Quantification of Ferritin from Staple Food Crops

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Ferritin—iron has been shown to be as bioavailable as ferrous sulfate in humans. Thus, biofortification to breed crops with high ferritin content is a promising strategy to alleviate the global iron deficiency problem. Although ferritin is present in all food crops, its concentration varies between species and varieties. Therefore, a successful ferritin biofortification strategy requires a method to rapidly measure ferritin concentrations in food crops. The objective of this study was to develop a simple and reliable ELISA using an anti-ferritin polyclonal antibody to detect ferritin in various crops. Crude seed extracts were found to have 10.2 ± 1.0, 4.38 ± 0.9, 1.2 ± 0.3, 0.38 ± 0.1, and 0.04 ± 0.01 μg of ferritin/g of dry seed in red beans, white beans, wheat, maize, and brown rice, respectively. Although the measured absolute concentrations of ferritin values were low, the presented method is applicable for rapid screening for the relative ferritin concentrations of large numbers of seeds to identify and breed ferritin-rich crops.

KEYWORDS: Ferritin; iron bioavailability; ELISA; staple crops

INTRODUCTION

Iron deficiency anemia (IDA) afflicts an estimated 2 billion people worldwide (*1*) and causes an array of serious adverse health outcomes with socioeconomic implications (*2*). Inadequate dietary intakes as well as low bioavailability of iron are the major causes of IDA (*3*). In many populations, inadequate iron intake is a result of a lack of dietary diversity and overreliance on staple food crops. This is a problem because staple food crops contain relatively low levels of nonheme iron and high levels of dietary factors such as phytic acid and polyphenols that inhibit human iron absorption (*4*).

Iron supplementation and food fortification programs have been implemented to combat IDA (*5*). However, these programs are challenging to sustain due to the lack of infrastructure, economics, and centralized facilities (*3*). Biofortification, the use of conventional breeding techniques or genetic engineering to enhance the micronutrient content of staple food crops, is thought to be a sustainable and cost-effective strategy to complement these intervention programs (*6*). Biofortification techniques are currently being used for the generation of staple crops (beans, rice, maize, and wheat) with enhanced levels of iron and/or increased bioavailable iron (*7*). The main current strategy is to increase total iron content, and increasing ferritin and reducing phytate concentrations are also possible strategies but not practical to pursue at this time (*8, 9*). Iron-biofortified

rice was the first to be examined for efficacy and was reported to improve the iron stores of nonanemic Filipino women (*10, 11*).

Ferritin is a stable iron storage protein consisting of a 24-subunit shell around a 4500-atom iron core (*12*). The protein has a molecular mass ranging from 460 kDa in mammals to 540 kDa in plants with a subunit size of approximately 28 kDa (*13*). It has been speculated that ferritin resists denaturation during gastrointestinal digestion and protects iron from chelators during human digestion, thus improving iron absorption in humans (*14*−*16*). Reports on the bioavailability and the mechanism of ferritin-iron absorption are conflicting. Earlier studies using extrinsic labeling show a low iron absorption from soybean meal (*17*). Contrary results were reported by another study (*16*) using intrinsic labeling. In human studies, ferritin-iron was shown to be as bioavailable as $FeSO_4$ (*15, 18*). Researchers from the same group also reported that ferritin is absorbed by endocytosis or micropinocytisis (*19*) and that ferritin-iron bioavailability was less affected by the factors that have an effect on $FeSO_4$, assuming ferritin is not digested or digested at higher than normal gastric pH 2 (*20*). Two other groups with recombinant pea ferritin (*21*) or purified pea protein (*22*) showed that iron is released from ferritin under in vitro gastrointestinal digestion and its bioavailability is affected by factors similar to those affecting inorganic iron. Thus, ferritin absorption may be dependent on the degree of gastric digestion, and it is possible that ferritin absorption may be slightly higher than, if not similar to, the highly bioavailable $FeSO_4$. Therefore, ferritin may serve as an efficient source of bioavailable iron.

