

Recombinant Pru p 3 and natural Pru p 3, a major peach allergen, show equivalent immunologic reactivity: A new tool for the diagnosis of fruit allergy

Araceli Díaz-Perales, PhD,^a María L. Sanz, MD, PhD,^b Gloria García-Casado, PhD,^a Rosa Sánchez-Monge, PhD,^a Francisco J. García-Selles, MD,^c Manuel Lombardero, PhD,^d Florentino Polo, PhD,^d Pedro M. Gamboa, MD, PhD,^e Domingo Barber, PhD,^d and Gabriel Salcedo, PhD^a Madrid, Pamplona, Murcia, and Bilbao, Spain

Background: The peach lipid transfer protein Pru p 3 has been identified as a major allergen from this fruit. Homologous cross-reactive allergens have been found in several plant foods and pollens. Recombinant Pru p 3 has been recently produced in the yeast *Pichia pastoris*.

Objective: We sought to evaluate the potential role of recombinant Pru p 3 as a novel tool for the diagnosis of fruit allergy.

Methods: Circular dichroism analysis was used to compare the protein folding of natural Pru p 3 and recombinant Pru p 3. IgE binding by both molecular forms was quantified by means of ELISA and ELISA inhibition assays, and their biologic activity was estimated by using basophil activation, histamine release, and sulphidoleukotriene production tests. Individual sera or blood samples from patients with peach allergy (up to 17) were used in the assays.

Results: A nearly identical circular dichroism spectra was shown by using natural Pru p 3 and recombinant Pru p 3, indicating that both protein forms are similarly folded. No difference was detected in the IgE-binding capacity of the 2 molecular versions. Basophil activation and induction of sulphidoleukotriene production were positive in 9 of 10 patients, and histamine release was induced in at least half of the patients, with similar effects of the natural and recombinant forms in the 3 assays.

Conclusion: Recombinant Pru p 3 shows a strong immunologic activity equivalent to that of its natural counterpart, and therefore it can be a useful tool for diagnosis (and future immunotherapy) of fruit allergy. (*J Allergy Clin Immunol* 2003;111:628-33.)

Key words: Recombinant allergen, fruit allergy, lipid transfer protein, diagnosis, IgE binding, basophil activation, histamine release, sulphidoleukotriene production

Allergy to fruits, usually associated with birch, grass, or mugwort pollinosis, is on the increase.¹ Most patients with pollen-food syndrome present isolated oral symptoms (the oral allergy syndrome) caused by homologous labile allergens in fresh fruits and pollens (ie, Bet v 1 homologues and profilins).¹ In the Mediterranean area Rosaceae fruits, mainly peach, are among the most frequent causes of food allergy in adult subjects.^{2,3} These patients are not allergic to birch pollen, and the main reactions are not directed to Bet v 1 homologues or profilins but to lipid transfer proteins (LTPs).^{2,4-6} Furthermore, allergic symptoms involving LTPs are more likely to be severe, including systemic reactions, in addition to the oral allergy syndrome. Interestingly, the strong resistance of LTPs to heat and simulated gastric fluid treatments^{7,8} point to LTPs as severe food allergens.

Plant LTPs constitute a family of 9-kD polypeptides that is widely distributed in plant foods and pollens and is involved in plant defense mechanisms against pathogens.⁹ Members of this family are folded in an all- α -type compact structure, with 4 α -helices connected by 4 disulfide bridges and a long C-terminal tail.⁹ LTPs have been identified as relevant allergens either in plant foods, such as peach (Pru p 3), apple, apricot, cherry, maize, and chestnut,^{5,6,10-12} or in pollens, such as *Parietaria* and *Artemisia* species.^{12,13} Cross-reactions of Pru p 3 with other allergenic homologues from foods and pollens have been demonstrated both in vitro¹² and in vivo.¹⁴ On the other hand, the high rate (91%) of positive skin prick tests (SPTs) to Pru p 3 in patients allergic to peach¹⁴ suggests that it could be a useful tool for diagnosis of Rosaceae fruit allergy. The cDNA coding for Pru p 3 has been isolated, and the recombinant allergen (rPru p 3) is expressed in the yeast *Pichia pastoris* to produce high amounts of such a tool and to allow its future manipulation.¹⁵

