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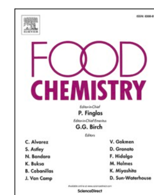

Peptides recovery from egg yolk lipovitellins by ultrafiltration and their *in silico* bioactivity analysis

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A B S T R A C T

The lipoproteins that remain after the extraction of phosvitin from the egg yolk granular fraction possess low industrial applicability. In this study, these lipoproteins were hydrolysed using trypsin, and the bioactivity of the resulting peptides was assessed by *in silico* analysis. In addition, in order to isolate the most valuable previously detected peptides, their transmission through a polyethersulfone (PES) membrane and a stabilised cellulose (SC) based membrane was also evaluated at several pHs. A pH of 4.0 gave the highest observed transmission of peptides through both membranes for every peptide identified in the permeate streams. Regarding the PES membrane, six peptide sequences detected in the permeate were predicted to be antihypertensive, although only one of them showed a bioactivity score higher than 0.5 according to Peptide Ranker. When the SC membrane was assessed, five peptides with a bioactivity score higher than 0.5 were detected in the permeate streams and eight peptides were predicted as antihypertensive. The *in silico* analysis performed showed that K.VQWGIIPSWIK.K was the most promising antihypertensive peptide found in the permeates.

1. Introduction

Egg yolk is consumed all around the world because of its nutritional and functional properties. It can easily be separated by centrifugation into two fractions, the egg yolk granules and plasma fractions. In comparison with the plasma fraction, the granular fraction has a low content of lipids and cholesterol and a high content of proteins; on the other hand, the lipid-rich plasma fraction has gelling and emulsifying properties similar to those of whole egg yolk (Kiosseoglou & Paraskevopoulou, 2005; Le Denmat, Anton, & Beaumal, 2000). Therefore, taking into consideration their different composition and functional properties, it would be advantageous to increase the value of whole egg yolk by making different use of each of its fractions. In this sense, while the egg yolk plasma could, to some extent, be used as a substitute for whole egg yolk, the range of applications for the granules is more reduced.

The egg yolk granular fraction is mainly composed of globular proteins, namely lipovitellins or high-density lipoproteins, linked to the phosvitin by phosphocalcium bridges. Phosvitin is the most phosphorylated protein found in nature, and shows remarkable metal chelating, antioxidant, emulsifying and antimicrobial properties (Yilmaz & Ağagündüz, 2020) and owing to these capacities, it has the potential for incorporation as a functional ingredient by the food industry (Marcet, Sáez-Orviz, Rendueles, & Díaz, 2022). However, the use of the remaining lipovitellins after phosvitin extraction has scarcely been studied. In fact, only the work of Chalamaiah, Esparza, Hong, Temelli, and Wu (2018), who studied the functional properties of the lipovitellins remaining after phosvitin extraction, was found in the scientific literature about this issue. Bearing in mind that these remaining lipovitellins could be a valuable source of bioactive peptides and, considering that the different phosvitin extraction procedures may exert different effects on the structure of these proteins, affecting their digestibility by proteases, the study of the bioactive peptides produced from these remaining lipovitellins by enzymatic hydrolysis could contribute to increasing the value of phosvitin extraction and hence of the egg yolk granular fraction.

Regarding the identification of bioactive peptides from the whole egg yolk, Eckert, Zambrowicz, Bobak, Zabłocka, Chrzanowska, and Trziszka (2019) identified two peptides with strong antioxidant and antihypertensive properties using delipidated egg yolk and two different proteases from yeast, whilst Park, Jung, Nam, Shahidi, and Kim (2001) found two peptides with strong antioxidant properties using lecithin-

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free egg yolk and alcalase, and Zambrowicz et al. (2015) isolated several peptides with antioxidant, antidiabetic and antihypertensive activities after the hydrolysis of previously delipidated egg yolk with a proteinase from Asian pumpkin and pepsin. However, one investigative pathway that has not been studied is the identification of bioactive peptides using trypsin and, as was commented above, the subfraction of lipovitellins remaining after phosvitin extraction.

Trypsin is an intensively studied serin protease with very interesting properties. Firstly, it is already extensively used in the food industry to decrease the allergenicity of food proteins. For example, the protease complex produced by Neova®, which contains trypsin from porcine pancreas, has been recently approved by the EFSA to be used as an ingredient in infant formulae (EFSA, 2021). Moreover, considering that many anti-hypertensive peptides are constituted of hydrophobic or cationic amino acids at the C-terminal position, and that trypsin shows a very high specificity for hydrolysing peptide sequences with arginine or lysine at *P*1, this protease has been frequently used to produce peptides with antihypertensive properties from milk proteins, rice bran protein, smooth-hound viscera proteins, egg white protein and porcine skeletal muscle proteins among others (Lee & Hur, 2017; Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018). Moreover, according to Lee et al. (2017), trypsin is considered an optimal enzyme for producing antihypertensive peptides from animal products, and is also widely employed to produce many other bioactive peptides (Bhat, Kumar, & Bhat, 2015; Mada, Ugwu, & Abarshi, 2020).

In any case, after obtaining the bioactive peptides, their separation from the reaction mixture is usually carried out by ultrafiltration, due to its scalability to an industrial scale, the use of mild operation conditions, and the low energy required (Ratnaningsih, Reynard, Khoiruddin, Wenten, & Boopathy, 2021). However, the membrane transit characteristics of these peptides is very difficult to predict, since there are interactions between the peptides and the surface of the membrane, interactions with different or similar peptides in the hydrolysis solution, and also between the peptides and the cake produced by fouling of the membrane, all of which may hinder their movement across the membrane.

In this work, and with the aim of expanding the range of applications of the granular fraction, for the first time the phosvitin-free lipovitellins subfraction from egg yolk was extracted and hydrolysed with trypsin, as trypsin is one of the enzymes most commonly used to produce bioactive peptides (Korhonen & Pihlanto, 2006) and it has never been used for their identification in either egg yolk or egg yolk subfractions. After the hydrolysis, the resulting peptides were identified, and their bioactivity was assessed *in silico.* Finally, since the commercial exploitation of bioactive peptides usually requires a first separation step by ultrafiltration, also for the first time for any bioactive peptide obtained from egg yolk, the mixture of peptides previously obtained by tryptic hydrolysis was separated using two different ultrafiltration membranes at several pHs and the transmission of each peptide found in the permeate was finally evaluated.

2. Materials and methods

2.1. Materials and reagents

Medium size eggs (50–60 g) were purchased from a local market. The following chemicals were purchased in Sigma-Aldrich (St. Louis, USA): NaCl (ref. S9888), NaOH (ref. S5881), HCl (ref. 320331), trypsin (E.C. 3.4.21.4, from porcine pancreas, ref. T7409), acetone (ref. 179124), Coomassie blue (ref. 1.15444), triton X-100 (ref. 93443), aluminium nitrate (ref. 237973), acetonitrile and formic acid 0.1% (v/v) (ref. 900686), H_3PO_4 (ref. 345245), glycine (ref. G7126), triglycine (ref. G1377), hexaglycine (ref. G5630), vitamin B12 (ref. V2876) and ribonuclease A (ref. R6513), bovine serum albumen (BSA, ref. A7030) and TrizMa® hydrochloride solution pH 8.0 (ref. T2694). Absolute ethanol (ref. 85651.360) was purchased from VWR (Pennsylvania, USA).

2.2. Obtaining isolated lipovitellins

Eggs were broken and the egg yolk was manually separated from the egg white. The surface of the egg yolks was carefully dried using blotting paper, and then the vitelline membrane was broken with a pair of tweezers. Afterwards, the egg yolk was poured into a beaker and diluted with water (1:1.5, v/v), the mixture was stirred for 20 min and subsequently centrifuged at 10000 × g for 50 min. The sediment, which is the granular fraction, was recovered.

