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Abstract
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Keywords
Iron bioavailability; heme iron; soy root nodules; leghemoglobin

Comments
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Iron Bioavailability of Hemoglobin from Soy Root Nodules Using a Caco-2 Cell Culture Model

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Heme iron has been identified in many plant sources—most commonly in the root nodules of leguminous plants, such as soy. Our objective was to test the effectiveness of soy root nodule (SRN) and purified soy hemoglobin (LHb) in improving iron bioavailability using an in vitro Caco-2 cell model, with ferritin response as the bioavailability index. We assessed bioavailability of iron from LHb (either partially purified (LHb_A) or purified (LHb_D)) with and without food matrix and compared it with that from bovine hemoglobin (BHb), ferrous sulfate ($FeSO_4$), or SRN. Bioavailability of each treatment was normalized to 100% of the $FeSO_4$ treatment. When iron sources were tested alone (100 ug iron/mL), ferritin synthesis by LHb_D and BHb were 19% ($P > 0.05$) and 113% ($P < 0.001$) higher than $FeSO_4$, respectively. However, when iron sources were used for fortification of maize tortillas (50 ppm), LHb_A and BHb showed similar bioavailability, being 27% ($P < 0.05$) and 33% ($P < 0.05$) higher than $FeSO_4$. Heat treatment had no effect on heme iron but had a significant reduction on $FeSO_4$ bioavailability. Adding heme (LHb_A) iron with nonheme ($FeSO_4$) had no enhancement on nonheme iron absorption. Our data suggest that heme iron from plant sources may be a novel value-added product that can provide highly bioavailable iron as a food fortificant.

KEYWORDS: Iron bioavailability; heme iron; soy root nodules; leghemoglobin

INTRODUCTION

Iron deficiency is a major nutritional problem, affecting over 2 billion people (*1*). Fortification of foods with iron has been a successful strategy for improving iron content of foods; however, the bioavailability of iron fortificants is often decreased due to the presence of inhibitors within the food matrix. Iron bioavailability is higher from heme iron sources because of lack of inhibition from chelating compounds including polyphenols and phytate and because of its intact absorption by pathways different than those of nonheme iron (*2*). Heme iron in the human diet is generally present in animal sources as a part of hemoglobin and myoglobin but is also found in many invertebrates, bacteria, fungi, and widely distributed in the plant kingdom (*3*). However, these heme proteins currently do not provide a significant amount of iron in human diets. Plant hemoglobins are most commonly found in nodulating legumes as part of the symbiotic nitrogen fixation pathway; however, nonsymbiotic hemoglobins also exist in many plants with diverse and yet undescribed roles in plant physiology (*4*).

Leghemoglobin (LHb), a symbiotic hemoglobin, is a monomeric heme protein originally identified in soybean root nodules and has been studied extensively (*5*, *6*). Because of its high affinity for oxygen, LHb makes less oxygen available, enhancing the nitrogen fixation process. The nitrogenase enzyme produced by symbiotic bacteria within legume roots requires an anaerobic environment, and therefore, the plant produces heme protein that is capable of scavenging oxygen within the cytosol of the root, resulting in a low-oxygen environment ideal for nitrogen fixation (*7*). Leghemoglobin accumulates iron in roots creating a large iron store. Iron levels of up to 2.5 mg total iron/g dry weight basis have been measured in soy nodules, with up to 26% of the total iron in heme form in the unpurified root (*8*). Most researchers are interested in the physiological role and structure of hemoglobin in the plant, but to our knowledge no studies have been reported on the use of plant hemoglobins for improving iron bioavailability of human diet.

Caco-2 cells are human intestinal adenocarcinoma cells exhibititing enterocyte-like biochemical and morphological characteristics and have been used widely for nonheme iron bioavailability studies (*9*, *10*). Heme bioavailability in Caco-2 cells is not well studied, but recent work has shown that these cells synthesize enzymes involved in heme uptake and metabolism, in particular hemeoxygenase (*11*), and that the mechanisms of heme transport are similar between humans and Caco-2 cell models (*12*). This evidence makes the Caco-2 cell model appealing for its potential in evaluating heme iron bioavailability. The objective of this study was to determine the iron bioavailability of crude soy root nodule extract (SRN) and two purified soy leghemoglobins and to compare their bioavailability with that of bovine hemoglobin (BHb) using the Caco-2 model. The underlying objective of this study is to introduce the concept of using plant hemoglobin as a heme iron source in diets that are consumed by humans and to promote further research into this area.

