Antioxidant activity and peptidomic analysis of porcine liver hydrolysates using alcalase, bromelain, flavourzyme and papain enzymes

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Abstract:

Porcine liver can be used to prepare hydrolysates with antioxidant activity employing proteolytic enzymes such as alcalase, bromelain, papain and flavourzyme. In this study, the antioxidant activity of these four porcine liver hydrolysates was evaluated by DPPH, ABTS, FRAP and ORAC assays and the identification of bioactive peptides was carried out by SWATH-MS technology. According to the SDS-PAGE pattern, the proteolysis index and the free amino acids amount, the protein degradation was clearly different among the studies enzymes. Indeed, alcalase enzyme produced the release of small peptides, meanwhile flavourzyme produced higher level of free amino acids. The heatmap analysis showed a peptidomic pattern more differentiated for alcalase than for the other enzymes. The peptides most abundant and correlated with antioxidant capacity were APAAIGPYSQAVLVDR from uncharacterized protein, GLNQALVDLHALGSAR, ALFQDVQKPSQDEWGK and LSGPQAGLGEYLFER from ferritin and LGEHNIDVLEGNEQFINAAK from trypsinogen. The production and characterization of biopeptides is a new challenge of meat industry.

Keywords: proteolytic enzymes, antioxidant peptides, swine industry, peptide mapping, mass spectrometry

1. Introduction

Large amount of wastes is generated by meat industry becoming a severe problem for sustainability. Therefore, the improvement and use of these meat by-products is a major goal for worldwide society. Among animal by-products, the edible fraction (accounting for 55% of the production) can be used and re-process for human consumption, meanwhile the rest of the fraction could be employed for agricultural and industrial applications (Alao, Falowo, Chulayo, & Muchenje, 2017). This meat offal are rich sources of proteins resulting a suitable raw material for preparing protein hydrolysates. Animal by-products such as blood, bones, collagen and organs have been employed as source of protein hydrolysates (Borrajo et al., 2019). Food peptides from parent proteins can reach the intestine as peptide or be liberated after digestion. This highly valuable protein hydrolysates are rich in biopeptides, with high digestibility and bio-absorption as well as different biological activities such as antihypertensive, antioxidant or antithrombotic among others (Yu, Hsu, Chang, & Tan, 2017). These bioactive peptides have small molecular weight (400-2000 Da) with sequences of approximately 4-16 amino acids. Accordingly, it has been demonstrated that the bioactivity of the smaller peptides showed the higher activity antioxidant, although this activity is also large extent determined by the amino acid sequence (Liu, Xing, Fu, Zhou, & Zhang, 2016).

All these factors result in a very complex issue which can be studied by an Omic strategy. Peptidomic together with proteomic approach can provided a comprehensive vision of proteins/peptides and their bioactivity in a complex food matrix. A main objective of peptidomics is to map of endogenous peptides resulting from food-processing and protein digestion, as well as bioactive

peptides identification (Dallas et al., 2015). Within animal products, peptidomic studies revealed a great influence of proteolysis on peptide bioactivity from beef, pork, chicken and turkey during digestion (Martini, Conte, & Tagliazucchi, 2019; Zhao et al., 2019).

Protein hydrolysates can be obtained from enzymatic, chemical and microbial hydrolysis, but the former one is the most widely selected process for food and pharmaceutical industries to produce bioactive peptides (O'Sullivan, Lafarga, Hayes, & O'Brien, 2017). The most frequently used enzymes come from animal tissues (pepsin and tripsin), plants (papain, ficin, and bromelain), and microbial sources (alcalase®, flavourzyme®, neutrase®, collagenase, or proteinase K) (Marzia, Santillo, Mariangela, Antonella, & Rosaria, 2017). Papain, pepsin or alcalase have been reported as the most successful enzymes, releasing antioxidant and anti-inflammatory biopeptides from animal tissues (O'Sullivan et al., 2017). For instance, myofibrillar proteins of porcine muscle have demonstrated antihypertensive activity through angiotensin-I converting enzyme inhibitory mechanisms (Katayama et al., 2008) as well as antioxidant activity (Saiga, Tanabe, & Nishimura, 2003). Recent investigations have proved that by-products from porcine organs can be used to prepare appealing hydrolysates. In fact, porcine liver protein hydrolysates from enzymatic hydrolysis with several enzymes such as papain, alcalase, pepsin and trypsin (Verma, Chatli, Kumar, & Mehta, 2019) or microbial suspension of *Monascus purpureus* (Yu et al., 2017) have showed a significant antioxidant capacity.

Therefore, there is a great interest on functional foods field by food and nutraceutical industries. Indeed, these biopeptides could be used both as preservatives in food and beverage providing a functional purpose, due to

antioxidant peptides might be protect human body from damage of oxidative stress and reduce the risk of degenerative diseases (Liu et al., 2016).

The aim of the present study is to assess the antioxidant activity of four porcine liver hydrolysates produced by alcalase, bromelain, papain and flavourzyme, identifying the bioactive peptides sequences by SWATH-MS methodology.

2. Materials and Methods

2.1. Porcine liver samples and chemical composition

A total of eight fresh porcine livers were purchased at a local meat market (Cárnicas M. Boo, Ourense). The assessment of moisture, protein, fat and ash was carried out in accordance with Franco & Lorenzo, (2014). The carbohydrate content was calculated based on the difference. The proteolysis index was calculated as the ratio: (non−protein nitrogen/nitrogen total) × 100. Total nitrogen content was determined with Kjeldahl method, meanwhile non-protein nitrogen was determined following protocol described by Lorenzo, García Fontán, Franco, & Carballo, (2008). The liver amino acid profile was performed after protein hydrolysis employing high performance liquid chromatography with fluorescence detector according to Franco & Lorenzo, (2014). Similarly, the same protocol was employed to determinate the total free amino acids without the hydrolysis step.

