Letter to the Editor

Enhanced Pru p 3 IgE-binding activity by selective free fatty acid-interaction

To the Editor:

Nonspecific lipid transfer proteins (nsLTPs) are major cross-reactive allergens identified in most plant-derived foods as well as pollen from diverse plants, and are often associated with severe symptoms in food allergy.[1] Currently, Pru p 3 (major food allergen from peach [*Prunus persica*]) is regarded as the primary sensitizer for nsLTP-caused allergies.[2] Pru p 3 shares the physicochemical characteristics of the nsLTP family. It is a small (9187 Da) basic protein, with a highly conserved 3-dimensional structure provided by 8 conserved cysteine residues forming 4 disulfide bridges. The common feature of nsLTPs is a hydrophobic cavity throughout the whole molecule that can host ligands such as fatty acids. However, this lipid-binding capacity varies among different nsLTP members, and depends on the specific characteristics of their tertiary fold as revised by Liu et al.[3] There is limited knowledge about the binding capacity of Pru p 3. So far, interactions with lauric acid,[4] cis-parinaric, palmitic, and linoleic acids[5] have been reported. Several structures of nsLTPs with and without ligands have been determined including peach[4] and hazelnut[6] and suggested a plasticity of the cavity when binding to ligands as compared with an unliganded molecule. However, whether the apo- or holoform of Pru p 3 has an impact on the IgE-binding activity is yet unknown. Therefore, we studied a range of saturated and mono-/poly-unsaturated fatty acids and their interaction with Pru p 3 and investigated whether Pru p 3-ligand interaction is able to affect IgE recognition in sera from peach-allergic patients. To address this question, we first performed nuclear magnetic resonance (NMR) experiments and molecular dynamic analyses. Subsequently, the IgE-binding activity was investigated by ELISA and basophil activation test (BAT) assays.

Details for materials, methods, and patients' data (Table E1) are provided in this article's Online Repository at www.jacionline.org.

Natural and recombinant Pru p 3 were purified and characterized (see Fig E1 in this article's Online Repository at www.jacionline.org). Preincubation of Pru p 3 with individual unsaturated and saturated free fatty acids induced a dose-dependent reduction of 1-anilinonaphthalene-8-sulfonic acid (ANS) binding. Unsaturated fatty acids induced a larger reduction in ANS binding compared with saturated fatty acids at all tested concentrations (10, 20, 50, and 100 μM). The largest reduction in the signal was observed for oleic acid (OLE [72.6%]) followed by linoleic acid (72%) and elaidic acid (68.6%) at a protein:ligand ratio of 1:10. In contrast, the saturated fatty acid stearic acid (STE) induced a reduction of 39.1% in ANS binding. In addition, saturated fatty acids with shorter C chains, such as lauric acid (C12), bind to the internal cavity of Pru p 3 more effectively than do saturated fatty acids with longer chains, that is, STE (C18) (Fig 1, *A*; see Table E2 in this article's Online Repository at www.jacionline.org).

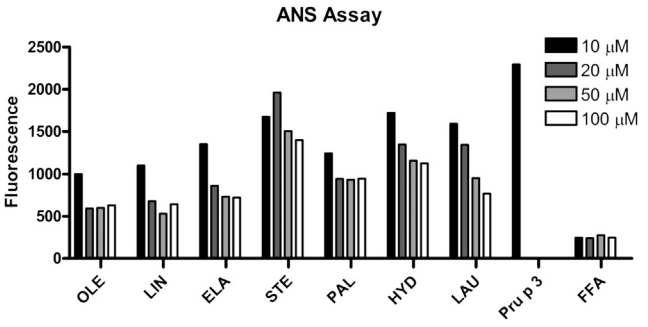

FIG 1. Pru p 3 displays differential ligand binding. Concentration-dependent reduction in ANS binding to Pru p 3 preincubated with FFA. Positive control: natural Pru p 3; Negative control: FFA. *ELA*, Elaidic acid; *FFA*, free fatty acid; *HYD*, 16-hydroxypalmitic acid; *LAU*, lauric acid; *LIN*, linoleic acid; *PAL*, palmitic acid.

To support the results from the ANS assay, we decided to perform water-ligand observed via gradient spectroscopy (W-LOGSY) experiments, a frequently applied 1-dimensional ligand-observation NMR technique for the detection of protein-ligand interactions. W-LOGSY experiments were acquired for OLE and STE because they showed the highest and the lowest binding capacity, respectively. NMR experiments confirmed the binding capacity observed in the ANS assay (see Fig E2 in this article's Online Repository at www.jacionline.org). Comparison of these 2 spectra indicates that recombinant Pru p 3 (rPru p 3) was able to bind OLE as shown by an inversion of the signal. In contrast, no interaction of rPru p 3 with STE was detected.

Because pH changes influence the presence of the OLE protonation state, we decided to investigate whether both OLE and its anion (OLE^-) affect the 3-dimensional structure of Pru p 3. Molecular dynamic analyses suggest changes in protein structure due to binding of OLE or OLE^-. Binding of OLE^- leads to a substantial conformational change in the C-terminal fragment of the protein. Because of the interaction between OLE^- and Pru p 3, the C-terminal loop is moved out toward the surface of the molecule, while the same region of Pru p 3 alone is closer to the core of the molecule (Fig 2, *A*). These changes are caused by binding of OLE^- between the third α-helix (h3) and the C-terminal loop of Pru p 3, which is stabilized by hydrogen bonds with Arg32 (Fig 2, *A*). Analysis of intramolecular contacts between Pru p 3 and OLE^- along the stable part of the molecular dynamic trajectory (10-150 ns; see Figs E3, *A*, and E4, *A*, in this article's Online Repository at www.jacionline.org) shows that the ligand interacts with polar residues on the surface of the protein (Arg32, Asn35, Arg44) and hydrophobic side chains in the upper part of the hydrophobic cavity (Ile31, Pro70, Ile77; Fig E4, *A*). This interaction with OLE^- affects residues 75 to 85, leading to an exposure of Pro78 and Tyr79 outside the hydrophobic cavity (Fig 2, *A*). The Pru p 3-OLE complex has a different structure, as the charge-neutral ligand immerses almost completely in the hydrophobic cavity (Figs E3, *B*, and E4, *B*) and the key residues making contacts with the ligand are now Leu10, Ile31, Asn35, Leu51, and Ile81. OLE penetrates the cavity in a more stretched conformation than does OLE^-, and in consequence, the bottom of the cavity opens via the movement of helix 3 away from helix 4 (see Figs E4, *B*, and E5 in this article's Online Repository at www.jacionline.org).

