Clinical role of a lipid transfer protein that acts as a new apple-specific allergen

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Background: Allergy to apple is commonly associated with birch pollinosis because the two share homologous allergens. However, some patients have apple allergy but no birch pollinosis, suggesting that there are allergens that do not cross-react with birch.

Objective: The aim of the study was to evaluate the IgE reactivity pattern to an apple extract in subjects with allergic reactions to apple, with and without birch hay fever.

Methods: Forty-three patients with oral allergy syndrome for apple and positive open food challenge, skin prick test, and serum specific IgE antibodies to apple were admitted to the study. Thirty-two had birch pollinosis (documented by specific IgE for birch) and 11 were not allergic to birch. The IgE reactivity pattern to apple extract was identified by SDS-PAGE and immunoblotting. The consistent allergen, a 9-kd protein, was then purified by HPLC and characterized by periodic acid–Schiff staining, isoelectric point, and N-terminal amino acid sequencing.

Results: The sera from 28% of patients allergic to apple with birch pollinosis, but from all patients allergic only to apple, recognized the 9-kd protein. This protein has an isoelectric point of 7.5 and is not glycosylated. Determination of its partial amino acid sequence showed that it belongs to the family of lipid transfer proteins, which act as major allergens in Prunoideae fruits.

Conclusions: These results indicate that a lipid transfer protein is an important allergen in patients allergic to apple but not to birch pollen. The prevalent IgE reactivity to this allergen in subjects with no birch pollinosis and the physicochemical characteristics of this protein suggest that sensitization may occur through the oral route. (J Allergy Clin Immunol 1999;104:1099-106.)

Key words: *Oral allergy syndrome, vegetable allergens, apple, birch pollinosis, immunoblotting, lipid transfer protein*

Abbreviations used

AU: Activity units
LTP: Lipid transfer proteins
MW: Molecular weight
OAS: Oral allergy syndrome
PAS: Periodic acid–Schiff
SPT: Skin prick test

Allergic reactions to apple are usually associated with birch hay fever, and up to 75% of birch-allergic patients have clinical reactions when they eat apple.[1] The most common picture of hypersensitivity to apple, and in general to fresh fruits and vegetables, is the so-called oral allergy syndrome (OAS), which involves local symptoms, with or without systemic manifestations, when the culprit food comes into contact with the oral mucosa.[2]

The first reports of the apple-birch syndrome date back to the first half of the century, when Dannfeldt in Sweden, as cited by Dreborg and Foucard,[3] and Tuft and Blumstein[4] in the United States described this kind of allergy. This was further illustrated by Mowat et al,[5] who collected a number of allergic reactions to apple, carrot, and potatoes in subjects with birch hay fever, most of them allergic to apple.

This association was found to be caused by homologous structures in apples and birch pollen.[6] The major allergens so far identified in apple extract (Mal d 1 and Mal d 2) are proteins with a molecular weight (MW) of 18 and 14 kd, structurally homologous to the birch pollen major allergens Bet v 1 and Bet v 2, belonging, respectively, to the family number 10 of the pathogenesis-related proteins[6] and to the family of profilins.[7] However, in Southern Europe allergy to apple—like to fresh fruits and vegetables in general—is not always associated with birch hay fever, and a number of patients are allergic to apple, peach, and other fruits, without any pollinosis.[1] This was reported in a study from Spain, which described patients allergic to Rosaceae fruits including apple, peach, and pear who had no pollinosis.[8] These clinical data, together with our own finding that the major allergen of peach is a 9-kd lipid transfer protein (LTP), the only allergen recognized by patients allergic to peach

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without birch pollinosis,[9] led us to check apples for allergens different from those involved in cross-reactivity with birch.

Thus the aim of the current study was to verify whether an LTP allergen was involved in causing reactions to apple in patients with allergic manifestations to apple but not to birch. We therefore compared the IgE-binding reactivity to apple of subjects allergic to this fruit with or without birch pollinosis by studying a large group of patients with demonstrated clinical sensitivity to apple.

