Bioactivity and peptide profile of whey protein hydrolysates obtained from Colombian double-cream cheese production and their products after gastrointestinal digestion

Bioactivity and peptide profile of whey protein hydrolysates obtained from Colombian double-cream cheese production

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ABSTRACT

Peptides obtained from whey proteins have been associated with different biological properties, which should be retained during the digestive process. In this work, hydrolysates were prepared from a whey protein concentrate obtained by ultrafiltration of Colombian traditional cheese whey and subsequent enzymatic hydrolysis using alcalase, chymotrypsin and flavourzyme. The antioxidant and ACE inhibitory properties were evaluated, and peptide profile was determined by UHPLC-MS/MS. Changes in biological properties and peptide profile after simulated gastrointestinal digestion were investigated. The alcalase hydrolysate showed higher ABTS radical scavenging capacity. An inhibition greater than 85% of ACE activity in the alcalase and chymotrypsin hydrolysates was exhibited. Different changes in biological activity after gastrointestinal digestion were observed: ABTS radical scavenging ability in all hydrolysates was increased and; in contrast, ACE inhibitory capacity decreased significantly in the alcalase and chymotrypsin hydrolysates. The antioxidant and ACE inhibitory properties in the flavourzyme hydrolysate remained stable after *in vitro* digestion. Differences in peptide profile in the hydrolysates were found. Tripeptides and tetrapeptides were mainly formed by alcalase, and medium and long peptides by chymotrypsin and flavourzyme. Resistance percents of peptides initially identified in the alcalase, chymotrypsin and flavourzyme hydrolysates were 65, 21 and 29%; respectively, after *in vitro* digestion under simulated conditions. Therefore, the alcalase hydrolysate obtained from ultrafiltrated whey protein concentrate showed antioxidant and ACE inhibition capacities and higher survivability of peptides after *in vitro* gastrointestinal digestion.

Keywords: Whey protein, Enzymatic hydrolysis, Antioxidant, ACE inhibition, Peptides, *In vitro* digestion

INTRODUCTION

Whey proteins are an important source of biologically active peptides that must be released by proteolytic processes such as enzymatic hydrolysis to exert a function. This process can be affected mainly for enzyme specificity that determines the number, size and biological activity of the peptides produced (Power, Jakeman, & FitzGerald, 2013). Although some peptides can be released naturally during digestion, the use of different enzymes could generate other fragments with a wide range of activities (Tavano, 2013). Proteases of different origins have been used in the production of bioactive peptides. These peptides can express diverse biological activities, such as antioxidants and ACE inhibitors, which are associated to the amino acid composition and its order in sequence of the peptides (Adjonu, Doran, Torley, & Agboola, 2013; Shazly et al., 2017; Zhang, Wu, Ling, & Lu, 2013).

Peptides with antioxidant activity can inactivate reactive oxygen species (ROS), act as free radical scavengers, inhibit the lipid peroxidation or chelate metal ions. ROS and free radicals can promote the development of cardiovascular diseases (Power et al., 2013; Sarmadi & Ismail, 2010). ACE inhibitory peptides have received considerable attention for their ability to prevent hypertension. ACE is an enzyme that converts angiotensin I to the potent vasoconstrictor, angiotensin II, and inactivates the vasodilator bradikinin (Norris & FitzGerald, 2013). In addition, hypertension is a risk factor for cardiovascular diseases that are the leading cause of death globally (World Health Organization, 2017).

The structure of peptides and consequently their activity can be affected *in vivo* by gastrointestinal digestion and subsequent absorption where proteolytic processes are involved which could cause degradation and inactivation of these peptides. The applicability of bioactive peptides depends on their ability to resist these processes and reach the target organ in an active form (Fu, Young, Dalsgaard, & Therkildsen, 2015; Sarmadi & Ismail, 2010). For example, peptides with low molecular weight could remain stable to these barriers

and exert their activity at the tissue level, while long-chain peptides are probably degraded by brush-border or cellular peptidases causing a loss of their function (Guo et al., 2018).

