

Effects of ascorbic acid, phytic acid and tannic acid on iron bioavailability from reconstituted ferritin measured by an *in vitro* digestion–Caco-2 cell model

Fuxia Jin^{1,2*}, Charles Frohman¹, Theodore W. Thannhauser¹, Ross M. Welch¹ and Raymond P. Glahn¹

¹USDA-ARS, The Robert W. Holley Center for Health and Agriculture, Tower Road, Ithaca, New York 14853, USA

²Onology, Viticulture and Natural Product Laboratory, Department of Chemistry, Appalachian State University, Boone, NC 28608, USA

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The effects of ascorbic acid (AA), phytate and tannic acid (TA) on Fe bioavailability from Fe supplied as reconstituted ferritin were compared with FeSO₄ using an *in vitro* digestion–Caco-2 cell model. Horse spleen apoferritin was chemically reconstituted into an animal-type ferritin (HSF) and a plant-type ferritin (P-HSF) according to the typical ratios of Fe:P found in these molecules. In the presence of AA (Fe:AA molar ratio of 1:20), significantly more Fe was absorbed from FeSO₄ (about 303 %), HSF (about 454 %) and P-HSF (about 371 %) when compared with ferrous sulfate or ferritin without AA. Phytic acid (PA; Fe:PA molar ratio of 1:20) significantly reduced Fe bioavailability from FeSO₄ (about 86 %), HSF (about 82 %) and P-HSF (about 93 %) relative to FeSO₄ and the ferritin controls. Treatment with TA (Fe:TA molar ratio of 1:1) significantly decreased Fe bioavailability (about 97 %) from both FeSO₄ and the ferritin samples. AA was able to partially reverse the negative effect of PA (Fe:PA:AA molar ratio of 1:20:20) on Fe bioavailability but did not reverse the inhibiting effect of TA (Fe:TA:AA molar ratio of 1:1:20) on Fe bioavailability from ferritin and FeSO₄. Overall, there were no significant differences in bioavailable Fe between P-HSF, HSF or FeSO₄. Furthermore, the addition of AA (a known promoter) or the inhibitors, PA and TA, or both, did not result in significant differences in bioavailable Fe from ferritin relative to FeSO₄. The results suggest that Fe in the reconstituted ferritin molecule is easily released during *in vitro* digestion and interacts with known promoters and inhibitors.

Ferritin: Iron bioavailability: Phytic acid: Tannic acid: Ascorbic acid

Ferritin is the principal Fe storage protein in all living aerobic organisms. It is a spherical, cage-like protein with nanocavities formed by multiple polypeptide subunits (for example, twenty-four protein subunits for horse spleen ferritin) arranged in 432 symmetry to give a hollow shell with a capacity of storing up to 4500 Fe(III) atoms. In legumes, such as peas and soybeans, a large amount (about 90 %) of Fe is stored in seeds within the ferritin molecule. This form of Fe may be useful in biofortification efforts as it can be selected via traditional breeding methods and engineered into plants via biotechnology^(1,2).

Recent human studies have demonstrated that reconstituted plant (P-HSF) and animal (HSF) forms of ferritin-Fe are equal in bioavailability to FeSO₄^(3,4). These studies were conducted in the absence of a food matrix. It appears from these studies that the protein shell of the ferritin molecule does not hinder Fe bioavailability. The next logical step in assessing ferritin-Fe bioavailability is to address the effect of promoters (for example, ascorbic acid (AA) and meat) and inhibitors (for example, phytic acid (PA), polyphenolics) of Fe bioavailability on ferritin-Fe. Studies with intrinsically ⁵⁵Fe-labelled soybeans that were known to have about 49 % of Fe in

phytoferritin demonstrated reasonably high Fe bioavailability (about 28 %) in non-anaemic, Fe-depleted women, and these samples contained a reasonably high level of PA (i.e. 2 % PA).

There are very few reports of direct testing of promoters and inhibitors on ferritin-Fe bioavailability. During the preparation of the present paper, the authors realised that Bejjani *et al.* published their results on purified pea ferritin for the effect of AA and PA⁽⁵⁾. The Fe core of pea ferritin was released under gastric conditions and became accessible to both AA and PA, which was in turn shown as increased or reduced Fe availability, respectively. In another recent study⁽⁶⁾, Fe absorption from reconstituted ferritin and ferrous sulfate was affected similarly by both promoters and inhibitors after *in vitro* digestion at pH 2. Fe from reconstituted ferritin was found to be less susceptible to the effect of promoters and inhibitors except tannins when the *in vitro* digestion was done at pH of 4. It is thus important to understand how a range of promoters and inhibitors can affect Fe availability from ferritin. Therefore, the objective of the present study was to utilise an *in vitro* digestion–Caco-2 cell model to assess the effects of AA, meat (cooked fish fillet), PA and tannic acid (TA) on ferritin-Fe bioavailability.

Abbreviations: AA, ascorbic acid; HSF, animal-type ferritin; ICP, inductively coupled plasma; MALDI-TOF, matrix-assisted laser desorption/ionisation time of flight; MWCO, molecular weight cut-off; PA, phytic acid; P-HSF, plant-type ferritin; TA, tannic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.

* **Corresponding author:** Dr Fuxia Jin, fax +1 828 262 6558, email jinff@appstate.edu

Materials and methods

Chemicals, enzymes and hormones

Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Experimental design. Four sets of experiments were conducted.

Experiment 1. The objectives of this experiment were: (1) to test the integrity of the starting sample, apoferritin and its reconstitution product; (2) to remove any unbound or surface-bound Fe after the reconstitution process; (3) to estimate ferritin digestibility during each stage of the *in vitro* digestion process. The analysis of surface-bound Fe²⁺, i.e. protein coat-sequestered Fe²⁺, as well as mineral core Fe, was accomplished using a spectrophotometer^(7–9).

Experiment 2. The objective of this experiment was to measure Fe release from the reconstituted ferritin during the *in vitro* digestion process under different conditions. The reconstituted ferritin was tested alone, with the promoter AA, with the inhibitors PA and TA, or with a combination of both inhibitors and promoters.

