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Meat and Ascorbic Acid Can Promote Fe Availability from Fe–Phytate but Not from Fe–Tannic Acid Complexes

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This study utilized an in vitro digestion/Caco-2 cell model to determine the levels of ascorbic acid (AA) and "meat factor" needed to promote Fe absorption from Fe complexed with phytic acid (PA) or tannic acid (TA). AA reversed the inhibition of Fe absorption by PA beginning at a molar ratio of 1:20:1 (Fe:PA:AA) but essentially had no effect on the Fe complexed with TA. Fish also reversed the inhibition of Fe uptake by PA but not by TA. TA and fish decreased total Fe solubility. Iron in the presence of PA was highly soluble. AA, but not fish, increased the percentage of soluble Fe as Fe^{2+} in the presence of both inhibitors. The results indicate that monoferric phytate is a form of Fe that can be available for absorption in the presence of uptake promoters. In contrast, a TA–Fe complex is much less soluble and unavailable in the presence of promoters.

KEYWORDS: Phytic acid; tannic acid; iron bioavailability; in vitro digestion; Caco-2

INTRODUCTION

Plant-based foods derived from mature legume seeds and whole cereal grains often contain high levels of potent inhibitors of nonheme Fe absorption, such as phytic acid (PA) and phenolic compounds (*1*). As a result, vegetarians and populations consuming mainly plant-based diets are at risk of developing Fe deficiency.

Development of strategies to increase the amount of bio-available Fe from staple food crops, i.e., biofortification, could prove to be an effective alternative to Fe supplementation or fortification of processed foods. Biofortification should be a more sustainable approach to improving Fe status and preventing Fe deficiency in developing countries as well as some Western nations; however, there are some serious obstacles to overcome for this approach to be effective (*2*). Breeding crops with higher Fe levels in their edible portions may not improve their quality as an Fe source, as analytical and in vitro evidence indicates that inhibitors, such as PA, are in considerable molar excess relative to Fe and may increase proportionately with Fe content (*3*). The alternative strategy of breeding to lower the levels of inhibitors may contribute to the risk of chronic disease, as PA is linked to various anticancer effects and other health benefits (*4*). Polyphenolic compounds are potent antioxidants and, as such, are also linked to prevention of many forms of cancer as well as heart disease (*5, 6*). Furthermore, lowering phytate significantly in staple food crops may lead to decreased crop yield (*7*). Seeds with significantly reduced phytate levels may also result in increased risk of mycotoxin formation during seed storage (*8*).

The form of inositol phosphate most common in staple food crops is inositol hexaphosphate (IP6). Significant amounts of inositol pentaphosphate (IP5) can also be present (*9, 10*). Although lesser-phosphorylated forms of inositol, such as inositol triphosphates and inositol tetraphosphates (IP3 and IP4), may inhibit Fe absorption (*11*), IP5 and IP6 are the primary forms of interest in staple crops.

PA in foods such as wheat complexes Fe through its phosphate ester groups and is considered to be a major inhibitor of Fe bioavailability (*12*). Monoferric phytate comprises the majority of seed Fe in cereal crops such as wheat, rice, and maize and is both soluble and bioavailable to rats, dogs, and humans (*13–16*). In all three species, Fe as monoferric phytate appears to be absorbed to the same extent as unbound nonheme dietary Fe, using both $FeCl_3$ or $FeSO_4$ as an Fe source. However, it is important to note that in these studies the molar ratio of phytate to Fe was much lower than that typical of most plant foods. In vitro and in vivo observations indicate that molar ratios of phytate to Fe similar to those observed in staple food crops (approximately 1:20 Fe:phytate) provide soluble Fe that is of low availability, presumably because the molar excess of PA complexes the Fe and limits interaction of the Fe with the brush border surface (*17, 18*). Given the above in vivo and in vitro observations, it is relevant to investigate how solubility and bioavailability of monoferric phytate change in the presence of whole foods and meal conditions and to quantify the amount of promoting factors needed to improve Fe availability. Overall, it appears to be incorrect to simply say that monoferric phytate is a poorly available form of Fe, as there are other contributing factors in the food matrix.

The strong inhibitory effects of polyphenolic compounds on Fe absorption have been documented in both human and in vitro studies (*1, 17*). Tannic acid (TA) is a hydrolyzable tannin that

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binds Fe in very stable complexes (*19*). This property may be related to its content of galloyl groups (*20*) and/or hydroxyl groups (*21*); however, the exact mechanism whereby polyphenolics inhibit Fe absorption by intestinal cells is not known.

Two major promoters of nonheme Fe absorption are ascorbic acid (AA) and meat (*18*). AA promotes Fe uptake by reducing ferric Fe to ferrous Fe, as the Fe^{2+} ion is highly bioavailable for uptake at the physiological conditions of the small intestine. Meats (i.e., cooked animal tissue) such as fish, chicken, and beef are also known to promote nonheme Fe availability. This enhancing effect is known as the "meat factor", although the precise mechanism by which meat enhances nonheme Fe bioavailability is not fully understood. Many research efforts have focused on the potential promotional effects of proteins; however, no specific Fe uptake enhancing protein or peptide has been isolated and identified (*22–24*). Recent research using cell cultures indicates that the meat factor effect may be the result, in part, of specific sulfated glycosaminoglycan carbohydrates present in the muscle tissue (*25*).

Several studies indicate that it is possible to overcome phytate and polyphenolic inhibition of Fe absorption by addition of reasonable levels of absorption enhancers (i.e., promoters). South and Miller reported that Fe binding by TA could be reduced by addition of AA in vitro, although this effect was dependent on the sequence of addition (*19*). Several human studies have also provided evidence that the effects of phytate and polyphenolics can be at least partially overcome by the addition of AA and meat (*18*, *26*, *27*).