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MATERIALS AND METHODS

LHb Preparation. Soybean plants (cultivar − OAC Bayfield) were field raised in sandy loam soil at Cambridge Research Station, University of Guelph, during the 2002 growing season on cropland used for potatoes in the prior two growing seasons. Seed was inoculated with Hi Stick Prep *Rhizobium japonicum* (Becker Underwood Canada, Saskatoon, Saskatchewan) at 1.8 g inoculant per 1 kg seed application rate. Fields were irrigated as needed. The root nodules were mechanically harvested at R7 maturity, removed from root structures, and lyophilized. Dried nodules were ground to pass a 30 mesh screen. Crude SRN fraction was prepared by reconstitution of dried nodule powder with water 1:5 w/w, followed by centrifugation (5000*g* for 30 min). Supernatant was collected, lyophilized, and stored at −20 °C until use. The aqueous extract was also used to prepare partially purified LHb (referred to as LHb_A) by using 50−80% ammonium sulfate ($(NH_4)_2SO_4$) precipitation, followed by desalting by dialysis with water. The desalted protein extract was lyophilized and stored at −20 °C for further use. Ion exchange chromatography with DEAE Sepharose (GE Healthcare, Piscataway, NJ) was used to further purify the LHb obtained from $(NH_4)_2SO_4$ precipitation. The column was equilibrated to pH 7.0 and ran with a linear gradient elution starting with deionized (DI) water and ending with 1 mol/L NaCl at pH 7.0. Eluants that were in the 405 nm absorbance peak were pooled, desalted by dialysis, and lyophilized (referred to as LHb_D). LHb_D was used in the initial studies without food matrix, and LHb_A was used in all tortilla fortification studies. Total iron was determined in samples by nitric acid digestion, and nonheme iron was determined by trichloroacetic acid protein precipitation method with a modification using ferrozine as a chromogen (*13*, *14*). Purity of protein in extracts and degradation of globin during in vitro digestion were assessed by SDS−PAGE using 10−20% polyacrylamide Tris-Tricine precast gels (Biorad, Hercules, CA) in a Ready Gel Cell (Biorad), and molecular weight was estimated by comparison with standards (Biorad). Gels were fixed using 5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA), followed by staining in 10% (v/v) aqueous acetic acid with 0.025% Coomassie Brilliant Blue G250 (Biorad) and destaining with 10% (v/v) aqueous acetic acid. Protein purity was estimated by gel optical density using QuantOne software (BioRad).

Tortilla Preparation. Unfortified masa harina (Ultrawhite #1 tortilla flour, Cargill Foods, Paris, IL) was fortified with 50 ppm iron with one of the following: SRN, LHb_A, BHb (98% pure, Sigma Aldrich, St. Louis, MO) or ferrous sulfate ($FeSO_4$) (Sigma Aldrich). Masa harina (200 g) was mixed with fortificants, sealed in a plastic container, and thoroughly mixed by shaking 2 min. Masa harina was reconstituted with DI water (1:2 w/w), weighed to 25 g portions, flattened by tortilla press, and fried at 200 °C on a Teflon pan, 2 min on each side. Tortillas were lyophilized and ground to pass a 30 mesh screen, sealed, and stored in the dark at room temperature. Iron content of lyophilized tortilla was verified using the method described above for total iron, and the biovailability was assessed using Caco-2 cells.

