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Iron-binding peptides from whey protein hydrolysates: Evaluation, isolation and sequencing by LC–MS/MS

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ABSTRACT

Iron–peptide complexes have been considered a promising source of more bioavailable iron, with reduced side effects as compared to iron salts. Whey protein isolate (WPI) hydrolyzed by alcalase, pancreatin or flavourzyme was ultrafiltered (cut off 5 kDa) and their fractions – retentates and filtrates – were evaluated for iron-binding capacity. The Fe–hydrolysate complexation reaction resulted in a dramatic increase in iron solubility at pH 7.0, from 0% to almost 100%. This result was obtained regardless of the molecular mass profile or the enzyme used to obtain the samples. Fractions from hydrolysate obtained with pancreatin (HP) were chosen to continue the study. The complexes formed with both fractions from HP were stable under simulated gastric digestion (50.8–89.4%). To identify the peptides with iron-binding capacity, the HP fractions were isolated by IMAC-Fe^{3+}, and the retentate showed higher relative concentrations of iron-binding peptides than the filtrate. Iron-binding peptide sequencing, accomplished by LC–MS/MS, showed Glu and/or Asp in all the sequences, and their carboxylic groups were amongst the main iron-binding sites. WPI hydrolysis with pancreatin yields peptides that can form iron complexes with the potential to increase iron bioavailability and reduce its pro-oxidant effect.

1. Introduction

Iron is an essential micro-mineral acting as a cofactor for a wide variety of cellular processes such as cellular respiration, the tricarboxylic acid cycle, lipid metabolism, synthesis of metabolic intermediates, gene regulation, and DNA replication and repair (Puig, Askeland, & Thiele, 2005). It is a component of cytochromes, hemoglobin, myoglobin and enzymes involved in oxygen and electron transport and peroxide breakdown (Wang, Huang, & Jiang, 2013). Despite the abundance of this mineral, its bioavailability is low due to its insolubility at physiological pH values (Puig et al., 2005) and it is strongly influenced by iron absorption enhancers and/or inhibitors in the diet (Zhu, Glahn, Nelson, & Miller, 2009).

Food supplementation with iron salts is still a challenge in the food industry, since various metal salts may result in changes in the physical and sensory properties of foods (Guo et al., 2014). Iron, a transition metal, may react with other components inducing lipid oxidation, sedimentation and sensory defects in the products to which it is added (Sugiarto, Ye, & Singh, 2009). In addition, when ingested in the form of salts, iron has low bioavailability, may promote the formation of reactive oxygen species (ROS) and may be responsible for gastric mucosa damages (Chaud et al., 2002). Radicals can start the peroxidation of lipids in biological membranes, enzyme inactivation and damage to the DNA structure (Saiga, Tanabe, & Nishimura, 2003). Metal chelating peptides have been identified as potential functional ingredients to improve bivalent mineral bioavailability (Guo et al., 2014). Iron–peptide complexes have been considered as an alternative to mitigate the problems related to iron fortification, and have been considered as one of the best choices to replace iron supplements (Wang et al., 2013).

Regarding the mechanism of iron–peptide complexation, Reddy and Mahoney (1995) suggested that the net charge, side chain length and functional groups of the amino acids and peptides seem to be directly related to the extent of complex formation with iron. Studies with iron–peptide complexes show that the major iron binding site corresponds primarily to the carboxyl groups (Chaud et al., 2002; Lee & Song, 2009), although the ε-amino nitrogen of lysine, the guanidine nitrogen of arginine, and the imidazole nitrogen of histidine may also have been involved in iron–peptide bonding (Reddy & Mahoney, 1995). Glycine and proline could also be involved in iron complexation (Storcksdieck, Bonsmann, & Hurrell, 2007). Nevertheless, proteins with iron-binding sites do not necessarily yield peptides with high iron-binding capacity. The position of iron-binding sites within the peptide

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sequence seems to be crucial to the iron-binding capacity. Besides the presence of specific amino acids, the choice of proteolytic enzyme is very important, because its specific action will influence the final composition of the hydrolysis products, mainly regarding the average peptide length and exposition of the side chains (Adler-Nissen, 1986).

Peptides from whey proteins have been widely studied in the last years due to their bioactive properties, including the promotion of iron absorption (Zhou et al., 2012). It has been demonstrated that whey protein hydrolysates enhance iron solubility in the small intestine (Nakano, Goto, Nakaji, & Aoki, 2007), and promote the reduction of ferric iron to the more soluble and bioavailable ferrous form (Argyri, Miller, Glahn, Zhu, & Kapsokefalou, 2007). These effects are related to some specific iron binding sites and/or the presence of relatively low MM (<10 kDa) peptides (Ou et al., 2010).

Iron–WPI peptide complexes formed prior to digestion could be an alternative not only to improve iron bioavailability, but also to decrease the pro-oxidant effect of the mineral, preventing the side effects related to free iron. Thus, the aims of this study were to obtain peptides from whey proteins using different enzymes, and to study their iron-binding capacity and the amino acid sequences involved in iron chelation.

2. Material and methods

2.1. Material

Whey protein isolate (WPI) PROVON® was obtained from Glanbia Nutritionals (Kilkenny, Ireland). The protein content of the WPI (87.6 ± 0.4% protein), determined by the micro-Kjeldahl method (AOAC, 2006), was calculated from the nitrogen content by multiplying the total nitrogen content by a factor of 6.38 (conversion factor).

Flavourzyme (produced by *Aspergillus oryzae*) was donated by Novozymes® Latin America Ltda. (Araucária, PR, Brazil); alcalase (produced by *Bacillus licheniformis*) and pancreatin (from porcine pancreas) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). The enzyme pepsin used for gastric digestion was purchased from Sigma-Aldrich® (St. Louis, MO, USA).