Double cream cheese is a Colombian stretched-curd cheese traditionally made from raw cow milk, which represents more than 30% of total cheese consumption in Colombia, with increasing production in recent years (Herrera, García-López, & Santos, 2016). Whey is a co-product obtained in this process that contains a variety of components with potential application in food industry. In this work, whey obtained in the manufacture of double cream cheese was ultrafiltered to produce a protein concentrate. Subsequently, whey protein concentrate was hydrolyzed with alcalase, chymotrypsin and flavourzyme; and the antioxidant and ACE inhibitory properties and peptide profile of the hydrolysates, before and after simulated gastrointestinal digestion made according to the standardised INFOGEST protocol (Minekus et al., 2014), were investigated.

MATERIALS AND METHODS

Materials

Double cream cheese whey was obtained from the Laboratory of Dairy Products of the Universidad Nacional de Colombia, Medellín. Alcalase 2.4L (endoproteinase from *Bacillus licheniformis*) and Flavourzyme 1000L (peptidase from *Aspergillus oryzae*) were supplied by Novozymes (Bagsvaerd, Denmark) and α-Chymotrypsin from bovine pancreas (≥ 40 units/mg protein) was purchased from Sigma-Aldrich (St.Louis, MO, USA). All other chemicals and reagents used were of analytical grade obtained from either Sigma–Aldrich or Merck.

Preparation of a whey protein concentrate (WPC)

Whey (500 mL) was subjected to ultrafiltration by using a tangential ultrafiltration module Vivaflow 50R equipped with a 10 kDa regenerated cellulose membrane (Hydrosart) and an

active membrane area of 50 cm^2 (Sartorius, Goettingen, Germany). The sample was concentrated 20-fold, lyophilized and stored at -20°C.

Enzymatic hydrolysis of WPC

WPC was resuspended in deionized water at a concentration of 1% (w/v) on a protein basis and the desired solution pH was adjusted by 1 M NaOH to obtain the optimal value for each enzyme. The hydrolysis of WPC was performed following the parameters reported in Table 1. The enzyme/substrate ratio and time of hydrolysis were previously optimized. Samples were heated at 85°C for 10 min and placed in an ice bath to inactive the enzymes. Insoluble solids were removed by centrifugation at 5000 *g* for 10 min and supernatants were adjusted to pH 7.0, lyophilized and stored a -20 °C until further analyses.

Degree of hydrolysis

The degree of hydrolysis (DH) was determined using the *o*-phthaldialdehyde (OPA) method proposed by Spellman, McEvoy, O'Cuinn, & FitzGerald, (2003).The OPA reagent (100 mL) was prepared by combinating the following solutions: 10 mL of 50 mM OPA (in methanol) and 10 mL of 50 mM N-Acetyl-L-Cysteine (NAC), 5 mL of 20% (w/v) sodium dodecyl sulfate (SDS) and 75 mL of borate buffer (0.1 M; pH 9.5). The reagent was protected from light and stirred at least 1 h before use. For assay, 10 µL of sample (or standard) was mixed with 1.2 mL of OPA reagent and the absorbance was recorded at 340 nm after 2 min. A calibration curve was prepared using leucine (0–2 mg/mL). The degree of hydrolysis (DH) was calculated according to the methodology described by Anzani et al., (2018).

Determination of protein and free amino acid content

The protein content of WPC and the resulting hydrolysates was determined by the Kjeldahl method of the AOAC (AOAC INTERNATIONAL, 2002). To determine the free amino acid content, 95 µL of each hydrolysate (10 mg/mL) were mixed with 5 µL of 5 mM Norleucine, as internal standard. A 1.25 mM amino acid standard solution (Sigma-Aldrich, St. Louis, MO, USA) with Norleucine was also prepared. Then, 10 µL of sample and standard were

derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate (AQC) using an AccQ-Fluor reagent kit (Waters WAT052880). The derivatized sample or standard were diluted with 100 µL of deionized water and analyzed by UPLC-ESI-MS system with the following conditions: column, RP Acquiry UPLC BEH C18 (1.7 µm, 150 x 2.1 mm, 300 Å); elution system: eluent A, water with 0.1% formic acid, eluent B, acetonitrile with 0.1% formic acid; gradient, 7 min 100% A, 21 min lineal from 100% A to 75.6% A, plus washing step and reconditioning; column temperature, 35 °C; sample temperature, 18 °C; injection volume, 4 µL; flow rate, 0.2 mL/min. MS parameters: ionization type, ESI + (positive ions); capillary voltage, 3.2 kV; cone voltage, 30 V; source block temperature, 150 °C; desolvation temperature, 300 °C; cone gas flow, 100 L/h; desolvation gas flow, 650 L/h. MassLynx™ V4.0 software was used for data processing (Waters Co., Milford, MA, USA).

