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Effects of protease-assisted aqueous extraction on almond protein profile, digestibility, and antigenicity

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ABSTRACT

Almonds (*Prunus dulcis*) are one of the most consumed tree nuts worldwide and have been recognized as a healthy and nutritious food. Nevertheless, almonds are also a source of allergenic proteins that can trigger several mild to life-threatening allergic reactions. The effects of selected extraction conditions (aqueous vs. protease-assisted aqueous extraction) on the protein profile determined by proteomics analysis of excised SDS-PAGE gel bands, *in vitro* protein digestibility, and immunoreactivity of almond protein extracts, were evaluated. Proteolysis altered almond protein sequential and conformational characteristics thus affecting digestibility and antigenicity. Proteomics analysis revealed that enzymatic extraction resulted in the reduction of allergen proteins and epitopes. While complete hydrolysis of Prunin 1 and 2 α-chain was observed, Prunin 1 and 2 β-chains were more resistant to hydrolysis. Protein *in vitro* digestibility increased from 79.1 to 88.5% after proteolysis, as determined by a static digestion model. The degree of hydrolysis (DH) and peptide content of enzymatically extracted proteins during gastric and duodenal digestion were significantly higher than the ones from unhydrolyzed proteins. Proteolysis resulted in a 75% reduction in almond protein immunoreactivity as determined by a sandwich enzyme-linked immunosorbent assay and a reduction in IgE and IgG reactivities using human sera. The present study shows that moderated hydrolysis (7% DH) using protease can be used as a strategy to improve almond protein digestibility and reduce antigenicity. This study's findings could further enhance the potential use of almond protein hydrolysates in the formulation of hypoallergenic food products with improved nutritional quality and safety.

1. Introduction

The growing demand for plant-based protein sources has been driven by the need to feed an increasing world population with sustainable and nutritious foods. To that end, the development of plant-based protein ingredients that rival or have improved functional and biological properties (e.g., improved digestibility and reduced allergenicity) compared to the ones from traditional animal protein ingredients is critical (Akharume et al., 2021).

Tree nuts (e.g., almonds, walnuts, and cashews, among others) are an important source of protein and lipids, ranking high among the healthiest snacks (Geiselhart et al., 2018). Despite their dense nutritional content, tree nuts are one of eight food groups that account for most food-induced allergies, with their consumption being associated with several mild to life-threatening immunoreactions in sensitive groups (Sicherer et al., 2003; Tiwari et al., 2010).

Almonds (*Prunus dulcis*) are one of the most abundant tree nuts produced in the world with a forecast production of 2.80 billion pounds in 2022 (USDA, 2022). They are also one of the most consumed tree nuts worldwide, being highly appreciated for their pleasant taste, abundance of nutritional compounds (lipids, proteins, vitamin E, and polyphenols) (Sathe, 1993; Yada et al., 2011), and ease of application in a wide range of products (i.e., snacks, dairy alternatives, gluten-free flours), being particularly attractive as a source of protein for vegetarian and vegan

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diets (Tomishima et al., 2021). However, the desirable techno-functional, nutritional, and textural properties of almonds that allow such applications are highly dependent on the almond protein characteristics (Dias and de Moura Bell, 2022; Wolf and Sathe, 1998). Proteins are of great importance in food processing and product development as they impart many of the functional and nutritional properties that can drive consumers' acceptance of the product. Despite the attractive properties of almond proteins, almond-induced allergies are the third most reported tree nut allergy in the United States, with a prevalence of 0.7% in the population (Gupta et al., 2019). Therefore, the development of processing strategies to improve the almond protein utilization potential is of great interest.

Processing strategies involving the use of blanching, roasting, heat treatments, high pressure processing and irradiation have been evaluated with respect to their potential to reduce the allergenicity of almond products (Bargman et al., 1992b; Mandalari and Mackie, 2018; Verhoeckx et al., 2015a,b). For almonds, studies using immunoblotting with sera from almond allergic patients have shown that blanching and roasting can reduce the IgE binding of a 15–17 kD band (Bargman et al., 1992a; Verhoeckx et al., 2015a,b). Among the almond products available in the market, almond milk has arisen as an important non-dairy beverage alternative. Almond milk is defined as colloidal dispersion obtained by disintegrating almonds with water (Dhakal et al., 2014). Therefore, studies aiming at reducing almond protein reactivity in those products are of great interest to minimize potential life-threatening reactions. Dhakal et al. (2014) reported decreased immunoreactivity of almond protein Pru du 6 after high-pressure treatment in almond milk, whereas no significant change in immunoreactive was observed after application of the thermal treatment (Su et al., 2004). demonstrated no changes in the immunoreactivity of Pru du 6 after γ-irradiation and γ-irradiation plus thermal treatment. Overall, studies have shown that almond proteins in food products are stable during dry-heat treatment at temperatures below 250 °C, while the combined effect of heat, pressure and presence of water resulted in a significant change in almond protein immunoreactivity (Mandalari and Mackie, 2018).

Environmentally friendly strategies such as aqueous and enzymatic aqueous extraction processes have been used to simultaneously extract lipids, proteins, and soluble carbohydrates from almond flour, avoiding the upstream use of mechanical pressing and/or flammable solvent extraction to produce defatted flours for protein extraction (Dias and de Moura Bell 2022; Dias et al., 2020, 2022). While the benefits of using enzymes to assist with the extraction have been evaluated regarding the overall extractability of oil and protein from almond flour and almond cake (Almeida et al., 2019; Dias et al., 2020; Souza et al., 2019) as well as the functional properties of the almond protein (Dias and Bell, 2022; Amirshaghaghi et al., 2017; Sze-Tao and Sathe, 2000), the impact of enzymatic extraction on the digestibility and allergenicity of almond proteins has yet to be evaluated.

Though it is common practice to utilize proteolysis to produce hypoallergenic dietary products from different protein sources, this process can cause protein structural modifications that might alter their functional, nutritional, and biological properties (de Souza et al., 2020; Dias et al., 2020, 2022; Dias and de Moura Bell, 2022). Thus, the overall goal of this study was to determine the effects of proteolysis through enzymatic extraction (EAEP) of full-fat almond flour on the protein profile of excised gel bands by liquid chromatography-tandem mass spectrometry (LC-MS/MS), *in vitro* protein digestibility (total protein digestibility, degree of hydrolysis, and peptide quantification kinetics), and almond protein allergen quantification (Sandwich enzyme-linked immunosorbent assay, ELISA) and antigenicity (IgE and IgG Western blotting). Elucidation of the impact of sustainable solvent-free extraction methods (i.e., aqueous vs. enzyme-assisted aqueous extraction) on the digestibility and allergenicity of the extracted proteins is critical for the development of a bio-guided process that will deliver more nutritious and safer food ingredients for subsequent applications.

2. Materials and methods

2.1. Materials

Commercial almond flour (obtained from a mix of Californian *Prunus dulcis* varieties) was kindly provided by Blue Diamond Growers (Sacramento, CA, USA). Whole almonds and screenings were blanched, deskinned, and then sieved through a US#12 mesh (1.70 mm sieve size) (ultra-fine granulometry), with a minimum recovery of 85%. The particle size distribution of the flour used is described as follows: D [4,3] was 245 μm and the D (10), D (50), and D (90) were 0.4, 146, and 714 μm, respectively (Mastersizer 3000E - Malvern Panalytical Inc., Westborough, MA, USA). The almond flour proximate composition was 42.6 ± 0.6% of oil, 27.9 ± 0.8% of carbohydrates, 21.7 ± 0.6% of protein, 5.3 ± 0.1% of moisture, and 2.4 ± 0.1% of ash. Moisture, fat, and ash were determined according to AOAC methods 925.09, 989.05, and 920.125, respectively (AOCS, 1990). Protein content was determined by the Dumas combustion method using a conversion factor of 5.18 (Vario MAX cube, Elementar Analysensysteme GmbH, Germany). Carbohydrates were determined by difference (100 − the sum of other components) (Ghribi et al., 2015). Each analysis was performed in triplicate and data were reported as the mean ± standard deviation.

A neutral endoprotease from *Bacillus subtilis* (5.5–9.5 optimum pH range and 30 to 70 °C optimum temperature range, and 2×10^6 PC/g of activity) was kindly supplied by Bio-Cat (Bio-Cat Inc., Virginia, NY, USA). Sodium dodecyl sulfate (SDS), casein, and 1-anilino-8-naphthalenesulfonate (ANS) were acquired from VWR Inc. (Chicago, IL, USA). Pepsin from porcine gastric mucosa (3706 U/mg), pancreatin from porcine pancreas (100 U/mg), amylase from porcine pancreas (1005 U/mg), mucin, bile salts, L-serine, o-phthaldialdehyde (OPA), trifluoroacetic acid, and iodoacetamide were purchased from Millipore Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Soybean protein isolated powder was acquired in a local grocery store (Davis, CA, USA). Tris buffer, β-mercaptoethanol, Laemmli sample buffer, Coomassie Blue G250, and Dual-color standard (10–250 kDa) were purchased from Bio-Rad (Hercules, CA, USA). Trypsin (sequencing grade) was purchased from Promega (Madison, WI, USA). Methanol and acetonitrile were of LC-MS grade. All other chemicals were of analytical grade.

2.2. Almond protein extraction methods

Almond protein extracts were produced by aqueous (AEP - unhydrolyzed) and enzymatic extraction processes (EAEP - hydrolyzed) from full-fat almond flour, as described by (Dias and de Moura Bell, 2022). For the AEP, 700 g of almond flour was dispersed into water to achieve a 1:10 solids-to-liquid ratio (w/v) in a 10-L jacketed glass reactor (CG-1965-610M - Chemglass Life Sciences LLC, Vineland, NJ, USA). The extraction was performed at pH 9.0, 50 °C, for 60 min under constant stirring (120 rpm). For the EAEP, 0.5% (w/v) (weight of enzyme per weight of almond flour) of Neutral Protease was added to the slurry, and extractions were performed as described for the AEP. After the extraction, the slurry was centrifuged at 3000 × g for 30 min at 25 °C to remove the insoluble fraction. The liquid fraction was placed back into the glass reactor and allowed to separate overnight at 4 °C into the protein-rich phase (protein extract) and oil-rich phase (cream). AEP and EAEP protein extracts were stored at −20 °C until subsequent analysis. Each extraction process was performed in triplicate. The proximate composition of AEP and EAEP protein extracts, determined as described in item 2.1, was 57.3 and 59.2% protein (db., dry basis), 8.1 and 7.1% lipids (db.), 12.2 and 9.7% ash (db.), 18.7 and 20.9% carbohydrates (db.) respectively (Dias and de Moura Bell, 2022).

