Functional, nutritional, antioxidant and sensory properties of faba bean (*Vicia faba*, L.) seed protein hydrolysates and fortified apple juice, and comparative peptidomic profile

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Abstract

Enzymatic hydrolysis of plant-derived proteins can improve their quality offering opportunities for food applications. In this study, three proteolytic enzymes (pepsin, trypsin, alcalase) were used, alone or combined, to produce faba bean protein hydrolysates (PHs). Their functional, nutritional and antioxidant properties were evaluated, and the peptidomic profile was assessed by LC-ESI-QO-MS/MS. Hydrolysis improved solubility of faba proteins at acidic and neutral pH, as well as their antioxidant properties. Peptidomic analysis identified 2031 peptides in the different PHs. Among them, 9 showed 100% homology with previously known antioxidant peptides and several others include in their sequences antioxidant motifs. Sensory data analysis showed that after addition of PHs to apple juice, no significant differences were perceived between control and some of the PHs. This study demonstrates that enzymatic hydrolysis enhances the functional and antioxidant properties of faba bean proteins. Specifically, hydrolysates can be used as functional food ingredients to produce fortified beverages.

Keywords

Faba proteins; enzymatic hydrolysis; mass spectrometry; bioactive peptides; apple juice; functional food; sensory analysis.

Abbreviations

AA: ascorbic acid; AAeq: ascorbic acid equivalents; AAS: amino acid score; ABTS: 2,2,-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid; BV: biological value; DPPH: 1,1-DiPhenyl-2-PicrylHydrazyl; EAAI: essential amino acid index; (E/T): essential amino acids to total amino acids FC: foaming capacity; FS: foaming stability; MW: molecular weight; PAGE: polyacrylamide gel electrophoresis; PE: Faba bean protein extract; PER: protein efficiency ratio; PHs: Faba bean protein hydrolysates; SDS: sodium dodecyl sulfate; TSS: total soluble solids; WHO: World Health Organization.

1. Introduction

The faba bean (*Vicia faba,* L.) is an annual legume that grows in different climatic zones from Europe to Africa and Asia. Consumed as a food in many countries, it is noteworthy for its low cost and valuable nutritional properties – high in proteins, carbohydrates, vitamins, minerals, and dietary fibers (Multari, Stewart & Russel, 2015). The ever-increasing demand for substitutes for animal-based proteins caused by population growth is driving the scientific community's interest toward this legume. Tha faba beans protein content ranges from 27% to 34% of the dry weight (depending on the variety and growing conditions), with the globulin storage proteins, vicilin, and legumin, comprising about 80% (Vioque, Alaiz & Giron-Calle, 2012). (In fact, research is ongoing to increase the quality and quantity of its proteins even further through genetic selection and agricultural conditions.) Compared to the FAO profile of required amino acids, faba beans amino acid composition is low for the sulphur amino acids cysteine and methionine, as well as tryptophan (Kaldy & Kasting, 1974). One way to increase the use of faba as a valuable protein source is by combining it with cereals, which are high in those amino acids, since fava is reach in lysine, which cereals lack, thus guaranteeing a complete amino acid supply for the human diet. Another way to increase faba consumption, even in countries where it is not part of the food tradition, is to transform the protein fraction into high-quality functional food components. Typically, this transformation is accomplished through microbial fermentation or enzymatic hydrolysis (Rizzello, Tagliazucchi, Babini, Rutella, Saa & Gianotti, 2016; Wouters, Rombouts, Fierens, Brijs & Delcour, 2016). The second method, enzymatic hydrolysis, is more widespread, because of the mild processing conditions, easily controlled reaction, and minimal formation of by-products. Both these proteolytic treatments break down the primary sequence, producing active amino acids and peptides. Protein hydrolysis can create new food applications by modifying the biological, nutritional, or functional properties of proteins. The most significant biological benefits that have been reported are antioxidant, anti-hypertensive, antimicrobial and anti-carcinogenic activities (Rizzello et al., 2016). In particular, a recent work showed angiotensin-converting enzyme (ACE)-inhibitory, antioxidant and lipoxygenase-inhibitory

activities of faba bean proteins after fermentation with *Lactobacillus plantarum 299v* (Jakubczyk, Karas, Złotek, Szymanowska, Baraniak & Bochnak, 2019). Pepsin treatment of a faba bean protein extract significantly increased its antioxidant properties (Ali, 2019). It is worth noting that proteolysis has improved the biological properties of other foods as well. For example, lima bean (*Phaseolus lunatus*, L.) protein hydrolysates produced with sequential pepsin-pancreatin hydrolysis had high ACE-inhibitory activity (Chel-Guerrero, Dominguez-Magana, Martinez-Ayala, Davila-Ortiz & Betancur-Ancona, 2012). Moreover, the hydrolysis of other plant proteins such as rice, rice bran, and hemp seed with proteolytic enzymes (neutrase, pepsin, alcalase, and pancreatin) has produced peptides with antioxidant activity (Rizzello et al., 2016).

Second, the ability to modify nutritional properties offers the significant advantage of eliminating anti-nutritional compounds. For faba beans, in particular, this means removing the favism-inducing glycosides, vicine and convicine (Vioque et al., 2012).

Lastly, the third advantage of proteolysis is improving the functional properties of plant proteins. For example, the bioavailability of plant proteins is often limited because of their low solubility in aqueous media (Wouters et al., 2016). However, it was recently demonstrated that the enzymatic treatment and ultrafiltration of faba bean protein extract significantly increased its protein solubility, foaming and oil-holding capacity (Eckert, Han, Swallow, Tian, Jarpa-Parra & Chen, 2019). Additionally, alcalase hydrolysis of a faba bean protein isolate increased the physical and oxidative stability of oil/water emulsions, and markedly reduced lipid oxidation during storage (Liu, Bhattarai, Mikkonen & Heinonen, 2019). Thus, enzymatic hydrolysis can create new products with enhanced bioactivity and superior nutritional and physicochemical properties compared to the original proteins.

The aim of the present study was: first, to evaluate the nutritional, functional, antioxidant and sensory properties of faba bean protein hydrolysates (PH) obtained using different enzymes; and second, to assess the sensorial properties of apple juice enriched with the PH. The peptidomic profiles of the PH were determined by high-resolution mass spectrometry to correlate biological activity with the released bioactive peptides. Apple juice was chosen since the apple (*Malus domestica*) is a leading

fruit variety in terms of world production, and its most important industrial application is the creation of juice. Adding hydrolyzed faba bean proteins addition to natural apple juice is an innovative approach to the goal of introducing a new viable protein source, increasing the commercial value of this legume while producing a healthy new drink.

2. Materials and Methods

2.1 Raw material and chemicals

The dried and completely matured Faba bean seeds (variety of Barkat) were bought from Provincial Agricultural Organization at Gorgan (Iran). Reagents were analytical grade, from Sigma (Saint Louis, MO, USA) and Merck (Darmstadt, Germany). Pre-cast gels, the MW marker for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bovine serum albumin (BSA), mass spectrometry solvents and related reagents were from Bio-Rad (Hercules, CA, USA).

2.2 Preparation of faba bean protein extract (PE)

Faba seeds were ground using a mill 1000 Asan Tus (Iranian Model) and passed through a 50-mesh sieve. The powder was defatted 3 times with hexane in the ratio of 1:3. The saturated solvent was replaced every 2 h. The sample was dried at room temperature and then stored at -18°C. The defatted flour was dispersed in distilled water (1:10), the pH was adjusted to 11 and the mixture was stirred for 60 min at room temperature. After centrifugation at 10518 *g* for 20 min, the supernatant was collected, and the pH was adjusted to 3. The precipitated proteins were recovered by centrifugation at 10518*g* for 20 min and then freeze-dried in an FDB 5503 dryer (Operon, Korea).

2.3 Preparation of faba bean protein hydrolysates (PHs) by protease treatments

The freeze-dried PE was dissolved at 4% (w/v) concentration in 100 mM phosphate buffer. The hydrolysis with single enzymes was performed using 3% (w/v) enzyme concentration, 180 min reaction time, and pH and temperature conditions optimal for each enzyme (respectively: for alcalase

8.5 and 50°C; for trypsin 7 and 37°C; for pepsin 2 and 37°C). The digestion reaction was stopped by inactivating the enzyme at 85°C for 15 min. After centrifugation at 10518*g* for 10 min, the supernatant was freeze-dried and stored at -18°C until use. Hydrolysis with two enzymes was performed sequentially, heat inactivating the first enzyme before addition of the second one. Nine faba bean protein hydrolysates were obtained, and named with the codes: **P** (PE hydrolyzed with pepsin 3%); **T** (PE hydrolyzed with trypsin 3%); **A** (PE hydrolyzed with alcalase 3%); **PT** (PE hydrolyzed with pepsin 1.5% and trypsin 1.5%); **TP** (PE hydrolyzed with trypsin 1.5% and pepsin 1.5%); **TA** (PE hydrolyzed with trypsin 1.5% and alcalase 1.5%); **AT** (PE hydrolyzed with alcalase 1.5% and trypsin 1.5%); **TAd** (PE hydrolyzed with trypsin 3% and alcalase 3%); **ATd** (PE hydrolyzed with alcalase 3% and trypsin 3%).

2.4 Protein pattern analysis by SDS-PAGE

Protein pattern of PE and PHs was analyzed on hand-cast 14% (v/v) SDS-polyacrylamide gels using Mini-PROTEAN® equipment from Bio-Rad (Hercules, CA, USA). The Precision Plus Protein Standard from the same company was selected as MW marker.

2.5 Protein concentration by Bradford and Kjeldahl assays

Soluble protein concentration and total protein content of the PE and PHs were analyzed using Bradford and Kjeldahl methods, respectively. The first assay was performed using the Quick Start Bradford Protein Assay kit from Bio-Rad (Hercules, CA, USA). The standard curve was obtained with BSA from 0.5 to 10 μg/mL. Total protein amount was determined by the Kjeldahl method (Schuman, Stanley & Knudsen, 1973) by mineralizing 1.0 g (d.w) of sample with 10 mL of 95:5 (v/v) sulphuric acid:phosphoric acid (H_2SO_4:H_3PO_4) mixture at 420°C for 180 min and subsequent distillation with 32% (v/v) sodium hydroxide (NaOH) and titration with 0.1 N H_2SO_4.