Experiment 3. The objective of this experiment was to compare Fe bioavailability from FeSO₄ and reconstituted ferritin when samples were present alone, in the presence of the promoter AA at an Fe:AA ratio of 1:20 (i.e. FeSO₄, FeSO₄+AA, reconstituted ferritin and reconstituted ferritin+AA), in the presence of PA (Fe:PA at 1:20) and TA (Fe:TA at 1:1), or in the presence of PA+AA (Fe:PA:AA at 1:20:20) and TA+AA (Fe:TA:AA at 1:1:20).

Experiment 4. The objective of this experiment was to compare the effectiveness of AA and fish meat on reversing the inhibitory effect of PA and TA on Fe bioavailability from reconstituted P-HSF.

Ferritin reconstitution

Horse spleen apoferritin was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA, USA) with less than 0.03 Fe atoms/subunit. Reconstituted horse spleen ferritin was prepared by mixing a solution of horse spleen apoferritin in 0.15 M-HEPES (pH 7.0) with a freshly made FeSO₄ solution (about 500 Fe atoms/ferritin molecule). The reaction was then allowed to take place for 24 h in a manner similar to Davila-Hicks *et al.*⁽³⁾ and Rohrer *et al.*⁽¹⁰⁾, but for a longer period of time so as to ensure that the reconstitution process went to completion. Reconstituted P-HSF was prepared by mixing a solution of apoferritin in 0.15 M-HEPES (pH 7.0) with a freshly made FeSO₄ solution (about 500 Fe atoms/ferritin molecule) with the addition of an aqueous solution of K₂HPO₄ at a P:Fe ratio of 1:4. The reaction was allowed to proceed for 24 h. The ferritin molecule reconstituted in a similar manner by other researchers was shown to have the same structure as the native molecule from results of X-ray absorption spectroscopy, Mössbauer spectroscopy and electron microscopy^(10–14). Fe incorporation into the reconstituted ferritins was confirmed through inductively coupled plasma (ICP)-atomic emission spectroscopy measurement of Fe concentration in the filtrate and retentate samples after the reconstituted ferritins were filtered using ultra-membrane filtration and microcentrifugation (10 000

Da molecular weight cut-off (MWCO)) from Amicon Microconcentrators, Amicon (Billerica, MA, USA).

Cell cultures

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and used in experiments at passage 29–32. Cells were seeded at a density of 50 000 cells/cm² in collagen-treated six-well plates (Costar Corp., Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 10 % (v/v) fetal bovine serum (Gibco) and 25 mM-HEPES (Sigma, St Louis, MO, USA). Cells were maintained at 37°C in an incubator with a 5 % CO₂–95 % air atmosphere with constant humidity, and the medium was replaced every 2 d. Cells for each study were used 14 d post-seeding. Under these conditions, the amount of cell protein measured in each well was found to be highly consistent from well to well within each culture plate.

In vitro digestion–Caco-2 cell culture method

In vitro digestion and enzyme preparation methods have been described in detail elsewhere⁽¹⁵⁾. Briefly, samples were combined in 50 ml tubes. To initiate the gastric phase of digestion, 10 ml of 140 mM-NaCl, 5 mM-KCl (pH 2) solution was added to each 50 ml tube. After pH adjustment to 2 with 1 M-HCl, 0.5 ml of pepsin solution was added to each tube, and the mixtures were incubated for 1 h at 37°C on a rocking platform (model RP-50; Laboratory Instruments, Rockville, MD, USA). After incubation, the pH was raised to 5.5–6.0 with 1 M-NaHCO₃ and 2.5 ml pancreatin–bile solution was added to each mixture. The pH was then adjusted to approximately 7.0, and the volume contained within each tube was adjusted by weight to 15 ml using a 140 mM-NaCl, 5 mM-KCl (pH 6.7) solution. At this point, the mixtures were referred to as 'digests'.

To initiate the intestinal digestion period, a 1.5 ml sample of the digest was placed into the upper chamber of a two-chambered system formed by placing well inserts fitted with 15 000 Da MWCO dialysis membranes into plate wells containing Caco-2 cell monolayers. Plates were covered and incubated at 37°C for 2 h on a rocking platform (approximately twelve oscillations/min). Following the 2 h incubation, the inserts were carefully removed and an additional 1 ml of minimum essential medium was added to each well. The cell plates were then replaced into the incubator to allow time for ferritin formation. Cells were harvested after 22 h (24 h from the start of the intestinal digestion).

For Fe-release studies, a 1.0 ml sample of sample solution was transferred to 1.5 ml microcentrifuge tubes at the following times during the *in vitro* digestion process: (1) after the sample solution was adjusted to pH 2; (2) after 1 h of peptic digestion; (3) after the samples were adjusted to the intestinal pH 7. Microconcentrators equipped with MWCO membranes (100 000 Da MWCO) were used to centrifuge a sample solution taken from each digestion stage at 3000 g for 30 min. The filtrate was analysed for Fe concentration and the retentate were stored for further ferritin digestibility studies.

Caco-2 cell ferritin, protein and iron assays

All glassware used in sample preparation and analyses was rinsed with 10 % HCl and 18 M Ω deionised water before use. Caco-2 cell ferritin formation served as a marker of cell Fe uptake from sample digest. Caco-2 cell protein content of samples was measured, after solubilisation in 0.5 M-NaOH, using a Bio-Rad DC protein assay kit, which is a commercial semi-micro adaptation of the Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Serum Ferritin Assay; Ramco Laboratories, Houston, TX, USA). A 10 μ l sample of the sonicated Caco-2 cell monolayer, harvested in 2 ml, was used for each ferritin measurement.

Analyses of total Fe content of the experimental solutions, samples, and digests were conducted using an ICP emission spectrometer (ICAP Model 61E Trace Analyser; Thermo Jarrell Ash Corporation, Franklin, MA, USA) after wet-ashing with HNO₃ and HClO₄. Soluble Fe concentration in sample solution from each stage of digestion was analysed using an ICP mass spectrometer (ICP-MS, Agilent 7500c ICP-MS; Agilent Technologies, MA, USA). Specifically, 400 μ l of each sample solution were filtered using a 100 kDa MWCO microconcentrator (Amicon, Billerica, MA, USA). The filtrate was collected and a sample was diluted with 2 % HNO₃ for determining Fe concentration via ICP-MS.