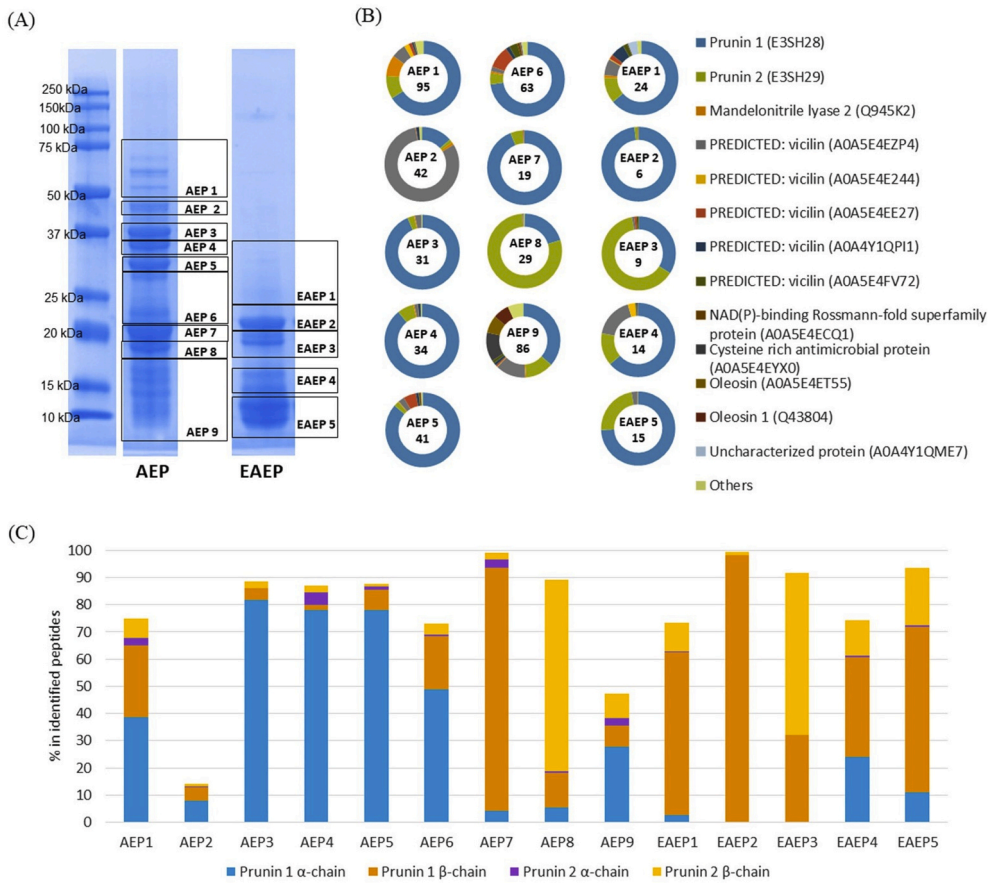

Fig. 1. SDS-PAGE protein profile of AEP and EAEP almond samples indicating the gel slices used for proteomics analysis (black squares) (A). Relative abundance of protein identifications (numbers in the middle of pie charts represent the number of proteins identified) (B) identifications of α- and β-chains in prunin 1 and prunin 2 (C) analyzed by LC-MS/MS-based proteomics analysis.

2.3. Proteomics analysis of excised gel bands

2.3.1. Protein electrophoresis-based separation (SDS-PAGE)

Proteins from AEP and EAEP extracts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent (β-mercaptoethanol) as described by Laemmli (1970). Samples were extracted using Laemmli buffer (1:1, v/v) for 5 min at 95 °C. Samples were then cooled at room temperature and loaded onto a precast 12% acrylamide gel. Electrophoretic separation was carried out at 200 V at room temperature for 1 h. Coomassie Blue G250 was used to stain the gel and a dual-color standard (10–250 kDa) was used as the molecular weight marker. The gel image and polypeptide distribution for the protein gels were obtained using the Gel DOC™ EZ Imager system and Image Lab software (Bio-Rad, Hercules, CA, USA). Gel bands were cut into 9 sections for AEP and 5 sections for EAEP extracts as shown in Fig. 1A. The excised gel bands were then placed in a 1.7 mL tube containing 150 μL of reverse osmosis water and stored at 4 °C until analysis.

2.3.2. Trypsin digestion

Each gel band section was diced into small pieces and placed in a 1.5 mL tube. In-gel digestion on the gel pieces was conducted as described by Gundry et al. (2009). Briefly, the gel pieces were destained with a water-methanol mixture (1:1, v/v), washed with water, and dehydrated with acetonitrile. Disulfide bonds were reduced with 100 μL of 10 mM dithiothreitol at 55 °C for 45 min and free cysteines were alkylated by 100 μL of 55 mM iodoacetamide at room temperature for 30 min. The gel pieces were washed with 25 mM ammonium bicarbonate in 50% acetonitrile (v/v) and dehydrated with acetonitrile. Trypsin digestion was performed by adding 10 μg/mL trypsin prepared in 25 mM ammonium bicarbonate to cover the gel pieces, incubating at 4 °C for 1 h and then at 37 °C overnight. The released peptides were collected by extracting the gel pieces with 50% acetonitrile and 1% trifluoroacetic acid in water (v/v/v). The peptide extract was dried using a centrifugal evaporator (MiVac Quattro, Genevac Ltd., Ipswitch, Suffolk, UK).

2.3.3. Peptide sample cleanup

The tryptic peptide sample was re-dissolved in 100 μL of 0.1% trifluoroacetic acid in water (v/v) and loaded to a C18 solid-phase extraction column (Discovery DSC-18, 500 mg, 3 mL tube, Millipore Sigma, St. Louis, MO, USA) preconditioned with 5 mL of acetonitrile followed by 5 mL of 0.1% trifluoroacetic acid. The column was washed with 6 mL of 0.1% trifluoroacetic acid. Peptides were recovered by flushing the column with 6 mL of a solution composed of 80% acetonitrile and 0.1% trifluoroacetic acid in water (v/v/v). The purified peptide sample was dried using a centrifugal evaporator, re-dissolved in 50 μL of 2% acetonitrile in water (v/v), and subsequently analyzed by LC-MS/MS.

2.3.4. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis

Purified tryptic peptides were analyzed by LC-MS/MS on a Q Exactive Plus Orbitrap Mass spectrometer in conjunction with Proxeon Easy-nLC 1200 HPLC (Thermo Scientific, Waltham, MA, USA) and Proxeon nanospray source. A volume containing 1 μg of peptides was loaded onto a 100 μm × 25 mm Dr. Maisch 100 Å 5U reverse-phase trap where the peptides were desalted online before being separated using a 75 μm × 150 mm Dr. Maisch 200 Å 3U reverse-phase column. Peptides were

eluted using an 80-min gradient with a flow rate of 300 nL/min. The mobile phase was composed of 0.1% formic acid in water (A) and acetonitrile (B). The gradient was programmed as follows: 0–48 min: 2–20% B; 48–60 min: 20–35% B; 60–62 min: 35–100% B; 62–64 min: 100% B; 64–65 min: 100–2% B; 65–80 min: 2% B. An MS survey scan was obtained for the mass-to-charge ratio (*m/z*) range 300–1600; MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to high-energy collisional dissociation (HCD). An isolation mass window of 1.6 *m/z* was used for the precursor ion selection, and normalized collision energy of 27% was used for fragmentation. A 5-s duration was used for the dynamic exclusion.

2.3.5. Data analysis

The LC-MS/MS data were analyzed by PEAKS Studio X+ (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Peptides and proteins were identified through a database search using almond (*Prunus dulcis*) protein sequences downloaded from the UniProt database (https://www.uniprot.org/, accessed 3/10/2020), including both Swiss-Prot and TrEMBL. The error tolerance was 10.0 ppm and 0.02 Da for the precursor and fragment ions, respectively. Semispecific digestion using trypsin as the enzyme with three maximal missed cleavages was used for predicting the precursor peptides. The variable modifications included deamidation on asparagine and glutamine, phosphorylation on serine, threonine, and tyrosine, oxidation on methionine, and carbamidomethylation on cysteine. Additional unspecific modifications and mutations were found by using the PEAKS PTM followed by the SPIDER function. Peptide identifications were filtered with the criterion of $-10\lg P \geq 35$ and protein identifications with $-10\lg P \geq 50$ as well as ≥ 5 unique peptides. Due to the existence of protein isoforms and homologous regions among different proteins in the UniProt protein database, manual curation was conducted to avoid redundant protein identifications by combining the proteins being identified mainly based on the same set of peptides into one protein identification.