Recombinant allergens can help to solve problems in the diagnosis and potential therapy of food allergy. This includes standardization of samples used in serologic and cutaneous tests, development of diagnostic microchips, and production of hypoallergenic variants by means of

From ^aUnidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Madrid; ^bDepartamento de Alergología e Inmunología Clínica, Clínica Universitaria de Navarra, Pamplona; ^cSección de Alergia, Hospital Virgen de la Arrixaca, Murcia; ^dDepartamento de I+D, ALK-Abelló, Madrid; and ^eServicio de Alergia, Hospital de Basurto, Bilbao. Supported by grant BMC2002-00196 (Ministerio de Ciencia y Tecnología, Spain).

Received for publication July 9, 2002; revised August 28, 2002; accepted for publication September 16, 2002.

Reprint requests: Gabriel Salcedo, Unidad de Bioquímica, Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.75

TABLE I. Clinical data of the patients allergic to peach

Patient	Age (y)/sex	Symptoms	Total IgE (kU/L)	Specific IgE to peach (kU/L)	SPT to peach (mm ²)
1	19/F	U, FA	69	9.2	22
2	27/F	U, FA	294	3.7	26
3	24/F	U, A	367	3.6	44
4	24/F	U, FA	1656	39.5	56
5	17/M	U	425	31.9	59
6	19/M	U, A	138	10.6	51
7	24/M	U, GE, V, AS	233	29.3	63
8	23/F	U, A	161	57.0	42
9	28/F	U, A	211	71.0	40
10	38/F	U, A	537	9.2	234
11	37/M	A	812	1.8	63
12	27/F	U, A	261	2.3	176
13	34/F	A	64	9.8	136
14	17/M	U, OAS	221	3.7	42
15	24/F	U	1000	4.1	100
16	28/F	U, A	80	5.8	66
17	23/F	U, A	192	7.3	156

U, Urticaria; FA, facial angioedema; A, angioedema; GE, glottis edema; V, vomiting; AS, asthma; OAS, oral allergy syndrome.

Abbreviations used

- CAST: Antigen-specific sulphidoleukotriene production test
- CD: Circular dichroism
- FAST: Flow cytometric cellular allergen stimulation test
- HRT: Histamine release test
- LTP: Lipid transfer protein
- MALDI: Matrix-assisted laser desorption/ionization mass spectrometry
- SPT: Skin prick test

protein engineering to be used for safer treatments.¹⁶⁻¹⁸ Moreover, purified allergens are also essential tools in basic research to elucidate the molecular mechanisms underlying the allergic reactions. However, recombinant allergens might be inaccurately processed and folded, thus showing a different allergenic potency than its natural counterpart. Therefore a prerequisite for the use of recombinant forms is to ensure that their physicochemical and immunologic properties are equivalent to those of their natural counterparts. Unfortunately, studies directly comparing the recombinant and natural forms of food allergens in the context of allergenic properties are rare.¹⁷ We summarize here such a study for the major peach allergen Pru p 3.

METHODS

Patients and sera

Seventeen patients with allergy to peach (Table I) were selected during prospective studies carried out at Hospital Virgen de la Arrixaca, Murcia (patients 1-7), and Hospital de Basurto, Bilbao (patients 8-17). All patients had immediate hypersensitivity to peach according to their clinical history, specific IgE levels to peach of greater than 1.5 kU/L, and positive SPT responses to peach extract (70 µg/mL nPru p 3 in the final prick sample, ALK-Abelló). All 17 patients had systemic reactions to peach, with a total of 33 accumulated systemic reactions (range, 1-4) in the patients studied.

Five healthy control subjects with negative SPT responses and no specific IgE to peach were also selected. Written informed consent from all subjects included in this study was obtained.

Total and specific IgE levels were determined by using the Pharmacia CAP system FEIA (Pharmacia Diagnostics). SPTs were performed with commercial glycerinated preparations (ALK-Abelló) or with purified natural Pru p 3 (nPru p 3; 20 µg/mL) by using conventional procedures. Histamine dihydrochloride (10 mg/mL) and 0.9% NaCl were used as positive and negative controls, respectively. Wheal responses 7 mm² greater than those produced by the saline control were considered positive.

