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Effects of different proteases on iron absorption property of egg white hydrolysates

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ABSTRACT

One of the causes of iron deficiency in human is poor absorption of non-heme iron from the diet. While proteins from meats have been reported in the literature to enhance the absorption of non-heme iron, other proteins, such as those from egg, are known to inhibit iron absorption. The objective of this study is to investigate non-heme iron binding property of egg white proteins hydrolyzed using pepsin and a combination of bacterial/fungal proteases. The iron bioavailability of non-heme iron, in the presence of egg white (EW) hydrolysates, was evaluated in vitro using a tissues culture model system - rat intestinal epithelial cells (IEC-6). In the first treatment condition, EW was digested in the presence of ferrous gluconate (FeGluc), producing a peptide-FeGluc complex. In the second treatment, EW was digested in the absence of FeGluc followed by the addition of the non-heme iron. In both treatments, the resulting EW hydrolysates were further separated into >0.1–0.5 kDa and >6–8 kDa peptide fractions using dialysis. The hydrolysate and FeGluc complex or mixtures were applied to the IEC-6 cells and iron absorption was measured after 2 h or 16 h. Results showed that the peptide-FeGluc complex digested with a combined proteases from *Bacillus licheniformis* (SDAY) and from *Aspergillus mellus*s (PP) increased the in vitro iron-binding property but did not enhance iron uptake by the in IEC-6 cells ($p < 0.05$). Peptide-FeGluc complex digested with pepsin alone (>0.1–0.5 kDa) resulted in significantly higher iron uptake in IEC-6 cells compared with the higher molecular weight complex (>6–8 kDa) produced using the same hydrolysis treatment. Similarly, enhanced iron uptake was observed with the complexes produced with the combined SDAY and PP enzymatic treatments (>0.1–0.5 kDa and >6–8 kDa) ($p < 0.05$). On the other hand, the enhanced iron absorption effect was not observed when pre-hydrolyzed free peptides were added to FeGluc. Overall, this study suggests that low molecular weight fractions of egg white protein hydrolysates can enhance the bioavailability of non-heme iron. Furthermore, the method by which the egg white proteins are being prepared, i.e., in the presence or absence of FeGluc, can affect the bioavailability of the non-heme iron.

1. Introduction

Iron deficiency is one of the most widespread micronutrient disorders, and is the leading cause of anemia in both developed and developing countries, affecting 1.62 billion people (McLean, Cogswell, Egli, Wojdyla, & de Benoist, 2008). A primary cause of iron deficiency is poor intestinal absorption of iron from the diet (Alleyne, Horne, & Miller, 2008). Absorption of dietary iron within the small intestine is influenced by its form, i.e., heme or non-heme iron (Hallberg, 1981). Heme iron is present in hemoglobin and myoglobin in meats, typically being absorbed at 5–35% in the intestine (Hurrell, 1997), compared with 2–10% for non-heme iron derived from plants, e.g., cereals, vegetables, and beans (Hurrell, 1997). Non-heme iron absorption is strongly influenced by the solubility of iron salts and the presence of absorption inhibitors such as phytates, polyphenols, and tannins (Hurrell, 1997).

There has been much investigation of the effect of food protein hydrolysates on non-heme iron absorption using gastrointestinal digestion enzymes. Enhanced non-heme absorption has been observed in diets that combine plant foods with meat proteins (Bæch et al., 2003; Cook & Monsen, 1976). The exact reason for this "meat factor" remains obscure, although it has been suggested that amino acids (e.g., cysteine and histidine) and polypeptides released upon digestion of meat proteins may be related to enhancing the bioavailability of non-heme iron (Mulvihill, Kirwan, Morrissey, & Flynn, 1998; Etcheverry, Hawthorne, Liang, Abrams, & Griffin, 2006).