2.2. Enzymatic hydrolysis of porcine liver

Livers were cleaned of fat and connective tissues and subsequently were cut into small cubes and frozen at –20 ºC with the aim of reduce liver viscosity. Subsequently, a homogenization mixing with ice (1:1 liver/ice) using a cutter

machine (Talsa K3, Valencia, Spain) was achieved. Enzymatic hydrolysis of liver was carried out using four different enzymes (Papain 6000 USP, Bromelain 2000 U/g and bioprotease LA 660 (alcalase) supplied by Biocon, Spain) and flavourzyme 1000L by (Novozymes, Bagsvaerd Denmark). Liver solutions (1:1, w/w) were preincubated for 30 minutes at the optimal temperature for each protease before addition of the enzyme. The conditions were: (37 ºC and pH=6) for papain, (40 ºC and pH=6) for bromelain, (50 ºC and pH08) for alcalase, and (50 ºC and pH=5.5) for flavourzyme. Enzymes were added in an enzyme-substrate ratio of 1:100 (w/w). Enzymatic hydrolysis was carried out in an orbital shaker-incubator at 125 rpm for seven hours, adjusting the above pH optimal by addition of NaOH or HCL 1N. After this time, enzymes were heat deactivated at 95 ºC for 3 min and the liver hydrolysates were cooled to room temperature using an ice bath. Afterward, they were centrifuged at 10.000g for 10 min using an Allegra X-22R Centrifuge (Beckman Coulter) and supernatants were filtered by 0.45 µm and subsequently lyophilized until further analysis.

2.3. SDS-PAGE Analysis

Peptide extracts from liver hydrolysates were separated under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fifteen micrograms were loaded on 10% pre-cast gels using a Mini-Protean Tetra Cell equipment (Biorad Lab., Hercules, CA, USA). The Laemmli buffer (62.5 mM TrisHCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 100 mM DTT) was used to dissolve and denature the samples (5 min, 95 °C). Staining was carried out using Coomassie Brilliant Blue G-250 solution. The images were acquired using the Gel Doc XR+ system (Bio-Rad Laboratories) and analysed by Image Lab™ software (Biorad Lab., Hercules, CA, USA).

2.4. Protein extraction and digestion

Lyophilized powder (50 mg) of liver hydrolysates was homogenised in RIPA buffer [200 mmol/L Tris/HCl (pH 7.4), 130 mmol/L NaCl, 10% (v/v) glycerol, 0.1% (v/v) SDS, 1% (v/v) Triton X-100, 10 mmol/L $MgCl_2$] and adding anti-proteases and anti-phosphatases (Sigma-Aldrich, St. Louis, MO, USA) using a TissueLyser II (Qiagen, Tokyo, Japan). Afterwards, the hydrolysate was centrifuged at 14.000g at 4 ºC for 20 min to obtain peptide solutions. The concentration was quantified by RC-DC kit (Biorad Lab., Hercules, CA, USA). The peptides were concentrated in a gel single in band of 10% SDS-PAGE and excised into pieces. The gel pieces were washed with Milli-Q and 50 mM ammonium bicarbonate in 50% methanol followed by dehydration with ACN by a vacuum centrifuge. The resulting peptide extracts were reduced by 10 mM DTT in 50 mM ammonium bicarbonate (60 ºC, 30 min) and then alkylated with 55 mM iodoacetoamide in 50 mM ammonium bicarbonate in darkness (room temperature, 30 min). Finally, the peptides were digested with 20 ng/μL trypsin (Promega, Madison, USA) in 20 mM ammonium bicarbonate (37 ºC, 16 h) and dissolved in 0.1% formic acid until analysis.

2.5. Generation of the reference spectral library

To obtain a pooled sample of each group, 4 μg of peptides from each sample were mixed and combined. The resulting solutions were analysed by shotgun data-dependent acquisition (DDA) approach using micro-LC-MS/MS. To separation of the peptides, the equipment used was a micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) and an YCM-TriartC18 column (150μm × 0.3 mm, 12 nm, s-3 μm) (YMC CO, Japan) at a flow rate of 5 μL/min. Solvent A (water, 0.1% formic acid) and solvent B (ACN, 0.1% formic acid) were used to

157 prepare mobile phases in the liquid chromatography. The gradient elution was
158 produced by 5% to 95% B for 30 min, 5 min at 90% B and other 5 min at 5% B
159 for column equilibration, for a total time of 40 min. A quadrupole-TOF mass
160 spectrometer of model Triple TOF 6600 (SCIEX, Framingham, MA, USA) working
161 with data-dependent acquisition system in positive ion mode was used. The
162 selected parameters were: a 250 ms survey scan was performed from 400 to
163 1250 m/z and MS/MS experiments from 100 to 1500 m/z (25 ms of acquisition
164 time) for a total cycle time of 2.8 s. The fragment ion mass spectra of the identified
165 peptides was used to generate the spectral library for SWATH-MS peak
166 extraction using ProteinPilot software v.5.0.1. (SCIEX, Framingham, MA, USA)
167 through Uniprot Swiss-Prot database.

168 ***2.6. Quantification by SWATH and data analysis***

169 Data independent acquisition (DIA) data from SWATH-MS analysis was
170 PeakView v.2.2. (SCIEX, Framingham, MA, USA) matching the reference
171 spectral library (Section 2.5.). Twenty-five samples from 5 groups (control,
172 alcalase, bromelain, papain and flavorzyme) with two technical replicates in each
173 case were analysed. Each peptide sample (4 µg) was analysed by LC as
174 described above with the following settings: acquisition time of 50 ms in a total
175 cycle time of 6.3 s and a cycle consisted of the acquisition of 65 scans per
176 SWATH window of variable width (1 m/z overlap) covering the 400 to 1250 m/z
177 mass range. All DIA files were loaded using the following settings: extraction
178 window (5 min); ion library mass tolerance (30 ppm); ten peptides per peptide;
179 six transitions per peptide; 95% peptide confidence; exclusion of modified and
180 shared peptides. A score and a false discovery rate (FDR) for each assigned
181 peptide were calculated by the software and only peptides with an FDR less than

1% were used for protein quantification. To compare the groups, a Student's t-test was used based on peptides with a p-value scoring above 0.05 and fold change of 1.5 as cut-off.

2.7. Determination of antioxidant capacity

2.7.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging method was carried out according to Brand-Williams, Cubelier, & Berset, (1995) with slight modifications. Aliquots of 100 µL of samples were added to 3900 µL of DPPH solution (60 µM in methanol) and incubated during 10 min at 37 ºC. Then the absorbance was measured in an UV-180 UV spectrophotometer spectrophotometer (Shimatzu, Kyoto, Japan) at 515 nm. Each liver hydrolysates extract was analysed in triplicate and its antioxidant activity was determined using trolox as standard, expressing the results as µg Trolox equivalents (TE)/g sample.

