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ARTICLE

Enzymatic treatment to decrease the allergenicity of Pru p 3 from peach

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Ana P. Tobajas[a,c], Ana Agulló-García[b,c], José L. Cubero[b,c], Carlos Colás[b,c,d], Alba Civera[a,c], Clara Esteban[a], Lourdes Sánchez[a], María D. Pérez[a,c*]

Pru p 3, a member of the Lipid Transfer Protein family, is considered a major allergen from peach as it often induces serious allergic reactions in peach allergic individuals. The high resistance of Pru p 3 to processing treatments and to digestion or enzymatic hydrolysis is probably the cause of the severity of this fruit allergy. The aim of this study was to determine the effect of treatment with a high number of proteases from different origins (vegetal, animal and microbial) on the degradation and allergenicity of Pru p 3. To perform this study, Pru p 3 was previously isolated using cation exchange chromatography and ultrafiltration, and purified protein was incubated with proteases under different conditions. Results showed that only two of fifteen proteases assayed were able to degrade efficiently the protein at acid pH, as determined by SDS-PAGE. These two commercial acid proteases, derived from *Aspergillus niger*, decreased more than 95% the immunoreactivity of Pru p 3 by ELISA using specific rabbit IgG, giving peptides lower than 3.2 kDa as determined by MALDI-TOF mass spectrometry. The hydrolysates obtained showed a decrease in reactivity of IgE respect to untreated Pru p 3 higher than 70% using three pools of sera from peach allergic individuals. Furthermore, when hydrolysates were tested by the prick test, more than 90% of peach allergic patients significantly decreased the average size of the wheal between 72% and 85%. Results suggest that acid protease from *Aspergillus niger* could be used to obtain novel hypoallergenic products more tolerable for peach sensitized individuals.

1. Introduction

Food allergy is estimated to affect up to 8% of children and infants and about 2-5% of the adult population and its prevalence have been increased in the last two to three decades.[1] Specifically, fruits are considered to be among the primary elicitors of food allergy in humans. In a comprehensive review spanning the years 2009 to 2023, it has been estimated that the global prevalence of fruit allergy ranges between 0.029% and 8%. Besides, variation among regions of fruit allergy is influenced by dietary habits, local fruit varieties and patterns of sensitization.[2]

Allergy to fruits presents two clinical patterns. One that results from a primary sensitization to labile pollen allergens, which usually induces local oropharyngeal reactions, and another that results from a primary sensitization with fruit allergens, which frequently induces systemic reactions.[3]

A wide variety of fruits has been reported to trigger allergic reactions that are listed in the database (www.allergen.org) of World Health Organization and International Union of Immunological Societies (WHO/IUIS). Besides, some of the most prevalent and widely studied are reactions to fruits of the Rosaceae family, which peach being a notable example.[4]

The nonspecific lipid transfer proteins (LTP) from the Rosaceae fruits have been recognized as major allergens for allergy non related to pollen.[5] The proteins of the LTP family are considered to be true allergens because of their high resistance to digestive enzymes, as well as their ability to induce oral sensitization and produce severe and systemic symptomatology, often being life-threatening by producing anaphylactic reactions.[6]

Pru p 3 from peach belongs to the LTP family and it has a molecular weight of 9 kDa and a basic isoelectric point. Its main structural motif is represented by a compact α-helix domain where four helices are connected by short loops. The protein contains eight cysteine residues that form four disulfide bridges, which confer it a high resistance to processing.[5] In addition, it has been shown that although technological treatments applied to Pru p 3 induce a certain degree of denaturation, the protein maintains its allergenic potential as determined by binding of IgE or skin prick test in peach allergic individuals. Thus, Pru p 3 shows a high resistance to heat treatments up to 100-120 °C (for 15 seconds to 2 hours) maintaining their allergenicity after heat processing.[7–9] Likewise, it has been shown that Pru p 3 subjected to other processing technologies like high pressure,

a Departamento de Producción Animal y Ciencia de Los Alimentos. Facultad de Veterinaria. Instituto Agroalimentario de Aragón (IA2) (Universidad de Zaragoza-CITA). Spain. E-mail: dperez@unizar.es
b Servicio de Alergología, Hospital Clínico Universitario Lozano Blesa, Zaragoza, Spain.
c Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain.
d Departamento de Medicina, Psiquiatría y Dermatología. Facultad de Medicina. Universidad de Zaragoza. Spain.
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pulsed electric fields or ultrasound maintain its allergenic potential.[9–12] These findings suggest that LTP proteins maintain their allergenic capacity in processed fruit products.

