Ferritin-Iron Is Released during Boiling and In Vitro Gastric Digestion¹⁴⁻³

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Abstract

Biofortification of staple foods with iron in the form of ferritin-iron is a promising approach to fighting iron-deficiency anemia in developing countries. However, contradictory results regarding iron bioavailability to humans from ferritin are not yet fully clarified. Furthermore, the question has been raised whether ferritin can potentially survive gastric passage intact and be absorbed via a ferritin-specific uptake mechanism. We studied changes of ferritin-iron and protein during cooking and in vitro gastric digestion. Water soluble, native ferritin-iron, measured in different legumes, represented 18% (soybeans) up to maximally 42% (peas) of total seed iron. Ferritin-iron was no longer detectable after boiling the legumes for 50 min in excess water. When the same cooking treatment was applied to recombinant bean ferritin propagated in Escherichia coli, some ferritin-iron remained measurable. During in vitro gastric digestion of recombinant bean ferritin and red kidney bean extract, ferritin-iron was fully released from the protein and dissolved at pH 2. Stability tests at varying pH at 37°C showed that the release of ferritin-iron starts at pH 5 and is complete at pH 2. We concluded that ferritin-iron is efficiently released from the ferritin molecule during cooking and at gastric pH and that it should be absorbed as efficiently as all other nonheme iron in food. J. Nutr. 138: 878–884, 2008.

Introduction

Anemia is still one of the major public health challenges on a global scale with an estimated 2 billion people affected worldwide (1); 1 billion of these suffer from iron-deficiency anemia (IDA). The major consequences of IDA are poor pregnancy outcome, reduced cognitive and motor development in infants, decreased immune function, and tiredness and reduced work capacity (2–4). An insufficient supply of bioavailable iron to the body is a major factor in the etiology of IDA. Supplementation with pharmaceutical iron preparations, food fortification, and dietary diversification are possible strategies to combat iron deficiency and IDA (5). An alternative strategy currently being explored is biofortification, which involves increasing the iron content of staple crops either by conventional plant breeding or genetic engineering (6). Biofortification can reach all population groups, including people with low incomes. It is potentially more sustainable than other approaches because recurrent costs are low, following a 1-time investment of developing suitable seed varieties (7,8).

Increasing the ferritin content of seeds is currently considered the most promising approach to biofortifying staple foods, such as rice, wheat, cassava, or beans, with iron (9). Ferritin serves as an iron storage protein in virtually all living organisms, including bacteria, plants, and animals. It consists of 24 subunits that are assembled into a spherical shell that can store up to 4500 Fe(III) atoms in its inner cavity in the form of an iron oxyhydroxide-phosphate mineral. Lines of transgenic rice and maize that express soybean ferritin (Glycine max) or common bean ferritin (Phaseolus vulgaris) have already been developed (10–12). However, a basic requirement for the successful implementation of a biofortified crop is an adequate bioavailability of the respective nutrient. For ferritin-iron, contradictory results have been published from several human studies, either showing good (13–16) or poor (17–21) iron absorption from ferritin. These differences have been associated with isotopic labeling of ferritin-iron. It has been suggested that labeling of animal ferritin under inflammatory conditions, as employed in the studies showing poor iron bioavailability, may have led to a ferritin molecule that releases iron less readily (9). Recently, it was proposed that ferritin is resistant to digestion in the gastrointestinal tract and that it might even be absorbed intact by the mucosal cell (14).

¹ Supported by ETH grant TH-35/03-3.
² Author disclosures: M. Hoppler, A. Schönächler, L. Meile, R. F. Hurrell, and T. Walczyk, no conflicts of interest.
³ Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.
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Materials and Methods

Materials. Dry peas, red kidney beans, white beans, and soy beans were purchased at local supermarkets in Zurich, Switzerland.

We have described the expression and isolation of recombinant bean (P. vulgaris) ferritin in detail elsewhere (24). Briefly, Escherichia coli cells were transformed with the expression plasmid PET 28a(+) (Novagen) containing ferritin cDNA from P. vulgaris (25). The bacterial culture was grown overnight at 37°C in M9 medium containing kanamycin (30 mg/L) and chloramphenicol (34 mg/L). Expression of ferritin was induced by adding isopropyl β-D-thiogalactopyranoside to the medium to a final concentration of 1 mmol/L together with iron at a concentration of 0.2 mmol/L for incorporation into ferritin. Recombinant bean ferritin was isolated by a 2-step procedure by ion exchange chromatography using a diethylaminoethyl (DEAE) Sepharose column (10 by 1.6 cm; GE Healthcare) followed by gel filtration using a Sephacryl S-300 column (2.6 by 60 cm). The purified ferritin was stored in 50% glycerol at –20°C. The protein concentration of the ferritin solution was determined by the Bradford method (26).

