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Simulated gastrointestinal digestion of Pru ar 3 apricot allergen: Assessment of allergen resistance and characterization of the peptides by ultra-performance liquid chromatography/electrospray ionisation mass spectrometry

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RATIONALE: Non-specific lipid transfer proteins (ns-LTPs) are major food allergens of the Rosaceae family. The severity of allergic reactions often relates to resistance of the allergen to digestion. Thus, it is important to evaluate the digestibility of these proteins and characterise the peptides generated in the gastrointestinal tract.

METHODS: Simulated gastrointestinal digestion of purified allergen Pru ar 3 was performed using pepsin for the gastric phase in aqueous HCl at pH = 2 and chymotrypsin and trypsin for the intestinal phase in aqueous NH₄HCO₃ at pH = 7.8. The peptide mixture obtained was analysed by ultra-performance liquid chromatography/electrospray ionisation mass spectrometry (UPLC/ESI-MS). Peptide sequences were identified by comparing their molecular mass to that obtained by *in silico* digestion, and were confirmed by the ions obtained by in-source fragmentation. Semi-quantification was performed for the intact protein by comparison with internal standards.

RESULTS: The resistance to gastrointestinal digestion of Pru ar 3 allergen was evaluated to be 9%. This value is consistent with that found for grape LTP, but much lower than the resistance found for peach LTP (35%). All the peptides generated were identified by ESI-MS on the basis of their molecular mass and from the ions generated from in-source fragmentation. Apart from low molecular mass peptides, five high molecular mass peptides (4500–7000 Da) containing disulphide bridges were identified. ESI-MS of the intact protein indicated a less compact folded structure when compared to that of the homologous peach LTP.

CONCLUSIONS: An extensive characterisation of the peptides generated from the gastrointestinal digestion of Pru ar 3 allergen was performed here for the first time via UPLC/ESI-MS analysis. The digestibility of the allergen was evaluated and compared with that of other LTPs, demonstrating that only a small amount of undigested protein remains, and that specific proteolytic action involves immunodominant epitopes. These data might explain the lower allergenicity of apricot LTP compared to peach LTP, despite their high sequence homology. Copyright © 2012 John Wiley & Sons, Ltd.

Allergy is a hypersensitivity reaction initiated by immunological mechanisms and the antibody responsible for an allergic reaction typically belongs to the IgE isotype (IgE-mediated allergy).^[1] A food allergy is an adverse immune response to a food protein. When some predisposed individuals eat a food allergen, they might present symptoms ranging from respiratory, gastrointestinal, cutaneous and cardiovascular, up to anaphylactic shock.^[2]

Allergy to Rosaceae fruits is often seen in association with birch pollinosis,^[3] but severe cases exist in which Rosaceae allergy can be observed in patients without this cross-reaction.^[4] In these cases, the major allergens belong to the

family of non-specific lipid-transfer proteins (ns-LTPs).^[5] Lipid-transfer proteins are small (molecular weight (MW) = 9–10 kDa) and basic (pI = 8.8–10.0) proteins that have been purified from various plant sources.^[6] They have a tridimensional structure characterized by an α -helical compact domain comprised of four helices connected by eight cysteine residues,^[7] with a tunnel-like hydrophobic cavity able to accommodate lipophilic ligands.^[8] ns-LTPs are involved in plant defense mechanisms against phytopathogenic bacteria and fungi and possibly in the assembly of hydrophobic protective layers of surface polymers, such as cutin.^[5] There is a high cross-reactivity among ns-LTPs from different plant species,^[9] due to their sequence and resulting epitope similarity.^[10] The resistance to proteolysis of ns-LTPs is well known:^[11] apple,^[12] cherry,^[13] grape,^[14] and peach^[15] have already been subjected to *in vitro* digestion in order to assess their digestibility. Apricots are widely grown in Europe, and allergic reactions are becoming more common, especially oral allergy syndrome.^[16]

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Apricot Pru ar 3 has a high sequence homology (91%) to peach Pru p 3; however, it shows a different rate of sensitization: all patients allergic to apricot are also allergic to peach, whereas not all peach allergic subjects are allergic to apricot, which appears to be less allergenic.^[17]

Since resistance to gastrointestinal digestion has not yet been evaluated for apricot LTP, the aim of this work was to evaluate the resistance of Pru ar 3 to proteolytic enzymes of the digestive tract; the peptides generated from this *in vitro* digestion were identified by using UPLC/ESI-MS and the undigested protein was semi-quantified using a non-digestible peptide-like compound as internal standard.

