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Immunoassay To Quantify the Major Peach Allergen Pru p 3 in Foodstuffs. Differential Allergen Release and Stability under Physiological Conditions

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Pru p 3 is a lipid transfer protein (LTP) that has been identified as the major peach (*Prunus persica*) allergen. However, little is known about the amount present in both raw and processed foodstuffs. Moreover, the in vivo release upon consumption of peach-containing foods remains unclear. We have developed a sensitive monoclonal antibody-based ELISA for Pru p 3. The method has been applied to measure the allergen levels in foodstuffs and the allergen release under different physiological conditions. A significant variability in all raw peaches and peach-containing foods tested has been detected. The allergen was extracted more efficiently at a low pH, and it was highly resistant to pepsin. This ELISA will be very useful in controlling the allergen concentration in diagnostics, in evaluating threshold levels in provocation tests, and in detecting hidden allergens in processed foods and cosmetics.

KEYWORDS: Peach; lipid transfer protein; allergen; food allergy; *Prunus persica*; Pru p 3; immunoassay; monoclonal antibody

INTRODUCTION

One of the last panallergens incorporated into the plant allergen portfolio is lipid transfer proteins (LTPs) (*1*, *2*). This family is composed of an unknown number of individual proteins that are widely distributed throughout the plant kingdom. Members of this family are low-molecular weight proteins, typically around 90−95 residues, with a single polypeptide chain that shows a characteristic pattern of eight cysteine residues, forming four disulfide bridges. The three-dimensional structure of some LTPs has been determined, and a general model has been postulated, which consists of a four-helix bundle formed by two antiparallel α-helix sets. The overall structure, stabilized by the four disulfide bridges, is very straightforward, the helices and connecting loops together creating a spiral with an internal hydrophobic cavity that can harbor lipidic components. LTPs are extremely stable, resisting either thermal denaturation or pepsin digestion (*3*). Processed foods such as beer (*4*) and wine (*5*) have been reported to cause allergic systemic reactions associated with specific LTPs.

Major allergens related to the LTP family have been detected in soybean hulls (Gly m 1) (*6*), *Parietaria* and *Artemisia* pollen (Par j 1, Art v 3) (*7*), *Rosaceae* fruits (Pru p 3 from peach, Mal d 3 from apple, etc.) (*1*, *2*), and other plant sources (*3*). The sequence homology ranges from 20%, between Gly m 1 and Pru p 3, maintaining the disulfide bond pattern, to almost a sequence identity (96%), i.e., between Pru p 3 and Mal d 3.

Fruit extracts used for allergy diagnosis may lack sensitivity (*8*) due to the variable content of allergenic activity. One way to characterize allergen extracts is to measure their major allergen levels by means of monoclonal antibody specific assays. In addition, these assays would facilitate the control of the allergen content of processed fruit derivatives and the assignment of risk threshold values linked to specific provocation tests.

In this report we describe the development of a specific mAb-based enzyme-linked immunosorbent assay (ELISA) to quantify the major peach allergen Pru p 3. This 9000 protein was previously purified and characterized, and the presence of specific IgE to it was demonstrated in all the sera assayed from a population of patients allergic to *Rosaceae* fruits (*1*). The assay has been used to investigate the allergen content in food and cosmetics containing processed peach, the content of Pru p 3 in different fresh fruit batches, and the release profile under different extraction conditions, including gastric-juice simulation.

MATERIALS AND METHODS

Chemicals and Supplies. Bovine serum albumin, Fraction V, was obtained from Boehringer Mannheim (Mannheim, Germany). Complete and incomplete Freund's adjuvant, pepsin,and Tween 20 were from

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Sigma Chemical Co. (St. Louis, MO). ELISA polystyrene plates of 96-well were from Costar (EIA/RIA 3590, Cambridge, MA). ELISA plates were developed with *o*-phenylendiamine (OPD, DAKO, Glostrup, Denmark), and the optical density was measured at 490 nm with a 650 nm reference filter in an Emax microplate reader (Molecular Devices Co., Sunnyvale, CA). Salts and other chemical reagents were supplied by Merck (Darmstadt, Germany).

Pru p 3 Release Kinetics. To study Pru p 3 release kinetics, two peaches were ground in the extraction buffer at a 10% w/v ratio, and incubated at 37 °C in a shaking water bath. Three different extraction solutions were used: 0.03 mol/L NaCl, 0.01 mol/L sodium phosphate, pH 7.0; 0.03 mol/L NaCl, pH 1.2; and simulated gastric fluid (SGF; 0.32% (w/v) pepsin in 0.03 mol/L NaCl, pH 1.2). Samples were taken after 1, 8, 15, and 30 min and 1, 2, 3, and 24 h and quenched by neutralization with a 0.375 volume of 0.15 mol/L Na_2CO_3. Samples were centrifuged, and the supernatant was filtered through 0.22 μm Minisart filters (Sartorius AG, Göttingen, Germany) and frozen until use.

