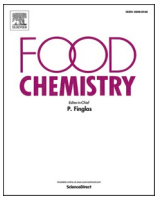
Food Chemistry 423 (2023) 136351

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Valorisation of pork by-products to obtain antioxidant and antihypertensive peptides

María López-Pedrouso [a], José M. Lorenzo [b,c], Ricard Bou [d], José Antonio Vazquez [e], Jesús Valcarcel [e], Mònica Toldrà [f], Daniel Franco [g,*]

[a] Department of Zoology, Genetics and Physical Anthropology, University of Santiago de Compostela, Santiago de Compostela, 15872 A Coruña, Spain
[b] Centro Tecnológico de la Carne de Galicia, Rúa Galicia Nº 4, Parque Tecnológico de Galicia, San Cibrao das Viñas, 32900 Ourense, Spain
[c] Área de Tecnología de los Alimentos, Facultad de Ciencias de Ourense, Universidad de Vigo, 32004 Ourense, Spain
[d] Food Safety and Functionality Program, Institute of Agrifood Research and Technology (IRTA), Finca Camps i Armet s/n, 17121 Monells, Spain
[e] Group of Recycling and Valorization of Waste Materials (REVAL), Marine Research Institute (IIM-CSIC), C/Eduardo Cabello, 6, Vigo 36208, Galicia, Spain
[f] Institute of Food and Agricultural Technology (INTEA), XIA (Catalonian Network on Food Innovation), Escola Politècnica Superior, University of Girona, C/Maria Aurèlia Capmany 61, 17003 Girona, Spain
[g] Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

ARTICLE INFO

Keywords:
Meat by-products
Porcine liver
Autolysis
Proteolysis
Bioactive peptides
Peptidomic
Endogenous enzymes (proteases)

ABSTRACT

The porcine liver could be used for the extraction of zinc-protoporphyrin (ZnPP) as a natural red meat pigment. During the autolysis process, porcine liver homogenates was incubated at pH 4.8 and 45 °C under anaerobic conditions to obtain insoluble ZnPP. After incubation, the homogenates were readjusted at pH 4.8, and at pH 7.5 before being centrifuged at 5500 × *g* for 20 min at 4 °C and the resulting supernatant were compared with the obtained at pH 4.8 at the beginning of the incubation. The molecular weight distributions of the porcine liver fractions at both pHs were very similar, however, eight essential amino acids were more abundant in fractions obtained at pH 4.8. Regarding the ORAC assay, porcine liver protein fraction at pH 4.8 showed the highest antioxidant capacity but antihypertensive inhibition was similar for both pHs. Peptides with strong bioactivity potential from aldehyde dehydrogenase, lactoylglutathione lyase, SEC14-like protein 3 and others were identified. The findings have demonstrated the potential of the porcine liver to extract natural pigments and bioactive peptides.

1. Introduction

Current trends in the meat industry indicate a rapid development of large-scale production due to the growing world population. Global consumption of meat proteins is expected to increase by 14% by 2030, particularly 13.1% for pork protein, compared to the average from 2018 to 2020 (OECD/FAO, 2021). The meat industry generates large amounts of by-products that require proper treatment and handling systems (Toldrá, Reig, & Mora, 2021). For sustainable development of the meat industry, by-products should be utilized and the extraction of high-added-value components such as biopeptides or pigments is a suitable strategy. Animal by-products including liver, heart, spleen, kidney, blood, fat, and meat trimmings can be used for human consumption or animal feed. In the porcine industry, these by-products account for about 30% of the live weight, consequently, there is a great concern to use them for reducing the livestock environmental impact. Specifically, in a pig between 60–75 kg of live weight, the liver weight is around 1.15–1.66 kg (Lynch, Mullen, O'Neill, Drummond, & Álvarez, 2018). The liver is widely incorporated in sausages and pates but also offers a good opportunity to obtain valuable components for food applications. It has a high nutritional value due to its high protein content, but it is also a good source of vitamins A and B12 and other nutrients such as iron, zinc and copper (Ockerman, Basu, & Toldrá, 2017).

The meat-product curing process generally includes sodium chloride and a nitrate/nitrite source generating an excellent taste and flavour. Particularly, nitrite (E250) produces reddish-pink colour as well as protection against lipid and protein oxidation. Regarding its antimicrobial activity, nitrites inhibit the growth of foodborne pathogens, particularly *Clostridium botulinum* spores. Only a few studies considered that nitrite-free meat products are microbiological safe. New

* Corresponding author at: Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain
E-mail address: daniel.franco.ruiz@usc.es (D. Franco).

https://doi.org/10.1016/j.foodchem.2023.136351
Received 11 November 2022; Received in revised form 6 May 2023; Accepted 8 May 2023
Available online 12 May 2023

technologies are still being investigated to reduce or even eliminate the concentration of nitrite maintaining the food safety in cured products (Fraqueza, Laranjo, Elias, & Patarata, 2021). However, nitrites could transform into carcinogenic compounds (nitrosamines) from the reaction of nitrites with free amino acids and amines under specific conditions. For this reason, nitrites should be totally or partially replaced in meat products using other more healthy additives (Stoica et al., 2022). A very promising strategy is the use of zinc protoporphyrin IX (ZnPP), as a natural pigment in meat dry-cured products, which is originated from haem proteins by replacement of iron with zinc in the heme group. This reaction seems to be mainly determined by the action of the enzyme ferrochelatase which catalyses the substitution on Fe(II) with Zn(II) in cured meat products (De Maere et al., 2018). Recently, Wakamatsu, Murakami, and Nishimura (2015) suggested that meat color can be improved in the presence of ZnPP, which can be formed by the addition of sources of ferroquelatase usually found in internal organs such as liver with a high ability for ZnPP formation. In the same line, during the curing process of nitrite-free dry-cured hams, it was demonstrated that the formation of ZnPP was related to salt content, water activity, lipolysis, and redness (Bou, Llauger, Arnau, & Fulladosa, 2018; Bou, Llauger, Arnau, Olmos, & Fulladosa, 2022).

Major research was carried out on ZnPP-forming properties of various porcine internal organs, and it was concluded that the liver had the greatest ability to form ZnPP (De Maere et al., 2018; Wakamatsu et al., 2015). Consequently, liver which is considered a low commercial value co-product from the meat industry can be valorised through the obtainment of alternative red meat pigments. After the extraction of ZnPP a residual soluble protein fraction that could be further reused to produce high-value ingredients rich in proteins and peptides with several bioactivities (e.g. antioxidant, antimicrobial or antihypertensive). In this regard, liver protein hydrolysates showed a marked antioxidant activity measured by DPPH, ABTS, FRAP and ORAC assays after exposure to enzymatic reactions with exogenous enzymes (alcalase, bromelain, flavourzyme and papain) at different incubation times. From a complex mixture of peptides, those originating from the following proteins (i.e. trypsinogen, ferritin, keratin, carboxylic ester hydrolase and globin domain-containing), showed the highest antioxidant properties (López-Pedrouso, Borrajo, Amarowicz, Lorenzo, & Franco, 2021). In addition to the enzymatic procedure, an alkaline extraction process assisted by ultrasounds has been developed with success to generate protein isolates from the porcine liver (Zou et al., 2018).

Even though enzymes can be one of the main costs in the enzymatic process along with downstream separation stages, they are preferable due to their specificity, hence novel processes based on low-price edible offal and endogenous enzymes should be considered. In this sense, there are limited studies based on the obtention of peptides with antioxidant and antihypertensive activity from liver hydrolysates using endogenous enzymes. Therefore, this study aimed, for the first time, the characterization of peptides with antioxidant and antihypertensive activities in a protein fraction discarded in the ZnPP pigment production from porcine liver.

2. Materials and methods

2.1. Preparation and liver homogenization. Autohydrolysis conditions

Porcine livers were prepared similarly as previously described to obtain ZnPP (Wakamatsu et al., 2015).Various modifications were introduced to optimize ZnPP formation, and the final weight of liver homogenates consisted of 20% (w/w) ground liver dispersed in an aqueous solution containing acetic acid and ascorbic acid to obtain a final concentration of 0.25% (v/v) and 0.1% (w/w), respectively (Llauger et al., 1903).

To conduct the experiments, twelve porcine livers (pH 6.3 ± 0.20) were collected the same day of slaughter from local slaughterhouses (Costa Brava SA, Girona, Spain; Friselva Girona, Spain). They were immediately vacuum-packed and frozen at – 20 °C until needed. Within a month, porcine livers from each slaughterhouse were partially thawed (≤0 °C), cut into dices, and trimmed of veins and connective tissue. Subsequently, four livers in dices were ground in a meat cutter bowl at ≤ 4 °C until a fine pooled liver paste was obtained. The ground pooled liver paste was vacuum-packed in metalized bags with polyethylene terephthalate/polyethylene packaging material (oxygen permeability of 1.5 mL/m^2/24 h and low water vapor permeability of 1 g/m^2/24 h) and stored at – 20 °C until further use within two months. In each experiment, 1 kg of ground liver was homogenized with 4 L of the aqueous solution (adjusted to pH 4.2) using an UltraTurrax T25 model disperser (IKA Werke GmbH & Co. KG, Staufen, Germany) at 9500 × *g* for 1 min. Then, the pH of the liver homogenates was checked and, if necessary, adjusted to pH 4.8 ± 0.02 with NaOH or HCl 1 N and incubated for 24 h in a reactor immersed in a water bath at 45 °C and under a continuous flow of nitrogen to maintain anaerobiosis conditions. Incubation temperatures were monitored during the incubation using a data logger (Pico Vacq PT, TMI Orion). Under these conditions, microbial growth was maintained below 3 log CFU/mL and after centrifugation the formed ZnPP remained in the insoluble protein fraction. The supernatant contains soluble proteins and peptides mainly resulting from the activity of endogenous proteases (Canduri et al., 1998).