2.7.2. ABTS Radical Scavenging Activity

This method was determined according to the procedure previously described by Re et al. (1999) with some modifications. This assay is based on the ability of antioxidants to quench the long-lived ABTS ((2,2-azinobis-(3-ethyl-benzothiazoline-6-sulphonate) radical cation, a bluish-green chromophore with specific absorption line at 734 nm. This radical was prepared mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate, keeping the mixture in darkness at room temperature for 12-16 h. Prior to use, the ABTS stock solution was diluted with distilled water to achieve an absorbance of 0.70 at 734 nm, being equilibrated at 30 ºC. A solution of 980 mL was added to an aliquot of 20 mL of each hydrolysate. Afterward, absorbance was measured in an UV-180 UV spectrophotometer at 734 nm. Each hydrolysate was analysed in triplicate and

its antioxidant activity was determined using a standard curve of ascorbic acid (AA), expressing the results as mg AA/100 g sample.

2.7.3. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay is based on Benzie & Strain (1996) method with some modifications. This test is based on capability of the antioxidants species to reduce iron (III) to the ferrous form iron (II) in an acid medium. The FRAP reagent was prepared using 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3$ x6H_2O solutions. This three solutions were mixed in a ratio of 10:1:1 (v:v:v). Afterward, 900 µL of this resultant FRAP solution was added to 30 µL of properly diluted sample and to 90 µL distilled water. The mixture was heated at 37 °C and left to react for 20 min at this temperature. After this time, the absorbance was measured in an UV-180 UV spectrophotometer at 593 nm. Each hydrolysate was analysed in triplicate and its antioxidant activity was determined using a standard curve of $FeSO_4$, expressing the results as µmol Fe^{+2}/100g sample.

2.7.4. Oxygen radical absorbance capacity assay (ORAC)

The ORAC assay was assayed according to the protocol of Huang, Majumder, & Wu (2010) with some modifications. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), being 200 µL the final volume of the reaction mixture. 25 µL of dilute sample and 150 µL of 0.8 µM fluorescein (oxidizable substrate) was added into the internal wells of a black 96-well microplate (FluoroNunc™ F96-MicroWell™ plate) and was immediately incubated at 37 ºC for 30 minutes in the own fluorescence instrument. Then, 25 µL of AAPH 184 mM (2,2-azobis (2-methylpropionamidine) dihydrochloride) solution was added rapidly to each well using the injectors of the fluorescence device, to start the reaction in the

microplate reader. The fluorescence was recorded with excitation and emission filters of 485 nm 528 nm, respectively. Samples were stirred prior to each reading. Trolox reagent was used as a standard reference compound and phosphate buffer was used as blank. Results were calculated based on the differences of areas under the curves of fluorescence decay of the fluorescein between the blank and the sample (net area under the curve). They were expressed as mg Trolox Equivalent (TE)/g sample.

2.8. Statistical analysis

Statistical analysis of the obtained data was conducted using the IBM SPSS Statistics 23.0 program (IBM Corporation, Somers, NY, USA). Normal distribution and homogeneity of variance were previously tested (Shapiro-Wilk). For each liver hydrolysate antioxidant capacity, one-way analysis of variance was applied to all assessed antioxidants tests (DPPH, ABTS, FRAP and ORAC) and the results were depicted expressed as mean. The least square means (LSM) were separated using Duncan's post hoc test. All statistical test of LSM were performed for a significance level $P<0.05$. Correlations among antioxidants tests ($P<0.01$) and identified and quantified peptides were determined using the Pearson´s linear correlation coefficient. From all set of peptides identified and quantified only those which showed at least one antioxidants test with $P<0.01$ and a coefficient of correlation higher than 0.5 were showed in the manuscript.

A hierarchical clustering of identified peptides based on the protein quantifications was generated by XLSTAT 2.01 (Addinsoft SARL, Paris, France) through a heat map using Euclidean distances.

3. Results and discussion

3.1. Proximate composition of pork liver

The average values of chemical composition of pork liver are displayed in Table S1. The moisture (74.29%), fat (3.32%), ash (1.41%) and protein (18.84%) content were similar to those values reported in other studies (Alfaia et al., 2020; Verma et al., 2019). These findings indicated that liver has a high nutritional content. Additionally, liver is a rich source of vitamin A, vitamin B and nicotinic acid (Ahmad, Imran, & Hussain, 2018).

Within protein content, results obtained from this study showed higher values than those reported in chicken liver (Seong et al., 2015) and beef liver (Seong et al., 2014). Moreover, the protein content of liver is higher than those from other pork by-products such as heart, lung, stomach, intestine, spleen, uterus and pancreas (Seong et al., 2014). Liver samples of the present study have a high level of essential amino acids such as threonine (5.11±0.39%), valine (6.9±0.54%), isoleucine (5.33±0.43%), leucine (9.21±0.78%), phenylalanine (5.60±0.46%), lysine (9.25±1.40%) and histidine (3.82±0.20%). These findings are in agreement with those reported by Seong et al. (2014), except for tyrosine content which it could be due to methodology analytical differences.

Therefore, the election of liver as rich source of proteins with an appealing amino acid profile seems adequate for the preparation of the protein hydrolysates. Moreover, it has also been reported that liver hydrolysates showed more antioxidant capacity than other pork tissues such as colon, pancreas and appendix (Damgaard, Otte, Meinert, Jensen, & Lametsch, 2014).

3.2. Characterization of the pork liver hydrolysates by action of alcalase, bromelain, flavourzyme and papain

The protein profile of the enzymatic hydrolysis was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic profile of hydrolysate obtained of pork liver from four different enzymes is displayed in Figure 1. The control lane showed seven main bands at 100, 76, 64, 54, 49, 41 and 23 kDa. The treatment using alcalase, bromelain, flavourzyme and papain resulted in disappearance of most bands in the range of 10-250 kDa confirming the enzymatic hydrolysis of these proteins. Additionally, it should be noted that these enzymes degraded differently liver protein. Indeed, alcalase lane showed a single band at 54 kDa, bromelain two bands at 54 and 39 kDa, flavourzyme 5 bands at 68, 60, 54, 41, and 33 kDa and papain 4 bands at 68, 58, 54, and 15 kDa. In addition, it can be observed that control band of 54 kDa resulted more difficult protein band to degrade than other bands (100, 76, 64, 49, 41 and 23 kDa) as well as a decrease in the intensity of total control bands and the presence of new bands by the effect of these enzymes. It could be clearly concluded that these enzymes affected liver protein differently. This result is in agreement with data previously reported by Fu, Liu, Hansen, Bredie, & Lametsch, (2018) who proved that other pork tissues subjected to an enzymatic degradation using these enzymes produced hydrolysates with different sensory properties due to variations in the peptides composition.