Allergen Extracts and Purified Allergens. Peaches and peach-containing products were obtained from local stores. Solid or semisolid products were homogenized with the extraction buffer (0.03 mol/L NaCl, pH 1.2) at a 10%w/v ratio and extracted for 2 h at 4 °C. After centrifugation (12000*g* for 20 min at 4 °C), the supernatants were filtered through 0.22 μm Minisart filters and stored frozen at −30 °C until use. Samples of peach-containing liquid products were processed for ELISA analysis by simply diluting them in the dilution buffer (PBS containing 1% BSA and 0.1% Tween 20).

Other fruit and pollen extracts used were supplied by ALK-Abelló (Madrid, Spain).

The protein concentration in peach extracts was determined according to the method of May et al. (*9*).

Pru p 3 was purified as described earlier (*1*). Briefly, skins of fresh peaches were ground and then homogenized with PBS buffer (1:10 (w/v)) and extracted for 1 h at 4 °C. After centrifugation, the supernatant was dialyzed against distilled water and freeze-dried. The material obtained was then subjected to gel-filtration chromatography on an Econo-Pac P6 desalinization column (Bio-Rad, Hercules, CA). The fractions corresponding to the protein peak were pooled, freeze-dried, and subsequently defatted with cold acetone and then with ethanol: ether (1:3 (v/v)). The dried residue was fractionated by reverse-phase HPLC on a preparative Vydac-C4 column (The Separations Group, Hesperia, CA), and the fractions containing the pure allergen were pooled, dialyzed against distilled water, and freeze-dried. Quantitative amino acid analysis of Pru p 3 was carried out using reagents (AccQ.Tag) and equipment from Waters (Milford, MA).

Production of Antibodies. For the production of monoclonal antibodies, BALB/c mice were immunized with Pru p 3. Fusion of spleen cells with P3-X63.Ag8.653 myeloma cells was performed according to the method of Galfré and Milstein (*10*). Ten days after the fusion, cell culture supernatants were screened for specific anti-Pru p 3 antibodies by an ELISA with purified Pru p 3 on the solid phase. The P3-X63.Ag8 mouse myeloma culture supernatant was used as a negative control. The positive hybrids were cloned and subcloned by limiting dilution.

Polyclonal antibodies against Pru p 3 were produced by immunization of New Zealand rabbits with Pru p 3 in Freund's adjuvant (complete for the first injection and incomplete for the rest). Injections were repeated every 15 days, and sera were collected 10 days after each injection. Anti-Pru p 3 antibodies titer was measured in each bleeding, and those sera with the highest titers were pooled and stored frozen at −80 °C in small aliquot fractions.

The OECD principles of good laboratory practices were followed for the care and use of animals.

Purification and Characterization of mAbs. Thirteen mAbs of IgG class were purified from culture supernatants by GammaBind Plus Sepharose affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). The specificity of these mAbs was demonstrated by an ELISA with Pru p 3 on the solid phase and by immunodetection after SDS−PAGE of a peach extract carried out essentially as reported earlier (*4*).

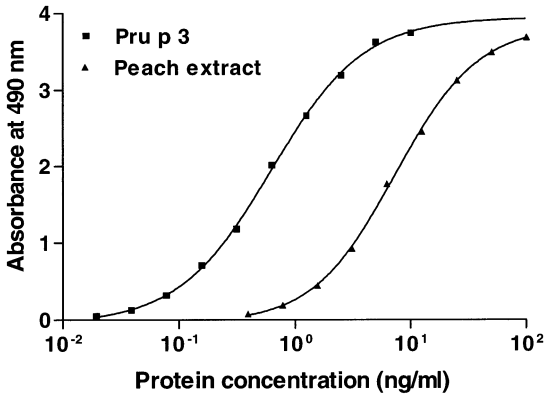

Figure 1. Dose–response curves obtained in the mAb-based Pru p 3 ELISA for the purified allergen and a peach extract. On the *x*-axis, the protein concentration, measured by amino acid analysis for Pru p 3 and by the method of May et al. (*9*) for the peach extract, is indicated. Absorbance values on the *y*-axis are expressed in absorbance units.