MATERIAL AND METHODS

Patients

Subjects with a history of OAS from apple, with or without systemic reactions, were recruited for this study from those referred to the Allergy Center of the Third Division of General Medicine of the University of Milan and to the Bizzozzero Division of the Niguarda Ca' Granda Hospital of Milan. Patients admitted to the in vitro part of the study had to present positive skin prick test (SPT) results to apple by the prick + prick technique,[3] specific IgE antibodies (CAP System, Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden), and a positive open oral challenge (unless they had had life-threatening reactions) with fresh apple, made by chewing increasing amounts of the fruit, from 4 to 64 g, as previously reported.[10] The challenge was stopped when symptoms arose. OAS symptoms were classified in 4 grades of increasing severity: grade 1, only oral mucosa symptoms; grade 2, oral mucosa and gastrointestinal symptoms; grade 3, oral mucosa symptoms plus systemic symptoms (urticaria, rhinoconjunctivitis, or asthma); grade 4, oral mucosa symptoms plus life-threatening symptoms (laryngeal edema, anaphylactic shock). The history of OAS to other vegetables was carefully evaluated in each patient.

The presence of pollinosis was established on the basis of the relationship between the occurrence of symptoms and the flowering period of the plants (February-April for birch and hazel pollen, May-June for grasses, April-July for *Parietaria*, July-September for mugwort, and August-October for ragweed) and by positive SPT to the respective pollen extracts (Bayer-DHS, Milan, Italy). The extracts used were Alphatests, which are standardized in activity units (AU) and controlled to a label claim of 400 AU/mL. Alphatests are registered for diagnostic use in the United States and in several European countries.

Blood was drawn from all patients and sera were collected and stored at –80°C until tested in vitro to identify apple allergens. The in vitro IgE reactivity of patients with OAS from apple, with or without birch pollinosis, and in patients with mugwort pollinosis was compared.

In vitro tests

Apple extract. The extract was prepared in our laboratory according to the method of Björksten et al.[11] A given amount of apple peels (Golden Delicious strain) were mixed 3:1 (wt/vol) in PBS, 10 mmol/L, pH 7, with 2% solid polyvinylpolypyrrolidone, 2 mmol/L EDTA, 10 mmol/L sodium diethyl dithiocarbamate, and 3 mmol/L sodium azide. After it was homogenized and centrifuged at 16,000 revolutions/min at 4°C for 30 minutes, the supernatant was dialyzed against PBS 10 mmol/L, pH 7, with 3 mmol/L sodium azide for 48 hours at 4°C, with the buffer changed at 16- to 18-hour intervals. Visking dialysis tubing (Serva Boehringer Ingelheim, Heidelberg, Germany) was used with a MW cutoff value of 8000 d. The extract was centrifuged at 3000 revolutions/min for 5 minutes and stored frozen at –20°C until used. For each experiment we handled about 300 g of peels in 100 mL of PBS, thus obtaining about 100 mL of final extract each time, with a protein content ranging from 6 to 9 mg/mL as assessed by the colorimetric method of Lowry et al[12] with use of Folin reagent and reading with a spectrophotometer at 700 nm. This extract was used for SDS-PAGE analysis but was too diluted for allergen purification; it was therefore concentrated by centrifugation at 3000 revolutions/min at 4°C for 10 hours with a Centricon 3 concentrator (Amicon) with a MW cutoff value of 3000 d. The extract was concentrated 15 times to achieve a final concentration of about 90 to 100 mg/mL protein, calculated by Lowry's method.