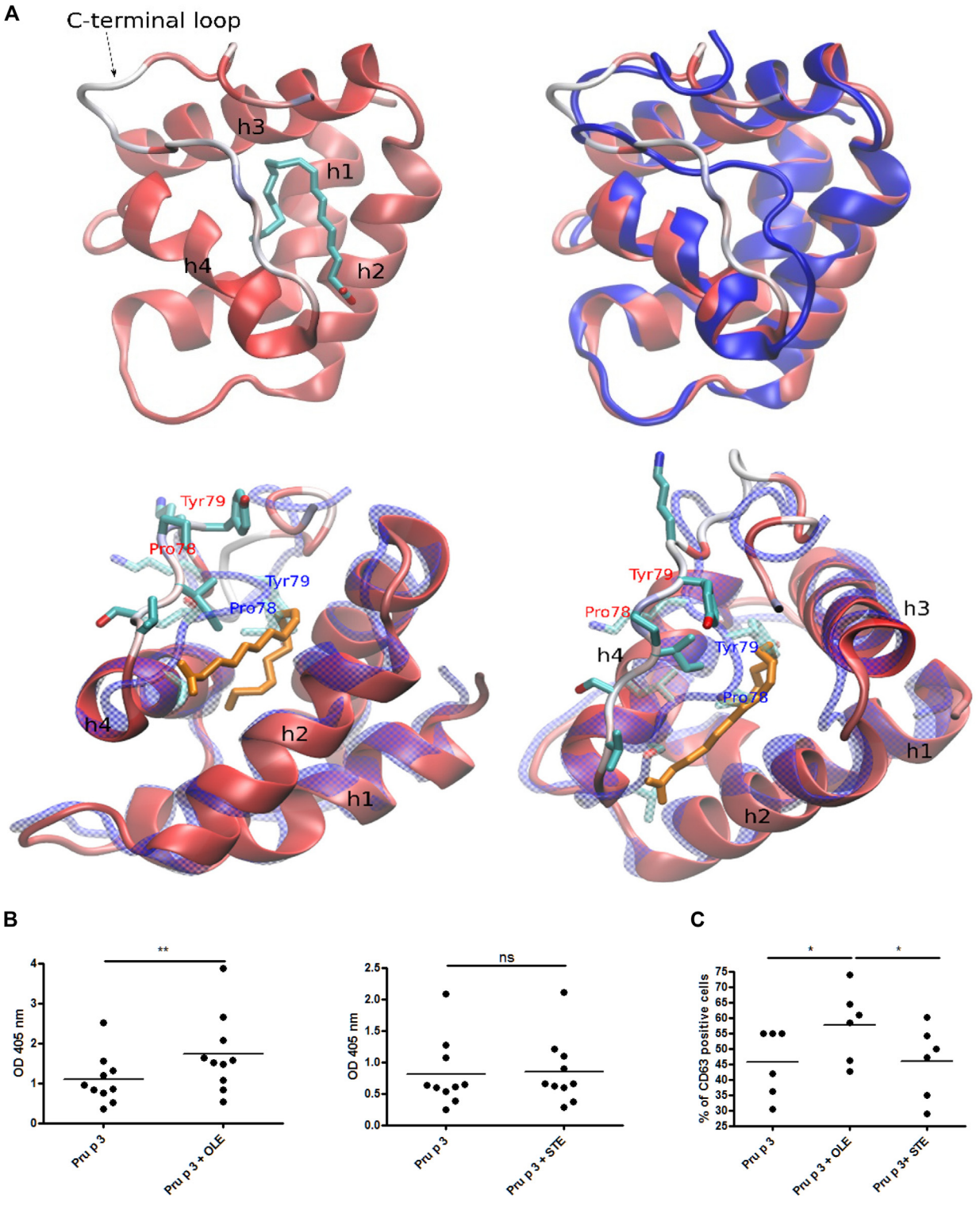

FIG 2. Pru p 3-FFA interaction and impact on the IgE binding. **A,** Molecular dynamic analysis. Pru p 3 with OLE^{-} *(upper left)*; Cα superposition of Pru p 3-apo (blue) and with OLE^{-} (red). The white ribbon of the Pru p 3-OLE^{-} complex represents the region affected by the OLE binding *(upper right)*; residues 75 to 80 of Pru p 3 affected by OLE^{-} binding *(lower panel)*. **B,** ELISA; data are representative of 10 patients' sera tested in triplicate with rPru p 3 alone and with OLE or STE. **C,** Basophil activation test assay. Percentages of $CD63^{+}$ basophils after stimulation with allergen at a concentration of 1 ng/mL and an allergen:FFA ratio of 1:10 in 6 patients sensitized to Pru p 3. The *P* value refers to the comparison of the median between allergen alone and in the presence of ligand. *FFA*, Free fatty acid; *ns*, nonsignificant. $*P \leq .05$ (unpaired 2-tailed *t* test). $**P < .01$.

