Iron absorption from soybean ferritin in nonanemic women\textsuperscript{1–3}

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ABSTRACT

Background: Dietary ferritin, a protein cage around an iron mineral, is an underestimated source of bioavailable iron. Plant ferritin, the most common dietary ferritin, has not been studied. Iron from animal ferritin is absorbed as well as is iron from FeSO\textsubscript{4} in women.

Objective: The objective was to examine iron absorption from purified soybean ferritin.

Design: Healthy, nonanemic women ($n = 16$) were fed a standardized meal (bagel, cream cheese, and apple juice) containing 1 $\mu$Ci $^{59}$Fe/meal as FeSO\textsubscript{4} or (extrinsically labeled) as iron-free soybean ferritin reconstituted with the high phosphate characteristic of plant ferritin (iron:phosphorus = 4:1). Iron-free, apo-soybean ferritin was prepared (with the use of thioglycolic acid and extensive dialysis) from purified ferritin. In a randomized crossover design, the other labeled meal, which contained FeSO\textsubscript{4} or ferritin, was given after 4 wk. The subjects received 140 $\mu$g Fe as ferritin (2.5 mg) or as FeSO\textsubscript{4}. After 28 d, whole-body $^{59}$Fe and $^{59}$Fe in red blood cells were measured before and after dosing.

Results: There was no significant difference in whole-body iron absorption from soybean ferritin (29.7 ± 19.8%) and that from FeSO\textsubscript{4} (34.3 ± 23.6%) or in iron absorption calculated from red blood cell incorporation (33.0 ± 20.1% for soybean ferritin and 35.3 ± 23.4% for FeSO\textsubscript{4}), which confirmed previous results with animal ferritin that was mineralized and labeled similarly. An inverse relation was observed between serum ferritin and iron absorption from both ferritin and FeSO\textsubscript{4}, which suggested that sensors regulating iron absorption respond similarly to iron provided as ferrous salts or as ferritin mineral.

Conclusion: Iron from soybean ferritin is well absorbed and may provide a model for novel, utilizable, plant-based forms of iron for populations with a low iron status. Am J Clin Nutr 2006;83:103–7.

KEY WORDS Iron absorption, soybean, ferritin, nonanemic women

INTRODUCTION

Ferritin is a storage form of iron in plants, particularly legumes (1), and an increasing target for improving seed iron composition (2–4). Ferritin iron is a large mineral (thousands of iron and oxygen atoms), inside a protein nanocage, that is very stable to temperature, heat, and protein denaturants (1, 5, 6) and is nature’s “iron concentrate.” In plants, the ferritin–iron mineral forms with the high phosphate content characteristic of prokaryotes (7–9). Plant breeding techniques have the potential of yielding high ferritin cultivars. Additionally, genetic engineering can be used to express high amounts of ferritin in staple foods (10), eg, rice (2, 3). Because legumes and other plants make up a considerable part of the diet in many populations in developing countries, iron in ferritin could contribute to improving iron status, provided that it is well utilized.

The bioavailability of iron from ferritin has been a matter of controversy. Several studies have shown that ferritin iron and soy iron, which is largely in ferritin (11–13), is well utilized (13–15), even when the phytate content is high (9). However, 2 studies of soy—in which the ferritin and phytate contents were high—suggest poor bioavailability (16, 17). Largely, the controversy appears to relate to the isolate labeling techniques used, and, to some extent, how the ferritin was produced. Ferritin is an unusual protein that can be produced in high concentrations as a stress response, such as during inflammation, and it consists of a large multimeric protein surrounding the iron mineral containing core “stress ferritin” (6, 18–21). Iron in ferritin cores is a solid mineral and, therefore, is not easily accessible for extrinsic labeling. Reconstituted ferritin minerals are the same as native minerals according to a variety of methods, such as X-ray absorption spectroscopy, Mössbauer spectroscopy, and electron microscopy (7, 9, 22–24). In early studies, in which extrinsic labeling was used to study ferritin iron or soy iron bioavailability in humans, it is likely that the iron label did not represent endogenous mineralized ferritin iron, which accounts for most of the iron in soy. In experiments in which intrinsically labeled animal ferritin iron is sought, not only is there the problem of endogenous mineralized ferritin iron, but there is also the problem that stimulants of ferritin synthesis, such as inflammation and stress, can also cause changes in the ferritin protein and iron contents. Note that estimated equilibration times for isotopically labeled iron with unlabeled forms of iron in the body are estimated to 1 y (25). A study in humans that used intrinsically labeled soybeans showed that iron was as well utilized as was iron from FeSO\textsubscript{4}, even though the diet fed was high in phytate, which normally inhibits iron absorption (4). Because a major part of soybean iron is bound to ferritin (11, 12), these results strongly...
suggest that iron in soybean ferritin is highly bioavailable. Animal feeding studies have also shown that iron in soybeans is well utilized (4).