EXPERIMENTAL

Reagents and solvents

Deionized water was obtained from a Alpha Q-Waters (Millipore, Billerica, MA, USA) purification system. Pepsin from porcine gastric mucosa (385 units/mg solid), α -chymotrypsin from bovine pancreas (61.75 units/mg solid), trypsin from porcine pancreas (1900 units/mg solid), formic acid ($\geq 99.9\%$) and acetonitrile were purchased from Sigma-Aldrich (Stockholm, Sweden). Ammonium bicarbonate was purchased from Fluka (Buchs, Switzerland). Hydrochloric acid (37% v/v) was purchased from Carlo Erba (Milan, Italy). Peptide Nucleic Acid (PNA, sequence H-GTAGATCACT-NH₂) was synthesized in our laboratories, as previously described.^[18]

Extraction and purification of apricot Pru ar 3

Extraction and purification of the apricot 9 kDa LTP, Pru ar 3, was performed as described previously.^[19] Briefly, apricot peel was homogenized (1:5 w/v) in potassium phosphate buffer (10 mM, pH 7) containing polyvinylpyrrolidone (20 g/L), diethyldithiocarbamate (1.6 g/L) and ethylenediaminetetraacetic acid (0.654 g/L). The extraction was performed at 4°C for 2 h. The homogenate was centrifuged at 40,000 g for 30 min. The concentrated raw extract was loaded onto a Resource S column (column dimensions 6.4 × 30 mm, particle size 15 μ m, pore size 200–10 000 Pharmacia Biotech, Uppsala, Sweden); the mobile phase was 30 mM sodium citrate dehydrate (pH 2.2) (eluent A) and 30 mM sodium citrate dehydrate plus 1 M NaCl (pH 2.2) (eluent B). The gradient length was 15 CV (column volume) with a flow rate of 3 mL/min; absorbance was monitored at 280 nm. The eluted fraction was further

separated by gel filtration followed by size-exclusion chromatography on a Superdex 75 HR column; elution buffer was 30 mM sodium citrate dehydrate and the flow rate was 0.7 mL/min. Absorbance was monitored at 280 nm.^[20]

SDS/PAGE-immunoblotting

The purity of Pru ar 3 was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described elsewhere.^[21] Briefly, SDS-PAGE was carried out essentially as described by Neville^[22] with a polyacrylamide stacking gel (6%) overlaying a gradient separation gel (7.5–20%). The sample was diluted 1:2 with buffer containing 3% 2-mercaptoethanol and was heated at 100°C for 5 min. Subsequently, the separated protein was electroblotted onto a nitrocellulose membrane (0.45 μ m; Amersham, Buckinghamshire, UK) using a trans-blot cell from BIORAD at 0.45 A and 100 V for 4 h at 4°C (transfer buffer was 25 mM Tris, 192 mM glycine, 20% methanol). The unoccupied protein-binding sites were blocked by incubation with phosphate-buffered saline (PBS 1×, pH 7.4) and 0.5% Tween-20 and the membrane was incubated with a pooled serum from three patients that were selected by means of both their clinical history and concentration of anti-apricot, anti-peach and anti-Pru p3 IgE-specific levels as measured by immunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) and expressed in kUA/L. Table 1 shows the demographic, clinical and immunological characteristics of the patients. IgE binding was detected by incubation with ¹²⁵I-labeled anti-human IgE antibodies and exposure on X-ray film at –70°C. Each serum was tested by immunoblot analysis that showed an IgE-binding profile against the purified Pru ar 3. Figure 1 shows the IgE immunoblotting profile of the purified 9 kDa Pru ar 3 with pooled serum.

Simulated gastrointestinal digestion of Pru ar 3

For the experiments to calculate the proportion of undigested protein, 46.7 μ L purified Pru ar 3 (30 μ g/mL) were added to 10 μ L of 10 mM PNA and the mixture was dried under nitrogen flux. For experiments to identify the generated peptides, 400 μ L purified Pru ar 3 (30 μ g/mL) were added to 10 μ L of 10 mM PNA and dried under nitrogen flux.