The present study is part of the HarvestPlus biofortification program, which includes the major objective of breeding staple food crops with significant levels of bioavailable Fe in their edible portions. Part of HarvestPlus's core research plan is to utilize an in vitro digestion/Caco-2 cell model to determine the levels of Fe absorption promoters needed to overcome the levels of inhibitors present in staple food crops (*28*). As a secondary effort, this in vitro model was used in the present study in specific hypothesis testing of the mechanisms by which phytate and polyphenolics inhibit Fe absorption.

This study represents the initial efforts to characterize (using an in vitro Caco-2 cell model) the mechanisms of inhibition of Fe absorption by PA and polyphenolic compounds and to determine the levels of promoters required to offset their inhibitory effects on Fe bioavailability from staple food crops in the diet. This in vitro research is an important first step toward laying a foundation for understanding the more complex interactions that occur during absorption of Fe from a complete diet matrix.

MATERIALS AND METHODS

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

Experimental Design. Four sets of experiments were conducted. The in vitro digestion/Caco-2 cell culture assay was used to assess Fe bioavailability (*29*). Iron solubility and oxidation state [i.e., Fe(II) and Fe(III)] were also determined.

Experiment 1. The objective of this experiment was to determine the minimum ratio of AA to PA necessary to promote Fe uptake in the presence of PA. A constant 1:20 Fe:PA molar ratio was maintained among digests, as this is a ratio commonly observed in most staple crops. AA was added to achieve Fe:AA ratios of 1: 0, 1, 5, 10, 20, 40, and 100. Soluble ferrous Fe and total soluble Fe were measured for all of the above conditions in digests identical to those used for cell Fe uptake experiments.

Experiment 2. The objective of this experiment was to determine the minimal level of AA necessary to promote Fe uptake in the presence

of TA. A constant 1:1 Fe:TA molar ratio was maintained. AA was added to achieve Fe:AA ratios of 1: 0, 1, 20, 100, 500, and 1000. Soluble ferrous Fe and total soluble Fe were measured for all of the above conditions in digests identical to those used for cell Fe uptake experiments.

Experiment 3. The objective of this experiment was to determine the minimum amount of fish (i.e., meat factor) required to promote Fe absorption in the presence of PA and TA. Samples containing a constant level of either PA (20 μmol/15 mL) or TA (1 μmol/15 mL) received 0, 0.5, or 1 g of lyophilized fish powder. Soluble ferrous Fe and total soluble Fe were measured for all of the above conditions in samples taken directly from digests prepared for iron availability studies.

Experiment 4. The objective of this experiment was to compare the inhibitory effects of PA and TA on Fe absorption using either $FeCl_3$ or $FeSO_4$ as the Fe source. Molar ratios of Fe:PA (1: 0, 1, 5, 10, and 20 Fe:PA) and Fe:TA (1: 0, 1, 5, 10, and 20 Fe:TA) were prepared while maintaining a concentration of 50 μmol/L of either $FeCl_3$ or $FeSO_4$.

Iron Uptake Experimental Design. Six independent replications were performed in all experiments. All samples were formulated to contain a total Fe concentration of 67 μmol/L (i.e., 1 μmol Fe/15 mL of sample mixture). Each experiment included a control treatment of $FeCl_3$ alone as well as a positive control reference treatment containing $FeCl_3$ combined with AA at low pH to produce a 1:20 molar ratio of Fe to ascorbate. Inserts containing only minimal essential medium (MEM) were used as a quality control to monitor cell baseline ferritin content, which ranged from 8.3 to 19.7 ng ferritin/mg cell protein.

Iron Uptake Sample Preparations. Stock solutions of $FeCl_3$ of 1 μmol/250 μL (4 mmol/L) (experiments 1–3) and 0.1 μmol/250 μL (0.4 mmol/L) (experiment 3) were prepared by dilution of a 1.04 g Fe/L (18.6 M) solution in 0.27 mol/L HCl (Sigma #1-9011). All PA and TA stock solutions were prepared by dissolution in water at concentrations of 20 μmol/250 μL (80 mmol/L) and 1 μmol/250 μL (4 mmol/L), respectively.

Ascorbate was prepared in water immediately prior to each experiment to obtain a 100 μmol/500 μL (200 mmol/L) solution (experiments 1 and 2) and was shielded from light by covering with aluminum foil to reduce photooxidation of the ascorbate.

Fish muscle tissue was chosen as a source of the meat factor for experiment 3 because of its low intrinsic Fe content. Icelandic haddock fillets were purchased from a local supermarket (Wegman's, Ithaca, NY). Skin and visible fat were removed, and the fish was cut into 1.5–2.5 cm cubes. Beakers containing approximately 200 g of fish and 50 mL of water were heated in a microwave oven at the "high" setting for 1.5 min and then stirred briefly. They were then heated for an additional 1.5 min and stirred three more times during this interval. After cooling, the fish mixture was frozen overnight, lyophilized, and ground with a coffee grinder (KRUPS type 203, Medford, MA). The intrinsic Fe content of fish was determined to be 5.91 μg Fe/g fish.

For experiments 1 and 2, 250 μL each of stock solutions of Fe and either PA or TA was combined first. Then, AA was added second using varying amounts of stock AA solution. Differences in volume were accounted for by addition of an appropriate volume of water. In general, 500 μL of AA or water was added per tube.

To formulate the iron uptake solutions in experiment 3, 250 μL of $FeCl_3$ (0.4 mmol/L) was combined with 250 μL of PA or TA at approximately pH 2. The lyophilized fish powder was then weighed and added directly to appropriate tubes in the amounts of 0, 0.5, or 1 g. Each mixture then received an additional amount of 18.6 M Fe solution in 0.27 mol/L HCl. In other words, to maintain a constant Fe concentration among samples, varying amounts of Fe solution were added to tubes containing fish. For example, fish was estimated to contain 4 μg intrinsic Fe/g fish. Thus, tubes containing no fish received 5.6 + 50.4 μg of $FeCl_3$, tubes with 0.5 g of fish received 5.4 + 48.6 μg of $FeCl_3$, and tubes with 1 g of fish received 5.2 + 46.8 μg of $FeCl_3$. After PA or TA, Fe, and fish were combined, 10 mL of 140 mM NaCl, 5 mM KCl, pH 2, solution was added to tubes, initiating the in vitro digestion process.

