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Synthesis of whey peptide-iron complexes: Influence of using different iron precursor compounds

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ABSTRACT

Iron-binding peptides are an alternative for increasing the bioavailability of iron and to decreasing its pro-oxidant effect. This study aimed to synthesize and characterize peptide-iron complexes using $FeCl_2$ or $FeSO_4$ as the iron precursor compounds. Whey protein isolate (WPI), WPI hydrolyzed with pancreatin, and its fractions obtained via ultrafiltration (cut-off 5 kDa) were used as ligands. The fluorescence intensity of the ligands significantly decreased as the iron concentration increased as a result of metal coordination with the iron-binding sites, which may have led to changes in the microenvironment of tryptophan. For both iron precursor compounds, the primary iron-binding site was carboxylate groups, and the linkage occurred via a bidentate coordination mode with two vibrational modes assigned to the COO−Fe linkage. However, infrared spectroscopy and thermal analysis results showed that the dynamics of the interaction is different for the iron precursor. The iron source may be of great importance because it may impact iron absorption and the pro-oxidant effect of the mineral.

1. Introduction

Iron is an essential micronutrient and an intrinsic component of hemoglobin, myoglobin, and cytochromes (Luo & Xie, 2012). This mineral acts as a regulator, activator, and controller of several enzymatic reactions. Among iron's functions, the formation of red blood cells, O_2 and CO_2 transport, electron transfer, redox reactions and cellular energy production can be highlighted (Lieu, Heiskala, Peterson, & Yang, 2001). Therefore, iron deficiency is responsible for several diseases and is one of the primary nutritional problems worldwide. Many efforts have been made to combat this deficiency, mainly regarding food fortification. However, this practice is still a challenge due to the questions associated, such as low bioavailability, digestive problems, such as stomach ache or diarrhea, and even changes in the flavor and appearance of food products (Sugiarto, Ye, & Singh, 2009).

Peptide-iron complexes can be an alternative to mitigate these problems. Under certain conditions, iron binding with an organic compound promotes the formation of complexes, which protects the micronutrient against the chemical reactions that occur during the digestive process (Gligic, Palic, & Nikolovski, 2004). Iron-peptide binding is based on the interaction between an electron donator group at the ligand surface (in this case, peptide) and a Lewis acid (transition metal ion). Peptides may present one or more accessible coordinating sites, ensuring that the metal atom becomes part of a biologically stable structure through coordinate covalent bonding. In this way, the complexed mineral is less prone to interactions with the chemical neighborhood (Miquel & Farré, 2007).

Ferrous or ferric salts, such as $FeCl_2$, $FeCl_3$, and $FeSO_4$, have been used to synthesize peptide-iron complexes and to study the iron-binding capacity of peptides (Caetano-Silva, Bertoldo-Pacheco, Paes-Leme, & Netto, 2015; Chaud et al., 2002; Huang, Ren, & Jiang, 2011; Kim et al., 2007; O'Loughlin, Kelly, Murray, FitzGerald, & Brodkorb, 2015; Ueno, Urazono, & Kobayashi, 2014; Zhou et al., 2012). Nevertheless, to the best of our knowledge, no effort has been made to study the effect of iron sources on complex formation, even though the role of different ions on peptide and protein structure and interactions is

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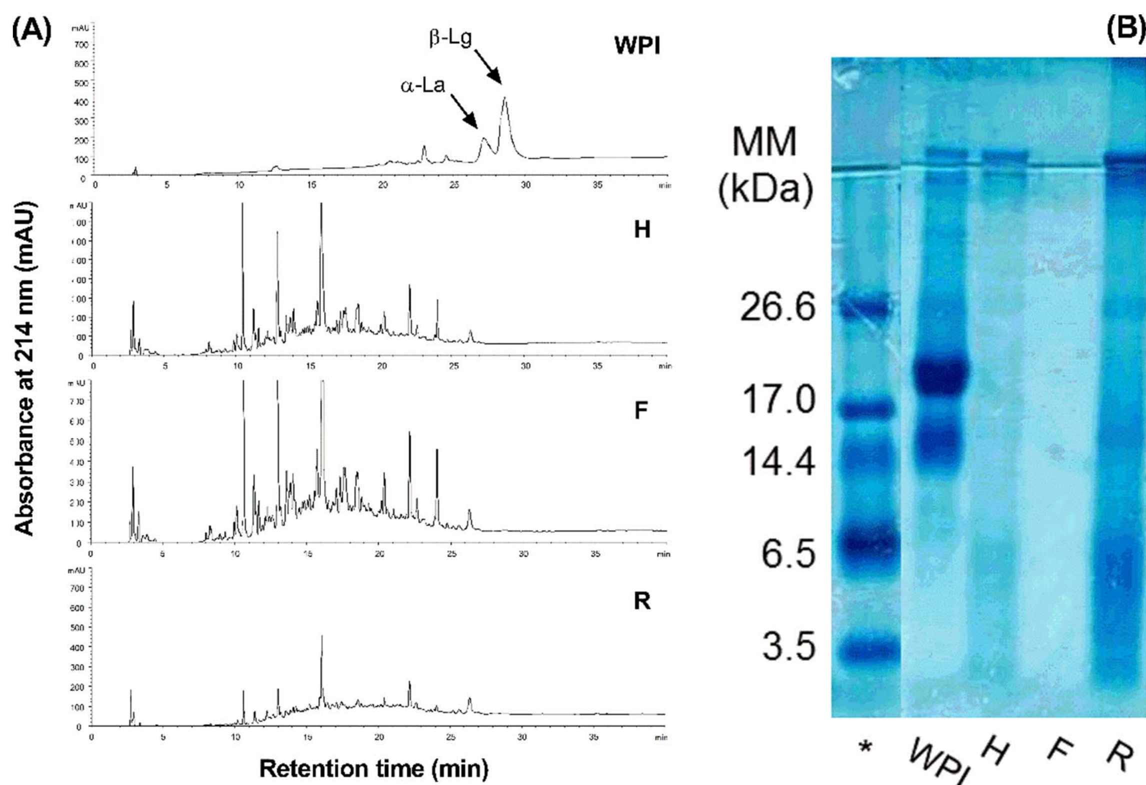

