Histidine Content of Low-Molecular-Weight Beef Proteins Influences Nonheme Iron Bioavailability in Caco-2 Cells

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ABSTRACT The objective of this study was to isolate and characterize beef muscle proteins that enhance nonheme iron bioavailability. Beef sirloin was cooked, lyophilized and reconstituted with water before in vitro digestion. After centrifugation, the digest supernatant was sequentially ultrafiltered using 10- and 1-kDa molecular weight cut-off membranes. Nonheme iron bioavailability was assessed by Caco-2 cell monolayer 59Fe uptake using an extrinsic labeling method. All ultrafiltration fractions significantly (P < 0.001) increased iron solubility at pH 6.0, compared with the blank. However, iron uptake was significantly (P < 0.001) greater than the blank only in the presence of the 1-kDa retentate (1KR). Therefore, the 1KR was chosen for further analysis. Immobilized metal affinity chromatography (IMAC) of the 1KR yielded four fractions, i.e., three distinct fractions (F1, F3, F4) and one fraction (F2) comprised of a few closely associated peaks. All four IMAC fractions resulted in significantly (P < 0.001) greater (two- to fivefold) iron solubility at pH 6.0, compared with the blank. Iron uptake with F2 and F4 was significantly greater than the blank (P < 0.001 and P < 0.05, respectively). Gel electrophoresis and matrix-assisted laser desorption/ionization analysis illustrated that histidine concentration increased progressively from F1 to F4, corresponding to a general, but not parallel increase in iron solubility and uptake. Our results suggest that the enhancement of nonheme iron absorption by beef may be due to peptides produced during gastrointestinal digestion and that histidine content may be important.

KEY WORDS: beef proteins • nonheme iron • bioavailability • Caco-2 cells • histidine

Iron absorption in humans depends on an individual's iron status (1), iron intake (2) and its bioavailability (3,4). Nonheme iron typically constitutes the major fraction of daily iron intake; unlike heme iron, however, its absorption is influenced by various dietary components (4–8). Many plant factors, such as phytate (9–11), polyphenols (9,12) and soy protein (13,14) inhibit, whereas some animal tissues (5,15–16) and ascorbic acid (17,18) enhance nonheme iron absorption in humans.

The enhancing effect of meat is attributed to unknown factor(s), usually referred to as “meat factor(s)”; these may also maintain iron in a soluble form, thereby increasing its bioavailability (4). Not all animal proteins have the same enhancing effect. For example, beef, lamb, liver, pork, poultry and fish enhance nonheme iron absorption, but egg, cheese, milk and ovalbumin proteins do not (5,15,19,20). The influence of meat on nonheme iron absorption was attributed initially to the stimulation of gastric acid secretion (21), but there is no strong evidence for this effect. Reducing components (22), stearic acid (23,24), certain amino acids (25–28) and peptides released during proteolytic digestion (29,30) have also been suggested to play a role in the enhancement of nonheme iron absorption.

Data concerning the factor(s) in meat responsible for the enhancement of nonheme iron absorption are inconclusive, possibly because of differences in the methodologies employed. In vivo, rat models have been used to study the influence of meat on nonheme iron absorption (31), but extrapolation of data from rats to humans is questionable because some factors shown to influence nonheme iron absorption in humans have been shown to have only a marginal or no effect in rats (9).

Caco-2 cell iron uptake is of increasing interest in nutritional studies assessing iron bioavailability. Caco-2 cells spontaneously differentiate into polarized monolayers with well-developed brush borders and exhibit many of the morphological and biochemical characteristics of enterocytes (32). Caco-2 cell iron uptake is a physiologic measure of iron absorption and has been shown to correlate well with human iron absorption when many dietary factors are tested (33). Recent studies using this cell line to study iron bioavailability,
including the enhancing effect of meat on nonheme iron uptake (33–36), prompted us to use this in vitro model to assist in isolating and characterizing beef proteins responsible for enhancing nonheme iron bioavailability.

**MATERIALS AND METHODS**

All of the enzymes and chemicals were obtained from Sigma Chemical (St. Louis, MO) unless noted otherwise. Deionized water was used throughout the study unless noted otherwise.