Physicochemical analysis

Natural and recombinant Pru p 3 were isolated by using the same chromatographic methods and conditions described by Díaz-Perales et al.¹⁵ The purified proteins were initially quantified by the method of Bradford,¹⁹ and a more exact quantification was then carried out by using amino acid analysis after acid hydrolysis. The purity of the isolated proteins was checked with SDS-PAGE and matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis, as previously described.^{5,15}

MALDI analysis was carried out by using a Biflex III spectrometer with delayed extraction (Bruker-Franzen).

Circular dichroism (CD) spectra were performed at 0.3 mg/mL purified protein in 10 mmol/L sodium phosphate buffer (pH 7.0) and recorded at 15°C with 0.1-cm path-length cells in an OLIS DSM 10 spectropolarimeter. The content of secondary structure elements was estimated from CD data by using the CDNN neural network analysis program.²⁰

Specific IgE binding to Pru p 3 and ELISA inhibition assays

A serum pool and individual sera from patients 1 to 7 (Table I) were used in these assays. Specific IgE binding to nPru p 3 and rPru p 3 was determined by using polystyrene 96-well microtiter plates (Costar 3590, Corning) coated with 50 µL of purified protein at 2 µg/mL in PBS buffer (0.1 mol/L sodium phosphate [pH 7.0] and 0.15 mol/L NaCl) for 1 hour at 37°C. The coated wells were blocked with 1% BSA in PBS buffer and then incubated with 50 µL of 1:3 serial dilutions of the serum pool (range, 1:1-1:3⁵ dilution) for 14 hours at 25°C. After washing with 0.1% Tween-20 in PBS buffer, wells were incubated with a peroxidase-labeled antihuman

IgE (Dako A/S) for 1 hour at 25°C. Plates were washed again and then developed with 50 µL of peroxidase substrate buffer (Dako, code S2045). After 30 minutes, the reaction was stopped with 50 µL of 4N H₂SO₄, and the OD was measured at 492 nm. PBS buffer with 1% BSA was tested as a negative control for each solid phase. Additionally, a serum from a patient not allergic to peach showed an OD of less than 0.05 units when tested with both Pru p 3 forms. Individual sera were assayed by using a single (1:3) dilution. Tests were performed in triplicate.

ELISA inhibition assays were carried out by using a procedure similar to that described above, with 25 µL of the serum pool previously preincubated with 75 µL of serial dilutions of nPru p 3 or rPru p 3 (range, 2–0.002 µg/mL) for 3 hours at 25°C. Inhibition values were determined as follows:

$$\text{Inhibition (\%)} = 100 \times (1 - [(OD - OD_{100\%}) / (OD_{0\%} - OD_{100\%})]),$$

with *OD* defined as the value with Pru p 3 as an inhibitor, *OD*_{100%} defined as the blank value of the assay, and *OD*_{0%} defined as the value with BSA as an inhibitor (negative control). All tests were performed in triplicate.

Data on specific IgE binding to nPru p 3 and rPru p 3 were compared by using a paired *t* test in the case of individual sera and by using a 2-way ANOVA for the serum pool (factors: dilution of serum pool and Pru p 3 form). ELISA inhibition results were evaluated by means of multiple regression, with percentage inhibition as the dependent variable and concentration (log transformed) and the Pru p 3 form as predictor variables in the model.

Flow cytometric cellular allergen stimulation test

Blood from patients 8 to 17 (Table I) and from control subjects were used for the flow cytometric cellular allergen stimulation test (FAST), the antigen-specific sulphidoleukotriene production test (CAST), and the histamine release test (HRT). A total volume of 10 mL of blood was used, with 5 mL for FAST and CAST and 5 mL for HRT. The blood was stored at 4°C, and tests were carried out within 24 hours of blood sampling.