The samples were reconstituted with 50 μ L of 10 mM HCl (pH 2), and 1 μ L of 1 mg/mL pepsin and stirred at 37°C for 3 h. After the gastric phase, 50 μ L of 100 mM NH₄HCO₃ (pH 7.8) was added to the samples. After the addition of 1 μ L

Table 1. Clinical and immunological characteristics of the patients selected as donors of the sera used for the immunoblotting study

Age	Sex	Peach		Peach recombinants		Apricot	
		Symptoms	Immuno CAP	Pru p 1	Pru p 3	Symptoms	Immuno CAP
39	F	3	91.5	55.2	100	1	4.91
50	F	3	70.3	10.7	37.1	3	49.7
23	M	3	53.5	26.3	51.8	1	24.0

1= Oral Allergy Syndrome (OAS)

3= OAS plus vomiting, hypotension and urticaria

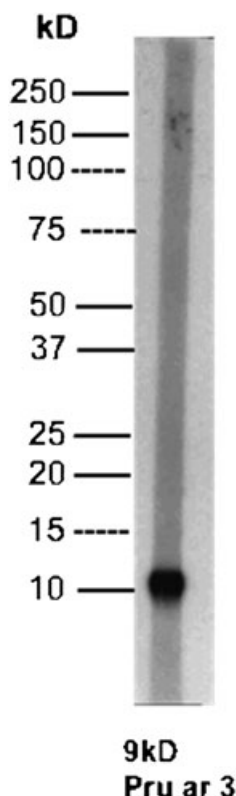


Figure 1. IgE immunoblotting of apricot LTP with a pooled serum of apricot allergic patients.

trypsin (1 mg/mL) and 1 μ L chymotrypsin (1 mg/mL), the samples were stirred for 4 h at 37°C. A blank sample was prepared, using all the buffers and enzymes but without the purified protein, to exclude peptides possibly deriving from autolytic cleavages of the enzymes. A negative control was also carried out: 46.7 μ L of purified Pru ar 3 (30 μ g/mL) were added to 10 μ L of 10 mM PNA and this sample underwent all *in vitro* processes, except enzymatic digestion. After the simulated gastrointestinal digestion, all samples including controls were dried under nitrogen flux.

UPLC/ESI-MS analysis

The mixtures obtained from enzymatic cleavage were separated by a reversed-phase (RP) column (ACQUITY UPLC BEH 300 C18 1.7 μ m 2.1 $\times$ 150 mm for peptide identification) in a UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters Acquity Ultraperformance) using a gradient elution. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min 100% A, 7–50 min from 100% A to 50% A, 50–52.6 min 50% A, 52.6–53 min from 50% A to 0% A, 53–58.2 min 0% A, 58.2–59 min from 0% A to 100% A, 59–72 min 100% A. The samples were analyzed with UPLC/ESI-MS in the full scan mode (flow 0.2 mL/min; analysis time 72 min; column temperature 35°C; sample temperature 6°C; injection volume 5 μ L; acquisition time 7.0–58.2 min; ionization type positive ions; scan range 100–2000 *m/z*; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 100°C; desolvation temperature 200°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h). To calculate the semi-quantification values, the characteristic ions of the protein and of the internal standards were extracted (obtaining eXtract Ion Chromatograms, XICs) and integrated via the MassLynx software.

RESULTS AND DISCUSSION

Pru ar 3 digestibility

After purification from apricot and immunological characterization, the Pru ar 3 protein was analyzed by UPLC/ESI-MS with or without proteolytic digestion (details in the Experimental section). The XIC is shown in Fig. 2: the characteristic ions of the protein ($[M+6H]^+ = 1529.6$; $[M+7H]^+ = 1311.3$; $[M+8H]^+ = 1147.6$; $[M+9H]^+ = 1020.0$) and of the internal PNA standard ($[M+3H]^+ = 909.9$; $[M+4H]^+ = 682.7$; $[M+5H]^+ = 546.4$; $[M+6H]^+ = 455.6$) were extracted and integrated using MassLynx software.

