Gastric digestion of pea ferritin and modulation of its iron bioavailability by ascorbic and phytic acids in caco-2 cells

Satyanarayana Bejjani, Raghu Pullakhandam, Ravinder Punjal, K Madhavan Nair

AIM: To understand the digestive stability and mechanism of release and intestinal uptake of pea ferritin iron in caco-2 cell line model.

METHODS: Pea seed ferritin was purified using salt fractionation followed by gel filtration chromatography. The bioavailability of ferritin iron was assessed using coupled in vitro digestion/Caco-2 cell model in the presence or absence of ascorbic acid and phytic acid. Caco-2 cell ferritin formation was used as a surrogate marker of iron uptake. Structural changes of pea ferritin under simulated gastric pH were characterized using electrophoresis, gel filtration and circular dichroism spectroscopy.

RESULTS: The caco-2 cell ferritin formation was significantly increased ($P < 0.001$) with FeSO$_4$ (19.3 ± 9.8 ng/mg protein) and pea ferritin (13.9 ± 6.19 ng/mg protein) compared to the blank digest (3.7 ± 1.8 ng/mg protein). Ascorbic acid enhanced while phytic acid decreased the pea ferritin iron bioavailability. However, either in the presence or absence of ascorbic acid, the ferritin content of caco-2 cells was significantly less with pea ferritin than with FeSO$_4$. At gastric pH, no band corresponding to ferritin was observed in the presence of pepsin either on native PAGE or SDS-PAGE. Gel filtration chromatography and circular dichroism spectroscopy revealed a pH dependent loss of quaternary and secondary structure.

CONCLUSION: Under gastric conditions, the iron core of pea ferritin is released into the digestive medium due to acid induced structural alterations and dissociation of protein. The released iron interacts with dietary factors leading to modulation of pea ferritin iron bioavailability, resembling the typical characteristics of non-heme iron.

INTRODUCTION

Iron deficiency anemia (IDA) is a serious public health problem worldwide, affecting about two billion people globally$^{[1,2]}$. In India, the major etiology for the alarming prevalence of iron deficiency is poor density and bioavailability of iron from commonly consumed cereal/pulse based diets$^{[3,4]}$. Therefore, food fortification is considered as a measure to increase the iron intake of the general population$^{[5,6]}$. Nevertheless, it is often difficult due to traditional consumption of whole grains such as rice, whose chemical fortification is not possible.

Ferritins are iron storage proteins ubiquitously present in all organisms. Like mammals, plants (particularly legumes) store iron in their seeds in the form of ferritin$^{[7]}$. Therefore, increasing the expression level of ferritin in plants is thought to increase the iron density of the staple grains and thereby the iron intake of iron deprived populations as well$^{[8]}$. Nevertheless, iron bioavailability from these sources remains a concern due to the presence of high concentrations of phytates and tannins, which are known to inhibit iron absorption.

Iron bioavailability studies on purified ferritin or soybean, a rich source of ferritin, are conflicting$^{[9-15]}$. Non-anemic women fed on reconstituted soybean ferritin as the iron source showed improvement in iron status$^{[16]}$. These observations along with the high stability of ferritin protein against denaturants and complex chemistry of ferritin iron led to the conclusion that either ferritin or its released iron core is directly absorbed by the enterocytes. This is in contrast with the reported low bioavailability of iron from legumes$^{[17]}$. Additionally, the transgenic maize over
expressing soybean ferritin gene together with phytase resulted in improved bioavailability of iron\(^{[18]}\). Apparent discrepancy between these observations has been explained by various procedures such as iron labeling of ferritin, source of ferritins \textit{in vitro} or \textit{in vivo} methods and the physiological and nutritional status of the subjects\(^{[16,19]}\). Thus, there is a need to understand the digestive stability of plant ferritin, the possible mechanism(s) of iron release and the influence of dietary factors on ferritin iron uptake in the gut. These studies are of specific interest in the context of ferritin-mediated biofortification as a strategy to improve bioavailable iron in plant food.

Caco-2 cell based screening methods have been used extensively in recent years to assess iron and carotenoid bioavailability from various foods and meals\(^{[20-24]}\). The model consists of sequentially simulating gastric and intestinal digestion of a test sample and adding it to differentiated cultures of caco-2 cells. Furthermore, ferritin formation in the caco-2 cells exposed to digests containing iron has been used as a surrogate marker of cellular iron status and is therefore, proportional to the bioavailability of iron\(^{[25]}\). The objective of the present study was to evaluate modulation of purified pea ferritin iron bioavailability by ascorbic and phytic acids using \textit{in vitro} digestion/Caco-2 cell model and its digestive stability, and the possible mechanism of iron release.