2.4. In vitro protein digestibility

AEP and EAEP protein extracts were subjected to *in vitro* digestion to assess the impact of the extraction methods employed (aqueous vs. enzymatic extraction) on total protein digestibility and the effects of the simulated gastrointestinal digestion process on protein molecular weight profile, degree of hydrolysis, and peptide content. The *in vitro* simulated gastrointestinal digestion was performed as described by Bornhorst and Singh (2013) and de Souza, Dias, Oliveira, de Moura Bell, & Koblitz, 2020 using simulated saliva (SSF), gastric (SGF), and intestinal (SIF) fluids to mimic the oral-gastro-duodenal digestion. The composition of the simulated fluids is presented in Supplementary material A Table 1S-A. Casein and soybean isolated protein powders were used for comparison purposes. For the oral phase, 5 mL of each sample was mixed with 3.33 mL of SSF and vortexed for 30 s. Subsequently, the simulated oral bolus was mixed with 6.66 mL of SGF. The pH was adjusted to 3.0 and the gastric digesta was incubated for 120 min at 37 °C and 120 rpm. The simulated gastric digesta was mixed with 10 mL of SIF. The solution pH was adjusted to 7.0 and the simulated digesta was incubated for 120 min at 37 °C and 120 rpm. To stop the digestion, samples were heated in a water bath at 85 °C for 3 min. A 24% (w/v) trichloroacetic acid (TCA) solution was added to the samples in a 1:1 (v/v) proportion and the samples were centrifuged at 4000 rpm for 30 min at 4 °C. Total *in vitro* protein digestibility was calculated using Equation (1). Total nitrogen (NT) and nonprotein nitrogen (NPN - soluble fraction after TCA precipitation) were assessed by the Dumas combustion method (Vario MAX cube, Elementar Analysensysteme GmbH, Germany).

$$Protein\ digestibility\ (\%) = 100\ x \left(\frac{NPN_{after} - \left(NPN_{before} - NPN_{blank}\right)}{NT_{before} - NPN_{before}} \right) \quad \text{(Eq. 1)}$$

where: NPN_{after} = nonprotein after digestion, NPN_{before} = nonprotein before digestion, NPN_{blank} = enzyme blank and NT_{before} = total protein before digestion.

2.5. Protein molecular weight profile

SDS-PAGE was used to evaluate changes in the molecular weight of almond proteins in the AEP and EAEP extracts due to oral, gastro, and duodenal digestion. Aliquots of AEP and EAEP samples were collected at 0, 0.5, 30, 60, 90, 120, 150, 180, 210, and 240 min of digestion and placed in a water bath at 85 °C for 3 min to stop the digestion process. The protein molecular weight profile was assessed as described in item 2.3.1. The gel was imaged using a Gel Doc™ EZ Imager system and Image Lab software.

2.6. Degree of hydrolysis

The degree of hydrolysis (DH) of the aliquots from the digestibility kinetics was evaluated by the o-phthaldialdehyde method (OPA) as described by Nielsen et al. (2001). Briefly, 400 μL of a 2% (weight of freeze-dried protein extract powder/volume) solution was added to 3 mL of OPA reagent, the mixture was vortexed and let stand for 2 min at room temperature, and the absorbance was measured at 340 nm. An L-serine solution (0.9516 meqv/L) was used as standard. A blank solution was prepared with distilled water instead of sample and used as the reaction control. The protein percentage in the protein extracts was obtained by the Dumas method (conversion factor 5.18), with the equipment Vario MAX cube (Elementar Analysensysteme GmbH, Germany).

The DH was determined as follows:

$$\text{DH}\ (\%) = \frac{h}{h_{tot}}\ x\ 100 \quad \text{(Eq. 2)}$$

Where h is the number of hydrolyzed bonds. h_{tot} is the total number of peptide bonds per protein equivalent (7.58) (Liu et al., 2016).

2.7. Peptide quantification

Aliquots from the digestion kinetics were precipitated using ice-cold ethanol (2:1) (ethanol:sample), incubated for 2 h at −20 °C, and centrifuged at 4000×*g* for 30 min at 4 C. The supernatant was separated and used for the analysis. Briefly, 20 μL of diluted samples were pipetted in a 96-well plate followed by the addition of 200 μL of Fluoraldehyde™ o-phthaldialdehyde (OPA) reagent solution (Thermo Fisher Scientific-Waltham, MA, USA). A blank was made by adding water instead of sample to the solution. The microplate was agitated for 5 min in a shaker at 300 rpm. The sample's fluorescence was determined at 340 nm (excitation) and 455 nm (emission) using a microplate reader (SpectraMax iD5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, California, USA). BSA standard curve was prepared at 0, 4, 8, 16, 20, and 40 μg (r^2 = 0.995) and used for the peptide quantification. Samples were analyzed in triplicate.

2.8. Sandwich ELISA for almond immunoreactivity

Almond immunoreactivity of AEP (unhydrolyzed) and EAEP (hydrolyzed) samples was initially determined by the Veratox kit for almond allergen (Neogen, Lansing, MI, USA). Samples were prepared as recommended by the manufacturer and a rabbit antibody-based inhibition sandwich ELISA assay was used for detecting and quantifying the presence of amandin (AMP), a major allergenic protein in almonds. An AMP calibration curve at concentrations of 0, 2.5, 5, 10, 15, 20 and 25 mg/L (r^2 = 0.9997) was used. The samples (6 replicates for each AEP and EAEP extract) were diluted to fall within the AMP standard curve and read at 450 nm in a microplate reader (SpectraMax iD5 Multi-Mode

Microplate Reader).

2.9. Immunoreactivity by western blotting

2.9.1. Initial screening

Five human blood (P4C, P35C, P78C, P38, and P196b) sera from patients showing strong IgE reactivity to almonds were used for the initial screening. Sample BB12, from a patient showing no reactivity to almonds, was used as a negative control. AEP and EAEP samples (270 μg) were loaded in a 12% Bis/Tris preparative gel, which was run for 45 min before being transferred to a 0.45 μm nitrocellulose membrane according to the method described by Towbin et al. (1979). The five human sera samples previously known to be immunoreactive to almonds were tested at three dilutions (1:10, 1:20, and 1:40) along with a control sample with no known allergy (BB12). The sera were incubated overnight with the nitrocellulose strips at room temperature. The strips were then washed and incubated with mouse anti-Human IgE Fc HRP secondary antibody at 1:10,000 for detection. Those preliminary blots (Supplementary material – Fig. 1S A and B) showed that only Human sera P4C and P196b exhibited reactivity with bands above 60 kDa and below 20 kDa. Moreover, it was determined that a 1:20 dilution of human sera and a 1:5000 dilution (per the manufacturer's recommendation) of secondary antibody would be sufficient to show differences in reactivity to the protein samples.

2.9.2. IgE immunoblotting

Two samples were chosen to be run by Western blotting: P196b and P4C. In each of these blots, 26 μg of each almond extract (AEP and EAEP) was reduced and run on a 12% Bis/Tris 15 well gel for 45 min before being transferred to a nitrocellulose membrane. Samples P4C and P196b were used at a 1:20 dilution for IgE. The mouse anti-Human IgE Fc secondary antibody dilution was adjusted to 1:5000. Three extraction replicates for each extraction process (AEP and EAEP) were evaluated (A1–B3 for AEP and B1–B3 for EAEP). Band relative quantification was performed using Image J (Schneider et al., 2012).

2.9.3. IgG immunoblotting

Samples P196b and P4C were again chosen to be tested by Western blot. In each of these blots, 26 μg of each almond extract was reduced and run on a 12% Bis/Tris 15 well gel for 45 min before being transferred to a nitrocellulose membrane. Samples P4C and P196b were used at a 1:200 dilution. Goat anti-Human IgG secondary antibody was used at a dilution of 1–10,000. Three extraction replicates for each extraction process (AEP and EAEP) were tested (A1–A3 for AEP and B1–B3 for EAEP).

2.10. Statistical analysis

The results are given as the means ± one standard deviation. Data were analyzed in the Statistica™ Software (TIBCO Software Inc, Palo Alto, CA, US) using a one-way ANOVA and Tukey's post hoc with $p < 0.05$.

3. Results and discussion

3.1. Effects of enzymatic hydrolysis on the protein profile by proteomics analysis of the excised gel bands

The SDS-PAGE protein and peptide gels (Fig. 1A) showed that the use of neutral protease during the extraction significantly affected the composition and molecular weight profile of almond proteins. To better understand the impact of proteolysis on almond protein composition, LC-MS/MS-based proteomics analysis was carried out to identify the specific proteins of interest. Selected protein bands from the SDS-PAGE (Fig. 1A) were in-gel digested using trypsin, and the resulting peptide pool was analyzed.

Detailed information about the proteins identified (ranked by the total peak area of the tryptic peptides generated from each protein) from each gel slice, including protein accession, protein name, sequence coverage, total peak area, and relative abundance, was reported in Supplementary material B (Tables 1S-B to 13S-B). Peak areas of the peptides belonging to the same protein identification were summed up to estimate the relative abundance of the identified proteins in each gel slice. Protein identifications including at least five unique peptides, and having a total peak area of above 1.0×10^6 or a sequence coverage above 25%, were reported in (Tables 1S-B to 13S-B).

Fig. 1B summarizes the number of proteins identified and the major proteins (above 1% relative abundance) identified in the gel slices. For the AEP and EAEP samples, each gel slice included 19–95 and 6–24 proteins, respectively. Identification of fewer proteins in EAEP gel slices indicated that a significant portion of the proteins had already been hydrolyzed into low molecular-weight peptides by the enzyme used during the extraction, and that those small peptides were not captured in the SDS-PAGE gel.

Among the AEP gel slice samples, AEP 1 (~50–78 kDa), AEP 6 (~22–29 kDa), and AEP 9 (~9–18 kDa) had the highest number of proteins identified (95, 63, and 86, respectively), likely because these gel samples contained different proteins with a broader molecular weight range. In addition, the three gel slice samples only included the less intense bands, which might also enable the identification of lower abundance proteins. Regarding the relative abundance of different proteins, prunin 1 (in AEP 1, 3, 4, 5, 6, 7, and 9 and EAEP 1, 2, 4, and 5) or prunin 2 (in AEP 8 and EAEP 3) was the most abundant protein in most gel slices (Fig. 1B). Prunin 1 and prunin 2 are the components of the hexameric seed storage protein amandin; they are each composed of two polypeptides, the acidic α-chain and the basic β-chain, that are linked by a disulfide bridge (Garcia-Mas et al., 1995). In almonds, amandin accounts for ~65% of water-extractable proteins and is also considered the major almond protein allergen (Wolf and Sathe 1998). Therefore, the identification of ways to reduce amandin allergenicity is of great interest.

