergy to plant-derived food and a serum sample from a nonallergic individual were probed with apple, kiwi, and celery CAPs after preincubation with the nitrocellulose-blotted 60 kd cross-reactive mugwort allergen (+) and with empty nitrocellulose strips (0 = negative control). Bound specific IgE is displayed in kilounits antigen per liter (KUA/L). The degree of inhibition is displayed as percent reduction of IgE binding.

(i.e., Protein Identification Resource, Genpept, and Swiss Protein databases).

Digestion of mugwort pollen extract with trypsin

Mugwort pollen extract was digested with 20 µg of trypsin (Boehringer Mannheim) per 50 mg of protein in the extract at 37° C for 2 hours. Digested protein extract was analyzed by SDS-PAGE²⁴ and Coomassie Brilliant Blue staining.²⁵ To allow a comparison of antibody reactivities with trypsinized and untreated mugwort pollen extracts, equal amounts of proteins were incubated with and without trypsin at 37° C before electrophoresis and blotting.

IgE-inhibition experiments

Sera from three patients with IgE reactivity to 60 to 69 kd components in various pollens, fruits, and vegetables who had a well-defined case history of food allergy (i.e., at least one of the following clinical symptoms: perioral erythema; swollen lips, tongue, and throat; itching in the mouth; rhinitis; urticaria; or angioedema after ingestion of apples, nuts, peanuts, and/or celery) were selected for immunoblot experiments. IgE inhibitions were done with the 60 kd mugwort allergen isolated by trypsinization of mugwort pollen extract and subsequent preparative Western blotting. Excised nitrocellulose containing the 60 kd protein (approximately 10 µg of protein per 0.5 ml serum) was then used to preadsorb allergic patients' sera overnight at 4° C. Preadsorbed sera were allowed to bind to nitrocellulose-blotted birch pollen, mugwort pollen, timothy grass pollen, peanut, apple, and celery extracts overnight at 4° C. Bound IgE was detected with 1:10 diluted ¹²⁵I-labeled anti-human IgE antibodies (Pharmacia). Sera from three additional patients with food allergy and a nonallergic control subject were preadsorbed with the purified nitrocellulose-blotted 60 kd mugwort allergen or with empty nitrocellulose sectors (negative control). The remaining IgE specific for apple, kiwi, and celery was then determined by CAP system (Pharmacia) measurements (Table I).

RESULTS

Patients allergic to pollen with food intolerance display IgE reactivity to allergens of approximately 60 kd in pollen and plant-derived food

The IgE reactivity of four representative patients allergic to mugwort pollen with a well-documented case history of food allergy and multiple sensitization to different pollens with nitrocellulose-blotted birch pollen, mugwort pollen, timothy grass pollen, peanut, apple, and celery proteins is shown in Fig. 1. Each of these patients showed IgE binding to bands of 60 to 69 kd in all extracts, with the exception of peanut, in which the binding was less pronounced. Additional IgE binding to a number of bands between 97 kd and 10 kd was also observed. By IgE inhibition experiments, the low-molecular-weight band (10 to 16 kd) could be identified as Bet v 2 (profilin) homologous protein. Most of the IgE reactivity to 30 kd allergens in timothy grass pollen is due to group I and group V allergens.^{27, 28} Nonallergic patients' sera, which were used as negative controls, showed no IgE binding to any of the extracts (data not shown).

Proteins of 60 kd present in pollens, fruits, and vegetables show similar amino acid compositions

Fig. 2, *A* shows that the 60 kd allergen represents a rather abundant protein in pollen, fruit, and vegetable extracts. The comparison of the amino acid compositions of the 60 kd bands in mugwort pollen, birch pollen, timothy grass pollen, peanut, apple, and celery is displayed in Fig. 2, *B*. The most significant similarities were observed among the pollen allergens and between mugwort and celery,