Native gel electrophoresis

Two different native PAGE protocols were applied to cross-examine and confirm the integrity and digestibility of ferritin molecules under the experimental conditions. Native horse spleen ferritin was purchased from Sigma (about 91 mg ferritin/ml) and horse spleen apoferritin was purchased from Calzyme (San Luis Obispo, CA, USA). To check for ferritin protein molecular integrity, native PAGE analysis of ferritin was first performed using NativePAGE™ Novex® 4–16 % Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). Ferritin stock solution was diluted with NativePAGE™ sample buffer, NativePAGE™ 5 % G-250 sample additive and deionised water, and 10 μ l of the sample was loaded into each well to ensure that each well contained a minimum of 1 μ g ferritin protein. Gels were run at 150 V constant voltages at a starting and final current of 20 and 3.95 mA, respectively. The gels were removed, rinsed thoroughly with 18 M Ω deionised water and placed in 100 ml fix solution of 40 % methanol–10 % acetic acid which was then microwaved on high (950–1100 W) for 45 s. The gel was incubated in the fix solution at room temperature and shaken on an orbital shaker for 30 min before the fix solution was decanted. This step was repeated and the gel was stained by shaking on an orbital shaker overnight in 100 ml of stain solution made from the Colloidal Blue Staining Kit (20 ml Stainer A, 5 ml Stainer B, 20 ml methanol and 55 ml deionised water). The stain solution was decanted the next morning and the gel was shaken on an orbital shaker for 5 min in 100 ml destain solution (8 % acetic acid). Finally, the destain solution was decanted and the gel was washed in 100 ml deionised water by shaking on an orbital shaker until the desired background was obtained.

Ferritin was also analysed for the effect of *in vitro* digestion on protein molecule integrity by running a Native PAGE with

a 7.5 % 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl ready gel (catalogue no. 161–1100; Bio-Rad, Hercules, CA, USA), with a Tris–glycine running buffer (25 mM-Tris–192 mM-glycine; pH 8.3). Then 20 μ l of ferritin solution in a sample buffer of 62.5 mM-Tris-HCl (pH 6.8), 25 % glycerol and 0.01 % bromophenol were loaded into each well. Gels were run at 200 V for 35 min with a starting current of 50 mA/gel and a final current of 30 mA/gel. The gels were removed, rinsed thoroughly with 18 M Ω deionised water and washed in 18 M Ω deionised water on a rocking plate for 15 min (water was changed every 5 min). A colloidal Coomassie blue solution (0.12 % Coomassie G-250, 10 % ammonium sulfate, 10 % phosphoric acid and 20 % methanol) was used to stain the gels for over 1 hr. The stained gels were again washed with 18 M Ω deionised water on a rocking plate for 15 min before the images were scanned.

Gel filtration chromatography

Econo-Pac 10DG columns (Bio-Rad, Hercules, CA, USA) were used to test for any free Fe or surface-bound Fe from the reconstituted ferritin solutions. These columns are packed with Bio-Gel P-6 DG gel, which excludes solutes greater than 6000 Da. The columns were first equilibrated with 20 ml of the eluting buffer (20 mM-Tris (pH 7.5), 150 mM-NaCl) before the loading of a 3.0 ml sample of the reconstituted ferritin solutions. The entire sample was then allowed to enter the column and the first 3.0 ml of effluent were discarded. The reconstituted ferritin solutions were eluted with 15 ml of buffer, while 1.0 ml fractions were collected from the column. The Fe concentration in each fraction was measured by ICP-MS. The ferritin concentration in each fraction was measured by the protein assay described above.

Ferritin mineral core integrity

Measurement of surface-bound Fe(II), protein coat-sequestered Fe(II) and total Fe in reconstituted ferritin molecules was done with a spectrophotometer after consultation with Dr Elizabeth Theil at Children's Hospital Oakland Research Institute (CA, USA). Reconstituted ferritin solution was prepared as stated in earlier sections and allowed for mineral core formation for over 24 h. The spectrophotometric method was similar to that discussed in a previous report with some minor modifications⁽⁸⁾. Briefly, bathophenanthroline disulfonic acid (BPDS) was used as the Fe-chelating agent for Fe(II) and a series of FeSO₄ solutions was used to establish calibration curves. For the measurement of surface-bound Fe(II), ferritin sample solution was mixed with an excess amount of BPDS in a pH 4.0 sodium acetate buffer solution and diluted with double deionised water. In order to measure the protein coat-sequestered Fe(II), ferritin was first denatured by heating in 1.0 M-HCl at 100°C for 30 min to release the protein coat-sequestered Fe(II). The process then proceeded in the same manner as that utilised for the measurement of surface-bound Fe(II). Total ferritin-Fe was analysed by first reducing the ferritin mineral core with sodium disulfite and then mixing the ferritin solutions with BPDS and sodium acetate buffer solution in deionised water.

Statistical analyses

Data were analysed using the software package GraphPad Prism (GraphPad Software, San Diego, CA, USA). ANOVA with Tukey's *post hoc* test was used to compare differences among means. Differences among means were considered significant at $P \leq 0.05$.

Results

The efficiency of Fe reconstitution and the structure of the final reconstitution product were well characterised and established⁽¹⁶⁾. When the solutions were visually checked, no precipitated Fe was observed during the reconstitution process and after storage. A parallel reconstitution experiment was run by adding ferrous sulfate solution to the reconstitution buffer (no apoferritin present) and precipitated Fe was seen immediately after the first drop of ferrous sulfate solution was added. The apoferritin solution, however, lacked any precipitated Fe during and after the reconstitution process. The lack of rust colour from gel filtration columns after reconstituted ferritin samples were eluted was an indication of no precipitated Fe trapped inside the gel filtration column. Furthermore, the eluted fractions did not contain any Fe when the gel filtration column was washed with 0.01 M-HCl afterwards. This again shows that there was no precipitated Fe trapped inside the gel filtration column. During the early stages of the present study, a membrane of 10 kDa MWCO was used and it took an extremely long time to finish micro-filtration. It was then decided that 100 kDa membranes were to be used for the later part of the experiments.

Integrity of apoferritin as well as the reconstituted ferritin molecules was checked through native gel electrophoresis. Both apoferritin and reconstituted ferritins showed bands at similar positions to those of native horse spleen ferritins at molecular weights of 480 kDa (monomer) and 720 kDa (aggregate molecule) Fig. 1 (A)). The stained bands in native gels were typically digested and analysed by matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF)-MS for a comparison of the tryptic fragments with the National Center for Biotechnology Information mass spectra library using a peak-matching program (data not shown) developed by Dr Xiaolong Yang at Cornell University Functional and Comparative Proteomics Center (Ithaca, NY, USA). The mass peaks from protein bands at mass of 480 and 720 kDa Fig. 1 (A)) matched the typical fragmentation peaks observed for the horse spleen ferritin molecule. Other parts of the digested gel except the very top pieces did not have matching mass peaks with horse spleen ferritin fragments.