**Sample preparation.** A total of 1.5 kg of lean beef, top sirloin steak, was obtained fresh from a local supermarket. All visible fat and connective tissues were removed; the remaining lean meat was cut into small, thin pieces, placed in 450 mL boiling water, and simmered for 30 min. After overnight chilling, all visible fat was removed and the sample homogenized in a blender (Osterizer, Sunbeam-Oster Household Products, Laurel, MS) for 5 min. The slurry was then rehomogenized using a Polytron PT-3000 Homogenizer (PT-DA 3012/2S generator; Brinkmann Instruments, Westbury, NY) for 5 min at 25,000 rpm. The slurry was lyophilized in Ziploc bags and stored under nitrogen at −80°C. Total protein of the powder was determined by a micro-Kjeldahl procedure (37). Total iron was measured by atomic absorption spectrophotometry (Allied Analytical Systems, Waltham, MA) after dry-ashing. Nonheme iron in the beef powder was determined by the modified method of Torrance and Bothwell (38) using ferrozine instead of bathophenanthroline disulfonic acid as the ferrous chromogen.

**In vitro digestion.** Lyophilized beef powder (20 g) was rehydrated with 110 mL water and the resulting slurry homogenized using a Polytron Homogenizer (PT-DA 3007/2 generator; Brinkmann Instruments) for 45 s at 18,000 rpm. The pH of the homogenate was slowly adjusted to 2.0 with 5 mol/L HCl (Fisher Scientific, Fair Lawn, NJ) and 4 mL pepsin solution [80 g pepsin/L (2800 U/mg protein) in 0.1 mol/L HCl] was added before incubation for 1 h at 37°C in a shaking water bath (Precision Scientific, Chicago, IL) at 90 rpm to simulate gastric digestion. After incubation, the pH was slowly raised to 6.0 by the dropwise addition of NaHCO₃ (1 mol/L), and 20 mL pancreatin solution [4 g pancreatin (4 × USP activity)/L in 0.1 mol/L NaHCO₃] was added. The sample was then incubated for 30 min at 37°C in a shaking water bath at 90 rpm to mimic duodenal digestion. The homogenate was then removed from the water bath, chilled on ice and centrifuged at 10,200 × g, 4°C for 30 min (Beckman Instruments, Palo Alto, CA) to collect the digest supernatant (DS). Protein (39) and nonheme iron concentration (38) of the digest supernatant were measured.

**Ultrafiltration.** The digest supernatant was subjected to sequential ultrafiltration in an Amicon stirred cell unit (Model 8200, SUC 200 mL; Amicon, Beverly, MA) above a magnetic stirrer using 10-kDa molecular weight cut-off (MWCO) membrane disks first followed by 1-kDa (Omega TM) low protein-binding modified polyethersulfone; Pall/Gelman Sciences, Ann Arbor, MI) at 4°C under nitrogen at 0.38 MPa. Preliminary studies showed that the use of a 5-kDa membrane was unnecessary because a minimal amount of protein was retained in the 5-kDa retentate fraction (unpublished data). To achieve sufficient filtration, 150 mL nanopure, analytical grade, 18.0 MΩ-cm, water (Barnstead/Thermolyne, Dubuque, IA), was added to resuspend the remaining solution after 90% of the total volume of each solution had filtered. This was repeated three times per membrane. The three fractions [10-kDa retentate (10KR), 1-kDa retentate (1KR) and 1-kDa filtrate (1KF)] collected were stored under nitrogen at −80°C.

**Im mobilized metal affinity chromatography (IMAC).** The procedure is similar to that described by Lönnertal et al. (40) to purify lactoferrin using chelating sepharose consisting of highly cross-linked agarose beads coupled to imino-diaceitic acid (Amersham Pharmacia Biotech, Piscataway, NJ). The column (10 × 2.5 cm) was charged with 0.1 mol/L CuSO₄ (IMAC, immobilized metal affinity chromatography; MALDI, matrix-assisted laser desorption/ionization analysis; MES, 2-[4-morpholino]-ethane sulfonic acid; MWCO, molecular weight cut-off; NTA, ferric:nitrilotriacetic acid (1:5 molar ratio); W, IMAC wash; 1KF, 1-kDa MWCO membrane filtrate; 1KR, 1-kDa MWCO membrane retentate; 10KR, 10-kDa MWCO membrane retentate.