The reagents tricine, sodium dodecil sulfate (SDS), and o-phthaldialdehyde (OPA) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Orthophenanthroline was obtained from Synth (São Paulo, SP, Brazil). Trifluoroacetic Acid (TFA), β-mercaptoethanol, Coomassie Brilliant Blue G250, sodium hydroxide and urea were obtained from Merck (Hohenbrunn, Germany). Bis-acrylamide was purchased from Amresco (Solon, Ohio, USA); and acrylamide and Tris base were from Bio-Rad (Hercules, CA, USA). Ferrous chloride ($FeCl_2 \cdot 4H_2O$) was from J. T. Baker (Center Valley, PA, USA). All other chemicals and reagents used were of analytical or chromatographic grade.

2.2. Production of enzymatic hydrolysates and their fractions

WPI was hydrolyzed under the following conditions of pH, temperature and enzyme:substrate ratio (E/S): (1) with alcalase: pH 8.0, 60 °C, E/S 1%; (2) with pancreatin: pH 8.0, 40 °C, E/S 4%; and (3) with flavourzyme: pH 6.7, 55 °C, E/S 4%. For this process the WPI (10% w/v) was dissolved in deionized water and the reaction occurred in an Automatic Titrator — pH Stat (model DL 21 Grafix; Mettler-Toledo, Schwerzenbach, Switzerland), with stirring and controlled temperature. During hydrolysis, the pH was maintained at the established value by the addition of 2 mol/L NaOH. The degree of hydrolysis (DH) was calculated according to Eq. (1) (Adler-Nissen, 1986). The hydrolysis processes were carried out in triplicate.

$$DH\ (\%) = B \times Nb \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100 \quad (1)$$

where: B = base consumption (mL); N_b = normality of the base; α = average degree of dissociation of the α-NH groups; MP = mass of protein (g); and h_{tot} = total number of peptide bonds in the protein substrate (meqv/g protein) — 8.8 for whey protein (Adler-Nissen, 1986).

After cooling to room temperature, the hydrolysates were centrifuged (17,000 ×g for 15 min). The supernatants were filtered through a microfilter with a pore size of 0.45 μm (Schleicher & Schüll, Dassel, Germany), and fractionated in an Ultrafiltration System Prep/Scale™ — TFF (Millipore, Bedford, MA, USA) (cut off 5 kDa). The fractions – retentates (H/r) and filtrates (H/f) – were freeze dried. The hydrolysates obtained with alcalase (A), pancreatin (P) and flavourzyme (F) gave rise to the following fractions: HA/r, HP/r and HF/r, with MM > 5 kDa; and HA/f, HP/f and HF/f, with MM < 5 kDa.

2.3. Total and free amino acid profiles and free amino groups

The total and free amino acid compositions of the hydrolysate fractions were determined after derivatization with phenylisothiocyanate (PITC) by HPLC, according to the method described by White, Hart, and Fry (1986), and Hagen, Augustin, Grings, and Tassinari (1993), respectively. Tryptophan was determined according to Spies (1967).

Free amino groups were determined by the o-phthaldialdehyde (OPA) method, as described by Nielsen, Petersen, and Dambmann (2001). A serine solution (0.9516 meqv/L) was used as the standard, and absorbance readings were taken at 340 nm. The assays were carried out in triplicate. The free amino group contents were calculated according to Eq. (2):

$$\text{Free amino groups}\left(\frac{\text{meqv}}{\text{L}}\right) = \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs standard} - \text{Abs blank}} \times 0.9516 \frac{\text{meqv}}{\text{L}} \times 0.1 \times \frac{100}{w} \times P \quad (2)$$

where: w = sample weight (g); P = % protein; and 0.1 = sample volume (L).

2.4. Electrophoresis

The electrophoretic profiles were determined using the SDS-PAGE tricine system, using separating (6 mol/L urea), resolving and stacking gels containing 16%, 10% and 4% of acrylamide, respectively, all containing 3% of bis-acrylamide (Schagger, 2006). The samples were dissolved (1% protein w/v) in the sample buffer (0.5 mol/L Tris–HCl, pH 6.8, 10% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.1% Coomassie Blue G250), heated at 90 °C/5 min and loaded onto the gels (5 μL). The gels were fixed for 1 h using a methanol/acetic acid/water (5:1:4) solution, stained using 0.025% (w/v) Coomassie Blue G250 in 10% acetic acid, and destained in 10% acetic acid solution.

2.5. Iron-binding capacity

The complexation reaction was carried out with a protein:iron ratio of 40:1 (w/w). The hydrolysates were rehydrated in deionized water (4% protein w/v) and the pH adjusted to 7.0 with 0.5 mol/L NaOH. $FeCl_2 \cdot 4H_2O$ was then added to the hydrolysate solutions to a final concentration of 0.1% Fe (w/v). The mixture was allowed to react while stirring at room temperature (25 ± 2 °C), and the pH adjusted every 15 min. After 60 min, the solutions were centrifuged (5000 ×g/20 min, 25 °C). The iron content in the supernatant was determined by atomic absorption spectrometry (AAS), according to Boen, Soeiro, Pereira-Filho, and Lima-Pallone (2008), using a Perkin-Elmer AAnalyst 300 spectrometer (Massachusetts, USA) equipped with a deuterium lamp background corrector. The standard curve ranged from 0.2 to 2.6 mg of Fe/L. All experiments were carried out in triplicate. Iron-binding capacity was evaluated considering the

percentage of solubilized iron after complexation ($Fe_{supernatant}$) in relation to that added initially ($Fe_{initial}$), according to Eq. (3):

$$\text{Iron-binding capacity } (\%) = \frac{\text{Fe supernatant}}{\text{Fe initial}} \times 100. \quad (3)$$