In order to remove the remaining plasma fraction from the sediment, it was dispersed in three times its weight of a solution of 0.16 M NaCl, and the resulting solution was centrifuged at 10000 × g for 50 min and then this procedure was repeated one more time. The washed granules were dissolved in 1.2 M NaCl (3% w/v) and the pH of the solution was adjusted to 3.0 using 1.0 N HCl; at this pH the lipovitellins precipitate and the phosvitin remains in the supernatant. The lipovitellins were recovered in the sediment after centrifugation at 10000 × g for 50 min and lyophilized before being delipidated using absolute ethanol at a proportion of 1:20 (w/w) for 3 h at 40 °C under gentle stirring. The delipidated lipovitellins were recovered by vacuum filtration and dispersed in acetone at a proportion of 1:20 (w/w) for 5 min. Finally, the delipidated lipovitellins were recovered by vacuum filtration again and dried in an oven at 40 °C overnight. The amount of protein contained in the granular fraction, the lipovitellins and the delipidated lipovitellins fraction was assessed by the combustion method of Dumas using a CNHS/O Elementar Vario El Analyzer (Elementar, Germany). The conversion factor used was 6.25 mg protein/mg of nitrogen.

In order to evaluate the degree of extraction of the phosvitin from the granular fraction, electrophoresis (SDS-PAGE) was performed using a stacking and a resolving gel with 3% and 12% of acrylamide, respectively. A specific staining phase for phosphoproteins consisting of Coomassie blue 0.05% (w/v), ethanol 0.025% (v/v), acetic acid 10% (v/v), Triton X-100 1% (v/v), aluminium nitrate 3.75% (w/v) and distilled water 40% (v/v) was used. In addition, pre-stained SDS-PAGE Molecular Weight Standards GangNam-STAIN^{TM} (iNtRON Biotechnology, South Korea) was employed.

2.3. Enzymatic hydrolysis and peptide identification

The enzymatic hydrolysis of the isolated lipovitellins was carried out using a bioreactor with an automatic system of temperature and pH adjustment (pH-burette 24 2S, Crison, Spain). For that purpose, 3 g of isolated lipovitellins was dispersed in 400 mL of distilled water under constant stirring at 37 °C, and the pH of this solution was maintained at pH 8.0 with 1.0 N NaOH. These pH and temperature values are customary operating values found in the literature for trypsin (Amorim et al., 2019; Tu et al., 2017). The hydrolysis started when the trypsin was added at the enzyme to substrate ratio of 75,000 U/g protein. This proportion was used after performing preliminary tests at several enzyme to substrate ratios, from 40,000 U/g protein to 100,000 U/g of protein, with the objective of achieving a high degree of hydrolysis in a short time whilst using a reasonable amount of enzyme. The reaction time was 6 h, and at the end, the enzyme was thermally inactivated in a water bath at 90 °C for 10 min. Finally, the reaction mixture was centrifuged at 10000 × g for 60 min and the supernatant was recovered.

The degree of hydrolysis was assessed according to the pH-Stat method (Adler-Nissen, 1986). This method correlates the amount of NaOH consumed during the hydrolysis in order to maintain the pH at 8.0 with the degree of hydrolysis by the following equation:

$$DH(\%) = \frac{h}{h\mathrm{tot}}\ \mathrm{x}\ 100 = \frac{100\mathrm{xVB\ xNB}}{\alpha\mathrm{x}M\mathrm{P\ x\ htot}} \quad (1)$$

where V_B and N_B are the volume (mL) and the normal concentration of the base (NaOH, 1 N), α is the degree of dissociation of the amine groups and is calculated according to Equation (2), M_p is the mass of the protein

(g), *h* is the number of peptide bonds hydrolysed (meq/g protein) and *htot* is the total number of peptide bonds in the protein (meq/g protein). The value of h_{tot} for egg yolk protein was assumed to be 8, based on a suggestion found in a reference (Wang & Wang, 2009).

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \quad (2)$$

The pK value used for the hydrolysis procedure is calculated approximately using the following equation of Guadix (2000).

$$pK = 3.80 + 0.45pH \quad (3)$$

Peptides were identified by reversed-phase ultra-high-performance chromatography coupled to tandem mass spectrometry (RP-UPLC-MS/MS) with a Dionex Ultimate 3000 RS UHPLC (Thermo Fisher Scientific, USA) connected in line to a Q-ToF Bruker Impact II mass spectrometer (Bruker, USA). The column used was a Bruker Intensity Trio C18 (50 × 2.1 mm, 3 μm). The solvent "A" was composed of ultrapure water MilliQ and formic acid 0.1% (v/v). Solvent "B" was composed of acetonitrile and formic acid 0.1% (v/v). The gradient of solvent "B" used was 2% for 1 min, followed by an increase from 2% to 35% over a period of 30 min, 35% for 1 min, from 35% to 80% over 4 min, and finally 80% of B for 1 min. The flow rate was 0.15 mL/min and the injection volume was 2 μL. The separation was performed at 30 °C and the mass spectra were obtained in the ion positive mode, using a fragmentation of 120 V with a range of exploration of 50–3000 *m/z*. Nitrogen was used as drying gas at the flow velocity of 6.0 L/min and at 300 °C, and as nebulizer gas at a pressure of 0.31 MPa. The capilar voltage was adjusted to 4500 V and only the peaks with an intensity higher than 10% of the most intense peak were considered for the analysis. The database and the software provided by MASCOT were used to identify the peptide masses obtained after the LC-MS/MS analysis. In this case, the mass tolerance limit was 0.6 Da, and the minimum peptide extension considered was three amino acids.

In order to verify whether the identified peptide sequences had been previously described as bioactive, these sequences were investigated by searching on the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008) and their isoelectric point and molecular weight were obtained using the ExPASy ProtParam bioinformatic tool (http://www.expasy.ch/tools/protparam-doc.html). To predict the bioactivity and antihypertensive properties of the detected peptides, AHTpin software (http://crdd.osdd.net/raghava/ahtpin/) (Kumar et al., 2015) and the Peptide Ranker program (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php) (Mooney, Haslam, Pollastri, & Shields, 2012) were consulted.

2.4. Membrane separation

Two different membranes were used, the first was a polyethersulfone membrane (PES) with a nominal molecular weight cut-off (NMWCO) of 1 kDa and a filtration area of 0.1 m^2 (PES1, Sartorius, Germany), the second was a stabilized cellulose-based membrane (SC) with a 2 kDa NMWCO and a filtration area of 0.1 m^2 (Hydro2, Sartorius, Germany).

The membranes were each assembled in a mini cassette Pellicon 2 (Millipore, USA). Before their first use, the pure water flux (J_w) of both membranes was tested, and after every test they were cleaned with 1.0 M NaOH and 2% H_3PO_4. The cleaning protocol includes 10 min of washing without permeate recirculation followed by 30 min of recirculation at 40 °C and at a constant transmembrane pressure (TMP) of 1.5 bar. The degree of recovery of the membranes was assessed after every cleaning by measuring the J_w parameter, achieving at the end of the cleaning procedure J_w values of at least 95% of the initial J_w.

The ultrafiltration of the hydrolysates was always carried out at the same TMP (2 bar), temperature (37 °C), and on full recirculation mode, in a similar way to Arrutia, Fernández, Menéndez, González, and Riera (2017). In the case of the PES and SC membranes the average permeate flux (J_p) was 9.0 ± 0.2 L/m^2h and 12.6 ± 0.5 L/m^2h, respectively. In order to study the effect of the pH on the selective permeation of the peptides, the ultrafiltration was performed at pH 4.0, 7.0 and 10.0 by adjusting the pH of the hydrolysate using 1.0 N HCl and 1.0 M NaOH. Before sampling, the ultrafiltration system was equilibrated for 15 min, and all membrane experiments were performed in triplicate.

The observed transmission (Tr_{obs}) of every peptide was calculated using the following equation:

$$\text{Trobs}(\%) = \frac{\text{Api}}{AHi} \text{x}100 \quad (4)$$

where A_{pi} and A_{Hi} are the area of the peptide peak i from the chromatograms of the permeate and the hydrolysate respectively.