The ratio $A_{\text{protein}}/A_{\text{PNA}}$ was found to be 0.0087 in the digested sample and 0.0970 in the negative control, indicating the proportion of undigested protein to be about 9%. This

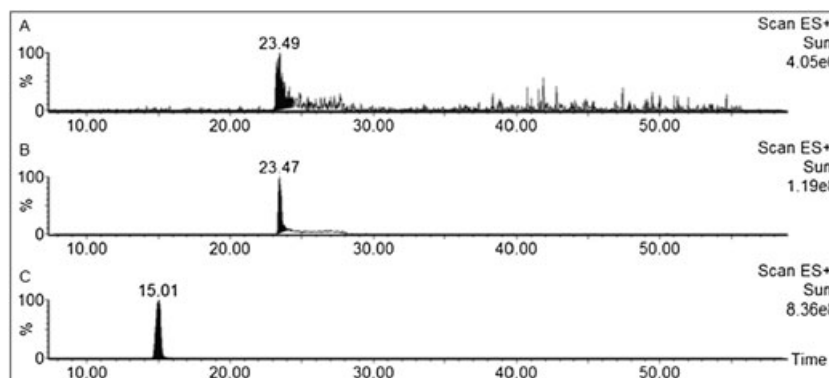


Figure 2. EXtract Ions Chromatograms of (A) Pru ar 3 subjected to simulated gastrointestinal digestion (sum of $[M+6H]^+ = 1529.6$; $[M+7H]^+ = 1311.3$; $[M+8H]^+ = 1147.6$; $[M+9H]^+ = 1020.0$), (B) the same amount of protein not digested (sum of $[M+6H]^+ = 1529.6$; $[M+7H]^+ = 1311.3$; $[M+8H]^+ = 1147.6$; $[M+9H]^+ = 1020.0$), and (C) PNA used as internal standard (sum of $[M+3H]^+ = 909.9$; $[M+4H]^+ = 682.7$; $[M+5H]^+ = 546.4$; $[M+6H]^+ = 455.6$).

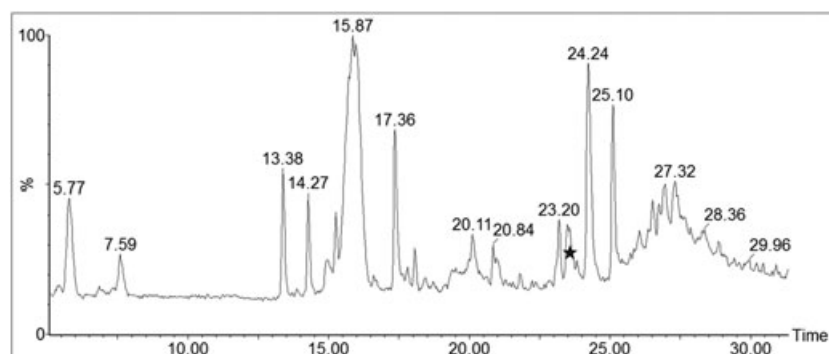


Figure 3. UPLC/ESI-MS chromatogram of digested Pru ar 3: the star indicates the intact protein.

proportion is consistent with that found for grape LTP (9%),^[14] but considerably smaller than that for peach (35%).^[15] The breakdown of Pru ar 3 determined in this experiment is more extensive than that obtained for Pru p 3 in a previous study^[15] and this might partially explain its lower allergenicity; however, other additional factors that play a role in the physiological digestion of a complex food matrix (instead of a purified protein) might also have an influence.

Molecular characterization of the peptides generated

Peptide identification after protein digestion was carried out by ESI-MS. As shown in Fig. 3 and Table 2, all the major peaks in the chromatogram were identified with UPLC/ESI-MS on the basis of their molecular mass and ions derived from in-source fragmentation. In-source fragmentation was obtained with the same mass spectrometer parameters (capillary 3.2 kV, cone voltage 30 V) used for the determination of Pru ar 3 digestibility. The peptides identified are reported in Table 2. No MS/MS experiments were performed to identify peptides generated, since, given the purity of the protein digested, a comparison with the theoretical cleavage sites of the enzymes used was sufficient. The theoretical peptide sequences obtained from *in silico* digestion were confirmed by ions generated by in-source fragmentation.