2.6 Analysis of functional properties

2.6.1 Protein solubility

Solubility was determined by the method of Klompong, Benjakul, Kantachote & Shahidi (2007). Briefly, 5 mg of protein hydrolysate were dispersed in 500 µL of deionized water and well mixed for 10 min. Samples with pH values from 2 to 12 were obtained by addition of HCl or NaOH. After correction of the pH, each sample was shaken for 30 min and centrifuged at 27440 *g* for 5 min. Protein content in the supernatant was determined using the Bradford method, while total protein content in the sample was determined using Kjeldahl method.

Protein solubility (%) was calculated according to the following equation:

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein in sample}} \times 100$$

2.6.2 Foaming capacity (FC) and foaming stability (FS)

FC and FS of protein extract and protein hydrolysates were determined according to the method of Jamdar, Rajalakshmi, Pednekar, Juan & Sharma (2010), with some modifications. Aliquots (20 mL) of 0.5% (w/v) sample solution were adjusted to pH 4, 6, 8 and 10, and then transferred into a 50 mL cylinder for homogenization with Ultra-Turrax T25 (IKA-Werke, Germany), at 16.000 rpm, for 2 min at room temperature. The total volume was read after 30 sec. The FC (%) was calculated as follows:

$$\text{FC (\%)} = \frac{(A_0 - \text{B})}{\text{B}} \times 100$$

where A_0 and B are the volumes (mL), respectively, after and before whipping.

The whipped sample was left stand at 25°C for 10 min and the volume of whipped sample was then recorded. The FS (%) was calculated as follows:

$$\text{FS (\%)} = \frac{(A_t - \text{B})}{\text{B}} \times 100$$

where A_t is the volume after standing (mL) and B is the initial volume, before whipping (mL).

184 **2.7 Amino acid analysis and evaluation of nutritional parameters**

185 A standard oven acidic hydrolysis of the proteins at high temperature was applied for the analysis of
186 the amino acid composition (Weiss, Manneberg, Juranville, Lahm & Fountoulakis, 1998).

187 Five mg of PE and PHs were accurately transferred in an ampule and phenol was added at 0.5% (w/v)
188 concentration. After addition of 0.6 mL HCl 6 M, the mixture was ultrasonicated for 15 min until
189 complete dissolution. The ampule was sealed and placed in the oven for hydrolysis at 110°C for 22
190 h. The sample was then neutralized by addition of 0.8 mL 6 M KOH, and transferred into a 5 mL
191 volumetric flask, brought to volume with 0.1 M potassium borate buffer (pH 8.5), and finally filtered
192 with a 0.45 μm syringe filter. For derivatization, a 90 μL aliquot of the hydrolyzed sample was
193 transferred into a 1.5 mL vial in the presence of 10 μL of internal standard (Norvaline, IS) solution.
194 After addition of 100 μL aliquot of 0.1 M potassium borate buffer (pH 8.5) and 200 μL aliquot of the
195 derivatization reagent solution 9-fluorenylmethylchloroformate (FMOC-Cl, 20 mM in acetonitrile),
196 the mixture was stirred and kept at room temperature for 20 min. Finally, formic acid (250 μL, 2%
197 w/v) was added to terminate the derivatization reaction. The final solution was filtered through a 0.45
198 μm syringe filter, before injection (20 μL) on a Phenomenex Kinetex Core-shell 5 μm C18 column
199 (150 x 4.6 mm i.d.). Separations were performed using a Jasco Model LG-980-02S gradient unit, a
200 Jasco PU-1580 pump and a Jasco UV-1575 UV/Vis detector (Jasco corporation, Tokyo, Japan) under
201 a ternary gradient elution using aqueous ammonium formate at two different pH values (5.5 and pH
202 7.5) and in the presence of formic acid (0.1%)/acetonitrile, 10/90 (v/v). The flow rate was 1.2 mL/min
203 and the detection wavelength was 265 nm (Themelis, Gotti, Orlandini & Gatti, 2019).

204 Nutritional properties of PHs were determined based on their amino acid profiles. Amino acid score
205 (AAS) was calculated using the FAO/WHO/UNU (1973) reference pattern.

206 $$AAS = \frac{\text{mg of amino acid in 1g total protein}}{\text{mg of amino acid in requirement pattern}} \times 100$$

207 Essential Amino Acid Index (EAAI) was measured by using the amino acid composition of the whole
208 egg protein as standard (Amza, Balla, Tounkara, Man & Zhou, 2013).

$$\mathrm{EAAI} = \sqrt[9]{\frac{(\mathrm{Lys} \times \mathrm{Thr} \times \mathrm{Val} \times \mathrm{Met} \times \mathrm{Ile} \times \mathrm{Leu} \times \mathrm{Phe} \times \mathrm{His} \times \mathrm{Trp})\mathrm{a}}{(\mathrm{Lys} \times \mathrm{Thr} \times \mathrm{Val} \times \mathrm{Met} \times \mathrm{Ile} \times \mathrm{Leu} \times \mathrm{Phe} \times \mathrm{His} \times \mathrm{Trp})\mathrm{b}}}$$

In this equation, "a" represents the content of amino acids specified in the formula in test sample and "b" the content of the same amino acids in egg standard protein (%), respectively.

Biological value (BV) and Protein efficiency ratio (PER) values were calculated according to Amza, Balla, Tounkara, Man & Zhou (2013).

$$\mathrm{BV} = 1.09 \times (\mathrm{EAAI}) - 11.7$$

$$PER = \begin{cases} Eq.1 = -0.684 + 0.456\ (Leu) - 0.047(Pro) \\ Eq.2 = -0.468 + 0.453(Leu) - 0.04(Tyr) \\ Eq.3 = -1.816 + 0.435(Met) + 0.780(Leu) + 0.211(His) - 0.944(Tyr) \\ Eq.4 = 0.08084\ (Thr + Val + Met + Ile + Leu + Phe + Lys) - 0.1094 \\ Eq.5 = 0.06320\ (Thr + Val + Met + Ile + Leu + Phe + Lys + His + Arg + Tyr) - 0.1539 \end{cases}$$

The proportion of essential amino acids to total amino acids (E/T) of the test protein was calculated as follow (Wani, Sogi, Singh & Shivhare, 2011):

$$E/T\% = \frac{(Ile+Leu+Lys+Met+Cys+Phe+Tyr+Thr+Trp+Val+His)}{(Ala+Asp+Arg+Gly+Glu+His+Ile+Leu+Lys+Met+Cys+Phe+Tyr+Pro+Ser+Thr+Trp+Val)} \times 100$$

2.8 *In vitro* antioxidant activity assays

The antioxidant activity assays were performed on a microplate scale and absorbance was measured using SPARK 10M microplate reader (TECAN, Mannedorf, CH). Results were expressed as mean values of three replicates.

The ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity was determined according to the procedure of Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans (1999). Briefly, an ABTS stock solution (7 mM in 2.45 mM $K_2S_2O_8$) was diluted with sodium acetate 20 mM pH 4.5, to an absorbance at 734 nm of 0.70 ± 0.02. The solution was mixed with the sample and incubated for 30 min in the dark. The absorbance was measured at 734 nm, and value was corrected with a water blank. Activity was expressed as mg ascorbic acid (AA) eq/L by means of a calibration curve, with AA from 0 to 5 mg/L.

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according to the method of Sharma & Bhat (2009). An aliquot of sample was mixed with 50 µM DPPH solution in methanol and the mixture was incubated for 30 min in the dark. The absorbance was read at 517 nm and corrected with a water blank. Activity was expressed as mg AA eq/L by means of a dose-response calibration curve (from 0 to 5 mg/L) of AA.

Ferrous ion-chelating activity was measured according to the method reported in Tang, Kerry, Sheehan & Buckley (2002). Sample aliquot was mixed with 50 µM ferrous sulfate ($FeSO_4$) and 300 µM ferrozine. After incubation for 10 min, the absorbance was measured at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

$$\text{Ferrous ion} - \text{chelating activity}\ (\%) = (A_0 - A_s)/A_0 \times 100$$

where A_0 is absorbance of the control and A_s is absorbance in the presence of sample.

2.9 Sensory evaluation of protein hydrolysates and fortified juices

Samples were evaluated by a panel of trained judges for selecting, among PHs, the most promising to be tested diluted in apple juices. Subsequently, the selected samples were used to fortify apple juices at 1% (w/v) to be tested by consumers, by comparing them with the sole apple juice (control sample). Study protocol followed the ethical guidelines of the sensory laboratory, approved by the University of Bologna, and a written informed consent was obtained from each participant before they entered the first test.

Samples were firstly evaluated asking to a panel to compare each of the PHs, diluted in water with one (T) randomly selected as control. The panel consisted of 11 trained members from different sections of the Food Science Department, Cesena, Italy and the test was conducted in a sensory laboratory. Trained judges (n=11, 7 females, 4 males, age from 21 to 60; students and employees of the University of Bologna) served as panelists. Each sample was prepared by adding 0.5% (w/v) of the PHs to distilled water, and 15 mL of this solution were distributed to the assessor, in a white plastic cup. Water and unsalted breadsticks were provided for cleaning mouth during tests. After this

preliminary test served as screening, 30 consumers (19 female, 11 males; age from 22 to 58) were recruited to participate a test in which they were firstly encouraged to describe if they normally consumed apple juices and/or products enriched in antioxidant compounds. Then they were asked to assess the juices with an addition of PHs of 1% (w/v) and to express and to mark on a 9-point hedonic scale the previously selected attributes of: sourness, sweetness, salty taste, apple aroma and overall liking. Apple juice without any addition of the PHs was used as control. Samples were randomly distributed in 10 mL white plastic cups. The pH of the fortified apple juices was determined by a pH Meter (AMEL 33-B, Italy) and a refractometer (Kruss DR-301, Germany) was used to determine the total soluble solids (TSS) at room temperature.