From our knowledge, there is very limited information about the effect of proteolytic enzymes on Pru p 3. Brenna et al. (2000)[7] studied the effect of acid proteases from *Rhizopus* spp. and *Aspergillus saitoi* on Pru p 3 and found that the protein band was observed by SDS-PAGE after 60 min of reaction with both enzymes. Wijesinha-Bettoni et al. (2010) [13] performed a simulated gastrointestinal digestion of Pru p 3 using pepsin, trypsin and chymotrypsin. They observed that the protein is resistant to gastric digestion and that only two of the 14 potential tryptic and chymotryptic cleavage sites were cleaved in the simulated gastroduodenal digest. Thus, after initial cleavage by chymotrypsin between Tyr79 and Lys80, the resulting 7940 Da polypeptide is cleaved by trypsin between Arg39 and Thr40. In the study of Cavatorta et al., (2010) [14] carried out using simulated gastrointestinal digestion on Pru p 3, authors showed that about 35% of the protein remains still intact after extensive digestion. Furthermore, they identified by Liquid Chromatography/Mass Spectrometry (LC/MS) the generated peptides of low and high molecular weight and after separation of them by LC, dot blotting analysis of fractions showed that the intact protein and the high molecular weight peptides were recognized by sera of peach allergic patients, whereas the small peptides were not reactive.

The aim of this study was to evaluate the effect of a high number of proteases from different origins on the degradation and allergenicity of Pru p 3. The extent of degradation was determined by SDS-PAGE and by sandwich ELISA using specific rabbit antibodies to Pru p 3, and the size of peptides was determined by MALDI-TOF mass spectrometry (MALDI-TOF MS). Allergenicity was determined "in vitro" by a competitive enzyme linked fluorescent immunoassay (ELFIA) using three pools of sera from peach allergic individuals, and "in vivo" using the skin prick test.

2. Materials and methods

2.1. Purification of Pru p 3

Peach (*Prunus persica*) extract was prepared from fresh peel from the variety Spanish indigenous "amarillo tardío", clone Calante. Peach peels were homogenised at a ratio 1:2 (w:v) with 10 mM sodium phosphate buffer (pH 5.6) containing 10 mM sodium diethyldithiocarbamate (DIECA), 2 % solid polyvinylpolypyrrolidone (PVPP), 2 mM ethylenediamine tetraacetic acid (EDTA) and 3 mM sodium azide using an ultraturrax. The mixture was kept under agitation for 2 h at 4 °C and centrifuged at 12,000 x g for 30 min at 4 °C. The supernatant was applied onto a SP-Sepharose column (5 × 2 cm) and after washing with 10 mM sodium phosphate buffer, pH 5.6, retained proteins were eluted using the same buffer containing 1 M NaCl. Fractions eluted were mixed and subjected to ultrafiltration using a membrane of 30 kDa. The permeate obtained, containing Pru p 3, was concentrated using a membrane of 3 kDa and subjected to SDS-PAGE. The degree of purity of Pru p 3, determined by densitometry of the stained gel, was higher than 95%.

2.2. Obtention and conjugation of anti-Pru p 3 antibodies

Purified Pru p 3 was inoculated in rabbits to obtain antisera as previously described.[15] All procedures carried out with animals were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project Licence PI 30/19) and are in compliance with the Spanish policy RD53/2013 for the correct use and care of animals, which meets the European Union's requirements (EU Directive 2010/63).

Specific antibodies anti-Pru p 3 were isolated by affinity chromatography using a HiTrap NHS activated HP column (GE Healthcare, Farfield, Connecticut, USA) coupled with Pru p 3 as previously described.[12] Then, purified antibodies were labelled with horseradish peroxidase (HRP) using the Lighting-link HRP conjugation Kit (Innova Biosciences, Cambridge, UK).

2.3. Enzymatic treatment of Pru p 3

Pru p 3 protein (1 mg/mL) and enzymes were prepared in McIlvaine buffer, containing 0.2 M disodium phosphate and 0.1 M citric acid, which were mixed to obtain pHs of 7.0, 6.0 and 3.3.[16] The enzymatic preparations used were protease from *Rhizopus* spp, protease from bovine pancreas, pronase from *Streptomyces griseus*, chymotrypsin from bovine pancreas, alcalase from *Bacillus licheniformis*, papain from papaya latex, bromelain from pineapple stem, ficin from fig tree latex, chymosin from calf stomach, pepsin from porcine gastric mucosa (Sigma-Aldrich, Poole, United Kingdom) and animal rennet from ruminant stomach, vegetal rennet from *Rhizomucor miehei* and vegetal rennet from *Cynara cardunculus* (Laboratorios Arroyo, Cantabria, Spain). Commercial food grade NATUZYM® AP (NAP) and Acid Stable Protease (ASP), both derived from *Aspergillus niger*, were kindly supplied by WeissBioTech GmbH (Ascheberg, Germany) and by Bio-Cat (Troy, Va, EEUU), respectively.

The hydrolysis treatments were carried out at conditions of pH, temperature and time indicated in Table 1, which included optimal pH and temperature of enzymes, among others. The enzymes were added at the ratio of 1/40 (w/v) except for NAP and ASP that were added at 3% (v/v) and 1% (w/v), following manufacturer's instructions. After incubation, the enzymes were inactivated by heating at 100 °C for 5 min in a thermostatic bath. A control containing the protein without enzymes was maintained at the same temperatures and pHs for the duration of the hydrolysis reaction.

Table 1. Proteases and conditions assayed to determine the degradation of Pru p 3.