SDS-PAGE

The apple extract was separated in a discontinuous buffer system in a SDS-PAGE gradient gel with 6% stacking and 7.5% to 20% separation gradient gel, essentially according to Neville.[13] The sample, at the protein concentration of 7 mg/mL, was diluted 1:2 in sample buffer containing TRIS adjusted to pH 6.1 with concentrated sulfuric acid, 10% SDS, 2-mercaptoethanol, 50% glycerol, and 1% bromphenol blue. The samples were denatured at 100°C for 5 minutes and centrifuged at 10000 revolutions/min for 5 minutes. Reference markers (Pharmacia Biotech-Uppsala, Sweden) at known MWs, 94, 67, 43, 30, 20.1, and 14.4 kd, were run in the same gel; 0.280 mg of apple extract per centimeter of gel was run at 0.6 mA for 16 hours in a Bio-Ras Protein IIxi vertical electrophoresis slab cell (Bio-Rad Labs, Richmond, Va).

After separation, part of the gel, containing the low MW marker and the apple extract, was fixed and stained with Coomassie brilliant blue R-250 (Pharmacia) and another part was used for immunoblotting studies.

Immunoblotting

Allergens separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane (Amersham 0.45 μm) with use of a transblot cell from Bio-Rad at 0.45 A, 100 V, for 4 hours at 4°C. The unoccupied protein binding sites in the nitrocellulose membrane were blocked by a 30-minute incubation at 37°C with PBS, pH 7.4, with 0.5% Tween 20. The nitrocellulose was then cut into strips, which were incubated overnight at room temperature with the serum of each single patient. IgE binding by specific antibodies was detected by incubation with iodine 125–labeled anti-human-IgE diluted 1:4 in blocking solution for 6 hours at room temperature and exposure on x-ray film at –70°C for 3 days.

A 9-kd protein was identified as a relevant allergen and was purified. Immunoblotting of the raw apple extract was done with use of pooled serum from nonallergic patients as a negative control.

Purification of apple 9-kd allergen by HPLC cationic exchange

The 9-kd protein from apple was isolated and purified by analytical cationic exchange chromatography with an HPLC system (AKTA Purifier, Pharmacia Biotech, Uppsala, Sweden) injecting 10 mL of the raw extract with use of a 10-mL Superloop (Pharmacia Biotech) after dilution to reach the correct pH and molarity in a Resource-S (6 mL) column (Pharmacia-Biotech). The mobile phase was sodium citrate dehydrate, buffer A, 0.03 mol/L, pH 6, and sodium citrate dehydrate 0.03 mol/L, plus sodium chloride 1 mol/L, pH 6, buffer B. The gradient was 20 column volumes long, with a flow rate of 6 mL/min. Absorbance was monitored at 280 nm. After the peak corresponding to the unbound material, only one further peak was detected on the chromatogram. Analyzed by SDS-PAGE, this fraction contained the 9-kd protein with other higher MW impurities, and we therefore had to collect large amounts of the fraction by repeating the procedures to achieve further resolution by gel filtration.

Gel filtration

Cationic exchange concentrated fractions were separated on a Superdex 75 column equilibrated and eluted with 15 mmol/L sodium chloride in 30 mmol/L of sodium citrate buffer, pH 6, at a flow rate of 0.7 mL/min. Before the first run a calibration curve was plotted by measuring the elution volumes of various standard substances at known MW: ribonuclease 13.7 kd, chymotrypsinogen A 25 kd, ovalbumin 43 kd, blue dextran 2000 67 kd (Pharmacia-Biotech). The injection volume was 200 μL and absorbance was monitored at 280 nm. The chromatogram showed 4 peaks. The fractions corresponding to the peaks were concentrated and analyzed by SDS/PAGE, and their protein content was measured by the method of Warburg and Christian,[14] reading by spectrophotometer at 260 and 280 nm. They were stored at –20°C.

To confirm the purity of 9-kd protein, we did SDS-PAGE according to the method described.

IgE binding capacity of 9-kd apple protein and its inhibition by crude apple extract

IgE immunoblotting was done with use of pooled sera from patients No. 33 to 43 to investigate the IgE binding capacity of the 9-kd purified protein from apple. To confirm that this was the 9-kd component detected in raw apple extract, we conducted an immunoblotting-inhibition experiment. Briefly, 500-μL aliquots of the pooled sera used for the 9-kd protein were inhibited with 500 μL of undiluted 9-kd purified protein (0.45 mg of protein). After incubation the inhibited sera were matched with a nitrocellulose strip of the blotted SDS-PAGE of the raw apple extract. A nitrocellulose strip was also matched with uninhibited pool serum. Then the experiment followed the steps described above.

