

Quantification of Peach Fruit Allergen Lipid Transfer Protein by a Double Monoclonal Antibody-based Sandwich ELISA

Zhong-shan Gao¹ · Ying-tao Ma^{1,2} · Xiang Zhou¹ · Zhao-wei Yang¹ · Hui-juan Jia¹ ·
Ling Gao¹ · Shan-dong Wu¹ · Lu-yang Han¹ · Xue-ying Yi¹ · Hui-ying Wang³ ·
Jaap H. Akkerdaas⁴ · Ronald van Ree⁵

Received: 14 April 2015 / Accepted: 26 July 2015
© Springer Science+Business Media New York 2015

Abstract Fruit is one of the most commonly reported food allergy sources in China, and peach lipid transfer protein (Pru p 3) has been identified as the major allergen inducing systematic symptoms. Crude allergen extracts and single component allergens have been used in food allergy diagnosis and immunotherapy. Reliable and sensitive analytical methods to quantify Pru p 3 content in fruit will help to identify low-allergenic cultivars among the abundant peach genetic resources in China. In this study, we developed a sensitive sandwich ELISA method to measure Pru p 3 in peach fruit based on two monoclonal antibodies with high sensitivity and linearity. Significant variability was observed in peach cultivars, with a much higher Pru p 3 level in peach peel than in pulp. This method will be very useful to select peach varieties with low Pru p 3 content and as a diagnostic product to detect hidden allergens in processed food.

Keywords Lipid transfer protein · Monoclonal antibodies · Peach allergen · Protein quantification · Sandwich ELISA

Introduction

Fruit allergy has been ranked as one of the most common food allergies in schoolchildren and adults (Gao and Gilissen 2011; Kavaliunas et al. 2012; Burney et al. 2014). In China, the number of people affected by fruit allergy has also increased with the growth in fruit consumption over the last two decades. Peach is widely planted and consumed in China, especially in the northern part, and is considered to be an important component of a healthy diet. However, it is one of the most frequently reported allergic fruit (Gao and Gilissen 2011). The majority of allergens in Rosaceae fruit have been reported to belong to four families: pathogenesis-related protein 10 (PR-10 protein, birch allergen Bet v 1 homologues), thaumatin-like proteins (TLP, PR-5 proteins), nonspecific lipid transfer proteins (nsLTPs, PR-14 proteins), and profilins (PRF) (Breiteneder and Ebner 2000). In peach, these four allergen families are denoted as Pru p 1 (PR-10), Pru p 2 (TLP), Pru p 3 (LTP), and Pru p 4 (profilin) (Chen et al. 2008). Allergy to

Zhong-shan Gao and Ying-tao Ma contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s12161-015-0272-x) contains supplementary material, which is available to authorized users.

✉ Zhong-shan Gao
gaozhongshan@zju.edu.cn

✉ Hui-ying Wang
marywang@zju.edu.cn

¹ Department of Horticulture/Allergy Research Center, Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, College of Agriculture and Biotechnology, Zhejiang University, 310058 Hangzhou, China

² Life Science College, Luoyang Normal University, Luoyang 471022, China

³ Department of Allergy, the Second Affiliated Hospital of Zhejiang University, School of Medicine, Zhejiang 310009, People's Republic of China

⁴ Department of Experimental Immunology, Academic Medical Center-University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands

⁵ Department of Otorhinolaryngology and Experimental Immunology, Academic Medical Center-University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands

peach shows distinct clinical characteristics: oral allergy syndrome (OAS) only, closely associated with birch pollinosis, and OAS plus severe systemic symptoms, mostly provoked by lipid transfer proteins (LTPs) (Zuidmeer et al. 2005; Egger et al. 2010). The geographical distribution of peach allergy is highly specific. In central and northern Europe, allergic reactions to peach are usually due to sensitization to the major birch pollen allergens Bet v 1 and the Bet v 1 homologous protein (Pru p 1 in peach). In contrast, in the Mediterranean area, which is virtually free of birch pollen, peach allergy seems to be the result of primary sensitization to the Pru p 3 (LTP) (Fernandez-Rivas et al. 2003; Asero and Pravettoni 2013). In China, the LTP has been demonstrated to be the major allergen causing peach allergenic reaction (some patients may even have anaphylaxis), especially in the north, and has been shown to cross-react with mugwort LTP (Gao et al. 2013; Ma et al. 2013). Peach consumer preference is strikingly different in the geographic regions in China: in the south, people prefer soft melting peach at full ripening stage so the peel can be removed easily, while in the north, people prefer crisp sweet peach. Some peach allergy patients reported their early tolerance to nectarine or normal peach without peel. A few studies have reported differences in allergenicity in peach cultivar, but no conclusive evidence has been provided.

Members of the plant nsLTP family are low molecular weight proteins, typically around 90–95 amino acids. These proteins contain eight cysteine residues forming four disulfide bridges (Kader 1996; Pasquato et al. 2006) and a highly conserved structure shared by most nsLTP molecules, which is why these allergens cross-react in different plants (Asero et al. 2000; Diaz-Perales et al. 2000; Ballmer-Weber 2002). The nsLTPs have been shown to be extremely resistant to thermal denaturation and pepsin digestion, as well as food processing steps such as homogenization, heating, and high-pressure treatment (Breiteneder and Ebner 2000; Vassilopoulou et al. 2006; Gaier et al. 2008; Garino et al. 2012), which enable these allergens to induce severe systemic reactions. Sequencing and characterization of the LTP-encoding gene in different peach cultivars have showed that these genes are completely conserved (Ma et al. 2014), but differentially expressed (Yang et al. 2011).