2.5. Size exclusion chromatography (SEC)

The size and the relative amount of the peptides produced by the enzymatic hydrolysis and the size and relative amount of the peptides found in the permeate after the ultrafiltration procedures were determined by *SEC* with an ÄKTA FPLC system (Amersham Biosciences, United Kingdom) equipped with a Superdex™ Peptide 10/300 GL column. The column was calibrated using several weight markers acquired from Sigma-Aldrich: Glycine (75.10 Da), Triglycine (189.17 Da), Hexaglycine (360.32 Da), vitamin B12 (1355.37 Da) and ribonuclease A (13700 Da) and the elution volume of these markers as well as the standard curve are shown in Figure S1A and S1B. The elution buffer used was a 1% TrizMa® hydrochloride solution pH 8.0, all samples were previously filtered using 0.45 μm PVDF filters, the elution flow selected was 0.7 mL/min and the peptides were detected using an UV detector at 214 nm. In every case, the area of the chromatograms obtained was divided into several ranges of molecular size, according to the calibration performed previously, and using the Unicorn 5.1 software provided with the FPLC device. In addition, to estimate the concentration of peptides after the trypsin hydrolysis, a calibration curve with several BSA concentrations was performed (Figure S2).

2.6. Molecular docking

Docking calculations between the angiotensin-converting enzyme (ACE) and nine peptides with predicted antihypertensive activity were carried out using the AutoDock CrankPep (ADCP) program (Zhang & Sanner, 2019) which generates and scores docking poses utilizing a grid-based semiempirical scoring function (Huey, Morris, Olson, & Goodsell, 2007) that combines Van der Waals (vdW), electrostatic and dehydration contributions and incorporates intramolecular energies and empirical entropy corrections. According to a recent benchmark study of docking programs (Weng et al., 2020), ADCP achieves the best performance in folding and docking peptides (up to 20 amino acids) within previously-defined active sites.

For the ACE, starting coordinates were obtained from the PDB ID:1O86 crystal structure (Natesh, Schwager, Sturrock, & Acharya, 2003) after removing all the water molecules and the lisinopril inhibitor. Missing residues were not built in this initial ACE structure. Particularly, the unresolved Ser_{435}-Glu_{436}-Gly_{437}-Gly_{438} sequence was handled by capping both ends with *N*-methyl and acetyl terminal residues, respectively. Missing atoms and hydrogens were added with the tLeap program and structurally relaxed using the SANDER program included in the Amber21 suite (Case, 2021)

The default docking parameters, excepting some atomic charges of the catalytic Zn(II) ion and its ligands, were used, a procedure similar to those employed in previous studies to improve the accuracy of the scoring function for zinc-enzymes (Hu & Shelver, 2003; Santos-Martins, Forli, Ramos, & Olson, 2014). Thus, a fractional charge for Zn (+1.19) that corresponds to the electrostatically-fitted atomic charge of the Zn ion in a small cluster model, $[Zn(methyl\text{-}imidazole)_2(acetate)]^+$, and

using the B3LYP/6-31G(d) level of theory (Becke, 1988; C. Lee, Yang, & Parr, 1988) as implemented in the Gausian09 program (Frisch et al., 2009), was employed. The heavy-atom coordinates were taken from the 1O86 structure and augmented with H-atom coordinates. Only the H atoms were relaxed with the Gaussian09 program. To preserve charge integrity, a + 0.81 charge was distributed evenly throughout the Zn-coordinated residues. In the ADCP calculations, initial coil conformations were selected for the ligand peptides. For each peptide, 64 independent Monte Carlo simulations (replicas) with 2.5 million evaluations of the scoring function per amino-acid and replica were run. The docking box to evaluate grid affinity maps was centred at the position of the zinc ion and it extended to 30 Å along each dimension. Cutoffs of 3.0 Å for the distance between heavy atoms and 8.0 Å for the distance between the centre of mass of the hydrophobic groups were defined to characterize hydrogen bonds and van der Waals contacts, respectively.

2.7. Statistical analysis

Experiments were performed in triplicate and are shown as the mean value ± standard deviation of three independent experiments (n = 3). Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using Statgraphics® V.15.2.06 statistical software.

3. Results and discussion

3.1. Obtaining isolated lipovitellins

After the separation of the granular fraction, the yield of the washed granules was 21.2 ± 3.2% (w/w) of the total dry matter of the whole egg yolk, which is in accordance with the 22% (w/w) egg yolk granules content reported by other authors using similar separation procedures (Motta-Romero, Zhang, Tien Nguyen, Schlegel, & Zhang, 2017). Afterwards, the granules were dissolved in a 1.2 M NaCl solution and the pH was decreased from 7.0 to 3.0, producing the aggregation of the granular fraction lipoproteins, which were separated from the phosvitin by centrifugation. In Figure S3A, the SDS-PAGE of the phosvitin extraction process is presented, showing in lane 1 the band profile of the granular proteins. In this lane the bands of 110 kDa, 78 kDa, 55 kDa and 31 kDa correspond to apoproteins that conform the lipovitellins, while the band of 45 kDa corresponds to phosvitin (Guha, Majumder, & Mine, 2019; Le Denmat et al., 2000). The same bands can be found in lane 2 prior to the centrifugation process, after decreasing the pH of the solution from 7.0 to 3.0. After centrifugation, in the supernatant only the band of phosvitin can be found (lane 3), while in the precipitate the rest of the bands of the granular fraction can be identified (lane 4). Finally, this precipitate was recovered, delipidated and dried, resulting in the white powder shown in Figure S3B. The protein content of the washed granular fraction and the lipovitellins collected before and after the delipidation process, all dried in an oven at 40 °C overnight, was 60.0 ± 1.1%, 57.2 ± 4.3% and 89.1 ± 1.0%, respectively.

3.2. Egg yolk granular lipovitellins hydrolysis

The DH of the isolated lipovitellins treated with trypsin over the reaction time is shown in Fig. 1A.

The DH value rose abruptly during the first 30 min of hydrolysis and then stabilised until almost reaching a plateau, as was observed by other authors (Deng, van der Veer, Sforza, Gruppen, & Wierenga, 2018;