To assist in ascertaining the protein constitutions in each gel slice, the total peak areas of the identified tryptic peptides originated from the α- and β-chain regions of prunin 1 and prunin 2 (Garcia-Mas et al., 1995) were calculated separately (Fig. 1C). AEP 1 (~50–78 kDa) contained 66.0% of prunin 1 and 10.0% of prunin 2; these prunin bands were primarily the prunin 1 (61.0 kDa) and the prunin 2 (55.9 kDa) precursor polypeptides, which haven't been turned into mature protein by post-translational modification, as revealed by the detection of the α- and β-chain regions both in high abundance. AEP 3 (~39 kDa) and AEP 4 (~34 kDa) consisted of primarily prunin 1 (93.5 and 89.2%, respectively) and prunin 2 (2.9 and 7.1%, respectively), with a stronger contribution of the α-chain regions. According to the above results and the molecular weight reported by Garcia-Mas et al. (1995), prunin 1 α-chain (40.1 kDa) appeared to be the dominant protein constituent in AEP 3 and AEP 4, while prunin 2 α-chain (34.5 kDa) was present yet in a small portion in AEP 4. For AEP 5 (~30 kDa), the high abundance of prunin 1 (86.4%), especially its α-chain region (78.0%), indicated that this gel band might be associated with prunin 1 α-chain, although the molecular weight was slightly lower than the value cited in the literature (40.1 kDa). AEP 7 (~21.5 kDa) contained 93.8% of prunin 1, mainly belonging to the β-chain of the protein. In comparison, AEP 8 (~19 kDa) included 78.6% of prunin 2 and 20.3% of prunin 1, also pertaining to the β-chain of prunin 2. Therefore, AEP 7 and AEP 8 were mainly composed of prunin 1 β-chain (20.9 kDa) and prunin 2 β-chain (21.4 kDa), respectively. For AEP 2 (~46 kDa), AEP 6 (~22–29 kDa), and AEP 9 (~9–18 kDa), the sum of the percentages of prunin 1 and prunin 2 was near 15, 80, and 50%, respectively. The molecular weights of the three gel slice samples were dissimilar to either the precursor or the α- and β-chains of prunin 1 and prunin 2. A previous study also showed that several minor bands, other than the above-mentioned major polypeptides, were observed on the SDS-PAGE gel of almond extracts (Sathe

Table 1
Identification of allergen proteins in bands from SDS-PAGE of AEP and EAEP samples by LC-MS/MS analysis of tryptic peptides produced by in-gel protein digestion, coupled with commercial software-based protein identification.

Band	UniProt accession	Protein	Allergens	MW (kDa)	Number of AAs	−10lgP	Coverage (%)	Number of peptides
AEP 1	E3SH28	Prunin 1	Pru du 6.0101	63.1	551	496.20	83	232
	Q43607	Prunin 1	Pru du 6	63.0	551	493.85	80	225
	Q43608	Prunin 2 (Fragment)	Pru du 6	57.1	504	368.24	59	64
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57.0	504	366.23	62	66
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61.2	563	339.11	50	61
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46.9	431	263.06	44	18
AEP 2	E3SH28	Prunin 1	Pru du 6.0101	63.1	551	462.80	70	138
	Q43607	Prunin 1	Pru du 6	63.0	551	460.18	73	141
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61.2	563	306.37	36	31
	Q43608	Prunin 2 (Fragment)	Pru du 6	57.1	504	302.14	43	24
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57.0	504	300.72	44	26
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46.9	431	279.64	52	22
AEP 3	E3SH28	Prunin 1	Pru du 6.0101	63.1	551	585.21	86	360
	Q43607	Prunin 1	Pru du 6	63.0	551	573.74	86	356
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57.0	504	358.60	59	54
	Q43608	Prunin 2 (Fragment)	Pru du 6	57.1	504	352.32	53	49
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46.9	431	285.39	53	22
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61.2	563	273.63	36	26
AEP 4	Q43607	Prunin 1	Pru du 6	63017	551	534.47	83	268
	E3SH28	Prunin 1	Pru du 6.0101	63052	551	533.61	83	267
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	391.59	66	78
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	379.54	60	73
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	259.22	35	22
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	153.61	11	4
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	171.86	31	7
AEP 5	E3SH28	Prunin 1	Pru du 6.0101	63052	551	577.89	89	350
	Q43607	Prunin 1	Pru du 6	63017	551	570.30	89	347
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	367.77	68	63
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	364.50	62	60
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	259.06	34	22
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	204.12	47	11
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	202.36	33	10
AEP 6	E3SH28	Prunin 1	Pru du 6.0101	63052	551	513.70	82	259
	Q43607	Prunin 1	Pru du 6	63017	551	509.53	81	257
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	337.12	53	44
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	334.88	56	45
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	289.24	34	29
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	214.49	50	15
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	267.59	45	18
AEP 7	E3SH28	Prunin 1	Pru du 6.0101	63052	551	600.56	90	343
	Q43607	Prunin 1	Pru du 6	63017	551	591.88	88	339
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	488.00	75	113
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	470.56	65	103
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	219.98	45	12
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	213.20	23	12
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	108.38	8	3
AEP 8	E3SH28	Prunin 1	Pru du 6.0101	63052	551	506.61	87	243
	Q43607	Prunin 1	Pru du 6	63017	551	492.43	85	239
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	488.41	72	231
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	439.33	66	194
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	212.65	38	11
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	181.35	25	10
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	141.29	18	6
AEP 9	E3SH28	Prunin 1	Pru du 6.0101	63052	551	545.32	84	246
	Q43607	Prunin 1	Pru du 6	63017	551	536.21	82	237
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	433.10	74	111
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	419.01	72	99
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	395.81	74	54
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	277.60	52	18
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	277.39	43	26
	B6CQR7	PR-10	Pru du 1	17,636	160	182.58	40	6

(continued on next page)

Table 1 (*continued*)

Band	UniProt accession	Protein	Allergens	MW (kDa)	Number of AAs	−10lgP	Coverage (%)	Number of peptides
	Q8GSL5	Profilin	Pru du 4, Pru du 4.0101, Pru du 4.0102	14061	131	56.15	10	1
EAEP 1	E3SH28	Prunin 1	Pru du 6.0101	63052	551	475.5	67	144
	Q43607	Prunin 1	Pru du 6	63017	551	461.55	69	145
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	316.32	38	36
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	307.9	41	36
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	263.89	31	18
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	212.63	37	11
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	190.75	24	8
EAEP 2	E3SH28	Prunin 1	Pru du 6.0101	63052	551	544.71	59	292
	Q43607	Prunin 1	Pru du 6	63017	551	538.75	60	295
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	312.09	48	39
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	280.98	38	34
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	110.47	13	3
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	92.56	6	3
EAEP 3	E3SH28	Prunin 1	Pru du 6.0101	63052	551	546.9	74	237
	Q43607	Prunin 1	Pru du 6	63017	551	538.47	76	236
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	521.84	62	220
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	492.24	62	195
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	187.12	29	7
EAEP 4	Q43607	Prunin 1	Pru du 6	63017	551	568.8	83	299
	E3SH28	Prunin 1	Pru du 6.0101	63052	551	567.59	85	298
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	449	65	87
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	431.8	59	80
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	253.92	43	21
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	150.79	14	7
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	70.07	5	2
EAEP 5	E3SH28	Prunin 1	Pru du 6.0101	63052	551	613.12	83	387
	Q43607	Prunin 1	Pru du 6	63017	551	571.58	85	385
	E3SH29	Prunin 2 (Fragment)	Pru du 6, Pru du 6.0201	57006	504	434.04	69	139
	Q43608	Prunin 2 (Fragment)	Pru du 6	57052	504	409.93	65	118
	A0A516F3L2	Cysteine-rich antimicrobial protein	Pru du 8, Pru du 8.0101	31068	264	311.52	56	19
	Q945K2	Mandelonitrile lyase 2	Pru du 10	61158	563	123.33	8	4
	P82952	γ-Conglutin 1	Pru du γ-conglutin	46945	431	71.83	5	2

et al., 2002). The compositions of the three samples' α- and β-chain regions were close to AEP 1 which contained the precursor polypeptides (Fig. 1C), suggesting that AEP 2, AEP 6, and AEP 9 may include the protein fragments of both prunin 1 and prunin 2 within the respective molecular weight ranges.

The EAEP samples contained 74.9–99.5% of prunin 1 and prunin 2, which is similar to the percentages in most of the AEP gel samples (76.0–96.4%, excluding AEP 2). However, all of the EAEP gel samples were dominated by the β-chain regions of prunin 1 and prunin 2 (Fig. 1C), demonstrating the higher resistance of the β-chain against the proteolysis by the protease used. EAEP 1 (~25–35 kDa) consisted of some faint smeared bands, which included 63.8% of prunin 1 (2.6% α-chain and 66.0% β-chain) and 11.1% of prunin 2 (0.2% α-chain and 10.7% β-chain). Because the AEP's gel bands above 25 kDa were mostly not seen in the EAEP extracts, EAEP 1 should mainly contain partially hydrolyzed products from these proteins and any unhydrolyzed proteins that maintained their original molecular weight. Besides the high abundance of the β-chain regions, EAEP 1 also contained some vicilins (predicted) with various sequences (Fig. 1B). Because the molecular weight of intact vicilin protein chains is much higher than the upper range of EAEP 1 (e.g., A0A5E4EZP4: 90.4 kDa; A0A5E4FV72: 57.6 kDa), the vicilins present in EAEP 1 should be hydrolyzed products of vicilin proteins. EAEP 2 (~21–22 kDa) contained 98.0% of the β-chain region and possessed a similar molecular weight to prunin 1 β-chain (20.9 kDa), indicating that EAEP 2 was mainly the intact prunin 1 β-chain. EAEP 3 (~18.5–20 kDa) also consisted of mostly the β-chain regions of prunin 1 (31.7%) and prunin 2 (59.6%), which were suggested to be the identities of the two major bands on EAEP 3. EAEP 4 (~15–17 kDa) and EAEP 5 (~9–14 kDa) had 77.9% and 97.1%, respectively, of the combination of Prunin 1 and prunin 2, with the majority belonging to the β-chain region. Similar to the AEP 9, the molecular weight ranges of EAEP 4 and EAEP 5 samples were below the sizes of the precursor polypeptides and the α- and β-chains of prunin 1 and prunin 2. Thus, these gel bands contained mainly the proteolyzed products from prunin 1 and prunin 2. Overall, in all the EAEP gel slices, only EAEP 4 and EAEP 5, representing the lower molecular weight range, contained a small portion of the α-chain region of prunin 1. Prunin 2 α-chain region only accounted for 0.0–0.6% of the abundance in the EAEP gel slices. These results reveal that the α-chain regions were easier to be hydrolyzed by the neutral protease than the β-chain regions. Despite prunin 1 α-chain being observed in high abundance in the AEP samples, during EAEP, it was likely broken down into low-molecular-weight peptides that cannot be detected by the SDS-PAGE.