Simulated gastrointestinal digestion

WPC and their hydrolysates were subjected to *in vitro* digestion implementing the standardised INFOGEST protocol (Minekus et al., 2014). 100 µL of sample (75 mg/mL) were mixed with 100 µL of simulated salivary fluid containing 1500 U/mL amylase for 2 min (oral phase). Then, 200 µL of simulated gastric fluid with 25000 U/mL pepsin were added and incubated for 2 h (gastric phase). Finally, 400 µL of simulated intestinal fluid containing 800 U/mL pancreatin were added and incubated for 2 h (gastrointestinal phase). All incubations were performed to 37 °C under agitation on a horizontal shaker. The digestion was terminated by inactivating the enzymes at 100 °C for 15 min, then the samples were centrifuged (3220 *g*, 45 min, 4 °C) to remove insoluble solids. The blanks were also prepared in the same way using deionized water instead of sample. The antioxidant and ACE inhibitory activities were determined by collecting samples at the end of the gastric (G) and gastrointestinal (GI) digestion. LC-MS/MS was used to identify the peptides.

ABTS radical scavenging activity

The antioxidant capacity was measured by ABTS radical scavenging activity according to the method described by Re et al., (1999) with some modifications. ABTS radical cation was generated mixing 10 mL of 2 mM ABTS with 100 µL of 70 mM sodium potassium persulfate and the mixture was kept at room temperature in darkness for 16 h. The diluted ABTS solution was prepared with 10 mM phosphate buffer (pH 7.4) to an absorbance of 0.70±0.02 at 734 nm. Then, 200 µL of sample (or standard) were mixed with 1.8 mL of diluted ABTS solution. After 1 h in the dark, the absorbance was registered at 734 nm and the results were reported as Trolox equivalents (µmol TE/g sample) using a Trolox calibration curve (10-90 µM).

Angiotensin converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity was assessed as described by Dellafiora et al. (2015) with minor modifications. WPC and their hydrolysates were prepared in deionized water to 10 mg/mL. The digested samples were used directly after simulated gastrointestinal digestion. For assay, 40 µL of sample were mixed with 100 µL of hippuryl-L-histidyl-L-leucine (HHL) substrate (5 mM, in 100 mM potassium phosphate buffer containing 300 mM NaCl, pH 8.3) and 10 µL of ACE solution (1 U/mL, in 10 mM potassium phosphate buffer with 500 mM NaCl, pH 7.0). The mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 125 µL of 1 N HCl. The hippuric acid (HA) released was measured by HPLC-UV on an Alliance 2695 separation system (Waters, Billerica, MA, USA), equipped with dual λ absorbance detector model 2487 (Waters) with the following conditions: column, RP JUPITER C18 (5 µm, 250 x 2 mm, 300 Å, Phenomenex); elution system: eluent A, water with 0.1% (v/v) of formic acid and 0.2% (v/v) of acetonitrile, eluent B, acetonitrile with 0.1% (v/v) of formic acid; gradient, 0-10 min 100% A, 10-25 min linear from 100% A to 33% A, 25-26 min linear from 33% A to 0% A, 26-27 min linear to 100% A, 27-35 100% A; column temperature, 35°C; sample temperature, r.t.; injection volume, 10 µL; acquisition time, 35 min; flow rate, 0.2 mL/min; UV detector: λ = 228. Empower software was used for data

analysis (Waters Co., Milford, MA, USA). For the control, potassium phosphate buffer (pH 8.3) instead of sample was used. For the control blank and sample blank, potassium phosphate buffer (pH 7.0) was added to the reaction mixture instead of enzyme. The inhibition (%) was calculated as follows:

$$Inhibition\ (\%) = [1 - \frac{\left([\mathrm{HA}]_{\mathrm{sample}} - [\mathrm{HA}]_{\mathrm{sample\ blank}}\right)}{\left([\mathrm{HA}]_{\mathrm{control}} - [\mathrm{HA}]_{\mathrm{control\ blank}}\right)}] \text{ x } 100\%$$

Where, $[HA]_{control}$ is the maximum concentration of the released HA in the absence of the inhibitors, $[HA]_{sample}$ is the minimal concentration of the released HA in the presence of the inhibitors, $[HA]_{control\ blank}$ is the concentration of the released HA in the absence of the inhibitors by auto-hydrolysis of HHL and $[HA]_{sample\ blank}$ is the concentration of the released HA in the presence of the inhibitors by auto-hydrolysis of HHL or the contribution of some interfering substances.