Amino acid sequencing

Protein and peptide sequence analysis was done on an Applied Biosystems 470A gas-phase sequencer (Applied Biosystems, Foster City, Calif) equipped with a 120A phenylthiohydantoin–amino acid derivative analyzer. All chemicals were from Applied Biosystems.

Isoelectrofocusing

The 9-kd purified protein was focused by a Pharmacia-LKB Phast System Ready using Phast Gel, pH gradient 3 to 9. The gel was fixed and stained with Coomassie brilliant blue R-250 and run according to the manufacturer's instructions.

Periodic acid–Schiff stain

Periodic acid–Schiff (PAS) staining was done to detect glycosylation of proteins. Ten microliters of purified 9-kd protein, corresponding to a protein content of 0.7 mg/mL, was run into Minigel, blotted onto a Problot membrane (Perkin-Elmer, Applied Biosystems), as described by Towbin and Gordon,[15] at 16 V, constant for 60 minutes except that methanol and SDS were omitted from the buffer. Two identical membranes were blotted at the same time, one stained with Coomassie R-250 and the other used for PAS staining for glycoproteins. For PAS staining the membrane was fixed in 12% trichloroacetic acid for 1 hour, tested with a 7% trichloroacetic acid solution containing 2% potassium metaperiodate and kept at 4°C for 1 hour. The membrane was then immersed in Schiff's reagent and kept in the dark at 4°C overnight. The background was destained in methanol; glycoproteins appeared as purple bands. Milk whey proteins were used as control.

RESULTS

Patients

Forty-three subjects, 24 women and 19 men, aged 15 to 55 years (median 28 years), were recruited for the in vitro study. All reported OAS and 9 systemic symptoms. Their demographic data, the specific IgE measurements (CAP system) for apple and birch pollen, the provocative dose of fresh apple, the OAS grading after challenge, and other pollens and foods causing symptoms are reported in Table I. The open challenge was done in 38 patients; in 6 it was not feasible because of previous life-threatening laryngeal edema. All provoked patients had OAS symptoms, 3 had urticaria (which was present in the historical reaction), and 1 had laryngeal edema (not reported in the history) in response to 32 g of fruit.

SDS-PAGE/immunoblotting

Coomassie brilliant blue–stained profiles of apple proteins showed different protein components with apparent MWs ranging from 9 to 67 kd. Fig 1 depicts the IgE immunoblotting in 43 apple-allergic patients (No. 1-32 birch positive, No. 33-43 birch negative). Birch-positive patients had IgE binding to proteins with MWs of 9 kd (9 patients, 28%), 15 kd (16 patients, 50%), 18 kd (29 patients, 91%), 28 kd (2 patients, 6%), 31 kd (13 patients, 41%), 43, 51, and 84 kd (11 patients, 34%), and 60 kd (7 patients, 22%). All birch-negative patients had IgE binding only to the 9-kd allergen. IgE binding to the 9-kd allergen was also observed in 6 of the 7 patients with mugwort pollinosis (86%).

In the immunoblotting inhibition experiment the 9-kd band of the total raw extract was completely inhibited by our purified 9-kd protein, confirming the total cross-reactivity (data not shown).

Purification of apple 9-kd allergen by HPLC (cationic exchange/gel filtration)

Fig 2, *A*, shows the chromatographic profile from the cationic exchange column. Besides the first peak, corresponding to the unbound material, there was one more peak. Analysis of this second fraction by SDS-PAGE (data not shown) indicated that the 9-kd protein was not pure and other proteins at higher MWs were also present. We collected 25 mL of this second fraction (protein content of 0.03 mg/mL) by repeated runs and concentrated it to a final volume of 1 mL, which had a protein content of 0.9 mg/mL. This concentrated fraction was injected onto a gel filtration column, obtaining the chromatographic profile shown in Fig 2, *B*. Then the peak corresponding to the purified 9-kd protein was collected, concentrated again to a final volume of 1.5 mL with a protein content of 0.2 mg/mL, and analyzed in SDS-PAGE (Fig 3). IgE immunoblotting of this purified protein is shown in the same figure.