FAST was performed essentially as previously described.²¹ Briefly, cells from the layer on top of the red blood cells were collected by means of centrifugation and resuspended in HEPES calcium buffer (20 mmol/L HEPES, 133 mmol/L NaCl, 5 mmol/L KCl, 7 mmol/L CaCl₂, 3.5 mmol/L MgCl₂, and 1 mg/mL BSA [pH 7.4]) containing IL-3 (2 ng/mL) and 10 µL of heparin (5000 IU/mL). Allergens and control solutions (50 µL) were added to the wells of polystyrene microtiter U plates (Greiner Microtron) mixed with 50 µL of each patient's cell suspension and incubated at 37°C for 40 minutes. The reaction was stopped by adding 100 µL of HEPES buffer without Ca²⁺ or Mg²⁺ but containing 0.27 mmol/L EDTA (washing buffer), and then plates were centrifuged. The basophils from the pellet were double labeled with anti-CD63 phycoerythrin-labeled and anti-IgE FITC-labeled antibodies (1:80 and 1:60 dilutions, respectively; Caltag Laboratories) and incubated for 30 minutes at 4°C, and then 4 mL of erythrolytic reagent (Ortho Diagnostic System) was added. Cell lysis was stopped with washing buffer, and after centrifugation, supernatants were diluted with 500 µL of the same buffer. Flow cytometric analysis of basophil surface markers was performed at 488 nm on a FACScan flow cytometer equipped with a 15-nW argon ion laser (Becton Dickinson) by using Cell Quest Software. In each assay at least 500 basophils were assessed. The parameters analyzed on the identified basophils were anti-IgE labeled and anti-CD63 labeled.

Natural and recombinant Pru p 3 were tested at 10, 0.33, and 0.033 µg/mL. These final concentrations were selected after preliminary assays with a pool of blood from control subjects to ascertain the lack of unspecific activation. A monoclonal Le27 anti-IgE

antibody (1 µL/mL, Bühlmann) was used as a positive control, and basal values without stimulation were evaluated with HEPES calcium buffer.

FAST responses were considered positive when both the stimulation index (percentage of activated basophils with Pru p 3/percentage of activated basophils in basal conditions) was equal to or greater than 2 at any of the 3 concentrations of Pru p 3 used and the allergen-specific activation was greater than 10%.

Statistical analysis of FAST, as well as CAST and HRT, data was carried out by using a nonparametric test for comparison of means: the Mann-Whitney test for independent groups and the Wilcoxon test for paired groups.

Antigen-specific sulphidoleukotriene production

Allergen-induced sulphidoleukotriene ELISA quantification was performed according to the instructions of the assay's manufacturer (CAST-ELISA, Bühlmann Laboratories). The amounts of nPru p 3 and rPru p 3 tested were the same as in the FAST. These concentrations were selected after preliminary assays with a pool of blood from control subjects to confirm the absence of nonspecific leukotriene release.

Results were considered positive when both the stimulation index (percentage of sulphidoleukotriene released after Pru p 3 stimulation/percentage of sulphidoleukotriene released without stimulus) was greater than 2 at any of the 3 Pru p 3 concentration tested and the antigen-specific sulphidoleukotriene production was greater than 100 pg/mL.

Histamine release test

HRTs were performed according to the method of Siraganian²² with a Brän-Lubbe autoanalyser, according to the modifications for whole blood previously described.²³ Polyclonal anti-IgE antibodies (Tago, Caltag) at 4.2 µg/mL were used as positive controls. Histamine release was measured as follows:

$$\text{Pru p 3-induced histamine release (\%)} = 100 \times (\text{Histamine in Pru p 3 stimulated supernatant} - \text{Basal value}) / (\text{Total histamine} - \text{Basal value}),$$

with *basal* defined as spontaneous histamine release in supernatant without stimulus and *total* defined as histamine content in the blood sample measured after treatment with 2.5% perchloric acid. Values of greater than 10% were considered positive.

RESULTS

Recombinant Pru p 3 was produced in *P. pastoris* with an added N-terminal glutamic acid residue for proper extracellular expression of the protein.¹⁵ Previous evidence, including an expected N-terminal amino acid sequence and recognition by anti-nPru p 3 antibodies,¹⁵ suggested the correct processing of the recombinant form. MALDI analysis of nPru p 3 and rPru p 3 showed a single sharp peak in each case, with a molecular mass of 9117 and 9251 d, respectively (not shown). The difference of 134 d between both allergen forms fully agrees with the expected mass increase (138 d) produced by the attachment of an N-terminal glutamic acid, thus providing additional support for the proper processing of rPru p 3. Furthermore, the recombinant form showed a nearly identical CD spectrum as that of the natural allergen (Fig 1), suggesting that rPru p 3 is correctly folded. The spectra presented minimum values at approximately

208 nm and a shoulder at 222 nm, which is indicative of an ordered structure with dominating α -helix content (49% and 54% for nPru p 3 and rPru p 3, respectively).

