2008

Method development for the quantification of ferritin in staple food crops

Rebecca Jane Lukac
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Medical Nutrition Commons, and the Nutrition Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/14916

This Thesis is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Method development for the quantification of ferritin in staple food crops

by

Rebecca Jane Lukac

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Nutritional Sciences

Program of Study Committee:
Manju B. Reddy, Major Professor
Kevin Schalinske
M. Paul Scott

Iowa State University
Ames, Iowa
2008
# TABLE OF CONTENTS

## LIST OF FIGURES

iv

## LIST OF TABLES

v

## ACKNOWLEDGEMENTS

vi

## CHAPTER 1. GENERAL INTRODUCTION

1

### INTRODUCTION

1

### THESIS ORGANIZATION

2

### REFERENCES

2

## CHAPTER 2. LITERATURE REVIEW

3

### IRON

3

- Importance of Iron
- Iron Metabolism
  - Iron Distribution and Utilization
  - Regulation of Iron Metabolism
- Iron Requirements
- Iron Deficiency
  - Prevalence and Causes
  - Adverse Effects
- Strategies to Alleviate Iron Deficiency
  - Supplementation
  - Fortification
  - Biofortification
  - Biotechnology
- Iron Bioavailability
  - Dietary Iron Absorption
  - Heme and Nonheme Iron
- Factors Affecting Nonheme Iron Bioavailability

33

### PHYTOFERRITIN

33

- Ferritin Structure
- Role of Phytoferritin
- Regulation of Phytoferritin Synthesis
- Iron and Ferritin Accumulation in Plants
- Analyzing Ferritin in Food Crops

40

### REFERENCES

41

## CHAPTER 3. ELISA METHOD DEVELOPMENT FOR THE QUANTIFICATION OF FERRITIN IN STAPLE FOOD CROPS

49

### ABSTRACT

49

### INTRODUCTION

50
LIST OF FIGURES

Figure 2.1. Classification of major iron-containing proteins 4
Figure 2.2. Distribution of total body iron 6
Figure 2.3. Regulation of transferrin receptor and ferritin expression 8
by iron regulatory protein and iron regulatory element
Figure 2.4. Iron homeostasis with hepcidin 9
Figure 2.5. Dietary iron uptake 21
Figure 2.6. Representation of spherical ferritin protein 33
Figure 2.7. Role of nicotianamine as an iron chelator in the plant cell 38
Figure 3.1. Immunoblots of seed ferritins using polyclonal and 69
monoclonal antibodies
Figure 3.2. Densitometry of western blots to quantify ferritin in red beans 70
Figure 3.3. Determination of protein working range for ferritin 71
quantification in red beans using indirect enzyme-linked
immunosorbant assay
Figure 3.4. Ferritin content for red beans, white beans, wheat, maize, 72
and brown rice determined using indirect enzyme-linked
immunosorbant assay
Figure 3.5. Ferritin and iron concentrations in red bean varieties 73
## LIST OF TABLES

| Table 2.1. | Several functions of iron-sulfur clusters | 5 |
| Table 2.2. | Recommended daily allowance for iron among different life stage groups according to dietary iron bioavailability | 11 |
| Table 2.3. | Risk factors for iron deficiency | 13 |
| Table 2.4. | Iron compounds used for iron fortification in order of preference | 16 |
| Table 2.5. | Dietary substances that influence iron bioavailability and their major dietary sources | 23 |
| Table 2.6. | Summary of ferritin and soybean iron bioavailability studies | 30 |
| Table 2.7. | Comparison of genetic target and signals that regulate ferritin synthesis in plants, animals, and bacteria | 36 |
| Table 3.1. | Sequence alignment of a 30-amino acid region in maize, production | 68 |
| Table 3.2. | Percent extracted protein | 68 |
ACKNOWLEDGEMENTS

I give my sincere thanks to my major professor, Dr. Manju Reddy, for her guidance and support throughout my graduate school experience. I am grateful for the opportunity to have worked with her on my research and thesis; it has been an invaluable experience.

I acknowledge Drs. Kevin Schalinske and M. Paul Scott for serving on my Program of Study Committee and for their review of my thesis.

I acknowledge Maneesha Aluru as a collaborator on the study. Her insightful contributions, patience, and willingness to teach me various purification and immunoblotting techniques were much appreciated.

A big thank you to my lab mates for their friendships and assistance with my study; to Amy Proulx, for reviewing my thesis and for her insights on life and research. I am fortunate to have worked with such inspiring people.

With gratitude, I acknowledge the International Food Policy Research Institute (IFPRI)/HarvestPlus for their financial support.

I thank Matthies Hoppler from the Institute of Food Science and Technology, Human Nutrition Lab, Zurich, Switzerland, for kindly donating recombinant pea ferritin.

I acknowledge Dr. Matthew Nonnenmann, Assistant Professor, University of Texas Health Science Center at Tyler, for review of my thesis.

Lastly, a big thank you to my friends and family for their enduring love and support.
CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Iron deficiency (ID) is the most prevalent nutritional deficiency worldwide, afflicting people of all ages and populations with an array of health complications and accompanying socioeconomic implications (Provan, 1999). The consequences of iron deficiency to both individuals and nations are considerable (Micronutrient Initiative, 2004). Several strategies such as supplementation and food fortification have been used to combat this problem (Zimmermann and Hurrell, 2007). However, these efforts have fallen short of making any significant improvements in the iron status of the global population (Micronutrient Initiative, 2004).

The use of biofortification to nutritionally enhance staple food crops through traditional breeding or genetic modification may provide a high source of bioavailable iron (Theil, 2004). As a highly sustainable strategy with the potential to reach even remote populations, biofortification shows potential as a complementary strategy for combating ID. Increasing the ferritin content of these food crops may help accumulate iron in the seed in a relatively bioavailable form (Zimmermann and Hurrell, 2007). However, an efficient method of measuring ferritin in the staple food crops is necessary prior to the implementation of biofortification. Therefore, the aim of this study was to develop a method to quantify ferritin in a large number and variety of food crops. Furthermore, the development of this method would allow for convenient screening and development of ferritin-rich crops.
THESIS ORGANIZATION

The thesis consists of a review of literature on the importance of iron, iron requirements, iron deficiency, iron bioavailability, and phytoferritin, and a paper entitled, “ELISA development for the quantification of ferritin in staple food crops.” This study involved the development of a method to quantify ferritin in a large number and variety of food crops using a crude ferritin extraction procedure, antigen selection for antibody production, immunoblotting, and enzyme-linked immunosorbant assay (ELISA) development.

REFERENCES


CHAPTER 2. LITERATURE REVIEW

IRON

Importance of Iron

Iron is an essential element involved in a variety of biochemical processes in the human body (Provan, 1999). Some of these processes include electron transfer reactions, gene regulation, cell growth and differentiation, oxygen binding and transport, immune defense, enzyme reactions, and neurotransmitter and protein synthesis (Provan, 1999; Beard, 2001). Since iron is a highly reactive, potentially toxic metal in its free form (McCord, 1998), it must be transported, stored and utilized in proteins to minimize damage to cells (Ponka, 1999). The major iron-containing proteins may be categorized into four classes (Beard, 2001): iron-containing nonenzymatic proteins (transferrin, ferritin, and hemosiderin), iron-sulfur (Fe-S) enzymes, hemoproteins, and nonheme noniron-sulfur iron-containing enzymes (Figure 2.1). Iron functions as a cofactor for several enzymes, wherein iron commonly exists in the form of heme or Fe-S clusters.

Heme iron is an oxygen carrier in hemoglobin and myoglobin for the transport and storage of oxygen. Hemoglobin consists of four globin chains, each containing a heme group that facilitates oxygen transport throughout the body. Myoglobin is a single globin chain protein that facilitates the transport of oxygen in myocytes. Other hemoproteins include oxidases, peroxidases and catalases for immune defense, and cytochromes in the electron transport chain (ETC) for ATP production (Atamna et al., 2002).
Iron exists in either ferrous ($\text{Fe}^{2+}$) or ferric ($\text{Fe}^{3+}$) oxidation states in the body. With its oxidation-reduction potential, iron can bind ligands (oxygen, nitrogen, and sulfur atoms) and participate in electron transfer reactions (Beard, 2001). Nonheme iron in the form of Fe-S clusters has several functions (Table 2.1) (Johnson et al., 2005). These functions include: electron transfer (ferredoxins), substrate binding/activation, gene regulation, enzyme activation (Johnson et al., 2005), and energy metabolism (aconitase and proteins of the ETC) (Beinert and Kiley, 1999). Iron is also important for porphyrin metabolism, collagen synthesis, lymphocyte and granulocyte function (FAO and WHO 2002), and required by enzymes for various brain functions involving myelination (Ortiz et al., 2004) and neurotransmitter synthesis. Specifically, iron is required for the synthesis of the
neurotransmitters serotonin (tryptophan hydroxylase) (Martinez et al., 2001) and dopamine (tyrosine hydroxylase) (Nagatsu, 1995).

Table 2.1. Several functions of Fe-S clusters. From Johnson et al. (2006).

<table>
<thead>
<tr>
<th>Function</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron transfer</td>
<td>Ferredoxins; redox enzymes</td>
</tr>
<tr>
<td>Coupled electron/proton transfer</td>
<td>Rieske protein; Nitrogenase</td>
</tr>
<tr>
<td>Substrate binding and activation</td>
<td>(de)Hydratases; Radical SAM enzymes; Acetyl-CoA synthase; Sulfite reductase</td>
</tr>
<tr>
<td>Fe or cluster storage</td>
<td>Ferredoxins; Polyferredoxins</td>
</tr>
<tr>
<td>Structural</td>
<td>Endonuclease III; MutY</td>
</tr>
<tr>
<td>Regulation of gene expression</td>
<td>SoxR; FNR; IRP; IscR</td>
</tr>
<tr>
<td>Regulation of enzyme activity</td>
<td>Glutamine PRPP amidotransferase; Ferrochelatase</td>
</tr>
<tr>
<td>Disulfide reduction</td>
<td>Ferredoxin:thioredoxin reductase; Heterodisulfide reductase</td>
</tr>
<tr>
<td>Sulfur donor</td>
<td>Biotin synthase</td>
</tr>
</tbody>
</table>

*Abbreviations used: SAM, S-adenosylmethionine; acetyl-CoA, acetyl coenzyme A; FNR, fumarate and nitrate reduction; IRP, iron-regulatory protein; IscR, iron-sulfur cluster assembly regulatory protein; PRPP, phosphoribosylpyrophosphate.

Iron Metabolism

The average adult requires about 3-5 g of iron, but this may vary depending on the physiological state, age, weight, and gender of the individual (Aisen, 2001). Iron homeostasis in the body is determined by the uptake, transport, management, storage, and export of iron. The regulation of these mechanisms is important for maintaining adequate iron for metabolic functions and to prevent iron overload and subsequent oxidative damage (Hentze et al., 2004).

Iron Distribution and Utilization

Following export from enterocytes, iron is immediately taken up into carrier proteins or transferrins (Tf) to be distributed to various tissues or compartments (Figure 2.2).
Transferrin proteins only account for less than 1 percent of the total body iron, but they transport up to about 20 to 25 mg of iron each day. For cellular iron uptake, transferrin-bound-iron binds transferrin receptor-1 (TfR1) on the surface of the cell and complexes clathrin-coated pits for endocytosis (Aisen et al., 2001). The acidic pH of the endosome releases the iron from Tf, which is eventually released back into the circulation for another cycle of iron transport. The major destination for transferrin is the bone marrow, because iron is required for the synthesis of hemoglobin. Therefore, erythrocytes contain about two-thirds of the total iron in the body (Beard, 2001). When the erythrocytes are degraded, the iron is reused by the reticuloendothelial system and again cycled back to Tf (Fairbanks, 1998). A smaller fraction of the body iron is used by other tissues for iron storage in
myoglobin and in a variety of enzymes. Excess iron is stored as ferritin and hemosiderin in the liver, spleen, bone marrow, and other tissues and can be utilized to meet daily requirements when dietary iron intake is insufficient (Aisen et al., 2001).