PAS stain

PAS staining on the 9-kd protein purified from apple and on a positive control excluded any glycosylation.

Isoelectrofocusing

Isoelectrofocusing was done on the 9-kd protein, which gave a mean isoelectric point of 7.5 after Coomassie brilliant blue R-250 staining.

TABLE I. Characteristics of Patients

Patient No.	Sex	Age (y)	Apple CAP system (kU/L)	Birch CAP system (kU/L)	Apple provocative dose to challenge
Patients allergic to apple and birch					
1	F	21	9.18	>100	8
2	F	35	16.5	92.4	16
3	F	22	1.75	27.3	8
4	M	17	4.86	18.6	16
5	F	41	10	77.4	NT
6	M	21	25.8	>100	16
7	F	15	11.1	98.8	8
8	F	44	6.16	90	NT
9	F	41	8.7	>100	NT
10	M	17	6.1	16.6	4
11	F	50	17.6	74.5	16
12	M	21	22.3	>100	8
13	M	25	1.76	45.2	8
14	M	13	31.2	91.2	NT
15	M	35	34.5	>100	8
16	F	55	2.96	58.6	16
17	M	35	1.69	9.99	16
18	M	19	60.3	>100	4
19	M	19	3.19	9.15	4
20	M	48	9.50	92.6	32
21	F	45	11	46	32
22	M	21	9.33	92	32
23	F	34	74.1	>100	NT
24	M	27	5.53	21.4	4
25	M	41	5.12	>100	16
26	F	34	21.8	>100	32
27	F	16	8.23	92.4	16
28	F	31	6.30	24.4	8
29	F	28	20.6	96	NT
30	M	37	2.81	9.04	8
31	F	42	2.39	30	16
32	M	37	4.03	>100	4
Patients allergic to apple but not to birch					
33	F	24	8.29	<0.35	8
34	F	29	15.8	<0.35	8
35	F	25	15.86	<0.35	16
36	F	15	3.82	<0.35	16
37	F	16	7.82	<0.35	32
38	F	22	3.22	<0.35	16
39	F	21	0.883	<0.35	8
40	M	18	58.5	<0.35	16
41	M	31	45.4	<0.35	8
42	F	32	0.367	< 0.35	8
43	M	39	5.34	<0.35	16

OAS was graded in response to challenge as follows: grade 1, only oral mucosa symptoms; grade 2, oral mucosa and gastrointestinal symptoms; grade 3, oral mucosa symptoms plus systemic symptoms (urticaria, rhinoconjunctivitis, or asthma); grade 4, oral mucosa symptoms plus life-threatening symptoms (laryngeal edema, anaphylactic shock). *F,* Female; *M,* male; *NT,* not tested because of life-threatening reactions (in these patients OAS was graded by history).
*According to severity by history.

Amino acid sequencing

The N-terminal sequence of the 9-kd protein was analyzed on the protein in solution and after SDS-PAGE and Western blotting on Problot membranes. In both cases the result was Ile-Thr-Cys-Gly-Gln-Val-Thr-Ser-Ser-Leu-Ala-Pro-Cys-Ile-Gly-Tyr-Val-Arg-Ser-Gly-Gly-Ala-Val-Pro-Pro-Ala-Cys-Cys-Asn-Gly-Ile-Arg-Asn-Ile-Asn-Gly. A database search revealed a very high degree of homology with several proteins belonging to the LTP family, especially peach, almond, sunflower, and bean.

