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Diversified strategies for improving iron bioavailability of maize

by

Amy Katheryn Proulx

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutrition Sciences

Program of Study Committee:
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Iowa State University

Ames, Iowa

2007

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Dedication

To my mother, who said,
"Whatever you do, be happy."
I am happy indeed.

To my father, who said,
"Whatever you do, have fun!
(and don't be a politician)"
I have fun imagining a better world through the science that we do.

To my grandmother, who said,
"Well, isn't that something, you will be a doctor one day!"
It was a very long journey, but I am there now.

To my loving husband, who said,
"Whatever you do, follow your heart."
He lives this message every day of his life.
This is one way in my life I lived this ideal.

And to my daughter, who smiled,
and sat on my knee as I wrote.
All my love for you.

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Abstract

Iron deficiency is the most common nutritional deficiency worldwide, affecting over 2 billion people, especially women and children. Populations consuming maize as a staple food are particularly prone to iron deficiency because of the low iron bioavailability (FeBA). The overall objective of this three-part study is to enhance FeBA in foods made of maize through novel fortification techniques, processing modifications, and genetic modifications.

The first study focused on the fortification of plant heme iron to improve FeBA in tortillas. Plants, particularly soy roots can produce heme iron as part of nitrogen fixation. Our objective was to test the effectiveness of soy root nodule (SRN) and purified soy hemoglobin (LHb) for FeBA using the in vitro Caco-2 cell model compared to bovine hemoglobin (BHb), and ferrous sulfate ($FeSO_4$). When iron sources were tested alone, the FeBA values of LHb and BHb were 19% ($P > 0.05$) and 113% ($P < 0.001$) higher than $FeSO_4$, respectively. However, when iron sources were used for fortification of maize tortillas (50 ppm), LHb and BHb showed similar FeBA, being 27% ($P < 0.05$) and 33% ($P < 0.05$) higher than $FeSO_4$. Our data suggest that heme iron from plants may be a novel value-added food fortificant with enhanced FeBA.

The second study evaluated the effects of maize processing on FeBA, with the intent to modify processing to enhance FeBA. Representative maize processing techniques were used: heating (porridge), fermentation (ogi), nixtamalization (tortillas), and decortication (arepas). The phytate content of maize products was significantly reduced by decortication (25.6%, $p = 0.003$) and nixtamalization (15%, $p = 0.03$), and

iron content was reduced by decortication (29.1%, $p = 0.002$). The relative bioavailability (RBA, compared to 100% FeBA of porridge with $FeSO_4$) of ogi was significantly higher than that of all other products when fortified with $FeSO_4$ ($p < 0.001$) or reduced iron ($p < 0.001$). Addition of lactic acid (6 mg/g of maize) significantly increased iron solubility and increased FeBA by about 2-fold ($p < 0.01$), especially in tortillas. Consumer panel results showed that lactic acid addition did not affect the organoleptic characteristics of tortillas and arepas ($p = 0.166$ and 0.831, respectively). These results suggest that fermentation, or the addition of lactic acid to unfermented products, may improve FeBA of maize products. Lactic acid addition may be more feasible than using highly bioavailable but expensive fortificants.

The last study involved genetic modification by overexpression of *Zea mays* hemoglobin (ZmHb) in maize endosperm tissue. A first vector was developed by cloning a high expression 27γ zein promoter from maize, and recombining it with a ZmHb gene fused to Green Fluorescent Protein (GFP), and *nos* terminator. Constructs with further modifications based on this first vector model were also produced: one without GFP and the other using 27γ zein terminator. Maize callus was bombarded with the first vector, regenerated to plants which produced first generation (F1) seed. High GFP expressing seed, as determined by excitation fluorescence spectrophotometry, was shown to have 4.2-4.9 ug ZmHb/g maize based on immunoassay with ZmHb antibody. Heme pyrolle incorporation into the protein was shown by iron catalysed chemilluminescence. Preliminary studies on two high GFP expressing lines show that FeBA of ZmHb enhanced maize, determined by the Caco-2 cell assay, was 68–125% higher than the

untransformed inbred maize variety, HiII ($p < 0.01$). Based on estimates from these assays, delivery of 1.6-2.5 μg heme iron per 100g serving of maize is possible. Our preliminary results in developing transgenic and intragenic maize suggest that overexpression of ZmHb in endosperm is feasible and may significantly enhance FeBA of maize by adding highly bioavailable heme iron.

General Introduction

It is well known in the nutrition field that iron deficiency is the most prevalent nutritional deficiency worldwide, affecting over 2 billion people (WHO, 2007). And many strategies for alleviation of iron deficiency exist, including supplementation, fortification, and dietary diversification. Still, rates of iron deficiency in populations are not declining as rapidly as one would hope, at less than 0.5% per year (Micronutrient Initiative, 2006). New strategies must be developed if iron deficiency rates are going to decrease at a rate that makes a significant impact on populations.

Maize is the staple food of many populations that are experiencing the most severe iron deficiency. In Central America and many parts of Sub Saharan Africa maize can comprise greater than 1500 kcal per day in the diet (FAOSTAT, 2004). With maize playing such an integral part of the diet, the micronutrient quality must be well understood. Maize has a very high level of phytate, which acts as an inhibitor of iron absorption, leaving a very low percentage of total iron absorbed. Attempts at iron fortification in foods made of maize have been only minimally successful, because the food matrix effects of maize are far too prominent. But perhaps it is the food matrix effects of maize foods that have been under-looked in nutrition studies evaluating bioavailability of maize fortificants. More effort is needed to evaluate how foods made of maize affect the absorption of iron, to define better practices in processing and fortification.

Hemoglobin has long been known to be a highly bioavailable iron source. The primary strategy for alleviation of iron deficiency through the diet is to eat more heme iron. Incorporation of meat into the diet, however, is often economically impossible, or

culturally inappropriate. It is now known that plants produce hemoglobin proteins with similar structural characteristics to animal hemoglobins. The use of plant sourced hemoglobins has not been evaluated in a nutritional context. Either using naturally derived hemoglobins from plants that produce large quantities, such as soy root nodules, or using biotechnology based strategies to overexpress hemoglobin proteins in seed tissues of plants, such as maize, have not been explored.

Rationale

Given the massive impact of iron deficiency on the world, new strategies for alleviating iron deficiency are warranted. Maize, as the staple grain of populations seriously affected by iron deficiency, requires a particular level of attention in this regard. This dissertation focuses on three strategies to improve iron bioavailability in maize: fortification, processing and biotechnology. With these strategies, this study shows proof of concept for new technology targeted at enhancing nutritional quality of iron.

Research Questions

This dissertation was designed to address the following gaps in understanding of iron bioavailability in maize: can understanding the food matrix effects of maize in its diverse food forms help us find better ways of processing and fortifying maize? Can hemoglobins from plants be a novel means of increasing bioavailable iron? And lastly, can we use biotechnology based approaches to use maize genetics to enhance iron bioavailability?

Organization of the study

This dissertation is divided into three main studies. The first study, in part 3, is entitled, "Iron Bioavailability of Hemoglobin from Soy Root Nodules Using a Caco-2 Cell Culture Model". This study is the first to evaluate the bioavailability of plant based hemoglobin proteins, to compare their bioavailability to animal sourced hemoglobin. This study is also the first to apply plant derived hemoglobins as a food fortificant. This study has been published in the Journal of Agricultural and Food Chemistry in 2006.

The second study, "Fermentation and lactic acid addition enhance iron bioavailability of maize" is a comprehensive study evaluating the effects of the predominant maize processing techniques on iron bioavailability. It also used a retrospective/prospective approach to processing, in that once the effects of typical processing were understood, the maize products were reformulated, incorporating the factors known to enhance bioavailiability, in this case acidification with lactic acid. With this second reformulation, much greater impacts on improving iron bioavailability were seen. This study was published in the Journal of Agricultural and Food Chemistry in 2007.

The last study, entitled "Overexpression of *Zea mays* hemoglobin in maize endosperm tissue can increase iron bioavailability" is the first study involving overexpression of hemoglobins for a nutritional purpose. The biotechnology based approach described in the study is novel for the use of a reporter gene, green fluorescent protein, in the first round of studies. This study is a long term research project, which will be completed in several years. The novel strategy of using intragenic transformation, described in this case as the use of only maize genes in recombination, is a very exciting

development. Use of only maize genetics to enhance iron bioavailability would be a very new strategy in the realm of biofortification. This study has been prepared with the intention to submit to the Journal of Nutrition upon evaluation and analysis of the F2 seed, a more robust data set. This is to be conducted in the Autumn of 2007.

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Literature Review

Introduction

Iron deficiency is the world's most prevalent nutritional deficiency, and perhaps is the nutritional deficiency which has been the most challenging to overcome. Iron has a complex absorption pathway confounded by extraneous factors in the diet. The challenge with bioavailability is one of the main reasons that iron deficiency remains as the most prevalent nutritional deficiency worldwide.

Globally, most iron is consumed from staple crops, plant-based foods which have very low bioavailability. Nutritional interventions to increase iron content in foods by fortification are the most cost effective method of delivering iron to populations but have been challenged by the complexities of food chemistry and food matrix effects. Addressing these challenges, and reducing iron deficiency could improve the health, wellbeing and productivity of billions of people worldwide.

Iron Deficiency

The Global Prevalence of Iron Deficiency

Based on current estimates, iron deficiency (ID), and associated iron deficiency anemia (IDA) affects over 2 billion people worldwide (WHO, 2007; Micronutrient Initiative, 2006). It has been suggested that 40 to 60% of infants between 6 and 24 months of age globally have reduced neural development, that 600,000 women die annually during childbirth, and up to 2% of GDP in developing countries is lost because of iron deficiency (Micronutrient Initiative, 2006).

Because of the global significance of iron deficiency, goals for iron deficiency alleviation have been set by international organizations. In 1990, the World Summit for Children declared the goal of "virtual elimination of iodine and vitamin A deficiency and a reduction of the prevalence of iron-deficiency anaemia in women by one third" by the year 2000 (UNICEF, 1990). This goal was continued in 1992 at the International Conference on Nutrition hosted by FAO and WHO, with the consensus "to reduce substantially . . . other important micronutrient deficiencies, including iron." (FAO and WHO, 1992). The challenge that was encountered with this approach was that many country-based strategies focused on a single intervention, rather than accounting for and rectifying the diverse causes of iron deficiency. As such, there was little change in iron deficiency rates during this ten-year time, with most global regions seeing reductions in iron deficiency in women (both pregnant and not) of less than 0.5% per year (Micronutrient Initiative, 2002).

At the 27th General Assembly Special Session Declaration, "A World Fit for Children" in 2002, there was a slight change in strategy, to incorporate the concept of a diversified strategic mission to "reduce by one third the prevalence of anaemia, including iron deficiency, by 2010; and accelerate progress towards reduction of other micronutrient deficiencies, through dietary diversification, food fortification and supplementation." (UNICEF, 2002). This slight change in the goal specifically noted the importance of diverse strategies to alleviate iron and other micronutrient deficiencies. Despite setting and working towards goals, only minor progress has been made to date.

Iron deficiency and IDA are health issues that are correlated to diet quality and public health, and these are usually tied to economic and social development (Horton,

2006) and in particular have the most devastating impact on women, infants and children (Darton-Hill et al, 2005). The scope of this research is not able to address the socioeconomic challenges of micronutrient deficiency, but strongly identifies with the principle that through socioeconomic development, nutrition and wellbeing can be improved.

Importance of Iron

Iron is essential for growth and development because of its importance in a wide variety of biochemical processes involved in maintaining cellular function. These include electron transport (cytochromes, iron-sulfur proteins), handling of molecular oxygen (peroxidase, catalase), oxygen transport and storage (hemoglobin and myoglobin, respectively), porphyrin metabolism, collagen synthesis, lymphocyte and granulocyte function, and neurotransmitter anabolism and catabolism (Cammack et al. 1990, Pollitt and Leibel 1976).

Body iron commonly exists in two oxidative states, the divalent ferrous and the trivalent ferric. Because of its oxidative states, heme iron functions in the respiratory cascade as an electron carrier as part of cytochromes in mitochondria and as a ligand for O_2 and CO_2 transport between tissues in the form of hemoglobin and myoglobin. Iron is also contained in myoglobin, which stores oxygen in the tissues. Aerobic metabolism requires iron in the Kreb's Cycle as a cofactor of some enzymes, such as aconitase and succinate dehydrogenase. The nonheme iron present in these enzymes is tightly bound to the sulfur atoms of cysteine residues. The complexes are often referred to as "iron-sulfur proteins" or "iron-sulfur clusters" if more than one such complex exists. Other nonheme

iron-containing enzymes are ribonucleotide reductase, needed for deoxyribonucleic acid (DNA) synthesis, xanthine dehydrogenase, necessary for catabolism of the purine ring, and NADH dehydrogenase and coenzyme-Q reductase, required in the respiratory cascade (Brody 1994).

Iron deficiency in infants and children has been associated with poor cognitive and behavioral development and these consequences are reviewed extensively (McCann and Ames, 2007). There still remains some controversy over the nature of a true causal relationship between iron deficiency and cognitive development since there are other confounding factors such as socioeconomic and nutritional factors. It has been suggested for example that iron deficient children typically belong to lower income groups, and may not have the stimulation and maternal care that is received by children in higher income settings (Grantham-McGregor and Ani, 2001). It is also suggested that iron deficiency due to diet is rarely seen alone and multi-nutrient deficiencies may be aggravating the proper development of cognition (Grantham-McGregor and Ani, 2001). More recent work however has suggested role of iron in neural tissue development and that iron deficiency in infancy affects neurometabolism, myelination, and neurotransmitter function (Lozoff et al, 2006).

Iron deficiency anemia has also been associated with fatigue and reduced work output due to reduced aerobic capacity and oxygen transport (Haas and Brownlie, 2001,). The effect of iron on fatigue and output has been measured in terms of reduced aerobic capacity (Brownlie et al, 2002), reduced muscle endurance (Brutsaert et al, 2003), and lower economic productivity (Gopaldas and Gujral, 2003).

It is also suggested that iron deficiency and in particular severe anemia (Hemoglobin (Hb) < 7.0g/dL) is a significant risk factor for increased morbidity and mortality during childbirth. The assumption is that the etiology comes from the combination of increasing blood volume during pregnancy, blood losses during childbirth, coupled with the high potential for uterine hemorrhaging in the early antenatal period. The epidemiological or longitudinal evidence is seriously lacking in this regard, however it is strongly understood that improved iron status mitigates much of this burden (Viteri, 1994; Rush, 2000)

The positive effects of iron supplementation on weight for age and weight for height are well documented in severely iron deficient children, but not in subclinical iron deficiency (Sachdev et al, 2006) suggesting the importance of iron in growth and development .

Iron Requirements

When considering iron deficiency, one must take into account iron intake and losses, and their equilibrium. Metabolic iron need is based on the physiological state such as pregnancy and growth. When iron losses or needs exceed the intake, iron deficiency occurs. Iron losses occur in menstruation, from infection (as in the case of intestinal parasites, or malaria), or from other trauma causing low level chronic or acute massive blood losses (for example childbirth, or hemorrhaging). The amount of total iron in the body varies between 2 and 4 g, and varies based on size, age, gender and physiological state. Total iron in the body is distributed in several compartments (**Figure 2.1**.). Most iron in the body (65%) is present in the erythrocytes, with a significantly

lesser proportion in circulating iron bound to transferrin (<1%). Tissue iron, in the form of myoglobin forms the largest store (20%) however a small proportion is present (2%) in tissues as enzymes such as aconitase or cytochromes. The body's store of iron is highly conserved (Fairbanks, 1998).

Iron losses in healthy individuals are typically 1 mg in men and 1.5 mg in women daily (Cook et al, 1992). These losses come from desquamation of the intestinal mucosa, hemoglobin in fecal matter, bile secretions, and losses of epithelial cells (Haurani and Marcolina, 1994). Menstruation is a significant source of iron loss, and accounts for the large variation of iron requirements between men and women of childbearing age (Fairbanks, 1998).

The amount of dietary iron that needs to be absorbed on a daily basis ranges from 1-1.5 mg/d to account for the obligatory losses of iron described above. Current recommendations for iron allowance are for 8 mg/d for males and postmenopausal women, and 18 mg/d for women of childbearing age. This is based on an assumption that 10% of iron is absorbed from the diet. How much iron absorbed is dependant on three factors: the total iron content, the bioavailability, and the iron status of the individual. The iron content of diets in most developing countries is low to moderate, however the low bioavailability of the predominantly plant based diet trumps the iron content, rendering iron unavailable for absorption.

Dietary Iron Absorption

Dietary iron exists in heme and nonheme forms, which form the two pools in the gut: nonheme and heme iron. Heme iron is derived from heme proteins, in particular

hemoglobin and myoglobin from animal sources. Plants are also capable of producing heme iron in very low concentrations. Heme iron is a highly bioavailable iron form, as the heme pyrrole acts as a protective clathrate structure, preventing interference from iron chelators, such as polyphenols or phytate (Andrews, 2005). Secondly, heme iron is absorbed by a unique pathway, different from nonheme iron. The pathways of iron absorption are described further in this paper. Heme iron contributes a minor fraction of the total iron in the diet, 10-15%, however because of the bioavailability, it accounts for approximately one third of the total iron absorbed (Hallberg and Rossander, 1982). Roughly 60-75% of iron in meat, fish and poultry is heme iron, which is approximately 30% available (Monsen, 1988). Extensive work has been done characterizing animal heme iron, however only one study reported in this dissertation shows that plant hemoglobin has similar bioavailability characteristics to heme iron from animal sources (Proulx and Reddy, 2006).

Nonheme iron is the predominant iron form in plant foods and makes up about 25% of the iron from animal foods. Nonheme iron is liberated from the food matrix, and ionized during gastric digestion and absorbed predominantly in the proximal duodenum (Dunn et al, 2007). In staple grains, absorption of iron tends to be very low, ranging from 1-7% (Monsen, 1988). Nonheme iron interacts with dietary inhibitors, such as phytate and polyphenols, or dietary enhancers such as ascorbic acid or the putatative polypeptide based "Meat Factor" (Zijp et al, 2000; Swain at al, 2002). As nonheme iron is absorbed into the intestinal epithelium by a transporter that is also responsible for transport of calcium and other divalent cations, there is competitive transport for iron with high concentration of divalent cations (Garrick et al, 2003). Ascorbic acid acts as an iron

chelator with high stability under a variety of pH conditions which enhances iron solubility (Teucher et al, 2004). Phytic acid acts as a potent chelator of iron and other cations because of its high negative charge density from multiple phosphate groups (Zhou and Erdman, 1995). Polyphenols and tannins similarly act to chelate iron through binding with negatively charged groups (Scalbert et al, 2005).

Iron Uptake and Utilization

Iron absorption has a complex uptake pathway, based on the type of iron being transported, as shown in **Figure 2.2**. Ferric iron is reduced to ferrous form by duodenal cytochrome b (Dcytb) (McKie et al, 2001), or another uncharacterized ferric reductase (Gunshin et al, 2005). Ferrous iron is transported through the apical membrane into the enterocyte by a divalent metal transporter 1 (DMT1), in a saturable and competitive manner. DMT1 is also responsible for proton transport, and transport of other metal cations, including zinc, calcium and magnesium (Gunshin et al, 1997). Heme iron is postulated to be transported into the enterocyte by heme carrier protein 1 (HCP1) (Shayeghi et al, 2005), however current knowledge of the transport mechanism is being debated, as it is postulated that HCP1 is not a heme transporter, rather a folate transporter (Qui et al, 2006). HCP1, based on current understanding is internalized by receptor-mediated endocytosis, followed by heme degradation by heme oxygenase 1 at the surface of the endoplasmic reticulum, liberating ferrous iron into the enterocytes (Shayeghi et al, 2005). The labile iron pool (LIP) is postulated to not be "free iron" rather iron in chaperone proteins, for example ferritin (Theil, 2003), or low molecular weight peptide chelates thereby limiting Fenton reaction production of free radicals. Iron is transported

through the basolateral membrane by ferroportin 1 (FPN1) (Donovan et al, 2000), with oxidation to ferric form by hephastin (Hp) followed by transport within the blood by transferrin (Vulpe et al, 1999). It is understood that under normal physiological conditions, FPN1 is negatively regulated by hepcidin. Hepcidin, an iron regulatory hormone expressed iron replete conditions, binds with FPN1, causing its internalization and degradation (Fraenkel et al, 2005). As only FPN1 has been implicated in iron transport to the circulation, it is postulated that FPN1 is the transporter implicated in limiting iron uptake during iron replete status (Donovan et al, 2000). Iron is transported in the serum by serum transferrin (Tf), and taken up by the cells by transferrin receptor (TfR) binding to the iron bound transferrin, then internalizing Tf by receptor mediated endocytosis. When cellular need for iron increases, TfR expression is upregulated, hence increasing iron uptake (**Figure 2.3**). It is suggested that TfR may act as a sensor of transferrin bound iron, influencing expression of hepcidin, a regulatory hormone affecting the FPN1 mediated release of iron from the enterocytes, macrophages and hepatocytes (Fleming and Bacon, 2005).