2.6. Fe–peptide complexes stability under simulated gastric conditions

The supernatants of the complexation reactions with HP/r and HP/f, containing the Fe–peptide complexes, were freeze dried and identified as Fe–HP/r and Fe–HP/f. These samples were then submitted to simulated gastric digestion. In vitro gastric digestion was accomplished according to Martos, Contreras, Molina, and López-Fandiño (2010) with adaptations. The freeze-dried supernatants containing the iron–peptide complexes were solubilized in simulated gastric fluid (35 mmol/L NaCl pH 2.0), the pH adjusted to 2.0 with 1 mol/L HCl, and the mixture incubated at 37 °C for 15 min in a Thermomixer Comfort (Eppendorf, Hamburg, Germany). The pepsin solution was then added (enzyme:substrate ratio 1:20 w/w), and the mixture incubated for an additional 60 min at 37 °C with stirring (400 rpm). The pH was adjusted to 7.0 with 1 mol/L NaOH and the volume adjusted to reach a final concentration of 3.9 mg protein/mL. As a control, a simulated gastric digestion with and without pepsin addition was carried out with $FeCl_2$ added at the same concentration as in the test samples. The samples were centrifuged (5000 ×*g*/20 min) and the iron content in the supernatant determined by atomic absorption spectrometry (AAS) according to Boen et al. (2008). All experiments were carried out in triplicate. The stability of the Fe–peptide complexes was evaluated according to Eq. (4):

$$\text{Stability of the Fe–peptide complexes } (\%) = \frac{\text{Fe supernatant}}{\text{Fe initial}} \times 100 \quad (4)$$

where: $Fe_{supernatant}$ = soluble iron content at pH 7.0 after simulated gastric digestion; and $Fe_{initial}$ = iron content initially added to simulated gastric digestion mixture.

2.7. Immobilized metal affinity chromatography (IMAC-Fe^{3+})

Iron-binding peptides were isolated from the selected hydrolysate fractions by IMAC-Fe^{3+} according to the methodology described by Lv et al. (2009) with adaptations, using a FPLC (ÄKTA prime plus FPLC system, GE Healthcare Bio-Sciences, Björkgatan, Sweden). A XK 16 column was packed with 20 mL of Iminodiacetic Acid Sepharose® (IDA-Sepharose) (Sigma-Aldrich®; St. Louis, MO, USA), obtaining bed dimensions of 20 mm × 78 mm. The column was washed with 6 bed volumes of purified water and incubated with 200 mmol/L $FeCl_3$ (30 mL) for iron charging. It was then washed with 8–9 bed volumes of purified water to remove the unbound iron, and the weakly bound iron removed by washing the column with 5–6 bed volumes of 50 mmol/L acetic acid buffer pH 4.0. The column was then treated with the equilibrating buffer prepared with 50 mmol/L acetic acid buffer at pH 5.5 with 0.1 mol/L NaCl (Lv et al., 2009). 2 mL of the sample solution (50 mg protein/mL) were loaded onto the column at a flow rate of 1 mL/min and detection was at 280 nm. The bound peptides were eluted with 20 mmol/L Na_2HPO_4. The fractions containing the iron-binding peptides obtained from the retentate of the pancreatin hydrolysate (HP/r*ib) and from the filtrate of the pancreatin hydrolysate (HP/f*ib) were collected and freeze-dried.

After elution of the iron-binding peptides, the column was washed with purified water and regenerated with 50 mmol/L EDTA. The experiments were carried out at room temperature.

2.8. Identification of the iron-binding peptides by LC–MS/MS

Prior to the LC–MS/MS analysis, the samples collected by IMAC-Fe^{3+} were desalted in a Sep-Pak C_{18} column with a 100 mg cartridge, particle size 55–105 μm (WAT023590, Waters Corporation, MA, USA). The peptides were separated on a C_{18} column (100 μm × 100 mm) (Waters Corporation, Milford, MA, USA) using a nano Acquity Ultra Performance LC (Waters Corporation, Milford, MA, USA) coupled with nano-electrospray tandem mass spectrometry on a Quadrupole Time-of-flight (Q-Tof) Ultima mass spectrometer (MicroMass/Waters Corporation), at a flow rate of 0.6 mL/min. The gradient was 0–90% acetonitrile in 0.1% formic acid for 60 min. The instrument was operated in the MS positive mode, and the data were acquired in continuum mode over the m/z range of 100–2000 at a scan rate of 1 s and an interscan delay of 0.1 s.

The spectra were acquired using the software MassLynx v.4.1 and the raw data files converted to a peak list format (mgf) without summing up the scans by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ldt.), and searched against the *Bostaurus* database, containing 51,274 sequences and 19,497,864 residues using the Mascot engine v.2.3.01 (Matrix Science Ltd.) with a tolerance of 0.1 Da for both precursor and fragment ions.

2.9. Statistical analysis

The results were expressed as the mean ± standard deviation and compared by the analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant at $P < 0.05$. The statistical analysis was carried out using the SPSS software program (SPSS 16.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Production, characterization and iron-binding capacity of WPI hydrolysate fractions

Alcalase showed the greatest hydrolytic action in the first minutes of hydrolysis followed by pancreatin and flavourzyme. In order to obtain hydrolysates with similar DH values of 16.8 and 16.4%, respectively, the hydrolysis time was set at 105 min for alcalase and 180 min for pancreatin. For flavourzyme, the hydrolysis time was set at 180 min, obtaining a DH value of 9.1%, lower than that obtained with alcalase and pancreatin in a shorter reaction time. Flavourzyme showed low hydrolytic activity, and no increase in the DH value was observed after 60 min of hydrolysis.