In Vitro Digestion. For experiments conducted without food matrix, SRN, LHb_D, BHb, and $FeSO_4$ were weighed to provide 1 mg iron in 10 mL final volume (100 ug/mL). Initially the iron sources were dissolved in 1 mL of a 140 mmol/L NaCl, 5 mmol/L KCl solution prior to digestion. Fortified tortilla samples were weighed to provide 200 μg total iron (~3.5 g to represent 50 ppm iron) and suspended in 5 mL of DI water. Pepsin was prepared by solubilizing 0.2 g of porcine pepsin A (1:60000) in 5 mL of 0.1 mol/L HCl. Pancreatin and bile solution were prepared by dissolving 0.05 g of porcine pancreatin (4 × USP) and 0.3 g of bile extract in 25 mL of 0.1 mol/L sodium bicarbonate ($NaHCO_3$). Trace minerals were removed from pepsin and pancreatin mixtures by treatment with Chelex-100 (BioRad) for 30 min, filtered through a separation column, and re-eluted with 5 mL of 0.1 mol/L HCl or 10 mL of 0.1 mol/L $NaHCO_3$, respectively (*15*). The pH of all samples, either with or without food matrix, was adjusted to pH 2.0 with 0.5 mol/L HCl, pepsin was added (0.5 mL), and the digest was incubated at 37 °C for 1 h on an orbital shaker at 200 rpm. The pH was then adjusted to 6.5 with 1.0 mol/L $NaHCO_3$ solution, and 2.5 mL of pancreatin solution was added. The samples were again incubated with shaking at 37 °C for 15 min and centrifuged, and the supernatant was then heat treated for 4 min at 100 °C in a boiling water bath to inactivate proteolytic activity as described by other researchers (*16*). Samples in aqueous matrix were adjusted to a final 10 mL volume with 140 mmol/L NaCl and 5 mmol/L KCl to provide a final concentration of 100 μg/mL. Samples with tortilla matrix were adjusted to final volume of 20 mL using DI H_2O to provide a 10 μg/mL iron. All digests were centrifuged at 5000*g* for 5 min, and supernatants were used for cell bioavailability experiments.

Cell Culture. All reagents for cell culture work were from Sigma Aldrich or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Caco-2 cells were purchased at passage 17 from American Type Culture Collection (Rockville, MD). The following experiments were conducted during passages 20−26. Cells were grown in a culture flask with Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids, and 1% v/v antibiotic−antimycotic solution. Cells were maintained at 37 °C in an incubator with 5% CO_2. Media was changed 3 times weekly. At 7 d, the cells were rinsed with Earle's Balanced Salt Solution (EBSS), trypsinised to dissociate the cells, centrifuged at 22.6 *g* for 5 min. The cells were seeded at a density of 5.6×10^3 cells/cm^2 in a 75 cm^2 culture flask for continued growth, or seeded on collagenized (Type 1 Rat tail collagen) 12-well cell culture plates (Corning Costar) at a density of 5×10^4 cells/cm^2 for iron bioavailability experiments. The cell culture plates were maintained under incubator conditions similar to those of the cell culture flask. Iron bioavailability experiments were conducted 15 d post seeding after rinsing with EBSS.

Iron Bioavailability by Caco-2 Cells. Bioavailability of heme sources was determined using ferritin concentration as an index of bioavailable iron in response to iron uptake (*15*). Serum free media modified from a published study (*14*) [DMEM with 1% v/v nonessential amino acids, 1% v/v antibiotic−antimycotic solution, 10 mmol/L PIPES (piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L), and epidermal growth factor (20 g/L)] was applied to the cell culture (0.5 mL) before adding an equal volume (0.5 mL) of sample supernatants and was incubated for 2 h. An additional 0.5 mL of serum-free media was added to the initial 1 mL, followed by a further incubation for 22 h. After 24 h total incubation, the samples were removed by aspiration from the cell culture wells, and cells were rinsed with 1 mL of EBSS. The cells were then lysed by addition of 0.5 mL of deionized water to each well and sonicated with a probe-type sonic dismembrator at lowest setting (<1 W output) for 15 s. Total cellular protein was determined in the lysates by the Bradford Coomassie Assay, (Pierce Laboratories, Rockford, IL). Ferritin in the lysates was determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford, TX) and measured using Cobra-II Gamma Counter with SpectraWorks software (Packard BioSciences, Meriden, CT). After normalizing ferritin concentration to cell protein concentration, the values were normalized, defined as percentages compared to $FeSO_4$, and expressed as relative biological values (RBV).