Currently, the most effective treatment for food allergy is strict avoidance of the allergen-containing food. However, total avoidance is usually difficult for the allergic patient since ingredients in processed food products may contain allergenic components. Currently, another approach is being used to investigate allergen-specific immunotherapy (SIT) (Toda et al. 2011); however, there is limited information on the amount of allergen present in raw and processed foodstuffs. The food industry is confronted with the need for reliable analytical methods to detect and quantify particular allergens in food to ensure compliance with food labeling and to improve consumer protection (Kirsch et al. 2009). Several technical

possibilities have been developed to detect potential allergens in food products, either targeting the allergen (protein) itself or using a marker that indicates the presence of the allergenic ingredient (Kirsch et al. 2009). Protein-based methods usually refer to enzyme-linked immunosorbent assay (ELISA) and more recently with mass-spectrometric methods which are quantitative methods (Kirsch et al. 2009). Thanks to its high precision, simple handling and good potential for standardization, the ELISA technique is most commonly used in laboratories of the food industry and official food control agencies. Two ELISA approaches are available to quantify allergens or proteins of potentially allergenic food: competitive ELISA and sandwich ELISA. The latter is the most common type of immunoassay for detecting potential food allergens and has been developed for several of them, with numerous test kits made commercially available in this format during the last decade. Sandwich ELISA has been used to quantify the peach allergen (Asturias et al. 1999; Ahrazem et al. 2007). In most sandwich ELISAs, a monoclonal antibody is usually used as the first antibody and a polyclonal as the detection antibody (Carnes et al. 2002). To date, there has been no report of two monoclonal antibodies, and sandwich ELISA test kit for peach LTP has been reported for commercial production. Our study focused on a sandwich ELISA method based on two monoclonal antibodies with specificity to peach fruits, which can also be applied for measuring LTPs in other fruits of plum, apricot, and cherry.

Materials and Methods

Preparation of Protein Extracts

Eight peach cultivars from different parts of China were collected. Peel (1 mm thickness) and pulp were carefully separated. Twelve other species (apricot, cherry, plum apple, hawthorn, loquat, kumquat, grape, muskmelon, strawberry, mango, and jujube) were bought from a local fruit market. For protein extraction, 1 g of homogenized material was ground to a powder in liquid nitrogen, and the powder diluted 1:5 (w/v) in Coca's solution (0.1 M Na₂CO₃, 0.1 M NaHCO₃, 0.1 M NaCl, 2 mM EDTA-Na₂, 20 mM sodium diethyldithiocarbamate trihydrate) (Grevil and Mehra 1975). After 1 h extraction at 4.0 °C with continuous stirring, the mixture was centrifuged at 12,000 rpm for 1 h, and the supernatant stored at −20 °C prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Purification of Peach LTP

Peach LTP was purified from a peel extract of the cultivar 'Hujingmili.' The supernatant was concentrated 10 times by ultrafiltration using a 30-kDa YM30 membrane (Amicon

Corp., Danvers, MA, USA). During ultrafiltration, the medium was replaced by a sodium acetate buffer (0.1 M), pH 4.5, containing 5 mM EDTA. Concentrates were separated on a HiPrep 16/10 SP-FF cation exchange column (GE Healthcare), eluted with 1 M NaCl. The purified natural peach LTP was verified for molecular weight by mass spectrometry (Applied Biosystem, 4800).

Protein Content Analysis and SDS-PAGE

The protein content was measured as mg/albumin equivalent using the Bradford method, reading absorbance values at 595 nm in a spectrophotometer (Biophotometer, Thermal Fisher). The extracted and purified proteins were analyzed by SDS-PAGE with a total polyacrylamide concentration of 10 %. Twenty microliters protein extract for each cultivar was diluted with 0.3 volumes of 1.33 M Tris-HCl buffer, pH 7.4, containing 8 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, and 40 % (w/v) glycerol, heated at 100 °C for 5 min, and then cooled on ice for 3–4 min. Ten microliters of each sample was loaded on the gel. Electrophoresis was at 30 V for 30 min and then at 100 V (Mini-PROTEAN Tetra cell, BIO-RAD, America) for 1 h. Gels were stained with Coomassie Brilliant Blue.

Production and Purification of Pru p 3-specific Antibodies

For the production of monoclonal antibodies (mAbs), eight Bal b/c mice (experimental animal use permission, SCXK(HU)2012-002, China) were immunized subcutaneously with 10–80 µg (interval of 10 µg) of rPru p 3, expressed in yeast as described by Zuidmeer (Zuidmeer et al. 2005) in complete Freund's adjuvant (Hua An Bioscience Technology, Hangzhou, China). Immunization was followed by five subcutaneous boosters (at intervals of 3 weeks, 2 weeks, 2 weeks, 3 weeks, and 3 weeks) with 40 µg rPru p 3 in incomplete Freund's adjuvant. Four days later, the spleen cells were mixed with SP2/O myeloma cells according to the reported method (Köhler and Milstein 1975). Antibody-producing hybridoma cells were screened by ELISA and Western blotted with rPru p 3 and peach peel extract. At the time, we screened the antibody and production, natural Pru p 3 was not available.

For antibody purification, hybridoma culture supernatants were purified on HiTrap Protein A and equilibrated in 50 mM phosphate buffer pH 7.4. The adsorbed IgG was eluted from the column using 0.2 M glycine-HCl buffer, pH 3.0. Peak fractions were collected and pooled in 5 mM phosphate-buffered saline (PBS, pH 7.4).