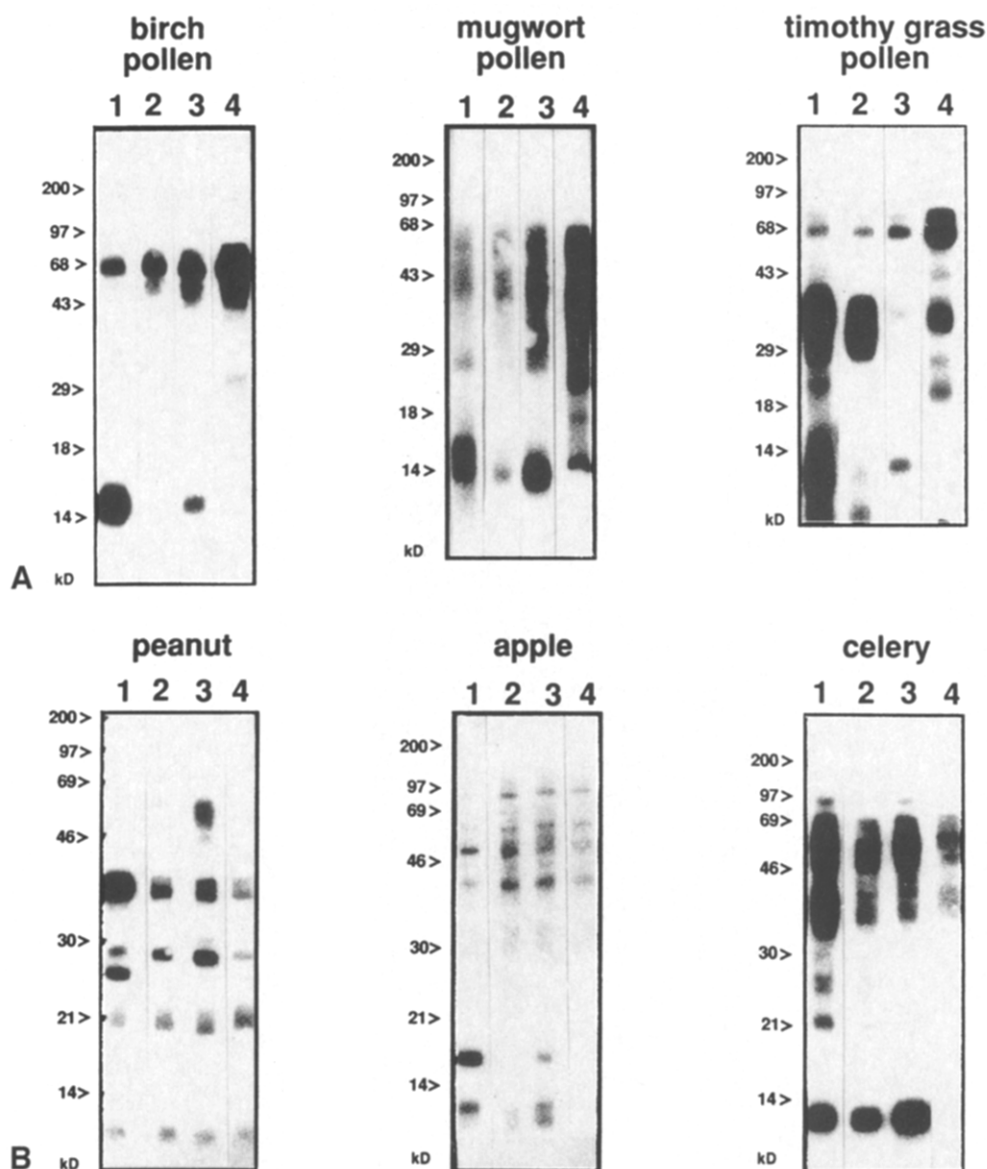


FIG. 1. IgE reactivity of four representative patients allergic to mugwort pollen displaying intolerance to different pollens (**A**) and plant extracts (**B**). Sera from four patients (lanes 1 to 4) were tested for IgE binding to birch pollen (*Betula verrucosa*), mugwort pollen (*Artemisia vulgaris*), timothy grass pollen (*Phleum pratense*), peanut (*Arachis hypogaea*), apple (*Malus domestica*), and celery (*Apium graveolens*).

respectively. The composition of the peanut allergen showed the least similarity.