Effect of Heat on Fortificant Bioavailability. By adding the iron fortificant to the masa harina (subjected to heating) or to freeze-dried unfortified tortilla powder (subjected to no heating), the effect of heat treatment on iron bioavailability was assessed by using Caco-2 cells as described above.

Effect of Heme on Nonheme Iron Bioavailability. Tortillas were made by fortifying masa harina to a total of 50 ppm iron using blends comprised of 0, 25, 50, 75, or 100% iron from LHb, with the remaining iron from $FeSO_4$. Each fortification blend was then evaluated for iron bioavailability using the Caco-2 cells.

Statistical Analysis. Differences in RBV among treatments was determined using ANOVA with Tukey's multiple comparison test. For assessing the effect of heat treatment on bioavailability, Student's *t* test was used to compare the bioavailability of fortification before and after heat treatment. All mean differences were deemed significant at $P \leq 0.05$. Analyses were performed using GraphPad software (GraphPad Prism version 4.02 for Windows, San Diego, CA).

RESULTS AND DISCUSSION

As per the SDS−PAGE gel (**Figure 1**), the LHb extract had the largest band at ~14 kDa, which is the reported molecular

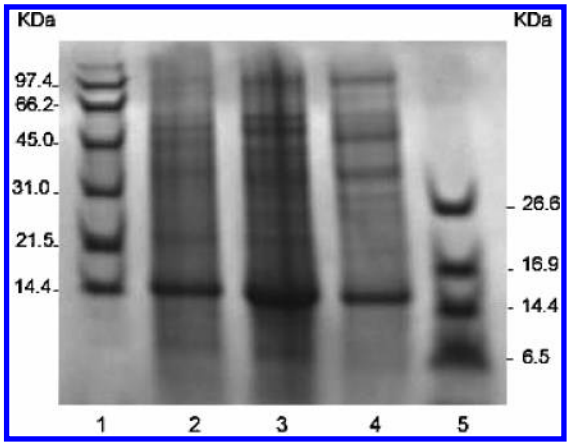

Figure 1. SDS–PAGE purity analysis of LHb protein fractions (Each lane contained 0.25 mg of protein.) Lane 1: standard 1 (14.4–97.4 kDa). Lane 2: soy root nodule (SRN) extract. Lane 3: 80% $(NH_4)_2SO_4$ precipitated fraction of the SRN extract (LHb_A). Lane 4: DEAE purified fraction following $(NH_4)_2SO_4$ precipitation (LHb_D). Lane 5: standard 2 (6.5–26.6 kDa).

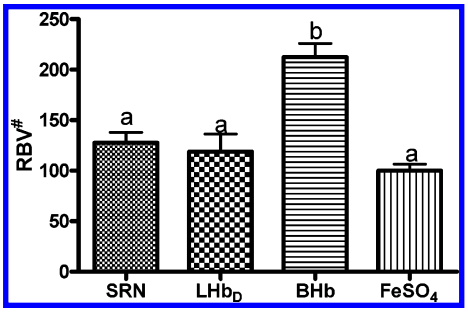

Figure 2. Iron bioavailability of SRN (soy root nodule), LHb_D (DEAE purified fraction of soy leghemoglobin), BHb (bovine hemoglobin), and $FeSO_4$ in aqueous solutions. #Relative biological value (RBV) = ng ferritin/μg cellular protein relative to the mean $FeSO_4$ value. Bars (mean ± SE, n=5) with similar letters are not significantly different ($P > 0.05$) based on ANOVA with Tukey's multiple comparison test.

weight of LHb (*6*). LHb purity increased from 21% in the SRN extract to 54% in the LHb_A and to 73% in the LHb_D extracts, respectively, as determined by optical densitometry comparing the density of the 14 kDa bands to the total lane density. No 14 kDa band was found after the pepsin digestion with SDS−PAGE for either LHb_A or BHb, suggesting globin from these proteins is completely degraded (data not shown).

