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RESEARCH ARTICLE

In vitro gastrointestinal digestion of the major peach allergen Pru p 3, a lipid transfer protein: Molecular characterization of the products and assessment of their IgE binding abilities

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A simulated gastrointestinal digestion has been carried out on purified peach lipid transfer protein, one of the main allergens among the population of the Mediterranean area and the major allergen of peach allergic patients. The percentage of intact protein, after extensive digestion, measured by comparison with a non-digestible peptide analogue used as internal standard, was found to be about one-third of the original protein content. The peptides formed in digested fraction were characterized by means of LC/MS. The products of the digestion essentially derived from trypsin action, whereas the protein appeared to be resistant to pepsin and chymotrypsin. The identified peptides could be classified as low molecular weight and high molecular weight peptides. The latter consisted of the full protein, with the disulfide bridges still intact, deprived of the smaller peptides. The different digestion products, including the high and low molecular weight peptides, were purified by LC and assessed, together with the intact protein, by dot-blot analysis with sera of allergic patients, allowing to estimate their potential allergenicity. The intact protein and the high molecular weight peptides were found to be recognized by patients' sera, whereas the small peptides were found to be not reactive.

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1 Introduction

Non-specific lipid transfer proteins (LTPs) are the most important allergens for non-pollen-related allergies to Rosaceae fruits [1]. LTP proteins are members of the prolamin family of plant allergens and are low molecular weight (LMW) proteins (from 90 to 95 amino acids) with eight cysteine residues forming four disulfide bridges, which are responsible for LTP compact folding and for a hydrophobic tunnel in their central cavity. [2] This fold includes four α-helices separated by short turns and a flexible and non-structured *C*-terminal coil. [3] These common structural features shared by LTPs from different fruits are thought to be responsible for the observed clinical cross-reactivity. [4, 5] Peach LTP is considered the prototypic member of this family of pan-allergens.

The stable tertiary conformation of LTPs makes these compounds extremely resistant to thermal or enzymatic degradation and is thought to be one of the most important

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Abbreviations: HMW, high molecular weight; **LMW,** low molecular weight; **LTP,** lipid transfer protein; **PNA,** peptide nucleic acid; **PVDF,** polyvinylidenedifluoride

factor contributing to the severe systemic reactions often observed in allergic patients [6] In particular, their known resistance to pepsin digestion [7] would allow the allergens to maintain their immunogenic and allergenic motifs and thus to interact with the immune system associated with the gastrointestinal epithelia, thereby inducing both sensitization and the systemic symptoms observed in allergic patients [8, 9].

These characteristics make them true food allergens, since they can sensitize the patient directly in the gastrointestinal tract [10] without cross-reacting with pollen allergens [11]. For example, sensitization to LTP has been recognized as a risk factor inducing severe reactions in non-birch related hazelnut [12] and apple [13] allergies. Although the sensibilization pathways of LTPs *via* the gastrointestinal tract are not yet fully understood, resistance to pepsinolysis has been incorporated into the decision tree assessment for potential allergenic risks posed by novel foods [14].

Simulated enzymatic digestion has been carried out for other LTPs, such as apple [7], cherry [9] and grape [15], but data concerning the resistance of the peach LTP to the subsequent action of both gastric and intestinal enzymes, about the peptides formed during the digestion and their potential allergenicity are still very scarce.

In this work, we evaluated in a model system the effects of the gastro-intestinal digestion of the peach LTP Pru p 3 by means of LC/MS. We quantified the extension of the proteolysis in artificially simulated gastrointestinal conditions, characterized the peptides produced and studied their IgE-binding capacity after their purification by semi-preparative HPLC.