Several attempts to obtain sufficient amounts of peptide fragments from the 60 kD mugwort allergen for sequencing failed. Treatment of Immobilon blots or gel slices with trypsin did not cleave the protein sufficiently. No N-terminal amino acid sequence could be obtained because of a blocked amino terminus of the protein. In gel digestion of the 60 kD protein with endoproteinase, Lys-C finally resulted in a nonapeptide, which, after

high-pressure liquid chromatography purification, could be sequenced. When compared with the Swiss-Prot library, the nonapeptide showed 77% homology with a peptide (amino acid 67-75) from subtilisin inhibitor (*Streptomyces albogriseolus*)^{29,30} (SWISS PROT accession number: P01006) (Fig. 3).

Art v 1 is difficult to digest with trypsin

The difficulties in digestion of Art v 1 with trypsin and the homology of a nonapeptide to a

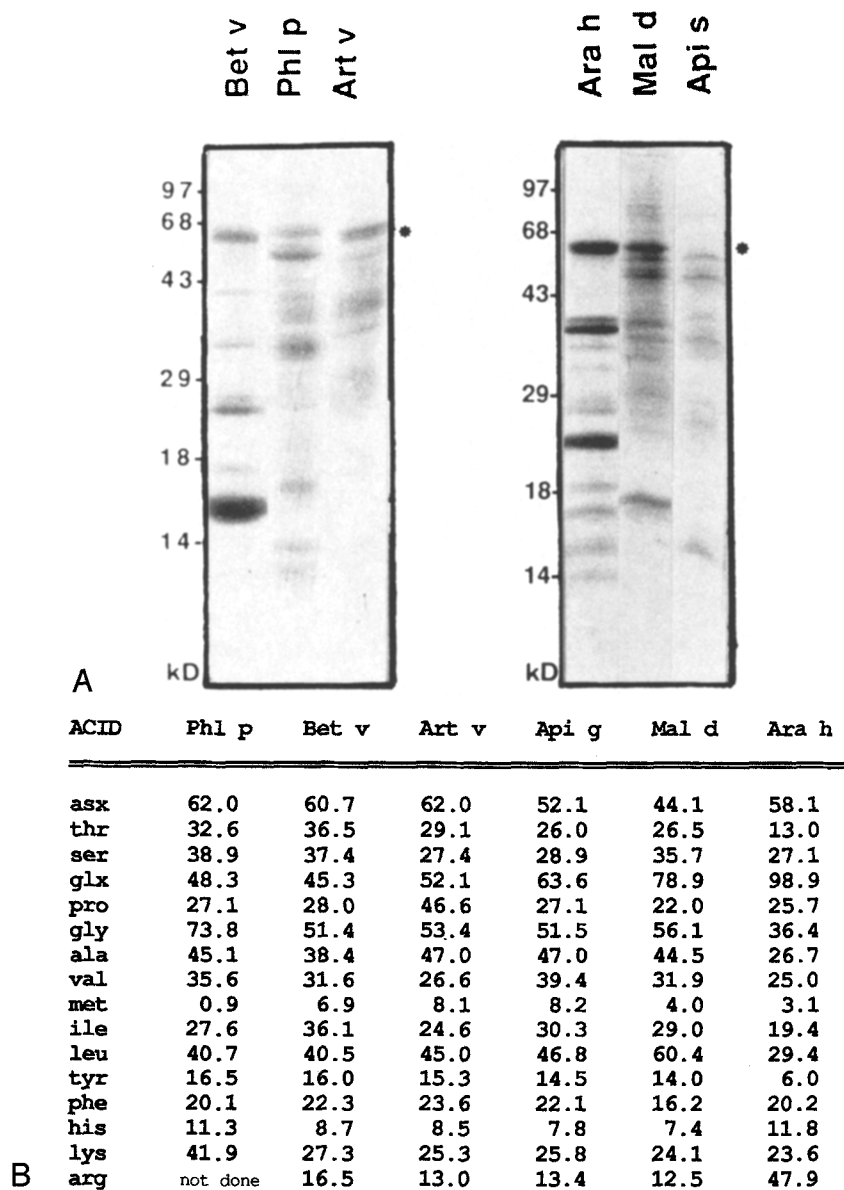


FIG. 2. A, Coomassie Brilliant Blue-stained SDS-PAGE containing pollen, fruit, and vegetable extracts. Positions of the bands in birch pollen (Bet v), timothy grass pollen (Phl p), mugwort pollen (Art v), peanut (Ara h), apple (Mal d), and celery (Api g) extracts, which were analyzed for their amino acid composition in **B**, are indicated with *asterisks*. The celery band migrates at a slightly lower molecular weight. **B**, Comparison of amino acid compositions of the 60 kd mugwort pollen allergen with cross-reactive allergens in different plant extracts. Values reflect number of residues.

subtilisin inhibitor from *S. albogriseolus* indicated that the 60 kd mugwort allergen might represent a protein that is rather resistant to trypsin treatment. Mugwort pollen extract was digested with trypsin and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Fig. 4 shows that after trypsin treatment of mugwort pollen extract, only a 60 kd component and a 35 to 40 kd component re-

mained. The band at 25 kd, indicated by an asterisk, represents trypsin.

To provide evidence that the remaining 60 kd protein represents an allergen, the IgE reactivity of three representative patients allergic to mugwort pollen with nitrocellulose-blotted trypsinized and untreated mugwort pollen extract was studied (Fig. 5). IgE binding of the sera to profilin at 14 kd and