As shown in Fig. 1, three different supernatants were collected from the same pooled liver homogenates during their processing: 1) before incubation (control), 2) at the end of the incubation and readjusting the pH to 4.8 with HCl 1 N when required (acid hydrolysate), 3) at the end of the incubation and after adjusting the pH to 7.5 with tetrasodium pyrophosphate ($Na_4P_2O_7$) (basic hydrolysate). Through the process, aliquots of the supernatants were separated in each step after centrifugation at 5500 × *g* for 20 min at 4 °C. Afterwards, the supernatant fractions were placed on a tray and froze at −70 °C overnight. Then samples were lyophilized in a laboratory scale freeze-dryer (VirTis model Unitop HL, Gardiner, NY, USA), under standard freeze-drying conditions, which consisted of a primary drying for 24 h (at −10 °C and 100 mT) followed by 6 h of secondary drying (+15 °C and 100 mT). Lyophilized samples were vacuum packed in polyethylene bags and stored at −80 °C until physicochemical and peptide characterization. The experiment was fully triplicated.

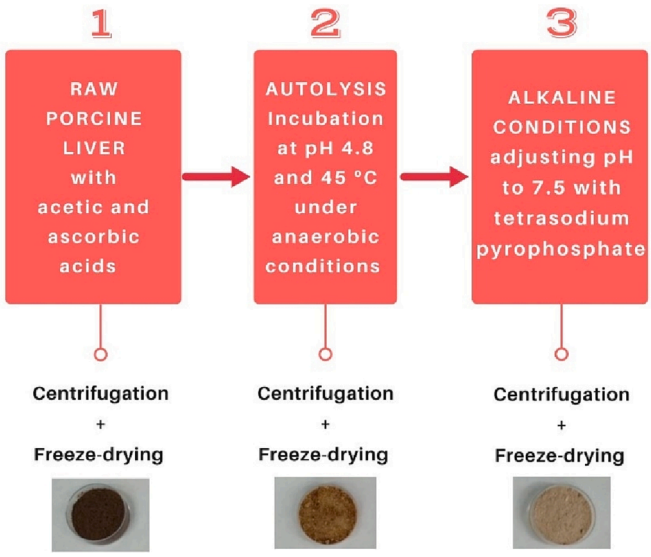


Fig. 1. Workflow of sample processing for Zn-protoporphyrin (ZnPP) extraction from porcine liver and their respective supernatant protein fractions for obtaining bioactive peptides.

2.2. Physicochemical characterization of porcine liver protein fractions. Chemical composition and colour parameters.

Standard methods were used to analyse the proximate composition of freeze-dried liver protein fractions. Moisture, ash, protein, and fat contents were determined by the procedures of the Association of Official Analytical Chemists. For moisture determination, 5 g of liver after the treatment was dried in the oven (Memmert UFP 600, Schwabach, Germany) at 105 °C until constant weight and the moisture percentage was calculated as sample weight loss. For total fat, 1 g of liver after the treatment was subjected to a liquid/solid extraction employing petroleum ether in an extractor machine (AnkomHCI Hydrolysis System XT10, Macedon, NY, USA) at 90 °C for 60 min, successively, the fat quantity was assessed by calculating the gravimetric difference. Kjeldahl nitrogen method was employed to determine protein content, where the total nitrogen content was multiplied by a conversion factor of 6.25. Finally, for the determination of ash content, a sample (3 g of liver hydrolysate and control supernatant) within a porcelain capsule was maintained in a muffle furnace (Carbolite® RWF 12–13, Hope Valley, UK) at 600 °C until constant weight. At this point, the ash percentage was obtained to calculate the sample weight loss. All measurements were determined at least in duplicate for each sample and the mean value was kept as a single measurement.

After grounding the freeze-dried liver protein fractions samples were placed on a petri dish and colour parameters were measured using a portable CM-600d colorimeter (Konica Minolta, Osaka, Japan) with the following conditions: pulsed xenon arc lamp, standard illumination D65, angle of 10° viewing angle geometry and aperture size of 8 mm. Data were acquired by the colour data software SpectraMagicTM Nx CM-S100w 2.90.0007 (Konica Minolta, Osaka, Japan) and expressed in the CIELAB space as lightness (L*), redness (a*) and yellowness (b*). Measurements were made in triplicate for each sample and the mean value was kept as a single measurement.

2.3. Free amino acids released during autohydrolysis

The extraction of free amino acids was carried out as follows: lyophilized liver hydrolysate (0.5 g) was homogenized with 25 mL of HCl 0.1 M for 8 min employing a disperser (Ika, T 25 digital Ultra-Turrax®, Staufen, Germany). Afterwards, it was cooled and centrifuged at 5000 × *g* for 20 min and 200 μL of the supernatant was mixed with 800 μL of acetonitrile to precipitate possible unhydrolyzed proteins. Finally, centrifugation at 5000 × *g* for 5 min was performed and an aliquot was frozen at −20 °C and stored until analysis. Derivatization and separation by HPLC were carried out according to (Franco & Lorenzo, 2014). Free amino acids were identified by retention time using an amino acid standard and expressed in mg/100 g of lyophilized supernatant.

2.4. Antioxidant in vitro activity of porcine liver hydrolysate

The lyophilized liver was dissolved in distilled water at a concentration of 0.2 g/mL. This solution was employed for all antioxidant tests.

2.4.1. DPPH radical scavenging activity

The DPPH assay was performed as follows: 0.1 mL of liver hydrolysate, previously dissolved was a mixture with 3.9 mL of DPPH solution (60 μM in methanol) and incubated for 10 min at 37 °C. Absorbance was measured at 515 nm (Shimadzu spectrophotometer, Kyoto, Japan). Trolox reactive was the standard used and results were expressed as μg Trolox equivalents (TE)/g sample.

2.4.2. ABTS radical scavenging activity ABTS radical

Cation Decolorization was performed as follows: ABTS was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and maintaining the mixture in the dark at room temperature for 12–16 h before its utilization. Before use, the ABTS stock solution was diluted with distilled water to achieve an absorbance of 0.70 at 734 nm, and was equilibrated at 30 °C. The solution (980 mL) was added to an aliquot of 20 mL of liver hydrolysate, previously dissolved. Absorbance was measured after 10 min in the darkness. Ascorbic acid was the standard used and results were expressed as mg ascorbic acid/100 g sample.

2.4.3. Ferric reducing antioxidant power assay (FRAP)

The FRAP test was carried out as follows: FRAP reagent was freshly prepared from mixing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ in the ratio of 10:1:1 (v:v:v). An aliquot of 900 mL of FRAP solution was mixed with 30 μL of proper liver hydrolysate, previously dissolved in 90 μL of distilled water. After incubation for 20 min at 37 °C in the darkness, the absorbance was determined at 593 nm. The $FeSO_4$ was the standard used and results were expressed as μmol Fe^{+2}/100 g sample.

2.4.4. Oxygen radical absorbance capacity assay (ORAC)

The ORAC test was performed as follows: The reaction was carried out in 75 mM phosphate buffer (pH 7.4), Twenty-five μL of liver hydrolysate, previously dissolved and 150 μL of 0.8 μM fluorescein (oxidizable substrate) was added into the internal wells of a black 96-well microplate (Biotek, Synergy H1, Winooski, VT, USA) and which was immediately incubated at 37 °C for 30 min in the fluorescence instrument. Afterwards, 25 μL of 2,2-azobis (2-methylpropionamidine) dihydrochloride 184 mM solution was added rapidly to each well to begin the reaction in the microplate. The fluorescence was measured with excitation and emission filters of 485 nm and 528 nm, respectively. The phosphate buffer and Trolox were used as the blank and standard reference, respectively. The results were estimated based on the differences of areas under the curves of fluorescence decay of the fluorescein between the blank and the sample and expressed as mg Trolox Equivalent (TE)/g sample.