	37°C	**Bovine pancreas**	**Pronase**
pH 7	50°C	Chymotrypsin	Alcalase
pH 6	50°C	Ficin	Bromelain
	65°C	Papain	
pH 3.5	37°C	Chymosin	Microbial rennet
		Pepsin	Animal rennet

	Vegetable rennet	
50°C	Acid Stable Protease (ASP)	*Rhizopus* spp.
	NATUZYM® AP (NAP)	

2.4. SDS-PAGE

Hydrolysates obtained after treatment with proteases were analysed by SDS-PAGE under reducing conditions according to Laemmli (1970) using 4–20% precast polyacrylamide gels on a Mini PROTEAN Tetra Cell (Bio-Rad, Milan, Italy).[17] Gels were stained with Coomasie Brillant Blue R-250.

2.5. Sandwich ELISA

The concentration of immunoreactive Pru p 3 in untreated and treated samples was determined by a sandwich ELISA previously developed.[12] Briefly, wells of microtiter plates were coated with 120 µl of anti-Pru p 3 antibodies (1 µg/ml) and incubated overnight at 4 °C. After washing, wells were blocked with 300 µl of ovalbumin at 3% (w/v) for 2 h. For the assay, wells were incubated with 100 µl per well of standards or samples diluted in 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 mM KCl and 0.14 M NaCl buffer, pH 7.4 (PBS) containing 0.1% bovine serum albumin (BSA) and 5% sucrose for 1 h. After washing with PBS containing 0.05% Tween 20 (PBST), wells were incubated with 100 µl of an appropriate dilution of anti-Pru p 3 antibodies labelled with peroxidase After washing again, 100 µl/well of a commercial peroxidase substrate containing tetramethylbenzidine (TMB) was added. After 30 min, the enzymatic reaction was stopped by adding 50 µl per well of 2 M H_2SO_4 and the absorbance was read at 450 nm. All samples and standards were analysed by triplicate.

2.6 Mass determination of proteins and peptides by MALDI-TOF MS

The hydrolysates obtained with ASP were analysed by MALDI-TOF MS in the Proteomic Service of the Instituto Aragonés de Ciencias de la Salud (Zaragoza, Spain). Samples were firstly treated with 0.1% trifluoroacetic acid (TFA). Then, they were mixed with the matrix (a saturated solution of sinapinic acid at a concentration of 10 mg/mL prepared in 50% acetonitrile containing 0.1% trifluoroacetic acid) and deposited in duplicate on a MALDI-Opti-Tof plate 384 (Sciex, Framingham, MA, EEUU).

The analysis was performed in positive linear mode with an accelerating voltage of 20 kV, a mass range of 1000-20000 Da, focus 9000, 1000 shots/spectrum and a laser intensity of 4800. The equipment was calibrated with a mixture of protein standards (Proteo Mass Protein MALDI-MS Calibration Kit MSCAL3, Sigma).

2.7. Patients and skin prick test

A number of 21 adult allergic patients to peach were voluntary recruited at the Allergy Department of the Hospital Clínico Universitario Lozano Blesa of Zaragoza (Spain). Patients who met the criteria for inclusion had a clinical diagnosis of LTP allergy, characterized by experiencing symptoms upon consuming peach and exhibiting a positive prick test reaction to peach LTP (ALK-Abelló S.A., Madrid, Spain) or possessing specific IgE levels to Pru p 3 greater than 0.35 kU/L detected using the ImmunoCAP FEIA system (ThermoFisher Scientific/Phadia, Uppsala, Sweden). All patients were previously given a questionnaire and signed an informed consent for using the results of this study. The study protocol was approved by the clinical research ethic committee of Aragón (CEICA) (Projects PI15/0323 and PI17/0351) in accordance with the principles contained in the Declaration of Helsinki and within the framework of current legal regulations for biomedical research studies with biological samples (Law 14/2007 on Biomedical Research, Directive 2004/23/EC of March 31 and RD 411/1996). All samples were tested in a unique session for each patient.

The skin prick test was performed according to the EAACI recommendations [18] with samples of purified Pru p 3 untreated and subjected to enzymatic treatment and with commercial peach LTP (ALK-Abelló S.A., Madrid, Spain) commonly used for LTP allergy detection. Negative and positive controls of saline solution and histamine hydrochloride (10mg/mL) respectively, were also tested in each patient. The major and minor diameters of the wheal were measured and their product was calculated. A positive result is considered when one of the diameters is greater than 3 mm or greater than that produced by the positive control. The percentage of increase or decrease in the product of the diameters of hydrolysed samples respect to the untreated protein was estimated for each patient. After performing the prick test to each patient, a blood sample was extracted and serum, obtained after clotting and centrifugation, was stored at – 20 °C until used.

In addition, patients were distributed into three groups based on their symptoms: at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak (ALOS), an oral allergy syndrome (OAS), or severe symptoms such as anaphylactic shock (ANS).

2.8. Competitive and non-competitive inhibition enzyme linked fluorescent immunoassay (ELFIA)

The presence of specific IgE (sIgE) against Pru p 3 was determined in all individual sera and in the three pools of sera from patients allergic to peach using Pru p 3 ImmunoCAP (Reference f420, ImmunoCAP FEIA system, ThermoFisher Scientific/Phadia, Uppsala, Sweden) in a Phadia 100 system. Assays were performed using a non-competitive format following manufacturer's instructions for sIgE determination.