Direct and Sandwich ELISA Test

Microtiter plates (Maxisorp, NUNC, Denmark) were coated with 100 µl 5 µg/ml rPru p 3 or 50 µg/ml fruit extracts

in 0.1 M bicarbonate buffer (pH 9.6). After blocking with 100 µl 1 % BSA and 0.05 % Tween 20 in PBS (PBS-BT), 100 µl mAbs (concentration of 2 µg/ml) were added and the mix incubated at 37 °C for 1 h. Detection was carried out using sheep anti-mouse IgG coupled with horseradish peroxidase (HRP). All the mAbs were biotinylated for sandwich ELISA, using peach peel extract to select the most sensitive mAb pair. To further test the stability of these monoclonal antibodies, we stored the selected antibody pair at 4 °C and room temperature, respectively, for 20 days, and then determined the efficiency of these two batches in the ELISA test.

Quantity Assessment of LTP in Peach Extracts Using ELISA

Microtiter plates (Maxisorp, NUNC, Denmark) were coated at 4 °C overnight with 100 µl of the B10-5 mAb at 2 µg/ml in coating buffer (PBS, pH 7.4). After blocking with 100 µl 1 % skimmed milk and 0.05 % Tween-20 in PBS, wells were incubated with 100 µl (allergen) extracts, with purified rPru p 3 and nPru p 3 used as the standard. Detection was carried out 100 µl biotinylated mAb 4-1 (2 µg/ml) in conjunction with horseradish peroxidase (HRP)-conjugated streptavidin (Amersham Biosciences, Little Chalfont, UK). References and reagents were both diluted in PBS buffer, all incubations were carried out at 37 °C for 1 h, and washing was completed with PBS/0.05 % Tween 20. Finally, the plates were incubated in the dark at room temperature for 10 min with the 100 µl substrate TMB (3, 3', 5, 5'-tetramethylbenzidine) and the reaction was stopped by addition of 100 µl 2 M hydrochloric acid. Serial dilutions of the samples were assayed in duplicate. LTP concentrations were calculated using Origin v 8.0 software.

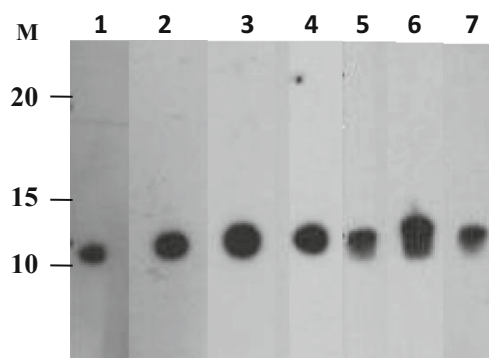


Fig. 1 Immunoblot analysis of seven monoclonal antibodies with rPru p 3 allergen. Note for lanes 1–7 with different antibodies and dilutions. 1: 4-1 (1:1000); 2: 4-6 (1:1000); 3: 7-1 (1:1000); 4: A7-2 (1:1000); 5: 6-F7 (1:5000); 6: B10-5 (1:5000); 7: 11-B7 (1:1000). Marker sizes are indicated on the left side

Table 1 mAbs binding of selected fruit extracts

mAb	ELISA activity (A450nm)												
	Peach	Apricot	Cherry	Plum	Apple	Hawthorn	Loquat	Kumquat	Grape	Muskmelon	Strawberry	Mango	Jujube
4-1	0.73	0.37	0.31	0.36	0.26	0.23	0.11	0.45	0.10	0.13	0.08	0.25	0.12
4-6	0.77	0.37	0.31	0.38	0.24	0.25	0.13	0.45	0.10	0.11	0.07	0.26	0.12
A7-1	0.85	0.16	1.16	0.26	0.18	0.22	0.11	0.42	0.09	0.07	0.06	0.24	0.11
A7-2	1.44	0.17	1.20	0.26	0.18	0.22	0.09	0.44	0.09	0.08	0.06	0.25	0.12
B10-5	1.31	0.61	0.44	0.29	0.34	0.13	0.16	0.33	0.09	0.06	0.06	0.08	0.10
6-F7	1.30	0.47	0.69	0.28	0.47	0.14	0.16	0.31	0.10	0.06	0.06	0.08	0.13
11-B7	1.31	0.26	0.48	0.26	0.45	0.14	0.16	0.29	0.08	0.06	0.05	0.09	0.11

Results

Selection of mAbs

Screening by ELISA against peach recombinant Pru p 3 resulted in seven LTP-reactive mAbs, which were also confirmed by immuno-dot-blotting against rPru p 3 (Fig. 1). The mAbs 4-1 and 4-6 are IgG2a isotype, while A7-1, A7-2, B10-5, 6-F7, and 11-B7 are all IgG1 isotype. These seven mAbs had higher binding capacity to peach extract (cv. Hujingmilu) than the other 12 fruit extracts (Table 1). With BSA as control, the OD was below 0.1. Preliminary tests with seven biotinylated mAbs for sandwich ELISA indicated that the seven mAbs could be classified into three groups with similar binding capacity pattern: group 1 with 4-1 and 4-2; group 2 includes A7-1 and A7-2; and group 3 includes B10-5, 6-F7, and 11-B7 (Tables 1 and 2). The mAb 4-1 was suitable as the detector antibody tested further with other mAbs (Table 2). Further combination tests with four mAb pairs showed that the mAbs in group 3 were quite similar and much more sensitive than A7-1, so we selected mAb B10-5 and 4-1-bio as the pair for sandwich ELISA (Fig. 2). The specificity of this antibody combination was studied by analyzing a batch of extracts from different fruits. The extracts from stone (genus *Prunus*) fruits (peach, apricot, cherry, and plum) gave