UHPLC-ESI-MS/MS analysis

In order to identify the peptides, samples was analyzed by LC-MS/MS using an ultra-HPLC (UHPLC) Ultimate 3000 (Dionex, Sunnyvale, CA, USA) coupled to a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionisation (ESI) source as described previously by Prandi et al., (2019). Peptide identification was performed manually. A list of peptide sequences from the principal whey proteins (α-lactoalbumin and β-lactoglobulin) and their theoretical fragmentation pattern were obtained using FindPept tool (https://web.expasy.org/findpept/) and a fragment ion calculator (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html), respectively. The peptide sequences were obtained by comparing the theoretical fragmentation pattern with the MS/MS spectra.

Statistical analysis

All data were reported as mean ± standard deviation (SD). Results were compared using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post

hoc test using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). Differences between means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Production of whey protein hydrolysates

Whey protein was concentrated by ultrafiltration of double cream cheese whey to a protein concentration of 52.07 ± 0.51% (w/w). The powder that contains not less than 25% protein after recovery and concentration of whey protein by physical separation techniques (membrane filtration) and drying processes is considered a whey protein concentrate (Kilara & Vaghela, 2018). Commercially, the protein content of a whey protein concentrate can vary from 35% to 80% (Kelly, 2019). Therefore, the ultrafiltration process used was adequate to recover whey proteins and obtain a product that can be referred as WPC.

WPC was subsequently hydrolyzed using 3 individual commercial enzymes derived from bacterial (alcalase), animal (chymotrypsin) and fungal (flavourzyme) sources, already used in several studies for the production of protein hydrolysates from food matrices (Adjonu et al., 2013; Shazly et al., 2017; Zhang et al., 2013). Alcalase (ALC) is an endopeptidase from *Bacillus licheniformis* with a broad specificity (Corrochano et al., 2019); chymotrypsin (CHY) is a digestive enzyme that cleaves at the C-terminal region of aromatic or bulky nonpolar residues (Phe, Tyr, Trp, Leu, Met) (Gauthier & Pouliot, 2003) and flavourzyme (FLA) is an enzymatic complex from *Aspergillus orysae* composed of eight enzymes: three endopeptidases, two aminopeptidases, two dipeptidyl peptidases and one amylase (Merz et al., 2015). The extent of whey protein hydrolysis was estimated by measuring the degree of hydrolysis (DH) with the OPA/NAC method (Spellman et al., 2003). As shown in Table 2, a high DH was observed in the ALC (37.66 ± 0.43%) and FLA (46.69 ± 0.02%) hydrolysates. This can be attributed to the broad specificity of these enzymes. FLA presented the highest

DH, which may be due to both its endopeptidase and exopeptidase activities. Similarly, Dryáková, Pihlanto, Marnila, Čurda, & Korhonen, (2010) prepared four whey protein hydrolysates using different enzymes including FLA and ALC with an enzyme/substrate ratio of 1/100 (w/w). These authors reported a higher DH for FLA (35.2%) followed of ALC (11.3%) after 180 min of hydrolysis. The differences in DH between the two studies can be explained by the physico-chemical conditions applied during enzymatic hydrolysis such as the reaction time and the enzyme/substrate ratio that can affect the enzymatic activity (Table 1). Embiriekah, Bulatović, Borić, Zarić, & Rakin, (2018) showed that increasing enzyme levels (pepsin and trypsin) and a longer time of hydrolysis affected positively the DH of a whey protein hydrolysate. The DH results here obtained were comparable to those of Peng, Xiong, & Kong, (2009), where a DH between 35-36% was obtained after 5h of hydrolysis of a whey protein isolate (WPI) treated with ALC. Several authors have obtained lower DH values compared to the present study during the enzymatic hydrolysis of whey protein ranging from 20 to 36% for ALC (Li-jun, Chuan-he, & Zheng, 2008; Peng et al., 2009; Zhang et al., 2013), 12 to 14% for CHY (Adjonu et al., 2013; Théolier, Hammami, Labelle, Fliss, & Jean, 2013) and 15 to 35% for FLA (Dryáková et al., 2010; Souza et al., 2019). These differences could also be due to the extent of denaturation of whey protein increasing the sensitivity of the enzyme to previously inaccessible regions in the protein and, as a result, an increase in the DH can be observed (Souza et al., 2019; Tavano, 2013). In this study, WPC was obtained by ultrafiltration before enzymatic treatment, which could induce the denaturation of the native protein. Belmejdoub, Rabiller-Baudry, Delaunay, & Gésan-Guiziou (2012) reported that globular proteins (β-Lactoglobulin, bovine serum albumin) underwent structural modifications by mechanical forces (shear stress) after ultrafiltration process.