Native horse spleen ferritin itself is commonly used as a molecular marker compound for protein gel electrophoresis. Lane a in both Figs. 1 (B) and (C) thus served as a molecular-weight marker to show if reconstituted ferritins have been denatured or not during both the reconstitution and *in vitro* digestion processes. Reconstituted ferritin of both animal type and plant type showed similar bands to those of native horse spleen ferritin even after storage for a long period of time (about 10 months) at low temperatures of -20°C Figs. 1 (B) and (C)). Native horse spleen ferritin is frequently used as a molecular marker compound for protein gel electrophoresis. Lane a in both Figs. 1 (B) and (C) thus

served as a molecular-weight marker to show if reconstituted ferritins have been denatured or not during both the reconstitution and *in vitro* digestion processes. There were no bands that showed up in the native gel after the reconstituted ferritins were digested in low pH saline solutions alone, with subsequent pepsin digestion, or with further pancreatic digestion Figs. 1 (B) and (C)). Protein and Fe analysis of the gel filtration fractions revealed that $> 95\%$ of the added Fe in the reconstitution process was associated with the ferritin protein.

Caco-2 cell ferritin formation from Fe in the digested samples containing ferrous sulfate and reconstituted ferritins was generally the same Fig. 2 (A)). The addition of AA (Fe:AA molar ratio of 1:20) to samples of P-HSF, HSF or FeSO_4 resulted in a similar increase of about 3–4-fold in Caco-2 cell ferritin formation as compared with samples not treated with AA Fig. 2 (A)).

When PA was present in the sample digest at an Fe:PA molar ratio of 1:20, bioavailable Fe in reconstituted P-HSF (about 93%), HSF (about 82%) and FeSO_4 (about 86%) was significantly decreased Fig. 2 (B)). Furthermore, addition of TA (Fe:TA molar ratio of 1:1) to the digests significantly decreased Fe bioavailability (about 97%) from ferrous sulfate and the reconstituted ferritin samples Fig. 2 (C)). The addition of AA at an Fe:AA molar ratio of 1:20 to samples when PA was present (Fe:PA molar ratio of 1:20) partially reversed the negative effects of PA on Fe bioavailability and restored cell-ferritin formation from reconstituted P-HSF (about 43%), HSF (about 79%) and FeSO_4 (about 67%) as compared with control treatments without PA Fig. 2 (B)). The inhibitory effect of TA (Fe:TA molar ratio of 1:1) was not reversed by AA additions even at an Fe:AA molar ratio of 1:1000 Fig. 2 (C)).

The interaction of reconstituted ferritins with PA, TA, AA and cooked fish meat was further investigated using P-HSF as the ferritin-iron source Figs. 3 (A) and (B)). Fish was able to partially reverse the adverse effect of PA (Fe:PA molar ratio of 1:20) on bioavailable Fe from P-HSF and resulted in an increase of Caco-2 cell ferritin formation when 0.5 g fish (about 35%) and 1.0 g fish (about 52%) was present in 15 ml of sample digest with an Fe concentration of $42.3\ \mu\text{mol/l}$ Fig. 3 (A)). Increases in bioavailable Fe were seen when AA (Fe:AA molar ratios of 1:5, 1:20 and 1:100, respectively) was added to the sample digest of P-HSF plus PA (Fe:PA molar ratio of 1:20). A 78% increase in cell ferritin formation was observed when AA was present at an Fe:PA:AA molar ratio of 1:20:100, when compared with samples containing only P-HSF Fig. 3 (A)). Interestingly, neither fish nor AA was able to reverse the inhibitory effect of TA on bioavailable Fe from P-HSF when TA was present at an Fe:TA molar ratio of 1:1 Fig. 3 (B)).

Most of the Fe was released during the pH 2.0 and peptic digestion stage Tables 1 and 2). Soluble Fe concentration was significantly lower after 2 h of intestinal digestion for both reconstituted HSF and P-HSF; however, the amount of released Fe was lower for the P-HSF relative to the HSF (i.e. 52 v. 100%). The presence of AA did not seem to affect the soluble Fe concentration for the reconstituted ferritin. PA made the released ferritin-Fe soluble even at the intestinal stage. However, the PA-Fe complex was so stable that this Fe was of lower availability regardless of its solubility. Reconstituted ferritin molecules have very low amounts of surface-bound Fe

