

Original article

Peach profilin: cloning, heterologous expression and cross-reactivity with Bet v 2

Background: Peach is among the main foods causing allergic reactions in the Mediterranean adult population. Only a single peach allergen, named Pru p 3, has been characterized. However, a potential role of profilin has also been suggested in grass pollen-associated allergy to peach.

Methods: Complementary DNA clones for two different peach profilin isoforms were obtained by reverse transcriptase polymerase chain reaction using non-degenerated primers. Expression of recombinant peach profilin was performed in *Escherichia coli*, and confirmed using rabbit polyclonal antibodies to sunflower pollen profilin. Twenty-nine individual sera from patients with peach allergy proved by double-blind, placebo-controlled food challenges (DBPCFC), either with ($n = 15$) or without ($n = 14$) specific IgE to Bet v 2, were used in immunodetection assays to test recombinant peach profilin reactivity.

Results: Each peach profilin cDNA included an open reading frame coding for a 131 amino acid protein. The peach profilin isoforms, designated Pru p 4.01 and Pru p 4.02, showed 80% of amino acid sequence identity, and were very similar (>70% identity) to allergenic profilins from plant foods and pollens. Recombinant Pru p 4.01 was expressed in *E. coli* as a nonfusion protein, displaying the expected molecular size and reacting with anti-profilin antibodies. rPru p 4.01 was recognized by all sera (15 of 15) with specific IgE to Bet v 2, whereas no sera (zero of 14) without IgE to this birch allergen reacted with rPru p 4.01.

Conclusions: Peach profilin Pru p 4 is very closed to other allergenic profilins from plant foods and pollens. A complete correlation between reactivity to rPru p 4 and rBet v 2 has been found in sera from peach allergic patients.

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Key words: cross-reactivity; food allergy; peach; profilin; recombinant allergen.

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Accepted for publication 28 February 2003

Immunoglobulin E (IgE)-mediated allergy to peach (*Prunus persica*) and to other Rosaceae fruits, usually associated with pollinosis, is common in the European adult population (1). Particularly, in the Mediterranean area, peach seems to be the most frequent cause of plant food allergy in adult patients (2, 3). Two main different patterns of sensitization to Rosaceae fruits have been found. In Central and North Europe and in North America, it is often linked to birch pollinosis. Fruit homologues to the major birch pollen allergen Bet v 1, are the major allergens in these population (4–6). Fruit profilins homologous to birch Bet v 2, and isoflavone reductases, seem to be also involved in the pollen–fruit cross-reactions (5, 6).

By contrast, most of the Mediterranean area is virtually free of birch trees, and, therefore, its patients sensitized to Rosaceae fruits do not show usually specific IgE to Bet v 1 (7). Lipid transfer proteins (LTPs), such as peach Pru p 3, are the major Rosaceae fruit allergens in this population, as it has been proved both *in vitro* and *in vivo* (8–12). Profilin and cross-reactive carbohydrate

determinants can also represent relevant IgE-binding components, specially in those patients with an associated grass pollen allergy (7).

Pru p 3 is the only peach allergen that has been isolated and characterized. Its recombinant form has been also obtained (13), and its cross-reactivity with other allergenic LTPs from plant foods and pollens has been reported (12, 14). According to the previous data mentioned above, peach profilin (hereafter designated as Pru p 4) is a potential relevant allergen of this fruit, which merits to be studied. Profilins are 14–17 kDa proteins found in all eukaryotic phyla, that bind to actin and to phosphatidylinositol 4,5-bisphosphate, thus likely being a link between signal transduction cascades and cytoskeleton organization (15). Allergenic plant profilins with a broad spectrum of cross-reactivity, are widely distributed in pollens and foods (5, 6, 16).

In the present study, the complete amino acid sequences of two Pru p 4 variants were obtained from their corresponding cDNA sequences. One of these variants was expressed in *Escherichia coli* as a non-fusion

protein, and its cross-reactivity with birch Bet v 2 was investigated.

Material and methods

Patients and sera

Twenty-nine patients with allergy to peach (Table 1) were selected during prospective studies carried out at the Allergy Unit of the Fundación Hospital Alcorcón (Madrid). All patients showed immediate hypersensitivity to peach after ingestion or contact according to their clinical history, and positive skin prick test (SPT) to peach ($>7 \text{ mm}^2$) by the prick-prick method (17). Peach allergy was confirmed by DBPCFC and the symptoms of contact urticaria by rub test. A pool of sera from three healthy subjects with negative SPT and no specific IgE to peach was used as negative control. Written informed consent from all patients and controls included in this study was obtained.