The protein content and the protein yields of the enzymatic reactions were also determined (Table 2). The protein content decreased in the ALC and FLA hydrolysates and no

statistically significant differences were found in the CHY hydrolysate in comparison to WPC. Respect to the protein yields, ALC (84.51 ± 0.00%) and CHY (88.49 ± 0.41%) had a higher protein extraction ability that FLA (52.80 ± 0.15%) indicating that there was greater protein solubilisation with these enzymes. ALC presented high DH and protein yield, which could indicate that nitrogen released after enzymatic hydrolysis, is in form of free amino acids and smaller peptides. In contrast, CHY had lower DH and higher protein yield showing that the nitrogen released is mainly in form of medium and long peptides. The free amino acid (FAA) content was also determinated as shown in Figure 1. The FAA content was 1.28 ± 0.03, 0.313 ± 0.002, 14.4 ± 0.1 and 0.0454 ± 0.0013 g AA/100 g sample in the ALC, CHY and FLA hydrolysates and WPC; respectively. The FAA content in the hydrolysates can be related to the DH, a high DH correspond to an elevated FAA content in the hydrolysates. FLA showed the highest DH which was consistent with a major release of amino acids at the end of hydrolysis. According to the results, a positive correlation between the DH and FAA content can be suggested as also reported by Embiriekah et al., (2018).

The hydrolysates were then analyzed by UHPLC-ESI-MS/MS to identify possible sequences of peptides (Table 3). 17 peptides were identified in the ALC hydrolysate. Small fragments containing between 3 and 7 amino acid residues were obtained with a molecular weight (MW) < 900 Da, 65% of the peptides had a MW < 500 Da and 35% ranged from 500 to 900 Da.

24 peptides containing between 3 and 21 amino acid residues were identified in the CHY hydrolysate, 12% of the fragments had a MW < 500 Da, 42% with a MW that ranged from 500 to 1000 Da and 46% with a MW > 1000 Da.

14 peptides in the FLA hydrolysate were identified. The peptides contained between 4 and 23 amino acid residues. 14% of the fragments had a MW < 500 Da, 43% with a MW between 500 to 1000 Da and the remaining one of MW > 1000 Da. The ALC hydrolysate was mainly composed of tri and tetra-peptides. In contrast, medium and long peptides (7-23 amino acid

residues) constituted the CHY and FLA hydrolysates. CHY hydrolysates showed the major amount of longer peptides, this is also consistent with lower DH observed. Morais et al., (2013) reported that whey protein hydrolysates obtained with ALC had a higher content of di and tri-peptides and lower of peptides with more than 7 amino acid residues. In addition, studies have reported that alcalase produces short peptides due to its broad specificity (O'Keeffe & FitzGerald, 2014; Sarmadi & Ismail, 2010; Shazly et al., 2017).

Antioxidant and ACE inhibitory activities of whey protein hydrolysates before and after simulated gastrointestinal digestion

Antioxidant activity was investigated by the ABTS cation radical decolorization method and reported as Trolox equivalents (TE). As shown in Figure 2, the ALC, CHY and FLA hydrolysates showed more potent antioxidant activity compared to WPC, any variations depending on the enzyme used during the hydrolytic process. Statistical differences were found in the antioxidant activity between the hydrolysates. The ALC hydrolysate displayed the greatest ABTS radical scavenging capacity (479.1 ± 2.9 µmol TE/g sample) followed of the CHY (400.7 ± 0.6 µmol TE/g sample) and FLA (287 ± 5.8 µmol TE/g sample) hydrolysates. Previous studies conducted by Mann et al., (2015) and Souza et al., (2019) reported higher antioxidant activity of a whey protein hydrolysate obtained after hydrolysis with ALC compared to the FLA digestion. Similarly, Dryáková et al., (2010) demonstrated that whey protein concentrates treated with ALC were the most effective in scavenging the ABTS cation radical compared to FLA, neutrase and protamex. Additionally, Athira et al., (2015) reported that whey protein hydrolysates prepared with ALC exhibited a potent ABTS radical scavenging activity.