Cell Cultures. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 29–32. Cells were seeded at a density of 50000

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cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's modified Eagle medium (DMEM,[3] Gibco, Grand Island, NY) with 10% v/v fetal bovine serum (FBS, Gibco) and 25 mmol/L HEPES (Sigma). Cells were maintained at 37 °C in an incubator with a 5% CO$_2$, 95% air atmosphere with constant humidity, and the medium was replaced every 2 days. Cells for each study were used 14 days postseeding. 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 (*29*). Briefly, iron uptake solutions and/or fish preparations were combined in 50 mL tubes as described above. 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 N 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). After incubation, the pH was raised to 5.5–6.0 with 1 M NaHCO$_3$ and 2.5 mL of 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 aliquot of the digest was placed into the upper chamber of a two-chambered system formed by placing well inserts fitted with 15000 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 12 oscillations/min). Following the 2 h incubation, the inserts were carefully removed and an additional 1 mL of MEM 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 all experiments under these conditions, the cell monolayers remained viable, healthy, intact, and attached to the surface of the plate. Because of volume requirements for pH adjustment of digests containing fish in experiment 3, the final volume of the intestinal digests following the in vitro digestion process was 17 mL. Aliquots of 1.7 mL instead of 1.5 mL were applied to cells in the Fe uptake portion of this study to maintain a constant Fe concentration.

Iron Assays. All glassware used in sample preparation and analyses was rinsed with 2.7 mol/L HCl and 18 MΩ deionized water before use. Caco-2 cell ferritin formation served as a marker of cell Fe uptake. The Caco-2 cell protein content of samples was measured, after solubilization in 0.5 mol/L NaOH, using a Bio-Rad DC protein assay kit, which is a commercial semimicroadaptation of the Lowry assay (Bio-Rad Laboratories, Hercules, CA). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Serum Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 μL 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 inductively coupled argon plasma emission spectrometer (ICAP model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA) after wet-ashing with HNO$_3$ and HClO$_4$.

Iron Solubility and Oxidation State Experimental Design. A colorimetric assay using ferrozine (5 μg/mL in water) as the colorimetric reagent was used to quantitate total soluble Fe and soluble ferrous Fe present in digests by spectrophotometric measurement at 562 nm (see discussion below for details of the assay). Six independent replicates of each sample digest were tested. For each replicate, four measurements were taken as follows: soluble Fe^{2+} and total soluble Fe at the beginning of the intestinal incubation period and soluble Fe^{2+} and total soluble Fe after a 2 h incubation at 37 °C and pH 7.

Iron Solubility and Oxidation State Procedures and Analyses. Procedures for soluble Fe determination consisted of a modified version of those reported by Kapsokefalou and Miller (*30*). Total soluble Fe and soluble ferrous Fe were measured at the start of the intestinal digestion period and at the end of the 2 h intestinal digestion period. For this assay, 1.5 mL aliquots of each digest were collected and centrifuged at 15000*g* for 10 min to remove insoluble Fe. Supernatant

aliquots of 200 μL from each sample were added to each of two microcuvettes. Of these, one received 100 μL of "nonreducing" solution (2.7 mol/L HCl), leaving soluble Fe^{2+} unaffected. The other received 100 μL of "reducing" solution (2.7 mol/L HCl, 0.15 mol/L hydroxylamine), thus converting all soluble Fe to the reduced Fe^{2+} form. To measure Fe^{2+} concentrations, 75 μL of ferrozine (5 mg/mL water) and 600 μL of HEPES (0.3 N, pH 9.9) were added to cuvettes containing the sample and either reducing or nonreducing solution. Absorbance at 562 nm was read after approximately 3 s.

Soluble Fe^{2+} values were measured within 30 min of centrifugation. Samples measured for total soluble Fe were incubated overnight in reducing solution to allow for reduction of all ferric Fe. After the addition of 75 μL of ferrozine, samples were incubated for an additional 24 h before measurement immediately following addition of 600 μL of HEPES. The iron concentration was determined by comparison to Fe standards prepared using 1040 μg/mL FeCl$_3$ (18.6 mol/L) solution in 0.27 mol/L HCl.

Statistical Analyses. Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). Analysis of variance with Tukey's post-test was used to compare differences among means. Unpaired *t*-tests were used to compare iron solubility before and after the 2 h incubation. Where appropriate, data were transformed in order to achieve equal sample variances. Differences among means were considered significant at $P \leq 0.05$.

RESULTS

In general, levels of total soluble Fe and soluble Fe^{2+} measured before and after the 2 h intestinal digestion period were not significantly different; therefore, only postdigestion solubility results are presented in **Figures 1–3**.

The total soluble Fe ranged from approximately 90–100% throughout the intestinal digestion period (**Figure 1A**). Thus, the increasing amounts of AA had a minimal effect on total Fe solubility but significantly increased the percentage of Fe as soluble Fe^{2+}, resulting in approximately 85–90% of the total soluble Fe being in the ferrous form throughout the intestinal digestion period.

The 1:20 molar ratio of FeCl$_3$ to PA decreased Caco-2 cell ferritin formation by 91% in comparison to the control (**Figure 1C**). At this molar ratio of Fe:PA, an equimolar amount of AA (i.e., a 1:20:1 ratio of FeCl$_3$:PA:AA) increased cell ferritin formation by 180%. Additional AA increased cell ferritin formation, but the effect was maximal at a 1:20:10 molar ratio of FeCl$_3$:PA:AA. Clearly, the AA was able to partially reverse the effects of phytate inhibition under these conditions.