Ferritin gene expression has been demonstrated in a variety of plants including *Arabidopsis*, soybeans, beans, cowpeas, peas, and maize (*23*−*28*). On the basis of sequence comparisons, animal and plant ferritins appear to be derived from a common

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evolutionary ancestor. However, there are a number of differences between animal and plant ferritins. On the other hand, plant ferritin gene organization is the same in both monocots and dicots, and there is a high degree of conservation among plant ferritin genes (*29*). Approximately 85−90% amino acid sequence identity exists among monocots and 65−80% identity between monocots and dicots.

The ferritin protein has been isolated and characterized from pea, soybean, French bean, alfalfa, and maize seeds. Ferritin concentrations of these seeds ranged from 8 to 80 μg/g of seed, with the ferritin concentrations in peas and soybeans reportedly among the highest. Legumes generally have high levels of iron and ferritin (*30, 31*), whereas, cereal grains such as rice, wheat, and maize have low ferritin levels (*32*). Western blot analyses performed with polyclonal antisera raised against purified pea ferritin show that the antibody cross-reacts with soybean, pea, maize, *Arabidopsis*, and alfalfa ferritins (*23, 33−35*). However, the pea antibody shows no immuno-cross-reactivity with ferritins from other agronomically important plants, such as rice and wheat (*36*). Although animal and plant ferritins share extensive sequence homology, there is no immuno-cross-reactivity between them.

Ferritin is known to be a major source of iron for the development of humans, animals, and plants, and ferritin-iron has been shown to have a bioavailability similar to that of ferrous sulfate, a highly bioavailable iron source (*15, 16, 18*). Therefore, ferritin-biofortified crops have the potential to provide a significant source of bioavailable iron to combat IDA. However, a convenient method to quantitatively measure ferritin concentrations in crops is currently lacking. In this paper, we report the development of a universal and reliable assay to quantify ferritin from major staple food crops.

MATERIALS AND METHODS

Materials. Red and white beans (*Phaseolus vulgaris*), soft white winter wheat (*Triticum aestivum*), and brown rice (*Oryza sativa*) were obtained from a local supermarket, and maize (*Zea mays* cv. Northrup King 60-B6, 2004 harvest) was obtained from the Iowa Grain Quality Laboratory. Recombinant bean ferritin (rFerr; *P. vulgaris*) that was used as a standard for immunoblot analyses and ELISA experiments was kindly donated by the Institute of Food Science and Technology, Laboratory of Human Nutrition (Zurich, Switzerland). Detailed synthesis and isolation procedures were described in a recent publication by that group (*37*). All other products and chemicals were obtained from Sigma Aldrich (St. Louis, MO) and Fisher Scientific Co. (Fair Lawn, NJ) unless otherwise stated.

Partial Purification of Ferritin from Seeds. Crude extracts were prepared from seeds using methods described by Barceló et al. (*35*) and Laulhere et al. (*34*) with modifications described below. Red beans (2 g), white beans (2 g), maize (5 g), wheat (5 g), and brown rice (5 g) were soaked in water for approximately 20 h at 4 °C. The seeds were homogenized on ice in 4 volumes of extraction buffer (10 mM sodium phosphate buffer, 100 mM sodium chloride, 2% polyvinylpyrolidone, and 1 mM phenylmethanesulfonyl fluoride, pH 7.2) using a Polytron PT10/35 (Brinkmann Instruments, Westbury, NY) at medium speed until the homogenate was smooth. The slurry was centrifuged at 15000*g* for 10 min at 4 °C. Magnesium chloride was slowly added to the supernatant at a final concentration of 0.7% (v/v), and following a 10 min incubation on ice, the samples were centrifuged at 26000*g* for 5 min at 4 °C. The resulting supernatant was then adjusted to 1% (v/v) with MgCl₂, and after an incubation of 1 h at 4 °C, 2% of sodium citrate (v/v) was added. The samples were incubated again for 20 min on ice prior to centrifugation at 26000*g* for 50 min at 4 °C. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.2, and stored at −20 °C until used for further analysis.