Based on the understanding of iron metabolism, the measures of iron status were developed. A wide variety of iron status biomarkers have been evaluated for ability to reflect stages of iron deficiency. Similar to the complexity of iron absorption, iron status measures are complex. Infections, inflammations and other nutrient deficiencies affect iron status, and as such a battery of tests are needed for specific iron assessment. The current iron status biomarkers used in clinical evaluation are listed in **Table 2.1**.

Alleviating iron deficiency

Improvement of iron status is currently based on three primary strategies, food fortification, supplementation, and dietary diversification. These strategies have recently been expanded to incorporate biofortification.

The success of all schemes to alleviate iron deficiency is evaluated by characterizing a variety of factors including the bioavailability, the efficacy and effectiveness of the food or supplement for alleviating and attenuating deficiency in affected individuals and populations. This also includes evaluating the acceptability of the intervention in terms of nutrient stability, cost, and organoleptic influence.

Assessment of Iron Bioavailability

Bioavailability is the relative measure of how well a nutrient is absorbed by the human body and becomes available for metabolic use. It is measured either indirectly using in vitro or animal studies, or directly in humans. In vitro methods, including solubility assays, and cell culture methods are commonly used as screening techniques for bioavailability. It is difficult to directly extrapolate the results from in vitro bioavailability measurements to human absorption and nutrient utilization, although they are efficient and cost effective methods of screening relative bioavailability. Animals may be more physiologically similar to humans than *in vitro* models for assessing bioavailability; however, for many nutrients, biological need differs from animal to human, or utilization differs due to variation in absorptive or synthetic pathways. Animal studies, like in vitro studies, are most practical as screening methods for relative bioavailability of fortificants, and in many circumstances may not be appropriate. In the case of rats, dietary factors differ in their ability to enhance iron bioavailability (Reddy

and Cook, 1991). Most effectively, human bioavailability studies are used to evaluate bioavailability and efficacy of fortification schemes.

Bioavailability is typically assessed by measuring absorption of radio or stable isotopes of nutrients by measuring change in metabolite synthesis, or excretion. Bioavailability is a useful tool in developing fortification schemes, in that it is predictive of the potential efficacy, and can approximate what dose level one would receive with long term consumption. Most studies conducted to date to evaluate fortification schemes have been bioavailability studies either in vitro, or using animals or humans.

Bioavailability studies are inherently limited in their capacity to evaluate how a fortified food would improve general population health, as is determined in efficacy and effectiveness studies. Human bioavailability studies are usually conducted on normal, healthy adults, which in the case of iron may not accurately correlate to absorption rates in iron deficient subjects, as iron absorption is upregulated in iron deficiency (Fairbanks, 1998).

The use of cell culture models for iron bioavailability assessment has greatly increased in the past fifteen years. The Caco-2 cell line is derived from a human colonic adenocarcinoma which spontaneously differentiates in cell culture to form a polarized epithelial monolayer with many of the characteristics of enterocytes (Pinto et al, 1983). This cell line has been shown to correlate reasonably well with human bioavailability models (Au and Reddy, 2000) at a fraction of the cost and time commitment. Recent work in the characterization of heme iron uptake pathways using Caco-2 cells suggests that beyond the well described application for non-heme iron bioavailability, this cell line may also be applicable to heme iron bioavailability (Uc et al, 2004; Follett et al, 2002).

The use of radioisotope based uptake, or ferritin as an index of bioavailability is well established (Au and Reddy, 2000; Glahn et al, 1998).

Iron Intervention Trials

In efficacy studies, iron fortified food or supplements are given for several months to a targeted group of individuals who exhibit iron deficiency, and changes in iron status are followed over time. The study design includes randomization of treatments, double-blinding to the treatment received, and using a placebo to control for effects unrelated to treatment factors. By controlling the food and nutrient intake of subjects, the inherent variability that a free living subject would experience in their food consumption is not observed. Researchers can however, through use of appropriate biomarkers, make estimates on nutrient bioavailability, for example using serum ferritin and transferrin receptor (Cook et al, 2003), and can show clinical evidence of alleviation of nutrient deficiency through administration of fortified foods or supplements.

Effectiveness studies are the final determinant of an intervention's performance, through delivery of a fortified food or supplement in a free living population. In an effectiveness study compliance, economic limitations, and factors affecting purchasing, distribution, consumption, absorption, and utilization of the nutrients can be under scrutiny within the context of alleviating nutrient deficiency. Effectiveness studies introduce fortified food or supplement into a population through the usual domestic or institutional channels, with the food or supplement subjected to variable consumption patterns, environmental and home preparation variables. Efficacy is defined as "the extent to which a specific intervention, procedure, regimen, or service produces a

beneficial effect under ideal conditions", whereas effectiveness has to do with the extent to which the same "specific intervention, procedure, regimen or services when deployed in the field does what it is intended to do for a defined population" (Last, 1998). In many circumstances, effectiveness studies carry the most credible weight for justifying the implementation of programs to policy makers. Unfortunately, effectiveness and efficacy studies are very expensive, and as such these studies in maize fortification schemes are rare.

Intervention Strategies to Alleviate Iron Deficiency

Supplementation is a useful mechanism for quickly alleviating iron deficiency through delivery of a controlled quantity of iron in a pharmacological format. Because of the high dose of iron given in supplementation, compliance may be low due to the side effects of gastric discomfort. In addition, supplementation requires an intensive public health system to provide proper availability and distribution, although it is typically a short term intervention (e.g. several months during pregnancy, or to alleviate clinical iron deficiency anemia). Recommendations for universal supplementation in iron replete women and children under the age of 5 may be causing unnecessary oxidative stress, while iron supplementation in protein energy malnutrition may increase morbidity, and in certain infections such as malaria increase mortality (Shumann et al, 2007).

Improvement of dietary diversity is a long term strategy that can effectively improve not only iron status, but most nutrient statuses through encouraging consumption of nutrient dense foods. The challenge of dietary diversification is that it requires a very strong political, social, and economic will within national and community level

organizations. Changing peoples' diets to improve nutritional quality is a long-term challenge that requires a strong educational component to show the potential benefits to the individual and the population. Since this strategy relies on natural food products, it has greatest opportunity for self sustainability. Dietary diversification is prone to failure during economic crisis, changes in agronomic conditions such as drought, flooding or introduction of plant or animal diseases, or changes in industrial or economic development, as in changing from self-sustenance to a trade oriented food supply (Tontisirin et al, 2002).

Dietary diversification can also be expanded to incorporate modifications in how foods are processed prior to incorporation into the diet. A moderate body of work is available suggesting how basic household or industrial processing can affect iron bioavailability in foods made of maize, in particular focusing on phytate reduction (Hotz and Gibson, 2007).

Fortification has traditionally been termed as the addition of nutrients in low amounts to industrially prepared staple foods targeted to the vulnerable population. Recent innovations have transitioned the concept of fortification into a broader context, including household fortification using self-administered "Sprinkles" mixed into household prepared meals (Zlotkin et al, 2004). It also includes co-fortification with non-nutritive components such as EDTA to enhance bioavailability (Hettiarachchi et al, 2004), and biofortification (Hotz and McClafferty, 2007). Industrial fortification uses commercial food processing and distribution channels for delivery of a nutrient in a staple food that is consumed in a self-limiting manner. Household-level fortification uses nutrient premixes formulated to the needs of the regional population and added to home

prepared foods. Biofortification is the process where nutrients are added by biological means to staple foods (mostly grains and starchy plant foods), through traditional or molecular crop breeding. Bioavailability enhancement can include addition of non-nutrients to enhance bioavailability (eg EDTA addition), or processing is modified to change the bioavailability characteristics (eg phytate reduction).

There no consensus regarding best practices in fortification, especially in maize. This lack of consensus is based on the lack of food matrix characterization and its effect on fortification. Given that there are few effectiveness studies, there is only minimal political will to legislate mandatory fortification or provide incentives for voluntary fortification. A strong advocacy campaign has been conducted by various non-governmental organizations in the attempts to promote the international nutritional goals outlined at the beginning of this review, especially alleviation of iron deficiency.

Iron fortification of maize food products

Of all the nutrients used for maize fortification, none provides as much challenge for success as iron. For this reason, a substantial proportion of the literature on bioavailability and maize deals with iron. The challenge of iron fortification, as in many fortification interventions, is the optimization of the fortification scheme in terms of bioavailability, acceptability and cost (Hurrell, 2002). Iron compounds have a variety of issues in regards to all of the above criteria, and particularly with respect to bioavailability in that it is highly variable based on the solubility of the iron itself, and is compounded by interactions with fiber, phytate and proteins in the food matrix, all of

which inhibit the absorption of the iron. On the other hand, food matrix interactions may enhance iron availability through chelating agents, protein binding, and pH effects.

Food matrix interactions

A number of factors in the food matrix impact iron in maize foods, including fiber, zein protein, and pH. A study was conducted to evaluate the food matrix interactions of maize tortillas on iron solubility from ferrous and ferric iron. In this study, it was found that increased fiber or zein protein (the dominant maize protein) content in tortillas significantly reduced ferrous iron solubility at pH 6 or higher, but increased ferrous solubility at pH < 5. Ferric iron solubility was markedly reduced between pH 4.5 and pH 7.5, and similarly reduced with increasing zein and fiber content (Reinhold, et al, 1984).

Fortification iron has a potential negative effect on organoleptic quality of maize products, in that soluble forms of iron can cause oxidative rancidity. As part of an accelerated storage study, fortification with either 40mg iron/lb (88ppm), or 200mg iron/lb (441ppm) as ferrous sulfate caused rancid odour after 28 days storage at 49°C in coarse maize grits, fine grits, and degerminated cornmeal. Reduced iron caused a subnormal odour in both fine and coarse grits under the same conditions. Flavor scores were lower in the products with odour defects. Reduced iron, which is known to have minimal solubility, had only minimal changes in odour and flavor after 28 days and 56 days storage (Anderson, et al., 1976). Significant changes in nutrient composition occur during the physical transformation of maize to tortillas. The grinding implements used for tortilla manufacture have shown to increase iron and zinc content, in particular the

combined use of traditional mortars and pestles and hand powered disc mills (Krause, et al., 1992; Krause, et al., 1993).

Bioavailability of iron fortification

A study assessing the intrinsic iron bioavailability of various traditional Latin American diets indicated that there is significant inhibition of iron absorption from many traditional foods - especially from maize based diets, and suggested that fortification would be a suitable method for overcoming the minimal iron absorbed from the diet (Acosta, et al. 1984). A similar study, examining the intrinsic iron of Latin American diets indicated that a diet of tortillas and beans had a significantly lower iron bioavailability than a diet to which a source of ascorbate (orange juice) and animal protein (eggs) had been added. The iron absorption in the maize tortilla based diet was 7.6% as compared to 21.3% in the enhanced diet (Maisterrena, et al. 1973). A study looking at the bioavailability of iron from traditional maize foods of Benin found that absorption rates were very low, ranging from 1.2% to 3.2% in maize-based meals evaluated by extrinsic radiolabeling method (Galan et al, 1990).

Maize foods have been studied extensively for appropriateness of iron fortificants, and the summary of some major studies can be found in **Table 2.2**. In the fortification bioavailability studies described in **Table 2.2**, a variety of food products were characterized, including maize porridges, nixtamalized tortillas, and maize based complementary foods. This information is summarized in **Table 2.3**. It is challenging to make best practices recommendations based on the diversity of products listed, especially without descriptions of processing and composition information.

Ferrous sulphate, because of its exceptionally high solubility, tends to have high bioavailability in maize foods; however, as described above, ferrous sulphate can impart negative organoleptic qualities to maize foods, especially dry flours that are stored for extended periods. Ferrous fumarate and ferric EDTA have very good bioavailability in maize foods because of their high solubility, and in the case of ferrous EDTA minimized inhibition from dietary factors, however they have higher monetary cost. The bioavailability of less soluble iron compounds, for example reduced iron, is very difficult to assess because of the difficulties of manufacturing radiolabeled iron. Attempts have been made to assess the true bioavailability without intrinsic tag labeling, but have shown minimal success (Hurrell, et al. 2002a). Reduced iron, the least expensive type of iron fortificant, has marginal bioavailability in humans (Walter, et al. 2004).

Beyond bioavailability, the use of fortificants is strongly influenced by cost effectiveness. A theoretical cost analysis of nixtamalized corn flour fortification with iron is summarized in **Table 2.4**, with ferrous sulphate not included because of its poor stability in maize.

Ferrous fumarate has been designated the most appropriate iron fortificant for maize foods by a number of groups, including the Venezuelan government. Iron fortification in Venezuela with 20 or 50 mg Fe as ferrous fumarate per kilogram flour in precooked yellow and white maize flours, along with a vitamin mix resulted in a significant decrease in iron deficiency rates, particularly in low socioeconomic groups. The percentage of subjects with low serum ferritin concentration decreased from 37% to 15% in children 7, 11 or 15 y in Caracas, Venezuela, over a 1 year period of iron fortification, and anemia rates in the same population decreased from 19% to 10% over

the same time period (Layrisse, et al. 1996). The evidence presented indicates that after a dramatic reduction in 1994, the prevalence of anemia returned to the 1992 level, probably due to deterioration of quality food consumption, and dengue epidemic. Whether the effect of is due to changing the iron fortification mandate from ferrous fumarate alone to ferrous fumarate and elemental iron (60:40 w/w for delivered iron) combination is difficult to determine. Maize flour consumption decreased during this time period, with an increase of wheat flour consumption. Regardless, iron fortification was shown to stabilize iron stores in the population.

The Venezuelan study demonstrates that a small increase in total dietary iron can have a significant effect on reducing iron deficiency rates in that population. The effectiveness of increasing dietary iron in deficient populations was further examined in a separate study, which showed that iron absorption from a labeled, ferric trisglycinate, fortified maize porridge was greater in iron deficient subjects than in iron replete subjects (Bovell-Benjamin, et al. 2000). The efficiency of absorption increases with decreased iron status, which may have effects on apparent absorption rates of iron fortificants. Most of the studies described in **Table 2.1** were conducted on healthy, iron replete subjects, which may indicate lower iron absorption rates. Studies conducted on anemic rats are not reliable predictors of human iron absorption, because of phytase activity in the gastrointestinal track and lack of ascorbic acid requirement (Lopez, et al. 1998).

Elemental Iron

Elemental iron is the most common iron fortification form. There have been no published human efficacy studies using elemental iron in maize foods. Elemental iron,

while having a low bioavailability, has had high level of use in the United States, Latin America, and Western Europe in cereal products. Bioavailability assays using reduced iron are inherently difficult to conduct because of difficulties in producing radiolabeled or stable isotopes of reduced iron forms that resemble commercial form sufficiently enough for meaningful comparisons. Reduced iron is desirable from a food processing perspective as a fortificant because of its low reactivity and low cost; however reduced iron has very low bioavailability. Reduced iron has been shown to have minimal negative organoleptic effects on fortified maize foods (Anderson, et al. 1976). Recent research has indicated that there are significant differences in iron bioavailability from reduced iron powders based on surface area and particle size, where smaller, higher surface area particles have better bioavailability in rat hemoglobin repletion tests, and higher solubility at pH 1.0, and 1.7 (Swain, et al. 2003). In studies summarized above (Walter, et al. 2004), the bioavailability of a laboratory produced, labeled, reduced iron isotope was assessed at 3.8% in tortillas fed to children. Rats consuming fortified masa flour displayed no difference in iron utilization value from the unfortified masa flour (Hernández, et al. 2003). Laboratory produced reduced iron powders are often not comparable to industrially processed reduced iron. However, when considered in optimized iron fortification schemes, reduced iron may have benefits in certain settings and for certain food types. More research on reduced iron must be conducted before recommendations for its use can be elaborated (Turner, 2002).

EDTA

Sodium Iron EDTA is currently the recommended form of fortification for maize foods. Sodium Iron EDTA, or ferric EDTA as a fortificant has been shown to have high bioavailability. Ferric EDTA has high solubility, and can overcome phytate inhibition (Mendoza, et al. 2001). Several studies have indicated that ferric EDTA has higher bioavailability than other iron forms, and addition of sodium EDTA may influence the bioavailability of soluble iron forms. When ferric EDTA was fortified into a maize porridge it displayed 10.5% bioavailability as compared to 4.7% from ferrous sulphate (Layrisse, et al. 2000a). Addition of sodium EDTA to nixtamalized maize tortillas fortified with ferrous fumarate in a 2:1 molar ratio increased fumarate bioavailability significantly (Walter, et al. 2003), however this improvement was not shown when sodium EDTA was added to tortillas fortified with reduced iron (Walter, et al. 2004). Overall, iron EDTA fortification is highly beneficial, however is currently limited because of the high cost as compared to other iron fortificants (**Table 2.4**).

Organic acids and iron fortification

Use of organic acids to enhance iron bioavailability has been suggested as another strategy to enhance bioavailability (Teucher et al, 2004). Limited studies have evaluated this idea in maize. Co-fortification of ascorbate in particular with iron has been considered as a method of enhancing iron bioavailability because of the enhancing effect of ascorbate. Wheat bread supplemented with phytate rich maize bran showed increased iron absorption when an ascorbate solution was applied to the bread post-baking before consumption (Siegenberg, et al. 1991). Ascorbic acid fortification was shown to enhance intrinsic iron absorption in a maize tortilla meal from 1.2% to 3.4%; however, dietary

inclusion of ascorbate-rich foods resulted in more pronounced increase in iron absorption than that observed with the ascorbate fortification (Hallberg and Rossander, 1984). The functional and organoleptic properties of maize food products fortified with iron and ascorbate have not been discussed, however in wheat flour foods, high levels of ascorbate have distinctive effects on dough rheology and product quality. Additionally, ascorbate is not heat stable; therefore fortification of foods prior to cooking may not provide significant increases in iron absorption. Social marketing and promotion of consumption of ascorbate rich foods may play a more vital role in influencing iron absorption rates than ascorbate co-fortification however, this too is being debated (Cook and Reddy, 2001). Other organic acids and their effects on iron bioavailability have not been evaluated.

Fermentation

Fermentation has been suggested as a means to enhance iron bioavailability through its potential role in reducing phytate from exogenous phytase in the fermentative organisms. As a point of interest, traditional home fermented maize beer produced in iron containers has very high iron bioavailability. The iron absorption from maize beer is high enough to put regular maize beer consumers at risk of iron overload. And conversely, beers fermented in non-iron containers have much lower iron content than those fermented in iron containers (Derman, et al. 1980). No studies have been conducted to identify if lactic acid bacteria fermented porridges have higher iron content and iron bioavailability when fermented in iron containers. The beer study did indicate that maize solids content decreased iron bioavailability, despite increased iron content,

suggesting a fiber or protein based interaction with iron. There is a lack of information on the iron bioavailability of traditional lactic acid fortified maize foods consumed in Latin America and Sub Saharan Africa, nor is there information on the efficacy of fortification in these same foods. One study identified that maize-soy tempe flour (70:30 w/w), which was fermented with *Rhizopus oryzae*, increased its iron solubility from 2.46% to 5.51% as evaluated by the dialysis method (Tchango Tchango, 1995). One could postulate that fermentation, through acidification and action of bacterial phytases acts to improve bioavailability, however no research to elucidate the mechanisms has been published. These hypotheses regarding maize processing and fermentation have led to the research conducted in this dissertation in part 4.