The proteolysis index which is correlated with protein hydrolysis produce small peptides and free amino acids contributing to texture changes and flavour development (López-Pedrouso, Lorenzo, Zapata, & Franco, 2019). As expected and in line with our electrophoretic data, the proteolysis index (Figure 2 A) in liver was different for each enzyme, increasing from 22.86% in control to 82.56%, 70.37%, 63.55% and 59.20% for alcalase, bromelain, flavourzyme and papain,

306 respectively. In agreement with our findings, in other studies alcalase has proved
307 a higher capability for hydrolysis compared to papain (Ahmadifard, Murueta,
308 Abedian-Kenari, Motamedzadegan, & Jamali, 2016). On the contrary, alcalase
309 (2593.37 mg aa/100 g liver) was not the enzyme which released the greatest
310 amount of total free amino acids, which was achieved by flavourzyme (6121.97
311 mg aa/100 g liver; Figure 2 B). This fact suggests that alcalase release mostly
312 small peptides, meanwhile flavourzyme produce a higher amount of free amino
313 acids. Additionally, it has been demonstrated that alcalase can even hydrolyze
314 proteins with hydrophobic amino acids at the end of peptide chain. These
315 hydrophobic amino acids raise the solubility in lipids, increasing the interaction
316 with hydrophobic radical species and hydrophobic polyunsaturated fatty acids
317 (Yu & Tan, 2017).

318 However, information provided by proteolysis index and total free amino acids is
319 not enough accurate, because food peptides are converted into di-, tri- and tetra-
320 peptides during digestion and they can easily pass into the bloodstream
321 complicating the identification of bioactive peptides. This family of small peptides
322 is very appreciated in terms of bioactivity (Panchaud, Affolter, & Kussmann,
323 2012), but their identification is a technological challenge because they can
324 belong to many proteins.

325 ***3.3. Identification of peptides from pork liver hydrolysates***

326 The peptidomic changes in pork liver hydrolysates were investigated using Label-
327 free mass spectrometry. For the hierarchical clustering, only data from peptides
328 identified and differentially abundance was processed. A heatmap was used for
329 providing information of abundance with a colour code (Figure 3). As can be
330 observed in this figure a total of 73 differentially abundant peptides were identified

among the four hydrolysates from enzymatic hydrolysis (alcalase, bromelain, papain and flavourenzyme). All liver samples analyzed were grouped into nine different clusters due to their quantification pattern and the heatmap visualization indicated a distribution of liver hydrolysates among analyzed peptides. The cluster analysis clearly showed more marked differences in mixtures of peptides obtained by alcalase treatment, which were grouped on the right side of Figure 3 in two clusters separated from the remaining 36 peptide samples. The nine peptide samples from papain treatment were also distributed in two clusters, meanwhile other nine samples from bromelain were grouped in another cluster. Finally, six samples of flavourzyme were grouped in two clusters. Overall, peptides from alcalase treatment are the more differentiated but the other peptides from the three enzymes also were quite different.

A possible explanation for the wide difference of alcalase in the peptide mixture could be due to its higher hydrolysis capacity, because the other three enzymes act in a more similar way producing more similar peptide mixtures but not equals. This greater proteolysis index can be observed in the monodimensional gels (Figure 1) by the disappearance of most bands, as well as in Figure 2 A. The findings of the current study are consistent with other authors who found that alcalase hydrolysates had a higher degree of hydrolysis than papain or pepsin hydrolysates obtained from animal (Lafarga, Álvarez, & Hayes, 2017) and vegetal (Wu et al., 2016) tissues.

3.4. Antioxidant capacity of liver porcine hydrolysates

The antioxidant capacity of the liver porcine hydrolysates was carried out using four tests (DPPH, ABTS, FRAP and ORAC) that have also been used to assess the antioxidant effect of peptides in other studies (Du et al., 2019). On the other

hand, SWATH method afforded a label-free quantification of peptide mixtures obtained by alcalase, bromelain, papain and flavourzyme (Table 1). Over 2000 peptides were identified and quantified in each sample, but only significantly different peptides among the four enzymatic treatments were considered. Among these 73 peptides, correlations between quantification and antioxidant activity were performed, resulting 35 peptides with significant correlations ($P<0.01$) and higher correlation coefficient than 0.5 that are depicted in Table 2.

The most abundant peptides were APAAIGPYSQAVLVDR from uncharacterized protein (1,694.9; 46,689.5; 57,847.3 and 54,070.6); GLNQALVDLHALGSAR from ferritin (27,606.1; 27,524.9; 10,359.4 and 24,380.3); ALFQDVQKPSQDEWGK from ferritin (18,525.8; 18,502.5; 5,832.1 and 18,931.3); LSGPQAGLGEYLFER from ferritin (24,637.8; 22,074.5; 9,058.8 and 19,753.6) and LGEHNIDVLEGNEQFINAAK from trypsinogen (92,134.4; 51,491.8; 30,478.2 and 36,997.2) for alcalase, bromelain, flavourzyme and papain, respectively. Other peptides were correlated with antioxidant capacity as showed in Table 2, but their quantity was not so high, hence they will not be described in the discussion. Additionally, the gastrointestinal digestion further degrades these peptides in smaller peptides. Three of these peptides were obtained from ferritin which is a main storage protein of iron in vertebrates located in liver and a peptide from trypsinogen which is the proenzyme precursor of trypsin. The iron intake contributes to maintain normal physiological process in human body as well as oxygen transportation, storage and synthesis of cytochromes and metalloenzymes (Heeney & Andrews, 2004).

As shown in Table 2, the APAAIGPYSQAVLVDR peptide displayed an important negative correlation for DPPH, ABTS and FRAP (-0.523, -0.724 and –0.562,

respectively). Thus, the degradation of this peptide may be increased the radical scavenging activity of the pork liver hydrolysate. In this sense, dipeptides which includes amino acid tyrosine (Y) has been proved to be effective radical scavenging because its aromatic amino acid could be contributing to stabilize them (Du et al., 2019). On the contrary, peptides as GLNQALVDLHALGSAR, ALFQDVQKPSQDEWGK and LSGPQAGLGEYLFER from ferritin showed a strong and positive correlation with ORAC assay (0.743, 0.605 and 0.682, respectively). It is possible to hypothesise that the increase in concentration of these peptides could increase an absorption of oxygen capacity and this finding is easily understandable due to ferritin protein can manage iron and oxygen through ferroxidase sites and substrates of iron and oxygen (Liu, Hintze, Lonnerdal, & Theil, 2006). Finally, the peptide LGEHNIDVLEGNEQFINAAK from trypsinogen also demonstrated a high and positive correlation with ABTS, FRAP and ORAC assays (0.789, 0.592 and 0.619, respectively). The ORAC method measures the loss of fluorescence of a protein (β-phycoerythrin) due to a change of its conformation by oxidative damage caused by peroxyl radicals, hence is the most relevant method from a biological perspective integrating both degree and time of antioxidant reaction (Zulueta, Esteve, & Frígola, 2009). This study produced results which corroborate the findings that proved antioxidant peptides from meat products which can protect cells and organisms from oxidative damage as reviewed (Liu et al., 2016).