2.10 Peptide identification and sequencing by LC-ESI-QO-MS/MS analysis

The PHs were submitted to high-resolution LC-ESI-QO-MS analysis for peptide identification. The LC-ESI-QO-MS analysis was performed with a UHPLC Ultimate 3000 coupled to a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA), using the C18 column (Zorbax SB-C18 Reversed phase, 2.1 × 50 mm, 1.8 μm particle size, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of (A) H_2O/formic acid (99.9:0.1, v /v) and (B) acetonitrile. The sample (10 μL, 20-fold diluted) was loaded into the column at a flow rate of 0.3 mL/min. The gradient started at 2% B, and grew to 3% B in 2 min. The mobile phase composition was raised to 27% B in 19 min and then to 90% in 4 min. The mass spectrometer was tuned and calibrated according to the manufacturer's instructions. The, MS/MS spectra were then converted to .mgf files and the peptides were identified by using the Swiss-Prot database through MASCOT (Matrix Science, Boston, MA, USA) protein identification software. The following parameters were considered: enzyme, none; peptide mass tolerance, ± 5 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. Only peptides with a best expected value lower than 0.05 that corresponded to $P<0.01$ were considered. For the analysis of

short peptides (< 5 amino acids length), the .mgf files were analyzed by a de novo peptide sequencing approach performed by Pepnovo software (http://proteomics.ucsd.edu/ProteoSAFe/) using the same parameters as reported above. The assignment process was complemented and validated by the manual inspection of MS/MS spectra. The peptides identified in PHs samples were investigated in relation to their bioactivity by comparison with previously identified bioactive peptides using the BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba & Darewicz, 2008).

2.11 Statistical analysis

All analyses were performed in triplicate. Statistical tests were performed using Microsoft Excel statistical software XLSTAT version 2018.5 and SPSS software (SPSS16, Inc, USA). Statistical significance of differences among several means was determined using one-way analysis of variance ANOVA with Tukey test, with a significant level of $P<0.05$. Sensory data were analyzed with LSD Fisher test, $P<0.05$.

3. Results and discussion

3.1 Preparation of PHs

Protein hydrolysates were produced by three proteases, alcalase, pepsin and trypsin, alone or in combination. Alcalase is an endoprotease that has been widely used for the generation of protein hydrolysates, for its high activity and low cost. Alcalase-derived hydrolysates are also more resistant to digestive enzymes (Sarmadi & Ismail, 2010). Pepsin and trypsin, which are endoproteases as well, are generally used to simulate human digestion.

The efficacy of the hydrolytic process was checked comparing the protein/peptide profile of hydrolysates to that of the substrate on SDS-PAGE (Fig. 1). The results indicate that all of the enzymatic treatments were able to degrade high MW proteins to smaller peptides. Pepsin, alone or in combination with trypsin (samples P, PT and TP), was particularly active, producing peptides with MW lower than 15 kDa. In the alcalase hydrolysate, peptides with low MW were strongly

predominant. In all the other samples, some non-hydrolyzed or partially hydrolyzed proteins were still present (bands with MW between 20 and 37 kDa), indicating a lower hydrolytic efficacy.

3.2 Solubility, foaming capacity and stability

Solubility is one of the most important functional property of proteins. The high solubility of a protein-based product is necessary for its application in manufactured foods, especially for rheological properties such as foaming capacity. The solubility of PE and PHs at pH values in the range of 2-12, is reported in Table 1. The results indicated that solubility of both PE and PHs was pH-dependent. In the pH range of 3-7, faba bean proteins had very low solubility (from 0.07 to 1.45%), but after enzymatic hydrolysis solubility significantly increased, especially in pepsin and trypsin hydrolysates (sample P, values from 41.52 to 57.54%; sample T from 8.43% to 44.03%, $P<0.05$). Similarly, solubility of faba bean proteins notably increased after enzymatic hydrolysis with pepsin and neutrase at pH 5 and 7 (Eckert et al., 2019). Hydrolysis of faba bean proteins by alcalase at pH 8 increased solubility of about 6-10% (Liu et al., 2019). A similar trend was also observed for peanut proteins, where hydrolysis improved the solubility up to 80%, in the pH range of 4–6 (Jamdar et al., 2010). In alkaline conditions, solubility of PE increased, as well as in pepsin and trypsin hydrolysates with maximum values at pH 12 for samples PE and T (75.75% and 56.07%, respectively) and at pH 11 for sample P (66.85%). For alcalase hydrolysate, the maximum solubility (45%) was obtained at pH 8 and 12. For all the other samples, resulted from sequential hydrolysis with two peptidases, the solubility remained significantly lower in all the pH-range evaluated ($P>0.05$). These results indicated that hydrolysis with selected enzymes was a useful method to increase solubility of faba bean protein extract in the pH range of 3-7. Generally, proteins solubility depends on several factors such as pH, polarity, molecular size and hydrophilic sites. The peptides produced through hydrolysis have smaller molecular masses and less tertiary structure than parental proteins. Besides, hydrolysis liberates ionizable groups that can interact with water molecules. All these factors can improve proteins solubility (Wouters et al., 2016). The different solubility observed

for different PHs can be due to the specific peptide profile (molecular size and exposure of hydrophilic or hydrophobic groups) generated by each enzyme or combination of enzymes. Samples obtained by sequential hydrolysis with alcalase and trypsin (samples AT, ATd and TAd), which had low solubility, showed indeed a protein pattern with lower degree of hydrolysis on SDS-PAGE (Fig. 1).

In association to solubility, another important characteristic of proteins is the ability to create stable foams. Foaming capacity provides unique texture in various foods including bread, cakes and ice cream. Foaming capacity and stability of PE and PHs are shown in Table 1. At pH 4 and 6 the PE showed poor foaming capacity, with values of 25 and 50%, respectively, which are similar to those reported for faba bean at pH 5 and 7 (31 and 67%) by Eckert et al. (2019). In acidic conditions, all PHs had higher foaming capacity than PE. Most plant proteins have limited foaming properties due to their compact structure or low solubility. Hydrolytic treatments disrupt the compact tertiary structure of native proteins and decrease their MW, facilitating their diffusion and adsorption to air-water interface, resulting in higher foaming capacity (Wouters et al., 2016; Eckert et al., 2019). The highest effect was observed for TP treatment (250% at pH 4 and 200% at pH 6), but values about three times higher than those of PE were observed for sample PT (170% at pH 6), TA (155% at pH 4), AT (155% at pH 4) and ATd (160% at pH 4). At pH values of 8 and 10, the foaming capacity of PE increased to 125 and 150% respectively. Similar values were observed for PHs, with the exception of samples PT (180% at pH 8 and 230% at pH 10) and TA (200% at pH 8). Hydrolysis of faba bean proteins with different enzymes is therefore recommended for increasing the foaming capacity at low pH values. The stability of foam containing PE after 10 min was low at pH 4 and 6 (15 and 20% respectively), but increased up to 100 and 125%, at pH 8 and 10. Hydrolysis generally improved foam stability at pH 4 and 6, particularly for samples PT (60 and 140%), TP (170 and 125%) and AT (135 and 100%). In basic solution, the stability of PHs was lower or in the range of PE determinations. Therefore, pH had significant effect on foaming stability, with a significant increase at acidic pH values. Reported data already confirmed that hydrolysis increased foaming stability in other plant

360 protein sources, like amaranth, bean, pumpkin, rice bran, lupin protein and corn glutelin (Wouters et
361 al., 2016, and references therein).
362
363 **3.3 Amino acid composition and nutritional properties**
364 The amino acid composition of a food protein source is an essential feature in determining its
365 nutritional value. Seeds are known to be a rich source of proteins, but they are in general deficient in
366 some essential amino acids, which compromise their nutritional quality. For example, legumes are
367 generally deficient in sulfur-containing amino acids (cysteine and methionine), and cereals in lysine
368 and tryptophan. Amino acid contents of PE and PHs (in %, w/w), are reported in Table 2. The amino
369 acid content of PE was referred to all the possible sources of amino acids in the extract (i.e. soluble
370 and insoluble proteins, peptides and free amino acids). Whereas, the amino acid content of PHs was
371 referred to the amino acid sources that are soluble after hydrolysis, and thus available in fortified food
372 prepared by the addition of these hydrolysates. The amino acid profile of PE was similar to that
373 reported from various authors on different faba cultivars (Kaldy et al., 1974 and references therein;
374 Palander, Laurinen, Perttila, Valaja & Partanen, 2006; Vioque et al., 2012; Hendawey & Younes,
375 2013; Eckert et al., 2019; Tab S1, supplementary material). The PE was rich in acidic aspartate
376 (18.28%) and glutamate (22.23%) residues, which are the most abundant amino acids of globulins,
377 the main proteins of the seed. All the essential amino acids, with the exception of tryptophan, were
378 present in the extract. Of these, methionine was the only limiting amino acids, while all the others
379 were present at concentrations higher than the WHO values, with leucine being the most concentrated
380 (9.27%). Among conditionally essential amino acids, arginine was the most abundant (12.55%) while
381 cysteine was not detected, as well as the non-essential amino acids glutamine and asparagine.
382 Hydrolysis had not an appreciable impact on the percentage content of most amino acids. An
383 exception, common to all PHs, was the strong decrease in alanine concentration, from 14.87 to values
384 of 3.86-4.77%. Besides, the concentration of aspartic acid was almost halved in all the hydrolysates.
385 This amino acid, together with glutamic acid, arginine, and leucine was anyway the most abundant