Overall, SDS-PAGE protein gels (Fig. 1A) demonstrated that enzymatic extraction can significantly alter the molecular weight profile of the proteins in the extracts. While AEP proteins were mainly composed of two polypeptide fragments of 38 kDa (amandin α-subunit) and 22 kDa (amandin β-subunit), proteolysis resulted in complete degradation of the basic subunit of amandin while no significant changes were observed for the acidic subunit (22 kDa).

Table 2
Relative abundance (‰)[a] of epitope sequences in prunin 1 and prunin 2 in SDS-PAGE gel spot samples.

Epitope sequence[b]	AEP 1	AEP 2	AEP 3	AEP 4	AEP 5	AEP 6	AEP 7	AEP 8	AEP 9	EAEP 1	EAEP 2	EAEP 3	EAEP 4	EAEP 5
Prunin 1 α-chain														
118 SSQQGRQQEQEQERQ 132	4.534[c]	0.518	8.611	5.644	10.425	1.262	0.451	1.005	4.392	0.035	0	0.003	4.934	1.528
145 QQEQQQERQGRQQGR 159	7.609	1.184	14.493	10.907	19.739	2.165	1.732	4.499	1.974	0.071	0	0	0.057	0
161 QQEEGRQQEQQQGQQ 175	47.034	14.084	104.519	98.924	132.942	57.731	4.896	3.212	11.409	0.546	0.006	0.011	0.066	0.027
225 LFHVSSDHNQLDQNP 239	2.470	0.630	7.652	7.852	5.544	5.579	0.396	0.497	4.632	0.389	0.004	0.011	1.447	7.381
281 QQEQQGSGNNVFSGF 295	0.020	0.000	0.147	0.017	0.036	0.073	0.005	0.013	0.066	0.000	0	0.000	0.001	0.013
Prunin 1 β-chain														
510 RALPDEVLANAYQIS 524	5.260	0.858	1.340	1.177	2.804	3.658	38.079	5.066	2.820	13.485	31.368	18.367	18.931	25.413
Prunin 2 α-chain														
17 FGQNKEWQLNQLEAR 31	0	0	0	0	0	0	0	0	0	0	0	0	0	0
105 DSQPQQFQQQQQQQQ 119	0.165	0.011	0.000	0.147	0.042	0.014	0.026	0	0.090	0	0	0	0.038	0
121 RPSRQEGGQGQQQFQ 135	0.245	0.006	0.033	0.419	0.160	0.100	2.627	0.424	1.428	0.031	0.001	0.036	0.358	1.088
185 QNQLDQVPRRFYLAG 199	1.071	0.100	0.074	3.116	0.523	0.219	0.733	0.094	1.159	0.017	0.002	0.026	0.047	0.157
209 QQGRQQQQQQQGQQG 223	0.874	0.078	0.053	2.386	0.369	0.169	0.500	0.073	0.949	0.013	0.002	0.013	0.035	0.067
225 GNNIFSGFDTQLLAQ 239	0.004	0.000	0	0.002	0.002	0.000	0.001	0.000	0.030	0	0	0	0	0
281 RGDQERQQEEQQSQR 295	0.545	0.123	0.032	0.335	0.027	0.153	0.129	0.021	0.050	0	0	0	0.001	0
Prunin 2 β-chain														
465 QNAFRISRQEARNLK 479	0.545	0.200	0.581	1.156	0.174	0.425	0.299	15.808	3.679	1.166	0.213	14.189	1.383	6.075

High to low

[a] Relative abundance was expressed in the total peak area of the epitope region/total peak area of the identified peptides in the same gel spot.
[b] Epitope sequences were reported by Willison et al., 2011.
[c] Background color from red to green represents abundance from high to low.

3.2. Identification of protein allergens

To date, several protein allergens in almonds, including Pru du 1, Pru du 2, Pru du 3, Pru du 4, Pru du 5, Pru du 6, Pru du 8, Pru du 10, and Pru du AP, have been reported (Supplementary material A-Table 2S A). By comparing the UniProt accession numbers of the protein allergens with the proteomics data from the gel slice samples, protein allergens present in each gel slice were identified and listed in Table 1. Pru du 1, Pru du 4, Pru du 6, Pru du 8, Pru du 10, and Pru du AP were found in at least one gel slice. Among these, Pru du 1 (PR-10) and Pru du 4 (profilin) were exclusively identified in AEP 9. As mentioned above, prunin 1 and prunin 2, which belong to the allergen Pru du 6 (amandin), were found in all the gel slice samples with a significant sequence coverage. Pru du 10 (mandelonitrile lyase 2) and Pru du 8 (cysteine-rich antimicrobial protein) were the other two protein allergens found, which had ≥1% relative abundance in at least one gel slice. Because all the bands in AEP 1 through AEP 5 (~30–78 kDa) were almost imperceptible on the SDS-PAGE gel of EAEP, the proteins in this range, including the precursor polypeptides and the α- and β-chains of Pru du 6 as well Pru du 10, were likely broken down into smaller fragments by the neutral protease during EAEP, which could indicate a reduction in the antigenicity potential of this sample.

Mandelonitrile lyase 2 (Pru du 10), with a theoretical molecular weight of 60.0 kDa (for the mature protein without modifications) and four N-glycosylation sites (Dreveny et al., 2001), is a recently identified protein allergen (Kabasser et al., 2021) in almonds. Dreveny et al. (2001) showed that mandelonitrile lyase 2 isolated from almond flour appeared as a single band on SDS-PAGE gel at ~60 kDa, which is similar to the theoretical value. Thus, mandelonitrile lyase 2 was expected to be found in the AEP 1 gel cut sample (~50–78 kDa). According to the proteomics analysis, mandelonitrile lyase 2 accounted for 9.2% of the protein abundance in AEP 1, with a sequence coverage of 50%. Because the molecular weight of mandelonitrile lyase 2 is close to the molecular weight of the prunin 1 precursor polypeptide, it is difficult to annotate the exact band for mandelonitrile lyase 2 in the AEP 1 sample. However, the absence of bands on the EAEP gel in the same molecular weight region (~60 kDa) indicated the destruction of mandelonitrile lyase 2 during enzymatic extraction (EAEP). Mandelonitrile lyase 2 was also found in other AEP gel slice samples with a sequence coverage ranging from 23 to 43%, but the relative abundance in those samples was low (0.0–1.3%). This protein was detected in some of the EAEP gel slice samples with a sequence coverage of 6–31%. The highest sequence coverage (31%) and the highest relative abundance (1.3%) were found in EAEP 1. As the bands in EAEP 1 were extremely faint, it can be concluded that mandelonitrile lyase 2 was drastically decreased by the neutral protease used in EAEP.

Cysteine-rich antimicrobial protein, which was initially speculated to be 2S albumin based on sequence similarity (Poltronieri et al., 2002) and later to be vicilin (Garino et al., 2015), was recently identified as a new family of allergen proteins (Che et al., 2019), named Pru du 8 in the WHO/IUIS database. Che et al. (2019) showed that the recombinant cysteine-rich antimicrobial protein band located at ~31 kDa on an SDS-PAGE gel. This protein was found to be present in the AEP 9 gel slice (~9–18 kDa) with a significant abundance (12.6%) and had the highest sequence coverage (74%; 84% when excluding the signal peptide sequence f(1–30)) (Supplementary Material Figs. 2S–A) and the greatest number of identified peptides (54 sequences) than all the other gel slice samples. Although it was also identified in other AEP gel slices (e.g., AEP 5 and AEP 6), the relative abundance was low (<0.5%). The primary location of cysteine-rich antimicrobial protein on an SDS-PAGE gel found in the present study appeared to agree with the band location of its natural form at 12 and 13 kDa reported by Poltronieri et al. (2002) and Kabasser et al. (2021), respectively. Poltronieri et al. (2002) found that the isolated 12 kDa IgE-binding protein contained the N-terminal region of cysteine-rich antimicrobial protein, whereas Kabasser et al. (2021) identified the purified 13 kDa protein as a C-terminal fragment of the same protein. Interestingly, in the current study, peptide identifications for AEP 9 gel cut covered both the N- and C-terminal regions (Supplementary Material Figs. 2S–A). The lower molecular weight of the natural protein compared with the recombinant protein might be related to uncharacterized post-translational proteolytic processing (Che et al., 2019). It is possible that the two previous studies (Poltronieri et al., 2002; Kabasser et al., 2021) isolated the N- and C-terminal fragments, respectively, of cysteine-rich antimicrobial protein due to the use of different purification techniques. Because no prior purification was conducted in the current study, both fragments were identified in the gel

Table 3
Low-molecular-weight peptides[a] identified by LC-MS containing epitope sequences from prunin 1 and prunin 2 found in the AEP and EAEP skims.