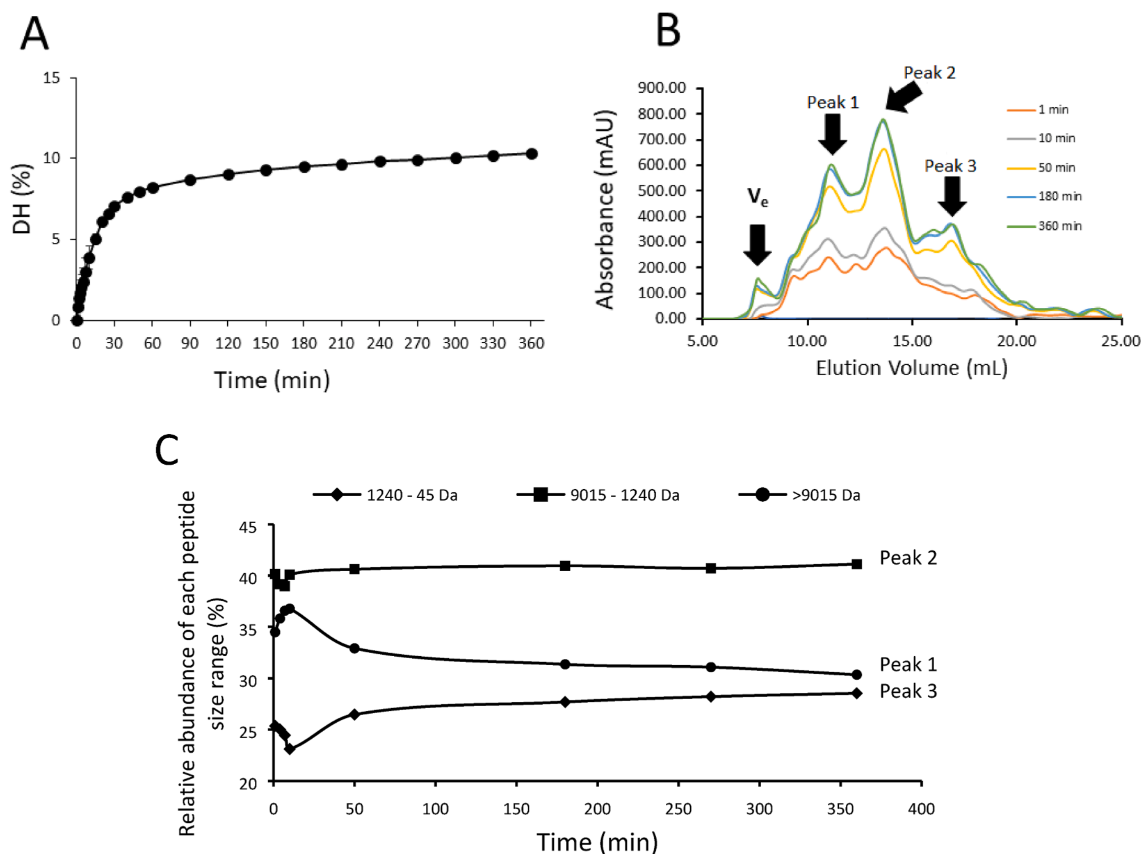

Fig. 1. A) Degree of hydrolysis (DH) over the time of reaction resulting from the tryptic hydrolysis of the isolated lipovitellins subfraction (enzyme/substrate ratio of 75,000 U/g protein). B) *SEC* chromatograms of the solubilised peptides at various reaction times, arrows indicate the volume exclusion (Ve) and the three main peaks detected. C) Relative abundance of each peptide size range obtained from the analysis of the area of the peaks detected in Fig. 1B over the time of reaction.

Ketnawa, Benjakul, Martínez-Alvarez, & Rawdkuen, 2017). In this case, the DH value reached 10.31 ± 0.12% after 360 min. In a previous study, Marcet, Alvarez, Paredes and Diaz (2014), using trypsin and delipidated whole granules at a ratio 1:25 (enzyme/substrate), reached a DH of 6.9 ± 0.3%, which is a noticeably lower value than that obtained in the present work. This difference could be due to the extraction of the phosvitin, which, being the most highly phosphorylated protein found in nature, shows a high resistance to trypsin hydrolysis (Yilmaz et al., 2020). Therefore, the presence of phosvitin in the protein mixture could lead to lower DH values, as occurred in the previous study of Marcet et al. (2014). The amount and size of the peptides solubilised from the isolated lipovitellins over the time of reaction was analysed by size exclusion chromatography (Fig. 1B). According to these results and as was expected, the greater the time of reaction, the higher was the concentration of solubilised peptides, achieving a peptide concentration of 5.24 mg/mL after 180 min of hydrolysis and 5.4 mg/mL after 360 min, which represents the solubilisation of 80% of the total protein treated with trypsin.

In order to determine the effect of the reaction time on the size of the peptides obtained, the three mean peaks obtained in the chromatograms were numbered in Fig. 1B. In peak 1 were found those peptides with a size greater than 9015 Da, in peak 2 those peptides with a size between 9015 and 1240 Da, and in peak 3 those with a size between 1240 and 45 Da. The area under these three main peaks of the chromatograms was calculated so that the relative abundance of the peptides in each of the three size ranges could be plotted against reaction time (Fig. 1C). During the first 30 min of reaction, the relative amount of the peptides of every size range varied slightly, but after 50 min the peptide populations stabilised. According to these results, the most abundant peptides in the mixture during the whole reaction time were those with a size between 9015 and 1240 Da, which correspond to those obtained from peak 2 in Fig. 1B, making up approximately 40% of the whole peptide population at every time tested. After 360 min of reaction, peptides with a size larger than 9015 Da and with a size range between 1240 and 45 Da represented 30.0% and 28.5%, respectively.

These peptides were detected by LC-MS and their sequence, isoelectric point and molecular weight are shown in Table 1. In this sense, the peptides identified in the hydrolysate were found within the amino

Table 1
Molecular weight[a], bioactivity score (BS)[b], antihypertensive activity prediction (AAP)[c] and isoelectric point (pI)[a] of the identified peptides.

VITELLOGENIN-2

MW (Da)	Peptide sequence	BS	AAP	pI
844.9	K.FEYSSGR.I	0.35	Non-AHT	6.35
871.0	K.LDSMSYR.Y	0.40	Non-AHT	6.19
886.0	K.TVVEPADR.N	0.06	Non-AHT	4.38
896.1	K.LIGEHEAK.I	0.07	Non-AHT	5.41
922.0	R.YVIQEDR.K	0.07	Non-AHT	4.38
958.2	R.NIGELGVEK.R	0.08	Non-AHT	4.54
961.2	K.VSTELVTGR.F	0.15	AHT	6.36
973.2	R.MVVALTSPR.T	0.18	Non-AHT	9.50
988.21	K.ELLQQVMK.T	0.10	Non-AHT	6.10
1025.3	R.FAGHPAAQVK.L	0.36	Non-AHT	9.11
1031.2	R.EETEIVVGR.H	0.07	AHT	4.26
1048.4	R.LPLSLPVGPR.I	0.65	AHT	10.11
1074.4	R.VEGLADVIMK.R	0.23	Non-AHT	4.38
1094.4	R.TMFPSAIISK.L	0.23	Non-AHT	9.11
1114.3	K.IQLEIQAGSR.A	0.12	Non-AHT	6.36
1114.4	R.NIGELGVEKR.T	0.10	Non-AHT	6.49
1134.4	K.MTPPLTGDFR.L	0.45	AHT	6.19
1295.7	R.GILNMFQMTIK.K	0.58	Non-AHT	9.11
1342.6	R.NIPFAEYPTYK.Q	0.50	AHT	6.35
1401.6	R.IANADNLESIWR.Q	0.48	Non-AHT	4.38
1418.8	R.LSQLLESTMQIR.S	0.22	Non-AHT	6.36
1436.7	R.DASFIQNTYLHK.L	0.27	Non-AHT	7.09
1445.8	R.VGATGEIFVVNSPR.T	0.20	Non-AHT	6.36
1458.8	K.ILGQEVAFININK.E	0.30	Non-AHT	6.35
1465.7	K.QSDSGTLITDVSSR.Q	0.10	AHT	4.21
1559.9	K.LEISGLPENAYLLK.V	0.37	Non-AHT	4.54
1560.8	R.SPQVEEYNGVWPR.D	0.62	AHT	4.54
1560.9	K.ELPTETPLVSAYLK.I	0.20	AHT	4.54
1582.0	K.TPVLAAFLHGISNNK.K	0.33	Non-AHT	9.11
1591.9	R.GSAPDVPMQNYGSLR.Y	0.67	Non-AHT	6.19
1595.2	R.FPAVLPQMPLQLIK.T	0.69	Non-AHT	9.11
1664.1	R.WLLSAVSASGTTETLK.F	0.13	AHT	6.35
1778.1	K.EALQPIHDLADEAISR.G	0.22	Non-AHT	4.31
1802.3	K.LMANSAGSVADLVEVGIR.V	0.21	Non-AHT	4.38
1947.4	K.KTGGLQLVVYADTDSVRPR.V	0.14	Non-AHT	6.31
1973.5	R.NSIAGQWTQPVWMGELR.Y	0.66	Non-AHT	6.36
2023.4	R.SYLYNYEGSMLNGLQDR.S	0.23	Non-AHT	4.38
2075.6	K.KTGGLQLVVYADTDSVRPR.V	0.13	Non-AHT	8.93
2236.8	R.QVYQISPFNEPTGVAVMEAR.Q	0.3	Non-AHT	4.54
2240.8	K.TVQGYLIQILADQSLPPEVR.M	0.17	Non-AHT	4.38
2569.2	R.SVVEWFYEFVPGAAFMLGFSER.M	0.22	AHT	4.26
2608.3	K.FLPISSSSAADIPVHIQIDAITALK.K	0.1	Non-AHT	5.22
2663.4	K.IVLMPVHTDADIDKIQLEIQAGSR.A	0.11	Non-AHT	4.66
2736.5	K.FLPISSSSAADIPVHIQIDAITALKK.I	0.22	Non-AHT	7.09
2784.3	R.IVETLQHIVLNNQQDFHDDVSYR.F	0.03	Non-AHT	4.64