Specific IgE to peach and to rBet v 2 were determined by the Pharmacia CAP system FEIA (Pharmacia Diagnostic, Uppsala, Sweden).

The SPTs were performed with purified natural Pru p 3 at a concentration of 20 $\mu\text{g/ml}$, as previously published (12). Histamine

hydrochloride (10 mg/ml) and saline solution were used as positive and negative controls, respectively. Wheals areas 7 mm^2 greater than that produced by the saline control were considered as positive.

The selected patients showed two different patterns according to the CAP and SPT responses: one group ($n = 15$) exhibited positive CAP to rBet v 2 and negative SPT to Pru p 3, and a second group ($n = 14$) showed negative CAP to rBet v 2 and positive SPT to Pru p 3 (Table 1).

Isolation of cDNAs encoding Pru p 4

Total RNA of peel from mature peaches was obtained by the method of Lagrimini et al (18). The reverse transcription was carried out with 5 μg of total RNA using the oligonucleotide primer 5'-TCGACTCTAGAGGATCCGAATTCAAGC(T)₁₅-3', and the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Piscataway, NY).

The cDNA obtained was amplified by polymerase chain reaction (PCR) using the oligonucleotide 5'-primer 5'-ATGTCGTGG CAGGCGTACG-3'. This primer was designed from the 5' nucleotide sequence of cDNAs for cherry, apple and pear profilins (accession numbers AF129425 for cherry, AF129426, AF129427, AF129428 for apple and AF129424 for pear). For the reverse direction the 3'-primer 5'-CTGCAGGTCGACTCTAGAGG ATCC-3' (complementary to that used for reverse transcription) was used for clon 1.2 (Pru p 4.02) and the 3'-primer 5'-TTACA GACCCTGCTCGATAAG-3' was used for clon 1.1 (Pru p 4.01). The latter was designed from the 3' nucleotide sequence of the cDNAs for cherry, apple and pear profilins. The amplification was performed with Taq DNA polymerase (Ecogen, Barcelona, Spain) as recommended by the manufacturer. The PCR program was 5-min denaturation at 95°C, followed by 10 cycles each of 1-min denaturation at 95°C, 30 s annealing at 59°C and 30 s extension at 72°C, followed by 25 cycles each of 1-min denaturation at 94°C, 1-min annealing at 55°C and 1-min extension at 72°C, and final extension for 5 min at 72°C. Reamplification was carried out using the same program, except annealing at 60°C for the first 10 cycles and 56°C for the last 25 cycles.

The PCR products were cloned into pGEM T Easy vector (Promega, Madison, WI). These constructs were used to transform *E. coli* DH5 α F' cells, and the plasmidic DNA from several clones with each construct, was sequenced using the ABI PRISM Dye Terminator Kit and an ABI 377 sequencer (Perkin-Elmer Biosystem, Warrington, UK).

Expression of Pru p 4.01 in *E. coli* cells

The coding region of the Pru p 4.01 cDNA was amplified by PCR with Taq DNA polymerase, a sense primer 5'-TATACATATG TCGTGGCAGGCG-3' (Nde I restriction site is underlined) and an antisense primer 5'-CTGCAGTTACAGACCCTGCTC-3' (Pst I restriction site is underlined). The PCR product was purified, digested with Nde I/Pst I restriction enzymes and inserted into the same sites of Prset B expression vector to transform *E. coli* BL21 cells.

The cells were grown in LB medium with 50 $\mu\text{g/ml}$ carbenicillin to an absorbance of 0.6 at 600 nm. Expression of Pru p 4.01 was induced by adding isopropyl-D-thiogalactopyranoside (IPTG) to LB medium at a final concentration of 2 mM, and the incubation was continued for 4 h at 37°C. Cells were harvested by centrifugation, and resuspended in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA. After two cycles of freeze-thawing, cells were sonicated to obtain the lysate. Insoluble material was removed by centrifugation (20 400 g for 5 min).