**Regulation of Iron Metabolism**

Preservation of iron homeostasis is largely achieved by the body’s ability to regulate the rate of intestinal iron absorption to accommodate the body iron needs. A change in the iron absorption rate generally occurs in response to the body’s iron stores, level of erythropoietic activity in the bone marrow, hemoglobin concentration, blood oxygen content, and presence of inflammatory cytokines (Miret et al., 2003). Any indication of low iron status, such as low iron stores, initiates an increase in iron absorption (Andrews, 1999). Therefore, iron absorption is the means by which the body replaces the 1-2 mg iron losses that occur each day (Andrews, 1999). Alternatively, iron absorption is reduced in response to inflammation or iron overload (Lynch, 2007).

Iron regulatory proteins (IRPs) control multiple aspects of iron metabolism through the posttranscriptional regulation of proteins involved in iron export (ferroportin), iron uptake (Tf), iron storage (ferritin), erythroid heme synthesis (eLAS), and the citric acid cycle (aconitase) (Hentze et al., 2004). The mRNAs of these proteins have stem-loop structures called iron response elements (IREs) that play a key role in the regulation of translation. In the absence of iron or with iron depletion, IRPs have an open cap-like confirmation that allows it to bind IREs on the mRNA. The mRNAs either have multiple IREs at the 3’-untranslated region (UTR) or a single IRE at the 5’-UTR (Figure 2.3). Transferrin receptor is an example of a protein that has multiple IREs in 3’-UTR of mRNA, where the binding of IRPs stabilizes the strand by preventing ribonuclease degradation. On the other hand, when
IRPs bind the IREs in 5’-UTR of ferritin mRNA (for example), it prevents translation. If there is excess iron in the cells, IRP binds iron and becomes inactive, which results in the degradation of TfR mRNA and the increased expression of ferritin to respectively minimize the cellular uptake of iron and sequester iron into storage.

**Figure 2.3.** Regulation of TfR and ferritin expression by IRE/IRP. From Pantopoulos (2004).

Hepcidin is a key hormone for iron regulation (**Figure 2.4**). It is an antimicrobial peptide that is regulated similarly to intestinal iron absorption in that its expression is controlled by iron stores, erythropoietic activity, hemoglobin, oxygen content, and inflammation (Flemming and Bacon, 2005). Hepcidin is secreted from the liver in response to high levels of circulating iron to negatively regulate ferroportin-1 and functions by preventing the release of iron from macrophages, hepatocytes, and enterocytes (Nemeth et al., 2004).
Iron Requirements

Iron requirements depend on the amount of iron lost and the metabolic need for iron.

Daily iron losses occur from the sloughing of cells from the skin, intestines, urinary tract, and lungs; amounting to an average iron loss of about 14 µg/kg body weight/day (Green, 1968).
Therefore, a 55 kg non-menstruating woman requires about 0.8 mg Fe/day and a 70 kg man requires about 1 mg Fe/day to accommodate for daily iron losses. Additional iron is required during various physiological stages of life to accommodate increases in demand for metabolic function, which often occur during child and adolescent growth and development, pregnancy/lactation, and menstrual blood loss.

A newborn has about 75 mg Fe/kg body weight, which is sufficient for the infant during the first 4-6 months of life. However, premature infants often miss the high supply of iron offered during the last trimester of pregnancy and therefore require supplemental iron (FAO/WHO, 2002). The need for body iron doubles after 4-6 months and doubles again from 1-6 years (FAO/WHO, 2002). Iron is especially important during these early stages of life for brain development (Beard, 2001).

Adolescents also have increased iron needs due to spurts of rapid growth; however, requirements are highly variable, often depending on growth rate and gender (Rossander-Hulthen and Hallberg, 1996). Pubescent girls that are menstruating and still growing may have iron needs that reach up to 3.2 mg/day. Requirements are also high for pubescent boys due to increases in blood volume, muscle mass, and myoglobin (CDC, 1998; Wharton, 1999). Menstrual blood loss varies for each woman, but based on average menstrual iron losses and basal iron loss, the total iron requirement is about 1.5 mg/day for menstruating women, but may reach up to 2.8 mg/day (Hallberg and Rossander-Hulthen, 1991). Postmenopausal women have the same iron requirements as men (FAO/WHO, 2002).

Pregnancy requires an increase of iron from 0.8 mg/day to about 10 mg/day, primarily during the final trimester to increase blood volume and hemoglobin concentration (Hallberg, 1992). Maintaining adequate iron levels during pregnancy through diet alone is
difficult for mothers (FAO/WHO, 2002). Therefore, the mother must build iron stores prior to pregnancy and take iron supplements to prevent depletion of iron. Lactating women lose about 0.3 mg/day of iron in the milk, so based on these losses and the basal iron loss, the total iron required for lactating women is about 1.1 mg/day (Hallberg, 1992).

Recommendations for dietary iron intake are based on the iron needs of the individual, but vary depending on the bioavailability of iron in the meal and the iron status of the individual. The recommended daily allowance (RDA) for individuals at various life stages are summarized in Table 2.2, at three levels of iron bioavailability (FAO/WHO, 2002).

Table 2.2. Recommended daily allowance (mg/d) for iron among different life stage groups according to dietary iron bioavailability. From FAO/WHO (2002).

<table>
<thead>
<tr>
<th>Life Stage Group</th>
<th>Age (years)</th>
<th>Dietary Iron Bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15%</td>
</tr>
<tr>
<td>Children</td>
<td>0.5-1\textsuperscript{a}</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7-10</td>
<td>6</td>
</tr>
<tr>
<td>Males</td>
<td>11-14</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>9</td>
</tr>
<tr>
<td>Females</td>
<td>11-14\textsuperscript{b}</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>11-14</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>20</td>
</tr>
<tr>
<td>Post Menopause</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Lactation</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Highly variable bioavailability
\textsuperscript{b} Non-menstruating
Iron Deficiency

Iron deficiency occurs in three progressive stages: iron depletion, iron deficient erythropoiesis, and iron deficiency anemia (WHO, 2001). Iron depletion is defined by a depletion of iron stores. Iron deficient erythropoiesis occurs with the depletion of iron stores and reduced transport of iron, characterized by an increase in transferrin receptor concentration. Iron deficiency anemia (IDA) occurs with severe ID and is characterized by both depleted iron stores and a low hemoglobin concentration (WHO, 2001).

Prevalence and Causes

Iron deficiency is the most prevalent nutritional problem, affecting an estimated 4 to 5 billion people worldwide (WHO, 2004). IDA is a major problem in developing countries, where about 90 percent of the anemic population resides. Even in the United States, IDA afflicts about one-third of the low-income pregnant women and about three-fourths of adolescent females do not meet dietary iron requirements (CDC, 1998). By reducing the work capacity in adults by 10-15 percent, IDA has also been estimated to have a global economic effect in the billions of dollars, causing an estimated 1.5 percent loss in gross domestic product (FAO, 2002).

Iron deficiency and ultimately IDA generally develop because of increased iron requirements or losses and inadequate iron intake, absorption, or utilization (Table 2.3) (Stang and Story, 2005). The increased iron requirements for infants, children, adolescents and pregnant women due to increased growth and development are previously discussed. Blood loss is the main cause of iron loss, generally due to heavy bleeding during menses, hemorrhage, or parasitic infection (hookworms and flukes), which are common in developing countries (Harvey et al., 2005; Loukas et al., 2006).
In developing countries, the major cause of ID is an inadequate iron intake. The high cost and inaccessibility of iron-rich foods, such as meat and fish, may limit the diet to consist primarily of staple food crops, such as wheat, maize, and rice in certain populations (Welch and Graham, 2004). Rice alone provides up to 80 percent of the caloric intake for half of the global population (Meng et al., 2005). Such a diet is not diversified enough to provide all of the nutrients necessary for good health. Staple food crops are generally considered to be poor sources of iron due to the low iron content and bioavailability (WHO, 1996). This poor bioavailability is due to the inhibition of nonheme iron absorption by dietary factors such as phytic acid and polyphenols, which are commonly found in staple crops (Hallberg et al., 1987).

### Table 2.3. Risk factors for iron deficiency. Modified from Stang and Story (2005); Andrews (1999).

<table>
<thead>
<tr>
<th>Inadequate iron intake/absorption/stores</th>
<th>Increased iron requirements/losses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetarian and vegan diets</td>
<td>Rapid growth</td>
</tr>
<tr>
<td>Excess bran, tannin, phytates, or starch in diet</td>
<td>Pregnancy (recent or current)</td>
</tr>
<tr>
<td>Low intake of meat, fish, poultry or iron fortified foods</td>
<td>Participation in endurance sports</td>
</tr>
<tr>
<td>Low intake of foods rich in ascorbic acid</td>
<td>Gastrointestinal blood loss (ulcer, tumor, parasitosis, inflammatory bowel disease, diverticulosis)</td>
</tr>
<tr>
<td>Loss or dysfunction of absorptive enterocytes</td>
<td>Heavy/lengthy menstrual periods</td>
</tr>
<tr>
<td>Bowel resection</td>
<td>Excessive blood donations</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>Infection</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>Cancer</td>
</tr>
<tr>
<td>Intrinsic enterocytes defects</td>
<td></td>
</tr>
</tbody>
</table>

**Adverse Effects**

Given the wide array of physiological functions that require iron during various stages of life, a deficiency of iron consequently causes an array of adverse health effects. Impaired cognitive development, growth, and behavior may occur among infants and children from ID during the early stages of life (final trimester of pregnancy to the age of two) (McCann and Ames, 2007). School children may be adversely affected by ID, which is
believed to decrease motor activity and school performance (Sachdev et al., 2005). Iron
deficiency may also increase the risk of heavy-metal (lead and cadmium) toxicity in children
(Zimmermann et al., 2006), due to an increased absorption capacity.

Iron deficiency anemia afflicts more than 500 million women each year and
contributes to more than 60,000 deaths during childbirth (Micronutrient Initiative, para. 1).
During pregnancy, IDA increases the risk for premature birth, low birth weight, prenatal and
perinatal neonatal loss, and maternal mortality (Schorr and Hediger, 1994). In people of all
ages, ID can impair immune function, leading to an increased risk of infection, morbidity,
and mortality. Fatigue is also a common symptom of IDA that may result in reduced
physical capability, work performance, and productivity (Baynes and Bothwell, 1990), which
may consequently have socioeconomic implications (Provan, 1999). Altered hormone
production and metabolism that affect neurotransmitter synthesis and thyroid hormones
ultimately impairs neurological, muscular, and temperature-regulatory systems (Beard,
2001).

Strategies to Alleviate Iron Deficiency

Reducing the incidence of ID would require the integration of several strategies,
including: dietary education, modification, and diversification to improve iron intake and
bioavailability; control over parasitic infections; supplementation; fortification of food
products; and biofortification or biotechnology to enhance the iron content in staple crops
(Zimmermann and Hurrell, 2007; Welch and Graham, 2003). Improving iron intake and
bioavailability through dietary modification and diversification would be among the most
sustainable and the ideal strategies for preventing ID (FAO/ILSI, 1997). However,
consumption of highly bioavailable iron-containing foods is often not financially feasible for many population groups and there are difficulties associated with changing traditional dietary practices (Zimmermann and Hurrell, 2007). As a treatment option, dietary modification and diversification would also require improvements in the processing, production, and distribution of a variety of foods (Huma et al., 2007; Marfo et al., 1990).

**Supplementation**

Iron supplementation is generally used as a short-term strategy for controlling or preventing ID, especially in high-risk groups such as pregnant women (Cogswell et al., 2003). For individuals with IDA, supplementation has been found to be highly effective for improving iron nutritional status (Cavalli-Sforza et al., 2005). The iron compounds commonly used in supplements (ferrous sulfate and ferrous gluconate), are both low cost and highly bioavailable (Zimmermann and Hurrell, 2007). However, there are several challenges associated with using supplementation as an ID reduction intervention. Some include, distribution, dosage compliance, and adverse side effects (e.g., nausea, abdominal pain) (Stolzfus and Dreyfus, 1998).