Total soluble Fe was significantly reduced in the presence of TA (**Figure 2A**). The addition of AA to digests of TA did not change the total soluble Fe but converted most of the soluble Fe in the digest to the Fe^{2+} oxidation state.

Caco-2 cell ferritin formation was significantly inhibited by 98% as compared to the control in the presence of a 1:1 ratio of Fe to TA (**Figure 2C**). An increase in ferritin formation in response to increasing levels of AA was only observed at a ratio of 1:1:1000 Fe:TA:AA.

Digests in experiment 3 were formulated to contain an Fe concentration of 59 μmol/L (3.29 μg/mL) because of volume requirements for pH adjustment of the digests containing fish. As in the previous experiments, Fe was highly soluble with only PA present in the digest, whereas approximately a 40% loss in Fe solubility was observed with the addition of TA (**Figure 3A**). In the presence of PA, this soluble Fe was entirely in the ferric form, whereas in the presence of TA a small amount of Fe (approximately 8–10% of the total) was in the Fe^{2+} oxidation state. The addition of fish to the digests decreased total Fe solubility by 25–30% in the digests with PA and completely eliminated soluble Fe in the digests with TA added (**Figure**
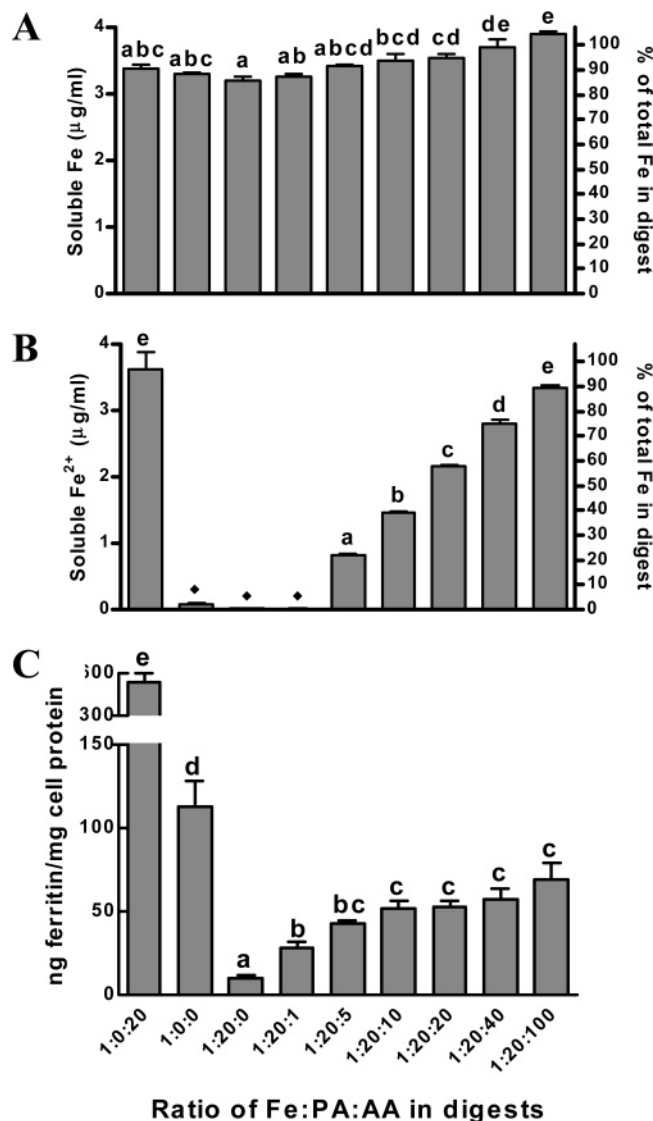
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Figure 1. (**A**) Total soluble Fe in digests, (**B**) soluble Fe^{2+} in digests, and (**C**) Caco-2 cell ferritin formation in response to digests. Digests contained a constant level of PA (1:20 Fe:PA) and increasing levels of AA. The digest iron concentration was 67 μmol/L. Values are means ± SEM. For solubility assays (**A** and **B**), values refer to analysis of digests after the 2 h intestinal digestion period. Bars with no letters in common are significantly different ($p < 0.05$). ◆ Represents an undetectable value. The absorbance of 0.02 ([Fe] = 0.137 μg/mL) was used as a cutoff point for reliable determination of iron concentration.

3A). The presence of 1 g of fish in the digests approximately doubled Fe uptake from the PA treatments (**Figure 3C**).

Iron absorption was maximally inhibited by PA at a molar ratio of 1:5 Fe:PA when $FeCl_3$ was used as an Fe source. Maximal inhibition occurred at 1:10 Fe:PA when Fe was added as Fe^{2+} (i.e., $FeSO_4$) (**Figure 4**). At the 1:1 Fe:PA molar ratio, ferritin formation was increased in the presence of Fe^{2+} supplied as $FeSO_4$ but decreased in the presence of Fe^{3+} as $FeCl_3$.