Among the enzymes used for the simulated gastrointestinal digestion (pepsin, chymotrypsin and trypsin), the main proteolytic action on Pru ar 3 is due to trypsin: from the 56 cleavage sites observed, 34 were due to trypsin action and only 14 to pepsin or chymotrypsin action. It is interesting to note that 8 cleavages were not specific, representing breakage of the peptide bond after Asn residues. The four disulphide bonds remained intact through the entire digestion process, leading to the formation of five high molecular mass (HMW) peptides (shown in Fig. 4). HMW peptide sequences were obtained by their molecular masses, as derived from mass spectra, and compared to the theoretical molecular masses obtained by excising the shorter peptides previously identified in the digestion mixture from the full intact protein. The missing sequences in the HMW peptides were found as short peptides (identified from a comparison between *in silico* digestion and molecular weight found), as

shown in Fig. 5. It can be observed that many cleavages were not complete, so that some overlapping peptides were found amongst the digestion products, either as short or long peptides.

Molecular comparison between Pru p 3 and Pru ar 3: allergenicity implications

An alignment between Pru p 3 (Q9LED1) and Pru ar 3 (P81651) indicates the high similarity of the two sequences (Fig. 6), with only seven differing amino acid residues (known epitopes for Pru p 3^[23] are underlined). Pru ar 3 contains two epitopes with different amino acid sequences: a glycine instead of a proline in one sequence and an asparagine replacing a serine in the other. This difference could influence the recognition of such epitopes by IgE of allergic patients and might also contribute to the different allergenicity observed. Although both these epitopes are partially broken down during digestion, due to the presence of cleavage sites in their sequences and the resulting deletion of some amino acids. This is not sufficient to exclude their allergenicity, as demonstrated previously for Pru p 3,^[15] also considering that part of the protein remains undigested and that residual allergenicity of the five HMWpeptides cannot be excluded.

A previous study^[24] concerning the Pru p 3 allergen identified two immunodominant T-cell-reactive regions: PCI-PYVRGGGAVPPAC (12–27) and, predominantly, SYPGVNP-NNAALPGK (57–72). The corresponding regions in Pru ar 3 are PCIGYVRGGGAVPPAC (with an amino acid substitution at position 15) and SISGVNPNNAALPGK (with amino acid substitutions at positions 58 and 59). The first sequence contains a cleavage site for pepsin and chymotrypsin (a tyrosine residue) and for trypsin (an arginine residue) that during digestion causes the break of the sequence because of the removal of the dipeptide VR. The sequence corresponding to position 12–27 is present in this digested form in all the HMW peptides identified, in which the portions PCIGY and GGGAVPPAC are present in two different subunits, bound together through a disulphide bridge involving the two cysteine residues (Fig. 4). The second sequence is, however, intact in the HMW peptide 3 (not visible in the chromatogram of total ion current reported in Fig. 3

Table 2. Peptides derived from simulated gastrointestinal digestion of Pru ar 3; the amino acid preceding the cleavage site is in brackets. Fragments ions derive from peptidic bond rupture: b are the N-term fragments while y are the C-term fragments; number in brackets represents the amino acid position at which the fragmentation occurred