These changes, although not affecting the overall structure, could be important regarding B-cell epitope exposure. Because the region affected by conformational changes of Pru p 3 is the one that was identified as the major IgE epitope responsible for severe reactions,[7] we decided to investigate whether conformational changes due to ligand binding lead to increased IgE-binding capacity. To test whether interaction of Pru p 3 with free fatty acids influences its IgE-binding capacity, we used sera from 10 peach-allergic patients sensitized to Pru p 3. Based on our results from ANS and W-LOGSY experiments, the 2 ligands with highest (OLE) and lowest (STE) binding activity to Pru p 3 were selected. Preincubation of rPru p 3 with OLE significantly ($P = .002$) increased the IgE-binding capacity of all sera tested by ELISA assay when compared with the allergen alone (Fig 2, *B*; see Table E3 in this article's Online Repository at www.jacionline.org). The largest differences were observed with sera nos. 2 and 6, with an increase of 118% and 89%, respectively. According to our data, STE did not have a considerable influence on the IgE-binding capacity of rPru p 3 ($P > .05$) (Fig 2, *B*; Table E3).

Furthermore, we investigated whether the binding of a lipid ligand also increases the activation of effector cells. Pru p 3 preincubated with OLE significantly increased the number of $CD63^+$ cells as compared with Pru p 3 alone ($P = .031$). The percentages of $CD63^+$ basophils after stimulation with Pru p 3 preincubated with OLE were also significantly ($P = .011$) increased when compared with $CD63^+$ basophils after stimulation of Pru p 3 preincubated with STE. In addition, comparison of Pru p 3 preincubated with STE and Pru p 3 alone showed that this ligand did not have any impact on basophils' activation ($P > .05$) (Fig 2, *C*; Table E3).

In conclusion, our ligand-binding assays provided interesting results regarding the binding specificity of Pru p 3, preferably binding poly- and mono-unsaturated fatty acids as compared with saturated ones. In parallel, using an *in silico* approach, we showed conformational changes of the cavity and a shift in the tertiary structure of Pru p 3 as a consequence of OLE and OLE^- binding. Notably, the IgE ELISA performed with sera from 10 peach-allergic patients confirmed our hypothesis that exposure of the C-terminal loop due to the OLE/OLE^- binding could indeed enhance the IgE-binding activity. In contrast, binding of STE was unable to increase the IgE-binding capacity. This corroborates our idea that only those ligands that shift the tertiary structure of Pru p 3, thus exposing IgE epitopes, could increase its allergenic potential. This observation is consistent with previous studies indicating that no synthetic peptides covering areas of helix 3 or loop 3 were identified among the most reactive peptides in the SPOT analysis, probably because most of this region is mainly buried inside the compact 3-dimensional structure of Pru p 3.[7] Consistent with the data obtained by ELISA, OLE, but not STE, had a significant impact on the basophil activation. This is in line with a study on grape nsLTP where the presence of phosphatidylcholine in an *in vitro* digestion assay had not only a protective effect on the allergen but also increased the ability of the allergen to induce basophil histamine release and to elicit skin reactions in 4 patients with grape allergy.[8] It becomes clear that lipids, in addition to their role as direct immune modulators, can influence the allergenicity of proteins[9] by modifying the allergen structure and biochemical properties. Among unsaturated fatty acids, OLE is abundantly found in peach fruits, but it is also endogenously present in the lipid bilayer of the human gut. Pru p 3 seems to be presented in its holoform to the allergic individual throughout exposure and digestion. This, in turn, explains that this immunodominant B-cell epitope is conserved and surface exposed. In this context, the application of Pru p 3 in diagnostic tests should be reconsidered, using Pru p 3 together with OLE for component-resolved diagnosis to increase assay sensitivity. Furthermore, for designing low allergenic Pru p 3 variants for immunotherapy, the amino acid residues relevant for OLE interaction are the targets for mutations and thus reduce the risk of side effects during immunotherapy.

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This work was funded by the Austrian Science Fund (FWF): grant number DK W 1248-B13 to P.D. and grant number SFB F4603 to K.H.-S. Further support came from the Horizon 2020 Programme of the European Union (iNEXT grant, project no. 653706). This research was also supported in part by PL-Grid Infrastructure and by the Marie-Curie project CARAMEL within the 7th European Community Framework Programme (grant no. 626572 to R.A.).

Disclosure of potential conflict of interest: R. Aina has received grants from the Marie-Curie project CARAMEL, 7th European Community Framework Programme (grant no. 626572). B. Bohle has received grants from the Austrian Science Fund (SFB grant nos. F4610, W1212, and W1248) and the Christian Doppler Research Organization and is a board member for Allergen Online Database, Paul Ehrlich Institute, and the Christian Doppler Research Organization. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. Allergy Asthma Clin Immunol 2010;6:1.
2. Vereda A, van Hage M, Ahlstedt S, Ibañez MD, Cuesta-Herranz J, van Odijk J, et al. Peanut allergy: clinical and immunologic differences among patients from 3 different geographic regions. J Allergy Clin Immunol 2011;127:603-7.
3. Liu F, Zhang X, Lu C, Zeng X, Li Y, Fu D, et al. Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis. J Exp Botany 2015;66:5663-81.
4. Pasquato N, Berni R, Folli C, Folloni S, Cianci M, Pantano S, et al. 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 2006;356:684-94.
5. Abdullah SU, Alexeev Y, Johnson PE, Rigby NM, Mackie AR, Dhaliwal B, et al. Ligand binding to an allergenic lipid transfer protein enhances conformational flexibility resulting in an Increase in susceptibility to gastroduodenal proteolysis. Sci Rep 2016;6:30279.
6. Offermann LR, Bublin M, Perdue ML, Pfeifer S, Dubiela P, Borowski T, et al. Structural and functional characterization of the hazelnut allergen Cor a 8. J Agricultural Food Chem 2015;63:9150-8.
7. García-Casado G, Pacios LF, Díaz-Perales A, Sánchez-Monge R, Lombardero M, García-Selles FJ, et al. Identification of IgE-binding epitopes of the major peach allergen Pru p 3. J Allergy Clin Immunol 2003;112:599-605.
8. Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, et al. Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. J Allergy Clin Immunol 2006;118:473-80.
9. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? J Allergy Clin Immunol 2014;134:521-9.

http://dx.doi.org/10.1016/j.jaci.2017.06.016

METHODS

Chemicals and reagents

All reagents were purchased from Sigma-Aldrich (St Louis, Mo) unless stated otherwise.