386 in PHs, similarly to what observed for PE. Methionine, which was present at low concentration before
387 hydrolysis (0.68%) and which is considered a limiting amino acid of faba bean (Kaldy et al., 1974;
388 Hendawey et al., 2013), was not detected in the hydrolyzed samples, probably because of oxidation
389 processes. Other significant variations were the strong increases in histidine content in pepsin
390 hydrolysate (sample P, from 3.54 to 9.92%) and of tyrosine in hydrolysates obtained with double
391 concentrations of combined alcalase and trypsin (samples ATd and TAd, from 3.54 to 5.34 and
392 6.67%, respectively). The different amino acid profile of PHs can be attribute to the specificity of
393 catalytic site and reaction mechanism of enzymes, generating peptides with different solubility.
394 Nutritional parameters of PE and PHs expressed as E/T (%), AAS (%), EAAI (%), BV (%) and PER
395 are reported in Table 2. All PHs had E/T values higher compared to PE, overcoming the recommended
396 value by FAO/WHO/UNU (36%), especially samples P, ATd and TAd (40.9, 41.6 and 41.7%,
397 respectively), with the only exception of sample T (35.8%). The amino acid score (AAS) in PE and
398 TAd was approximately equal (119%), and close to that recently reported by other authors (127%,
399 Eckert et al., 2019). All the other PHs had AAS higher than 100%. EAAIs of PHs were in the range
400 of 70.0–94.9%, while the BVs were in the range of 91.8 to 64.5%, with the highest values for pepsin
401 hydrolysate (sample P, 94.9 and 91.8%). Usually, a protein source with BV between 70-100% and
402 EAAI above 90% is assumed to be of good nutritional quality (Amza et al., 2013). All PHs, with
403 exception of AT and ATd samples, have proven to be useful and high-quality food.
404 Protein efficiency ratio (PER) is a quality index ranging from 0 (low protein quality) up to 2 and
405 above (high protein quality) (Amza et al., 2013). The PER values of PE and most of PHs were higher
406 than 2. Most of PHs (except for TA, ATd and TAd) turned out to be of good (samples PE, P, A, PT,
407 TP) or intermediate (T and AT) quality.
408 All the above results indicate that faba bean PHs represent an interesting protein supply, rich in some
409 essential amino acids, even if not adequately balanced for human diet. They could become a strategic
410 alternative to other more consumed plant protein sources, especially in the formulation of new

functional products with improved nutritional value. In particular, the high content of lysine suggests a possible use as supplement to cereal based diets and products, which lack in this amino acid.

3.4 Antioxidant properties

The antioxidant properties of PE and its hydrolysates were analyzed for their ABTS- and DPPH-radical scavenging activities and ferrous ion-chelating ability. The first two methods exploit the scavenging capacity of hydrogen donating-antioxidants towards the free radicals $ABTS^{\cdot+}$ and $DPPH^{\cdot}$. The third one measures the ability of antioxidants to chelate transition metal ions like Fe^{2+}. Results are reported in Fig. 2 and are expressed as mg AA eq/g of protein for ABTS and DPPH assays, and as IC_{50} (mg/L) values for ferrous ion-chelating ability assay.

Radical scavenging activity of all hydrolysates was generally higher than that of the original substrate (Fig. 2, A and B), as recently observed for a faba bean pepsin hydrolysate (Ali, 2019). Among single enzyme hydrolysates, the most active was the one obtained by alcalase ($P< 0.05$). ABTS value of this sample was 55.9 mg AA eq/g of protein, while the DPPH value was 26.2 mg AA eq/g of protein, ten and five times higher than PE, respectively. The higher sensitivity of ABTS method is due to the preferred interactive reaction between ABTS radical and hydroxylated aromatic compounds present in the peptides sequences (Li, Shen, Deng, Li & Ding, 2014). The differences may be related to the changes in protein composition and surface hydrophobicity values for the respective hydrolysates. Thus, peptides in hydrolysates might differently scavenge the two $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals. The combination of alcalase with trypsin, even in double concentration, and independently on the sequential order the enzymes were added to the substrate (samples TA, AT, ATd, TAd), produced hydrolysates with strongly lower antioxidant properties, with respect to the alcalase hydrolysate ($P<0.05$). These results could be correlated to the lower degree of hydrolysis observed for these samples on SDS-PAGE. Peptide bioactivity is in fact dependent on the MW of peptides, besides on the amino acid composition and sequence, being higher for smaller peptides (Rizzello et al., 2016). The highest radical scavenging activity in double enzyme hydrolysates, were obtained with the

combination of pepsin and trypsin (sample TP, ABTS value 59,7 mg AA eq/g of protein, and sample PT, DPPH value 28.2 mg AA eq/g of protein) enhancing the effect produced by the single enzyme hydrolysis (P<0.05). Similar results were reported in many other studies. For instance, date protein hydrolysates produced with a mixture of enzymes (alcalase and thermolysin) had higher DPPH scavenging activity than hydrolysates produced with each enzyme separately (Ambigaipalan, Al-Khalifa & Shahidi, 2015).

Transition metal ions are known to stimulate lipid oxidation producing reactive peroxyl and alkoxyl radicals. Chelation of transition metal ions like Fe^{2+} by antioxidative peptides would prevent oxidation (Klompong et al., 2007). The IC_{50} values reported in Fig. 2C, indicate that enzymatic hydrolysis increased the Fe^{2+} chelating property of the undigested substrate up to fifty times (P<0.05). The lowest IC_{50} values (meaning higher chelating activity) were observed for alcalase hydrolysates (sample A, 4.19 mg/L) and for combined pepsin and trypsin hydrolysates (samples PT and TP, 3.50 and 3.88 mg/L, respectively). Hydrolysates prepared with trypsin alone or combined with alcalase had the lowest Fe^{2+} chelating activity (samples T and AT, 8.62 and 7.86 mg/L, respectively) (P<0.05). Similar results were obtained by other authors on different substrates. Flaxseed protein hydrolysates produced with papain, trypsin, pancreatin, alcalase and flavourzyme were more effective as Fe^{2+} chelators than the protein isolate (Karamac, Kosinska-Cagnazzo & Kulczyk, 2016). Date seed flour protein hydrolysates prepared using a mixture of alcalase and flavourzyme had the highest chelating activity compared to single enzymes (Ambigaipalan et al., 2015). During hydrolysis, peptide bond cleavage increase metal ions binding due to an increased concentration of carboxylic and amino groups from acidic and basic amino acids, respectively. This metal ions binding ability may lead to a reduction of prooxidative metal ions from the system (Liu, Kong, Xiong & Xia, 2010).

The above results indicate that enzymatic hydrolysis significantly improved the antioxidant potential of faba bean proteins in terms of both radical scavenging activity and transition metal ion chelation. These effects were greatly affected by the type of protease, the proteases combination as well as the

462 sequential order they were added to the substrate. Alcalase and the combination of pepsin and trypsin
463 produced the most bioactive hydrolysates.
464
465 **3.5 Peptidomic profile of PHs**
466 The peptidomic profile of the different hydrolysates was analyzed by means of high-resolution mass
467 spectrometry. The complete list of identified peptides together with the MS data is reported in
468 supplementary material (Table S2). A total of 2031 unique peptides were identified in the different
469 hydrolysates. The highest amount of peptides was found in the hydrolysates obtained with pepsin and
470 trypsin in combination (PT and TP) with 656 and 659 identified peptides, respectively. According to
471 the SDS-PAGE results, hydrolysis by combination of trypsin and alcalase (samples TA, AT, TAd and
472 ATd) resulted in a lower amount of identified peptides respect to pepsin/trypsin combinations.
473 Among the hydrolysates obtained after incubation with the 3 individual enzymes, alcalase was found
474 to have the highest hydrolytic efficiency, as already proved by electrophoresis analysis.
475 As expected, Venn diagram (Figure S1, supplementary material) revealed great differences in the
476 peptidomic profile of the sample hydrolyzed with the 3 different enzymes. Only 2 peptides
477 (corresponding to the 0.2% of peptides identified in the three samples) were found commonly released
478 by the three enzymes. No more peptides were found in common between peptic and tryptic
479 hydrolysates whereas 18 peptides were commonly found in peptic and alcalase hydrolysates and 16
480 peptides in tryptic and alcalase hydrolysates. A comparison between the peptidomic profiles of PT
481 and TP samples indicated that the order of addition of the enzymes was of paramount importance in
482 the release of specific peptide fragments with just 272 peptides (corresponding to the 26.1% of total
483 peptides) commonly found in the two samples (Figure S1, supplementary material). The same
484 consideration can be made for the hydrolysates obtained by combination of trypsin and alcalase with
485 only 220 peptides (25.2% of total peptides) in common between TP and PT samples (Figure S1,
486 supplementary material).

Sequence matching suggested that most of the released peptides were included in the sequence of faba bean vicilin and legumin B-types (Figure S2, supplementary material). The highest number of peptides originated from vicilin and legumin B-types was found in the samples hydrolyzed with both the combination of pepsin and trypsin (PT and TP samples) suggesting that the combined use of these enzymes cleaved more efficiently both the proteins.