Recent studies suggest that egg, milk and soy proteins, which are known iron absorption inhibitors (Hallberg, 1981; Hurrell, Lynch, Trinidad, Dassenko, & Cook, 1989; Kapsokefalou & Miller, 1991; Lynch, Dassenko, Cook, Juillerat, & Hurrell, 1994), can increase non-heme iron absorption in the small intestine when hydrolyzed with enzymes such as Alcalase® and Flavozyme® (Etcheverry et al., 2004; Abeyrathne, Lee, Jo, Nam, & Ahn, 2014; Zhang, Huang, & Jiang, 2014; Palika, Mashurabad, Nair, Reddy, & Pullakhandam, 2015). Enzymatic

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hydrolysates of egg protein have a variety of functions, such as antiviral, anti-hypertensive, and anti-oxidant activates (Mine, 2007). However, there are few studies on hydrolysates of egg white proteins in relation to iron absorption. Abeyrathne et al. (2014) hydrolyzed ovalbumin using various enzymes (pepsin, pepsin + papain, pepsin + Alcalase®, Alcalase® + trypsin and α-chymotrypsin) followed by comparing the iron-binding property of the hydrolysates and their antioxidant capability. They found that a two-enzyme treatment using Alcalase® and trypsin had the highest iron-chelating activity among treatments described above. However, the nature of peptide-iron interactions was not elucidated. Palika et al. (2015) identified an iron-binding fragment derived from ovalbumin ($DKLPGFGDS^{(PO)}_4$ IEAQ, 61–73 residues) digested using porcine pepsin and pancreatin. Since dephosphorylation of a synthetic peptide corresponding to the identified iron-binding peptide sequence inhibited iron-binding activity, it was suggested that ligation of the peptide with a phosphoseryl group might have prevented ferric iron from aggregating at neutral pH, enhancing iron absorption in the small intestine. Similarly, an iron-absorption-promoting effect has been reported for phosphopeptides derived from β-casein (Bouhallab et al., 2002).

Enzymatic hydrolysis of protein produces peptides of different characteristics (e.g., molecular weight, charge, and hydrophobicity) depending on the enzyme (e.g., *endo*peptidase versus *exo*peptidase) and the hydrolysis conditions (e.g., pH, temperature, concentration, reaction time) used (Adler-Nissen, 1986). In the present study, it is hypothesized that treating egg white proteins with different proteases could result in hydrolysates of variable degree of hydrolysis (DH), affecting iron binding property and therefore bioavailability of non-heme iron within the small intestine. To test the hypothesis, rat intestinal epithelium cells (IEC-6) tissue cultures were used as a model system to investigate the effects of hydrolysates produced from different enzymes (porcine, bacterial and fungal) on the absorption of non-heme iron. A rat intestinal epithelium cell (IEC-6) tissue culture model is being used in this study because it has been shown to express divalent metal transporter 1 (DMT1) and hephaestin, both of which are involved in iron absorption and transport in the human body (Kane & Miller, 1984; Thomas & Oates, 2002). The objectives of this study are to: 1) investigate the effects of porcine pepsin, or bacterial and fungal enzymes, on hydrolysis of EW; (2) study the effects of EW hydrolysates on bioavailability of non-heme iron using IEC-6 tissue culture.

2. Materials and methods

2.1. Materials

Crude albumin from chicken egg white (62–88% purity) was obtained from Sigma Aldrich Co. (Oakville, ON). The proteases used were donated by Amano Enzyme Co. (Nagoya, Japan): Thermoase® PC10F (THERMO) from *Bacillus stearothermophilus*; Protin NY100® (NY) from *Bacillus amyloliquefacients*; Protin SD-AY10® (SDAY) from *Bacillus licheniformis*; ProteAX® (PAX) from *Aspergilus oryzae*; Protease A® (PA) from *Aspergilus oryzae*; Protease M® (PM) from *Aspergilus oryzae*; Protease P® (PP) from *Aspergillus mellus*; and Peptidase R® (PR) from *Rhizopus oryzae*. Porcine pepsin (PEP) and ferrous gluconate (FeGluc) was obtained from Sigma Co. (Oakville, ON). All other reagents were of the highest grade available commercially. The combinations of materials used in each treatment are described in the following sections and summarized in Table 1.