Purification of natural Pru p 3 and expression and purification of rPru p 3

Natural Pru p 3 (nPru p 3) was extracted and purified from peach (*P persica*) peel as previously described by Gaier et al.[E1] Briefly, the peel of 1 kg of peach fruits was ground in liquid nitrogen and transferred in the extraction buffer (46 mM sodium phosphate, 2 mM EDTA, 20 mM sodium diethyldithiocarbamate, 3 mM NaN_3, 3% w/v PVPP, pH 7.0). The mixture was stirred for 1 hour at 4°C and then centrifuged (10,000*g* for 10 minutes, 4°C). The supernatant was precipitated with 95% w/v ammonium sulphate, resuspended, and filtered through a 0.22 μM filter. nPru p 3 was then purified with cation exchange chromatography (Mono S column, GE Healthcare, Little Chalfont, United Kingdom) followed by gel filtration (Superdex 200 column, GE Healthcare).

The recombinant counterpart of nPru p 3 was produced in the yeast *Pichia pastoris*. The protein sequence of mature Pru p 3 was retrieved from Genebank (Acc. no CAB96876) and optimization for *P pastoris* codon usage as well as prediction of glycosylation sites was performed. The plasmid construct pPIC-ZαA-Pru p 3 (ThermoFisher Scientific, Waltham, Mass) was linearized with SacI (New England Biolabs, Ipswich, Miss) and used to transform GS115 *P pastoris* cells (ThermoFisher Scientific) by electroporation. Transformed cells were grown on yeast extract peptone dextrose medium plates containing 100 μg/mL zeocin (Invivogen, San Diego, Calif) at 28°C for 5 days. Multicopy screening was performed by replica plating of the positive cells on yeast extract peptone dextrose with increasing zeocin concentration (up to 2000 μg/mL). Positive transformants expressing rPru p 3 with highest yield were cultivated in 200 mL minimal glycerol medium containing histidine for 1 day at 28°C under shaking conditions until the culture reached an OD_{600} value of 2 to 3. Cells were harvested and transferred to minimal methanol + histidine medium (24°C, 140 rpm), containing 1% of methanol to induce protein expression. Methanol (at a final concentration of 1%) was also added to the liquid culture every 12 hours. After 5 days of incubation, the culture supernatant was collected by centrifugation (6000*g* for 20 minutes at 4°C) and stored at −20°C.

For subsequent purification, 200 mL of supernatant was dialyzed against binding buffer (20 mM sodium acetate, pH 6.5). After filtration through a 0.22 μm filter, the protein solution was applied to a 1 mL Mono S column (GE Healthcare). Bound proteins were eluted by a linear concentration gradient of NaCl (0-0.5 mol/L) at a flow rate of 1 mL/min. In the second step, samples containing protein of interest were applied on an RP-HPLC Jupiter C5 analytical column (Phenomenex, Torrance, Calif). The column was equilibrated with the mobile phase (10% acetonitrile, 90% water, 0.1% trifluoroacetic acid) and eluted by increasing concentration of acetonitrile (up to 50%) with flow rate 1 mL/min. nsLTP-containing fractions were analyzed by 15% SDS-PAGE and the concentration of the purified rPru p 3 was determined using the bicinchoninic acid assay according to the manufacturer's protocol (ThermoFisher Scientific). Samples were stored at −20°C.

Protein structure characterization

The N-terminal sequences of both recombinant and natural Pru p 3 were determined using an Applied Biosystems Procise 491 sequencer (Applied Biosystems, Foster City, Calif). Purified proteins (100 pmol) were adsorbed onto a Prosorb cartridge and subjected to sequence analysis. For intact mass determination, nonreduced protein samples of purified nPru p 3 and rPru p 3 were spotted in a ratio of 1:1 with matrix (α-cyano-4-hydroxycinnamic acid) onto a ground steel matrix-assisted laser desorption & ionization target plate and measured in linear mode on a matrix-assisted laser desorption & ionization time-of-flight mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany). Secondary structure analysis of purified Pru p 3 was performed by CD spectroscopy (Jasco International Co., Hachioji, Tokyo). CD spectra of natural and recombinant Pru p 3 were measured from 190 to 260 nm at 25°C and pH 7.5 using 10 mM sodium phosphate buffer in 2-mm path length quartz cell. Spectra represent the average of 4 accumulations collected at 100 nm/min with a 2-second time constant, 0.5 nm resolution, and sensitivity of ±100 mdeg. Spectra are represented as molar circular dichroism (with respect to moles of amide bonds).

Lipid-binding assays

ANS displacement assay. The probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) is nonfluorescent in water but fluorescent when binding to the hydrophobic cavity of Pru p 3, with a maximum emission wavelength at 456 nm. Natural and recombinant Pru p 3 (10 μM) were incubated overnight at 4°C with different ligands: 3 unsaturated fatty acids, OLE (C18:1), elaidic (or trans-OLE; C18:1), and linoleic (C18:2), and 3 saturated fatty acids, stearic (STE; C18:0), lauric (C12:0), and palmitic (C16:0), at the following molar ratios: 1:1, 1:2, 1:5, and 1:10. In addition, to test whether the hydroxyl group influences the binding of free fatty acids to Pru p 3, 16-hydroxypalmitic acid (C16OH) was tested as described above. Binding of ligands was monitored by adding 10 μM 1,8-ANS and measuring the decrease in 1,8-ANS fluorescence. All samples were analyzed in triplicate. Purified nPru p 3 with ANS and ligands with ANS served as controls.