3.5.1 Identification of antioxidant peptides in PHs

Several peptides with previously demonstrated antioxidant activity or sharing homology of sequence with known antioxidant peptides were identified in the different hydrolysates (Table 3). Nine peptides, sharing 100% of homology with peptides previously characterized for their antioxidant properties, were identified in the different hydrolysates. The peptides TETWNPNHPEL and FVPH have been previously reported as responsible for the antioxidant properties of chickpea protein hydrolysates (Torres-Fuentes, Contreras, Recio, Alaiz & Vioque, 2015). The remaining identified antioxidant peptides were di-peptides with a Y or W residue in their sequences. The presence of Y and/or W residues in the sequence of peptides is considered of paramount importance in determining the antioxidant effect of a peptide, because of their strong ability to donate a proton (Rival, Boeriu & Wichers, 2001; Tagliazucchi, Helal, Verzelloni & Conte, 2016). Their occurrence in the sequences of antioxidant di- and tri- peptides is actually recognized as responsible for their antioxidant activity. Several other peptides depicting structural homology with previously described antioxidant peptides were detected in the different faba beans hydrolysates. For example, as reported in Table 3, 17 and 26 peptides shared the antioxidant sequences TETWNPNHPEL and ALEPDHR, respectively. All of these peptides were from the hydrolysis of faba bean legumin B-types. Both these sequences were identified as antioxidant peptides in chickpea protein hydrolysates (Torres-Fuentes et al., 2015). The antioxidant propertied of ALEPDHR may be due to the presence of the basic amino acids H and R. The imidazole and guanidine group of H and R, respectively, may act as donors or acceptors of

512 protons, especially when they were at the C-terminus position (Suetsuna, Ukeda & Ochi, 2000;
513 Wallner, Hermetter, Mayer & Wascher, 2001).
514 Additional 17 peptides, released after the hydrolysis of faba bean vicilin shared in their structure the
515 antioxidant sequence VIPAGYP. This peptide was identified as responsible for the antioxidant
516 properties of a hydrolysate from soybean β-conglycinin prepared with protease S (Chen, Muramoto
517 & Yamauchi, 1995).
518 Peptides displaying the sequence TETWNPNHPEL can be the most relevant to the total antioxidant
519 activity of faba bean proteins hydrolyzed with pepsin and trypsin (PT). In fact, sample PT was
520 characterized for the presence of 15 out of 17 peptides sharing this sequence. Sample PT, which was
521 characterized for the highest DPPH radical scavenging activity, also contained the antioxidant
522 sequences TETWNPNHPEL, FVPH, LY, IY, VY and YV. The sample hydrolyzed with trypsin and
523 pepsin (TP), instead, showed the highest ABTS radical scavenging activity. This sample contained
524 both the antioxidant peptides TETWNPNHPEL and FVPH as well as the antioxidant di-peptides YV.
525 Indeed, it contained 8 and 7 peptides sharing the antioxidant sequences TETWNPNHPEL and
526 VIPAGYP, respectively. Moreover, it was also the richest in peptides containing the antioxidant
527 sequences PHW and IY (Saito et al., 2003; Beermann, Euler, Herzberg & Stahl, 2009). Faba beans
528 proteins hydrolyzed with alcalase (sample A) also released peptides with high ABTS and DPPH
529 radical scavenging activities. This sample did not contain peptides with 100% of homology with
530 previously known antioxidant peptides. However, this sample was rich in VIPAGYP- and PHY-
531 containing peptides (Saito et al., 2003). Most of these peptides also contained the antioxidant
532 sequence YVE (Tian, Fang, Jiang, Guo, Cui & Ren, 2015).
533
534 **3.5.2 Identification of other bioactive peptides in PHs**
535 Additional 31 peptides with previously demonstrated bioactivities have been identified in the
536 different hydrolysates prepared from faba bean protein extract (Table S3, supplementary material).
537 The majority of the peptides (13 peptides) were both dipeptidyl-peptidase-IV (DPP-IV)- and

angiotensin-converting enzyme (ACE)-inhibitors whereas 12 and 6 peptides were exclusively DPP-IV- or ACE-inhibitor, respectively. The hydrolyzed samples with the highest number of identified bioactive peptides were PT and ATd, followed by sample TA. All of the three samples contained the potent ACE-inhibitory peptide VY (IC_{50} = 7 μmol/L), previously isolated from brewed sake, sardine muscle proteins hydrolysates and *in vitro* digested milk. Interestingly, this dipeptide was effective also *in vivo* by decreasing blood pressure in spontaneously hypertensive rats and mild hypertensive human subjects (Saito, Wanezaki, Kawato & Imayasu, 1994; Kawasaki et al., 2000; Tagliazucchi, Martini, Shamsia, Helal & Conte, 2018). Sample PT and TA also included the dipeptide VK, which showed very low IC_{50} value (13 μmol/L). Indeed, the peptide AW (IC_{50} = 10 μmol/L) was found exclusively in ATd sample. Moreover, these three samples were also characterized for the highest number of DPP-IV-inhibitory peptides.

3.6 Sensory properties of PHs and fortified orange juice

The literature reports that bioactive hydrolysates can produce off-flavors and bitter taste (Moller, Scholz-Ahrens, Roos & Schrezenmeir, 2008). So, to set the maximum amount of PHs to be possibly added to the apple juices, a preliminary sensory evaluation by a trained panel (11 assessors) was performed and showed that there was no significant difference between the diverse PHs solutions in water at 0.5% (P<0.05, data not reported). Since no off flavors were detected by the panelist at this dilution, all the PHs were tested in apple juice to check the sensory consequences of their addition in a real case. As PE had no satisfactory solubility, this sample was not tested. Assuming a masking effect of the apple juice and considering the need of adding a higher amount of PHs to reach a fortifying effect, the test with the consumers was realized preparing juices added with 1% of PHs.

Sensory evaluation demonstrated that the use of samples with different PHs for enrichment of apple juices, caused low turbidity with some insoluble particles (Table 4). The only exception is represented by apple juice fortified with P, which showed more sour taste without any significant difference in terms of turbidity, when compared to control (P>0.05). Apple juices added with TA and AT were

564 rated as the highest in sweetness (6.1 and 6, respectively) and significantly different to the samples
565 obtained by pepsin, that showed the lowest intensity of sweet attribute (P<0.05). The instrumental
566 evaluation of the acidity (pH) did not show significant differences with control sample (P<0.05). The
567 salty taste increased, by adding PHs, but only the juice added with AT showed a significant difference
568 if compared to the others (P<0.05). In all apple juices, except those fortified with TP, TA and AT, no
569 significant differences were reported (P>0.05). The main problem, highlighted, only for a number of
570 the supplemented apple juices, during the sensory evaluation, was the perception of bitterness, often
571 reported by the consumers in the comments (Table 4). In particular, this was evident for juices added
572 with P, A (reported as bitterness) and TP, AT and ATd (reported as low bitterness). The overall liking
573 of juices fortified with ATd was even higher than control (even if not significantly), while P, TAd
574 and A showed no significant difference with control apple juice (P>0.05). The TSS of fortified apple
575 juices were increased by adding PHs but they were not significantly different if compared to control
576 apple juice (P>0.05). Khairallah, Hettiarachchy & Rayaprolu (2016) reported that no significant
577 differences were perceived between the freshly prepared control juice and the samples prepared by
578 adding peptide fraction into orange juice (0.3%). The pH of the orange juice alone and with peptide
579 fractions did not show any significant changes demonstrating that orange juice can be a potential
580 vehicle for application of bioactive peptide fractions (Khairallah et al., 2016). Results described in
581 the present work indicate that apple juice, similarly to the orange one, can be effectively used to
582 incorporate hydrolysates of faba proteins as a source of amino acids and peptides with antioxidant
583 properties.
584
585 **4. Conclusions**
586 This study showed that faba beans are a suitable source of proteins of high nutritional quality,
587 containing all essential amino acids (except tryptophan) at concentrations higher than the WHO
588 values. It is particularly rich in leucine and arginine. Enzymatic hydrolysis improved some nutritional
589 parameters, overcoming the problem of faba protein's poor solubility. Moreover, it produced low

590 MW peptides with increased solubility at acidic and neutral pH, improving the rheological properties
591 such as foaming capacity and stability. All PHs showed antioxidant properties in terms of radical
592 scavenging activity and ferrous ion chelation capacity. A complete peptidomic analysis identified
593 several peptides with previously demonstrated antioxidant activity or sharing sequence homology
594 with known antioxidant peptides. In order to test consumer acceptability, we conducted a sensory
595 analysis of apple juice supplemented with faba hydrolysates, which revealed no significant
596 differences compared to the majority of the other hydrolysates tested. Hydrolysates obtained with
597 pepsin (alone or combined with trypsin) or alcalase might be preferable for application as food
598 ingredients, due to their combination of nutritional, functional and bioactive properties. Importantly,
599 the pepsin hydrolysate has the additional advantage of not altering the sensory acceptability of apple
600 juice. These faba protein hydrolysates could be therefore an innovative ingredient in the preparation
601 of functional foods due to their amino acid content and antioxidant properties.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Supplementary data

Supplementary data associated with this article can be found in the online version.

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Figure captions

Fig. 1. SDS-PAGE of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (ATd). pST: peptide MW marker; St: MW marker.

Fig. 2. ABTS radical scavenging activity (mg AAeq/g protein, **A**) DPPH radical scavenging (mg AAeq/g protein, **B**) and Ferrous ion-chelating activity (IC_{50}, mg/L, **C**), of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (ATd). Means followed by the same letter did not differ significantly (Tukey test, $P>0.05$).

Table 1. Functional properties (solubility, foaming capacity and foaming stability %) at different pH of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (TAd).