To compare the binding of sIgE to untreated and enzymatic treated Pru p 3 samples, a competitive assay was carried out using Pru p 3 ImmunoCAP. To this end, untreated and treated Pru p 3 samples (1/40 in PBS) were mixed with the pool of sera (1/1, v/v in PBS) and sIgE was determined as described by Tobajas et al., (2020)[12].

Changes in IgE-binding to Pru p 3 induced by enzymatic treatments (sIgE sample) with respect to untreated sample (sIgE 100%) and negative control (buffer, sIgE 0%) was estimated as follows:

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$$\text{IgE binding (\%)} = \frac{\text{sIgE sample} - \text{sIgE 0\%}}{\text{sIgE 100\%} - \text{sIgE 0\%}} \, x \, 100$$

2.9. Statistical analysis

Data from the prick test were statistically analysed using the SPSS version 15.0 for Windows (Statistical Package for the Social Sciences (SPSS) Inc., Chicago, IL, USA). To summarize the data, descriptive statistics, frequencies, percentages, as well as graphs and charts were used. The data were found to have a non-normal distribution using the Kolmogorov-Smirnov and Shapiro-Wilk W tests, and therefore non-parametric tests were used for the analysis. The Wilcoxon signed-rank test was used to determine differences between control and treated samples. A significance level of $p < 0.05$ was predefined in all cases.

For Pru p 3 concentration determined after enzymatic treatment by ELISA, data were analysed for statistical significance with GraphPad Prism 8 software, using a one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test.

3. Results and discussion

3.1 Effect of proteolysis on degradation of Pru p 3

The effect of treatment with different proteases on the degradation of Pru p 3 was determined by SDS-PAGE. In the case of those enzymes in which a considerable decrease in the intensity of the Pru p 3 band was observed when incubating under acidic pH conditions, which corresponds to that of fruit extracts, the hydrolysates were further analysed. These analysis included the determination of the loss of reactivity with rabbit antibodies specific to Pru p 3 using a sandwich ELISA previously developed [12], and the characterization of hydrolysates by MALDI-TOF MS.

The effect of treatment of purified Pru p 3 with different proteases on the degradation of the protein by SDS-PAGE is shown in Figure 1. These treatments were performed at the optimum pH, temperature and enzyme-substrate ratio of the corresponding enzymes and some additional conditions were also assayed for proteases from *A. niger*. Untreated protein showed a major band of 9 kDa which corresponds to the molecular weight of Pru p 3.[5] As can be observed, Pru p 3 was not affected by papain, bromelain, ficin, alcalase, chymosin, and animal, microbial and vegetable rennets, as no decrease in the intensity of the Pru p 3 band was observed. For pronase and chymotrypsin, a decrease in the intensity of the Pru p 3 band was observed besides the appearance of a band of lower molecular weight indicating a considerable degradation of Pru p 3. When assaying NAP and ASP, both derived from *Aspergillus niger*, a marked disappearance of Pru p 3 band was obtained and it could not be visualized after incubation with protease from bovine pancreas. Therefore, it was assumed that peptides with low molecular weight were produced by these enzymes that could have escaped from the gel.

Other bands of high molecular weight present only in hydrolyzed samples correspond to either proteases or other proteins present in enzymatic preparations as some of them are extracts of animal or vegetal origin and other are produced by fermentation of different fungi.

As the pH of peach is about 3.5, the proteases to be used during juice processing to degrade Pru p 3 should be effective at that acidic condition. Therefore, some additional experiments were performed using protease from bovine pancreas, whose optimum pH and temperature are 7.5 and 37 °C, to know if it could maintain its enzymatic activity at lower pHs. Results showed that its activity decreased markedly with the decrease of pH to 6.0 and 5.0, being necessary very long

