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In vitro iron absorption of α-lactalbumin hydrolysate-iron and β-lactoglobulin hydrolysate-iron complexes

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ABSTRACT

To study the feasibility of promoting iron absorption by peptides derived from α-lactalbumin and β-lactoglobulin, the present work examined the transport of iron across Caco-2 monolayer cell as in vitro model. Caco-2 cells were seeded in bicameral chambers with α-lactalbumin hydrolysate-Fe (α-LAH-Fe) complex and β-lactoglobulin hydrolysate-Fe (β-LGH-Fe) complex, α-LAH and iron mixture, β-LGH and iron mixture, $FeSO_4$ and ascorbic acid mixture, and $FeSO_4$. In addition, the cytotoxicity of α-LAH-Fe and β-LGH-Fe complexes were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The iron absorption and ferritin content were assessed using the coupled in vitro digestion/Caco-2 cell model. Results support that peptide-iron complexes can promote ferritin formation and it is possible to apply β-LGH-Fe complexes as iron-fortified supplements with high iron absorbability.
Key words: iron absorption, α-lactalbumin hydrolysate-iron complex, β-lactoglobulin hydrolysate-iron complex, Caco-2 cells, ferritin

INTRODUCTION

Iron is an essential trace mineral that plays an important role in physical metabolism, including oxygen transport, DNA synthesis, and electron transport in human bodies (Lieu et al., 2001). It can be found in a broad variety of foods. Heme iron, which only comes from hemoglobin and myoglobin in animal food, possesses high bioavailability (de Castro Cardoso Pereira and dos Reis Baltazar Vicente, 2013). Non-heme iron, which is present in dark green leafy vegetables, can be severely impaired by iron absorption inhibitors such as phytate (Hurrell et al., 1992; Abizari et al., 2012) and some phenolic compounds (Petry et al., 2010).

Iron deficiency leads to iron deficiency anemia, the most common and widespread nutritional disorder, according to the World Health Organization (Geneva, Switzerland). Iron fortification is used to increase dietary iron intake. Soluble iron salts (e.g., ferrous sulfate) are not good candidates due to their negative effects on product stability, undesired colored complexes with polyphenol, and adverse organoleptic changes in high-fat or polyphenol-containing food. The free solubilized iron is also a prooxidant (Letelier et al., 2010). Poorly water-soluble iron compounds are more inert than solubilized iron, but they tend to have lower bioavailability, as the iron must be released first by the action of gastric juice (Walczyk et al., 2013). Therefore, the development of iron supplement and iron-absorption-promoting ingredients is critical.

Mineral-binding proteins in foods that are rich in acidic clusters and with specific binding sites for minerals are able to sequester a large amount of divalent cation (Hettiarachchy et al., 2012). The mineral-binding peptides derived from food protein could increase the solubility of minerals at intestinal pH (Erba et al., 2002; Meisel and FitzGerald, 2003). The formation of soluble peptide-mineral complexes and resistance to proteolysis of peptides in the intestinal lumen as mineral carriers contributed to the bioavailability of minerals (FitzGerald, 1998). Whey proteins have been widely applied for commercial interest as an emulsifier and whipping agent in food and nutraceuticals with nutritive value (Yamauchi et al.; 1980; Marshall, 2004; Marinova et al., 2009). Consumption of cow milk by infants and toddlers has adverse effects on their iron stores, which is probably due to its low iron content (Ziegler, 2011). It also has been reported that iron absorption was reduced by native milk protein and protein hydrolysis lessened this negative effect (Hurrell et al., 1989; Kibangou et al., 2005). Milk protein hydrolysate could improve iron uptake in the Caco-2 cells model (Argyri et al., 2009). The major organ for digestion and absorption of nutrients is the small intestine. Caco-2 cells, which derived from a human colon adenocarcinoma, spontaneously differentiate into cells with enterocyte morphology (Levy et al., 1995). As Caco-2 cells exhibit several morphological

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and functional characteristics of mature enterocytes, the monolayers are suitable as an in vitro model for absorption and have been applied for the assessment of iron absorption from food (Au and Reddy, 2000; Hendricks et al., 2001; Argyri et al., 2007). Ferritin in Caco-2 cells is involved in iron absorption, which is a protein polymer with a hollow shell structure that can store iron (Vanoaica et al., 2010). Ferritin formation could be used as an indicative index of cell iron uptake (Glahn et al., 1998). Ascorbic acid [vitamin C (**Vc**)], which possesses reducing and chelating properties, has been reported to increase iron absorption and hemoglobin and plasma ferritin concentrations (Monárrez-Espino et al., 2011).