**MATERIALS AND METHODS**

**Materials**

Dried pea (\textit{Pisum sativum}) seeds were procured from the local market. Ferrous sulfate (FeSO\(_4\)·7H\(_2\)O) was obtained from Sigma Chemical Co. (St. Louis, MO). Purification of ferritin from pea seeds was performed as described by Laulhere \textit{et al}\(^{[26]}\). Briefly, 150 g powdered pea seed was suspended and homogenized in 3 volumes of 10 mmol/L phosphate buffer at pH 7.5, containing 1 mmol/L EDTA. The suspension was filtered through three layers of muslin cloth and further clarified with centrifugation at 10,000 \(\times\) g for 5 min. Protein concentration in the cell lysates was estimated using BCA protein assay kit.

**Ferritin measurement**

A human ferritin sandwich ELISA method developed in-

**Assessment of iron bioavailability in caco-2 cells**

Caco-2 cells were obtained from the National Center for Cell Sciences (Pune, India) and used in experiments at passage 15-20. Cells were seeded at a density of 50,000 cells/cm\(^2\) in 6-well plates (Corning, India). The cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS), 10% non-essential amino acids and 0.4 mmol/L glutamine and 1% antibiotic-antimyotic solution. The cells were maintained at 37°C in an incubator with a 5% CO\(_2\)/95% air atmosphere at constant humidity. Once the cells reached confluency, the concentration of FBS in the medium was reduced to 10% and it was changed every 2 d. These cells were used for iron bioavailability experiments at 12-14 d after seeding.

Immediately prior to the intestinal digestion, the growth medium was removed from culture wells and the monolayer washed thrice with MEM (MEM was used because it contains less amount of endogenous iron compared to the DMEM). A fresh 2 mL aliquot of MEM (5% chelex-100 treated) covered the cells during the experiment. A sterilized insert ring fitted with a dialysis membrane (12,000 MW cut-off, Spectrapore) using an adaptor was introduced into the wells, thus creating a two-chamber system. An aliquot of 2 mL intestinal digest was pipetted into the upper chamber. The plate was covered and incubated in humidified CO\(_2\) incubator at 37°C for a period of 2 h with regular mixing at 15 min intervals. After the intestinal digestion, the upper chamber was removed and the plates were further incubated for a period of 22 h. At the end of this the cells were washed thrice with ice-cold saline and collected by scraping into 400 \(\mu\)L saline. The contents were sonicated for 20 s using a probe sonicator and centrifuged at 5000 \(\times\) g/min for 5 min. Protein concentration in the cell lysates was estimated using BCA protein assay kit.

**In vitro digestion**

The simulated \textit{in vivo} digestion was performed as described previously by Glahn \textit{et al}\(^{[23]}\) with minor modifications. Briefly, reagents required for thedigest such as saline, 40 g/L pepsin (0.1 mol/L HCl), pancreatin-bile salt mixture (0.05 g pancreatic and 0.3 g bile extract/25 mL, 0.1 mol/L NaHCO\(_3\)) were prepared freshly and mixed with 5% Chelex-100 and incubated for 30 min at room temperature to remove the endogenous iron present in these solutions. The Chelex-100 was removed from these solutions by passing it through a sintered glass funnel.

Peptic digestion was carried out in 50 mL screw-cap culture tubes. One hundred microlitre of stock purified pea ferritin (780 \(\mu\)g of iron/2.5 mg protein/mL) was diluted to 9 mL with saline in the presence and absence of 1.5 and 1:1 molar ratios of ascorbic acid and phytic acid (inositol hexaphosphorate dodeca sodium salt isolated from rice, Sigma Cat#P3168), respectively. The pH of the samples was adjusted to 2.0 with 6 mol/L HCl and 0.5 mL of the pepsin solution was added and the volume adjusted to 10 mL. The tubes were immersed in a shaking water-bath at 37°C for 60 min. For intestinal digestion, the pH of the sample (also referred to as the “digesta”) was increased to 6 with 1 mol/L NaHCO\(_3\), 2.5 mL of pancreatin-bile extract mixture added and further adjusted to pH 7 with 1 mol/L NaOH. The final volume made up to 15 mL with saline and the digestion was carried out in the upper chamber of the transwell plate for 2 h as described below. Saline and ferrous sulfate treated similarly were fed to cells and run simultaneously as control and reference, respectively.
house and validated against recombinant ferritin (94/572) obtained from WHO International Laboratory for Biological Standards (NIBSC, UK) was used to estimate the caco-2 cell ferritin content\[26\]. Ferritin content was estimated either in neat or diluted caco-2 cell lysate using human liver ferritin IgG-HRP and the substrate system orthophenylene diamine-H\(_2\)O\(_2\). The color intensity was measured using ELISA plate reader (Multiskan Accent, Lab systems, USA). Iron and inorganic phosphate content of purified pea ferritin was determined by bathophenanthroline method\[27\] and micro-method of Chen et al\[28\], respectively. Purified pea ferritin (1 mg/mL) was electrophoresed (Biofocus 3000, Bio-Rad) in an uncoated silica capillary column (50 \(\mu\)m ID, 375 \(\mu\)m OD, 50 cm length) using 10 mmol/L phosphate buffer pH 2.3 at 20 kV and detected at 215 nm.