The nitrogen content of the dry ground seeds were determined using the Dumas method (*38*) with a Rapid NIII Analyzer (Elementar

Americas, Inc., Mt. Laurel, NJ) and the total protein content in the seeds was calculated from the percent nitrogen, using a conversion factor of 6.25. The protein concentrations of the crude ferritin extracts from the initial and final phases of extraction were determined using the Bradford method (*39*). The percent protein extracted from each seed was calculated on the basis of the amount of soluble protein in the extraction buffer after the first centrifugation step relative to the total protein content of the seeds.

Generation of Anti-Ferritin Antibodies. Ferritin protein sequences for beans, wheat, maize, and rice were obtained from GenBank (www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw/) to find highly conserved sequences among the divergent seed ferritin sequences. A common 30 amino acid sequence corresponding to residues 105−134 of maize ferritin (accession no. CAA58147) was found to be 100% identical between maize, rice, and wheat ferritins and approximately 97% identical with ferritins from common beans. A highly immunogenic 22 amino acid sequence, within this 30 amino acid sequence, corresponding to residues 113−134 in maize ferritin was used for the generation of anti-ferritin polyclonal antibodies (ProSci Inc., Poway, CA). Antibodies specific for the ferritin protein were made by affinity purification against the 22 amino acid peptide used to generate the antibodies.

Immunoblot Analyses. Crude ferritin extracts containing 10−30 μg of protein were electrophoresed on a 12% SDS-PAGE (*40*). For immunoblot analyses, the separated proteins were transferred onto nitrocellulose membranes (Whatman, Maidstone, Kent, U.K.) according to the method described by Towbin et al. (*41*). After blocking in 5% blocking solution [5% nonfat dry milk dissolved in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 (v/v)] for 3 h at room temperature (RT), the membranes were incubated with anti-ferritin antibody (1:3000 dilution in 2% blocking solution) for 16−18 h at RT and washed three times for 10 min each with TBST [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 (v/v)]. Following incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 in 2% blocking solution) at RT for 3 h, the membranes were again washed three times for 10 min each with TBST. The membranes were then flooded with chemiluminescent substrate (Pierce, Rockford, IL) for 1 min and exposed to photographic film (Blue Lite Autorad Film, ISC BioExpress, Kaysville, UT).

ELISA Development. An indirect ELISA protocol was adapted from methods described by Sigma Aldrich and Flowers et al. (*42*) with modifications. Crude ferritin protein was extracted from the seeds as described above. Dilutions of the samples and standards were made with bicarbonate buffer, pH 9.6. Working ranges were based on the range of protein concentrations found to be optimal for the quantification of ferritin.

Nunc Immuno Plates (Sigma Aldrich M9410) were coated with 100 μL/well sample or standard. After incubation of the plate for 15−20 h at 4 °C, wells were washed twice with 400 μL/well of phosphate-buffered saline and 0.1% Tween 20 (PBST) and blocked with 200 μL/well of 50% Stabilcoat Immunoassay Stabilizer (Surmodics, Eden Prairie, MN) for 4 h at 25 °C. The plates were incubated with 100 μL/well of anti-ferritin antibody (1:500) in 25% Stabilcoat at 25 °C for 90 min and washed three times with PBST. Following an incubation with HRP-conjugated goat anti-rabbit antibodies in 25% Stabilcoat (100 μL/well; 1:5000) at 25 °C for 1 h, the wells were washed three times with PBST, and the reaction with chemiluminscent substrate (10 mL of citrate phosphate buffer, pH 5, 10 mg of *o*-phenylenediamine, 30 μL 30% hydrogen peroxide; 100 μL/well) was allowed to proceed at 25 °C for 30 min in the dark. After the reaction had been stopped with 25% sulfuric acid (25 μL/well), absorbance was read at 490 nm with 560 nm as a reference wavelength using Microplate Reader ELx808 (Bio-Tek Instruments, Inc., Winooski, VT) equipped with KC Junior software (version 1.14). Ferritin concentrations in the seeds were measured against the rFerr standard curve. All ELISA experiments were repeated with three separate extractions and assayed in triplicate from each extraction.