Solubility (%)										
pH	PE	P	T	A	PT	TP	TA	AT	ATd	TAd
2	33.58±0.29^{b}	41.95±0.16^{a}	11.02±0.16^{d}	16.73±0.12^{c}	8.01±0.17^{d}	7.99±0.12^{d}	10.25±0.06^{d}	2.96±0.16^{e}	3.81±0.12^{e}	4.66±0.04^{e}
3	1.445±0.05^{d}	41.52±0.12^{a}	8.43±0.64^{c}	12.39±0.02^{b}	3.20±0.18^{d}	7.87±0.06^{c}	9.26±0.04^{bc}	1.05±0.12^{d}	2.36±0.08^{d}	3.21±0.13^{d}
4	0.23±0.39^{e}	43.74±0.23^{a}	13.50±2.19^{b}	12.94±0.36^{b}	6.85±0.08^{cd}	11.56±0.07^{bc}	7.49±0.15^{cd}	1.06±0.10^{e}	2.71±0.14^{de}	3.28±0.11^{de}
5	0.07±0.62^{g}	55.27±0.06^{a}	13.78±0.02^{cd}	24.66±0.06^{b}	12.57±0.25^{cd}	16.73±0.24^{c}	15.80±0.27^{c}	3.97±0.15^{fg}	9.50±0.80^{de}	6.41±0.28^{ef}
6	0.93±0.01^{f}	44.31±0.23^{a}	25.94±0.09^{b}	18.31±0.33^{c}	16.92±0.05^{cd}	16.13±0.22^{cd}	27.63±0.36^{b}	10.15±0.08^{e}	11.55±0.04^{e}	13.76±0.11^{de}
7	1.05±0.28^{g}	57.54±1.15^{a}	44.03±0.10^{b}	23.90±0.27^{d}	19.54±0.17^{de}	19.95±0.00^{de}	32.19±0.28^{c}	14.16±0.02^{f}	17.53±0.01^{ef}	15.36±0.19^{ef}
8	18.32±0.15^{de}	47.88±0.24^{a}	48.44±1.26^{a}	45.42±0.22^{a}	21.43±0.06^{c}	20.72±0.15^{cd}	35.44±0.16^{b}	13.59±0.08^{f}	18.03±0.02^{de}	15.82±0.16^{ef}
9	48.85±0.04^{a}	46.67±0.38^{a}	41.58±0.69^{b}	25.42±0.83^{d}	23.05±0.45^{d}	19.66±0.19^{e}	35.79±0.14^{c}	13.88±0.19^{f}	18.23±0.19^{e}	18.34±0.00^{e}
10	24.02±0.49^{d}	49.53±0.02^{a}	43.80±0.15^{b}	26.44±0.52^{d}	23.51±0.03^{d}	20.00±0.00^{e}	36.10±0.04^{c}	13.95±0.13^{f}	18.98±0.14^{e}	17.30±0.01^{ef}
11	66.14±0.18^{a}	66.85±0.07^{a}	45.71±0.27^{b}	29.38±1.44^{d}	22.20±0.72^{e}	19.53±0.13^{e}	35.11±0.01^{c}	14.26±0.00^{f}	20.47±0.19^{e}	18.59±0.18^{e}
12	75.75±0.30^{a}	55.43±0.18^{b}	56.07±0.07^{b}	43.54±1.07^{c}	23.85±0.16^{d}	20.45±0.21^{de}	43.45±1.73^{c}	14.26±0.27^{f}	19.35±0.05^{def}	15.50±0.31^{ef}
Foaming capacity (%)										
4	25±2.50 ^{i}	70±14.00 ^{g}	140±20.00 ^{d}	65±7.00 ^{h}	80±5.00 ^{f}	250±15.00 ^{a}	155±15.00 ^{c}	155±5.00 ^{c}	160±12.00 ^{b}	105±5.00 ^{e}
6	50±5.00 ^{i}	125±10.00 ^{e}	140±14.00 ^{c}	125±5.00 ^{e}	170±15.00 ^{b}	200±12.00 ^{a}	90±5.00 ^{g}	130±12.00 ^{d}	120±6.00 ^{f}	85±10.00 ^{h}
8	125±12.00 ^{g}	105±5.00 ^{h}	125±8.00 ^{g}	140±10.00 ^{e}	180±5.00 ^{b}	125±5.00 ^{g}	200±20.00 ^{a}	135±9.00 ^{f}	150±10.00 ^{d}	160±7.00 ^{c}
10	150±10.00 ^{d}	150±20.00 ^{d}	145±12.00 ^{e}	125±5.00 ^{f}	230±20.00 ^{a}	125±10.00 ^{f}	145±10.00 ^{e}	170±5.00 ^{b}	155±15.00 ^{c}	125±5.00 ^{f}
Foaming stability after 10 min (%)										
4	15±3.00 ^{j}	25± 4.00 ^{i}	80±11.00 ^{e}	35±5.00 ^{h}	60±5.00 ^{g}	170±20.00 ^{a}	90±7.00 ^{d}	135±13.00 ^{b}	130±10.00 ^{c}	65±5.00 ^{f}
6	20±2.50 ^{h}	35±5.00 ^{f}	45±7.00 ^{e}	35±4.00 ^{f}	140±15.00 ^{a}	125±8.00 ^{b}	25±4.00 ^{g}	100±15.00 ^{c}	85±10.00 ^{d}	45±11.00 ^{e}
8	100±7.00 ^{b}	20±2.50 ^{f}	40±5.00 ^{e}	60±6.00 ^{d}	100±10.00 ^{b}	40±5.00 ^{e}	100±15.00 ^{b}	80±9.00 ^{c}	80±5.00 ^{c}	105±7.00 ^{a}
10	125±10.00 ^{b}	55±6.00 ^{f}	60±7.0 ^{e}	40±5.00 ^{h}	135±9.00 ^{a}	45±2.50 ^{g}	55±7.00 ^{f}	120±10.00 ^{c}	105±11.00 ^{d}	30±5.00 ^{i}

Means followed by the same letter did not differ significantly (Tukey test, P>0.05).

Table 2. Total amino acid composition (%, w/w) and nutritional parameters (PER: protein efficiency ratio; E/T: essential to total amino acids; AAS: amino acid score; EAAI: essential amino acid index; BV: biological value), of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): Pepsin 3% (P); Trypsin 3% (T); Alcalase 3% (A); Pepsin 1.5% and Trypsin 1.5% (PT); Trypsin 1.5% and Pepsin 1.5% (TP); Trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and Trypsin 1.5% (AT); Alcalase 3% and Trypsin 3% (ATd); Trypsin 3% and Alcalase 3% (TAd).

g AA/ 100 g protein	Symbol	PE	P	T	A	PT	TP	TA	AT	ATd	TAd	WHO*
Arginine	Arg	12.55±3.02^{a}	11.5±3.91^{a}	13.12±3.51^{a}	11.48±4.82^{a}	11.75±0.52^{a}	11.69±3.55^{a}	11.39±0.11^{a}	11.27±0.02^{a}	10.31±0.20^{a}	11.45±0.42^{a}	
Serine	Ser	5.86±2.67^{a}	4.77±5.63^{ab}	4.77±2.28^{ab}	3.95±1.29^{ab}	4.03±5.20^{ab}	3.96±1.89^{ab}	3.14±4.68^{ab}	2.86±0.70^{b}	2.39±3.15^{b}	2.48±3.16^{b}	
Aspartic acid	Asp	18.28±1.49^{a}	9.37±3.09^{bc}	10.33±2.61^{b}	9.22±2.54^{bc}	8.77±4.27^{bcd}	8.86±3.77^{bcd}	7.46±1.64^{bcd}	6.90±0.24^{cd}	6.07±0.13^{d}	6.48±0.87^{cd}	
Glutamic acid	Glu	22.23±2.23^{d}	23.89±1.73^{bcd}	28.82±4.93^{a}	25.42±4.65^{bc}	25.78±0.16^{b}	25.28±1.25^{bc}	25.14±0.09^{bc}	24.41±2.22^{bcd}	22.65±0.17^{cd}	25.19±0.53^{bc}	
Threonine	Thr	5.45±2.65^{a}	3.86±3.37^{a}	4.17±1.64^{a}	3.76±4.11^{a}	3.50±0.63^{a}	3.58±1.61^{a}	3.92±0.18^{a}	3.70±0.44^{a}	3.49±0.61^{a}	4.00±0.27^{a}	2.3
Glycine	Gly	4.77±1.16^{a}	4.41±3.66^{a}	5.16±1.23^{a}	4.70±2.94^{a}	4.21±2.41^{a}	4.15±2.61^{a}	4.51±0.16^{a}	4.37±0.94^{a}	4.05±0.66^{a}	4.58±1.61^{a}	
Alanine	Ala	14.87±1.83^{a}	4.59±3.48^{b}	4.77±3.42^{b}	4.51±0.29^{b}	4.21±3.24^{b}	4.15±1.30^{b}	4.32±0.44^{b}	4.20±0.72^{b}	3.86±0.09^{b}	4.58±1.14^{b}	
Tyrosine	Tyr	3.00±4.55^{bc}	2.38±5.92^{c}	3.77±4.23^{bc}	3.20±3.85^{bc}	2.45±2.88^{bc}	2.26±5.74^{c}	4.71±1.77^{abc}	3.87±2.52^{abc}	5.34±0.35^{ab}	6.67±0.71^{a}	
Proline	Pro	5.45±1.51^{a}	6.06±4.36^{a}	5.76±1.24^{a}	4.70±4.99^{a}	4.38±5.48^{a}	4.52±5.01^{a}	5.10±5.14^{a}	4.88±3.97^{a}	4.41±4.46^{a}	5.15±4.82^{a}	
Methionine	Met	0.68±4.08^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	1.6
Valine	Val	5.32±2.33^{a}	5.14±1.77^{a}	5.56±4.46^{a}	5.27±4.57^{a}	4.73±0.86^{a}	4.71±1.89^{a}	5.10±0.88^{a}	5.21±0.28^{a}	4.78±0.65^{a}	5.34±0.55^{a}	3.9
Phenylalanine	Phe	4.77±3.78^{a}	5.33±4.74^{a}	5.16±2.59^{a}	5.46±3.46^{a}	5.08±4.28^{a}	5.09±3.44^{a}	4.91±0.40^{a}	5.05±1.15^{a}	4.78±1.00^{a}	5.15±1.45^{a}	
Isoleucine	Ile	5.04±2.49^{a}	3.86±5.15^{a}	4.77±3.19^{a}	4.51±4.10^{a}	4.21±0.62^{a}	3.96±3.27^{a}	4.51±0.11^{a}	4.54±0.74^{a}	4.23±1.64^{a}	4.58±0.98^{a}	3.0
Leucine	Leu	9.27±1.78^{a}	9.19±2.40^{a}	7.75±2.81^{a}	7.90±1.72^{a}	7.36±1.78^{a}	7.73±0.98^{a}	7.85±0.89^{a}	8.24±1.00^{a}	7.55±1.35^{a}	8.20±1.68^{a}	5.9
Histidine	His	3.54±5.6^{b}	9.92±5.63^{a}	4.77±6.05^{b}	4.70±6.22^{b}	4.38±6.47^{b}	4.15±5.95^{b}	4.12±5.09^{b}	3.53±3.55^{b}	3.86±5.41^{b}	4.00±5.07^{b}	1.5
Lysin	Lys	5.32±2.88^{a}	5.14±2.64^{a}	4.57±2.01^{a}	5.46±3.09^{a}	6.31±2.12^{a}	5.28±2.16^{a}	5.30±2.66^{a}	5.21±2.55^{a}	4.23±2.86^{a}	4.96±2.36^{a}	4.5
Tryptophane	Trp	0	0	0	0	0	0	0	0	0	0	0.6