Proteins from animal tissues has also the advantage of a high content methylhistidine and hydroximethyllysine and other essential amino acids in high bioavailability. Moreover, hydrophobic amino acids such as alanine (A), isoleucine (I), leucine (L), proline (P), phenylalanine (F) and tyrosine (Y) which

406 are included in these peptides increase the solubility in aqueous solution,
407 enhancing scavenge free radicals (Yu & Tan, 2017). This fact also suggests that
408 these peptides showed antioxidant capacity even higher after digestion.

409 Several of these antioxidant biopeptides contain other dipeptides or tripeptides,
410 which have also been found in the Biopep database (Minkiewicz, Iwaniak, &
411 Darewicz, 2019). Indeed, thirteen peptides contain dipeptides or tripeptides
412 described by another authors (Table 3). Therefore, it can be assumed that these
413 thirteen peptides are contributing to increase the antioxidant capacity of the pork
414 liver hydrolysate. In addition, it can be suggested that the bioactive peptides could
415 be contained in the sequence of parent peptides released by gastric digestion. It
416 is important to highlight that the ferritin peptides GLNQALVDLHALGSAR and
417 ALFQDVQKPSQDEWGK available in large quantities of alcalase, bromelain and
418 papain mixtures included antioxidant peptides as LH, LHA, KP and WG (Chen,
419 Muramoto, Yamauchi, & Nokihara, 1996; Huang et al., 2010; Saito et al., 2003).
420 Another peptides as IYVVDVGTEPR, GGPVQVLEDQELK and
421 AADGTWEPFALGK, which have an intermediate concentration in bromelain,
422 flavorenzyme and papain also included IY, EL, LK, GTW and TW described by
423 other authors as potent antioxidants (Beermann, Euler, Herzberg, & Stahl, 2009;
424 Liu et al., 2015; Suetsuna, Ukeda, & Ochi, 2000). In the remaining cases, the
425 dipeptides or tripeptides were not identified most likely because these databases
426 are not very extensive yet.

427 **4. Conclusions**

428 This study showed the great value of pork liver for preparing hydrolysates due to
429 its high protein content and adequate amino acid profile. Additionally, the
430 proteolytic enzymes assayed (alcalase, bromelain, papain and flavourzyme)

differentially affected the hydrolysis, suggesting complex mixtures of biopeptides as final products with antioxidant activity. In this regard, the peptidomic map from alcalase treatment were more differentiated from the other enzymes. The APAAIGPYSQAVLVDR, GLNQALVDLHALGSAR, ALFQDVQKPSQDEWGK, LSGPQAGLGEYLFER and LGEHNIDVLEGNEQFINAAK peptides from an uncharacterized protein, ferritin and trypsinogen are main candidates to explain the antioxidant capacity of the pork liver. Evidences from this study suggest that porcine liver hydrolysates could be an appealing base for developing health foods, but further studies are needed to quantify the bioactive peptides as well as to determine their *in vivo* bioavailability.

Conflicts of Interest: The authors declare no conflict of interest.

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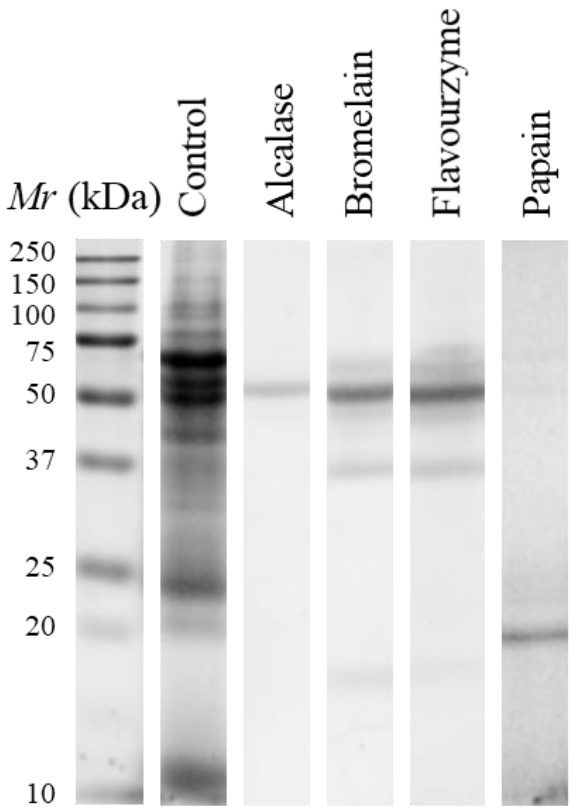
CAPTION TO FIGURES

Figure 1. SDS-PAGE separation of proteins of pork liver samples obtained from control and enzymatic treatments (alcalase, bromelain, flavourzyme and papain).

Figure 2. Free amino acids content (A) and proteolysis index (B) of pork liver samples obtained from control and enzymatic treatments (alcalase, bromelain, flavourzyme and papain).