VITELLOGENIN-1

MW (Da)	Peptide sequence	BS	AAP	pI
859.0	K.ANLVDVTK.S	0.09	Non-AHT	6.19
960.	R.NIEDLAASK.M	0.09	Non-AHT	4.38
1066.4	K.EFLMSGLAAK.Y	0.25	Non-AHT	6.35
1158.4	R.LTELLNSNVR.L	0.09	AHT	6.36
1197.5	K.MFGQELLFGR.L	0.82	Non-AHT	6.36
1228.4	K.SNIEEVLLALK.A	0.20	Non-AHT	4.53
1326.7	K.VQWGIIPSWIK.K	0.77	AHT	9.11
1384.7	R.IRSEVEISGIGPK.L	0.16	AHT	6.49
1437.8	K.VAGNVQAQITPSPR.S	0.14	Non-AHT	10.11
1439.6	R.AANEENYESVWK.Q	0.23	Non-AHT	4.26
1439.8	R.YLLDLLPAAASHR.S	0.36	AHT	7.09
1469.8	K.IQVTIQAGDQAPTK.M	0.10	Non-AHT	6.19
1482.9	K.LTQALTGQLSIPIK.F	0.22	Non-AHT	9.11
1533.9	K.FLPGYAAGASELPLK.V	0.70	Non-AHT	6.35
1566.9	R.NVPLYNAIGEHALR.M	0.41	Non-AHT	7.10
1640.1	R.MSFKPVYSDVPIEK.I	0.25	AHT	6.41
1654.2	K.MTPVLLPEAVPDIMK.M	0.40	Non-AHT	4.38
1720.3	K.VNAHVPVNVVATIQMK.E	0.23	Non-AHT	8.73
1841.3	K.VDVQLVVVQLAETNWK.A	0.16	Non-AHT	4.38
1843.3	R.YVPGVALVLGFSEAHQR.N	0.40	Non-AHT	7.10
1980.5	R.NFLGDVIPPGITIVAQAVR.S	0.35	Non-AHT	6.19
2023.4	K.VYTYNYESILFSGIPEK.G	0.12	AHT	4.54
2118.5	R.LQDLVETTYEQLPSDAPAK.A	0.07	AHT	3.92
2144.7	R.VADPIEVGIAAEGLQEMFVR.G	0.53	Non-AHT	4.01
2194.8	K.YFVLNNAGSLIPTMAVSQLR.T	0.42	Non-AHT	9.10
2261.7	R.LDKDTLQNVLQVWYGPDEK.I	0.20	Non-AHT	4.23
2265.7	R.IHSIEAAEYNGIWPTSSFSR.S	0.29	AHT	5.41
2304.9	R.GSLQYQFGSELLQLPVHLFK.I	0.78	Non-AHT	7.10

[a] : Calculated with ExPASy ProtParam (http://www.expasy.ch).
[b] : Calculated with Peptide Ranker (http://bioware.ucd.ie/).
[c] : Calculated with AHTpin software (http://crdd.osdd.net/).

acid sequence of the two main laying hen vitellogenins (type I and II), which are the HDLs and phosvitin precursor proteins synthesized in the liver and transported to the oocytes (Li, Leghari, He, Zeng, Mi, & Zhang, 2014). A higher number of peptides in the hydrolysate were identified within the vitellogenin II amino acid sequence, which is because vitellogenin type II is synthesised in the liver in larger amounts than type I (Evans, Silva, & Burch, 1988). In addition, as expected, no phosvitin-related amino acid sequences were identified in the hydrolysate.

The bioactivity of these peptides was analysed *in silico* with the Biopep, Peptide Ranker and AHTpin programs. None of the peptides obtained in this investigation had been previously identified as bioactive on the Biopep database, which is not unexpected, since there is no work in the literature which studied the trypsin hydrolysis of the lipoprotein subfraction from the egg yolk granular fraction.

Regarding Peptide Ranker, several authors have set the threshold bioactivity score at 0.5, so peptides with predicted scores higher than this value are more likely to be bioactive (Ding, Liang, Yang, Sun, & Lin, 2020; Tu et al., 2018). As is shown in Table 1, 16% of all identified peptides showed a score equal to or higher than 0.5, which suggests that this hydrolysate could be a valuable source of bioactive peptides. In addition, 26% of the peptides shown in Table 1 were identified as antihypertensive peptides by AHTpin. It has been proved that the models used in AHTpin show an average accuracy of 78.58% when detecting peptides capable of inhibiting the ACE (Basith, Manavalan, Hwan Shin, & Lee, 2020).

Finally, according to Lapointe, Gauthier, Pouliot, and Bouchard (2005), the identified peptides could be broken down into three categories: acidic peptides (isoelectric point lower than 5.0), neutral peptides (isoelectric point between 5.0 and 8.0) and basic peptides (isoelectric point higher than 8.0). In total, 39.13% of the peptide population found in the hydrolysate were acid, 15.94% basic, and 44.92% neutral.

3.3. Membrane separation

3.3.1. Concentration and size of the peptides detected in the permeate streams

The hydrolysates obtained after treating the isolated lipovitellins with trypsin for 180 min were ultrafiltered at several pHs using the PES and SC membranes. Prior to ultrafiltration, the obtained hydrolysate was diluted by half in order to minimise membrane fouling. The permeates were analysed by *SEC* and the chromatograms are shown in Fig. 2A and 2C. The relative abundance of peptides of the different size ranges after the ultrafiltration process is shown in Fig. 2B and 2D. As can be seen in these chromatograms, only a small part of the peptides found in the hydrolysate passed through the membranes. In any case, as expected, the highest peptide concentration found in the permeate streams was that with a size range lower than 1240 Da, while peptides within the highest range of size were almost undetected (Fig. 2B and 2D).

In this respect, according to the manufacturer, the SC membrane used in these experiments has a NMWCO that is double that for the PES membrane. However, regarding the concentration of peptides in the permeate at every pH tested, the performance of the two membranes was similar (Table 2). This could be because in the end, protein–protein and protein-membrane interactions will occur, and the resulting fouling may imply the total or partial blocking of the membrane pores, and thus, it is possible for membranes that have different NMWCOs but are made from different materials to have similar performance. In addition, the difference in pore size between PES and SC could well be accurate when the substance and the ultrafiltration conditions used by the manufacturer to characterise these membranes are used, but this NMWCO could differ to some extent when other compounds are being assessed.

Regarding the pH and with reference to Table 2, at pH 4.0 the concentration of peptides in the permeate was noticeably higher than at pH 7.0 and 10.0 for both membranes. This relatively high ability of the peptides to cross the membranes at pH 4.0 could be explained by considering the characteristics of both the membranes used and the peptides, and bearing in mind that electrostatic interactions between membrane and peptides are one of the major parameters that drive