Epitope sequence[b]	Peptide sequence	
	AEP	EAEP
Prunin 1 α-chain		
118 SSQQGRQQEQEQERQ 132	–	–
145 QQEQQQERQGRQQGR 159	–	–
161 QQEEGRQQEQQQGQQ 175	GRQQQEEGRQQEQQQGQQGRPQ, RQQQEEGRQQEQQQGQQGRPQQ, GRQQQEEGRQQEQQQGQQGRPQQ, GRQQQEEGRQQEQQQGQQGRPQQQ, GRQQQEEGRQQEQQQGQQGRPQQQQ, GRQQQEEGRQQEQQQGQQGRPQQQQQ, GRQQQEEGRQQEQQQGQQGRPQQQQQFRQ	–
225 LFHVSSDHNQLDQNP 239	LFHVSSDHNQLDQNPRK, YNDGDQELVAVNLFHVSSDHNQLDQNPRK, WSYNDGDQELVAVNLFHVSSDHNQLDQNPRK, YNDGDQELVAVNLFHVSSDHNQLDQNPRKFY, YWSYNDGDQELVAVNLFHVSSDHNQLDQNPRK, VAYWSYNDGDQELVAVNLFHVSSDHNQLDQNPRK, VAYWSYNDGDQELVAVNLFHVSSDHNQLDQNPRKF	– – –
281 QQEQQGSGNNVFSGF 295	–	–
Prunin 1 β-chain		–
510 RALPDEVLANAYQIS 524	LRALPDEVLANAYQISREQ, FLRALPDEVLANAYQISREQ, LRALPDEVLANAYQISREQARQ, FLRALPDEVLANAYQISREQARQ, LRALPDEVLANAYQISREQARQL, LRALPDEVLANAYQISREQARQLK, FLRALPDEVLANAYQISREQARQLK, LRALPDEVLANAYQISREQARQLKY, FLRALPDEVLANAYQISREQARQLKY	–
Prunin 2 α-chain		
17 FGQNKEWQLNQLEAR 31	–	–
105 DSQPQQFQQQQQQQQ 119	–	–
121 RPSRQEGGQGQQQFQ 135	FRPSRQEGGQGQQQFQGEDQQDRHQK	–
185 QNQLDQVPRRFYLAG 199	–	–
209 QQGRQQQQQQQGQQG 223	LAGNPQDEFNPQQQGRQQQQQQQGQQGNGNNIFSGFDTQ	–
225 GNNIFSGFDTQLLAQ 239	–	–
281 RGDQERQQEEQQSQR 295	–	–
Prunin 2 β-chain		
465 QNAFRISRQEARNLK 479	–	–

[a] Low-molecular-weight peptides were identified from AEP and EAEP extracts by Huang et al. (2022).
[b] Epitope sequences were reported by Willison et al. (2011).

slice AEP 9. Cysteine-rich antimicrobial protein possibly contains several disulfide bridges, which might make the protein more resistant to proteolysis. Indeed, the cysteine-rich antimicrobial protein was detected in EAEP 4 and EAEP 5, which covered most of the AEP 9 molecular weight range. Nonetheless, the lower relative abundance (0.5% and 0.1%, respectively), sequence coverage (43% and 56%, respectively), and the number of peptides identified (21 and 19 sequences, respectively) in the EAEP 4 and EAEP 5 gel cuts, compared with AEP 9, suggested that, during the EAEP, the enzyme partially damaged the linear structure of this protein allergen. Overall, the use of protease to assist the extraction of almond proteins led to the reduction of the presence of allergen-related proteins, which could indicate a potential reduction in its antigenicity.

3.3. Impact of extraction conditions on prunin 1 and prunin 2 epitope sequences

Proteolysis reduces the molecular weight of proteins by breaking them down into smaller fragments. SDS-PAGE and proteomics analysis results for the gel slice samples revealed a decrease in several protein allergens after the use of neutral protease during the extraction step. However, epitopes eliciting allergenic responses, especially linear epitopes, might still be intact after partial proteolysis. Therefore, further investigations on whether immunoreactive epitopes are affected by the use of protease are necessary.

Willison et al. (2011) identified six and eight IgE-reactive epitope sequences from prunin 1 (Pru du 6.01) and prunin 2 (Pru du 6.02), respectively, using overlapping peptides and a pooled serum from almond allergic patients. To better understand the impact of using neutral protease during extraction on the epitope sequences in almond proteins, the relative abundance of these sequences was estimated by calculating the proportion of the epitope sequence regions among all the identified tryptic peptides (Table 2). The results showed that the epitope sequence of prunin 1 f(161–175) represented a significant abundance in the gel cuts AEP 1 and AEP 3–6 (47‰−133‰). The abundance of the same epitope sequence in the EAEP gel cuts was much lower than in the AEP ones, demonstrating that the enzyme greatly destroyed this epitope at the protein level. Overall, lower epitope abundances in the EAEP samples were observed for several epitope sequences, including prunin 1 f(145–159), prunin 2 f(185–199), prunin 2 f(209–223), and prunin 2 f (281–295) compared with AEP samples. These epitope sequences are all located in the α-chain region, which suffered extensive proteolysis during EAEP (Fig. 1C).

In contrast, the epitope sequences in the β-chain, including prunin 1 f (510–524) and prunin 2 f(465–479), generally had similar or higher relative abundance in the EAEP gel samples compared to the AEP. AEP 7 and EAEP 2, which were the main bands containing prunin 1 β-chain, had comparable relative abundances of prunin 1 f(510–524) (38‰ and 31‰, respectively). Similarly, AEP 8 and EAEP 3, which consisted of mainly prunin 2 β-chain, contained similar relative abundances of prunin 2 f(465–479) (16‰ and 14‰, respectively). This reveals that a significant portion of the two epitope sequences was still encrypted in the β-chain despite the use of the enzyme in the EAEP. The resistance of the epitope sequences of prunin 1 f(510–524) and prunin 2 f(465–479), located in the β-chain regions, is in agreement with the resistance of the β-chains against hydrolysis by the neutral protease. Because the protease substantially reduced the α-chains during EAEP, the β-chains became dominant in all the EAEP gel slices (Fig. 1C). This could, in turn, explain the higher relative abundance of prunin 1 f(510–524) and prunin 2 f (465–479) from β-chains in EAEP 1, EAEP 4, and EAEP 5 (Table 2).

In addition to being encrypted in proteins and larger peptides that can be detected by Tris-glycine SDS-PAGE (~6–400 kDa), linear epitopes may also exist in low-molecular-weight peptides (<5 kDa). Low-molecular-weight peptides can be naturally occurring or generated by enzymatic hydrolysis. Our previous study characterized low-molecular-weight peptides present in the almond skim fraction (protein-rich extract) generated by AEP and EAEP with LC-MS/MS by searching against the UniProt protein database (Huang et al., 2022). In that study, a total of 554 and 1219 low-molecular-weight peptides were identified from AEP and EAEP extracts, respectively, with the majority originating

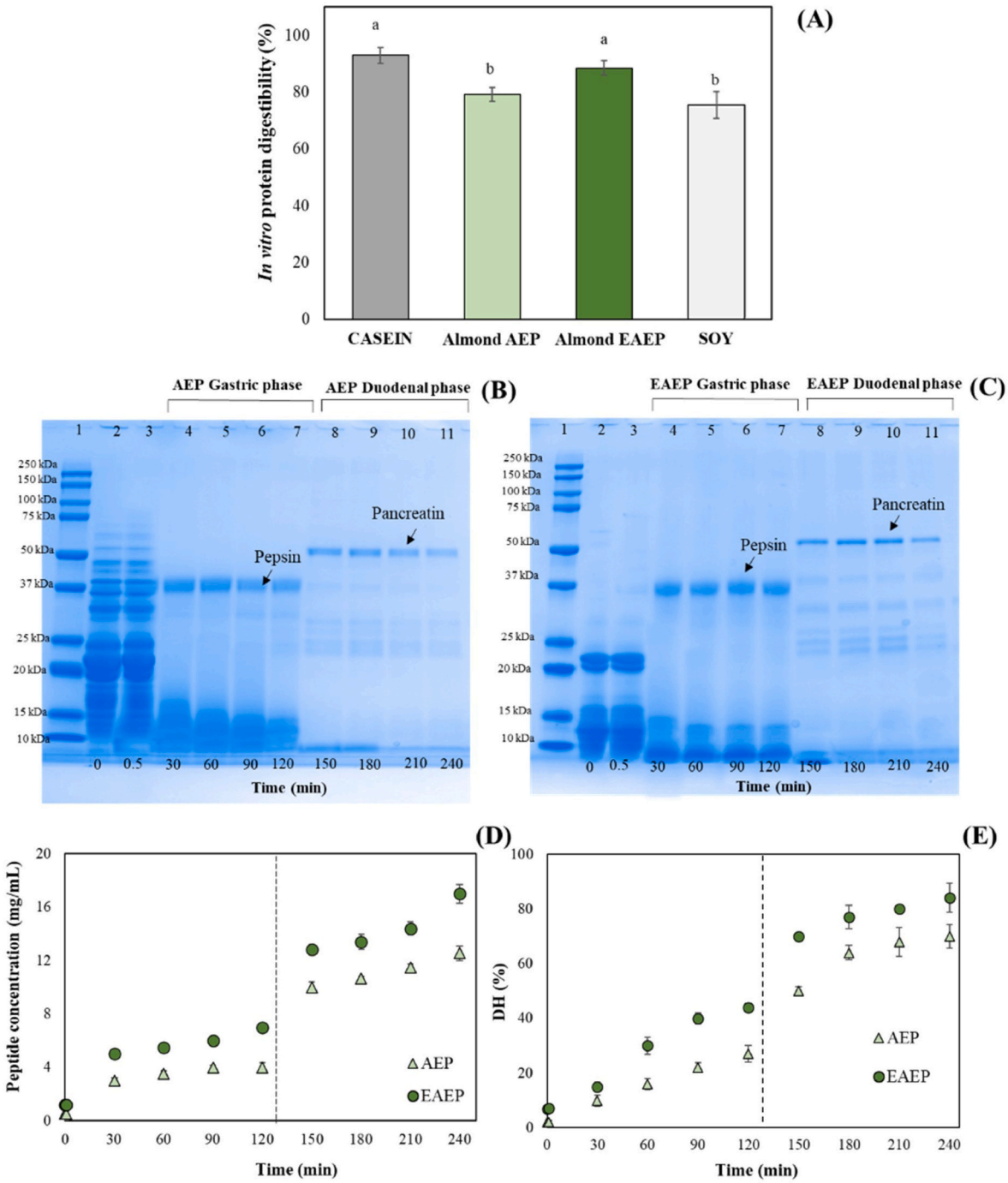

Fig. 2. Total *in vitro* protein digestibility of casein, unhydrolyzed (AEP) and hydrolyzed (EAEP) almond protein extracts and soybean isolated protein. Different letters indicate a significant difference between samples at $p < 0.05$ (A). SDS-PAGE of digestion kinetics for the AEP (B) and EAEP (C) almond proteins samples, arrows indicate the pepsin in the gastric phase and the pancreatin in the intestinal phase. Peptide concentration (D) and degree of hydrolysis (DH) (E) of AEP and EAEP proteins samples.

from prunin 1 (193 and 194 sequences, respectively) and prunin 2 (96 and 133 sequences, respectively). By searching the IgE-reactive epitope sequences reported by Willison et al. (2011), 25 peptide sequences in the AEP extract were found to contain the full sequence of any of the IgE-reactive epitopes (Table 3). The IgE-reactive epitope sequences involved in the 25 peptides include prunin 1 f(161–175) (seven peptides), prunin 1 f(225–239) (seven peptides), prunin 1 f(510–524) (nine peptides), prunin 2 f(121–135) (one peptide), and prunin 2 f(209–223) (one peptide). Conversely, none of the peptides present in the EAEP samples contain any IgE-reactive epitopes in a full sequence, demonstrating that proteolysis disrupted IgE-reactive epitopes encrypted in low-molecular-weight peptides.

