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

Elsevier Editorial System(tm) for Food Chemistry or its open access mirror
Manuscript Draft

Manuscript Number: FOODCHEM-D-20-01048R1

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

Article Type: Research Article (max 7,500 words)

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

Corresponding Author: Professor Tullia Gallina Toschi,

Corresponding Author's Institution:

First Author: Seyedeh Parya Samaei

Order of Authors: Seyedeh Parya Samaei; Mohammad Ghorbani; Davide Tagliazucchi; Serena Martini; Roberto Gotti; Thomas Themelis; Federica Tesini; Andrea Gianotti; Tullia Gallina Toschi; Elena Babini

Abstract: Enzymatic hydrolysis of plant-derived proteins can improve their quality by 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, and 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 had antioxidant motifs in their sequences. 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.

*Response to Reviewers

Reviewer #1: Overall comments

The manuscript used different enzymes to hydrolyse faba bean proteins to improve its functional properties and bioactivity. The peptide sequences in the hydolysates were identified. Adding these hydrolysates to apple juice could improve its the nutritional and functional property without compromising on the taste. However, this study is lack of novelty and enzymatic hydrolysis has been done substantially as a way to improve the functional property of proteins. In addition, considering the price of the hydrolysates, practical application in apple juice might be a problem.

Reply: We agree with the reviewer that protein hydrolysis by proteases has been frequently carried out in the past as a means to improve the functional properties of food proteins. This is clearly not the innovative aspect of our work. However, the novelty of this work lies in the integrated approach which combines the proteolysis with the analysis of technological, nutritional, functional, and sensorial properties of the hydrolysates as well as the identification of functional compounds by high resolution mass spectrometry. The combination of all these aspects gave us the opportunity to identify the hydrolysate(s) which can have the most impact on human health without prejudging the sensorial aspects of apple juice.

Concerning the problem of the cost of hydrolysates, this is certainly an important assessment to consider for future commercial applications. We did not deal with this aspect because, after establishing the protocol for preparation of hydrolysates and evaluation of their properties, it could be possible to optimise the production process in a scale-up phase, reducing the costs. Different business reports (https://www.businesswire.com/news/home/20200121005546/en/Global-Protein-Hydrolysates-Market-Estimated-Grow-CAGR; https://www.industryarc.com/Research/Protein-Hydrolysates-Market-Research-504438; https://www.gminsights.com/industry-analysis/protein-hydrolysate-market) indicate that the protein hydrolysate market will increase during the next years. For this reason and also considering faba as a very promising source, we believe there is space for applications of these hydrolysates in food and drink supplementation.

Also, the introduction is not in a good flow and logic and English needs to be improved.

Reply: The introduction was modified, and the entire paper was carefully corrected by a native English language speaker.

The detailed comments are listed below:

Major comments and suggestions:

(1) L211, Comment: The authors might want to check the equation of EAAI. The following reference might be help: FAO (1991). Protein Quality Evaluation, Report of the Joint FAO/WHOExpert Consultation. Rome: FAO Food and Nutrition Paper No. 51. Also, the reference for calculation of EAAI should be added

Reply: the equation was reported in Amza, Balla, Tounkara, Man & Zhou, 2013, and thus this reference for calculation of EAAI was added to the text.

(2) L369-422, comment: I am curious the value of this measurement. Since the hydrolysates were produced from the same protein source, the amino acid compositions and some nutritional properties should be similar as hydrolysis only release the peptides or amino acid rather than convert one type of amino acid to another.

Reply: Only in some of the samples were the AAs amounts found to be significantly different. Moreover, the amino acid content of PE is referred to all the possible sources of amino acids in the extract (i.e. soluble and insoluble proteins, peptides and free amino acids), whereas the amino acid content of PHs is referred to the amino acid sources that are soluble after hydrolysis (see lines 362-364). The discrepancies found are explained as reported in text at 374-385.

(3) Q: Many bioactive peptides with antioxidant activity were identified, is that possible to quantify them based on their signal intensity? This information is important as both amino acid composition and their proportions contribute to the final bioactivity.

Reply: The ionization of specific peptides in mass spectrometry experiments is a major limitation in quantitative analysis with electrospray ionization mass spectrometry (ESI-MS). The relative ionization of individual peptides is dependent on intrinsic and extrinsic factors. The most important extrinsic factor is the

so-called "matrix effect" which is caused by the co-elution of matrix components (typically salts, ions, highly polar compounds and carbohydrates) that may alter (either suppressing or enhancing) the ionization of the target analyte (Furey, Moriarty, Bane, Kinsella, & Lehane 2013). The intrinsic factors are related to the amino acid sequence of the peptides. Some amino acids, such as basic or hydrophobic amino acids, are more efficiently ionized and give more intense signals in ESI-MS experiments (Cech, & Enke, 2000).
Given these considerations, it is clear that, to overcome these limitations, each single peptide needs to be quantified by using a specific synthetic standard. Furthermore, to obtain reliable quantitative data, specific experimental conditions for each peptide should be set in the mass spectrometer. Finally, the scope of our experimental plan was to obtain a qualitative picture of the peptidomic profile of the samples.
***Cech**, N. B., & Enke, C. G. (2000). Relating electrospray ionization response to nonpolar character of small peptides. Analytical Chemistry, 72, 2717-2723.*
***Furey**, A., Moriarty, M., Bane, V., Kinsella, B., & Lehane, M. (2013). Ion suppression a critical review on causes, evaluation, prevention and applications. Talanta, 115, 104-122.*

(4) Q: How the hydrolysis time was decided for each enzyme or its combination? Is that based on degree of hydrolysis or something else? Such information might be required
Reply: preparation of protein hydrolysates for food application is usually obtained by enzymatic reactions in the range of 1 to 5 h, and other papers reporting that maximum activity was reached after 2 hours (for example, Tang et al., 2009). Ali (2019) reported that the antioxidant activity of faba bean protein cultivar Giza3, studied by using pepsin, was increased with increasing time of hydrolysis from 0 to 180 min (best antioxidant activity in the range of 60-180 min). The higher degree of hydrolysis was observed at reaction time of 60-180 min as well (the maximum at the 180 min). The same trend was observed during the hydrolysis of rapeseed protein isolates, whey protein, red tilapia fish protein, and rice bran protein concentrate using alcalase, chymotrypsin, flavourzyme, neutrase thermolysin, protamex, and papain enzymes (Chabanon et al., 2007; Karamac and Rybarczyk, 2008; Dryakova et al., 2010; Daud et al., 2013; Wisuthiphaet et al., 2015; Ahmadifard et al., 2016). Once the optimal enzyme or enzyme combination was found, capable of producing the hydrolysate with highest bioactivity, functional and sensory properties, evaluation of the effect of different hydrolysis times, these properties could be evaluated.
References:
***Tang**, S. Z., Kerry, J. P., Sheehan, D., & Buckley, D. J. (2002). Antioxidative mechanisms of tea catechins in chicken meat systems. Food Chemistry, 76, 45-51. https://doi.org/10.1016/S0308-8146(01)00248-5.*
***Ali**, M. (2019). Functional properties of faba bean protein and effect of enzymatic hydrolysis on its antioxidant activity. Zagazig Journal of Agricultural Research, 46(1), 2019.*
***Chabanon**, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. (2007). Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. Process Biochemistry, 42, 1419-1428.*
***Karamac**, M., Kosinska-Cagnazzo, A., & Kulczyk, A. (2016). Use of different Proteases to Obtain Flaxseed Protein Hydrolysates with Antioxidant Activity. International Journal of Molecular Sciences, 17(7), 1027. https://doi.org/10.3390/ijms17071027.*
***Dryakova**, A., Pihlanto, A., Marnila, P., Curda, L., & Korhonen, H. J. (2010). Antioxidant properties of whey protein hydrolysates as measured by three methods. European Food Research and Technology, 230, 865-874.*
***Daud**, N. A., Babji, A. S., and Yusop, S. M. (2013). Antioxidant Activities of Red Tilapia (Oreochromis niloticus) Protein Hydrolysates as Influenced by Thermolysin and Alcalase. AIP Conf. Proc. AIP. DOI: 10.1063/1.4858734*
***Wisuthiphaet**, N., Kongruang, S., & Chamcheun, C. (2015). Production of fish protein hydrolysates by acid and enzymatic hydrolysis. Journal of Medical and Bioengineering, 4.*
***Ahmadifard**, N., Murueta, J. H. C., Abedian-Kenari, A., Motamedzadegan, A., & Jamali, H. (2016). Comparison the effect of three commercial enzymes for enzymatic hydrolysis of two substrates (rice bran protein concentrate and soy-been protein) with SDS-PAGE. Journal of Food Science and Technology, 53, 1279-1284.*

Minor comments and suggestions:

(1) L142, add "by" in front of "inactivating"
Reply: Correction was made. See lines 130-131.

(2) L153, Q: Protein pattern analysis of what? Need to put the sample names, such as PE or its hydrolysates in this section
Reply: Sample names were added.

(3) L159-161, Comment: Might need to add the protein concentration of which sample, see above comment. The part is not clear, might need to be in more detail
Reply: It is now specified in Par 2.5 that the soluble protein concentration and total protein content were measured on PE and PHs using the Bradford and Kjeldahl methods, respectively. The Bradford method was not described in more detail as the kit used from Sigma has a basic and easy protocol that can be reproduced by simply following the instructions of this kit. For Kjeldahl, the description is essential but data necessary to reproduce the method are all available (amount of sample, solution descriptions reaction times and temperatures).

(4) L169-170, "shaken very well" change to "well mixed"
Reply: This sentence was changed. See lines 158-159.

(5) L171, change "HCl and 1N or 10 N NaOH" to "HCl or NaOH"
Reply: Correction was made. See line 159.

(6) L183, "0" should be subscript in equation of FC, same for "t" in FS
Reply: Correction was made. See lines 173 and 177.

(7) L305, "P" should be italic
Reply: Correction was made. See line 287.

(8) Figure 1. The unit KDa should be added somewhere in the Figure
Reply: Correction was made.

(9) Ll350, change "more" to "higher"
Reply: Correction was made. See line 336.

(10) Table 1, comment: add the standard deviation and statistic analysis for foaming capacity and stability.
Reply: Table 1 was completed.

Reviewer #2:
The manuscript provides a comparative assessment of How sorghum genotypes affect the functional and sensory properties of whole meal expanded extrudates for which little prior information has been reported. However, the authors of the manuscript need enlightening substantially the results presented. Several clarifications are required for contribute with the manuscript, as follow:
-Stat of art, of the topic developed in this work, as well as the novelty and the contribution to knowledge, should be highlighted and included in the Introduction.
-The authors should be deepening the discussion about the results obtained in this manuscript.
-The quality of study (scientific novelty) could be improve if the results observed being compared with other studies.
-Authors measured the antioxidant activity using two different protocols. What is the rationale of using two different protocols? Do they provide different information?
-The conclusion of the study presented does not reflect the experimental findings.
Reply: It seems that there has been a mistake as the Reviewer's comments relate to another work. Therefore, the comments of this reviewer were not taken into consideration.

Reviewer #3

The reading of paper has been enough easy to understand the work and there are data about faba protein hydrolysates used to fortify fruit juices but I think they could improvement it.