Pru p 3 ELISA. ELISA plates were coated overnight at 4 °C with 100 μL of anti-Pru p 3 mAb 1.1.1 at 5 μg/mL in PBS. After blocking with PBS containing 1% BSA and 0.1% Tween 20 (dilution buffer), wells were sequentially incubated with samples and references, anti-Pru p 3 rabbit serum (dilution 1:20 000), and goat anti-rabbit IgG antibodies conjugated with peroxidase (DAKO, dilution 1:2000). Samples, references, and reagents were diluted in dilution buffer. All incubations were carried out for 1 h at room temperature, with intermediate washes using PBS containing 0.1% Tween 20 between successive steps. Finally, the wells were incubated in the dark for 30 min at room temperature with a solution of OPD, and the color reaction was stopped by adding 2 N HCl. Serial dilutions of the samples (factor 2) were assayed in duplicate. Samples were diluted at least 1:10 to avoid potential effects from the sample matrix. The Pru p 3 concentration of samples was calculated by interpolating from a standard curve constructed with serial 2-fold dilutions of Pru p 3, starting from 10 ng/mL.

RESULTS

ELISA for Pru p 3 Quantification. After testing the different antibody combinations, the best results in terms of sensitivity and linearity for a sandwich ELISA to quantitate Pru p 3 were found when using mAb 1C1 as the capture antibody and an anti-Pru p 3 rabbit serum as the second antibody. The dose−response curves obtained in the ELISA with purified Pru p 3 and peach extracts were parallel (Figure 1), allowing us to accurately measure the LTP content in these extracts by comparison of the curves against a standard Pru p 3 curve. The model that best describes the relationship between the absorbance and the concentration of antigen is a four-parameter logistic curve (*11*). The limit of detection of the ELISA, determined from the values obtained for the ordinate at the origin and the standard deviation in 10 different assays, is very low, only 0.1 ng/mL, and the practical working range lies between 0.2 and 5.0 ng/mL. Moreover, the reproducibility of the method is very high (intra- and interassay coefficients of variation of 3.1 and 12.2%, respectively).

The selectivity of the assay was studied by analyzing a battery of extracts from different fruits and pollens. Only extracts from some related fruits belonging to the *Rosaceae* family (cherry, almond, and apple) gave a positive response, although these extracts had to be tested at a concentration about 1000-fold higher than peach extracts to produce a detectable signal. Furthermore, the curves obtained with these extracts were not parallel to the standard curve with purified Pru p 3, indicating that the antibodies used in the assay have different affinities for the homologous proteins in other extracts.

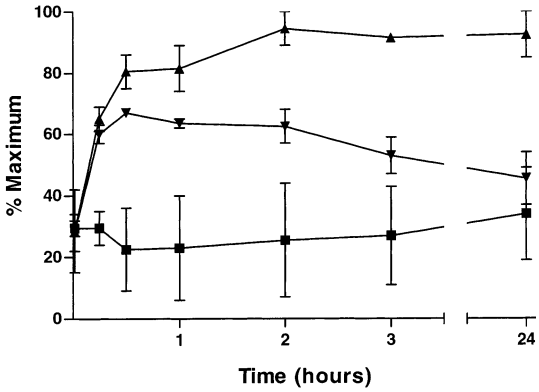

Figure 2. Pru p 3 extraction kinetics. Ground fresh peaches were extracted in 0.03 M NaCl, pH 7.0(■); 0.03 M NaCl, pH 1.2 (▲); and SGF: 0.32% (w/v) pepsin in 0.03 M NaCl, pH 1.2(▼), as described in Materials and Methods. Samples were taken at different times, and the Pru p 3 content in the solution was measured with the mAb-based ELISA. Results are expressed as percentages of the maximum concentration reached. Points represent mean values of extractions of two different peaches from the same batch. Bars indicate the standard error of the estimate of mean value.

Release Profile of Pru p 3. The solubilization kinetics of Pru p 3 under physiological conditions was studied using phosphate buffer and acid pH solutions with (SGF) or without pepsin.

Figure 2 summarizes the results of these experiments. Of the highest concentration of Pru p 3, 35% is detected during the first minutes under all conditions. However, acid pH solutions are more effective for its solubilization. Pepsin only starts to digest Pru P 3 after 2 h of incubation. Even after 24 h, 40% of the allergen has not been processed.

Quantitation of Pru p 3 in Fresh Fruits and Processed Foods. Nine different batches of fresh peaches, consisting of at least four pieces, were processed and tested. The mean amount of Pru p 3 per kilogram of fruit was 16.50 mg, with a CV of 43% (range 3.8−23.9 mg). Pru p 3 was about 10-fold more abundant in peel than in pulp.

