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ARTICLE

Comparing Soluble Ferric Pyrophosphate to Common Iron Salts and Chelates as Sources of Bioavailable Iron in a Caco-2 Cell Culture Model

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Iron bioavailability from supplements and fortificants varies depending upon the form of the iron and the presence or absence of iron absorption enhancers and inhibitors. Our objectives were to compare the effects of pH and selected enhancers and inhibitors and food matrices on the bioavailability of iron in soluble ferric pyrophosphate (SFP) to other iron fortificants using a Caco-2 cell culture model with or without the combination of *in vitro* digestion. Ferritin formation was the highest in cells treated with SFP compared to those treated with other iron compounds or chelates. Exposure to pH 2 followed by adjustment to pH 7 markedly decreased $FeSO_4$ bioavailability but had a smaller effect on bioavailabilities from SFP and sodium iron(III) ethylenediaminetetraacetate (NaFeEDTA), suggesting that chelating agents minimize the effects of pH on iron bioavailabilty. Adding ascorbic acid (AA) and cysteine to SFP in a 20:1 molar ratio increased ferritin formation by 3- and 2-fold, respectively, whereas adding citrate had no significant effect on the bioavailability of SFP. Adding phytic acid (10:1) and tannic acid (1:1) to iron decreased iron bioavailability from SFP by 91 and 99%, respectively. The addition of zinc had a marked inhibitory effect on iron bioavailability. Calcium and magnesium also inhibited iron bioavailability but to a lesser extent. Incorporating SFP in rice greatly reduced iron bioavailability from SFP, but this effect can be partially reversed with the addition of AA. SFP and $FeSO_4$ were taken up similarly when added to nonfat dry milk. Our results suggest that dietary factors known to enhance and inhibit iron bioavailability from various iron sources affect iron bioavailability from SFP in similar directions. However, the magnitude of the effects of iron absorption inhibitors on SFP iron appears to be smaller than on iron salts, such as $FeSO_4$ and $FeCl_3$. This supports the hypothesis that SFP is a promising iron source for food fortification and dietary supplements.

KEYWORDS: Soluble ferric pyrophosphate; ferritin; ascorbic acid; pH; divalent metal ions; Caco-2 cells; iron bioavailability

INTRODUCTION

Iron deficiency affects one-third of the world's population (*1*, *2*). Poor bioavailability of dietary nonheme iron is considered a major factor contributing to the problem (*3*). Many different forms of iron ranging from iron salts to iron chelates to elemental iron powders have been approved as iron sources for food fortification (*4*). In many cases, the efficacy of these fortificants is strongly influenced by iron absorption enhancers and inhibitors in the diet (*5*, *6*). For example, ascorbic acid (AA) can reduce Fe^{3+} to the more soluble Fe^{2+} and, thus, is a potent enhancer of nonheme iron absorption (*7*, *8*). On the other hand, polyphenols, such as phytic acid and tannins, found in plant-based diets inhibit iron bioavailability by forming stable iron chelates prior to or during epithelial uptake, thereby preventing the iron from passing through intestinal epithelial cells (*9*−*11*).

Soluble ferric pyrophosphate (SFP) is a ferric iron chelate, which was used in the United States as a hematinic in the early 1900s but is no longer prescribed for treatment of anemia. Recently, related research on iron compounds showed that SFP is uniquely compatible with lipid emulsions and may be a well-tolerated, bioavailable source of iron intravenously when administered as an additive to hemodialysates and total parenteral nutition admixtures (*12*). The iron core of SFP is tightly chelated to citrate and pyrophosphate ligands. Unlike the similarly named ferric pyrophosphate, which is essentially insoluble in aqueous solutions, SFP is very soluble in aqueous solutions over a wide range of pH (pH 2−8). Food-grade SFP is quite inexpensive and

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is allowed as an iron fortificant in Europe (*40*) but currently has no similar applications in the United States.