Table 2 Monoclonal antibody pair matching test for sandwich ELISA (OD at A450nm)

mAb	4-1	4-6	A7-1	A7-2	11-B7	B10-5	6-F7
4-1-bio	1.07	0.83	2.24	2.46	2.58	2.63	2.60
4-6-bio	0.76	0.71	2.18	2.35	2.44	2.69	2.55
A7-1-bio	2.44	2.52	0.61	0.55	0.90	0.65	0.64
A7-2-bio	2.54	2.62	0.71	0.65	1.19	1.28	0.90
11-B7-bio	0.45	0.55	0.25	0.19	0.33	0.22	0.40
B10-5-bio	0.60	0.82	0.22	0.15	0.35	0.29	0.39
6-F7-bio	1.53	1.78	0.18	0.17	0.55	0.45	0.46

Coated with 50 μ l of 2 μ g/ml mAb, peach protein extract 50 μ l of 5 μ g/ml, detector mAb 50 μ l of 2 μ g/ml

a positive response with the same soluble protein extracts (Fig. 3), but not other genus in the same Rosaceae family of fruits (apple, loquat, and strawberry). These two mAbs were stable and valid after storage at room temperature for 20 days.

Purification of Peach LTP

Natural LTP, isolated from peel extracts of the peach cultivar 'Hujingmilu,' was a protein in the molecular mass range of around 9 kDa. The observed mass of this protein was 9124.48 Da (Fig. 4), which is close to the theoretical mass, 9136, of peach Pru p 3 (Q9LED1). This natural Pru p 3 had binding potency to IgE in pooled sera of peach Pru p 3 sensitive patients (informed consent was obtained from all individual participants in this study and this research was approved by the local ethics committee no. 2011-R-1, Second Affiliated Hospital, College of Medicine, Zhejiang University) and to the mAbs produced with rPru p 3 (data not shown). The purified natural LTP was used for the standard curve in the sandwich ELISA.

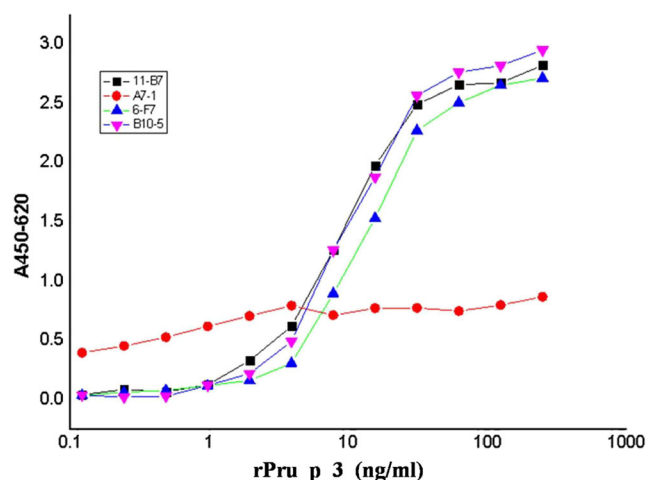
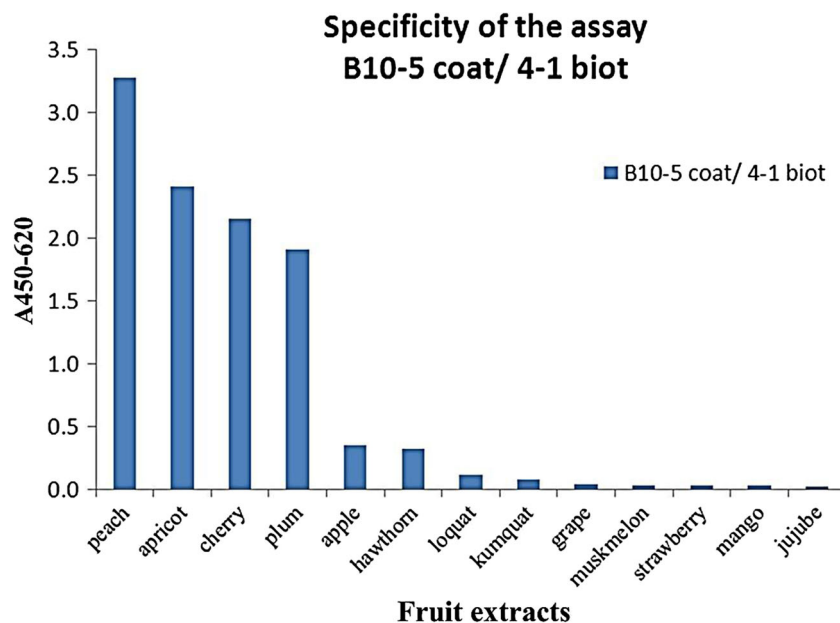
**Fig. 2** Dose-response curves of rPru p 3 in sandwich ELISA with four mAbs combinations. mAb 4-1 as the detector antibody, OD values refer to A450-A650

Fig. 3 Specificity of a selected pair of sandwich ELISA with different fruit protein extracts. All extracts were diluted to 150 ng/ml. The values are the mean of three independent experiments