### Native and SDS–PAGE

To study the digestive stability, purified pea ferritin (1 mg/mL) at pH 7.2 (10 mmol/L phosphate buffer) and at gastric pH 2 (saline-HCl) was incubated in the presence and absence of 1.6 mg/mL of pepsin at 37°C for 1 h. At the end of the incubation, the pH of the solution was adjusted to pH 7.2 with 1 mol/L sodium bicarbonate. Aliquots of the reaction mixtures were immediately mixed with sample buffer with or without SDS. The samples were analyzed on 6% native PAGE and 12.5% SDS-PAGE. The gels were stained for iron by Prussian blue staining method\[29\] or for protein by the coomassie brilliant blue method.

### Gel filtration chromatography

Gel filtration chromatography of purified pea ferritin was performed using TSK-2000 SW (Altex) column connected to HPLC (Agilent, Model: 1100). Briefly, 200 \(\mu\)g of purified pea ferritin was incubated at pH 7.2 or pH 2 for 20 min and then subjected to size fractionation using 10 mmol/L phosphate buffered saline pH 7.5, at a flow rate of 1 mL/min and the elution was monitored at 280 nm for protein and at 420 nm for protein bound iron.

### Circular dichroism spectra analysis

Purified pea ferritin (1 mg/mL) was incubated for 20 min at room temperature either in 10 mmol/L phosphate buffer saline pH 7.2 or in saline HCl pH 2. Circular dichroism (CD) was measured with a spectropolarimeter (JASCO-810) using 1 mm cell at 0.2 nm intervals and 1 nm bandwidth. Spectra were signal averaged by adding five accumulations. The baseline was corrected by subtracting the spectra of respective buffer blanks obtained under identical conditions. Percentage of secondary structure was calculated using the web-based program K2D (http://www.embl-heidelberg.de/~andrade/k2d).

### Statistical analysis

Statistical analysis was performed using the software package SPSS-7. Each experiment was conducted in triplicate and analyzed in duplicate. The ferritin data were log transformed and the means were compared using one-way ANOVA followed by least significant difference (LSD) post-hock test. The results were considered significant if \(P\) value was < 0.05.

### RESULTS

#### Purification and characterization of pea seed ferritin

Purification of pea ferritin using MgCl\(_2\) precipitation followed by gel filtration on Sepharose-6B column resulted in reddish-brown colored protein fractions. Capillary zone electrophoresis of pooled fractions showed a single peak suggesting high purity of the protein (Figure 1A). Native PAGE of this protein followed by staining for protein (Figure 1B) and iron (data not shown) revealed a single band and intense reaction with iron confirming the identity of ferritin. Upon SDS-PAGE, the purified ferritin showed two bands at 28 and 26 kDa regions (Figure 1C). The iron and phosphate concentrations of pea ferritin were 325 and 375 \(\mu\)g/mg protein, respectively. The yield of purified protein was 13-16 mg protein/kg of seeds using the above purification procedure.

#### Effect of ascorbic acid and phytic acid on pea ferritin iron bioavailability

The bioavailability of iron from both FeSO\(_4\) and pea ferritin was assessed using simulated \textit{in vitro} digestion/ Caco-2 cell model either in the presence or absence of ascorbic acid (1:5 molar ratio) and ferritin in the presence of phytic acid (1:1 molar ratio) (Figure 2). The caco-2 cell ferritin formation was significantly increased (\(P < 0.001\)) with iron from FeSO\(_4\) (19.3 ± 9.8 ng/mg protein) or pea...
ferritin (13.9 ± 6.19 ng/mg protein) compared to the blank digest (3.7 ± 1.8 ng/mg protein). Addition of ascorbic acid along with FeSO\(_4\) (188.8 ± 82.8 ng/mg protein) or pea ferritin (125.1 ± 55.49 ng/mg protein) resulted in marked increase in the ferritin formation compared to controls without ascorbic acid (P < 0.001). Addition of phytic acid along with pea ferritin reduced the caco-2 cell ferritin formation compared to pea ferritin alone (4.45 ± 2.2 ng/mg protein) (P < 0.001). However, the ferritin content of caco-2 cells exposed to FeSO\(_4\) was significantly higher than pea ferritin either in the presence or absence of ascorbic acid (P < 0.004).