2 Materials and methods

2.1 Chemicals and reagents

Polyvinyl-polypirrolidone, Tris-HCl, NaCl and Tween-20 were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Ammonium hydrogen carbonate (NH_4HCO_3) was purchased from Fluka (Sigma). Modified trypsin was purchased from Promega (Madison, WI, USA). Doubly deionized water was obtained using a MilliQ system (Millipore, Bedford, MA, USA); Amicon filter devices with nominal MW cut-off of 5 kDa, and polyvinylidenedifluoride (PVDF) filter were purchased from Millipore. Formic acid 99% purity was purchased from Acros Organics. PVDF membrane, OPTI-4-CN kit and Ovalbumin were purchased from BIO-RAD. HPLC grade ACN was purchased from VWR International (Batavia, IL, USA). The Peptide Nucleic Acid (PNA) was synthesized in our laboratory as previously described [16]. Sera of patients allergic to peaches were provided by Niguarda Ca' Granda Hospital (Milano) and were tested with ImmunoCap® Specific IgE Fluoro-enzyme immunoassay for recombinant allergens Pru p 1, Pru p 3, Pru p 4, Bet v 1 and Bet v 2.

2.2 Chromatographic equipments

The chromatographic system consisted of a Waters ALLIANCE 2695 HPLC system (Milford, MA, USA) with a Waters 996 PhotoDiode Array detector followed by a MICROMASS ZMD single quadrupole mass spectrometer. Instrument control was accomplished by Waters MassLynx v4.0. The analytical column was a Jupiter Phenomenex C18 (300 Å, 250 × 4.6 mm, 5 μm). The mass spectrometer operated in the following conditions: ESI positive, capillary voltage 3 kV, cone voltage 30 V, source temperature 100°C, desolvation temperature 150°C, spraying gas (N_2) 100 L/h, desolvation gas (N_2): 400 L/h, full scan acquisition from 150 to 1500 m/z in continuum mode and 2.9 s of scan time.

2.3 Protein extraction

The Pru p 3 protein was extracted and purified from peels of peach (*Prunus persica*) variety Italia K2, as previously described [17]. Briefly, peach peel was obtained by cutting 2–3 mm from the external part of peaches. Ground peels were homogenized in a phosphate-buffered saline solution (PBS, 0.01 M sodium phosphate and 0.137 M NaCl, pH 6.8), at 25% w/v ratio and extracted for 2 h at 4°C under continuous stirring after addition of 4% w/w polyvinyl-polypirrolidone. An almost clear extract was obtained after centrifugation, filtration and dialysis.

2.4 Peach LTP purification

The Pru p 3 protein was purified by means of two steps, as previously described [17], the first on a semi-preparative C8 in a RP-HPLC system equipped with a UV analyzer set at 240 nm and the second directly on the analytical C18 column on the HPLC-PDA-ESI/MS system. Purification was efficiently achieved by means of a T-split before the single quadrupole analyzer: the collection time of the eluate was defined by monitoring the characteristic ions of the protein (following the eXtract Ion Current). Protein concentration was determined according to the Bradford method [18] by using a Perkin Elmer Lambda 20 Bio UV absorbance detector.

2.5 Simulated gastrointestinal digestion of Pru p 3 protein

Eleven microliters of a solution containing 5.5 μg/μL of Pru p 3 were mixed to 50 μL of 0.1 N HCl solution at pH 2, then 2 μL of a pepsin solution (0.5 μg/μL in water), and 11.6 μL of a 15-mer PNA (MW 4238 Da) solution (2.5 μg/μL in water) were added. The PNA was used as a non-digestible internal standard. The solution was maintained for 3 h at 37°C at pH

2 to simulate gastric digestion. Then, 40 μL of a 50 mM ammonium bicarbonate buffer (pH 7.8), 1 μL of trypsin solution (1 μg/μL in 50 mM acetic acid) and 2 μL of a α-chymotrypsin solution (0.5 μg/μL in 50 mM acetic acid) were added. The mixture was maintained for 4 h at 37°C and pH 7.8 to simulate intestinal digestion. A control experiment was also performed by substituting the enzymes with equal volumes of buffers. The peptide mixture was analyzed by LC/ESI-MS (system described in Section 2.2). The flow rate was 1 mL/min, eluent A was 0.1% formic acid+0.2% CH_3CN in Milli Q H_2O and eluent B was 0.1% formic acid+ 0.2% Milli Q H_2O in CH_3CN. Gradient: 5 min of eluent A isocratic, 35 min of linear gradient to 40% of eluent B, 10 min of isocratic 40% B, 10 min of non-linear gradient to 100% B and reconditioning. The injection volume was 30 μL. Peptides were characterized by means of ''Expasy Peptide Cutter'' software simulations, measured molecular weights and spectral fragments obtained by in-source collision.