1. The number of authors is too large for the hard work carried out by the ten authors in the trials. It is necessary to justify it.
Reply: The Credit Author Statement submitted with this paper has been now modified, adding the role played by each author.

2. In the lines 88 and 89. The phrase "Many examples are reported in literature" could be deleted.
Reply: Introduction was modified, and the sentence was removed.

3. The formule of Foaming capacity (FC), it is Ao not AO.
Reply: Correction was made. See line 173.

4. Line 415: blank is left over
Reply: Correction was made. See line 398.

5. The title needs to be changed to Sensory properties of PHs and fortified apple juice
Reply: The title was changed to "Functional, nutritional, antioxidant, sensory properties and comparative peptidomic profile of faba bean (Vicia faba, L.) seed protein hydrolysates and fortified apple juice".

6. Could you tell me why you use Duncan test and not Tukey test?
Reply: The method of Duncan can be applied in the same conditions of the Tukey's test, but with a significance (experiment wise) less cautionary, and then a power to a single test (or comparison wise) greater. According to the Reviewer's suggestion, the Duncan test was replaced by Tukey test.

7. I suggest to be careful with with the requested format by the editor both in the significance values ($P<0.05$ o $P>0.05$) and in the references (there is mix).
Reply: The format of significance values and References were checked.

8. I don not found the reference FAO/WHO/UNU (1981).
Reply: The reference was added.

As far as I'm concerned, that's all I can observe.

Reviewer #4: Manuscript ID: FOODCHEM-D-20-01048

Title: "Functional, nutritional, antioxidant and sensory properties of faba bean (Vicia faba, L.) seed protein hydrolysates and comparative peptidomic profile"
General comments
The paper analyses the nutritional, functional, antioxidant and sensory quality of faba bean protein hydrolysates obtained by different enzymes.
The basic idea of the manuscript is good, and it could be of practical interest.
However, there is information that should be completed

MATERIAL AND METHODS
I think that it is necessary to specify details of the sample. For example, environmental conditions, maturity stage, etc. can condition the composition in the faba bean and its functional properties.
Answer: Faba beans were dried and completely matured. This information was added at the beginning of Par. 2.1. See lines 111-112. No other information was available as this was a commercial product.

Lines 231, 238, 246: How were those samples prepared? (mg/mL?)

Answer: Paragraph 2.8 was reduced to answer to the request of one of the Reviewer. Sample solutions were in mg/mL, this information was added in lines 218-219.

Why is IC50 used in some antioxidant capacity determinations and not in others?
Answer: The expression of the data as equivalent of a standard compound is the best way to present data when the plot absorbance value vs standard concentration is linear such as in the ABTS and DPPH assays. However, the Fe2+-chelating assay is a binding assay where the absorbance data are converted in a % of binding inhibition. In this case, the plot absorbance value vs % of inhibition shows a sigmoidal trend rather than a linear one. With this type of behaviour, it is inappropriate to express data as standard equivalent since they are strongly dependent on the amount of sample. Therefore, the correct way is to carry out the assay by using different amounts of sample and, through the building of the sigmoidal plot, calculate the IC50 (i.e. the concentration of sample needed to inhibit the binding by 50%).

pH and total soluble solids are physical parameter and not sensory determination.
What is the unit of soluble solids? ºBrix?
Answer: The title of the table was corrected and the unit of TSS was added.

REFERENCES
Check references: different format in journal, volume, …
Answer: References were checked and edited.

TABLES
Put in the footnote what the statistics letters mean
Reply: Correction was made.

Reviewer #5: FOODCHEM-D-20-01048
This work studies the different properties of faba bean (Vicia faba, L.) seed protein hydrolysates and comparative peptidomic profile. The study is interesting in the aims that it sets out but it needs some correcting before being published.

-In section 2.3 it states that the reaction time between the enzymes and PE is 180 min. for the obtainment of peptides. Normally, in proteomics, the enzyme action takes a much longer period of time (overnight). The authors should explain on what basis they have come to use 180 min.
Reply: preparation of protein hydrolysates for food application is usually obtained by enzymatic reactions in the range of 1 to 5 h, and other papers report that maximum activity is reached after 2 hours (for example, Tang et al., 2009). Ali (2019) displayed that the antioxidant activity of faba bean protein cultivar, Giza3 studied by using pepsin enzyme was increased with increasing the time of hydrolysis from 0 to 180 min (better antioxidant activity in the range of 60-180 min). The higher degree of hydrolysis was observed at a reaction time of 60-180 min as well (the maximum at the 180 min). The same trend was observed during the hydrolysis of rapeseed protein isolates, whey protein, red tilapia fish protein and rice bran protein concentrate using alcalase, chymotrypsin, flavourzyme, neutrase thermolysin, protamex, and papain enzymes (Chabanon et al., 2007; Karamac and Rybarczyk, 2008; Dryakova et al., 2010; Daud et al., 2013; Wisuthiphaet et al., 2015; Ahmadifard et al., 2016). Once found the enzyme or enzyme combination, capable of producing the hydrolysate with highest bioactivity, functional and sensory properties, evaluation of the effect of different hydrolysis times, on these properties, could be evaluated.
References:
***Tang**, S. Z., Kerry, J. P., Sheehan, D., & Buckley, D. J. (2002). Antioxidative mechanisms of tea catechins in chicken meat systems. Food Chemistry, 76, 45-51. https://doi.org/10.1016/S0308-8146(01)00248-5.*
***Ali**, M. (2019). Functional properties of faba bean protein and effect of enzymatic hydrolysis on its antioxidant activity. Zagazig Journal of Agricultural Research, 46(1), 2019.*

***Chabanon**, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. (2007). Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. Process Biochemistry, 42, 1419-1428.*
***Karamac**, M., Kosinska-Cagnazzo, A., & Kulczyk, A. (2016). Use of different Proteases to Obtain Flaxseed Protein Hydrolysates with Antioxidant Activity. International Journal of Molecular Sciences, 17(7), 1027. https://doi.org/10.3390/ijms17071027.*
***Dryakova**, A., Pihlanto, A., Marnila, P., Curda, L., & Korhonen, H. J. (2010). Antioxidant properties of whey protein hydrolysates as measured by three methods. European Food Research and Technology, 230, 865-874.*
***Daud**, N. A., Babji, A. S., and Yusop, S. M. (2013). Antioxidant Activities of Red Tilapia (Oreochromis niloticus) Protein Hydrolysates as Influenced by Thermolysin and Alcalase. AIP Conf. Proc. AIP. DOI: 10.1063/1.4858734*
***Wisuthiphaet**, N., Kongruang, S., & Chamcheun, C. (2015). Production of fish protein hydrolysates by acid and enzymatic hydrolysis. Journal of Medical and Bioengineering, 4.*
***Ahmadifard**, N., Murueta, J. H. C., Abedian-Kenari, A., Motamedzadegan, A., & Jamali, H. (2016). Comparison the effect of three commercial enzymes for enzymatic hydrolysis of two substrates (rice bran protein concentrate and soy-been protein) with SDS-PAGE. Journal of Food Science and Technology, 53, 1279-1284.*

-In section 2.7 it is not easy to understand how they have analyzed the amino acid content of PE without having previously made an enzyme hydrolysis of the proteins in order to liberate amino acids. This is also related to lines 375-378.
Reply: A standard oven acidic hydrolysis of the proteins at high temperature was applied for analysis of the amino acid composition as highlighted in the revised version of the manuscript (experimental details were also reported), see lines 604-605. An additional reference, supporting the reason for applying this conventional method, was introduced (Weiss, M., Manneberg, M., Juranville, J. F., Lahm, H. W., & Fountoulakis, M. (1998). Effect of the hydrolysis method on the determination of the amino acid composition of proteins. Journal of Chromatography A, 795, 263-275. https://doi.org/10.1016/S0021-9673(97)00983-7).

-The name of the reactive of the derivation FMOC-CL should be specified.
Reply: Correction was made, see line 191.

-In the formulae E/T% why are the amino acids asparagine and glutamine not taken into account in the denominator?
Reply: The formula was according to Wani, et al. (2011); this Reference was added to the paper. In our samples the amount of asparagine and glutamine was zero.

-Section 2.8 is excessively long. The different methods to calculate the antioxidant activity should be explained in one single section. Likewise, the Material and Methods section is excessively long.
Reply: Subparagraphs of Section 2.8 were removed, and the entire Materials and Methods section was reduced, trying to keep all essential information to ensure reproducibility of the methods.

-It is necessary to put the significant figures of the measures and the error margins correctly on the Tables.
Reply: Tables were checked.

-In the discussion of Table 1, there should be some comment made on why at the same pH exists the big difference of solubility between the different hydrolysates and why the solubility is generally less in AT, ATd and TAd.
Reply: the different solubility observed for different PHs is due to the specific peptide profile (molecular size and exposure of hydrophilic or hydrophobic groups) generated by each enzyme or combination of enzymes. Samples AT, ATd, and TAd showed on SDS-PAGE lower hydrolysis, which might explain their lower solubility. A comment was added to the text (lines 326-330).

-In Table 3 there is a peptide of one amino acid (W: tryptophan).
Reply: We apologize for the typo and wish to thank the reviewer for his comment. We deleted W from Table 3 since, of course, it is not a peptide.

-It should be explained how it is known that the sequence ALEPDHR is an antioxidant peptide.
Reply: We apologize for the missing information, some discussion about the antioxidant properties of ALEPDHR were added in the manuscript. See lines 501 to 506.

-Table 2 does not show the data of protein efficiency ratio (PER).
Reply: PER values are reported in Table 2 (PER-EQ1 to PER-EQ.5). The title of Table 2 was corrected.

-Some conclusions should be reached on which sample of the nine faba bean protein hydrolysates obtained (P, T, A, PT, TP, TA, AT, TAd, ATd) is the best for enriching apple juice.
Reply: A comment on the best samples for application of protein hydrolysates as ingredients for apple juice has been added to conclusions (see lines 589-591).

Highlights

- Faba bean proteins and hydrolysates had good nutritional value
- Enzymatic hydrolysis improved functional properties of faba proteins
- Faba protein hydrolysates showed antioxidant activity
- Antioxidant peptides were identified by peptidomic analysis
- Apple juice supplemented with hydrolysates had acceptable sensory properties

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

Seyedeh Parya Samaei[a], Mohammad Ghorbani[a], Davide Tagliazucchi[b], Serena Martini[b], Roberto Gotti[c], Thomas Themelis[c], Federica Tesini[d], Andrea Gianotti[d], Tullia Gallina Toschi[d] *, Elena Babini[d]

[a] Gorgan University of Agricultural Sciences and Natural Resources, Department of Food Science, Shahid Beheshti Avenue, Gorgan, Iran

[b] University of Modena and Reggio Emilia, Department of Life Sciences (DSV), Via Amendola 2, 42122 Reggio Emilia, Italy

[c] *Alma Mater Studiorum* - University of Bologna, Department of Pharmacy and Biotechnology, Via Belmeloro 6, 40126 Bologna, Italy

[d] *Alma Mater Studiorum* - University of Bologna, Department of Agricultural and Food Sciences, Piazza Goidanich 60, 47521 Cesena, Italy

*Corresponding author: Tullia Gallina Toschi

Tel. +39 051 2096010

Tel. +39 0547 636121

Fax. +39 051 2096017

E-mail address: tullia.gallinatoschi@unibo.it (Tullia Gallina Toschi)

Authors e-mails:

Seyedeh Parya Samaei: samaeiparya@gmail.com

Mohammad Ghorbani: moghorbani@yahoo.com

Davide Tagliazucchi: davide.tagliazucchi@unimore.it

Serena Martini: serena.martini@unimore.it

Roberto Gotti: roberto.gotti@unibo.it

Thomas Themelis: thomas.themelis2@unibo.it

Federica Tesini: federica.tesini@unibo.it

Andrea Gianotti: andrea.gianotti@unibo.it

Elena Babini: elena.babini2@unibo.it

Abstract

Enzymatic hydrolysis of plant-derived proteins can improve their quality by 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–MS/MS. Hydrolysis improved solubility of faba proteins at acidic and neutral pH, and 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 had antioxidant motifs in their sequences. 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-ethylbenzothiazoline-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, as it is high in proteins, carbohydrates, vitamins, minerals, and dietary fiber (Multari, Stewart & Russel, 2015). The ever-increasing demand for substitutes for animal-based proteins caused by population growth is directing the scientific community's interest towards this legume. The protein content of faba bean ranges from 27% to 34% of 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). Compared to the FAO profile of required amino acids, the amino acid composition of faba beans is low in the sulfur-containing amino acids cysteine and methionine, as well as tryptophan (Kaldy & Kasting, 1974).