Table 1. Clinical data, specific IgE levels and SPT responses of the selected patients with allergy to peach

Patient	Age (years)/sex	Symptoms	Pollen allergy	Specific IgE (kU/l)		SPT Pru p 3
				Peach	rBet v 2	
1	23/F	OAS	Yes	—	8.6	—
2	22/F	OAS	Yes	0.6	13.2	—
3	34/M	OAS,CU	Yes	3.0	—	+
4	69/F	A,CU	No	2.3	—	+
5	19/M	OAS	Yes	—	1.7	—
6	10/F	OAS,CU	No	—	3.1	—
7	18/F	OAS,CU	Yes	0.8	3.7	—
8	9/M	U,CU	Yes	6.9	—	+
9	37/M	OAS,CU	Yes	6.9	—	+
10	29/F	OAS	No	—	—	+
11	23/F	OAS,CU	Yes	—	2.6	—
12	16/F	U	Yes	—	10.3	—
13	16/F	OAS,CU	Yes	—	1.8	—
14	19/F	OAS,CU	Yes	3.5	—	+
15	13/F	U,CU	Yes	1.2	—	+
16	27/M	OAS	Yes	0.9	—	+
17	29/M	OAS	Yes	3.0	20.0	—
18	17/M	U	Yes	—	7.0	—
19	25/F	OAS	Yes	1.6	1.9	—
20	22/M	U,CU	Yes	3.6	—	+
21	20/F	A	No	0.9	—	+
22	25/M	OAS	Yes	17.9	—	+
23	17/F	OAS	Yes	—	2.3	—
24	41/F	OAS,CU	Yes	—	2.9	—
25	15/M	OAS	Yes	—	2.1	—
26	17/F	U,CU	Yes	1.0	6.7	—
27	27/F	CU	Yes	—	—	+
28	23/F	U,CU	Yes	12.7	—	+
29	24/F	OAS,CU	Yes	1.7	—	+

M, male; F, female; IgE, immunoglobulin E; SPT, skin prick test; OAS, oral allergy syndrome; A, anaphylaxis; CU, contact urticaria; U, urticaria; —, negative result ($<0.35 \text{ kU/l}$ or $<7 \text{ mm}^2$).

Immunodetection

Protein content of cell lysates were determined by the method of Bradford (18), and then separated (25 µg of protein) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (19). Replica gels were electrotransferred onto polyvinylidene difluoride membranes, as previously reported (20). After washing and blocking, membranes were incubated (16 h at room temperature) with a serum pool or individual sera (1 : 2 dilution) from peach allergic patients, and then with alkaline phosphatase-conjugated monoclonal anti-human IgE (clon GE-1; Sigma, St Louis, MO; 1 : 500 dilution) and revealed by adding 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution.

To identify recombinant Pru p 4.01, blocked membranes were incubated with rabbit polyclonal antibodies to sunflower pollen profilin Hel a 2 (kindly provided by Dr A. Martinez, Bial-Aristegui, Bilbao, Spain; 1 : 5000 dilution) (21), and then treated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1 : 500 dilution) and finally with a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution.

Results

Immunodetection after SDS–PAGE separation of a phosphate buffer saline (PBS) extract from peach fruit

with antibodies to sunflower pollen profilin Hel a 2 (21) did not detect any reactive band (not shown). This result suggested that peach profilin Pru p 4 was present at very low levels (at least in the PBS extract), considering the wide recognition pattern of anti-Hel a 2 antibodies against profilins from different pollens and plant foods (21, 22). Thus, cloning of cDNAs coding for Pru p 4, and the heterologous expression of the allergen were attempted, instead of to approach the characterization of Pru p 4 at the natural protein level.

The PCR amplification using nondegenerated oligonucleotide primers based on the known cDNA sequences corresponding to other Rosaceae fruit profilins, allowed to isolate cDNA fragments of around 700 bp. Three independent clones rendered identical coding sequences corresponding to Pru p 4.01 (Fig. 1). Furthermore, a genomic cloned with the same coding sequence (exons) and two introns, was also obtained (not shown). The Pru p 4.02 sequence (Fig. 1) was confirmed in two independent clones. The cDNAs comprised a 393 bp open reading frame coding for a protein of 131 amino acid residues. The calculated molecular mass for Pru p 4.01 polypeptide was 14 004 Da and its isoelectric point 4.67, whereas those for Pru p 4.02 variant were 13 989 Da and 4.73,