Phytate and Fiber in Bioavailability Reduction

Fiber from maize is known to be high in phytate, which is a potent bioavailability inhibitor due to its tendency to chelate divalent metals, including calcium, iron and zinc. Phytate content is related to total fiber, in that maize fiber (pericarp and germ) has higher concentrations of phytate than endosperm. Industrial processing, which is optimized to minimize dry matter losses, may increase phytate content by more efficiently milling maize bran for its inclusion in maize foods rather than remove it as a waste stream as in the traditional household methods. Nixtamalization has been shown to marginally reduce phytate levels from 1113 mg/100 g to 727 mg/100 g after processing with 1.2% calcium carbonate (Urizar Hernández and Bressani, 1997). These reductions in phytate are not sufficient to increase iron bioavailability, as reductions to levels less than 30 mg per entire meal are needed to see improvement in iron bioavailability (Hurrell, et al. 1992).

Industrially processed tortillas had significantly more phytate than home processed tortillas made from maize grown in the same region (Garzon, et al. 1984), likely due to increased net fiber content from inclusion of more pericarp in the final product. No significant differences in phytate content, or iron absorption from maize porridge or pancake were found regardless of different industrial processing techniques, including extrusion, roller drying, amylase treatment, or home prepared commercial product (Hurrell, et al. 2002b).

Most fiber in maize is insoluble (Nyman, et al, 1984) Industrially processed tortillas were found to contain 6.60% neutral detergent fiber, 3.75% acid detergent fiber, and 2.89% hemicellulose (Reinhold and Garcia, 1979). Diets with increasing amounts of fiber from a maize and bean mixture displayed lower relative biological value of iron assessed in rats compared to ferrous sulphate control (Garcia-Lopez and Wyatt, 1982), however this data may not directly correlate to humans as rats can metabolize phytate.

The role of phytase, either through introduction of a commercial enzyme, or through fermentation or germination has been explored in relation to iron and zinc bioavailability. Dephytinized maize porridge produced using commercial enzyme had 8.9% iron absorption versus 1.8% in its native phytate counterpart (Hurrell, et al. 2003). Home dephytinization has been developed for maize foods, either through addition of commercial phytase to a 1:4 w/w maize:water slurry, followed by settling and decantation of supernatant (Manary, et al. 2002); or by soaking pounded maize flour in water in a 1:4 w/w ratio, followed by settling and draining prior to utilization (Hotz et al. 2001). Home addition of phytase caused a phytate reduction of 93%, while soaking without added enzymes caused a reduction of 52%. Fermentation of a 70:30 maize:soy

mixture by *Rhizopus oryzae* was found to decrease phytate content by 46% (Tchango Tchango, 1995). Hydrolysis of phosphate groups from phytate to create a lower level of inositol phosphates is the goal, as they are less inhibiting to iron uptake (Sandberg, et al. 1999), and zinc bioavailabilty.

Recommendations for Iron Fortification of Maize

Recommendations exist for the fortification of maize flour with iron. Sodium Iron EDTA has been recommended as the ideal fortificant for nixtamalized maize flour at 20 mg/kg, with the next preference being ferrous fumarate, at 40 mg/kg, followed by microencapsulated ferrous sulphate or ferrous fumarate (No authors, 2002). Ferrous sulphate, followed by ferrous fumarate are the iron fortificants of choice for degermed, sifted or bolted maize products, with double quantity fine mesh (<45um) electrolytic iron and microencapsulated ferrous sulphate or ferrous fumarate as secondary choices (PAHO et al. 2002). Development of low cost ferrous sulphate microencapsulation could change recommendations, as ferrous sulphate typically displays the best iron bioavailability, but without microencapsulation can cause negative changes in organoleptic quality. Iron compounds should be added to the level up to which they do not change the organoleptic qualities of the food, usually between 20ppm and 40ppm (PAHO et al. 2002). The use of fine mesh electrolytic iron, despite having low bioavailability, has high stability and low cost, and may be more practical for some settings.

<u>Biofortification for improving crops</u>

The concept of using agronomic approaches to enhancing the nutritional value of crops is both one rooted in history, and yet a very modern approach to affecting micronutrient nutrition. Historically, crop breeding was conducted to enhance yield and general nutritional quality, along with agronomic and economic adaptability. Modern biofortification is defined as "the development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology." (Nestel et al, 2006) to target enhancement of specific nutritional attributes, in particular micronutrient content and bioavailability. Since the advent of the first major biofortification efforts, with theoretical work beginning in the 1980's with studies into enhancing amino acid quality in seeds by transforming tobacco to express a brazil nut protein (Altenbach et al, 1988) through the widely publicized golden rice enhanced with b-carotene through expression of phytoene synthase from daffodils in the rice endosperm (Burkhardt et al, 1997), many crops have been enhanced with various traits to enhance nutritional quality by biotechnology.

Because of the nature of biofortification, which is the development of a self perpetuating means to provide a more nutrient rich diet, in theory, seed could be distributed with the intention of self perpetuation regrowing the seed each year. This would eliminate the bulk of recurrent costs seen in fortification and supplementation initiatives and reduce the need for recurring intervention by governments or international development agencies (Bouis, 2003).

There is some controversy, however, regarding the utilization of biofortified crops. While biofortification has been developed using either traditional crop breeding techniques, it has also incorporated modern genetic engineering. The acceptability of

genetically modified crops is still highly variable, because of various fears about genetic transfer, environmental impact, and changes in seed distribution modes affecting economic systems. Proponents of genetic modification technology however suggest that consumers and legislative groups will be more willing to adopt genetically modified crops when a clear and well documented benefit to the consumers and producers can be seen (King, 2002).

From a technical standpoint, biofortified crops have been suggested to be infinitely self perpetuating. Genetically modified traits may not be readily passed on from generation to generation without significant monitoring programs, as some traits are not physiologically advantageous to the crops. For example, phytate reduction for enhancing iron and other trace minerals bioavailability has been suggested as a means to improve bioavailability in maize (Mendoza et al, 1998), however phytate reduction has been shown to have a significant impact on yield, and germination quality (Pilu et al, 2005). Suggestions that biofortified crops will be self perpetuating without continuous evaluation and monitoring have been made (Graham and Welch, 1996; Graham et al, 2001), however genetic traits bred in low resource areas could and should be suspect for genetic drift.

Proof of concept studies

Several crops have been evaluated for their potential modification to enhance iron content and bioavailability. Germplasm of several staple crops has been evaluated for potential to breed for higher iron content without targeting specific genes. This includes beans (Welch et al, 2000), and maize (Oikeh, et al, 2003) where the beans showed

fractional absorption of 53 to 76% of total iron when assayed in an extrinsically labeled rat-based uptake assay, and the maize showed variation between 4% lower to 49% higher than the reference control as evaluated using the Caco-2 assay. Non-specific increases in iron content do not evaluate the speciation of the iron within the seed, and as such may be increasing unavailable iron. While the studies listed evaluated cofactors influencing iron bioavailability, including phytate and polyphenols, there is no consideration regarding environmental variability, and genetic segregation in the varieties suggested.

One controversial study evaluated iron enhanced rice (IR68144–2B-2–2-3) (Gregorio et al, 2000) for its ability to improve iron status in Filipino women. Enhanced rice (3.21 mg/kg Fe) was compared to typical rice (0.57 mg/kg Fe) over 9 months feeding. In anemic women, no significant changes in iron status (hemoglobin, serum ferritin or total body iron) were seen, while in non-anemic women serum ferritin and total body iron increased. As rice made up ~50% of the women's' caloric intake, it was anticipated that greater increases in iron status would have been seen, in particular with anemic women whose iron uptake should be upregulated. It however has been postulated that the anemic women may have had multiple nutrient deficiencies or other health issues counteracting any benefit from the rice (Haas et al, 2005).

Transgenic methods for enhancing iron content and bioavailability have also been developed for various crops. Soybean ferritin has been overexpressed in rice, which when evaluated in a rat hemoglobin repletion model had bioavailability similar to rice fortified with ferrous sulphate (Murray-Kolb et al, 2002). Human lactoferrin has also been expressed in rice, also showing similar bioavailability to ferrous sulphate fortified rice (Lonnerdal and Bryant, 2006). Maize has specifically been modified to enhance iron

bioavailability by reducing phytate content. Mutants of phytate have been evaluated, and shown to have 49% higher iron bioavailability as tortillas, and 50% as porridge, however differences between the normal and low phytate lines were significant when products were fortified (Mendoza et al, 2001, Mendoza et al, 1998). One last strategy was to express transgenic soybean ferritin and *Aspergillus niger* phytase which showed approximately 3 fold higher bioavailability as assessed using the Caco-2 assay as compared to the wild type maize (Drakakaki et al, 2005).

The idea of conducting genetic modification of plants using only coding sequence from the plant to be modified itself is gaining a good deal of interest, as it could potentially circumvent much of the negative impressions about transgenic modification. As discussed earlier, heme iron is a highly bioavailable source of iron (Proulx and Reddy, 2006), and most plants have genes coding for hemoglobin proteins (Kundu et al, 2003). The overexpression of maize hemoglobin in maize is a proposed means to biofortify maize with highly bioavailable iron which will be discussed in this dissertation in part 5.

Plant Hemoglobins

The discovery that plants produce hemoglobin is not new, rather it was more than 60 years ago that the first research into soy leghemoglobin was done (Kubo, 1939). Legumes have been shown to produce hemoglobin in response to infection with *Rhizobium spp.* as part of the nitrogen fixation process. This is termed symbiotic hemoglobin. The bacteria, which are facultative aerobes require oxygen transfer through the root tissues, while the nitrogenase enzyme responsible for conversion of atmospheric N_2 to nitrate functions better in a low oxygen environment (Wittenberg et al, 1974). The

presence of a hemoglobin acts as an oxygen diffusion and scavenging mechanism combined, based on the reversible oxygen binding of the hemoglobin. More recent work has shown that many, and perhaps almost all plants have genetic coding for other hemoglobins not associated with nitrogen fixation. The purpose of these non-symbiotic hemoglobins is not certain, however it is suggested that they may play a part in tissue differentiation in the xylem (Ross et al, 2001), anoxia (Taylor et al, 1994), flood tolerance (Taylor et al, 1994), light deprivation (Lira-Ruan et al, 2001) or nitric oxide metabolic pathways (Dordas et al, 2003). The genetics and physiological significance of plant hemoglobins is reviewed in several articles (Garrocho-Villegas et al, 2007; Hebelstrup et al, 2007).

As heme proteins, plant hemoglobins have been postulated to have similar bioavailability characteristics as their animal sourced proteins, making them a potential source of highly available iron. This research is part of the first research project in this dissertation (part 3), showing how hemoglobin from soy root nodules compares in bioavailability to animal sourced hemoglobin. Secondly, as most plants have genetic code for hemoglobin, the hemoglobin gene could be used for intragenic transformation of plants, in particular maize which has many nutritional factors inhibiting iron bioavailability. This strategy is used for the research project in part 5 of this dissertation.

Conclusion

Iron deficiency is an enormous nutritional concern globally, and the development of new strategies for its alleviation is vital. Food based strategies, in particular fortification, process modification, and biofortification have been shown to have strong

potential to be sustainable and cost effective means to alleviate iron deficiency. Maize, and its food products are particularly problematic for fortification, as there is a lack of consensus for best fortification practices because of the diversity of maize processing technologies. Novel strategies to improve iron bioavailability in maize foods should be developed, and this dissertation aims to describe three novel approaches to improving iron bioavailability in maize products. This includes fortification of maize tortillas with plant hemoglobins, processing maize in ways to better enhance bioavailability of intrinsic or fortification iron, and lastly biofortification using intragenic overexpression of maize hemoglobin genes to increase the amount of bioavailable iron in maize. With the advent of a variety of new technologies, hopefully iron deficiency can be alleviated in populations around the world.

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Tables

Table 2.1. Iron Status Indicators
From Zimmerman and Hurrell, (2007)

	Selected cutoff values to define iron deficiency	**Comments**
Hemoglobin (g/L)	6 months–5 years <110 6 years–11 years <115 Non-pregnant women <120 Pregnant women <110	When used alone, it has low specificity and sensitivity
Mean corpuscular volume (MCV) (cu μm)	Children older than 11 years and adults <82	A reliable, but late indicator of iron deficiency Low values can also be due to thalassemia
Reticulocyte hemoglobin content (CHr) (pg)	In infants and young children <27.5 In adults ≤28.0	A sensitive indicator that falls within days of onset of iron-deficient erythropoiesis False normal values can occur when MCV is increased and in thalassemia. Wider use is limited because it can only be measured on a few models of analyzer
Transferrin saturation	<16%	It is inexpensive, but its use is limited by diurnal variation in serum iron and by many clinical disorders that affect transferrin concentrations

Table 2.1 (continued)

Erythrocyte zinc protoporphyrin (ZPP) (μmol/mol haem)	5 years or younger >70 Children older than 5 years >80 Children older than 5 years on washed red cells >40	It can be measured directly on a drop of blood with a portable haematofluorometer A useful screening test in field surveys, particularly in children, in whom uncomplicated iron deficiency is the primary cause of anaemia Red cells should be washed before measurement because circulating factors, including serum bilirubin, can spuriously increase values Lead poisoning can increase values, particularly in urban and industrial settings
Serum ferritin (SF) (μg/L)	5 years or younger <12 Children older than 5 years <15 In all age groups in the presence of infection <30	It is probably the most useful laboratory measure of iron status; a low value of SF is diagnostic of iron deficiency anaemia in a patient with anaemia In healthy individuals, SF is directly proportional to iron stores: 1 μg/L SF corresponds to 8–10 mg body iron or 120 μg storage iron per kg bodyweight As an acute-phase protein, SF increases independent of iron status by acute or chronic inflammation; it is also unreliable in patients with malignancy, hyperthyroidism, liver disease, or heavy alcohol intake

Table 2.1 (continued)

	Selected cutoff values to define iron deficiency	Comments
Serum transferrin receptor (sTfR)	Cutoff varies with assay, and with patient age and ethnic origin	Main determinants are the erythroid mass in the bone marrow and iron status; thus, sTfR is increased by enhanced erythropoiesis and iron deficiency sTfR is not substantially affected by the acute-phase response, but it might be affected by malaria, age, and ethnicity Its application limited by high cost of commercial assays and lack of an international standard
sTfR-to-SF ratio		This ratio is a quantitative estimate of total body iron; the logarithm of this ratio is directly proportional to the amount of stored iron in iron-replete patients and the tissue iron deficit in iron deficiency In elderly people, this ratio might be more sensitive than other laboratory tests for iron deficiency This ratio cannot be used in individuals with inflammation because SF might be high independent of iron stores This ratio is assay specific Although it is only validated for adults, this ratio has been used in children

Table 2.2. Iron fortification in maize foods – percent iron bioavailability

Study	Subjects	Food Matrix	Iron Form and Dose	Bioavailability*
(Björn-Rasmussen et al. 1972)	25 males, 21-27a	Wholemeal maize Chapatis, intrinsically labeled with ^{59}Fe	4.6mg Fe as ferric chloride	-Intrinsic Fe 1.78% -Ferric chloride 1.73%
(Ashworth and March, 1973)	14 healthy infants, 7-21mo	Corn-Soy-Milk complementary food	4.6mg Fe as ferrous fumarate	-Ferrous fumarate 6.0%
(Ashworth, et al. 1973)	42 healthy children 5-24mo	Whole maize meal slurry intrinsically labeled with ^{59}Fe	0.5mg Fe as intrinsic maize iron	-Intrinsic Fe 4.3%
(Morck, et al. 1981)	27 healthy males, 18-45y	Corn-Soy-Milk (CSM) complementary food, and Corn-Soy Blend (CSB) complementary food	9mg Fe as ferrous fumarate	-CSM 0.57-1.40% -CSB 0.92%
(Davidsson, et al. 2002)	33 healthy adolescent girls, 12-13y	Corn tortillas and refried black beans	2mg Fe as ferrous fumarate, or ferrous sulphate, with or without Na_2EDTA	-Ferrous fumarate 5.5 – 6.2% -Ferrous fumarate + Na_2EDTA 1:1 total iron 6.7% -Ferrous fumarate + Na_2EDTA 1:1 fortification iron 5.8% -Ferrous sulphate 5.5% -Ferrous sulphate+ Na_2EDTA 1:1 fortification iron 9.0%

*Human bioavailability is compared to 40% ferrous ascorbate absorption

Table 2.2 (continued)

Study	Subjects	Food Matrix	Iron Form and Dose	Bioavailability*
(Martinez-Torres, et al. 1979)	Rural Venezualens -27 males, 80 females	Golfio: 70% toasted maize flour, with spices, mixed with fortificant syrup Tequiche: 500g maize flour mixed with 400g fortificant syrup, blended with milk from 2 coconuts	3mg ^{59}Fe in sugar syrup as Fe(III)EDTA	-Golfio 8.1% -Tequiche 9.9%
(Mendoza, et al. 1998)	14 healthy males, 19-35y	Nixtamalized maize tortilla, either normal or low phytate varietals	-intrinsic iron measured against ^{59}Fe tracer	-normal phytate 5.48% -low phytate 8.15%
(Mendoza, et al. 2001)	14 healthy females, 19-42y	Nixtamalized maize porridge, either normal or low phytate maize varietials, with 3.4mg intrinsic iron per serving	1mg Fe as ferrous sulphate, or Fe (II)EDTA in low or normal phytate porridge	-Normal phytate with ferrous sulphate 1.69% -Normal phytate with Fe(II)EDTA 5.73% -Low phytate with ferrous sulphate 1.91% -Low phytate with Fe(II)EDTA 5.40%
(Walter, et al. 2003)	15 healthy females, 31-50y	Nixtamalized maize tortillas with 15mg/kg intrinsic iron	30mg/kg Fe as ferrous fumarate, ferrous fumarate 2:1 mol with Na_2EDTA, ferrous bis-glycinate, or NaFeEDTA, or in a rice and beans accompanying meal	-Intrinsic Fe 0.69% -Ferrous fumarate 0.87% -ferrous fumarate with Na_2EDTA 2.98% -ferrous bis-glycinate 1.27% -NaFeEDTA 5.30% -Native Fe with meal 3.19%

Table 2.2 (continued)

Study	Subjects	Food Matrix	Iron Form and Dose	Bioavailability*
(Walter, et al. 2004)	15 healthy children, 5-7y	Nixtamalized maize tortillas	3mg/100g Fe as reduced iron ^{58}Fe isotope, in gelatin capsule embedded in tortilla, with or without Na_2EDTA 1:2 molar ratio	-Reduced iron isotope 3.8% -Reduced iron with Na_2EDTA 5.1%
(Layrisse, et al. 2000)	18 males, 56 females, 15-50y, all healthy, low socioeconomic status	100g precooked maize flour porridge with 50g cheese and 10g margarine, with 1.6mg intrinsic iron	3mg Fe as ferrous sulphate, ferrous bisglycinate, or FeEDTA	-Native iron 3.2% -Ferrous sulphate 4.7% -Ferrous bisglycinate 8.4% -FeEDTA 10.5%
(Bovell-Benjamin, et al. 2000)	10 healthy males	Whole maize meal porridge	11mg/kg Fe as ferrous sulphate or ferrous bisglycinate	-Ferrous sulphate 1.7% -Ferrous bisglycinate 6.0%
(Björn-Rasmussen and Hallberg, 1974)	30 healthy adults, 19-35y	Maize flour porridge	5mg Fe as labeled ferrous sulphate, mixed with 12.5, 25, 50, 100 and 200mg ascorbic acid	-no ascorbate 5.68% -12.5mg ascorbate 9.32% -25mg ascorbate 13.05% -50mg ascorbate 19.50% -100mg ascorbate 20.83% -200mg ascorbate 9.83%

Table 2.3. Summary of fortification of maize foods with iron fortificants based on Table 2.2 studies

Food type	Fortificant	Percent absorption (%)
Complementary Foods	Ferrous fumarate	0.92 – 6.0
Breads	Ferric chloride	1.73-1.78
Porridges	Ferrous sulphate	1.7
	Ferrous sulphate + ascorbate	5.69 – 20.83
	NaFeEDTA	8.1 – 9.9
	Ferrous bisglycinate	6.0
Nixtamalized foods	Ferrous fumarate	5.5 – 6.2
	Fumarate + NaEDTA	6.7
	Ferrous sulphate	1.69 – 4.7
	Ferrous sulphate + NaEDTA	9.0
	NaFeEDTA	5.30 - 10.5
	Reduced iron	3.8
	Reduced iron +NaEDTA	5.1
	Ferrous bisglycinate	1.27 – 8.4

Table 2.4. Theoretical Effectiveness and Cost Analysis of Nixtamalized Corn Flour Fortification in Central America -modified from Dary (2002).