Nutritional parameters	PE	P	T	A	PT	TP	TA	AT	ATd	TAd
PER-EQ.1	3.27^{a}	3.50^{a}	2.85^{a}	2.92^{a}	2.67^{a}	2.84^{a}	2.89^{a}	3.07^{a}	2.75^{a}	3.05^{a}
PER-EQ.2	3.51^{a}	3.60^{a}	2.89^{a}	2.98^{a}	2.77^{a}	2.94^{a}	2.90^{a}	3.11^{a}	2.73^{a}	2.98^{a}
PER-EQ.3	3.62^{ab}	5.19^{a}	1.67^{bcd}	2.32^{bc}	2.53^{bc}	2.95^{abc}	0.73^{cde}	1.70^{bcd}	-0.15^{de}	-0.87^{e}
PER-EQ.4	2.78^{a}	2.52^{a}	2.47^{a}	2.50^{a}	2.41^{a}	2.34^{a}	2.44^{a}	2.47^{a}	2.24^{a}	2.49^{a}
PER-EQ.5	3.31^{a}	3.41^{a}	3.08^{a}	3.11^{a}	2.99^{a}	2.91^{a}	3.12^{a}	3.04^{a}	2.91^{a}	3.28^{a}
E/T%	33.53^{f}	40.93^{ab}	35.78^{ef}	38.62^{bcde}	37.60^{cde}	37.00^{de}	39.84^{abcd}	40.06^{abc}	41.60^{a}	41.74^{a}
AAS%	119.31^{a}	107.18^{cd}	109.79^{bc}	109.21^{bc}	103.31^{e}	100.15^{f}	111.48^{b}	110.01^{bc}	105.65^{de}	119.47^{a}
EAAI%	91.48^{b}	94.92^{a}	88.31^{cd}	88.74^{bc}	86.94^{cd}	85.62^{d}	87.11^{cd}	75.39^{e}	69.95^{f}	88.34^{cd}
BV%	88.01^{b}	91.76^{a}	84.54^{c}	85.02^{c}	83.06^{cd}	81.62^{d}	83.25^{cd}	70.47^{e}	64.54^{f}	84.59^{c}

*: Essential amino acid recommendation by FAO/WHO/UNU (1981) for weaned (10–12 years old) children.
Means followed by the same letter did not differ significantly (Tukey test, P>0.05).

Table 3. Peptides identified in the different faba bean protein hydrolysates that share structure homology with previously described antioxidant peptides

Peptide	***Sample[a]***
Antioxidant peptides (100% homology)	
TETWNPNHPEL	PT, TP, AT, ATd, TAd
FVPH	PT, TP, AT
LY	PT, ATd, TAd
IY	PT, ATd, TAd
VY	PT, TA, AT, ATd
YV	PT, TP, TA, AT, TAd
LW	AT, ATd
IW	AT, ATd
AW	ATd
Peptides sharing the antioxidant TETWNPNHPEL sequence	
RLDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	ATd
LDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**R	T, PT, AT, ATd
LDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
DNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
NIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT
N**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
ALEPDHRVESEAGL**TETWNPNHPEL**	PT
LEPDHRVESEAGL**TETWNPNHPEL**	PT
EPDHRVESEAGL**TETWNPNHPEL**	PT, AT, ATd
VESEAGL**TETWNPNHPEL**	PT, AT, ATd
SEAGL**TETWNPNHPEL**	PT, TP, ATd
AGL**TETWNPNHPEL**	PT, TP, AT, ATd
GL**TETWNPNHPEL**R	PT
GL**TETWNPNHPEL**	PT, TP, AT, ATd
L**TETWNPNHPEL**R	TP
L**TETWNPNHPEL**	PT, TP
TETWNPNHPELR	TP
Peptides sharing the antioxidant ALEPDHR sequence	
RLDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	ATd
RLDNIN**ALEPDHR**VE	A, TA, AT, ATd
LDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**R	T, PT, AT, ATd
LDNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
LDNIN**ALEPDHR**VESEAGLTET	PT
LDNIN**ALEPDHR**VESEAGLTE	AT, ATd
LDNIN**ALEPDHR**VESEAGLT	AT, ATd
LDNIN**ALEPDHR**VESEAGL	PT, TP, AT, ATd
LDNIN**ALEPDHR**VESEAG	PT, TP
LDNIN**ALEPDHR**VESEA	PT, AT, ATd
LDNIN**ALEPDHR**VESE	AT, ATd
LDNIN**ALEPDHR**VE	TA, ATd
LDNIN**ALEPDHR**	AT, ATd
DNIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
DNIN**ALEPDHR**VESEAG	PT
DNIN**ALEPDHR**VE	A, TA, AT, ATd
NIN**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT
N**ALEPDHR**VESEAGL**TETWNPNHPEL**	PT, AT, ATd
N**ALEPDHR**VESEAGL	PT, TP, AT, ATd
N**ALEPDHR**VE	AT, ATd, TAd
N**ALEPDHR**	AT
ALEPDHRVESEAGL**TETWNPNHPEL**	PT
ALEPDHRVESEAGL	P, PT, TP
ALEPDHRVESEAG	TP

ALEPDHRVESE	A, TP, AT, ATd
ALEPDHRVE	A, TA, AT, ATd, TAd

Peptides sharing the antioxidant VIPAGYP sequence

QVQNYKAKLSPGDVL**VIPAGYP**VAIK	T
KAKLSPGDVL**VIPAGYP**VAIKA	A
KAKLSPGDVL**VIPAGYP**VAIK	A
AKLSPGDVL**VIPAGYP**VAIKASSNLNLVGFGINAENNQR	T, A
AKLSPGDVL**VIPAGYP**VAIK	T, TP, AT
AKLSPGDVL**VIPAGYP**VA	TP
LSPGDVL**VIPAGYP**VAIKASSNLNLVGFGINAENNQR	T, A, TP
LSPGDVL**VIPAGYP**VAIKA	A
LSPGDVL**VIPAGYP**VAIK	T, A, TP, AT
LSPGDVL**VIPAGYP**VAI	AT, ATd
L**VIPAGYP**VAIKASSNLNL	P
L**VIPAGYP**VAIK	TP
L**VIPAGYP**VA	P
VIPAGYPVAIKASSNLNL	TP
VIPAGYPVAIKASSNL	TP
VIPAGYPVAIKA	A
VIPAGYPVAIK	TP, A

Peptides sharing the antioxidant PHW sequence

LYRNG**IY**A**PHW**NINANSLL	TP
LYRNG**IY**A**PHW**NINANSL	TP
LYRNG**IY**A**PHW**NINA	TP
YRNG**IY**A**PHW**NINANSLL	TP, P
YRNG**IY**A**PHW**NINANSL	TP
YRNG**IY**A**PHW**NINAN	PT
YRNG**IY**A**PHW**NINA	TP, P
YRNG**IY**A**PHW**NIN	A
YRNG**IY**A**PHW**	P, TP
RNG**IY**A**PHW**NIN	AT, ATd
A**PHW**NINAN	PT

Peptides sharing the antioxidant PHY sequence

VN**YVE**INEGSLLL**PHY**NSR	TA
VN**YVE**INEGSLLL**PHY**NS	AT
VN**YVE**INEGSLLL**PHY**N	A
YVEINEGSLLL**PHY**NSR	TA, TAd, A
YVEINEGSLLL**PHY**NS	AT
YVEINEGSLLL**PHY**N	TA, A
VEINEGSLLL**PHY**NSRAIV	P
VEINEGSLLL**PHY**NSR	A
VEINEGSLLL**PHY**	TP, P
INEGSLLL**PHY**NSR	A
EGSLLL**PHY**NSR	A
L**PHY**N	A

Peptides sharing the antioxidant YVE sequence

VN**YVE**INEGSLLL**PHY**NSR	TA
VN**YVE**INEGSLLL**PHY**NS	AT
VN**YVE**INEGSLLL**PHY**N	A
VN**YVE**INEGSLLLPH	TAd, A
VN**YVE**IN	A
VN**YVE**	TA, ATd
YVEINEGSLLL**PHY**NSR	TA, TAd, A
YVEINEGSLLL**PHY**N	TA, A
YVEINEGSLLLPH	TAd, A
YVEINE	A
YVEIN	TA, ATd, TAd

Peptides sharing other antioxidant sequence	
E**TW**NPNHPEL	TAd
E**TW**NPNHPE	A, TAd
TWNPNHPEL	A, AT, TAd
TWNPNHPE	A, TAd
TWNPN	TAd
PGCPQT**YQEP**R	PT
T**YQEP**RSS	A
T**YQEP**R	AT, ATd
YQEPR	TP
LYVIR	A
GT**TY**	A
S**AY**	TAd
AYGE	AT
AYE	TP
V**TY**	TAd
S**TY**	TAd
Peptides fragment of antioxidant sequence	
YVR	A, TA
AVPY	T, PT, TA, AT
YF	T
HR	PT, TA, TAd
YN	PT, AT
FY	TA
NY	TA
WT	AT
SY	AT