**Trace metal removal.** The IMAC wash and eluent fractions were made metal free with Chelex-100 (200–400 mesh, sodium form; Bio-Rad Laboratories, Hercules, CA) resin treatment before electrophoresis and amino acid composition analysis to aid in separation and because even trace amounts of metals may interfere with the detection of some amino acids, respectively.

**Concentration and desalting.** Microsep™-1-kDa MWCO centrifugal devices (Omega TM, low protein–binding modified polyether sulfone; Pall/Gelman Sciences) were used to simultaneously concentrate and desalt the IMAC wash and eluent fractions. Samples were centrifuged at 7500 rpm for 30 min and the supernatants collected were stored under nitrogen at −80°C.

**Cell culture.** Caco-2 cells were purchased at passage 17 from American Type Culture Collection (Rockville, MD). Experiments were conducted at passages 33–36. Cells were grown in Dulbecco’s modified Eagle medium with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acids (Gibco BRL, Grand Island, NY), and 1% (v/v) antibiotic-antimycotic solution (Gibco BRL). Cells were maintained at 37°C in an air incubator with 5% CO₂. The media were changed every 3 d. At 90–100% confluence, the cells were rinsed with Earle’s balanced salt solution (EBSS; Gibco BRL), dissociated using Trypsin-EDTA (Gibco BRL), and centrifuged for 6 min at 22.6 × g (Fisher Scientific). Cells were then seeded in a 75-cm² culture flask (Corning Costar, Cambridge, MA) at a density of 5.6 × 10⁶ cells/cm² for continued growth. For iron uptake experiments, cells were seeded onto 24-well (15.5-mm diameter) cluster plates (Corning Costar) treated (41) with collagen (Type 1 Rat Tail Collagen; Collaborative Biomedical Products, Bedford, MA) at a density of 5 × 10⁶ cells/cm². Cells were used for iron uptake experiments at 15 d postseeding. Protein concentration was determined (39) in the cells after solubilization (0.5 mol/L NaOH; Fisher Scientific) and sonication of the monolayers.

**59Fe cell uptake procedures.** Uptake solutions were prepared in EBSS with 10 mmol 2-[4-morpholino]-ethane sulfonic acid (MES; Fisher Scientific) buffer at pH 6.0 immediately before use. Uptake solutions containing 20 μmol/L iron (FeCl₃ · 6H₂O) were prepared using EBSS/MES with ascorbic acid (ASC) (ferri:ascorbate; 1:20 molar ratio), nitrilotriacetic acid (NTA) (ferric:NTA; 1:5 molar ratio), no added ligand (blank) or test solutions (1 or 2 g of beef protein/L). The ASC served as a positive, NTA as a negative control and FeCl₃ as a blank. Test samples refer to DS, 10KR, 1KR, 1KF and IMAC wash (W) or eluent fractions (F1–F4). A trace amount (0.48 ng: 277.5 Bq of 59Fe in 10 mmol/L HCl) of radioactive iron (59FeCl₃ · DuPont/NEN, Boston, MA) was used in each of the uptake solutions. The radioisotope was added to the 20 μmol/L iron at pH 2.0; buffer (pH 6.0) was added, followed by the addition of the sample containing the protein. This allowed the radioisotope to exchange with cold iron at acidic pH. Uptake solutions were then immediately vortexed and used in the cell uptake procedures. Measurement of radioiron uptake was performed by modification of the method outlined by Glahn and Van Campen (28) with the exception of using EBSS/MES at pH 6.0 instead of Hank’s balanced salt solution/piperazine-N,N’-bis-[2-ethanesulfonic acid] (HBSS/PIPES) at pH 6.7. Fifteen days after seeding, growth medium was removed from each well and the cell monolayers were rinsed twice with 500 μL of EBSS/MES at 37°C and pH 6.0. A 300-μL aliquot of cell uptake solution containing 59Fe was then placed on the cell monolayer and another 300-μL aliquot was used to measure initial radioactivity. The monolayers were then placed in an incubator at
37°C with 5% CO₂ for 1 h. After incubation, iron uptake was terminated by removing the uptake solution and immediately rinsing the monolayers three times with 500 μL of stop solution [140 mmol/L NaCl, 5 mmol/L KCl (Fisher Scientific) and 10 mmol/L MES] at 20°C and pH 6.0. Nonspecifically bound iron was removed using the method described by Glahn and Van Campen (28) by applying a 500-μL volume of removal solution (stop solution plus 1 mmol/L bathophenanthroline disulfonic acid and 5 mmol/L sodium hydrosulfite) at 20°C and pH 6.0 to each well for 10 min. The cells were then rinsed again twice with 500 μL of stop solution. Monolayers were solubilized in 1 mL of 0.5 mol/L NaOH and placed along with other solutions collected in scintillation tubes for 59Fe counting using a gamma scintillation counter (Auto-Gamma™, Packard Instrument Company, Meriden, CT).