The results of the iron bioavailability study using aqueous solutions of SRN, LHb_D, and BHb are shown in **Figure 2**. The relative biological values (RBV, compared to 100% with $FeSO_4$) were 28 ± 10%, 19 ± 17%, and 113 ± 13% higher than $FeSO_4$, respectively, (mean ± SEM) for SRN, LHb_D, and BHb. The iron bioavailability of BHb was 2-fold higher than all other samples ($P \leq 0.001$), but the iron bioavailability of SRN and LHb_D was similar to that of $FeSO_4$. Since the bioavailability of SRN and LHb_D was similar, LHb_A, we found no advantage using the pure fraction. Hence, the partially purified LHb_A fraction was used for tortilla fortification studies.

Unlike the previous results without food matrix, the RBV for 50 ppm fortified tortillas with SRN was 19% lower than that of $FeSO_4$, but was not significantly different **(Figure 3)**. The LHb_A and BHb tortillas exhibited 27 ± 6% and 33 ± 10% higher bioavailability than $FeSO_4$ ($P < 0.05$) and with no difference between them. Although based on dry weight, total iron content varied for SRN, LHb_A, and BHb (1.42, 1.7, and 2.9 mg/g, respectively), and weight adjustment provided equal amounts of total iron in all the treatments prior to in vitro

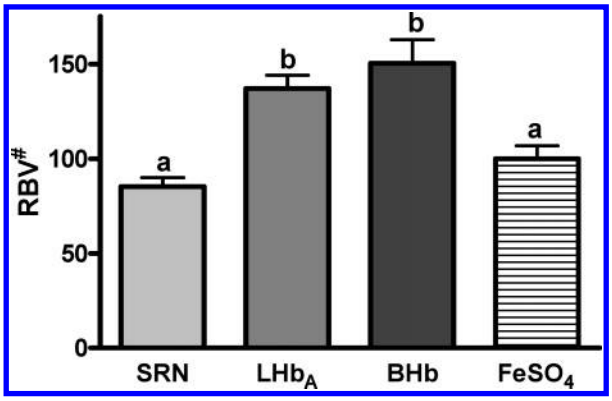

Figure 3. Iron bioavailability of tortillas fortified with SRN (soy root nodule), LHb_A ($(NH_4)_2SO_4$ purified soy leghemoglobin), BHb (bovine hemoglobin), and $FeSO_4$ with 50 ppm iron. #Relative biological value (RBV) = ng ferritin/μg cellular protein relative to the mean $FeSO_4$ value. Bars (mean ± SE, n = 9–10) with similar letters are not significantly different ($P > 0.05$) based on ANOVA with Tukey's multiple comparison test.

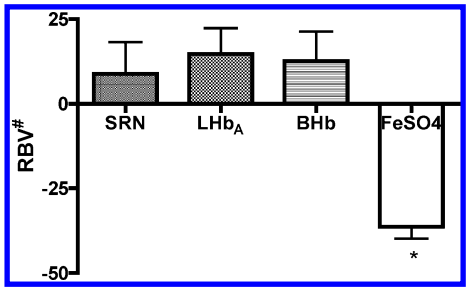

Figure 4. Effect of heat treatment on iron bioavailability of fortified tortillas (heated − not heated). Bioavailability was measured from iron sources added before (heated) and after making tortillas (not heated). #Relative biological value (RBV) = ng ferritin/μg cellular protein relative to the respective $FeSO_4$ value ran simultaneously with each treatment. Student t test was used to compare heated and not heated treatments (mean ± SE, n = 9–10) for each iron source. *$P < 0.05$. SRN = soy root nodule extract, LHb_A = $(NH_4)_2SO_4$ purified soy leghemoglobin, BHb = bovine hemoglobin.

digestion. The heme iron content also varied based on dry weight basis, 1.0, 1.4, and 2.3 mg/g for SRN, LHb_A, and BHb, respectively, but the 74−83% of added iron was in heme form in all the treatments.