60 kD mugwort pollen protein:

APGPSXXNP

|||||

subtilisin inhibitor :

67 APGPSGTHP 75

FIG. 3. A nonapeptide digested and purified from the 60 kd mugwort allergen showed homology with a subtilisin inhibitor from *S. albogriseolus*.

to a 28 to 30 kd allergen was completely abolished after trypsin treatment, whereas IgE reactivity against the 60 kd component was less affected. Nitrocellulose-blotted trypsin present in the trypsin-treated mugwort pollen extract bound the ^{125} I-labeled anti-human IgE antibodies at 25 kd, as indicated with an asterisk in Fig. 5.

Monoclonal antibodies raised against the major mugwort allergen, Art v 1, cross-react with homologous proteins in fruit and vegetables

The monoclonal antibodies 2B3, 2G12, 3A4, and 4D1 raised against the major mugwort pollen allergen, Art v 1, cross-reacted with proteins of 60 to 69 kd in fruit and vegetable extracts (Fig. 6). A 60 kd component was detected in apple and celery extracts; however, it was less pronounced in peanut. Additional binding to proteins between 46 and 12 kd was observed.

The 60 kd mugwort allergen inhibits IgE binding to homologous allergens in pollen and plant-derived food

IgE-inhibition experiments were done to investigate the cross-reactive nature of the trypsin-resistant allergen. Preincubation of three representative sera (P1, P2, and P3) (Fig. 7, A and B) with the 60 kd mugwort allergen led to a significant reduction of IgE binding to mugwort allergens of molecular weights between 30 to 80 kd in patients P2 and P3. Likewise, IgE binding to a number of bands with comparable molecular weights in birch pollen, timothy grass pollen, peanut, apple, and celery was substantially reduced. The IgE reactivity of patient P1 was less affected by the preadsorption, indicating that additional cross-reactive epitopes were lost by the trypsin treatment of the competitor. The RAST inhibition shown in Table I supports our contention that the 60 kd mugwort allergen shares IgE epitopes with plant food allergens. In certain sera, up to 60% of IgE binding to apple, kiwi, and celery could be preadsorbed with the 60 kd mugwort allergen.