Iron chelation is known to be both a benefit and a concern in iron fortification (*13*, *14*). In the case of SFP, the shield of pyrophosphate and citrate ligands prevents direct exposure of the ionic iron to the food matrix and to cells and tissues. Therefore, SFP is likely less toxic than simple iron salts that lack this structural feature. On the other hand, chelating ligands have the potential to bind the iron so tightly that it is not sufficiently bioavailable for use by cells (*15*, *16*). The route of iron uptake has been studied extensively. It is well-established that the first step in the absorption of dietary iron is the transport of iron from the intestinal lumen into the enterocytes and that this transport involves a membrane protein called divalent metal transporter (DMT-1) (*17*−*19*). DMT-1 appears to be specific for divalent cations; therefore, Fe^{3+} must be reduced, possibly by a ferric reductase called Dcytb, to Fe^{2+} before being transported into the cell (*20*, *21*). However, it is still unclear whether iron in chelated forms is transported across the intestinal mucosal barrier via the DMT-1 mechanism and whether iron is released from its chelating ligand prior to or during absorption (*22*−*24*).

In the present study, Caco-2 cells were used to evaluate iron uptake from SFP under various experimental conditions. The Caco-2 cell line is a human adenocarcinoma cell line that has proven to be a useful model for studying iron bioavailability from foods and iron fortificants (*25*, *26*). The cells differentiate into polarized monolayers with characteristics such as a brush border membrane containing the enzymes present in normal absorptive epithelial enterocytes. Ferritin formation by Caco-2 cells following exposure to an iron source is used as a marker for iron uptake (*27*, *28*).

The specific aims of the present study were to assess the bioavailability of SFP and probe the mechanisms of cellular uptake of SFP. These aims were achieved by addressing the following questions: (1) How does the bioavailability of SFP compare to that of conventional Fe fortificants, such as $FeSO_4$, NaFeEDTA, and ferrous bisglycinate (Ferrochel)? (2) What effect does solution pH have on the bioavailability of SFP as compared to the bioavailability of other Fe sources? (3) To what extent is the bioavailability of SFP enhanced by ascorbic acid, cysteine, or citrate or inhibited by phytate or polyphenols? (4) How is the bioavailability of SFP influenced by other divalent cations? (5) Does the incorporation of SFP in food matrices change its bioavailability?

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) unless stated otherwise. All reagents were prepared with ultrapure water (resistivity of 18.2 MΩ). Glassware and utensils were soaked in 10% HCl for no less than 4 h and rinsed with deionized water prior to use. Three test samples of SFP were characterized analytically and provided by BioLink Life Sciences, Inc. (Cary, NC) for Caco-2 cell studies. Three lots of SFP were evaluated: (1) SFP1, Lot BLS512305-SFPTC, iron content, 8.6% by weight (w/w); (2) SFP2, Lot BLS512426-SFPTC, iron content, 8.6% (w/w); (3) SFP3, Lot BLS511913-SFPDC, iron content, 10.2% (w/w).

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) at passage 17 and used in experiments at passages 29−35. Cells were seeded at a density of 50 000 cells/cm^2 in collagen-treated 24-well plates (Costar Corp., Cambridge, MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37 °C in an incubator with a 5% CO_2 and 95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 1% antibiotic/antimycotic solution, 25 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 10% fetal bovine serum. A total of 2 days before the experiment, the growth medium was removed from each culture well, and the cell layer was washed and maintained with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/L piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 1% antibiotic/antimycotic solution, 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 34 μg/L triiodothyronine, and 20 μg/L epidermal growth factor. This enriched MEM contained less than 80 μg of iron/L. All ingredients and supplements for cell culture media were obtained from GIBCO (Rockville, MD). Iron uptake experiments were conducted 13 days post-seeding.

Protocol for Direct Application of Samples on Caco-2 Cell Monolayers. Caco-2 cells were seeded and maintained as described above. Solutions of the SFP samples with corresponding treatments were mixed with MEM and placed directly on the cell monolayers. The final concentration of all iron fortificant solutions was 20 μmol/L, unless stated otherwise. The treated Caco-2 cells were incubated for about 20 h before harvesting. Cellular ferritin and total protein were then analyzed and compared among all treatments. This protocol was used in experiments 1−4 described in the Results.