Variable	Reduced Iron	Ferrous Fumarate	Ferrous Bisglycinate	NaFeEDTA
Maximum Iron Load (mg Fe/kg)	30	30	15	15
Iron absorption in 200g flour (mg)	0.12	0.36	0.27	0.33
Relative absorption	0.33	1.00	0.75	0.92
Cost of the compound (US$/kg)	2.00	3.00	25.00	10.50
Percentage iron in compound (%)	97	33	20	14
Cost per kilogram iron (US$/kg)	2.06	9.09	125.00	75.00
Relative cost per kilogram iron	0.23	1.00	13.75	8.25
Iron fortification cost (US$/MT)	0.06	0.27	1.87	1.13
Relative iron fortification cost	0.22	1.00	6.93	4.19
Multiple fortification cost (US$/MT)	0.66	0.87	2.47	1.73
Percentage of price over US$45/kg (%)	0.15	0.19	0.55	0.38
Relative multiple fortification cost	0.76	1.00	2.84	2.00
Total annual cost per million persons ($)	48,180	63,510	207,320	126,290

Figures

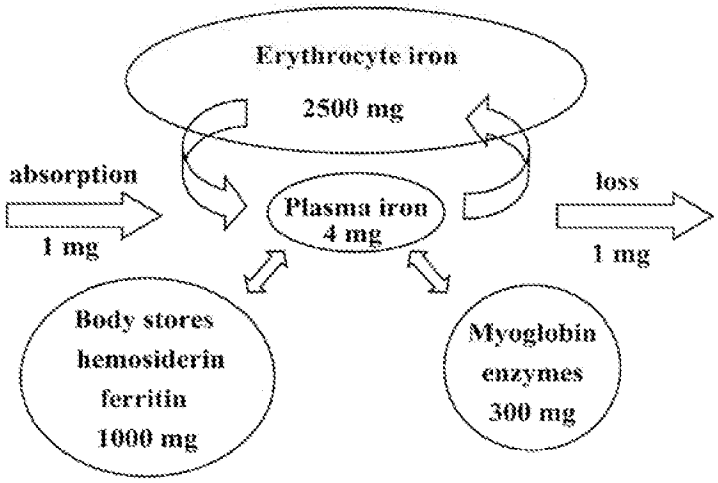


Figure 2.1. Body stores of Iron

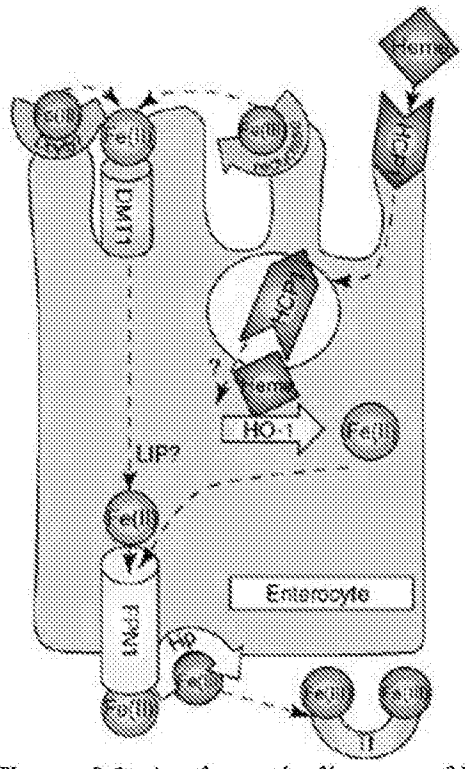
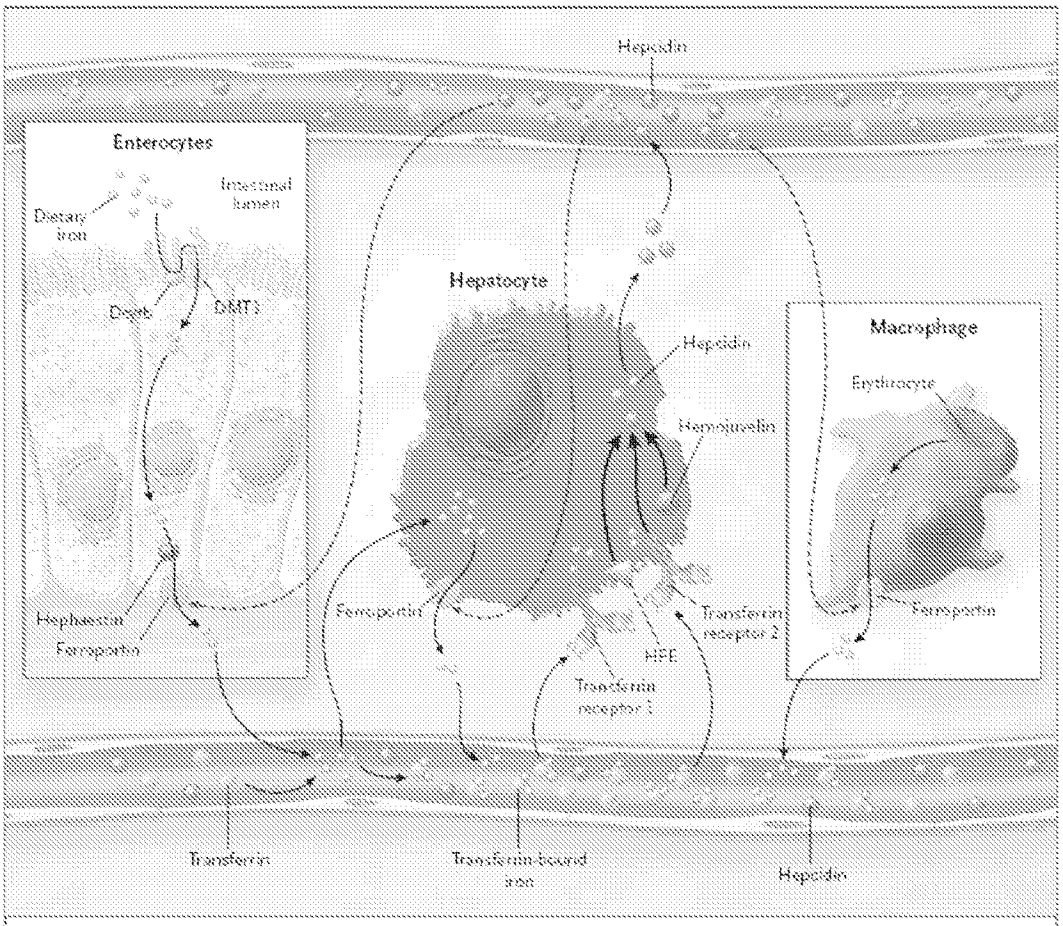


Figure 2.2. A schematic diagram of iron uptake in intestinal epithelial cells. From Dunn et al. (2006).



Interplay of Key Proteins in Iron Homeostasis.
In the duodenal enterocyte, dietary iron is reduced to the ferrous state by duodenal ferric reductase (Dcytb), transported into the cell by divalent metal transporter 1 (DMT1), and released by way of ferroportin into the circulation. Hephaestin facilitates enterocyte iron release. Hepatocytes take up iron from the circulation either as free iron or transferrin-bound iron (through transferrin receptor 1 and transferrin receptor 2). Transferrin receptor 2 may serve as a sensor of circulating transferrin-bound iron, thereby influencing expression of the iron regulatory hormone hepcidin. The hepcidin response is also modulated by HFE and hemojuvelin. Hepcidin is secreted into the circulation, where it down-regulates the ferroportin-mediated release of iron from enterocytes, macrophages, and hepatocytes (dashed red lines).

Figure 2.3 Interplay of Key Proteins in Iron Homeostasis, From Fleming et al (2005).

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

AK Proulx, MB Reddy, J. Agric. Food Chem. **2006,** 54, 1518-1522

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Abstract

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

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

Introduction

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

Leghemoglobin (LHb), a symbiotic hemoglobin, is a monomeric heme protein originally identified in soybean root nodules and has been studied extensively (*5*, *6*). Because of its high affinity for oxygen, LHb makes less oxygen available, enhancing the nitrogen fixation process. The nitrogenase enzyme produced by symbiotic bacteria within legume roots requires an anaerobic environment, and therefore, the plant produces heme protein that is capable of scavenging oxygen within the cytosol of the root, resulting in a

low-oxygen environment ideal for nitrogen fixation (*7*). Leghemoglobin accumulates iron in roots creating a large iron store. Iron levels of up to 2.5 mg total iron/g dry weight basis have been measured in soy nodules, with up to 26% of the total iron in heme form in the unpurified root (*8*). Most researchers are interested in the physiological role and structure of hemoglobin in the plant, but to our knowledge no studies have been reported on the use of plant hemoglobins for improving iron bioavailability of human diet.

Caco-2 cells are human intestinal adenocarcinoma cells exhibititing enterocyte-like biochemical and morphological characteristics and have been used widely for nonheme iron bioavailability studies (*9*, *10*). Heme bioavailability in Caco-2 cells is not well studied, but recent work has shown that these cells synthesize enzymes involved in heme uptake and metabolism, in particular hemeoxygenase (*11*), and that the mechanisms of heme transport are similar between humans and Caco-2 cell models (*12*). This evidence makes the Caco-2 cell model appealing for its potential in evaluating heme iron bioavailability.

The objective of this study was to determine the iron bioavailability of crude soy root nodule extract (SRN) and two purified soy leghemoglobins and to compare their bioavailability with that of bovine hemoglobin (BHb) using the Caco-2 model. The underlying objective of this study is to introduce the concept of using plant hemoglobin as a heme iron source in diets that are consumed by humans and to promote further research into this area.

Materials and Methods

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

using 10-20% polyacrylamide Tris-Tricine precast gels (Biorad, Hercules, CA) in a Ready Gel Cell (Biorad), and molecular weight was estimated by comparison with standards (Biorad). Gels were fixed using 5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA), followed by staining in 10% (v/v) aqueous acetic acid with 0.025% Coomassie Brilliant Blue G250 (Biorad) and destaining with 10% (v/v) aqueous acetic acid. Protein purity was estimated by gel optical density using QuantOne software (BioRad).

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

In Vitro Digestion. For experiments conducted without food matrix, SRN, LHbD, BHb, and $FeSO_4$ were weighed to provide 1 mg iron in 10 mL final volume (100 ug/mL). Initially the iron sources were dissolved in 1 mL of a 140 mmol/L NaCl, 5 mmol/L KCl solution prior to digestion. Fortified tortilla samples were weighed to provide 200 g total iron (~3.5 g to represent 50 ppm iron) and suspended in 5 mL of DI water. Pepsin was prepared by solubilizing 0.2 g of porcine pepsin A (1:60000) in 5 mL

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

Cell Culture. All reagents for cell culture work were from Sigma Aldrich or Gibco BRL (Grand Island, NY) unless otherwise mentioned. Caco-2 cells were purchased at passage 17 from American Type Culture Collection (Rockville, MD). The following experiments were conducted during passages 20-26. Cells were grown in a culture flask with Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% v/v nonessential amino acids, and 1% v/v antibiotic-antimycotic solution. Cells were maintained at 37 °C in an incubator with 5% CO_2. Media was

changed 3 times weekly. At 7 d, the cells were rinsed with Earle's Balanced Salt Solution (EBSS), trypsinised to dissociate the cells, centrifuged at 22.6 *g* for 5 min. The cells were seeded at a density of 5.6 x 10^3 cells/cm^2 in a 75 cm^2 culture flask for continued growth, or seeded on collagenized (Type 1 Rat tail collagen) 12-well cell culture plates (Corning Costar) at a density of 5 x 10^4 cells/cm^2 for iron bioavailability experiments. The cell culture plates were maintained under incubator conditions similar to those of the cell culture flask. Iron bioavailability experiments were conducted 15 d post seeding after rinsing with EBSS.

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

TX) and measured using Cobra-II Gamma Counter with SpectraWorks software (Packard BioSciences, Meriden, CT). After normalizing ferritin concentration to cell protein concentration, the values were normalized, defined as percentages compared to $FeSO_4$, and expressed as relative biological values (RBV).

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

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

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

Results and Discussion

As per the SDS-PAGE gel (**Figure 3.1**), the LHb extract had the largest band at 14 kDa, which is the reported molecular weight of LHb (*6*). LHb purity increased from 21% in the SRN extract to 54% in the LHbA and to 73% in the LHbD extracts,

respectively, as determined by optical densitometry comparing the density of the 14 kDa bands to the total lane density. No 14 kDa band was found after the pepsin digestion by SDS-PAGE for either LHbA or BHb, suggesting globin from these proteins is completely degraded (data not shown).

The results of the iron bioavailability study using aqueous solutions of SRN, LHbD, and BHb are shown in **Figure 3.2**. The relative biological values (RBV, compared to 100% with $FeSO_4$) were 28 ± 10%, 19 ± 17%, and 113 ± 13% higher than $FeSO_4$, respectively, (mean ± SEM) for SRN, LHbD, and BHb. The iron bioavailability of BHb was 2-fold higher than all other samples ($p < 0.001$), but the iron bioavailability of SRN and LHbD was similar compared to that of $FeSO_4$. Since the bioavailability of SRN and LHbD was similar, LHbA, we found no advantage using the pure fraction. Hence, the partially purified LHbA fraction was used for tortilla fortification studies.

Unlike the previous results without food matrix, the RBV for 50 ppm fortified tortillas with SRN was 19% lower than that of $FeSO_4$, but was not significantly different **(Figure 3.3)**. The LHbA and BHb tortillas exhibited 27 ± 6% and 33 ± 10% higher bioavailability than $FeSO_4$ ($P < 0.05$) and with no difference between them. Although based on dry weight, total iron content varied for SRN, LHbA, and BHb (1.42, 1.7, and 2.9 mg/g, respectively), and weight adjustment provided equal amounts of total iron in all the treatments prior to in vitro digestion. The heme iron content also varied based on dry weight basis, 1.0, 1.4, and 2.3 mg/g for SRN, LHbA, and BHb, respectively, but the 74-83% of added iron was in heme form in all the treatments.

Heat displayed no significant impact on bioavailability other than on $FeSO_4$ bioavailability **(Figure 3.4)**. The bioavailability of $FeSO_4$ was 36 ± 6% lower in samples

fortified before cooking ($p < 0.001$), indicating that iron bioavailability was decreased by heating the fortificant during cooking. Although not significant, SRN, LHb, and BHb showed increases in bioavailability with heating. Nonheme iron bioavailability was not affected by adding heme iron from the LHbA at any concentration raging from 25 to 75% (data not shown).

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

Currently heme iron in the human diet is almost exclusively from animal sources, and its intake has been shown to have a positive correlation with iron status (*19*, *20*). Indeed, one major recommendation for improving iron status in populations is to incorporate sources of heme iron in the diet because of its high iron bioavailability (*21*). However, the incorporation of animal-sourced heme iron is often unfeasible because of economic costs or because cultural and religious barriers forbid the consumption of meat in populations where iron deficiency is prevalent.

While it has long been known that plants produce heme proteins, they have not been extensively studied in human iron nutrition. A recent rat hemoglobin repletion study by one of the authors showed a bioavailability of 59% with SRN compared to $FeSO_4$ which was similar to a 60% bioavailability with BHb (*8*). Unlike human studies, the lower bioavailability of BHb compared to $FeSO_4$ raises some concern of this model for

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

There are a number of reasons why a discrepancy exists in the bioavailability results in **Figures 3.2** and **3.3**. The first possible explanation is the difference in the iron concentrations in the uptake solutions, which was 10-fold higher in aqueous solution experiments (**Figure 3.2**), and the presence of solid milieu of food affecting uptake. Since we reported the values relative to $FeSO_4$, it is also important to consider how $FeSO_4$ bioavailability is affected by the food matrix when comparing the results from different experiments. Differences in $FeSO_4$ bioavailability greatly influence the RBV of the treatments. Hence, the low RBV of BHb in **Figure 3.3** should not be viewed as a reduction of bioavailability in the presence of food. Iron chemistry and solubility is highly dependent on the digestive milieu. Lower LHbD bioavailability compared to that

of BHb in aqueous solution (**Figure 3.2**) might also be attributed to the differences in globin fractions of those two proteins. Although structural homology between these two proteins is high (*5*), the amino acid sequence of bovine α or β globin compared to soy globin shows low sequence homology when compared with BLOSUM-62 (NCBI Protein-Protein BLAST). Globin protein has been shown to increase the bioavailability of heme iron (*12*), and the presence of hydrophobic peptides hydrolyzed from the globin protein during digestion is known to affect the absorption of heme iron (*23*). It is possible that globin degradation may be different between LHb and BHb and the solubility of iron may be better maintained in BHb with its globin degradation products. It is also possible that there may be a lipid enhancing effect in the BHb which is not present in the LHb. Since the BHb was derived from bovine reticulocytes, it may have trace amounts of lipids which in the aqueous environment may influence iron bioavailability (*24*). Our results suggest that LHb may not be anymore beneficial than $FeSO_4$ if it is used as a supplement. However, our goal was to assess bioavailability with food, which is more applicable for determining the use of LHb for food fortification.

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

similar to that of BHb (**Figure 3.3**), suggesting the usefulness of this fraction in food fortification.

The RBV of BHb decreased, from 113% to 27% of $FeSO_4$, when heme iron was fortified into tortillas compared to without food matrix. Besides the lower concentration of fortificant in this experiment, the lower bioavailability of BHb with the food matrix might be due to the influence of calcium in tortillas due to nixtamalization treatment with calcium hydroxide. Calcium has been shown to decrease the bioavailability of heme iron bioavailability as well as nonheme iron (*25*, *26*). Another explanation might be that $FeSO_4$ availability is low in aqueous solution due to its low solubility at neutral pH without any chelating agents. Since the results are expressed as RBV to $FeSO_4$, the decrease in bioavailability of BHb should be viewed as higher bioavailability of $FeSO_4$ in the presence of tortillas compared to without food matrix. Unlike the results in **Figure 3.2**, BHb and LHb bioavailability is similar in **Figure 3.3**, suggesting that there might be differences in iron solubility or the effect of calcium.

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

The effect of heat on the bioavailability of heme iron sources is minimal, unlike $FeSO_4$ which decreases in bioavailability during heating. Although not significant, the

heat treatment tends to improve the bioavailability of heme iron, which may be due to denaturation of the globin proteins and enhancement of hydrophilic interactions. However, the decrease in $FeSO_4$ bioavailability during heating might be due to interaction of iron with maillard browning products (*29*) or from oxidation of ferrous iron to the less bioavailable ferric form (*30*). It appears that the heme pyrolle provides a protective effect against the heat effect.

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

Conclusion

Our results showing the bioavailability of hemoglobin from soy root nodules similar to that of heme iron from animal sources when added with tortillas provide a unique alternative fortificant. However, no advantage of using LHb as a supplement was found because its bioavailability is similar to commonly used $FeSO_4$. Because of the known minimal inhibition of heme iron, unlike nonheme iron, by dietary factors and because of the high heat stability of this iron form, plant hemoglobins may have great potential to be used as a fortificant for improving nutrition. Soy root nodules have no current use in agriculture production and as such may provide a novel value-added product for soy producers. Given optimal nodulation conditions, 2-3 plants would

provide sufficient LHb to meet 25% of the recommended daily requirement of iron, assuming 25% of heme iron absorption. The use of plant hemoglobins in iron nutrition warrants further study of sensory and safety issues related to fortification to make this a feasible reality.