2.5. Antihypertensive in vitro activity of pork liver hydrolysates

The determination of the antihypertensive activity of porcine liver hydrolysate was carried out using N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate following the protocol modified by (Estévez et al., 2012). Angiotensin I-converting enzyme (ACEI) inhibitory activity (I_{ACE}) of hydrolysates (prepared at a concentration of 5 g/L) was calculated as a relationship of the average slope of decrease in Absorbance over time and expressed in terms of the percentage of enzyme inhibition, as follows:

$$I_{ACE}(\%) = \left(1 - \frac{rA_h}{rA_c}\right) \times 100 \quad (1)$$

where I_{ACE} is the ACE-inhibitory capacity (%), rA_h is the slope of decrease in Absorbance at 340 nm in the presence of inhibitor (hydrolysate), and rA_c is the slope decrease in Absorbance at 340 nm in the absence of inhibitor (control). The protein-hydrolysate concentration that generates 50% of I_{ACE} (IC_{50}) was calculated by adjusting the dose–response relationship between I_{ACE} *vs.* hydrolysate concentration to a Weibull equation (Amado, González, Murado, & Vázquez, 2016):

$$I_{ACE} = K\left\{1 - \exp\left[-\ln 2\left(\frac{C}{IC_{50}}\right)^{a}\right]\right\} \quad (2)$$

where K is the maximum I_{ACE} (%), C is the protein-hydrolysate concentration (g/L), IC_{50} is the concentration for semi-maximum I_{ACE} (g/L), and a is the form parameter related to the maximum slope of the function (dimensionless).

2.6. Molecular weight of porcine liver protein fractions by gel permeation chromatography

The molecular weight distributions of liver fractions were obtained by Gel Permeation Chromatography (GPC) (Vázquez et al., 2020). The system (Agilent 1260 HPLC) consisted of a quaternary pump, injector, column oven, refractive index, diode array and dual-angle light scattering detectors. The samples were eluted with 0.15 M ammonium acetate / 0.2 M acetic acid (pH 4.5) at 1 mL/min after a 100 μL injection. Separation was achieved with a set of four Proteema columns (PSS, Germany): precolumn (5 μm, 8x50 mm), 30 Å (5 μm, 8x300 mm), 100 Å (5 μm, 8x300 mm), and 1000 Å (5 μm, 8x300 mm) kept at 30 °C. Detectors were calibrated with a polyethylene oxide standard of average weight molecular weight of 106 kDa (polydispersity index 1.05) from PSS (Mainz, Germany). Absolute molecular weights estimation was made with refractive index increments (dn/dc) of 0.185.

2.7. Peptidomic analysis by sequential window acquisition of all theoretical mass spectra (SWATH-MS)

2.7.1. Extraction and digestion of total peptides from porcine liver hydrolysates

Porcine liver hydrolysates (50 mg) before incubation (control), after incubation (pH = 4.8) and after adjusting the pH (pH = 7.5) were homogenized using RIPA buffer consisting of 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 finally adding antiproteases and anti-phosphatases (SigmaAldrich, St. Louis, MO, USA). Samples were centrifuged at 14.000 × *g* (4 °C, 20 min) to collect the supernatant. The quantification of peptides and proteins in samples was carried out with an RC-DC kit (Biorad Lab., Hercules, CA, USA). The peptide mixture was concentrated using 10% SDS-PAGE in a single band which was excised and cut into small pieces. The pieces of gels were washed with Milli-Q water and secondly 50 mmol/L ammonium bicarbonate in 50% methanol and dehydrated with acetonitrile in a vacuum centrifuge. Subsequently, peptides were reduced (10 mmol/L DTT in 50 mmol/L ammonium bicarbonate at 60 °C during 30 min) and alkylated (55 mmol/L iodoacetamide in 50 mmol/L ammonium bicarbonate in darkness at room temperature during 30 min). Finally, the digestion was carried out with 20 ng/μL trypsin (Promega, Madison, USA) in 20 mmol/L ammonium bicarbonate at 37 °C for 16 h. The resulting peptides were stored in 0.1% formic acid.

2.7.2. Generation of the reference spectral library

Liver hydrolysates of each treatment (Control, pH 4.8, pH 7.5) were mixed in equal amounts (4 μg for each sample) and the resulting mixture was subjected to shotgun data-depent acquisition (DDA) by micro-LC-MS/MS analysis in triplicate to build the spectral library. For liquid chromatography, a micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) using a YCM-Triart C18 column (150 μm × 0.3 mm, 12 nm, s-3 μm) (YMC CO, Japan) at a flow rate of 5 μL/min was used to separate the peptides. To prepare the mobile phase, solvent A (water, 0.1% formic acid) and solvent B (ACN, 0.1% formic acid) eluting from 5% to 95% B for 30 min, 5 min at 90% B and another 5 min at 5% B for column equilibration, resulting in a total time of 40 min. Regarding mass spectrometry, a quadrupole-TOF mass spectrometer of model Triple TOF 6600 (SCIEX, Framingham, MA, USA) was used in a 250 ms survey scan performed from 400 to 1250 *m/z* followed by MS/MS experiments from 100 to 1500 *m/z* (25 ms of acquisition time) for a total cycle time of 2.8 s. The fragment ion mass spectra of the resulting peptides were included in the spectral library for SWATH-MS peak extraction generated by ProteinPilote software from Uniprot Swiss-Prot database search.

2.7.3. Quantification of peptides and data analysis

This SWATH-MS analysis consists of data-independent acquisition (DIA) using the PeakView v.2.2. (SCIEX, Framingham, MA, USA) and the reference spectral library from the previous section. From three groups (control, pH = 4.5, pH = 7.5), a total of nine samples were analyzed with two technical replicates for each 9 samples. An amount of 4 μg for each sample was separated by LC as described previously. In the mass spectrometry, an acquisition time of 50 ms in a total cycle time of 6.3 s was carried out. A cycle consisted of the acquisition of 65 scans per SWATH window of variable width (1 *m/z* overlap) covering the 400 to 1250 *m/z* mass range. False discovery rate (FDR) and score were calculated for each peptide, and only peptides with an FDR of less than 1% were used for protein quantification. A Student's *t*-test was used to compare the groups based on peptides with a p-value above 0.05 and a fold change of 1.5 as the cut-off.

2.8. Statistical analysis

For the statistical analysis of the results (chemical composition, colour parameters, free amino acids, antioxidant and antyhypetensive capacity) an analysis of variance (ANOVA) one-way using the SPSS package (SPSS 23.0, Chicago, IL, USA) was performed. The least squares mean (LSM) were separated using Duncan's *t*-test. All statistical tests of LSM were performed for a significance level of $\alpha = 0.05$. Correlations between peptides and antioxidant and antyhypetensive assays were determined using Pearson's linear correlation coefficient with the above statistical software package mentioned.

Fitting procedures of antihypertensive data and parametric estimations were calculated by minimizing the sum of quadratic differences between the observed and model-predicted values, using the non-linear least-squares (GRG non-linear) method provided by the macro-'Solver' of the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's *t* test) and consistence of mathematical models (Fisher's F test) were evaluated by "SolverAid" macro (Levie's Excellaneous web-site: https://www.bowdoin.edu/~rdelevie/excellaneous).

The prediction of bioactive peptides was carried out by the BIOPEP-UWM database available at https://www.uwm.edu.pl/biochemia/index.php/pl/biopep (Minkiewicz, Iwaniak, & Darewicz, 2019). The occurrence of frequency (A) of bioactive peptides was calculated as A = a/N, where a = number of bioactive peptides and N = total number of amino acid residues in the protein chain.

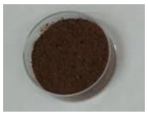
3. Results and discussion

3.1. Physicochemical characterization of porcine liver protein fractions

The activity of ferrochelatase in the liver is higher than in muscle, and thus the amount of Zn formed in liver mitochondria is greater (Wakamatsu et al., 2015). The formation of ZnPP was optimized to pH 4.8 but for their use as a food colouring ingredient, the pH of the liver homogenate can be adjusted to 7.5, after the incubation period. The pH for the formation of ZnPP is in agreement with previous findings also reported optimal ZnPP production in porcine liver at pH of 4.5 (Wakamatsu et al., 2015). As ZnPP mainly remains in the insoluble protein fraction after 24 h of incubation regardless of the pH (4.5 and 7.5), the red–purple pigment concentrate was collected after centrifugation and the resulting supernatants were recovered and analysed for their valorisation as side streams. As can be seen in Table 1, the luminosity (L*) of the freeze-dried supernatants raised from 33.12 in the raw liver to 76.37 after incubation and adjusting the pH to 7.5. The same effect was observed in the redness (a*) decreasing from 8.15 to 3.19 likely because of the Fe-porphyrin transformation into insoluble ZnPP. At pH 4.8, the colour properties of the freeze-dried protein hydrolysate showed intermediate values (61.51 and 8.54, for L* and a* values, respectively) suggesting that substantial changes (e.g., proteolysis) have occurred during the incubation.