3.4. *Effects of protein hydrolysis on in vitro protein digestibility*

Extraction conditions, especially proteolysis, can significantly affect the protein *in vitro* digestibility of the extracted protein. Protein hydrolysates have been reported as a suitable source of protein for human nutrition, as their gastrointestinal absorption seems to be more effective than that of intact proteins (Grimble 1991).

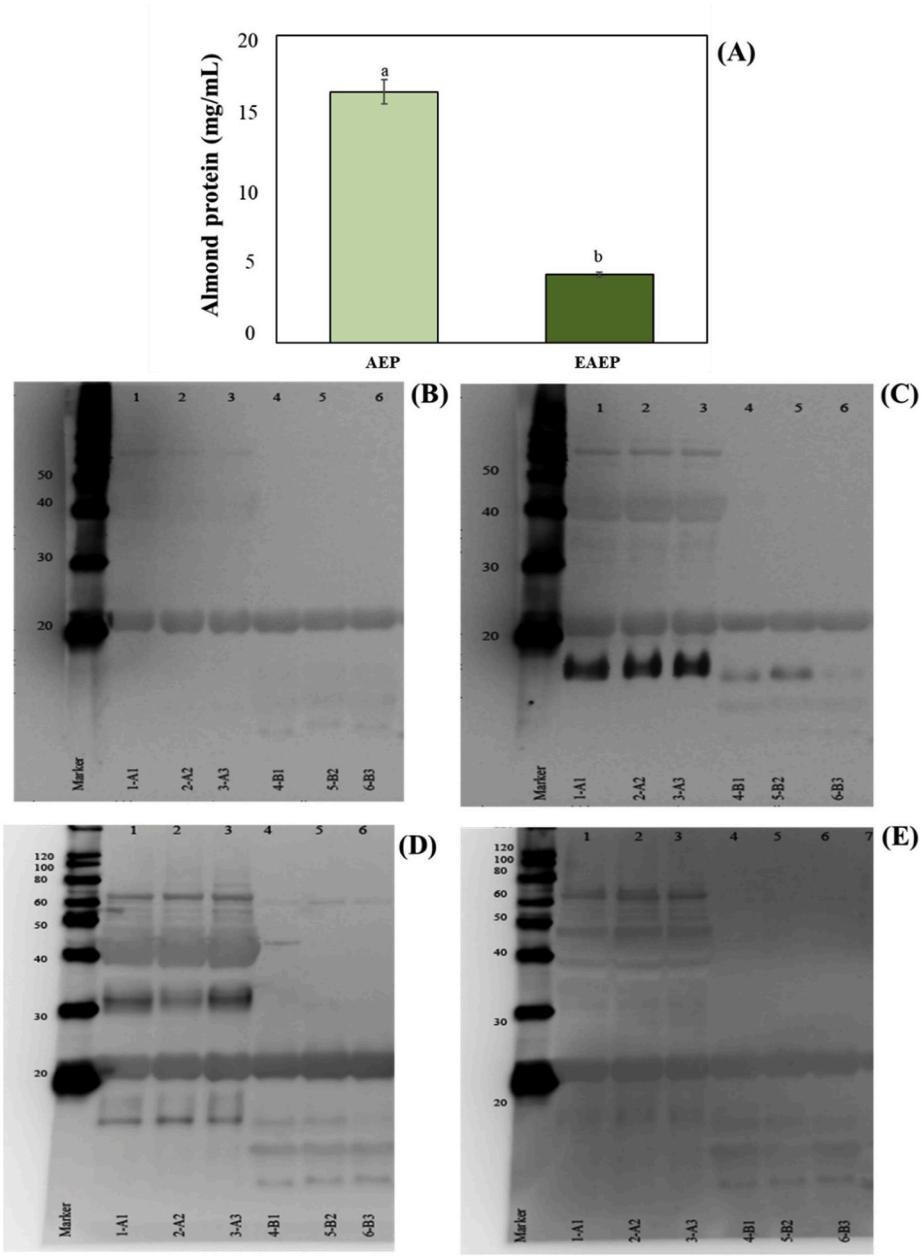

Fig. 3. Total almond protein immunoreactivity by sandwich ELISA (A). Different letters indicate a significant difference between samples at $p < 0.05$. Western blot of AEP (unhydrolyzed samples - A1 to A3) and EAEP (B1 to B3): Human serum sample P4C, Primary: human sera 1:20, Secondary: mouse anti-Human IgE Fe 1:5000 (B); Human serum sample P196b, Primary: human sera 1:20, Secondary: mouse anti-Human IgE Fc 1:5000 (C); Human serum sample P4C, Primary: human sera 1:200, Secondary: goat anti-Human IgG Fc 1:10,000 (D); Human serum sample P196b, Primary: human sera 1:200, Secondary: goat anti-Human IgG Fe 1:10,000 (E).

The *in vitro* digestibility of almond proteins from the unhydrolyzed (AEP) and hydrolyzed samples (EAEP) are shown in Fig. 2A. Casein and soy protein isolated powder were also evaluated for comparison purposes. Proteolysis significantly improved the *in vitro* protein digestibility of almond protein samples from 79.1 ± 2.4% to 88.5 ± 3.6%. While casein and soy protein exhibited the highest and lowest digestibility values (92.9 ± 2.7% and 72.3 ± 4.3%, respectively), casein digestibility was not significantly different from that of the almond hydrolysates (EAEP). Casein is commonly used as a reference protein for *in vitro* protein digestibility assays, and the value herein reported agrees with the ones reported for casein digestibility (92–99%) (Alonso et al., 2000; El-Aal et al., 1986). The higher protein digestibility of the EAEP samples can be attributed to the partial breakdown (moderate degree of protein hydrolysis) of large protein bodies into smaller sizes by the protease (He et al., 2015), which can facilitate the access of digestive enzymes (pepsin and pancreatin) to the protein sites due to reduction in steric hindrance, leading to an improvement in the protein hydrolysis during digestion.

Similar findings have been shown for air-classified pea protein-enriched flour where protein digestibility increased from 84 to 89% after hydrolysis by papain (degree of hydrolysis (DH) of 11%) (Konieczny et al., 2020). The present study's results differ from the ones reported by de Souza, Dias, Oliveira, de Moura Bell, & Koblitz, 2020 in that a decrease in the *in vitro* protein digestibility from 73 to 64% was observed after the use of alkaline protease to assist the extraction of protein and oil from the almond cake. It is important to highlight that besides the difference in starting material (full-fat almond flour in the present study vs. almond cake in the cited literature), the aforementioned study used a different protease (alkaline protease) to assist the extraction and the extracted protein had a significantly higher degree of hydrolysis (7% in the present study vs. 23% in the literature) before digestion. Extensive hydrolysis could entail fewer attack sites available for the digestive enzymes (pepsin and pancreatin), which could have underestimated this parameter (Souza, Dias, Oliveira, de Moura Bell, & Koblitz, 2020).

The protein profile of AEP and EAEP samples during oral (0.5 min), gastric (30, 60, 90, 120 min), and duodenal (150, 180, 210, and 240

min) digestion was evaluated by SDS-PAGE (Fig. 2B and C). The oral phase did not affect the protein profile of AEP and EAEP samples as expected. However, after 30 min of digestion, significant proteolysis was observed for both samples, with AEP and EAEP samples having the majority of protein bands <18 kDa and <14 kDa, respectively. These results agree with the ones reported by Sathe (1993), where only peptides <20 kDa were found after 30 min of gastric digestion of the almond flour proteins and demonstrate that proteolysis before digestion can enhance protein hydrolysis by gastric enzymes. Similar results were reported by Souza et al., 2019), where faster digestion of almond cake hydrolysates by pepsin was observed during *in vitro* digestion.

From 60 to 90 min of gastric digestion the protein profile remained unchanged. At 120 min, the AEP protein profile was slightly more hydrolyzed than after 90 min. After 30 min of duodenal digestion (total digestion time of 150 min), only a small band at ~10 kDa can be seen for both AEP and EAEP samples, indicating that all proteins were broken down into protein fragments and peptides <10 kDa. This band gets fainter with the increase of duodenal digestion time, indicating that the protein sites susceptible to proteolysis are accessible to the digestive enzymes (pepsin and pancreatin) in almond proteins extracted by both AEP and EAEP. Similar results were reported by Souza et al., 2019) for almond cake proteins where no bands above 10 kDa were observed in the SDS-PAGE after intestinal digestion. It is important to mention that the bands found in the gastric phase for both AEP and EAEP samples at ~38 kDa and the bands found in the intestinal phase from 25 to 55 kDa are related to the pepsin and pancreatin enzymes, respectively (Fig. 3 B, C and Supplementary material A Fig. 3S–A).

