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Gastrointestinal digestion enhances the endothelium-dependent vasodilation of a whey hydrolysate in rat aortic rings

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ABSTRACT

Whey proteins present encrypted biofunctional peptides that need to be released from the native protein to exert their biological activity. Antihypertensive whey peptides are the most studied ones, which can be explained by high prevalence of this chronic degenerative disease. The present study investigated whether the molecular changes occurred during the gastrointestinal digestion of a whey protein hydrolysate could modulate its vasorelaxant potential in rat aortic rings. Spectrophotometric data and SDS-PAGE gel showed a small degree of hydrolysis during the gastric phase and intense intestinal proteolysis. RP-HPLC revealed the formation of a large peptide profile. During the simulated digestion, 198 peptides were generated and identified and, left-shifted the concentration-response curve of the endothelium-dependent vasorelaxation, as recorded for the digested hydrolysates. In conclusion, gastrointestinal digestion of the whey hydrolysate leads to the generation of bioactive peptides with enhanced vasodilatory potency, reinforcing the relevance of whey-derived products in blood pressure regulation.

1. Introduction

Digestion is a complex process in which foods, after ingestion, pass through different mechanical and enzymatic transformations to micro and macronutrients that can be used by the body for numerous functions, such as growth, energy, cells maintenance and replacement (Guerra et al., 2012). Among macronutrients, proteins present encrypted fragments with biological activity, named bioactive peptides. These peptides are not active within the parent protein and need to be released during food processing and/or gastrointestinal digestion to be further absorbed and reach the target organs (Nongonierma & Fitzgerald, 2015).

Whey proteins present several of these encrypted peptides, being associated with different health benefits, including antihypertensive, antithrombotic, anticarcinogenic and immunomodulatory effects (Bulut Solak & Akin, 2012; Nongonierma & Fitzgerald, 2015; O'Keeffe & FitzGerald, 2018). Antihypertensive whey peptides are subject of various studies, which is justified by the high prevalence of this chronic degenerative disease. Moreover, along the years people are becoming more health conscious and tendentious to replace allopathic medicines for natural treatments and functional foods (Beltrán-Barrientos, Hernández-Mendoza, Torres-Llanez, González-Córdova, & Vallejo-Córdoba, 2016). Whey peptides may lower blood pressure (BP) through different mechanisms, such as inhibition of angiotensin-I converting enzyme (ACE), renin and pro-renin, blockage of calcium channels and AT1 receptors, and stimulation of nitric oxide production (Udenigwe & Mohan, 2014). In this sense, our research group started to investigate the relationship between different technological conditions, including scaling up the process and the comparison of drying methods with the preservation of the vasorelaxant activity of whey peptides (Mellinger-Silva et al., 2015; Ozorio, Pereira, et al., 2019; Ozorio, Silva, et al., 2019).

These byproducts of the dairy industry, however, are rarely commercialized as bioactive products, highlighting the need to continue the studies in this field to confirm its biological effect.

However, one subject that still deserves attention concerns the effects of food processing and digestion on the release of the antihypertensive peptides. Indeed, bioactive peptides may be released from

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the parent protein through chemical or enzymatic hydrolysis but their profile may completely change after gastrointestinal digestion (Phelan & Kerins, 2011; Sinha, Radha, Prakash, & Kaul, 2007). Studying the release of bioactive peptides during gastrointestinal digestion directly in humans would be the more effective and relevant approach. However, studying multistage processes *in vivo* is very complex, costly and involves several restrictions for ethical reasons (Guerra et al., 2012; Ménard et al., 2014). The INFOGEST network on food digestion proposed an international consensus for *in vitro* digestion (Minekus et al., 2014) that is being currently validated with other *in vitro* and *in vivo* data (Egger et al., 2016a, 2016b; Sanchón et al., 2018) and may provide us some immediate answers about the modifications that occur with these peptides during digestion. In this sense, the present study aimed to investigate the hypothesis that biochemical transformations occurring in whey protein hydrolysate during gastrointestinal digestion may result in different profiles of vascular effect. To explore this hypothesis, the whey protein hydrolysate was subjected to a simulated gastrointestinal digestion, and the vascular activity of the resulting products was evaluated in rat aortic rings maintained in organ baths (Mellinger-Silva et al., 2015). Our results disclosed a new piece of evidence regarding the vasorelaxant potential of whey peptides.

2. Materials and methods

2.1. Materials

Bovine whey protein concentrate 88% of protein (WPC88) was donated by Alibra Ingredientes Ltda (Campinas, SP, Brazil) and was used as substrate. Commercial pepsin (E.C. 3.4.23.1) from porcine gastric mucosa was donated by Bela Vista Produtos Enzimáticos Ind. e Com. Ltda. (Bela Vista, SC, Brazil). A commercial kit (bile acid kit, 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany) was used to determine the concentration of bile salts in the bile. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany).