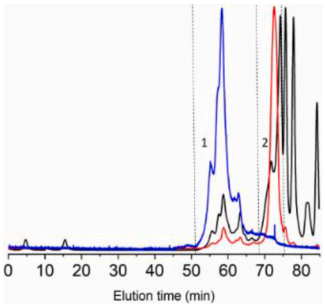
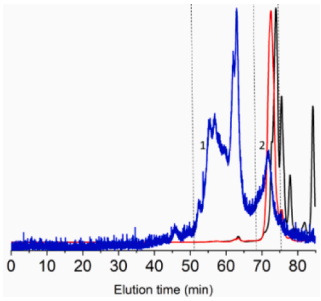
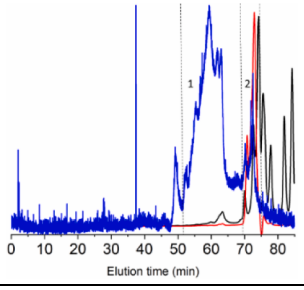
The chemical composition of porcine liver protein fraction (liver before autohydrolysis) had a high content of protein (63.07%) and a low

Table 1
Chemical composition (mean ± SD, n = 3) and colour parameters of freeze-dried control and porcine liver protein hydrolysates at two pH (4.8 and 7.5).

	Control (non-hydrolyzed liver protein fraction)	Liver protein hydrolysates pH 4.8	Liver protein hydrolysates pH 7.5	SEM	p-value
					
Colour parameters					
Luminosity (L*)	33.12 ± 0.87 [a]	61.51 ± 2.62[b]	76.37 ± 0.80[c]	4.36	<0.0001
Redness (a*)	8.15 ± 0.56[b]	8.54 ± 0.87[b]	3.19 ± 0.50 [a]	0.60	<0.0001
Yellowness (b*)	14.07 ± 0.63 [a]	30.39 ± 1.47[c]	21.19 ± 0.88[b]	1.63	<0.0001
Chemical composition					
Moisture (g/100 g)	13.31 ± 0.54	14.33 ± 0.51	14.19 ± 3.42	0.46	0.650
Fat (g/100 g)	0.95 ± 0.30 [a]	0.51 ± 0.28 [a]	5.95 ± 0.88[b]	0.61	<0.0001
Ash (g/100 g)	11.16 ± 0.66[b]	8.60 ± 1.15 [a]	38.54 ± 1.40[c]	3.29	<0.0001
Protein (g/100 g)	63.07 ± 0.41[b]	67.52 ± 1.22[c]	36.71 ± 2.13 [a]	3.31	<0.0001

SEM = standard error of mean; Duncan test α = 0.05; [a-c]Means in the same row with different letters differ significantly (P < 0.05).

Table 2
Mean molecular weight (Da) and associated standard error of porcine liver protein hydrolysates determined by GPC. Mw: weight average molecular weight; Mn: number average molecular weight; PDI: polydispersity index. Different superscript letters mean statistical significance within columns at α = 0.05 (n = 3). Peak area % based on refractive index detector data. At right, it is depicted the GPC eluograms of porcine liver hydrolysates. A: Control; B: hydrolysates at pH 4.8; C: hydrolysates at pH 7.5. Blue line: right angle light scattering detector; red line: refractive index detector; black line: DAD detector (232 nm). Vertical line: Number average molecular weight (Mn).

	Mn (Da)	Mw (Da)	PDI	Peak Area (%)	
Control (non-hydrolyzed liver protein fraction)					
Peak 1	75053 ± 3442[b]	96264 ± 2065[b]	1.283	16.5 ± 1.6[b]	
Peak 2	791 ± 52[B]	811 ± 52[B]	1.026	83.5 ± 1.6 [A]	
Liver protein hydrolysates at pH 4.8					
Peak 1	52191 ± 2481 [a]	78950 ± 1594 [a]	1.513	3.8 ± 0.1 [a]	
Peak 2	429 ± 22 [A]	480 ± 31 [A]	1.119	96.2 ± 0.1[B]	
Liver protein hydrolysates at pH 7.5					
Peak 1	54226 ± 3759 [a]	81417 ± 1613 [a]	1.501	2.8 ± 0.2 [a]	
Peak 2	389 ± 23 [A]	569 ± 95 [B]	1.463	97.2 ± 0.2[B]	

[a-b]Means in the same column for peak 1 with different letters differ significantly (P < 0.05). [A-B]Means in the same column for peak 2 with different letters differ significantly (P < 0.05). Mn = molecular weight in number; Mw = molecular weight.

Table 3
Free amino acid profile (mean ± SD, n = 3; mg/100 g liver) of freeze-dried control and porcine liver protein hydrolysates at two pH (4.8 and 7.5).

	Control (non-hydrolyzed liver protein fraction)	Liver protein hydrolysates pH 4.8	Liver protein hydrolysates pH 7.5	SEM	p-value
Essential					
His	303.90 ± 45.21 ^{a}	1215.36 ± 192.46^{c}	809.95 ± 104.40^{b}	94.84	<0.001
Ile	206.00 ± 30.02 ^{a}	1423.93 ± 279.94^{c}	816.91 ± 93.23^{b}	126.41	<0.001
Leu	588.43 ± 85.10 ^{a}	3312.33 ± 666.16^{c}	1812.33 ± 562.44^{b}	292.45	<0.001
Lys	414.13 ± 86.41 ^{a}	2501.98 ± 478.83^{c}	1109.30 ± 230.33^{b}	221.49	<0.001
Met	197.91 ± 28.91 ^{a}	886.78 ± 94.17^{c}	508.76 ± 54.29^{b}	69.81	<0.001
Phe	267.11 ± 28.00 ^{a}	1515.16 ± 307.90^{c}	989.06 ± 75.41^{b}	130.57	<0.001
Thr	225.23 ± 32.70 ^{a}	934.66 ± 142.76^{c}	630.86 ± 73.17^{b}	73.52	<0.001
Val	267.93 ± 40.31 ^{a}	1523.56 ± 221.93^{c}	918.86 ± 106.65^{b}	128.37	<0.001
Total EAA	2470.66 ± 364.68 ^{a}	13313.80 ± 2209.65^{c}	7596.06 ± 735.20^{b}	1115.62	<0.001
Non-Essential					
Arg*	93.16 ± 15.89 ^{a}	1096.86 ± 126.35^{c}	359.16 ± 128.27^{b}	105.53	<0.001
Ala	510.98 ± 81.95 ^{a}	1597.48 ± 210.71^{c}	883.81 ± 209.63^{b}	116.21	<0.001
Asp	242.71 ± 52.59 ^{a}	1055.66 ± 196.58^{c}	534.63 ± 151.77^{b}	87.77	<0.001
Cys	324.30 ± 53.14 ^{a}	1646.16 ± 208.47^{c}	933.41 ± 115.38^{b}	134.68	<0.001
Glu	1005.85 ± 189.97 ^{a}	1833.26 ± 350.40^{b}	1219.23 ± 200.59 ^{a}	102.42	<0.001
Gly	602.83 ± 87.88 ^{a}	871.21 ± 75.63^{b}	618.93 ± 66.77 ^{a}	34.36	<0.001
Pro	228.41 ± 44.88 ^{a}	755.63 ± 112.44^{b}	333.18 ± 126.57 ^{a}	59.63	<0.001
Ser	439.03 ± 73.94 ^{a}	1845.98 ± 305.52^{c}	989.23 ± 216.95^{b}	148.65	<0.001
Tau	220.96 ± 32.80^{c}	164.11 ± 22.06^{b}	92.56 ± 10.38^{c}	13.77	<0.001
Tyr	290.18 ± 36.51 ^{a}	1514.66 ± 157.39^{c}	993.93 ± 75.45^{b}	123.80	<0.001
Total NEAA	3958.45 ± 649.05 ^{a}	12381.06 ± 1043.94^{c}	6958.11 ± 641.38^{b}	863.76	<0.001

*Arginine is considered as semi-essential amino acids; SEM = standard error of mean; Duncan test α = 0.05; $^{a\text{-}c}$Means in the same row with different letters differ significantly (P < 0.05).

content of fat (0.95%). This protein percentage was lower than those reported for liver of cattle and chicken (70% and 75%, respectively), but higher than shown in the fish liver (45%) (Zou et al., 2021). For this reason, it is so desirable to prepare protein hydrolysates from porcine liver protein supernatant.

The chemical composition of liver hydrolysate at a pH of 4.8 showed a slight increase in protein (67.52%) and a decrease in fat percentage (0.51%). This increase can be attributed to the hydrolysis caused by endogenous liver enzymes (autolysis). It has been demonstrated that this autolysis produces the solubilization of the protein fraction of porcine and bovine by-products to the same extent as Alcalase and papain in a dosage of 0.02% (2 h) (Lapeña, Vuoristo, Kosa, Horn, & Eijsink, 2018). Therefore, these findings suggest that the use of endogenous enzymes in the porcine liver is a low-cost process and easy to handle achieving suitable hydrolysis.

Subsequently, tetrasodium pyrophosphate was added until a pH of 7.5 to the liver homogenates and then centrifuged to obtain the hydrolysed/autolysed supernatant at this pH. In this case, the chemical composition of hydrolysates changed significantly (P < 0.05) in terms of the content of protein (36.71%) and fat (5.95%). The addition of tetrasodium pyrophosphate seems to cause significant protein precipitation because of the pH and salinity change. In this regard, it has been demonstrated that phosphates function as buffers, precipitants and ion exchange in the meat industry. Indeed, in the case of muscle tissue, the addition of pyrophosphate produces the dissociation of actin and myosin increasing the water-holding capacity and modifying other textural characteristics. For instance, tetrasodium pyrophosphate is added to dairy products to flocculate the protein (Lampila, 2013). Accordingly, with other authors, the extraction of ferritin from the chicken liver was obtained in the supernatant after centrifugation at pH 4.8, however, the liver protein was precipitated in phosphate buffer solution at pH 7.2 with saturated ammonium sulfate (Zou et al., 2021).