Pru p 4.01	atg	tcg	tgg	cag	gcg	tac	gtc	gat	gac	cac	ttg	atg	tgc	gat	att	gac	ggc	aac	cgc	ctc	60
Pru p 4.02							a	c		t	c			a		a		a			
Pru p 4.01	M	S	W	Q	A	Y	V	D	D	H	L	M	C	D	I	D	G	N	R	L	20
Pru p 4.01														E		E		H	L		
Pru p 4.01	acc	gcc	gcg	gca	atc	ctc	ggc	caa	gac	ggc	agc	gtt	tgg	tct	cag	agc	gcc	acc	ttt	cct	120
Pru p 4.02	t t		c	c		a		c					g c				t	c			
Pru p 4.01	T	A	A	A	I	L	G	Q	D	G	S	V	W	S	Q	S	A	T	F	P	40
Pru p 4.02	S					I		H						A							
Pru p 4.01	gcg	ttt	aag	cct	gaa	gag	ata	gct	gcc	att	ctg	aaa	gat	ttc	gac	caa	ccc	gga	acg	ctt	180
Pru p 4.02	ca	g				a	g g	a	g			t	c		a t	g	t	t a			
Pru p 4.01	A	F	K	P	E	E	I	A	A	I	L	K	D	F	D	Q	P	G	T	L	60
Pru p 4.02	Q	L					V	T	G			N			N	E			S		
Pru p 4.01	gcc	cca	act	ggt	tta	ttc	ctt	ggt	ggg	aca	aag	tat	atg	gtg	atc	caa	ggt	gaa	gcc	ggt	240
Pru p 4.02				g	g	at		a	c			c		c		a	a	g c a	a		
Pru p 4.01	A	P	T	G	L	F	L	G	G	T	K	Y	M	V	I	Q	G	E	A	G	80
Pru p 4.02						Y												P			
Pru p 4.01	gct	gtc	att	cga	gga	aag	aag	ggc	tct	ggt	ggc	att	act	gtt	aag	aag	acc	aat	cag	gcc	300
Pru p 4.02				a				a c	a		t	g		c			g	c	tt	t	
Pru p 4.01	A	V	I	R	G	K	K	G	S	G	G	I	T	V	K	K	T	N	Q	A	100
Pru p 4.02								P				V					S	T	L		
Pru p 4.01	ctc	atc	att	ggt	ata	tat	gat	gag	cct	ttg	act	cca	ggg	cag	tgc	aac	atg	att	gtc	gaa	360
Pru p 4.02	t g c g			c	t				a a			t	c	a					t		
Pru p 4.01	L	I	I	G	I	Y	D	E	P	L	T	P	G	Q	C	N	M	I	V	E	120
Pru p 4.02		L								M											
Pru p 4.01	agg	ctt	ggc	gat	tac	ctt	atc	gag	cag	ggt	ctg	taa	396								
Pru p 4.02			t		t	g g	t				c	g									
Pru p 4.01	R	L	G	D	Y	L	I	E	Q	G	L	-	131								
Pru p 4.02						V															

Figure 1. Nucleotide and deduced amino acid sequences of cDNA coding regions for peach profilin variants Pru p 4.01 and Pru p 4.02. Numbers indicated nucleotide (upper) or amino acid (lower) positions. Only nucleotide or amino acid differences of Pru p 4.02 sequences with those of Pru p 4.01 are included. The nucleotide sequences have been submitted to EMBL Database under accession numbers AJ491881 (Pru p 4.01) and AJ491882 (Pru p 4.02).