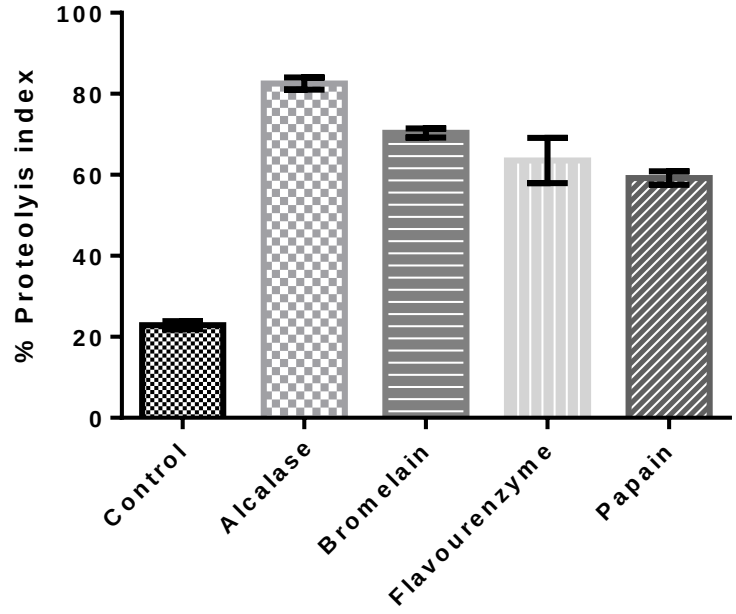
Figure 3. Heat map analysis of 73 differentially abundant peptides (logarithmic scale in base 2) of from control and enzymatic treatments of pork liver samples. Green and red colors represent relatively high and low values of peptide abundance, respectively.



623

624 **Figure 1.**

625



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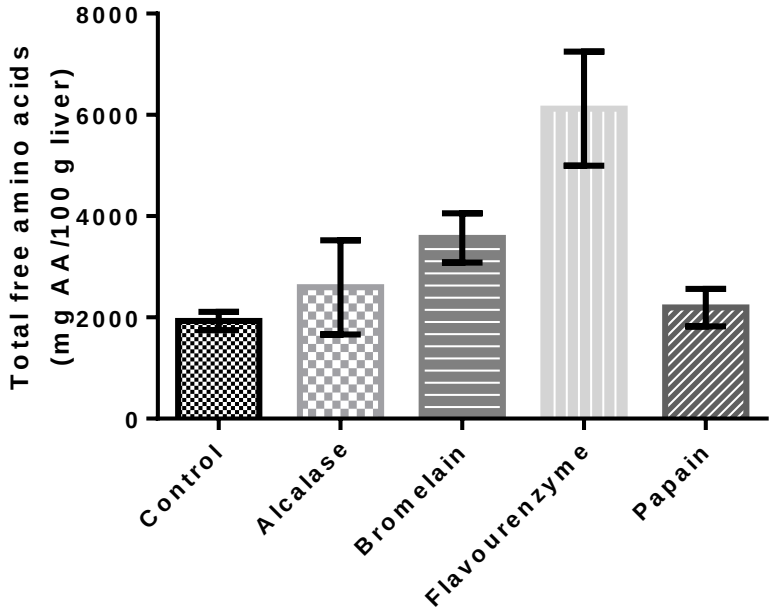

B

626 **Figure 2.**

627

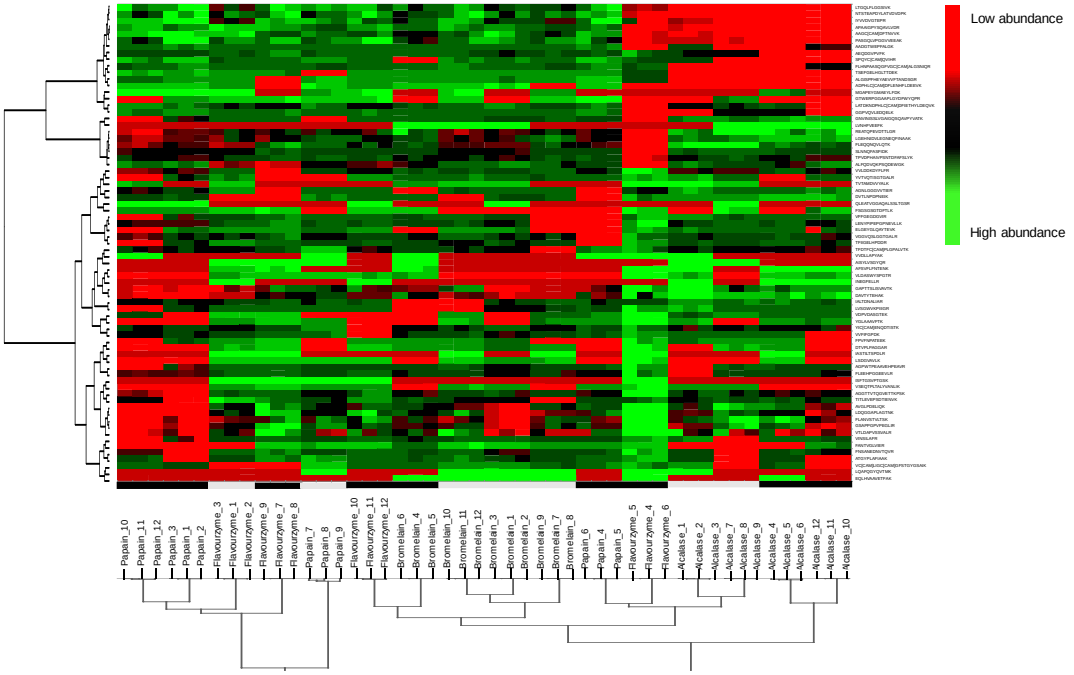
Low abundance
High abundance
Papain_10
Papain_11
Papain_12
Papain_3
Papain_1
Papain_2
Flavourzyme_3
Flavourzyme_1
Flavourzyme_2
Flavourzyme_9
Flavourzyme_7
Flavourzyme_8
Papain_7
Papain_8
Papain_9
Flavourzyme_10
Flavourzyme_11
Flavourzyme_12
Bromelain_6
Bromelain_4
Bromelain_5
Bromelain_10
Bromelain_11
Bromelain_12
Bromelain_3
Bromelain_1
Bromelain_2
Bromelain_9
Bromelain_7
Bromelain_8
Papain_6
Papain_4
Papain_5
Flavourzyme_5
Flavourzyme_4
Flavourzyme_6
Kcalase_1
Kcalase_2
Kcalase_3
Kcalase_7
Kcalase_8
Kcalase_9
Kcalase_4
Kcalase_5
Kcalase_6
Kcalase_12
Kcalase_11
Kcalase_10

Figure 3.