3.2. Effectiveness of autolysis on liver hydrolysates

3.2.1. Molecular weight distribution of porcine liver protein fractions

Livers from different animal sources have great potential as a source of protein and bioactive peptides for pharmaceutical or food industry applications. In this regard, the release of peptides with an antioxidant activity using alcalase, bromelain, flavourzyme or papain from the porcine liver was already assayed (López-Pedrouso et al., 2021). However, the effects of endogenous enzymes to obtain liver hydrolysates were not already assessed. The analysis of the molecular weight performed by GPC was very similar for both hydrolysates obtained. Two main size profiles were defined in those cases: i) A low proportion (2.8–3.8%) of a high protein fraction (>79 kDa quantified as Mw), and ii) a great proportion (>96%) of peptides lower than 0.6 kDa (Table 2). The presence of those high molecular weight protein fractions may be due to the aggregation of a small amount of particulate protein or to the existence of a limited non-hydrolyzable protein. The values of the average molecular weight number (Mn) for the hydrolysates were also determined, ranging in the interval of 390–430 Da (Table 2). For control samples, these values were higher: 16.5% of a protein fraction of almost 100 kDa and 83.5% of average-size peptides of 811 KDa. This indicated that autolysis had a major impact on liver proteins and the molecular weight was considerably reduced by endogenous enzymes after incubation. However, the pH adjusting to 7.5 had a modest effect on the molecular weight composition of this supernatant when compared to that at pH 4.8.

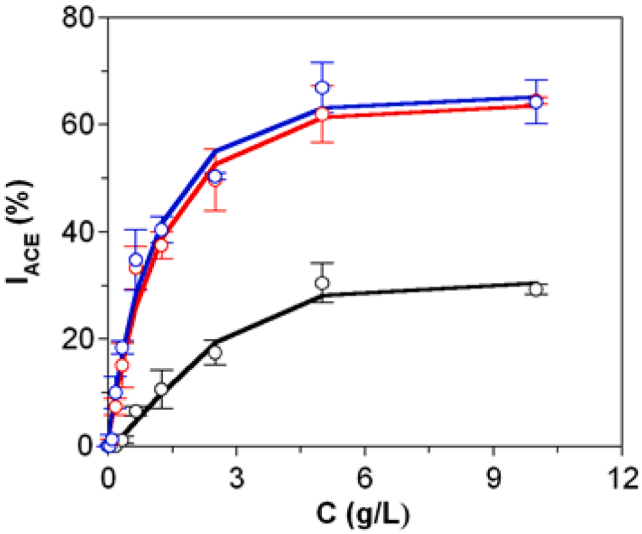
The use of endogenous enzymes has been particularly studied in the production of fish silage using visceral digestive enzymes and lowering the pH to activate the proteases (Aspevik et al., 2017), but fewer studies have been conducted in meat by-products. Hydrolysates from meat by-products, employing endogenous and exogenous (alcalase and papain) enzymes were compared, resulting in the latter ones being more effective in generating hydrolysates (Lapeña et al., 2018). Therefore, the use of endogenous enzymes has the advantage of an economic and safe process, but optimization is required to improve the specificity and reproducibility.

3.2.2. Free amino acid profile of porcine liver protein fraction

In food processing wastes, amino acids from protein hydrolysates are of special interest in the field of biotechnological and pharmaceutical research. The hydrolysis of liver protein fractions largely resulted in free amino acids, as shown in Table 3. A total of 25.67 g/100 g of amino acids were produced after incubation at pH 4.8. It is well-known that several factors, including temperature, time, and pH affect the level of hydrolysis. Specifically, after adjusting the pH to 7.5, the total amount of

Table 4
Antioxidant and antihypertensive activity of freeze-dried control and porcine liver protein hydrolysates at two pH (4.8 and 7.5) (Values expressed in mean ± SD, n = 3).

	Control liver protein fraction	Liver protein hydrolysates pH 4.8	Liver protein hydrolysates pH 7.5	SEM	p-value
Antioxidant tests					
DPPH (μg trolox/g)	45170.18 ± 3046.60 ^{c}	33001.39 ± 3639.06 ^{b}	15701.88 ± 1221.31 ^{a}	4352.14	<0.001
ABTS (mg AA acid/100 g)	4199.67 ± 213.04 ^{a}	4085.66 ± 238.18 ^{a}	6328.51 ± 39.72 ^{b}	368.60	<0.001
FRAP (μmol Fe^{+2}/100 g)	36711.35 ± 892.89 ^{c}	23377.78 ± 524.42 ^{b}	13638.31 ± 532.66 ^{a}	3349.37	<0.001
ORAC (mg Trolox/g)	31.33 ± 8.49 ^{a}	99.40 ± 30.62 ^{b}	64.27 ± 7.08 ^{ab}	11.22	0.013
DPPH IC_{50} (mg/mL)	4.73 ± 0.45 ^{a}	5.42 ± 0.93 ^{a}	13.57 ± 0.13 ^{b}	1.43	<0.001
Antihypertensive test-ACE					
ACE inhibition (%)	30.48 ± 1.41 ^{a}	63.93 ± 1.64 ^{b}	65.42 ± 1.30 ^{b}	5.71	<0.001
IC_{50} of ACE inhibition (g/L)	1.88 ± 0.15 ^{b}	0.90 ± 0.06 ^{a}	0.81 ± 0.03 ^{a}	0.17	<0.001



SEM = standard error of mean; Aa = ascorbic acid; TE = Trolox equivalent; Duncan test α = 0.05; $^{a\text{-}c}$Means in the same row with different letters differ significantly (P < 0.05).
Dose-response curves showed experimental data of ACE inhibition at different concentrations of porcine liver hydrolysates (points) and data fitted to Weibull Eq. (2) (continuous lines). Control (black) and liver hydrolysates at pH 4.8 (red) and pH 7.5 (blue). Error bars are, in all cases, the confidence intervals for 3 samples from individual batches and α = 0.05.

amino acids decreased to 14.55 g/100 g which seems related to the precipitation or aggregation effect. Regarding the free amino acid profile, eight essential amino acids were detected and quantified resulting in all of them being significantly more abundant in liver hydrolysates obtained at pH 4.8.

Proteins are broken down into amino acids and small peptides (di- and tri-peptides) during food processing and gastric digestion for protein synthesis, to carry out most of life's principal functions. Proteins are formed by 20 different amino acids divided into nine essential amino acids which cannot be synthesized *de novo* (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and must be provided from foods, and the rest are non-essential amino acids. Raw liver showed a high content of essential amino acids in comparison with other pork by-products such as the pancreas, uterus, and small and large intestines (Seong et al., 2014). Although only a small amount (14.55 to 25.67%) became free amino acids from protein, they strongly contribute to sensory quality. The amino acids can develop tastes from bitter (Trp, Ile, Leu, Cys, Lys, Tyr, Phe, Arg, Val, Met, His) to sweet (Gly, Ala, Thr, Gln, Pro, Asn and Ser) and umami (Glu, Asp) (Zamuz et al., 2019). The umami taste providing a savoury/brothy taste has a key role in the sensorial features of pork products, and consequently, the behaviour of umami compounds is studied during the food process (Rotola-Pukkila, Pihlajaviita, Kaimainen, & Hopia, 2015). All free amino acid concentrations including Asp and Glu were higher in liver hydrolysates at pH 4.8 suggesting that these hydrolysates would have more sensorial variations.

3.3. Bioactivity of porcine liver protein fraction

3.3.1. Antioxidant capacity

The antioxidant activity of liver hydrolysate was assessed by DPPH, ABTS, FRAP and ORAC as well as DPPH IC_{50} (Table 4) assays. All of these antioxidant tests were previously used to assess in vitro antioxidant potential of the porcine liver (Borrajo, López-Pedrouso, Franco, Pateiro, & Lorenzo, 2020). The in *vitro* antioxidant methods evaluate different antioxidant activities, depending on the transference of electrons or hydrogens, hence comparisons are not necessarily easy. Regarding DPPH and FRAP, the control (non-hydrolyzed liver samples) showed greater values in comparison with the liver hydrolysates obtained at pH 4.8. Even this antioxidant capacity decreased drastically in the case of the liver hydrolysates at pH 7.5. In the case of DPPH and FRAP, the antioxidant activity is measured as single electron transfer-based assays. Raw liver is a source rich in vitamins, endogenous enzymes and other molecules that could exhibit antioxidant capacity,

Table 5
Correlations between antioxidant capacity and peptide quantification obtained from freeze dried protein fractions of porcine liver non-hydrolysed and hydrolysates at pH 4.8 and 7.5.