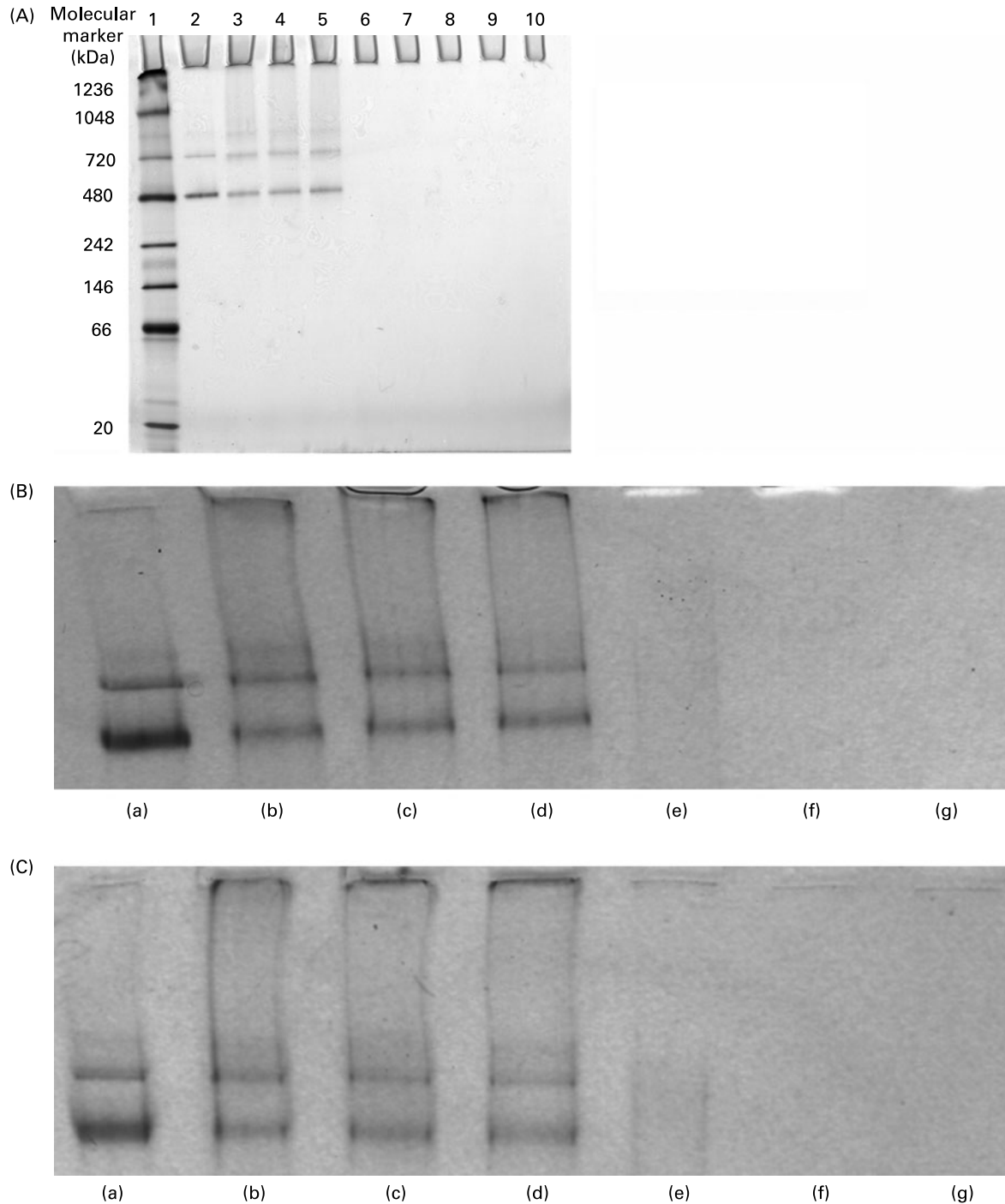


Fig. 1. (A) Native PAGE image of ferritin using NativePAGE™ Novex® 4–16 % Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The gels were stained with the Colloidal Blue Staining Kit. All photographs show the start line at the top with migration in a vertical direction towards the anode. 1, Native protein standard marker; 2, native horse spleen ferritin (1 g); 3, horse spleen apoferritin (1 g); 4, reconstituted animal-type ferritin (1 g); 5, reconstituted plant-type ferritin (1 g); 6–10, blanks (sample buffer only). (B) Electrophoresis in 7.5 % 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl polyacrylamide gel of horse spleen ferritins stained with Coomassie blue. All photographs show the start line at the top and migration in a vertical direction towards the anode. (a) Native horse spleen ferritin standard showing α - and β -bands. (b) Horse spleen apoferritin showing α - and β -bands. (c) Freshly prepared reconstituted horse spleen ferritin (HSF) showing α - and β -bands. (d) Reconstituted horse spleen ferritin (HSF) after storage showing α - and β -bands. (e) HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0). (f) HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion. (g) HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion. (C) Electrophoresis in 7.5 % Tris-HCl polyacrylamide gel of horse spleen ferritins stained with Coomassie blue. All photographs show the start line at the top and migration in a vertical direction towards the anode. (a) Native horse spleen ferritin standard showing α - and β -bands. (b) Horse spleen apoferritin showing α - and β -bands. (c) Freshly prepared reconstituted plant-type ferritin (P-HSF) showing α - and β -bands. (d) Reconstituted reconstituted plant-type ferritin (P-HSF) after storage showing α - and β -bands. (e) P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0). (f) P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion. (g) P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion.