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Figures

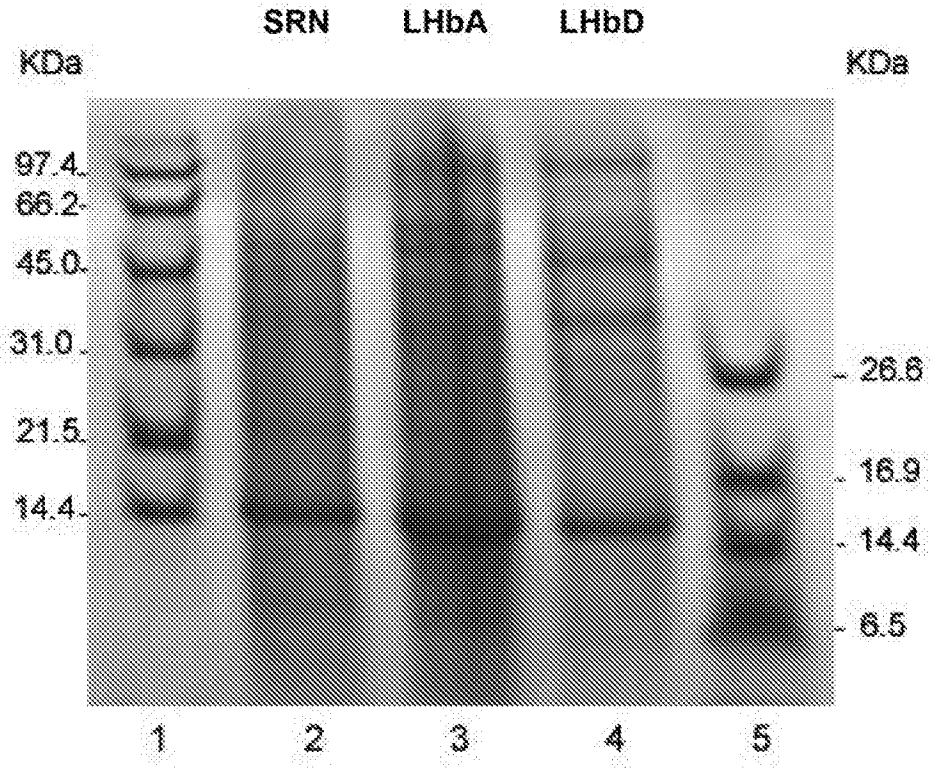

Figure 3.1. SDS-PAGE purity analysis of LHb protein fractions (Each lane contained 0.25 mg of protein.) Lane 1: standard 1 (14.4 - 97.4 kDa). Lane 2: soy root nodule (SRN) extract. Lane 3: 80% $(NH_4)_2SO_4$ precipitated fraction of the SRN extract (LHbA). Lane 4: DEAE purified fraction following $(NH_4)_2SO_4$ precipitation (LHbD). Lane 5: standard 2 (6.5 - 26.6 kDa).

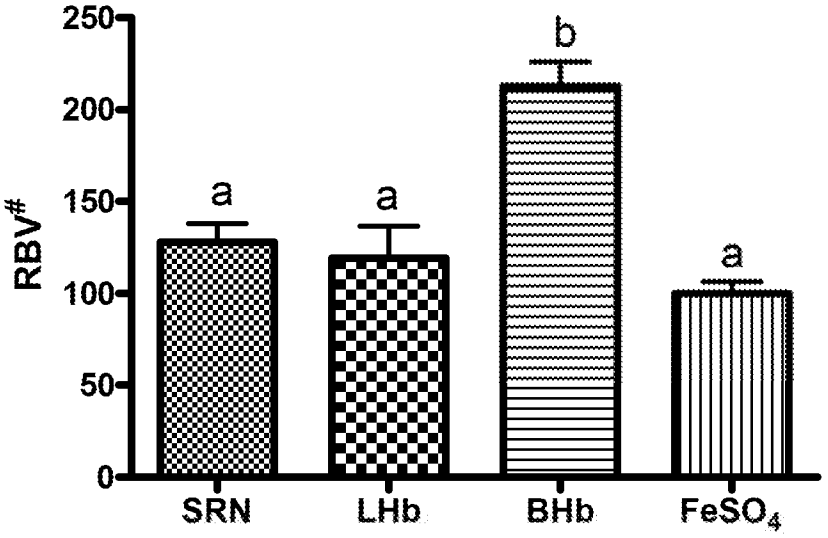

Figure 3.2. Iron bioavailability of SRN (soy root nodule), LHbD (DEAE purified fraction of soy leghemoglobin), BHb (bovine hemoglobin), and $FeSO_4$ in aqueous solutions. # Relative biological value (RBV) = ng ferritin/ μg cellular protein relative to the mean $FeSO_4$ value. Bars (mean ± SE, n=5) with similar letters are not significantly different ($p > 0.05$) based on ANOVA with Tukey's multiple comparison test.

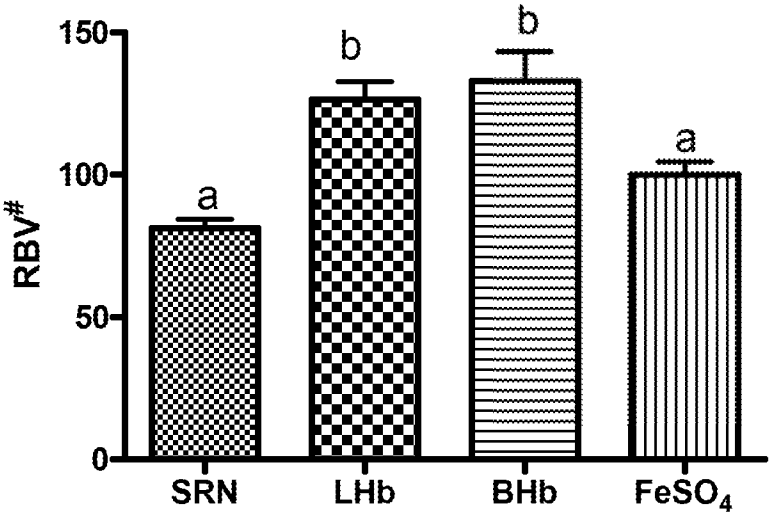

Figure 3.3. Iron bioavailability of tortillas fortified with SRN (soy root nodule), LHbA ($(NH_4)_2SO_4$ purified soy leghemoglobin), BHb (bovine hemoglobin), and $FeSO_4$ with 50 ppm iron. #Relative biological value (RBV) = ng ferritin/µg cellular protein relative to the mean $FeSO_4$ value. Bars (mean ± SE, n) 9-10) with similar letters are not significantly different ($p > 0.05$) based on ANOVA with Tukey's multiple comparison test.

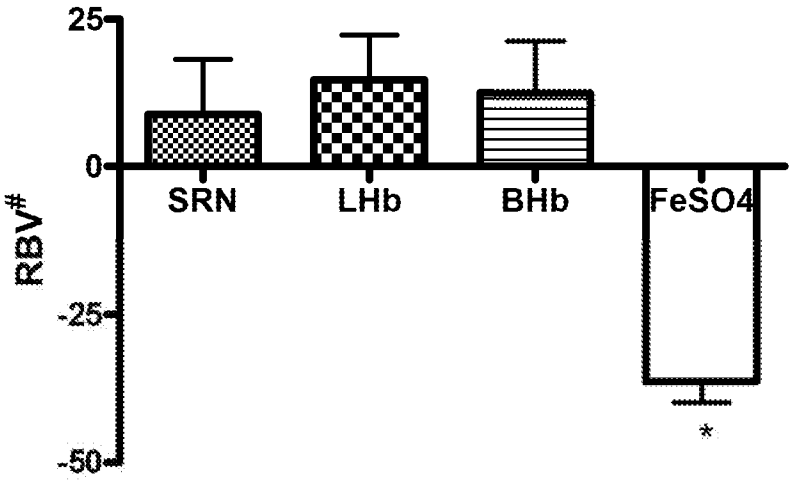

Figure 3.4. Effect of heat treatment on iron bioavailability of fortified tortillas (heated minus not heated). Bioavailability was measured from iron sources added before (heated) and after making tortillas (not heated). #Relative biological value (RBV) = ng ferritin / μg cellular protein relative to the respective $FeSO_4$ value run simultaneously with each treatment. Student t test was used to compare heated and not heated treatments (mean ± SE, n = 9-10) for each iron source. *$p < 0.05$. SRN = soy root nodule extract, LHbA = $(NH_4)_2SO_4$ purified soy leghemoglobin, BHb = bovine hemoglobin.

Fermentation and Lactic Acid Addition Enhance Iron Bioavailability of Maize

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Abstract

Maize is one of the most important cereal crops for human consumption, yet it is of concern due to its low iron bioavailability. The objective of this study was to determine the effects of processing on iron bioavailability in common maize products and elucidate better processing techniques for enhancing iron bioavailability. Maize products were processed to represent different processing techniques: heating (porridge), fermentation (ogi), nixtamalization (tortillas), and decortication (arepas). Iron and phytate contents were evaluated. Iron bioavailability was assessed using the Caco-2 cell model. Phytate content of maize products was significantly reduced by decortication (25.6%, $p = 0.003$) and nixtamalization (15%, $p = 0.03$), and iron content was reduced by decortication (29.1%, $p = 0.002$). The relative bioavailability (RBA, compared to 100% bioavailability of porridge with $FeSO_4$) of ogi was significantly higher than that of other products when fortified with $FeSO_4$ ($p < 0.001$) or reduced iron ($p < 0.001$). Addition of lactic acid (6 mg/g of maize) significantly increased iron solubility and increased bioavailability by about 2-fold ($p < 0.01$), especially in tortillas. The consumer panel results showed that lactic acid addition does not significantly affect the organoleptic characteristics of

tortillas and arepas ($p = 0.166$ and 0.831, respectively). The results suggest that fermentation, or the addition of small amounts of lactic acid to unfermented maize products, may significantly improve iron bioavailability. Lactic acid addition may be more feasible than the addition of highly bioavailable but expensive fortificants. This approach may be a novel means to increase the iron bioavailability of maize products to reduce the incidence of iron deficiency anemia.

KEYWORDS: Fermentation; nixtamalization; Caco-2 cells; iron bioavailability

Introduction

Iron deficiency is the most prevalent nutritional deficiency worldwide, affecting over 2 billion people (*1*). Maize ranks as the largest cereal crop produced globally, and the third most important grain for human consumption, with 116 million tons consumed as food in 2003 (*2*). Due to the low iron bioavailability of maize, research is mainly focused on fortification strategies appropriate to maize products (*3*). Little attention, however, has been paid to food matrix effects in maize, except for phytate content. Because of the complexity and variation in maize processing, we can expect changes in the food matrix other than changes in phytate that will affect iron bioavailability.

Fortification is a common practice for increasing iron content of maize products. However, the highly bioavailable iron fortificant, ferrous sulfate ($FeSO_4$), is highly unstable and induces oxidative rancidity; on the other hand, elemental iron is very stable but has low bioavailability. Sodium iron EDTA has been proposed as the most effective fortificant, but its use is minimal due to its high cost (*4*). The fortification of maize with

iron has been minimally successful at combating iron deficiency because of the high level of phytate in maize, which significantly inhibits absorption of both intrinsic and added iron (*5*).

Traditional maize processing and its industrial adaptations are highly diverse and differentiated by cultural preferences. In general, traditional maize processing can be broadly categorized by four main processing techniques: heating, nixtamalization, decortication, and fermentation (*6*). Many of the contradictory results with maize iron fortification studies may be due to the use of products developed through different processing methods, thereby not accounting for strong food matrix effects. A great level of interest is being given to defining best practices for maize product fortification (*4*); however, only a few studies have focused on the specific effects of processing on iron content and solubility (*7*, *8*), and none to our knowledge have taken a systematic approach comparing the different processes described above across the same starting material, especially including a bioavailability assessment.

The hypothesis of this study is that different processing techniques will affect the iron bioavailability of maize products, and, in particular, fermentation or lactic acid addition may beneficially affect iron bioavailability. The objective of this study is to evaluate the effect of the primary traditional processing techniques for maize, heating, nixtamalization, fermentation, and decortication, on phytate and iron content, iron solubility, and iron bioavailability. It is anticipated that by understanding the effects of processing, appropriate techniques can be transferred from one traditional process to another to improve iron bioavailability.

Experimental Methods

Maize (cv. Northrup King 60-B6, 2004 harvest) was obtained from the Iowa Grain Quality Laboratory and cleaned by manual sorting to remove extraneous material and broken seeds. All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Mass balance technique, conducted in duplicate, was used to determine iron and phytate losses. Deionized water was used throughout the study.

Porridge Preparation (Heat). Maize (200 g) was ground in a coffee grinder to pass a 30-mesh screen (maize meal). The maize meal was mixed with 300 mL of H_2O and heated in a glass pot with stirring until the maize became stiff (porridge).

Arepa Preparation (Decortication). Maize (200 g) was tempered to raise the moisture content to 50%. Bran and germ were separated by using a blunt blade blender and mixed with water, and the floating fraction was collected (bran + germ). The endosperm fraction was run repeatedly through a KitchenAid grinder until smooth (arepa starch). Water was added to make the starch material malleable. Cakes of 50 g (1 cm thick) were formed and dry-fried in a Teflon-coated pan at 200 °C for 5 min per side (arepa).

Tortilla Preparation (Nixtamalization). Calcium hydroxide (400 mL of a 1% solution) was mixed with 200 g of maize in a glass pot. The maize mixture was cooked at 80 °C for 30 min and then left to steep at room temperature for 12 h. The steep liquid was drained, and the maize was rinsed three times with H_2O. The steep and rinse waters were retained for analysis (nejayote). The maize was run through the KitchenAid grinder repeatedly until smooth (masa). Water was added to make the masa malleable. Balls (25

g) were formed, pressed flat in a tortilla press, and dry-fried in a Teflon-coated pan at 200°C for 2 min per side (tortilla).

Ogi Preparation (Fermentation). Maize (200 g) was steeped in 300 mL of H_2O at 25 °C for 24 h. The maize and steep water were blended in a Waring blender until smooth and then fermented spontaneously over 24 h at 25 °C (ogi, uncooked). The fermented maize was then boiled in a glass pot until stiff (ogi, cooked).

All maize samples were lyophilized, ground in a coffee grinder, passed through a 30-mesh sieve, and stored frozen at -20 °C for further use.

Iron Content. Total iron content in maize products and fractions was measured by a wet ashing method, followed by a colorimetric assay (*9*, *10*). Briefly, 1 g of sample was wet-ashed with nitric acid in a microwave at 250 W until the resulting liquid was clear. The sample was diluted to 10 mL, and the iron concentration was measured colorimetrically using ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt] as a chromogen in a microplate assay.

Phytate Content. Phytate was measured using anion exchange chromatography (*11*). Samples (1 g) were subjected to extraction with 2.4% HCl, centrifugation, and filtration. Supernatant was mixed with 0.75 mol/L NaOH and a 0.1 mol/L Na_2EDTA solution and run on an AG1-X4, 100-200 mesh, chloride form ion-exchange resin (Bio-Rad, Hercules, CA) in a 1.5 x 30 cm column. Phytate was eluted with a 0.7 mol/L NaCl solution, heat digested with H_2SO_4 and HNO_3, and then reconstituted with H_2O. Phosphorus content was measured using ammonium molybdate and 4-amino-3-hydroxy-1-naphthalenedisulfonic acid in 0.15 mol/L Na_2SO_3 and 0.92 mol/L $NaHSO_3$, and inositol hexaphosphate equivalents were calculated to estimate phytate concentration.

In Vitro Digestion. After preparation of the products with maize, freeze-dried products were fortified with 50 ppm of iron by adding $FeSO_4$ or reduced iron (RedFe, American Ingredients, Kansas City, MO) prior to digestion. Samples were prepared for bioavailability assessment as described earlier (*12*). Fortified samples were weighed to deliver 200 ug of total iron (~3.5 g), mixed with water, and adjusted to pH 2.0 using 5 M HCl. Pepsin solution [porcine pepsin A (1:60000) in 0.1 mol/L HCl] was added and incubated at 37 °C with shaking for 1 h. Following the pH adjustment to 6.0, pancreatin and bile solution were added [porcine pancreatin (4xUSP) and bile extract in sodium bicarbonate solution], and incubation was continued for 15 min. Samples were heat-treated (4 min at 100 °C) to inactivate proteolytic enzyme activity (*13*) and centrifuged, and the supernatant was used for soluble iron and bioavailability experiments.

Lactic Acid Addition. To determine the effect of lactic acid addition on iron solubility and bioavailability, 6 mg/g lactic acid was added to arepa, tortilla, and porridge prior to in vitro digestion. This acidification level was chosen on the basis of reported values of maize products fermented over 24 h (*14*).

Cell Culture for Iron Bioavailability. All reagents for cell culture work were from Sigma Aldrich or Gibco BRL (Grand Island, NY) unless otherwise mentioned. The following experiments were conducted in Caco-2 cells at passages 31-36 using the previously described method (*12*). Cells were grown in a culture flask with Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 1% v/v nonessential amino acids, and 1% v/v antibiotic-antimycotic solution. Cells were maintained at 37 °C in an incubator with 5% CO_2. Trypsinized cells were seeded to collagenized (type 1 rat tail collagen) 12-well cell culture plates (Corning Costar) at a density of 5×10^4 cells/cm^2

for iron bioavailability experiments. The plates were maintained at 37 °C and 5% CO_2. Iron bioavailability experiments were conducted 15 days postseeding after a rinse with Earle's Balanced Salt Solution. Serum free medium was prepared with DMEM with 1% v/v nonessential amino acids, 1% v/v antibiotic-antimycotic solution, 10 mmol/L piperazine-*N,N*□-bis[2-ethanesulfonic acid] (PIPES), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 *u*g/L), triiodothyronine (34 *u*g/L), and epidermal growth factor (20 ug/L), as modified from Glahn et al. (*15*). Serum-free medium (0.5 mL) and 0.5 mL of the supernatant of each digest were added to the cell culture well and incubated for 2 h. A subsequent 0.5 mL of serum-free medium was added after the 2 h incubation, followed by further incubation for 22 h. After 24 h total of incubation, the samples were removed by aspiration from the cell culture wells, and the cells were rinsed with 1 mL of Earle's Balanced Salt Solution. The cells were lysed in H_2O by sonication. Total lysate protein was determined according to the Bradford Coomassie assay (Pierce Laboratories, Rockford, IL). Ferritin in the lysates was determined by using a radioimmunoassay kit (Fer-Iron II, Ramco Laboratories, Stafford, TX) and a Cobra-II gamma counter with SpectraWorks software (Packard BioSciences, Meriden, CT). After ferritin concentration is normalized to cell protein concentration, the values are expressed as relative bioavailability (RBA) as compared to porridge fortified with $FeSO_4$.

Iron Solubility. Supernatant from the in vitro digest was mixed 1:1 v/v with 20% w/v trichloroacetic acid in 6 mol/L HCl, incubated for 20 h at 65 °C, and then subjected to centrifugation at 5000*g* for 10 min (*8*). Iron in the supernatant was measured using the colorimetric method described under Iron Content.

Sensory Analysis. Because lactic acid addition may change the organoleptic characteristics of food, we conducted a sensory analysis on lactic acid added products. Differences between untreated and lactic acid treated samples were assessed by using the triangle test (*16*). The protocol was approved for exemption by the human subjects review committee at Iowa State University. The products were prepared using commercial mixes that best emulated the processed products prepared in the above sections, either with or without food grade lactic acid (6 mg of powdered lactic acid; Purac, Lincolnshire, IL). All products were prepared as per the manufacturers' directions. Arepas (Harina PAN, Refinadora de Maiz Venezolana, C.A., Aragua, Venezuela) were shaped into cakes, 10 cm in diameter and 1 cm thick, and fried in a dry pan until crisp and browned both sides. Masa for tortillas (MASECA, Azteca Milling, City of Commerce, CA) was prepared, pressed in a tortilla press, and fried on both sides in a dry pan until the tortilla puffed with steam. Porridge (Bob's Red Mill Cornmeal, Coarse Ground, Milwaukie, OR) was prepared by boiling cornmeal with H_2O. Untrained panelists (44 total, 4 male and 40 female, ages 18-63) tested each of the three food products in a random sequence, receiving three samples (two identical, one different treatment). Ballots were scored for panelists' ability to identify the product that was different.

Statistical Analysis. Differences in iron and phytate content between processing steps and between with and without lactic acid treatment in the same food product were determined using Student's *t* test. Differences in RBA and iron solubility among the processed products (grouped by fortification) were determined using ANOVA with Tukey's multiple-comparison test. To assess the interactive effect of processing and

fortification, two-way ANOVA was used. Binomial probability distribution analysis was used for sensory analysis triangle test scores. All mean differences were deemed to be significant at $p < 0.05$. Analyses were performed using GraphPad software (GraphPad Prism version 4.02 for Windows, GraphPad Software, San Diego, CA), except for two-way ANOVA, which was performed with SAS (SAS 9.0 for Windows, Cary, NC).