			Correlations with antioxidant and antihypertensive activities				
Peptide	**Protein name**	**Uniprot**	**DPPH**	**ABTS**	**FRAP**	**ORAC**	**ACE**
FSSETWQNLGTLHR	AB hydrolase-1 domain-containing protein	A0A4X1SEA1	0.676*	-0.940**	0.614	0.053	-0.305
SKFDNLYGC[CAM]R	Adenosylhomocysteinase	D0G0C3	0.777*	-0.979**	0.687*	0.205	-0.352
VAFTGSTQVGK	Aldehyde dehydrogenase 1 family member A1	I3LRS5	0.820**	-0.533	0.923**	-0.757*	-0.983**
ILDLIESGK	Aldehyde dehydrogenase 1 family member A1	I3LRS5	0.808**	-0.48	0.916**	-0.744*	-0.997**
YC[CAM]AGWADK	Aldehyde dehydrogenase 1 family member A1	I3LRS5	0.842**	-0.534	0.939**	-0.711*	-0.994**
WC[CAM]TISNQEANK	Beta-1 metal-binding globulin	A0A4X1TVN6	0.924**	-0.913**	0.899**	-0.189	-0.703*
YNSFATALK	Carbonic anhydrase	A0A4X1UCS1	0.870**	-0.971**	0.770*	0.075	-0.478
IIPPGSGIIHQVNLEYLAR	Citrate hydro-lyase	I3LJW4	0.909**	-0.880**	0.832**	-0.033	-0.606
VLLEAAIR	Citrate hydro-lyase	I3LJW4	0.842**	-0.776*	0.899**	-0.417	-0.793*
LHGSGDQEAWQR	Cysteinylglycine-S-conjugate dipeptidase	A0A480XC77	0.824**	-0.868**	0.828**	-0.246	-0.629
FLQEIYNSNNQK	Fibrinogen gamma chain	I3LJW2	0.288	-0.693*	0.114	0.698*	0.28
ETTIQGLDGLSER	Fructose-bisphosphate aldolase	A0A4X1VHB8	0.797*	-0.509	0.906**	-0.732*	-0.973**

(*continued on next page*)

supporting the findings of the present study. Among others, ORAC assay as a hydrogen atom transfer-based assay is the preferred choice for complex food matrices and biological systems in general due to multiple interactions and complex reaction kinetics, even though its antioxidant activity is only assessed against peroxyl radicals (Tan & Lim, 2015; Schaich, Tian, & Xie, 2015).

Regarding the biological activity of protein hydrolysates, it has been demonstrated that the antioxidant capacity of porcine liver hydrolysates increased with the level of proteolysis as demonstrated by ORAC assay. The highest antioxidant capacity is produced by liver hydrolysates at pH 4.8 (99.40 mg Trolox/g). In the next step, the precipitation of protein fraction is produced as suggested in Table 3. Liver hydrolysates at pH 4.8 were enriched in amino acids in comparison to other hydrolysates. In general, proteins or peptides of liver hydrolysates are differentially precipitated by their distinctive isoelectric points in the pH gradient. These efficient extraction methods are also safe to use as edible proteins (Zou et al., 2021). Overall, the dissolved proteins and peptides produced a great impact on the antioxidant capacity of liver protein fraction according to our results.

3.3.2. *ACE-I inhibitory activity*

Angiotensin I-converting enzyme (ACEI) is a zinc protease which needs zinc and chloride for its activity playing an important role in regulating blood pressure. ACEI converts an inactive form of a decapeptide (angiotensin I) into an octapeptide (angiotensin II) which is a potent vasoconstrictor and inactivates the catalytic function of bradykinin. Although ACEI activity was widely observed in all the samples, liver hydrolysates at pH 4.8 and 7.5 were higher and similar as displayed in Table 4. The control sample, a non-hydrolyzed liver, mainly composed of fibrils of connective tissue, showed lower activity due in part to the difficulty of its dissolution in water, even though soft thermal treatment and vigorous agitation, even ultrasound processing was tried. In this case, the dose–response curves for control showed very low maximum ACEI values (K = 30.4 ± 3.4%) with an IC_{50} = 1.88 ± 0.43 g/L. The hydrolysates led to the highest results: larger K data (63.7 ± 7.8% and 65.3 ± 8.4%, at pH 4.8 and 7.5, respectively) and higher activities (0.886 ± 0.310 g/L and 0.804 ± 0.303 g/L, at pH 4.8 and 7.5, respectively). However, the differences between both hydrolysates were not statistically significant ($p > 0.05$).

ACEI peptides could be obtained from vegetal sources, such as soybean (M. Li, Xia, Zhang, & Li, 2018) or mushrooms (Zhang, Roytrakul, & Sutheerawattananonda, 2017) by enzymatic hydrolysis, but also it was demonstrated that marine sources such as macroalga or jellyfish produce antihypertensive peptides (Etemadian et al., 2021). Other animal proteins from cooked beef, pork, chicken and turkey have been employed to isolate peptides with ACE-inhibitory activity after in vitro gastrointestinal digestion (Martini, Conte, & Tagliazucchi, 2019). Thus, these peptides could be used and consumed as nutraceuticals due to their antioxidant and antihypertensive peptides.

3.4. *Potential antioxidant and ACE-inhibitory peptides based on peptidomic approach*

The peptidomic identification and quantification were carried out using SWATH-MS technology. In this sense, a total of 1716 peptides were detected and quantified among the nine samples (non-hydrolyzed liver and hydrolysates at pH 4.8 and 7.5). Apart from these nine biological samples, two technical replicates were carried out in each sample. A correlation analysis between quantification and antioxidant and antihypertensive values is displayed (Table 5). Most of the peptides, in particular 36 peptides, were correlated using Pearson's correlation analysis with antioxidant capacity, and 20 of the peptides were associated with both antioxidant and antihypertensive activity, respectively. Consequently, this suggests that these are potential bioactive peptides and further validation should be required.

It must be emphasized that five peptides from two isoforms of aldehyde dehydrogenase (I3LRS5, ALDH1A1) and four peptides from fructose bisphosphate aldolase (A0A4X1VHB8, ALDOB) were correlated with both activities. It is clear to understand the high biological activity of peptides from ALDH1A1 considering its function. Aldehydes derived

Table 5 (*continued*)

ALQASALK	Fructose-bisphosphate aldolase	A0A4X1U5U2	0.907**	-0.813**	0.815**	-0.091	-0.632
ALANC[CAM]QAAK	Fructose-bisphosphate aldolase	A0A4X1VHB8	0.809**	-0.469	0.891**	-0.685*	-0.959**
GILAADESTGSIAKR	Fructose-bisphosphate aldolase	A0A4X1U5U2	0.317	-0.667*	0.116	0.741*	0.274
VSEEIEDIIK	Gamma-glutamylcyclotransferase	A0A5G2R9Y8	0.249	-0.667*	0.106	0.668*	0.277
LLDTAGANLK	Glyoxylate and hydroxypyruvate reductase	F1ST73	0.923**	-0.715*	0.978**	-0.514	-0.932**
VGDKVLLPEYGGTK	10 kDa heat shock protein	Q6WSP6	0.29	-0.672*	0.1	0.679*	0.288
NVAEVDLSTSVLGQR	Hydroxyacid oxidase 1	I3LVF1	0.867**	-0.59	0.931**	-0.607	-0.939**
AAWAFSR	Lactoylglutathione lyase	I3LDM7	0.818**	-0.505	0.921**	-0.727*	-0.990**
DGLILTSR	Maillard deglycase	Q0R678	0.855**	-0.584	0.948**	-0.688*	-0.983**
AGAGSATLSMAYAGAR	Malate dehydrogenase	A0A5G2RGL7	0.797*	-0.957**	0.717*	0.133	-0.412
VIVVGNPANTNC[CAM]LTASK	Malate dehydrogenase	A0A5G2QPX2	0.934**	-0.776*	0.980**	-0.551	-0.912**
YGLAAAVFTK	Mitochondrial aldehyde dehydrogenase 2	B2ZF47	0.856**	-0.579	0.940**	-0.697*	-0.973**
TLPIDGDYFSYTR	Mitochondrial aldehyde dehydrogenase 2	B2ZF47	0.850**	-0.542	0.901**	-0.605	-0.925**
SPWWLR	N-acyl-L-amino-acid amidohydrolase	A0A4X1SEK8	-0.697*	0.784*	-0.651	-0.058	0.458
VALNMLAR	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	A0A5G2QMY5	-0.895**	0.972**	-0.795*	-0.094	0.499
VLSSMTDAVLAR	Ornithine carbamoyltransferase	F1RXQ9	0.883**	-0.949**	0.852**	-0.101	-0.616
VLGTAGTEEGQNIVLQNGAHE VFNHR	Quinone oxidoreductase	Q19QT8	0.946**	-0.803**	0.978**	-0.477	-0.890**
INYGGEIPK	SEC14-like protein 3 isoform 1	A0A286ZQ63	0.930**	-0.695*	0.989**	-0.588	-0.964**
VLDASWYSPGTR	Sulfurtransferase	F1SKL2	0.766*	-0.494	0.885**	-0.727*	-0.958**
ETLPFPIIDDK	Thioredoxin domain-containing protein	A0A4X1VDC5	0.738*	-0.763*	0.730*	-0.32	-0.57
AYQDQKPGTSGLR	Uncharacterized protein	A0A4X1TAN5	0.835**	-0.526	0.932**	-0.745*	-0.995**
VLTPTQVK	Uncharacterized protein	F1RKG8	0.865**	-0.608	0.960**	-0.696*	-0.984**
ELLSGPNR	Uncharacterized protein	A0A4X1TAN5	0.786*	-0.790*	0.746*	-0.285	-0.584
C[CAM]DEPILSNR	Uncharacterized protein	F1RKG8	0.923**	-0.831**	0.967**	-0.502	-0.862**