The percentage of undigested protein after enzymatic treatment was calculated by measuring the semiquantitative ratio of the protein area and the PNA area in the control experiment (taken as reference) and in the simulated digestion.

2.6 Purification of peptides obtained by simulated GI digestion

The peptides were purified by an analytical LC coupled to a single quadrupole mass spectrometer, by monitoring their characteristic ions in order to selectively collect the different peptides in a pure form during their elution. Their characteristic ions were selectively monitored during the LC run, allowing to recover the pure peptides when exiting from the chromatographic system. Thus, all the main products derived from the digestion of the protein were separately recovered, dried under a N_2 flux and stored frozen. The three high molecular weight (HMW) peptides were collected together due to their very close elution times. The pure peptides were redissolved in the TBS (Tris Buffer Saline) buffer immediately before the blotting procedure.

2.7 Dot blotting

Buffers utilized for this step were prepared as follows: TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5); TBS-T buffer (0.05% Tween-20 in TBS); blocking buffer: 1% Ovalbumin in TBS-T.

Allergic sera were prepared by the Unit of Allergology and Clinical Immunology, Niguarda Cá Granda Hospital, Milan, Italy. The serum utilized for the reported experiment gave negative (inferior to 0.1 kUA/L) response to Pru p 1, Pru p 4, Bet v 1 and Bet v 2, positive (9.41 kUA/L) response to Pru p 3. Another serum with lower specific IgE to Pru p 3 was also tested.

Peptides were redissolved in 5 μL of PBS buffer and spotted on the activated PVDF membrane. The membrane was then washed in TBS-T for 5 min, incubated with blocking buffer for 2 h, washed twice in TBS-T for 5 min, incubated with serum of allergic patients (1:50 in blocking buffer) for 2 h, washed twice in TBS-T for 5 min, incubated with Rabbit-anti-human IgE antibodies (1:3000 in TBS-T) for 1 h, washed twice with TBS-T for 5 min and incubated with Goat-anti-rabbit-HRP antibodies (1:3000 in TBS-T) for 2 h. The complex formed was detected by the OPTI-4-CN detection kit, incubating the substrate for 30 min and stopping the reaction by washing with Milli-Q water for 15 min. The blotted strip was acquired on a Densitometer GS-800 (BIO-RAD).