Results and Discussion

Changes in Iron Content during Processing. Iron content was found to not significantly change during processing except in arepa, where iron decreased 30.3% during decortication ($p = 0.003$), with a net decrease of 29.1% in the final product ($p = 0.002$) (**Figure 4.1**). It was not surprising to see the decrease in iron content with the physical removal of bran and germ, as iron content is high in the germ and, to a lesser extent, in the bran layer. Defatted germ is reported to contain 36.7 mg/100 g (*17*) and 2.79 mg/100 g for crude maize bran compared to 2.38 mg/100 g for whole maize (*18*). Because of a lack of waste streams, changes in iron content for porridge and ogi preparation were not significant. Because of the small weight of the waste stream in tortilla processing (neyajote), there was a negligible impact on iron content. Iron losses in steep water are low during nixtamilization because iron solubility is low due to the high pH associated with alkali treatment. However, in most traditional tortilla processes, the bran and germ layer are removed post-steeping, which should correspond to iron losses similar to decortication.

Changes in Phytate Content during Processing. The total phytate content of maize products was significantly affected by fermentation in ogi preparation (-25.3%, $p =$

0.002) and decortication in arepa preparation (-34.5%, $p = 0.01$) (**Figure 4.1**); however, only arepa and tortilla showed a significant net decrease of phytate compared to the starting material (-25.6%, $p = 0.003$, and -16.6%, $p = 0.02$, respectively). Decortication would physically decrease the phytate content due to bran and germ removal, whereas fermentation would induce phytase activity. Fermentation, soaking, and germination are known to induce both endogenous phytases in seeds and exogenous phytases from microbial sources (*19*). Because endogenous phytase activity is low in maize [9 mIU/g as compared to 440 mIU/g for triticale (*20*)], phytate reduction in ogi is likely due to the microflora growth during fermentation.

More successful reductions in maize phytate have been seen from the addition of exogenous phytase from other sources, including the use of bacterial phytase (99.2%) (*21*), wheat phytase (88%), and germinated sorghum (72%) (*22*). Phytate was shown to be reduced 42% in fermented maize gruel and 60 and 98% in fermented maize gruel with 10 or 50 mg of wheat phytase, respectively (*8*). A 60% reduction in maize phytate after 96 h of germination at 32 °C was shown (*23*), but this level of reduction may have a minimal impact in improving iron bioavailability (*21, 24*). Whereas germination has been implicated in reducing phytate content in maize porridges (*25*), the changes in starch functionality may limit its practical application in other maize products (arepa and tortilla).

Heating primarily reduced the phytate content of tortillas (11.7%, $p = 0.07$) but did not affect other maize products. The differences might be due to the higher heat achieved in dry heating in the tortilla preparation. The reduction of phytic acid content

varies in the nixtamalization process on the basis of the conditions, such as alkali concentration, steeping time, and temperature (*7*).

It has been suggested that phytate has to be reduced by >90% to see a 2-fold improvement in iron bioavailability (*21*). The phytate to iron molar ratio in all of the unfortified and fortified products is high, ranging from 17 to 30 for unfortified products and from 4.9 to 6.8 in fortified products (**Table 4.1**). It has been shown that the phytate to iron molar ratio should be <1, and preferably 0.4, to observe a significant increase in bioavailability (*26*). None of our products achieved this low ratio.

Iron Solubility and Bioavailability. To compare the iron bioavailability of all maize products, results were normalized to porridge with $FeSO_4$ values and referred to as RBA. The bioavailability of unfortified maize products was low as compared to fortified maize products, ranging from 37 ± 13 to 46 ± 16% RBA (**Figure 4.2**). As iron content could not logically be matched between fortified and unfortified products, these data should be interpreted with caution; however, they serve to show the net improvement in RBA with fortification.

As anticipated, on average, the bioavailability of fortified products is about 2-fold higher than that of their unfortified counterparts except ogi with $FeSO_4$, which was 6.6-fold higher (RBA of 37 ± 13 versus 245 ± 16%). When $FeSO_4$ fortified products were compared, the RBA of ogi was 2.5-fold higher than those of the other products (RBA of 96 ± 6, 92 ± 11, and 100 ± 4% for tortillas, arepas, and porridge, respectively) ($p < 0.001$). When products were fortified with RedFe, the RBA values of ogi (142 ± 6%) and porridge (111 ± 6%) were higher than those of tortilla (79 ± 6%) and arepa (84 ± 6% ($p < 0.001$ and $p < 0.01$ for ogi and porridge, respectively).

The increase in iron bioavailability in ogi cannot be solely attributed to phytate reduction, as phytate levels were similar across all products, but can be attributed to increased iron solubility due to the acidic food matrix developed during lactic acid fermentation. Increased iron solubility was reported in various other lactic acid fermented products (*8*, *27-31*), with the assumption that high-soluble-iron foods have higher bioavailability.

When the bioavailability data were analyzed with two-way ANOVA, both fortificant ($p < 0.0001$) and product ($p = 0.0158$) had a significant effect on iron bioavailability. A significant interaction between fortificant and product was also observed ($p = 0.008$). The effect of fortificant was not surprising, as it is well-known that $FeSO_4$ has high bioavailability as compared to RedFe (*32*, *33*); however, the strong effect of product indicates a processing and food matrix effect on bioavailability. Our results suggest that choosing optimal fortification and processing techniques is important to achieve significant improvement in iron bioavailability. The iron bioavailability of ogi is higher than that of other maize products, suggesting that manipulation of the food matrix is an important strategy to enhance iron bioavailability in other products. Given the lactic acid production during the fermentation in ogi, it is feasible to add lactic acid to other products without further changing the processing conditions.

Overall, addition of lactic acid to unfermented products increased bioavailability on average 1.7-fold (**Figure 4.3**). In the unfortified products, tortilla had an increase of 2.1-fold ($p < 0.01$) and porridge and increase of 1.8-fold ($p < 0.0001$) with lactic acid addition. In $FeSO_4$-fortified products, RBA increased 2.0-fold in tortillas ($p < 0.001$), whereas in products with RedFe, the RBA increased 2.1-fold in tortillas ($p < 0.0001$) and

1.7- fold in arepas ($p < 0.0001$). Regardless of fortification, the RBA of tortilla was improved with lactic acid addition. Given the low iron solubility of tortillas as compared to other products (**Table 4.2**), much of this improvement in bioavailability is likely attributed to the increase of iron solubility accompanying the acidification; however, it cannot be solely attributed to iron solubility. The low solubility of iron in all of the tortilla products is likely due to the high pH attributed to the alkali treatment during processing. As expected, the pH of the tortillas (pH 7.8) was higher than those of arepa and porridge (both pH 6.1) and ogi (pH 4.0). The addition of lactic acid to arepa and porridge reduced the pH to 3.9-4.1 and 4.6 with tortillas (data not shown). As shown in **Table 4.2**, lactic acid addition significantly improved iron solubility in tortillas ($p < 0.001$), regardless of fortification, but not in the other products, suggesting the importance of an acidic environment on iron solubility and bioavailability.

Lactic acid has been shown to enhance iron bioavailability through an enhancing effect on ferric iron transport across the intestinal epithelium (*34*), suggesting its effectiveness is greater with ferric than ferrous iron. Our results, showing greater effectiveness of lactic acid in arepa fortified with RedFe as compared to $FeSO_4$, support the previous study. The effect of lactic acid on improving bioavailability in maize food products is most likely from two different effects: pH reduction preventing the formation of insoluble oxides, most evidently observed in high-pH foods (tortillas) and, to a lesser extent, enhancement of absorption of ferric iron because the enhancement was less with moderate-pH foods (arepa and porridge). Given the very high phytate level in the porridge, acidification may have been insufficient to overcome the phytate inhibition in porridge.

Effect of Lactic Acid on Organoleptic Characteristics. Untrained panelists were capable of detecting a difference in organoleptic characteristics between lactic acid and typical porridges ($p < 0.0001$); however, they were not capable of detecting a difference in tortillas ($p = 0.166$) and arepas ($p = 0.831$). These results are summarized in **Table 4.3**.

Consumer testing suggested that the levels of lactic acid we used to improve iron bioavailability may not affect product quality except for porridge. The study was, however, limited, in that the consumer population is not a typical maize foods consuming population. It is possible that a maize-consuming population may be more sensitive to changes in formulation. On the other hand, the foods in this study were presented without any of the traditional seasonings and accompaniments that may act to mask off-flavors.

At the household level, the addition of lactic acid culture or acid steeps may be a useful technique in certain product formulations. In addition, given the availability of powdered lactic acid, flours could be supplied with added lactic acid. This may be a cost-effective means to increase the bioavailability of low-bioavailable fortificants such as RedFe rather than including high-cost, high-bioavailable fortificants. However, long-term storage studies would be necessary to evaluate the impact on nutrient stability and organoleptic acceptability of lactic acid enhanced maize products.

This study supports the premise that fortification alone is not sufficient to improve iron status in populations; rather, an effort to improve the bioavailability of traditional foods using appropriate technology is necessary for successful fortification strategies.

Conclusion

The effects of processing on iron bioavailability in maize are significant, especially with fermentation increasing bioavailability. The enhancing effect of fermentation may be attributed mostly to acidification from natural lactic acid production rather than phytate reduction. Lactic acid addition greatly improved iron bioavailability of low-bioavailable maize products, especially tortillas, without affecting organoleptic quality. The role of lactic acid and fermentation may have a beneficial impact on current iron fortification schemes, increasing iron solubility and bioavailability. Hence, the addition of lactic acid may be a novel means to increase the iron bioavailability of maize products.

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Figures

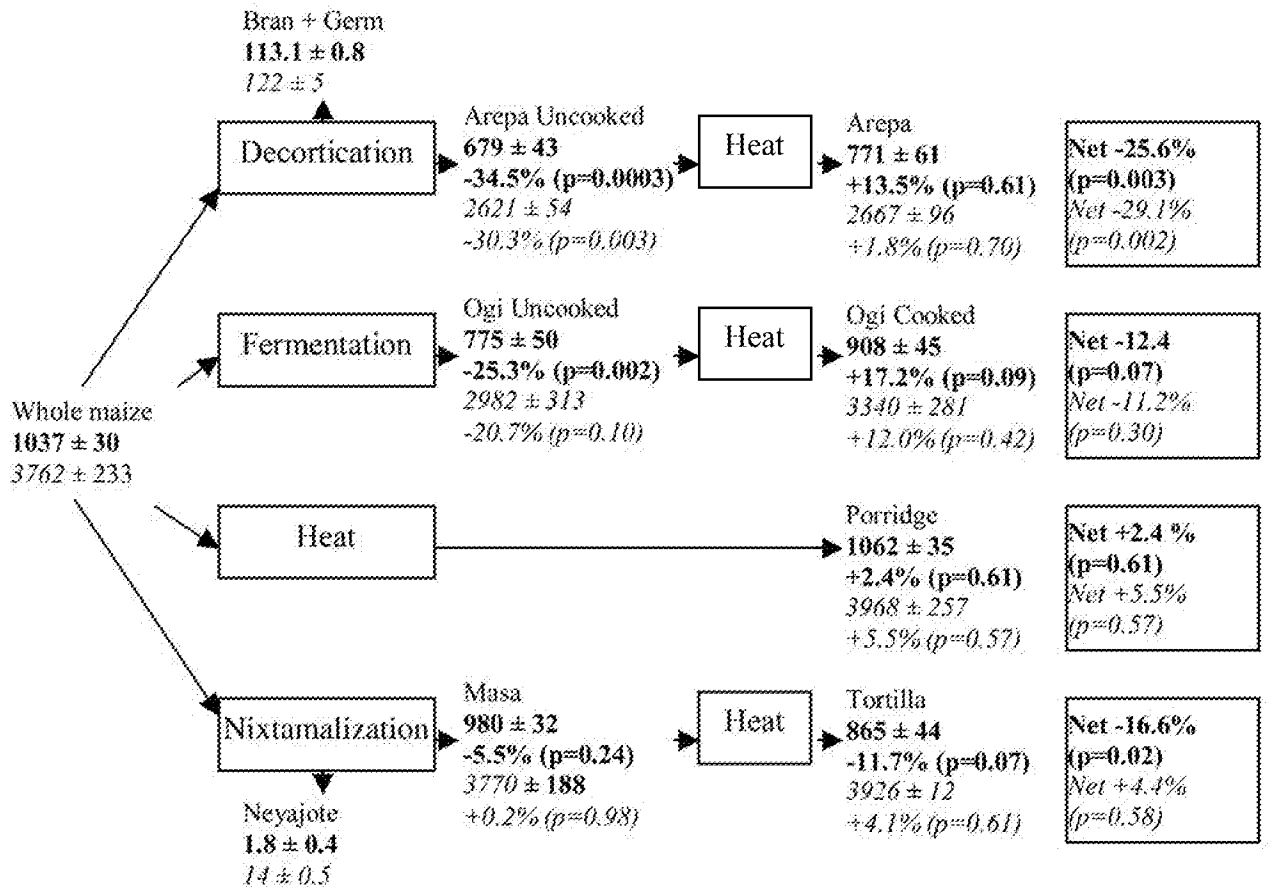

Figure 4.1. Phytate and iron content of maize during processing. Phytate in bold expressed as milligrams of phytate per 200 g, dwb, starting sample weight (mean ±SE); iron in italics, expressed as micrograms of Fe per 200 g, dwb, starting sample weight (mean ±SE). Differences were determined between samples using Student's t test, n = 4-8.

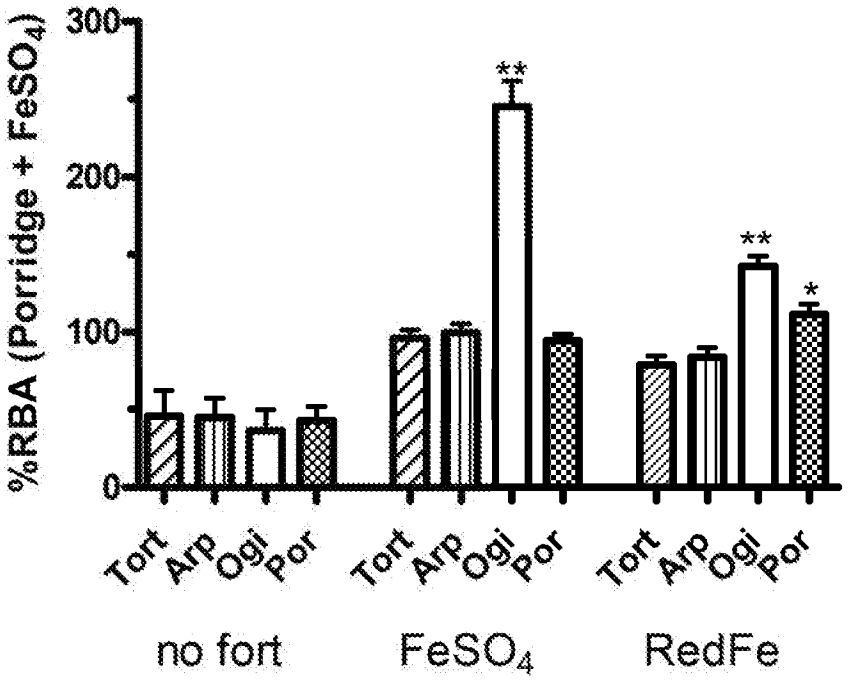

Figure 4.2. Relative bioavailability [RBA (ferritin per milligram of cell protein) normalized to porridge with $FeSO_4$] of maize products without fortification (no fort) or fortified with ferrous sulfate ($FeSO_4$) or reduced iron (RedFe). Tort, tortilla; Arp, arepa; Por, porridge. Columns with stars are significantly different within similarly fortified products as determined by ANOVA with Tukey's multiple-comparison test: *, $p < 0.05$; **, $p < 0.001$; $n = 9\text{-}14$.

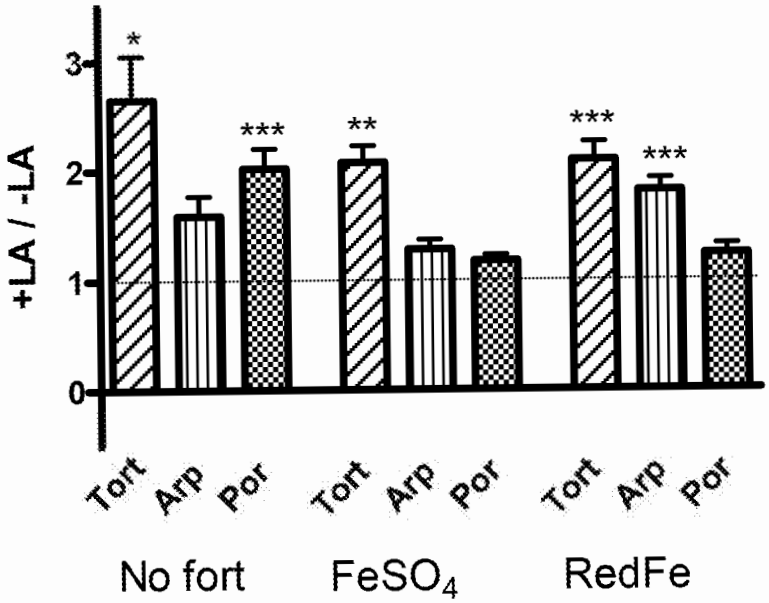

Figure 4.3. Relative bioavailability ratios [RBA (ferritin per milligram of cell protein) normalized to porridge with $FeSO_4$] of products with or without added lactic acid (+LA/-LA). Dotted line represents no effect of LA. Tort, tortilla; Arp, arepa; Por, porridge; RedFe, reduced iron. Student's t test was used to compare RBA of +LA and –LA products: *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$; n = 5-14.

Tables

Table 4.1. Phytate to Iron Molar Ratios for maize products

	Tortilla	**Arepa**	**Ogi**	**Porridge**
No fortification	17.1 ± 1.3	20.7 ± 3.0	21.9 ± 2.6	30.0 ± 6.9
With fortification	4.9 ± 0.3	5.1 ± 0.6	5.6 ± 0.4	6.8 ± 0.7

Mean ± SD, n =4-8.

Table 4.2. Percent soluble iron in processed maize products following in vitro digestion

	Tortilla	**Arepa**	**Ogi**	**Porridge**
No fortification	-0.7 ± 0.9^{a}	6.7 ± 2.6^{ab}	14.9 ± 2.4^{b}	7.1 ± 4.3^{b}
+ lactic acid	$18.4 \pm 0.5^{**a}$	$26.8 \pm 2.0^{**b}$		$24.7 \pm 0.5^{*b}$
+ $FeSO_4$	-0.6 ± 0.2^{a}	30.6 ± 0.6^{c}	21.2 ± 1.5^{b}	28.4 ± 1.5^{c}
+ $FeSO_4$ + lactic acid	$3.9 \pm 0.1^{**a}$	27.4 ± 2.2^{b}		25.6 ± 1.3^{b}
+ Red Fe	-0.6 ± 0.2^{a}	14.6 ± 0.6^{b}	13.8 ± 2.0^{b}	14.3 ± 0.4^{b}
+ Red Fe + lactic acid	$6.5 \pm 0.9^{**a}$	13.6 ± 1.5^{b}		12.7 ± 0.6^{b}

%soluble iron is expressed relative to total iron in the sample. Mean ± SD, n = 4-8. Difference among processing treatments was determined by ANOVA with Tukey's Multiple comparison. Means in the same row with the same superscript are not significantly different. Lactic acid effect for each fortificant and treatment was determined individually by Student's t test, * $p < 0.01$, ** $p < 0.001$.

Table 4.3. Number of respondents able to correctly differentiate lactic acid added products from typical products using triangle testing

		p value*
Arepa	14 / 44	0.831
Tortilla	19 / 44	0.166
Porridge	32 / 43	<0.0001

*Binomial probability distribution

Overexpression of *Zea mays* Hemoglobin in maize endosperm tissue can increase iron bioavailability

Intended to be published in Journal of Nutrition upon evaluation of the F2 maize.

