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Small peptides from enzymatic whey hydrolyzates increase dialyzable iron

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ABSTRACT

Iron fortification of food to compensate for dietary deficiency of this micronutrient is beset with problems of low iron bioavailability. Such problems may be mitigated using iron-chelated peptides. Small peptides from whey were obtained by hydrolysis with pancreatin, Alcalase, and Flavourzyme, and were evaluated for their effect on iron availability using the iron dialyzability method, and for iron binding capacity using iron (III)-immobilized metal ion affinity chromatography. The small peptides showed iron binding capacity to form chelates, the contribution of which to iron stability and solubility was shown by iron dialyzability enhancement.

1. Introduction

Iron-fortified food has been an alternative used by various countries to cope with dietary deficiency of this micronutrient (WHO/FAO, 2006). Problems associated with this practice, such as low iron bioavailability and sensory changes in food, have been continuously addressed. Some reports have indicated that iron-chelated peptides increase the stability, absorption, and bioavailability of iron (Ashmead, 1991; Bouhallab et al., 2002; Miquel, Alegría, Barberá, & Farré, 2006).

In the current study, whey proteins were utilized to get low molecular weight peptides after enzymatic treatment with pancreatin, Alcalase, or Flavourzyme. These enzymes are known to promote a high degree of hydrolysis (DH) when used for hydrolyzing whey proteins (Kim et al., 2007). The peptides obtained were evaluated for their effect on iron availability through dialyzability method, and iron binding capacity by Iron (III)-immobilized metal ion affinity chromatography [IMAC-Fe(III)].

2. Materials and methods

2.1. Material

Whey protein isolate (WPI; PROVON® Protein) was provided by Glanbia Nutritionals (Kilkenny, Ireland). Pepsine, pancreatin, and bile extract were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock standard solution of iron (1000 mg mL^{-1}) as $FeCl_3$ was purchased from Merck KGaA (Darmstadt, Germany). Solvents used were HPLC grade, and all other chemicals were of analytical grade. IMAC resin Sepharose High-Performance was supplied by GE Healthcare Bio-Science AB (Uppsala, Sweden).

2.2. Preparation of whey protein isolate (WPI) enzymatic hydrolyzates, degree of hydrolysis, and fractionation

The WPI was hydrolyzed by Alcalase (from *Bacillus licheniformis*, activity 2.4 AU g^{-1} protein) and Flavourzyme 1000L (from *Aspergillus oryzae*, activity 500 leucine aminopeptidase unit [LAPU] g^{-1} protein), both from Novozymes, (Novozymes Latin America Limited, Araucaria, PR, Brazil), and pancreatin (from porcine, activity 4 × United States Pharmacopeia units) from Sigma (Sigma–Aldrich, Saint-Quentin-Fallavier, France) using a Mettler-Toledo DL21 pH-stat (Mettler-Toledo Inc., Columbus, OH, USA) at 10% (w/v) substrate concentration. Degree of hydrolysis (DH) was calculated as recommended by Adler-Nissen (1986). In this method the DH is proportional to the base consumption required to maintain the pH constant during hydrolysis.

The best conditions for hydrolysis were obtained using an experimental Rotatable Central Composite Design (2^2 RCCD) employing three central points in a set of 11 trials, fixing pH and the enzyme/substrate (E/S) ratio as independent variables. The measured variable response was DH.

Fractionation of the WPI enzymatic hydrolyzates was accomplished using ultrafiltration system and Prep/Scale™-TFF Cartridges

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with nominal cut-off of 5 kDa (Pellicon® Millipore, Bedford, MA, USA). The permeated material, fraction <5 kDa, was lyophilized and stored at −20 °C.