The electrophoretic profiles of the hydrolysates and their fractions are shown in Fig. 1. The profiles of HA, HP and their respective fractions (Fig. 1, lanes 3 and 4; 6 and 7) showed that the main WPI proteins, α-La

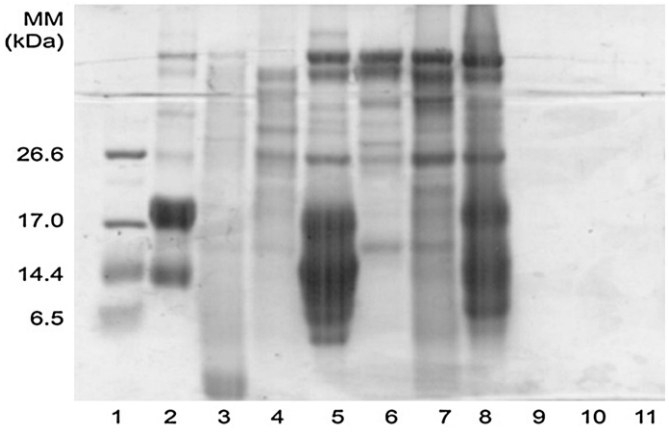

Fig. 1. SDS-PAGE gel of hydrolysates and their fractions (SDS-PAGE/tricine under reducing conditions). (1) Molecular mass standard; (2) WPI; (3) hydrolysate with alcalase (HA); (4) hydrolysate with pancreatin (HP); (5) hydrolysate with flavourzyme (HF); (6 to 8) retentates (>5 kDa) from HA, HP and HF, respectively; (9 to 11) filtrates (<5 kDa) from HA, HP and HF, respectively.

(14.2 kDa) and β-Lg (18.4 kDa) were hydrolyzed, confirmed by the disappearance of their corresponding bands. On the other hand, intense bands in the region of approximately 6 to 19 kDa and also bands of non-hydrolyzed α-La and β-Lg were observed in the profiles of both the hydrolysate HF and its fraction HF/r (Fig. 1, lanes 5 and 8). No bands were observed in the profiles of the filtrates (Fig. 1, lanes 9–11), suggesting the predominance of small peptides (<3 kDa), which may not have been stained by the Coomassie Blue (Krohn, 2001). The presence of bands with MM below 6.5 kDa in the profiles of the retentates (Fig. 1, lanes 6–8) suggests that the separation by ultrafiltration was not complete. The ultrafiltration process was carried out at a pH value around neutrality which favors aggregate formation and may have contributed to the presence of small peptides in the retentate.

The amino acid composition of the hydrolysate fractions (results not shown) showed high contents of Asp and Glu, 8.4–11.3 and 18.4–22.5 g/100 g protein, respectively, whose carboxylic groups are amongst the most important iron-binding sites (Chaud et al., 2002; Lv et al., 2009). Histidine and proline, which also have a role in iron-binding, were present at concentrations of 1.2–1.9 and 5.0–6.6 g/100 g protein, respectively.

Since free amino acids have lower iron-binding capacity than peptides (Huang, Ren, & Jiang, 2011), both the free and total amino acid compositions were evaluated. In all the fractions, only about 2% of the total Asp and Glu were shown to be in the free form, suggesting that the main iron-binding sites are in peptide sequences.

The different hydrolytic actions of the enzymes also resulted in different free amino group contents and compositions of the hydrolysate fractions (Fig. 2). The exopeptidase activity of flavourzyme (Nchienzia, Morawicki, & Gadang, 2010) was responsible for the presence of 60% of free amino groups coming from free amino acids in the fraction HF/f. The endopeptidase activity of alcalase (Smyth & Fitzgerald, 1998) led to a low free amino acid content in both fractions (retentate and filtrate). The fractions from HP showed intermediate results, in accordance with the presence of endo- and exopeptidases in this enzymatic system (Yamamoto, 1975).

The iron–peptide complexation was carried out at pH 7.0 since the coordinated binding is favored when the ionizable electron donating groups of the amino acid residues are partially deprotonated (Porath, 1990). At this pH, deprotonation of the majority of the aspartic acid (pK β-COOH = 3.86) and glutamic acid (pK -COOH = 4.25) side chains is to be expected, and the same pH was used to determine the peptide iron-binding capacity by other authors (Eckert, Bamdad, & Chen, 2014; Lee & Song, 2009; Sugiarto et al., 2009; Zhou et al., 2012). The iron-binding capacity of the fractions is shown in Fig. 3. In the control assay carried out only with $FeCl_2$, iron solubility at pH 7.0 was below 0.5% of the initial content, confirming the insolubility of iron at this pH

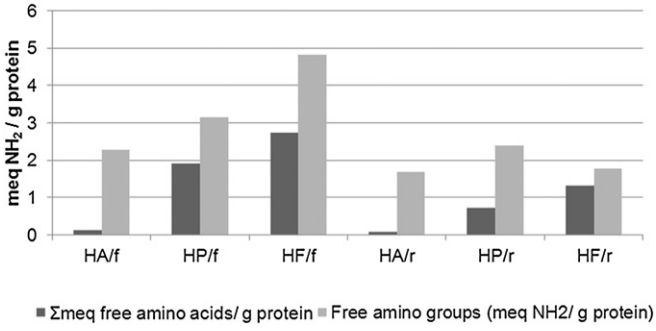

Fig. 2. Content of free amino acid (Σmeq NH_2/g protein) and free amino groups (meq NH_2/g protein) of hydrolysates fractions. HA/r and HA/f: retentate (>5 kDa) and filtrate (<5 kDa) from alcalase hydrolysate; HP/r and HP/f: retentate (>5 kDa) and filtrate (<5 kDa) from pancreatin hydrolysate; HF/r and HF/f: retentate (>5 kDa) and filtrate (<5 kDa) from flavourzyme hydrolysate. Free amino groups: determined by OPA method; Σmeq NH_2/g protein = Σ(aa / eq aa) × 1000, where aa is each amino acid concentration (g/g protein) and eq aa is the NH_2 equivalent of each amino acid.