Water-ligand observed via gradient spectroscopy. As saturation-transfer difference approach, W-LOGSY is based on a transient nuclear Overhauser effect experiment, and implies transfer of magnetization via an intermolecular nuclear Overhauser effect and spin diffusion. Nonbinders and binders are easily discriminated from each other because they give W-LOGSY signals of opposite sign.[E2] W-LOGSY NMR experiments were acquired for OLE and STE dissolved in H_2O with 10% (v/v) of $(CD_3)_2SO$ (hexadeuterodimethyl sulfoxide). rPru p 3 stock solution was prepared in 20 mM sodium acetate buffer, pH 6.5, containing 0.1 mol $NaCl/D_2O$ 9:1. The allergen was mixed with tested ligands at a molar ratio of 1:20, to a final protein concentration of 5 μM. High-resolution NMR experiments were carried out using an Avance 700 Bruker spectrometer equipped with a cryo probe, operating at a proton resonance frequency of 700 MHz (11.7 Tesla) at 298 K. The experiments were performed with a 180° inversion pulse applied over the water signal at approximately 4.7 ppm by means of a Gaussian-shaped selective pulse of 10 ms. Each W-LOGSY spectrum was acquired with 512 scans.

Molecular dynamic analysis

Initial coordinates of Pru p 3 were taken from PDB:2B5S, and the AMBER force field ff03.r1 was applied to the protein. Molecular force fields of OLE and its anion (OLE^-) were described with the general AMBER force field. Ligands were first optimized in extended conformations in vacuum at the B3LYP/6-31G(d,p) level and then the electrostatic potential around them was computed at the level that is consistent with the general AMBER force field, that is, HF/6-31G(d). Quantum chemical computations were performed with Gaussian 09 suite of programs.[E3] From the electrostatic potential RESP atomic charges were fitted with the use of antechamber and RESP programs from the AmberTools package.[E4] The ionization state of Pru p 3 residues was tested with the PROPKA 3.1 software and for pH 7 all residues were predicted to be in their standard ionization state (N- and C-termini all LYS, ARG, and ASP charged). The N-terminal Met residue of the Pru p 3 structure was removed, as it was added to the protein because of its heterologous expression in a bacterial host. The protein molecule was subsequently placed in a periodic box filled with explicit water molecules described with the TIP3P model and appropriate number of chloride anions (7 for the unliganded protein and its complex with OLE, 6 for the Pru p 3/OLE^- complex) to obtain a charge-neutral system. Size of the box was such that its edge was 10 Å away from the protein surface in each direction. The system was first minimized in 3 steps: 5,000 steps with protein atoms restrained with 500 kcal/mol $Å^2$ harmonic constant; 5,000 steps with 10 kcal/mol $Å^2$ harmonic restraint on protein; and then 10,000 steps of an unrestrained minimization. After minimization, the system was heated up from 0 to 300 K during a 50 ps NV dynamics and then its density was equilibrated in a 0.5 ns NPT dynamics. Subsequent unrestrained NPT (T = 300 K, P = 1 atm) production dynamics

simulation spanned 150 ns for each system, with a snapshot saved every 10 ps. Integration time step used in molecular dynamics simulations was 2 fs. The SHAKE algorithm was used to constrain bonds of hydrogen atoms, and temperature and pressure were controlled with the Langevin dynamics and isotropic position scaling algorithm, respectively. Snapshots of the last 10 ns of simulations were clustered with the average linkage algorithm applied to Cα carbons of the protein backbone. Representative structures of dominating clusters were used for structure comparisons.

Patients

Serum samples were obtained from a well-defined group of 10 peach-allergic patients (7 females and 3 males with a mean age of 29.7 years) and 4 controls from nonatopic donors. The study was approved by the Ethic Committee of the Medical University of Vienna (EK1263/2014), and written informed consent was obtained from all subjects. Peach-specific and Pru p 3–specific IgE values were evaluated by means of CAP/RAST (ThermoFisher, Uppsala, Sweden). IgE values and clinical symptoms to peach of all patients are summarized in Table E1. After consumption of peach, all patients developed systemic reactions, such as urticaria, angioedema, or rhinitis and some of them also suffered from oral allergy syndrome. In addition, all patients positively responded to skin prick test with commercial peach extract as well as to prick-to-prick test with fresh peaches.

IgE ELISA

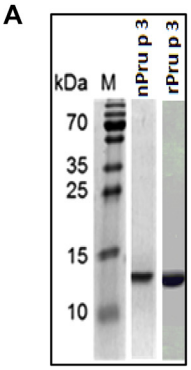
To ensure the binding of the protein in the native state to the ELISA plate, Thermo Scientific Nunc Immobilizer Amino surface plates were used. Twenty micrograms of rPru p 3 (2 μM) in 100 mM sodium carbonate, pH 9.6, was incubated at room temperature for 1 hour to enable reaction with amine and thiol functions. After coupling to the surface, remaining Nunc Immobilizer electrophilic groups were quenched by reaction with 10 μM ethanolamine. A total of 60 μM of OLE or STE was added and incubated overnight with protein at 4°C. Subsequent steps were performed as described previously.[E1] As negative controls, normal human serum, STE, and OLE, respectively, were tested in parallel; the mean value of the negative controls was subtracted.