Protocol for Combined *in Vitro* Digestion/Caco-2 Cell Culture. The method of Glahn et al. (*25*) was used with slight modification. Briefly, the pH of each sample was adjusted to pH 2.0 with 5.0 mol/L HCl. An aliquot of pepsin solution was added at the concentration of 0.5 mL of pepsin/10 mL of sample. This mixture was placed on a rocking shaker in an incubator set at 37 °C for 1 h (55 oscillations/min). The pH of the sample was raised to pH 5.8 with 1 mol/L $NaHCO_3$ before the addition of pancreatin-bile extract (2.5/10 mL sample). The pH was then adjusted to pH 7.0 with NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl and 5 mmol/L KCl. The growth medium was removed from each cell well and replaced with a fresh 1 mL aliquot of MEM. A sterilized insert ring, fitted with a dialysis membrane, was then inserted into the well, thus creating a two-chamber system. A 2.0 mL aliquot of the intestinal digest was pipetted into the upper chamber. The plate was returned to the incubator and incubated for 120 min with 6 oscillations/min rocking speed. When the intestinal digestion was terminated, the insert ring and digest were removed. The solution in the bottom chamber was allowed to remain on the cell monolayer, and an additional 1 mL of MEM was added to each well. The cell culture plate was then returned to the incubator for an additional 20 h, after which the cells were harvested for analysis. This protocol was used in experiment 5 described in the Results.

Harvesting of Caco-2 Cells for Ferritin Analysis. The procedures used in the determination of ferritin and total protein contents of Caco-2 cells were similar to those described by Etcheverry et al. (*27*, *29*). Briefly, growth medium was first removed from the culture well by aspiration, and the cells were washed twice with a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES at pH 6.8. The cells were harvested by adding an aliquot of deionized water and placing in a sonicator (Lab-line Instruments, Melrose Park, IL) at 4 °C for 15 min. Cells were frozen at −20 °C immediately after harvest until later analysis. The ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular iron concentration. Therefore, the ratio of ferritin/total protein, expressed as nanograms of ferritin/milligram of protein, was used as an index of the cellular iron uptake.

Statistical Analysis. Each treatment was performed in triplicate. Data were tested for normality and analyzed by analysis of variation (ANOVA) with Tukey's paired comparison using Minitab Release 15 software (Minitab, Inc., State College, PA), depending upon the specific test needed. In all comparisons, significant differences were defined at $p < 0.05$.

RESULTS

All experiments included a set of "blanks", in which the baseline ferritin formation was assessed using harvested untreated Caco-2 cells that were incubated with fresh MEM for the same duration in each experiment. This value was about 2 ng