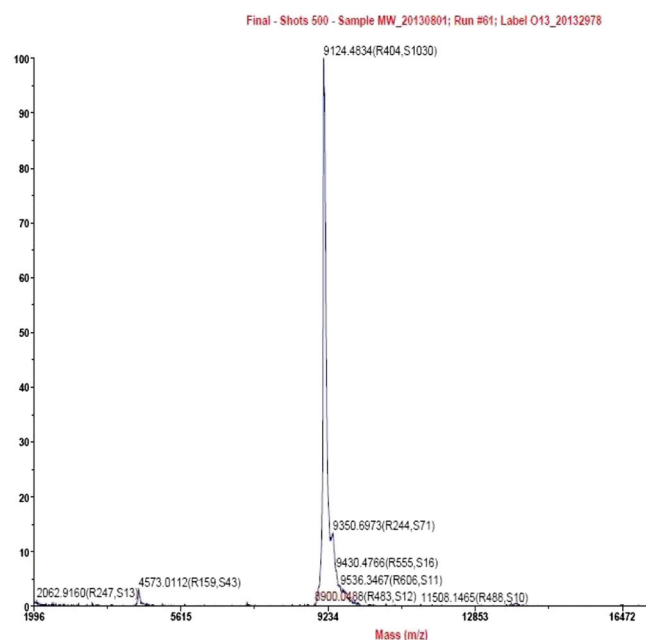
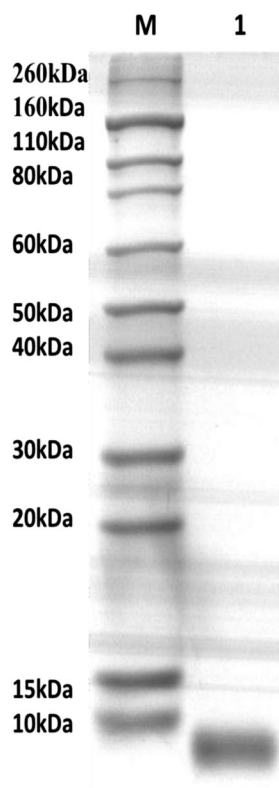


ELISA for Pru p 3 Quantification

When the antibody combination of mAbs B10-5 and 4-1 biot antibody combination was used to quantify the natural Pru p 3 in peach cultivars, a four-parameter logistic dose response curve in the ELISA with purified nPru p 3 and recombinant Pru p 3 was obtained (Fig. 5). There is a clear

discrepancy between these two curves: the curve with nPru p 3 was chosen as the standard as the purified natural protein is closer to the allergen in fruits. The limit of detection of the ELISA was 0.5 ng/ml. The intra- and inter-assay coefficients of variation of this method were 2.9 and 13.3 %, respectively, which indicates good reproducibility (Tables 3).

Fig. 4 SDS-PAGE analysis of purified natural Pru p 3 and mass characterization. Lanes: *M*—protein markers; *I*—purified natural Pru p 3



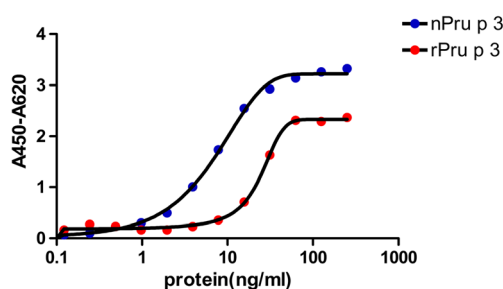


Fig. 5 Dose–response standard curve of nPru p 3 and rPru p 3 in ELISA with mAb B10-5 as the capture antibody and biotinylated mAb 4-1 as the detector antibody

Quantification of Pru p 3 in Peach Peel and Pulp

The peel and pulp of eight peach cultivars were tested for the content of Pru p 3. The mean for Pru p 3 in the peel was 48.85 μg per gram fresh weight, ranging from 14.09 to 67.06 $\mu\text{g/g}$, and in the pulp, 3.12 $\mu\text{g/g}$, ranging from 0.57 to 5.02 $\mu\text{g/g}$ (Table 4). Among those cultivars investigated, Hongshanhu (HSH) had the highest content in the peel, 67.06 $\mu\text{g/g}$, while the lowest, 14.09 $\mu\text{g/g}$, was found in the peel of Yanfeng (YF). The variation of Pru p 3 content in the peel was not consistent with that found in the pulp.

Discussion

In recent years, crude allergen extracts have been used for allergy diagnosis and immunotherapy; however, the allergen composition and allergenic potency may differ from extract to extract, as well as in different cultivars (Sancho et al. 2008). A reliable analytical method with high sensitivity and specificity is urgently needed to quantify particular potential allergens in natural sources and food products. Here, we report on a monoclonal antibody-based sandwich ELISA with a sensitivity threshold of 0.5 ng/ml Pru p 3, which is lower than the result for the Pru p 3 allergen detected using sandwich ELISA based on mixed mono- and polyclonal antibodies (Duffort et al. 2002). This is comparable to Hel a 2 (Asturias et al. 1998).