Direct ELISA assays were performed with a serum pool (from patients 1-7 of Table I) and each individual serum from patients with peach allergy (Fig 2) to compare the IgE-binding capacity of both Pru p 3 forms. Overlapping curves were found for nPru p 3 and rPru p 3 with the serum pool, and similar IgE-binding levels for the 2 allergen forms were found with each individual serum. The equivalence in IgE-binding capacity of both Pru p 3 forms was further confirmed with ELISA inhibition experiments (Fig 3), which reflected the similar inhibitory behavior of the natural and recombinant allergen. No significant statistical differences were observed in the binding of IgE to nPru p 3 and rPru p 3 in the direct and ELISA inhibition assays.

The biologic activity of nPru p 3 and rPru p 3 was analyzed by using basophil activation (FAST), sulphidoleukotriene production (CAST), and HRTs. Blood from 10 patients with peach allergy (patients 8-17 in Table I) was used. All 10 patients had positive SPT responses to nPru p 3 (20 μ g/mL; median, 45 mm²). Table II summarizes the results obtained with the allergen concentrations that produced maximal basophil activation or mediator release in most blood samples. Nine of 10 patients had positive FAST and CAST responses both with nPru p 3 and rPru p 3. HRT responses were considered positive in 5 and 7 patients for nPru p 3 and rPru p 3, respectively. None of the control subjects had positive results in any test at any of the concentrations assayed. There were no statistically significant differences when comparing the results of FAST, CAST, or HRT at the same concentration of nPru p 3 and rPru p 3.

DISCUSSION

The accurate diagnosis and safe immunotherapy of food allergy is greatly constrained by the lack of standardization of the food extracts used in serologic or skin tests. The identification and characterization of major allergens and their production at high yields as recombinant proteins can provide useful tools to circumvent the diagnostic constraints.^{16,17} A previous step for the use of recombinant allergens is to prove that their immunologic properties are equivalent to those of their natural counterparts. A recent revision by Lorenz et al¹⁷ has shown that a very limited number of recombinant food allergens have been “very well characterized” regarding their allergenic capacity, including not only specific IgE-binding tests but also mediator release assays.

Pru p 3, the major allergen and LTP from peach, is a good candidate as a diagnostic tool for allergy to this fruit. Peach is a prominent agent causing allergic reactions after ingestion of food in the European adult population, and it reaches first place among plant foods in Spain.^{2,3} Pru p 3 exerts greater than 90% of positive SPT responses in Mediterranean patients with a well-established clinical history of allergy to peach.¹⁴ However, it

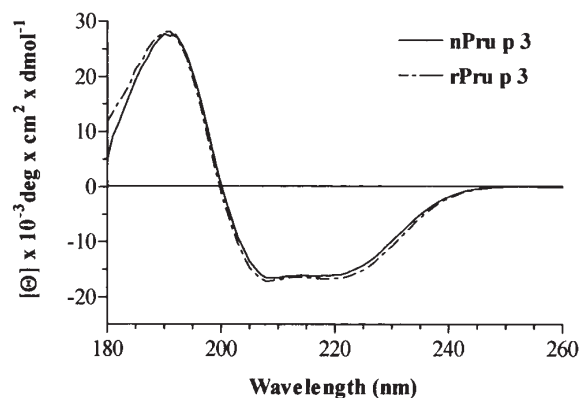


FIG 1. CD spectra of nPru p 3 and rPru p 3.

should be stated that sensitization to Pru p 3 and other Rosaceae fruit LTPs is rather rare among the Central and Northern European population. On the other hand, allergenic Pru p 3 homologues are present in several foods and pollens, and cross-reactions between members of this allergen family have been demonstrated.^{8,12,14} Additionally, rPru p 3 has been produced and isolated.¹⁵