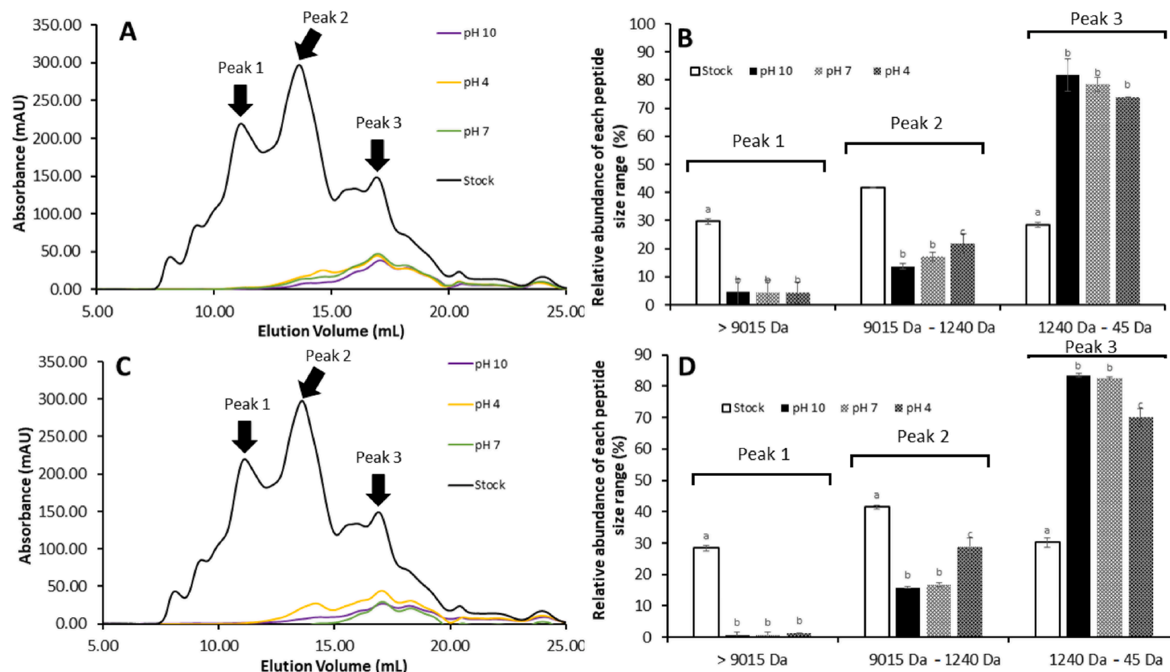

Fig. 2. A) *SEC* chromatograms of the hydrolysate and the permeate streams at several pHs using the 1 kDa NMWCO polyethersulfone (PES) membrane. B) Relative abundance of each peptide size range obtained from the analysis of the area of the peaks detected in Fig. 2A. C) *SEC* chromatograms of the hydrolysate and the permeate streams at several pHs using the 2 kDa NMWCO stabilized cellulose (SC) membrane. D) Relative abundance of each peptide size range obtained from the analysis of the area of the peaks detected in Fig. 2C. Different lowercase letters in the same range of size indicate significant differences ($p < 0.05$).

Table 2
Peptide concentrations ordered by range of sizes found in the permeate streams obtained after ultrafiltration of the tryptic hydrolysate at different pHs.

	Peptide concentration (mg/mL)					
	SC 2 kDa			PES 1 kDa		
	pH 4.0	pH 7.0	pH 10.0	pH 4.0	pH 7.0	pH 10.0
1240–45 Da	0.20 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	0.20 ± 0.03	0.15 ± 0.03	0.13 ± 0.01
9015–1240 Da	0.08 ± 0.02	0.03 ± 0.01	0.02 ± 0.02	0.06 ± 0.02	0.04 ± 0.01	0.02 ± 0.01
>9015 Da	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
Total	0.29 ± 0.03	0.19 ± 0.01	0.15 ± 0.01	0.27 ± 0.03	0.20 ± 0.01	0.16 ± 0.03
Stock solution*	2.6 ± 0.1			2.6 ± 0.1		

*: Concentration of peptides in the tryptic hydrolysate.

membrane fouling (Persico et al., 2020; Persico, Dhulster, & Bazinet, 2018). First of all, according to Susanto & Ulbricht (2005) PES and SC membranes have a relatively small negative surface charge of around −5 mv at pH 4.0, and this negative charge becomes greater as the pH of the medium increases, reaching values at pH 10.0 of around −20 mv and −35 mv for the SC and PES membrane, respectively. Therefore, considering the range of pH tested, at pH 4.0 the negative membrane surface charge was the lowest, and the attractive and repulsive forces established with the peptides could be minimised by the low charge contribution of the membrane.

This importance of the surface charge of the membranes may explain the results observed in this study, and the low negative charge of the surface of the membranes at pH 4.0 may be less disruptive for the passage of the peptides across them, favouring at this pH the transmission of non-charged (acidic peptides) or positively charged peptides (neutral and basic peptides). In fact, Persico et al. (2020) studied the composition of the fouling produced by peptides on several PES membranes when in contact with a whey hydrolysate at pH 7.0, and found that the fouling was composed of only 15% positively charged peptides, while negatively charged peptides formed the major part of the peptides adhered to the surface of the membrane, despite the fact that, under the testing conditions, the PES membranes were also strongly negatively charged. These authors conclude that other factors may be intervening to explain why positively charged peptides did not contribute more to fouling the negatively charged PES membranes. These results of Persico et al. (2020) may imply that the negatively charged peptides were more poorly transmitted through these organic membranes, perhaps because they are more prone to establish interactions with the membrane surface. In this sense, according to the peptide permeability results shown in Fig. 3, every peptide identified in the permeates was more easily transmitted through both membranes at pH 4.0, with basic peptides being found in the permeate at pH 4.0, whilst no acidic peptides were found at pH 10.0. In the case of the neutral peptides, they permeate more at pH 4.0 than at pH 10.0, which supports, in this case, the importance of the low negative surface charge of the membranes at pH 4.0 to better transmit non-charged or positively charged peptides.

3.3.2. Sequencing and in silico bioactivity of the peptides detected in the permeate streams

Among the acidic peptides detected in the permeate when the PES membrane was tested (Fig. 3A), R.IANADNLESIWR.Q has the highest bioactivity score of 0.48 but has no antihypertensive properties, and K.QSDSGTLITDVSSR.Q was predicted to be an antihypertensive peptide. Regarding the neutral peptides, the peptide K.MTPPLTGDFR.L was predicted to be antihypertensive and its bioactivity value was 0.45, the highest among the neutral peptides. In addition, peptides R.WLLSAVSASGTTETLK.F, K.VSTELVTGR.F and R.LTELLNSNVR.L were also predicted to be antihypertensive. Regarding the basic peptides, five were detected in the permeate streams, and among them, R.MVVALTSPR.T showed the best transmission rates at every pH tested, it being the smallest and with the most hydrophobic residues of all the basic peptides. However, this peptide showed a low bioactivity score of 0.18 and no antihypertensive properties. In fact, the only basic peptide with predicted antihypertensive properties found in the permeate was R.LPLSLPVGPR.I, which, according to Peptide Ranker also showed the highest bioactivity score (0.65) among all the peptides found in the different permeate streams when this membrane was used. Finally, as was also the case for acidic and neutral peptides, the basic ones were best transmitted through the membrane at pH 4.0.

Regarding peptide transmission when the SC was assessed, similar behaviour to that shown for the PES membrane was observed, and every peptide detected was most effectively transmitted through the membrane at pH 4.0 (Fig. 3B). Furthermore, the peptides transmitted through the SC membrane were mainly the same ones that were able to pass through the PES membrane, except for four new neutral peptides that were identified in the permeate streams. Two of these four new neutral peptides, R.NIPFAEYPTYK.Q and R.YLLDLLPAAASHR.S, were predicted as antihypertensive, and K.MFGQELLFGR.L showed the highest bioactivity score among all the peptides identified in the hydrolysate (0.82). The population of acidic peptides decreased from seven to only two different peptides, and none of them showed *in silico* antihypertensive activity or a Peptide Ranker score higher than 0.5. Furthermore, the number of basic ones identified decreased from five to three, and the sequence K.VQWGIIPSWIK.K showed a bioactivity score of 0.77 and antihypertensive properties. This decrease in the number of identified acidic and basic peptides between PES and SC membranes may be attributed to the greater hydrophilic nature of the regenerated cellulose, which could have a greater tendency to produce strong hydrogen-bond interactions between the membrane and the biopolymers, hindering the transmission of some hydrophilic compounds through the membrane (Yamamura, Okimoto, Kimura, & Watanabe, 2014). In this sense, a decrease in the transmission of some selected peptides was previously referred to by other authors ultrafiltering a tryptic digest when the performance of an SC membrane was compared with that of a PES membrane (Fernández & Riera, 2012).