Screening of Various Red Bean Varieties Using ELISA. Thirteen different red bean varieties were obtained from the International Center for Tropical Agriculture (CIAT; Cali, Colombia) and screened for

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Table 1. Extraction of Total Protein

seed	protein[a] (g/100 g)	extracted protein[b] (%)
red beans	25.5	64.5 ± 0.12
white beans	23.0	54.6 ± 0.00
wheat	11.1	32.0 ± 0.07
maize	6.7	33.1 ± 0.04
brown rice	8.1	20.4 ± 0.02

[a] Total protein determined using nitrogen analyzer ($n = 2-3$). [b] Mean ± SD. Extracted protein represents the fraction of total protein extracted with buffer from soaked seeds, measured using the Bradford assay ($n = 2-4$).

Figure 1. Immunoblot analysis of ferritin from staple food crops. Molecular weight (MW) standards are shown on the left (in kDa) to approximate ferritin molecular weight. Partially purified ferritin extracts from seeds were loaded onto a 12% SDS-PAGE and probed with an anti-ferritin polyclonal antibody as described under Materials and Methods. Lanes: Std (standard, rFerr), 80 ng; RB (red beans), 10 μg; WB (white beans), 30 μg; Wt (wheat), 15 μg; Mz (maize), 10 μg; BR (brown rice), 25 μg.

ferritin content using the aforementioned ELISA method. Differences among the bean varieties were assessed using ANOVA followed by Dunnett's multiple-comparison test to compare all of the values against a high bean variety. The non-heme iron content of the red bean varieties was also measured using the method described by Torrance and Bothwell (*43*) and modified by Proulx and Reddy (*44*). The Pearson correlation between iron and ferritin concentrations among the red bean varieties was also determined. Statistical analyses were performed using Graph-Pad Prism, version 4.02 for Windows (San Diego, CA).

RESULTS AND DISCUSSION

Purification of Ferritin from Seeds of Staple Food Crops. Previous studies have shown that ferritin is present in very low quantities (approximately 0.001-0.005%) in seeds of food crops (*33-35*). Therefore, it would not have been feasible to measure ferritin concentrations from total protein extracts without at least partially purifying it from seeds. As the first approach to develop a simple and rapid assay to quantify ferritin, we partially purified the protein from seeds of various staple crops using the method of Laulhere et al. (*34*), with some modifications. The total protein in the dry seeds ranged from 8 to 26% of dry seed, with maize seeds having the lowest amount of protein, whereas beans had the highest amount of total protein (**Table 1**). On the basis of these observations, we decided to vary the range of the starting material for each seed. For instance, we used approximately 1 g of beans and 5 g of maize to extract sufficient amounts of ferritin for quantitative analyses.

We observed somewhat low levels of total protein in the extracts with the percent of protein extracted from each seed following the first extraction ranging from 20 to 65% (**Table 1**). The low levels of protein in the extract may suggest that not all of the protein in the seed is readily extractable. For example, zein and glutelin make up approximately 72% of the protein in maize and are shown to be insoluble in neutral buffers (*45*). Given this information, the first extraction step from our crude extraction procedure may have extracted most of the soluble protein including ferritin (with 33% total protein extracted from maize seeds). In an effort to expedite the crude extraction procedure described in the methods, we tried to quantify ferritin using the supernatant from the first centrifugation, known as the first extract. However, we were unable to detect ferritin with immunoblot and/or indirect ELISA using this extract. On the basis of previous results, our hypothesis is that ferritin concentrations were too low relative to the total protein concentration in the crude extract for the ferritin to be detected (*33-35*). Further purification of the seed extracts was required to quantify ferritin using indirect ELISA.