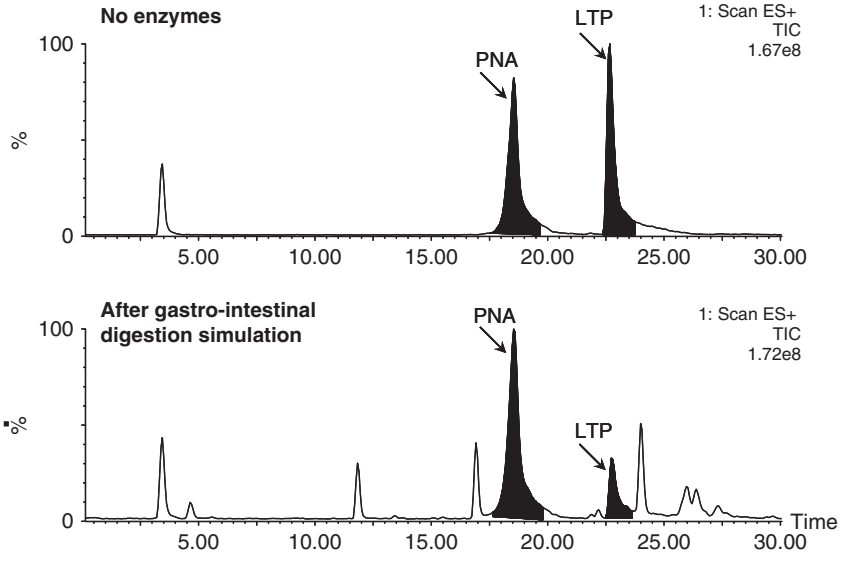

Figure 1. LC/ESI-MS analysis of LTP treated with the proteolytic enzymes simulating gastrointestinal digestion and control experiment. The PNA is not recognized by the enzymes so it can be used as internal standard in order to relatively quantify the changes in the LTP content.

Table 1. Identified peptides generated from Pru p 3 digestion

Rt (min)	MW (Da)	Sequence	LTP position	MS spectra characteristic ions (*m/z*)
4.7	273	VR	17–18	274.2(MH^+), 175.1(y_1)
11.8	588	TTPDR	40–44	589.4(MH^+), 488.4(y_4), 387.3(y_3), 290.2(y_2), 175.1(y_1)
13.5	472	NLAR	36–39	473.4(MH^+), 359.4(y_3), 246.2(y_2)
16.9	799	NVNNLAR	33–39	800.5(MH^+), 400.9(MH_2^{2+}), 587.4(y_5), 473.3(y_4), 246.2(y_2)
22.5	1663	SASVPGVNPNNAAALPGK	55–72	832.7(MH_2^{2+}), 555.0(MH_3^{3+}), 660.5(y_{14}^{2+})
22.8	9135	Intact LTP protein	1–91	1306.0(MH_7^{7+}), 1142.9(MH_8^{8+}), 1016.3(MH_9^{9+})
24.0	1904	QLSASVPGVNPNNAAAPGK	53–72	953.1(MH_2^{2+}), 636.1(MH_3^{3+})
26.0	5712	HMW LTP peptide 1	(discussed in text)	1143.4(MH_5^{5+}), 953.0(MH_6^{6+}), 817.1(MH_7^{7+})
26.3	5585	HMW LTP peptide 2 5712-K_{91}	(discussed in text)	1117.9(MH_5^{5+}), 931.8(MH_6^{6+}), 798.8(MH_7^{7+})
27.3	5386	HMW LTP peptide 3 5585-$T_{89}V_{90}$	(discussed in text)	1078.1 (MH_5^{5+}), 898.8 (MH_6^{6+}), 770.5(MH_7^{7+})

Ions listed in the last column, identified with standard peptide fragment notations, allow the correct identification of the peptide sequences and are also those monitored during purification of peptides.

