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

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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 an anaerobic environment, and therefore, the plant produces a 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 exhibiting 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.
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 extraction was prepared by reconstitution of dried nodule powder with water 1:5 w/v, followed by centrifugation (5000g 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 with Hi Stick Prep (GE Healthcare, Piscataway, NJ) to further purify the LHb obtained from (NH4)2SO4 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 with water. The desalted protein extract was lyophilized and stored at -20 °C for further use. Ion exchange chromatography with DEAE Sepharose (GE Healthcare) was used for potatoes in the prior two growing seasons. Seed was inoculated with Durisco’s Modified Eagle 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% CO2. 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 × 104 cells/cm² in a 75 cm² 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 × 104 cells/cm² 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 FeSO4, 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 using Caco-2 cells as described above.

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
purified fraction of soy leghemoglobin), BHb (bovine hemoglobin), and fraction of the SRN extract (LHb A ). Lane 4: DEAE purified fraction following (NH₄)₂SO₄ precipitation (LHb D ). Lane 5: standard 2 (6.5–26.6 kDa).

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% (NH₄)₂SO₄ precipitated fraction of the SRN extract (LHb A ). Lane 4: DEAE purified fraction following (NH₄)₂SO₄ precipitation (LHb D ). Lane 5: standard 2 (6.5–26.6 kDa).

weight of LHb (6). LHb purity increased from 21% in the SRN extract to 54% in the LHbD and to 73% in the LHbD 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 LHbA 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, LHbD, and BHb are shown in Figure 2. The relative biological values (RBV) compared to 100% with FeSO₄ were 28 ± 10%, 19 ± 17%, and 113 ± 13% higher than FeSO₄, respectively, (mean ± SEM) for SRN, LHbA, and BHb. The iron bioavailability of BHb was 2-fold higher than all other samples (P ≤ 0.001), but the iron bioavailability of SRN and LHbD was similar to that of FeSO₄. Since the bioavailability of SRN and LHbD was similar, LHbA, we found no advantage using the pure fraction. Hence, the partially purified LHbA 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 FeSO₄, but was not significantly different (Figure 3). The LHbA and BHb tortillas exhibited 27 ± 6% and 33 ± 10% higher bioavailability than FeSO₄ (P < 0.05) and with no difference between them. Although based on dry weight, total iron content varied for SRN, LHbA, and BHb (1.42, 1.7, and 2.3 mg/g, respectively), and weight adjustment provided equal amounts of total iron in all the treatments prior to in vitro digestion. The heme iron content also varied based on dry weight basis, 1.0, 1.4, and 2.3 mg/g for SRN, LHbA, 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 FeSO₄ bioavailability (Figure 4). The bioavailability of FeSO₄ 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 LHbD at any concentration ranging 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. 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 FeSO₄ which was similar to a 60% bioavailability with BHb. Unlike human studies, the lower bioavailability of BHb compared to FeSO₄ 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, 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. 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. Heme iron absorption in Caco-2 cells was shown to be affected by hemeoxygenase induction, more importantly by iron status similar to humans. Our results showing higher bioavailability of BHb compared to FeSO₄ 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.

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 FeSO₄, it is also important to consider how FeSO₄ bioavailability is affected by the food matrix when comparing the results from different experiments. Differences in FeSO₄ bioavailability greatly influence 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 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 BLAST (ncbi Protein-Protein BLAST). Globin protein has been shown to increase the bioavailability of heme iron, and the presence of hydrophobic peptides hydrolyzed from the globin protein during digestion is known to affect the absorption of heme iron. 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. Our results suggest that LHb may not be anymore beneficial than FeSO₄ 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 LHbA or BHb. Since we have to use a 30% higher amount of this fraction to get equal amounts of heme iron compared with LHbA, its use in food fortification may be limited due to organoleptic problems. Because of high purity, we can use a lesser amount of LHbA to avoid acceptability problems, but it adds higher cost for preparation. However, LHbA obtained from (NH₄)₂SO₄ fractionation had a reasonable level of purity as well as biological activity 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 FeSO₄, 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 FeSO₄ 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 FeSO₄, the decrease in bioavailability of BHb should be viewed as higher bioavailability of FeSO₄ 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 FeSO₄ catalyzed lipid peroxidation and changes in color of the food itself. As such, FeSO₄ is not currently recommended for maize foods fortification, but our results with LHbA showing bioavailability higher than FeSO₄ with food offer a promising new alternative iron fortification scheme.

The effect of heat on the bioavailability of heme iron sources is minimal, unlike FeSO₄ 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 FeSO₄ 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 pyrrole provides a protective effect against the heat effect.

No beneficial effect of heme/nonheme iron combinations on bioavailability, compared to both iron 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 FeSO₄. However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used FeSO₄. However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used FeSO₄. However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used FeSO₄. However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used FeSO₄.

LITERATURE CITED


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