**Effect of gastric pH and pepsin on stability of pea ferritin**

The digestive stability of purified pea ferritin was studied in the presence and absence of pepsin at gastric pH. At pH 7.2, pea ferritin predominantly migrated as a single high molecular weight protein band on native PAGE. However, no band was observed either in the absence or presence of pepsin at gastric pH, no other band except a protein band at 36 kDa region (pepsin) in the presence of pepsin.

**Effect of gastric pH on pea ferritin quaternary structure**

The quaternary structural changes of pea ferritin as a function of pH were studied using gel filtration chromatography on TSK-2000 column. At pH 7.2, pea ferritin eluted at 4.8 min as a single peak having absorption at 280 (protein) and 420 nm (protein bound iron). Pea ferritin incubated at pH 2 eluted at 9.5 min predominantly as low molecular weight protein when monitored at 280 nm and only minor fraction of protein eluted at 4.8 min. However, the peak due to absorption of protein bound iron at 420 nm was completely absent at pH 2.

**Effect of gastric pH on pea ferritin secondary structure**

The secondary structural changes of pea ferritin as a function of pH were studied using far UV CD spectral analysis. At native pH, purified pea ferritin showed a typical α-helical spectrum, and acidification led to loss of secondary structure. Analysis of the CD spectrum indicated high content of α-helix (76%) in the protein followed by β-sheet (2%) and random coil (22%) conformation at pH 7.2. However, on acidification α-helical content of pea ferritin decreased (28%) while β-sheet (23%) and random coil (49%) conformations increased.

**DISCUSSION**

On a chemical basis, dietary iron is classified as non-heme iron of plant origin (inorganic ferric or ferrous iron) and heme iron of animal origin. Ferritins are ubiquitous iron transporters in the body, storing iron in a safe and slow-releasing form. The stability and bioavailability of ferritin in the presence of ascorbic and phytic acid were studied to understand the implications for digestion and absorption in the gut. The results show that ascorbic acid enhances ferritin formation, while phytic acid reduces it, indicating the role of these compounds in regulating iron availability.

The digestive stability study revealed that at pH 7.2, pea ferritin remains stable, migrating as a high molecular weight protein band. However, at pH 2, it dissociates under gastric conditions and releases bound iron, evident from the elution profile. The CD spectrum analysis further confirmed the structural changes, showing a decrease in α-helical content with an increase in β-sheet and random coil structures on acidification.

These findings are crucial for understanding how dietary iron is managed in the gut, particularly in the context of digestive conditions like peptic ulcer disease where gastric pH can vary. The implications for iron absorption and the potential for dietary strategies to enhance or reduce iron bioavailability are significant.
Ascorbic acid to the digest. Interestingly, pea ferritin iron from FeSO₄ and phytic acids are known to promote and inhibit iron absorption. The purified pea ferritin showed a typical α-helical spectrum similar to the observations reported with horse spleen ferritin. However, on acidification (pH 2) there was a significant loss of α-helical structure and increase in the β-sheet and random coil conformation, implying severe secondary structural alternations in the protein at gastric pH. Collectively, the findings from PAGE, gel filtration and CD spectroscopic studies on pea ferritin strongly suggest loss of quaternary and secondary structures of the protein at gastric pH. Thus, under these conditions it is unlikely that the denatured pea ferritin protein retains its iron binding ability.

Interestingly, these observations contrast with the human studies using either purified reconstituted soybean or horse spleen ferritin. These studies have concluded that the relative bioavailability of ferritin iron is close to 1.0 when compared with FeSO₄. Based on studies in rats, it was proposed that ferritin iron is bioavailable in the presence of dietary inhibitors. However, studies in women with low iron stores suggest a lower bioavailability (~ 0.46) of iron from intrinsically labelled soybean when compared to FeSO₄, which is comparable to the results of the present study. Other possible reasons for the relatively high bioavailability of ferritin iron in human subjects (30%-35%)...
could be the variations in gastric conditions and iron status prevailing in the study subjects. It is also noteworthy that unlike the in vitro digestion model, gastric digestion and emptying in humans is a dynamic process. Therefore, the released ferritin iron has equal chances of interaction with enterocytes or chelators/reducers in the food, and thereby the observed high bioavailability of ferritin iron in humans. The other likely reason may be subtle differences between pea and soy ferritins.

In conclusion, purified pea ferritin under gastric conditions loses its quaternary and secondary structural elements and undergoes peptic digestion leading to release of protein bound iron. The released iron is accessible to ascorbic and phytic acids to bring about reduction or chelation reflecting as increased or reduced bioavailability of pea ferritin iron, the characteristic properties of non-heme iron pool. Therefore, apart from increasing the iron density through plant ferritin, ensuring substantial reduction in phytate concentrations appears to be necessary for biotechnological approaches to use plant ferritin as a tool in crop-biofortification.

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