Abstract

Biofortification has been suggested as a novel means to improve iron content or bioavailability in staple crops. Maize is a staple crop in many parts of the world but the bioavailability is low due to its high phytate content. Our hypothesis is that through over-expression of the *Zea mays* hemoglobin (ZmHb) gene in maize endosperm we can deliver iron in a highly bioavailable form, and circumvent the inhibitory effect of phytate. A gene construct was developed by transcriptionally fusing ZmHb to the translational fusion product of *Zea mays* 27 zein promoter with a green fluorescent protein (GFP). This construct was stably transformed into maize, and F1 seeds were evaluated for the presence of ZmHb and GFP by immunoassay, for the presence of heme pyrrole incorporation by chemilluminescence, and analysed for iron bioavailability using the Caco-2 cell culture model. Expression of ZmHb-GFP fusion product was confirmed by using separate ZmHb and GFP antibodies. Two lines, P65 and P26 that are positive for ZmHb and GFP, expresssed 4.2 and 4.9 ug ZmHb / g seed, respectively. Line P26 ($p < 0.001$) had 125% and line P65 had 68% ($p < 0.01$) higher iron bioavailability than HiII, the untransformed line. These preliminary studies suggest that ZmHb overexpression in endosperm may be a successful strategy to significantly increase bioavailable iron in

maize. Future studies with animal models using later generations of seeds are necessary to confirm these results.

Introduction

Iron deficiency has been long identified as the most prevalent nutrient deficiency worldwide (Stolzfus, 2003). Despite a variety of efforts and international mandates for reductions of iron deficiency, minimal progress has been made because of the diverse challenges. Alleviating iron deficiency can improve health, wellbeing (Zimmerman and Hurrell, 2007) and economic status (Horton, 2006) but challenges do exist in implementing iron supplementation and fortification programs in some of the most isolated and resource poor regions of the world where iron deficiency is most prevalent. Maize is a common staple crop in many developing countries where iron deficiency is very high, and is problematic nutritionally because of its high phytate content causing low iron bioavailability. Fortification of maize has been only marginally successful, and processing-based strategies have had limited dissemination in low resource settings. Novel strategies are necessary to make a large impact on reduction of iron deficiency in populations consuming maize, and agronomic-based technologies may provide such a novel approach.

Biofortification, the improvement of nutritional quality of staple crops by traditional and biotechnology based crop breeding, is considered a novel, cost efficient, and self perpetuating means of improving the nutritional quality of staple crops (Nestel et al, 2006). Transgenic modification for improving iron bioavailability has been previously developed in several staple crops, including rice, and maize. Transgenic rice

expressing soybean ferritin, when evaluated in a rat hemoglobin repletion model, had bioavailability similar to rice fortified with $FeSO_4$ (Murray-Kolb et al, 2002). Recently, rice expressing transgenic human lactoferrin bioavailability was also shown to be equivalent to $FeSO_4$ fortified rice in young women (Lonnerdal and Bryant, 2006).

Specifically referring to maize, several strategies have been explored for biofortification. This has included screening of maize germplasm for high iron bioavailable lines (Oikeh et al, 2003) and identifying mutants with reduced phytate levels, designated as *lpa* for low phytic acid (Raboy, 2001). These low phytate transformants have shown improve iron bioavailability from porridge and tortillas (Mendoza et al, 2001; Mendoza et al, 1998). Maize which expressed both transgenic soybean ferritin and *Aspergillus niger* phytase also had significant increases of bioavailable iron (Drakakaki et al, 2005).

There is a complex series of challenges to face with the previous strategies for improving maize iron bioavailability. While low phytate maize has shown promising results with iron absorption, low yield of this maize (Raboy et al., 2000) and other negative effects on the seed (Pilu et al., 2005) limits its commercial production. Expression of human proteins in staple foods, as in the case of lactoferrin expressing rice, poses ethical challenges in incorporating foreign (in this case, human) genes. This approach however may be useful for the development seed lines for pharmaceutical applications rather than open sourced seed for food use. Merely increasing non-heme iron content, either as total iron, or as ferritin iron may not be effective, as the phytate content has to be significantly reduced by at least 90% to see a meaningful increase in iron absorption (Hurrell et al, 2003).

Hemoglobin iron absorption from animal sources is not affected by the dietary factors, icluding phytate ((Lynch et al, 1985). Most plants also have genes coding for hemoglobin proteins (Kundu et al, 2003) and the hemoglobin genes in maize have been characterized (Arechaga-Ocampo et al, 2001). Since we have shown in our earlier study that plant hemoglobin is as bioavailable as bovine hemoglobin (Proulx and Reddy, 2006), the role of plant hemoglobin for nutrition looks promising. Overexpression of hemoglobin in the endosperm of maize through genetic modification may be ultimately possible to develop grain with highly available iron. Our hypothesis is that through overexpression of the *Zea mays* hemoglobin gene in maize endosperm we can improve iron bioavailability by delivering iron in a highly bioavailable form, and circumventing the inhibitory effect of phytate. Our ultimate goal would be to use only maize DNA sequences to produce this enhancement through intragenic recombination.

Materials and Methods

Construct Development

All constructs were based on the original plasmid pAct1IsGFP-1 (Cho et al, 2000) which has the modified green fluorescent protein (GFP) gene sGFPs65T (Genbank accession #ABB59985) and nos terminator sequence (modified from Genbank accession V00087). This plasmid was modified to include the promoter sequence from the 27 kDa gamma zein (27zn) (Genbank accession #EF061093) which was shown to produce high expression of GFP within maize endosperm tissue (Shepherd et al, 2007). The cDNA for *Zea mays* hemoglobin (ZmHb) was obtained from the Iowa State University Expressed Sequence Tag Library (Genbank accession # BM333948).

For the construct used for maize transformation (**Figure 5.1**), a PCR product was amplified using Pfu polymerase (Stratagene, La Jolla CA) from the ZmHb EST (forward primer CGCCCTTCCATGGCACTCGCGGAGGCC; reverse CCATGGCATCGGGCTTCATCTCCC), and subcloned to pCR 2.1 Topo Vector (Invitrogen Corporation, Carlsbad CA) for amplification in *E. coli* XL1 Blue (Stratagene, La Jolla CA). The ZmHb was restriction digested from pCR 2.1 vector by NcoI, and was inserted into the 27zn GFP vector at the NcoI restriction site such that ZmHb and GFP formed a gene fusion product (27zn ZmHb GFP nos). The sequence of the construct was verified by DNA sequencing at the Iowa State University DNA facility prior to transformation.

Stable Transformation

Stable transformation of gene constructs into maize plants was accomplished at the Plant Transformation Facility at Iowa State University, by gold particle bombardment of HiII Type II callus (Frame et al, 2000). Callus was bombarded with the plant hemoglobin expression vector (**Figure 5.1**), and cobombarded with a plasmid containing the *bar* gene for bialaphos resistance (Gordon-Kamm et al, 1990) T0 callus was raised on bialaphos selective media, and resistant callus was screened for the presence of the gene of interest (27zn ZmHb GFP nos) by PCR using GoTaq (Promega, Madison, WI) (27zn ZmHb GFP nos – forward primer #1 CCGATCGACACCATGGCACTCGCGGAG, reverse #1 CTTGCTCACCATGGCATCGGGCTTCATC; forward primer #2 GATGAAGCCCGATGCCATGGTGAGCAAG, reverse #2 CTGCAGCCGGGCGGCCGCTTTACTTG). Transformed callus that was positive for

insertion of the construct was regenerated to plants in the Plant Transformation Facility Greenhouse, and crossed to the inbred B73 to create F1 kernels.

ZmHb Antibody Production

The coding sequence for ZmHb (Genbank accession #AY005818) was also inserted into a pET28 plasmid for expression in *E. coli* BL21 Star DE3 (Invitrogen Corporation, Carlsbad CA) so that the insertion resulted in a transcriptional fusion with a c-terminal 6x His tag. The resulting protein was purified using metal affinity chromatography as previously described (Smagghe et al, 2006). Purified transgenic ZmHb was utilized for polyclonal antibody production in rabbits (ProSci Incorporated, Poway, CA) for immunoassay based analyses described below.

Screening Transgenic Maize Seed for GFP Expression:

Every F1 seed, grouped by transformation event, from the 27zn ZmHb GFP nos transformation was screened for the presence of GFP. The first screening was a visual evaluation of GFP expression. This was accomplished by placing intact kernels endosperm up and germ down into a black 48 well cell culture plate, and measuring fluorescence with spectrofluorometer (Tecan, Mannedorf-Zurich, Switzerland) at 485 nm excitation and 535 nm emission wavelengths. GFP positive seeds were selected by a combination of spectrofluorometer readings 4 times higher than the untransformed negative control, combined with a secondary manual screen conducted by visual inspection through an orange filter using a hand-held Dark Reader lamp (Clare Chemical Research Inc. Dolores, CO).

Seeds that were positive for GFP were secondarily screened by immunoassay for the presence of both GFP and ZmHb. Ground maize (50 mg) was suspended in 500 uL

of Lammeli buffer, boiled, centrifuged, and 15 uL supernatant loaded on 15% SDS-PAGE gels. Separated proteins were then transferred to a nitrocellulose membrane, blocked overnight with 5% milk in Tris Buffered Saline with Tween (TBST). Membranes were probed for 3h either with anti-GFP monoclonal antibody (Living Colors, Clontech, Mountain View, CA), or with anti-ZmHb polyclonal antibody (described above) with 1:25,000 dilution in 2% milk in TBST. Membranes were then probed for 1h with either goat anti-rabbit or goat anti-mouse antibody conjugated with horseradish peroxidase, both diluted 1:50,000 in 2% milk in TBST. Immunoreactive bands were visualized by chemilluminescence and abundance of GFP and ZmHb proteins was estimated by densitometry (QuantOne, Biorad, Hercules CA) against purified recombinant ZmHb standard or GFP standard (Clontech, Mountain View, CA, USA).

Measurement of the incorporation of the iron pyrrole into the transgenic ZmHb was accomplished using the pseudoperoxidase capacity of iron associated with the heme pyrrole, coupled with luminol based chemilluminescence, modified from Bonfils et al, (1995). Briefly, 200 mg of ground maize, positive for ZmHb based on immunoassay, was extracted for 30 min with shaking in 1 mL 100 mM Tris, pH 6.8, and centrifuged. The supernatant was concentrated by ultrafiltration through a 10,000 MWCO filtration device (Millipore Ultrafree MC), then the entire filter retentate (50 uL ± 5 uL) was mixed with 200 uL of luminol buffer (1mL 0.1 mol/L luminol, 1 mL 0.02 mol/L 4-iodophenol, and 20 uL 30% hydrogen peroxide in 50 mL phosphate buffered saline, pH 7.4). Chemilluminescence was measured (Synergy 4, BioTek, Winooski VT) and heme iron concentrations were estimated by comparing with hemoglobin standards using log-log curve fitting.

Iron Content

Total iron content in maize was measured using the modified AOAC method 944.02. Briefly, 0.1 g of sample was mixed with 2.0 mL nitric acid and ashed at 250 W in a microwave digester (Milestone Sci, Shelton, CT) until the resulting liquid was clear. The digested sample was diluted to 5 mL with deionized H_2O. Iron content was measured as described previously (Proulx and Reddy, 2006) using ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid Sodium salt) and measuring absorbance at 562 nm in a microplate reader.

In Vitro Digestion

Maize samples were prepared for bioavailability assessment using the method described by Glahn et al 1998 and Proulx and Reddy, 2006. Maize samples (0.5 g) were mixed with 0.7 mL deionized water, 1.0 mL of 0.1 mol/L HCl, and adjusted to pH 2.0 with 1.mol/L HCl. Pepsin solution (0.2 mL of 0.2 g porcine pepsin A (1:60000) in 10 mL 0.1 mol/L HCl) was added, and the sample was incubated at 37° C with shaking (500 rpm) to mimic gastric digestion. The pH was then adjusted to 6 with 1 mol/L sodium bicarbonate, and 0.715 mL of pancreatin and bile solution was added (0.05 g porcine pancreatin (4xUSP) and 0.3 g bile extract in 25 mL of 0.1 mol/L sodium bicarbonate solution). To mimic duodenal digestion, the samples were again incubated at 37° C with shaking for 15 min. The digests were heat treated for 4 min at 100° C to inactivate proteolytic activity as suggested previously (Jovani et al, 2001) and centrifuged at 5000 x g for 10 min. The resulting supernatant was used for measuring bioavailable iron.

Iron Bioavailability in Caco-2 Cells

All reagents for cell culture work were from Sigma Aldrich (St Louis MO) or Gibco BRL (Grand Island NY) unless otherwise mentioned.

Caco-2 cells were obtained at passage 17 from American Type Culture Collection (Rockville, MD). The following experiments were conducted with cell passages 23-26 as described by Proulx and Reddy (2007). Cells were grown in a culture flask with Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal bovine serum (FBS), 1 % v/v nonessential amino acids and 1% v/v antibiotic-antimycotic solution. Cells were maintained at 37° C in an incubator with 5 % CO2. Media was changed 3 times weekly. At 7 d, the cells were rinsed with Earle's Balanced Salt Solution (EBSS), trypsinised to dissociate the cells, and centrifuged at 22.6 x g. The cells were seeded at a density of 5.6 x 10^3 cells/cm^2 in a 75cm^2 culture flask for continued growth, or seeded on collagenized (Type 1 Rat tail collagen) 48 well cell culture plates (Corning Costar) at a density of 5 x 10^4 cells/cm^2 for iron bioavailability experiments. The cell culture plates were maintained at 37° C and 5 % CO_2. Iron bioavailability experiments were conducted 15 d post seeding after rinsing the cell monolayer with EBSS. Serum free media, prepared with DMEM with 1 % v/v nonessential amino acids, 1 % v/v antibiotic-antimycotic solution, 10 mmol/L PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L) and epidermal growth factor (20 μg/L) (Glahn et al, 1998), was used during cell iron uptake. Serum free media (0.125 mL) and 0.125 mL of the supernatant of each digest were added to the cell culture well, and incubated for 2 h. A subsequent 0.125 mL serum free media was added after the 2 h incubation, followed by a further incubation for 22 h. After 24 h total incubation, the samples were removed by aspiration from the cell culture wells, and cells

rinsed with 0.25 mL EBSS. The cells were then lysed by addition of 0.125 mL deionised water to each well, and sonicated with a probe-type sonic dismembranator at lowest setting (< 1 W output) for 15 s. Total cellular protein was determined in the lysates by the Bradford Coomassie Assay, (Pierce Laboratories, Rockford Illinois). Ferritin in the lysates was determined by radioimmunoassay (Fer-Iron II, Ramco Laboratories, Stafford TX), and measured using a Cobra-II gamma counter with SpectraWorks software (Packard BioSciences, Meriden CT). Ferritin concentration was normalized to cell protein concentration as well as corrected for differences in iron content and expressed as relative biological availability (RBA) of the control line, HiII.

Statistical Analysis

All maize sample analyses were performed in 2 replicates, except for bioavailability measurements in which were analysed in 3 replicates. The samples were analysed in 3 repeated measures, except for bioavailability. Five to six repeated measurements were carried out on each replicate for assessing cell iron bioavailability. Differences among the treatments were determined using one way ANOVA with Tukey's post hoc testing (GraphPad Prism 4.0, San Diego, CA).

Results

Construct Building and Plant Transformation

The construct developed for transformation incorporated the 27 kDa gamma zein promoter previously cloned from the maize inbred Va26 (Shepherd et al, 2007). The use of this promoter was based on its previous evidence showing good protein expression and tissue specificity within maize endosperm. The 27 kDa gamma zein promoter was

transcriptionally fused to the ZmHb coding sequence, which was translationally fused to GFP by elimination of the stop codon within the ZmHb gene. The predicted fusion protein product would have a molecular weight of 40 kDa, 15kDa contributed from ZmHb and 25kDa contributed by GFP as shown in **Figure 5.2**, and this molecular weight was confirmed by SDS-PAGE combined with an immunoassay (**Figure 5.3**). In the transformed maize we were not able to detect the ZmHb fusion product because a native protein in high abundance is also 40kDa (**Figure 5.2**). Because of the ubiquitous nature of heme compounds within cells, it was assumed that the ferrochelatase activity required for heme pyrrole insertion into the expressed ZmHb would be accomplished without need of further genetic modification, and this was evaluated in the chemilluminescent assay results described below. The Nos terminator was chosen because of its common use in biotechnology based applications.

Eighty bialophos resistant callus transformation events were screened for the presence of transgene DNA by PCR. The primer sets selected were designed to identify the artificial sequence arrangement between the ZmHb coding sequence and the 27 zein promoter, as both are originally derived from maize genetic code. The first primer set covered the region where the 27 zein promoter was fused to the ZmHb coding sequence, through the coding region where ZmHb and GFP were translationally fused. The second primer set covered the ZmHb and GFP fusion region through to the end of the GFP coding region, thereby identifying only synthetic regions of DNA. This approach eliminated any ambiguity that may have arisen from amplification of native ZmHb or 27 zein promoter code.

Of the 80 calli screened, 20 were positive for both regions of the construct. The transgene-positive calli were regenerated to plants which were used to pollinate the non-transgenic inbred line B73 to produce F1 seeds. Fifteen out of 20 transformation events were positive for GFP, implying that as a fusion product, ZmHb should also be expressed. Given the small number of seeds available in the F1 generation, two lines with containing sufficient number of seeds were selected for further analysis. These lines are referred to as P26 and P65.

Seed Screening

Whole ground seeds were analysed for the presence of GFP and ZmHb proteins by western blot immunoassays. Both transgenic lines accumulated a product of approximately 40 kDa, approximately the estimated molecular weight of the ZmHb-GFP fusion product. This product reacted both with antibodies specific for GFP and ZmHb (**Figure 5.3**). There was no cross-reactivity of the ZmHb antibody against untransformed lines, B73 and Hill suggesting low concentration of native Hb concentrations in maize. Densitometry of the immunoassay using the ZmHb antibody indicates that the concentration of the fusion protein was approximately 4.2 ug ZmHb per g maize in line P26, and 4.9 ug per g of maize in line P65. The GFP was expressed at approximately 25.7 ug and 36.7 ug per g maize in P26 and P65, respectively as per the immunoassay. This suggests a molar ratio of ZmHb to GFP in line P26 of 1:3.6, and in line P65 a ratio of 1:4.5.

To ensure that expressed ZmHb accumulated heme pyrrole, we made an attempt to quantitate heme pyrrole with chemilluminescence in two transgenic maize lines.

The transgenic line P26 had 6.8 μg Hb /g in the seed, which was higher than the HiII seeds ($p<0.001$), whereas line P65 was not distinguishable from untransformed HiII. This may indicate that despite the presence of the heme protein, incorporation of the heme pyrrole does not occur in all transgenic lines.

Iron Bioavailability

In the Caco-2 bioavailability assay, significant variation among the samples was observed. The bioavailabilty was assessed as ferritin response to the digests, normalized to total cell protein. Since the iron content was varied among the seeds (33.4 ug/g in HiII, 20.4 ug/g in P26 and 23.1 ug/g in P65), ferritin values were further corrected to iron content of the seeds (**Figure 5.4**). Mean bioavailability values for the two transgenic lines and the non-transgenic hybrid HiII were significantly different. P26 had 125% higher bioavailability than HiII (0.95 ± 0.08 vs 0.42 ± 0.02 (ng ferritin/ug protein)/(mg Fe/g maize) $p < 0.001$). The measure of bioavailability was based on the standard ferritin versus cellular protein content normalized to the iron content of the maize. The P65 line had 0.67 ± 0.05 (ng ferritin/ug protein/mg Fe, which was 68% higher than HiII ($p < 0.01$).

Discussion

Maize has a significant amount of phytate, which is a potent inhibitor of iron absorption. Even by doubling the quantity of iron in maize, there is ample phytate to radically inhibit most iron absorption. Thus it is important to develop other strategies that improve iron bioavailability rather than merely increasing iron content. The strategy of overexpressing ferritin has received attention recently because plant ferritins have

shown absorption as high as $FeS0_4$ in rat models (Murray-Kolb et al, 2002) and in human models (Lonnerdal et al, 2006). However it is now understood that ferritin is degraded during digestion and iron associated with the protein will enter the common non heme iron pool, thus making it susceptible to phytate inhibition (Bejjani et al, 2007).