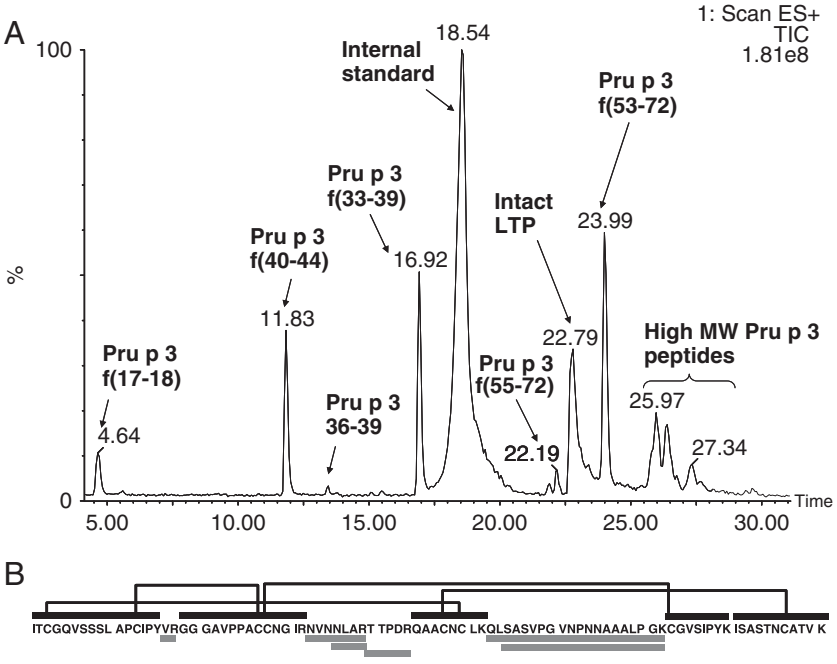

Figure 2. LC/ESI-MS chromatogram of the mixture of peptides produced by the simulated gastrointestinal digestion (A) and identification of the released Pru p 3-derived peptides mapped onto the protein sequence (B): in black, the HMW peptide 5712; in grey, LMW peptides.

3 Results and discussion

3.1 Simulated gastrointestinal digestion of Pru p 3: Resistance to proteolysis

The purified Pru p 3 protein, obtained as previously described [17], underwent a proteolytic treatment simulating a gastrointestinal digestion. The main proteolytic events taking place during the gastrointestinal digestion were explored in this work: pepsin action during gastric digestion and a combination of trypsin and chymotrypsin during intestinal digestion. Incubation times were chosen according to the model of Vermeirssen [19] as 3 h for the gastric phase and 4 h for the intestinal phase were set.

First, the relative percentage of the non-digested protein was evaluated. A protease resistant oligonucleotide analogue [16] was added as an internal standard. As shown in Fig. 1, a significant percentage of the protein was still intact even after this extensive digestion protocol. A comparison to the Pru p 3 signal present in the control non-digested sample was done by normalizing the chromatographic peak area against the PNA and showed that 35% ($\pm$5%) of the peach allergen Pru p 3 remained intact during the enzymatic treatment (Fig. 1). These data indicate that a considerable percentage of the intact protein can survive digestion and get in contact with the intestinal mucosa. However it is important to underline that the food matrix effects might also modify the *in vivo* behavior, either increasing or decreasing the protein resistance.

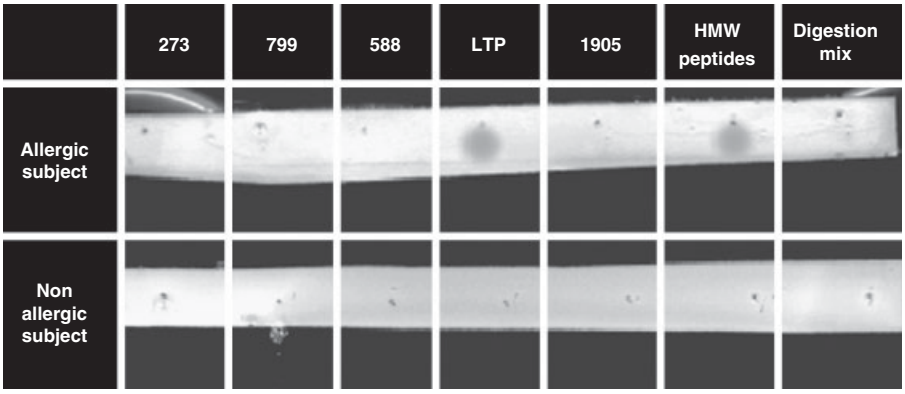

Figure 3. IgE immunodetection of purified peptides blotted with the serum of a patient allergic to peach and with the serum of a non-allergic subject. Peptides are described with their MW. Intact LTP is also present.

3.2 Identification of peptides resulting from digestion

Several peptides were released by Pru p 3 digestion, as it appears in Fig. 1, and were analyzed by LC/ESI-MS. Most abundant peptides were completely identified by MW, mass spectral fragments and database search (http://us.expasy.org/tools/peptidecutter/) and are listed in Table 1 and in Fig. 2A.

Six LMW peptides released by proteolysis were clearly identified, together with the intact undigested Pru p 3 protein. Three HMW peptides were also detected.