2.2. Whey enzymatic hydrolysis

The enzymatic hydrolysis of the WPC88 was performed in pilot scale (100 L) according to Ozorio, Silva, et al. (2019). Briefly, the WPC88 was suspended at a concentration of 1.25% (w/v), and the pH was adjusted to 2 with 1 M HCl. Pepsin (5.34 $\mu U \cdot g^{-1}$ of protein) was added and the hydrolysis occurred at constant stirring for 3 h at 37 °C. The enzymatic activity of pepsin was measured according to Anson (1938). The pH and the temperature were monitored during the entire experiment. The reaction was stopped by increasing the temperature to 80 °C for 5 min. Whey hydrolysate was then spray dried (Niro atomizer, Copenhagen, Denmark; inlet temperature: 170 °C; outlet temperature: 90 °C) and the hydrolyzed powder was maintained frozen (−20 °C) until further analysis.

2.3. Whey hydrolysate in vitro digestion

The *in vitro* gastrointestinal digestion of the whey hydrolysate was performed according to the harmonized INFOGEST protocol (Minekus et al., 2014) with slight modifications. Briefly, a whey hydrolysate solution (8% w/v) was prepared and added to a simulated gastric fluid containing pepsin from porcine gastric mucosa (2000 $U \cdot mL^{-1}$, EC 3.4.23.1, Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 50:50 (v/v). The gastric digestion occurred for 2 h in a water bath (Grant, OLS 200, Cambridgeshire, England) under constant stirring (100 rpm). Samples were collected at 1, 10, 30, 60 and 120 min and added to microtubes containing 0.5 $mg \cdot mL^{-1}$ of the protease inhibitor pepstatine A (P5318, Sigma-Aldrich, St. Louis, MO, USA). After 120 min, the pH of the gastric mixture was adjusted to 7 with 2 M NaOH to perform the intestinal digestion and the solution was mixed to a simulated intestinal fluid (50:50 v/v) containing trypsin (100 $U \cdot mL^{-1}$ of final mixture), chymotrypsin (25 $U \cdot mL^{-1}$ of final mixture) and bile (10 $mmol \cdot L^{-1}$ of final mixture). All enzyme activities and bile salts concentration were determined as described by Minekus et al. (2014). Samples were withdrawn at 1, 10, 30, 60 and 120 min and added to microtubes containing the protease inhibitor pefabloc (0.1 M, 76307, Sigma-Aldrich, St. Louis, MO, USA). All samples were maintained at −20 °C until further analysis. As the samples investigated in this study contain high protein/peptide concentration, authors chose not to perform the oral phase of the *in vitro* digestion.

2.4. Degree of hydrolysis

The release of free amino groups (NH_2) was measured according to the OPA (orthophtaldialdehyde) method described by Darrouzet-Nardi, Ladd, and Weintraub (2013) with slight modifications. The OPA reagent was prepared with ethanol and 700 μL of 1 M DTT (DL-Dithiothreitol, D0632, Sigma) instead of methanol and β-mercaptoethanol, respectively. The assay was carried out by adding 100 μL of the OPA/DTT reagent to 50 μL of the samples which were previously diluted (1:40 v/v). The OPA when reacting with free amino groups and with a SH-compound forms a compound that absorbs light at 340 nm. The 96-well plate was measured after 10 min of incubation (Multiscan Go, Thermo Scientific). A standard curve was prepared using methionine standard solutions (Sigma-Aldrich) and the degree of hydrolysis was expressed as mg $NH_2 \cdot g^{-1}$ of sample, subtracting the controls. The assays were performed in triplicate and the results were submitted to a one-way analysis of variance (ANOVA) followed by *t*-test, using the XLSTAT software (Addinsoft, 2019). A p value < 0.05 was considered statistically significant.

2.5. Protein electrophoresis

The SDS-PAGE protein electrophoresis was performed according to Laemmli (1970). Samples were diluted (1:50 v/v) in Invitrogen NuPAGE LDS sample buffer 4× (Thermo Fisher Scientific, California, USA) and applied to the NuPAGE™ 4–12% Bis-TRIS gels. Besides the whey hydrolysate digested and undigested samples, two control samples were used: pepsin in gastric simulated fluid; trypsin and chymotrypsin in intestinal simulated fluid, both fluids prepared as described by Minekus et al. (2014). The electrophoretic run occurred throughout 90 min at 50 mA and 100 V using a Bio-Rad Mini PROTEAN® Tetra Cell (California, USA). Gels were fixed (fixing buffer: 40% ethanol; 10% acetic acid) for 30 min, then stained overnight (BioRad Bio-Safe Coomassie G-250). After discoloration, gels were scanned using G:BOX by Syngene (Cambridge, UK).

2.6. Reversed phase – High performance liquid chromatography (RP-HPLC)

The WPC88, the whey hydrolysate and the digested whey sample solutions were analyzed using an analytical HPLC unit from Jasco (Jasco Corporation, Japan) coupled with a Hypersil BDS C18 column (100 × 4.6 mm, particle size 2.4 μm, Thermo Scientific, USA), according to Ozorio, Pereira, et al. (2019). Samples were injected in a total volume of 20 μL and the chromatographic runs were of 40 min with a flow rate of 1.0 $mL \cdot min^{-1}$ at room temperature (approximately 20 °C). Solutions of 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v) and 0.1% TFA in acetonitrile (ACN) (v/v) were used as solvents A and B, respectively. Hydrolysates were eluted as follows: 0–2 min, 5% B; 2–15 min, 5–20% B; 15–20 min, 20–30% B; 20–25 min, 30–40% B; 25–28 min, 40–50% B; 28–32 min, 50–60% B; 32–34 min, 60–70% B; 34–36 min, 70–80% B; 36–38 min, 80–5% B; 38–40 min, 5% B. Peptides were detected at 216 nm.