Apple OAS grade after challenge	Other pollens causing symptoms	Other foods causing symptoms
1		Cherry
1		Cherry
1	Grass	Cherry, apricot, peach, pear
1	Grass	Cherry, apricot, plum, banana, almond, walnut, hazelnut
4*	Grass, ragweed, mugwort	Peanut, hazelnut, kiwi, tomato, peach, apricot, strawberry, cucumber, eggplant, banana, pear, melon, watermelon, pineapple, orange
1	Grass, *Parietaria*, mugwort	—
1	Grass	Cherry, apricot, peach, banana
4*	Grass, mugwort	Cherry
4*	Grass	Peach, almond
1	Grass	Cherry, apricot, peach, almond, hazelnut
1	Grass, *Parietaria*	—
1	Grass	—
3	Grass, *Parietaria*, mugwort	Tomato, banana
4*		Cherry, pear, banana, carrot, celery, fennel
1		—
2	Grass, *Parietaria*	Peach, tomato, parsley
1		Cherry, apricot, plum, peach
1	Grass	Peach, banana
1	Grass	Cherry, apricot, peach
4		—
1	Grass	Apricot, peach, plum, pear, carrot, celery, fennel, walnut, peanut
1	Grass	—
4*	Grass	Cherry, apricot, plum, peach, carrot, fennel, kiwi, melon, pear
1		Peach, plum, almond
1	*Parietaria*	Apricot
1	Grass	Cherry, apricot, plum, peach
1	Grass	—
1		Peach
4*		Cherry, apricot, peach, pear, banana, melon, tomato, kiwi, carrot, fennel, hazelnut, peanut, pea, chestnut
3		—
1		—
1		—
2	Mugwort	Apricot, almond, banana, pear, orange, tomato, celery
1		Almond, walnut, orange, lettuce
1	Mugwort, ragweed	Cherry, apricot, plum, peach, kiwi
1		Cherry
1		Cherry
3		Cherry
1		Cherry, plum, peach, pear, peanut
1	Mugwort, ragweed	Peanut, almond, hazelnut, walnut, broad bean, pea, strawberry
1	*Parietaria*	Banana, tomato
1		Peach
1		Apricot, cherry

DISCUSSION

In this study we evaluated 43 patients allergic to apple, 32 of whom were clinically sensitive to birch pollen and 11 not sensitive. Except for a few not challenged because of severe reactions in the past, all patients were recruited on the basis of OAS symptoms in response to challenge with apple, confirming that OAS is the major clinical picture in apple allergy. However, during challenge systemic symptoms also developed in 4 patients, and 1 had laryngeal edema, indicating that OAS cannot be considered merely a local allergic reaction but is a

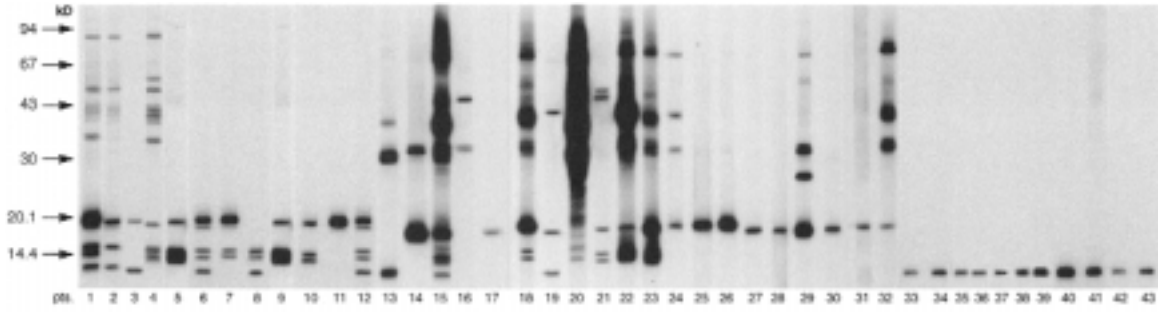

FIG 1. IgE reactivity patterns to crude apple extract in 43 patients with OAS. Patients No. 1 to 32 had birch pollinosis, No. 33 to 43 no birch pollinosis.