The inhibitory effect of TA at a 1:1 TA:Fe molar ratio was also attenuated when Fe was supplied as Fe^{2+} (i.e., $FeSO_4$). At higher levels of TA, Fe absorption was maximally inhibited regardless of the oxidation state of Fe present. The addition of equimolar amounts of AA and PA to digests containing $FeCl_3$ (PA:AA:Fe 20:20:1 and 10:10:1) increased Fe uptake to levels equivalent to that of digests containing a 1:1 molar ratio of Fe to PA (**Figure 4**).
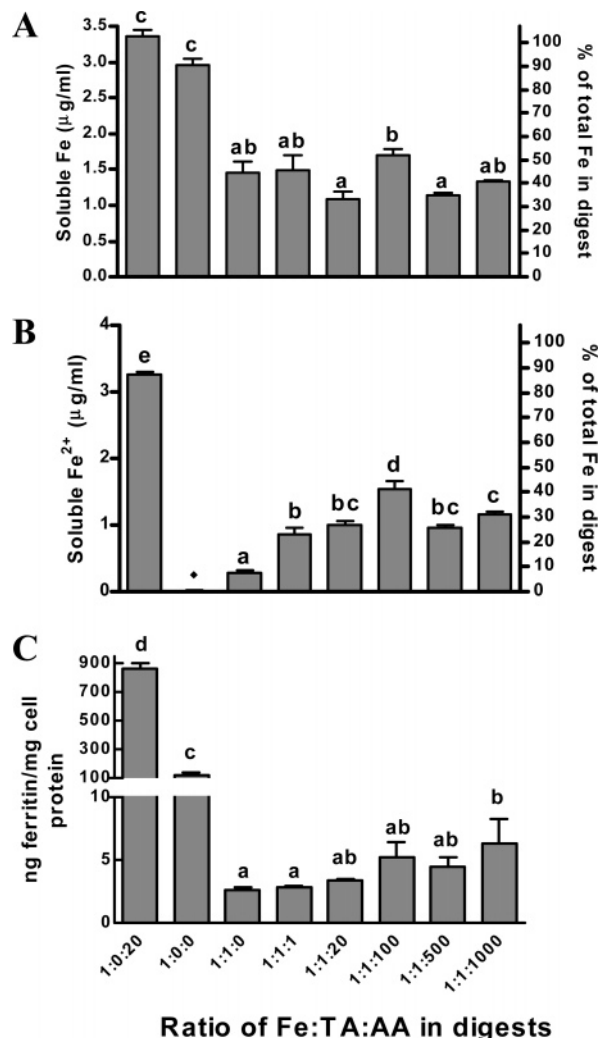

Figure 2. (**A**) Total soluble Fe in digests, (**B**) soluble Fe^{2+} in digests, and (**C**) Caco-2 cell ferritin formation in response to digests. Digests contained a constant level of TA (1:1 Fe:TA) and increasing levels of AA. The digest iron concentration was 67 μmol/L. Values are means ± SEM. For solubility assays (**A** and **B**), values refer to analysis of digests after the 2 h intestinal digestion period. Bars with no letters in common are significantly different ($p < 0.05$). ◆ Represents an undetectable value. The absorbance of 0.02 ([Fe] = 0.129 μg/mL) was used as a cutoff point for reliable determination of iron concentration.

DISCUSSION

For nonheme Fe to be absorbed by the enterocyte, it must first be soluble at the luminal pH of the intestine. At pH values above 3, ferric Fe rapidly becomes insoluble unless complexed with a soluble ligand. In the upper duodenum, the region of the intestine where most Fe absorption is thought to occur, luminal pH values range between 5 and 6.5 depending on the luminal contents and time postingestion of a meal (*31*). Ferrous Fe in the free form is more soluble at the luminal pH of the duodenum and, therefore, more available for absorption (*32*). Thus, the primary factors affecting Fe solubility are pH and complexation by compounds such as organic acids, polyphenolic compounds, and proteins. Furthermore, the strength of the binding, solubility, and concentration of the complex in the food or meal play a major role in Fe bioavailablity, as these factors influence the degree to which Fe can interact with other compounds and the luminal iron transporter. For example, binding of Fe by ethylenediaminetetraacetic acid (EDTA) is relatively stable at gastric pH and thereby prevents interaction of Fe with inhibitors
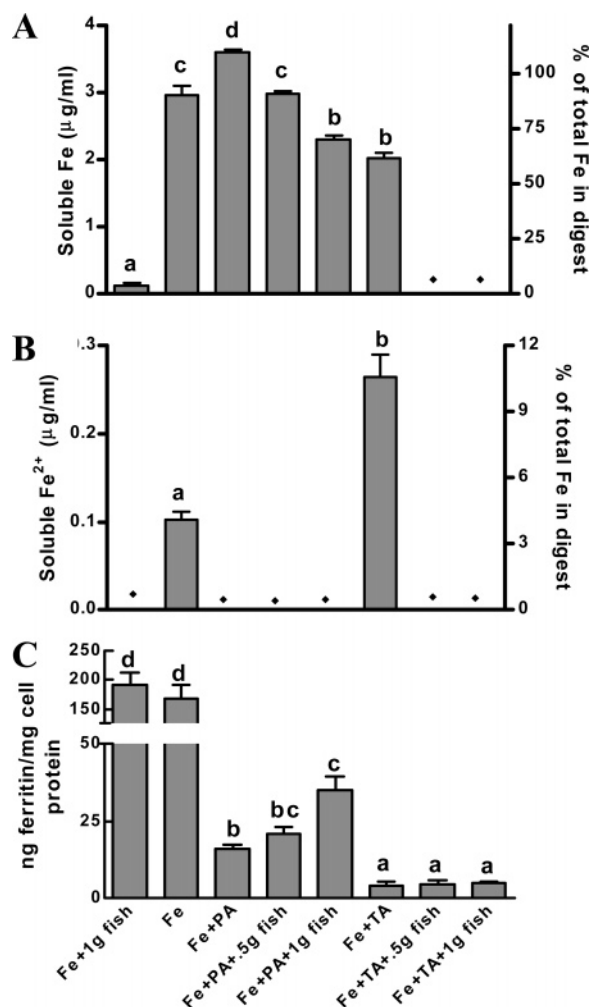
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Figure 3. (**A**) Total soluble Fe in digests, (**B**) soluble Fe^{2+} in digests, and (**C**) Caco-2 cell ferritin formation in response to digests. Digests contained a constant level of PA (1:20 Fe:PA) or TA (1:1 Fe:TA) and addition of 0, 0.5, or 1 g of fish. The digest iron concentration was 59 μmol/L. Values are means ± SEM. For solubility assays (**A** and **B**), values refer to analysis of digests after the 2 h intestinal digestion period. Bars with no letters in common are significantly different ($p < 0.05$). ◆ Represents an undetectable value. The absorbance of 0.02 ([Fe] = 0.088 μg/mL) was used as a cutoff point for reliable determination of iron concentration.