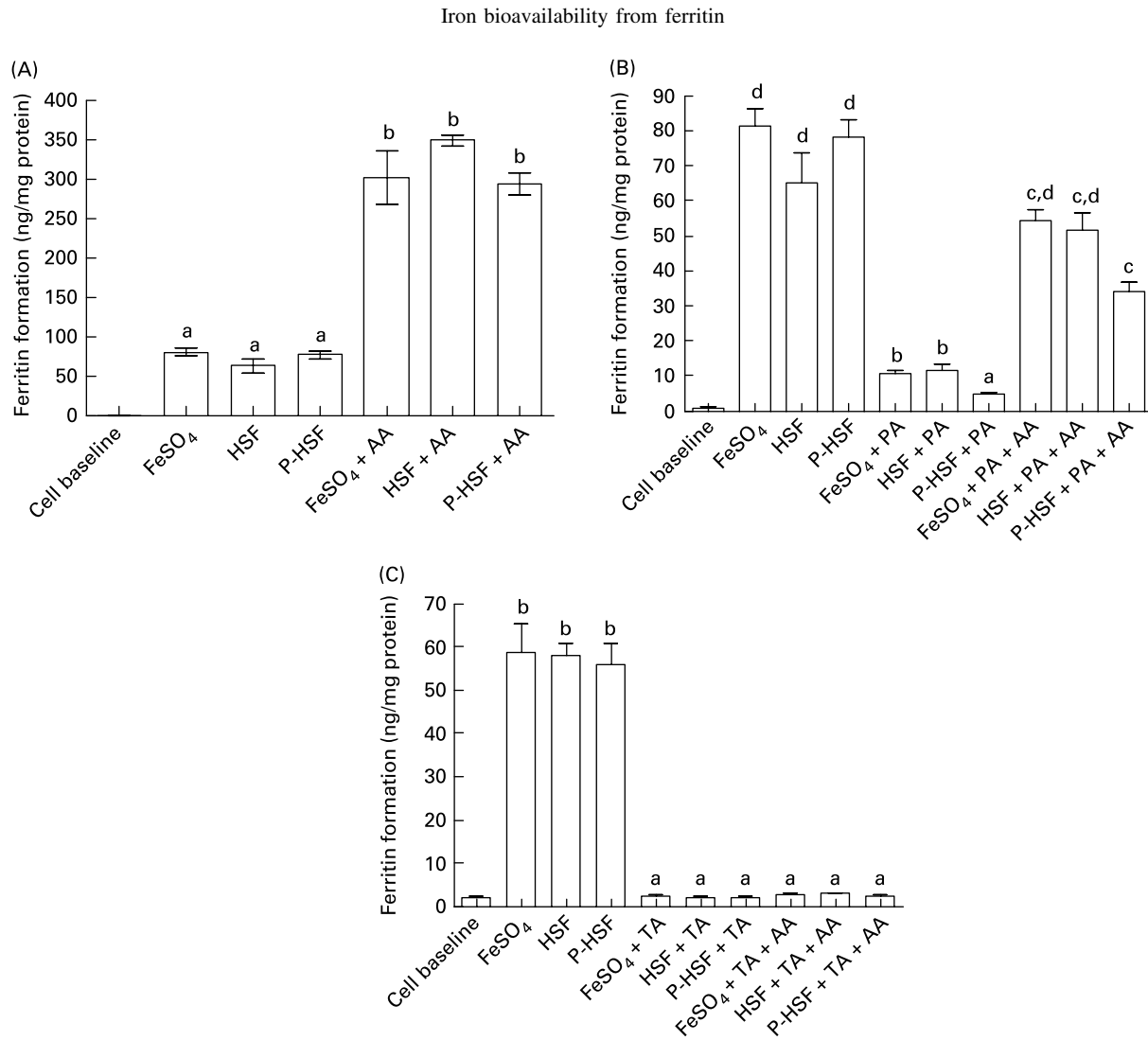


Fig. 2. (A) Caco-2 cell ferritin formation in response to *in vitro* digests. Digests contained a constant Fe concentration of 42.3 $\mu\text{mol/l}$ and a constant molar ratio of Fe:ascorbic acid (AA) molar ratio of 1:20. HSF, reconstituted horse spleen ferritin; P-HSF, reconstituted phytoferritin. Values are means (n 6), with standard errors represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P \leq 0.05$). (B) Caco-2 cell ferritin formation in response to the inhibiting effect of phytic acid (PA) (Fe:PA molar ratio of 1:20 when PA was present) and the promoting effect of AA (Fe:AA molar ratio of 1:20 when AA was present). Digest Fe concentration was 42.3 $\mu\text{mol/l}$. Values are means (n 6), with standard errors represented by vertical bars. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P \leq 0.05$). (C) Caco-2 cell ferritin formation in response to the inhibiting effect of tannic acid (TA) (Fe:TA molar ratio of 1:1 when TA was present) and the promoting effect of AA (Fe:AA molar ratio of 1:1000 when AA was present). Digest Fe concentration was 42.3 $\mu\text{mol/l}$. Values are means (n 6), with standard errors represented by vertical bars. ^{a,b} Mean values with unlike letters were significantly different ($P \leq 0.05$).

(< 5 %) and the protein coat-sequestered Fe was essentially non-detectable (Table 3). The majority of the Fe entered the protein mineral core in the form of Fe(III). The fact that all detectable Fe in the retentate of digest solutions eluted together with the protein on gel filtration chromatography proved that Fe precipitation did not affect Fe recovery during the Fe-release experiments.

Discussion

Fe bioavailability from the crystalline mineral-Fe core inside the protein coat of ferritin has been re-evaluated during the past decade. In contrast to the earlier studies^(17–21) which suggested that ferritin was a poor source of bioavailable Fe, the Fe in the ferritin molecule has been recently reported to have high bioavailability in both animal and human studies^(3,4,22,23). The discrepancies between these results

could be due to poor equilibration of the labelled Fe atoms with the Fe atoms in the solid mineral core of ferritin in the earlier studies^(22,24). In an effort to avoid errors caused by both extrinsic and intrinsic labelling when studying ferritin-Fe or soyabean Fe bioavailability, reconstituted ferritin was only tested after Fe depletion from the original protein molecule and subsequent reconstitution of the labelled Fe into the protein mineral core was performed. The reconstituted ferritin molecule was shown to be the same as the native molecule from results obtained using X-ray absorption spectroscopy, Mössbauer spectroscopy and electron microscopy^(10–14). Fe from reconstituted horse spleen ferritin and Fe from soyabean ferritin were both shown to have the same bioavailability as FeSO₄ when fed to non-anaemic women^(3,4).

In the present study, apoferritin and reconstituted ferritin protein had similar bands as those of the horse spleen ferritin standard in the native gels; thus the integrity of the

apoferritin and reconstituted ferritin protein was demonstrated. The reason for more than one single component showing up in the native gels was due to the heterogeneity in the ferritin protein. Both apoferritin and ferritin may show more than one single component in the ultracentrifuge⁽²⁵⁾ and on