Quantification of total iron. The iron content of legumes and ferritin were analyzed by graphite furnace atomic absorption spectrophotometry (GF-AAS, AA240Z, Varian). Legumes were ground under liquid nitrogen and then suspended in a 10-fold volume of ice-cold phosphate buffer (50 mmol/L sodium phosphate, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulphonyl fluoride, 1% polyvinylpolypyrrolidone, pH 7). Proteins were extracted after homogenization with a Polytron (PT1200 E, Kinematica). After centrifugation at 20,000 × g for 20 min, the supernatant was separated from the sediment. For cooking and digestion experiments, the recombinant bean ferritin solution and legume extracts were analyzed for ferritin-iron content by size exclusion chromatography using a Superdex 200 column with a fractionation range of 10–600 kDa (1 × 30 cm, GE Healthcare) and phosphate buffer for elution (0.15 mmol/L sodium phosphate, 0.15 mmol/L NaCl, pH 7). Protein elution was monitored spectrophotometrically at 280 nm. Fractions of 4 mL were collected and iron content was measured in each fraction by GF-AAS. Ferritin-iron eluted from the column ~20 min after sample injection. For pea extract, it was verified whether all the iron collected in this fraction was ferritin-bound. The collected fraction was desalted and further purified by ion exchange chromatography using a DEAE column (GE Healthcare) with a NaCl gradient of 0–0.3 mol/L. Iron containing fractions after ion exchange chromatography were pooled and concentrated by ultrafiltration. The concentrated sample was applied to native PAGE. Iron in gels was stained as Prussian blue [2% K₃Fe(CN)₆ solution in 2% HCl]. For phosphorus quantification in recombinant bean ferritin, the phosphate buffer was removed and exchanged with 18 MΩ water (NANOpure system, Barnstead/Thermolyne) by using ultrafiltration spin columns (Vivaspin, Sartorius AG) and passing 18 MΩ water 5 times through the columns. Phosphorus content was determined by multi-collector inductively coupled plasma MS (Neptune, Thermo Scientific) by external calibration using a gravimetrically prepared standard.

Ferritin stability during cooking. To investigate the stability of ferritin during cooking, recombinant bean ferritin and different legume seeds were boiled in a 5-fold volume of 18 MΩ water for 50 min (recombinant bean ferritin, dry peas, red kidney beans, and soybeans) or 100 min (white beans) until tender. Cooking experiments were repeated with 1 mmol/L EDTA (pH 7) in the cooking water. EDTA was added to allow the detection of ferrous or ferric iron in the soluble fraction. In the absence of a chelator, free iron was poorly soluble at neutral pH and would precipitate. The legumes were cooled down after cooking and a phosphate buffer was added to 10-fold the sample volume. Cooked legumes were blended using a hand blender and homogenized with a Polytron. The samples were centrifuged at 20,000 × g for 20 min at 4°C and the supernatant was loaded onto the gel filtration column for ferritin separation.

Ferritin stability at different pH and in the presence of EDTA. To assess ferritin stability during digestion, recombinant bean ferritin was exposed to a pH range that could be expected in the human stomach. Ferritin was warmed up to 37°C and pH adjusted to 7, 3, 3.5, and 2, respectively, by the addition of 0.1 mol/L HCl. Samples were flushed with argon to prevent oxidation and were incubated in a shaking water bath at 37°C in the dark. Aliquots were taken after 15, 30, 60, and 120 min, respectively, and pH adjusted to 7 using 0.1 mol/L NaOH. After centrifugation at 20,000 × g for 20 min at 4°C, the supernatant was applied to the gel filtration column for ferritin separation. For experiments with EDTA, ferritin stability in the presence of EDTA was verified by incubating recombinant bean ferritin at neutral pH in a shaking water bath at 37°C in a 1 mmol/L EDTA solution. Aliquots were taken after 15, 30, 60, and 120 min and ferritin was separated as described earlier.