Figure 4. Caco-2 cell ferritin formation in response to digests containing PA (**A**) or TA (**B**) and iron as $FeSO_4$ or $FeCl_3$. * Denotes digests that contained $FeSO_4$ as an iron source. All other digests were formulated with $FeCl_3$. The digest iron concentration was 50 μmol/L. Values are means ± SEM. Bars with no letters in common are significantly different ($p < 0.05$, $n = 6$). Within each graph, upper case letters are used to represent analysis of PA studies, while lower case letters refer to analysis of TA studies. Ferritin formation (mean = 1227.6 ± 24.3, $n = 6$) from positive control [AA:Fe (50 μmol/L) = 20:1] was not included in graph A. Ferritin formation (mean = 1505.8 ± 44.8, $n = 6$) from positive control [AA:Fe (50 μmol/L) = 20:1] was not included in graph B.

such as polyphenolics. The Fe(III)−EDTA complex is less stable at the pH of the intestine, thus allowing some uncomplexed Fe to be available for absorption (*33*).

Phytate has been known to inhibit Fe absorption in humans for more than six decades (*34−37*). Numerous other studies over the years have investigated the role of phytate in Fe bioavailability in various models and food matrices; yet, despite all of these efforts, the mechanism of phytate's inhibitory effect on Fe bioavailability has not been clearly defined.

Under conditions such as in the present study, in which no foods were present in the in vitro digests, we propose that phytate primarily inhibits nonheme Fe absorption by binding the Fe and thereby restricting exchange or interaction of Fe with the brush border surface and the Fe^{2+} transporter. Several observations support this hypothesis. First, a previous in vitro study (using the same model as the present study) demonstrated that increasing Fe dialyzability corresponding to an increasing molar excess of phytate was also associated with a decrease in iron availability (*17*). The results of the present study are similar
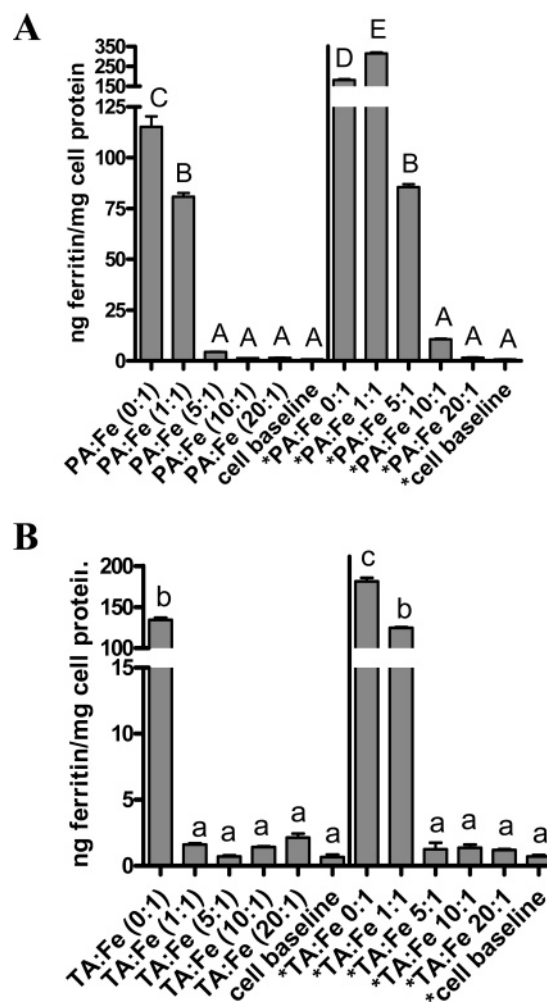
and clearly indicate that Fe bound to PA is highly soluble but of relatively low bioavailability at a 1:20 molar ratio of Fe: phytate (**Figure 1**). Moreover, the observed decrease in Fe bioavailability with increasing phytate (**Figure 3**) supports the complexation hypothesis of Fe inhibition, as a similar loss of Fe bioavailability with increasing levels of the an Fe(III) complex has also been demonstrated for citric acid and nitrilotriacetic acid (*38*).

The observation that AA only partially reversed the inhibitory effect of phytate, even at molar excess to phytate, can also be explained by a complexation mechanism of inhibition. AA is known to reduce Fe^{3+} to Fe^{2+} at low pH and only complexes Fe weakly. Therefore, in the absence of any other compounds that may bind or oxidize Fe, the Fe is highly bioavailable (**Figures 1−4**; *28*). Under the conditions of the present study, more ferrous Fe was formed by AA reduction of ferric Fe. However, the 20-fold molar excess of phytate relative to Fe was likely the dominant factor binding Fe and thereby reducing

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access of free Fe to interaction with the iron transporter or membrane ferric reductase (**Figure 1**).

Interestingly, Fe bioavailability from $FeSO_4$ was significantly enhanced in the presence of an equimolar amount of phytate, whereas an equimolar level of phytate to Fe(III) inhibited Fe(III) absorption from $FeCl_3$ (**Figure 4**). However, both forms of Fe showed decreased bioavailability with increasing amounts of phytate, further demonstrating the effect of phytate complex formation on Fe bioavailability. The enhancing effect of an equimolar amount of phytate is a significant observation and was probably due to increased Fe solubility of the $FeSO_4$– phytate complex relative to the $FeSO_4$. One could speculate that the equimolar amount of phytate relative to Fe was probably just enough to improve solubility but not to induce a mass action effect and inhibit Fe uptake. In other words, a 1:1 molar ratio of $FeSO_4$:phytate is a form of Fe that remains readily exchangeable and available for absorption, as demonstrated previously in vivo (*14*).