2.2. Pepsin digestion

EW was suspended at 1.5 mg/mL in deionized distilled water. The pH of the EW suspension was adjusted to 2 with 1 N HCl followed by the addition of PEP (PEP to protein ratio of 1:100 (*w*/w); 3200–4500 units/mg protein enzymatic activity) and 200 μM FeGluc. The PEP digestion was carried out for 2 h at 37 °C in a shaking water bath (BS-21 model, Jeio-Tech Lab companion, Seoul, Korea). Following hydrolysis, the solution was heated at 90 °C for 15 min to deactivate the protease, and cooled to room temperature. Subsequently, the solution was centrifuged at 3000×*g* for 10 min. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) was used to qualitative comparisons of the extent of hydrolysis among treatments.

Table 1
Description of samples.

PEP	Pepsin
SDAY	Protin SD-AY10
SDAY + PP	A two-enzyme combination treatment: SDAY followed with PP
PEP (0.1)	Hydrolysates prepared with pepsin and separated with 0.1–0.5 kDa MWCO dialysis membrane
PEP (6–8)	Hydrolysates prepared with pepsin and separated with 6–8 kDa MWCO dialysis membrane
SDAY + PP (0.1)	Hydrolysates prepared with SDAY and PP and separated with 0.1–0.5 kDa MWCO dialysis membrane
SDAY + PP (6–8)	Hydrolysates prepared with SDAY and PP and separated with 6–8 kDa MWCO dialysis membrane

2.3. Single enzyme treatment

For single enzyme treatments, EW was suspended at 1.5 mg/mL in a 20 mM buffer at the optimal pH for each enzyme: sodium carbonate (pH 10.0) for SDAY; Tris-HCl (pH 8.0) for PP, PR and THERMO; Tris-HCl (pH 7.0) for PAX, PA, NY; and sodium acetate (pH 4.5) for PM. Optimal conditions were adapted as the manufacturer recommended. The protease was added at 1:25 (*w*/w) enzyme:protein ratio. In the first treatment, EW was digested in the presence of 200 μM ferrous gluconate (FeGluc), to produce peptide-FeGluc complex. On the other hand, in the second treatment, EW was digested without the addition of FeGluc. The digestion mixtures were incubated at optimal temperatures (45 °C for PP & PR; 50 °C for PAX, PM, & PX; 55 °C for NY; 60 °C for SDAY; 70 °C for THERMO) for 4 h for bacterial enzymes or 8 h for fungal enzymes. Following the hydrolysis treatment, the solution was heated at 90 °C for 15 min to deactivate the protease, and then cooled to room temperature. Subsequently, the solution was centrifuged at 3000×g for 10 min, and the supernatant was used to analyze the qualitative extent of hydrolysis using SDS-PAGE.

2.4. Combined two-enzyme treatment

Among the enzymes used in the preliminary experiment, PM, PP, and SDAY showed complete hydrolysis of the ovotransferrin band and partial hydrolysis of the ovalbumin band. Therefore, these three enzymes were used in the two-enzyme hydrolysis experiment. SDAY was used in the first enzyme treatment, followed by either PP or PM. Protein concentration was adjusted to 1.5 mg/mL and pH was adjusted to 10.00 ± 0.5 with NaOH. An aliquot of 200 μM FeGluc was added into the protein solutions. SDAY was added into the solutions at 1:25 enzyme:protein ratio (*w*/w). The solution was incubated at 60 °C, with constant shaking, for 4 h. Samples were taken out at each 1 h interval to determine the DH and subjected to SDS-PAGE analysis. After incubation of 4 h with SDAY, pH of the sample was adjusted to 8.0 for PP or 4.5 for PM. PP or PM was added into the solution at 1:25 enzyme:protein ratio (*w*/w). The sample was digested for 16 h with constant shaking at 50 rpm at 45 °C for PP or 50 °C for PM. Following hydrolysis, the solution was heated at 90 °C for 15 min to deactivate the protease, and then cooled to room temperature. Subsequently, the solution was centrifuged at 3000×*g* for 10 min, and the supernatant was used to analyze the qualitative extent of hydrolysis using SDS-PAGE.