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Characterization of iron-binding phosphopeptide released by gastrointestinal digestion of egg white

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ABSTRACT

Binding and solubilization of ferric iron by food peptides, released during digestion, facilitate intestinal iron absorption. In the present study, we investigated the release of iron-binding peptides during *in vitro* gastrointestinal digestion of chicken (*Gallus gallus*) egg white. The iron-binding activity of the egg white protein increased upon gastrointestinal digestion. The iron-binding fraction of egg white digesta was purified by gel filtration chromatography followed by reverse phase HPLC. Subsequently, this fraction was identified as an internal fragment of ovalbumin (DKLPGFGDS$^{(PO)}_4$IEAQ, 61–73 residues, GenBank AAB59956.1) by MALDI-MS/MS followed by *de novo* sequencing. The synthetic peptide corresponding to the identified iron-binding peptide sequence bound and increased the ^{59}Fe-iron uptake. Further, the synthetic peptide also stimulated the iron-induced ferritin synthesis in intestinal Caco-2 cells. While, dephosphorylation of synthetic peptide completely inhibited the iron-binding activity, methyl-esterification of its carboxyl groups partially inhibited the activity. These results suggest that food derived peptides modulate intestinal iron absorption and that the isolated iron-binding egg peptide could be a potential nutraceutical for improving iron absorption.

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1. Introduction

Iron exists in ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms in nature. Dietary iron is available mainly in two forms, the heme- and non-heme iron. Heme-iron is better absorbed than non-heme iron (Baker, Anderson, & Baker, 2003). Dietary non-heme iron present predominantly in ferric form is reduced by duodenal cytochrome-B (DcytB) prior to its intestinal absorption *via* divalent metal ion transporter 1 (DMT1) (Sharp & Srai, 2007). The intestinal absorption of ferric-iron is limited by its poor solubility at near neutral pH, and chelation improves its solubility and intestinal absorption (Hurrell et al., 2004; Palika et al., 2013). Phytic acid (inositol-hexaphosphate, IP6) and polyphenols, the major secondary metabolites of plant foods, are potent inhibitors of non-heme iron absorption (Hurrell, 2002; Nair & Iyengar, 2009). Therefore, the observed high prevalence of iron deficiency anemia in vegetarians could be due to poor density and bioavailability of iron (Nair & Iyengar, 2009).

Therapeutic iron supplementation and food fortification are potential strategies to prevent iron deficiency anemia (Hurrell, 2002; Hurrell et al., 2004; Nair & Iyengar, 2009). However, oral iron therapy is associated with free radical damage, and the addition of iron to food results in unacceptable organoleptic properties due to the pro-oxidant nature of iron (Casanueva & Viteri, 2003; Douglas, Rainey, Wong, Edmonson, & LaCroix, 1981). Although more inert (encapsulated) or chelated forms of iron increase the shelf life of products, the iron bioavailability from such sources remains a serious concern (Hurrell et al., 2004). Therefore, alternate strategies such as selection of iron fortificants with higher iron solubility/bioavailability or addition of components that increase iron solubility and hence absorption are needed.

Studies over the past two decades have demonstrated that iron bioavailability from protein rich foods, particularly from animal sources, is high (Cook & Monsen, 1976; Hurrell, 2002; Hurrell et al., 2004; Nair & Iyengar, 2009). Histidine or cysteine-rich peptides released during the

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digestion of dietary protein are reported to stimulate intestinal iron absorption (Hurrell, Reddy, Juillerat, & Cook, 2006; Storcksdieck & Hurrell, 2007; Swain, Tabatabai, & Reddy, 2002; Taylor, Martinez-Torres, Romano, & Layrisse, 1986). Indeed, casein phosphopeptides (CPP) isolated from *in vitro* digests of milk have been demonstrated to improve mineral bioavailability including that of iron (Bouhallab & Bougle, 2004). Iron-binding peptides have also been isolated from soybeans, whey protein and yeast (de la Hoz et al., 2014; Lv et al., 2009). These observations suggest wide-spread occurrence of iron-binding peptides in foods that are released during digestion. However, the quantity of such peptides present or released during digestion is a subject of investigation. Further, the nature of the iron-binding peptides and their precursor proteins remains to be characterized from different food sources. Hence, isolation of these peptides in pure form and characterization of their primary structure shall aid immensely in understanding the nature of iron-binding food peptides and associated mineral binding mechanisms.

Eggs, a rich source of protein, are widely consumed. Peptides with antiviral, anti-hypertensive, anti-microbial, anti-cancer and anti-oxidant activities that have been identified in the enzymatic hydrolysates of egg proteins (Mine, 2007). Studies in humans and animal models have reported the inhibitory effect of eggs on iron bioavailability (Grengard, Sentenac, & Mendelsohn, 1964; Rose, Vahlteich, & Macleod, 1934). The egg yolk phosphovitin, an extensively phosphorylated protein, binds iron and prevents its release during gastric and intestinal phases of digestion (Grengard et al., 1964). However, iron-binding peptides if any, released during the digestion of egg white proteins and their effect on intestinal iron absorption are not studied yet. Recently Abeyrathne, Lee, Jo, Nam, and Ahn (2014) reported the release of iron and copper binding peptides during enzymatic digestion of ovalbumin. However, the nature of the specific peptides involved in mineral binding and associated mechanisms remains to be studied.

The present study examined the effect of gastric and intestinal digestion of egg white protein on the iron-binding capacity and attempted to decipher the molecular identity of specific peptide(s) involved in iron-binding. Further, the effect of the isolated iron-binding peptide on iron uptake and iron induced ferritin synthesis was also assessed in intestinal cells.