Coomassie stain

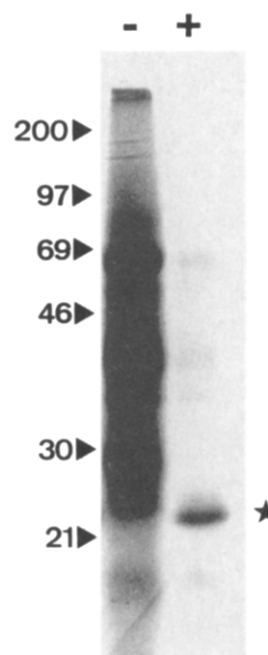


FIG. 4. Substantial amounts of the 60 kd mugwort pollen allergen remain after trypsin treatment of mugwort pollen extract. Coomassie Brilliant Blue staining of SDS-PAGE separated untreated (-) and trypsinized (+) mugwort pollen extract. Star indicates the trypsin band at 25 kd.

DISCUSSION

Structural similarities of proteins from different sources provide an explanation for a number of clinically observed cross-reactivities in type I allergy. For an accurate understanding of the molecular basis underlying the cross-sensitization of patients with allergy, these structures need to be defined. As yet, only two cross-reactive allergens, Bet v 1 and profilin (Bet v 2), have been identified as significant contributors to allergic reactions against pollen and plant-derived food.¹¹⁻²⁰ In this study a third cross-reactive plant allergen was described in pollen and plant-derived foods, which may represent another component in cross-reactivities in respiratory and food allergy.

Patients allergic to mugwort who showed IgE

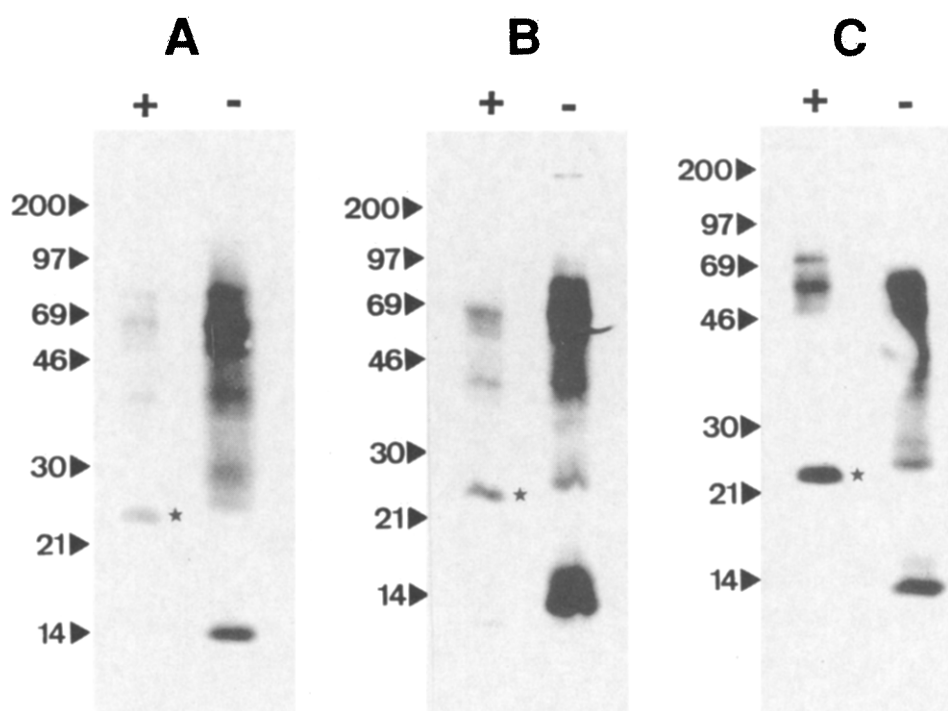


FIG. 5. IgE reactivity of sera from patients allergic to mugwort pollen with nitrocellulose-blotted, untreated mugwort pollen extract (–) and trypsinized mugwort pollen extract (+). Stars indicate the position of trypsin.