2.3. Immobilized-metal affinity chromatography. Isolation of iron-binding peptides

IMAC-Fe(III) is a technique which allows to select iron-binding proteins or peptides efficiently. Affinity chromatography IMAC-Fe(III) consisted of iron(III) immobilized on Sepharose High Performance (HP) gel, and was used for selecting the peptides with high iron affinity from hydrolyzates <5 kDa. The method described by De la Hoz et al. (2014) was followed here. Briefly, a column was packed with IMAC Sepharose HP (10 mL) and charged with Fe^{3+} (5 mL of 200 mmol L^{-1} $FeCl_3$). A 50 mmol L^{-1} sodium acetate-acetic acid buffer (NaAc/HAc), pH 3.6, was used as equilibration and as adsorption buffer. A 100 mmol L^{-1} $NH_4H_2PO_4$, pH 4.5 buffer was used to elute the bound peptides. The absorbance of the eluates was monitored at 280 nm. Regeneration of the column was achieved with 50 mmol L^{-1} EDTA overnight. All chromatographic experiments were carried out at room temperature (~25 °C).

2.4. Determination of amino acid profile

Amino acids were determined by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Shimadzu HPLC system (Shimadzu Corporation, Tokyo, Japan) equipped with UV detector (254 nm) and a C18 column Luna-Phenomenex (250 mm × 4.6 mm, 5 μ; Phenomenex Inc., Torrence, CA, USA). Amino acid quantification was performed following the methods described by Hagen, Frost, and Augustin (1989) and White, Hart, and Fry (1986).

2.5. In vitro dialyzability

In vitro dialyzability was used to predict iron bioavailability of hydrolyzates (fractions <5 kDa) following the procedure described by Argyri et al. (2009, 2011). Briefly, the fractions <5 kDa of lyophilized WPI hydrolyzates were dissolved in milli-Q water (4 mg mL^{-1} protein) and had their pH adjusted to 2.8 with 6 mol L^{-1} HCl. A solution of 0.075 mol L^{-1} piperazine -N, N′- bis [2-ethane-sulfonic acid] disodium salt (PIPES) buffer, pH 5.7, was used as control. An aliquot of 0.1 mL of iron as iron (II) sulfate ($FeSO_4$) in aqueous solution was added to 1.9 mL of each one of the sample solutions to bring the final concentration to 0.20 mmol L^{-1}. It was assumed that, under these conditions, the formation of iron-chelates begins immediately. In order to compensate for endogenous iron in the samples, a blank with no $FeSO_4$ was run for each one of the hydrolyzates. The samples were submitted to a simulated gastrointestinal digestion using a six-well plate and glass inserts (homemade) with 6–8000 Da membranes fastened with o-rings enabling digest fractioning by dialysis. The iron dialyzability was expressed as a percentage of the total iron content and was calculated as:

$$\text{Iron dialyzability}(\%) = [(\text{dialyzable iron})/(\text{total iron})] \times 100$$

Dialyzable iron was the iron content of the dialyzate, and the total iron was the amount of iron added to the enzymatic hydrolyzates prior to digesting (final concentration of 0.2 mmol L^{-1}).

2.6. Determination of iron content

Iron content in hydrolyzates and dialyzates solutions was measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX, Varian, Mulgrave, Australia). All the samples were submitted to hydrochloric acid treatment and filtered through 0.2 μm syringe filters, prior to their application in ICP.

2.7. Statistical analysis

Analyses were performed in triplicate. Data were expressed as means ± standards deviations (SD), and compared by analysis of variance (ANOVA) and the Tukey test. Statistical analysis was performed using the STATISTICA 7 software package for Windows (StatSoft, Inc., Tulsa, OK, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Enzymatic hydrolyzates, degree of hydrolysis

The best conditions to hydrolyze the WPI were defined by performing a 2^2 RCCD experiment, and the results are shown in Table 1.

3.2. Dialyzable iron as index of bioavailability

Even though the iron dialyzability does not define the iron absorption level, it is an indicative of an absorbable condition. In vitro dialyzability is a simple tool for predicting iron availability in food or meals and for studying dietary factors influencing absorption (Argyri et al., 2011; Fairweather-Tait et al., 2005).