One 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 study demonstrated 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

84 properties (Ali, 2019). It is worth noting that proteolysis can improve the biological properties of
85 other foods as well. For example, lima bean (*Phaseolus lunatus*, L.) protein hydrolysates produced
86 with sequential pepsin-pancreatin hydrolysis have high ACE-inhibitory activity (Chel-Guerrero,
87 Dominguez-Magana, Martinez-Ayala, Davila-Ortiz & Betancur-Ancona, 2012). Moreover, the
88 hydrolysis of other plant proteins such as rice, rice bran, and hemp seed with proteolytic enzymes
89 (neutrase, pepsin, Alcalase, and pancreatin) has produced peptides with antioxidant activity
90 (Rizzello et al., 2016).
91 Proteolysis may also modify nutritional properties offering the significant advantage of eliminating
92 anti-nutritional compounds. For faba beans, in particular, this means removing the favism-inducing
93 glycosides, vicine, and convicine (Vioque et al., 2012).
94 A third advantage of proteolysis is to improve the functional properties of plant proteins. For
95 example, the bioavailability of plant proteins is often limited because of their low solubility in
96 aqueous media (Wouters et al., 2016). However, it was recently demonstrated that the enzymatic
97 treatment and ultrafiltration of faba bean protein extract significantly increased its protein solubility,
98 foaming, and oil-holding capacity (Eckert, Han, Swallow, Tian, Jarpa-Parra & Chen, 2019).
99 Additionally, Alcalase hydrolysis of a faba bean protein isolate increased the physical and oxidative
100 stability of oil/water emulsions, and markedly reduced lipid oxidation during storage (Liu,
101 Bhattarai, Mikkonen & Heinonen, 2019). Thus, enzymatic hydrolysis can create new products with
102 enhanced bioactivity and superior nutritional and physicochemical properties compared to the
103 original proteins.
104 The aim of the present study was firstly to evaluate the nutritional, functional, antioxidant, and
105 sensory properties of faba bean protein hydrolysates (PH) obtained using different enzymes; and
106 second, to assess the sensorial properties of apple juice enriched with the PH. The peptidomic
107 profiles of the PHs were determined by high-resolution mass spectrometry to correlate biological
108 activity with the bioactive peptides released. Apple juice was chosen since the apple (*Malus*
109 *domestica*) is a leading fruit 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 with 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

Dried and completely mature faba bean seeds (variety of Barkat) were bought from Provincial Agricultural Organization at Gorgan (Iran). Reagents were analytical grade, from Sigma (Saint Louis, MO) 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).

2.2 Preparation of faba bean protein extract (PE)

Faba seeds were ground using a 1000 Asan Tus mill (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 10,518 *g* for 20 min, the supernatant was collected, and the pH was adjusted to 3. The precipitated proteins were recovered by centrifugation at 10,518 *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. Hydrolysis with individual enzymes was performed using 3% (w/v) enzyme concentration, 180 min reaction time, and pH and temperature conditions optimal for each enzyme (for Alcalase pH 8.5 and

50 °C; for trypsin pH 7 and 37 °C; for pepsin pH 2 and 37 °C). The digestion reaction was stopped by inactivating the enzyme at 85 °C for 15 min. After centrifugation at 10,518 *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. 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). 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). 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) sulfuric 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, and Shahidi (2007). Briefly, 5 mg of protein hydrolysate were dispersed in 500 µL of deionized water and 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 27,440 *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, and 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 before homogenization with Ultra-Turrax T25 (IKA-Werke, Staufen, Germany) at 16,000 rpm, for 2 min at room temperature. The total volume was read after 30 s. The FC (%) was calculated as follows:

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

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. *FS* (%) was calculated as follows:

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

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

183 **2.7 Amino acid analysis and evaluation of nutritional parameters**

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

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

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

Essential amino acid index (EAAI) was measured by using the amino acid composition of the whole 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, and 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 follows (Wani, Sogi, Singh, and 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

Antioxidant activity assays were performed on a microplate scale and absorbance was measured using SPARK 10M microplate reader (TECAN, Mannedorf, CH). Aliquots of each sample solution (mg/mL) were used in each assay. Results were expressed as mean values of three replicates.

The ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity was determined according to the procedure of Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999). The absorbance was measured at 734 nm, and 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.

226 The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according
227 to the method of Sharma and Bhat (2009). The absorbance was read at 517 nm and corrected with a
228 water blank. Activity was expressed as mg AA eq/L by means of a dose–response calibration curve
229 (from 0 to 5 mg/L) of AA.
230 Ferrous ion-chelating activity was measured according to the method reported in Tang, Kerry,
231 Sheehan, and Buckley (2002). Sample aliquot was mixed with 50 μM ferrous sulfate ($FeSO_4$) and
232 300 μM ferrozine. After incubation for 10 min, the absorbance was measured at 562 nm. The
233 percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

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

234 where A_0 is absorbance of the control and A_s is absorbance in the presence of sample.
235
236 **2.9 Sensory evaluation of protein hydrolysates and fortified juices**
237 Samples were evaluated by a panel of trained judges for selecting, among PHs, the most promising
238 to be tested by dilution in apple juice. Subsequently, selected samples were used to fortify apple
239 juices at 1% (w/v) to be tested by consumers, by comparing them with apple juice alone (control
240 sample). The study protocol followed the ethical guidelines of the sensory laboratory, approved by
241 the University of Bologna, and written informed consent was obtained from each participant before
242 they entered the first test.
243 Samples were firstly evaluated asking to a panel to compare each of the PHs, diluted in water with
244 one (T) randomly selected as control. The panel consisted of 11 trained members from different
245 sections of the Food Science Department, Cesena, Italy and the test was conducted in a sensory
246 laboratory. Trained judges (n = 11, 7 females, 4 males, ages from 21 to 60; students and employees
247 of the University of Bologna) served as panelists. Each sample was prepared adding 0.5% (w/v) of
248 the PHs to distilled water, and 15 mL of this solution were distributed to the assessor in a white
249 plastic cup. Water and unsalted breadsticks were provided for cleaning the mouth during tests. After
250 this preliminary screening test, 30 consumers (19 female, 11 males; ages from 22 to 58) were

recruited to participate in a test in which they were firstly encouraged to describe if they normally consumed apple juices and/or products enriched in antioxidant compounds. They were then asked to assess the juices with an addition of PHs of 1% (w/v) 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 addition of the PHs was used as a 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, AMEL S.r.l., Milan, Italy) and a refractometer (DR-301; Krüss GmbH, Hamburg, 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 an Ultimate 3000 UHPLC coupled using an electrospray interface (ESI) to a Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, MA), using a C18 column (Zorbax SB-C18 reversed phase, 2.1 × 50 mm, 1.8 μm particle size; Agilent Technologies, Santa Clara, CA). 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 increased to 3% **B** in 2 min. The mobile phase composition was increased 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 peptides were identified by using the Swiss-Prot database through MASCOT (Matrix Science, Boston, MA) 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); the maximal number of post-translational modifications permitted in a single peptide was 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 in length),.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; IBM, Armonk, NY). Statistical significance of differences among several means was determined using one-way analysis of variance ANOVA with Tukey test, with a significance level of $p < 0.05$. Sensory data were analyzed with LSD Fisher test with a $p < 0.05$ considered as significant.

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 generation of protein hydrolysates, given its high activity and low cost. Alcalase-derived hydrolysates are also more resistant to digestive enzymes (Sarmadi & Ismail, 2010). Pepsin and trypsin, which are also endoproteases, 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 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 properties 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 the 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 by about 6–10% (Liu et al., 2019). A similar trend was also observed for peanut proteins, where hydrolysis improved 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 other samples after sequential hydrolysis with two peptidases, solubility remained significantly lower in all the pH ranges evaluated ($p > 0.05$). These results indicated that hydrolysis with selected enzymes is a useful method to increase solubility of faba bean protein extract in the pH range of 3–7. Generally, protein solubility depends on several factors such as pH, polarity, molecular size, and hydrophilic sites. The peptides produced by hydrolysis have smaller molecular masses and less tertiary structure than the parental proteins. In addition, hydrolysis liberates ionizable groups that can interact with water molecules. All these factors can improve protein solubility (Wouters et al., 2016). The different solubility observed for

Comment [j1]: Do you mean <?

329 different PHs can be due to the specific peptide profile (molecular size and exposure of hydrophilic
330 or hydrophobic groups) generated by each enzyme or combination of enzymes. Samples obtained
331 by sequential hydrolysis with Alcalase and trypsin (samples AT, ATd and TAd), which had low
332 solubility, yielded a protein pattern with a lower degree of hydrolysis on SDS-PAGE (Fig. 1).
333 In addition to solubility, another important characteristic of proteins is the ability to create stable
334 foams. Foaming capacity provides unique texture in various foods including bread, cakes, and ice
335 cream. Foaming capacity and stability of PE and PHs are shown in Table 1. At pH 4 and 6 the PE
336 showed poor foaming capacity, with values of 25 and 50%, respectively, which are similar to those
337 reported for faba bean at pH 5 and 7 (31 and 67%, Eckert et al. (2019)). In acidic conditions, all
338 PHs had higher foaming capacity than PE. Most plant proteins have limited foaming properties due
339 to their compact structure or low solubility. Hydrolytic treatments disrupt the compact tertiary
340 structure of native proteins and decrease their MW, facilitating their diffusion and adsorption to the
341 air–water interface, resulting in higher foaming capacity (Wouters et al., 2016; Eckert et al., 2019).
342 The highest effect was observed for TP treatment (250% at pH 4 and 200% at pH 6), but values
343 about three times higher than those of PE were observed for sample PT (170% at pH 6), TA (155%
344 at pH 4), AT (155% at pH 4) and ATd (160% at pH 4). At pH values of 8 and 10, the foaming
345 capacity of PE increased to 125 and 150% respectively. Similar values were observed for PHs, with
346 the exception of samples PT (180% at pH 8 and 230% at pH 10) and TA (200% at pH 8).
347 Hydrolysis of faba bean proteins with different enzymes is thus recommended to increase the
348 foaming capacity at low pH values. The stability of foam containing PE after 10 min was low at pH
349 4 and 6 (15% and 20%, respectively), but increased up to 100% and 125%, at pH 8 and 10,
350 respectively. Hydrolysis generally improved foam stability at pH 4 and 6, particularly for samples
351 PT (60% and 140%, respectively), TP (170% and 125%, respectively) and AT (135% and 100%,
352 respectively). In basic solution, the stability of PHs was lower or in the range of PE determinations.
353 Therefore, pH had significant effect on foaming stability, with a significant increase at acidic pH
354 values. Previous data already confirmed that hydrolysis increased foaming stability in other plant