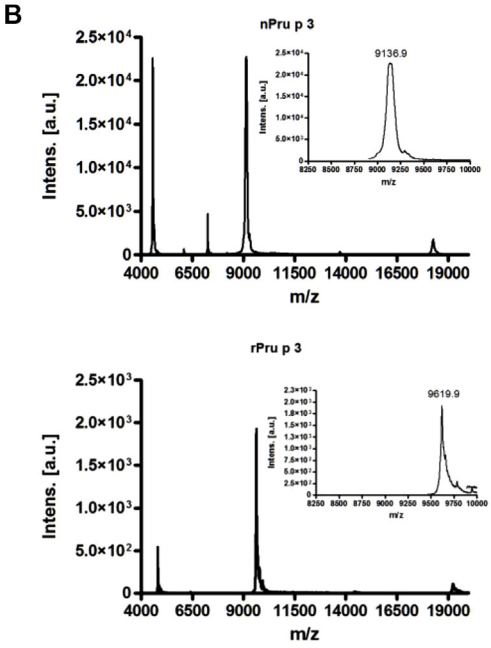
Basophil activation test assay

PBMCs were isolated from peripheral blood of non–peach-sensitized donors by density gradient separation with Ficoll-Hypaque (ThermoFisher). Receptor-bound IgE was removed from basophils by incubation in lactic acid (pH 3.9) as previously described.[E5] Stripped basophils were then passively sensitized by incubation in human serum from 6 peach-sensitized donors for 60 minutes at 37°C. Subsequently, they were stimulated with titrated concentrations of rPru p 3 (10 μg/mL, 1 μg/mL, 100 ng/mL, 1 ng/mL, 100 pg/mL, 1 pg/mL, 0.1 pg/mL) that had been incubated with different dilutions of STE or OLE (ratio 1:1, 1:5, 1:10, 1:100) overnight at 4°C. The ratio of protein:-ligand (1:10) with a final rPru p 3 concentration of 1 ng/mL or 1 μg/mL was selected after preliminary optimization, and only these data are shown. Basophils were labeled with CCR3 (eBioscience, Santa Clara, Calif) and CD123 (Biolegend, San Diego, Calif) and their activation was presented as percentage of $CD63^+$ basophils.

REFERENCES

E1. Gaier S, Marsh J, Oberhuber C, Rigby NM, Lovegrove A, Alessandri S, et al. Purification and structural stability of the peach allergens Pru p 1 and Pru p 3. Mol Nutr Food Res 2008;52:S220-9.
E2. Dalvit C, Fogliatto G, Stewart A, Veronesi M, Stockman B. WaterLOGSY as a method for primary NMR screening: practical aspects and range of applicability. J Biomolecular NMR 2001;21:349-59.
E3. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, et al. Gaussian 09, Revision E.01. Wallingford, CT: Gaussian, Inc; 2016.
E4. Case DA, Darden TA, Cheatham TE, Simmerling CL, Wang J, Duke RE, et al. AMBER 12. San Francisco: University of California; 2012.
E5. Santos AF, James LK, Bahnson HT, Shamji MH, Couto-Francisco NC, Islam S, et al. IgG4 inhibits peanut-induced basophil and mast cell activation in peanut-tolerant children sensitized to peanut major allergens. J Allergy Clin Immunol 2015;135:1249-56.





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                10          20          30          40          50
nPru p 3: ITCGQVSSAL APCIPYVRGG GAVPPACCNG IRNVNNLART TPDRQAACNC
       60          70          80          90
LKQLSASVPG VNPNNAAALP GKCGVHIPYK ISASTNCATV K

                10          20          30          40          50
rPru p 3: EAEFITCGQV SSALAPCIPY VRGGGAVPPA CCNGIRNVNN LARTTPDRQA

       60          70          80          90
ACNCLKQLSA SVPGVNPNNA AALPGKCGVH IPYKISASTN CATVK
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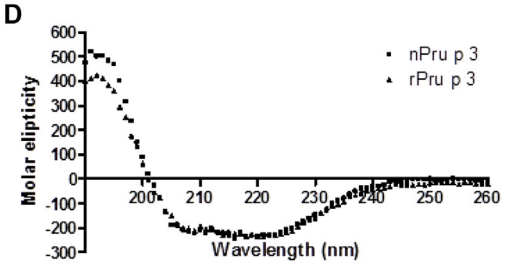

FIG E1. Comparison of purified nPru p 3 and rPru p 3. **A,** Coomassie stained 15% SDS-PAGE. **B,** Matrix-assisted laser desorption & ionization time-of-flight mass spectrum analysis. **C,** Amino acid sequences. Cysteines, responsible for disulfide bonds, are marked in red; additional residues from *P pastoris* cleavage marked in green; N-terminal amino acid residues verified by sequencing are underlined. **D,** Far-ultraviolet circular dichroism spectra. *Intens.*, Intensity.