The antioxidant activity in the different hydrolysates may be related to the presence of peptides with specific structural characteristics. The peptide profile of each hydrolysate is governed mainly by the enzyme specificity that cleaves at a specific site and generates peptides with different size, sequence and bioactivity (Contreras, Hernández-Ledesma,

Amigo, Martín-Álvarez, & Recio, 2011; Power et al., 2013; Sarmadi & Ismail, 2010). ALC produced a hydrolysate containing peptide fragments with a MW < 900 Da indicating that antioxidant activity could be associated with low MW peptides. Dryáková et al. (2010) observed greater effectiveness to scavenge the ABTS radical in the ALC hydrolysates containing peptides with shorter chain lengths. Shazly et al. (2017) reported that the highest antioxidant activity in casein hydrolysates was shown in the fraction with low molecular weight peptides (< 1 kDa). Additionally, the prevalence of some amino acids and their order in the sequence can affect the antioxidant activity (Li, Li, He, & Qian, 2011; Power et al., 2013; Sarmadi & Ismail, 2010). It is known that the presence of aromatic amino acids such as Tyr (Y) improved the antioxidant activity of the peptides due to their ability to stabilize radicals by donation of electrons or hydrogens. Also, basic and acidic amino acid residues can act as hydrogen donors, and hydrophobic residues facilitate the accessibility to hydrophobic radical species (Sarmadi & Ismail, 2010; Shazly et al., 2017). Studies have reported that hydrophobic amino acids such as Ala (A), Pro (P), Val (V), Ile (I), Leu (L), Phe (F), Tyr (Y) can contribute on antioxidant properties (Athira et al., 2015; Contreras et al., 2011). As shown in Table 3, one or more of these amino acids are found in the peptides detected in the hydrolysates. Li, Li, He, & Qian, (2011) studied the structure-antioxidant activity relationship of peptides obtained from different food proteins by QSAR modeling. They found that the following characteristics may contribute to the antioxidant properties of peptides: presence of a hydrophobic amino acid at the N-terminus; Trp (W), Glu (E), Leu (L), Ile (I), Met (M), Val (V) and Tyr (Y) frequently present as C-terminal residue; and an acid, basic or hydrophilic residue in the amino acid next to the C-terminal that is possibly the major contributor to antioxidant activity. Most of the peptides identified in the hydrolysates of ALC (82%), CHY (92%) and FLA (64%) possessed some of the characteristics mentioned above. INY (α-La f(120-122)) in the ALC hydrolysate, IRL (β-Lg f(173-175)), IVTQTM (β-Lg f(18-23)), VLDTDY (β-Lg f(110-115)) and LIVTQTM (β-Lg f(17-23)) in the CHY hydrolysate,

and VEEL (β-Lg f(59-62)) in the FLA hydrolysate were consistent with the three structural features of antioxidant peptides. None of these sequences have been reported as antioxidants in the Milk Bioactive Peptide Database (Nielsen, Beverly, Qu, & Dallas, 2017) and BIOPEP Database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008).

Finally, hydrolysates were subjected to simulated gastrointestinal digestion with the INFOGEST protocol in order to evaluate the effect of the gastric (G) and gastrointestinal (GI) phases on the antioxidant activity (Figure 2). The antioxidant activity of all hydrolysates increased after the G and GI digestions. With respect to the undigested hydrolysates, the ABTS radical scavenging capacity increased 1.5, 1.9 and 1.6-fold after G phase and 1.3, 2.3 and 1.2-fold after the GI phase in the ALC, CHY and FLA hydrolysates, respectively. A greater proteolysis in the hydrolysates could cause an increase in antioxidant proprieties due to the production of smaller peptides and the release of free amino acids. Previous studies have shown that the biological activity obtained after GI digestion can be either persisted, decreased or increased (Quirós, Contreras, Ramos, Amigo, & Recio, 2009; Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014). For example, the antioxidant capacity of whey protein hydrolysates by the ORAC method was maintained or decreased after *in vitro* digestion (Power-Grant et al., 2015). You, Zhao, Regenstein, & Ren, (2010) found that the ABTS radical scavenging ability of a loach protein hydrolysate decreased slightly after gastric digestion and then increased during subsequent intestinal digestion.