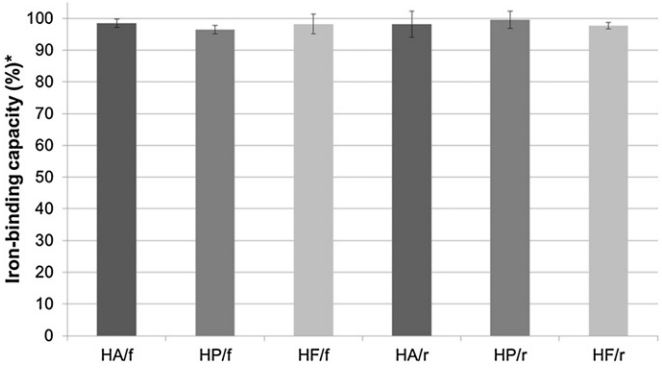

Fig. 3. Iron-binding capacity (%) of hydrolysates fractions. HA/r and HA/f: retentate (>5 kDa) and filtrate (<5 kDa) from alcalase hydrolysate; HP/r and HP/f: retentate (>5 kDa) and filtrate (<5 kDa) from pancreatin hydrolysate; HF/r and HF/f: retentate (>5 kDa) and filtrate (<5 kDa) from flavourzyme hydrolysate. (*) Soluble iron content in the supernatant after complexation reaction at pH 7.0, in relation to the initially added. Values are mean of 6 determinations; values with same letters do not differ statistically ($P < 0.05$).

value. Iron solubility increased after the iron–peptide complexation reaction, reaching iron-binding capacity values ranging from 96.5 to 99.5%, regardless of the enzyme or fraction used for complexation (Fig. 3). According to Zhu et al. (2009), chelating ligands protect the iron from binding with water, thereby hindering the formation of ferric hydroxides and increasing iron solubility. This infers that an iron–peptide interaction had occurred since free iron is insoluble at neutral pH, and the peptide profiles and enzyme used did not affect the results, in agreement with the results obtained with barley protein hydrolysates (Eckert et al., 2014). These results are possibly due to multiple binding sites of the different peptides released during enzymatic hydrolysis showing different affinities. High free amino acid contents, as presented by the fraction HF/f, did not affect the iron-binding capacity, although amino acids show lower affinity for minerals than for peptides (Huang et al., 2011).

All the fractions obtained from the hydrolysates produced by the enzymes tested showed the same ability to improve iron solubility at pH 7.0. These results are of interest because increasing iron solubility at intestinal pH may result in greater availability of iron for absorption, and iron solubility under the conditions of the gastrointestinal tract is considered to be a predictive parameter of iron bioavailability (Swain, Newman, & Hunt, 2003; Ueno, Urazono, & Kobayashi, 2014). Therefore, for the continuity of this study, the fractions obtained from the hydrolysate obtained with pancreatin were selected. This choice was made because pancreatin is an endogenous enzymatic system that releases small amounts of large peptides and large amounts of di- and tripeptides (Silvestre et al., 2013), which are more absorbable than longer peptides (Clemente, 2000).

3.2. Fe–peptide complex stability under simulated gastric conditions

The stability of Fe–peptide complexes obtained with the pancreatin hydrolysate fractions, Fe–HP/f and Fe–HP/r, was evaluated under simulated gastric conditions. This experiment was based on the following premises (Puig et al., 2005): if the gastric pH and/or the enzyme activity promote the breaking of the Fe–peptide binding, or if the iron is weakly bound, the iron released during digestion will precipitate when the pH is adjusted to 7.0. Otherwise, if the complexes are stable to gastric digestion conditions, the iron will remain soluble at pH 7.0, which is the intestinal pH value. Thus, soluble iron was determined after simulating gastric digestion and neutralization (pH 7.0), to evaluate the stability under conditions simulating gastric digestion.

The low iron solubility (0.8%) in the control digestion assay ($FeCl_2$) carried out without pepsin confirmed the insolubility of iron at pH 7.0 (Fig. 4). Iron solubility increased to 10.9% when the control digestion

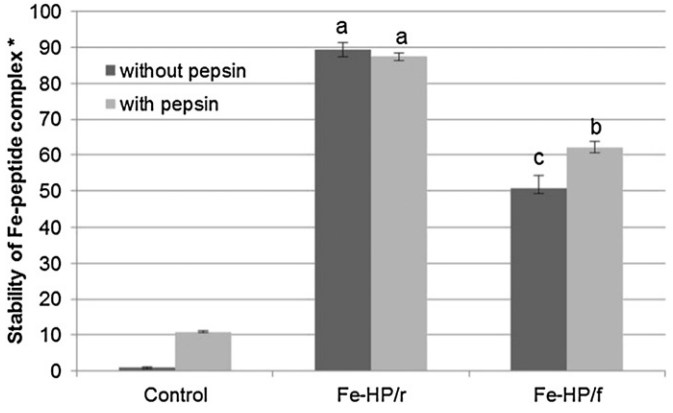

Fig. 4. Stability of Fe–peptide complex under simulated gastric conditions. Control: assay with $FeCl_2$; Fe–HP/r: Fe–peptide complex from retentate (>5 kDa) and Fe–HP/f: Fe–peptide complex from filtrate (<5 kDa) from hydrolysate with pancreatin. (*) Soluble iron content in the supernatant at pH 7.0 after simulated gastric digestion, in relation to the initially added. Values are mean of 6 determinations; values with same letters do not differ statistically ($P < 0.05$).