355 protein sources, such as amaranth, bean, pumpkin, rice bran, lupin protein, and corn glutelin
356 (Wouters et al., 2016, and references therein).
357
358 **3.3 Amino acid composition and nutritional properties**
359 The amino acid composition of a food protein source is an essential feature in determining its
360 nutritional value. Seeds are known to be a rich source of proteins, but they are in general deficient
361 in some essential amino acids, which compromise their nutritional quality. For example, legumes
362 are generally deficient in sulfur-containing amino acids (cysteine and methionine), and cereals in
363 lysine and tryptophan. Amino acid contents of PE and PHs (in %, w/w), are reported in Table 2.
364 The amino acid content of PE was referred to all the possible sources of amino acids in the extract
365 (i.e. soluble and insoluble proteins, peptides, and free amino acids). The amino acid content of PHs
366 was referred to the amino acid sources that are soluble after hydrolysis, and thus available in
367 fortified food prepared by the addition of these hydrolysates. The amino acid profile of PE was
368 similar to that reported by various authors on different faba cultivars (Kaldy et al., 1974 and
369 references therein; Palander, Laurinen, Perttila, Valaja & Partanen, 2006; Vioque et al., 2012;
370 Hendawey & Younes, 2013; Eckert et al., 2019; Tab S1, supplementary material). The PE was rich
371 in acidic aspartate (18.28%) and glutamate (22.23%), which are the most abundant amino acids of
372 globulins, the main proteins of the seed. All essential amino acids, with the exception of tryptophan,
373 were present in the extract. Of these, methionine was the only limiting amino acid, while all the
374 others were present at concentrations higher than the WHO values, with leucine being the highest
375 (9.27%). Among conditionally essential amino acids, arginine was the most abundant (12.55%),
376 while cysteine was not detected and nor where the non-essential amino acids glutamine and
377 asparagine.
378 Hydrolysis did not have an appreciable impact on the percentage content of most amino acids. An
379 exception, common to all PHs, was the strong decrease in alanine concentration, from 14.87 to
380 values of 3.86-4.77%. Additionally, the concentration of aspartic acid was almost halved in all

hydrolysates. This amino acid, together with glutamic acid, arginine, and leucine was the most abundant in PHs, similarly to what was observed for PE. Methionine, which was present at a low concentration before hydrolysis (0.68%) and which is considered a limiting amino acid of faba bean (Kaldy et al., 1974; Hendawey et al., 2013), was not detected in hydrolyzed samples, probably because of oxidation processes. Other significant variations were strong increases in histidine content in pepsin hydrolysate (sample P, from 3.54 to 9.92%) and tyrosine in hydrolysates obtained with twice the concentrations of combined Alcalase and trypsin samples (ATd and TAd, from 3.54 to 5.34 and 6.67%, respectively). The different amino acid profile of PHs can be attributed to the specificity of catalytic site and reaction mechanism of enzymes, generating peptides with different solubilities.

Nutritional parameters of PE and PHs expressed as E/T (%), AAS (%), EAAI (%), BV (%), and PER are reported in Table 2. All PHs had E/T values higher compared to PE, exceeding the recommended value by FAO/WHO/UNU (36%), especially samples P, ATd and TAd (40.9, 41.6 and 41.7%, respectively), with the only exception being sample T (35.8%). The amino acid score (AAS) in PE and TAd was approximately equal (119%) and close to that recently reported by other authors (127%, Eckert et al., 2019). All other PHs had AAS higher than 100%. EAAIs of PHs were in the range of 70.0–94.9%, while the BVs were in the range of 91.8 to 64.5%, with the highest values for pepsin hydrolysate (sample P, 94.9 and 91.8%). Usually, a protein source with BV between 70–100% and EAAI above 90% is assumed to be of good nutritional quality (Amza et al., 2013). All PHs, with exception of AT and ATd samples, have been shown to be high-quality food sources.

Protein efficiency ratio (PER) is a quality index ranging from 0 (low protein quality) up to 2 and above (high protein quality) (Amza et al., 2013). The PER values of PE and most of PHs were higher than 2. Most PHs (except for TA, ATd and TAd) were of good (samples PE, P, A, PT, TP) or intermediate (T and AT) quality.

406 All the above results indicate that faba bean PHs are an interesting supply of proteins, rich in some
407 essential amino acids, even if not adequately balanced for the human diet. They could become a
408 strategic alternative to other more widely consumed plant protein sources, especially in the
409 formulation of new functional products with improved nutritional value. In particular, the high
410 content of lysine suggests a possible use as a supplement to cereal-based diets and products, which
411 lack this amino acid.
412
413 **3.4 Antioxidant properties**
414 The antioxidant properties of PE and its hydrolysates were analyzed for their ABTS- and DPPH-
415 ~~radical scavenging~~radical scavenging activities and ferrous ion-chelating ability. The first two
416 methods exploit the scavenging capacity of hydrogen-donating antioxidants towards the free
417 radicals $ABTS^{\cdot+}$ and $DPPH^{\cdot}$. The third measures the ability of antioxidants to chelate transition
418 metal ions like Fe^{2+}. Results are reported in Fig. 2 and are expressed as mg AA eq/g of protein for
419 ABTS and DPPH assays, and as IC_{50} (mg/L) values for ferrous ion-chelating ability assay.
420 Radical-scavenging activity of all hydrolysates was generally higher than that of the original
421 substrate (Fig. 2, A and B), as recently observed for a faba bean pepsin hydrolysate (Ali, 2019).
422 Among single enzyme hydrolysates, the most active was that obtained by Alcalase ($p < 0.05$). The
423 ABTS value of this sample was 55.9 mg AA eq/g of protein, while the DPPH value was 26.2 mg
424 AA eq/g of protein, which were 10 and 5 times higher than PE, respectively. The higher sensitivity
425 of the ABTS method is due to the preferred interactive reaction between ABTS radical and
426 hydroxylated aromatic compounds present in the peptide sequences (Li, Shen, Deng, Li & Ding,
427 2014). The differences may be related to changes in protein composition and surface
428 hydrophobicity values for the respective hydrolysates. Thus, peptides in hydrolysates might
429 differently scavenge the two $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals. The combination of Alcalase with
430 trypsin, even at twice the concentration, and independent of the sequential order in which the
431 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, in addition to the amino acid composition and sequence, being higher for smaller peptides (Rizzello et al., 2016). The highest ~~radical scavenging~~radical scavenging activity in double enzyme hydrolysates was 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 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 50 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 enzyme preparations (Ambigaipalan et al., 2015). During hydrolysis, peptide bond cleavage increased metal ions binding due to an increased concentration of carboxylic and amino groups from acidic and

458 basic amino acids, respectively. This metal ion binding ability may lead to a reduction of
459 prooxidative metal ions from the system (Liu, Kong, Xiong & Xia, 2010).
460 The above results indicate that enzymatic hydrolysis significantly improved the antioxidant
461 potential of faba bean proteins in terms of both ~~radical scavenging~~radical scavenging activity and
462 transition metal ion chelation. These effects were greatly affected by the type of protease, the
463 protease combination, and the sequential order in which they were added to the substrate. Alcalase
464 and the combination of pepsin and trypsin produced the most bioactive hydrolysates.
465
466 **3.5 Peptidomic profile of PHs**
467 The peptidomic profile of different hydrolysates was analyzed by high-resolution mass
468 spectrometry. The complete list of identified peptides together with the MS data is reported in
469 Supplementary Material (Table S2). A total of 2031 unique peptides were identified in the different
470 hydrolysates. The highest number of peptides was found in the hydrolysates obtained with pepsin
471 and trypsin in combination (PT and TP) with 656 and 659 identified peptides, respectively.
472 According to the SDS-PAGE results, hydrolysis by combination of trypsin and Alcalase (samples
473 TA, AT, TAd and ATd) resulted in a lower amount of identified peptides with respect to
474 pepsin/trypsin combinations. Among the hydrolysates obtained after incubation with the 3
475 individual enzymes, Alcalase was found to have the highest hydrolytic efficiency, as already shown
476 by electrophoresis analysis.
477 As expected, a Venn diagram (Figure S1, Supplementary Material) revealed large differences in the
478 peptidomic profile of the sample hydrolyzed with the 3 different enzymes. Only 2 peptides
479 (corresponding to the 0.2% of peptides identified in the three samples) were found to be commonly
480 released by the three enzymes. No additional peptides were found in common between peptic and
481 tryptic hydrolysates, whereas 18 peptides were common to peptic and Alcalase hydrolysates and 16
482 peptides to tryptic and Alcalase hydrolysates. A comparison between the peptidomic profiles of PT
483 and TP samples indicated that the order of addition of enzymes was of paramount importance in the

484 release of specific peptide fragments with just 272 peptides (corresponding to 26.1% of the total
485 peptides) commonly found in the two samples (Figure S1, Supplementary Material). The same
486 consideration can be made for the hydrolysates obtained by combination of trypsin and Alcalase
487 with only 220 peptides (25.2% of total peptides) in common between TP and PT samples (Figure
488 S1, Supplementary Material).
489 Sequence matching suggested that most of the released peptides were included in the sequence of
490 faba bean vicilin and legumin B-types (Figure S2, Supplementary Material). The highest number of
491 peptides originated from vicilin, and legumin B-types were found in the samples hydrolyzed with
492 both the combination of pepsin and trypsin (PT and TP samples) suggesting that the combined use
493 of these enzymes more efficiently cleaved both proteins.
494
495 **3.5.1 Identification of antioxidant peptides in PHs**
496 Several peptides with previously demonstrated antioxidant activity or sharing homology of
497 sequence with known antioxidant peptides were identified in the different hydrolysates (Table 3).
498 Nine peptides, sharing 100% homology with peptides previously characterized for their antioxidant
499 properties, were identified in the different hydrolysates. The peptides TETWNPNHPEL and FVPH
500 have been previously reported to be responsible for the antioxidant properties of chickpea protein
501 hydrolysates (Torres-Fuentes, Contreras, Recio, Alaiz & Vioque, 2015). The remaining identified
502 antioxidant peptides were dipeptides with a Y or W residue in their sequences. The presence of Y
503 and/or W residues in the sequence of peptides is considered of paramount importance in
504 determining the antioxidant effect of a peptide, because of their strong ability to donate a proton
505 (Rival, Boeriu & Wichers, 2001; Tagliazucchi, Helal, Verzelloni & Conte, 2016). Their occurrence
506 in the sequences of antioxidant di- and tripeptides is recognized to be responsible for their
507 antioxidant activity.
508 Several other peptides having structural homology with previously described antioxidant peptides
509 were detected in the different faba bean 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 generated by 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 properties 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 protons, especially when they were at the *C*-terminal position (Suetsuna, Ukeda & Ochi, 2000; Wallner, Hermetter, Mayer & Wascher, 2001).