**Fortification**

Iron fortification is a long-term, cost-effective intervention to reduce ID incidence (WHO/FAO, 2006). However, problems exist when using fortification with iron compounds. Specifically, identifying bioavailable iron compounds that will be compatible with the food vehicle is challenging (Mehansho, 2006). Many of the highly bioavailable forms of iron often react with food components in the food vehicle, causing peroxidation or color and flavor changes that are not well accepted by the consumer (Trowbridge and Martorell, 2002).
Consequently, the less bioavailable forms of iron have been used in food fortification. The iron compound chosen for fortification often depends on the diet of the target population and the food vehicle (Table 2.4). Part of the practicality of food fortification is the ability to incorporate iron into foods that are commonly consumed by populations at risk of ID. Some of the common food fortification vehicles being used globally, include: soy sauce (China), fish sauce (Vietnam), wheat and maize flour (South Africa), powdered milk (Chile), curry powder (South Africa), low-grade salt (Africa), rice (India), and wheat flour (worldwide) (Zimmermann and Hurrell, 2007). There are also self-added fortification products in the form of powders or sprinkles, crushable tablets, and fat-based spreads that have been found to be effective treatments (Nestel et al., 2003; Zlotkin et al., 2003). Other challenges of fortification are measuring the effectiveness of the products and ensuring financial affordability and accessibility of the products (Hurrell, 2002).

Table 2.4. Iron compounds used for iron fortification in order of preference. Modified from Zimmermann and Hurrell (2007).

<table>
<thead>
<tr>
<th>Most foods (cereal flours)</th>
<th>High phytate cereal flours/High peptide sauces</th>
<th>Liquid milk products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulfate</td>
<td>NaFeEDTA*</td>
<td>Ferrous biglycinate</td>
</tr>
<tr>
<td>Ferrous fumarate</td>
<td></td>
<td>Micronised dispersible</td>
</tr>
<tr>
<td>Encapsulated ferrous sulfate or fumarate</td>
<td></td>
<td>ferric pyrophosphate</td>
</tr>
<tr>
<td>Electrolytic iron</td>
<td></td>
<td>Ferric ammonium citrate</td>
</tr>
<tr>
<td>Ferric pyrophosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaFeEDTA*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Ethylenediaminetetraacetic acid

Biofortification

Biofortification involves the use of selective plant breeding techniques to improve the micronutrient content of staple food crops (wheat, bean, cassava, maize, rice, and yam). As a
long-term strategy for combating ID, biofortification is believed to be both highly sustainable and cost-effective (Meenakshi et al., 2007). After research, development, breeding, and dissemination of iron-rich seeds there are few recurrent costs. This is primarily because once the seeds are distributed to farmers, the crops could be locally grown; a useful means for reaching remote areas where there is limited access to other intervention strategies. Since biofortification is based on improving foods that are commonly consumed, there would be little need for any behavioral changes of the consumers (Nestel et al., 2006). Therefore, compliance would not be an issue in this strategy. An important benefit of this strategy, especially in regards to farmer acceptance, is that the high iron status of the plant seemed to improve crop yields (Graham et al., 2001). Disease resistance and stress tolerance were also improved in the seedlings of iron-rich plants, even in iron-poor soils (Welch and Graham, 1999).

There is evidence that it is physiologically feasible to increase the micronutrient levels of staple foods through breeding (Graham et al., 2001). When several different cultivars of common beans, wheat, and rice were grown in the same field, the seeds were found to have a wide genetic variation in the total iron content (Welch and Graham, 2004). For example, cultivars of common beans (Phaseolus vulgaris) grown in the same field were found to have iron in the range of 34-89 µg/g (Beebe et al., 2000). This large variation suggests that it should be possible to increase the iron content through the selective breeding of food crops (Welch and Graham, 2004). Additional evidence exists that the variations in iron concentrations among rice cultivars were mostly due to genetic components rather than environmental (Gregorio et al., 1999). Conversely, maize had a small range of iron concentrations among the cultivars (Bunziger and Long, 2000), indicating that selection for
iron-rich maize cultivars may not be feasible using traditional breeding alone. The constancy of seed iron-density across a variety of environmental conditions should also be considered for the effective implementation of biofortification as a global strategy (Welch and Graham, 2004).

Both rice (7-23 mg Fe/kg) and wheat (25-56 mg Fe/kg) may have increased iron content through selective breeding. However, the milling process for these seeds results in a loss of almost all of the iron, because most of the iron in cereal grains is concentrated in the bran (Resurreccion et al., 1979; Widdowson, 1975). Therefore, wheat and rice may not be the best crops for biofortification, unless there was an endosperm-specific iron improvement. This is due to the difficulty of increasing the iron concentration of milled wheat up to 40 mg/kg, which is the concentration of iron fortified wheat flour (Zimmermann and Hurrell, 2007). A proof of concept study to improve iron status of Filipino women by providing an extra 1.4 mg Fe/day through the consumption of iron biofortified rice, resulted in a moderate increase of iron stores (Haas et al., 2005). This study reveals that iron biofortified crops may improve the iron status of women and contribute to meeting the RDA for iron. However, there is concern that biofortification using conventional breeding techniques may only provide moderate increases in iron content, limiting its capacity to make a significant contribution to iron intake (Haas et al., 2005). The variety of culturally unique preparation and processing techniques that reduce the iron content or bioavailability in seeds also remains a major challenge and concern for biofortification (Welch and Graham, 2002). As a strategy to combat ID, biofortification must be able to increase the iron content high enough to see a beneficial effect on the iron status of the population.
Another issue with biofortification of food crops is the question of iron bioavailability. Increasing the iron content of the crops for nutritional enhancement would be futile if the iron is not bioavailable. Cereals and legumes have high concentrations of phytate and polyphenols that are known inhibitors of iron absorption (Sayers et al., 1973). Therefore, one strategy to improve iron bioavailability is to reduce phytate concentrations in the iron-dense varieties (Bouis, 2002). Low-phytate mutants, with a two-thirds reduction of phytate, have been identified for maize, barley, and rice genotypes (Raboy, 2000). However, a 90% reduction of phytate may be required to see significant improvements in iron absorption, which may not be achieved through conventional breeding (Hurrell et al., 1999). Phytate reduction may also cause anomalies in the yield, germination and growth characteristics of the plant (Pilu et al., 2005).

Alternatively, some research has found little effect of phytate on iron absorption with the long-term consumption of soybeans (Murray-Kolb et al., 2003). With about 50% of the soybean iron distributed to proteins (ferritin), as measured by radioactive label, the good iron bioavailability may be attributed to the high ferritin concentrations in these soybeans (Murray-Kolb et al., 2003). Although the bioavailability of ferritin is still in question, there is interest in increasing the ferritin concentration of plants to improve the iron content and possibly iron bioavailability (Theil, 2004).

**Biotechnology**

Most biofortification efforts have generally involved conventional breeding techniques, due to the poor acceptance and political barriers associated with genetically modifying food crops (Jauhar, 2006). However, biotechnology may be a useful strategy for improving iron content and bioavailability where conventional breeding is ineffective.
Several approaches, including the expression of lactoferrin, hemoglobin, phytase, and ferritin, have been attempted to enhance the iron content and bioavailability in a variety of plants. Human lactoferrin was expressed in rice and found to have similar bioavailability as ferrous sulfate-fortified rice (Lonnerdal and Bryant, 2006). The over-expression of maize hemoglobin as a highly bioavailable iron source is still at its early stages (Proulx et al., unpublished). Temperature-resistant *Aspergillus niger* phytase was inserted in rice endosperm, but was not able to tolerate the heat from cooking and did not significantly reduce phytic acid in rice (Lucca et al., 2001). Lucca et al. (2002) was able to increase the iron content of rice by two fold, by introducing a *Phaseolus vulgaris* ferritin gene and a heat-resistant phytase from *Aspergillus fumigatus* that was able to degrade the phytate in the seeds to produce a potentially bioavailable and high source of iron. Murray-Kolb et al. (2002) also produced rice over-expressing ferritin and found that the transgenic rice was as effective as ferrous sulfate for improving the iron status of iron-deficient rats.

Some of the challenges associated with enhancing the iron in the food crops are related to overcoming the homeostatic control of iron in the plant tissue. The potential barriers of biotechnology are related to: the absorption of iron from the soil into the root, the translocation of iron from the xylem and phloem sap to the seed or edible part of the plant, and the distribution of the iron to different tissues so that the seed is able to accumulate nontoxic bioavailable iron for human consumption (Lucca et al., 2006). For example, Goto et al. (2005) reported that the accumulation of iron in the high ferritin-expressing rice may have been limited by the iron uptake and transport rather than influenced by the expression of ferritin. The study of transgenic crops and their potential to complement traditional plant breeding warrants attention as a means for combating ID.
Iron Bioavailability

The absorption of dietary iron largely depends on the iron status of the individual and the amount and availability of the iron consumed (Hentze et al., 2004). The bioavailability for a nutrient represents the ability of the nutrient to be taken up or absorbed and used by the body.

Dietary Iron Absorption

Following ingestion of a meal, digestion breaks up the food to release nutrients for absorption (Figure 2.5). The mechanism and efficiency of iron absorption largely depends on the solubility of the iron, the form it is in when it reaches the intestinal lumen, and the presence of other dietary components ingested with the meal (Dunn et al., 2007).

Figure 2.5. Dietary iron uptake. From Zimmermann and Hurrell (2007).
The two major forms of iron in foods are heme and nonheme iron. Before dietary nonheme iron can be absorbed, Fe (III) must be reduced to Fe (II) by ferrireductase duodenal cytochrome-b (Dcytb). Fe (II) can then enter the cell through the apical membrane via divalent metal transporter-1 (DMT-1). Heme iron binds to heme carrier protein-1 (HCP-1), is internalized by receptor-mediated endocytosis and sent to the endoplasmic reticulum where the iron is released from heme by heme oxygenase-1 (HO-1) (Shayeghi et al. 2005). The iron from either source makes up the intracellular iron pool, which is delivered to the basolateral membrane to be exported by ferroportin-1, and oxidized by ferroxidase hephaestin (Hp) to Fe(III) for binding to transferrin (Donovan et al., 2005; Chen et al., 2004).

**Heme and Nonheme Iron**

The iron in our food generally exists in the organic form as heme or as ferric or ferrous compounds referred to as nonheme iron. Heme and nonheme iron have different mechanisms for intestinal absorption, but they ultimately contribute to the same intracellular iron pool. Heme iron primarily comes from the breakdown of hemoglobin and myoglobin in meat, fish, and poultry and makes up about 10-15 percent of the total iron in Western diets (Hallberg and Rossander, 1982). Heme iron is directly absorbed into the enterocytes with virtually no influence by dietary factors, so the bioavailability of heme iron may reach up to 30 percent (Fairbanks, 1994). Nonheme iron is primarily found in grains, vegetables, and some animal products. Although, about 85-90 percent of the total dietary iron consumed is nonheme iron (Hallberg and Rossander, 1982), the absorption of nonheme iron is commonly affected by dietary factors that chelate the iron and either enhance or inhibit absorption (Table 2.5) (Hallberg and Hulthen, 2002). The result is a bioavailability that may range from
only 1-10 percent (Hallberg and Rossander, 1982), hence nonheme iron makes a smaller
correction to the total iron absorbed compared to heme iron.

Table 2.5. Dietary substances that reduce or promote iron bioavailability and their major dietary
sources. Modified from Graham et al. (2001).