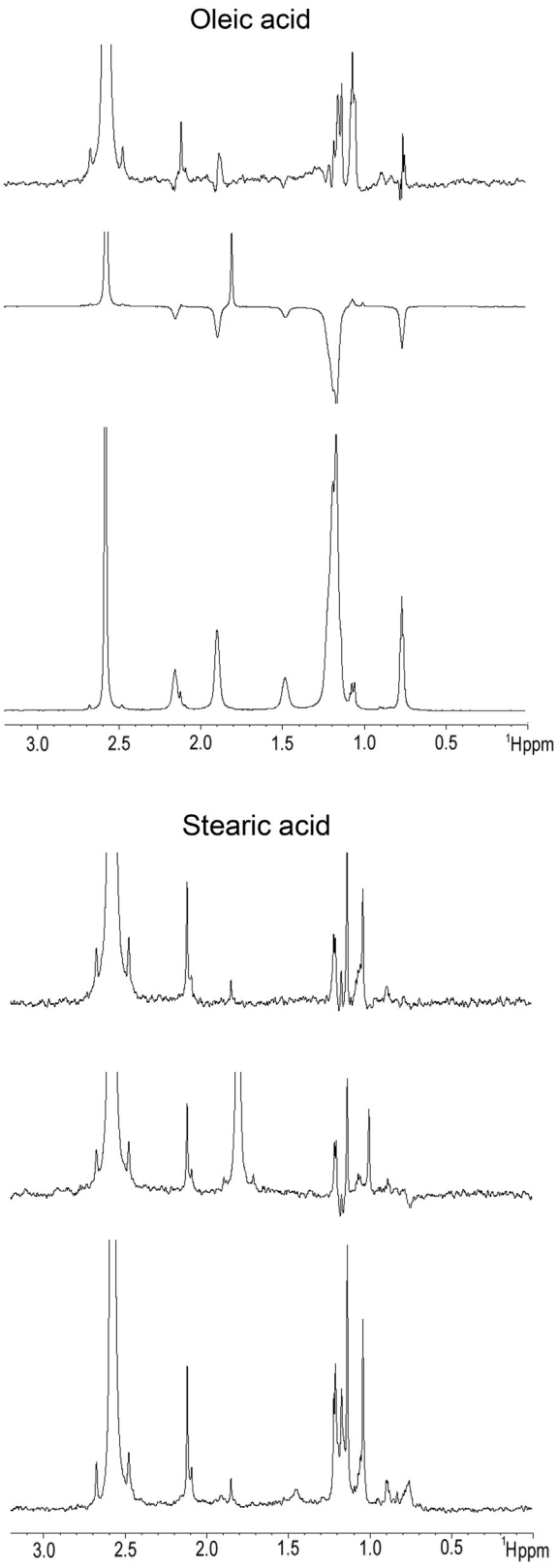

FIG E2. Pru p 3 displays differential ligand binding. 1-Dimensional W-LOGSY spectrum of 80 μM OLE and STE alone (upper), in the presence of Pru p 3 (8 μM) (middle); 1-Dimensional 1H spectrum of OLE and STE (lower).

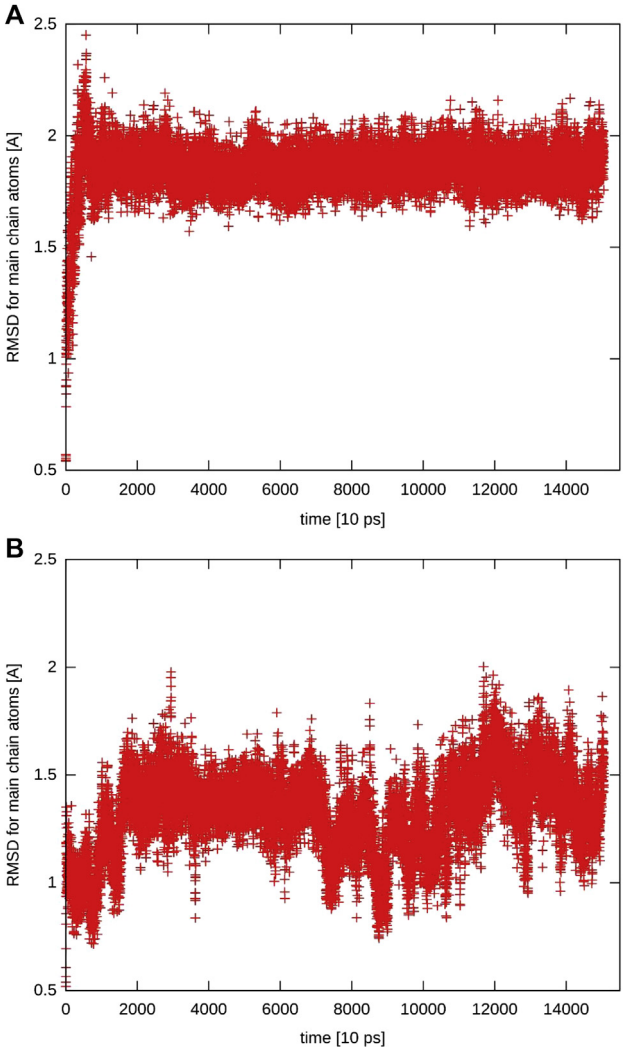

FIG E3. RMSD versus time plot for molecular dynamic simulation **A,** Pru p 3–OLE^{-} complex; **B,** Pru p 3–OLE complex. *RMSD,* Root-mean-square deviation of atomic positions.