Table 3 Accuracy, precision, and reproducibility test of ELISA

Spiked nPru p 3 (ng/ml)	Intra-assay ($n=6$)				Inter-assay ($n=3$)			
	Measured	SD	CV	Recovery rate	Measured	SD	CV	
	(ng/ml)	(ng/ml)	(%)	(%)	(ng/ml)	(ng/ml)	(%)	
10	9.84	0.14	1.42	98.4	9.78	0.17	3.46	
5	4.79	0.14	2.92	95.8	5.02	0.16	1.64	
2	2.07	0.05	2.42	103.5	2.07	0.12	5.67	
0.5	0.52	0.018	3.46	104	0.51	0.03	4.9	

Table 4 Pru p 3 content of eight peach cultivars

Cultivar	$\mu\text{g/g}$ of fresh weight (mean $\pm$ SD)	
	Peel	Pulp
Hongshanhu	67.06 $\pm$ 0.04	1.73 $\pm$ 0.10
Jinxiuhuangtao	66.04 $\pm$ 0.04	5.02 $\pm$ 0.16
Xinhong	61.05 $\pm$ 0.18	0.57 $\pm$ 0.04
Hujingmilu	53.80 $\pm$ 0.14	4.24 $\pm$ 0.06
Jinxia	51.93 $\pm$ 0.22	3.03 $\pm$ 0.25
Qiufen	49.07 $\pm$ 0.22	5.15 $\pm$ 0.06
Xinyu	27.72 $\pm$ 0.16	2.69 $\pm$ 0.08
Yanfeng	14.09 $\pm$ 0.03	2.56 $\pm$ 0.04

The reproducibility of our sandwich ELISA was confirmed by assessing the inter- and intra-assay variability of the standard curve with natural Pru p 3 covering the confident linear range from 0.5 to 15.6 ng/ml (Fig. 5). Additionally, when different fruit extracts were tested, only peach, apricot, cherry, and plum extracts showed high reactivity, with peach as the most reactive. The specificity of our monoclonal antibodies to prune fruits may be explained by the amino acids that are conserved in LTPs of stone fruit but different from other Rosaceae fruits. Candidate amino acids include 7S, 19G, 36N, 55S, and 68A (Supplementary Fig. 1), the residue N36 was also reported as part of a mimotope of Pru p 3 able to recognize IgEs from sera of patients displaying systemic reaction to peach (Pacios et al. 2008). Specific double mAb-based ELISA was also reported in apple LTP (Mal d 3) (Zuidmeer et al. 2005).

As for the major allergen in a limited number of peach cultivars, significant differences have already been found, but the range of values in our research was similar to that of Spanish and US cultivars (Ahrazem et al. 2007). Further research is planned on more cultivars of core peach collections of both Chinese and foreign cultivars (Li et al. 2013) with this ELISA assay, and we hope to select out low Pru p 3 content varieties for clinical trial on peach allergy patients.

In our study, purified natural Pru p 3 and recombinant Pru p 3 were used at the same time for the dose–response standard curve; however, the Pru p 3 content calculated from these two curves differed, with the recombinant Pru p 3 about four times higher than that of purified natural Pru p 3 (Fig. 5). It was very surprising to see that although the mice were boosted with rPru p 3, the reactions to the antibodies were greater with nPru p 3. A very similar phenomenon also has been reported when quantifying peach profilin, demonstrating that the use of a recombinant protein isoform as standard might overestimate the profilin content of samples (Asturias et al. 1999). One explanation may be that recombinant proteins are not correctly folded as natural Pru p 3, and also lack of natural ligands, such as lipids which may affect affinity (Bublin et al. 2014). This

has been demonstrated in apple Mal d 3, olive tree profilin, and sunflower pollen profilin (Asturias et al. 1997, 1998; Zuidmeer et al. 2005). But the recombinant Pru p 3 (by yeast system) was demonstrated to have similar immunologic reactivity (Diaz-Perales et al. 2003).

Plant lipid transfer proteins are considered to be involved in many biological processes: cutin formation and embryogenesis, defense reactions against pathogens, adaptation of plants to various environmental conditions and bacterial symbiosis with plants (Kader 1996). As well as the rapid and accurate evaluation of LTP content in peach extracts, the ELISA procedure we describe here could also be very useful for other research, including the involvement of LTP in the lipid pathway and formation of aroma in peach (Li et al. 2015) and other *Prunus* stone fruits.

Conclusion

We developed a reliable and sensitive sandwich ELISA method to measure lipid transfer protein (LTP) in peach (Pru p 3 allergen), based on two monoclonal antibodies. We recommend using natural rather than recombinant protein as the standard to calculate the true content. These antibodies and reference peach LTP can be used to screen large sets of peach germplasm to identify low allergenic cultivars.

Compliance with Ethical Standards

Funding This study was funded by the National Natural Science Foundation of China (grant nos. 31272131 and 31372040).

Conflict of Interest ZS Gao and HJ Jia have received research grants from China Natural Science Foundation (grant nos. 31272131 and 31372040). All other authors declare that they have no conflict of interest.

Ethical Approval The use of mice for antibody production was approved by experimental animal use permission (SCXK-HU-2012-002, China), and Zhejiang province guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the local ethical standards (Zhejiang Ethics Committee, authorization no. 2011-R-1, Second Affiliated Hospital, College of Medicine, Zhejiang University) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