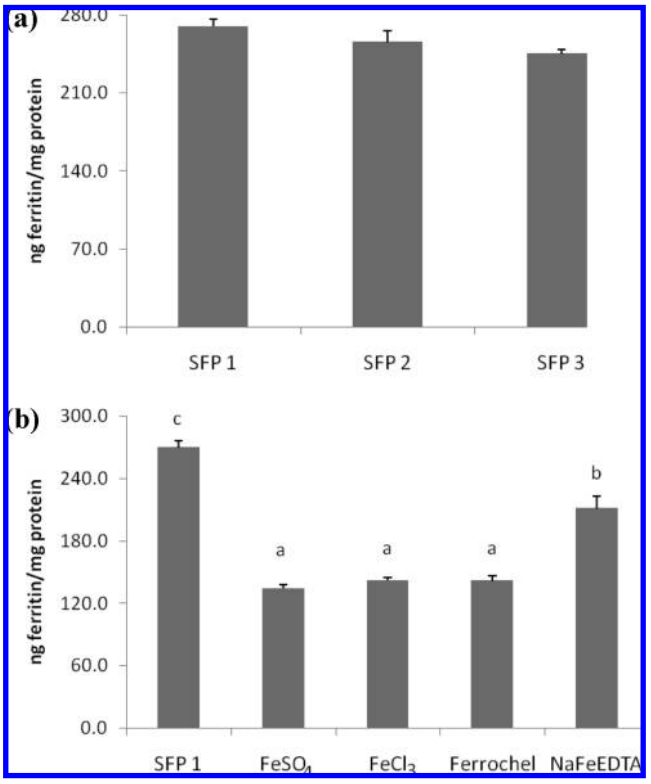

Figure 1. Bioavailability of iron fortificants. In each experiment, the final concentration of iron was 20 μmol/L. Two independent experiments were performed on separate days using cells from different batches with either four or three replicates; data collected from the same treatment were pooled. (a) Iron uptake from SFP samples collected from three production lots. No significant differences were found among the samples, and SFP1 was selected randomly to represent all SFPS in the ensuing experiments. (b) Bioavailability of SFP in comparison to other iron salts or chelates. Different letters on bars indicate significant differences ($p < 0.05$) using one-way ANOVA. Each column represents the mean ferritin formation value + standard error of the mean (SEM) ($n = 7$).

of ferritin/mg of protein and was consistent throughout the study. Because of limitations in the scale of each graph, this baseline value is only shown in **Figure 3b**.

Experiment 1: Bioavailability of SFP in Caco-2 Cells. The bioavailability of each of the three SFP samples was assessed (**Figure 1a**). No significant differences in ferritin formation were found in the three samples ($p = 0.084$). As a result, SFP1 was randomly selected to represent all SFPs in the ensuing experiments (experiments 1−5). The bioavailability of SFP1 was then compared to that of conventional Fe compounds or chelates, such as ferrous sulfate ($FeSO_4$), ferric chloride ($FeCl_3$), ferrous bisglycinate (Ferrochel), and sodium iron(III) ethylenediaminetetraacetate (NaFeEDTA) (**Figure 1b**). Ferritin formation in cells exposed to SFP1 was twice the amount of that in cells treated with simple iron salts, such as $FeSO_4$ ($p < 0.001$). NaFeEDTA also induced 1.6-fold higher ferritin formation in Caco-2 cells compared to $FeSO_4$.

Experiment 2: SFP Bioavailability at Selected Acidic and/or Neutral pH Levels. The bioavailability of SFP was compared to that of $FeSO_4$ and NaFeEDTA with or without a pretreatment of acidification and neutralization (**Figure 2**). This process mimics pH changes during digestion and was used to test the stability of an iron compound or chelate across a wide range of physiological pH conditions along the gastrointestinal (GI) tract. The SFP solution was cloudy when first dissolved at pH 2 and turned clear when pH was gradually adjusted to 7; however, the color of

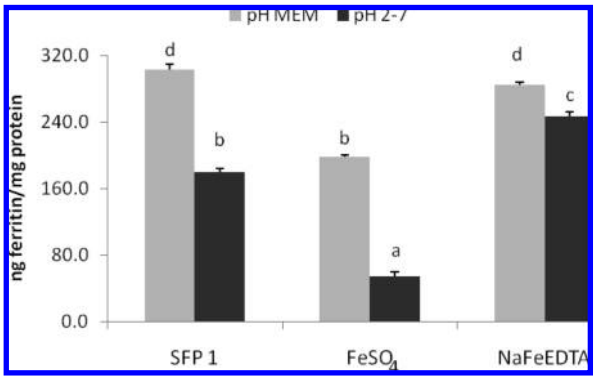

Figure 2. Bioavailability of SFP was compared to $FeSO_4$ and NaFeEDTA at two pH conditions. [Lighter bars ("pH MEM")] Iron sources were directly added to fresh MEM (pH 7.0), mixed, and applied on cells. [Darker bars ("pH 2−7")] Iron sources were first dissolved in pH 2 buffer and incubated on a rocking shaker (55 oscillations/min) for 1 h. The pH of these solutions was then gradually adjusted to 7.0 with NaOH before being mixed with fresh MEM and placed directly on cells. In either case, the final concentration of iron from all three sources was 20 μmol/L. Bars with no letters in common are significantly different (one-way ANOVA, $p < 0.05$). Bars represent ferritin formation values + SEM ($n = 5$).

the solution was darker than if dissolved at pH 7 (indicated as "pH MEM"). As shown in **Figure 2**, in general, adjusting the pH from 2 to 7 significantly lowered the bioavailability of SFP1, $FeSO_4$, and NaFeEDTA by 40.6, 72.6, and 13.4%, respectively. However, chelated forms of iron sources (SFP and NaFeEDTA) were less affected by the change of pH than iron in $FeSO_4$.