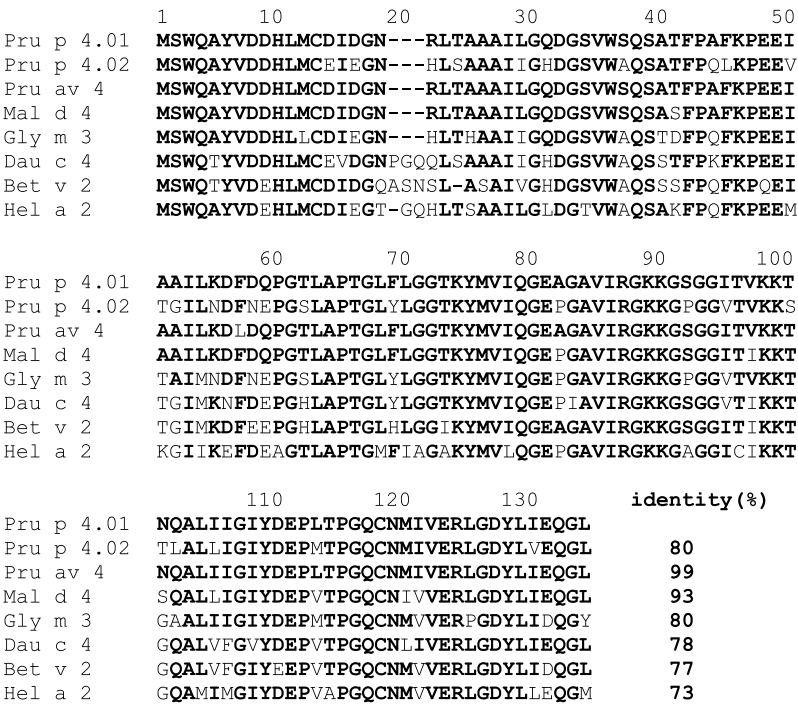


Figure 2. Alignment of amino acid sequences of Pru p 4.01 and Pru p 4.02 with those of allergenic profilins from cherry (Pru av 4, accession number AF129425), apple (Mal d 4; accession number AF129428), soybean (Gly m 3. 0101; accession number AJ223982), carrot (Dau c 4; accession number AF456482), and birch (Bet v 2; accession number M65179) and sunflower (Hel a 2; accession number Y1521O) pollen. Gaps (-) to obtain the proper sequence alignment are indicated. Percentages of sequence identity with respect to Pru p 4.01 are included at the bottom right of figure.

respectively. The deduced amino acid sequences of both Pru p 4 variants showed 80% identity, thus confirming their closed relationship. Pru p 4.01 was even closer (> 90% of sequence identity) to allergenic profilins from other Rosaceae fruits, such as cherry (Pru av 4) and apple (Mal d 4) (Fig. 2). High levels of sequence identity (> 70%) were also found between Pru p 4.01 and members of the same allergen family from seeds (soybean Gly m 3), vegetables (carrot Dau c 4), and pollens (birch Bet v 2 and sunflower Hel a 2) (Fig. 2).

As a previous step to study the reactivity of Pru p 4.01 to IgE of sera from peach allergic patients, the Pr set B expression vector carrying the Pru p 4.01 cDNA coding sequence was used to transform *E. coli* BL21 cells. Expression of rPru p 4.01 after induction with IPTG (4 h) was achieved (Fig. 3). Most of rPru p 4.01 was recovered as soluble protein in the supernatant fraction (not shown), but, by contrast with other plant food recombinant profilins expressed in *E. coli* (23, 24), a low amount of rPru p 4.01 was found (see lanes 1A and 2A of Fig. 3). However, a specific expression of the recombinant allergen was clearly obtained (see lanes 1B and 2B of Fig. 3).

The supernatant fraction from *E. coli* lysates expressing rPru p 4.01 was IgE-immunodetected with 29 individual sera from peach allergic patients (Fig. 4). The rPru p 4.01 was recognized by all sera (15 of 15) with specific IgE to

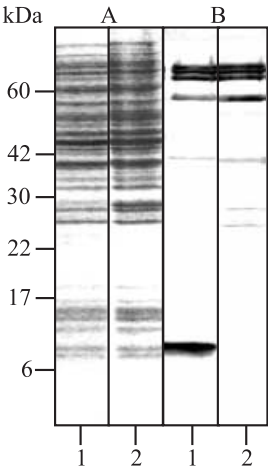


Figure 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis separation of the supernatant fraction from transformed *Escherichia coli* lysates with Pr set B vector carrying the Pru p 4.01 cDNA (1) and with empty Pr set B vector (2). (A) Coomassie Blue staining; (B) immunodetection with polyclonal antibodies to Hel a 2 profilin.

rBet v 2 (see Table 1). On the contrary, no serum (14 of 14) without specific IgE to birch profilin reacted with rPru p 4.01. No reaction was found with sera from control subjects.