An additional 17 peptides, released after the hydrolysis of faba bean vicilin shared in their structure the antioxidant sequence VIPAGYP. This peptide was identified to be responsible for the antioxidant properties of a hydrolysate from soybean β-conglycinin prepared with protease S (Chen, Muramoto & Yamauchi, 1995).

Peptides displaying the sequence TETWNPNHPEL can be the most relevant to the total antioxidant activity of faba bean proteins hydrolyzed with pepsin and trypsin (PT). In fact, sample PT was characterized by the presence of 15 of 17 peptides sharing this sequence. Sample PT, which was characterized by the highest DPPH ~~radical scavenging~~radical scavenging activity, also contained the antioxidant sequences TETWNPNHPEL, FVPH, LY, IY, VY, and YV. The sample hydrolyzed with trypsin and pepsin (TP), on the other hand, showed the highest ABTS ~~radical scavenging~~radical scavenging activity. This sample contained both the antioxidant peptides TETWNPNHPEL and FVPH as well as the antioxidant dipeptides YV. Indeed, it contained 8 and 7 peptides sharing the antioxidant sequences TETWNPNHPEL and VIPAGYP, respectively. Moreover, it was also the richest in peptides containing the antioxidant sequences PHW and IY (Saito et al., 2003; Beermann, Euler, Herzberg & Stahl, 2009). Faba beans proteins hydrolyzed with Alcalase (sample A) also released peptides with high ABTS and DPPH ~~radical scavenging~~radical scavenging activities. This sample did not contain peptides having 100% homology with previously known antioxidant peptides. However, this sample was rich in VIPAGYP- and PHY-containing

peptides (Saito et al., 2003). Most of these peptides also contained the antioxidant sequence YVE (Tian, Fang, Jiang, Guo, Cui, and Ren, 2015).

3.5.2 Identification of other bioactive peptides in PHs

An additional 31 peptides with previously demonstrated bioactivities were identified in the different hydrolysates prepared from faba bean protein extract (Table S3, Supplementary Material). The majority of 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 protein hydrolysates, and *in vitro* digested milk. Interestingly, this dipeptide is also effective *in vivo* in decreasing blood pressure in spontaneously hypertensive rats and mild hypertensive human subjects (Saito, Wanezaki, Kawato, and Imayasu, 1994; Kawasaki et al., 2000; Tagliazucchi, Martini, Shamsia, Helal, and Conte, 2018). Samples PT and TA also included the dipeptide VK, which showed a very low IC_{50} value (13 μmol/L). Indeed, the peptide AW (IC_{50} = 10 μmol/L) was found exclusively in sample ATd. Moreover, these three samples were also characterized by 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). Thus, to establish the maximum amount of PHs to be added to apple juice, a preliminary sensory evaluation by a trained panel (11 assessors) was performed, which showed that there was no significant difference between the diverse PH solutions in water at 0.5% ($p < 0.05$; data not reported). Since no off flavors were detected by the panelists at this dilution, all PHs were tested in apple juice to examine the sensory consequences of their

addition. As PE did not have satisfactory solubility, this sample was not tested. Assuming a masking effect of apple juice and considering the need to add a greater amount of PHs to reach a fortifying effect, the test with consumers was carried out by preparing juices supplemented with 1% PHs.

Sensory evaluation demonstrated that the use of samples with different PHs for enrichment of apple juices caused some turbidity with insoluble particles (Table 4). The only exception was apple juice fortified with P, which showed some sour taste without any significant difference in terms of turbidity, compared to control ($p > 0.05$). Apple juices with TA and AT were rated as the highest in sweetness (6.1 and 6.0, respectively) and significantly different to PH samples obtained by pepsin, which showed the lowest intensity of sweetness ($p < 0.05$). The instrumental evaluation of the acidity (pH) did not show significant differences with control samples. The salty taste increased by adding PHs, but only the juice with AT showed a significant difference compared to the others ($p < 0.05$). In all apple juices, except those fortified with TP, TA, and AT, no significant differences were reported. The main problem observed, but only in some of the supplemented apple juices during the sensory evaluation, was the perception of bitterness, often referred to by consumers (Table 4). In particular, this was evident for juices with P, A (reported as bitterness) and TP, AT and ATd (reported as low bitterness). The overall liking of juices fortified with ATd was higher than the control (even if not significantly), while P, Tad, and A showed no significant difference with control apple juice. The TSS of fortified apple juices were increased by adding PHs but they were not significantly different compared to control apple juice. Khairallah, Hettiarachchy, and Rayaprolu (2016) reported that no significant differences were perceived between freshly prepared control juice and samples prepared by adding peptide fraction into orange juice (0.3%). The pH of orange juice alone and with peptide fractions did not show any significant changes, demonstrating that orange juice can be a potential vehicle for use of bioactive peptide fractions (Khairallah et al., 2016). The results described in the present study indicate that apple juice, similar to orange juice,

can be effectively used to incorporate hydrolysates of faba proteins as a source of amino acids and peptides with antioxidant properties.

4. Conclusions

This study demonstrates that faba beans are a suitable source of proteins with high nutritional quality, containing all essential amino acids (except tryptophan) at concentrations higher than the WHO values and which are particularly rich in leucine and arginine. Enzymatic hydrolysis improved some nutritional parameters, overcoming the problem of poor solubility of faba protein. Moreover, it produced low MW peptides (PHs) with increased solubility at acidic and neutral pH, improving rheological properties such as foaming capacity and stability. All PHs showed antioxidant properties in terms of ~~radical scavenging~~radical scavenging activity and ferrous ion chelation capacity. A complete peptidomic analysis identified several peptides with previously demonstrated antioxidant activity or sharing sequence homology with known antioxidant peptides. In order to test consumer acceptability, we conducted a sensory analysis of apple juice supplemented with faba hydrolysates to achieve a fortification at 1% (w/v), which revealed no significant differences compared to the majority of the other hydrolysates tested. Hydrolysates obtained with pepsin (alone or combined with trypsin) or Alcalase might be preferable for application as food ingredients, due to their combination of nutritional, functional, and bioactive properties. These latter faba protein hydrolysates could thus be an innovative ingredient in the preparation of functional foods, due to their amino acid content and antioxidant properties.

606 **Funding**

607 Seyedeh Parya Samaei was supported by a research grant from the Iranian Ministry of Science,
608 Research and Technology.

609

610 **Acknowledgements**

611 The authors acknowledge the Fondazione Cassa di Risparmio di Modena for funding the HPLC-
612 ESI-Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer system at the Centro
613 Interdipartimentale Grandi Strumenti (CIGS).

614

615 **Conflict of interest**

616 The authors declare that there are no conflicts of interest.

617

618 **Supplementary data**

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

620 **References**

621

622 Ali, M. (2019). Functional properties of faba bean protein and effect on enzymatic hydrolysis on its
623 antioxidant activity. *Zagazig Journal of Agricultural Research*, 46, 99-114. doi:
624 10.21608/zjar.2019.40325.

625

626 Ambigaipalan, P., Al-Khalifa, A. S., & Shahidi, F. (2015). Antioxidant and angiotensin, I
627 converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using
628 Alcalase, Flavourzyme and Thermolysin. *Journal of Functional Foods*, 18, Part B, 1125-1137.
629 https://doi.org/10.1016/j.jff.2015.01.021.

630

631 Amza, T., Balla, A., Tounkara, F., Man, L., & Zhou, H. M. (2013). Effect of hydrolysis time on
632 nutritional, functional and antioxidant properties of protein hydrolysates prepared from gingerbread
633 plum (*Neocarya macrophylla*) seeds. *International Food Research Journal*, 20(5), 2081-2090.
634 https://doi.org/10.1038/sj.leu.2401972.

635

636 Beermann, C., Euler, M., Herzberg, J., & Stahl, B. (2009). Anti-oxidative capacity of
637 enzymatically released peptides from soybean protein isolate. *European Food Research and*
638 *Technology*, 229, 637-644. https://doi.org/10.1007/s00217-009-1093-1.

639

640 Betancur-Ancona, D., Sosa-Espinoza, T., Ruiz-Ruiz, J., Segura-Campos, M., & Eckert, L. (2014).
641 Enzymatic hydrolysis of hard-to-cook bean (*Phaseolus vulgaris l.*) protein concentrates and its
642 effects on biological and functional properties. *International Journal of Food Science and*
643 *Technology*, 49, 2-8. doi: 10.1111/ijfs.12267. https://doi.org/10.1111/ijfs.12267.

644

645 Chel-Guerrero, L., Dominguez-Magana, M., Martinez-Ayala, A., Davila-Ortiz, G., & Betancur-
646 Ancona D. (2012). Lima bean (*Phaseolus lunatus*) protein hydrolysates with ACE-I inhibitory
647 activity. *Food and Nutrition Science*, 3, 511-521. http://dx.doi.org/10.4236/fns.2012.34072.
648
649 Chen, H. M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides
650 from soybean β-conglycinin. *Journal of Agricultural and Food Chemistry*, 43, 574-578.
651 https://doi.org/10.1021/jf00051a004.
652
653 Eckert, E., Han, J., Swallow, K., Tian, Z., Jarpa-Parra, M., & Chen, L. (2019). Effects of enzymatic
654 hydrolysis and ultrafiltration on physicochemical and functional properties of faba bean protein.
655 *Cereal Chemistry*, 96, 725-741. https://doi.org/10.1002/cche.10169.
656
657 FAO/WHO/UNU. (1973). Energy and protein requirements: Report of a joint FAO/WHO ad hoc
658 expert committee. Rome and Geneva: FAO Nutrition Meetings Report Series No. 52, WHO
659 Technical Report Series No. 522. https://apps.who.int/iris/handle/10665/41042.
660
661 FAO/WHO/UNU. (1981). Energy and protein requirements: Report of a Joint FAO/WHO/UNU
662 Expert Consultation (held in Rome from 5 to 17 October 1981). World Health Organization.
663 https://apps.who.int/iris/handle/10665/39527.
664
665 Hendawey, M., & Younes, A. (2013). Biochemical Evaluation of Some Faba Bean Cultivars under
666 Rainfed Conditions at El-Sheikh Zuwayid. *Annals of Agricultural Sciences*, 58, 183-193.
667 https://doi.org/10.1016/j.aoas.2013.07.010.
668
669 Jakubczyk, A., Karas, M., Zlotek, U., Szymanowska, U., Baraniak, B., & Bochnak, J. (2019).
670 Peptides obtained from fermented faba bean seeds (*Vicia faba*) as potential inhibitors of an enzyme