assay was carried out with pepsin, possibly due to interactions between iron and available functional groups in the enzyme. When the Fe–peptide complexes Fe–HP/r and Fe–HP/f were submitted to gastric conditions, with or without pepsin, the iron remaining in solution at pH 7.0 was 5- to 9-fold higher than in the control assay with pepsin, thereby evidencing the stability of Fe–peptide complexes to gastric digestion conditions. The addition of pepsin did not significantly affect ($P < 0.05$) the stability of the complex Fe–HP/r at pH 7.0. However, the stability of the complex Fe–HP/f increased from 50.8 to 62.2% due to the presence of pepsin. Part of the iron initially soluble at pH 7.0 became insoluble after simulated gastric digestion, possibly because metallic ions weakly bound to the peptides were released and precipitated when the pH was raised from 2.0 to 7.0 due to a rearrangement of loosely chelated ligands during the pH shift (Zhu et al., 2009). The Fe–HP/r sample showed greater stability than the Fe–HP/f one, suggesting that the presence of peptides with higher MM may have contributed to the stability of the complex during gastric digestion. These results are important because stable complexes potentially hinder iron pro-oxidant effects, preventing the side effects related to free iron (Sugiarto, Ye, Taylor, & Singh, 2010), and protecting the gastrointestinal mucosa from damage caused by free iron (Chaud et al., 2002).

3.3. Affinity chromatography isolation of iron-binding peptides and sequencing by LC–MS/MS

To characterize the peptides involved in the complexation with iron, the isolation of iron-binding peptides from the fractions of the pancreatin hydrolysate (HP/r and HP/f), was carried out by IMAC, which has been widely used for this purpose (De La Hoz et al., 2014). Iron-binding peptides were eluted between 70 and 90 min (Fig. 5). The relative concentration (area of peak/Σ area of peaks) of the iron-binding peptides (second peak) was 60% for sample HP/r and 40% for sample HP/f (Fig. 3).

The isolation of iron-binding peptides was carried out at pH 5.5 because at this pH the ionizable groups of the Asp and Glu residues, with pK values of 3.86 and 4.25, respectively, are deprotonated, which favors the binding with iron (Bresolin, Miranda, & Bueno, 2009). However, the imidazole ring of His (pK 6.0) is still mostly protonated, which is not favorable to iron interaction. Thus under these conditions, the Asp and Glu residues may have higher iron-binding capacities than histidine (Ramadan & Porath, 1985), which also has an important role in iron

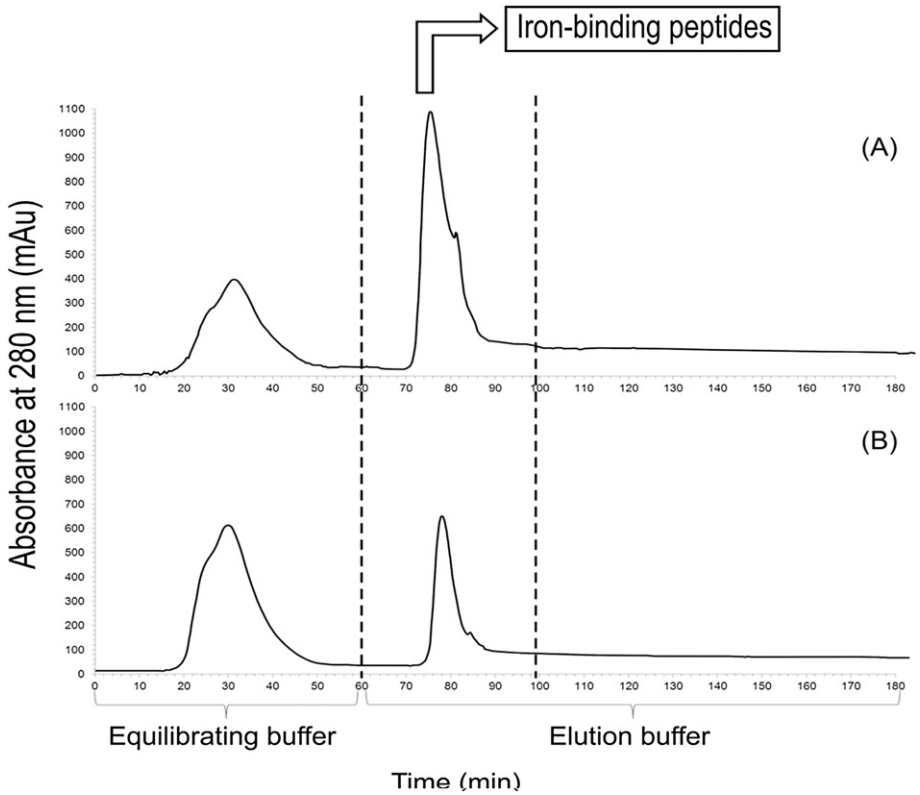

Fig. 5. Chromatographic profile (IMAC-Fe^{3+}) of fractions of WPI hydrolysate with pancreatin. (A) HP/r — retentate (>5 kDa) from hydrolysate with pancreatin and (B) HP/f — filtrate (<5 kDa) from hydrolysate with pancreatin. Analysis conditions: IDA-Sepharose 6B resin; flow rate 1 mL/min; monitoring at 280 nm; equilibrating buffer: sodium acetate buffer 50 mmol/L, pH 5.5 with 0.1 mol/L NaCl/elution buffer: Na_2HPO_4 solution 20 mmol/L.

complexation in affinity chromatography (Lv et al., 2009). In the samples evaluated, the Asp and Glu concentrations were 6- to 11-fold higher than that of His, which emphasizes the importance of these acidic amino acids in the iron-binding capacity.