Pepsin/HCl digestion. Gastric digestion of recombinant bean ferritin and red kidney bean extract was simulated in vitro using a modification of the procedure of Miller et al. (27). For digestion, 40 mg pepsin G protein (pepsin from porcine stomach mucosa, Sigma-Aldrich) were added and pH was adjusted to 2 by adding 0.1 mol/L HCl. The mixture was incubated in a shaking water bath at 37°C for 2 h. After incubation, pH was adjusted to 7 using an aqueous NaOH solution. EDTA was then added to a final concentration of 1 mmol/L in the digest to prevent precipitation of solubilized iron at neutral pH. After centrifugation at 20,000 × g for 20 min at 4°C, the supernatant was applied to the gel filtration column for ferritin separation.

Kinetics of ferritin degradation during simulated pepsin/HCl digestion. Recombinant bean ferritin and raw and cooked peas were subjected to an in vitro procedure that simulated gastric conditions. Pepsin was added in the same concentration as described above. The acidity of the solution was lowered stepwise to mimic a transient change, as reported for the human stomach during gastric digestion (28–30). Digestion was initiated by lowering the pH to 5 (t = 0) to imitate the higher pH due to the postprandial buffering effect of a meal in the stomach (29). The pH was then lowered in 3 steps by adding 0.1 mol/L HCl to pH 3.5 after 15 min, to pH 2.5 after 30 min, and to pH 2 after 60 min, respectively. Experiments were terminated at 120 min. Samples were incubated at 37°C in a shaking water bath in the dark and were kept under argon to protect samples from oxidation. Aliquots for ferritin iron determination were taken after 15, 30, 60, and 120 min. After digestion, pH was adjusted to 7 using 0.1 mol/L NaOH to mimic pH conditions in the duodenum as the predominant age of intestinal iron absorption. Samples were centrifuged at 20,000 × g for 20 min at 4°C and applied to the gel filtration column for ferritin separation.

Results

Characterization of recombinant bean ferritin. P. vulgaris ferritin was expressed in E. coli and used to study ferritin stability during cooking and digestion. Purity and characteristic properties of the recombinant ferritin were the same as previously described (24), as assessed by SDS PAGE and native PAGE stained for protein and iron. In the presence of FeSO₄ in the bacterial growth medium, purified recombinant bean ferritin contained ~1,000 Fe atoms per ferritin molecule. This degree of iron saturation of ferritin is well within the range found in various plant ferritins (31–33). Phosphorus quantification showed a molar iron to phosphorus ratio of 4.2:1, which is in the typical range reported for plant ferritins (34,35). When purified recombinant bean ferritin was separated on a Superdex 200 column, it was recovered in fraction A3 ~20 min after injection (Fig. 1A).
An amount of >95% of total iron of the purified recombinant bean ferritin solution was recovered in this fraction.

**Method for quantification of ferritin-iron.** Gel filtrations of recombinant bean ferritin and iron measurements were performed in 3 independent runs to assess the reproducibility of the method. Iron concentration (mean ± SD) in ferritin-containing fraction A3 (Fig. 1A) was 36.5 ± 0.9 μg Fe/g injected recombinant ferritin solution, yielding good reproducibility as judged by the resulting CV of 2.5%. The data presented for cooking and digestion studies were based on single experiments. After incubation of recombinant bean ferritin at pH 5, the elution profile changed and an additional peak was observed 16 min after injection (Fig. 1B, fraction A2). Because molecules of higher molecular weight elute earlier from a gel filtration column, this fraction can be expected to correspond to aggregated ferritin molecules. Ferritin aggregation has already been observed and described in earlier studies (36,37). For the ferritin-iron quantification in legumes, we found that fraction A3 after gel filtration of pea extract contains only ferritin-iron: during separation of fraction A3 over a DEAE column, all iron quantitatively eluted into fractions at NaCl concentrations of 0.15–0.25 mol/L, which is typical for ferritin (32,38–40). The pooled iron containing fractions showed 1 single band in native PAGE when stained for iron. This band displayed the same mobility as pure recombinant bean ferritin (Supplemental Fig. 1).

**Ferritin stability during cooking.** Iron speciation was studied in recombinant bean ferritin and different legumes by gel filtration before and after cooking. Soybeans showed the lowest soluble ferritin-iron content of 18% of total seed iron, and peas the highest at 42% (Table 1). After cooking for 50–100 min in boiling water, the soluble ferritin-iron content of the 4 analyzed legumes was below the detection limit of the applied method of 5% relative to total iron content of the seeds. Similar observations were made for solutions of recombinant bean ferritin after cooking. Ferritin-iron that was recovered after cooking was found to be 8% for recombinant bean ferritin.