To determine whether we could extract more of the soluble protein from seeds, we performed repeated extractions from the first pellet using the same volume of buffer as for the first extraction. The supernatants from each extraction were then pooled together, and the rest of the purification steps were completed according to the protocol (refer to Materials and
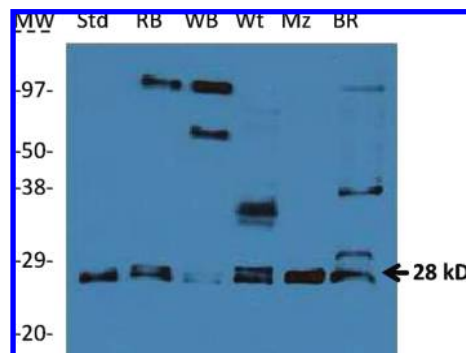
Methods). Although repeated protein extractions from the first pellet did increase the concentration of total proteins by 45-72% among the seeds, no significant impact on ferritin concentrations in the extracted sample was found, suggesting that remaining ferritin may not be not extractable (data shown). We also applied various dry seed grinding techniques in an attempt to improve the overall extraction of the protein. However, the percent protein extracted was low for most of the seeds compared to the original Polytron grinding method using soaked seeds (data not shown). Our results suggest that ferritin is best extracted from water-soaked whole seeds than dry seeds. In addition, further purification may be useful for measuring absolute concentrations, but the partial purification of ferritin was adequate to develop a rapid and reliable assay and to measure relative concentrations.

Immunoreactivity of Anti-Ferritin Antibody with Ferritins from Various Staple Crops. Our hypothesis was that by using a highly conserved peptide sequence to generate an anti-ferritin antibody, we would be able to develop a common method for measuring ferritin concentrations from various staple food crops. Hence, we generated an anti-ferritin polyclonal antibody using a highly immunogenic 22 amino acid peptide sequence that is significantly conserved among all plant ferritin sequences (**Table 2**). This amino acid sequence is part of the α-helix, helix turn, and the L-loop regions of the protein that have little secondary structure, thus making it more accessible to antigen−antibody interactions. This region of the protein also corresponds to amino acids 39−60 of horse ferritin L chain (accession no. PO2791), providing more evidence that the sequence is conserved across plants and animals (*47*).

Immunoblot analyses show that anti-ferritin polyclonal antibody cross-reacts with seed ferritins from beans, maize, wheat, and rice (**Figure 1**). Different amounts (10−30 μg) of protein were required to detect ferritin from various seeds depending on the seed type. Differences in the apparent reactivity of each sample with the antibody might be related to the type of protein or percent of total protein as ferritin in the extracts. Immunoblot analyses also revealed dual banding patterns for red beans, white beans, and wheat, but a single protein band for maize and brown rice, with molecular weights of the protein bands in the approximate range of 26−28 kDa. Previous studies have reported similar molecular weights for ferritins from soybeans (*33, 47*), alfalfa seeds (*35*), and bean, pea, and maize (*34, 48*). Goto et al. (*49*) reported that the 28 kDa peptide can be cleaved, releasing its 1.5−2 kDa extension peptide to produce both 26.5

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Table 2. Sequence Alignment of a 30 Amino Acid (AA) Region in Maize, Wheat, Rice, and Common Bean Ferritins Containing a Highly Immunogenic 22 AA Region Used for Polyclonal Antibody Production

plant	GenBank accession no.	sequence
maize 1	CAA58147	YHSLFAYFDRDNVALKGFAKFFKESSDEER
maize 2	CAA43664	YHSLFAYFDRDNVALKGFAKFFKESSDEER
maize 3	P29036	YHSLFAYFDRDNVALKGFAKFFKESSDEER
maize 4	CAA43664	YHSLFAYFDRDNVALKGFAKFFKESSDEER
wheat 1	AAW68440	YHSLFAYFDRDNVALKGFAKFFKESSDEER
wheat 2	AAT67051	YHSLFAYFDRDNVALKGFAKFFKESSDEER
rice 1	AAM74943	YHSLFAYFDRDNVALKGFAKFFKESSDEER
rice 2	AAQ74385	YHSLFAYFDRDNVALKGFAKFFKESSDEER
rice 3	AAM74942	YHSLFAYFDRDNVALKGFAKFFKESSDEER
bean 1	AAU08208	YHSLFAYFDRDNVALKGFAKFFKESSEEER
bean 2	P25699	YHSLFAYFDRDNVALKGFAKFFKESSEEER

and 28 kDa polypeptide subunits, whereas Masuda et al. (*50*) reported that the 26.5 and 28 kDa proteins were found to have different amino acid sequences and, therefore, represent two different ferritin subunits.