The isolated iron-binding peptides (HP/r*ib and HP/f*ib) were subsequently analyzed by LC–MS/MS. The peptide sequence of the parent proteins and the identified sequences are presented in Table 1. Of the 34 sequences identified, 28 were from β-Lg while only 4 were from

Table 1
Identified fragments in the samples HP/r*ib and HP/f*ib and the complete sequence of the parent proteins.

Parent protein						
β–Lactoglobulin	Complete sequence of parent protein					
	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENDECAQKKIIAEKTKIPA VFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEE QCHI					
	Identified peptides					
	HP/r*ib			HP/f*ib		
	Sequence	m/z	Charge	Sequence	m/z	Charge
	^{125}TPEVDDE^{131}	804.3	+1	^{125}TPEVDDE^{131}	804.3	+1
	^{43}VEELKPTPE^{51}	521.3	+2	^{43}VEELKPTPE^{51}	521.3	+2
	^{43}VEELKPT^{49}	408.2	+2	^{127}EVDDEALEK^{135}	524.2	+2
	^{42}YVEELKPTPE^{51}	602.8	+2	^{126}PEVDDEALEK^{135}	572.7	+2
	^{125}TPEVDDEALEK^{135}	623.2	+2	^{125}TPEVDDEALEK^{135}	623.3	+2
	^{94}VLDTDYK^{100}	427.2	+2	^{44}EELKPTPEGDLE^{55}	678.8	+2
	^{123}VRTPEVDDE^{131}	530.2	+2	^{125}TPEVDDEALEKF^{136}	696.8	+2
	^{43}VEELKPTPEGDLEI^{56}	784.9	+2	^{124}RTPEVDDEALEK^{135}	701.3	+2
				^{127}EVDDEALEKFDK^{138}	719.3	+2
				^{43}VEELKPTPEGDLE^{55}	728.3	+2
				^{125}TPEVDDEALEKFD^{137}	754.3	+2
				^{126}PEVDDEALEKFDK^{138}	512.2	+3
				^{43}VEELKPTPEGDLEI^{56}	784.8	+2
				^{44}EELKPTPEGDLEIL^{57}	791.9	+2
				^{42}YVEELKPTPEGDLE^{55}	809.9	+2
				^{125}TPEVDDEALEKFDK^{138}	545.9	+3
				^{43}VEELKPTPEGDLEIL^{57}	784.9	+2
				^{41}VYVEELKPTPEGDLE^{55}	859.4	+2
				^{42}YVEELKPTPEGDLEI^{56}	866.4	+2
				^{42}YVEELKPTPEGDLEIL^{57}	922.9	+2
α–lactoalbumin	Complete sequence of parent protein					
	EQLTKCEVFRELKDLKGYGGVSLPEWVCTAFHTSGYDTQAIVQNNDSTEYGLFQINNKIWCKDDQNPHSSNICNISC DKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWLCEKL					
	Identified peptides					
	HP/r*ib			HP/f*ib		
	Sequence	m/z	Charge	Sequence	m/z	Charge
	^{82}DDDLTDDI^{89}	921.4	+1	^{80}FLDDDLTDDIM^{90}	656.8	+2
	^{61}CKDDQNPH^{68}	478.7	+2	^{79}KFLDDDLTDDIM^{90}	720.8	+2
BSA (bovine serum albumin)	Complete sequence of parent protein					
	DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVA SLRETYGDMADCCEKEQPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLY ANKYNGVFQECCQAADKGACLLPKIETMREKVLTSSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKL VTDLTKVHKECCHGDLLECADDRADLAKYICBBZBTISSKLKECKDPCLLEKSHCIAEVEKDAIPEDLPPLTADFAEDKD VCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYTSVFDKLKHLVDEPQNLIKZBC BZFEKLGEYXXXALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVESK VTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENF VAFVDKCCAADDKEACFAVEGPKLVVSTQTALA					
	Identified peptides					
	HP/r*ib			HP/f*ib		
	Sequence	m/z	Charge	Sequence	m/z	Charge
	^{11}FKDLGEEH^{18}	487.7	+2	–	–	–
	^{106}KDDSPDLPK^{114}	507.7	+2	–	–	–

HP/r*ib: fraction collected by IMAC referring to the peptides with iron-binding capacity (second peak) of retentate (>5 kDa) from WPI hydrolysate with pancreatin.
Hp/f*ib: fraction collected by IMAC referring to the peptides with iron-binding capacity (second peak) of filtrate (<5 kDa) from WPI hydrolysate with pancreatin.
Sequences highlighted correspond to peptides identified in fraction Hr/Pib; sequences in bold correspond to peptides identified in fraction Hf/Pib; sequences highlighted and in bold correspond to peptides identified in both samples.
A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val; W = Thr; Y = Tyr.