Heat displayed no significant impact on bioavailability other than on $FeSO_4$ bioavailability **(Figure 4)**. The bioavailability of $FeSO_4$ was 36 ± 6% lower in samples fortified before cooking ($P < 0.001$), indicating that iron bioavailability was decreased by heating the fortificant during cooking. Although not significant, SRN, LHb, and BHb showed increases in bioavailability with heating. Nonheme iron bioavailability was not affected by adding heme iron from the LHb_A at any concentration raging from 25 to 75% (data not shown).

Heme iron bioavailability has long been known to have higher bioavailability (18−29%) than nonheme iron (<10%) (*17*), because heme iron has a different uptake pathway than nonheme iron. Heme is released from the globin protein and iron is absorbed intact with porphyrin into the mucosal cells (*18*). High bioavailability of heme can be partly attributed to this different pathway and partly to the lack of inhibition from dietary factors such as phytate and polyphenols which strongly inhibit nonheme iron absorption (*2*, *17*).

Currently heme iron in the human diet is almost exclusively from animal sources, and its intake has been shown to have a positive correlation with iron status (*19*, *20*). Indeed, one major

recommendation for improving iron status in populations is to incorporate sources of heme iron in the diet because of its high iron bioavailability (*21*). However, the incorporation of animal-sourced heme iron is often unfeasible because of economic costs or because cultural and religious barriers forbid the consumption of meat in populations where iron deficiency is prevalent.

While it has long been known that plants produce heme proteins, they have not been extensively studied in human iron nutrition. A recent rat hemoglobin repletion study by one of the authors showed a bioavailability of 59% with SRN compared to $FeSO_4$ which was similar to a 60% bioavailability with BHb (*8*). Unlike human studies, the lower bioavailability of BHb compared to $FeSO_4$ raises some concern of this model for measuring heme iron absorption; however, the similarity in bioavailability between LHb and BHb promoted this current study. Since the rat is not shown to be the most reliable model to assess human bioavailability (*22*), we have used a cell culture model to further evaluate LHb iron bioavailability studies. The Caco-2 cell model is appealing because of its low cost, reliability, and wide use for nonheme iron bioavailability (*9*, *10*). Interest in the use of this model for studying heme bioavailability and metabolism has been limited, but is increasing, as is described in recent studies (*11*, *12*). Heme iron absorption in Caco-2 cells was shown to be affected by hemeoxygenase induction, more importantly by iron status similar to humans (*12*). Our results showing higher bioavailability of BHb compared to $FeSO_4$ alone or with food further promote that the Caco-2 cell model may indeed be useful for evaluating heme iron bioavailability. Since iron within the epithelial cells has a similar metabolic fate regardless of its source, either heme or nonheme, ferritin may be a useful index of bioavailability, regardless of the source of the iron (*15*).

There are a number of reasons why a discrepancy exists in the bioavailability results in **Figures 2** and **3**. The first possible explanation is the difference in the iron concentrations in the uptake solutions, which was 10-fold higher in aqueous solution experiments (**Figure 2**), and the presence of solid milieu of food affecting uptake. Since we reported the values relative to $FeSO_4$, it is also important to consider how $FeSO_4$ bioavailability is affected by the food matrix when comparing the results from different experiments. Differences in $FeSO_4$ bioavailability greatly influence the RBV of the treatments. Hence, the low RBV of BHb in **Figure 3** should not be viewed as a reduction of bioavailability in the presence of food. Iron chemistry and solubility is highly dependent on the digestive milieu. Lower LHb_D bioavailability compared to that of BHb in aqueous solution (**Figure 2**) might also be attributed to the differences in globin fractions of those two proteins. Although structural homology between these two proteins is high (*5*), the amino acid sequence of bovine α or β compared to soy globin shows minimal sequence homology when compared with BLOSUM-62 (NCBI Protein-Protein BLAST). Globin protein has been shown to increase the bioavailability of heme iron (*12*), and the presence of hydrophobic peptides hydrolyzed from the globin protein during digestion is known to affect the absorption of heme iron (*23*). It is possible that globin degradation may be different between LHb and BHb and the solubility of iron may be better maintained in BHb with its globin degradation products. It is also possible that there may be a lipid enhancing effect in the BHb which is not present in the LHb. Since the BHb was derived from bovine reticulocytes, it may have trace amounts of lipids which in the aqueous environment may influence iron bioavailability (*24*). Our results suggest that LHb may not be anymore beneficial than $FeSO_4$ if it is used as a supplement. However, our goal was to assess bioavailability with food, which is more applicable for determining the use of LHb for food fortification.