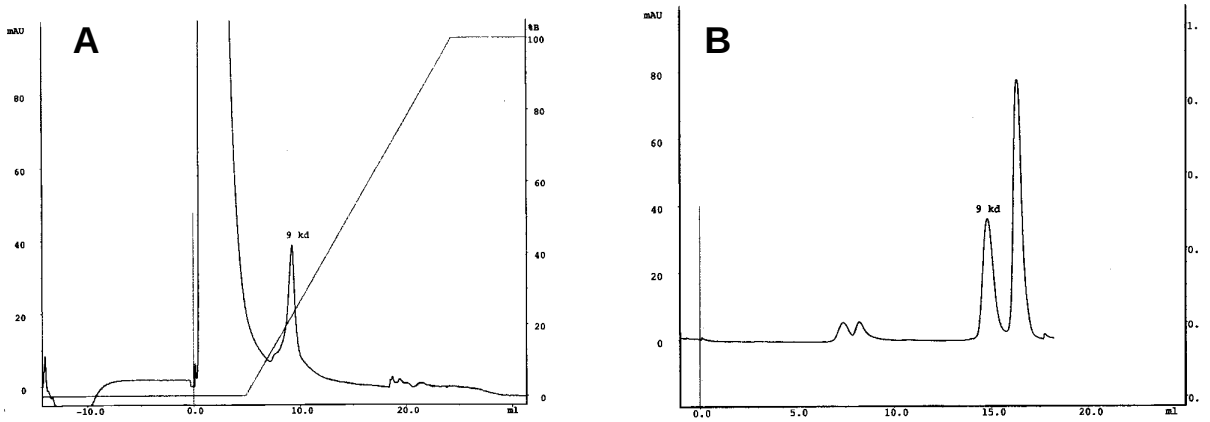

FIG 2. Cationic exchange of crude apple extract fractionated over HPLC Resource S **(A)**. Gel filtration of peak in cationic exchange chromatography **(B)**.

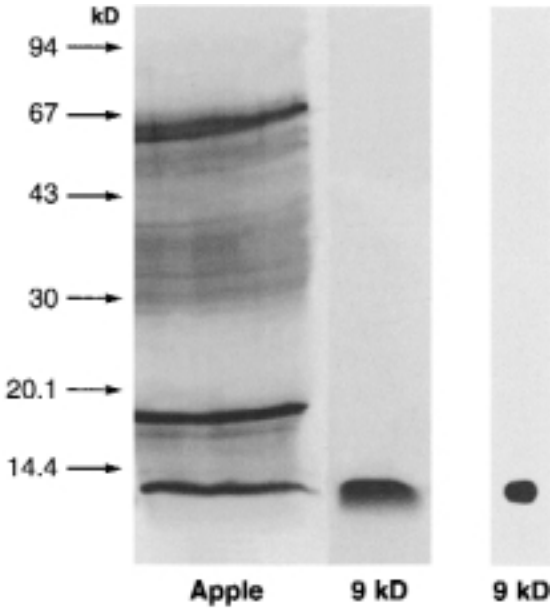

FIG 3. SDS-PAGE staining with Coomassie brilliant blue R-250 of crude apple extract and of 9-kd purified protein and IgE immunoblotting of 9-kd purified protein.

true food allergy with different levels of severity that need to be rated as we tentatively did in this study, to enable the allergist to establish the level of OAS for practical purposes, although from a pathophysiologic point of view laryngeal edema in OAS could be considered an extended local reaction.