Spectrometric analysis (MALDI and CD) of nPru p 3 and rPru p 3 supported the equivalence of their structural properties. The difference of molecular masses between both forms indicates a correct processing of rPru p 3 (see the “Results” section), and comparison of their CD spectra (Fig 1) strongly suggests a similar folding. Interestingly, the high degree of α -helix content in the secondary structures deduced from the spectra fully agree with the 4- α -helix model shown by other LTPs,⁹ such as that from maize.²⁴

Two different lines of evidence point to the close immunologic similarity of nPru p 3 and rPru p 3. First, direct IgE-binding determinations and ELISA inhibition assays (Figs 2 and 3) with pooled and individual sera from patients with peach allergy indicated that the 2 Pru p 3 forms display the same specific IgE-binding capacity. Second, basophil activation, sulphidoleukotriene tests, and HRTs (Table II) showed a high stimulatory activity of both natural and recombinant allergens. Positive FAST and CAST responses were obtained in nearly all patients (9/10) either for nPru p 3 or rPru p 3. The lower number of positive HRT responses (7/10 for rPru p 3 and 5/10 for nPru p 3) can probably be explained because the histamine release method is clearly less sensitive than FAST and CAST under the experimental conditions and restraints applied.²¹ The rPru p 3 and nPru p 3 forms displayed equivalent stimulatory activity in all 3 tests measuring basophil activation and mediator-induced release. These assays, performed for the first time with allergenic LTPs, as far as we know, suggest that cell activation models are a valid alternative to human in vivo testing (SPT) of recombinant allergens, which has strong legal restrictions and ethical limitations in basic research.

Together, the experimental evidence discussed above strongly indicates that rPru p 3 and nPru p 3 have similar

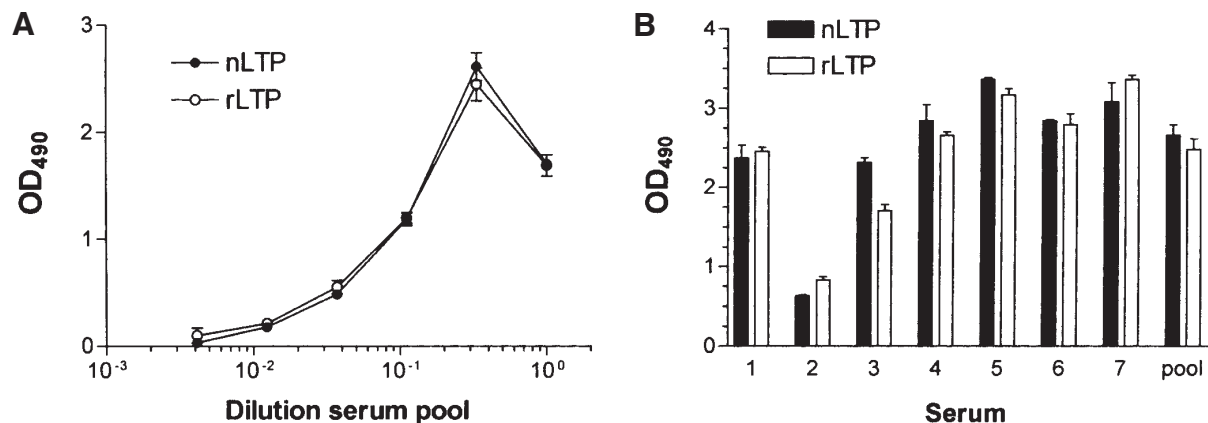


FIG 2. IgE binding to nPru p 3 and rPru p 3 by using a serum pool (A) or each individual serum (B). Individual sera (1-7) were used at 1:3 dilution. Means ($n = 3$) and SEs (bars) are represented. The statistical tests performed (see the "Methods" section) resulted in a not significant difference for the variable Pru p 3 form ($P = .447$ in A and $P = .572$ in B).