Experiment 3: Effects of Iron Absorption Enhancers and Inhibitors on SFP Bioavailability. Solutions of SFP or $FeCl_3$ were exposed to nonheme iron absorption enhancers, such as ascorbic acid (AA), cysteine (Cys), or citrate (Cit) (**Figure 3a**), or inhibitors, such as phytate (Phy) and tannic acid (Tan) (**Figure 3b**). In the presence of AA, ferritin formation increased 3.2- and 3.6-fold in cells treated with SFP1 and $FeCl_3$, respectively ($p < 0.001$ for both increases). In the presence of Cys, ferritin formation increased 2.2-fold in both SFP- and $FeCl_3$-treated cells ($p < 0.001$). When added at the same ratio (iron/enhancer = 1:20), AA was a more effective enhancer for the already highly bioavailable SFP1 than cysteine. Citrate at this ratio significantly enhanced $FeCl_3$ bioavailability but had no significant effect on SFP. Adding phytate at Fe/Phy = 1:10 or tannic acid at Fe/Tan = 1:1 molar ratio greatly and significantly inhibited iron uptake from both iron sources. Phytate decreased ferritin formation from SFP1 and $FeCl_3$ by 91 and 94%, respectively; and tannic acid caused a 99% decrease in ferritin formation from SFP1 and $FeCl_3$.

Experiment 4: Effects of Selected Divalent Cations on the Bioavailability of SFP. The bioavailability of SFP was assessed when divalent cations, such as Ca^{2+}, Mg^{2+}, or Zn^{2+}, were added to the SFP solution (**Figure 4**). These divalent cations were selected because individuals who are vulnerable to Fe deficiency are also likely to be vulnerable to Zn deficiency. Likewise, people who are vulnerable to Fe deficiency and have advanced renal disease use calcium and magnesium salts as phosphate binders. In addition, it is known that DMT-1 is not a specific transporter for ferrous ion (*2*, *30*). Many divalent metal cations, including Ca^{2+}, Mg^{2+}, and Zn^{2+}, can be taken up via DMT-1 and, therefore, can competitively inhibit ferrous iron uptake by this transporter (*30*). As the data in **Figure 4** show, when the effects of Zn, Ca, and Mg are compared, adding Zn (Fe/Zn = 1:1 molar) inhibited ferritin formation from SFP1 and $FeCl_3$ the most (95 and 98%, respectively). The inhibitory effect of Ca (Fe/Ca = 1:140 molar) on iron absorption was less than Zn but also significant, with a decrease