t_R (min)	MW (Da)	Ions [M+nH] ⁺ ⁿ⁺	Sequence	b ⁺	y ⁺	b ²⁺	y ²⁺	b ³⁺	y ³⁺
5.77	744.7	373.3 (2+) 249.3 (3+)	(R)TTPDRR	203.1 (2)	175.1 (1)		166.1 (2) 223.7 (3) 272.2 (4) 322.8 (5)	243.2 (6)	215.4 (5)
7.59	344.3	345.3 (+) 173.1 (2+)	(N)GIR	171.1 (2)	175.1 (1) 288.2 (2)				
13.38	472.4	473.4 (+) 237.2 (2+)	(N)NLAR		175.1 (1) 246.2 (2) 359.3 (3)	228.6 (4)	180.4 (3)		
14.27	719.7	720.7 (+) 360.8 (2+)	(Y)KISASTN	129.2 (1) 242.2 (2) 329.3 (3) 400.4 (4) 487.4 (5) 588.6 (6)	133.0 (1) 234.2 (2) 321.2 (3) 479.4 (5)				
15.25	591.5	592.5 (+)	(K)ISASTN		133.0 (1)				
17.36	799.6	800.6 (+) 400.8 (2+)	(R)NVNNLAR		587.6 (5)				
20.11	572.5	573.4 (+)	(R)NVNNL						
20.84	2263								
23.20	1654	828.1 (2+)	(L)SGSISGVNPN NAAALPGK		301.2 (3)		151.5 (3)		
23.57	9172	552.2 (3+) 1529.8 (6+) 1311.4 (7+) 1147.5 (8+) 1020.1 (9+)	intact protein		485.5 (5)				
25.10	1895	948.7 (2+)	(K)QLSGSISGV NPNNAALPGK		301.2 (3)				
26.06	5603	632.7 (3+) 935.0 (6+) 801.4 (7+) 701.5 (8+) 623.4 (9+)	HMW PEPTIDE 1	102.2 (1) C	623.7 (6) A	138.7 (3) C	547.5 (11) E 636.1 (14) B		217.2 (6) C
26.51	4901	981.1 (5+) 817.6 (6+) 701.1 (7+) 613.4 (8+)	HMW PEPTIDE 2						
26.95	4574	916.0 (5+) 763.4 (6+)	HMW PEPTIDE 3				182.2 (3) C		
27.32	6778	1130.8 (6+) 969.3 (7+) 848.3 (8+) 754.1 (9+)	HMW PEPTIDE 4	102.0 (1) C	1309.8 (14) C		102.0 (2) C		
28.36	4535	1134.3 (4+) 907.5 (5+)	HMW PEPTIDE 5	890.3 (8) C	907.9 (8) C	220.1 (6) B			

but detectable with the XIC) and in the two low molecular mass peptides with retention times 23.20 and 25.10 min (Table 2 and Fig. 3).

It is also noticeable that the amino acid substitutions in Pru ar 3 lead to a slightly different secondary structure compared to Pru p 3: the theoretical helical content of Pru ar 3, calculated using Predict Protein software, is 44.0%, slightly lower than that of Pru p 3 (46.2%) (Fig. 7). The regions that theoretically have a α -helix secondary structure are indicated with an

'H': it is interesting to note that the only two amino acid differences exactly fall within the regions previously identified as epitopes. The lower helical content of Pru ar 3 could contribute to its higher *in vitro* digestibility, due to its less compact structure. This hypothesis is further supported also by the mass spectra of the two proteins, shown superimposed in Fig. 8: the multicharge pattern of Pru p 3 is slightly richer in the higher *m/z* ions than Pru ar 3 (Table 3), indicating a more compact structure of the former.