<table>
<thead>
<tr>
<th>Dietary Substances</th>
<th>Major Dietary Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antinutrients</strong></td>
<td></td>
</tr>
<tr>
<td>Phytic acid</td>
<td>Whole legume seeds and cereal grains</td>
</tr>
<tr>
<td>Fiber (cellulose, hemicelluloses,</td>
<td>Whole cereal grain products (wheat, rice, maize, oat,</td>
</tr>
<tr>
<td>lignin, cutin, etc.)</td>
<td>barley, rye)</td>
</tr>
<tr>
<td>Tannins and polyphenols</td>
<td>Tea, coffee, beans, sorghum</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>Spinach leaves, rhubarb</td>
</tr>
<tr>
<td>Calcium</td>
<td>Dairy products and supplements</td>
</tr>
<tr>
<td>Haemagglutinins (lectins)</td>
<td>Most legumes and wheat</td>
</tr>
<tr>
<td>Heavy metals (Cd, Hg, Pb, etc.)</td>
<td>Contaminated leafy vegetables and roots</td>
</tr>
<tr>
<td><strong>Enhancing factors</strong></td>
<td></td>
</tr>
<tr>
<td>Organic acids (ascorbic acid,</td>
<td>Fresh fruits and vegetables</td>
</tr>
<tr>
<td>fumarate, malate, citrate)</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Animal meats</td>
</tr>
<tr>
<td>‘The meat factor’</td>
<td>Animal meats</td>
</tr>
<tr>
<td>Certain amino acids (met, cys, his,</td>
<td>Animal meats</td>
</tr>
<tr>
<td>lys)</td>
<td></td>
</tr>
<tr>
<td>B-carotene</td>
<td>Green and orange vegetables</td>
</tr>
</tbody>
</table>

Factors Affecting Nonheme Iron Bioavailability

*Phytic acid.* In plants, phytate is the major storage compound for phosphate and
functions to sequester iron in the seed for growth (Raboy et al., 2001). Found primarily in
seeds, cereal grains, legumes and nuts, phytate is an inhibitor of nonheme iron absorption
(Frossard et al., 2000). Following the ingestion of a meal, phytate is released via digestion
and complexes with nonheme iron in the intestinal lumen, making the iron unavailable for
absorption. The high consumption of phytate, such as with vegetarian diets, has been found
to increase the incidence of IDA (Shaw, 1995). An intake of 5-10 mg of phytate has the
potential to reduce the nonheme iron bioavailability by up to 50 percent (Zhou and Erdman,
1995).
Soybeans have the potential to be a high source of iron; however, they contain about 10-22.2 g/kg phytic acid (Reddy et al., 1982). The reduction of phytic acid in soybeans was found to improve the nonheme iron absorption (Graf and Eaton, 1990) and the addition of meat or ascorbic acid to the diet was found to trump the inhibitory effect of phytate (Hallberg et al., 1987).

**Polyphenols.** These are compounds that exist in a variety of foods, such as fruits and vegetables, but are most commonly found in herbal and black teas, coffee, red wine, and cocoa (Hurrell et al., 1999). Polyphenols, such as tannins, are major inhibitors of nonheme iron absorption. Beverages containing 100-400 mg of polyphenols were found to reduce iron absorption by 60-90 percent (Hurrell et al., 1999). It is thought that once the polyphenols are released during digestion, they chelate iron in the intestinal lumen, rendering iron insoluble and thereby reducing uptake. The daily consumption of polyphenols in the Spanish diet was found to average about 2.6-3 g per person (Saura-Calixto et al., 2007).

**Calcium.** It is fairly well accepted that calcium from dairy product or supplements reduces nonheme iron absorption (Cook et al., 1991; Hallberg et al., 1991); however, there is evidence that dietary calcium may not have an inhibitory effect on iron from a varied diet (Reddy and Cook, 1997). Otherwise, calcium appears to reduce iron availability by 50-60 percent when a dose of 40-300 mg of calcium is consumed with a food item (Hallberg et al., 1991). Inhibition is speculated to occur from the formation of a calcium phosphate complex that binds iron during digestion, subsequently reducing the iron bioavailability (Morck and Cook, 1981).

**Ascorbic acid.** With the ability to both reduce and form a stable complex with iron, ascorbic acid has been found to enhance nonheme iron absorption (Hallberg et al., 1986).
Ascorbic acid has a dose-relating effect, whereby a 25-1000 mg dose of ascorbic acid given in a formula meal with 4.1 mg iron, improved iron absorption by 0.8-7.1 percent (Cook and Monsen, 1977). Fortification attempts with ascorbic acid effectively improved iron bioavailability; however, there were problems with oxidation in certain foods. Vitamin C supplementation (Cook and Monsen, 1977) and ascorbic acid given as part of a complete meal were each found to only moderately improve iron status (Cook and Reddy, 2001).

**Meat.** It not only provides highly bioavailable heme iron, but meat also has the ability to enhance the absorption of all of the nonheme iron in a meal (Conrad and Umbreit, 2000). Populations with a low consumption of meat have a higher incidence of ID, even with the same total iron intake as meat eaters (Conrad and Umbreit, 2000). Meat apparently seems to be an important component of the diet as a useful source of iron in regards to iron absorption. However, the mechanism or factors in meat that influence nonheme iron absorption are still unknown, but it is thought that they may be related to the amino acid profile of the meat (Bjorn-Rassmussen and Hallberg, 1979) and the ability to solublize the iron to improve absorption (Swain et al., 2002).

**Ferritin.** Soybeans are considered to have high iron content, but the bioavailability of that iron is in question. The bioavailability of soybean iron largely depends on what form it is found, in ferric phytate or ferritin, and whether ferritin iron absorption is affected by dietary factors. Several earlier studies have reported a low bioavailability of iron from soybeans and have attributed this to the high concentration of phytate in these seeds (Layrisse et al., 1975; Lynch et al., 1984). However, despite its high phytate content, soybean iron has also been found to be well absorbed compared to ferrous sulfate in marginally iron deficient women (Murray-Kolb et al., 2003). Since iron absorption is negatively correlated with iron
status, the high absorption of soybean iron may be attributed to the poor iron status of the subjects. For example, when soybeans were fed as a soup to nonanemic men, the iron bioavailability was low compared to absorption from ferrous sulfate (Lynch et al., 1984). However, it cannot be ruled out that the high iron absorption may have been due to a high ratio of ferritin to phytate in the seeds or the good bioavailability of ferritin iron, because the soybeans used in the Murray-Kolb et al. (2003) study had high concentrations of ferritin with about 50 percent of the iron bound as ferritin.

Some of the first ferritin bioavailability studies in humans found that ferritin iron was poorly absorbed when fed either alone or with various food items (Kuhn et al., 1968; Layrisse et al., 1975; Lynch et al., 1984; Martinez-Torres et al., 1986; Skikne et al., 1997). Bioavailability of iron in these studies ranged from 0.5-3.8 percent compared to 8.2-24 percent bioavailability of ferrous sulfate, as measured by red blood cell (RBC) incorporation. Absorption was also affected by many of the same dietary factors (phytate, desferrioxamine, ascorbic acid, meat) as nonheme iron (Kuhn et al., 1968; Martinez-Torres et al., 1986; Skikne et al., 1997), even though ferritin iron often had a slightly different bioavailability than other traditional nonheme iron compounds. The problem with these studies (with the exception of Lynch et al., 1984) lies with the use of subjects with varying iron needs and iron statuses for the study of bioavailability, because this likely contributed to a highly variable iron absorption among the subjects in each study.

In contrast, some more recent ferritin bioavailability studies in humans (Sayers et al., 1973; Murray-Kolb et al., 2003) and rats (Beard et al., 1996; Chang et al., 1996) have reported good ferritin iron absorption with 20-33 percent bioavailability (RBC). In a few cases, ferritin iron was also observed to be as bioavailable as ferrous sulfate in humans.
(Davila-Hicks et al., 2004; Lonnerdal et al., 2006). Ferrous sulfate is highly soluble and generally found to have high bioavailability in foods, so any iron source with comparable bioavailability would be considered a good source of iron. The major ferritin and soybean iron bioavailability studies are summarized in Table 2.6. Some studies also include the effects of dietary factors on ferritin and/or soybean absorption.

There are several different factors that may have contributed to the contradictory results of the ferritin and soybean iron bioavailability studies. First of all, both the iron status and gender of the human subjects was variable in many of the studies (Skikne et al., 1997; Martinez-Torres et al., 1986; Layrisse et al., 1975; Cook et al., 1972; Kuhn et al., 1968), which can greatly affect iron absorption. Another major problem may be due to the method of radioactive labeling used. An early validity study of intrinsic versus extrinsic labeling of iron in soybeans reported little difference between the methods, with a 1.3 extrinsic to intrinsic ratio and no difference between ferrous or ferric forms of iron (Cook et al., 1972). However, it is currently believed that the iron used to extrinsically label ferritin in foods may not equilibrate with the natural iron in the protein, which would result in an inadequate representation of ferritin iron (Welch, 1993; House, 1999). There is also a reportedly reduced iron absorption observed with extrinsically labeling of a complete meal rather than a single food item (Reddy et al., 2000). Problems with intrinsic labeling arise when the ferritin production is induced in the animal, such as through inflammation, whereby the iron is again not representative of normal ferritin iron (Theil, 2004). The conflicting bioavailability results may also be due to the type of iron salt used as the source of radiolabel in a meal (Murray-Kolb et al., 2003), whereby ferric citrate is generally better absorbed than ferrous sulfate or ferric chloride that readily bind phytate (Lynch et al., 1984). Lastly, the meal in which the
soybean or ferritin iron was provided may influence the results due to a potential food matrix effect and competition with various meal components, such as phytate.

As stable proteins found to resist denaturation by heat (85°C), 6 M urea, and proteolytic enzymes at a neutral pH (Liu et al., 2003), ferritins are suspected to potentially resist denaturation during gastrointestinal digestion (Theil, 1987). However, the stability of ferritin during digestion remains highly controversial. With the efficacy of ferritin iron absorption relying strongly on the theory that the stable protective coat prevents the inhibitions of nonheme iron absorption, it is important to investigate the digestibility of ferritin and how this may modulate its iron bioavailability.

A recent study measured the uptake of iron from purified pea seed ferritin, digested in vitro, using a Caco-2 cell model (Bejjani et al., 2007). Despite the loss of higher order structure of the protein after digestion, the iron uptake was 13.9 ng/mg of protein from digested ferritin, which was comparable with that of ferrous sulfate (19.3 ng/mg protein) and correlated with previous bioavailability studies in humans. However, the iron uptake was significantly reduced with the addition of phytate (4.5 ng/mg protein) and enhanced with the addition of ascorbic acid (125 ng/ml protein).

A similar study compared the uptake of either intact or digested (pH 2 or pH 4) purified horse spleen ferritin using a Caco-2 cell model (Kalgaonkar and Lonnerdal, 2008). Ferritin alone showed an increase in cell uptake with greater digestion of the protein (from intact to in vitro digestion at pH 2). However, the pH 2 digested ferritin was also the most greatly influenced by dietary factors (phytic acid, ascorbic acid, tannic acid, calcium, and hemin), in a similar pattern as nonheme iron. Interestingly, the pH 4-digested ferritin was not
influenced by any of the dietary factors with the exception of tannic acid, which unexpectedly increased uptake.

Both studies verified that ferritin may not have the capability to prevent chelation of iron with dietary factors after digestion at pH 2. However, it is thought that as ferritin is normally consumed within a food matrix where pH within a bolus of food may not reach pH 2, ferritin may be partially protected from complete digestion (Kalgaonkar and Lonnerdal, 2008). Researchers have also reported that the rate of ferritin proteolysis may depend on the conformation of the protein and that iron-bound ferritin had reduced digestibility (pepsin, pH 2.5) by 2-3 times compared to that for apoferritin, with only a fractional loss of structure (Crichton, 1970).
Table 2.6. Summary of ferritin and soybean iron bioavailability studies in rats and humans and ferritin iron absorption studies in human Caco-2 cells