671 involved in the pathogenesis of metabolic syndrome. *LWT - Food Science and Technology*, 105,
672 306-313. https://doi.org/10.1016/j.lwt.2019.02.009.
673
674 Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., & Sharma, A. (2010). Influence of
675 degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitor activity of
676 peanut protein hydrolysate. *Food Chemistry*, 121, 178-184.
677 https://doi.org/10.1016/j.foodchem.2009.12.027.
678
679 Kaldy, M. S., & Kasting, R. (1974). Amino acid composition and protein quality of eight faba beans
680 cultivars. *Canadian Journal of Plant Science*, 54, 869-871. https://doi.org/10.4141/cjps74-155.
681
682 Karamac, M., Kosinska-Cagnazzo, A., & Kulczyk, A. (2016). Use of different Proteases to Obtain
683 Flaxseed Protein Hydrolysates with Antioxidant Activity. *International Journal of Molecular*
684 *Sciences*, 17(7), 1027. https://doi.org/10.3390/ijms17071027.
685
686 Kawasaki, T., Seki, E., Osajima, K., Yoshida, M., Asada, K., Matsui, T., & Osajima, Y. (2000).
687 Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle
688 hydrolyzate, on mild hypertensive subjects. *Journal of Human Hypertension*, 14, 519-523.
689 https://doi.org/10.1038/sj.jhh.1001065.
690
691 Khairallah, M. G., Hettiarachchy, N. S., & Rayaprolu, S. J. (2016). Stability and quality of a
692 bioactive peptide fraction incorporated orange juice. *Food Science and Technology*, 66, 523-529.
693 https://doi.org/10.1016/j.lwt.2015.10.013.
694
695 Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and
696 functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as

697 influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, 102, 1317-1327.
698 https://doi.org/10.1016/j.foodchem.2006.07.016.
699
700 Li, X., Shen, S., Deng, J., Li, T., & Ding, C. (2014). Antioxidant activities and functional properties
701 of tea seed protein hydrolysates (*Camellia oleifera* Abel.) influenced by the degree of enzymatic
702 hydrolysis. *Food Science Biotechnol*, 23, 2075-2082. https://doi.org/10.1007/s10068-014-0282-2.
703
704 Liu, C., Bhattari, M., Mikkonen, K. S., & Heinonen, M. (2019). Effects of Enzymatic Hydrolysis of
705 Fava Bean Protein Isolate by Alcalase on the Physical and Oxidative Stability of Oil-in-Water
706 Emulsions. *Journal of Agricultural and Food Chemistry*, 12, 6625-6632.
707 https://doi.org/10.1021/acs.jafc.9b00914.
708
709 Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of
710 porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, 118,
711 403-410. https://doi.org/10.1016/j.foodchem.2009.05.013.
712
713 Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., & Darewicz, M. (2008). BIOPEP database and
714 other programs for processing bioactive peptide sequences. *Journal of AOAC International*, 91,
715 965-980. https://doi.org/10.1093/jaoac/91.4.965.
716
717 Moller, N. P., Scholz-Ahrens, K. E., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and
718 proteins from foods: indication for health effects. *European Journal of Nutrition*, 47(4), 171-182.
719 doi: 10.1007/s00394-008-0710-2.
720

721 Multari, S., Stewart, D., & Russel, W. R. (2015). Potential of fava bean as future protein supply to
722 partially replace meat intake in the human diet. *Comprehensive Reviews in Food Science and Food*
723 *Safety*, 14(5), 511-522. doi:10.1111/1541-4337.12146.
724
725 Palander, S., Laurinen, P., Perttila, S., Valaja, J., & Partanen, K. (2006). Protein and amino acid
726 digestibility and metabolizable energy value of pea (*Pisum sativum*), faba bean (*Vicia faba*) and
727 lupin (*Lupinus angustifolius*) seeds for turkeys of different age. *Animal Feed Science and*
728 *Technology*, 127, 89-100. https://doi.org/10.1016/j.anifeedsci.2005.07.003.
729
730 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).
731 Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical*
732 *Biology and Medicine*, 26, 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3.
733
734 Rival, S. G., Boeriu, C. G., & Wichers, H. J. (2001). Caseins and casein hydrolysates. 2.
735 Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and*
736 *Food Chemistry*, 49, 295-302. https://doi.org/10.1021/jf0003911.
737
738 Rizzello, C. G., Tagliazucchi, D., Babini, E., Rutella, G. S., Saa, D. L. T., & Gianotti, A. (2016).
739 Bioactive peptides from vegetable food matrices: Research trends and novel biotechnologies for
740 synthesis and recovery. *Journal of Functional Foods*, 27, 549-569.
741 https://doi.org/10.1016/j.jff.2016.09.023.
742
743 Saito, K., Jin, D. H., Ogawa, T., Muramoto, K., Hatakeyama, E., Yasuhara, T., & Nokihara, K.
744 (2003). Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry.
745 *Journal of Agricultural and Food Chemistry*, 51, 3668-3674. https://doi.org/10.1021/jf021191n.
746

747 Saito, Y., Wanezaki, K., Kawato, A., & Imayasu, S. (1994). Structure and activity of angiotensin I
748 converting enzyme inhibitory peptides from sake and sake lees. *Bioscience, Biotechnology, and*
749 *Biochemistry*, 58, 1767-1771. https://doi.org/10.1271/bbb.58.1767.
750
751 Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides*,
752 31(10), 1949-1956. https://doi.org/10.1016/j.peptides.2010.06.020.
753
754 Schuman, G. E., Stanley, A. M., & Knudsen, D. (1973). Automated total nitrogen analysis of soil
755 and plant samples. *Soil Science Society of America Journal*, 37, 480-481.
756 https://doi.org/10.2136/sssaj1973.03615995003700030045x.
757
758 Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food Chemistry*, 113,
759 1202-1205. https://doi.org/10.1016/j.foodchem.2008.08.008.
760
761 Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical
762 scavenging activities peptides derived from casein. *Journal of Nutritional Biochemistry*, 11, 128-
763 131. https://doi.org/10.1016/S0955-2863(99)00083-2.
764
765 Tagliazucchi, D., Helal, A., Verzelloni, E., & Conte, A. (2016). Bovine milk antioxidant properties:
766 Effect of in vitro digestion and identification of antioxidant compounds. *Dairy Science &*
767 *Technology*, 96, 657-676. https://doi.org/10.1007/s13594-016-0294-1.
768
769 Tagliazucchi, D., Martini, S., Shamsia, S., Helal, A., & Conte, A. (2018). Biological activities and
770 peptidomic profile of in vitro-digested cow, camel, goat and sheep milk. *International Dairy*
771 *Journal*, 81, 19-27. https://doi.org/10.1016/j.idairyj.2018.01.014.
772

773 Tang, S. Z., Kerry, J. P., Sheehan, D., & Buckley, D. J. (2002). Antioxidative mechanisms of tea
774 catechins in chicken meat systems. *Food Chemistry*, 76, 45-51. https://doi.org/10.1016/S0308-
775 8146(01)00248-5.
776
777 Themelis, T., Gotti, R., Orlandini, S., & Gatti, R. (2019). Quantitative amino acids profile of
778 monofloral bee pollens by microwave hydrolysis and fluorimetric high performance liquid
779 chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, 173, 144-153.
780 https://doi.org/10.1016/j.jpba.2019.05.031.
781
782 Tian, M., Fang, B., Jiang, L., Guo, H., Cui, J. Y., & Ren, F. (2015). Structure-activity relationship
783 of a series of antioxidant tripeptides derived from β-lactoglobulin using QSAR modeling. *Dairy*
784 *Science and Technology*, 95, 451-463. https://doi.org/10.1007/s13594-015-0226-5.
785
786 Torres-Fuentes, C., Contreras, M. M., Recio, I., Alaiz, M., & Vioque, J. (2015). Identification and
787 characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry*, 180,
788 194-202. https://doi.org/10.1016/j.foodchem.2015.02.046.
789
790 Vioque, J., Alaiz, M., & Giron-Calle, J. (2012). Nutritional and functional properties of Vicia faba
791 protein isolates and related fractions. *Food Chemistry*, 132 (1), 67-72.
792 https://doi.org/10.1016/j.foodchem.2011.10.033.
793
794 Wallner, S., Hermetter, A., Mayer, B., & Wascher, T. C. (2001). The alpha-amino group of L-
795 arginine mediates its antioxidant effect. *European Journal of Clinical Investigation*, 31, 98-102.
796 https://doi.org/10.1046/j.1365-2362.2001.00771.x.

797 Wani, A. A., Sogi, D., Singh, P., & Shivhare, U. (2011). Characterization and Functional Properties
798 of Watermelon (Citrullus lanatus) Seed Protein Isolates and Salt Assisted Protein Concentrates.
799 *Food Science and Biotechnology*, 20, 877-887. https://doi.org/10.1007/s10068-011-0122-6.
800
801 Weiss, M., Manneberg, M., Juranville, J. F., Lahm, H. W., & Fountoulakis, M. (1998). Effect of the
802 hydrolysis method on the determination of the amino acid composition of proteins. *Journal of*
803 *Chromatography A*, 795, 263-275. https://doi.org/10.1016/S0021-9673(97)00983-7.
804
805 Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the
806 functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive*
807 *Reviews in Food Science and Food Safety*, 15, 786-800. https://doi.org/10.1111/1541-4337.12209.

Figure captions

Fig. 1. SDS-PAGE of faba bean protein extract (PE) and its hydrolysates with enzymes (enzyme concentration % w/v): ~~Pepsin~~pepsin 3% (P); ~~Trypsin~~trypsin 3% (T); Alcalase 3% (A); ~~Pepsin~~pepsin 1.5% and ~~Trypsin~~trypsin 1.5% (PT); ~~Trypsin~~trypsin 1.5% and ~~Pepsin~~pepsin 1.5% (TP); ~~Trypsin~~trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and ~~Trypsin~~trypsin 1.5% (AT); Alcalase 3% and ~~Trypsin~~trypsin 3% (ATd); ~~Trypsin~~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~~ 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~~pepsin 3% (P); ~~Trypsin~~trypsin 3% (T); Alcalase 3% (A); ~~Pepsin~~pepsin 1.5% and ~~Trypsin~~trypsin 1.5% (PT); ~~Trypsin~~trypsin 1.5% and ~~Pepsin~~pepsin 1.5% (TP); ~~Trypsin~~trypsin 1.5% and Alcalase 1.5% (TA); Alcalase1.5% and ~~Trypsin~~trypsin 1.5% (AT); Alcalase 3% and ~~Trypsin~~trypsin 3% (ATd); ~~Trypsin~~trypsin 3% and Alcalase 3% (ATd). Means followed by the same letter did not differ significantly (Tukey test, ~~*P*~~*p* > 0.05).