IgE immunoblotting confirmed the importance of the reported apple allergens: the 18-kd protein, probably Mal d 1, homologous with Bet v 1[16]; the 14-kd component, probably Mal d 2, a profilin[17]; and a 30-kd component, which probably corresponds to a thaumatin-like protein described by Hsieh et al.[18]

The main finding of the study is a previously unreported apple allergen eliciting IgE reactivity in 28% of apple-allergic patients, which was found to be a 9-kd protein, the only allergen recognized by patients allergic to apple but not to birch pollen. By contrast, patients allergic to apple and birch clearly also reacted to 18- and 14-kd proteins, corresponding to the Mal d 1 and Mal d 2 allergens. Thus people not allergic to birch pollen may be allergic to apple, but in this population the allergens involved are not the Bet v 1 or Bet v 2 homologs but a 9-kd protein that does not cross-react with homologous components in birch pollen. This

allergen has already been accepted by the World Health Organization/International Union of Immunological Societies Subcommittee on Allergen Nomenclature, with the denomination Mal d 3. Amino acid sequencing showed that it belongs to the recently discovered family of LTPs, present in many fruits and vegetables, where they play a very important defensive role. However, LTP has never been described in apple, so we do not know its biologic role.

An LTP is the most important allergen of Prunoideae, such as peach, apricot, plum, and cherry,[10] which in Italy, as in the rest of the Mediterranean area, are the foods most frequently involved in reactions in adults.[1,19] Most patients allergic to peach are not sensitized to birch pollen and their sera react in vitro against a 9-kd protein, subsequently identified as an LTP.[9] Peach, included in the subfamily of Prunoideae, belongs to the family of Rosaceae, which comprises apple. Analysis of the amino acid sequence showed homology higher than 90% between the LTP from apple and peach, clearly explaining the clinical cross-reactivity in patients not allergic to birch pollen. Of 11 patients with hypersensitivity to apple but not to birch, 10 were also allergic to one or more Prunoideae fruits.

This does not mean we can disregard the role of Bet v 1 as a cross-reacting allergen because peach is also the most frequent cause of food allergy in patients allergic to birch pollen, in whom the cross-reactivity is due to a Bet v 1 homologous protein.[10] Undoubtedly, the discovery of Bet v 1 and its homologs was a milestone in improving the knowledge of food allergy, but it cannot explain all the cross-reactions observed in clinical practice. We have now found that a new class of allergens, the LTP, is another important group of cross-reacting molecules, not only among Prunoideae but also among Rosaceae.

LTPs are important for defending plants against microbial attacks and are thus likely to be located in the most superficial layer of the fruits. They are highly resistant to environmental changes, including temperature and pH, and maintain their activity after incubation for 5 minutes at 90°C, probably because of several disulfide bridges in the tertiary structure.[20] It has long been known that physicochemical stability is the main requirement for a strong allergen, and this fits with the important allergenic role of this class of molecules. The resistance of this LTP, if also demonstrated for apple, could well explain the persistence of allergenic activity after digestion or salivary degradation, whereas Bet v 1 is sensitive to physicochemical degradation, so it is likely to be inactivated after oral passage.

This strongly suggests that sensitization to the Bet v 1 homologous apple allergen in birch-allergic patients occurs by inhalation, whereas sensitization to the 9-kd LTP, which does not cross-react with similar pollen allergens, can only develop by the oral route. Thus 2 clinical models of sensitization to apple are likely to exist, one—the most common—depending on sensitization to birch pollen, particularly Bet v 1, causing reactions on subsequent oral contact with the homologous allergen Mal d 1, and the other arising directly from ingestion of the LTP from apple.

The importance of birch pollinosis and apple consumption in different countries must be considered by analyzing the literature on apple allergy and planning further investigations. It seems clear that in countries where birch trees are abundant sensitization to apple mainly develops toward the Bet v 1 homolog, whereas in the Mediterranean area, where there are few birch trees—in some areas none—patients may become allergic through oral sensitization to the LTP. The sensitizing role of other pollens such as mugwort in relation to the presence of LTP, with significant homology with the apple LTP, also has to be investigated. Another major advance should come from assessing the presence of LTP in different apple strains, as done by Vieths for Mal d 1,[21] and how environmental factors influence the expression of these proteins.

In conclusion, we found that a 9-kd protein belonging to the family of LTP is a novel apple allergen. In view of the importance of apples in the human diet and of the presumed oral route of sensitization, apple LTP is likely to be an important allergen in countries where birch trees are not common.

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