The low bioavailability of SRN with tortilla may be due to the presence of nonhemoglobin fractions in the extract compared to LHb_A or BHb. Since we have to use a 30% higher amount of this fraction to get equal amounts of heme iron compared with LHb_A, its use in food fortification may be limited due to organoleptic probelms. Because of high purity, we can use a lesser amount of LHb_D to avoid acceptability problems, but it adds higher cost for preparation. However, LHb_A obtained from $(NH_4)_2SO_4$ fractionation had a reasonable level of purity as well as a bioavailability with tortillas similar to that of BHb (**Figure 3**), suggesting the usefulness of this fraction in food fortification.

The RBV of BHb decreased, from 113% to 27% of $FeSO_4$, when heme iron was fortified into tortillas compared to without food matrix. Besides the lower concentration of fortificant in this experiment, the lower bioavailability of BHb with the food matrix might be due to the influence of calcium in tortillas due to nixtamalization treatment with calcium hydroxide. Calcium has been shown to decrease the bioavailability of heme iron bioavailability as well as nonheme iron (*25*, *26*). Another explanation might be that $FeSO_4$ availability is low in aqueous solution due to its low solubility at neutral pH without any chelating agents. Since the results are expressed as RBV to $FeSO_4$, the decrease in bioavailability of BHb should be viewed as higher bioavailability of $FeSO_4$ in the presence of tortillas compared to without food matrix. Unlike the results in **Figure 2**, BHb and LHb bioavailability is similar in **Figure 3**, suggesting that there might be differences in iron solubility or the effect of calcium.

Ferrous sulfate is considered an ideal positive control for bioavailability assessment because of its high bioavailability in most food products. However it is not practical for maize foods fortification because of the adverse organoleptic effects caused by $FeSO_4$ catalyzed lipid peroxidation and changes in color of the food itself (*27*). As such, $FeSO_4$ is not currently recommended for maize foods fortification (*28*), but our results with LHb_A showing bioavailability higher than $FeSO_4$ with food offer a promising new alternative iron fortification scheme.

The effect of heat on the bioavailability of heme iron sources is minimal, unlike $FeSO_4$ which decreases in bioavailability during heating. Although not significant, the heat treatment tends to improve the bioavailability of heme iron, which may be due to denaturation of the globin proteins and enhancement of hydrophilic interactions. However, the decrease in $FeSO_4$ bioavailability during heating might be due to interaction of iron with maillard browning products (*29*) or from oxidation of ferrous iron to the less bioavailable ferric form (*30*). It appears that the heme pyrolle provides a protective effect against the heat effect.

No beneficial effect of heme/nonheme iron combinations on bioavailability, compared to both irons alone at the same concentration, was shown in our study, suggesting that heme does not enhance the bioavailability of nonheme iron. Therefore, the positive effects of meat on nonheme iron absorption may be due to factors in meat other than the hemoglobin fraction. The enhancing effect of animal tissue on nonheme iron bioavailability was generally attributed to the "meat factors" rather than hemoglobin (*31*−*32*).

Our results showing the bioavailability of hemoglobin from soy root nodules similar to that of heme iron from animal sources when added with tortillas provide a unique alternative fortificant, hence to improve the iron status of the population.

However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used $FeSO_4$. Because of the known minimal inhibition of heme iron, unlike nonheme iron, by dietary factors and because of the high heat stability of this iron form, plant hemoglobins may have great potential to be used as a fortificant for improving nutrition. Soy root nodules have no current use in agriculture production and as such may provide a novel value-added product for soy producers. Given optimal nodulation conditions, 2−3 plants would provide sufficient LHb to meet 25% of the recommended daily requirement of iron, assuming 25% of heme iron absorption. The use of plant hemoglobins in iron nutrition warrants further study of sensory and safety issues related to fortification to make this a feasible reality.

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