Fig. 1. (A) Chromatographic profile (RP-HPLC) and (B) electrophoretic profile (SDS-PAGE Tricine) of ligands. **WPI** – whey protein isolate; **H** - hydrolysate; **F** – filtrate (fraction < 5 kDa); **R** - retentate (fraction > 5 kDa). * Molecular mass standard.

widely known (Zhang & Cremer, 2006).

Our previous studies showed that fractions of WPI hydrolysate with pancreatin have the same iron-binding sites regardless of the molecular mass (MM) (Caetano-Silva et al., 2015); however, only complexes synthesized with low-MM peptides (MM < 5 kDa) showed a capacity to increase iron uptake in vitro compared with the iron salt form. Regarding the iron source, complexes synthesized with $FeCl_2$ led to greater ferritin synthesis than complexes synthesized with $FeSO_4$ ($p < 0.05$) (Caetano-Silva, Cilla, Bertoldo-Pacheco, Netto, & Alegría, 2017). Therefore, our results suggested that different iron precursors influence the functionality of the complexes due to differences in complex structure caused by the counter ions, chloride or sulfate. The aim of the present work was to test this hypothesis using peptide-iron complexes synthesized using two iron salts, $FeCl_2$ and $FeSO_4$, and different MM ligands to understand the effect of the iron source on the dynamics of the peptide-iron interaction, which may contribute to their future application for food fortification.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained from Glanbia Nutritionals (Kilkenny, Ireland). Pancreatin (4xUSP, from porcine pancreas, EC 232-468-9, P1750) was purchased from Sigma-Aldrich® (St. Louis, MO, USA). Iron precursors, $FeCl_2{\cdot}4H_2O$ and $FeSO_4{\cdot}7H_2O$, were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Sigma-Aldrich® (St. Louis, MO, USA), respectively. All other chemicals and reagents used were of analytical or chromatographic grade.

2.2. Preparation of hydrolysate and its fractions

The hydrolysate was obtained from enzymatic hydrolysis of WPI with the enzymatic system pancreatin under previously defined conditions (Caetano-Silva et al., 2015). Briefly, WPI was diluted in deionized water (10% protein w/v), and the reaction occurred at pH 8.0; it was monitored using an automatic titrator, pH Stat (Mettler-Toledo, model DL 21 Grafix, Schwerzenbach, Switzerland) with stirring and a controlled temperature (40 °C). After 180 min, the enzyme was deactivated by heating (85 °C for 15 min), and the reaction mixture was cooled in an ice bath.

The degree of hydrolysis (DH), determined according to Adler-Nissen (1986), was 16.2%. The pH was adjusted to 6.0 with 1 mol/L HCl, and a portion of the reaction mass was then freeze-dried; this sample was named hydrolysate (**H**). A portion of **H** was fractionated in a Pellicon® ultrafiltration system (Millipore, Bedford, MA, USA) with a cut-off membrane of 5 kDa (Cartridge Prep/Scale – TFF 6 ft^2). The fraction with an MM < 5 kDa was named filtrate (**F**), and the one with MM > 5 kDa was named retentate (**R**).

2.3. Characterization of the ligands

2.3.1. Chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed in an Agilent liquid chromatograph with a semi-preparative and analytical quaternary pump system and a diode array detector (DAD) (Agilent, Waldbronn, Germany) on a Microsorb – MV™ C_{18} column (4.6 mm × 250 mm; 5 μm particle size) (Rainin, Woburn, MA, USA). The solvent composition was solvent A – 0.04% de TFA in ultrapure water; solvent B – 0.03% TFA in acetonitrile. The gradient elution conditions were solvent B from 0 to 70% in 40 min, 100% in 5 min, and returning to 0% in 5 more min. Detection was at 214 nm, and the sample injection volume was 50 μL (3 mg protein/mL for **H**, **F**, and **R**, and 1 mg protein/mL for **WPI**). The proteins, α-lactoalbumin (α-La) and β-lactoglobulin (β-Lg), from bovine milk (Sigma-Aldrich®, St. Louis, MO, USA), were used as standards.

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