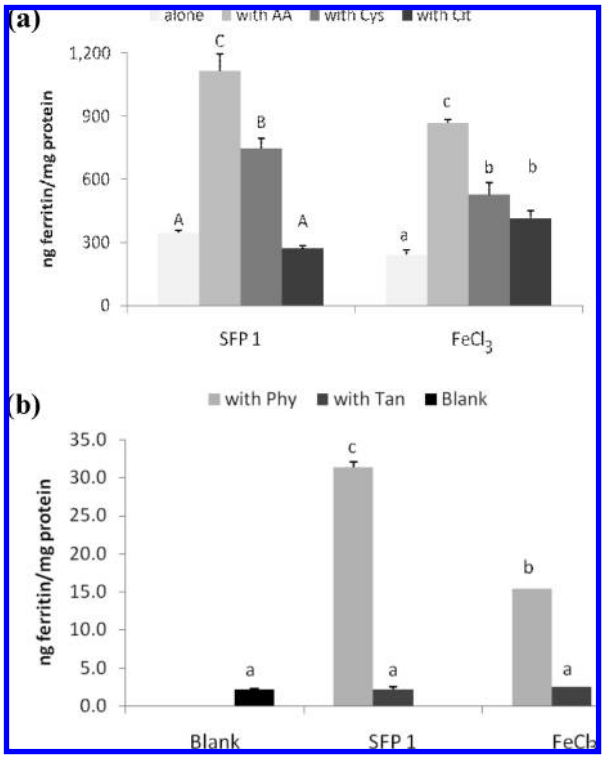

Figure 3. Ferritin response from SFP1 or $FeCl_3$, in the presence or absence of ascorbic acid (AA), cysteine (Cys), citrate (Cit), phytate (Phy), or tannic acid (Tan). The final concentration of iron from both sources was 20 μmol/L. The final concentration of each absorption enhancer or inhibitor was chosen based on papers by Glahn et al. (*30*, *37*). Specifically, the final concentration of AA was 400 μmol/L (Fe/AA = 1:20); the final concentration of cysteine was 400 μmol/L (Fe/Cys = 1:20); the final concentration of citrate was 400 μmol/L (Fe/Cit = 1:20); the final concentration of phytate was 200 μmol/L (Fe/Phy = 1:10); and the final concentration of tannic acid was 20 μmol/L (Fe/Tan = 1:1). Significant differences were determined as $p < 0.05$ (one-way ANOVA). In panel a, within each category, bars with no letters in common are significantly different and no comparison between SFP and $FeCl_3$ was performed. In panel b, across the three categories, bars with no letters in common are significantly different. Values represent the means of ferritin formation + SEM ($n = 3$).

of 61 and 36% in ferritin formation from SFP1 and $FeCl_3$, respectively. The effect of Mg (Fe/Mg = 1:80 molar) was the least, inhibiting ferritin formation from SFP1 by 17.6%.

Experiment 5: Effects of Selected Food Matrices on Fe Bioavailability Using an *in Vitro* Digestion/Caco-2 Model. The bioavailability of SFP1 was compared to that of $FeCl_3$ in the presence of rice (**Figure 5a**), and the bioavailability of SFP1 was compared to that of $FeSO_4$ and NaFeEDTA in the presence of nonfat dry milk (NFDM) (**Figure 5b**). Rice was chosen to expose SFP to foods containing ingredients such as phytate that could interfere with iron uptake. NFDM was chosen because of the interest in using SFP as an iron fortificant in formula and infant foods. The combined *in vitro* digestion/Caco-2 cell culture protocol was used to mimic digestion in the GI tract. As shown in **Figure 5a**, rice alone provided little bioavailable iron. Adding SFP1 or $FeCl_3$ significantly increased ferritin formation by 13.4- and 9.4-fold, respectively. Adding AA to the rice and iron mixture further enhanced ferritin formation by another 3.4-fold in the case of SFP1 and 6.8-fold in the case of $FeCl_3$. As shown in **Figure 5b**, the iron-free NFDM alone was shown to be a poor source of bioavailable iron. Adding SFP1 or $FeSO_4$ significantly increased ferritin formation, and the magnitude of increase was similar.