2.7. Tandem mass spectrometry

Mass spectrometry (MS) analysis was conducted as previously described by Deglaire et al. (2016). A nano-RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source was used. Digested samples were diluted 1000 times in the injection buffer and filtered (0.45 μm cut-off) before concentration on a μ-precolumn pepMap100 (C18 column, 300 μm i.d. × 5 mm length, 5 μm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) and separation on a PepMap RSLC column (C18 column, 75 μm i.d. × 250 mm length, 3 μm particle size, 100 Å pore size; Dionex).

Peptide separation was performed at a flow rate of 0.3 $\mu L.min^{-1}$ using solvents A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA] and B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA]. The elution gradient first rose from 5 to 35% solvent B over 40 min, then up to 85% solvent B over 3 min before column re-equilibration. The mass spectra were recorded in positive mode using the *m/z* range 250–2000. The resolution of the mass analyzer for *m/z* of 200 amu (atomic mass unit) was set in the acquisition method to 70,000 for MS and 17 500 for MS/MS. For each MS scan, the ten most intense ions were selected for MS/MS fragmentation and excluded from fragmentation for 20 s.

Peptides were identified from the MS/MS spectra using the X!TandemPipeline software (https://pubs.acs.org/doi/abs/10.1021/acs.jproteome.6b00632J. Proteome Res. 16, 2, 494–503) against a homemade database composed of major milk proteins to which was added the common Repository of Adventitious Protein (http://thegpm.org/crap). The possible post-translational modifications were serine or threonine phosphorylation, methionine oxidation. Peptides identified with an e-value < 0.05 were automatically validated. The peptide false discovery rate was less than 0.6%. Peptides with angiotensin-I-converting enzyme inhibitory activity, were identified using the Biopep database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

2.8. In vitro vascular relaxation

The procedures described in this study were approved by the Institutional Animal Care and Use Committee of Universidade Federal de Santa Catarina (Florianópolis, SC, Brazil; authorization number 5371190815). We used male Wistar rats (300–350 g), provided by our Central Vivarium, housing in ventilated cages under controlled temperature (22 ± 2 °C) and light/dark cycle (12/12 h), with water and chow *ad libitum*.

For these experiments, the animals (n = 6) were euthanized by anesthetic overdose using ketamine and xylazine (140 and 40 mg/kg) administered by intraperitoneal route and the thoracic aorta was immediately removed and placed in Petri dishes containing cold (~4 °C) physiological saline solution (PSS, composition in mM: 131.3 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4{\cdot}7H_2O$, 14.9 $NaHCO_3$, 5.5 D-glucose, 1.6 $CaCl_2{\cdot}2H_2O$, 0.08 EDTA, pH 7.4). The arteries were cleaned from adipose and connective tissues, cut in rings with 3–4 mm length, and placed in organ baths contained warmed PSS (37 °C) continuously bubbled with 95% O_2/5% CO_2. The vascular tone was detected by isometric transducers and recorded by a computer-coupled data acquisition hardware and software (PowerLab® and LabChart v. 7.4, respectively, both from AD Instruments, Castle Hill, Australia).

The aortic rings were allowed to stabilize for 60 min under a basal tone of 3 g, and were stimulated using a modified PSS containing 120 mM KCl (composition, in mM: 14.4 NaCl, 119.9 KCl, 1.6 $CaCl_2{\cdot}2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$, 0.5 D-glucose, 1.6 $CaCl_2{\cdot}2H_2O$, 0.08 EDTA, pH 7.4), in order to assess the contractile responses of each preparation, a procedure commonly used to verify the integrity of preparations studied in organ bath apparatus. A new interval was allowed, and the vasodilatory effect of acetylcholine (1 $\mu mol{\cdot}L^{-1}$) under the tonic contraction induced by phenylephrine (1 $\mu mol{\cdot}L^{-1}$) was evaluated. The experiments were performed in both endothelium-intact and endothelium-denuded vessels, which had the endothelial function disrupted by rubbing a small needle around the lumen. The effectiveness of endothelial removal was confirmed by the complete absence of vasodilatory responses to acetylcholine, and only vessels that reached a relaxation greater than 90% were used as endothelium-intact preparations. Following evaluation of endothelial function, the baths were washed for removal of vasoactive agents. After 60 min of rest, cumulative concentrations of the undigested (1, 3, 5 and 10 $mg{\cdot}mL^{-1}$) and digested (1, 3, 10, 30, 50 and 100 $\mu g{\cdot}mL^{-1}$) whey protein hydrolysates were added in phenylephrine-contracted preparations. The results were expressed as the mean ± standard error of the percentage of maximal relaxation induced by the hydrolysates in at least six aortic rings obtained from different animals. The data were subjected to two-way analyses of variance (ANOVA) followed by Tukey's post hoc analyses. A value of $p < 0.05$ was accepted as statistically significant. Graphs and statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