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Iron Form and Dose</th>
<th>Food Matrix</th>
<th>Bioavailability</th>
<th>General Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beard et al., 1996</td>
<td>8 rats (iron deficient)</td>
<td>Control: 30 mg Fe/kg diet FeSO₄: 30 mg/kg Ferritin (ft): 29 mg/kg Soybean meal (SBM): 26 mg/kg</td>
<td>AIN diet (Borel et al., 1991) or AIN Fe-deficient diet</td>
<td>Fe recovery (spleen) FeSO₄: 126% Ft: 97% SBM: 72%</td>
<td>89-100% recovery from anemia with all treatments</td>
</tr>
<tr>
<td>Chang et al., 2005</td>
<td>8 rats (iron deficient)</td>
<td>Control: 48 mg Fe/kg diet Horse spleen ft (HSF): 35 mg/kg Ft-producing recombinant yeast (FTY): 35 mg/kg Ferrous ammonium sulfate (FAS): 30 mg/kg, 50 mg/kg</td>
<td>AIN-76A diet or AIN Fe-deficient diet</td>
<td>Fe recovery (spleen) HSF: 103% FTY: 99% FAS (30): 85% FAS (50): 93%</td>
<td>Iron recombinant ferritin of yeast is bioavailable, indicating potential iron source as iron-fortified yeast</td>
</tr>
<tr>
<td><strong>Humans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kuhn et al., 1968</td>
<td>2-17 people, 11-60 y (nonanemic to iron deficient)</td>
<td>⁵⁷Fe intrinsically labeled soybeans (SB) and purified animal liver ferritin, ⁵⁹Fe extrinsically labeled ferrous ascorbate (FA)</td>
<td>Soybean patty with margarine or ferritin solution</td>
<td>Fe absorption from Food /FA SB/FA: 0.68 Ft/FA: 0.12</td>
<td>Composition of diet is important for bioavailability of dietary iron</td>
</tr>
<tr>
<td>Cook et al., 1972</td>
<td>11 people, 16-78 y (nonanemic to iron deficient)</td>
<td>⁵⁷Fe intrinsically labeled or ⁵⁹Fe extrinsically labeled soybeans</td>
<td>Boiled, mashed soybeans</td>
<td>RBC</td>
<td>Both labeling tags provide a valid measure of Fe absorption</td>
</tr>
<tr>
<td>Sayers et al., 1973</td>
<td>5 women</td>
<td>As FeSO₄ or intrinsically labeled bean ferritin</td>
<td>Biscuit</td>
<td>RBC</td>
<td>Ferritin Fe was well absorbed</td>
</tr>
<tr>
<td>Study</td>
<td>Subjects</td>
<td>Iron Form and Dose</td>
<td>Food Matrix</td>
<td>Bioavailability</td>
<td>General Conclusions</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>-------------------</td>
<td>-------------</td>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Layrisse <em>et al.</em>, 1975</td>
<td>7 people (nonanemic to iron deficient)</td>
<td>$^{59}$Fe or $^{59}$Fe extrinsically labeled purified rabbit liver ferritin and soybean iron and FeCl$_3$</td>
<td>Alone or with Fe labeled boiled soybean mush</td>
<td>RBC Ferritin: 1.9% Ferritin in soybean: 0.5%</td>
<td>Ferritin Fe absorption is reduced by dietary factors in soybeans</td>
</tr>
<tr>
<td>Lynch <em>et al.</em>, 1984</td>
<td>10 men, 18-20 y (nonanemic)</td>
<td>2.7 mg extrinsically labeled $^{59}$Fe or $^{59}$Fe + 1.3 mg added as FeCl$_3$ in soybean 3 mg $^{55}$Fe or $^{59}$Fe as FeSO$_4$</td>
<td>Soybean soup</td>
<td>RBC Soybean: 1.7% FeSO$_4$: 16%</td>
<td>Bioavailability of Fe in soybeans is low</td>
</tr>
<tr>
<td>Martinez-Torres <em>et al.</em>, 1986</td>
<td>10 men 53 women (nonanemic to iron deficient)</td>
<td>2 mg meat $^{59}$Fe, 2.8 mg vegetable $^{59}$Fe extrinsically labeled and $^{59}$Fe as rabbit ferritin or as 3 mg Fe ascorbate</td>
<td>Meat with rabbit ferritin or traditional meal (3 veg + meat)</td>
<td>RBC Ferritin + meat: 9% Ferritin + meat + vegetables: 3.0% Fe ascorbate: 32%</td>
<td>Bioavailability of Ferritin Fe is similar to nonheme iron</td>
</tr>
<tr>
<td>Skikne <em>et al.</em>, 1997</td>
<td>10 people, 23-41 y (nonanemic to iron deficient)</td>
<td>5 mg $^{59}$Fe as FeSO$_4$ or $^{59}$Fe intrinsically labeled as purified bovine ferritin</td>
<td>Bovine spleen and liver ferritin alone or with food</td>
<td>RBC FeSO$_4$(alone): 24% FeSO$_4$ (food): 8.2% Ft (alone): 3.8% Ft (food): 3.2% Ft (50 mg Fe): 0.6%</td>
<td>Ferritin Fe is poorly absorbed and not useful as source of pharmaceutical iron</td>
</tr>
<tr>
<td>Murray-Kolb <em>et al.</em>, 2003</td>
<td>18 women, 19-23 y (marginally iron deficient)</td>
<td>Intrinsically labeled $^{55}$Fe as FeSO$_4$ (3 mg Fe) or SB muffins (3 mg Fe) or soup (4.5 mg Fe) (49% of $^{55}$Fe in ferritin)</td>
<td>Soybean (SB) soup and muffins</td>
<td>RBC FeSO$_4$: 61% SB: 27%</td>
<td>Soybeans are good source of Fe</td>
</tr>
<tr>
<td>Davila-Hicks <em>et al.</em>, 2004</td>
<td>30 women (nonanemic)</td>
<td>1 mg $^{57}$Fe as FeSO$_4$ or extrinsically labeled purified horse spleen ferritin reconstituted with PO$_4$</td>
<td>Breakfast meal</td>
<td>Whole Body, RBC FeSO$_4$: 19%, 39% Ferritin: 22%, 28%</td>
<td>Fe from ferritin is as bioavailable as Fe from FeSO$_4$</td>
</tr>
<tr>
<td>Lonnerdal <em>et al.</em>, 2006</td>
<td>16 women (nonanemic)</td>
<td>1 mg $^{57}$Fe as FeSO$_4$ or extrinsically labeled purified soybean ferritin reconstituted with PO$_4$</td>
<td>Breakfast meal-bagel, cream cheese, apple juice</td>
<td>Whole Body, RBC FeSO$_4$: 34%, 35% Ferritin: 30%, 33%</td>
<td>No significant difference b/w Fe absorption from FeSO$_4$ and ferritin</td>
</tr>
</tbody>
</table>
Table 2.6. continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Iron Form and Dose</th>
<th>Food Matrix</th>
<th>Absorption</th>
<th>General Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalgaonkar and Lonnerdal, 2008</td>
<td>Human intestinal Caco-2 cells</td>
<td>Horse spleen ferritin (ft) intact or digested <em>in vitro</em> (pH 2 or pH 4), extrinsically labeled with $^{59}$Fe as FeSO$_4$</td>
<td>None</td>
<td>FeSO$_4$: 0.58</td>
<td>Ferritin is digested and ferritin Fe absorption is inhibited by phytate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ft (intact): 0.41 Ft (pH 4): 0.92 Ft (pH 2): 5.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FeSO$_4$ + phytate: 11% ↓ absorption Ft (intact) + phytate: no effect Ft (pH 2) + phytate: 35% ↓ absorption</td>
<td></td>
</tr>
<tr>
<td>Bejjani et al., 2007</td>
<td>Human intestinal Caco-2 cells</td>
<td>Purified pea seed ferritin digested <em>in vitro</em> (pH 2), Fe measured using bathophenonthroline method</td>
<td>None</td>
<td>FeSO$_4$: 19.3</td>
<td>Ferritin Fe absorption is inhibited by phytate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ft (pH 2): 13.9 Ft (pH 2) + phytate: 4.45</td>
<td></td>
</tr>
</tbody>
</table>

% absorption assumes 80-85% absorption of iron into erythrocytes
PHYTOFERRITIN

Ferritin Structure

Ferritin is a large (540-600 kDa) multi-subunit globular protein found in most organisms including plants, animals, and bacteria that serves as the primary storage site for iron. It consists of 24 peptide subunits (~28 kDa each), arranged into four helix bundles that form a hollow spherical shell (Figure 2.6) and an iron core with the capacity to store up to 4,500 iron atoms (Harrison and Arosio, 1996). The ferritin secondary and quaternary structures are highly conserved, but gene organization varies depending on the main function of ferritin. The amino acid sequences of plant and animal ferritins are about 39-49% identical (Andrews et al., 1992). Phytoferritins also have an extra peptide sequence at the amino terminus of each protein subunit compared to animal ferritins. The first part of this plant-specific sequence is the transit peptide, which is responsible for ferritin localization into cell plastids. The extension peptide makes up the second part of this sequence, and is believed to play a role in ferritin stability (Van Wuytswinkel et al., 1995) and possibly the molecular assembly of ferritin (Proudhon et al., 1989).

Figure 2.6. Representation of spherical ferritin protein (A); ferritin protein with open pores (B); and cross-section of ferritin cavity (C). From Liu and Theil (2005).
Ferritin subunits are generally categorized into two groups, either H- or L- (heart and liver or heavy and light) subunits, depending on their size, amino acid composition, surface charge, and immunoreactivity (Arioso et al., 1978). H-subunits are generally responsible for the uptake and rapid oxidation of Fe (II) and L-subunits play a role in core formation; together their functions are complementary (Van Wuytswinkel et al., 1995). The H- and L-ferritin subunits vary with cell type, species, and even physiological condition and may partially account for differences in the whole ferritin structure. In general, phytoferritins sequences are H/L-chain heteropolymers; for example, pea seed ferritins were reported to contain approximately 49% H-subunits and 40% L-subunits (Harrison and Arioso, 1996).

**Role of Phytoferritin**

As the primary storage protein for iron, ferritins play a major role in maintaining cellular iron homeostasis. Iron is important in plants for electron transfer reactions in respiration and photosynthesis, it is a cofactor for enzymes required for DNA synthesis, and involved in nitrogen-fixation pathways (Liu and Theil, 2004). However, in the presence of oxygen, free iron in the cell can produce free radicals by the Fenton reaction that can lead to cell damage via lipid peroxidation, protein oxidation, or DNA damage (Briat and Lebrun, 1999). Therefore, the storage of iron in ferritin also serves to protect the cells from oxidative damage from free iron.

To store excess iron in the cell, ferritin takes up iron in the form of Fe (II) via gated pores or channels at the junctions of H-chain subunits. The Fe (II) ions are subsequently oxidized at ferroxidase sites in the ferritin core for storage as Fe (III) oxyhydroxide crystals in the iron core. The reactions are shown in equations 1-4 below (Liu and Theil, 2005).
Molecular oxygen in the iron core serves as an electron acceptor to bind the iron atoms in ferroxidase reactions (Equations 1, 2) for the oxidation of ferrous ions into ferric oxyhydroxide and ultimately, the uptake into the protein cavity (Equation 4) (Liu and Theil, 2005).

Phosphate groups are also found in the crystalline arrangement mostly associated with the iron on the perimeter of the iron core, which are believed to help bind Fe (II) to the iron core (Cheng and Chasteen, 1991; Hequing et al., 1993) and improve the thermodynamic stability of the core (Watt et al., 1985). Phytoferritins have been found to have higher concentrations of phosphate in the iron core than animal ferritins, whereby the phosphate concentration in the core is believed to be a reflection of the composition of phosphate in the cell (Waldo et al., 1995).

When there is a demand for iron in the cell, it can be released from ferritin in a controlled manner through the unfolding of gated pores or more commonly through the lysosomal degradation of ferritin (Kidane et al., 2006). Iron (III) is subsequently reduced by various reductants to Fe (II) and becomes soluble in the presence of chelators (Dognin and Crichton, 1975).
Regulation of Phytoferritin Synthesis

Phytoferritin synthesis is regulated by the iron status of the cells, where phytoferritin is constantly being degraded and resynthesized to provide an available intracellular iron pool or a means of storage when excess iron is in the cell (Briat et al., 1995). The mechanism of ferritin synthesis regulation is quite different for plants and animals (Table 2.7). The expression of ferritin in animals is mostly coordinated and regulated by cellular iron, which influences IRP and IRE in ferritin mRNAs for the translational control of ferritin expression. However, there is no translational control of phytoferritins, which do not even have a 5′-IRE on the mRNA sequence (Lescure et al., 1991). Rather, control is at the transcriptional level, whereby phytoferritins genes are induced to accumulate mRNAs in response to iron overload and are repressed by ID (Briat et al., 1999).

Table 2.7. Comparison of the genetic target and signals that regulate ferritin synthesis in plants, animals, and bacteria. From Hintze and Theil (2006).