We previously used purified animal (horse spleen) ferritin to assess iron absorption in women (16). We extrinsically labeled the purified ferritin by removing the endogenous, unlabeled iron in the iron mineral by dialysis against thioglycolic acid and subsequently reminalizing the ferritin with iron containing the radiolabel to ensure that the labeled iron represented ferritin iron. We found that iron from animal ferritin was highly available, and its absorption was equal to that of iron from FeSO₄. Because ferritin in plants is more likely a form of dietary iron than animal ferritin in most populations, we isolated soybean ferritin and studied iron absorption from this iron source.

SUBJECTS AND METHODS

Recruitment and screening

Potential subjects were recruited by advertisement on bulletin boards at the University of California, Davis; provided an explanation of the study; and scheduled for screening. Before screening, written informed consent was obtained from each subject. The screening consisted of a brief health questionnaire to determine whether the subjects had a history of hematologic or gastrointestinal disorders, to assess iron status via a finger prick, and to perform a pregnancy test. The subjects were excluded from the study if they had a history of hematologic or gastrointestinal disorders, were anemic (hemoglobin < 90 g/L), were taking iron-containing supplements and refused to discontinue use for the duration of the study, or were pregnant. Sixteen women participated in the study. The study was approved by the Human Subjects Review Committee and the Radiation Use Authorization Committee at the University of California, Davis.

Iron sources

Soybean ferritin was isolated and purified as previously described by using a combination of seed extraction, fractionation by filtration, centrifugation, ammonium sulfate precipitation, and ion-exchange chromatography and analyzed by sodium dodecyl sulfate gel electrophoresis to determine purity (25). Iron was removed from the soybean ferritin by thioglycolic acid reduction and dialysis (25) and the iron mineral reconstituted (7) with the radiolabel present, as previously described (15). Reconstitution of ferritin with this procedure produces a mineral that, when measured by X-ray absorption spectroscopy and by Mössbauer spectroscopy, is essentially indistinguishable from ferritin isolated from natural tissue (7, 24). All steps were performed at 4 °C unless otherwise specified. To remove endogenous, unlabeled iron from soybean ferritin, the isolated protein was mixed with 2% thioglycolic acid in a 1:1 ratio, gently purged with nitrogen, and stored at 4 °C for 1 h. This mixture was then placed in dialysis tubing (molecular weight cutoff of 10 000, Spectra/Por CE; Spectrum, Rancho Dominguez, CA) and placed into a dialysate of 1% thioglycolic acid and 0.05 mol HEPES/L. The dialysate was changed every 8 h for 3 d. On day 4, the dialysate consisted of 0.05 mol HEPES/L only. The final dialysate change on day 4 consisted of 0.15 mol HEPES/L and 0.1 mol NaCl/L. The iron-free ferritin protein (<10 iron atoms/protein), often called apo-ferritin even though the iron is not a cofactor, was then stored at 4 °C until the mineral inside the ferritin protein was reconstituted.

The iron mineral in the iron-free ferritin protein was reconstituted with 59FeSO₄ (27.7 mCi/mg; Perkin Elmer, Boston, MA) to 480 iron atoms/protein molecule, with iron mineral (K₂HPO₄) for study use (18). A quantity of iron equal to that in ferritin in the form of 59FeSO₄ ferrous sulfate was used for comparison. Plant type high phosphate iron mineral was formed in the presence of K₂HPO₄ at a ratio of phosphate:59Fe:iron (4:1) to reconstitute a ferritin iron mineral similar to that found in plants such as soybeans.

Study protocol

Subjects were randomly assigned to begin the study with either the 59Fe-labeled soybean ferritin meal or the 59Fe-labeled FeSO₄ meal, each containing 1 mg Fe. The labeled ferritin or FeSO₄, which provided 1 μCi 59Fe to each subject, was administered in 60 mL apple juice. A plain, white-flour bagel (100 g) and full-fat cream cheese (15 g) was also provided to represent a realistic situation for subjects consuming a ferritin-containing meal. All meals were consumed after the subjects had fasted overnight for 12 h.