Table 1. Chemical composition and amino acid content of pork liver (n=8). Results expressed as mean ± standard error

	Mean	SD
Moisture (%)	74.29	0.43
Fat (%)	3.32	0.11
Ash (%)	1.41	0.03
Carbohydrate (%)	2.13	0.52
Protein (%)	18.84	0.05
Amino acid (% respect protein content)		
Asp	9.27	0.92
Ser	5.05	0.50
Glu	13.10	0.97
Gli	6.51	0.37
Ala	5.93	0.61
Pro	5.68	0.45
Tyr	2.68	0.24
NEAA	**48.22**	**3.36**
His	3.82	0.20
Arg	6.56	0.54
Thr	5.11	0.39
Val	6.90	0.54
Lys	9.25	1.40
Iso	5.33	0.43
Leu	9.21	0.78
Phe	5.60	0.46
EAA	**51.78**	**1.94**
EAA/NEAA	**1.07**	

NEAA: non-essential amino acids; EAA: essential amino acids

662

663 **Table 2**. Quantification of antioxidant peptides identified by SWATH obtained from alcalase, bromelain, flavourzyme and papain
664 treatments.

Peptide	Protein	Alcalase	Bromelain	Flavourzyme	Papain
AEQDGVPVFK	A0A480E686	29.1^{c}	159.7^{b}	279.6^{a}	128.1^{b}
ELGEYGLQAYTEVK	Mitochondrial aldehyde dehydrogenase (ALDH2)	97.3^{b}	100.7^{b}	346.9^{a}	57.0^{b}
IYVVDVGTEPR	Selenium binding protein 1 (SELENBP1)	407.8^{c}	1,955.3^{b}	1,839.9^{b}	3,268.6^{a}
GGPVQVLEDQELK	Selenium binding protein 1 (SELENBP1)	55.5^{c}	1,145.2^{b}	1,117.8^{b}	2,126.9^{a}
FLHNPAASQGFVGC[CAM]ALGSNIQR	Selenium binding protein 1 (SELENBP1)	22.4^{b}	715.8^{a}	665.3^{a}	707.2^{a}
NTSTEAPDYLATVDVDPK	Selenium binding protein 1 (SELENBP1)	90.9^{b}	555.9^{a}	516.0^{a}	667.8^{a}
LTGQLFLGGSIVK	Selenium binding protein 1 (SELENBP1)	292.1^{c}	2,149.6^{b}	2,013.6^{b}	3,315.7^{a}
SPQYC[CAM]QVIHR	Selenium binding protein 1 (SELENBP1)	16.3^{c}	157.1^{b}	110.5^{bc}	648.2^{a}
GAPTTSLISVAVTK	Aldehyde dehydrogenase 7 (ALDH7A1)	197.0^{a}	45.8^{b}	99.4^{b}	38.7^{b}
VLDASWYSPGTR	A0A481AX04	68.8^{b}	27.9^{b}	356.1^{a}	41.9^{b}
INEGFELLR	PKS_ER domain-containing protein (LOC100512795)	462.6^{a}	0.0^{b}	258.9^{a}	0.0^{b}
VINSILAFR	M20_dimer domain-containing protein	193.7^{ab}	140.7^{bc}	311.9^{a}	49.5^{c}
DAVTYTEHAK	A0A480KDB1	651.3^{a}	141.7^{b}	251.1^{b}	121.2^{b}
DVTLNPDPNEIK	Isopentenyl-diphosphate delta isomerase (IDI1)	234.7^{a}	27.9^{b}	52.1^{b}	35.3^{b}
APAAIGPYSQAVLVDR	Uncharacterized protein (RIDA)	1,694.9^{b}	46,689.5^{a}	57,847.3^{a}	54,070.6^{a}
AAGC[CAM]DFTNVVK	Uncharacterized protein (RIDA)	283.7^{c}	8,203.9^{b}	7,393.2^{b}	14,562.6^{a}

PASGQLVPGGVVEEAK	Uncharacterized protein (RIDA)	144.4[b]	2,194.0[a]	2,514.0[a]	3,229.1[a]
GLNQALVDLHALGSAR	Ferritin	27,060.1[a]	27,524.9[a]	10,359.4[b]	24,380.3[a]
ALFQDVQKPSQDEWGK	Ferritin	18,525.8[a]	18,502.5[a]	5,832.1[b]	18,931.3[a]
LSGPQAGLGEYLFER	Ferritin	24,637.8[a]	22,074.5[ab]	9,058.8[c]	19,753.6[b]
QNYSTEVEAFVNR	Ferritin	10,002.9[a]	8,683.5[ab]	3,332.0[c]	7,400.6[b]
DDVALEGVSHFFR	Ferritin	1,301.1[a]	1,185.9[a]	360.2[b]	1,107.8[a]
REATQPEVDTTLGR	Carboxylic ester hydrolase (CES3)	4,327.7[a]	1,907.7[b]	1,391.0[b]	1,328.8[b]
MGAPEYGMAEYLFDK	Ferritin (FTH1)	3.5[c]	29.2[bc]	93.0[ab]	126.1[a]
LVNHFVEEFK	Heat shock 70kDa protein (HSPA1A)	900.9[a]	185.2[b]	0.0[b]	0.0[b]
GNVINISSLVGAIGQSQAVPYVATK	Hydroxysteroid 17-beta dehydrogenase 14	392.4[a]	136.3[b]	118.5[b]	24.4[c]
AADGTWEPFALGK	A0A481CSP9	107.2[c]	1,611.5[b]	1,271.3[b]	2,910.8[a]
ALGISPFHEYAEVVFTANDSGR	A0A481CSP9	0.0[c]	543.0[ab]	373.6[b]	715.6[a]
TSEFGELHGLTTDEK	A0A481CSP9	0.0[c]	94.1[ab]	77.5[b]	136.2[a]
FLEEHPGGEEVLR	A0A480SB71	94.5[b]	106.8[b]	325.0[a]	74.7[b]
IALTDNALIAR	60S ribosomal protein L7	222.8[a]	40.1[c]	101.9[b]	64.7[bc]
LGEHNIDVLEGNEQFINAAK	Trypsinogen	92,134.4[a]	51,491.8[b]	30,478.2[c]	36,997.2[b][c]
FLEQQNQVLQTK	Keratin 1 (KRT1)	5,017.4[a]	2,310.5[b]	1,979.0[b]	2,030.0[b]
SLNNQFASFIDK	Keratin 1 (KRT1)	3,342.3[a]	1,623.3[b]	1,511.3[b]	1,519.6[b]
AGNLGGGVVTIER	A0A481CBE4	187.3[a]	71.9[b]	82.0[b]	34.3[b]

665 This table only includes the peptides identified by SWATH and correlated with antioxidant capacity (Pearson correlation coefficient higher than 0.5 at P<0.01).
666 Abundance mean values of these peptides with the significant differences noted by a-c upper letter (P<0.05) are showed.
667

668

669 **Table 3.** Correlations of antioxidant peptides identified by SWATH obtained from alcalase, bromelain, flavourzyme and papain
670 treatments determined by DPPH, ABTS, FRAP and ORAC assays.