<table>
<thead>
<tr>
<th>Protein Location*</th>
<th>Gene regulation</th>
<th>Signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals Cytoplasm, Mitochondria</td>
<td>Transcriptional, Translational</td>
<td>Fe, O₂, H₂O₂</td>
</tr>
<tr>
<td>Plants Plastid</td>
<td>Transcriptional</td>
<td>Fe³⁺(Cit)₆ or Fe³⁺ + ascorbate</td>
</tr>
<tr>
<td>Bacteria Cytoplasm, DNA complex</td>
<td>Transcriptional</td>
<td>Stress, Starvation</td>
</tr>
</tbody>
</table>

*All cells, varied with development/environment

It is believed that an increase of the iron storage capacity in plant cells may serve to signal or promote iron uptake into cells and sequester excess iron that flows into the cells (Van Wuytsuwinkel et al., 1999; Yoshihara et al., 2003). This relationship between iron and ferritin content has been investigated by over-expressing ferritin in transgenic crops. Goto et al. (1998) produced high-iron transgenic tobacco plants with a gene derived from soybean
ferritin cDNA and observed a positive correlation between iron and ferritin content. Goto et al. (2000) later produced transgenic lettuce with high expression of ferritin and observed a 1.2-1.7 fold increase of iron content and enhanced growth of the plant, which further endorsed the breeding of high iron crops through the introduction of a ferritin gene. Conversely, Qing et al. (2005) developed two ferritin hyper-expressing transgenic rice lines and observed little correlation between ferritin expression and iron concentrations in seeds. Apparently, the expression of a ferritin gene in plants has the capacity to increase the iron content in plants; however, iron accumulation may be limited by the plant’s ability to take up or transport iron, which may be affected by soil and genotype (Moraghan et al., 2002).

**Iron and Ferritin Accumulation in Plants**

When iron is transported into a plant, it is immediately chelated to prevent the production of oxygen radicals that cause damage to the cells. The primary chelator for free iron in plants is nicotianamine (NA), which is present in all tissues of higher plants (Scholz et al., 1992). It is also thought that NA may play a role in the transport of iron between and within cells. Although NA is the initial iron chelator, the main destination of iron is for functional purposes: as a cofactor for proteins with enzymatic functions, for iron-sulfur clusters and heme. And in the case of iron overload, it is stored as ferritin or iron precipitates in vacuoles and mitochondria (Figure 2.7) (Hell and Stephan, 2003).
Excess free iron in the cell induces phytoferritin synthesis (Briat et al., 1999). Phytoferritins are primarily found in plastids and seem to accumulate in developing tissues where there is generally a high demand for iron (Seckback, 1982). In young plants, ferritins are concentrated in roots and leaves to provide iron primarily for proteins involved in nitrogen fixation or photosynthesis (Lobreaux and Briat, 1991). As the plant matures, ferritins become concentrated in the seeds to provide an iron source for seed germination. After germination, ferritin is degraded for the release of iron; this is marked by the shortening of the 28 kDa subunits to 26.5 and 25 kDa polypeptides (Lobreaux and Briat, 1991). However, in dry seeds without germination the ferritin remains present in the seed.
Legumes, nodulating plants that require iron for nitrogen fixation, contain more iron in their seeds than cereal grains (Lynch et al., 1998). About 70-90% of the iron in beans was found to be bound to ferritin (Marentes and Grusak, 1998), whereas iron in wheat grain is primarily bound to phytate (May et al., 1980). The high concentration of iron in the nodulating plants may have played a role in inducing the synthesis of ferritin to sequester excess iron for storage and later use (Briat and Lebrum, 1999).

In the seeds of food crops, the majority of iron was found to be associated with either phytic acid or ferritin (Lott et al., 1995; Marenthes and Grusak, 1998), both of which can greatly affect the bioavailability of the seed iron in humans—phytic acid as an inhibitor of iron absorption and ferritin as a potentially good source of bioavailable iron. The distribution of iron and whether it binds to phytate or ferritin during seed development is poorly understood. The composition of these iron-binding seed components may play a role in iron distribution. Alternately, iron distribution may be determined by various mechanisms of iron homeostasis or a factor such as NA. The delivery of iron to the cell and to the plastids is highly dependent on NA, which is thought to be a mediator of iron storage and homeostasis. In NA-free tomato plant mutants iron precipitates were found in the plastids (Becker et al., 1995), but ferritin was not detected (Liu et al., 1998). Therefore, NA may play a role in iron storage and ferritin synthesis.

Investigating the speciation of iron in the seed is important to gain a better understanding of how various seed components may affect iron bioavailability, because the location and form of iron in the seed can greatly influence the value of the seeds as a source of iron. It is not only important to consider what molecule the iron is bound to, but where it is distributed in the seed. In general, plant iron is distributed between the seed coat and
embryo depending on the genotypic traits of the plant (Moraghan and Grafton, 2002). In common beans and soybeans, iron is concentrated in the seed coats, which was found to have higher bioavailability than iron in the embryos due to the higher solubility of iron in the seed coat (Moraghan et al., 2002; Lombardi-Boccia et al., 1995). However, some processing and preparation techniques for beans require the removal of the seed coat, thereby reducing the iron content of the food. This can be resolved by altering the preparation methods, but it emphasizes the importance of investigating iron distribution and bioavailability in seeds.

**Analyzing Ferritin in Food Crops**

The biochemical study of phytoferritins has mainly encompassed the purification and characterization of ferritin from a few different plants, primarily soybeans, peas, lentils, maize, and common beans (Barceló et al., 1997; Bescure et al., 1990; Sczekan and Joshi, 1987; Crichton et al., 1978; Laulhere et al., 1988). Ferritin in these seeds have been quantified (ranging from 8-80 µg/g of seed), but this procedure involves both the purification of ferritin from the seed and immunoblotting, which can be both time-consuming and laborious. As the secondary measure in these characterization studies, the current ferritin quantification methods are inadequate for rapidly measuring ferritin in seeds.

Since ferritin has gained positive attention due to its high iron binding capacity and iron bioavailability, it has created interest in the use of ferritin-biofortified staple food crops as a bioavailable source of iron for combating ID. However, a method for rapidly measuring ferritin in seeds is currently lacking. A simple and reliable assay would facilitate the rapid screening of large numbers of seeds that can be used by plant breeders to identify and breed ferritin-rich crops.
REFERENCES


Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. *J Nutr* 2001;131:568S-79S.


Crichton RR. Susceptibility to proteolysis as a probe of the conformation of ferritin and apoferritin in solution. *Biochem J* 1970;119:40P.


CHAPTER 3. ELISA DEVELOPMENT FOR THE QUANTIFICATION OF FERRITIN IN STAPLE FOOD CROPS

Rebecca J. Lukac\textsuperscript{1}, Maneesha Aluru\textsuperscript{2}, Manju B. Reddy\textsuperscript{1}

A paper to be submitted to the \textit{Journal of Agricultural and Food Chemistry}

ABSTRACT

Biofortification is a strategy for combating iron deficiency that involves the selective breeding of food crops to increase total bioavailable iron. Evidence showing ferritin as a good source of bioavailable iron has created interest for enhancing ferritin content in seeds. However, the successful implementation of ferritin biofortification requires a method to rapidly measure ferritin in staple food crops. The objective of this study was to develop an ELISA to quantify ferritin in red beans, white beans, wheat, maize, and brown rice. The following steps were taken for ELISA development. A common amino acid sequence among all seed ferritins was identified to produce an anti-ferritin antibody, ferritin was extracted from the seeds using a crude extraction procedure, and immunoblotting techniques were used to verify the immunoreactivity of the antibodies with the ferritin sample. Using our developed ELISA with the polyclonal antibody and recombinant pea ferritin standard, the quantity of ferritin by crude extraction was found to be 10.2±1.0, 4.38±0.9, 1.2±0.3, 0.38±0.1, and 0.04±0.01 µg/g in red beans, white beans, wheat, maize, and brown rice, respectively. There was no significant correlation between ferritin and iron in red beans;

\textsuperscript{1}Graduate student and Associate Professor, respectively, Department of Food Science and Human Nutrition, Iowa State University, Ames, IA

\textsuperscript{2}Collaborator, Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA
therefore the measurement of ferritin may be of value for improving the iron bioavailability in staple crops. This simple and reliable assay may facilitate the rapid screening of large numbers of seeds that can be used by plant breeders to identify and breed ferritin-rich crops.

**KEYWORDS:** ferritin, iron bioavailability, ELISA, staple crops

**INTRODUCTION**

As the most prevalent global nutrition problem, iron deficiency (ID) afflicts an estimated 4-5 billion people worldwide (1), causing an array of serious adverse health outcomes with socio-economic implications (2). The primary cause of ID is the insufficient intake of bioavailable iron (3). In many populations, this inadequate iron intake is the result of a lack of dietary diversity and over-reliance on staple food crops. Given that staple food crops contain relatively low levels of iron and high levels of dietary factors that inhibit human iron absorption (4).

Iron supplementation and food fortification programs have been implemented to combat ID (5). However, underdeveloped countries may not be able to afford and/or maintain supplementation and it is a challenge to produce foods fortified with highly bioavailable iron compounds which are also compatible with the food vehicle (3). Biofortification, the use of breeding techniques or biotechnology to enhance the micronutrient content of staple food crops, may be a sustainable and cost-effective strategy to complement these intervention programs (6). Biofortification techniques are currently being used for the development of staple crops (beans, rice, maize, and wheat) with elevated levels of iron or increased iron bioavailability (7). These strategies include reducing phytate, increasing total iron, and increasing ferritin content. Iron biofortified rice was the first to be
examined for efficacy and reportedly improved the iron stores of women with iron-poor diets (8).

Ferritin is the iron storage protein for plants, animals, bacteria, and archea, consisting of a 24-subunit protein shell around an iron core (9). With the capacity to store up to 4,500 atoms of iron, increased expression of ferritin and reduction of phytate may increase the concentration of bioavailable iron in seeds (10, 11). As a stable protein, ferritin may resist denaturation during gastrointestinal digestion; thereby protecting iron from chelators during human digestion, and improving absorption (12). Ferritin iron is reported to be well absorbed in humans (13-15). However, ferritin bioavailability studies report mixed results (16-20) and questions still exist about the mechanism of ferritin iron absorption. A recent study by Kalgaonkar and Lonnerdal reported that undigested ferritin is not affected by dietary factors and is believed to have a different mechanism of absorption than the iron from digested ferritin (21). Therefore, if ferritin is not completely broken down during digestion, it may serve as a significant source of bioavailable iron.

Ferritins are concentrated in the seeds (22), whereby the majority of purification studies have been performed with soybean ferritins (23-25). However, alfalfa seed (26), bean, maize (25), and pea (27) ferritins have also been experimentally isolated, purified and characterized. Ferritin concentrations of these seeds ranged from 8-80 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 (28, 29); whereas, cereal grains such as wheat have low ferritin levels (30).

Ferritin biofortified crops have the potential to provide a high source of bioavailable iron to combat ID. However, a convenient method to measure ferritin in crops is currently
lacking. Therefore, the purpose of this study was to develop a rapid reliable assay to quantify ferritin among staple food crops (beans, maize, wheat, and rice) to provide a convenient screening tool for the development of ferritin-rich 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 pea ferritin (rFerr) was kindly donated from the Institute of Food Science and Technology, Laboratory of Human Nutrition (Zurich, Switzerland) for use as a standard for immunoblot and ELISA experiments. All other products and chemicals were obtained from Sigma Aldrich (St. Louis, MO) and Fisher Scientific Co. (Fairlawn, NJ) unless otherwise stated.

**Antibody Production.** Ferritin protein sequences for beans, wheat, maize, and rice were obtained from GenBank (www.ncbi.nlm.nih.gov) and compared using ClustalW (http://www.ebi.ac.uk/clustalw/) to find multiple sequence alignments of the divergent seed ferritin sequences. A common 30-amino acid sequence corresponding to residues 105-134 of maize ferritin (Accession Number CAA58147) was found to be 100% identical between maize, rice and wheat ferritins and approximately 97% identical with ferritins from common beans. Within this 30-amino acid sequence a 22-amino acid region corresponding to residues 113-134 in maize ferritin was found to be highly immunogenic (Table 3.1). The region also corresponds to amino acids 39-60 of horse ferritin L chain (Accession No. PO2791), providing more evidence that the sequence may be conserved (31).
The antigen was selected from the conserved region in the ferritin sequences that would best elicit an immune response based on the predicted number of turns, hydrophilicity, antigenicity, and amino acid composition, using Custom Antigen Design Assistance (ProSci, Inc., Poway, CA). This amino acid sequence is part of the alpha-helix, helix turn and the L-loop regions of the protein that have little secondary structure, which should make it accessible to antigen-antibody interactions. The highly immunogenic 22-amino acid sequence was used for anti-ferritin polyclonal antibody production in rabbit and monoclonal antibody production in mice. Antibodies (Ab) were screened for immunoreactivity with seed ferritins using gel electrophoresis and western blot described in detail below. Both monoclonal and polyclonal Ab were affinity purified by ProSci prior to their use in the experiments.