3.1. Degree of hydrolysis and protein electrophoresis

The proteolytic activity during gastrointestinal digestion of the whey hydrolysate was estimated by the release of free amino groups. The results illustrated in Fig. 1 show an increase in the concentration of amino groups after gastrointestinal digestion, which ranged from 12.4 $mg{\cdot}g^{-1}$ to 24.2 $mg{\cdot}g^{-1}$. A small degree of hydrolysis was observed during the gastric phase, which was already expected, since pepsin had been previously used to hydrolyze the WPC88 and a more prominent digestion occurred during the intestinal phase. Even so, the release of these molecules was not very high, which can be an indicative of the formation of several peptides, instead of free amino acids. These results were confirmed by the SDS-PAGE gel (Fig. 2), which showed only a slight decrease in the β-lactoglobulin (β-lg) band that remained partially resistant to pepsin digestion. The resistance of β-lg to gastric digestion was previously reported by Bateman, Ye, & Singh, 2010 and Guo, Fox, Flynn, & Kindstedt, 1995, who attributed this finding to the β-lg stable globular configuration at low pH, as target amino acid residues are buried in the protein core. In contrast, an intense proteolytic activity was observed during the intestinal phase, leading to an intense β-lg hydrolysis in less than 10 min of intestinal digestion, which is possibly related to a conformational transition at pH above 7, exposing amino acid residues that are sensitive to trypsin and chymotrypsin hydrolysis (Diarrassouba et al., 2013; Sanchón et al., 2018).

3.2. RP-HPLC peptide profiles

The peptide profiles confirm what was aforementioned, showing the effects of processing and simulated gastrointestinal digestion in the hydrolysis of major whey proteins α-lactalbumin (α-la) and β-lg (Fig. 3B). In acid pH, α-la assumes a molten globule state, with a more flexible conformation during the early phases of the aggregation process, maintaining an intermediate folding with preservation of the alpha-helical domains and partial unfolding of the beta-sheet ones. Spontaneous rearrangements of the disulfide bonds occur forming α-la isomers and polypeptides. (de Laureto, De Filippis, Di Bello, Zambonin, & Fontana, 1995; Kamau, Cheison, Chen, Liu, & Lu, 2010; Kuwajima, 1996). Moreover, β-lg presents a stable globular conformation at pH 2, which was preserved during the hydrolysis process (Bateman et al., 2010). Following the simulated digestion, no significant changes were observed in the peptide profile of the gastric phase (data not shown), since pepsin was previously used to generate the whey hydrolysate. Then, after the intestinal phase, β-lg was hydrolyzed and a wide variety of peptides was obtained, including hydrophobic and hydrophilic

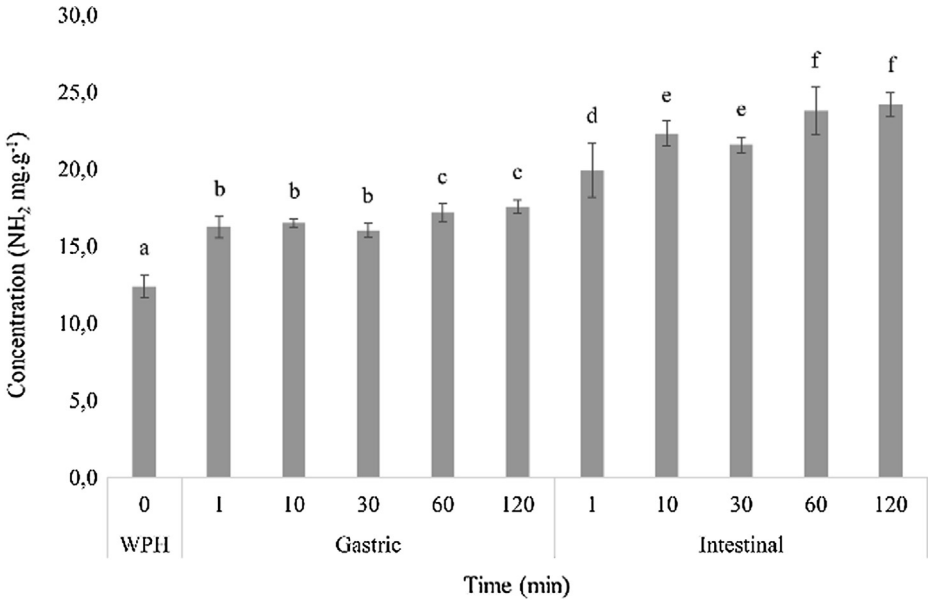

Fig. 1. Free amino acids release during whey hydrolysate *in vitro* gastrointestinal digestion. WPH – control sample, undigested whey hydrolysate. Letters indicate significant difference ($p < 0.05$).

molecules, as shown in Fig. 3C.