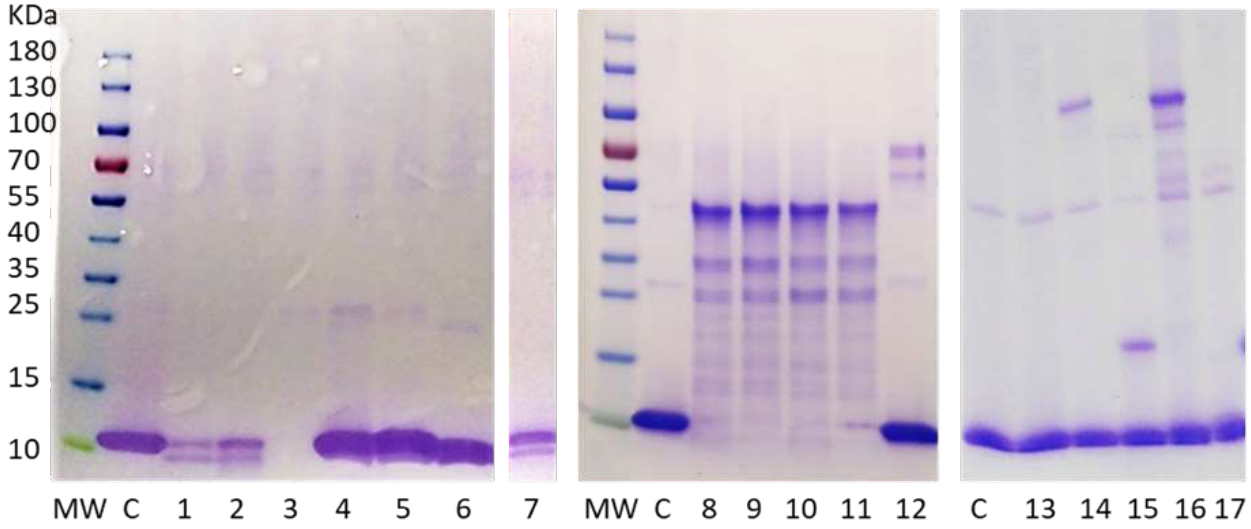

Figure 1. SDS-PAGE on polyacrylamide gel (4–20%) under reducing conditions of untreated and protease-treated Pru p 3. The pH, temperature (°C) and time (h) of the treatment are indicated in parentheses. Molecular weight marker (MW). Control untreated Pru p 3 (C). Lane 1, Pronase (7/37/2). Lane 2, Chymotrypsin (7/50/2). Lane 3, Bovine pancreas (7/37/2). Lane 4, Papain (6/65/2). Lane 5, Bromelain (6/50/2). Lane 6, Ficin (6/50/2). Lane 7, Alcalase (7/50/2) Lane 8, NATUZYM® AP (NAP) (3.5/50/2). Lane 9, NAP (3.5/25/24). Lane 10, Acid Stable Protease (ASP) (3.5/50/2). Lane 11, ASP (3.5/25/24). Lane 12, Rhizopus (3.5/50/2). Lane 13, Pepsin (3.5/37/2). Lane 14, Chymosin (3.5/37/2). Lane 15, Microbial rennet (3.5/37/2). Lane 16, Animal rennet (3.5/37/2). Lane 17, Vegetable rennet (3.5/37/2).

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times (72 h) at 25 °C to achieve a considerable degradation (see the ESI, Fig. S1†). These results indicate that protease from bovine pancreas is not suitable to be applied in the processing of peach derivatives, as the required pH conditions of the enzyme would induce undesirable browning of the product, since optimal pH for the action of polyphenol oxidase browning enzyme is 5.0–7.0, and it shows inhibition under acidic conditions.[19]

NAP and ASP were also assayed maintaining its optimum pH (3.5) but at different conditions of temperature and time (25 °C and 24 h), instead of at 50 °C for 2 h, as recommended by manufacturers. Results showed a similar degradation of Pru p 3 under both conditions (Figure 1). These findings indicate that the proteolysis treatment could be performed by adding the protease preparations sterilized by microfiltration after juice processing to carry out the hydrolysis during storage before going on the market.

Until now, a few studies have been performed to determine the effect of enzymatic treatment on Pru p 3. Brenna et al. (2000) [7] using SDS-PAGE determined the action of acidic proteases from *Aspergillus saitoi* and *Rhizopus* spp. at pH 3.4 and 50 °C on a semi-purified extract of Pru p 3 and found that the protein band was present after 60 minutes of reaction time, suggesting that both enzymes were not able to hydrolyze the protein.

Based on the results obtained in our study, the most efficient enzymes to degrade Pru p 3 were NAP and ASP and therefore, they were used in additional experiments. Furthermore, acid protease from *Rhizopus*, which showed to be ineffective to degrade Pru p 3 has been used as comparison.

The effect of NAP and ASP on the concentration of immunoreactive Pru p 3, determined using a specific sandwich ELISA previously developed [12], is shown in Figure 2. Assays performed with both proteases incubated with the purified protein at pH 3.5 under both tested conditions (50 °C, 2 h and 25 °C, 24 h) indicated that the concentration of immunoreactive Pru p 3 decreased markedly, by more than 90% compared to the untreated protein, whereas treatment with protease from *Rhizopus* showed an increase of immunoreactive Pru p 3 of about 20%, although differences were non-significant. Similar results were obtained when the incubation with NAP or ASP at 25 °C for 24 h was performed with peach extract or commercial peach juice, obtaining a decrease in IgG reactivity of more than 93% (results not shown). These findings support that the treatment with NAP and ASP induces a marked loss of epitopes recognized by IgG, which suggests an intensive degradation of the protein.

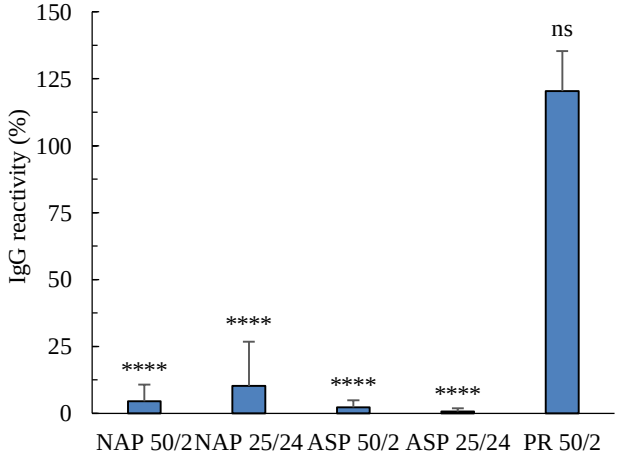

Figure 2. Degradation of Pru p 3 determined by sandwich ELISA treatment with NATUZYM® AP (NAP), Acid Stable Protease (ASP) and protease from Rhizopus (PR) at pH 3.5. Results are the mean ± standard deviation of data from two experiments analysed by triplicate and are expressed as percentage of control untreated protein (100%). Numbers correspond to the temperature (°C) and time (h) of treatment. Asterisks indicate significant differences with the control *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

The analysis of hydrolysates obtained with ASP by MALDI-TOF MS is shown in Fig. S2 a-e. Untreated Pru p 3 exhibits two peaks of molecular weights of 9134 Da and 4751 Da which correspond to the native Pru p 3 and to the molecular ion of the protein ionized with a +2 charge, respectively (see the ESI, Fig. S2†).