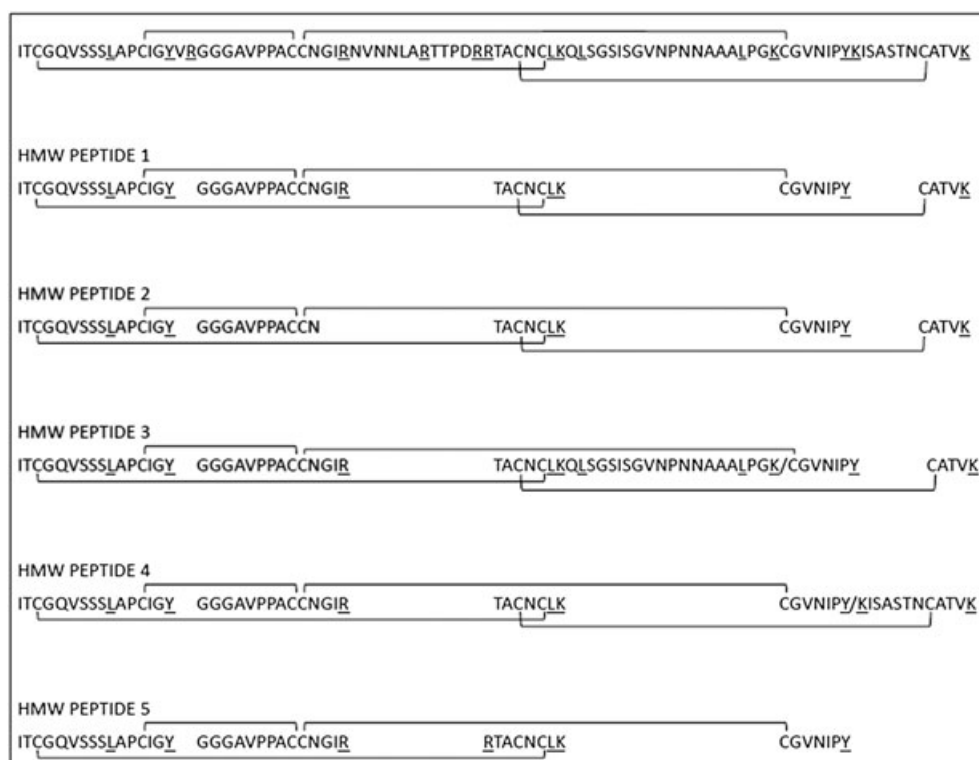


Figure 4. Amino acid sequence of Pru ar 3 (P81651) and of the five high molecular mass peptides released during digestion (amino acid residues recognized by trypsin or pepsin/chymotrypsin are underlined) and identified with UPLC/ESI-MS analysis.



Figure 5. The short peptides generated from the cleavage of Pru ar 3 and identified in the digestion mixture by UPLC/ESI-MS are underlined.

Q9LED1	ITCGQVSSSLAPCIPYVRGGGAVPPACNGIRNVNNLARTTPDRQAACNCLKQLSASVPG	60
P81651	ITCGQVSSSLAPCIGYVRGGGAVPPACNGIRNVNNLARTTPDRRTACNCLKQLSGSISG	60
	*****	*****
Q9LED1	VNPNNAALPGKCGVSIPYKISASTNCATVK	91
P81651	VNPNNAALPGKCGVNIPYKISASTNCATVK	91
	*****	*****

Figure 6. Alignment between Pru p 3 (Q9LED1) and Pru ar 3 (P81651); known epitopes are underlined. "*" means that the residues in that column are identical, ":" means that conserved substitutions have been observed and "." means that semi-conserved substitutions are observed.

CONCLUSIONS

In this work, the peptides generated from the gastrointestinal digestion of Pru ar 3 were identified through extensive use of UPLC/ESI-MS analysis: the structural core of the protein remains mostly bound by four disulphide bridges, generating high molecular mass peptides and some short peptides not involved in by disulphide bridges. The resistance to digestion of Pru ar 3 was similar to that of grape LTP, but lower than

that of Pru p 3, probably due to small differences in the secondary structures. According to the data obtained by MS analysis, the lower allergenicity of apricot LTP compared to peach LTP, despite their high sequence homology, seems to arise from a combination of factors: different amino acids in the epitope regions, a slightly less compact tertiary structure, higher accessibility to proteolytic enzymes during gastrointestinal digestion, and specific cleavage of immunodominant sequences.

Figure 7. Theoretical prediction of the secondary structure of Pru ar 3; α -helical regions are indicated with 'H'.

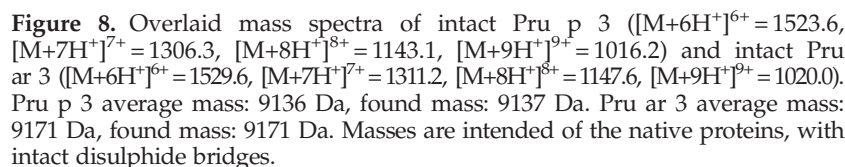


Figure 8. Overlaid mass spectra of intact Pru p 3 ($[M+6H]^{6+}=1523.6$, $[M+7H]^{7+}=1306.3$, $[M+8H]^{8+}=1143.1$, $[M+9H]^{9+}=1016.2$) and intact Pru ar 3 ($[M+6H]^{6+}=1529.6$, $[M+7H]^{7+}=1311.2$, $[M+8H]^{8+}=1147.6$, $[M+9H]^{9+}=1020.0$). Pru p 3 average mass: 9136 Da, found mass: 9137 Da. Pru ar 3 average mass: 9171 Da, found mass: 9171 Da. Masses are intended of the native proteins, with intact disulphide bridges.

Charge state	Prun 3 % base peak intensity	Prup 3 % base peak intensity
[M+6H] ⁶⁺	6.99	18.9
[M+7H] ⁷⁺	63.5	100
[M+8H] ⁸⁺	100	77.0
[M+9H] ⁹⁺	15.5	3.57

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