**Solubility assays.** Iron solubility was also measured in each uptake solution. After 300 μL was applied to the cell monolayer, 1.2 mL of each cell uptake solution containing 59Fe was placed into a microcentrifuge tube and incubated for 30 min at room temperature. Samples were then centrifuged for 15 min at 15,000 × g using a microcentrifuge (Brinkmann Instruments). A 500-μL portion of the supernatant was used for radioisotope counting. The percentage of iron solubility was calculated with respect to iron (20 μmol/L FeCl₃ in 10 mmol/L HCl) solubility at pH 2.0.

**Electrophoresis.** Separation of proteins and peptides present in digest supernatant, ultrafiltration fractions and the IMAC eluent fractions was performed by SDS-PAGE using Tris/Tricine/SDS buffer (Bio-Rad). A prestained gel consisting of 10–20% Tris-Tricine (NOVEX, San Diego, CA) was used for the DS and ultrafiltration fractions (1KR and 10KR). A prestained gel consisting of 16.5% Tris-Tricine (Bio-Rad) was used for the IMAC eluent fractions (F1–F4) to facilitate detection and separation of the smaller molecular weight peptides. Low range (Bio-Rad) and ultra-low range SDS-PAGE molecular weight markers were used for molecular weight estimations ranging from 1 to 100-kDa. Gels were fixed with 5% (v/v) glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) before staining with 0.25% (v/v) Coomassie Brilliant Blue G-250 (Eastman Kodak, Rochester, NY) in 10% (v/v) acetic acid (Fisher Scientific) for 1 h. Destaining was done using 10% (v/v) acetic acid (Fisher Scientific).

**Matrix-assisted laser desorption/ionization (MALDI) analysis.** The MALDI analysis was performed to determine the molecular weight(s) of components in the IMAC eluent fractions (F1–F4). Test solutions were comprised of 1 μL of sample solution (50 pmol protein/μL) mixed thoroughly with 24 μL of matrix solution (10 μg α-cyano-4-hydroxycinnamic acid/L). The test solution (1 μL) was applied to the target region of the sample grid and allowed to crystallize before MALDI analysis using a Voyager-DE PRO (PerSeptive Biosystems/Perkin Elmer, Foster City, CA), which is a linear time-of-flight mass spectrometer. Spectra were acquired at 50 shots/spectrum and are presented as mass/charge at a range of 0.5 to 20-kDa (Data Explorer software, version 3.4; PerSeptive Biosystems, Inc.).

**Amino acid composition analysis.** The complete amino acid composition of the IMAC wash (W) and eluent fractions (F1–F4) was determined at the University of Iowa (Molecular Analysis Facility, Iowa City, IA) by performic acid oxidation and hydrochloric acid hydrolysis before anion-exchange analysis using 2–4 g proteinate/L. Norleucine was added to each sample as an internal standard. Ion-exchange analysis (Beckman Instruments) was done with postcolumn derivatization using ninhydrin with separation on a 12-cm hydrolysate column using sodium citrate buffer in combination with a 3-step temperature program.

**Statistical analysis.** Data were analyzed using one-way ANOVA and each group mean was compared to the blank using Dunnett’s multiple comparison test (Prism software, version 3.02; GraphPad Software, San Diego, CA). The mean differences were considered significant at P ≤ 0.05. Unless otherwise noted, values are expressed as ratio means ± SEM.