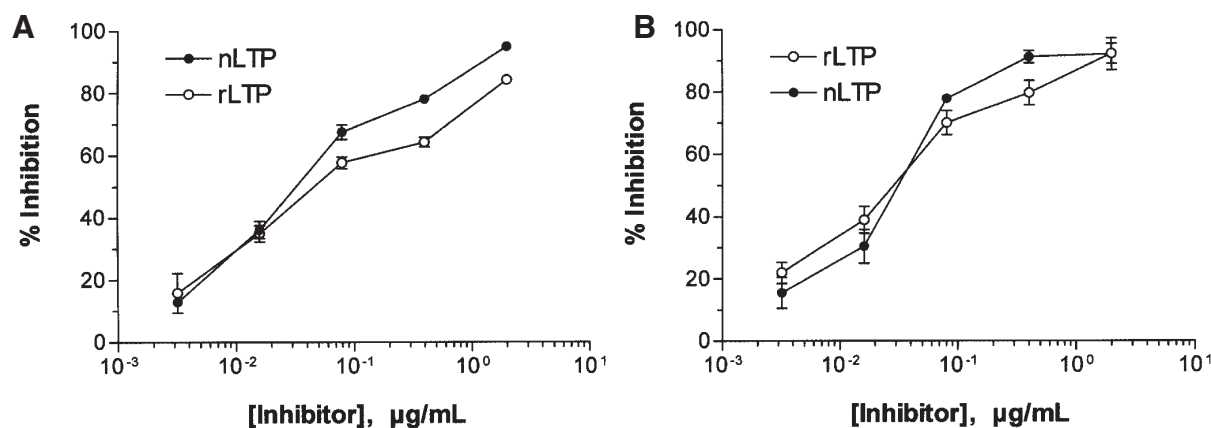


FIG 3. ELISA inhibition with nPru p 3 (A) or rPru p 3 (B) in the solid phase and nPru p 3 and rPru p 3 as inhibitors. Means ($n = 3$) and SEs (bars) are represented. The statistical tests performed (see the "Methods" section) resulted in a not significant difference for the variable Pru p 3 form ($P = .139$ in A and $P = .903$ in B).

TABLE II. FAST, CAST, and HRT responses induced by nPru p 3 and rPru p 3 in blood from patients allergic to peach

	FAST, % activated basophils (SI)†‡		CAST, pg/mL (SI)†‡		HRT, % induced histamine release‡	
	nPru p 3	rPru p 3	nPru p 3	rPru p 3	nPru p 3	rPru p 3
Patients*						
8	72.8 (34.6)	75.5 (35.9)	1438 (33.4)	1280 (29.7)	67.7 (+)	73.8 (+)
9	75.4 (3.2)	71.2 (3.1)	4727 (2.5)	4796 (2.6)	58.5 (+)	63.7 (+)
10	37.2 (33.8)	63.3 (26.8)	2270 (16.3)	3495 (25.4)	6.7 (-)	9.3 (-)
11	52.9 (5.6)	43.4 (4.6)	1014 (7.3)	1003 (7.2)	6.8 (-)	10.2 (+)
12	54.8 (49.8)	54.6 (49.6)	1681 (35.7)	1136 (24.1)	75.0 (+)	66.7 (+)
13	78.8 (20.2)	72.8 (18.6)	584 (4.2)	1353 (9.8)	8.2 (-)	19.7 (+)
14	21.1 (7.8)	18.8 (6.9)	2314 (15.4)	3875 (25.8)	0.0 (-)	0.0 (-)
15	73.5 (3.1)	65.9 (2.8)	574 (8.5)	8337 (124.4)	62.2 (+)	66.0 (+)
16	85.6 (4.8)	88.4 (5.0)	4852 (2.4)	4761 (2.4)	87.6 (+)	86.8 (+)
17	4.8 (3.2)	3.4 (2.2)	48 (-)	125 (-)	7.1 (-)	7.1 (-)
Control subjects ($n = 5$)						
Median	0.2	0.0	47	0.0	0.0	0.0
Range	0-0.5	0-0.6	0-272	0-182	0-1.1	0-1.1

SI, Stimulation index.

*Patient numbers are as shown in Table I.

†The stimulation index is shown as defined in the "Methods" section. FAST and CAST were performed at 0.33 µg/mL allergen and HRT at 1 µg/mL allergen.

‡The statistical analysis of data obtained with nPru p 3 and rPru p 3 resulted in a not significant difference in FAST ($P = .386$), CAST ($P = .241$), and HRT ($P = .161$) responses.

physicochemical and allergenic properties, thus allowing us to propose rPru p 3 as a useful tool for the diagnosis of peach allergy, particularly among the Mediterranean population.

We thank Dolores Lamoneda (ETSIA, UPM) for technical assistance and Linda Puebla (ALK-Abelló) for language revision.