Table(s)

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~~ pepsin 3% (P); ~~Trypsin~~ trypsin 3% (T); Alcalase 3% (A); ~~Pepsin~~ pepsin 1.5% and ~~Trypsin~~ trypsin 1.5% (PT); ~~Trypsin~~ trypsin 1.5% and ~~Pepsin~~ pepsin 1.5% (TP); ~~Trypsin~~ trypsin 1.5% and Alcalase 1.5% (TA); Alcalase 1.5% and ~~Trypsin~~ trypsin 1.5% (AT); Alcalase 3% and ~~Trypsin~~ trypsin 3% (ATd); ~~Trypsin~~ trypsin 3% and Alcalase 3% (TAd). ~~Means followed by the same letter did not differ significantly (Duncan test, p>0.05).~~

pH	PE	P	T	A	PT	TP	TA	AT	ATd	TAd
Solubility (%)										
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	1.44±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	25.00±2.50^{i}	70.00±14.00^{g}	140.00±20.00^{d}	65.00±7.00^{h}	80.00±5.00^{f}	250.00±15.00^{a}	155.00±15.00^{c}	155.00±5.00^{c}	160.00±12.00^{b}	105.00±5.00^{e}
6	50.00±5.00^{i}	125.00±10.00^{e}	140.00±14.00^{c}	125.00±5.00^{e}	170.00±15.00^{b}	200.00±12.00^{a}	90.00±5.00^{g}	130.00±12.00^{d}	120.00±6.00^{f}	85.00±10.00^{h}
8	125.00±12.00^{g}	105.00±5.00^{h}	125.00±8.00^{g}	140.00±10.00^{e}	180.00±5.00^{b}	125.00±5.00^{g}	200.00±20.00^{a}	135.00±9.00^{f}	150.00±10.00^{d}	160.00±7.00^{c}
10	150.00±10.00^{d}	150.00±20.00^{d}	145.00±12.00^{e}	125.00±5.00^{f}	230.00±20.00^{a}	125.00±10.00^{f}	145.00±10.00^{e}	170.00±5.00^{b}	155.00±15.00^{c}	125.00±5.00^{f}
Foaming stability after 10 min (%)										
4	15.00±3.00^{j}	25.00± 4.00^{i}	80.00±11.00^{e}	35.00±5.00^{h}	60.00±5.00^{g}	170.00±20.00^{a}	90.00±7.00^{d}	135.00±13.00^{b}	130.00±10.00^{c}	65.00±5.00^{f}
6	20.00±2.50^{h}	35.00±5.00^{f}	45.00±7.00^{e}	35.00±4.00^{f}	140.00±15.00^{a}	125.00±8.00^{b}	25.00±4.00^{g}	100.00±15.00^{c}	85.00±10.00^{d}	45.00±11.00^{e}
8	100.00±7.00^{b}	20.00±2.50^{f}	40.00±5.00^{e}	60.00±6.00^{d}	100.00±10.00^{b}	40.00±5.00^{e}	100.00±15.00^{b}	80.00±9.00^{c}	80.00±5.00^{c}	105.00±7.00^{a}
10	125.00±10.00^{b}	55.00±6.00^{f}	60.00±7.00^{e}	40.00±5.00^{h}	135.00±9.00^{a}	45.00±2.50^{g}	55.00±7.00^{f}	120.00±10.00^{c}	105.00±11.00^{d}	30.00±5.00^{i}

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

Formatted: Font: Italic

Table(s)

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). ~~Means followed by the same letter did not differ significantly (Duncan test, p>0.05).~~

g AA/ 100 g	Symbol	PE	P	T	A	PT	TP	TA	AT	ATd	TAd	WHO*
Arginine	Arg	12.55±3.02^{a}	11.50±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}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{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±5.60^{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
~~**W**~~	~~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

Formatted Table

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	4
E**TW**NPNHPE	A, TAd	
TWNPNHPEL	A, AT, TAd	5
TWNPNHPE	A, TAd	
TWNPN	TAd	6
PGCPQT**YQEP**R	PT	
T**YQEP**RSS	A	7
T**YQEP**R	AT, ATd	
YQEPR	TP	
LYVIR	A	8
GT**TY**	A	
S**AY**	TAd	9
AYGE	AT	
AYE	TP	10
V**TY**	TAd	
S**TY**	TAd	11
Peptides fragment of antioxidant sequence		12
YVR	A, TA	
AVPY	T, PT, TA, AT	13
YF	T	
HR	PT, TA, TAd	
YN	PT, AT	14
FY	TA	
NY	TA	15
WT	AT	
SY	AT	16

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

Table(s)

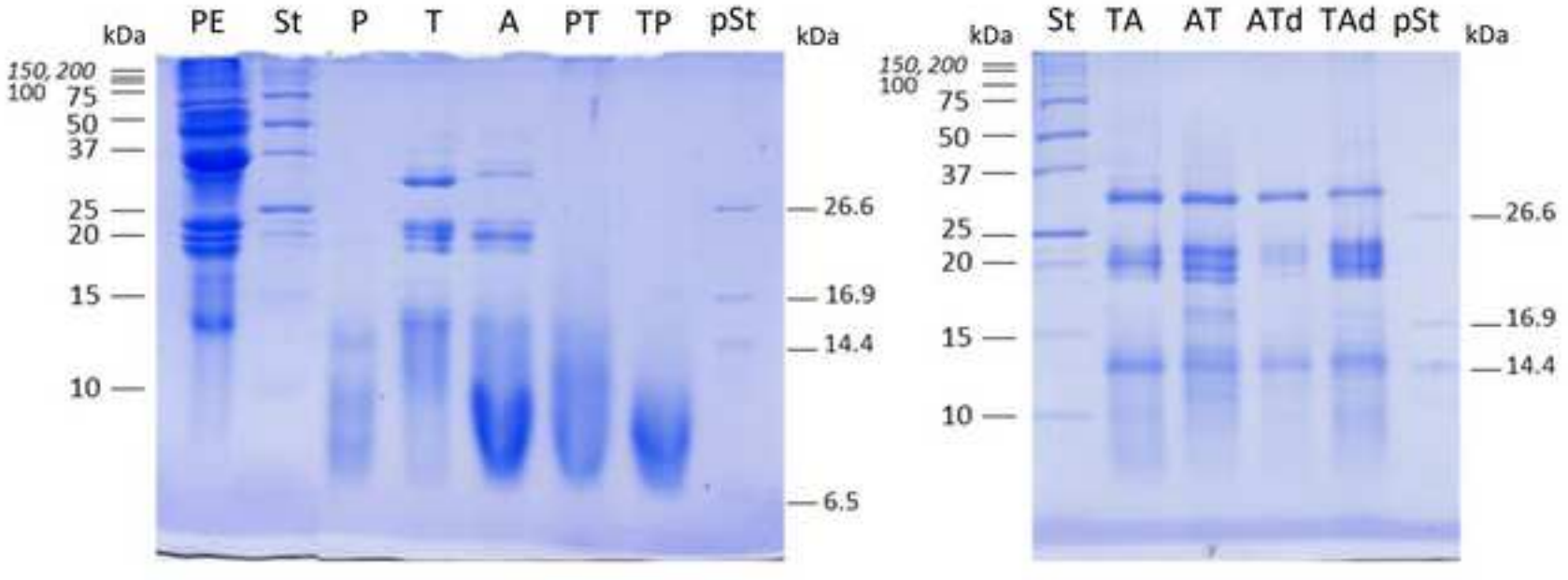
Table 4. Sensory properties, pH and Total soluble solids (TSS, ºBrix) of apple juice fortified with faba bean protein hydrolysates (PHs). P, T, A, PT, TP, TA, AT, ATd and TAd represent PE hydrolyzed with enzymes (%, w/v): pepsin 3%; trypsin 3%; Alcalase 3%; pepsin 1.5% and trypsin 1.5%; trypsin 1.5% 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). ~~Means followed by the same letter did not differ significantly (for sensory data, LSD Fisher test was applied, $p<0.05$; for pH and TSS values, Duncan test was applied, $p<0.05$).~~

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 fruit 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 fruit smell other than apple. Precipitate.
pH	3.4^{a}	3.2^{a}	3.7^{a}	3.7^{a}	3.9^{a}	3.2^{a}	4.1^{a}	4.1^{a}	4.0^{a}	4.1^{a}
TSS	11.2^{b}	12.4^{ab}	11.9^{ab}	13.0^{a}	11.8^{ab}	12.1^{ab}	11.9^{ab}	11.9^{ab}	12.1^{ab}	11.8^{ab}

Means in the same row followed by the same letter did not differ significantly (for sensory data, LSD Fisher test was applied: $p < 0.05$; for pH and TSS values, Tukey test was applied: $p < 0.05$).

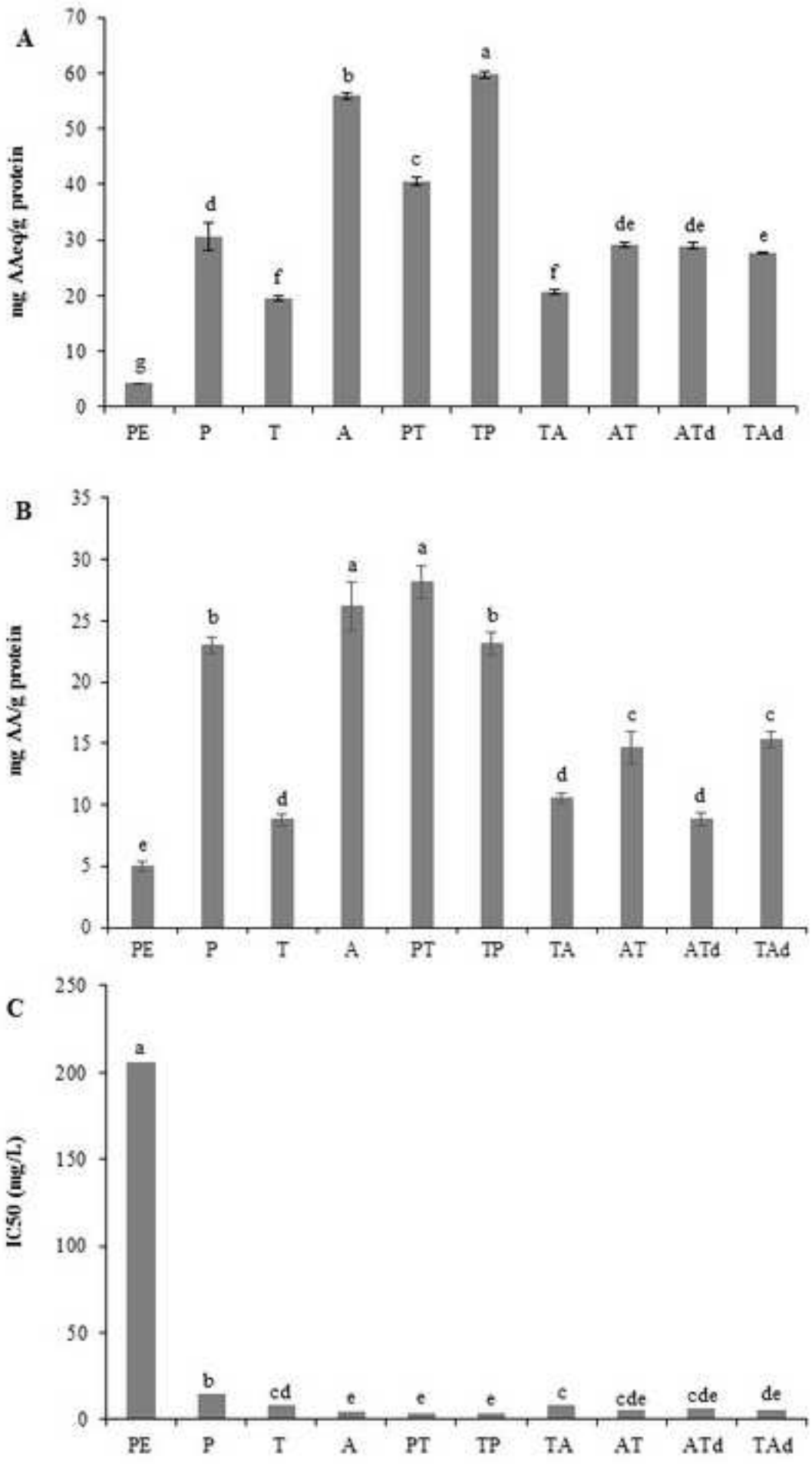
Figure(s)

Click here to download high resolution image



Figure(s)

Click here to download high resolution image



***Declaration of Interest Statement**

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

*Credit Author Statement

Author declaration

1. Conflict of Interest

X No conflict of interest exists.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

2. Funding

X Funding was received for this work.