The effect of WPI hydrolyzates on iron dialyzability is shown in Table 2. Since no dialyzable iron was detected in the blank samples, it was not necessary to correct the results for contribution of endogenous iron. Compared with control 0.075 mol L^{-1} PIPES, the three enzymatic hydrolyzates (fractions <5 kDa) significantly improved iron dialyzability. No statistical differences were observed among the enzymes.

The increase of dialyzable iron by hydrolyzates suggested the formation of iron-peptides chelates. It is known that the chelate-structure provides high stability and solubility for iron, preventing its oxidation and subsequent precipitation (Ashmead, 2001). These chelates provided greater amount of iron available in comparison to the Fe^{2+} in ferrous sulfate (control of PIPES solution).

3.3. Iron-binding capacity of peptides evaluated by iron (III)-immobilized metal ion affinity chromatography

Peptides with the highest capacity to bind iron were retained on the affinity chromatographic column, thus being separated from the bulk hydrolyzate <5 kDa. The peptides retained were eluted with $NH_4H_2PO_4$, pH 4.5 buffer, collected, lyophilized and characterized for amino acids profile analysis.

The amino acid profiles of each of the WPI hydrolyzates <5 kDa and the respective iron-chelating fractions isolated by IMAC were obtained by RP-HPLC (data not shown). Matching the amino acid composition of peptides in hydrolyzates and chelated peptides, for

Table 1
Conditions utilized for hydrolyzing whey protein isolate (WPI) using pH-Stat and degree of hydrolysis reached.

Enzyme	pH	Enzyme/substrate ratio (%)	Temperature (°C)	Degree of hydrolysis (%)
Alcalase	8.0	1.0	60	21.40
Pancreatin	8.0	4.0	40	23.73
Flavourzyme	6.7	2.6	55	16.28

Table 2
Dialyzability of iron affected by fraction of hydrolyzates <5 kDa from whey protein isolate (WPI) treated with Alcalase, pancreatin, and Flavourzyme.

Samples	Enzyme	Iron dialyzability[a] (%)
WPI hydrolyzate	Alcalase	42.4 ± 4.7[a]
	Pancreatin	38.6 ± 4.2[a]
	Flavourzyme	40.2 ± 0.9[a]
Control (0.075 M PIPES)		24.1 ± 2.4[b]

[a] Values are means ± standard deviation (SD) ($n = 3$); different superscript letters indicate a significant difference ($P < 0.05$) between data.

the same enzymatic treatment, revealed that chelated peptides from Alcalase were rich in Lys, Pro, Asp, and His residues, whilst those from pancreatin hydrolyzates were rich in Asp, Glu, and His. However, Pro and His were the predominant residues in Flavourzyme hydrolyzates. The presence of Asp and Glu residues in the chelates is indicative that coordinated covalent bindings between iron and carbonyl groups from those residues were favored.

Peptides from whey protein hydrolyzed by Neutrase, rich in Asp, Glu, and Pro residues among others, improve iron absorption during in vitro digestion combined with Caco 2 cell (Ou et al., 2010). Storcksdieck, Bonsmann, and Hurrel (2007) argue that the iron-binding peptides in enzymatic digests from myofibrillar protein, rich in aspartic and glutamic acid residues, are facilitators of iron solubility.

The presence of proline residues in the chelated peptides seems to be a characteristic feature of peptides with iron-binding capacity, probably because proline places constraints in angles of peptide backbone favoring the binding. Yaron, Naider, and Scharpe (1993) reported that Pro residues confer unique structural constraint on peptide chain, which could influence the susceptibility to protease activity.

4. Conclusions

Small peptides from whey enzymatic hydrolyzates showed iron binding capacity to forming chelates. The contribution of chelates in the iron stability and solubility could be noted by iron dialyzability enhancement. The results reflect an improvement of the iron availability by peptides in simulated gastro-intestinal digestion.