Regarding the *in vitro* digestion of WPC, no statistical differences in antioxidant activity were found during the G digestion and then a significant increase was observed during the subsequent GI digestion. Whey proteins are relatively stable to pepsin digestion, specifically β-lactoglobulin, due to its structural conformation. β-lactoglobulin is the most abundant whey protein that contains a hydrophobic core inside a β-barrel where are found the majority of hydrophobic and aromatic residues (pepsin cleavage sites), causing the highly resistance

to pepsin (Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, & Haertlé, 1995). The resistant of β-lactoglobulin to G digestion in static models has been reported previously in several studies (Miralles, del Barrio, Cueva, Recio, & Amigo, 2018; Picariello et al., 2010). After the GI phase, the TE values of the concentrate increased to 4.5-fold from 102.3 to 458.6 ± 40.6 μmol TE/g sample. In the same way, Power-Grant et al., (2015) demonstrated that the ORAC value of a whey protein concentrate after exposure to *in vitro* GI conditions increased to 2.5-fold from 13.66 ± 1.02 to 36.6 ± 3.4 μmol TE/100 g powder. Corrochano et al. (2019) also reported that the antioxidant activity measured by the ABTS radical scavenging ability, FRAP method and ORAC assay of a whey protein isolate was increased after simulated GI digestion. The increase in antioxidant properties after GI digestion can be attributed to the release of peptides and amino acids by the pancreatin enzyme.

Although the antioxidant activity of WPC at the end of the GI digestion was improved (4.5-fold), the ALC and CHY hydrolysates showed greater antioxidant activity in the same phase of digestion, 623.9 ± 53.1 and 903.9 ± 43.7 μmol TE/g sample; increasing 6.1 and 8.8-fold for ALC and CHY, respectively, compared to WPC. These results could suggest that enzymatic hydrolysis of WPC favor the antioxidant properties after digestion. Power-Grant et al., (2015) found higher ORAC values of two whey protein hydrolysates (44.5 ± 2.1 and 60.6 ± 4.5 μmol TE/100 g power) compared to the concentrate (36.6 ± 3.4 μmol TE/100 g power) after gastrointestinal digestion.

The ACE inhibitory activities of WPC and the resulting hydrolysates are presented in Figure 3. All hydrolysates inhibited greater than 40% ACE activity at a concentration of 10 mg/mL. The ALC and CHY hydrolysates showed higher ACE inhibitory capacity. No statistically significant differences were found between these hydrolysates (91.97 ± 0.36% for ALC and 88.33 ± 0.45% for CHY). Additionally, no ACE inhibitory activity was observed in WPC, which suggests that the enzymatic hydrolysis of whey proteins could be key for the release of peptides with ACE inhibitory potential. Fu, Young, Dalsgaard, & Therkildsen, (2015) found

that bovine collagen showed high ACE inhibitory capacity after hydrolysis with ALC and papain. Cheung, Aluko, Cliff, & Li-Chan, (2015) reported that a whey protein isolate exhibited the ability to inhibit ACE after hydrolysis with different enzymes and an inhibition higher than 50% in most hydrolysates was observed. Potential ACE inhibitory peptides generally possess short sequences (2-12 amino acid residues) and their bioactivity is influenced by the three last positions in the C-terminal region containing bulky, aromatic or branched hydrophobic amino acids (Corrêa et al., 2014; Norris & FitzGerald, 2013). The presence of Pro (P) in this region can increase the inhibitory activity of ACE (Lassoued et al., 2015). Tyr (Y), Phe (F), Trp (W), Leu (L), Ala (A) and Val (V) could also contribute to ACE inhibition (Norris & FitzGerald, 2013; Zenezini Chiozzi et al., 2016). The presence of hydrophobic amino acids in the C-terminal region can facilitate the binding of the peptide to the active site of ACE to act as an inhibitor (Otte, Shalaby, Zakora, & Nielsen, 2007). In the present study, small peptides (< 12 amino acid residues) were obtained in all hydrolysates and some of the amino acid residues mentioned above were found in the C-terminal region of the majority of peptides. In addition, RVY (β-Lg f(56-58)), HIRL (β-Lg f(172-175)), LIVTQ (β-Lg f(17-21)), VLDTDY (β-Lg f(110-115)) identified in the CHY hydrolysate; and GVSLPEW (α-La f(39-45)) in the FLA hydrolysate have previously been identified as ACE inhibiting peptides (Lacroix, Meng, Cheung, & Li-Chan, 2016; Matsufuji et al., 1994; Mullally, Meisel, & FitzGerald, 1996; Otte et al., 2007; Vallabha & Tiku, 2014). INY (α-La f(120-122)), IIAE (β-Lg f(87-90)), SLPE (α-La f(41-44)), SFNPT (β-Lg f(166-170)) and GGVSLPE (α-La f(38-44)) detected in the ALC hydrolysate have been found within the C-terminal region of peptides with ACE inhibitory activity (Nielsen, Beverly, Qu, & Dallas, 2017).