2. Material and methods

2.1. Materials

Porcine pepsin (Cat#P7012), pancreatin (Cat#P7545) and all other chemicals/reagents were obtained from Sigma Chemical Co. (Bangalore, India), unless otherwise specified. $^{59}FeCl_3$ (carrier free) was obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai, India. Chicken eggs were purchased from the local market. Phosphorylated synthetic peptide (DKLPGFGDS$^{(PO)}{}_4$IEAQ) was obtained from GenScript (NJ, USA) and its purity (>98%) was confirmed by MALDI-MS.

2.2. In vitro digestion and isolation of iron-binding peptides

2.2.1. Simulated gastrointestinal digestion

Chicken eggs (*Gallus gallus*, 4 Nos) were boiled in water for 20 min and the solid whites were collected, and washed with Milli Q water. The egg whites (112 g) were cut into small pieces, suspended in 250 mL normal saline and homogenized in a kitchen blender for 2 min at the maximum speed (18,000 rpm). The simulated *in vitro* gastric and intestinal digestion was carried out as described previously (Glahn, Lee, Yeung, Goldman, & Miller, 1998; Pullakhandam, Nair, Pamini, & Punjal, 2011), except that the digestion was carried out for 6 h to ensure complete digestion of the egg white protein. Briefly, the pH of the egg white suspension was adjusted to 2 with 6 N HCl followed by the addition of 1 g of porcine pepsin (≥2500 units/mg protein, dissolved in 10 mL of 1 N HCl). The pepsin digestion was carried out for 6 h at 37 °C in a shaking water bath. At the end of this step, the pH of sample was raised to 6.5 with 2 M $NaHCO_3$ followed by the addition of 1 g of porcine pancreatin (8X USP equivalents, dissolved in 0.01 mM $NaHCO_3$). The samples were incubated at 37 °C for 6 h to complete digestion. During different stages of digestion, an aliquot of the digestion mixture was drawn for testing the iron solubilization activity as described below. At the end of simulated gastrointestinal digestion, the samples were clarified by centrifugation at 10,000 *g* for 15 min at 4 °C and the supernatant is referred to as the 'digesta'.

2.2.2. Ultrafiltration

The digesta was subjected to ultrafiltration through 5 kDa cut-off centrifugal filters (Millipore) to collect <5 kDa peptides in the filtrate (5kF) and >5 kDa proteins/peptides in the retentate (5kR). The 5kF and 5kR fractions were frozen and stored at −20 °C, until further analysis.

2.2.3. Solid phase extraction

Solid phase extraction was performed on an octadecyl-silica (C-18) matrix (Discovery DSC 18, Sigma). Briefly, the glass column consisting 5 g of C-18 matrix was equilibrated with 2% acetonitrile (v/v) containing 0.1% trifluoroacetic acid (TFA). The 5kF fraction (10 mL containing 0.1% TFA) was loaded on the column, washed with 100 mL of 2% acetonitrile to remove unbound proteins. The bound proteins were then eluted with 10 mL of 50% acetonitrile, concentrated in a vacuum evaporator (Vacufuge Plus, Eppendorf). The dried fractions were stored at −20 °C, until further analysis. They were reconstituted in saline (0.9% NaCl, containing 0.1% TFA) prior to the analysis.

2.2.4. Iron solubilization assay

Ferric iron is not soluble at neutral pH unless it is reduced to ferrous form or is bound to soluble components. Therefore, estimation of soluble iron in the absence of reduction provides a direct measure of iron-binding activity. The ferric iron solubilization assay was performed as described previously (Palika et al., 2013). Briefly, 100 μL aliquots of the digesta, 5kF, 5kR, C-18 bound and flow-through fractions or synthetic peptide (0 to 50 μM) were diluted with 50 mM 2-(N-Morpholino) ethanesulfonic acid sodium salt (MES) buffer pH 6.5 to 1 mL and supplemented with 25 μM $FeCl_3$ (traced with 50 nCi $^{59}FeCl_3$), and incubated for 30 min at 37 °C. At the end of incubation, samples were centrifuged at 10,000 *g* at 4 °C for 15 min and the supernatant solution (800 μL) was mixed with 5 mL of Bray's mixture and counted in liquid scintillation counter (PerkinElmer; TRICARB 2900TR). The percent binding of iron was calculated considering the solubility of ^{59}Fe iron in 6 N HCl as 100%.

2.2.5. Ferric iron reduction assay

The reduction of ferric iron was measured as described previously (Palika et al., 2013). Briefly, the reaction mixture (500 μL) contained egg peptide fractions (100 μL), 25 μM $FeCl_3$ and 500 μM ferrozine (a chromogen for ferrous iron) in 50 mM MES pH 6.5. The reaction mixture was incubated for 120 min at 37 °C, and the absorbance was measured at 562 nm in a micro-plate reader (BioTek; Powerwave HT-1).

2.2.6. Gel filtration chromatography

A superdex-peptide (10/30, HR) column (Amersham Biosciences) connected to Akta-purifier module (Amersham Biosciences) was equilibrated with 5 column volumes of normal saline. The C-18 bound fraction reconstituted in 500 μL of saline containing 0.1% TFA was loaded on the above column and eluted with the equilibration buffer at a flow rate of 0.5 mL/min, while monitoring the absorption at 215 nm. A total of 29 fractions (1 mL each) were collected and used immediately in the iron solubilization assay. The fractions with iron solubilizing activity were concentrated by solid phase extraction on C-18 column as described above.

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