The peptide quantification and the degree of hydrolysis (DH) of AEP and EAEP samples during digestion were also measured at 0, 0.5, 30, 60, 90, 120, 150, 180, 210, and 240 min (Fig. 3 B and C). The hydrolyzed sample (EAEP) presented a higher peptide content (1.7 vs. 2.9 mg/mL) and a higher DH (1.5 vs. 7%) before digestion due to the action of the protease during the extraction process compared with the unhydrolyzed sample (AEP). Oral digestion did not affect the peptide content or the DH of both AEP and EAEP samples. During gastric digestion, a steady increase in the peptide concentration and DH was observed due to the action of pepsin. EAEP samples presented significantly higher peptide concentration and DH values compared with the unhydrolyzed samples (AEP) during the gastric phase. At the end of the gastric phase, the DH of the AEP and EAEP samples reached 21 and 41%, respectively. During the duodenal digestion, a further increase in the peptide concentration and DH was observed due to the action of pancreatic enzymes. While AEP and EAEP samples showed similar trends, EAEP samples were more extensively hydrolyzed through the course of duodenal digestion. The significant increase in the peptide concentration and DH observed after 30 min of intestinal digestion (150 min of total digestion time) can be attributed to the pepsin action during the gastric phase, which promoted the hydrolysis of the almond protein thus facilitating access to the protein sites for the pancreatin enzyme. The peptide concentration and the DH significantly increased within digestion time reaching values of 17 and 13 mg/mL for peptide concentration and 86 and 71% DH for EAEP and AEP samples, respectively. The peptide concentration and DH are in accordance with the SDS-PAGE protein profile observed for AEP and EAEP proteins. The higher peptide concentration and DH of the EAEP samples suggest a greater exposure of cleavage sites in the smaller protein fragments present in this sample. Those results are in congruence with the higher total *in vitro* digestibility of the almond hydrolyzed (EAEP) samples and reinforce the beneficial role of using selected proteases to assist the extraction of full-fat almond flour as an effective strategy to significantly enhance protein *in vitro* digestibility.

3.5. Effect of enzymatic hydrolysis on protein antigenicity

3.5.1. Sandwich ELISA for almond immunoreactivity

Protein hydrolysis has been used in the production of hypoallergenic food ingredients because of its effectiveness in disrupting sequential and conformational epitopes (Cabanillas et al., 2012; Verhoeckx et al., 2015). However, depending on the type of enzymes used and the hydrolysis conditions, peptides of different lengths may be obtained with different levels of allergenicity (Cabanillas et al., 2010a, Cabanillas et al., 2012; Clemente et al., 1999).

Aiming to understand the impact of enzymatic extraction on almond protein immunoreactivity, a preliminary assessment of the potential allergenicity of the almond protein extract was performed using a rabbit antibody-based inhibition ELISA assay to detect and quantify the presence of major almond allergenic protein (amandin). Due to the lack of manufacturer's information about the almond proteins against which the antibody is raised, the levels of immunoreactivity recorded for the almond protein sample were considered representative of the total allergenicity. Fig. 3A illustrates the ELISA results for AEP and EAEP almond protein samples. Enzyme hydrolysis promoted a 75% reduction in immunoreactivity against almond proteins as estimated by the ELISA. These results suggest that the use of a neutral protease during the extraction affected the structural conformation of almond proteins in a manner that reduced the detection of antibodies to the almond protein hydrolysates. These preliminary results indicate a potential reduction in the allergenic potential of the almond hydrolysates. Similar results were reported by Clemente et al. (1999) for chickpea protein where an 80% reduction in antigenicity was achieved after hydrolysis with the alkaline protease Alcalase as measured by an antibody-capture assay. Proteolysis was also described as an efficient strategy to reduce the antigenicity of lentils and peanut proteins accessed by ELISA using pooled serum from patients with a clinical allergy to lentils and peanuts (Cabanillas et al., 2010a; 2012).

3.5.2. Immunoreactivity of AEP and EAEP protein extracts by western blotting

To further explore the observed effects of proteolysis on the almond protein antigenicity, Western immunoblotting (IgE and IgG) using human sera from two previously selected patients (P4C and P196b) with known immunoreactivity to almonds (Fig. 3B–E) was conducted. The IgE immunoblot assay showed recognition of Prunin (60 kDa Pru du 6), Prunin α-chain (40 kDa), and Prunin 1 and 2 β-chains (21 kDa and 19 kDa) for the AEP samples (lanes A1–A3) for P4C and P196b, with the more intense response observed for P196b (Fig. 3C). Prunins are constituents of amandin, the almond major protein (Sathe et al., 2002). Because of its prevalence and heat stability, amandin has been considered an excellent marker for almond allergenicity (Roux et al., 2001). Although other proteins may also be implicated in almond food allergies for a particular patient, amandin appears to include the key IgE-reactive polypeptides in sera from patients with life-threatening almond food allergy (Roux, Teuber, Robotham and Sathe, 2001).

Proteolysis significantly reduced IgE recognition in both human sera (Fig. 3B and C). Compared with the unhydrolyzed samples (AEP), there was no recognition of proteins above 22 kDa, similar recognition of proteins at ~20 kDa, and a reduction in the recognition at 19 kDa in the hydrolysates (EAEP). However, smaller protein fragments in the EAEP samples were also recognized. Sample P196b showed more intense bands in comparison with human serum sample P4C for EAEP (lanes B1–B3) proteins. Proteins at ~20 kDa are likely more resistant to hydrolysis than other immunoreactive proteins as observed in the SDS-PGAE gel and proteomics assays (Fig. 1). Therefore, proteolysis resulted in important destruction of IgE-binding epitopes in the almond hydrolysates as shown by *in vitro* experiments. However, some allergenic proteins were still detected by sera from the two patients tested. A significant reduction in IgE reactivity was also reported for lentil (Cabanillas et al., 2010a) and peanut (Cabanillas et al., 2012) protein hydrolysates using both immunoblotting and ELISA analysis.

The IgG immunoblot assay (Fig. 3D and E) also showed recognition of similar proteins as the IgE assay for both AEP and EAEP samples, however, more bands above 40 kDa were reactive for the unhydrolyzed samples. Protein hydrolysis also promoted a reduction in IgG

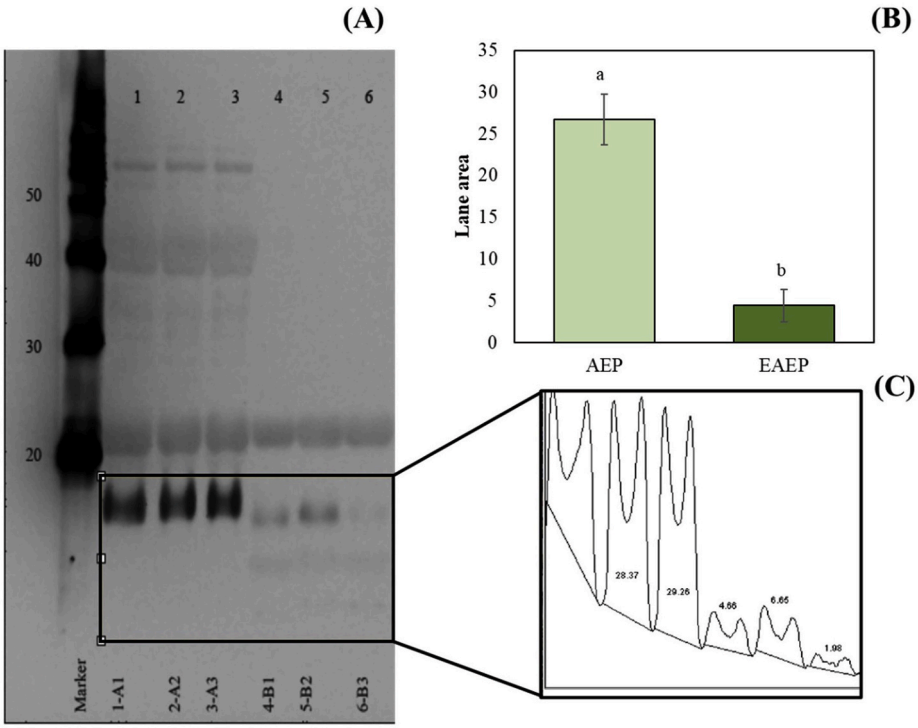

Fig. 4. Western blot of P196b IgE highlighting the more reactive bands (A); Average of the lane area for the AEP and EAEP samples (B), Integration of the highlighted bands by Image J (C).

recognition of almond proteins for both P4C and P196b human sera samples.

Due to its high reactivity, IgE immunoblot using serum sample P196b was selected to further investigate the reactivity of the proteins at 18–19 kDa, which showed higher immunoreactivity for both AEP and EAEP samples in the Western blot assay (Fig. 3B–E). The protein bands were subjected to integration using Image J software (Fig. 4A and C). A 74% reduction in the area value was observed for the hydrolyzed samples, in accordance with the ELISA results (Fig. 4B). Overall, the results indicate that the use of a neutral protease to assist the extraction of almond proteins resulted in structural protein changes that decreased both IgE and IgG recognition compared to the unhydrolyzed samples. Those results are in accordance with the proteomics results that reported partial destruction of the allergen protein epitopes and a reduction in its detection in the EAEP samples.

4. Conclusions

The present study demonstrates the applicability of a neutral protease in the extraction of proteins from full-fat almond flour and sheds light on its impact on the digestibility and allergenicity of the extracted proteins. The use of protease during the extraction process led to the complete hydrolysis of prunin α-chains and partial hydrolysis of prunin β-chains. Proteolysis also led to the formation of smaller protein fragments and peptides and a consequent reduction in the protein allergen epitopes identification. Importantly, protein hydrolysis also significantly improved the protein *in vitro* digestibility from 79.1 to 88.5%, as evidenced by the higher release of peptides and degree of hydrolysis during the gastric and duodenal digestion phases. A 74% reduction in immunoreactivity was observed for the hydrolyzed samples along with a reduction in the IgE and IgG recognition compared to the unhydrolyzed almond proteins. Enzymatic extraction of almond proteins led to the production of protein hydrolysates with improved digestibility and reduced antigenicity that could soon become an alternative option to the use of intact protein in the development of hypoallergenic food ingredients. Although further studies are needed to characterize the biological activity of the residual allergens and to assess the clinical relevance of these findings, this enzymatic procedure appears as a promising method to obtain hypoallergenic almond protein hydrolysates.

CRediT authorship contribution statement

Fernanda Furlan Goncalves Dias: Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Yu-Ping Huang:** Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Joseph Schauer:** Methodology, Formal analysis, Writing – review & editing. **Daniela Barile:** Supervision, Writing – review & editing. **Judy Van de Water:** Immune Study Design and, Supervision, Writing – review & editing. **Juliana Maria Leite Nobrega de Moura Bell:** Supervision, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100488.

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