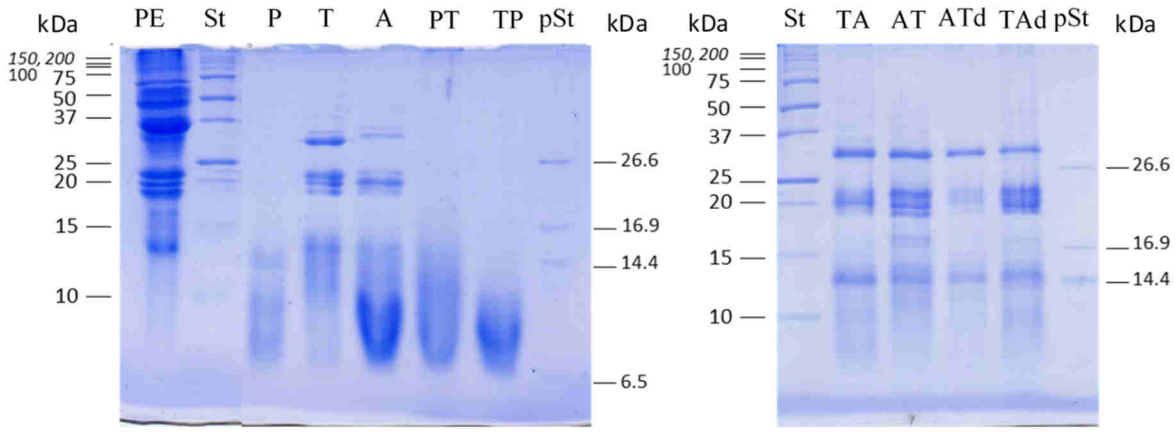
[a]Sample in which the peptide was identified (P: Pepsin 3%; T: Trypsin 3%; A: Alcalase 3%; PT: Pepsin 1.5% and Trypsin 1.5%; TP: Trypsin 1.5% and Pepsin 1.5%: TA: Trypsin 1.5% and Alcalase 1.5%; AT: Alcalase 1.5% and Trypsin 1.5%; TAd: Trypsin 3% and Alcalase 3%; ATd: Alcalase 3% and Trypsin 3%).

Table 4. Sensory properties, pH and Total soluble solids (TSS, ºBrix) of apple juice fortified with faba bean protein hydrolysates (PHs). P, T, A, PT, TP, TA, AT, ATd and TAd represent PE hydrolyzed with enzymes (%, w/v): Pepsin 3%; Trypsin 3%; Alcalase 3%; Pepsin 1.5% and Trypsin 1.5%; Trypsin 1.5% and Pepsin 1.5%; Trypsin 1.5% and Alcalase 1.5%; Alcalase 1.5% and Trypsin 1.5%; Alcalase 3% and Trypsin 3%; Trypsin 3% and Alcalase 3%. C = Control (apple juice).

Attribute	C	P	T	A	PT	TP	TA	AT	ATd	TAd
Sour	4.9±2.3^{ab}	5.8±1.8^{a}	3.8±2.2^{cd}	4.1±1.9^{bc}	3.6±1.8^{cd}	5.5±1.9^{a}	2.9±1.7^{d}	3.1±1.7^{cd}	3.1±1.8^{cd}	3.0±1.8^{d}
Sweet	5.4±1.7^{a}	4.1±1.9^{b}	5.4±1.8^{a}	5.5±1.9^{a}	5.9±1.8^{a}	4.4±1.9^{b}	6.0±1.9^{a}	6.1±1.8^{a}	5.8±1.8^{a}	5.9±1.7^{a}
Salty	2.1±1.2^{b}	3.0±1.9^{b}	2.6±1.4^{b}	2.3±1.6^{b}	2.6±1.8^{b}	4.1±2.5^{a}	2.7±1.9^{b}	2.7±1.9^{b}	2.5±1.8^{b}	2.8±1.9^{b}
Apple aroma	6.5±2.1^{a}	4.6±1.9^{c}	6.0±1.9^{ab}	5.6±1.9^{abc}	5.8±2.0^{abc}	4.7±2.0^{c}	5.7±1.9^{abc}	5.8±1.6^{abc}	6.9±5.2^{a}	5.0±2.1^{bc}
Overall acceptance	5.0±1.7^{ab}	5.3±1.5^{ab}	4.8±2.0^{bc}	4.8±2.1^{bc}	4.3±2.2^{bcd}	3.9±2.1^{cd}	3.7±2.2^{d}	3.4±2.1^{d}	6.0±2.1^{a}	5.0±2.0^{ab}
Comments	The most appreciated smell Clear	Bitterness Sour	The most turbid sample; Presence of precipitate	Presence of precipitate; Good smell, the most similar to apple; Low bitterness; Little black points inside	Low turbidity, with fruits pieces,	Low bitterness, Presence of insoluble particles	Low turbidity Unpleasant smell, Salty and sour	Low turbidity Low bitterness, Smell of yeast	Low astringency, low bitterness Turbidity, Flour-like smell	Low turbidity Artificial taste Presence of other fruit smell more than apple, Precipitate
pH	3.41^{a}	3.27^{a}	3.71^{a}	3.78^{a}	3.93^{a}	3.23^{a}	4.13^{a}	4.13^{a}	4.03^{a}	4.13^{a}
TSS	11.2^{b}	12.4^{ab}	11.9^{ab}	13^{a}	11.8^{ab}	12.1^{ab}	11.9^{ab}	11.9^{ab}	12.1^{ab}	11.8^{ab}

Means followed by the same letter did not differ significantly in the same raw (for sensory data, LSD Fisher test was applied, P>0.05; for pH and TSS values, Tukey test was applied, P>0.05).

Figure 1.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St
P
T
A
PT
TP
pSt
kDa
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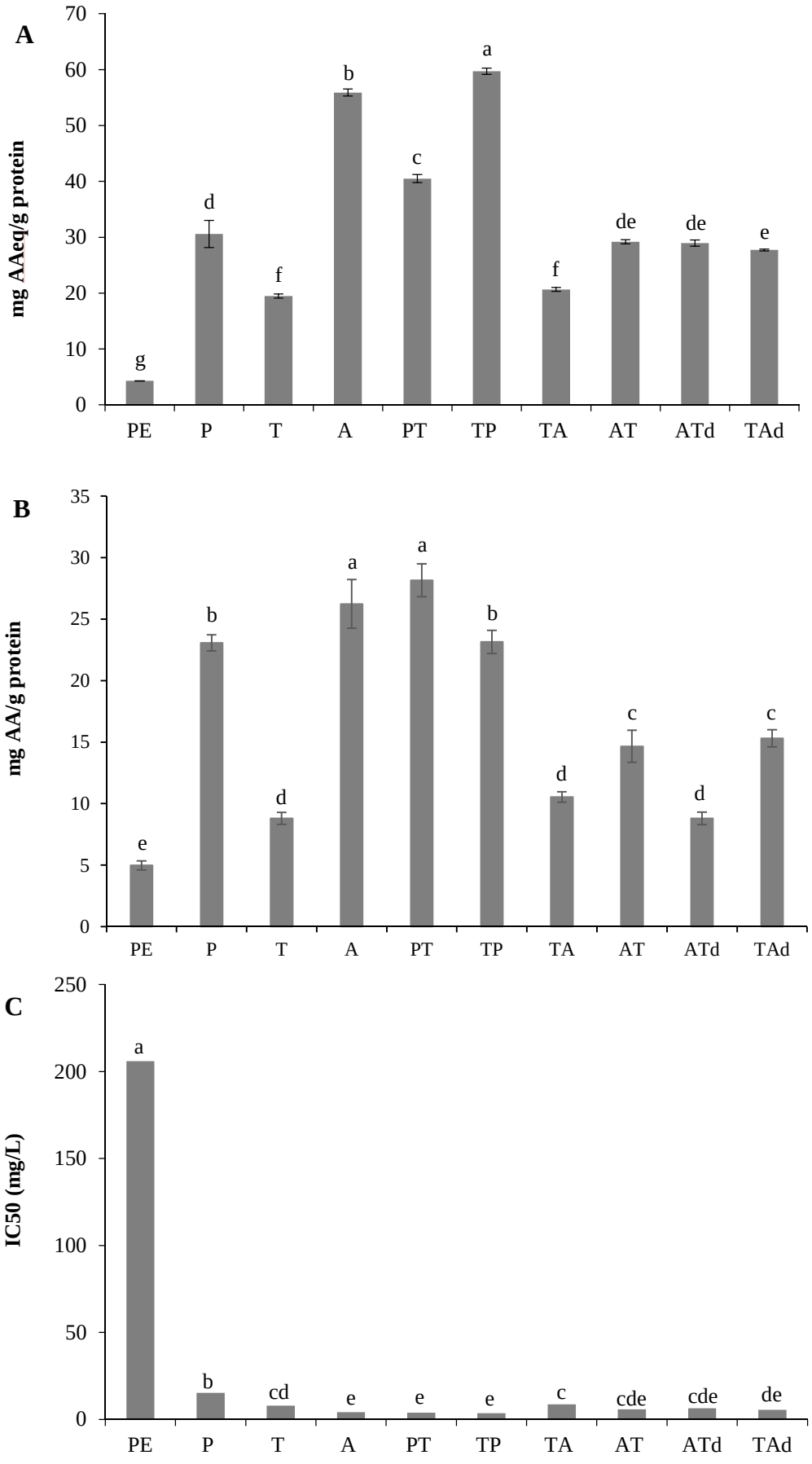
100
75
50
37
25
20
15
10
26.6
16.9
14.4
6.5
kDa
St
TA
AT
ATd
TAd
pSt
kDa
150, 200
100
75
50
37
25
20
15
10
26.6
16.9
14.4

Figure 2.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40
30
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g
d
f
b
c
a
f
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PE
P
T
A
PT
TP
TA
AT
ATd
TAd
B
mg AA/g protein
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30
25
20
15
10
5
0
e
b
d
a
a
b
d
c
d
c
PE
P
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A
PT
TP
TA
AT
ATd
TAd
C
IC50 (mg/L)
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0
a
b
cd
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cde
cde
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PE
P
T
A
PT
TP
TA
AT
ATd
TAd