3.3. Peptide sequences

Several new peptides generated during the *in vitro* gastrointestinal digestion process were identified by MS analysis. Fig. 4 presents a heat map comparing peptide profiles of the undigested samples with gastric and intestinal whey digested samples, presenting the frequency, in percentage, that each amino acid appears as part of a peptide sequenced from a determined protein. β-lg and α-la were chosen to be part of the graph because of they are the main whey proteins, as well as κ-casein and β-casein, remained from the cheese-making process. All other peptides sequenced are listed in the Supplementary Table S1.

The partial resistance of β-lg to enzymatic hydrolysis was also confirmed by the MS analysis, once peptides from different portions of the protein were sequenced in the three fractions analyzed, showing that only part has been hydrolyzed to small peptides and free amino acids undetectable by the method used. Notably, the peptide VLDTDYK, which was previously described as an *in vitro* inhibitor of ACE (Hernández-Ledesma, Recio, & Amigo, 2008; Mann, Athira, Sharma, Kumar, & Sarkar, 2019; Tavares & Malcata, 2013), was found among the 56 newly generated peptides of the intestinal phase. As previously discussed, α-lactalbumin assumes a molten globule state in acid pH, with possible formation of polypeptides which were not identified by the MS method used. Hence, -only 17 peptides were sequenced from the different fractions of the whey analyzed.

Among the identified peptides, DKVGINY, also previously described as an *in vitro* inhibitor of ACE (Tavares & Malcata, 2013; Egger et al.,

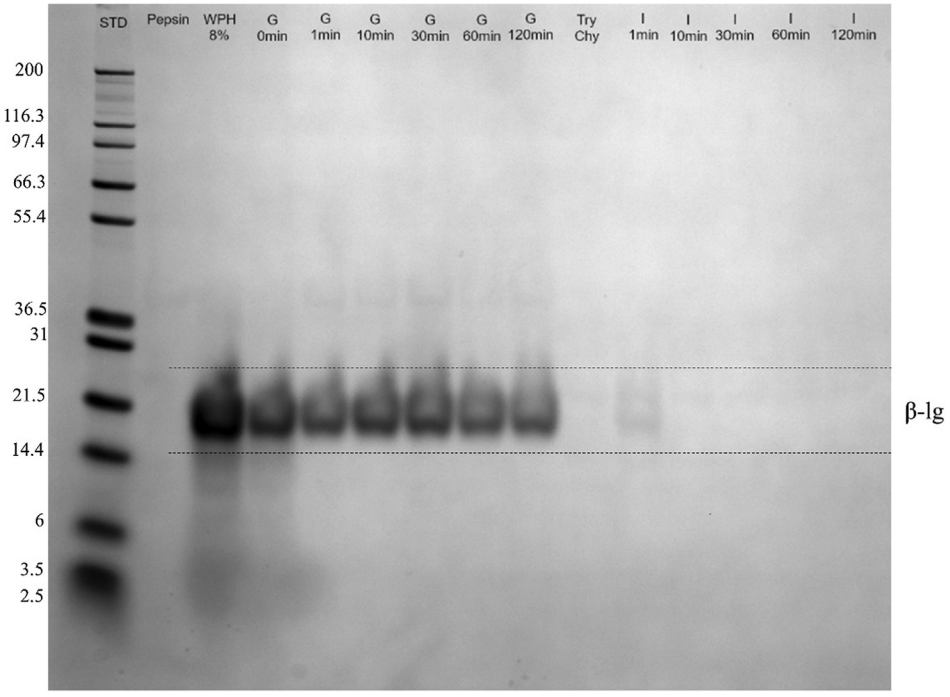

Fig. 2. Electrophoretic profile of undigested and digested whey hydrolysate samples. STD: standard solution (molecular weight in Daltons); Pepsin: pepsin diluted in gastric simulated fluid; Try, Chy: Trypsin and chymotrypsin diluted in gastrointestinal fluid; WPH 8%: whey protein hydrolysate 8% (w/v); G: Gastric phase; I: Intestinal phase; β-Lg: β-lactoglobulin; 0–120 min: time of digestion.