**RESULTS**

The cooked, lyophilized beef powder contained 90.0 ± 0.2% (n = 3) protein, 89.7 ± 3.4 μg/g (n = 2) total iron and 32.7 ± 5.5 μg/g (n = 2) nonheme iron. Enzymatic digestion of the beef powder resulted in solubilization of 65% of the protein and 42% of the nonheme iron. Gel electrophoresis confirmed that ultrafiltration using 10- and 1-kDa MWCO membranes was effective in separating DS proteins according to their molecular weight range (Fig. 1). The peptides contained in the 1KF fraction were not detectable on the gel (data not shown).

In Figure 2, values are presented as ratios to the blank containing iron alone. Radioisotopy solubility and uptake for the ASC and NTA controls and test solutions (2 g protein/L) from the DS and ultrafiltration fractions (1KR, 1KR and 1KF) are shown. The iron solubility at pH 6.0 was significantly higher (10- to 11-fold; P < 0.001) than the blank by the addition of ASC or NTA. Iron solubility in the presence of DS and the ultrafiltration fractions was also significantly higher (3- to 8-fold; P < 0.001) than the blank. Among the ultrafiltration fractions, the 1KR maintained a fivefold higher iron solubility. Iron uptake by the cells in the presence of ASC was 3.5 times higher (P < 0.001) and in the presence of NTA was one third that of the blank (P < 0.05). Of the ultrafiltration fractions, iron uptake was significantly (P < 0.001) greater than the blank only in the presence of the 1KR. Therefore, the 1KR was chosen for further analysis.

Analysis of the 1KR by IMAC yielded four fractions, i.e., three distinct fractions (F1, F3, F4) and one comprised of a few closely associated peaks (F2). The elution profile is shown in Figure 3. Iron solubility and uptake in the presence of the IMAC wash (W) and F1–F4 are shown in Figure 4; values are also presented as ratios to the blank containing iron alone. Due to low yield, only 1 g protein/L was used for the W and F1–F4 test solutions. The W and F1–F4 all conferred significantly (P < 0.001) greater iron solubility than the blank (~2- to 5-fold). Iron uptake in the presence of F2 and F4 increased significantly relative to the blank (P < 0.001 and P < 0.05, respectively), whereas it significantly (P < 0.001) decreased in the presence of W, assuring us that this fraction did not possess proteins that are capable of enhancing mucosal iron uptake. Although a 1.3-fold higher iron uptake was found with F3, the effect was not significantly different from the blank. No significant effect on iron uptake was found with F1.
Electrophoretic separation of F1–F4 revealed that each of F1–F4 contains a heterogeneous mixture of proteins/peptides with molecular weights ranging from 1 to 7 kDa (Fig. 5). To further distinguish proteins in each fraction, MALDI analysis was performed (Fig. 6). The results show that there are many proteins/peptides in each sample; they differ minimally in size, with molecular weights <7 kDa. Table 1 shows the amino acid composition, grouped according to charge and polarity of W and F1–F4. Because the elution profile was identical in each IMAC run, we pooled the samples from many (~10) different runs. The analyses revealed that histidine concentration of W further distinguish proteins in each fraction, MALDI analysis was performed (Fig. 6). The results show that there are many proteins/peptides with molecular weights ranging from ~1 to 7 kDa (Fig. 5). To
was 3.1 to 6.9% that of F2–F4 and that it increased progressively from F1 to F4, ranging from 1.2 to 16.0 mol/100 mol. Figure 7 shows the relationship between iron uptake and histidine content in W and F1–F4. The increase in iron solubility (Fig. 4) was generally consistent with the increase in histidine (Fig. 7) content of the IMAC samples with the exception of W. The increase in iron uptake was positively related with histidine content from W to F2, but reached a plateau from F3 to F4. No relationship was found with iron solubility and/or uptake and cysteine content, which was approximately threefold greater in F1 than in W and F2–F4. The glutamine and/or glutamic acid content was lower in F1–F4 than W and the profiles illustrated a weak trend with a progressive decrease of glutamine in F1–F4. A weak trend was also found in lysine content, which generally increased from F1 to F4 and was 13–81% greater than in W. The difference in other amino acids was not consistent among the fractions.