On day 1 of the study, the subjects arrived between 0700 and 0900 in a fasted state, and background whole-body radioactivity was determined with a whole-body counter (Institute of Toxicology and Environmental Health, University of California, Davis, CA) equipped with two 10 × 20-cm sodium iodide crystals and a multichannel analyzer (ND-66; Nuclear Data, Schaumburg, IL). The subjects were then given the first randomized labeled meal, and radioactivity was immediately recounted in the whole-body counter. The subjects were instructed to refrain from eating and from drinking any fluid except water for the next 4 h. Radioactivity was counted 14 and 28 d after consumption to assess retention of the previously consumed dose. On day 28, the subjects arrived in a fasted state for whole-body counting. After whole-body radioactivity was determined, a venous blood sample was drawn from an antecubital vein for the determination of 59Fe incorporation into red blood cells (RBCs). After the blood draw, the subjects were given the second randomized labeled meal, and radioactivity was recounted in the whole-body counter. Radioactivity in the subjects was counted in the whole-body counter 14 and 28 d after consumption of the second meal. A final blood sample was drawn on day 28. Whole-body iron absorption was calculated after the RBC count was made in a defined volume of blood (5 mL), assuming a blood volume of 71.4 mL/kg body weight and an incorporation of 85% into hemoglobin. All values were corrected for decay.

Blood samples were used to measure hemoglobin (HemoCue, Angelholm, Sweden) and ferritin (Ferritin IRMA; Diagnostic Products Corporation, Los Angeles, CA) concentrations.

Statistics

Two-factor repeated-measures analysis of variance was used to compare iron absorption and incorporation with method and type of iron as within-subject factors. The software package used was SAS release 8.02 (SAS Institute Inc, Cary NC). The absorption variables were log transformed to conform to the assumption that the error terms are normally distributed.
The iron status of the 16 women participating in the study was adequate, although one woman was slightly anemic (hemoglobin: 110 g/L) and 4 were iron deficient as defined by low serum ferritin concentrations (12 g/L). The mean (±SD) concentration of hemoglobin was 134 ± 12 g/L and of serum ferritin was 32.6 ± 33.5 g/L (Table 1). There was no significant interaction between type of iron and method (P = 0.58), nor was there any significant difference in iron absorption between the 2 forms of iron (P = 0.31) or the 2 methods (P = 0.28). Iron absorption was 29.9 ± 19.8% from soybean ferritin and 34.3 ± 23.6% from FeSO₄, as assessed by whole-body counting. A significant inverse relation between serum ferritin and iron absorption from soybean ferritin (r = −0.7673, P = 0.0008) and between serum ferritin and iron absorption from FeSO₄ (r = −0.7218, P = 0.0024) was found (Figure 1).

DISCUSSION

In our previous study (15), we found no significant difference in iron absorption from animal ferritin and FeSO₄. Because plant ferritin iron mineral has a higher phosphate content (phosphate:

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TABLE 1
Iron status of the subjects and iron absorption determined by 2 methods

Iron absorption as estimated from RBC incorporation of iron was 33.0 ± 20.1% from soybean ferritin and 35.3 ± 23.4% from FeSO₄. By this method, there was also an inverse correlation between serum ferritin and iron absorption, both for iron from soybean ferritin (r = −0.5408, P = 0.046) and from FeSO₄ (r = 0.6300, P = 0.021).

There was a close correlation between iron absorption from soybean ferritin determined by whole-body absorption and by the RBC incorporation method (r = 0.9267, P < 0.0001) and similarly for FeSO₄ (r = 0.9115, P < 0.001).

FIGURE 1. Correlation between iron absorption from soybean ferritin and serum ferritin (r = −0.7673, P = 0.0008) and between iron absorption from FeSO₄ and serum ferritin (r = −0.7218, P = 0.0024). Iron absorption was determined by whole-body counting.
Iron = 4:1) than does animal ferritin (phosphate:iron = 1:1), we prepared and used both high and low phosphate ferritin in that study. We found no significant difference in iron absorption between the 2 forms (16), which suggested that this difference in phosphate content of the iron mineral does not affect iron absorption. That a difference in ferritin protein could affect iron absorption could not be ruled out.