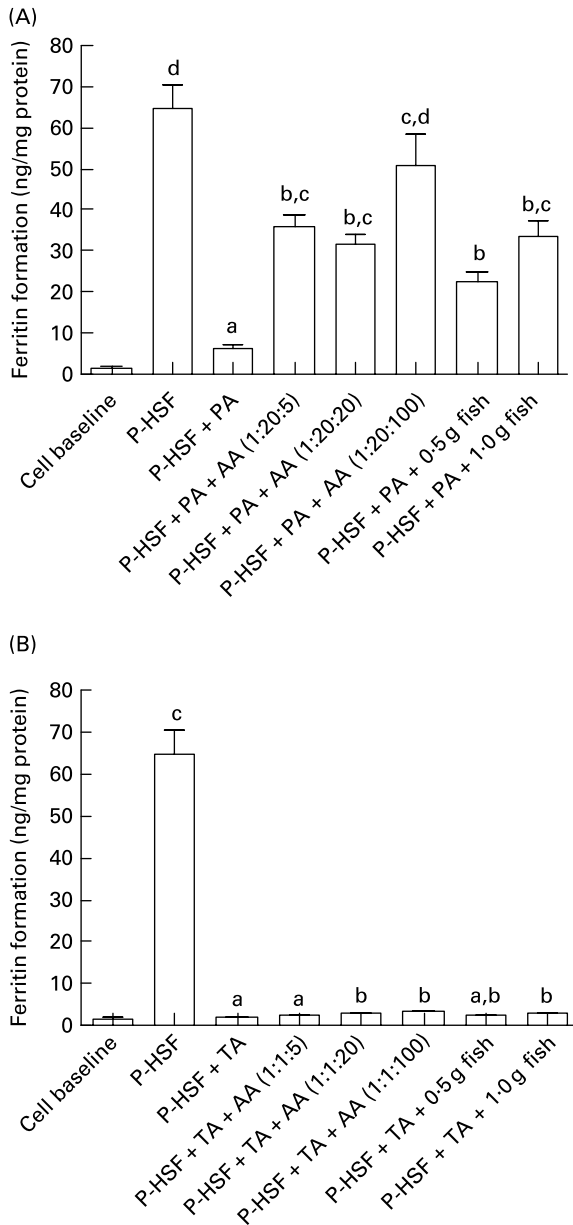


Fig. 3. (A) Inhibiting effect of phytic acid (PA) and the promoting effect of ascorbic acid (AA) and fish meat on Caco-2 cell ferritin formation in response to digests of phytoferritin (P-HSF) with PA added at an Fe:PA molar ratio of 1:20. AA was present at an Fe:AA molar ratio of 1:5, 1:20 and 1:100. Fish meat was supplied with an Fe:fish ratio of 42.3 $\mu\text{mol Fe/l}$:0.5g fish and 42.3 $\mu\text{mol Fe/l}$:1.0g fish, respectively. Digest Fe concentration was 42.3 $\mu\text{mol/l}$. Values are means (n 6), with standard errors represented by vertical bars. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P \leq 0.05$). (B) Effect of tannic acid (TA), AA and fish meat on Caco-2 cell ferritin formation in response to digests of P-HSF with TA supplied at an Fe:TA molar ratio of 1:1. AA was present at an Fe:AA molar ratio of 1:5, 1:20 and 1:100. Fish was supplied at an Fe:fish ratio of 42.3 $\mu\text{M-Fe}$:0.5g fish and 42.3 $\mu\text{M-Fe}$:1.0g fish, respectively. Digest Fe concentration was 42.3 $\mu\text{mol/l}$. Values are means (n 6), with standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P \leq 0.05$).

gel electrophoresis^(26,27). Williams & Harrison confirmed the possible presence of monomer, dimer, trimer, tetramer, pentamer and higher oligomers in ferritin⁽²⁸⁾. The amino acid analyses and electron microscopic evidence showed that the oligomer molecules are similar to monomers. Reconstituted ferritins also showed similar bands in native gel after long-time storage at low temperatures (-20°C), thereby indicating molecular stability. When placed in a saline solution of pH 2.0, the ferritins were denatured, which is consistent with the previous research showing that low pH readily dissociates ferritin molecules⁽²⁹⁾. Reconstituted ferritins were dissociated into subunits by *in vitro* digestion conditions and Fe mineral inside the protein shell was thus accessible to other components in the solution. These results confirmed the findings in a recent study⁽⁶⁾, in which reconstituted ferritin protein was dissociated into subunits at pH 2 during *in vitro* digestion conditions. Ferritin-Fe uptake by Caco-2 cells was thus significantly affected by promoters and inhibitors. Kalgaonkar & Lonnerdal also measured Fe uptake from intact undigested ferritin and compared with Caco-2 cell Fe uptake from ferrous sulfate. Their results basically showed that Fe from intact undigested ferritin has lower availability than ferrous sulfate Fe. In the present study, Caco-2 cell Fe uptake from intact undigested ferritin was compared with baseline control Caco-2 cells, which were treated with the culture media without any additional Fe supplementation. The fact that Fe uptake from intact undigested ferritin was about 60-fold higher than baseline suggested that Fe from intact ferritin could be available to Caco-2 cells arguably through some sort of endocytotic pathways⁽³⁰⁾. Thus the present results provide complementary rather than contradictory information from previous work.

The binding of Fe^{3+} to the surface of apo-, holo- and reconstituted horse spleen ferritins was weak with < eight Fe atoms per horse spleen ferritin under various conditions⁽³¹⁾. In the present study, the presence of only a low level of free Fe in the reconstituted ferritin solutions indicated that the Fe reconstitution process was very efficient and that the majority of Fe entered the protein to form the mineral core. The non-detectable amount of protein coat-sequestered Fe(II) provided further evidence that almost all of the added Fe(II) entered the protein mineral core. Therefore, it appears that the reconstituted ferritins represent integral protein molecules with Fe mineral inside the protein shell. Hence, the available Fe from the reconstituted ferritin solutions came from mineral-core Fe and not from free Fe or surface-bound Fe. From these observations, we can confidently conclude that the reconstituted ferritin samples function as natural ferritin.

The Fe-uptake results reported here using the Caco-2 cell culture model support the results from human studies⁽³⁾. Bioavailable Fe from reconstituted ferritins and FeSO_4 was the same regardless of the presence or absence of AA. These results, which are similar to previous results obtained in human studies^(3,4) which used reconstituted horse spleen ferritin and soyabean ferritin, support the recent observation in human subjects that ferritin-Fe can be highly bioavailable.

Fe deficiency is a major human nutritional disorder in the world. In developing countries where cereal grains are consumed as the staple food, Fe deficiency is a significant threat to human health, since grain-Fe content is either low or most of the Fe is removed during grain processing and milling. Recently, the soyabean ferritin gene was successfully

Table 1. Iron release from animal-type ferritin (HSF) during each stage of the *in vitro* digestion process (Mean values (*n* 3) with their standard errors)

Sample	% Fe*	
	Mean	SEM
HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	105.4 ^b	13.4
HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	99.5 ^b	1.6
HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	7.2 ^a	0.1
HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	96.0 ^b	5.6
HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	80.7 ^b	10.2
HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	6.9 ^a	3.3
HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	77.4 ^b	12.9
HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	69.9 ^b	6.2
HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	99.2 ^b	3.1
HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	94.9 ^b	9.7
HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	97.2 ^b	11.2
HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	91.3 ^b	0.9

AA, ascorbic acid; PA, phytic acid.

* Percentage of free Fe measured in the filtrate of a 100 kDa microconcentrator.

^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$).

transferred and expressed in elite indica rice grains having highly desirable agronomic and field-performance traits⁽³²⁾. Higher levels of both Fe and Zn were obtained even after polishing the rice grain. Since ferritin is an efficient Fe storage molecule for aerobic organisms, incorporation of ferritin into these crops through genetic engineering represents a potential strategy to combat Fe deficiency by loading more Fe into the edible portion of the crop.