The anti-ferritin antibody also recognized high molecular weight proteins in red and white beans (74 and 94 kDa), wheat (40 and 44 kDa), and rice (33, 48, and 94 kDa) (**Figure 1**). Similar higher molecular weight proteins, of unknown origin, were reported in pea seeds with immunoblot analyses (*33*). We used the affinity-purified antibody for immunoblot analyses, and BLAST (www.ncbi.nlm.nih.gov) searches revealed that the 22 amino acid region used to generate the antibody was highly specific for ferritin sequences from plants. Therefore, we hypothesize that the high molecular weight proteins may represent either partially degraded fractions of the holoprotein and/or cross-linked polymers of ferritin subunits (*33*). Nevertheless, our results indicate that the polyclonal antibody, as expected, showed wide-range immunoreactivity with ferritins from various staple food crops and is suitable for the development of a universal method for measuring ferritin concentrations in staple food crops.

Quantification of Ferritin from Seeds of Staple Food Crops Using ELISA. Although immunoblot analyses coupled with densitometry may be used for estimating ferritin concentrations, they are susceptible to gel-to-gel variations and pose problems with scalability. We therefore used the ELISA technique to develop a method for measuring ferritin concentrations in various staple food crops. Ferritin concentrations were calculated (*n* = 3 extractions with 3 triplicates each) using the recombinant ferritin (rFerr) as a standard (ranging from 5 to 500 ng/mL). Ferritin concentrations in the extracts were measured using a standard curve with a four-parameter curve fit.

Protein working ranges used for the quantification of ferritin were determined for each seed type and represent the protein range with both a linear increase in ferritin concentration (ng/mL) and the range where ferritin per gram of seed remains relatively constant (**Figure 2**). Without quenching, the ferritin content per gram of seed should be constant no matter how much sample is used in the ELISA. The optimal concentrations of protein to use in ELISA experiments were important to determine because of the limited protein-binding capacity on the ELISA plate. When excess protein was added to the well, the plate became oversaturated and a plateau or decrease in ferritin binding was observed. The protein-binding capacity of the wells appeared to be limited to approximately 20 μg/mL of protein according to several experiments performed for the determination of the working range. An example of the determination of the working range is demonstrated in **Figure**
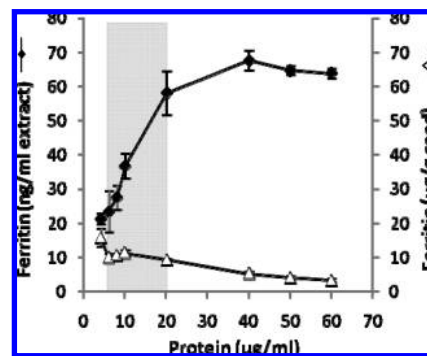

Figure 2. Determination of protein working range (6−20 μg/mL protein) for ferritin quantification in red beans (RB) using indirect ELISA and rFerr standard curve. The anti-ferritin polyclonal antibody was used at a final concentration of 1:500 for ELISA. Ferritin concentrations (ng/mL) were determined from 4, 6, 8, 10, 20, 40, 50, and 60 μg/mL of RB protein and used to calculate the ferritin content per gram of seed (μg/g). The working range (shaded area) represents the protein concentrations that provide a linear increase in ferritin concentration (ng/mL) with constant ferritin content (μg/g). Ferritin (μg/g seed) = [(ferritin (μg/mL) × dilution factor) × (volume of sample prior to protein measurement)/(g of dry seed used in extraction)].
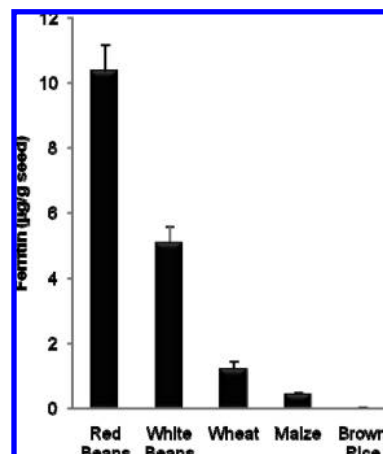

Figure 3. Ferritin content per gram of seed for red beans, white beans, wheat, maize, and brown rice. The values were determined using indirect ELISA and rFerr standard curve. Values represent the means ± SD of samples tested in the working range, *n* = 3 extractions measured in triplicate.