The absorption of iron from soybean ferritin was high, ≈30%, and equally high as from FeSO₄, as determined by either whole-body counting or RBC incorporation. These results are similar to those observed in previous studies that used hydroponically (intrinsically) labeled soybeans in women with borderline iron deficiency (13) or purified animal (horse spleen ferritin) in women with normal iron status (15). Although iron absorption from soybean ferritin, as determined by whole-body counting (29.9%), was somewhat higher than that from horse spleen ferritin in our previous study (21.9%) (15), it should be noted that iron absorption from FeSO₄ also was proportionally higher (34.3% compared with 16.7%). This finding is most likely explained by the smaller amount of iron given in the present study, because the difficulty in isolating large amounts of purified soybean ferritin, in contrast with ferritin from the unusually iron-rich horse spleen, restricted the amounts of purified soybean ferritin available and we had to limit the dose of iron given. It is well known that iron absorption is inversely correlated with the dose given and with iron status (26-28).

The isotope labeling technique used likely had a profound effect on the results obtained for iron absorption from ferritin. This is illustrated by the study of Skikne et al (18), who used both in vivo (intrinsic) and in vitro (extrinsic) labeling of bovine spleen ferritin and assessed iron absorption by the RBC incorporation method in men and women. They acknowledged the difficulties involved in preparing intrinsically labeled ferritin and, therefore, also attempted to add the radiolabel extrinsically. However, the extrinsic label was added to isolated ferritin, which already had unlabeled iron mineral and which appeared to have a very high iron:protein ratio suggestive of the presence of denatured ferritin or exposed iron core with different exchange rates than core inside native protein. They concluded that iron absorption from the extrinsically labeled ferritin, 20% (range: 2.6–58.7%), was inappropriately high and that the considerably lower results from intrinsically labeled ferritin (4.0%; range: 0.4–16.9%) were more likely to be valid. The wide variation could have resulted from inhomogeneity of native and denatured ferritin iron, which would not have been detectable by the methods used to analyze the protein alone. Moreover, the intrinsic ferritin used in their study was prepared by an elaborate procedure involving the coating of bovine RBCs with anti-bovine ferritin used in their study was prepared by an elaborate procedure.

The mechanism by which iron is absorbed from ferritin is not yet known. Although it originally was expected that ferritin is digested in the gastrointestinal tract and iron is taken up from the released iron mineral, this may not necessarily be correct. Ferritin in solution is resistant to proteolysis, protein denaturation, and low pH (29, 30), and immunoreactive soy ferritin has been detected both after heat treatment in solution, a standard procedure used to isolate ferritin from tissues (16), and after oven baking of soybeans (14). In addition, we showed, using in vitro digestive conditions with pepsin and pancreatic enzymes, that ferritin is relatively resistant (S Kalgaonkar, SL Kelleher, EC Theil, and B Lönnerdal, unpublished observations, 2004). Moreover, using monolayers of human intestinal Caco-2 cells in culture we showed that both iron and ferritin protein are taken up by the cell (31). Finally, plant ferritin would be protected during digestion by the cell wall and the plastid membrane and by the hull in some seeds (7). It is not yet known whether this occurs via a putative ferritin receptor pathway, as has been suggested for tissues such as the liver (32) and placenta (33), or by a more general pinocytic pathway, but it is possible that ferritin will facilitate the uptake of iron by the enterocyte. Once inside the cell, ferritin may be digested and iron released into a general intracellular iron pool. Further molecular studies are needed to explore these possibilities.

In our previous study in which we used animal (horse spleen) ferritin (15), we obtained higher iron absorption values by the RBC incorporation method for FeSO₄ than for horse spleen ferritin. This finding may have been related to different uptake mechanisms. We found no such differences in the present study; results from both methods were similar for soybean ferritin and for FeSO₄. The reason for the previous observation is not known, but it may relate to the relatively low number of subjects used (the difference was not significant) or to differences in ferritin preparations. Generally, we found a very good correlation between iron absorption from ferritin estimated by the RBC incorporation method and by whole-body counting (r = 0.9267). This finding agreed with the findings of previous studies by other investigators (34).

Iron status (as assessed by serum ferritin) appears to be inversely correlated with iron absorption, similar to what is known for nonheme iron. Because we hope that ferritin may become a more commonly used source of bioavailable iron in populations with iron deficiency, this observation is encouraging.

We are grateful to Christina Yi for technical assistance and to Korry Hintze for helpful discussions. BL designed the absorption study. BL and AB carried out the absorption study. XL and ECT purified the soybean ferritin. ECT developed the labeling technique. BL and ECT wrote the manuscript with input from the other authors. There were no conflicts of interest.

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