All of the sources of funding for the work described in this publication are acknowledged below:

Seyedeh Parya Samaei was supported by a research grant from the Iranian Ministry of Science, Research and Technology

3. Intellectual Property

X We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

4. Research Ethics

☐ We further confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

☐ IRB approval was obtained (required for studies and series of 3 or more cases)

☐ Written consent to publish potentially identifying information, such as details or the case and photographs, was obtained from the patient(s) or their legal guardian(s).

5. Authorship

The International Committee of Medical Journal Editors (ICMJE) recommends that authorship be based on the following four criteria:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND

2. Drafting the work or revising it critically for important intellectual content; AND

3. Final approval of the version to be published; AND

4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All those designated as authors should meet all four criteria for authorship, and all who meet the four criteria should be identified as authors. For more information on authorship, please see http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html#two.

X All listed authors meet the ICMJE criteria. We attest that all authors contributed significantly to the creation of this manuscript, each having fulfilled criteria as established by the ICMJE.

We believe these individuals should be listed as authors because:

Seyedeh Parya Samaei, for methodology, formal analysis, data curation, writing-review and editing.

Mohammad Ghorbani, for conceptualization, resources, methodology, investigation, supervision (coordinator of the first part of the project, performed at University of Gorgan).

Davide Tagliazucchi, for conceptualization, resources, methodology, investigation, supervision, writing-review and editing (peptide sequencing section).

Serena Martini, for methodology, formal analysis, data curation, writing-review and editing (peptide sequencing section).

Roberto Gotti, for conceptualization, resources, methodology, investigation, supervision, writing-review and editing (amino acid analysis section).

Thomas Themelis, for methodology, formal analysis, data curation, writing-review and editing (amino acid analysis section).

Federica Tesini, for methodology, formal analysis, data curation, writing-review and editing (evaluation of sensory properties of hydrolysates and fortified juices).

Andrea Gianotti, for conceptualization, resources, methodology, investigation, supervision (evaluation of bioactive properties).

Tullia Gallina Toschi, for conceptualization, resources, methodology, investigation, supervision, writing-review (evaluation of sensory properties of hydrolysates and fortified juices).

Elena Babini, for conceptualization, resources, methodology, investigation, supervision, writing-review and editing and project administration (coordinator of the project).

X We confirm that the manuscript has been read and approved by all named authors.

X We confirm that the order of authors listed in the manuscript has been approved by all named authors.

6. Contact with the Editorial Office

The Corresponding Author declared on the title page of the manuscript is:

Prof. Tullia Gallina Toschi

☐ This author submitted this manuscript using his/her account in EVISE.

X We understand that this Corresponding Author is the sole contact for the Editorial process (including EVISE and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

X We confirm that the email address shown below is accessible by the Corresponding Author, is the address to which Corresponding Author's EVISE account is linked, and has been configured to accept email from the editorial office of American Journal of Ophthalmology Case Reports:

tullia.gallinatoschi@unibo.it

X Someone other than the Corresponding Author declared above submitted this manuscript from his/her account in EVISE:

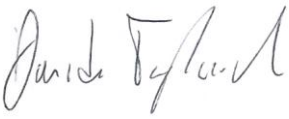
Elena Babini

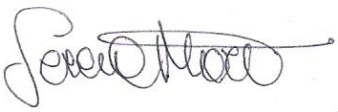
X We understand that this author is the sole contact for the Editorial process (including EVISE and direct communications with the office). He/she is responsible for communicating with the other authors, including the Corresponding Author, about progress, submissions of revisions and final approval of proofs.

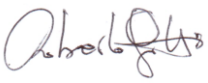
We the undersigned agree with all of the above.

	Author's name (Fist, Last)	Signature	Date
1.	Seyedeh Parya Samaei		26/12/2019

2. Mohammad Ghorbani  26/12/2019

3. Davide Tagliazucchi  26/12/2019

4. Serena Martini  26/12/2019

5. Roberto Gotti  26/12/2019

6. Thomas Themelis  26/12/2019

7. Federica Tesini  26/12/2019

8. Andrea Gianotti  26/12/2019

9. Tullia Gallina Toschi  26/12/201

10. Elena Babini  26/12/2019

Supplementary Material

Click here to download Supplementary Material: Table S1_May18.docx

Table S1. Comparison of the total amino acid composition (g of AA per 16 g of total nitrogen) of faba bean as reported by various authors

Amino acid	Clarke (1970)†	Nitsan (1971)†	Evans et al. (1972)† *cult. Columba*	Kaldy & Kasting (1974)†⊥	Palander et al. (2006)†	Hendawey & Younes (2013)† *cult. Giza 843*	Hendawey & Younes (2013)† *cult. Sakha 3*	Eckert et al., 2019††	Vioque et al, 2012††	Current research ††
Lysin	6.6	5.4	6.8	6.6	5.97	9.71	12.64	5.83	7.00	5.32
Histidine	2.6	2.1	2.8	2.6	2.61	2.90	7.77	2.48	2.80	3.54
Arginine	10.3	7.5	10.3	10.5	8.96	5.71	19.77	7.81	10.00	12.55
Aspartic acid	11.9	-	11.9	13.0	9.28	17.55	21.17	11.51*	13.30*	18.28
Threonine	4.0	4.1	3.9	3.3	2.96	5.04	7.01	4.11	3.70	5.45
Serine	5.5	-	5.3	4.2	4.28	6.46	7.45	6.54	6.30	5.86
Glutamic acid	19.7	-	18.3	20.3	15.67	23.87	17.99	15.30**	19.90**	22.23
Proline	-	-	4.6	4.1	4.27	6.58	18.40	5.52	3.40	5.45
Glycine	5.6	-	4.8	4.2	3.95	8.53	9.17	7.16	4.90*	4.77
Alanine	4.2	-	4.6	1.7	3.97	6.23	10.44	5.58	4.40	14.87
Cysteine (half)	0.8	1.4	4.8	3.9	1.26	-	-	1.21	0.50	-
Valine	4.9	5.7	4.8	3.9	3.41	5.88	8.67	5.93	4.10	5.32
Methionine	0.7	0.7	0.9	0.8	0.52	0.03	0.36	0.67	0.10	0.68
Isoleucine	4.4	4.6	4.3	4.3	3.67	4.58	8.09	4.81	3.80	5.04
Leucine	7.9	8.1	8.0	8.3	6.57	10.19	12.98	8.34	8.00	9.27
Tyrosine	3.9	-	3.3	2.8	3.16	2.52	5.01	2.96	2.60	3.00
Phenylalanine	4.6	3.7	4.5	4.4	3.98	4.51	9.89	3.95	4.90	4.77
Tryptophan	-	1.0	-	1.0	-	-	-	0.3	0.30	-

† AA evaluated on grounded seed

⊥ Average of eight cultivars

†† AA evaluated on the total proteins extracted from the seed

* Represents Asparagine and Aspartic acid

** Represents Glutamine and Glutamic acid

Supplementary Material

Click here to download Supplementary Material: Table S2_May18.xlsx

Table S2. Complete list of pe
proteins hydrolysed whit alc
proteins hydrolysed whit try
sample 9: faba bean seed pr

Sample
Sample 3
Sample 2
Sample 2
Sample 4
Sample 4
Sample 2
Sample 4
Sample 2
Sample 2, 4
Sample 4
Sample 3
Sample 2
Sample 3
Sample 4
Sample 3
Sample 2, 4, 7
Sample 6
Sample 2
Sample 4
Sample 4
Sample 4
Sample 2, 3, 4
Sample 2
Sample 4
Sample 4
Sample 1
Sample 5
Sample 6
Sample 2, 3, 4, 5
Sample 4
Sample 7
Sample 4
Sample 1, 5
Sample 3, 6
Sample 4
Sample 3
Sample 8
Sample 2, 4, 7, 8
Sample 4
Sample 3

Table S3. Peptides with previously demonstrated bioactivity identified in the different faba bean protein hydrolysates.

Peptide	***Sample***[a]	***Bioactivity***[b]	***IC_{50}***[c]
VVF	P, A, TP, TA	ACE-inhibitor	35 µmol/L
FDK	T	ACE-inhibitor	389 µmol/L
VAF	T, A, PT, TP, TA, ATd, TAd	ACE-inhibitor	36 µmol/L
YF	T	DPP-IV-inhibitor	n.a.
VAP	T, TP	ACE-inhibitor	2 µmol/L
VR	T, TP, TA, AT, TAd	ACE-inhibitor	53 µmol/L
		DPP-IV-inhibitor	826 µmol/L
VH	T	DPP-IV-inhibitor	n.a.
MV	T, TP, TA, ATd	DPP-IV-inhibitor	n.a.
VK	A, PT, TP, TA	ACE-inhibitor	13 µmol/L
		DPP-IV-inhibitor	n.a.
PQ	A, TA, AT, ATd	ACE-inhibitor	n.a.
		DPP-IV-inhibitor	n.a.
RPY	PT, TP	ACE-inhibitor	n.a.
HR	PT, TA	DPP-IV-inhibitor	n.a.
YN	PT, AT	ACE-inhibitor	51 µmol/L
		DPP-IV-inhibitor	n.a.
NR	PT	ACE-inhibitor	n.a.
		DPP-IV-inhibitor	n.a.
VY	PT, TA, AT, ATd	ACE-inhibitor	7 µmol/L
		DPP-IV-inhibitor	n.a.
YV	PT, TP, TA, AT, TAd	ACE-inhibitor	575 µmol/L
		DPP-IV-inhibitor	n.a.
VQ	PT, TP	DPP-IV-inhibitor	n.a.
QV	PT, AT, ATd	DPP-IV-inhibitor	n.a.
VT	PT	DPP-IV-inhibitor	n.a.
TV	PT	DPP-IV-inhibitor	n.a.
NY	TA	ACE-inhibitor	33 µmol/L
		DPP-IV-inhibitor	n.a..
VM	TA, ATd, TAd	DPP-IV-inhibitor	n.a..
WT	AT	DPP-IV-inhibitor	482 µmol/L
SY	AT	ACE-inhibitor	66 µmol/L
		DPP-IV-inhibitor	n.a..
VAV	ATd	ACE-inhibitor	260 µmol/L
NF	ATd	ACE-inhibitor	46 µmol/L
		DPP-IV-inhibitor	n.a..
AW	ATd	ACE-inhibitor	10 µmol/L
		DPP-IV-inhibitor	n.a..
VN	ATd	DPP-IV-inhibitor	n.a..

PV	ATd	DPP-IV-inhibitor	n.a..
VP	ATd	ACE-inhibitor	420 μmol/L
		DPP-IV-inhibitor	880 μmol/L
EK	TAd	ACE-inhibitor	n.a..
		DPP-IV-inhibitor	3200 μmol/L

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

[b]ACE: angiotensin-converting enzyme; DPP-IV: dipeptydil- peptidase-IV

[c]IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity. The values are from BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008).