**Ferritin stability at different pH and in the presence of EDTA.** When recombinant bean ferritin was incubated at 37°C at different pH conditions, a progressive degradation of the molecule over time could already be observed at pH 5, whereas

<table>
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<th>Legume and treatment</th>
<th>Total iron concentration1 μg/g</th>
<th>Ferritin-bound iron % of total Fe</th>
<th>Other soluble iron % of total soluble iron</th>
<th>Total soluble iron % of total soluble iron</th>
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<tr>
<td>Dry peas</td>
<td></td>
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<tr>
<td>Uncooked</td>
<td>47.8 ± 0.8</td>
<td>42</td>
<td>31</td>
<td>73</td>
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<td>Cooked</td>
<td>&lt;5²</td>
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<td>70</td>
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<td>White beans</td>
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<tr>
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<tr>
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<td>&lt;5²</td>
<td>51</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Cooked with EDTA</td>
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<td>61</td>
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<td>Red kidney beans</td>
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<tr>
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<td>44</td>
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<tr>
<td>Cooked with EDTA</td>
<td>8</td>
<td>85</td>
<td>93</td>
<td></td>
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</table>

1 Total iron concentrations based on 3 replicate measurements. Values are means ± SD.
2 Below the detection limit of the applied method.
3 ND, not determined.
4 Total soluble iron. Speciation of soluble iron was not further studied.

### Table 1

**Speciation of iron in different legumes before and after boiling for 50–100 min in H₂O at 100°C in the presence and absence of EDTA in the boiling water.

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ferritin stability was not affected at pH 7 under comparable conditions (Fig. 2). At pH 3.5, amounts of ferritin-iron were found to decrease progressively over 2 h. No ferritin-iron could be detected within 15 min after adjusting the pH of the solution to 2. At pH 5 and pH 3.5, the formation of ferritin oligomers occurred after 15 min that were detectable until termination of the experiments after 120 min (Fig. 2B,C). The formation of ferritin oligomers could not be observed at pH 2. EDTA (1 mmol/L) had no detectable effect on ferritin stability when ferritin was incubated over 2 h at 37°C (Fig. 2E).

**Pepsin/HCl digestion of ferritin.** The simulated gastric digestion of recombinant bean ferritin and red kidney bean extract indicates that iron is likely to be released from ferritin during gastric passage. After in vitro digestion, no iron eluted from the column at retention times typical for ferritin (Fig. 3). Iron was almost quantitatively eluted ~40 min after sample injection in the presence of EDTA. This was observed for both the recombinant bean ferritin and the red kidney bean extract (Fig. 3B,D). In a separate experiment, we found that Fe-EDTA solution applied to the same gel filtration column was also quantitatively recovered 40 min after injection (data not shown).

**Kinetics of ferritin degradation during simulated HCl-pepsin digestion.** Both recombinant bean ferritin and native pea ferritin in ground peas was fully degraded within 2 h during a simulated pepsin/HCl digestion (Fig. 4). Similar to the pH stability experiments, the formation of ferritin oligomers was observed. Released iron from ferritin could not be quantitatively detected in these experiments due to the poor solubility of iron at neutral pH. Therefore, EDTA was added in a separate experiment to prevent iron precipitation in the aqueous phase. Boiling and blending released almost 80% of the pea iron (Table 2). The remaining insoluble iron was solubilized and released from the pea matrix during in vitro digestion (Table 2). Our experiments show that any ferritin not affected by cooking can be expected to be degraded later during gastric passage.

**Discussion**

We designed this study to help clarify the contradictory findings on ferritin-iron bioavailability (13–15,17–20). Previous studies have measured iron absorption from in vivo or in vitro labeled ferritin. Contradictory findings from these human iron-absorption studies are possibly related to the complexity of conducting such studies and the challenge of isotopically labeling iron in the ferritin molecule. Recently it was suggested that ferritin-iron is protected from iron absorption inhibitors through the protein hull, possibly leading to increased iron absorption (41). In this study, we have addressed the fundamental question of ferritin stability during cooking and gastric passage. Monitoring changes of the ferritin molecule and its iron core during these processes could contribute to a better understanding of how ferritin-iron is absorbed. If ferritin does not survive meal preparation and gastric digestion to a significant extent, any ferritin-specific absorption pathway would contribute little to human iron nutrition.