**Partial Purification of Ferritin.** Crude extracts were prepared from all seeds using methods described by Barceló et al. (26) and Laulhere et al. (25) 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 hrs at 4°C. The seeds were removed from the water and homogenized on ice in 4 volumes of extraction buffer (10 mM sodium phosphate buffer, 100 mM sodium chloride, 2% polyvinylpyrolidone, and 1 mM phenylmethylsulphonylfluoride, pH 7.2) using a Polytron PT10/35 (Brinkmann Instruments, Westbury, NY) at medium speed until homogenate was smooth. The slurry was centrifuged at 15,000 x g for 10 min at 4°C. After slowly adding MgCl₂ to the supernatant to a final 0.7% (v/v) concentration, the sample was incubated on ice for 10 min and centrifuged at 26,000 x g for 5 min at 4°C. The supernatant was collected and MgCl₂ was slowly added to
a final 1% (v/v) concentration with 1 hr incubation on ice, followed by the addition of 2% sodium citrate (v/v) for 20 min incubation on ice, and a final centrifugation at 26,000 x g for 50 min at 4°C. The supernatant was removed and the entire pellet was resuspended with 10 mM sodium phosphate buffer, pH 7.2 to a total volume of 1 ml for bean varieties, 0.5 ml for wheat and maize and 0.2 ml for rice, and stored at -20°C.

**Protein Analysis.** The nitrogen content of the dry ground seeds was determined using the Dumas method (32) 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 nitrogen 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 (33). The percent protein extraction was calculated based on the protein content of the initial extract.

**Gel Electrophoresis.** Proteins in the sample extracts were separated in reducing conditions using 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (34). The gels (1.5 mm) were either stained and destained with Coomassie blue R-250 in fixative and 40% methanol/10% acetate to detect separated protein bands or used for western blot.

**Western Blot.** Proteins from the SDS gel were transferred to nitrocellulose membrane (Whatman, Maidstone, Kent, UK) at 350 mAmps in tris-glycine transfer buffer for 1.5-2 hrs according to the methods described by Towbin *et al.* (35) with modifications described below.
Following the transfer, the nitrocellulose membranes were incubated in 5% blocking solution (5% nonfat dry milk dissolved in tris buffered saline with 0.1% Tween 20; TBST) for 3 hrs at room temperature (RT) to reduce non-specific binding. The proteins were probed with anti-ferritin monoclonal Ab (1:10,000) or anti-ferritin polyclonal Ab (1:3,000) in 2% blocking solution overnight at RT. The membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:5,000) in 2% blocking solution for monoclonal Ab (goat anti-rabbit for polyclonal Ab) at RT for 3 hrs. TBST was used to wash the membranes 3 times (5-10 min each) following both antibody steps. Blots were soaked with chemiluminescent substrate (Pierce, Rockford, IL) for 1 min, with a 1 min exposure to photographic film (Blue Lite Autorad Film, ISC BioExpress, Kaysville, UT) to create an image of the antibody-bound ferritin from the blot. A broad range prestained SDS-PAGE ladder (Bio-Rad, Hercules, CA) was used as a molecular weight standard.

**Densitometry.** Varied concentrations of protein in the extract were prepared in triplicate along with rFerr, run simultaneously on two gels, and immunoblotted. A digital image of the blot was captured and analyzed by densitometry using Gel-Pro Analyzer (Media Cybernetics, Inc., Bethesda, MD) to quantify the antibody-bound ferritin in terms of the optical density of the band. The average intensity of each band was determined and the ferritin was calculated from the mean of 3 intensity measurements using the rFerr standard curve.

**ELISA.** An indirect ELISA protocol was adapted from methods described by Sigma Aldrich and Flowers *et al.* (36) with modifications. Protein was extracted from the seeds as described above. Dilutions of the samples and standards were made with bicarbonate buffer,
pH 9.6, as determined by several trial runs for each seed. 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 incubating the plate for 15-20 hrs at 4°C, plates were washed twice with 400 µl/well of phosphate buffered saline and 0.1% Tween 20 (PBST), and tapped dry on a paper towel. To block non-specific binding, 200 µl/well 50% Stabilcoat Immunoassay Stabilizer (Surmodics, Eden Prairie, MN) was added to the plate for 4 hrs at 25°C. The plate was emptied, tapped dry, and 100 µl/well anti-ferritin polyclonal Ab (1:500) in 25% Stabilcoat was added. Following incubation at 25°C for 90 min the primary antibodies were discarded and the plate was washed 3 times as before and tapped dry. HRP-conjugated goat anti-rabbit secondary antibodies in 25% Stabilcoat (100 µl/well; 1:5,000) were added to the plate and incubated at 25°C; after 1 hr the plate was emptied, washed 3 times, and tapped dry. Substrate (10 ml citrate phosphate buffer, pH 5, 10 mg o-phenylenediamine, 30 µl 30% hydrogen peroxide; 100 µl/well) was added and incubated at 25°C for 30 min in the dark until the reaction was stopped with 25% sulfuric acid (25 µl/well) and absorbance read at 490 nm wavelength and 560 nm reference using KC Junior software (version 1.14) and Microplate Reader ELx808 (Bio-Tek Instruments, Inc., Winooski, VT). Ferritin concentrations in the seeds were measured against the rFerr standard curve.

Application of ELISA to Screen Beans. Thirteen red bean varieties were obtained from the International Center for Tropical Agriculture (CIAT; Cali, Columbia) to be screened for ferritin content using the ELISA method described above. Samples were received as dry ground seed, so modifications to the method included homogenizing the seed in 6x volume
of extraction buffer without soaking of the seeds. Nonheme iron content of the dry ground seed was determined in duplicate using the method described by Torrance and Bothwell (37) and modified by Proulx and Reddy (38).

Statistics. The percent protein extracted from each seed was calculated based on the amount of soluble protein in the extraction buffer after the first centrifugation step relative to the total protein content of the seeds. All ELISA experiments were repeated three times, with three separate extractions and assayed in triplicate. Differences among the 13 red bean varieties were determined using ANOVA with Tukey’s multiple comparison test and considered to be statistically significant at $p \leq 0.05$. The Pearson correlation between iron and ferritin concentrations among the red bean varieties was also determined with significance threshold at $p \leq 0.05$. Statistical analyses were performed using Graph-Pad Prism, version 4.02 for Windows (San Diego, CA).

RESULTS AND DISCUSSION

Antibody specificity. Anti-ferritin polyclonal (Figure 3.1A) and monoclonal (Figure 3.1B) Ab were both immunoreactive with all of the seed ferritins for beans, maize, wheat, and rice, as verified with immunoblotting. Various amounts of protein were required to detect ferritin depending on the seed type and Ab used; protein required for detection ranged from 10-30 µg for polyclonal blots and 10-50 µg for monoclonal blots. Differences in the apparent reactivity of each sample with the Ab may be related to the type of protein or percent of total protein as ferritin in the extracts.

Western blot with polyclonal Ab revealed double bands for red beans, white beans, and wheat, but a single broad band for maize and brown rice, with molecular weights in the
approximate range of 26-28 kDa, corresponding to that of rFerr standard. Previous studies have reported similar molecular weights of ferritins from soybeans (23, 24), alfalfa seeds (26), bean, pea, and maize (25, 39). Goto et al. (40) reported that the 28 kDa peptide can be cleaved, releasing its 1.5-2 kDa extension peptide to produce both 26.5 and 28 kDa polypeptide subunits. Whereas, Masuda et al. (41) reported that the 26.5 and 28 kDa soybean ferritin peptides were found to have different amino acid sequences, and therefore represent two different ferritin subunits.

The polyclonal Ab also recognized high molecular weight (MW) proteins in red and white beans (74 and 94 kDa), wheat (40 and 44 kDa), and rice (33, 48, and 94 kDa). However, the monoclonal Ab was very specific for the 28 kDa polypeptide, whereby one polypeptide was recognized for each of the seeds with the exception of rice that had an additional 94 kDa band. The high MW proteins recognized by the Ab may represent polymers of ferritin subunits (24) or other proteins from the sample extract that share a similar epitope with the peptide sequence. If these high MW bands are polymers of ferritin subunits there is a chance that the monoclonal Ab could not identify the polymers, because the monoclonal epitope may not be completely exposed.

**Protein Extractability.** Total protein in the seeds ranged from 8-26 g per 100 g of seed, with the smallest percent of protein in maize and highest in beans (Table 3.2). Percent of protein extracted from each seed following the first extraction ranged from 20-65% among the seeds. 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 (Osborne and Mendel, 1914) and are believed to be insoluble to the
reagents used for our extraction. Given this information, the first extraction step from our crude extraction procedure may have extracted most of the soluble protein and ferritin in maize (with 33% total protein extracted). However, there are noticeable losses of ferritin at subsequent purification steps as revealed with western blot analysis (data not shown). Efforts were made to improve the initial extraction of protein from the seeds using various techniques described below.

To extract more protein from the seed, we attempted a double extraction, where the protein was extracted from both the seed and from the first pellet. The supernatant from each extraction was pooled together and the rest of the purification steps were completed according to the protocol. As expected, the double extraction method increased the amount of total protein extracted from the seeds; however, ferritin from these samples was undetectable with indirect ELISA. It is possible that the double extraction increased the concentration of proteins other than ferritin, which made the ferritin concentration relative to the total protein too low to detect. These data suggest that our initial extraction may be sufficient to extract the ferritin.

Various techniques for the grinding of dry seeds were also attempted to improve the overall extraction of the protein. A coffee grinder and Spex CertiPrep GenoGrinder 2000 (Metuchen, NJ) were both used to grind the seed into a fine powder for ferritin extraction. 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).

**Western Blot and Densitometry.** Monoclonal Ab were used for immunoblot quantification of ferritin, due to the high sensitivity and specificity of the monoclonal Ab
compared to the polyclonal Ab. The quantity of rFerr used (40-100 µg of ferritin) positively correlated with band intensity \((r = 0.991, \text{linear curve fit})\) with western blot densitometry (Figure 3.2A). Using this standard curve, we were able to calculate the ferritin values from the band intensities for each seed. An example of western blot ferritin quantification for RB (5-40 ng protein) is demonstrated in Figure 3.2B. Ferritin values of WB, Wt, and Mz were also determined with this method (data not shown), but rice ferritin was not determined due to the difficulty of obtaining a range of detectable bands. 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 not able to detect ferritin with western blot or indirect ELISA using this extract. We suspect that the ferritin concentration was too low or diluted relative to the total protein concentration in the crude extract for the ferritin to be detected. Further purification of the seed extracts were required to quantify ferritin using indirect ELISA. Western blot densitometry was not ideal for ferritin quantification due to the high gel-to-gel and day-to-day variability. However, western blotting may be useful as a semi-quantitative measure of ferritin for small numbers of seeds.

ELISA. Ferritin concentrations were calculated \((n = 3 \text{ extractions}; \text{polyclonal Ab})\) using rFerr standard curve \((r^2 = 0.9999)\) ranging from 5-500 ng/ml of ferritin with a 4-parameter curve fit (four parameters represent left asymptote, right asymptote, slope, and slope at the inflection point). With the use of monoclonal Ab, we were able to obtain a similar standard curve as with polyclonal Ab, but could not accurately and repeatedly
quantify ferritin. Therefore, the polyclonal Ab were used for all ELISA ferritin quantification experiments.

An important question to ask is why we were able to detect ferritin with the polyclonal Ab and not monoclonal Ab using ELISA and why the monoclonal Ab worked with western blot but not ELISA. One main difference between western blot and ELISA procedures is the sample preparation. Unlike the western blot, the protein samples used in ELISA are not reduced or separated by size. Even though the Ab were found to be highly immunogenic, if part of the epitope is imbedded within the ferritin structure, the binding of the highly specific monoclonal Ab may not occur. Since polyclonal Ab are often comprised of Ab that recognize several epitopes on the antigen, the Ab should have no problem binding to an antigen with a few unexposed residues. This may explain why the polyclonal Ab worked with ELISA when the monoclonal Ab did not.