REFERENCES

1. Sicherer SH. Clinical implications of cross-reactive food allergens. *J Allergy Clin Immunol* 2001;108:881-90.
2. Pastorello EA, Incorvaia C, Pravettoni V, Farioli L, Conti A, Vigano G, et al. New allergens in fruits and vegetables. *Allergy* 1998;53(suppl 46):48-51.
3. Rodriguez J, Crespo FJ, Lopez-Rubio A, Cruz-Bertolo J, Ferrando-Vivas P, Vives R, et al. Clinical cross-reactivity among foods of the Rosaceae family. *J Allergy Clin Immunol* 2000;106:183-9.
4. Pastorello EA, Ortolani C, Farioli L, Pravettoni V, Ispano M, Borgia A, et al. Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: an in vivo and in vitro study. *J Allergy Clin Immunol* 1994;94:699-707.
5. Sanchez-Monge R, Lombardero M, Garcia-Selles FJ, Barber D, Salcedo G. Lipid-transfer proteins are relevant allergens in fruit allergy. *J Allergy Clin Immunol* 1999;103:514-9.
6. Scheurer S, Pastorello EA, Wangorsch A, Kästner M, Hausteiner D, Vieths S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the in vitro diagnosis of cherry allergy. *J Allergy Clin Immunol* 2001;107:724-31.
7. Lindorff-Larsen K, Winther JR. Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Lett* 2001;488:145-8.
8. Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier MF, Ciurana CLF, et al. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000;122:20-32.
9. Kader JC. Lipid-transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* 1996;47:627-54.
10. Pastorello EA, D'Ambrosio FP, Pravettoni V, Farioli L, Giuffrida G, Monza M, et al. Evidence for a lipid transfer protein as the major allergen of apricot. *J Allergy Clin Immunol* 2000;105:371-7.
11. Pastorello EA, Farioli L, Pravettoni V, Ispano M, Scibola E, Trambaioli C, et al. The maize major allergen, which is responsible for food-induced allergic reactions, is a lipid transfer protein. *J Allergy Clin Immunol* 2000;106:744-51.
12. Diaz-Perales A, Lombardero M, Sanchez-Monge R, Garcia-Selles FJ, Pernas M, Fernández-Rivas M, et al. Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and *Rosaceae* fruits, with different IgE-binding capacities. *Clin Exp Allergy* 2000;30:1403-10.
13. Duro G, Colombo MA, Costa V, Izzo R, Porcasi R, Di Fiore G, et al. Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1. 0101. *Int Arch Allergy Appl Immunol* 1997;112:348-55.
14. Garcia-Selles FJ, Diaz-Perales A, Sanchez-Monge R, Alcantara M, Lombardero M, Barber D, et al. Patterns of reactivity to lipid transfer protein of plant foods and *Artemisia* pollen: an in vivo study. *Int Arch Allergy Immunol* 2002;128:115-22.
15. Diaz-Perales A, Garcia-Casado G, Sanchez-Monge R, Garcia-Selles FJ, Barber D, Salcedo G. cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. *Clin Exp Allergy* 2002;32:87-92.
16. Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000;106:409-18.
17. Lorenz AR, Scheurer S, Hanstein D, Vieths S. Recombinant food allergens. *J Chromatogr B* 2001;756:255-79.
18. Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM, Twardosz A, et al. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J* 2002;16:414-6.
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
20. Bohm G, Muhr R, Jaenicke R. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng* 1992;5:191-5.
21. Sanz ML, Sanchez G, Gamboa PM, Vila L, Uasuf C, Chazot M, et al. Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to *Dermatophagoides pteronyssinus* and *Lolium perenne*. *Clin Exp Allergy* 2001;31:1007-13.
22. Siraganian RP. Histamine secretion from mast cells and basophils. *Trends Pharmacol Sci* 1983;4:432-7.
23. Rosano A, Prieto I, Sanz ML, Oehling A. Reliability of histamine release test in dust mite allergy: influence of the sensitisation degree. *J Invest Allergol Clin Immunol* 1995;5:289-93.
24. Han GW, Lee JY, Song HK, Min CCK, Moon J, Shin DH, et al. Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography. *J Mol Biol* 2001;308:263-78.