Only showed those correlations significantly (**P < 0.01 and *P < 0.05) and a correlation coefficient higher than 0.5 with at least two antioxidant and antihypertensive tests. Those peptides in red significantly have correlations with both activities.

from the external environment and the internal metabolism of biomolecules are metabolized by ALDHs. ALDH1A1 has the main function of catalysing the oxidation of exogenous and endogenous aldehydes to carboxylic acids like other ALDHs. Reactive oxygen species (ROS) lead to elevated oxidative stress and cell damage provoking their cytotoxicity and carcinogenesis. The content of ALDH1A1 was related to numerous diseases such as alcoholic liver disease, Sjögren-Larsson syndrome (SLS), type II hyperprolinemia, hyperammonemia, Parkinson's disease, and cancers (Li, Yang, Liang, Jiang, & Ma, 2021; Singh et al., 2013).

The liver is an organ with a metabolic function controlled by insulin and other hormones. Important physiological process such as nutrient metabolism of carbohydrate, fat and protein occurs in the liver therefore its proteome includes proteins related to metabolic enzymes affected by growth, feed efficiency and other performance traits (Bovo, Luca, Galimberti, Dall'Olio, & Fontanesi, 2018). In the cytoplasm, glycolysis is whereby the glucose is converted into pyruvate, and subsequently, oxidized to generate ATP in the TCA cycle. In the case of ALDO, the main function is to catalyse the breakdown of the fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in a reversible reaction. This means that ALDO has a key role in glycolysis and gluconeogenesis pathways. Three isoforms of aldolase are differentially expressed in tissues, and ALDOB is more expressed in the liver and kidney. Even, it has been validated as a marker of human diseases and cancers (Chang, Yang, Tien, Yang, & Hsiao, 2018). Furthermore, two peptides from malate dehydrogenase must be highlighted. The first peptide AGAGSATLSMAYAGAR was only correlated with antioxidant activity (particularly ABTS), but other VIVVGNPANTNC[CAM]LTASK appears to be antioxidant and antihypertensive activities. Malate dehydrogenase is a ubiquitous enzyme which catalyses the oxidation of malate to oxaloacetate as part of metabolic pathways utilizing the NAD/NADH coenzyme system (Minárik, Tomaásková, Kollárová, & Antalík, 2002).

After this first selection (Table 5), liver-derived peptides were considered for in silico prediction of bioactive peptides assessed by the BIOPEP-UWM database (Minkiewicz et al., 2019). The parameter used to indicate the bioactivity is parameter A defined as the frequency of occurrence of bioactive fragments in a protein sequence and peptides that were considered in case A was above 0.5. This database indicated that the peptides with a higher probability of ACE-inhibitory activity were YGLAAAVFTK (mitochondrial aldehyde dehydrogenase),

INYGGEIPK (SEC14-like protein 3), GILAADESTGSIAKR (fructose-bisphosphate aldolase), VAFTGSTQVGK (aldehyde dehydrogenase), AGAGSATLSMAYAGAR (malate dehydrogenase), VGDKVLLPEYGGTK (10 kDa heat shock protein), AYQDQKPGTSGLR (uncharacterized protein), IIPPGSGIIHQVNLEYLAR (citrate hydro-lyase) and AAWAFSR (lactoylglutathione lyase). In the case of antioxidant activity, there are no available calculations by BIOPED for all the peptides in Table 5. At present, the use of computational tools does not provide definitive results of bioactivity, but they help us at the stage of search and selection (Coscueta, Batista, Gomes, da Silva, & Pintado, 2022).

In short, these facts suggest that peptides with strong potential for bioactivity from both points of view (correlations and bioinformatic analysis) such as AYQDQKPGTSGLR (uncharacterized protein), YGLAAAVFTK (mitochondrial aldehyde dehydrogenase), AAWAFSR (lactoylglutathione lyase), VAFTGSTQVGK (aldehyde dehydrogenase) and INYGGEIPK (SEC14-like protein 3) should be preferred candidates for further *in vitro* and *in vivo* validation tests.

4. Conclusions

To our knowledge, this work is the first study dealing with the production of bioactive hydrolysates, presenting antioxidant and antihypertensive properties, by autolysis of porcine liver protein fraction generated during the obtaining process of a natural pigment (zinc protoporphyrin IX). The hydrolysate prepared at pH 4.8 showed the highest content of protein and essential amino acids, the lowest values of fat and ash and the largest bioactivities. The most likely peptide carriers of biological activity were predicted and identified through peptidomic analysis. The present approach has been demonstrated to be an effective and sustainable way to fully valorise porcine liver by means of obtaining colouring ingredients, meat protein sources and bioactive peptides generating zero residues in a context of circular bio-economy.

Funding

The authors acknowledge the financial support from the "Ministerio de Economía y Competitividad (MINECO)" and "Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)" in Spain (Projects RTA2017-00024-C04-01, RTA2017-00024-C04-02, and RTA2017-00024-C04-04).

CRediT authorship contribution statement

María López-Pedrouso: Conceptualization, Formal analysis, Software, Writing - original draft. **José M. Lorenzo:** Conceptualization, Funding acquisition, Writing - review & editing. **Ricard Bou:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **José Antonio Vázquez:** Formal analysis, Methodology, Writing - review & editing. **Jesús Valcarcel:** Formal analysis, Methodology. **Mònica Toldrà:** Conceptualization, Writing - review & editing. **Daniel Franco:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgements

Daniel Franco and José M. Lorenzo belong to the competitive reference research group, FunMeat (Axencia Galega de Innovación, GAININ607A2019/01) and Daniel Franco and Jose M. Lorenzo are members of the HealthyMeat network, funded by CYTED (Ref.119RT0568). José Antonio Vázquez and Jesus Valcarcel want to thank the financial support of Xunta de Galicia (Grupos de Potencial Crecimiento, IN607B 2021/11).

References

Amado, I. R., González, M. P., Murado, M. A., & Vázquez, J. A. (2016). Shrimp wastewater as a source of astaxanthin and bioactive peptides. *Journal of Chemical Technology and Biotechnology, 91*(3), 793–805. https://doi.org/10.1002/jctb.4647

Aspevik, T., Oterhals, Å., Rønning, S. B., Altintzoglou, T., Wubshet, S. G., Gildberg, A., … Lindberg, D. (2017). Valorization of proteins from co- and by-products from the fish and meat industry. *Topics in Current Chemistry, 375*(3), 123–150. https://doi.org/10.1007/s41061-017-0143-6

Borrajo, P., López-Pedrouso, M., Franco, D., Pateiro, M., & Lorenzo, J. M. (2020). Antioxidant and antimicrobial activity of porcine liver hydrolysates using flavourzyme. *Applied Sciences (Switzerland), 10*(11), 1–14. https://doi.org/10.3390/app10113950

Bou, R., Llauger, M., Arnau, J., & Fulladosa, E. (2018). Zinc-protoporphyrin content in commercial Parma hams is affected by proteolysis index and marbling. *Meat Science, 139*(January), 192–200. https://doi.org/10.1016/j.meatsci.2018.01.027

Bou, R., Llauger, M., Arnau, J., Olmos, A., & Fulladosa, E. (2022). Formation of Zn-protoporphyrin during the elaboration process of non-nitrified serrano dry-cured hams and its relationship with lipolysis. *Food Chemistry, 374*, Article 131730. https://doi.org/10.1016/j.foodchem.2021.131730

Bovo, S., Luca, A. D., Galimberti, G., Dall'Olio, S., & Fontanesi, L. (2018). A comparative analysis of label-free liquid chromatography-mass spectrometry liver proteomic profiles highlights metabolic differences between pig breeds. *PLoS ONE, 13*(9), 1–19. https://doi.org/10.1371/journal.pone.0199649

Canduri, F., Ward, R. J., de Azevedo Jr, W. F., Gomes, R. A., & Arni, R. K. (1998). Purification and partial characterization of cathepsin D from porcine (Sus scrofa) liver using affinity chromatography. *IUBMB Life, 45*(4), 797–803. https://doi.org/10.1080/15216549800203222

Chang, Y. C., Yang, Y. C., Tien, C. P., Yang, C. J., & Hsiao, M. (2018). Roles of aldolase family genes in human cancers and diseases. *Trends in Endocrinology and Metabolism, 29*(8), 549–559. https://doi.org/10.1016/j.tem.2018.05.003

Coscueta, E. R., Batista, P., Gomes, J. E. G., da Silva, R., & Pintado, M. M. (2022). Screening of novel bioactive peptides from goat casein: In silico to in vitro validation. *International Journal of Molecular Sciences*, *23*(5). https://doi.org/10.3390/ijms23052439.