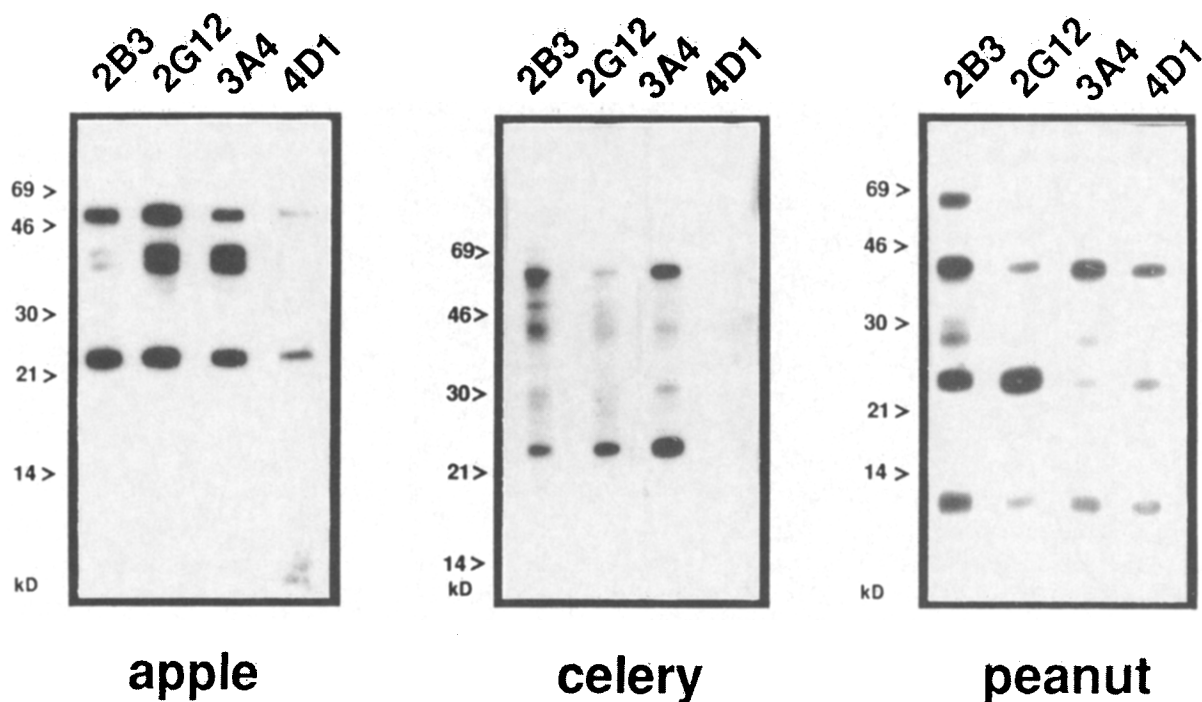


FIG. 6. Reactivity of monoclonal antibodies raised against the major mugwort pollen allergen, Art v 1, with different plant extracts. Four monoclonal antibodies raised against Art v 1 (2B3, 2G12, 3A4, and 4D1) were tested for reactivity with nitrocellulose-blotted apple (*Malus domestica*), celery (*Apium graveolens*), and peanut (*Arachis hypogaea*) proteins.