Ferritin is a fairly stable protein as the overall structure can tolerate the treatment of 6 M-guanidine⁽³³⁾ and heating at 60–85°C⁽³⁴⁾. However, the stability of seed or phytoferritin has not been studied greatly. A major finding of the present research is that Fe absorption promoters (AA and fish) and inhibitors (PA and TA) affected ferritin-Fe and FeSO₄ bioavailability in a similar manner. AA enhanced Fe uptake from both

reconstituted ferritins and FeSO₄ to comparable levels. Also, PA and TA inhibited Fe uptake from both reconstituted ferritins and FeSO₄. These observations indicate that ferritin-Fe from the reconstituted ferritin molecules is a highly exchangeable form and therefore it is not a form of Fe with uniquely high bioavailability. If it is exposed to the low pH conditions of the stomach, then the Fe in the protein appears to be released and interacts with other components in the meal; thus it is not necessarily protected from inhibitors. However, if sufficient intact protein survives passage through the stomach then there is potential for very high bioavailability. This potential is unknown but is probably dependent on the rate of intact molecule uptake, digestion via pancreatic enzymes and interaction with inhibitors present in the meal. The mechanism by which intact ferritin is taken up remains unknown. Based on

Table 2. Iron release from plant-type ferritin (P-HSF) during each stage of the *in vitro* digestion process (Mean values (*n* 3) with their standard errors)

Sample	% Fe*	
	Mean	SEM
P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	52.0 ^b	4.1
P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	52.4 ^b	11.8
P-HSF after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	5.9 ^a	0.9
P-HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	64.0 ^b	9.5
P-HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	52.8 ^b	3.4
P-HSF + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	3.2 ^a	1.3
P-HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	48.0 ^b	4.9
P-HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	57.0 ^b	0.8
P-HSF + PA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	61.4 ^b	3.2
P-HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0)	55.6 ^b	5.0
P-HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0) and 1.0 h of pepsin digestion	59.4 ^b	0.4
P-HSF + PA + AA after dilution by 150 mM-NaCl, 5 mM-KCl (pH 2.0), 1 h of pepsin digestion and 2 h of pancreatin–bile extract digestion	60.6 ^b	3.3

AA, ascorbic acid; PA, phytic acid.

* Percentage of free Fe measured in the filtrate of a 100 kDa microconcentrator.

^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$).

Table 3. Measurement of surface-bound Fe(II), protein coat-sequestered Fe(II) and total iron in native horse spleen ferritin and reconstituted ferritin molecules through the chelating of Fe(II) to bathophenanthroline disulfonic acid

Ferritin molecule	% Surface-bound Fe(II)*	% Protein coat-sequestered Fe(II)†
Native horse spleen ferritin	8.1	Non-detectable
Reconstituted animal-type ferritin (HSF)	4.9	Non-detectable
Reconstituted plant-type ferritin (P-HSF)	4.5	Non-detectable

* Calculated as the percentage of Fe(II) to the total Fe in protein molecules. Each value represents an average of a triplicate measurement with % relative SD being less than 10.

† Calculated as the percentage of protein coat-sequestered Fe(II) to the total Fe in protein molecules. There was no detectable protein coat-sequestered Fe(II) from the colorimetric reactions.

their work on another large Fe-binding protein, lactoferrin, with Caco-2 cells, Suzuki *et al.* proposed that some proteins could escape digestion and be taken up via endocytotic pathways⁽³⁰⁾. However, these researchers have doubts as to whether ferritin was taken up through a putative ferritin receptor pathway^(35,36) or by a more general pinocytotic pathway. The Fe-release study in the present paper indicated that Fe from ferritin was released when protein subunits unfolded under the low pH (2.0) conditions during the peptic digestion process. Moreover, under the present *in vitro* conditions, it was unlikely for whole ferritin molecules to pass through the dialysis membrane with a MWCO of 15 000 Da, which is well below the ferritin molecular weight (about 500 000 Da). However, unpublished results in this group showed a level of human ferritin formation in the Caco-2 cells that was 60-fold higher than the baseline (i.e. Caco-2 cells with only the growth media) when the cell monolayer was exposed to horse spleen ferritin samples without the application of *in vitro* digestion and MWCO membranes, (X Yang and TW Thannhauser, unpublished results). Thus, in addition to protein dissociation and mineral core Fe release, ferritin-Fe may also become available by way of whole molecule uptake.

Although, in general, reconstituted HSF, P-HSF and FeSO₄ showed similar Fe bioavailability in the present study, there are occasions where reconstituted P-HSF seems to contain less bioavailable Fe compared with the Fe in reconstituted HSF and FeSO₄. For example, P-HSF has about 50 % less bioavailable Fe than reconstituted HSF and FeSO₄ in the presence of PA (1:20; Fig. 2 (A)). Whether this is due to the different chemical environment (for example, more P) inside P-HSF is not yet known. The amount of soluble Fe released from ferritin during *in vitro* digestion was dependent on the ferritin type studied. P-HSF released > 30 % less soluble Fe compared with HSF or FeSO₄. At present, no clear explanation for these results can be offered.

AA could serve as a reducing agent to mobilise Fe from the crystalline core⁽³⁷⁾ when it is incubated with ferritin *in vitro*. However, AA is also readily oxidised to dehydroascorbate due to the short half-time (less than 2 h) of its reduced form⁽³⁸⁾. The presence of AA in the reconstituted ferritin digest thus did not have a noticeable effect on Fe solubility

after the pancreatin–bile extract digestion stage since the total time of AA in aqueous solutions is almost 4 h.

In summary, significant amounts of Fe from reconstituted ferritin molecules were shown to be solubilised during *in vitro* digestion and become available to Caco-2 cells. Whether Fe from the natural form of phytoferritin is affected similarly to FeSO₄ by promoters and inhibitors *in vivo* is not yet known. Such knowledge will require further studies with both animal and human trials. Although ferritin molecules were shown to be transcytosed across Caco-2 cells⁽³⁹⁾, it has not been determined if ferritin-bound Fe is taken up intact as a large molecule, or if most of the ferritin-bound Fe in food is released during the digestion process and only freed Fe is taken up. We infer from the present results that biofortification of the phytoferritin gene into staple crops will not necessarily lead to improved Fe availability although such efforts may result in the production of nutrient-dense seeds with Fe in the form of ferritin.

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