Protein working ranges used for the quantification of ferritin were determined for each seed 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. With no quenching, the ferritin content per gram of seed should be constant no matter how much sample is used. The optimal concentrations of protein to use in ELISA experiments were important to determine, because of the limited protein-binding capacity and lack of ferritin specificity of the ELISA plate. For indirect ELISA experiments, the sample is added to the wells first, so any protein in the sample extract can bind to the walls and potentially limit the capacity for ferritin to bind. When excess protein is added to the well, the plate becomes oversaturated and a plateau or decrease in ferritin binding may be observed.
The protein binding capacity of the wells appears to be limited to approximately 20 µg/ml of protein according to several experiments performed for the determination of the working range. Higher protein concentrations were found to cause a plateau effect on ferritin concentration. An example of the determination of the working range is demonstrated in Figure 3.3 for RB, whereby 6-20 µg/ml of protein was found to be the optimal protein range for RB ferritin quantification. The working ranges for WB, Wt, Mz, and BR were found to be 4-20, 4-10, 4-20, and 6-20 µg/ml of protein, respectively (data not shown).

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 RB, WB, Wt, Mz, and BR, respectively (n = 3 extractions, assayed in triplicate). As discussed earlier, we believe that we may have extracted most of the soluble protein including ferritin from the seeds. However, for the purpose of identifying ferritin-rich seed varieties an exact value is not needed.

Variations in the protein working ranges may be caused by differences in the food matrices of the seeds. Values in Figure 3.4 represent the mean ± SD of ferritin determined from protein in the working range. Both bean varieties were found to have significantly higher ferritin (p < 0.05) from the other seeds. However, ferritin values should not be compared between seed types, because of differences in protein extractability and quantification using a standard from a different species (pea ferritin).

**Application of ELISA to Screen for Ferritin**

Ferritin concentrations of thirteen red bean varieties were determined using 10 µg/ml of protein, which is within the working range for RB. Protein extractability of the red bean varieties was not measured; however, we would expect a similar percent protein extraction as
the RB variety obtained from the local market (~64.5%). Ferritin values of the red bean varieties ranged from 1.6-5.1 µg/g of seed (Figure 3.5), with significant differences between the two high and four low ferritin seeds ($p < 0.01$). This range of ferritin values at 1.6-5.1 µg/g is lower than what was determined for the market-variety RB (10.3 µg/g). We believe that these low ferritin values among the red bean varieties were the result of the poor extraction of ferritin from dry ground seed. Protein extractability of dry ground RB was found to be approximately 15% less than that of soaked seeds. Therefore, with less protein extracted, less ferritin was detected.

The total nonheme iron was also determined for the bean varieties to evaluate the relationship between iron and ferritin ($n = 3$). 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. Since we obtained the seeds from an international source where they were ground into a powder to avoid the shipment of viable seed, we cannot rule out the possibility of iron contamination. However, these values may represent the true iron concentrations in the seeds, whereby the unexpectedly high iron in the low ferritin varieties may be the result of the homeostatic control of iron in the plant (7). The regulation of iron accumulation is poorly understood; however, it is evident that the accumulation of iron in the seeds may not always correspond to the expression of ferritin. An important question to consider is whether increasing the expression of ferritin will increase the iron concentration of the seed. Qu et al. (43) produced transgenic rice with high ferritin expression, but found that the seeds did not always accumulate iron in response to the level of exogenous ferritin expressed; rather the iron accumulation may have been limited by iron uptake and transport. A possible mediator
of iron storage and homeostasis is nicotianamine (NA), which is known to deliver iron to cells and plastids. In NA-free tomato plant mutants, iron precipitates were found in the plastids (Becker et al., 1995), but no ferritin was detected (Liu et al., 1998). These studies with NA-free plants suggest that NA may play a role in iron storage and ferritin synthesis.

Although a few low ferritin red bean varieties were found to have high iron, the high ferritin beans had consistently high iron among all varieties. These results emphasize the importance of measuring ferritin in addition to iron, because the measurement of seed ferritin may function as a determinant for both iron content and bioavailability, whereas the measurement of iron is solely indicative of content. At the very least, ferritin may be an important supplemental measure to total iron in the seed, if not a substitute.

In conclusion, our indirect ELISA will facilitate the screening of a variety of staple food crops for ferritin that can be used by plant breeders to identify and breed ferritin-rich crops to serve as a potentially bioavailable iron source. Additionally, this ELISA method will be useful for transgenic plant studies for identifying ferritin-rich varieties. Future studies are needed to identify whether ferritin is useful as a biofortificant and whether the impact of ferritin biofortification is significant enough to improve the iron status of the population. Additionally, further bioavailability studies are needed, along with investigation of the impact of cooking on ferritin and iron content in foods and the validation of the relationship between ferritin and iron accumulation.

**LITERATURE CITED**


**Table 3.2.** Percent extracted protein.

<table>
<thead>
<tr>
<th>Seeds</th>
<th>GenBank Accession #</th>
<th>Total Protein $^1$ g/100 g</th>
<th>Extracted Protein $^2$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Beans</td>
<td>(CAA58147)</td>
<td>25.5</td>
<td>64.5±0.12</td>
</tr>
<tr>
<td>White Beans</td>
<td>(CAA43664)</td>
<td>23.0</td>
<td>54.6±0.00</td>
</tr>
<tr>
<td>Wheat</td>
<td>(P29036)</td>
<td>11.1</td>
<td>32.0±0.07</td>
</tr>
<tr>
<td>Maize</td>
<td>(CAA43664)</td>
<td>6.7</td>
<td>33.1±0.04</td>
</tr>
<tr>
<td>Brown Rice</td>
<td>(AAW68440)</td>
<td>8.1</td>
<td>20.4±0.02</td>
</tr>
</tbody>
</table>

$^1$Total protein determined using Nitrogen Analyzer (n=2-3)

$^2$Mean ± SD extracted protein represents the fraction of total protein extracted with buffer from soaked seeds, measured using the Bradford Assay (n=2-4)

**Table 3.1.** Sequence alignment of a 30 amino acid (AA) region in maize, wheat, rice, and common bean ferritins containing highly immunogenic 22 amino acid region used for antibody (Ab) production.

<table>
<thead>
<tr>
<th>Seeds</th>
<th>GenBank Accession #</th>
<th>30-AA Conserved Region (ClustalW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize 1</td>
<td>(CAA58147)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Maize 2</td>
<td>(CAA43664)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Maize 3</td>
<td>(P29036)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Maize 4</td>
<td>(CAA43664)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Wheat 1</td>
<td>(AAW68440)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Wheat 2</td>
<td>(AAT67051)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Rice 1</td>
<td>(AAM74943)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Rice 2</td>
<td>(AAQ74385)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Rice 3</td>
<td>(AAM74942)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Beans</td>
<td>(AAU08208)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
<tr>
<td>Beans</td>
<td>(P25699)</td>
<td>YHSLFAYFDRDNVALKGFAKFFKESSDEER</td>
</tr>
</tbody>
</table>

**22-AA Highly Immunogenic Region**

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER

YHSLFAYFDRDNVALKGFAKFFKESSDEER
FIGURES:

Figure 3.1. Immunoblots of seed ferritins using polyclonal (A; 1:3000) and monoclonal (B; 1:10,000) antibodies. Molecular weight (MW) standards are shown next to the blots to approximate ferritin MW in kDa. Extracts were prepared as described in the materials and methods and each extract was loaded with different amounts of protein. *Std* (standard, rFerr) 80 ng; *RB* (red beans) 10 µg (A), 30 µg (B); *WB* (white beans) 30 µg (A), 40 µg (B); *Wt* (wheat) 15 µg (A), 50 µg (B); *Mz* (maize) 10 µg (A, B); *BR* (brown rice) 25 µg (A), 30 µg (B).
Figure 3.2. Densitometry of western blots using monoclonal antibody (1:10,000) to quantify ferritin in red beans. Recombinant pea ferritin (A; linear fit, r = 0.991) was used as a standard curve to determine the ferritin content in the crude extracts of red bean ferritin (B; linear fit, r = 0.961).
Figure 3.3. Determination of protein working range (6-20 µg/ml protein) for ferritin quantification in red beans (RB) using indirect ELISA with polyclonal Ab (1:500) and rFerr standard curve (4-parameter fit). 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 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 3.4. Ferritin content per gram of seed for red beans, white beans, wheat, maize and brown rice determined using indirect ELISA with polyclonal antibody and rFerr standard curve. Values represent the means and SD of samples tested in the working range, n = 3 extractions measured in triplicate.
Figure 3.5. Ferritin (A) and iron (B) concentrations in red bean varieties. Values represent mean and SD of 3 extractions, assayed in triplicate. Differences among varieties were determined using ANOVA with Tukey’s multiple comparison test, $p \leq 0.05$. Seeds obtained from CIAT (International Center for Tropical Agriculture).
CHAPTER 4. GENERAL CONCLUSIONS

GENERAL DISCUSSION

We were able to successfully develop an ELISA for the quantification of ferritin in staple crops (beans, wheat, maize, and rice), which may be useful to plant breeders and researchers for the screening of large numbers of seeds and development of ferritin-rich crops. Future research should focus on expanding our understanding of how increasing phytoferritin concentrations influences iron accumulation in seeds. Prior to the implementation of ferritin biofortification, it is important to ensure that the strategy is beneficial to the population. Iron biofortification has already been shown to improve crop yields and grow in various climatic zones; however, more research is needed in the areas of iron bioavailability and its ability to improve iron status in populations (Welch and Graham, 2004). The implementation of this method for measuring ferritin has the potential to expedite the progression of the seed development for ferritin-rich crops and may ultimately aid in research to better our understanding of whether ferritin biofortification would have a significant impact on the population for combating iron deficiency.

REFERENCES

APPENDIX: ADDITIONAL GRAPHS

These tables and figures represent the “data not shown” in the manuscript.

Table 1. Percent extracted protein from soaked seeds (A), dry ground seed ground with coffee grinder (B), using double extraction (C), and dry seeds ground with GenoGrinder (D)

<table>
<thead>
<tr>
<th></th>
<th>Total Protein %</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Beans</td>
<td>25.5</td>
<td>71.45±0.19</td>
<td>57.20</td>
<td>140.16</td>
<td>50.47</td>
</tr>
<tr>
<td>White Beans</td>
<td>23.03</td>
<td>70.76±0.19</td>
<td>54.80</td>
<td>129.55</td>
<td>57.28</td>
</tr>
<tr>
<td>Wheat</td>
<td>11.1</td>
<td>35.17±0.09</td>
<td>23.01</td>
<td>59.93</td>
<td>35.79</td>
</tr>
<tr>
<td>Maize</td>
<td>6.73</td>
<td>36.93±0.09</td>
<td>28.36</td>
<td>65.78</td>
<td>6.90</td>
</tr>
<tr>
<td>Brown Rice</td>
<td>8.1</td>
<td>22.69±0.05</td>
<td>20.80</td>
<td>40.31</td>
<td>12.11</td>
</tr>
</tbody>
</table>

1 Total protein determined using Nitrogen Analyzer (n=2-3)
2 Mean ± SD extracted protein represents the fraction of total protein extracted with buffer, measured using the Bradford Assay (n=1-4)

Figure 1. Recombinant ferritin standard curve using polyclonal antibody in ELISA, 5-500 ng/ml of protein, 4-parameter fit, r=0.9999.
Figures 2. Determination of protein working ranges (shaded region) for ferritin quantification in white beans (A), wheat (B), maize (C), and brown rice (D), using indirect ELISA with polyclonal Ab (1:500) and rFerr standard curve.

A. White Beans

B. Wheat

C. Maize

D. Brown Rice
Figure 3. Densitometry of western blots using monoclonal antibody to quantify ferritin. Values represent the means and SD of the average intensity of 3-4 bands, corrected for differences in total protein concentration in the extracts and extrapolated to represent ferritin per gram of seed from 100% extraction. Differences were determined by ANOVA with Tukey’s multiple regression, $p \leq 0.05$; $n=3$. 