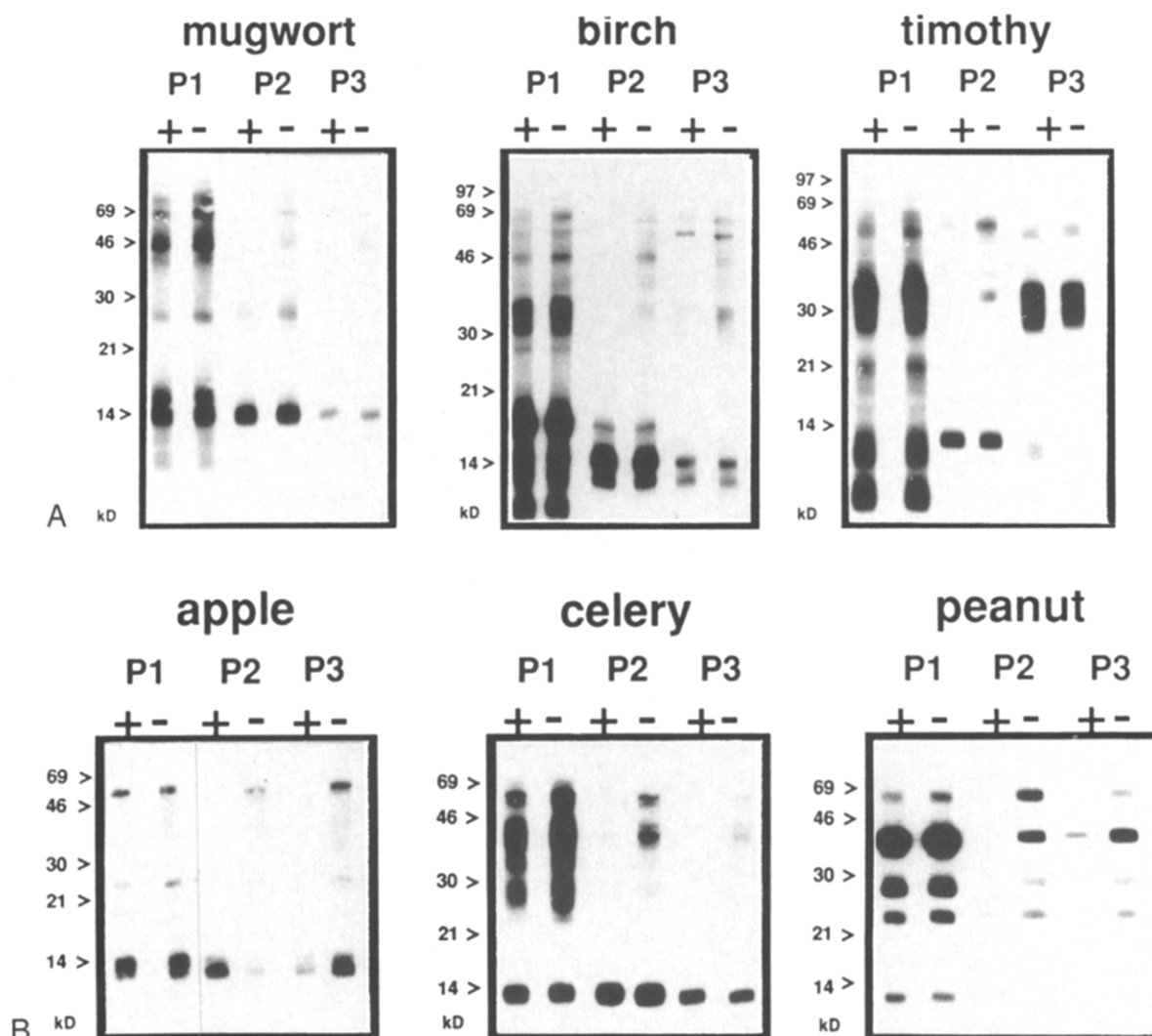


FIG. 7. The 60 kd trypsin-resistant mugwort allergen inhibits IgE binding to nitrocellulose-blotted homologous allergens in different pollen (A) and plant extracts (B). Sera from three representative patients allergic to mugwort with food intolerance (P1, P2, and P3) were preincubated without (–) or with the 60 kd trypsin-resistant mugwort pollen allergen (+). Preincubated sera were then tested for IgE binding to different plant extracts (mugwort pollen [*Artemisia vulgaris*], birch pollen [*Betula verrucosa*], timothy grass pollen [*Phleum pratense*], apple [*Malus domestica*], celery [*Apium graveolens*], and peanut [*Arachis hypogaea*]).

reactivity to the major mugwort pollen allergen Art v 1, a protein of 60 kd molecular weight and who according to case history were sensitized to tree pollen, grass pollen, weed pollen, and plant-derived food also displayed IgE binding to components of a comparable molecular weight in these sources. A panel of monoclonal antibodies raised against Art v 1²³ cross-reacted with allergens of similar molecular weight present in tree, grass, and weed pollen and fruit and vegetables.