De Maere, H., Chollet, S., De Brabanter, J., Michiels, C., Paelinck, H., & Fraeye, I. (2018). Influence of meat source, pH and production time on zinc protoporphyrin IX formation as natural colouring agent in nitrite-free dry fermented sausages. *Meat Science, 135*(September), 46–53. https://doi.org/10.1016/j.meatsci.2017.08.024

Estévez, N., Fuciños, P., Sobrosa, A. C., Pastrana, L., Pérez, N., & Luisa Rúa, M. (2012). Modeling the angiotensin-converting enzyme inhibitory activity of peptide mixtures obtained from cheese whey hydrolysates using concentration-response curves. *Biotechnology Progress, 28*(5), 1197–1206. https://doi.org/10.1002/btpr.1587

Etemadian, Y., Ghaemi, V., Shaviklo, A. R., Pourashouri, P., Sadeghi Mahoonak, A. R., & Rafipour, F. (2021). Development of animal/ plant-based protein hydrolysate and its application in food, feed and nutraceutical industries: State of the art. *Journal of Cleaner Production, 278*. https://doi.org/10.1016/j.jclepro.2020.123219

Franco, D., & Lorenzo, J. M. (2014). Effect of muscle and intensity of finishing diet on meat quality of foals slaughtered at 15 months. *Meat Science, 96*(1), 327–334. https://doi.org/10.1016/j.meatsci.2013.07.018

Fraqueza, M. J., Laranjo, M., Elias, M., & Patarata, L. (2021). Microbiological hazards associated with salt and nitrite reduction in cured meat products: Control strategies based on antimicrobial effect of natural ingredients and protective microbiota. *Current Opinion in Food Science, 38*, 32–39. https://doi.org/10.1016/j.cofs.2020.10.027

Lampila, L. E. (2013). Applications and functions of food-grade phosphates. *Annals of the New York Academy of Sciences, 1301*(1), 37–44. https://doi.org/10.1111/nyas.12230

Lapeña, D., Vuoristo, K. S., Kosa, G., Horn, S. J., & Eijsink, V. G. H. (2018). Comparative assessment of enzymatic hydrolysis for valorization of different protein-rich industrial byproducts. *Journal of Agricultural and Food Chemistry, 66*(37), 9738–9749. https://doi.org/10.1021/acs.jafc.8b02444

Li, B., Yang, K., Liang, D., Jiang, C., & Ma, Z. (2021). Discovery and development of selective aldehyde dehydrogenase 1A1 (ALDH1A1) inhibitors. *European Journal of Medicinal Chemistry, 209*, Article 112940. https://doi.org/10.1016/j.ejmech.2020.112940

Li, M., Xia, S., Zhang, Y., & Li, X. (2018). Optimization of ACE inhibitory peptides from black soybean by microwave-assisted enzymatic method and study on its stability. *LWT, 98*(August), 358–365. https://doi.org/10.1016/j.lwt.2018.08.045

Llauger, M., Arnau, J., Albano-Gaglio, M., Bover-Cid, S., Martín, B., & Bou, R. (1903). Utilization of porcine livers through the formation of Zn-protoporphyrin pigment optimized by a response surface methodology. *Foods, 12*(9). https://doi.org/10.3390/foods12091903

López-Pedrouso, M., Borrajo, P., Amarowicz, R., Lorenzo, J. M., & Franco, D. (2021). Peptidomic analysis of antioxidant peptides from porcine liver hydrolysates using SWATH-MS. *Journal of Proteomics, 232*(November). https://doi.org/10.1016/j.jprot.2020.104037

Lynch, S. A., Mullen, A. M., O'Neill, E., Drummond, L., & Álvarez, C. (2018). Opportunities and perspectives for utilisation of co-products in the meat industry. *Meat Science, 144*(June), 62–73. https://doi.org/10.1016/j.meatsci.2018.06.019

Martini, S., Conte, A., & Tagliazucchi, D. (2019). Comparative peptidomic profile and bioactivities of cooked beef, pork, chicken and turkey meat after in vitro gastro-intestinal digestion. *Journal of Proteomics*. https://doi.org/10.1016/j.jprot.2019.103500

Minárik, P., Tomaásková, N., Kollárová, M., & Antalík, M. (2002). Malate Dehydrogenases - Structure and function. *General Physiology and Biophysics, 21*(3), 257–265.

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences, 20*(23). https://doi.org/10.3390/ijms20235978

Ockerman, H. W., Basu, L., & Toldrá, F. (2017). Edible By-products. *Lawrie's Meat Science: Eighth Edition, 679–696*. https://doi.org/10.1016/B978-0-08-100694-8.00022-4

OECD/FAO. (2021). 6 Meat. *Agricultural Outlook 2021-2030*, 163–177.

Rotola-Pukkila, M. K., Pihlajaviita, S. T., Kaimainen, M. T., & Hopia, A. I. (2015). Concentration of umami compounds in pork meat and cooking juice with different cooking times and temperatures. *Journal of Food Science, 80*(12), C2711–C2716. https://doi.org/10.1111/1750-3841.13127

Schaich, K. M., Tian, X., & Xie, J. (2015). Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. *Journal of Functional Foods, 14*, 111–125. https://doi.org/10.1016/j.jff.2015.01.043

Seong, P. N., Park, K. M., Cho, S. H., Kang, S. M., Kang, G. H., Park, B. Y., … Van Ba, H. (2014). Characterization of edible pork by-products by means of yield and nutritional composition. *Korean Journal for Food Science of Animal Resources, 34*(4), 434–447. https://doi.org/10.5851/kosfa.2014.34.4.434

Singh, S., Brocker, C., Koppaka, V., Chen, Y., Jackson, B. C., Matsumoto, A., … Vasiliou, V. (2013). Aldehyde dehydrogenases in cellular responses to oxidative/electrophilic stress. *Free Radical Biology and Medicine, 56*, 89–101. https://doi.org/10.1016/j.freeradbiomed.2012.11.010

Stoica, M., Antohi, V. M., Alexe, P., Ivan, A. S., Stanciu, S., Stoica, D., … Stuparu-Cretu, M. (2022). New strategies for the total/partial replacement of conventional sodium nitrite in meat products: A review. *Food and Bioprocess Technology, 15*(3), 514–538. https://doi.org/10.1007/s11947-021-02744-6

Tan, J. B. L., & Lim, Y. Y. (2015). Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts. *Food Chemistry, 172*, 814–822. https://doi.org/10.1016/j.foodchem.2014.09.141

Toldrá, F., Reig, M., & Mora, L. (2021). Management of meat by- and co-products for an improved meat processing sustainability. *Meat Science, 181*(January). https://doi.org/10.1016/j.meatsci.2021.108608

Vázquez, J. A., Rodríguez-Amado, I., Sotelo, C. G., Sanz, N., Pérez-Martín, R. I., & Valcárcel, J. (2020). Production, characterization, and bioactivity of fish protein hydrolysates from aquaculture turbot (Scophthalmus maximus) wastes. *Biomolecules, 10*, 1–13. https://doi.org/10.3390/biom10020310

Wakamatsu, J. I., Murakami, N., & Nishimura, T. (2015). A comparative study of zinc protoporphyrin IX-forming properties of animal by-products as sources for improving the color of meat products. *Animal Science Journal, 86*(5), 547–552. https://doi.org/10.1111/asj.12326

Zamuz, S., Purriños, L., Galvez, F., Zdolec, N., Muchenje, V., Barba, F. J., & Lorenzo, J. M. (2019). Influence of the addition of different origin sources of protein on meat products sensory acceptance. *Journal of Food Processing and Preservation, 43*(5), 1–12. https://doi.org/10.1111/jfpp.13940

Zhang, P., Roytrakul, S., & Sutheerawattananonda, M. (2017). Production and purification of glucosamine and angiotensin-I converting enzyme (ACE) inhibitory peptides from mushroom hydrolysates. *Journal of Functional Foods, 36*, 72–83. https://doi.org/10.1016/j.jff.2017.06.049

Zou, Y., Bian, H., Li, P., Sun, Z., Sun, C., Zhang, M., … Wang, D. (2018). Optimization and physicochemical properties of nutritional protein isolate from pork liver with ultrasound-assisted alkaline extraction. *Animal Science Journal, 89*(2), 456–466. https://doi.org/10.1111/asj.12930

Zou, Y., Shahidi, F., Shi, H., Wang, J., Huang, Y., Xu, W., & Wang, D. (2021). Values-added utilization of protein and hydrolysates from animal processing by-product livers: A review. *Trends in Food Science and Technology, 110*(January), 432–442. https://doi.org/10.1016/j.tifs.2021.02.033