Unlike ferritin, hemoglobin is highly bioavailable iron source with a unique uptake pathway and transport mechanisms (Shayeghi et al, 2005). The clathrate-type structure around the iron is protective against the inhibitory effects of phytate and other non-heme iron inhibitors (Lynch et al, 1985). Therefore, expressing hemoglobin in plants is a novel strategy for biofortification. However, ethical reasons of using animal proteins in plants and also foreign gene affecting plant growth. Results of our previous study showed soy and bovine hemoglobins have similar bioavailability (Proulx and Reddy, 2006), suggesting that plant hemoglobins could be useful candidates for biofortification and could confer nutritional advantages of heme iron to plant foods. But secondly, by using plant based genetics, and in particular strategies involving only intragenic recombination, novel nutritionally enhanced staples such as maize could be developed without introduction of transgenic material.

The functional role of hemoglobin in plants is not completely certain, however it has been postulated to be either for gas binding to facilitate metabolism, to interact with other organic molecules to facilitate transport or synthesis, or to scavenge oxygen within cells (Arredondo-Peter et al, 1998). In leguminous plants, such as soybeans, the predominant hemoglobins are classified as symbiotic because of their exclusive expression by the plant in the presence of nitrogen fixing bacteria, facilitating a lower oxygen concentration for increased functionality of nitrogenase enzyme (Wittenberg et

al, 1975). In non-leguminous plants, the physiological function of hemoglobins is not well understood, and may be part of a stress-response mechanism, as mRNA transcripts and expression of hemoglobin protein increase under hypoxia (Taylor et al, 1994). A few studies evaluate transgenic expression of maize hemoglobins and their role in physiology, again suggesting a role in nitric oxide metabolism (Dordas et al, 2004) and hypoxia (Sowa et al, 1998). No studies of maize hemoglobin have been conducted in nutritional studies.

The use of the GFP fusion in the construct presented was specifically to facilitate easy visual screening in the first attempts to overexpress hemoglobin in maize endosperm. GFP is easy to detect using visual screening. Given the assumption that expression of GFP would be coupled to the expression of the ZmHb by the fusion of the gene sequences, a screening methodology for ZmHb could then be developed for utilization in future plant transformations overexpressing ZmHb without the GFP. It was unexpected that the molar ration of GFP to ZmHb was not 1:1, however immunoblotting is not an ideal quantitative method, suggesting that the error may be methodological.

Attempts to find native ZmHb in untransformed seed and seedling tissues showed no cross reactivity against the antibody developed, suggesting that either the antibody is specific to only overexpressed ZmHb or the native hemoglobin is not in detectable concentrations. The ZmHb presumed expressed in plant tissues were neither detected in roots, stems or leaves of 14d seedlings when exposed to 48h of oxygen reduced environment, or 250 mM NaCl (data not shown). When attempting to identify the band for expression of the ZmHb-GFP fusion product, it similarly was not identified in the

SDS-PAGE gel (**Figure 5.2**), suggesting the protein expression level is very low in the transgenic crop.

In the construct developed, it is assumed that there is heme pyrrole incorporation into the protein based on previous transgenic expression studies showing that mechanisms for heme incorporation into the globin protein was spontaneous and not requiring overexpression of enzymes responsible for heme synthesis (Smagghe et al, 2006). Secondly it is assumed that cytoplasmic accumulation of the protein would be sufficient for heme pyrrole incorporation based on evidence that *Arabidopsis thaliana* hemoglobin expression is targeted to the cytosol (Hebelstrup et al, 2007).

The chemilluminescent assay developed for evaluation of heme iron incorporation is based on the Fenton reaction where iron reacts with hydrogen peroxide forming hydroxyl radical, which catalyses the chemilluminescent reaction. Although we assumed that the catalytic activity comes from iron incorporated into ZmHb, other metalloproteins such as ferritin or low molecular weight iron, such as that bound to phytate, or in free ionic form could also catalyse this reaction. However, by using the 10,000 MWCO ultrafiltration device, we would have eliminated low molecular weight iron binding components which would have been eluted in the concentration process. The assay could be refined to sieve out other metaloproteins larger than ZmHb by using a two-stage molecular weight cutoff ultrafiltration,.

With the expression of 4.2-68 µg ZmHb/g maize in P26, and 4.9 µg ZmHb/g maize in P65, we can expect 1.6-2.5 µg Fe from heme per 100g serving of maize, given a 1:1 molar ratio of iron atom to 15 kDa protein. While the amount appears to be a small, we suspect that GFP may be interfering with the heme iron incorporation into the protein.

It is promising that the transformed line with confirmed heme incorporation (P26) had higher bioavailability than the P65 line. It is possible that this difference is due to environmental effects or differences in genetic background. Although Hi11 was the same genotype that was transformed, and used for comparison basis in this study, this genotype does not breed true because it is a hybrid. Thus, comparisons to this genotype are somewhat risky. We chose only two transformed lines in this study to assess iron accumulation and bioavailability based on the largest amount seed available for the assay rather than highest levels of ZmHb or GFP or based on how many copies of the gene construct were inserted. More definite conclusions can be made once we conduct studies when more seeds are available in the F2 generation..

Subcellular localization of the ZmHb protein within the endosperm was not investigated in this study. The localization of recombinant proteins in endosperm has been shown important for maximal protein functionality, and for minimization of unknown effects on other components within the seed (Drakakaki et al, 2006). It is known that the presence of transgenic hemoglobin can have significant effects on plant growth and can alter concentrations of a wide variety of metabolites (Holmberg et al, 1997). Compartmentalization for greater protein stability, however, may compromise heme pyrrole incorporation. In the case of ZmHb overexpression, while it is assumed that cytosolic expression is adequate, appropriate targeting may permit higher expression levels, or greater heme pyrrole insertion. It is suggested that the enzymes in plants responsible for heme synthesis and the insertion of iron into heme pyrroles are associated with the mitochondria and chloroplasts (Van-Lis et al, 2005; Masuda et al, 2003). It is not currently clear where heme pyrrole insertion into apohemoglobin takes place.

As mentioned, the data presented is preliminary, with a larger data set comprising 8 transformation events will be developed upon regeneration of F2 maize lines. By increasing the number of lines evaluated, and by increasing the sample size, the quality of the analysis will improve. With a very small sample size, seed to seed variation can play a highly significant effect on results. And with more transformed lines to select from, the best breeding material can be selected for future studies and applications.

Much interest has been recently placed on stacking traits to capitalize on synergistic improvements in crop quality. It could be possible in the future to cross maize lines which have different iron bioavailability enhancements, such as overexpressed ZmHb or ferritin, and low phytate mutants. There may also be interest in enhancing iron uptake regardless of targeting to a specific metalloprotein. This could be accomplished through manipulation of iron metabolic pathways, such as incorporation of muagenic acid, ferric reductase, or yellow stripe 1 mutants into the breeding lines.

It is the long term goal of this research to develop maize lines overexpressing ZmHb using only maize DNA. Given the development of maize lines utilizing the 27zn promoter, ZmHb, and Zein termination sequences all derived from maize genomic sequences, it will open a novel aspect of genetic breeding, and challenge regulatory definitions of genetic modification. Recombination, rather than transgenic modification may have more consumer acceptance because of the use of only native genes. Secondly, the strategy of eliminating transgenic material is important to eliminate the impact of GFP on protein synthesis and heme incorporation from the fusion product. It is anticipated that the non-fused ZmHb may have a higher iron pyrrole incorporation and therefore higher bioavailability, as it would be a protein in the native state.

Conclusion

Overexpression of *Zea mays* hemoglobin in the endosperm of maize has the potential to significantly increase the bioavailability of iron. Further breeding efforts will aim to increase the quantity of bioavailable iron within the endosperm tissue, and aim to use only maize derived genetic materials, to broaden the potential nutritional impact of this crop.

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Figures

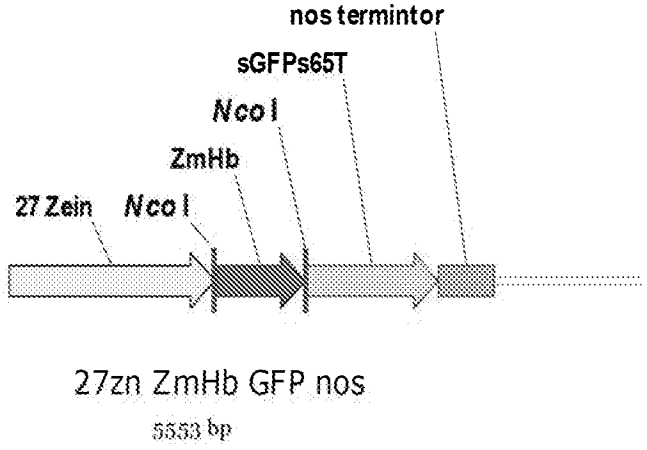


Figure 5.1. Schematic drawing of construct 27zn ZmHb GFP nos developed for overexpression of *Zea mays* hemoglobin in maize endosperm.

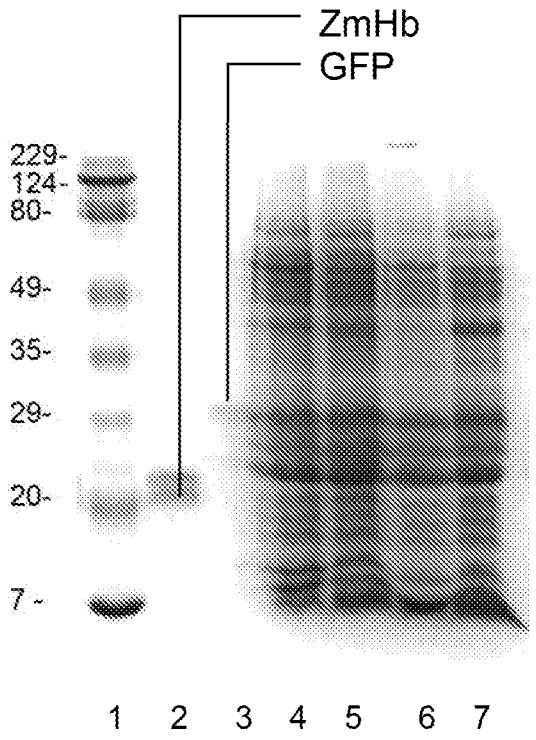


Figure 5.2. SDS PAGE gel stained in Coomassie, showing similarity of whole kernel protein profiles across all analysed maize lines. 1. Biorad Broadrange Prestained Ladder (all molecular weights in kDa); 2. ZmHb expressed in E. coli; 3. GFP; 4. P2P284-26, 5 P2P284-65, 6. untransformed HiII, 7. B73.

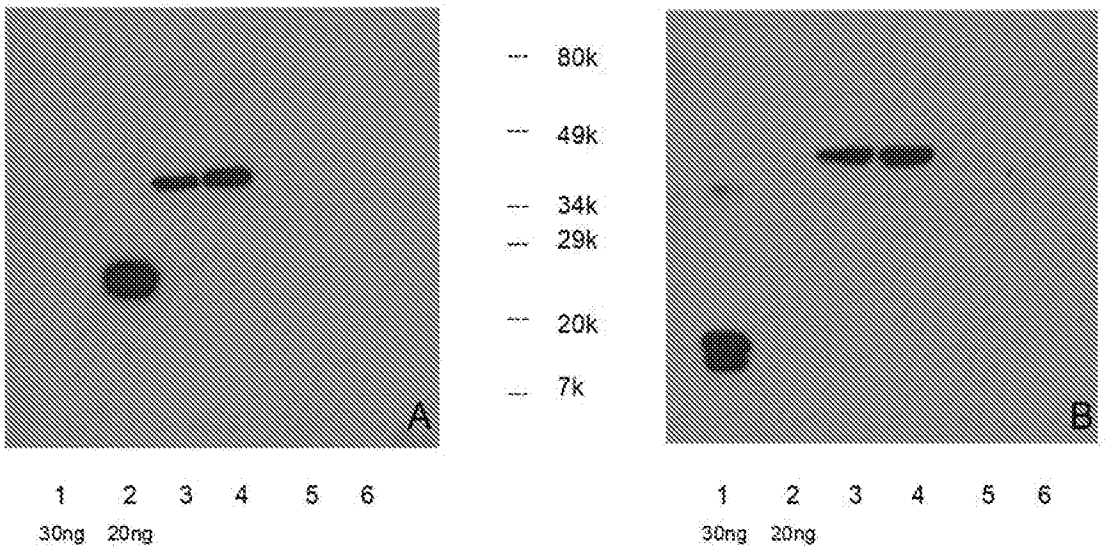


Figure 5.3. Western blot probing for GFP (A) and ZmHb (B) across all analysed maize lines. For all maize samples, 15uL was loaded from 50mg maize suspended in 500uL Lammeli buffer. Lane 1. Transgenic ZmHb from E. coli (30 ng); 2. GFP (20 ng); 3. P2P284-26 4, P2P284-65, 5;. untransformed HiII; 6. B73.

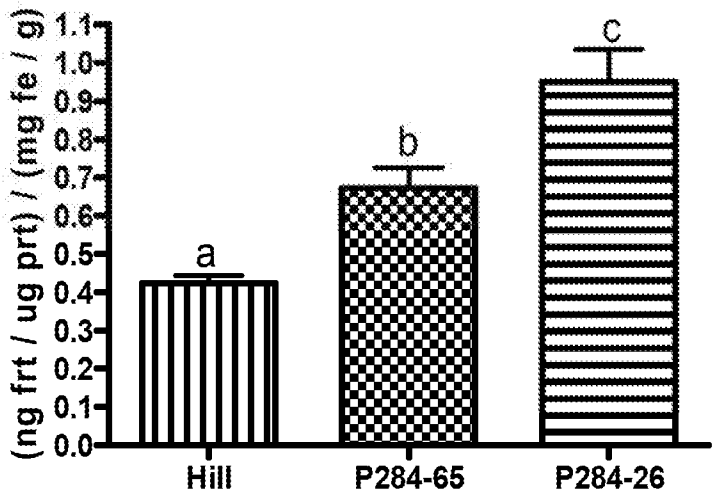


Figure 5.4. Iron bioavailability of genetically modified maize lines with *Zea mays* hemoglobin overexpression in endosperm. Bioavailability termed as (ng ferritin / ug protein) normalized over mg Fe per g maize. N=19 for P284-26, 23 for Hill, and 24 for P284-65. Columns with different letters are significantly different ($p<0.05$) as per one way ANOVA with Tukey's multiple comparison.

Appendix

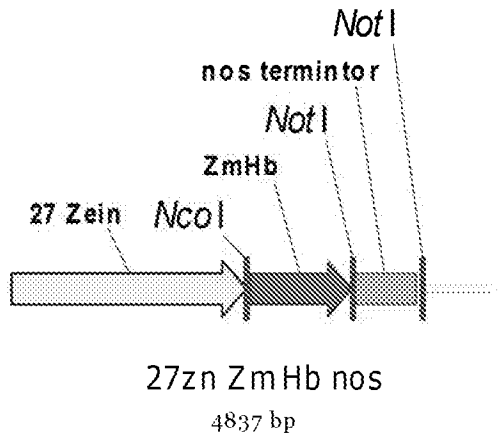
Materials and Methods not discussed in the Results section

In the second construct (**Figure 5.5-A**), a second PCR product was amplified using *Pfu* polymerase from the ZmHb EST with addition of a 5' NcoI (forward primer CGCCCTTCCATGGCACTCGCGGAGGCC; reverse CGCCCTTGCGGCCGCTACTAGGCATCGG), and subcloned to pCR 2.1 Topo Vector with amplification in *E. coli* XL1 Blue. The ZmHb was restriction digested from pCR 2.1 vector by NcoI and NotI. The 27zn GFP vector was subjected to digestion with NcoI and partial digestion with NotI to remove the GFP gene. ZmHb was ligated to the partially digested 27zn GFP vector to form the vector 27zn ZmHb nos. This construct has been stably transformed and screened for the presence of the gene of interest in undifferentiated callus.

The third construct (**Figure 5.5-B**) was developed using a putative zein terminator (Zntm) sequence determined from the 3' region after the stop codon sequence in the 27 gamma zein gene (Genbank Accession # X53514) and cloned by PCR using *Pfu* polymerase from genomic DNA from the *Zea mays* inbred line B73 (forward primer GCGGCCGCAAGAAACTATGTGCTGTAGTATAGCC; reverse GCGGCCGCAAGCTTAGCTTCAAGTCTTTAACT). The PCR product was subcloned to pCR 2.1 Topo vector, and amplified in *E. coli* XL1 Blue. The Zntm terminator sequence was digested from the pCR 2.1 vector with NotI. The 27zn ZmHb nos vector was digested with NotI, and the vector ligated with the Zntm product forming vector 27zn ZmHb Zntm. Prior to stable transformation into maize callus, the 27zn ZmHb Zntm

vector was linearized to remove non-maize genetic material, thereby having an intragenic transformation event. This construct is ready for transformation.

A



B

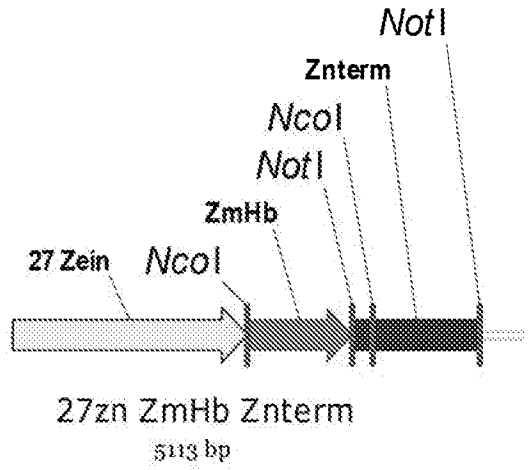


Figure 5.5. Schematic drawings of constructs developed for future work in overexpression of *Zea mays* hemoglobin in maize endosperm. A – 27zn ZmHb nos, B – 27zn ZmHb Znterm.

General Conclusions

Summary

As shown in the three studies of this dissertation, through novel methods of fortification, processing and biotechnology the bioavailability of maize can be increased significantly over the typical fortification or processing methods, or over the common inbred lines of maize. Improvements in iron bioavailability are possible, and could, in the case of the processing technologies, be applied immediately.

Recommendations and Future Research

The research studies presented have various levels of feasibility and practicality in the real world. The use of soybean leghemoglobin as a fortificant, while interesting, and potentially a high value added component for soy production, is highly inefficient at this point, and not cost effective in contrast to the standard fortificants. The soy leghemoglobin study is most useful as a proof of concept study, showing that plants indeed produce hemoglobin, and the hemoglobin is comparable to animal sourced hemoglobin in iron nutrition. As such, this proof of concept lends itself very well to the biotechnology based project, in that evidence was there showing that plant hemoglobin is bioavalable, prior to engaging in a long term plant transformation project.

The research presented in the fermentation and lactic acid enhancement paper is the most feasible research for immediate application. With a second human study, either a bioavailability or efficacy trial, evaluating the effect of lactic acid enhanced tortillas or arepas on iron status, this could have a strong effect on encouraging process

modification. Further research into maize product quality when enhanced with lactic acid, be it organoleptic, microbial, and process quality would be required. Acceptability within the consuming populations, both by processors (industrial and community scale) as well as by the consumers would also need to be evaluated.

With the development of hemoglobin expression in maize, a long term breeding program must be developed to select for highest expression of ZmHb. As alluded to in the appendix of Part 5, two further constructs have been developed for stable transformation, including one construct comprised of only maize coding sequences. The intention of this project in the long term is to develop intragenic transformations overexpressing maize hemoglobin. As such it would bring an entirely novel approach to biofortification in maize. Studies into evaluating the phenotype of the transformed maize would be of considerable interest, as would proteomic or transcriptomic studies to better characterize how heme synthesis or iron uptake pathways are being modified in the transformed maize. Once characterized, it could be better determined if a second transformation would be necessary to upregulate iron uptake, or incorporation into the protein. Lastly characterizing the processing stability of the hemoglobin enhanced maize would be vital to its application.

One consideration with the different strategies presented is whether there could be a synergistic effect of combining technologies, for example processing the biotechnology enhanced maize with lactic acid, or fermentation to achieve yet higher bioavailability.

Given the diversity of maize food products, and the diversity of consumers, the strongest approach to enhancing iron bioavailability in maize is a diversified approach.

With novel strategies, combined with the existing knowledge and technologies, a greater impact on decreasing iron deficiency could be realized.