The identified LMW peptides confirmed, as already known, that Pru p 3 is resistant to pepsin [7], since no specific peptide cleavages were observed in the molecule, even after a prolonged treatment (3 h). Pru p 3 protein proved to be very resistant also to α-chymotrypsin, since only one, possibly two, of the seven sites for this enzyme in the Pru p 3 sequence were cleaved: the specific bond between Leu_{54}-Ser_{55} and that between Tyr_{16} and Val_{17}, although the latter has already been observed when Pru p 3 was digested by trypsin only [17]. On the contrary, Pru p 3 was highly digested by trypsin, in all its specific cleavage sites (http://us.expasy.org/tools/peptidecutter/). Moreover, together with these specific peptides, the aspecific cleavage sites Tyr_{16}-Val_{17}, Ala_{88}-Thr_{89} and Val_{90}-Lys_{91} could also be attributed to an aspecific activity of trypsin.

The three main HMW peptides were identified as the core of the Pru p 3 protein after the release of the LMW peptides: the MW 5712 Da peptide corresponded to the protein still linked by the four disulphide bridges, after the release of the LMW peptides, further cleaved between Lys_{80} and Ile_{81}. The MW 5584 Da peptide was analogous to the previous peptide but without Lys_{91} and the MW 5384 Da peptide corresponded to the previous one without Tyr_{89} and Val_{90}. In all cases, the disulfide bridges were able to keep the different cleaved parts linked together.

The sequences of the peptides generated during the digestion, mapped onto the Pru p 3 structure, are shown in Fig. 2B.

3.3 Allergenicity of the purified peptides derived from simulated gastrointestinal digestion

To verify the allergenicity of the released peptides, a dot-blotting protocol [20] was performed on the purified proteolytic peptides (details of the purification in Section 2). After spotting the different purified products on the PVDF membrane, incubation with allergic patient serum was performed. A non-allergic patient serum was used as control. The results are shown in Fig. 3.

A further experiment with a different patient gave similar results. These experiments confirmed that only the intact Pru p 3 and the HMW peptides were recognized by the sera of allergic patients. This indicates that Pru p 3 has still the potential to be recognized by IgE, eliciting an allergic reaction after the gastrointestinal digestion. Moreover, even after the release of some peptides, the core of Pru p 3, still linked by the disulfide bridges, is able to elicit an allergic reaction.

The major reactive IgE-binding linear epitopes characterized up to now in the Pru p 3 allergen [21] are located in sequences 11–20, 31–40, 71–80. Anyway, according to the present results, the amino acidic sequence 11–20 is cleaved during gastrointestinal digestion in positions 16 and 18, and the amino acidic sequence 31–40 is also extensively hydrolyzed. The derived small peptides derived from these sequences were not recognized by IgEs. However, the Pru p 3 protein without these peptides was still recognized by the allergic patient serum, indicating that other epitopes are probably also important.

4 Concluding remarks

The present results show that an important percentage of the peach LTP protein (Pru p 3) can be found intact after a gastrointestinal digestion, confirming resistance toward pepsin and chymotrypsin. Several Pru p 3-derived small peptides were identified after the digestion, together with the intact Pru p 3 and few high MW peptides, consisting of the Pru p 3 depleted of the small peptides, with the different parts still linked by disulfide bridges.

A rapid on-line purification of the digestion products and a dot-blotting detection of allergenic peptides were performed, allowing an assessment of the IgE-binding characteristics of the digested peptides. The intact Pru p 3 and the high MW peptides were found to be recognized by sera of allergic patients, outlining the potential for a persistence of the allergenicity of intact Pru p 3 even after a gastrointestinal digestion and, most interestingly, a potential allergenicity of the high MW peptides, albeit deprived of several parts of the Pru p 3 sequence.

These results suggest that the allergen stability in the gastrointestinal tract might be a key feature in the induction of the allergic response to peach LTP.

The authors have declared no conflict of interest.

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