DISCUSSION

Identification of the factor(s) in meat that enhance nonheme iron absorption has been difficult because performing human studies for this type of investigation is often not an option due to the inconvenience and cost. Rat models are appealing for in vivo studies (31,42), but extrapolation of iron absorption data from rats to humans is questionable because meat has shown no effect on nonheme iron absorption in rats, unlike in humans (8). Iron uptake by Caco-2 cells offers a physiologic means to measure iron absorption, and nonheme iron uptake by the cells has shown significant correlation with human iron absorption when many dietary factors are tested (33). Based on the reliability of the Caco-2 cell system, we

<table>
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<th>F2</th>
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\(^1\) Amino acid analysis was performed only once on the pooled samples collected from –10 immobilized metal affinity chromatography (IMAC) runs with identical elution profiles, for each of IMAC W and F1–F4. Amino acid analysis was performed by performic acid oxidation and hydrochloric acid hydrolysis before anion-exchange analysis that involved postcolumn derivatization using ninhydrin with separation on a column using sodium citrate buffer. Detailed procedure is provided in Materials and Methods section.

\(^2\) Asx, asparagine and/or aspartic acid.

\(^3\) Glx, glutamine and/or glutamic acid.

\(^4\) Methionine sulfoxide.

\(^5\) Cysteic acid.
used this in vitro model to identify beef proteins that enhance nonheme iron bioavailability.

Although solubility is useful in predicting the enhancement of iron absorption (4,30), our data show that iron solubility alone does not reliably predict its uptake or bioavailability. For example, the solubility of iron in the presence of NTAs was 10% higher than ASC, but iron uptake by Caco-2 cells was 90% lower with NTAs compared with ASC. Because NTAs is a strong chelator of nonheme iron, it maintains iron solubility but does not donate its iron to the intestinal mucosa, whereas ASC solubilizes iron by reducing and/or forming a complex with iron, making it available for mucosal uptake. Therefore, the affinity of a compound for iron and the type of complex formed may be equally important in determining the effect on nonheme iron absorption. If bound tightly, donation of iron to the mucosal cell may not occur, whereas if weakly bound, iron may dissociate and precipitate at the pH of the small intestine. Many factors, including chelating and solubilizing components (22,43) and stearic acid (23,24), have been suggested to be responsible for the enhancement of iron absorption by meat. The effect of meat on nonheme iron absorption may be due to peptides released during proteolytic digestion, which are hypothesized to increase the solubility of inorganic iron (29,30). On the contrary, others have found that the factor in meat responsible for enhanced nonheme iron absorption may not be related to gastrointestinal digestion (44). Preliminary data in our laboratory (data not shown) suggested that although undigested meat may possess factor(s) capable of solubilizing iron, the concentration of their counterparts in the digested sample is much greater. Therefore, we utilized proteolytic digestion because it more closely simulates the physiologic condition and increases the yield of proteins of interest.

We used copper as a ligand in IMAC because ferric iron forms polymers and precipitates within the optimal pH range in which most proteins adsorb to the IMAC gel. Copper was used as a ligand in an earlier study to purify lactoferrin, an iron-binding protein (47) and transferrin (48); substitutions for these amino acids have been shown to be involved in iron binding in the active binding sites of a number of iron-containing enzymes and proteins of biological importance and often participate with other amino acids, such as lysine, within these sites. For example, interactions between histidine and lysine have been shown to be involved in iron binding in lactoferrin (47) and transferrin (48); substitutions for these amino acids decrease the affinity of these proteins for iron.

Among other amino acids, cysteine and reduced N-terminal cysteinyl-peptides were reported to have a positive effect on iron absorption in humans (27,28,34). However, our data do not support the finding that cysteine enhances nonheme iron absorption. Compared with F2–F4, the cysteine content of F1 was threefold higher, but iron uptake in the presence of F1 was at most 60% lower. Methodological differences may be responsible for the differences among the studies.

In conclusion, although our data do not confirm that histidine is solely responsible for increasing nonheme iron uptake by Caco-2 cells, our results suggest that the enhancing effect of beef on nonheme iron absorption may be due to a low-molecular-weight peptides produced during gastrointestinal digestion and that histidine content may be one of the important factors. Further studies to determine how histidine may play a role in the enhancement of nonheme iron absorption and whether its influence depends on its association with other amino acids would be useful to understand the mechanism involved in the enhancement of nonheme iron absorption by meat.

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LITERATURE CITED