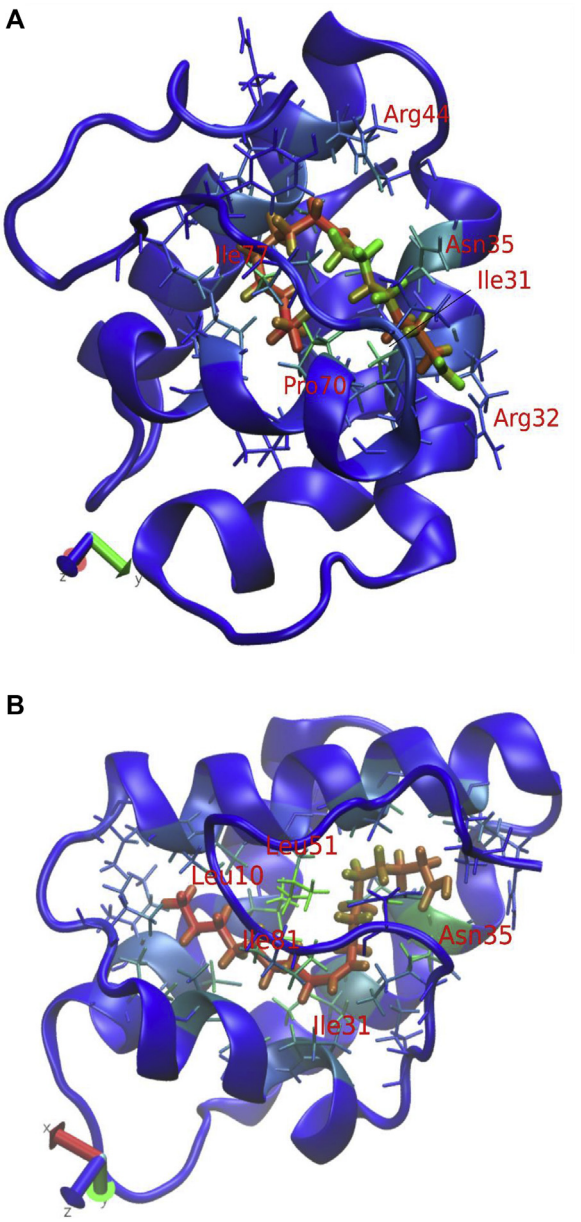

FIG E4. Intermolecular contacts in Pru p 3-OLE^{-} **(A)** and Pru p 3-OLE **(B)** complex from molecular dynamic simulation (10-150 ns). Color code from red (most frequent contacts), through green to blue (no contacts). Protein residues making most contacts are labeled.

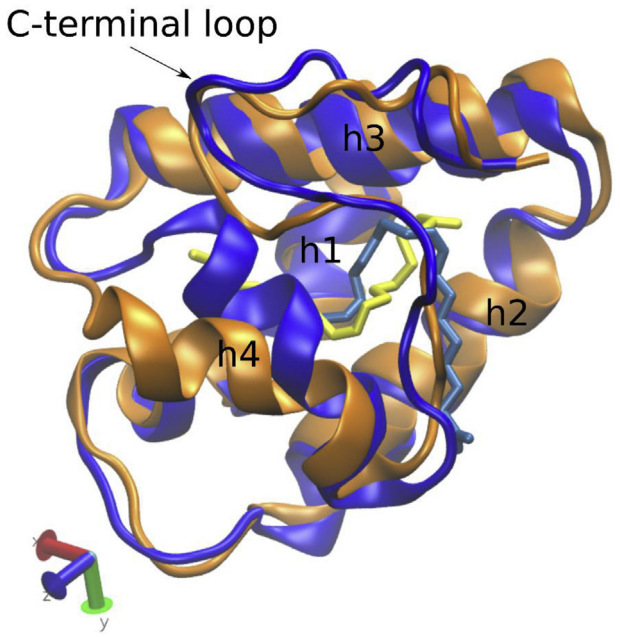

FIG E5. Superimposed structures of Pru p 3-OLE^{-} (in blue) and Pru p 3-OLE (in orange/yellow) complexes.

TABLE E1. Clinical data of the patient population

			IgE (kU/L) specific for:		Symptoms to peach*				Other sensitizations*	
Patient no.	**Sex**	**Age (y)**	**Peach**	**Pru p 3**	**OAS**	**URT**	**AE**	**RHIN**	**Respiratory**	**Food**
1	Female	16	44.0	54.0	+	+			G	eg, le, to
2	Female	23	26.0	29.6			+	+	A, J	le, me, to, wa
3	Female	44	0.8	3.1	+	+			G	ar, le, to, wa
4	Male	14	13.5	4.2		+			G, J, M	—
5	Male	46	0.6	0.5		+	+		G, J, M, O	ap, ha, pe
6	Male	30	2.9	1.3		+			J, T, O	ha
7	Female	26	6.4	4.0		+			G, T	f
8	Female	41	0.8	0.4				+	J	—
9	Female	26	4.8	6.8	+	+			A, D, G, J, M	ha, le, or, to
10	Female	31	7.5	1.9		+			G	ha, eg, pn

AE, Angioedema; *OAS*, oral allergy syndrome; *RHIN*, rhinitis; *URT*, urticaria.

*G, Grasses; eg, eggplant; le, lettuce; to, tomato; A, Alternaria species; J, cypress; me, melon; wa, walnut; ar, apricot; M, mugwort; O, olive; ap, apple; ha, hazelnut; pe, pear; T, plane; f, fish; D, mites; or, orange; pn, peanuts.

TABLE E2. Pru p 3 displays differential ligand binding

	ANS fluorescence reduction (%)			
Ligand	**10 μM**	**20 μM**	**50 μM**	**100 μM**
LAU (C12:0)	30.6	41.6	58.5	66.5
PAL (C16:0)	45.9	58.9	59.3	58.8
HYD (C16-OH)	25.0	41.4	49.7	51.1
STE (C18:0)	27.0	14.6	34.4	39.1
OLE (C18:1)	56.5	74.1	73.9	72.6
ELA (C18:1)	41.2	62.5	68.1	68.6
LIN (C18:2)	52.2	70.4	76.9	72.0

Concentration-dependent reduction in ANS binding to Pru p 3 preincubated with free fatty acid.
ELA, Elaidic acid; *HYD*, 16-hydroxypalmitic acid; *LAU*, lauric acid; *LIN*, linoleic acid; *PAL*, palmitic acid.

TABLE E3. Impact of interaction between free fatty acids and Pru p 3 on IgE binding and basophil activation

	ELISA		BAT	
Patient serum no.	**Pru p 3 + OLE**	**Pru p 3 + STE**	**Pru p 3 + OLE**	**Pru p 3 + STE**
1	+42.57	+54.69	+6.50	−1.30
2	+117.86	+2.64	+15.70	−1.50
3	+72.41	+3.10	+19.00	−5.10
4	+54.47	+18.60	+9.40	+5.20
5	+23.17	−4.81	+6.00	−0.80
6	+89.28	+68.23	+16.50	+5.20
7	+23.97	+9.93		
8	+54.13	+1.29		
9	+76.40	−1.43		
10	+57.96	−39.28		

BAT, Basophil activation test.
Values in the table correspond to differences in % between Pru p 3 preincubated with OLE or STE and Pru p 3 alone. (+) upregulation, (−) downregulation.