Some other authors have found bioactive peptides from egg yolk. Among these, Zambrowicz, Pokora, et al. (2015) performed the lipid extraction of whole egg yolk using ethanol and the by-product obtained was hydrolysed with pepsin in order to identify peptides with antioxidant, antihypertensive and antidiabetic peptides. They found that the sequence YIEAVNKVSPRAGQF, which belongs to the protein Apovitellenin-1 (https://www.uniprot.org/uniprot/P02659.fasta), an apoprotein present in the egg yolk plasma fraction, was the most antihypertensive peptide obtained. In a similar way, Zambrowicz, Eckert, et al. (2015) hydrolysed the same whole egg yolk delipidated by-product with a proteinase from Asian pumpkin (*Cucurbita ficifolia*) and among the peptides obtained they found that the sequence LAPSLPGKPKPD, which comes from a protein that is involved in the morphogenesis of the retina of chickens (https://www.uniprot.org/uniprot/Q9IAL1.fasta), was the most promising, showing the highest antioxidant and antidiabetic properties. In addition, Eckert et al. (2019) also hydrolysed proteins from ethanol-delipidated whole egg yolk but with a proteinase from *Yarrowia lipolytica* yeast, and found that the sequence QSLVSVPGMS showed the highest DPPH and antihypertensive properties. In this case, this peptide came from the chicken embryo protein *Aryl hydrocarbon receptor nuclear translocator-like protein 2* (https://www.uniprot.org/uniprot/Q8QGQ7). The bioactivity of all these sequences can hardly be compared with those found in the present work, since the protein source was delipidated whole egg yolk and not the isolated lipovitellins fraction, trypsin was not used in any case to hydrolyse the protein source, and none of the highlighted sequences come from the granular fraction of the egg yolk. In addition, the present paper is not only focused on obtaining bioactive peptides from the tryptic hydrolysis of the lipovitellin fraction, but also on their transmission rate across two

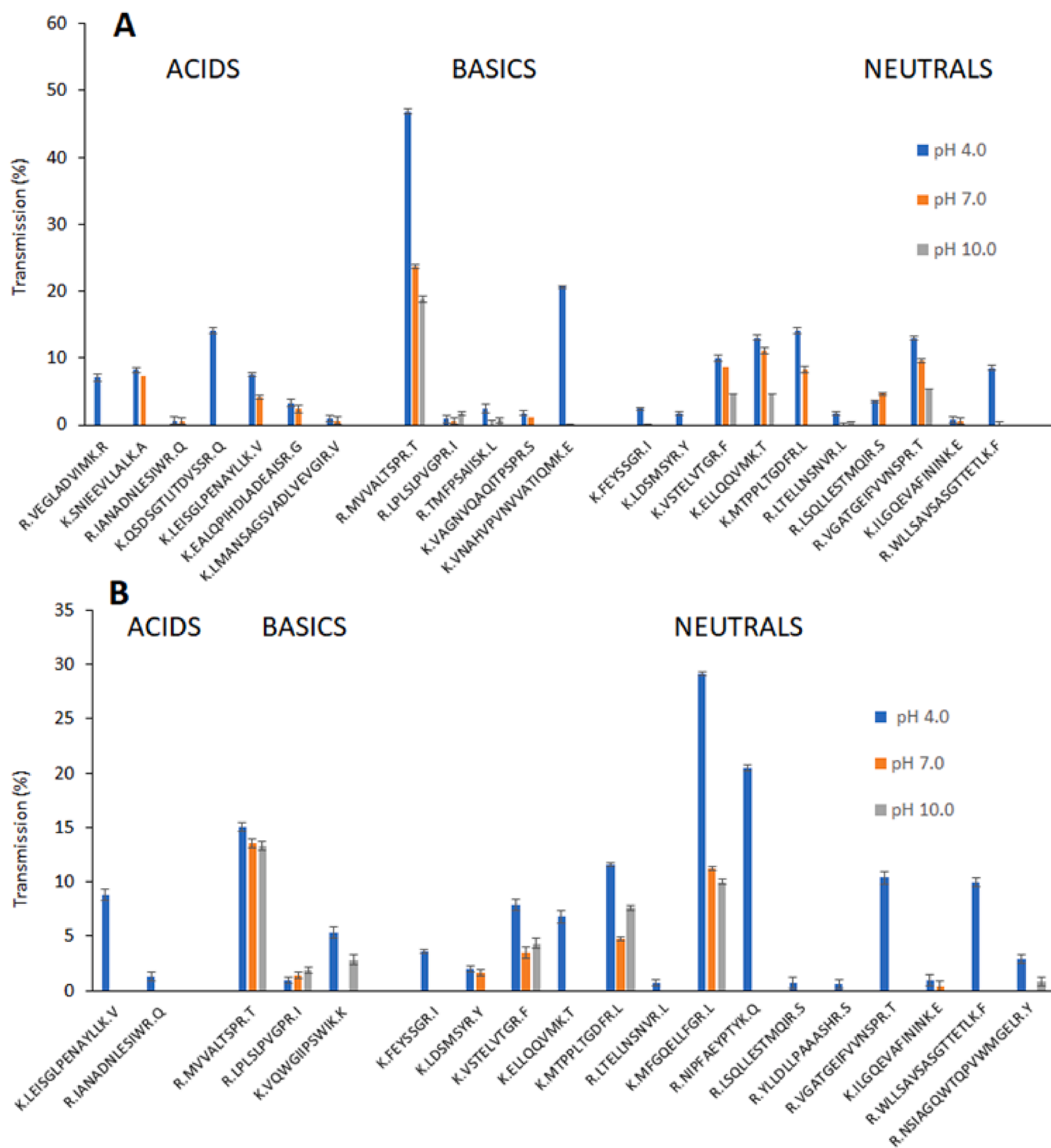

Fig. 3. Observed peptide transmission using the polyethersulfone (PES) membrane (A) and the stabilized cellulose (SC) membrane (B). The identified peptides were divided into three categories according to their pI values: acid peptides (pI < 5.0), neutral peptides (5.0 < pI < 8.0) and basic peptides (pI > 8.0) (Lapointe et al., 2005).

different ultrafiltration membranes at several pH values in order to pre-evaluate the best conditions to isolate them from the hydrolysate mixture. In this sense, some of the peptides highlighted by Zambrowicz, Eckert, et al. (2015) and Eckert, et al. (2019), such as LAPSLPGKPKPD and QSLVSVPGMS, are peptides that may not be isolated for commercial purposes after hydrolysing the whole egg yolk or its fractions because they come from proteins that cannot be found in the plasma nor in the granular subfractions, but from quantitatively minor proteins.

All in all, every peptide detected in the permeate streams was able to pass through the membranes, to a greater or lesser extent, when the ultrafiltration was performed at pH 4.0. Among all these peptides, nine were predicted to have antihypertensive properties, and of these nine peptides, eight could be found in the permeate when the SC membrane was used, while six of them were identified in the permeate when the ultrafiltration was carried out using the PES membrane. Therefore, considering the total number of different peptides identified in the permeate streams at pH 4.0 (Fig. 3A and 3B), the 42% and 27% of all the peptides found were predicted as antihypertensive when the SC and the PES membrane were used, respectively. In addition, when the PES membrane was used, no peptides were detected in any permeate with a bioactivity score higher than 0.5, but when the SC membrane was tested, the 26% of the peptide sequences detected showed a bioactivity score higher than 0.5.

3.3.3. Molecular docking

According to the AHTpin software, nine peptides found in the permeates at pH 4.0 were predicted as antihypertensive when the ultrafiltration experiments were carried out using the SC and PES membranes. In order to determine how these peptides are able to interact with the ACE active site, and to detect the peptide with the highest potential to be antihypertensive, molecular docking simulations between those peptides and ACE were conducted using the ADCP program, and for each peptide tested the four poses with the lowest docking score were considered (Table S1). In addition, in order to gain a deeper insight into

the binding mechanism between the most promising peptides obtained and the enzyme, the three different peptides with the pose with the lowest docking score were selected and further analysed (Fig. 4). According to the results obtained, these peptides were K.VQWGIIPSWIK.K, with a binding score of −29.4 kcal/mol; R.YLLDLLPAAASHR.S, with a binding score of −28.1 kcal/mol, and K.QSDSGTLITDVSSR.Q, with a binding score of −26.7 kcal/mol.