After *in vitro* GI digestion, a decrease in ACE inhibitory activity was observed in the ALC and CHY hydrolysates. The remaining activity was approximately 44% and 23% for ALC and CHY, respectively, which may be due to that some peptides with potential to inhibit ACE were degraded and converted to inactive molecules during digestion under simulated

conditions. In contrast, no statistically significant differences were found in ACE inhibitory activity of the FLA hydrolysate after the GI phase suggesting that the peptides present in this hydrolysate can resist the gastrointestinal digestion process or can be partially hydrolyzed to other smaller molecules without loss of biological function. Quirós et al. (2009) evaluated the effect of simulated gastrointestinal digestion on the stability of peptides obtained from β-casein with antihypertensive activity. The results showed that the peptides can resist the action of the digestive enzymes, be partially hydrolyzed or totally degraded causing that the activity may be maintained, increased or decreased. Similar results for peptides obtained by hydrolysis of protein whey with ACE inhibitory activity were reported by Tavares et al. (2011). Also, Fu et al. (2015) found that the ACE inhibitory activity of fractions obtained from a bovine collagen hydrolysate decreased after digestion with pepsin and pancreatin.

Low ACE inhibitory activity of WPC was exhibited after *in vitro* digestion indicating that the enzymatic hydrolysis prior to the digestion of the concentrate may be necessary for the production of peptides that counteract the ACE activity, as previously reported for antioxidant activity.

The peptides identified in the ALC hydrolysate resisted G digestion and 65% of the fragments were stable to GI digestion. The CHY and FLA hydrolysates were more sensitive to treatment with pepsin and pancreatin. 33% of the peptides in the CHY hydrolysate and 36% of the peptides in the FLA hydrolysate were digested during G digestion, and 79% of the fragments in the CHY hydrolysate and 71% of the fragments present in the FLA hydrolysate were degraded during GI digestion. The peptides in the ALC hydrolysate showed greater resistance to GI digestion, which may be related to the size of their sequences. Previous studies have reported that small peptides could resist hydrolysis by the enzymes involved in digestion and be easily absorbed in the intestine (Morais et al., 2013; Shazly et al., 2017). The following peptides survived the *in vitro* digestion and could

present potential antioxidant capacity: INY (α-La f(120-122)) in the ALC hydrolysate, and IRL (β-Lg f(173-175)) and VLDTDY (β-Lg f(110-115)) in the CHY hydrolysate. INY (α-La f(120-122)), IIAE (β-Lg f(87-90)), SFNPT (β-Lg f(166-170)) and GGVSLPE (α-La f(38-44)) in the ALC hydrolysate, IRL (β-Lg f(173-175)) and VLDTDY (β-Lg f(110-115)) in the CHY hydrolysate, and TPEV (β-Lg f(141-144)) in the FLA hydrolysate could be possible candidates to inhibit ACE. The INY (α-La f(120-122)) and VLDTDY (β-Lg f(110-115)) peptides could present antioxidant and ACE inhibitory properties suggesting the existence of multifunctional peptides as reported by Contreras et al. (2011).

CONCLUSION

In the present study, WPC was independently hydrolyzed with three different enzymes obtaining peptides of different sizes and sequences, which exerted antioxidant and ACE inhibitory activities. The enzymatic hydrolysis of WPC favored the biological properties after *in vitro* digestion under simulated conditions. Specifically, the peptides identified in the ALC hydrolysate were more resistant to the GI digestion process and, consequently, were still able to exert bioactive functions. In addition, each enzyme could produce a hydrolysate with different biological activities and peptides, and its choice will depend on the potential benefits desired in the final product or for a given application.

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