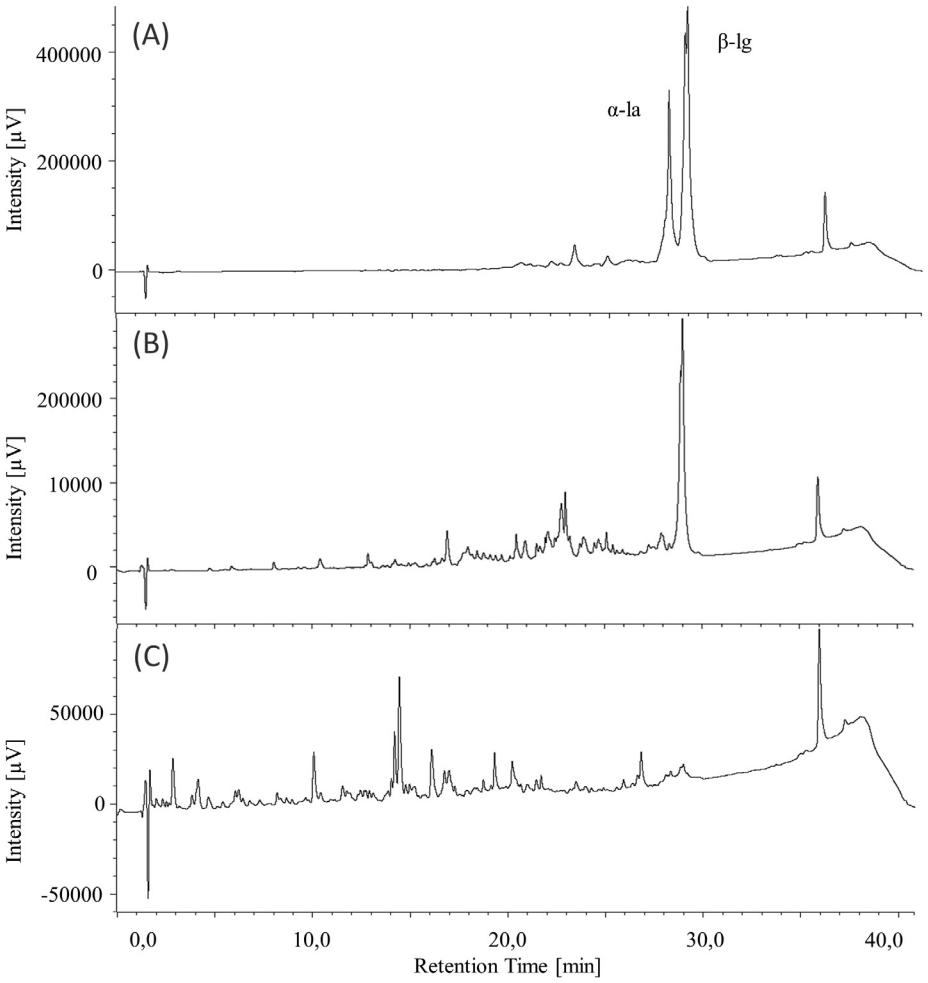

Fig. 3. RP-HPLC peptide profiles of (A) control sample – WPC88; (B) undigested whey hydrolysate; (C) intestinal digested whey hydrolysate. α-la: α-lactalbumin; β-lg: β-lactoglobulin.

2016a, 2016b) was found in the gastric digest. Nevertheless, DKVGINY was probably hydrolyzed by intestinal enzymes, once it could not be identified after enteric digestion.

Bovine milk presents endogenous proteases such as elastase, cathepsin D, cathepsin B, kallikrein, several carboxy- and aminopeptidases, and plasmin (Dallas, Murray, & Gan, 2015). Plasmin is capable of cleaving αS1-, αS2- and β-caseins on the carboxyl sides of lysine and arginine amino acid residues, releasing these casein peptides soluble in whey (Ismail & Nielsen, 2010; Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Moreover, during the cheese-making process κ-casein is cleaved between the Phe105-Met106 amino acids, being separated into two main fragments, the hydrophobic *para*-κ-casein (Glu1–Phe105) that

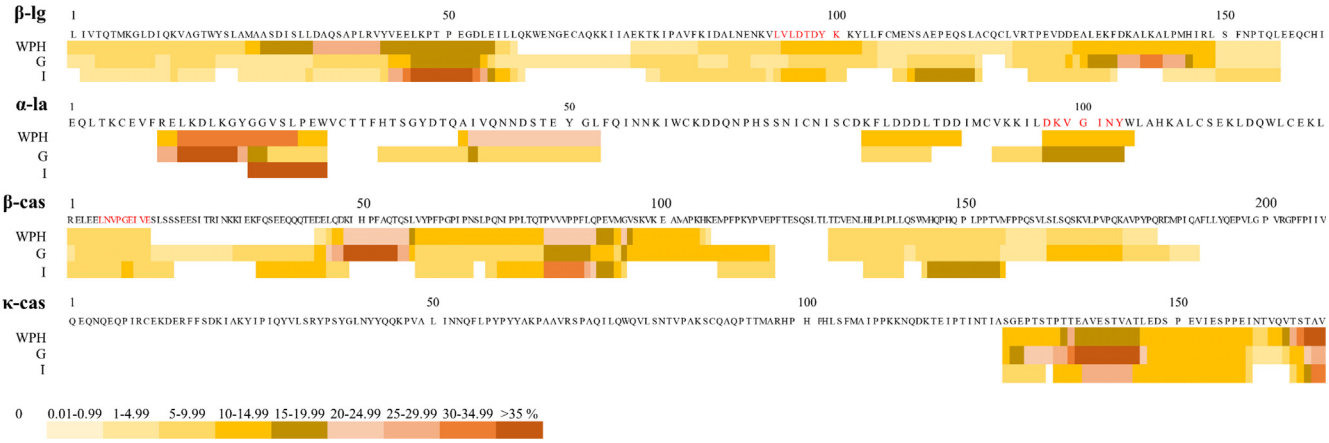

Fig. 4. Nano-RSLC – MS/MS heat map built with the frequency of appearance, in percentage, that each amino acid was identified as part of a peptide sequence from β-Lactoglobulin (β-lg), α-Lactalbumin (α-la), β-Casein (β-cas) and κ-Casein (κ-cas) after whey hydrolysate (WPH) *in vitro* gastrointestinal digestion. G: gastric phase; I: intestinal phase. White color represents no frequency and dark orange high frequency. Letters in red represent ACE inhibitory peptide sequences identified in the samples using the Biopep database. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