α-La and 2 from BSA, the latter being exclusively found in HP/f*ib. No sequence from lactoferrin, an iron-binding glycoprotein of the transferrin family, with great affinity for the iron ion (Ueno et al., 2014), was identified. This may be due to its low concentration in comparison to the main whey proteins.

For both samples, the sequences identified came from only three regions of β-Lg and two of α-La molecules (Table 1). The majority of the sequences were part of the regions ^{42}YVEELKPTPEGDLEIL^{57} and ^{124}RTPEVDDEALEK^{135} of β-Lg and ^{82}DDDLTDDI^{89} of α-La. These sequences were identified by Picariello et al. (2010) as extremely resistant to gastrointestinal digestion, which may suggest that they may exert a role in iron absorption in the small intestine by increasing its solubility (Ueno et al., 2014). Two sequences from two distinct regions of BSA, ^{11}FKDLGEEH^{18} and ^{106}KDDSPDLPK^{114} were only found in the fraction HP/r*ib, although, due to their low MM, it was to be expected they were also present in the HP/f*ib.

More than 80% of the sequences identified showed the presence of aspartic (D) and glutamic (E) acids, and all of them had from 2 to 5 Asp and/or Glu residues, which could explain the high iron-binding capacity of these peptides. Storcksdieck et al. (2007) and Wu, Liu, Zhao, and Zeng (2012) also found high levels of Asp and/or Glu in the sequencing of peptides from the in vitro digestion of muscle tissue from different meat sources and from anchovy muscle protein hydrolysates, respectively, these residues being amongst the main iron-binding sites. Lysine (K) and leucine (L) residues, which can also be involved in iron-binding (Chaud et al., 2002; Reddy & Mahoney, 1995), were found in more than 80% of the sequences identified, and proline (P), threonine (T) and valine (V) residues were found in more than 70%. The side chains of the aliphatic and aromatic amino acids can contribute to the metal binding capacity either through the thermodynamic stability or conformation of the peptide complexes, since they do not contain any donor atoms outside the peptide backbone (Sovago & Osz, 2006). It has been suggested that the role of proline in the iron-binding capacity is in the induction of structural bends in the proteins, which might aid the peptides in assuming a conformation that favors iron-binding (Storcksdieck et al., 2007). Argyri et al. (2007) reported that the proline rich hexapeptide PGPIPN enhances iron uptake in Caco-2 cells. The alcoholic –OH groups of serine or threonine are also reported as metal-binding sites of proteins or peptides (Wu et al., 2012).

Although the analysis covered the m/z range from 100 to 2000, the Mascot software identified larger peptides, but with a low score in HP/r*ib (results not shown). The greater the peptide length, the greater the difficulty in identifying them, because they may not be well ionized or fragmented during LC–MS/MS (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). Therefore peptides with MM above 2000 Da can only be identified if they have basic residues in their sequence, which give a charge to each peptide, diminishing the m/z ratio. The increase in the number of possible combinations of oligomer mass and charge, due to the increase in the MM and number of charges, leads to congestion and superposition of the peaks which are too close to each other on the m/z scale to be resolved by the analyzer (Fenn et al., 1989). This fact results in an effective decrease in resolution, thus hampering identification of the sequences. Although the sequences identified in HP/r*ib and HP/f*ib were similar, more sequences were identified in HP/f*ib than in HP/r*ib, possibly due to the abovementioned issues.

Peptides with iron-binding properties can also exert antioxidant action, either in food or in biological systems, hindering the pro-oxidant effect of free iron. This is because transition metals like iron, when free, catalyze the generation of reactive oxygen species (ROS), including the hydroxyl (•OH) and superoxide ($O_2^{\bullet -}$) radicals, leading to oxidation of unsaturated lipids and promoting oxidative damage at different levels (Saiga et al., 2003), although the complexed mineral is more stable and less prone to interactions. The in silico analysis of the peptides identified using the database of the BIOPEP software (Olsztyn, Poland) showed that some peptides have amino acid sequences with confirmed antioxidant activity, such as YVEEL (Pihlanto, 2006), identified in β-Lg, and KD (Suetsuna, 1999), LKP, LK, and LP (Huang, Majumder, & Wu, 2010), identified in proteins from other sources. The digestion process may promote the release of these sequences, which can exert antioxidant activity inside the organism.

Differences in iron absorption at the intestinal barrier may occur because this depends on the features of the ligands and also on the complexes formed (Kratzer & Vohra, 1986). Thus further studies are needed to make the application of Fe–peptide complexes feasible.

4. Conclusion

WPI hydrolysis with alcalase, pancreatin and flavourzyme yielded fractions with great capacity to increase iron solubility at pH 7.0 by the formation of Fe–peptide complexes. The sequencing of the iron-binding peptides by LC–MS/MS showed sequences from the three main whey proteins. The sequences were part of the regions ^{42}YVEELKPTPEGDLEIL^{57} and ^{124}RTPEVDDEALEK^{135} of β-Lg; ^{82}DDDLTDDI^{89} of α-La; or ^{11}FKDLGEEH^{18} and ^{106}KDDSPDLPK^{114} of BSA. The iron-binding capacity of the hydrolysate fractions could be due to the Asp and Glu residues, the main iron-binding sites, which were present in more than 80% of the sequences identified.

The iron–peptide complexes obtained from the hydrolysis of WPI with pancreatin were stable under simulated gastric digestion. Since iron–peptide bonding makes the metal less reactive, iron–peptide complexes can potentially hinder the side effects promoted by the pro-oxidant effect of free iron.

Complexation of these peptides with iron may exert a crucial role in obtaining products with higher bioavailability of this mineral and reduce its pro-oxidant effect. However, the possible effects of complexation need to be confirmed.

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