The optimum passage range of Caco-2 cells for experimental purposes was 28 to 65 passages. During these passages, the Caco-2 cells became increasingly columnar in appearance, the brush border became more apparent and more uniform, and intercellular spaces became more well defined after prolonged incubation (Briske-Anderson et al., 1997). Transepithelial electrical resistance (**TEER**) values of Caco-2 cells have been reported as a good indication of the tightness of junction structure (Lu et al., 1996; Briske-Anderson et al., 1997; Leonard et al., 2000). Cytotoxicity to Caco-2 cells can be used to evaluate the dose-dependent toxic potential with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (**MTT**) assay. The MTT assay, which is based on the conversion of MTT into formazan crystals by living cells, determines mitochondrial activity. The excess iron cannot be actively excreted by the human body. Excess of redox active iron leads to oxidative stress and tissue damage, as a result of the formation of free radicals (Okada, 1996; Papanikolaou and Pantopoulos, 2005). In addition, a higher concentration of Fe loading leads to disruption of the cell membrane and enhancement of genome instability and cancer risk (Huang, 2003; Prá et al., 2012).

β-Lactoglobulin and α-LA are the major proteins in whey protein. Our previous study showed that β-LG hydrolysate (**β-LGH**) obtained with alcalase exhibited the highest iron-binding capacity. Compared with β-LGH, the formed β-LGH-Fe complexes exhibited new absorption peaks in Fourier-transform infrared spectroscopy, which indicated the formation of peptide-iron complexes (Zhou et al., 2012). Whether the chelated complexes possess higher mineral absorbability still awaits further investigation. Therefore, the objectives of this study were to explore the feasibility of promoting iron absorption by α-LA hydrolysate (**LAH**)-Fe and β-LGH-Fe complexes, and compare the in vitro absorption efficiency of α-LAH-Fe and β-LGH-Fe complexes using Caco-2 monolayer cells.

MATERIALS AND METHODS

Materials

Alcalase [endoproteinase from *Bacillus licheniformis*; 2.4 Anson units (AU)/g] was donated by Novozymes North America Inc. (Franklinton, NC). Pepsin (EC3.4.23.1; 800–2,500 U/mg of protein) from porcine gastric mucosa and pancreatin from porcine pancreas [P7545; 8 × United States Pharmacopeia (USP) specification] were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bovine α-LA (Arla-20; protein 88–94%) was obtained from Arla Foods Ingredients (Viby, Denmark). Bovine β-LG isolate (97.6% protein on a dry basis, 93.2% purity) was provided by Davisco Foods International Inc. (Eden Prairie, MN).

Preparation of α-LAH and β-LGH

α-Lactalbumin (5%, wt/vol) and β-LG (5%, wt/vol) were hydrolyzed with alcalase [enzyme:substrate ratio (E/S) = 5%] at 50°C for 6 h, respectively. The pH of dispersion was adjusted to 8.0 and maintained with 1 *M* NaOH during the hydrolysis process. After hydrolysis, the solutions were heated in an 85°C water bath for 15 min to inactivate the enzyme. Then, the hydrolysates were cooled to room temperature and ultrafiltered using membrane with a 10,000-Da molecular weight cutoff (Millipore Corp., Bedford, MA) to remove the enzyme and unhydrolyzed protein. The permeated α-LAH and β-LGH were freeze-dried, sealed in plastic bags, and stored at 4°C for the in vitro experiment. The degree of hydrolysis of the obtained α-LAH and β-LGH was 18.53 and 23.78%, respectively.

Formation of α-LAH-Fe Complexes and β-LGH-Fe Complexes

The α-LAH-Fe complexes and β-LGH-Fe complexes were prepared according to Zhou et al. (2012). Binding of iron to the freeze-dried α-LAH and β-LGH was performed by mixing them with ferric chloride solution (hydrolysate:Fe mass ratio = 40:1) at 25°C and pH 7.0, respectively. The reaction was carried out in a shaker (DSHZ-300A; Taicang Experimental Instrument Co. Ltd., Jiangsu Province, China) for 30 min. Free iron was precipitated and removed by centrifugation at 3,000 × *g* for 20 min at 25°C after the binding process. The soluble peptide-bound iron complexes were collected in the supernatant.

Cell Cultures

Human epithelial colorectal adenocarcinoma Caco-2 cells [American Type Culture Collection (ATCC)] with