Attempts were made to obtain an N-terminal amino acid sequence or peptide sequences. The N-terminus appeared to be blocked, and it was

extremely difficult to digest the protein with trypsin. Cleavage with endoprotease Lys-C finally allowed us to obtain peptides, of which one could be sequenced. This nonapeptide showed sequence homology with a portion in subtilisin inhibitor from *Streptomyces*.^{29, 30} The observation that the 60 kd component could not be cleaved with trypsin and the sequence homology of the peptide with a protease inhibitor prompted us to investigate whether the 60 kd mugwort allergen might represent a protein with increased resistance to trypsin digestion.

The high degree of protease resistance of the 60

kd allergen was confirmed by the fact that it still bound IgE even after extensive trypsinization of the mugwort pollen extract. Although the binding of IgE antibodies directed against mugwort profilin at 14 kd and against a 30 kd allergen was completely abolished, the 60 kd allergen still bound substantial amounts of IgE. The observed partial reduction of IgE binding to the 60 kd allergen might be explained by a partial trypsin resistance of the allergen, or alternatively, by comigration of trypsin-sensitive allergens. IgE inhibition experiments performed with the 60 kd mugwort pollen allergen prepared by trypsin digestion showed that IgE binding to 60 kd components present in tree, grass, and weed pollens, as well as in fruit and vegetables (peanut, apple, and celery), could be significantly reduced. RAST inhibition studies indicated that substantial amounts (up to 60%) of apple-, celery-, and kiwi-specific IgE could be preadsorbed with the 60 kd mugwort pollen allergen.

The immunoblot inhibition studies revealed that the 60 kd allergen was able to block IgE binding to components of higher and lower molecular weight, suggesting a polymorphism of the cross-reactive structures. It is thus possible that the described cross-reactive allergen is composed of different subunits, as described for the major allergen of cat (Fel d 1),³¹ or that the allergen polymorphism is due to the occurrence of isoallergenic variants as described for other plant allergens.³¹⁻³⁵

The observed polymorphism might also be attributed to posttranslational modifications of the allergen, such as glycosylation. Because carbohydrates have been discussed as possible cross-reactive IgE-binding structures,³⁶ it is even possible that the cross-reactive IgE antibodies are directed against carbohydrate moieties.

Although the analysis of the amino acid compositions of the cross-reactive structures in birch pollen, timothy grass pollen, mugwort pollen, peanut, apple, and celery revealed a high degree of similarity and although the 60 kd proteins could be stained with Coomassie Brilliant Blue even after extensive trypsin treatment, we cannot exclude the possibility that IgE reactivity might be directed against bound carbohydrates.

On the other hand, deglycosylation experiments indicated that IgE binding to the 60 kd allergen is not significantly reduced (Fischer et al., Unpublished data), but as yet, no complementary DNA coding for the cross-reactive mugwort pollen allergen has been isolated by IgE immunoscreening. The difficulties in obtaining complete cDNA

clones for the cross-reactive allergen by antibody screening might be due to the intrinsic property of the protein, which may not be expressed in sufficient amounts in *Escherichia coli*, may not be correctly folded, or may consist of several subunits.

In summary, a cross-reactive 60 kd mugwort pollen allergen, presumably representing Art v 1, which is responsible for multivalent allergy to different pollens and foods of plant origin is described. Compared with other cross-reactive allergens (e.g., profilin), the 60 kd allergen showed a remarkable resistance against trypsin treatment and is expressed in unrelated plant species and different plant tissues.

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