References

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. London, UK: Elsevier Applied Science Publishers.

Argyri, K., Tako, E., Miller, D. D., Glahn, R. P., Komaitis, M., & Kapsokefalou, M. (2009). Milk peptides increase iron dialyzability in water but do not affect DMT-1 expression in Caco-2 cells. *Journal of Agricultural and Food Chemistry, 57*, 1538–1543.

Argyri, K., Theophanidi, A., Kapna, A., Staikidou, C., Pounis, G., Komaitis, M., et al. (2011). Iron or zinc dialyzability obtained from a modified in vitro digestion procedure compare well with iron or zinc absorption from meals. *Food Chemistry, 127*, 716–721.

Ashmead, H. D. (1991). Comparative intestinal absorption and subsequent metabolism of metal amino acid chelates and inorganic metal salts. In K. S. Subramanian, G. V. Iyengar, & K. Okamoto (Eds.), *ACS symposium series: Vol. 445. Biological trace element research* (pp. 306–319). Washington, DC, USA: American Chemical Society.

Ashmead, H. D. (2001). The chemistry of ferrous bisglycinate chelate. *Archives of Latinoamerican Nutrition, 51*(Suppl. 1), S13–S21.

Bouhallab, S., Cinga, V., Ait-Oukhatar, N., Bureau, F., Neuville, D., Arhan, P., et al. (2002). Influence of various phosphopeptides of caseins on iron absorption. *Journal of Agricultural and Food Chemistry, 50*, 7127–7130.

De la Hoz, L., Ponezi, A., Milani, R., Nunes da Silva, V., de Souza, A. S., & Bertoldo-Pacheco, M. T. (2014). Iron-binding properties of sugar cane yeast peptides. *Food Chemistry, 142*, 166–169.

Fairweather-Tait, S., Lynch, S., Hotz, C., Hurell, R., Abrahamse, L., Beebe, S., et al. (2005). The usefulness of in vitro models to predict the bioavailability of iron and zinc: a consensus statement from the Harvest-Plus Expert Consultation. *International Journal for Vitamin and Nutrition Research, 75*, 371–374.

Hagen, S. R., Frost, B., & Augustin, J. (1989). Pre-column phenylisothiocyanate derivatization and liquid chromatography of amino acids in food. *Journal of the Association of Official Analytical Chemists, 72*, 912–916.

Kim, S. B., Seo, I. S., Khan, M. A., Ki, K. S., Nam, M. S., & Kim, H. S. (2007). Separation of iron-binding protein from whey through enzymatic hydrolysis. *International Dairy Journal, 17*, 625–631.

Miquel, E., Alegría, A., Barbera, R., & Farré, R. (2006). Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding. *International Dairy Journal, 16*, 992–1000.

Ou, K., Liu, Y., Zhang, L., Yang, X., Huang, Z., Nout, M. J. R., et al. (2010). Effect of neutrase, alcalase, and papain hydrolysis of whey protein concentrates on iron uptake by Caco-2 cells. *Journal of Agricultural and Food Chemistry, 58*, 4894–4900.

Storcksdieck, S., Bonsmann, G., & Hurrell, R. F. (2007). Iron-binding properties, amino acid composition, and structure of muscle tissue peptides from in vitro digestion of different meat sources. *Journal of Food Science, 72*, S019–S029.

White, J. A., Hart, R. J., & Fry, J. C. (1986). An evaluation of the Waters Pico-Tag system for the amino-acid analysis of food materials. *Journal of Automatic Chemistry, 8*, 170–177.

WHO/FAO. (2006). *Guidelines on food fortification with micronutrients*. Geneva, Switzerland: World Health Organization and Food and Agriculture Organization of the United Nations.

Yaron, A., Naider, F., & Scharpe, S. (1993). Proline-dependent structural and biological properties of peptides and proteins. *Critical Reviews in Biochemistry and Molecular Biology, 28*, 31–81.