Certainly a key observation from the present study is that monoferric phytate is a form of Fe that can be available for absorption. It has been shown many times that monoferric phytate is a highly soluble and stable complex and that it is highly available to rats (*13*). Indeed, an often overlooked study using dogs was the first to demonstrate that monoferric phytate was equal in bioavailability to $FeSO_4$ and that the Fe from monoferric phytate exchanged as readily as $FeSO_4$ with other components of the meal (*14*). A mineral balance study in adult men consuming whole vs dephytinized wheat bran demonstrated that, after an initial adaptation period of approximately 5 days, the men consuming the whole wheat bran were in positive iron balance, significantly higher than the men consuming the dephytinized wheat bran (*37*). Clearly, this study indicates that the Fe in monoferric phytate can be absorbed by humans and that Fe complexed by phytate does not represent an irreversible, nonavailable form of Fe in the dietary pool. However, there may be some interactions of monoferric phytate with Ca and other polyvalent cations that could result in coprecipitation of Fe bound to insoluble Ca–phytate precipitates in certain food matrices relatively high in Ca or other polyvalent cations (*39*– *42*). Such interactions could make the Fe in mixed cation complexes of monoferric phytate unavailable for absorption.

Interestingly, Sandberg et al. found that degradation of phytate in the colon was decreased in pigs fed diets that were supplemented with $CaCO_3$ (*39*). The authors suggested that this effect could result from formation of Ca–phytate complexes that are soluble at low pH and insoluble at high pH. Thus, high colonic pH influenced by $CaCO_3$ buffering could lead to complex precipitation. In another study, Zn was shown to complex with Ca and phytate in vitro, with high levels of Ca potentiating Zn binding to inositol phosphates (*43*). As binding of Fe and Zn by phytate is similar, the formation of insoluble complexes with Ca and phosphate may be a factor in decreasing Fe absorption.

The present study of the interaction between phytate and Fe indicates that reasonable dietary levels of AA can partially reverse the inhibitory effects of phytate. Initiation of reversal of inhibition by phytate began at a molar ratio of 1:20:1 Fe: PA:AA, which is approximately equivalent to 475 mg of phytate and 6.3 mg of ascorbate, assuming 2 mg of Fe in an 80 g whole wheat roll (*44*). For comparison, 1 cup (248 g) of raw, unfortified orange juice contains approximately 124 mg of AA (*44*), yielding, in this case, an Fe:PA:AA molar ratio of 1:20: 20.

Human studies have also provided evidence that reasonable amounts of dietary AA could partially overcome inhibition of Fe absorption by phytate; however, it should also be noted that the ratio of phytate to Fe and ascorbate studied was somewhat lower (*18*, *26*). For example, Siegenberg et al. found that Fe absorption from a bread meal containing 3 mg of Fe and 58 mg of phytate doubled when 30 mg of ascorbate was added (*26*). This corresponds roughly to a final molar ratio of 1:1.6: 3.2 Fe:PA:AA.

Although these human studies have the advantage of measuring Fe bioavailability from whole foods, the 20-fold molar excess of phytate used in the present study is more typical of phytate levels found in staple food crops (*3*, *45*). Thus, investigation of the interaction between Fe, phytate, and AA is an important initial research step for the HarvestPlus program; however, as stated previously, other components of the food matrix may also interact with the Fe–phytate complex and alter the bioavailability of the Fe. Further in vitro research must now be conducted to investigate the effects on Fe bioavailability of additional components such as proteins, carbohydrates, and phytochemicals in the staple crop food matrix.

The Caco-2 cell model is particularly useful in this regard because large numbers of crop varieties can be cost effectively screened and ranked for bioavailable Fe, with promising varieties advanced for further breeding or in vivo comparisons depending on the yield characteristics, disease resistance, and in vitro assessment. The model takes into account the interactions between promoters and inhibitors as well as levels of intrinsic Fe, whereas without such a model, selection of promising varieties would be based solely on the content of these components. Screening staple crops based on content of Fe and other compounds is problematic as it is well-known in the field of Fe bioavailability that concentration of these components often does not correlate with bioavailability.

Developing low-phytate crop varieties and removing phytate from foods by enzymatic degradation have received attention as methods of achieving improved Fe bioavailability from staple crops and food products. However, there are several potential drawbacks to these approaches. First, because seed phytate content is highly influenced by edaphic factors (e.g., levels of available P in the soil), it is extremely difficult for plant breeders to use phytate concentration as a criterion for screening plant varieties (*46*). Furthermore, in both rat and Caco-2 cell models, no correlation has been found between seed phytate content and level of bioavailable Fe (*3*, *47*). In addition, despite numerous investigations of the role of phytate as an antinutrient, its various potential health benefits must also be considered (*48*). The anticarcinogenic activity of phytate has been demonstrated by both in vivo and in vitro studies, in which inositol hexaphosphate was associated with decreased proliferation of malignant cells (*4*). Evidence also exists for benefits relating to the antioxidant function of phytate and inhibition of hydroxyl radical formation (*49*).