In the hydrolysates generated with ASP, the 9 kDa peak belonging to Pru p 3 was not observed and the predominant peptides obtained had molecular weights of less than 3.2 kDa and 1.8 kDa for treatments at 50 °C for 2 h and 25 °C for 24h, respectively (Fig. S2 b and c). It should be noted that although optimum treatment temperature of the ASP is 50 °C, treatment at 25°C for 24 h was even more effective as it produced peptides of lower molecular weight. However, the sample incubated with protease from *Rhizopus* (Fig. S2 d) displays a similar chromatographic profile to that obtained with the native protein, indicating that it is not able to degrade Pru p 3.

The analysis of the ASP in buffer by MALDI-TOF MS gave peaks within the molecular weight range from 20 to 100 kDa, as it was observed in the electrophoretic profile (Fig. S2 e).

These findings suggest that Pru p 3 degradation could be performed adding the protease either during the juice clarification process with pectinolytic enzymes to decrease viscosity [20] that is performed at 50 °C for 2 hours before pasteurization, or sterilized by filtration into used package after heat treatment so that the enzyme could act during the storage period before going on the market.

This approach to hydrolyze a food component which cause adverse effects in sensitized individuals is already a standard practice in the lactose-free milk for lactose intolerant individuals. In these cases, lactase is usually aseptically added to the finished product, to hydrolyze lactose during the period that the product remains in the factory for quality control checking before being released to the market.[21]

According to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPHGAN) all peptides in hydrolysed formula milk for children allergic to milk proteins should have a size lower than 3 kDa and be dominated by peptides with a size of about 1.5 kDa, hence containing at maximum one linear epitope and thus should not be able to cross-link IgEs on the surface of tissue mast cells and blood basophils and cause allergic reactions.[22] Therefore, the

peptides obtained in the degradation of Pru p 3 using NAP and ASP would be within the molecular weight range indicated by ESPHGAN to consider a food product as hypoallergenic.

3.2 Effect of proteolysis on allergenicity of Pru p 3

The effect of proteolysis on allergenicity of Pru p 3 was studied using NAP and ASP, as they showed the most efficient degradation of the protein under acidic conditions. The impact of proteolysis on allergenicity of Pru p 3 was determined by "in vitro" and "in vivo" techniques.

The "in vitro" technique consists of a competitive ELFIA technique as previously described by Tobajas et al. (2020). [12] All sera were previously tested using a non-competitive ELFIA format. Three pools of sera from peach allergic patients that presented symptoms of different severity (oral allergy syndrome, at least one systemic symptom or anaphylactic shock) were assayed. To this end, serum from allergic individuals presenting an IgE level against Pru p 3 higher than 1.30 kU/L were mixed to prepare the pools. The specific IgE level in these three pools were 5.43, 6.84, and 9.86 kU/L, respectively.

Results obtained (Figure 3) showed that hydrolysates of Pru p 3 obtained with NAP and ASP incubated at 50 °C for 2 hours or at 25 °C for 24 hours induced a reduction in reactivity of the protein with IgE, which ranged between 75% and 88% for OAS pool, between 77% and 84% for ALOS pool and between 50% to 68% for AS pool, compared to untreated sample (100%). Likewise, experiments performed with hydrolysates obtained from a peach extract or a commercial peach juice incubated with ASP at 25 °C for 24 h using the AS pool showed a decrease in IgE reactivity higher than 80 % respect to untreated samples (100%). The higher loss of reactivity obtained when using these hydrolysates compared to those obtained with the purified protein could be possible due to the expected lower concentration of Pru p 3 present in the extract and juice (results not shown). By contrary, reactivity of Pru p 3 incubated with the protease from *Rhizopus* with the three pool of sera was similar to that of the untreated protein. These results suggest that treatment with proteases from *Aspergillus niger* markedly decrease the allergenic potential of Pru p 3 whereas the protein maintains allergenicity after treatment with protease from *Rhyzopus*.

It is noticeable that the decrease of reactivity of Pru p 3 with IgG obtained in this study is higher than that obtained with IgE, as this fact could be due to a comparative higher loss of protein epitopes recognized by IgG.

In the study of simulated gastrointestinal digestion of purified Pru p 3 performed by Cavatorta et al. (2010) [14], authors observed that the dot-blot analysis using sera of allergic patients showed that intact protein and the high molecular weight peptides obtained after treatment were found to be recognized by IgE, whereas the small peptides were no reactive. These findings indicate that Pru p 3 retains its ability to bind IgE after gastroduodenal digestion and hence its allergenic potential.