2 for red beans, where 6−20 μg/mL of protein was found to be the optimal protein range for ferritin quantification. The working ranges for white beans, wheat, maize, and brown rice were found to be 4−20, 4−10, 4−20, and 6−20 μg/mL of protein, respectively (data not shown). Variations in the protein working ranges may be caused by differences in the food matrices of the seeds.

Values in **Figure 3** represent the mean ± SD of ferritin determined from the protein working range. The values represent three extractions with triplicate assays from each extraction. The ferritin content of the crude extracts was found to be 10.3 ± 0.9, 5.1 ± 0.5, 1.2 ± 0.6, 0.4 ± 0.1, and 0.04 ± 0.01 μg/g of seed for red beans, white beans, wheat, maize, and brown rice, respectively, representing 0.004, 0.002, 0.001, and 001% and a negligible amount of ferritin in relation to total protein. These values are 5 times lower than the reported values in the ferritin purification studies, where more ferritin losses are expected (*33, 34*). Possible differences might be due to type of seed, time, and conditions for seed soaking, losses during $MgCl_2$ precipita-

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tion and interference being due to food matrices during ELISA measurement. Because longer soaking (~72 h) was reported to yield less ferritin due to insoluble ferritin crystalline arrays formed with reuptake of iron from the soaking water, we have used shorter soaking times as was suggested in the previous study (*33*). The shorter period (18 h), similar to our 20 h, of seed soaking is expected to result in minimal losses during partial purification. Consequently, we did not detect ferritin in the supernatant after $MgCl_2$ precipitation (data not shown). On the other hand, our values agree with the 13−16 μg/g pea seed ferritin reported in a recent study, which used the same method for extraction of seed ferritin (*22*). Absolute concentration of ferritin from each seed may well be higher than estimated by our method; however, relative concentrations rather than absolute concentrations of ferritin are important for identifying ferritin-rich lines from the same staple food crop. Although both red and white bean varieties were found to have higher ferritin levels when compared to rice, wheat, and maize seeds, caution should be taken in the comparison of ferritin levels between seeds from different crops. Differences in protein extractability and quantification using a standard from a different species might lead to variability in the estimation of ferritin concentrations. Although our method is useful to screen the seeds from the same crop for ferritin comparisons, whether differences in phytate, polyphenols, or other factors within the same species affect total protein or ferritin extractability needs further investigation.