- Ahratzem O, Jimeno L, Lopez-Torres G, Herrero M, Espada JL, Sanchez-Monge R, Duffort O, Barber D, Salcedo G (2007) Assessing allergen levels in peach and nectarine cultivars. *Ann Allergy Asthma Im* 99:42–47
- Asero R, Pravettoni V (2013) Anaphylaxis to plant-foods and pollen allergens with lipid transfer protein syndrome. *Curr Opin Allergy Clin Immunol* 13(4):379–385
- Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier MF, Ciurana CL, Verbeek E, Mohammadi T, Knul-Brettlova V, Akkerdaas JH, Bulder I, Aalberse RC, van Ree R (2000) Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arc Allergy Immunol* 122:20–32
- Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, Palacios R (1997) Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. *J Allergy Clin Immunol* 100:365–372
- Asturias JA, Arilla MC, Gomez-Bayon N, Aguirre M, Martinez A, Palacios R, Martinez J (1998) Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen. *Mol Immunol* 35:469–478
- Asturias JA, Arilla MC, Aguirre M, Gomez-Bayon N, Martinez A, Palacios R, Sanchez-Gascon F, Martinez J (1999) Quantification of profilins by a monoclonal antibody-based sandwich ELISA. *J Immunol Methods* 229:61–71
- Ballmer-Weber BK (2002) Lipid transfer protein as a potential panallergen? *Allergy* 57:873–875
- Breiteneder H, Ebner C (2000) Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 106:27–36
- Bublin M, Eiwegger T, Breiteneder H (2014) Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* 134(3):521–529
- Burney PG, Potts J, Kummeling I, Mills EN, Clausen M, Dubakiene R, Barreales L, Fernandez-Perez C, Fernandez-Rivas M, Le TM, Knulst AC, Kowalski ML, Lidholm J, Ballmer-Weber BK, Braun-Fahlander C, Mustakov T, Kralimarkova T, Popov T, Sakellariou A, Papadopoulos NG, Versteeg SA, Zuidmeer L, Akkerdaas JH, Hoffmann-Sommergruber K, van Ree R (2014) The prevalence and distribution of food sensitization in European adults. *Allergy* 69(3):365–371
- Carnes J, Fernandez-Caldas E, Gallego MT, Ferrer A, Cuesta-Herranz J (2002) Pru p 3 (LTP) content in peach extracts. *Allergy* 57:1071–1075
- Chen L, Zhang SM, Illa E, Song LJ, Wu SD, Howad W, Arus P, van de Weg E, Chen KS, Gao ZS (2008) Genomic characterization of putative allergen genes in peach/almond and their synteny with apple. *BMC Genomics* 9:543
- Diaz-Perales A, Lombardero M, Sanchez-Monge R, Garcia-Selles FJ, Pemas M, Fernandez-Rivas M, Barber D, Salcedo G (2000) 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* 30:1403–1410
- Diaz-Perales A, Sanz ML, Garcia-Casado G, Sanchez-Monge R, Garcia-Selles FJ, Lombardero M, Polo F, Gamboa PM, Barber D, Salcedo G (2003) 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. *J Allergy Clin Immunol* 111(3):628–633
- Duffort OA, Polo F, Lombardero M, Diaz-Perales A, Sanchez-Monge R, Garcia-Casado G, Salcedo G, Barber D (2002) Immunoassay to quantify the major peach allergen Pru p 3 in foodstuffs. Differential allergen release and stability under physiological conditions. *J Agr Food Chem* 50:7738–7741
- Egger M, Hauser M, Mari A, Ferreira F, Gadermaier G (2010) The role of lipid transfer proteins in allergic diseases. *Curr Allergy Asthma Rep* 10(5):326–335
- Fernandez-Rivas M, Gonzalez-Mancebo E, Rodriguez-Perez R, Benito C, Sanchez-Monge R, Salcedo G, Alonso D, Rosado A, Tejedor MA, Vila C, Casas ML (2003) Clinically relevant peach allergy is related to peach lipid transfer protein, pru p 3, in the Spanish population. *J Allergy Clin Immunol* 112:789–795