On the other hand, skin prick tests were carried out in individuals allergic to LTP from peach to determine the effect of enzymatic treatment on "in vivo" allergenicity. Samples tested included untreated Pru p 3 and hydrolysates obtained with NAP or ASP incubated at 50 °C for 2 h and at 25 °C for 24 h or acid protease from *Rhizopus* incubated at 50 °C for 2 h.

Table 2 shows the level of specific IgE to Pru p 3 of each patient serum, the symptoms indicated by patients and the product of the major and minor diameters of the wheals corresponding to untreated and enzymatic treated protein for each allergic individual. Results expressed as the product of diameters were preferred as some individuals presented an irregular area or a pseudopod in the prick test.[23] The percentage of cutaneous response related to the corresponding untreated protein (100%) is also given.

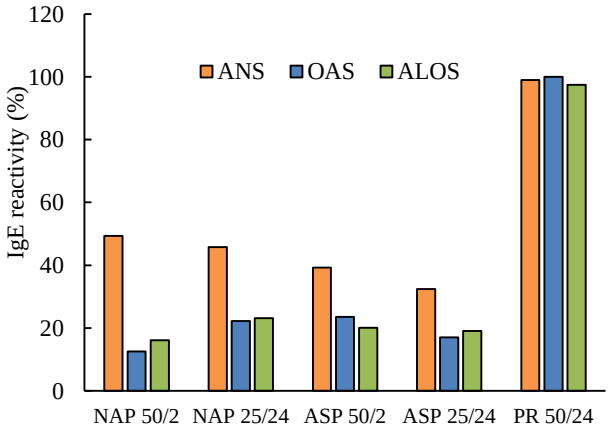

Figure 3. Effect of treatment with NATUZYM® AP (NAP), Acid Stable Protease (ASP) and protease from Rhizopus (PR) on the allergenicity of Pru p 3 determined by indirect competitive immunofluorescence assay (ELFIA). The three pools of sera assayed corresponded to patients that showed one of these three types of symptomatology: OAS, Oral allergy syndrome; ALOS, at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak; ANS, anaphylactic shock. Numbers correspond to the temperature (°C) and time (h) of the treatment. The results are expressed as percentage of the untreated sample (100%).

It should be noted that the relationship between the products of the diameters of the wheals and the specific IgE values gave a very low correlation coefficient (r^2 = 0.0087). Besides, it seems that there is not a relationship between the severity of the symptomatology and the product of the wheal or the level of specific IgE, which indicates that allergenicity of Pru p 3 should be evaluated on an individual manner.

The comparison of the mean ranges of the product of wheal diameter between untreated and treated Pru p 3 with NAP and ASP showed significant differences under all assayed conditions (Z=-4.015, p<0.0001). However, no significant differences were found for samples treated with protease from *Rhizopus* (Z = -0.313, p = 0.754).

Besides, when comparing Pru p 3 treated with NAP and ASP respect to untreated protein, a marked decrease of reaction (wheal diameter product between 15% and 28% compared to control untreated protein) was obtained in 95-100% of

patients (Table 3). Furthermore, no increase in the reaction was observed in any patient and no changes resulted in only one patient for the treatment with ASP at 50 °C during 2 h. However, when Pru p 3 was treated with protease from *Rhizopus*, 47.5% of patients showed a decrease of the wheal diameter product, with a mean value of 53.9%, whereas 47.5% of patients showed an increase, with an average value of 222%, and only 5% showed no changes.

Results of this study obtained after the treatment of Pru p 3 with proteases from *Aspergillus niger* (ASP and NAP) are very promising, as they indicate a notable decrease in the allergenic potential of the protein. Additionally, the impact of enzymatic treatment with proteases from *Aspergillus niger* on allergenicity of Pru p 3 should also be studied using cellular techniques such as the basophil activation test [24], in vivo animal models [25] as well as challenge tests in sensitized individuals, performed under medical supervision. These studies would allow us to know whether hydrolysed products are devoid of risk and whether they would be effective in improving tolerance in allergic patients.[26,27]

Furthermore, a sensorial analysis of peach products obtained by enzymatic treatment should also be carried out to determine consumer acceptance, although it is expected that flavour would not be substantially modified due to the low protein content present in fruit-derived products.

Table 2. Effect of treatment of Pru p 3 with NATUZYM® AP (NAP), Acid Stable Protease (ASP) and protease from Rhizopus (PR) on the allergenicity of Pru p 3 determined by the prick test in peach allergic patients. The results correspond to the product of the wheal diameters. Treatments were performed at 50 °C for 2 h or at 25 °C for 24 h. Changes in allergenicity are also expressed as the percentage of the wheal diameter product respect to untreated sample (100%). C, untreated control sample.