Our studies show that native legume ferritin is altered to a large extent during cooking. We measured ferritin-iron contents in different legumes before cooking (Table 1) and found ferritin-iron as a percentage of total iron to vary from 42% in dry peas to 18% in soybeans (Table 1). In a previous study where Mössbauer spectrometry was employed for iron speciation, 90% of soybean seed iron was found in a trivalent state and showed an isomer
The view that ferritin could protect its iron core from absorption inhibitors (41) was primarily based on the results of 2 iron absorption studies where intrinsically labeled soybeans were used (13,48). Those studies reported relatively high fractional absorption (20% and 27%, respectively) despite the presence of phytic acid in the test meals. When considering early and intermediate products formed during digestion. It is unlikely that these play a special role for iron uptake because they are undetectable by the end of gastric digestion. Our results (Figs. 2–4) are in agreement with 2 recent studies, which showed that after in vitro digestion at pH 2, the ferritin protein hull was fully dissociated or degraded (46,47). In the study by Kalgaonkar and Lönnertal (47), this effect was less pronounced when gastric digestion was simulated at pH 4 instead of pH 2. This result gives rise to the hypothesis that some ferritin may leak into the intestine in intact form at an early stage of gastric digestion. It can be argued, however, that these experiments were conducted with uncooked, intact ferritin. Ferritin-iron is mostly present in foods such as legumes that usually undergo long and harsh cooking procedures. As shown by our experiments, these conditions have a smaller impact on iron absorption than previously assumed. The view that ferritin could protect its iron core from absorption inhibitors (41) was primarily based on the results of 2 iron absorption studies where intrinsically labeled soybeans were used (13,48). Those studies reported relatively high fractional absorption (20% and 27%, respectively) despite the presence of phytic acid in the test meals. When considering early and
The extrinsic tag absorption. Even in the study of Sayers et al. absorption from the labeled legume-based meals was similar to administration. In all studies, the efficiency of intrinsic tag relative to total pea iron, respectively.

Recent intrinsic/extrinsic tag studies (48–51), however, it seems unlikely that ferritin-iron is better absorbed than other nonheme iron. In these studies, iron in cereals and legumes was labeled intrinsically by adding a radioiron tag during growth, presumably also labeling ferritin-iron intrinsically. An extrinsic radioiron tag in the form of FeCl3 was added shortly before test meal administration. In all studies, the efficiency of intrinsic tag absorption from the labeled legume-based meals was similar to the extrinsic tag absorption. Even in the study of Sayers et al. (48), which showed high fractional iron absorption, the extrinsic tag of FeCl3 was as well absorbed as the intrinsically labeled soybean iron (including ferritin-iron). An evaluation of the data by Murray-Kolb et al. (13) in this context is difficult because no extrinsic tag was given for comparison. Therefore, these experiments do not provide any indication that ferritin-iron is absorbed to a different degree than other nonheme iron. This is also in line with 2 recent human studies that showed that iron from in vitro labeled plant and animal ferritin is as equally well absorbed as iron from ferrous sulfate (14,15). Furthermore, Caco-2 cell studies have demonstrated that after in vitro digestion, uptake of ferritin-iron was influenced by dietary factors such as phytic acid, tannic acid, calcium, and ascorbic acid (46,47). Another Caco-2 cell study has shown that iron absorption from common beans is influenced by the polyphenol content of the beans (52), which likewise does not indicate that the ferritin-iron in the beans is protected from absorption inhibitors.

In conclusion, the results from our study show that most plant ferritin is altered during cooking, either by release of iron or denaturation of the protein hull. Any remaining ferritin-iron, either in soluble form as intact ferritin, as insoluble ferritin after denaturation, or as the naked mineral core, is then released and dissolved in a pH-dependent manner during gastric digestion. Such released and dissolved iron would be taken up by the divalent metal transporter-1 pathway for nonheme iron and would display similar susceptibility to iron absorption enhancers and inhibitors. At the present stage, we cannot exclude the possibility that small amounts of ferritin may survive cooking and gastric passage intact. However, our experiments indicate that these amounts can be safely expected to be very small and are therefore unlikely to change overall absorption efficiency of plant iron.

Acknowledgments
We thank Stefan Storcksdieck genannt Bonsmann and Christophe Zeder for their advice.

Literature Cited