Screening High and Low Ferritin Containing Red Bean Varieties with ELISA. As a first approach to assess the suitability of the ELISA method, we screened 13 different red bean varieties obtained from CIAT for ferritin content. Samples were received as dry ground seed, so modifications to the method included homogenizing the seed in a 6 times volume of extraction buffer without soaking the seeds. Ferritin concentrations of 13 red bean varieties were determined using 10 μg/mL of the protein extract, which is within the working range for red beans. Protein extractability of the red bean varieties was not measured, but a similar percent protein extraction reported earlier (~64.5%) was assumed for further measurements. **Figure 4A** shows the average ferritin concentrations of the red bean varieties using our method. The ferritin concentrations ranged from 1.6 ± 1.5 to 5.1 ± 1.0 μg/g of seed (mean = 3.02 μg/g; n = 2 extractions assayed in triplicate). Large variation was found in the concentrations, but the values were less variable from the same extraction, suggesting the extraction procedure is adding major variability. Although our study focused on developing the ELISA method for screening, future studies are needed to optimize the extraction procedure (soaked vs dry seeds) for improved precision. On the basis of our preliminary analyses for these 13 bean varieties, we identified 6 lines that are higher than average, and there was a 3-fold difference between low and high ferritin lines (5.12 vs 1.64 μg/g of seed). Despite the variation in ferritin content within the same variety, we were able to identify significant differences between high- and low-ferritin bean varieties. Ferritin concentration from bean variety 13 was significantly higher than those of three low-ferritin bean varieties (numbers 1−3, $P < 0.05$) based on ANOVA with Dunnett's multiple-comparison test. However, a large number of seeds need to be screened before recommendations can be provided to plant breeders on high-ferritin bean lines. This range of ferritin values at 1.6−5.1 μg/g is lower than our previously determined values for red bean (10.3 μg/g of seed dry weight). Our hypothesis is that these low ferritin values were the result of poor extraction of ferritin from dry ground
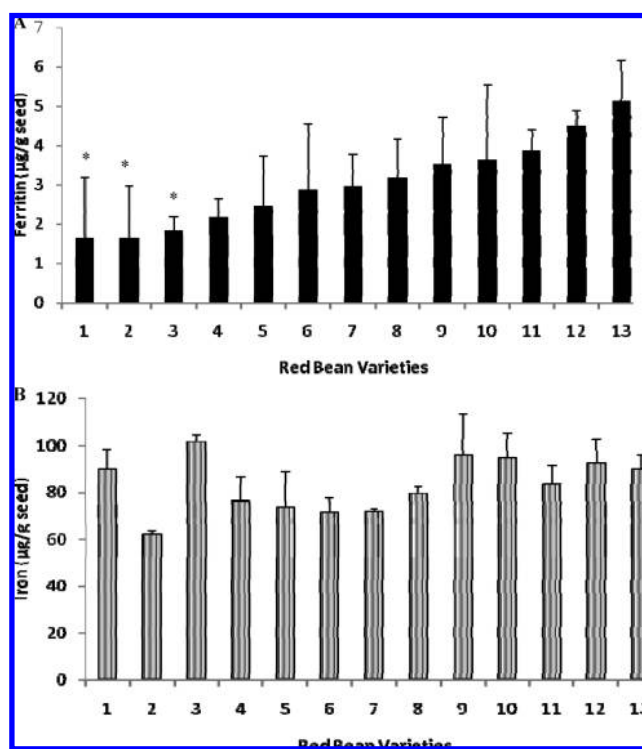

Figure 4. Ferritin (**A**) and iron (**B**) concentrations in red bean varieties. Ferritin values represent mean ± SD of two extractions, assayed in triplicate, and the iron values are from dry seeds assessed in duplicate from two independent samples. Seeds were obtained from CIAT (International Center for Tropical Agriculture). *, $P < 0.05$; significantly different from high-ferritin line 13 based on ANOVA with Dunnett's multiple-comparison test.

seed. Hence, we recommend using soaked whole seeds instead of dry seeds in future studies.

To determine whether ferritin levels correlated with the iron content of the seeds, we also measured non-heme iron content of the 13 red bean varieties (**Figure 4B**). The correlation between total iron and ferritin in the seed was not statistically significant ($r = 0.31$). The iron concentrations among the low-ferritin varieties were highly variable, with unexpectedly high iron among two of the varieties. However, we cannot rule out the possibility of iron contamination in these two lines. Although the regulation of iron accumulation is poorly understood, it is evident that the accumulation of iron in the seeds may not always correspond to the expression of ferritin. These results emphasize the importance of measuring ferritin, which is an important source of bioavailable iron similar to $FeSO_4$, rather than measuring iron content alone. Because of organoleptic and stability problems associated with $FeSO_4$, consumption of ferritin-rich crops may be a better and sustainable alternative to consumption of fortified foods.

In conclusion, our indirect ELISA method can be used to measure relative ferritin concentrations in a variety of staple food crops. Using this method, we have been able to detect low- and high-ferritin lines from various red bean varieties. Thus, this universal method can be used as a convenient tool for screening large numbers of seeds of the same crop and to identify ferritin-rich lines.

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