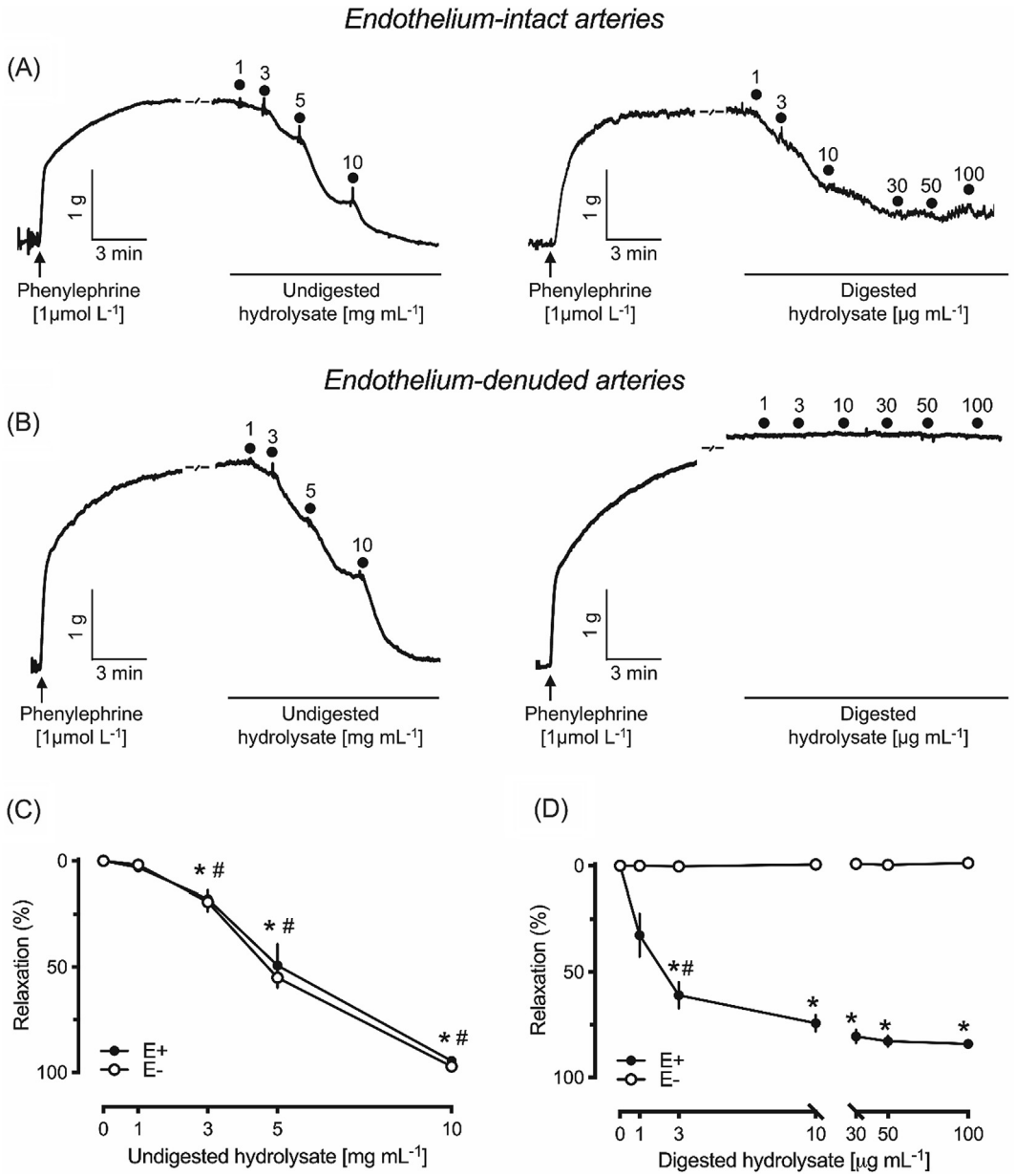

Fig. 5. Whey hydrolysates-induced vascular relaxation. Trace records of the vascular relaxation induced by undigested and digested whey hydrolysates in endothelium-denuded (A) and endothelium-intact (B) rat aortic rings. The mean ± SEM of relaxation of arteries from 6 different animals after incubation of the undigested and digested hydrolysates are presented in panels C and D, respectively. * indicates $p < 0.05$ compared with the maximal vascular tone before de addition of the hydrolysate. # indicates $p < 0.05$ compared with the previous concentration.