- Gaier S, Marsh J, Oberhuber C, Rigby NM, Lovegrove A, Alessandri S, Briza P, Radauer C, Zuidmeer L, van Ree R, Hemmer W, Sancho AI, Mills C, Hoffmann-Sommergruber K, Shewry PR (2008) Purification and structural stability of the peach allergens Pru p 1 and Pru p 3. *Mol Nutr Food Res* 52:S220–S229
- Gao ZS, Gilissen LJW (2011) In: Jenks MA, Bebeli PJ (eds) *Breeding of hypoallergenic fruits, in breeding for fruit quality*, Firstth edn. Wiley, Hoboken, pp 105–126
- Gao ZS, Yang ZW, Wu SD, Wang HY, Liu ML, Mao WL, Wang J, Gadermaier G, Ferreira F, Zheng M, van Ree R (2013) Peach allergy in China: a dominant role for mugwort pollen lipid transfer protein as a primary sensitizer. *J Allergy Clin Immunol* 131:224–226
- Garino C, Zitelli F, Travaglia F, Coisson JD, Cravotto G, Arlorio M (2012) Evaluation on the impact of sequential microwave/ultrasound processing on the IgE binding properties of Pru p 3 in treated peach juice. *J Agr Food Chem* 60:8755–8762
- Greval RS, Mehra YN (1975) Preparation of allergenic extracts. *Ind J Otolaryngol Head Neck Surg* 27(2):59–61
- Kader JC (1996) Lipid-transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:627–654
- Kavaliunas A, Surkiene G, Dubakiene R, Stukas R, Zagminas K, Saulyte J, Burney PGJ, Kummeling I, Mills C (2012) EuroPrevall survey on prevalence and pattern of self-reported adverse reactions to food and food allergies among primary schoolchildren in Vilnius, Lithuania. *Medicina-Lithuania* 48(5):265–271
- Kirsch S, Fourdrilis S, Dobson R, Scippo ML, Maghuin-Rogister G, De Pauw E (2009) Quantitative methods for food allergens: a review. *Anal Bioanal Chem* 395:57–67
- Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495–497
- Li XW, Meng XQ, Jia HJ, Yu MY, Ma RJ, Wang LY, Cao K, Shen ZJ, Niu L, Tian JB, Chen MJ, Xie M, Arus P, Gao ZS, Aranzana MJ (2013) Peach genetic resources: diversity, population structure and linkage disequilibrium. *BMC Genet* 14:84
- Li XW, Jiang J, Zhang LP, Yu Y, Ye ZW, Wang XM, Zhou JY, Chai ML, Zhang HQ, Arús P, Jia HJ, Gao ZS (2015) Identification of volatile and softening-related genes using digital gene expression profiles in melting peach. *Tree Genet Genomes*. doi:10.1007/s11295-015-0891-9
- Ma SK, Yin J, Jiang NN (2013) Component-resolved diagnosis of peach allergy and its relationship with prevalent allergenic pollens in China. *J Allergy Clin Immunol* 132:764–767
- Ma YT, Zhou X, Gao ZS, Li XW, Jia HJ, Wu HX, Xie RJ, Wang ZQ, Yu ML, Cao K, Chen SJ, Li YH, Wang HY, van Ree R (2014) The *PpLTP1* primary allergen gene is highly conserved in peach and has small variations in other *Prunus* species. *Plant Mol Biol Rep* 32:652–663
- Pacios LF, Tordesillas L, Cuesta-Herranz J, Compes E, Sánchez-Monge R, Palacin A, Salcedo G, Diaz-Perales A (2008) Mimotope mapping as a complementary strategy to define allergen IgE-epitopes: peach Pru p 3 allergen as a model. *Mol Immunol* 45(8):2269–2276
- Pasquato N, Berni R, Folli C, Folloni S, Cianci M, Pantano S, Helliwell JR, Zanotti G (2006) Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specific lipid transfer protein pan-allergens. *J Mol Biol* 356:684–694
- Sancho AI, van Ree R, van Leeuwen A, Meulenbroek BJ, van de Weg EW, Gilissen LJWJ, Puehringer H, Laimer M, Martinelli A, Zaccharini M, Vazquez-Cortes S, Fernandez-Rivas M, Hoffmann-Sommergruber K, Mills ENC, Zuidmeer L (2008) Measurement of lipid transfer protein in 88 apple cultivars. *Int Arch Allergy Imm* 146:19–26
- Toda M, Reese G, Gadermaier G, Schulten V, Lauer I, Egger M, Briza P, Randow S, Wolfheimer S, Kigongo V, Del Mar San Miguel Moncin M, Fötisch K, Bohle B, Vieths S, Scheurer S (2011) Protein unfolding strongly modulates the allergenicity and immunogenicity of Pru p 3, the major peach allergen. *J Allergy Clin Immunol* 128:1022–1030
- Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, Papadopoulos NG, Saxoni-Papageorgiou P, van Ree R, Mills C (2006) Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *J Allergy Clin Immun* 118(2):473–480
- Yang ZW, Ma YT, Chen L, Xie RJ, Zhang B, Lu MD, Wu SD, Gilissen LJWJ, van Ree R, Gao ZS (2011) Differential transcript abundance and genotypic variation of four putative allergen-encoding gene families in melting peach. *Tree Genet Genomes* 7:903–916
- Zuidmeer L, van Leeuwen WA, Budde IK, Cornelissen J, Bulder I, Rafalska I, Besoli NT, Akkerdaas JH, Asero R, Rivas MF, Mancebo EG, van Ree R (2005) Lipid transfer proteins from fruit: cloning, expression and quantification. *Int Arch Allergy Imm* 137:273–281

		↓	*	↓	20	*	↓	40	*
Pru p 3	:	ITCGQVSSSLAPCIEYVRGGGAVPPACCGIRNVNNLARTT	PDRQAACNC	:	50				
Pru ar 3	:	ITCGQVSSSLAPCIGYVRGGGAVPPACCGIRNVNNLARTT	EDRRTACNC	:	50				
Pru av 3	:	ITCGQVSSNLAPCIAYVRGGGAVPPACCGIRNVNNLAKTT	ADROTACNC	:	50				
Pru d 3	:	ITCGQVSSNLAPCINYYKGGGAVPPACCGIRNVNNLARTT	ADRRAACNC	:	50				
Mal d 3	:	ITCGQVTSSSLAPCIGYVRSGGAVPPACCGIRTINGLARTT	ADROTACNC	:	50				
Fra a 3	:	ITCGQVASNLSPCLTYVKS	GGAVEAACC	SGIRNLNGMAKTT	ADRQAACNC : 50				

		↓	60	↓	*	80	*
Pru p 3	:	LKQLSASVPGVNPNNAAALPGKCGVSI	IPYKISASTNCATVK	:	91		
Pru ar 3	:	LKQLSGSISGVNPNNAAALPGKCGVNI	IPYKISASTNCATVK	:	91		
Pru av 3	:	LKQLSASVPGVNPNNAAALPGKCGVNI	IPYKISASTNCATVK	:	91		
Pru d 3	:	LKQLSGSIPGVNPNNAAALPGKCGVNI	IPYKISASTNCATVK	:	91		
Mal d 3	:	LKNLAGSISGVNPNNAAALPGKCGVNV	IPYKISTSTNCATVK	:	91		
Fra a 3	:	LKQAAGGIKGLNPNI	AAGLPGKCGVSV	IPYKISTTTNCAAVK	: 91		