Considering the tradeoffs between these beneficial properties of phytate and the well-documented inhibitory effects of high phytate levels on Fe absorption, it may be possible to obtain some ideal ratio of Fe to phytate at which Fe absorption is not excessively inhibited and the health benefits of phytate are retained. For example, mutant low-phytate maize varieties have been identified (*50*). When fed to human subjects as tortillas, the low-phytate maize variety had significantly greater Fe availability (49%) than the normal phytate wild type (*51*). The molar ratio of PA to Fe was 8.4:1 in the tortilla prepared from the mutant (low-phytate) maize and 16:1 from the wild-type

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maize. Using the Fe concentration and bioavailability estimates from this human trial, one can estimate that approximately 50 μg more Fe would be absorbed per 100 g of low-phytate maize consumed. Whether this level of enhancement of Fe absorption could be nutritionally significant if incorporated into the diet over a prolonged period of time (i.e., months) is not known. However, a recent long-term human study involving rice, which is currently under review for publication, suggests that such an amount of available Fe is indeed beneficial in maintaining or improving Fe stores and preventing anemia in some individuals (Dr. Jere Haas, Cornell University, personal communication). In this long-term human study, Fe concentration in the diet was increased by using rice that was less polished (i.e., undermilled), which improved the Fe concentration of the rice by 7−9 μg/g and maintained the molar ratio of phytate to Fe. Clearly, more long-term studies need to be done to determine if simply increasing the Fe density of staple food crops can alleviate Fe deficiency anemia, and if so, the target levels for Fe density must be defined. Notably, increasing Fe density in the seeds may not lower the ratio of phytate:Fe, due to the wide variety of factors that can influence seed phytate levels.

The in vitro digestion/Caco-2 model used in this study should be a good predictor of the effects of phytate on Fe bioavailability as it has been shown to match human studies in a concentration−response manner. For example, Hallberg et al. investigated a range of PA levels on Fe absorption in human subjects (*18*). A similar study has also been done with this in vitro model (*17*). At a 1:1 phytate to Fe molar ratio, 69 and 70% inhibition of Fe uptake was observed in the human and Caco-2 studies, respectively, relative to Fe uptake in the absence of phytate. The highest molar ratio of phytate:Fe used in the human study was 5.16:1, which resulted in an 82% inhibition of Fe absorption relative to that where no phytate was present. Using the in vitro model, an 79 and 85% inhibition of Caco-2 cell ferritin formation (i.e., Fe uptake) was observed with phytate:Fe molar ratios of 3:1 and 10:1, respectively. Clearly, the in vitro results are in close agreement with the human study and lend confidence to the use of this in vitro tool to study food Fe bioavailability.

Glahn et al. showed that maximum inhibition by TA in vitro occurs beginning at a 1:0.1 molar ratio of Fe to TA; however, levels of TA in foods are generally higher (*1, 17*). In contrast to phytate, inhibition of cell Fe uptake by TA was generally not reversed by AA or meat additions under the conditions of this experiment. Although a statistically significant increase in Fe uptake in the presence of TA was seen at the highest ratio of AA to TA studied, this increase was very small in relation to the control and observed at a level of AA difficult to obtain through diet alone. It is also interesting to note that even though soluble ferrous Fe was increased by the addition of AA to the digest, no significant increase in Fe uptake was observed (**Figure 3**). This observation suggests that the AA merely reduced the Fe already complexed by the TA and did not release the Fe from the complex.

There is evidence that not all polyphenolics inhibit Fe absorption to the same extent as TA (*20*). At present, it appears that the condensed tannins are potent inhibitors of Fe availability, but the precise mechanism whereby polyphenolics inhibit Fe availability remains unknown. The order of combination of Fe with AA and TA has been shown to affect Fe-binding interactions of TA and AA (*19*). This may explain the results of Siegenberg et al., which suggested that AA could reverse TA inhibition in vivo even at a ratio of 1:4.6:2.6 Fe:tannate:AA (*26*). In this human study, Fe was ingested with a food vehicle separately from TA and AA (although at the same meal) and thus not allowed to interact with either until initiation of digestion. Absorption results may be different if Fe exists in association with TA or AA within a particular food before combination with other food components during digestion. Polyphenolics also appear to decrease Fe solubility, and this effect appears to occur both in the food and during interaction with Fe in the gastrointestinal tract.

Finally, as with phytate, it should be noted that polyphenolic compounds are beneficial compounds that play a role in prevention of both cancer and cardiovascular disease due to their antioxidant properties (*5, 6*). Decreasing the concentration of these compounds in foods to increase Fe bioavailability may also increase the risk of cancer and cardiovascular disease. This possibility should be considered in the context of the whole diet. Thus, to improve Fe bioavailability on a population basis, the wisest approach may be to increase the promoters of Fe absorption in the diet without dramatic reductions in the Fe absorption inhibitors.

The ability of fish to counteract the inhibitory effects of phytate but not TA further illustrates the difference in the Fe-binding properties of each compound. The decrease in Fe solubility upon addition of fish was lessened in the presence of phytate, an observation that is consistent with the complexation hypothesis presented above.

The results of this study suggest that the effects of the meat factor do not result from an increase in total Fe solubility or reduction of Fe to the ferrous form. Under these conditions, fish increased Fe absorption in the presence of phytate without an increase in Fe solubility or ferrous Fe level (**Figure 3**). These results are consistent with the findings of Huh et al., who also demonstrated that Fe in the presence of fish showed reduced dialyzability (solubility) but enhanced availability to cells (*25*). Although the exact mechanism of the meat factor effect is still unknown, its activity has been linked to the carbohydrate fraction of meat digests and thus may involve oligosaccharides present on the surface of muscle tissue (*25*). Identification of the precise factors in meat that promote Fe uptake should be explored further as this could be of relevance to improving Fe bioavailability from plant foods.

In summary, compounds that complex Fe do not always make the Fe bioavailable. The concentration of the Fe complex, the strength of the bond, and the interaction of the complex with other food ingredients are significant factors affecting Fe bioavailability. The primary reason that AA promotes ferric Fe bioavailability is its ability to reduce ferric Fe to free ferrous Fe that is readily transported by the Fe^{2+} transporters.

In the future, it will be important to investigate the interactive effects of plant proteins and carbohydrates, as well as other minerals such as Ca, on the mechanisms by which Fe absorption is enhanced or inhibited. This information may provide additional clues as to how plants can be modified for improved Fe bioavailability from diets high in foods prepared from cereal grains and legume seeds.

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