As reported by other authors, ACE has three main active site pockets with which the inhibitors interact, these being S1, which includes Ala354, Tyr523 and Glu384; S1′, which includes Glu162, and S2′, which includes Gln281, His353, Tyr520, Lys511 and His513 (Yu, Chen, Zhao, Li, Liu, & Chen, 2018). Considering their amino acid composition, these pockets have a high degree of hydrophobicity, so peptides with a high number of hydrophobic amino acids will be favoured for interaction with the ACE active centre, producing a higher degree of inhibition. According to Yu et al. (2018), peptides with Trp, Tyr, Phe, Pro, Ile, Ala, Leu and Met in the composition of the three amino acids at the end of the C-terminal have a high probability of possessing greater ACE inhibitory activity, and in fact, K.VQWGIIPSWIK.K showed the lowest docking score according to the results shown in Table S1. In any case, the three peptides with the lowest docking score were able to form hydrogen bonds with the ACE active site (Fig. 4), hydrogen bonds being considered to be one of the most important stabilizing interactions that can be formed between peptides with antihypertensive properties and this enzyme (Chaudhary, Vats, Chopra, Biswas, & Pasha, 2009). The peptide K.VQWGIIPSWIK.K was able to establish eight hydrogen bonds with the active centre of the enzyme, two of them with the S1 pocket, and one with the S2′ pocket; however, R.YLLDLLPAAASHR.S and K.QSDSGTLITDVSSR.Q were able to establish more hydrogen bonds with the ACE active site, and in particular, K.QSDSGTLITDVSSR.Q, was able to establish five interactions with S2′ and one with S1. In this sense, it has also to be considered that the peptide with the highest affinity for the enzyme, K.VQWGIIPSWIK.K, was able to establish two interactions with the zinc ion in the ACE active site, bearing in mind that the zinc ion plays a key role in the inhibition of the ACE activity (Fu et al., 2019). One of these two interactions was between the carbonyl group of the Ile10 and the zinc ion, but the other one was between the carboxylate group of the Lys11 and the zinc ion, the latter being stronger than the former. In addition, the other two peptides interacted with the zinc ion as well, but only through a carbonyl group. Finally, it has to be also considered that the scoring function of ADCP is a composition of two individual scores, one for the conformation of the peptide, and another one for the interaction between the peptide and the enzyme, and the summation of these two scores resulted in the lowest docking score for the K.VQWGIIPSWIK.K peptide.

All in all, K.VQWGIIPSWIK.K appears to be the most promising antihypertensive peptide produced by the tryptic hydrolysis of the lipovitellins and can be found in the permeates analysed when the SC membrane is used.

4. Conclusions

The hydrolysate resulting from the hydrolysis with trypsin of the isolated lipovitellins contained 26% of peptides with predicted antihypertensive activity, but after the ultrafiltration of the hydrolysate at pH 4.0 using the SC membrane, the percentage of peptides identified and predicted to have antihypertensive properties in the permeate stream was 42%. In addition, the peptide with the highest bioactivity score in

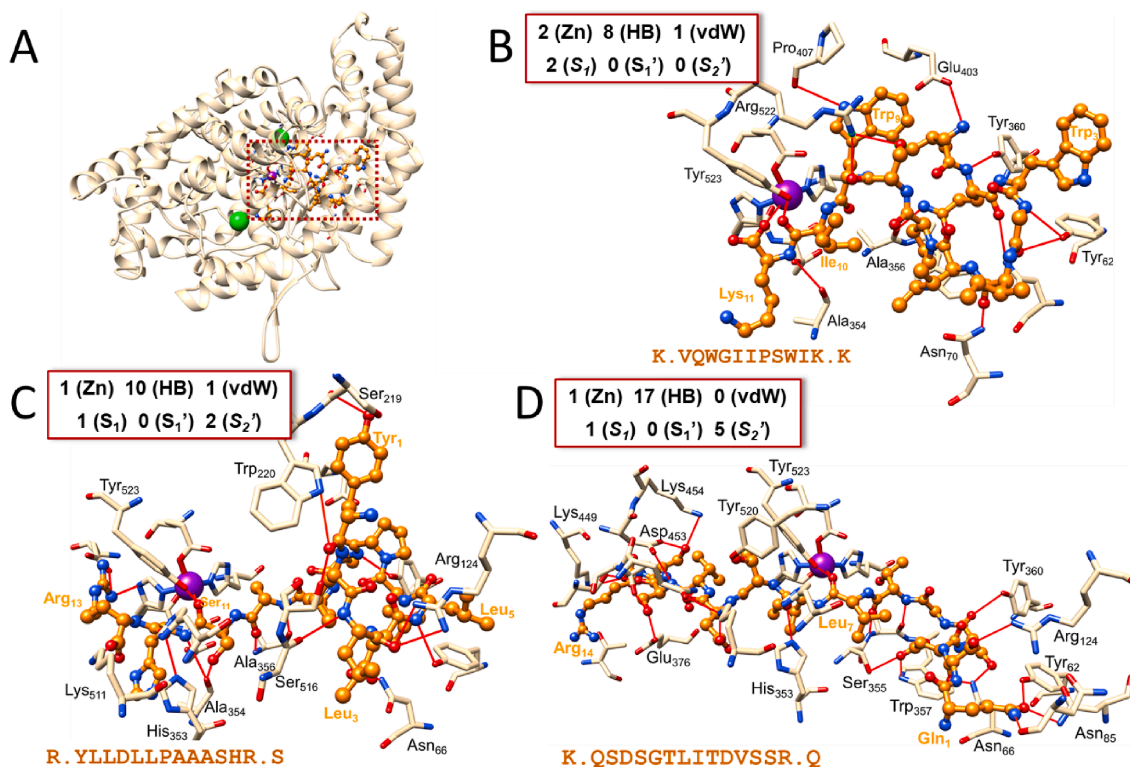

Fig. 4. A) Ribbon representation of the complex between ACE and the K.VQWGIIPSWIK.K peptide corresponding to the most favoured docking pose. Zinc (in magenta) and chloride (in green) ions are shown in CPK spheres. B) Close view of the enzyme-ligand contacts in the ACE/K.VQWGIIPSWIK.K complex. The ligand atoms are shown in ball-and-stick (C in orange, O in red and N in blue). C) and D) Close views of the most favoured ACE/R.YLLDLLPAAASHR.S and ACE/K.QSDSGTLITDVSSR.Q docking complexes, respectively. The number of ACE···peptide contacts is indicated for each complex (Zn: Zn···O interactions, HB: hydrogen bond and vdW: van der Waals). The number of contacts with the ACE residues at the S_1 (Ala$_{354}$, Glu$_{384}$, and Tyr$_{523}$), S_1′ (Glu$_{162}$), and S_2′ (Gln$_{281}$, His$_{353}$, Lys$_{511}$, His$_{513}$, and Tyr$_{520}$) binding sites are also included (residue numbering as in the 1O86 PDB structure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the hydrolysate was also found in the permeate. This underlines the importance of the composition of the membrane matrix and the relevance of the electrostatic interactions between the peptides and the membrane in the ultrafiltration process in order to isolate bioactive peptides. Furthermore, according to the results of the molecular docking tests performed, all the peptides identified in the permeate stream and predicted as antihypertensive were able to dock to the ACE active site. Finally, it should be noted that this work opens the door for future research with respect to trypsin hydrolysis of the lipovitellins fraction, since the permeate obtained using the SC membrane at pH 4.0 contains a mixture of peptides with promising antihypertensive properties and could therefore be tested *in vivo* as a crude product. Additionally, the peptides identified here with a higher transmission rate and/or with the highest predicted bioactive properties could be further purified in order to be tested *in vivo*.

CRediT authorship contribution statement

Ismael Marcet: Methodology, Investigation, Writing – review & editing, Formal analysis, Writing – original draft, Visualization. **Jaime Delgado:** Investigation, Formal analysis. **Natalia Díaz:** Methodology, Investigation. **Manuel Rendueles:** Writing – review & editing, Funding acquisition, Supervision, Resources. **Mario Díaz:** Funding acquisition, Supervision, Resources.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132145.

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