Patient				Wheal diameter product					Allergenicity (%)				
	S	kU/L	C	ASP 50/2	ASP 25/24	NAP 50/2	NAP 25/24	PR 50/2	ASP 50/2	ASP 25/24	NAP 50/2	NAP 25/24	PR 50/2
LTP01	ALOS	1.09	80	8	12	0	12	112	10	15	0	15	140
LTP02	ALOS	0.03	36	16	16	9	9	70	44	44	25	25	194
LTP03	ALOS	15.1	80	16	9	16	0	98	20	11	20	0	123
LTP04	ALOS	2.56	80	16	16	9	9	140	20	20	11	11	175
LTP05	ALOS	0.35	168	42	36	25	56	320	25	21	15	33	190
LTP06	OAS	9.98	260	16	12	16	20	84	6	5	6	8	32
LTP07	ANS	1.57	240	35	25	25	16	126	15	10	10	7	53
LTP08	OAS	2.59	112	56	20	20	20	56	50	18	18	18	50
LTP09	ANS	0.92	63	30	8	8	12	72	48	13	13	19	114
LTP10	ANS	2.03	180	36	16	16	25	90	20	9	9	14	50
LTP11	OAS	6.83	930	200	144	140	98	480	22	15	15	11	52
LTP12	ANS	0.75	168	128	60	98	35	390	76	36	58	21	232
LTP13	ALOS	0.12	63	12	0	0	0	72	19	0	0	0	114
LTP14	ALOS	0.6	144	0	0	16	20	56	0	0	11	14	39
LTP15	OAS	4.22	180	0	0	25	0	160	0	0	14	0	89
LTP16	ALOS	6.21	56	36	20	16	0	325	64	36	29	0	580
LTP17	ALOS	9.01	30	0	0	0	0	12	0	0	0	0	40
LTP18	ANS	4.99	36	35	30	16	12	120	97	83	44	33	333
LTP19	ALOS	1.34	192	0	0	0	0	112	0	0	0	0	58
LTP20	ANS	13.8	192	25	20	16	0	120	13	10	8	0	63
LTP21	ANS	18.6	126	35	0	64	56	160	28	0	51	44	127

Symptoms (S) correspond to oral allergy syndrome (OAS), at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak (ALOS) or anaphylactic shock (ANS); kU/L correspond to sIgE of each patient determined by immunoCap.

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From our knowledge, only two enzymatic modified products are available in the market for individuals sensitized to food proteins: hypoallergenic infant formulas for babies allergic to cow's milk proteins and gluten-free beer for individuals with celiac disease. The processing technology applied to manufacture these products is based on the use of enzymes that efficiently hydrolyse proteins present in those foods.[28,29] These products can be ingested without causing any adverse symptoms in most sensitive individuals. Therefore, the products developed using the enzymatic treatment applied in our study would be the first hypoallergenic fruit-based products on the current market.

Table 3. Number of peach-allergic patients (n) who showed an increase, no change or a decrease of allergenicity when Pru p 3 was assayed by skin prick test. X (%) corresponds to the average value of the percentages of the product of diameters for each group. Pru p 3 was treated with NATUZYM® AP (NAP), Acid Stable Protease (ASP) and protease from Rhizopus (PR) at pH 3.5. The temperature (°C) and time (h) of the treatment are indicated in parentheses.

	Group 1		Group 2		Group 3
Pru p 3 + Protease	**Decrease allergenicity**		**Increase allergenicity**		**No changes**
	X (%)	n	X (%)	n	n
ASP (50/2)	28.4	20	-	0	1
ASP (25/24)	15.1	21	-	0	0
NAP (50/2)	14.9	21	-	0	0
NAP (25/24)	15.2	21	-	0	0
PR (50/2)	53.9	10	199	10	1

Conclusions

In this study we have shown that enzymatic treatment with acid protease derived from *Aspergillus niger* induces a severe degradation of Pru p 3 producing peptides of molecular weight lower than 3 kDa. Furthermore, hydrolysis treatment is able to decrease the IgE binding of Pru p 3 "in vitro" and to markedly decrease the wheal reaction "in vivo" obtained by the prick test. Therefore, the processing with acid proteases from Aspergillus niger could be used as a procedure to elaborate fruit products with a decreased allergenicity. It would also be necessary to carry out studies with acid protease from *Aspergillus niger* on a pilot scale to verify if results obtained with purified Pru p 3 are reproducible in peach products under industrial conditions. Furthermore, studies should be performed in order to determine if proteins of the LTP family from other fruits are also effectively degraded by the acid protease applied in this study.

Author contributions

A. P. T.: Writing-original draft, Writing-review & editing, Methodology, Investigation, Data curation, Conceptualization. A. A.-G.: Writing-review & editing, Investigation, Data curation. J. L. C.: Writing-review & editing, Formal analysis, Investigation. C. C.: Writing-review & editing, Conceptualization, Methodology, Resources, Funding acquisition. A. C.: Writing-review & editing, Investigation. C. E.: Writing-review & editing, Investigation. L. S.: Writing-review & editing, Methodology. M. D. P.: Writing-review & editing, Conceptualization, Methodology, Resources, Funding acquisition, Project administration, Supervision

Ethical approval

The study protocol with humans was approved by the Clinical Research Ethic Committee of Aragón (CEICA) (Projects PI15/0323 and PI17/0351). The study protocol with animals were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project PI 30/19).

Conflicts of interest

There are no conflicts to declare.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article. Additional data that support the findings of this study are available from the corresponding author, MDP, upon reasonable request.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article. Additional data that support the findings of this study are available from the corresponding author, MDP, upon reasonable request.