Different marketed products (mainly foods and cosmetics) containing peach or peach flavors were also tested for Pru p 3 content. The detected concentrations are summarized in Table 1. In almost every group of products a significant variation of allergen concentration was observed. No Pru p 3 was detected in alcoholic peach liquors, tea, or cosmetics. On the other hand, significant amounts were detected in the vast majority of processed peach foods. The level of variability observed in each individual group is usually within the range detected in natural fruits. The strongest variability was observed in the case of juices, which could be due to the different manufacturing procedures.

Table 1. Quantification of Pru p 3 in Peach-Containing Products

product	manufacturer[a]	content	Pru p 3 (μg/ g)	
baby foods	1A	peach/banana	0.868	
	1B	peach/cheese	1.264	
	2	apple/peach/cereals	3.229	
yogurts	2A	peach purée	1.311	
	2B	peach	0.193	
	3A	peach	0.185	
	3B	peach/cheese	0.170	
jams	4	peach	1.004	
	1A	peach	0.240	
	1B	peach	0.376	
	5	peach	2.687	
	6	peach	0.472	
	7	peach	2.024	
energetic preparation	8	peach	5.53	
juices	7A	peach	2.537	
	7B	peach	3.698	
	9A	peach	2.358	
	9B	peach	2.418	
	3	peach/apricot	0.110	
	10	peach/grape	2.696	
	11	peach	3.358	
	12	peach	1.328	
syrup-preserved			fruit	syrup
peach	13	peach	0.182	0.123
	14	peach	1.885	1.274
	7	peach	0.338	0.054
	15	peach	1.104	0.871
	16	peach	0.174	0.027
alcohol-free beverages	17	peach liquor	0.051	
	7	peach liquor	0.023	
	18	tea with peach flavor	$<10^{-3}$	
	2	tea with peach flavor	$<10^{-3}$	
alcoholic beverages	7	sangría	$<10^{-3}$	
	19	sangría	$<10^{-3}$	
	20	peach liquor	$<10^{-3}$	
	21	peach liquor	$<10^{-3}$	
	22	peach liquor	$<10^{-3}$	
miscellaneous	23	shampoo	$<10^{-3}$	
	24	air-freshener	$<10^{-3}$	
	25	toothpaste	$<10^{-3}$	
	7	soap	$<10^{-3}$	

[a] Numbers indicate different manufacturers. Letters correspond to different batches of the same manufacturer. Since this is not an exhaustive study of products from each manufacturer (only one or two batches were analyzed), the identification of these has been omitted.

DISCUSSION

A sensitive and reproducible ELISA method based on a specific mAb has been developed to measure peach LTP (Pru p 3).

Crude allergen extracts, prepared from natural sources, are commonly used for the diagnosis of fruit allergy. The potency of these extracts may vary from batch to batch, because of the lack of an adequate standardization method. This can be due to the variability of the natural source, the extraction protocol used, or the stability of the different allergens involved. Consequently, fruit extracts used for diagnosis may have a low sensitivity (*8*). A good way to standardize allergenic extracts is to measure their major allergen content by immunoassays based on specific mAbs. The methodology has been widely applied in the case of inhalant allergens with very good results. In this sense, the Pru p 3 assay will be very useful in the standardization of peach extracts, increasing their diagnostic value.

Another important application of the Pru p 3 assay would be the determination of risk threshold allergen doses for the peach provocation test. In the different peach fruit batches tested, we have observed differences of about 10-fold in the LTP content. Clearly, the use of foods standardized in terms of major allergen content will reduce the uncertainty factor when extrapolating data from the patients selected for the challenge test to the entire population of individuals with the corresponding allergy.

Finally, the problem of hidden food allergens is of major concern both for the food industry and the food-allergic consumer. There is a clear need for analytical methods specific and sensitive enough to detect even trace amounts of food allergens, and in this sense, sandwich ELISAs based on specific mAbs are ideal. Due to its stability and resistance to pepsin digestion, LTP is a potentially severe food allergen and its presence should be included in the labeling of processed fruit derivatives. The high sensitivity of the ELISA here described,

which enables the detection of Pru p 3 at the level of ppb (ng/g), renders it very useful not only for quantifying this allergen in food preparations but also for detecting the presence of hidden peach LTP in foods.

Abbreviations Used. ELISA, enzyme-linked immunosorbent assay; LTP, lipid transfer protein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.

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