Peptide	Protein	DPPH	ABTS	FRAP	ORAC
AEQDGVPVFK	A0A480E686	-0.013	**-0.532****	-0.304*	**-0.529****
ELGEYGLQAYTEVK	Mitochondrial aldehyde dehydrogenase (ALDH2)	0.081	-0.292*	-0.215	**-0.537****
IYVVDVGTEPR	Selenium binding protein 1 (SELENBP1)	-0.466**	**-0.671****	**-0.567****	-0.363*
GGPVQVLEDQELK	Selenium binding protein 1 (SELENBP1)	-0.480**	**-0.667****	**-0.627****	-0.368
FLHNPAASQGFVGC[CAM]ALGSNIQR	Selenium binding protein 1 (SELENBP1)	-0.324*	**-0.721****$^{}$	**-0.507***	-0.436**
NTSTEAPDYLATVDVDPK	Selenium binding protein 1 (SELENBP1)	-0.383**	**-0.658****	**-0.527****	-0.361*
LTGQLFLGGSIVK	Selenium binding protein 1 (SELENBP1)	-0.481**	**-0.706****	**-0.584****	-0.374**
SPQYC[CAM]QVIHR	Selenium binding protein 1 (SELENBP1)	-0.405**	-0.444**	**-0.554****	-0.285*
GAPTTSLISVAVTK	Aldehyde dehydrogenase 7 (ALDH7A1)	**0.581****	**0.549****	**0.646****	0.220
VLDASWYSPGTR	A0A481AX04	0.209	-0.207	-0.159	**-0.526****
INEGFELLR	PKS_ER domain-containing protein (LOC100512795)	**0.506****	0.053	0.198	-0.253
VINSILAFR	M20_dimer domain-containing protein	**0.708****	-0.156	**0.647****	**-0.585****
DAVTYTEHAK	A0A480KDB1	**0.554****	**0.682****	**0.663****	0.323*
DVTLNPDPNEIK	Isopentenyl-diphosphate delta isomerase (IDI1)	0.416**	**0.749****	0.463**	0.298*
APAAIGPYSQAVLVDR	Uncharacterized protein (RIDA)	**-0.523****	**-0.724****	**-0.562****	-0.433**
AAGC[CAM]DFTNVVK	Uncharacterized protein (RIDA)	**-0.585****	**-0.592****	**-0.610****	-0.344*

PASGQLVPGGVVEEAK	Uncharacterized protein (RIDA)	-0.389**	**-0.533****	**-0.504****	-0.384**
GLNQALVDLHALGSAR	Ferritin	0.117**	0.417**	0.377**	**0.743****
ALFQDVQKPSQDEWGK	Ferritin	0.048	0.356*	0.364*	**0.605****
LSGPQAGLGEYLFER	Ferritin	0.147	**0.501****	0.438**	**0.682****
QNYSTEVEAFVNR	Ferritin	0.268	**0.607****	**0.607****	**0.768****
DDVALEGVSHFFR	Ferritin	0.089	**0.502****	0.405**	**0.718****
REATQPEVDTTLGR	Carboxylic ester hydrolase (CES3)	0.162	**0.774****	**0.532****	**0.558****
MGAPEYGMAEYLFDK	Ferritin (FTH1)	**-0.684****	-0.379**	**-0.514****	-0.405**
LVNHFVEEFK	Heat shock 70kDa protein (HSPA1A)	0.190	**0.646****	0.394**	0.393**
GNVINISSLVGAIGQSQAVPYVATK	Hydroxysteroid 17-beta dehydrogenase 14	0.339*	**0.768****	**0.590****	0.458**
AADGTWEPFALGK	A0A481CSP9	**-0.501****	**-0.668****	**-0.637****	-0.317*
ALGISPFHEYAEVVFTANDSGR	A0A481CSP9	-0.358*	**-0.566****	**-0.510****	-0.203
TSEFGELHGLTTDEK	A0A481CSP9	**-0.550****	**-0.601****	**-0.555****	-0.302*
FLEEHPGGEEVLR	A0A480SB71	0.038	-0.264	0.238	**-0.505****
IALTDNALIAR	60S ribosomal protein L7	0.467**	**0.749****	**0.585****	0.397**
LGEHNIDVLEGNEQFINAAK	Trypsinogen	0.366*	**0.789****	**0.592****	**0.619****
FLEQQNQVLQTK	Keratin 1 (KRT1)	0.309*	**0.686****	**0.628****	**0.591****
SLNNQFASFIDK	Keratin 1 (KRT1)	0.247	**0.633****	0.493**	0.483**
AGNLGGGVVTIER	A0A481CBE4	0.391**	**0.629****	**0.659****	0.418**

671 This table only includes peptides with at least one significant antioxidant test by Pearson correlation coefficient higher than 0.5 at
672 P<0.01 (bold)
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676 **Table 4.** Dipeptides and tripeptides (highlighted in bold) included in pork liver peptides generated from enzymatic treatments with
677 antioxidant capacity according to literature.

Peptide	References
ELGEYGLQ**AY**TEVK	(Suetsuna et al., 2000)
IYVVDVGTEPR	(Beermann et al., 2009)
GGPVQVLEDQ**E**LK	(Suetsuna et al., 2000); (W. Y. Huang et al., 2010)
F**LHN**PAASQ**GFVG**C[CAM] ALGSNIQR	(Cheng, Chen, & Xiong, 2010); (Saito et al., 2003);
VLDAS**WYS**PGTR	(Hernández-Ledesma, Amigo, Recio, & Bartolomé, 2007)
INEGF**ELLR**	(Suetsuna et al., 2000);
DAV**TY**TEHAK	(Cheng et al., 2010)
PASGQLVPGGVVE**EAK**	(Suetsuna et al., 2000)
GLNQALVD**LHA**LGSAR	(Chen et al., 1996); (Saito et al., 2003)
ALFQDVQ**KP**SQDE**WG**K	(W. Y. Huang et al., 2010); (Anna et al., 2016)
AAD**GTW**EPFALGK	(R. Liu et al., 2015)
TSEFG**ELHG**LTTDEK	(Cheng et al., 2010); (Suetsuna et al., 2000); (Saito et al., 2003)
FLEEHP**GGE**EVLR	(Bougatef et al., 2010)

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