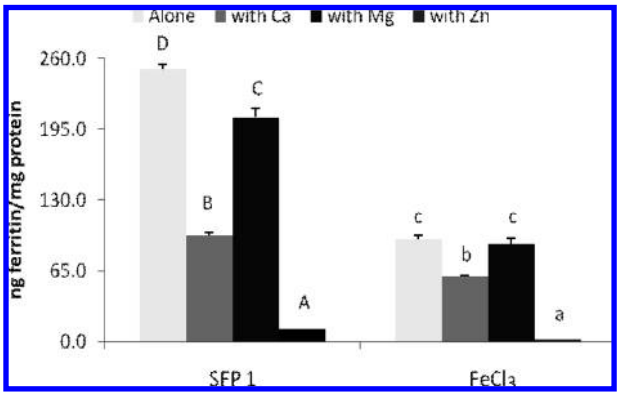

Figure 4. Ferritin formation from SFP1 or $FeCl_3$, in the presence or absence of three cations: Ca, Mg, and Zn. The final concentration of iron from SFP or $FeCl_3$ was 20 μmol/L. The concentrations of other divalent cations were chosen based on the recommended daily allowances (RDAs) for the various minerals (*38*) and a paper by Glahn et al. (*30*): (1) RDA for Ca (individual aged 19−50 years) is 1000 mg or 25 mmol, (2) RDA for Mg (adults) is 350 mg (average) or 14.6 mmol, (3) RDA for Zn (adults) is 10 mg (average) or 0.154 mmol, and (4) RDA for Fe (adults) is 10 mg (average) or 0.18 mmol. On these bases, in these experiments, the final concentration of calcium (Ca) was 2800 μmol/L (Fe/Ca = 1:140); the final concentration of magnesium (Mg) was 1600 μmol/L (Fe/Mg = 1:80); and the final concentration of zinc (Zn) was 20 μmol/L (Fe/Zn = 1:1). In each category, bars with no letters in common are significantly different (one-way ANOVA, $n = 4$, $p < 0.001$). Columns between SFP1 and $FeCl_3$ were not compared.

Ferritin formation from Caco-2 cells treated with NaFeEDTA-enriched NFDM was also significantly higher than the NFDM alone, but the magnitude of increase was only about 60% of the SFP-enriched ones.

DISCUSSION

SFP exhibited higher bioavailability than simple iron salts, such as $FeSO_4$ and $FeCl_3$. The bioavailability of SFP may be related to its iron core being shielded by the surrounding pyrophosphate and citrate ligands from being in direct contact with iron absorption inhibitors or competitors. On the other hand, these ligands may provide iron as a relatively low-molecular-weight entity that readily interacts with biological iron receptors, because polymeric iron compounds have significantly slower iron-transfer kinetics. The data indicate that the ligands do not present a physical barrier or chelate iron with sufficiently high affinity to inhibit iron uptake by the cells.

Changing pH may affect compound solubility as well as structural stability, which in turn can affect iron bioavailability. The chelating ligands protect the iron from binding with water, thereby decreasing the formation of ferric hydroxides and increasing the solubility and bioavailability of iron. The SFP solution was cloudy at pH 2 but turned to a darker, clear solution at pH 7, indicating a change of structure, although the bioavailability of SFP did not seem to be affected by this change. The mechanism involved in this observation is unclear. We hypothesize that the loosely chelated citrate ligands may rearrange during the shift of pH.

The bioavailability of SFP and $FeCl_3$ was affected similarly in the presence of iron absorption enhancers, such as AA and cysteine, and inhibitors, such as phytate and tannic acid, suggesting that the ferric iron from SFP is reduced to ferrous iron, which dissociates from the pyrophosphate and citrate ligands and is taken up as an ionic form of iron at the brush border site in the small intestine. This dissociation of ligands may be caused by the