forms the cheese curd and the hydrophilic caseinomacropeptide (CMP) (Met106–Val169), which remains in whey. During this process, the casein micelle is broken, and part of the caseins may disperse in whey (Hallén, Lundén, Allmere, & Andrén, 2010; Kastberg Møller, Rattray, Sørensen, & Ardö, 2012), which also justifies the identification of β-casein (β-cas) peptides in the different whey fractions analyzed. The evolution of the β-cas digestion can also be observed in the heat map (Fig. 4), by the increasing blank spaces and by the reduction and enhancement of the intensity of the colors in different areas. Even so, LNVPGEIVE, another peptide with inhibitory effect on ACE in *in vitro* assays was found in the undigested and gastric digested samples, and both VVVPPF and VYPFPGPI were identified encrypted within the structure of other β-cas peptides from the intestinal fraction. Regarding κ-casein, 45 peptides were sequenced from its hydrophilic CMP portion, as revealed in Fig. 4. Among the 45 peptides identified, 8 present ACE-inhibitory peptides encrypted within their structures. Although this study has not been designed to explore the structure–function relationship between the peptides and their effectiveness as specifically ACE inhibitors, it is reasonable to suggest that their presence in the digested hydrolysate reinforce the putative beneficial effects of whey against cardiovascular diseases. However, it is important to mention that blood pressure regulation is not limited to the inhibition of ACE, which is only a part of complex mechanisms involving several pathways.

3.4. Influence of digestion on whey-induced vascular relaxation

The experiments performed to evaluate the vascular effects of both undigested and intestinal digested whey hydrolysates revealed that both presented vasodilatory activity, as illustrated in the typical records showed in Fig. 5A, which shows the concentration-dependent relaxation induced by undigested and digested hydrolysates in endothelium-

intact arteries previously stimulated by the α1-adrenergic receptor agonist phenylephrine. As shown in Fig. 5B and 5C, the vasodilatory effect induced by the undigested hydrolysate does not depend on the endothelial function, since there was no difference between the vascular relaxation obtained in endothelium-intact (maximal effect of 94.6 ± 1.2%) and endothelium-denuded (maximal effect of 97.2 ± 1.6%) arteries. Nonetheless, the gastrointestinal digested hydrolysate resulted in a maximal relaxation of 84.2 ± 1.6% in endothelium-intact preparations, but it was completely ineffective in arteries without endothelial function (Fig. 5D). Importantly, if in one hand the gastrointestinal digestion resulted in a loss of activity in endothelium-denuded arteries, on the other hand, the concentrations of the digested hydrolysate required to induce vasodilation in endothelium-intact preparations were significantly reduced, compared with the undigested hydrolysate. This finding clearly indicate that *in vitro* gastrointestinal digestion of the whey protein hydrolysate did generate vasoactive compounds that are fully dependent on endothelial function. Moreover, the half maximal effective concentration for the vasodilatory effect, a parameter often used to compare the potency of different drugs was reduced from 5017 (4740–5270) $\mu g \cdot mL^{-1}$ for undigested to 1.07 (0.75–1.51) $\mu g \cdot mL^{-1}$ for the digested hydrolysate. Thus, the molecular changes occurred during gastrointestinal digestion, including the generation of 198 identified peptides in gastric and intestinal phases, resulted in a significant increase in the potency of the vasodilatory effect of the whey hydrolysate, as well the generation of peptides associated with the endothelium-dependent vasodilation. Nitric oxide has been described as the primary vasodilator produced by endothelial cells in different arteries, including conductance vessels such as the aorta. Nevertheless, several other mediators produced by the endothelium are also associated with regulation of the vascular tone, including products of cyclooxygenase, reactive oxygen species, and unidentified substances that remain classified as endothelium-derived hyperpolarizing factors. Importantly, endothelial dysfunction is the main component behind several cardiovascular diseases (Konukoglu & Uzun, 2016). Additional experiments are being carried out by our research group to further explore the involvement of nitric oxide and other endothelium-derived products in the vasodilatory effect of the digested whey protein hydrolysate.

4. Conclusions

The gastrointestinal digestion of the whey hydrolysate generated a diverse molecular profile, composed by hydrophobic and hydrophilic peptides. Among these molecules, 198 peptides were sequenced from the gastric and intestinal digested samples, some of them previously described as ACE inhibitors. The molecular changes occurred during gastrointestinal digestion potentiated the endothelium-dependent vasorelaxant activity of the whey hydrolysate. Although the results obtained in the present study are preliminary, they are very promising, since it was demonstrated that the digestion process enhanced the potential effects of the WPH on the cardiovascular system. New research is being carried out to best evaluate the mechanisms involved in the absorption of the whey peptides and in the vascular relaxation promoted by them. Studies evaluating their biological potential *in vivo* need to be performed so as it can be further used as a biofunctional ingredient by food and pharmaceutical industries.

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Authors' contributions

Luisa Ozorio executed all the experiments, drafted and interpreted the manuscript; Natália K. Matsubara and José Eduardo da Silva-Santos were responsible for the vasorelaxant activity assays in rat aortic rings; Gwenaële Henry helped with the *in vitro* digestion and degree of hydrolysis assays; Yann Le Gouar contributed with the SDS-PAGE electrophoresis; Julien Jardin performed the mass spectrometry analysis; Caroline Mellinger-Silva designed the study and interpreted the results; Lourdes M. C. Cabral supervised the study; Didier Dupont designed and supervised the study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109188.

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