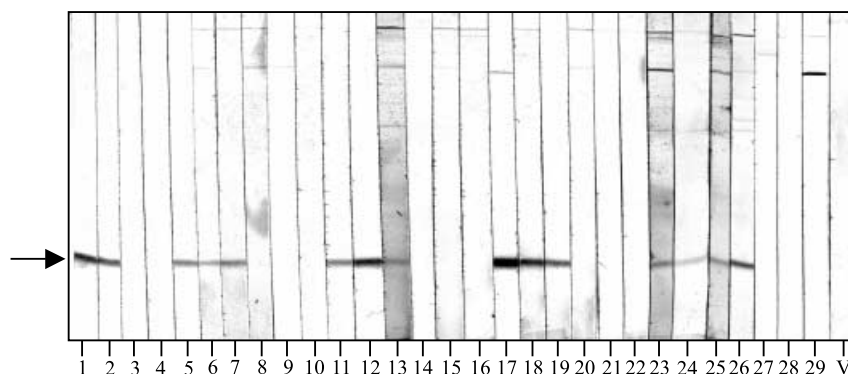


Figure 4. Immunoglobulin E-immunodetection with individual sera (1–29; numbers as in Table 1) from peach allergic patients. The supernatant fraction from *Escherichia coli* lysates expressing rPru p 4.01 (1–29) or transformed with empty vector (v; negative control immunodetected with a pool of sera 1–29) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, electrotransferred to polyvinylidene difluoride membranes and immunodetected with the corresponding serum.

Discussion

In spite of being peach among the main foods causing allergic reactions in the Mediterranean adult population (2, 3), only a single allergen, namely Pru p 3, had been previously characterized (9, 10, 13). However, a role of other allergens, particularly profilin, in grass pollen-associated allergy to peach, has been suggested (6, 7).

A very low level of Pru p 4 (profilin) seems to be present in peach fruit, thus hindering the isolation of the natural allergen. A similar restriction could explain the virtual lack of reports on purified natural profilins from plant foods, and to approach alternatively their characterization by cloning and heterologous expression of their recombinant forms (23, 24). The cDNAs coding for two different Pru p 4 isoforms have been isolated and sequenced. The complete deduced amino acid sequences showed 80% of identity, and their calculated molecular masses and isoelectric points were similar to those of other plant food profilins previously studied (6). Moreover, a closed relationship (over 70% of sequence identity) was found with members of the profilin family from fruits, seeds, vegetables and pollens (Fig. 2).

Pru p 4.01, the isoform closer to profilins of other Rosaceae fruits, such as cherry and apple, was expressed in *E. coli*, although apparently at low level. Most of the recombinant allergen was recovered in a soluble form, and was strongly recognized by anti-Hel a 2 antibodies (Fig. 3). The rPru p 4.01 also reacted with IgE of sera from peach allergic patients (Fig. 4). Interestingly, a survey of two different well defined groups of patients (see Table 1), rendered a clear cut scenario. A complete correlation between rPru p 4.01 recognition and specific IgE levels to rBet v 2 was obtained: all sera ($n = 15$) with positive CAP to recombinant birch profilin reacted with rPru p 4.01, whereas all sera ($n = 14$) with negative CAP

to rBet v 2 were also negative to rPru p 4.01. A high percentage of sera (80–92%) preselected for an IgE-reactivity to rBet v 2 also showed positive IgE-immunoblot to recombinant pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4) profilins, as previously reported (24). These results suggest that specific IgE to rBet v 2 can be a suitable diagnostic test for potential sensitization to Rosaceae fruit profilins.

The results obtained with both groups of peach allergic patients included in this study, suggest a different pattern of clinical characteristics. The group positive to Pru p 4.01 and with negative SPT to Pru p 3 showed a significantly ($P = 0.001$) lower IgE to peach, and a tendency to present clinical symptoms linked to oral allergy syndrome and less systemic reactions than the group negative to Pru p 4.01 and with positive SPT to Pru p 3. The high resistance of profilins to treatment with human saliva and their rapid degradation by simulated gastric fluids (22), in contrast with the strong stability of Pru p 3 in the latter treatment, can be on line with the different clinical patterns mentioned above. Furthermore, a recent survey of a larger population of peach allergic subjects has shown that the appearance of isolated oral symptoms, as well as sensitization to rBet v 2 (and, therefore, to Pru p 4), increase with age (M. Fernandez-Rivas et al., unpublished). By contrast, positive SPT to Pru p 3 decreases in patients with the older ages at onset of peach allergy.

Acknowledgments

We thank Dr A. Martinez (Bial-Aristegui, Bilbao) for kindly providing the anti-Hel a 2 antibodies, and J. García-Guijarro and D. Lamóneda (ETSIA, UPM) for technical assistance. Financial support was provided by Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, FIS (grant No. 00/0179) and by the KPMG-FHA 2000 award.

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