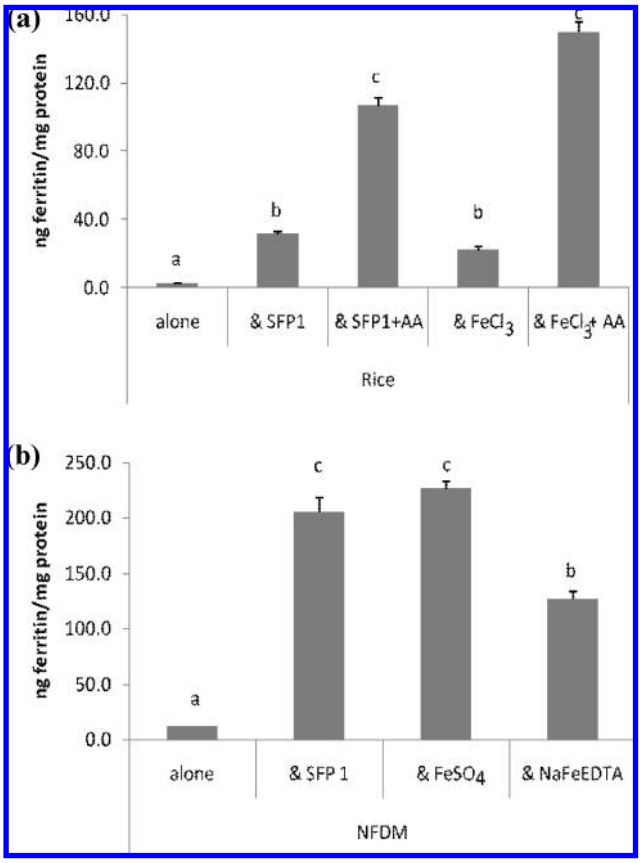

Figure 5. Effect of food matrices on iron bioavailability from various iron sources, assessed by an *in vitro* digestion/Caco-2 cell model. (a) Rice was added as Nshiki Rice reference, which had been cooked, freeze-dried, and then ground into fine powder. This rice powder had been determined to contain 2.7 ppm Fe and 2.86 μmol of phytate/g of rice (*39*). During *in vitro* digestion, 1 g rice was added to each treatment prior to pepsin digestion. The final concentration of iron (in the upper chamber) was 50 μmol/L, and if present, the final concentration of AA in the upper chamber was 1000 μmol/L (Fe/AA = 1:20). (b) In this experiment, 0.274 g of NFDM was added to each treatment prior to pepsin digestion. The final concentration of iron (in the upper chamber) was 100 μmol/L. In both panels, bars with no letters in common are significantly different (One-way ANOVA, $p < 0.001$). Values are mean ferritin formation + SEM ($n = 3$).

change of pH or by competitive bindings of other ingredients in food (*31*). The effect of citrate on iron absorption has been studied with mixed results (*15*, *32*). When added at a ratio of 20:1 (Cit/Fe), citrate may solubilize unprotected ferric ions in $FeCl_3$, thereby enabling transport via DMT-1 and possibly functions as an uptake enhancer. It is possible that the ratio of Cit/Fe in SFP is already at its optimum, because we observed that further addition of citrate did not affect iron uptake from SFP.

The bioavailability of SFP and $FeCl_3$ was affected similarly in the presence of other divalent metal ions. This finding, in conjunction with the findings in experiment 3, supports our hypothesis that iron from SFP dissociates from the chelating ligands prior to or during intestinal uptake, is reduced by DcytB, and is taken up via DMT-1. The addition of Zn ions inhibited ferritin formation the most, indicating that Fe and Zn directly compete for an uptake pathway, namely, the DMT-1 route. Ca and Mg only partially inhibited iron uptake, possibly because they can be taken up by several pathways (*33*−*36*), making DMT-1 less critically involved in their uptake and transport.

The bioavailability of nonheme iron can be greatly influenced and is usually decreased by incorporation into foods. This is seen in data from experiment 5 in comparison to those from experiment 1. For example, the bioavailability of SFP without rice (experiment 1, around 260 ng of ferritin/mg of protein) is much higher than that with rice (experiment 5, around 30 ng of ferritin/mg of protein). Adding AA to this diet further enhances iron bioavailability; however, values are still much lower than those from cells treated without rice. NFDM, on the other hand, is a different food matrix. Specifically, NFDM does not have prominent iron absorption inhibitors, such as the phytate present in rice, but it contains Ca (300 mg of Ca/23 g of NFDM), which may partially compete with iron for uptake as discussed in experiment 4. Adding SFP alone to NFDM brought ferritin formation levels to about 200 ng of ferritin/mg of protein, suggesting good bioavailability of SFP in dairy foods. However, this level is still lower than the ferritin formation of SFP without food (**Figure 1**), which may be due to the competition from Ca uptake. The bioavailability of SFP in NFDM was also significantly higher than that of NaFeEDTA, suggesting that chelating agents differ in their effect on iron bioavailability, especially in the presence of food. This may indicate an advantage of using SFP in fortifying milk protein-based products.

In conclusion, iron bioavailability of SFP to Caco-2 cells was as high as or higher than that of simple iron salts, such as $FeCl_3$ and $FeSO_4$. SFP is a promising agent for use in food fortification and especially in dairy or lipid-based fortification. *In vivo* studies designed to probe the mechanisms involved in the absorption and use of SFP iron as well as sensory and food-processing aspects of SFP applications are warranted.

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